

2014

Plasma insulin-degrading enzyme: Characterisation and evaluation as a potential biomarker for Alzheimer's disease

Michelle Tegg
Edith Cowan University

Follow this and additional works at: <https://ro.ecu.edu.au/theses>



Part of the [Biochemical Phenomena, Metabolism, and Nutrition Commons](#), [Diseases Commons](#), and the [Geriatrics Commons](#)

Recommended Citation

Tegg, M. (2014). *Plasma insulin-degrading enzyme: Characterisation and evaluation as a potential biomarker for Alzheimer's disease*. Edith Cowan University. Retrieved from <https://ro.ecu.edu.au/theses/1198>

This Thesis is posted at Research Online.
<https://ro.ecu.edu.au/theses/1198>

Edith Cowan University

Copyright Warning

You may print or download ONE copy of this document for the purpose of your own research or study.

The University does not authorize you to copy, communicate or otherwise make available electronically to any other person any copyright material contained on this site.

You are reminded of the following:

- Copyright owners are entitled to take legal action against persons who infringe their copyright.
- A reproduction of material that is protected by copyright may be a copyright infringement. Where the reproduction of such material is done without attribution of authorship, with false attribution of authorship or the authorship is treated in a derogatory manner, this may be a breach of the author's moral rights contained in Part IX of the Copyright Act 1968 (Cth).
- Courts have the power to impose a wide range of civil and criminal sanctions for infringement of copyright, infringement of moral rights and other offences under the Copyright Act 1968 (Cth). Higher penalties may apply, and higher damages may be awarded, for offences and infringements involving the conversion of material into digital or electronic form.

USE OF THESIS

The Use of Thesis statement is not included in this version of the thesis.

Plasma Insulin-Degrading Enzyme: Characterisation and Evaluation as a Potential Biomarker for Alzheimer's Disease

Michelle Tegg, B.Sc.

Student Number: 981167

Master of Science in Human Biology

Faculty of Computing, Health and Science

School of Medical Sciences

Edith Cowan University

Supervisors

Prof. Ralph Martins

Dr. Veer Gupta

Dr. Giuseppe Verdile

Dr. Simon Laws

ABSTRACT

Alzheimer's disease (AD) is increasing in prevalence due to increasing lifespan and altered lifestyle. It is the fourth major cause of death in Western countries, resulting in significant economic and social impact (Von Strauss, *et al.*, 1999; Goate, 1997). There are no blood biomarkers currently accepted for the diagnosis of AD, and the identification of suitable biomarkers would eventually reduce the necessity for invasive, expensive and slow diagnostic procedures, as well as facilitate prognostic studies. An AD blood test would decrease the need for delaying diagnosis due to ambivalent presentation, and allow therapeutic intervention to commence at an earlier and more functional stage for the sufferer, thereby maximising the benefits of treatment. It is also feasible that a blood biomarker would be of use in the development of therapeutic treatments, which are currently inadequate.

Numerous studies have suggested that hyperinsulinemia and type II diabetes (DM2) significantly increase the risk of developing Alzheimer's disease (AD). Therefore, much research interest has been aimed recently toward determining the putative common mechanisms of these conditions. One enzyme which has been implicated in both AD and DM2 is insulin degrading enzyme (IDE). This project focuses largely on the characterisation of plasma IDE expression and catalytic activity, to help determine potential role/s of IDE in the development of AD, and the suitability of IDE as an AD biomarker. Evidence is also provided to support the concept that IDE impairments may be the common factor that links AD, hyperinsulinemia and DM2.

DECLARATION

I certify that this thesis does not, to the best of my knowledge and belief:

i. incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education;

ii. contain any material previously published or written by another person except where due reference is made in the text of this thesis; or

iii. contain any defamatory material;

MICHELLE TEGG

ACKNOWLEDGEMENTS

There are a number of people who I wish to thank for their various contributions that have made this thesis possible.

First of all I would like to thank my supervisors; Professor Ralph Martins, Dr Veer Gupta, Dr Guiseppe Verdile and Dr Simon Laws, for giving me the opportunity to do this project, and for their assistance and guidance throughout the project. During the difficult times when I sometimes doubted my abilities, they provided encouragement and inspiration. I truly appreciate all their unwavering support, and the many hours that they spent guiding and teaching me. In addition, this project (and ECU) has provided me the chance to publicise my research to the wider Alzheimer's community by assisting me to attend an international conference held in Paris in 2011 (Alzheimer's Association International Conference – AAlC). This was an eye-opening experience for me as it was my first overseas trip, at which I had the amazing opportunity to meet and talk with some of the greatest researchers of Alzheimer's disease in the world.

My work has also given me the opportunity to meet and work with some amazing colleagues, who have supported me and some have provided some useful advice as well as practical assistance, in the lab and in general. In particular I would like to thank Karl De Ruyck, Andrea Wilson, Mengqi Chen, Steve Pedrini, Eugene Hone, Linda Wijaya, Mike Morici, Renae Barr, Sam Burnham, Pratishtha Chatterjee, Tenielle Porter, Belinda Brown and Stephanie Rainey-Smith.

I would also like to acknowledge that this research is supported by CSIRO, the Science and Industry Endowment Fund and "in kind" contributions from study partners. The study also received support from the National Health and Medical Research Council (NHMRC) Australia via the Dementia Collaborative Research Centres program (DCRC) and through a project grant (APP1009292) awarded to Professor Ralph Martins, Dr Simon Laws and Dr Veer Gupta. Furthermore I would like to acknowledge the financial support of the Cooperative Research Centre (CRC) for Mental Health, from the Australian Government. Pfizer International has contributed financial support to assist with analysis of blood samples and to further the AIBL research program. The

McCusker Alzheimer's Research Foundation Inc. contributed financial and "in kind" support to AIBL. I thank these contributing organizations, as well as all those who took part as subjects in the study for their commitment and dedication to helping advance research into the early detection and causation of AD.

Thanks also to Dr Scott Miners who graciously corresponded with me in the early stages of optimising the IDE activity assay. This assay was originally developed by Dr Miners, and he assisted me with suggestions on improving the protocol which enhanced the results dramatically. I would also like to thank Professor Erik Helmerhorst for his interesting conversations on my topic and for his advice on lab-based issues.

I would also like to thank PathWest Laboratory Medicine WA for providing me with detailed protocols of the methods used to collect data for the AIBL study; I am most grateful for your assistance.

Special thanks to David Neustein who provided me with countless hours of invaluable advice and support; and without whom I would never have had the courage to even apply for a Master's degree. Thanks to Heather Williams and other members of the ECU Graduate Research School also, who provided support and assistance to me for the duration of my degree, as well as workshops run by the SOAR Centre and of course, some amazing social networking get-togethers.

Finally, I would like to thank my partner Steve Grecian, as well as my daughters Jessica, Rachael, Jasmine and Chelsea, and of course my mum and dad. Without their support, patience, love and encouragement, I would not have had the opportunity or ability to undertake such a challenging and inspiring endeavour, and for this I am truly grateful.

PUBLICATIONS

Conference Publications (Posters)

1. **Tegg, M.**, Rainey-Smith, S., Gupta, V. B., & Martins, R. (2011). Insulin-degrading enzyme as a potential blood biomarker for Alzheimer's disease. *Alzheimer's & Dementia*, 7(4), S331.
2. Chen, M., **Tegg, M.**, Avdesh, A., Mondal, A., Lardelli, M., Verdile, G., & Martins, R. (2012). Analysis of insulin-degrading enzyme (IDE) expression and function in zebrafish embryos. *Alzheimer's & Dementia*, 8(4), P674.

TABLE OF CONTENTS

ABSTRACT	iii
DECLARATION	v
ACKNOWLEDGEMENTS.....	vii
PUBLICATIONS	ix
Conference Publications (Posters)	ix
TABLE OF CONTENTS	x
LIST OF FIGURES	xxii
LIST OF TABLES.....	xxviii
ABBREVIATIONS.....	xxx
CHAPTER 1 - Literature review/introduction.....	1
AD Introduction.....	1
1.1 AD symptoms and characteristics	2
1.1.1 Clinical Characteristics of AD	2
1.1.2 Macroscopic Characteristics	3
1.1.3 Pathological Characteristics.....	3
1.1.3.1 Neurofibrillary Tangles (NFT).....	4
1.1.3.2 Senile Plaques (SP).....	4
1.2 Beta Amyloid (A β) - A Key Molecule	5
1.2.1 Early onset and Late onset AD.....	5
1.2.2 Amyloid Precursor Protein (APP).....	6
1.2.3 A β Toxicity in AD	8
1.3 Aetiology of LOAD	9

1.4 Diagnostic Techniques.....	10
1.4.1 Imaging Techniques and Biopsy.....	11
1.4.2 AD Biomarkers	12
1.4.3 MCI.....	13
1.4.4 SMC.....	13
1.4.5 The need for better AD biomarkers.....	13
1.4.5.2 The “Ideal Biomarker for AD”	14
1.4.6 A β as a Biomarker for AD Diagnosis	15
1.4.6.1 A β and PET Imaging.....	15
1.4.6.2 A β in CSF.....	15
1.4.6.3 A β in Plasma	16
1.5 Apolipoprotein E.....	18
1.5.1 Association of <i>APOE</i> genotype with AD.....	18
1.5.2 Functions of ApoE	19
1.5.3 <i>APOE</i> and AD.....	20
1.5.4 ApoE in plasma.....	21
1.6 A β Degradation and Clearance.....	22
1.6.1 Insulin-Degrading Enzyme	23
1.6.1.1 Structure and Function of IDE.....	26
1.7 Type 2 Diabetes, Insulin Resistance Syndrome and AD	30
1.7.1 Insulin and AD	32
1.7.1.1 Hyperinsulinemia	32
1.7.1.2 Insulin and Cognition	33
1.7.1.3 Insulin and A β	34
1.7.1.4 Hypercholesterolemia, Insulin and IDE.....	35
1.8 Hyperinsulinemia, IDE and AD.....	37

1.8.1 IDE Catalytic Activity	40
1.8.2 IDE and A β Neurotoxicity	42
1.8.3 ApoE and IDE.....	43
1.9 IDE Levels in Plasma	44
1.10 Genetic Linkage and Association of IDE	45
1.11 Summary and Project Hypothesis	47
CHAPTER 2 – Materials and Methods.....	53
2.1 Materials.....	53
2.1.1 Antibodies	53
2.1.2 Suppliers of Reagents	56
2.1.2.1 Proteins.....	56
2.1.2.2 Other reagents.....	56
2.1.3 Suppliers of Equipment.....	58
2.1.3.1 Western Immunoblotting	58
2.1.3.2 ELISA/Fluorescent and BCA Protein Assay	58
2.2 Methods	59
2.2.1 The Australian Imaging, Biomarker and Lifestyle Flagship Study of Ageing (AIBL) Cohort.....	59
2.2.2 Sample preparation	62
2.2.2.1 Methods used for the measurement of biomarkers other than IDE.....	62
2.2.3 Determination of plasma total protein content.....	63
2.2.4 IDE levels in human plasma by Western immunoblotting	65
2.2.4.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation of proteins	65
2.2.4.2 LS-C55007 immunoblotting protocol	66
2.2.4.3 AB9210 immunoblotting protocol.....	66

2.2.4.4	ab28561 immunoblotting protocol.....	67
2.2.4.5	Stripping of antibodies from membrane	67
2.2.4.6	Ponceau S and Coomassie staining as a loading control	67
2.2.4.7	Test of EDTA spiking on IDE levels in lithium-heparin collected plasma	68
2.2.4.8	Test of cross-reactivity of LS-C55007 (and ab28561) to purified BSA and HSA .	68
2.2.4.9	Test of cross-reactivity of ab28561 to purified BSA and albumin-free blocking agent	69
2.2.4.10	Pre-incubation with recombinant human IDE blocking test for AB9210 for specificity	69
2.2.4.11	Pre-incubation with immunizing peptide blocking test for LS-C55007 for specificity	70
2.2.4.12	Pre-incubation with immunizing peptide blocking test for ab28561 for specificity	70
2.2.4.13	Pre-clearing IgG from plasma.....	71
2.2.4.14	Pre-clearing albumin from plasma.....	71
2.2.4.15	Preparation of A β ₄₂	72
2.2.4.16	Immunoprecipitation of IDE from plasma	72
2.2.4.17	Western Blotting image analysis and quantitation.....	73
2.2.5	IDE levels in human plasma by enzyme-linked immunosorbent assay (ELISA)	74
2.2.5.1	Kamiya IDE ELISA commercial kit.....	74
2.2.5.2	Indirect In-house ELISA for IDE	74
2.2.5.3	Sandwich ELISA for IDE.....	75
2.2.5.4	Low-level data analysis	76
2.2.6	Catalytic activity of plasma IDE by immunocapture-based IDE-specific fluorometric assay	77
2.2.6.1	Immunocapture-based IDE specific fluorometric assay	77
2.2.6.2	Inhibition of catalytic activity of IDE by IDE-specific antibodies and A β ₄₀	78
2.2.6.3	Inhibition of catalytic activity of IDE by A β ₄₂	78
2.2.6.4	Preparation of insulin.....	79
2.2.6.5	Inhibition of catalytic activity of IDE by insulin	79
2.2.6.6	Determination of fluorometric activity assay cross-reactivity with human (HSA) and bovine (BSA) serum albumin.....	79
2.2.6.7	Comparison of IDE activity from EDTA collected plasma and Lithium-Heparin (Li-Hep) collected plasma.....	79

2.2.6.8 Low-level data analysis	80
2.2.7 Statistical Analysis.....	81

CHAPTER 3 – Full-length plasma IDE Levels are higher in MCI in comparison to healthy controls or AD, as measured by Western Immunoblotting..... 83

3.1 Introduction.....	83
-----------------------	----

3.1.1 Aims	85
------------------	----

3.2 Experimental Summary	86
--------------------------------	----

3.2.1 Antibody characterisation: Pre-incubation of polyclonal IDE antibody Ab 1 with recombinant human IDE to check specificity for human plasma IDE.	86
--	----

3.2.2 Testing the batch-to-batch variability of IDE antibody Ab 1 on human plasma and rrIDE.	86
---	----

3.2.3 Test of alternate polyclonal IDE antibody Ab 2: comparison with IDE antibody Ab 1.....	86
--	----

3.2.4 Determination of the effect of EDTA in blood collection tubes on levels of human plasma IDE detected with IDE antibody Ab 2.....	87
--	----

3.2.5 Investigating potential cross-reactivity of IDE antibody Ab 2 with purified human and bovine albumin.....	87
---	----

3.2.6 Pre-incubation of IDE antibody Ab 2 with a synthesised immunogen peptide to assess specificity of antibody to IDE in human plasma.....	88
--	----

3.2.7 Test of alternate polyclonal IDE antibody Ab 3: assessment of its detection of rrIDE as well as full-length IDE in human plasma.....	88
--	----

3.2.8 Determination of the effect of EDTA in blood collection tubes on the detection of human plasma IDE using IDE antibody Ab 3.....	88
---	----

3.2.9 Testing IDE antibody Ab 3 for cross-reactivity with BSA, HSA and a non-albumin containing blocking agent.	89
--	----

3.2.10 Pre-clearing and albumin-removal from human plasma.	89
---	----

3.2.11 Immunoprecipitation (IP) of IDE from human plasma with polyclonal IDE antibodies Ab 4 and Ab 5.....	90
3.2.12 Testing the specificity of IDE antibody Ab 3 for IDE in human plasma by pre-incubation with a commercial blocking peptide.	90
3.2.13 Determination of full-length IDE levels in the plasma samples of the AIBL cohort by SDS-PAGE and Western immunoblotting, using IDE antibody Ab 3.	91
3.3 Results.....	92
3.3.1 Bands detected by IDE antibody Ab 1(a) (Batch 1) in human plasma are IDE.	92
3.3.2 Subsequent batches of polyclonal IDE antibody Ab 1 are unable to detect full-length IDE in human plasma.....	93
3.3.3 Comparison of IDE antibody Ab 1 (AB9210, Batch 1) and IDE antibody Ab 2 (LS-C55007).	95
3.3.4 EDTA has no effect on the intensity of the 115-120 kDa band detected with IDE Ab 2.....	99
3.3.5 IDE Ab 2 cross-reacts with HSA strongly, and BSA weakly, in Western immunoblotting.	101
3.3.6 Testing the specificity of IDE Ab 2 by pre-incubation blocking of the antibody with the immunising peptide.....	102
3.3.7 Test of IDE antibody ab28561 (Ab 3).	105
3.3.8 EDTA has no effect on the intensity of the 120-130 kDa band detected with IDE Ab 3 (ab28561).	108
3.3.9 Ab 3 (ab28561) cross-reacts with BSA, HSA and a non-albumin containing blocking agent.....	109
3.3.10 Immunoprecipitation of IDE from human plasma.....	112

3.3.11 Pre-incubation immunising peptide blocking test on IDE antibody Ab 3 (ab28561) for specificity.	115
3.3.12 Measurement of the 120-130 kDa band in plasma samples obtained from the AIBL cohort at baseline.....	116
3.3.13 Low-level data analysis.	117
3.3.14 Full-length plasma IDE levels are increased in MCI compared with HC in the total cohort.	126
3.3.15 Full-length plasma IDE levels are increased in females with MCI compared with HC and AD	131
3.3.16 Levels of full-length plasma IDE do not differ in <i>APOE</i> $\epsilon 4$ carriers compared with non-carriers.	132
3.3.17 Relationships between total plasma IDE levels and other biochemical data collected in the AIBL study.	137
3.3.17.1 The ratio of full-length IDE to insulin is not significantly different between classifications.....	145
3.3.17.2 The ratio of full-length IDE to total A β (42+40) tends to be higher in MCI compared with HC.	147
3.3.17.3 The ratio of full-length IDE to insulin + total A β_{42+40} is not different between clinical classifications.....	149
3.4 Discussion	151
3.4.1 Summary.....	165

CHAPTER 4 – Total plasma IDE Levels measured by enzyme-linked immunosorbent assay (ELISA) are decreased in MCI in comparison to healthy controls or AD. 169

4.1 Introduction.....	169
4.1.1 Aims	170
4.2 Experimental Summary	171
4.2.1 Test of commercial IDE ELISA kit for suitability in testing human plasma. .	171

4.2.2 Screening of eight commercial IDE antibodies to determine suitability for an in-house, indirect IDE ELISA for use on human plasma samples.....	171
4.2.3 Test of the three most suitable IDE antibodies for an IDE sandwich ELISA.....	171
4.2.4 Assay control tests.	171
4.2.5 Optimisation of coating conditions for the IDE sandwich ELISA.	172
4.2.6 Optimisation of sample and antibody diluents in the IDE ELISA.	172
4.2.7 Optimisation of detection antibody incubation in the IDE ELISA.....	172
4.2.8 Investigation of the upper detection limit of the IDE sandwich ELISA.....	173
4.2.9 Optimisation of coating antibody concentration and extension of standard curve for upper detection limit analysis in the IDE sandwich ELISA.	173
4.2.10 Test of non-albumin containing blocking agent to reduce background in the IDE sandwich ELISA.	173
4.2.11 Measurement of total plasma IDE levels in a subset of the AIBL cohort. .	174
4.2.12 Testing for potential cross-reactivity of BSA (standard and Fraction V) and HSA in the IDE sandwich ELISA.	174
4.2.13 Spike and recovery assessment of IDE sandwich ELISA.....	174
4.3 Results.....	175
4.3.1 The commercial IDE ELISA kit is unsuitable for the testing of human plasma samples.	175
4.3.2 Indirect ELISA detection is effective for purified rat IDE, but not for IDE in human plasma.	178
4.3.3 Optimisation of a sandwich ELISA for measuring plasma IDE in the AIBL cohort.....	188
4.3.4 Tests for non-specific binding of IDE sandwich ELISA reagents.	192
4.3.5 Optimising the coating conditions for the IDE sandwich ELISA.....	193

4.3.6 Optimising the sample and detection antibody diluent in the IDE sandwich ELISA.....	195
4.3.7 Improvements in detected signals in the IDE sandwich ELISA following changes to the detection antibody incubation time and temperature.	197
4.3.8 A trial IDE sandwich ELISA was linear to approximately 4000 ng/mL and upper detection limit was not reached at 64000 ng/mL.....	199
4.3.9 Coating concentration of Ab 3 in IDE sandwich ELISA produces the highest signal at 5 µg/mL and upper detection limit is not reached at 160000 ng/mL IDE.	201
4.3.10 Testing a non-albumin containing blocking agent in the IDE sandwich ELISA.	203
4.3.11 Low-level data analysis.	204
4.3.12 Total plasma IDE levels are reduced in MCI and AD compared with HC...	204
4.3.13 Total plasma IDE levels reduce (not significantly) with age.	214
4.3.14 <i>APOE</i> $\epsilon 4$ carriers do not have significantly different total plasma IDE levels compared with non-carriers.	215
4.3.15 Relationships between total plasma IDE levels and other biochemical data collected in the AIBL study	220
4.3.15.1 Total IDE levels correlate with age, A β , testosterone, and HDL cholesterol...	220
4.3.15.2 The ratio of total IDE to insulin is lower in MCI and AD individuals.	229
4.3.15.3 The ratio of total IDE to A β_{42+40} is significantly lower in MCI and AD compared with HC.	234
4.3.15.4 The ratio of total IDE to Insulin + A β_{42+40} is significantly lower in MCI and AD compared with HC.	238
4.3.16 Follow-up optimisation (1): Cell-culture grade BSA produces a dose-dependent signal in IDE sandwich ELISA but Fraction V BSA and HSA do not.	242
4.3.17 Follow-up optimisation (2): Spike and recovery assessment of IDE sandwich ELISA.....	244

4.4 Discussion	246
Analysing ELISA background problems	253
4.4.1 Summary	258
CHAPTER 5 –IDE enzyme activity in plasma is higher in MCI compared with HC and AD.....	259
5.1 Introduction.....	259
5.1.1 Aims	260
5.2 Experimental summary.....	261
5.2.1 Test of immunocapture-based fluorometric assay for the measurement of the catalytic activity of plasma IDE.....	261
5.2.2 Test of fluorometric IDE activity assay specificity	261
5.2.3 Comparison of EDTA-collected blood and lithium-heparin-(LiHep) collected blood: determining which is better in the IDE activity assay.	261
5.2.4 Determination of plasma IDE catalytic activity in the AIBL cohort using the IDE immunocapture-based fluorometric activity assay.	262
5.3 Results.....	263
5.3.1 PCP and rrIDE generate a time and dose-dependent signal in the fluorometric activity assay.....	263
5.3.2 Validating the new batch of coating antibody Ab 3 (ab28561).....	268
5.3.3 EDTA collected plasma produces a higher signal than Lithium-heparin collected plasma in the IDE fluorometric activity assay.....	273
5.3.4 Investigating the specificity of the IDE catalytic activity assay using the IDE inhibitors A β ₄₂ and insulin.	275
5.3.5 Optimisation Summary	277
5.3.6 Low-level data analysis.	278

5.3.7 Plasma IDE catalytic activity is higher in MCI compared with HC and AD...	279
5.3.8 Plasma IDE catalytic activity does not increase with age.	290
5.3.9 <i>APOE</i> $\epsilon 4$ genotype does not impact on plasma IDE catalytic activity.	291
5.3.10 The ratio of IDE activity to insulin levels in plasma is not significantly different between classifications.....	296
5.3.11 The ratio of IDE Activity: $A\beta_{42}$ + $A\beta_{40}$ levels increases in MCI and AD compared with HC.	302
5.3.12 IDE Activity/Insulin + $A\beta_{42+40}$ ratio increases in MCI compared with HC and AD.....	306
5.3.13 Correlation analyses of plasma IDE catalytic activity with multiple biochemical measures in the AIBL cohort.	310
5.4 Discussion	317
5.4.1 Summary	326
Chapter 6 – Final Discussion	329
6.1 Introduction.....	329
6.2 Assay development and validation	330
6.3 Potential significance of plasma IDE in AD pathology.....	333
6.3.1 Findings of IDE levels/activity measurements by classification	335
6.3.2 Impact of <i>APOE</i> $\epsilon 4$ genotype on IDE levels and activity	339
6.3.3 Associations between IDE levels and activity with biochemical variables measured in the AIBL cohort	340
6.3.3.1 Testosterone.....	340
6.3.3.2 IDE levels and plasma $A\beta$	341
6.3.3.3 Associations with gender.....	344
6.4 Limitations	346

6.5 Future Directions	350
6.6 Conclusions	353
REFERENCES	355
APPENDIX – Further optimisation tests for Fluorometric Activity Assay	425
A1 Determination of fluorometric activity assay cross-reactivity with human (HSA) and bovine (BSA) serum albumin	425
A2 Testing phosphate-based buffers against tris-based buffers as sample diluents in the IDE fluorometric activity assay.....	427

LIST OF FIGURES

Figure 1.1 Representation of the generation of A β	7
Figure 1.2 The IDE protein in complex with an A β peptide.	27
Figure 1.3 Representation of various factors associated with AD or DM2 which may affect IDE function.	49
Figure 1.4 Flowchart of how a reduction in IDE levels or activity may lead to the development of both AD and DM2.....	51
Figure 2.1 Diagram of IDE epitopes to which commercially-available antibodies bind.	55
Figure 2.2 Classification of AIBL cohort subgroups at baseline by diagnosis (n=1112).	61
Figure 3.1 Immunising peptide (protein) blocking experiment on IDE antibody AB9210 (Ab 1(a))......	93
Figure 3.2 Comparison of original batch of IDE antibody AB9210 (Ab 1(a) (Batch 1)) with a subsequent batch of AB9210 (Ab 1(b) (Batch 2)) on human plasma and rrIDE. .	94
Figure 3.3 (A) and (B) Comparison of polyclonal IDE antibody AB9210 (Ab 1(a) (Batch 1)) with LS-C55007 (Ab 2) on human plasma and rrIDE.	96
Figure 3.4 Investigating the effect of extended ECL development time on the detection of full-length human plasma IDE with Ab 2.	98
Figure 3.5 Plasma samples probed with Ab 2 (LS-C55007), using rrIDE as positive control, and developed using ECL™ Advance.	99
Figure 3.6: Investigating the effect of EDTA on IDE detection.	100
Figure 3.7 Test of cross-reactivity between Ab 2 (LS-C55007) and HSA or BSA.	102
Figure 3.8(A) Pre-incubation of IDE Ab 2(LS-C55007) with the immunising peptide to test antibody specificity (Test 1).	103
Figure 3.8(B) Pre-incubation of IDE Ab 2(LS-C55007) with the immunising peptide to test antibody specificity (Test 2).	104
Figure 3.8(C) Pre-incubation of IDE Ab 2 (LS-C55007) with the immunising peptide to test antibody specificity (Test 3).	105
Figure 3.9 Test of Ab 3 (ab28561) on human plasma, rat liver, human frontal cortex and recombinant IDE (rat and human).	107
Figure 3.10 Investigating whether EDTA in blood samples interferes with Ab 3 (Ab28561) detection of putative IDE protein bands.....	108
Figure 3.11(A) Test of cross-reactivity of IDE Ab 3 with HSA and BSA.....	110

Figure 3.11(B) Test of cross-reactivity of IDE antibody Ab 3 with BSA and ECL™ Advance Blocking Reagent.....	111
Figure 3.12(A) Immunoprecipitation of IDE from human plasma using IDE mouse polyclonal Ab 4 (250706).	113
Figure 3.12(B) Immunoprecipitation of IDE from human plasma using IDE antibody Ab 5 (AF2496).	114
Figure 3.13 Testing IDE Ab 3 (ab28561) specificity by blocking with the immunising peptide prior to adding to Western blots.	116
Figure 3.14 120-130 kDa band measured in human plasma samples obtained from the AIBL cohort (Ab 3).	117
Figure 3.15(A) Frequency histogram of overall data (IDE levels measured by Western immunoblotting) before transformation.	119
Figure 3.15(B) Frequency histogram of overall data (IDE levels measured by Western immunoblotting) after square root transformation.	120
Figures 3.16 (A)(B)(C) Frequency histograms of data (IDE levels measured by Western immunoblotting (WB)) stratified by classification (HC, MCI, AD, respectively) before transformation.	123
Figures 3.17 (A)(B)(C) Frequency histograms of data (IDE levels measured by Western immunoblotting) stratified by classification (HC, MCI, AD, respectively) after transformation by square root.	125
Figure 3.18 Levels of full-length plasma IDE are increased in MCI compared with HC and AD.	130
Figure 3.19 Full-length plasma IDE is higher in females compared with males.	131
Figure 3.20 Full-length plasma IDE levels are increased in MCI compared with HC and AD in females only.	132
Figure 3.21(A) Full-length plasma IDE levels are no different in $\epsilon 4$ non-carriers compared with carriers.	133
Figure 3.21(B) and (C) Full-length plasma IDE levels are not significantly different by clinical classification for $\epsilon 4$ non-carriers or carriers.	134
Figure 3.22 Full-length plasma IDE levels are significantly increased in MCI compared with HC and AD.	137
Figure 3.23(A) The ratio of full-length IDE to insulin is not significantly different between classifications.	146

Figure 3.23(B) The ratio of full-length IDE to A β ₄₂₊₄₀ is not significantly different between classifications.....	148
Figure 3.23(C) The ratio of full-length IDE to insulin + A β ₄₂₊₄₀ is not significantly different between classifications.....	150
Figure 3.24 Hypothetical representation of potential mechanism of batch-to-batch variability in polyclonal antibodies.	153
Figure 4.1 (A) Comparison of standard curves from Run 1 and Run 2, using the commercial IDE ELISA kit.....	176
Figure 4.1 (B) Test of commercial IDE ELISA kit with human plasma (Run 1 versus Run 2).	177
Figure 4.1 (C) Test of commercial IDE ELISA kit with human plasma – raw values (Run 1 versus Run 2).....	178
Figures 4.2 A (A) and (B) Screening of monoclonal IDE antibody MMS-282R (MAb 7).	180
Figures 4.2 B (A) and (B) Screening of polyclonal IDE antibody GTX111664 (Ab 8). ...	181
Figure 4.2 C (A) and (B) Screening of polyclonal IDE antibody AB9210 (Ab 1).	182
Figure 4.2 D (A) and (B) Screening of polyclonal IDE antibody 250706 (Ab 4).	183
Figure 4.2 E (A) and (B) Screening of monoclonal IDE antibody 5282-1 (MAb 9).....	184
Figure 4.2 F (A) and (B) Screening of polyclonal IDE antibody H00003416-A01 (Ab 6).	185
Figure 4.2 G (A) and (B) Screening of polyclonal IDE antibody LS-C55007 (Ab 2).	186
Figure 4.2 H (A) and (B) Screening of polyclonal IDE antibody ab28561 (Ab 3).	187
Figures 4.3 (A) and (B) Optimisation of IDE sandwich ELISA using Ab 3 and Ab 6.	189
Figures 4.4 (A) and (B) Comparison of the potential detection antibodies Ab 6 and Ab 2.....	191
Figure 4.5 Testing for non-specific binding/cross-reactivity in the IDE sandwich ELISA using ab28561 as coating antibody (1:400) and LS-C55007 as detection antibody (1:750).....	192
Figures 4.6 (A) and (B) Optimisation of coating conditions for the IDE sandwich ELISA.	194
Figures 4.7 (A) and (B) Optimising buffer constituents for the dilution of samples and detection antibody in sandwich ELISA.	196

Figures 4.8 (A) and (B) Optimisation of detection antibody incubation time and temperature.....	198
Figure 4.9 (A) and (B) Trial IDE sandwich ELISA to give an extended 11-point standard curve and measure five individual plasma samples.	200
Figure 4.10 Optimisation of coating conditions with Ab 3 and extension of standard curve to 160000 ng/mL.	202
Figure 4.11 Test of non-albumin containing blocking agent in the IDE sandwich ELISA.	203
Figure 4.12 (A) and (B) Sample standard curve and 10 sets of samples.....	206
Figure 4.13(A) and (B) Levels of total plasma IDE are decreased in MCI and AD compared with HC, and no different in males versus females.....	212
Figure 4.14 Total plasma IDE levels reduce (not significantly) with age.....	214
Figure 4.15(A) Total IDE levels are not significantly reduced, in <i>APOE</i> $\epsilon 4$ carriers compared with non-carriers.	216
Figure 4.15(B) and (C) Total IDE levels are reduced in MCI and AD (but not significantly), in <i>APOE</i> $\epsilon 4$ carriers but not in non-carriers.	218
Figure 4.16(A) AD and MCI individuals have a lower IDE/Insulin ratio than controls (overall).	230
Figure 4.16(B) and (C) MCI individuals that do not carry an <i>APOE</i> $\epsilon 4$ allele have a lower IDE/Insulin ratio than non- $\epsilon 4$ controls.....	232
Figure 4.17(A) The plasma IDE/ $A\beta_{42+40}$ ratio is significantly reduced in MCI and AD in comparison to HC.....	235
Figure 4.17(B) and (C) The plasma IDE/ $A\beta_{42+40}$ ratio is not significantly reduced in MCI and AD in comparison to HC when stratified by <i>APOE</i> status.	236
Figure 4.18(A) The plasma IDE/Insulin+ $A\beta_{42+40}$ ratio is significantly reduced in MCI and AD in comparison to HC.	239
Figure 4.18(B) and (C) The plasma IDE/Insulin+ $A\beta_{42+40}$ ratio is significantly reduced in MCI in comparison to HC in <i>APOE</i> $\epsilon 4$ non-carriers only.	240
Figure 4.19 Determination and comparison of potential cross-reactivity of different albumins in the IDE sandwich ELISA.	243
Figure 4.20 Assessment of the potential interference of plasma components in the IDE sandwich ELISA.....	245

Figures 5.1(A) and (B) Test of fluorometric IDE enzyme activity assay, using ab28561 (Ab 3) as coating antibody.	266
Figure 5.1(C). Test of immunocapture-based IDE fluorometric activity assay (with Ab 3) to analyse components of the assay.....	267
Figure 5.1(D). Further validation test of fluorometric activity assay using specific IDE antibodies and A β 40 as inhibitors of IDE-based cleavage of the fluorogenic peptide.	268
Figures 5.2(A) and (B) Test of fluorometric IDE activity assay using new batch of IDE Ab 3 as capture antibody.	270
Figure 5.3 Test of immunocapture-based IDE fluorometric activity assay (with new batch of Ab 3) to analyse components of the assay.....	273
Figure 5.4 Comparison of IDE activity in PCP originating from blood collected in EDTA tubes versus LiHep tubes.	275
Figure 5.5(A). Test of fluorometric activity assay specificity by inhibition with beta-amyloid isoform 42 (A β ₄₂).....	276
Figure 5.5(B). Test of fluorometric activity assay specificity by inhibition with insulin.	277
Figure 5.6(A) and (B) Sample standard curve and 10 sets of samples.....	280
Figure 5.7 Catalytic activity of plasma IDE is increased in MCI compared with HC and AD.....	285
Figure 5.8(A) Gender has no effect on the catalytic activity of plasma IDE overall. ...	286
Figure 5.8(B) and (C) Plasma IDE activity is increased (not significantly) in male MCI compared with HC.	288
Figure 5.9 Plasma IDE activity does not increase with age.....	290
Figure 5.10(A) Plasma IDE catalytic activity is not significantly different in <i>APOE</i> ϵ 4 carriers compared with non-carriers.	292
Figure 5.10(B) and (C) Plasma IDE catalytic activity is not significantly different between HC/MCI/AD classifications after dividing results into <i>APOE</i> ϵ 4 carriers and non-carriers.....	294
Figure 5.11(A) The IDE activity/insulin level ratio is not significantly different between classifications in the overall subgroup.....	297
Figure 5.11(B) The IDE activity/insulin level ratio is not significantly different between males and females in the overall subgroup.....	298

Figure 5.11(C) and (D) The IDE activity/insulin level ratio is not significantly different between classification when stratified by <i>APOE</i> $\epsilon 4$ status.....	300
Figure 5.12 (A) The IDE Activity/ $A\beta_{42+40}$ ratio is increased in MCI (not significantly) and AD (significantly) when compared with HC.	303
Figure 5.12 (B) and (C) The IDE Activity/ $A\beta_{42+40}$ ratio is increased in MCI and AD (not significantly) when compared with HC in <i>APOE</i> $\epsilon 4$ carriers only.....	304
Figure 5.13(A) IDE Activity/Insulin + $A\beta_{42+40}$ ratios are increased in MCI (significantly) and AD (not significantly) compared with HC.....	307
Figure 5.13(B) and (C) IDE Activity/Insulin + $A\beta_{42+40}$ ratios are no different by classification when stratified by <i>APOE</i> $\epsilon 4$ status.	308

LIST OF TABLES

Table 2.1 Commercial primary IDE antibodies.....	53
Table 2.2 Additional commercial non-IDE and secondary antibodies.....	54
Table 3.1 Values of skewness, kurtosis, Kolmogorov-Smirnoff (K-S) and Shapiro-Wilk (S-W) tests of distribution normality in the overall data before and after square root transformation.....	121
Table 3.2 Values of skewness, kurtosis, Kolmogorov-Smirnoff (K-S) and Shapiro-Wilk (S-W) tests of distribution normality in the data before transformation.....	124
Table 3.3 Values of skewness, kurtosis, Kolmogorov-Smirnoff (K-S) and Shapiro-Wilk (S-W) tests of distribution normality in the data after transformation by square root.	124
Table 3.4 Demographics of the AIBL cohort subset used for the Western immunoblotting IDE analysis.	128
Table 3.2 Correlation matrix of full-length plasma IDE levels with other variables.	139
Tables 3.3 (A) Correlation matrix of full-length plasma IDE levels with other biochemical variables (males only).....	140
Tables 3.3 (B) Correlation matrix of full-length plasma IDE levels with other biochemical variables (females only).....	141
Table 3.4 (A) Correlation matrix of full-length plasma IDE levels with other variables for <i>APOE</i> $\epsilon 4$ non-carriers only.....	143
Table 3.4 (B) Correlation matrix of full-length plasma IDE levels with other variables for <i>APOE</i> $\epsilon 4$ carriers only.....	144
Table 4.1 Basic demographics of the AIBL cohort subset of participants for whom plasma IDE levels were measured by ELISA.....	208
Table 4.2 Correlation matrix of total plasma IDE levels with other variables.	222
Tables 4.3 (A) Correlation matrix of total plasma IDE levels with other biochemical variables separately by gender (males only).	225
Tables 4.3 (B) Correlation matrix of total plasma IDE levels with other biochemical variables separately by gender (females only).	226
Table 4.4 (A) Correlation matrix of total plasma IDE levels with other variables for <i>APOE</i> $\epsilon 4$ non-carriers only.	227

Table 4.4 (B) Correlation matrix of total plasma IDE levels with other variables for <i>APOE</i> $\epsilon 4$ carriers only.....	228
Table 5.1 Basic demographics of the AIBL cohort subset of participants for whom plasma IDE catalytic activity was measured by fluorometric assay.	282
Table 5.2 Correlation matrix of plasma IDE catalytic activity with other biochemical variables measured as part of the AIBL study.....	312
Table 5.3(A) Correlation matrix of plasma IDE catalytic activity with other biochemical variables measured as part of the AIBL study after stratification for gender – males only.....	313
Table 5.3(B) Correlation matrix of plasma IDE catalytic activity with other biochemical variables measured as part of the AIBL study after stratification for gender – females only.....	314
Table 5.4(A) Correlation matrix of plasma IDE catalytic activity with other biochemical variables measured as part of the AIBL study after stratification for <i>APOE</i> $\epsilon 4$ status - $\epsilon 4$ non-carriers only (unadjusted).	315
Table 5.4(B) Correlation matrix of plasma IDE catalytic activity with other biochemical variables measured as part of the AIBL study after stratification for <i>APOE</i> $\epsilon 4$ status - $\epsilon 4$ carriers only (unadjusted).	316

ABBREVIATIONS

AA	Amino acid/s
A β	Beta-amyloid
Ab	Antibody
AD	Alzheimer's disease
Adj.	Correlation after adjustment (Bonferroni-Holm)
AIBL	Australian Imaging, Biomarker and Lifestyle Flagship Study of Ageing
<i>APOE</i>	Apolipoprotein E (Gene)
ApoE	Apolipoprotein E (Protein)
APP	Amyloid precursor protein
BBB	Blood-brain barrier
BSA	Bovine serum albumin
CNS	Central nervous system
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
E2/3/4	Epsilon 2/3/4
ECL	Enhanced chemiluminescence solution
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EOFAD	Early-onset familial AD
FAD	Familial AD
H ₃ PO ₄	Phosphoric acid

HCl	Hydrochloric acid
HDL	High-density lipoprotein
HEPES	N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid
HFIP	Hexafluoroisopropanol
HRP	Horseradish peroxidase
HSA	Human serum albumin
HSP	Heat shock protein
IDE	Insulin-degrading enzyme
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
IP	Immunoprecipitation
kDa	Kilodalton
LDL	Low-density lipoprotein
LDS	Lithium dodecyl sulphate
LiHep	Lithium-heparin anticoagulant
M(Ab)	Monoclonal antibody
MCI	Mild cognitive impairment
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MQ H ₂ O	Molecular-grade water
mRNA	Messenger RNA
MWM	Molecular weight marker

NMDA	N-methyl-d-aspartic acid
NFDM	Non-fat dairy milk powder
NFT	Neurofibrillary tangle/s
OD	Optical density
O/N	Overnight (~18 hours)
PBS	Phosphate-buffered saline
PBST	PBS + 0.05% Tween-20
PCP	Pooled control plasma
PET	Positron Emission Tomography
PI	Protease inhibitor
PiB	Pittsburgh Compound B
RA	Reducing agent
RNA	Ribonucleic acid
rhIDE	Recombinant human IDE
rrIDE	Recombinant rat IDE
RT	Room temperature (~ 25°C)
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SMC	Subjective memory complainers
SP	Senile plaque/s
TBS	Tris-buffered saline

TBST	TBS + 0.05% Tween-20
TGF	Transforming growth factor
TMB	3,3',5,5'-tetramethylbenzidine
TP	Total protein
Unadj.	Correlation prior to adjustment (Bonferroni-Holm)
WB	Western immunoblot

CHAPTER 1 - Literature review/introduction.

AD Introduction

Alzheimer's disease (AD) is a progressive, neurodegenerative disorder, and is the most common form of dementia, accounting for between 50% and 80% of all dementia cases (Alzheimer's disease facts and figures (2012)). Clinical symptoms include increasing memory loss and cognitive functioning, ultimately leading to severe disability and death. The prevalence of AD is rising globally, and the prevalence and incidence increases with age (Von Strauss, *et al.*, 1999; Gascón-Bayarri *et al.*, 2007). In Australia, the number of individuals with dementia is currently around 266,000, and is expected to exceed 940,000 by 2050 (Deloitte Access Economics, 2011). In the United States of America (USA), AD has been found to be the sixth highest cause of death (Alzheimer's disease facts and figures (2012)) and results in significant economic and social impact, yet research funding to date has been grossly inadequate.

One of the key hallmarks of AD pathology is the development of senile plaques in various regions of the brain, of which the core component is a 4kDa peptide termed beta-amyloid or A-beta ($A\beta$). It is widely believed that the initiation of AD pathology is triggered by abnormal increases in the steady-state levels of $A\beta$ in the brain, as described by the "amyloid cascade hypothesis" (Hardy & Higgins, 1992).

AD can be classified according to the age of onset, with the majority of cases occurring after the age of 65 years (around 95%). This type of AD is termed "late-onset AD" (LOAD), whereas AD with an onset occurring prior to 65 years of age is termed "early-onset AD" (EOAD). While most EOAD cases are known to be caused by mutations in one of three genes, the aetiology of LOAD is less clear. The accumulation of $A\beta$ in EOAD appears to be due primarily to increased production of $A\beta$ peptides, or a proportionate increase in the production of the longer, more amyloidogenic $A\beta_{1-42}$ peptide (Hardy, 1997), however evidence suggests that faulty $A\beta$ clearance mechanisms may also be implicated in the development of LOAD (Selkoe, 2001). At present, the most significant known genetic risk factor for LOAD is possession of the *Apolipoprotein E epsilon 4* allele (*APOE ϵ 4*).

Numerous studies have found an association between Type 2 diabetes (DM2) and AD; however the mechanisms that are common to these two diseases, and mechanisms of DM2 that cause an increased risk of AD are not well understood. One enzyme which is believed to have a role in both diseases is insulin-degrading enzyme (IDE): this enzyme can degrade A β therefore it is believed to play a major part in the clearance of A β . This chapter will explain the processes and key features of AD pathology, specifically focusing on recent literature and encompassing IDE-related A β clearance mechanisms as well as factors that link type-2 diabetes and LOAD.

1.1 AD symptoms and characteristics

1.1.1 Clinical Characteristics of AD

Clinically, individuals with AD present with amnesic memory deficits and usually one or more other symptoms such as aphasia (language impairment), agnosia (impaired ability to recognise objects, people, sounds, shapes or smells) or impairment of executive functioning (Cummings & Cole, 2002). These symptoms progressively worsen, leading to functional and behavioural difficulties in daily life activities and eventually severe disability and death. There are also a number of neuropsychiatric symptoms that are regularly seen in AD such as depression, anxiety, psychosis and hallucinations, among others (Cummings, 2001). In advanced AD, apraxia (inability to perform voluntary movements) also becomes apparent and the patient can be prone to secondary infection, which is the usual cause of death (McKhann, *et al.*, 1984). On average, the life expectancy after diagnosis of AD is approximately 3-10 years (Zanetti, *et al.*, 2009), and this is highly influenced by the age at which a person is diagnosed – for example, newly diagnosed patients in their 60s or 70s will live an average of 7-10 years, whereas a person diagnosed in their 90s is expected to have a life expectancy of around 3 years.

1.1.2 Macroscopic Characteristics

At a macroscopic level, severe atrophy occurs in the amygdala, the entorhinal cortex (EC) and the hippocampus, with the latter being one of the earliest affected brain regions (Duyckaerts, *et al.*, 2009; Scahill, *et al.*, 2002). These areas have essential roles in memory formation, spatial awareness, and emotional reaction, which are functions that are affected in the early AD stages. This atrophy appears to result from the degeneration of neurons and other cells, leading to loss of volume in the inferior temporal and superior and middle frontal gyri (Duyckaerts, *et al.*, 2009). The rate of the atrophy also appears to be accelerated in individuals with one or more *APOE ε4* alleles, in a dose-dependent manner (Chen *et al.*, 2007, Agnosta, *et al.*, 2009).

1.1.3 Pathological Characteristics

At a microscopic level, the primary pathological features of AD include extracellular deposits of amyloid called senile plaques (SP) and intracellular neurofibrillary tangles (NFT) that appear in cortical and limbic areas of the brain, and the number and distribution of these lesions increase as the disease progresses. As mentioned earlier, the main components of amyloid are aggregates of Aβ peptides. Another pathological feature frequently found in AD is cerebral amyloid angiopathy (CAA), sometimes called congophilic angiopathy (Glennner & Wong, 1984). This is characterised by the deposition of Aβ aggregates in the cerebrovascular walls in a regional pattern which is similar to the deposition of SP, leading to vascular damage (Thal *et al.*, 2008). Aβ deposition in the vasculature indicates peripherally circulating Aβ may influence AD pathogenesis.

There are other pathological features commonly seen in AD, including granulovacuolar degeneration (GVD) which involves intraneuronal double-membraned bodies that appear to be related to autophagic mechanisms (Funk, *et al.*, 2011). GVD is not only found in AD, but in other neurodegenerative disorders as well. Another feature, Hirano bodies (HB) - actin-related aggregates - are also found in neurons in AD, however are also considered to be common in normal ageing as well (Gibson & Tomlinson, 1977).

These disturbances cause neuronal dysfunction and synaptic loss, leading to brain atrophy and cognitive impairment.

1.1.3.1 Neurofibrillary Tangles (NFT)

Like HB, NFT are not just found in AD, but can be found in some specific regions of the brain in healthy ageing. NFT are also found in some other brain degenerative conditions (Nelson, *et al.*, 2012; Bouras, *et al.*, 1994). It has been shown that the number of NFT correlates with the level of cognitive impairment in AD (McKee *et al.*, 1991). It is widely believed that the processes leading to SP deposition may cause the subsequent formation of NFT which leads to neuronal death (Hardy & Allsop, 1991; Yamaguchi, *et al.*, 2001). NFT are insoluble aggregates that build up following the hyperphosphorylation of tau protein. Tau is normally associated with microtubule stability related to cytoskeletal structure, as well as the formation of axons and dendrites.

1.1.3.2 Senile Plaques (SP)

First recognised over 100 years ago by Alois Alzheimer (Stelzmann, *et al.*, 1995), the main component of SP is insoluble, fibrillar beta-amyloid ($A\beta$) which forms its core (Glenner & Wong, 1984), and plaques are often bordered by activated microglia and abnormal neurites (Zubenko, 2007). Plaques do contain other components, including ApoE (Barger, *et al.* 2008; Sheng, *et al.*, 1996), IDE (Bernstein, *et al.*, 1999) alpha1 antichymotrypsin, alpha2 macroglobulin, interleukin 6, complement proteins C1Q, C3, and C4, amongst others (Liao, *et al.*, 2004). SP form extracellularly and are often found in close proximity to neurons containing NFT (Masters, *et al.*, 1985).

The deposition of $A\beta$ fibrils and SP in specific regions of the brain appears to precede the onset of neuronal loss and synaptic dysfunction (Glenner & Wong, 1984; Masters, *et al.*, 1985). As $A\beta$ is the major component of SP, and abnormal accumulation of this peptide is thought to initiate the pathogenic cascade of processes seen in AD (Hardy & Selkoe, 2002), the regulation of $A\beta$ concentration and factors that may accelerate its aggregation are believed to be of vital importance.

1.2 Beta Amyloid (A β) - A Key Molecule

The A β molecule is a 39-43 amino acid 4kDa peptide, which is synthesised and detectable throughout the body under normal physiological circumstances, however its role is unclear (Masters *et al.*, 1985; Haass *et al.*, 1992; Seubert *et al.*, 1992). It is a breakdown product of a larger protein (see below), and is normally cleared quickly, however during pathological processes such as AD, there appears to be an imbalance between its production, and its degradation and/or clearance (Savage, *et al.*, 1998; Selkoe, 2001). This leads to the accumulation of A β , and as it is highly fibrillogenic, aggregation and deposition of amyloid fibrils occur in the brain in the form of SP as well as diffuse aggregates (Hardy & Selkoe, 2002; Suzuki *et al.*, 1994).

1.2.1 Early onset and Late onset AD

AD is broadly divided into two main subtypes, referred to as early-onset AD (EOAD) and late-onset AD (LOAD). EOAD, in which the onset of symptoms occurs before the age of 65 years, is caused by autosomal dominant mutations in either the presenilin 1 (*PS1*), presenilin 2 (*PS2*) or the amyloid precursor protein (*APP*) genes, and is often also termed early-onset familial AD (EOFAD) (Goate, *et al.*, 1991; Sherrington, *et al.*, 1995; Levy-Lahad, *et al.*, 1995). There is also a very small subgroup of sporadic EOAD. EOAD, however, only accounts for around 5% of AD cases, with LOAD accounting for the majority of cases (Cummings & Cole, 2002). However, some AD cases exhibit Mendelian inheritance in which no mutations in any of the recognised genes exist, indicating the involvement of genes which are yet to be identified (Goate, 1997).

A greater understanding of AD has come from studies of EOAD. The discoveries of the many *APP*, *PS1* and *PS2* genetic mutations that lead to AD highlight the central role of A β in AD, as A β peptide is a proteolytic product of APP, and the presenilins are proteins involved in the last proteolytic process that cleaves A β from the parent molecule. In EOAD, genetic mutations in *PS1*, *PS2* and *APP* have been demonstrated to cause the disease mostly via increased production of A β (Selkoe, 2001; Verdile, *et al.*, 2004; Hardy, 1997), and depending on the site of the *APP* mutation, can also alter A β 's affinity for fibrillogenesis (Walsh & Selkoe, 2007). The increased understanding of A β

properties associated with identifying these mutations initiated one of the most widely accepted hypotheses amongst AD researchers, “the amyloid cascade hypothesis” (Hardy & Allsop, 1991). The hypothesis contends that AD pathology is initiated by increased levels of A β , which leads to A β accumulation and aggregation, as well as a cascade of other events including NFT formation, oxidative stress, mitochondrial dysfunction (reviewed in Lin & Beal, 2006), inflammatory responses and vascular damage, culminating in neuronal loss (Hardy & Higgins, 1992). There is substantial and growing evidence which supports this hypothesis, however it remains controversial due to a number of discrepancies (Hardy & Selkoe, 2002; Selkoe, 2011; Karran, *et al.*, 2011). As yet, it has not been either proved or disproved, but regardless, A β is widely believed to be central in the pathogenesis of AD. In the case of LOAD, which comprises approximately 95% of AD cases, it appears that the accumulation of A β may be largely due to defective clearance and/or degradation mechanisms rather than overproduction (Selkoe, 2001). As such, proteins which are directly involved in A β degradation and clearance mechanisms are being studied to characterise AD neuropathology better, as well as to identify potential AD biomarkers.

1.2.2 Amyloid Precursor Protein (APP)

Through multi-step proteolytic processing, A β is generated from its parent molecule, the amyloid precursor protein (APP). A β is synthesised via sequential cleavage of APP (see Figure 1), a transmembrane protein which is ubiquitously expressed (Kinoshita *et al.*, 2003; Haass *et al.*, 1992). This process involves two enzymatic proteases, β -secretase (also referred to as “BACE”) and γ -secretase. APP is initially cleaved by BACE extracellularly, and the remaining membrane bound portion (termed “C99”) is then cleaved within the membrane by γ -secretase to liberate A β (LaFerla *et al.*, 2007). Because γ -secretase does not have sequence specificity, the intra-membrane cleavage site at the C-terminus can vary, producing A β peptides that range from 38-43 residues long (Wolfe *et al.*, 1999). The two main A β peptides in the human brain are 40 and 42 amino acids long, of which A β_{1-40} (A β_{40}) is the principal species in normal physiology (Seubert, *et al.*, 1992; Haass, *et al.*, 1992). A β_{42} appears to be the most conducive to self-association and fibrillogenesis (Jarrett, *et al.*, 1993; Burdick, *et al.*, 1992; Snyder, *et*

al., 1994), and has also been found to be more neurotoxic than the shorter species (Davis & Van Nostrand, 1996). APP can also be cleaved by α -secretase which cleaves within the A β sequence, at position 16, thus precluding the formation of the full length A β peptide.

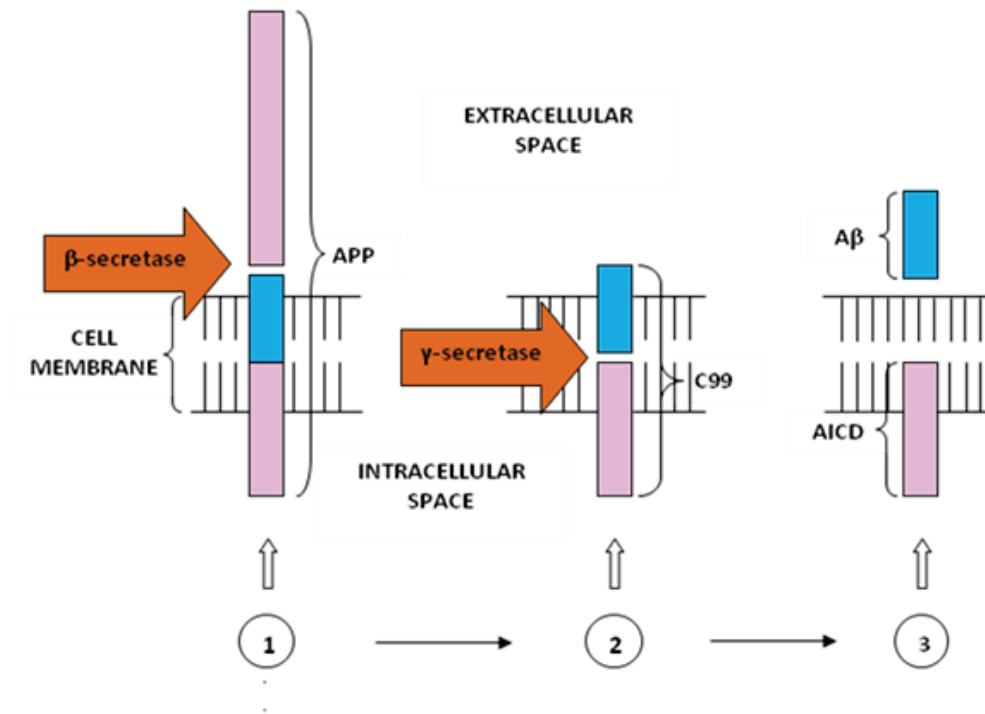


Figure 1.1 Representation of the generation of A β .

1. Cleavage of membrane-bound APP extracellularly by β -secretase (BACE).
2. Cleavage of remaining C99 portion of APP within the plasma membrane by γ -secretase.
3. Liberation of A β into the extracellular milieu. The remaining portion of APP (AICD) is released into the cytosol (intracellular space).

It should be noted that while A β is shown in Figure 1 to be released extracellularly, and this was originally the accepted location for its generation, further studies have shown that it can also be produced intracellularly (Wertkin, *et al.*, 1993; reviewed in La Ferla, *et al.*, 2007). There is also now considerable evidence that A β can accumulate intracellularly as well as extracellularly in the human brain (Gouras, *et al.*, 2000; Martin, *et al.*, 1995; Cabrejo, *et al.*, 2006) and that this precedes plaque formation (Gyure, *et al.*, 2001; Knobloch, *et al.*, 2007). This indicates that intracellular

accumulation of A β is an early event in AD pathogenesis and may be an upstream event to extracellular accumulation.

As well as intracellular accumulation, recent evidence suggests that A β accumulates within the mitochondria early in AD, and this appears to be due to its importation into the mitochondria from the cytosol (Manczak, *et al.*, 2006; Reddy, *et al.*, 2008; reviewed in Chen & Yan, 2010; Leal, *et al.*, 2013). Increased mitochondrial A β (*mitA β*) has recently been associated with the severity of both mitochondrial dysfunction and cognitive function in transgenic mouse models of AD (Dragicevic, *et al.*, 2010; Caspersen, *et al.*, 2005). Furthermore, the distribution of *mitA β* in AD brains is similar to the distribution of AD pathology. Therefore the regulation of *mitA β* as well as intracellular A β is crucial in preserving normal mitochondrial function and disruption of mitochondrial function may be an initiating factor in the development of AD.

1.2.3 A β Toxicity in AD

Monomers of A β self-associate to form oligomers, and subsequently fibrils, that appear to be associated with the level of toxicity and degree of neurodegeneration (Roher, *et al.*, 1996; Pike, *et al.*, 1991). These A β fibrils then aggregate into SP. Originally, it was thought that the neurotoxicity of A β could be primarily attributed to the insoluble amyloid fibrils present in SP, however further research has suggested that plaques are relatively inert, and it is more likely to be soluble, pre-fibrillar small A β oligomers which cause neuronal injury (Stine, *et al.*, 2003; Walsh & Selkoe, 2007; Pike, *et al.*, 1993; McLean, *et al.*, 1999; Lue, *et al.*, 1999). A β has been demonstrated to have neurotrophic properties *in vitro*, which are dependent on low peptide concentration, and reduced length of exposure time (Pike, *et al.*, 1991; Yankner, *et al.*, 1990; Pike, *et al.*, 1993). Similarly, oligomer and fibril formation, and the respective neurotoxicity of these aggregates, have been shown to be isoform-dependent, concentration-dependent and time-dependent, as well as dependent on certain metal ions such as Zn²⁺ or Cu²⁺ (Stine, *et al.*, 2003; Hartley, *et al.*, 1999). In addition, studies have uncovered an apparent intermediate between oligomers and mature fibrils, termed “protofibrils” which appear to be similar to oligomers regarding formation and neurotoxicity (Dahlgren, *et al.*, 2002; Hartley, *et al.*, 1999; Walsh, *et al.*, 1997; Stine, *et*

al., 2003). Oligomers of A β have also been found to inhibit long-term potentiation (LTP), which is associated with memory formation (Walsh, *et al.*, 2005; Wang, *et al.*, 2002). Therefore, the accumulation of A β , and in particular the A β_{42} isoform, may instigate the self-association of this peptide into soluble, pre-fibrillar forms, initiating the neurotoxicity that precedes plaque formation.

One mechanism which is believed to be partly responsible for the accumulation and aggregation of cerebral A β is the impairment of its degradation or clearance from the CNS.

1.3 Aetiology of LOAD

The aetiology of LOAD is complex and not completely understood. The major risk factor for AD is age. There have been a number of other risk factors reported, such as a family history of AD, which have also been shown to be significant (van Duijn, *et al.*, 1994; Lindsay, *et al.*, 2002). Other risk factors include female gender, low education level, head trauma, late maternal age and depression, among others, however the results of some studies have been conflicting (Launer, *et al.*, 1999; Lindsay, *et al.*, 2002; van Duijn, *et al.*, 1994; van Duijn, 1996).

The major genetic risk factor to date appears to be possession of one or more $\epsilon 4$ allele of the *APOE* gene, as mentioned previously (Goate, 1997; Corder, *et al.*, 1993; Strittmatter, *et al.*, 1993(a); Rebeck, *et al.*, 1993). However, around 50% of LOAD cases do not carry the *APOE* $\epsilon 4$ genotype and this indicates that other factors are involved in the aetiology of LOAD (Corder, *et al.*, 1993). Genetic studies have uncovered several other genes which appear to have some influence on the risk of LOAD, although not as great an influence as the *APOE* gene. It is highly probable that environmental and lifestyle factors also influence the risk of sporadic AD, and probably interact with relevant genetic factors (Roher, *et al.*, 2009).

1.4 Diagnostic Techniques

There are currently two diagnostic standards used for AD, the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) Alzheimer's criteria, and criteria outlined by the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision (DSM-IV-TR[®]) (American Psychiatric Association, 2000). These two standards are similar in nature. There are also several imaging techniques in use, which can complement these diagnostic standards.

NINCDS-ADRDA criteria categorises patients into one of four categories; "definite", "probable", "possible" or "unlikely" Alzheimer's disease. A diagnosis of "definite" AD requires histopathologic confirmation, via an autopsy or brain biopsy. For a diagnosis of "probable" AD, there must be impairment in at least two of the eight cognitive domains: "memory, language, perceptual skills, attention, constructive abilities, orientation, problem solving and functional abilities" (McKhann, *et al.*, 1984). A diagnosis of "possible" or "unlikely" AD indicates that there is dementia present with features that are atypical of AD, yet which cannot be explained by any other diseases that may cause dementia.

Similarly, the current DSM-IV-TR[®] diagnostic procedure involves initially ascertaining the presence of dementia, by the confirmation of memory impairment, accompanied by at least one other symptom such as aphasia, apraxia, agnosia and/or impairment of executive functioning. The severity of these deficits must be sufficient to impair functionality of daily living (DSM-IV-TR[®], American Psychiatric Association, 2000), and are evaluated using assessment tools such as the Mini-Mental State Exam (MMSE). The DSM-IV-TR[®] states that onset of the disease should be gradual, and that the decline in impairment must be progressive and distinguishable from the decline that occurs in the normal ageing process. Essentially, the diagnosis of Alzheimer's disease can only be made once all other causes of dementia and disorders with similar symptoms have been excluded, and consideration must be given to potentially confounding factors such as ethnicity, culture and educational history, all of which may have an effect on the outcome of the evaluation.

1.4.1 Imaging Techniques and Biopsy

Cerebral computerised tomography (CT) and/or magnetic resonance imaging (MRI) can also be used to confirm specific macroscopic changes that are characteristic of AD such as atrophy, particularly of the hippocampus, and to eliminate any other pathological cause of dementia which is not of the Alzheimer type. Less commonly, functional imaging techniques such as MRI, positron-emission tomography (PET) or single photon emission computed tomography (SPECT) have been used to assist in differential diagnosis. Although not part of the standard diagnostic screening, these imaging techniques have been shown to be very useful for differentiation of AD, and suggestions have been made for revision of the NINCDS-ADRDA to include them in the criteria, along with CSF biomarkers such as A β and tau which have also shown promise (Dubois *et al.*, 2007; Visser, *et al.*, 2009). In the last ten years advances have been made with the discovery of amyloid-imaging agents such as “Pittsburgh Compound-B” (PiB) which are used in conjunction with PET imaging (Klunk, *et al.*, 2004). PiB-PET allows quantification of amyloid deposition in the brains of living individuals. The deposition of A β amyloid (as well as synaptic loss and neuronal death) occurs for many years before symptoms manifest, therefore the PiB-PET imaging technique can also identify patients with no symptoms, yet who are likely to develop AD - people at a pre-clinical stage (Okello, *et al.*, 2009; Pike, *et al.*, 2007). Microscopic analysis from a biopsy may also reveal pathological hallmarks of AD such as SP and NFT, and similarly can help exclude or confirm a diagnosis of AD (DSM-IV-TR[®], 2000). However these techniques are costly and invasive, require an expert clinician or surgeon, and in the case of biopsy, there are considerable risks. Due to these factors, their usefulness in a clinical setting is limited.

As mentioned above, by the time AD symptoms appear, considerable synaptic and neuronal loss has occurred. For the foreseeable future, potential treatments are at best likely to stop or slow the pathogenesis of the disease, not reverse it. To maximise the effect of potential future treatments, early pre-clinical diagnosis is necessary. Thus it is clear that current standard diagnostic techniques for AD are insufficient, as they do not identify the disease at an early stage, and are definitive only after autopsy examination. Therefore, the identification of AD pre-clinical biomarkers would improve diagnostic efficacy.

While A β would seem to be an obvious candidate as a biomarker for AD, research findings have been inconclusive concerning its validity, and it is probable that a combination of biomarkers may be necessary for accurate diagnostics. While the mutations responsible for EOAD appear to alter A β concentration via increased production, particularly of the A β_{42} peptide (Hardy, 1997), increasing evidence suggests that impaired clearance of this peptide may be responsible for the majority of sporadic AD cases (Selkoe, 2001). As such, proteins or other biological substances that directly affect the regulation of A β levels via clearance mechanisms may have value as possible biomarkers.

1.4.2 AD Biomarkers

To date, there is no universally accepted biomarker or set of biomarkers, that has the level of specificity or sensitivity required for definitive diagnosis of AD (DSM-IV-TR[®], 2000; Consensus Report of the Working Group on: “Molecular and Biochemical Markers of Alzheimer’s Disease”, 1998). Although studies on CSF biomarkers such as A β and tau have shown potential for differentiating between AD, mild cognitive impairment (MCI), subjective memory complainers (SMC) and healthy controls (Blennow, 2004; Visser, *et al.*, 2009; Wallin *et al.*, 2006; Herukka *et al.*, 2007), the invasiveness of CSF collection and high costs of imaging make these techniques impractical for routine use in clinical practice. Current diagnostic techniques are largely based on a clinician’s subjective judgement, the quality of which may vary based on their experience. Knopman *et al.*, 2001, states that the two diagnostic standards described above have good reliability, with a sensitivity of 81% on average, but a specificity of only 70%, on average. These figures are based on thirteen studies which examined the accuracy of clinical diagnostic tools for AD. It is likely, however, that these figures are not likely to be representative of accuracy in the early stages of the disease (Frank *et al.*, 2003), and as previously stated, the only way to confirm the diagnosis of Alzheimer’s at present, is by autopsy examination.

1.4.3 MCI

Potential Alzheimer's patients in the very early stages of the disease may be diagnosed with MCI (Frank *et al.*, 2003). MCI is characterised by memory loss and other cognitive impairment, without meeting the neuropathologic criteria for dementia (Petersen *et al.*, 1999). When memory loss is the primary symptom, it is termed "amnesic" MCI, and is considered to be a particular risk factor for AD. It has been shown that at least 50% of MCI patients do progress to dementia (Schmidtke & Hermeneit, 2008; Gauthier, *et al.*, 2006), however some MCI patients either go on to develop disorders other than AD, show no progression of impairment, or even recover, so the ability to differentiate between these cases is of particular diagnostic importance.

1.4.4 SMC

Individuals with SMC have also been found to have an increased risk for developing dementia in a number of studies (O'Brien, *et al.*, 1992; Geerlings, *et al.*, 1999; Schmand, *et al.*, 1996). In a recent study, SMC individuals were found to have significantly lower scores on tests for cognitive impairment, such as the MMSE, compared with controls. In addition, the *APOE* $\epsilon 4$ allele was found to be over-represented in this cohort, in a dose-dependent manner (Laws, *et al.*, 2002). This may indicate that individuals with subjective memory complaints could be at a higher risk for developing AD, similar to MCI patients.

1.4.5 The need for better AD biomarkers

One of the problems with current diagnostics is the inability to identify individuals before or during the early stages of neurodegeneration that lead to clinical dementia. As mentioned earlier, it would be of enormous benefit to identify one or more biomarkers to assist AD diagnosis at the earliest possible stages, and preferably prior to the onset of the irreversible symptoms. This would allow treatment to commence at a more functional stage for the patient and slow or delay the progression of symptoms (Borroni, *et al.*, 2006). Quality of life could be maximised for sufferers and

their families, and economic benefits regarding healthcare would be significant (Brookmeyer *et al.*, 1998). Recently, recommendations were made by the National Institute of Ageing (NIH) and Alzheimer's Association for the revision of current diagnostic criteria (Jack, *et al.*, 2011). The recommendations from these working groups were in part to include scientifically validated biomarkers of AD in the standards that may detect AD at a preclinical stage, namely CSF levels of A β , total tau (T-tau) and phosphorylated tau (P-tau), increased brain amyloid load and reduced fluorodeoxyglucose uptake determined by positron-emission topography (PET) imaging (pattern-specific), and brain atrophy determined by structural magnetic resonance imaging (MRI). Searching for blood biomarkers as well as any biomarkers that can detect AD at the earliest possible stage is therefore of great importance and many potential biomarkers are currently in the process of being validated (Hampel, *et al.* 2010).

1.4.5.2 The “Ideal Biomarker for AD”

The Working Group on Molecular and Biochemical Markers of Alzheimer's Disease (The Ronald and Nancy Reagan Research Institute of the Alzheimer's Association and the National Institute on Ageing Working Group, 1998) has suggested that the “ideal biomarker” for AD should possess a number of features. Firstly it should be related to a pathological feature of AD, and be validated in confirmed AD cases. It should be sensitive enough to allow early diagnosis and to differentiate it from other dementias. In addition, the “ideal biomarker” should be reliable, biomarker screening should be inexpensive and simple, and should involve relatively non-invasive testing. The detection of biomarkers in easily accessible biological fluids is therefore an area of intense interest. Although CSF is likely to be the most accurate biological fluid for reflecting pathophysiological changes, as it is in direct contact with the brain (Blennow, 2004), techniques such as lumbar puncture for the analysis of CSF, are highly invasive, uncomfortable for the patient and often expensive, requiring an expert clinician to perform the procedure. Therefore, identification of one or more peripheral blood biomarkers would be highly useful for diagnostic, prognostic and therapeutic purposes.

A variety of different blood biomarkers could potentially be used together to improve the precision of the screening process. For example, studies have recently been

focusing on the analysis of protein expression patterns in plasma or serum, with positive results (Doecke, *et al.*, 2012; Soares, *et al.*, 2012; O'Bryant, *et al.*, 2010; Ray *et al.* 2007). In addition, the $A\beta_{42}/A\beta_{40}$ ratio has been shown in plasma to be potentially a promising biomarker for identifying persons at risk of developing MCI or AD (Okereke, *et al.*, 2009; Graff-Radford *et al.*, 2007). However, there is some controversy regarding the usefulness of plasma $A\beta$ as a reliable, pre-clinical biomarker (Rissman, *et al.*, 2012). The identification of additional biomarkers that could be used in conjunction with, or instead of the above potential biomarkers, would be highly beneficial. Regardless, $A\beta$ and/or proteins that interact with it may be the most useful due to their key roles in AD pathology. The use of $A\beta$ as a biomarker is discussed in detail below.

1.4.6 $A\beta$ as a Biomarker for AD Diagnosis

1.4.6.1 $A\beta$ and PET Imaging

Fibrillar amyloid deposits in the brain of living persons can be visualised by PET, using amyloid-imaging agents such as [18F]1,1-dicyano-2-[6-(dimethylamino)-2-naphthalenyl]-propene, more commonly known as ^{18}F -DDNP, and *N*-methyl-[^{11}C]2-(4'-methylaminophenyl)-6-hydroxybenzothiazole, known as PiB (Klunk, *et al.*, 2004; Nordberg, 2004). While PiB only detects amyloid burden (Ikonomovic, *et al.*, 2008), ^{18}F -DDNP is believed to detect both amyloid and NFT (Shin, *et al.*, 2008). Studies have demonstrated that AD patients in the early stages can be differentiated from healthy controls by PiB-PET imaging and an inverse correlation has been shown between PiB retention and glucose metabolism in the brain (Klunk, *et al.*, 2004).

1.4.6.2 $A\beta$ in CSF

Currently, the most promising biomarkers for the diagnosis of early AD appear to be CSF levels of $A\beta_{42}$ together with total tau (t-Tau) and hyperphosphorylated tau (tau phosphorylated at residue 181, P-tau181P), which are clearly linked to the different pathological hallmarks of AD. This combination of biomarkers has been shown to be able to differentiate AD from normal ageing and other diagnoses (Blennow, 2004), as well as identifying which individuals are likely to convert from SMC or MCI to AD

(Mattson, *et al.*, 2009; Diniz, *et al.*, 2008; Visser, *et al.*, 2009). Nevertheless again, the value of CSF biomarkers is limited clinically as a routine diagnostic test for AD, as the collection of CSF is highly invasive and side effects are common (Williams, *et al.*, 2008). Blood would be preferential for the identification of potential biomarkers as it is inexpensive and simple to collect, and is not particularly invasive.

1.4.6.3 A β in Plasma

A number of studies have focused on the detection of A β as a biomarker in blood, yet with inconsistent results (Graff-Radford, *et al.*, 2007; Fukumoto, *et al.*, 2003). Although plasma levels of A β have been shown to be increased in familial types of AD (Scheuner, *et al.*, 1996), this trend is less clear in sporadic forms, with considerable overlap between controls and AD cases. In addition it appears that levels of plasma A β increase with age, which may lend a confounding factor (Mayeux, *et al.*, 1999). Overall, it appears that plasma A β is elevated prior to amyloid deposition, though after some deposition occurs there is a concomitant, progressive reduction in plasma levels (Kawarabayashi, *et al.*, 2001; Kuo, *et al.*, 2000; Schupf, *et al.*, 2008). This suggests that peripheral A β may be a source for cerebral accumulation of the peptide, it may also mean that less cerebral A β is reaching the circulation. Longitudinal studies support the theory that elevated plasma A β is a risk factor for AD (Mayeux, *et al.*, 1999; Mayeux, *et al.*, 2003). The pattern of plasma A β levels over time may explain why various cross-sectional studies have found such a broad overlap between controls and sporadic cases (Fukumoto, *et al.*, 2003; Vanderstichele, *et al.*, 2000). It has also been shown that A β can pass from CSF to plasma and vice versa through the blood-brain barrier (BBB), however there is no correlation between A β levels in these biological fluids, indicating contributions from both CNS and peripheral tissues (Le Bastard, *et al.*, 2009; Mehta, *et al.*, 2000; GhersiEgea, *et al.*, 1996).

Several studies have investigated the plasma A β_{42} /A β_{40} ratio as a possible indicator of AD risk. In general, it appears that a low A β_{42} /A β_{40} ratio may be associated with increased risk of AD and/or MCI (Graff-Radford, *et al.*, 2007; Abdullah, *et al.*, 2007; Van Oijen, *et al.*, 2006; and reviewed by Koyama, *et al.*, 2012). Interestingly, it may be an increase of the A β_{40} species relative to A β_{42} that causes this decrease, as some studies have not found a change in A β_{42} levels (Van Oijen, *et al.*, 2006; Abdullah, *et al.*, 2007).

However, a recent meta-analysis showed that there was no change in $A\beta_{40}$ levels, while there was a slight reduction in $A\beta_{42}$ levels, albeit non-significant (Song, *et al.*, 2011). Furthermore, in longitudinal studies it was shown that baseline levels of $A\beta$ and the $A\beta_{42}/A\beta_{40}$ ratio were marginally higher in AD patients but again these results were non-significant. Another study found that the $A\beta_{42}/A\beta_{40}$ ratio was not affected by *APOE* genotype, contrary to individual concentrations of plasma $A\beta_{40}$ and $A\beta_{42}$ (Blennow, *et al.*, 2009). This may indicate that the $A\beta_{42}/A\beta_{40}$ ratio in plasma could be more beneficial as a biomarker than individual $A\beta_{42}$ and $A\beta_{40}$ levels, or could be used in conjunction with other biomarkers. In accordance with a number of other studies, findings from the AIBL study (see Chapter 2, Materials and Methods for details) have found lower levels of plasma $A\beta_{42}$, and thus a decrease in the $A\beta_{42}/A\beta_{40}$ ratio in AD patients when compared with healthy controls (HC) (Lui, *et al.*, 2010). In addition, it was found that the levels of $A\beta_{42}$ as well as the $A\beta_{42}/A\beta_{40}$ ratio were negatively correlated with brain $A\beta$ load. These results are consistent with findings from the AIBL follow-up at 18 months, which also showed a reduction of $A\beta_{42}$ in MCI individuals and healthy controls who converted to MCI during this period (Rembach, *et al.*, 2013). This suggests that although plasma $A\beta$ may have limited usefulness as a single biomarker for AD, it may have some clinical value for early detection of AD when used in conjunction with other biomarkers.

Although the current literature on plasma levels of $A\beta$ is inconsistent, this inconsistency may also be due to methodological difficulties and different techniques. Results from the AIBL study acknowledged differences in results when using different commercial kits to measure $A\beta$ levels, as well as various confounding covariates (Lui, *et al.*, 2010; Rembach, *et al.*, 2013). ELISA-based methods may fail to detect $A\beta$ which is bound to other proteins or differentiate between $A\beta$ oligomers and free $A\beta$, resulting in only a portion of the total peptide being measured (Portelius, *et al.*, 2008; Kuo, *et al.*, 1999; Abdullah, *et al.*, 2007). In a study by Xia, *et al.* (2009), using a specially designed ELISA for the purpose of detecting $A\beta$ oligomers, they found that oligomeric and monomeric levels of $A\beta$ were strongly associated. In a study by James & Mamo (2005), they demonstrated that the presence of lipids with $A\beta$ *in vitro* can alter the predominant form of $A\beta$, and sequester it, rendering it unavailable for detection by commonly used methods. In addition, it has been shown that in gel-based techniques,

denaturing conditions can liberate these oligomers or bound proteins, increasing total levels of measurable A β (Portelius, *et al.*, 2008). Various other methodological factors have also been demonstrated to confound results (Vanderstichele, *et al.*, 2000), indicating a need for a methodological standard for measurement of this protein (and likely other proteins) in plasma. It is more than likely that one blood protein marker will not be sufficient to diagnose pre-clinical AD, with adequate specificity and sensitivity. There are many plasma proteins that are being investigated for their potential as a biomarker. The following will be a discussion on those that are directly related to my project, ApoE, insulin and IDE.

1.5 Apolipoprotein E

Apolipoprotein E (ApoE) is a 34 kDa, 299-residue polymorphic protein that has been strongly implicated in the pathogenesis of AD (Sheng, *et al.*, 1996; Mahley, *et al.*, 2006; Strittmatter, *et al.*, 1993(a); Corder, *et al.*, 1993). The *APOE* gene on chromosome 19 that encodes ApoE has three alleles, ϵ 2, ϵ 3 and ϵ 4. The three ApoE protein isoforms differ by substitutions at residues 112 and 158, with ApoE2 containing cysteine at these positions, ApoE4 containing arginine at both positions, and ApoE3 having an arginine substitution at only residue 158 (Bentley, *et al.*, 2002). To date, ϵ 4 is still the major genetic risk factor for LOAD, being significantly overrepresented in LOAD cases compared with controls (Kaur & Balgir, 2006; Saunders, *et al.*, 1993; Strittmatter, *et al.*, 1993(a)). Generally, the ϵ 3 variant of *APOE* appears to be the “wild-type” allele, with a frequency of 60-70% in the general population, while the ϵ 4 variant has a frequency of only 15-20% (Mahley, *et al.*, 2006). The ϵ 2 allele seems to have some protective effect against AD, however in the general population, its frequency is only 5-10%.

1.5.1 Association of *APOE* genotype with AD

A number of studies have investigated the association of *APOE* genotype with AD. Most have found that the ϵ 4 allele is significantly associated with an increased risk for developing AD, in a dose-dependent manner (Kaur & Balgir, 2006; Corder, *et al.*, 1993;

Saunders, *et al.*, 1993). Corder, *et al.* (1993) found that the risk for AD increased from 20% for individuals without an $\epsilon 4$ allele, to 47% in individuals with one $\epsilon 4$ allele, and to 91% in individuals homozygous for the $\epsilon 4$ allele.

Not only is the risk for developing AD increased by the $\epsilon 4$ allele, but also the age of disease onset has been shown to be significantly decreased in a dose-dependent manner (Corder, *et al.*, 1993). Hence, $\epsilon 4/\epsilon 4$ homozygotes have been found to develop AD earlier than their heterozygotic $\epsilon 4/\epsilon 3$ and $\epsilon 4/\epsilon 2$ counterparts. For individuals without an $\epsilon 4$ allele, Corder, *et al.* (1993) found the mean age of onset was 84.3 years, while the ages of AD onset for $\epsilon 4$ heterozygotes and $\epsilon 4$ homozygotes were 75.5 years and 68.4 years, respectively. In addition, the $\epsilon 4$ allele has been associated with accelerated brain atrophy rates, in both cognitively normal individuals and AD patients, also in a dose-dependent manner (Chen, *et al.*, 2007; Agnosta, *et al.*, 2009). Rebeck, *et al.* (1993) also showed that $\epsilon 4/\epsilon 4$ AD patients had significantly greater amyloid plaque deposition when compared with $\epsilon 3/\epsilon 3$ AD patients.

1.5.2 Functions of ApoE

The ApoE protein is a normal component of certain lipoproteins, and as such has functional roles in lipid (including cholesterol and lipid soluble vitamins) transport and clearance in the body (Mahley, 1988), and in the brain it plays a role in neuronal growth, maintenance and repair (Ignatius, *et al.*, 1986; Cedazo-Mínguez, 2007). ApoE functions as a ligand in receptor-mediated endocytosis of lipoprotein particles. In plasma, ApoE proteins are found on lipoproteins in association with other apolipoproteins, whereas in the brain ApoE and two other apolipoproteins, ApoJ and ApoA-1, are predominantly found in high density lipoprotein (HDL)-like particles (Kim, *et al.*, 2009)

ApoE is generated predominantly in the liver and brain, but also in much smaller amounts in most other tissues (Bentley, *et al.*, 2002). The isoforms of ApoE have different affinities for the blood lipoproteins, with ApoE2 and ApoE3 showing preferential affinity for HDL while ApoE4 binds preferentially to VLDL (Weisgraber & Mahley, 1996).

1.5.3 APOE and AD

While the *APOE* $\epsilon 4$ genotype has been confirmed as a risk factor for AD, the mechanism of pathogenesis is less clear. The ApoE protein has been localised within both SP and NFT, indicating a direct interaction with components of these lesions (Sheng, *et al.*, 1996; Namba, *et al.*, 1991). Many other pathogenic changes observed in AD have also been associated with ApoE, including NFT formation, oxidative stress, and dysfunction of lipid regulation, synaptic plasticity and cholinergic function (reviewed by Cedazo-Mínguez, 2007), indicating how important ApoE may be in AD pathogenesis.

Abnormal cholesterol and lipid metabolism has been associated with the pathogenesis of AD, with high total plasma cholesterol suggested as a risk factor (Roher, *et al.*, 1999; Notkola, *et al.*, 1998), however some studies have shown a decrease in cholesterol and lipid levels in AD patients compared with controls (Siest, *et al.*, 2000). In addition, it has been reported that AD patients have higher serum levels of the detrimental LDL cholesterol, and lower levels of the beneficial HDL cholesterol than control cases (Roher, *et al.*, 1999; Kuo, *et al.*, 1998). Kuo, *et al.*, (1998) also demonstrated a positive correlation between total and LDL cholesterol levels, and cerebral levels of $A\beta_{42}$, independent of *APOE* genotype. It is feasible that either low levels or dysfunction of the ApoE protein may contribute to AD pathology independently of the $\epsilon 4$ allele, possibly due to abnormal lipid transport or metabolism.

Due to the discovery of ApoE in amyloid plaques and CAA, studies were carried out to ascertain whether ApoE could bind $A\beta$ directly, and it was found that ApoE can bind to, and facilitate the fibrillogenesis of $A\beta$ in an isoform-dependent fashion, suggesting a role as a “pathological chaperone” (Strittmatter, *et al.*, 1993(b); Wisniewski & Frangione, 1992, p237; Wisniewski, *et al.*, 1994; LaDu, *et al.*, 1994). The differences in amino acids between ApoE2, ApoE3 and ApoE4 appear to differentially affect the binding interactions within the protein, as well as to other molecules such as $A\beta$ (LaDu, *et al.*, 1994; LaDu, *et al.*, 1995), and there was *in vitro* evidence that ApoE4 could bind $A\beta$ peptides more avidly than the other two isoforms, potentially explaining why ApoE4 increases AD risk.

However, the association of ApoE with lipids is also a determinant of its binding affinity toward A β (Tokuda, *et al.*, 2000). Not only does the binding affinity increase following lipidation, it also changes the isoform-specific differences (LaDu, *et al.*, 1995). It was then found that the efficiency of complex formation between lipidated ApoE and A β follows the order of ApoE2 > ApoE3 >> ApoE4 (Tokuda *et al.*, 2000).

Since the binding efficiency of ApoE isoforms to A β correlates inversely with the risk of developing AD, it was then hypothesised that ApoE2 and ApoE3 may enhance the clearance of A β , compared to ApoE4 (Hone, *et al.*, 2003; Yang, *et al.*, 1999). To complicate the story further, later studies have since also found in AD-model transgenic mouse studies that a complete lack of ApoE results in no deposition of true plaques and much less A β deposition overall, which would not be expected if ApoE's major interaction with A β was to facilitate clearance. Other *in vivo* studies that involved crossing AD-model mice with ApoE knock-in mice demonstrated a clear isoform-dependent (E4>E3>E2) effect on A β accumulation (Bales *et al.*, 2009; Kim *et al.*, 2009). Due to such apparently conflicting results, it is still not clear whether increasing or decreasing brain ApoE levels would be therapeutic against AD development.

The ApoE isoforms also appear to have different neurotrophic properties, with E3 facilitating, and E4 inhibiting neurite outgrowth, respectively (Nathan, *et al.*, 1994). Studies have shown that ApoE, and in particular E4, may exacerbate the neurotoxicity of A β (Manelli, *et al.*, 2007), and may also be directly toxic to neurons when not associated with lipids (Cedazo-Mínguez, *et al.*, 2001). Overall, ApoE appears to be intimately involved with many of the pathogenic mechanisms which are characteristic of AD.

1.5.4 ApoE in plasma

As with A β , there has been contention regarding the levels of ApoE in plasma. Although it has been demonstrated that *APOE* genotype has an effect on serum levels of the ApoE protein, with ϵ 4 carriers having the lowest levels and ϵ 2 carriers the highest, it has been suggested that *APOE* genotype and protein levels may affect AD

risk independently (Schiele, *et al.*, 2000; Siest, *et al.*, 2000). Indeed, there does not appear to be consistency between reports on AD diagnosis and plasma ApoE protein levels. Studies have found both increased levels (Taddei, *et al.*, 1997) and decreased levels (Siest, *et al.*, 2000), and some have found no difference (Scacchi, *et al.*, 1999; Slooter, *et al.*, 1998). Preliminary studies of the AIBL cohort have been promising, with total plasma ApoE levels being able to differentiate SMC and MCI patients from either HC or AD patients, with the lowest levels occurring in *APOE* $\epsilon 4$ homozygotes and the highest in healthy controls (Martins, 2009). Moreover, plasma ApoE4 levels have also been measured, and could differentiate between AD patients, MCI patients and controls, with AD patients once again having the lowest levels (AD<MCI<HC). In addition, as previously mentioned, there was an inverse correlation between ApoE4 levels and $A\beta_{42}$ levels in the plasma regardless of disease status. These results indicate that plasma levels of total ApoE and ApoE4 may be good candidates as early diagnostic biomarkers, particularly together with other blood biomarkers. However, it should be taken into account that some independent factors may alter levels of plasma ApoE, such as geographical location (Schiele, *et al.*, 2000), levels of the cholesterol transporter ABCA1 (Hirsch-Reinshagen, *et al.*, 2009), and other biological factors such as age, gender and body mass index (BMI) and possibly certain medications (Vincent-Viry, *et al.*, 1998). In addition, methodological differences may have contributed to the inconsistencies in previous literature. Similar to the data for plasma $A\beta$, standardisation of methodological procedures for the measurement of ApoE in plasma would be highly beneficial. It has also been demonstrated that expression levels of ApoE correlate with the formation of SP and levels change with disease progression (Barger, *et al.*, 2008; Kuo, *et al.*, 2000).

1.6 $A\beta$ Degradation and Clearance

The removal of $A\beta$ from the CNS may occur via several mechanisms. Firstly, according to the well-characterised “peripheral sink hypothesis”, cerebral $A\beta$ has been shown to be transported across the blood brain barrier (BBB) and into the peripheral circulation, facilitated by the LDL receptor-related protein-1 (LRP-1) (Shibata, *et al.*, 2000; DeMattos, *et al.*, 2001). This efflux appears to be isoform-dependent, with studies

showing a slower rate of clearance across the BBB of A β ₄₂ in comparison to A β ₄₀ (Bell, *et al.*, 2007). Once in the periphery, A β -binding agents may bind to the peptide and prevent its return to the CNS (Sagare, *et al.*, 2007; Takata, *et al.*, 2007). However, free peripheral A β may enter the CNS due to the BBB's bi-directional nature, assisted by the receptor for advanced glycation end products (RAGE) (Deane, *et al.*, 2003). It is therefore probable that the degradation and/or binding of A β to other agents in the periphery could be of importance to its accumulation in the brain.

Another mechanism for cerebral A β clearance is by transport through perivascular drainage pathways into the CSF, and subsequently into the peripheral circulation via the blood-CSF barrier (Zlokovic, *et al.*, 2005; Preston, *et al.*, 2003). Failure of components of this mechanism may have a role in both vascular and parenchymal deposits of A β , and may be related to the development of CAA.

The elimination of A β from the CNS can also potentially occur via proteolytic degradation. A number of proteases have been shown to degrade A β including insulin-degrading enzyme (IDE), neprilysin (NEP), endothelin-converting enzyme (ECE), angiotensin-converting enzyme (ACE), the uPA/tPA-plasmin system, cathepsin D, gelatinase A, gelatinase B, matrix metalloendopeptidase-9, coagulation factor Xia, antibody light chain c23.5 and hk14, and α 2-macroglobulin complexes (Wang, *et al.*, 2006; Miners, *et al.*, 2008a). Of these, IDE has attracted considerable interest as a primary regulator of A β levels, and accumulating evidence suggests that it may have a role in AD pathology via this interaction with A β .

1.6.1 Insulin-Degrading Enzyme

Insulin-Degrading Enzyme (IDE) (EC 3.4.24.56, formerly known as EC 3.4.22.11), also known as insulysin, insulin protease and insulinase, is a 110 kDa thiolmetalloendopeptidase which consists of 1019 amino acid residues (Selkoe, 2001; Affholter, *et al.*, 1988). The high degree of evolutionary conservation in both exonic and intronic sequences of *IDE* suggests an important role in cellular function (Qiu & Folstein, 2006). It is a multi-functional protein, with some of its identified roles being 1) regulation of peripheral insulin levels, 2) processing of antigenic proteins destined

for the major histocompatibility complex (MHC) (Parmentier, *et al.*, 2010), 3) regulating cellular proteasome activity (Bennett, *et al.*, 2000(b)) and 4) playing a role in cell survival and differentiation (Tundo, *et al.*, 2012; Kayalar & Wong, 1989). It was also reported by Li and associates (2006) that IDE was a receptor for the varicella-zoster virus, however this has since been found to be controversial (Carpenter, *et al.*, 2010). Other important functions for IDE include regulation of steroid-related signalling (Kupfer, *et al.*, 1994), and also notably being responsible for the clearance of amyloidogenic peptides (Farris, *et al.*, 2003; and reviewed in Authier, *et al.*, 1996(a)).

IDE is expressed in almost all tissues of the body, including the brain, where it is highly expressed in cerebral neurons (Bernstein, *et al.*, 1999; Kuo, *et al.*, 1993). Studies have also shown that it is produced and secreted by microglial cells in association with exosomes (Tamboli, *et al.*, 2010; Qiu, *et al.*, 1997) as well as astrocytes (Jiang, *et al.*, 2008). IDE has been detected in most peripheral tissues, including the lung, skeletal muscle, skin, breast, testes and several blood cell types, as well as the liver and kidney which are believed to be the primary organs responsible for the clearance of peripheral A β (Kuo, *et al.*, 1993; Hone, *et al.*, 2003). In addition, its presence has been reported in endothelial and smooth muscle cells, and pericytes within cerebral microvessels; areas of A β deposition in cerebral amyloid angiopathy (CAA), which often co-exists with AD (Morelli, *et al.*, 2004; Gao, *et al.*, 2004). Interestingly, it has also been detected in AD senile plaques, indicating a role for IDE in plaque pathology (Bernstein, *et al.*, 1999).

At a subcellular level, IDE is mainly located in the cytosol (Akiyama, *et al.*, 1988; Authier, *et al.*, 1996(b)), however it has also been localised to peroxisomes (Kuo, *et al.*, 1994), endosomes (Hamel, *et al.*, 1991), exosomes (Bulloj, *et al.*, 2010), rough endoplasmic reticulum (RER) (Qiu, *et al.*, 1998), plasma membranes (Seta & Roth, *et al.*, 1997; Goldfine, *et al.*, 1984) and recently, lipid rafts associated with the plasma membrane (Bulloj, *et al.*, 2008). In addition, it has been detected within proteasome complexes (Bennett, *et al.*, 2000(b)), and has also been found secreted into the CSF (Qiu, *et al.*, 1998). A longer, mitochondrial isoform of IDE has also been confirmed *in vitro*, this mitochondrial isoform is generated from an alternative translation start site 41 amino acids upstream of the first Met of cytosolic IDE, and the additional amino acid sequence forms a mitochondrial targeting sequence (Leissring, *et al.*, 2004). This

isoform has been shown to exist in the human brain and recently has been demonstrated to regulate *mitAβ* (Leal, *et al.*, 2013). It is highly likely that IDE found in different subcellular locations of IDE controls different pools of Aβ; where mitochondrial IDE regulates *mitAβ*, cytosolic IDE regulates intracellular Aβ, and membrane-bound IDE most likely degrades extracellular Aβ.

Most of the expressed IDE has been reported to be the 110kDa isoform, with the membrane-bound isoform having a molecular weight of 115kDa (Vekrellis, *et al.*, 2000). However, some older studies have reported other molecular weights including 80kDa (Burghen, *et al.*, 1972), 150-160kDa (Kolb & Standl, 1980), 135kDa (Yokono *et al.*, 1981), 170kDa and 60kDa (Camberos, *et al.*, 2001). While these discrepancies may be due to methodological differences or analyses of different tissues and species, it is possible that multiple forms exist. A truncated isoform of IDE has also been identified which has been reported in the National Centre for Biotechnology Information (NCBI) database (Reference sequence NP_001159418.1, http://www.ncbi.nlm.nih.gov/protein/NP_001159418.1). This form is identical to the 464 amino acid residues on the C-terminal end of the full-length protein. This may correspond to the 60kDa protein reported. However, this isoform is unlikely to be active in degrading Aβ as it does not contain the catalytic site required for catabolism. In agreement with this, it has been shown experimentally that the C-terminal half of IDE is not catalytically active (Li, *et al.*, 2006). The molecular weights stated in the UniProtKB database (<http://www.uniprot.org/uniprot/P14735>) for the full-length and the truncated IDE isoforms are 117.968kDa and 54.240kDa, respectively. At the commencement of this project, there were no published results of IDE in blood or plasma so it was unknown what isoforms may be present in this biofluid. However, a paper published very recently is, to our knowledge, the first to detail IDE activity in human serum in AD and MCI patients (Liu, *et al.*, 2012(a)). The results of this study suggest that AD and MCI have lower IDE activity levels when compared with healthy controls (n = 216). However, only 8.7% of this cohort were MCI individuals (n = 19), and 7.4% were AD patients (n = 16), therefore this study may not have sufficient power to form a solid conclusion.

Farris and colleagues (2005) identified an alternative full-length human isoform of IDE which was generated as a result of alternative splicing, which they termed “15b-IDE”.

This isoform was similar to the canonical isoform (termed “15a-IDE”) with the exception of exon 15, and was shown to exist in both the cytosol and in the mitochondria of human brain and liver. The 15b-IDE isoform was demonstrated to have an impaired ability to degrade insulin and A β , when compared with 15a-IDE, and it was also found that both isoforms could exist intracellularly as either homodimers or heterodimers. Hence, an increase in the ratio of 15b-IDE:15a-IDE isoforms could potentially have a negative effect on A β and/or insulin degradation.

1.6.1.1 Structure and Function of IDE

IDE was first discovered around 60 years ago due to its ability to degrade the insulin B chain (Mirsky & Broh-Kahn, 1949), and has since been found to cleave a variety of other substrates, including amylin (Bennett, *et al.*, 2000(a)), β -endorphin (Safavi, *et al.*, 2006), insulin-like growth factors I and II (IGF-I and IGF-II) (Roth, *et al.*, 1984), glucagon (Duckworth & Kitabchi, 1974), atrial natriuretic peptide (ANP) (Müller, *et al.*, 1991), transforming growth factor-alpha (TGF- α) (Garcia, *et al.*, 1989), bradykinin, kallidin (Malito, *et al.*, 2008), somatostatin (Ciaccio, *et al.*, 2009) and the APP intracellular domain (AICD), which is cleaved from APP during A β processing by γ -secretase (Edbauer, *et al.*, 2002; Venugopal, *et al.*, 2007). However, AD researchers suddenly developed significant interest in this enzyme when it was discovered that IDE was also found to degrade A β (Kurochkin & Goto, 1994; Qiu, *et al.*, 1998; McDermott & Gibson, 1997; Chesneau, *et al.*, 2000) (Figure 1.2). Although IDE is known to bind and degrade a wide range of substrates, it has a preferential affinity for insulin (Qiu, *et al.*, 1998; Duckworth, 1988; Edbauer, *et al.*, 2002). However, unlike most enzymes which recognise substrates via sequence specificity, IDE appears to have conformational specificity for its substrates, many of which share a similar secondary structure – can form beta strands, and have a tendency toward amyloidogenesis (Kurochkin, 1998; Kurochkin, 2001; Bennett, *et al.*, 2000(a)). It is therefore possible that it may have a primary function in the prevention of amyloid formation and deposition by degrading potentially amyloidogenic peptides.

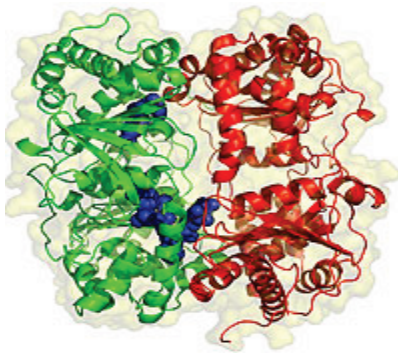


Figure 1.2 The IDE protein in complex with an A β peptide. (Shen, *et al.*, 2006)

The N-terminal domain of IDE is green, and C-terminal domain is red. The A β peptide (blue) is shown within the degradation chamber of the IDE protein.

IDE can be divided into two subunits, the N-terminal half and the C-terminal half (IDE-N and IDE-C, respectively) of approximately 55 kDa each, which are joined by a loop comprised of 28 amino acids (Shen, *et al.*, 2006). These subunits interact with each other to form a triangular crypt which engulfs and binds its substrates to the internal catalytic site before hydrolysing them at specific residues, favouring smaller substrates which are less than 6kDa with few positively-charged residues at the C-terminus (Duckworth, *et al.*, 1998; Shen, *et al.*, 2006). IDE can also be divided further into four domains, with domains 1 (AA 43-285) and 2 (AA 286-515) comprising IDE-N, and domains 3 (AA 542-768) and 4 (769-1016) comprising IDE-C (Shen, *et al.*, 2006). The active catalytic site of IDE is located in domain 1, and residues His108, Glu111, His112 and Glu189, which associate with a zinc ion, have been demonstrated to be crucial for normal activity of the enzyme (Shen, *et al.*, 2006; Perlman & Rosner, 1994; Song, *et al.*, 2005).

IDE displays allosteric kinetic properties with evidence suggesting that the enzyme usually exists in a closed conformation, but shifts to an open conformation when activated. This has been shown to occur by each half rotating away from each other. One proposed mechanism responsible for this appears to be a disruption in hydrogen bonding at the IDE-N and IDE-C interface; it has been confirmed that mutations in this region which dislocate this interface between domains 1 and 4, promote the open conformation and hence substantially increase the catalytic activity of the enzyme (Shen, *et al.*, 2006; Im, *et al.*, 2007). It has also been demonstrated that the open conformation can be initiated by the binding of nucleotide polyphosphate anion regulators such as ATP, to a cationic distal exosite located primarily in domain 4, within the catalytic chamber (Shen, *et al.*, 2006; Im, *et al.*, 2007; Noinaj, *et al.*, 2011). Although ATP was first reported to be an inhibitor, the regulation of IDE by nucleotide

polyphosphates appears to be concentration-dependent, and at physiological concentrations ATP activates the enzyme to increase hydrolysis of small peptide substrates (Camberos & Cresto, 2007; Song, *et al.*, 2004). It is possible that other anions may also regulate IDE in this way (Noinaj, *et al.*, 2011 (b)). IDE's ability to be activated selectively through the polyphosphate cationic exosite could be useful in developing drug targets for this mechanism, and synthetic compounds have been identified which can activate the enzyme nearly 7-fold in this manner (Cabrol, *et al.*, 2009).

In addition to the active site in domain 1, a distal site which binds peptide substrates has been identified in domain 2, also within the catalytic cavity. The binding of small peptides to this site is also thought to be involved in allosteric kinetics and activation. This is supported by studies of mutations in this region which have been found to eradicate these properties (Noinaj, *et al.*, 2011 (a)). However, the distal site appears to be a distinct site from the anion-binding site, as activation of IDE by the small peptide bradykinin was largely unaffected by an excess of either ATP or triphosphate (Song, *et al.*, 2004). Larger IDE substrates such as insulin and A β have been demonstrated to bind to this distal site via their N-terminus, while simultaneously binding to the active site, inducing a change in substrate conformation toward β -strands. This mechanism has been proposed as a way of positioning larger substrates for cleavage via the active site, and in addition may also indicate a means of locking IDE in the closed conformation due to electrostatic charges (Leissring, *et al.*, 2010). Smaller substrates less than 12 amino-acids in length are unable to bind to both sites simultaneously, however may act as heterotropic activators if they have a stronger affinity for the distal site than the active site, as in the case of bradykinin (Amato, *et al.*, 2009; Song, *et al.*, 2003; Noinaj, *et al.*, 2011 (a)). Binding to both the active site and distal site simultaneously by larger peptides such as insulin, has been shown to eliminate both allosteric behaviour and activation by ATP and small peptide substrates, possibly by either physical blocking of the cationic binding site/s and/or steric hindrance. Experimental evidence suggests that via peptide binding to this site, IDE can be selectively activated toward particular substrates; for example dynorphin B-9 increases the rate of A β ₁₋₄₀ degradation but inhibits insulin degradation (Song, *et al.*, 2003). Similarly, somatostatin, has been shown to positively regulate the degradation of A β

by IDE (Tundo, *et al.*, 2012; Ciaccio, *et al.*, 2009), and interestingly, reduced levels of somatostatin have been implicated both in the pathology of AD, and also as part of normal ageing (Ciaccio, *et al.*, 2009; Gavilán, *et al.*, 2007). This feature of selective activation by small peptides could offer a potential target for A β -reducing therapies for AD.

As well as activation by small peptides and phosphates, IDE activity has also been found to be regulated by calcium, manganese, magnesium and zinc ions (Duckworth, *et al.*, 1990; Ebrahim, *et al.*, 1991) with zinc being essential for its function. It was demonstrated recently that a single exposure of lead can impair IDE activity *in vitro*, and increase the intracellular accumulation of A β (Behl, *et al.*, 2009). One theory that was put forward to explain this mechanism involved lead replacing the zinc ion at the catalytic site, being a bivalent cation itself. Physiological levels of these cations, therefore, are likely to be influential in regulating IDE catalytic activity.

IDE also requires free thiol groups for its proteolytic activity and this seems to be related to its cysteine residues. Studies have shown that alkylation, oxidation, nitrosylation or glutathionylation may modify cysteine-related thiols and cause complete or partial inhibition of the enzyme (Neant-Fery, *et al.*, 2008; Shinall, *et al.*, 2005; Malito, *et al.*, 2008; Cordes, *et al.*, 2011). While alkylation of thiols is irreversible, nitrosylation and glutathionylation appear to be reversible modifications. Oxidation may inhibit IDE directly, or indirectly via oxidised glutathione. These effects could therefore provide another mechanism for IDE dysfunction in AD.

The aggregation status of IDE is also important in regulating its function, and IDE has been found to exist normally as a mixture of monomers, dimers and tetramers in equilibrium, with dimers and tetramers being the dominant species (Song, *et al.*, 2003; Song, *et al.*, 2004). The dimer is reportedly the most active form. Evidence suggests that ATP shifts the aggregation status of IDE towards the monomeric form, and increases its affinity for degrading smaller peptides such as bradykinin and dynorphin B-9, without effect on larger peptides like insulin and A β (Song, *et al.*, 2004). In addition, the monomeric variant of IDE does not appear to be activated by small peptides or ATP, and displays classic Michaelis-Menten kinetics rather than allosteric, indicating that the association of two or more subunits has a cooperative effect (Song,

et al., 2010). Thus the balance of these species is important in the overall function and enzymatic activity of IDE.

As mentioned previously, IDE appears to have many roles in human physiology, as well as disease pathology, one of them being its association with the development of type 2 diabetes, otherwise known as diabetes mellitus type 2 (DM2) (Furukawa, *et al.*, 2008; Karamohamed, *et al.*, 2003; Kwak, *et al.*, 2008). Numerous studies have also found an association between DM2 and AD (Ott, *et al.*, 1996; Ott, *et al.*, 1999; Muller, *et al.*, 2007). However, the putative mechanisms which may be common to both DM2 and AD, or mechanisms for DM2 increasing risk of AD have yet to be elucidated. This link between DM2 and AD will be discussed in more detail below.

1.7 Type 2 Diabetes, Insulin Resistance Syndrome and AD

One of the precursors to DM2 is the insulin resistance syndrome (also called “Metabolic Syndrome” or “Syndrome X”), which has also been implicated in the development of AD. Characteristics associated with DM2 and insulin resistance syndrome include insulin resistance, high peripheral levels of insulin (hyperinsulinemia), hyperglycaemia and impaired glucose tolerance, obesity, hypertension, and abnormal peripheral lipid and cholesterol concentrations, among others (Kuusisto, *et al.*, 1997; Chang, 2008; Wehr, *et al.*, 2006; and reviewed by Craft, 2006 and Mielke & Lyketsos, 2006). Accumulating evidence suggests that in particular, insulin resistance, hyperinsulinemia and DM2 are significant risk factors for AD (Ott, *et al.*, 1996; Ott, *et al.*, 1999; Kuusisto, *et al.*, 1997; Muller, *et al.*, 2007).

Cross-sectional studies have demonstrated that DM2 is considerably over-represented in AD patients in comparison with non-AD controls (Kuusisto, *et al.*, 1997; Ott, *et al.*, 1996; Stewart & Liolitsa, 1999). In addition, it has been shown in numerous large, population-based longitudinal studies that DM2 may significantly increase the risk of AD (Peila, *et al.*, 2002; Ott, *et al.*, 1999; Leibson, *et al.*, 1997; Xu, *et al.*, 2004; Arvanitakis, *et al.*, 2004). However, some studies have not found an association between DM2 and risk of AD, or the results may have demonstrated a trend but were non-significant (Luchsinger, *et al.*, 2001; MacKnight, *et al.*, 2002; Hassing, *et al.*, 2002).

A study by Peila *et al.* (2002) found that AD patients with co-morbid diabetes had an increase in the prevalence of SP, NFT and CAA, and especially in carriers of the *APOE* $\epsilon 4$ allele. In addition, both AD and DM2 have an increased prevalence with age (von Strauss, *et al.*, 1999; Davidson, 1979). This may suggest that a common pathological mechanism related to ageing may be implicated in both DM2 and AD.

Pathological similarities exist between DM2 and AD; cytotoxic amyloid is deposited in both conditions. While fibrillar A β is deposited in particular brain regions in AD (Glenner & Wong, 1984), most DM2 patients have similar amyloid deposits in the islets of Langerhans in the pancreas, composed of fibrillar islet amyloid polypeptide (IAPP), also known as amylin (Cooper, *et al.*, 1987; Westermark, *et al.*, 1987). Studies have shown that amylin may aggregate intracellularly in the β -cells prior to cell loss, and subsequently form extracellular deposits after the death of cells (O'Brien, *et al.*, 1995; O'Brien, *et al.*, 1994). It is possible that AD pathology follows the same pattern. In fact, several studies have shown that A β is produced intracellularly and over-production or reduced clearance may be significant in AD pathology (Wertkin, *et al.*, 1993; and reviewed by Gouras, *et al.*, 2010). Interestingly, islet amylin deposits contain ApoE akin to A β deposits, indicating a role for ApoE in amyloid formation in both conditions (Wisniewski & Frangione, 1992). Further evidence linking DM2 to AD comes from a study by Janson *et al.* (2004), which found that AD patients are more likely to have islet amyloid deposits than non-AD controls. The same study found that non-AD DM2 patients are not more likely to develop SP, although when diffuse or neuritic plaques were present, they correlated with the duration of DM2. The results are different to what has been found in other studies, as their data support the hypothesis that patients with Alzheimer disease are more vulnerable to DM2 (not the other way around). The results need to be replicated in other studies, using a larger cohort.

DM2 patients have been shown to be more susceptible to cognitive impairment, and to have lower MMSE scores than controls (van den Berg, *et al.*, 2005; Grodstein, *et al.*, 2001; Sinclair, *et al.*, 2000; Arvanitakis, *et al.*, 2004). Interestingly, it has been reported that medications that promote the secretion of insulin further increase the risk for AD in diabetics (Luchsinger, *et al.*, 2001; Ott, *et al.*, 1999; Ott, *et al.*, 1996). It is possible, therefore, that the link between DM2 and AD may be related to insulin.

1.7.1 Insulin and AD

Insulin, well known for its role in regulating peripheral glucose levels, is principally produced and secreted by β -cells in the pancreas, and insulin secretion occurs in normal physiology in response to elevated plasma glucose (Martini, 1998). It is also known that insulin plays an important role in the CNS, gaining access from the periphery by receptor-mediated transport across the BBB (Banks, *et al.*, 1997). It has been shown that insulin plays a role in long-term potentiation (LTP), a process which underlies memory formation (Craft, 2006). In addition, animal studies have demonstrated that neurons can generate insulin, and insulin receptors have been found in key areas of the brain involved with AD pathology and cognitive function (Devaskar, *et al.*, 1994; Schechter, *et al.*, 1988; Craft & Watson, 2004). Due to the role of insulin in glucose regulation, it is likely that insulin abnormalities impair cerebral glucose metabolism, a characteristic which has been demonstrated in both AD and MCI brains (De Santi, *et al.*, 2001). Elevation of peripheral insulin levels has also been shown to increase insulin concentration in the CNS (Wallum, *et al.*, 1987). However, it has been shown that insulin levels in the brain are actually lower in AD patients (Craft, *et al.*, 1998) and evidence suggests this can be caused by the presence of hyperinsulinemia-related insulin resistance in the periphery, causing a reduction in brain insulin uptake through the blood-brain barrier (Baura, *et al.*, 1996; Craft, *et al.*, 1998; Kaiyala, *et al.*, 2000). In addition, insulin resistance in the CNS may be associated with impairment of several molecules involved in the insulin signalling cascade (Talbot, *et al.*, 2012). Conversely, hyperinsulinemia could also inhibit A β degradation in the periphery itself through competitive inhibition of IDE, leading to A β accumulation both peripherally and then in the CNS by migration across the BBB.

1.7.1.1 Hyperinsulinemia

Hyperinsulinemia, referring to high peripheral levels of insulin, is an early feature of DM2 in many individuals, occurring well prior to altered glucose metabolism (Weyer, *et al.*, 2000). Chronic hyperinsulinemia leads to, correlates with, and therefore is used as a measure of insulin resistance. It is also used as a predictor of DM2 (Laakso, 1993;

Lundgren, *et al.*, 1990; Weyer, *et al.*, 2000). Research suggests that hyperinsulinemia is also a significant risk factor for AD (Luchsinger, *et al.*, 2004), and in contrast to the findings that brain insulin levels decrease in AD as mentioned above, numerous studies have demonstrated elevated serum insulin levels in AD subjects in comparison with controls (Kuusisto, *et al.*, 1997; Weyer, *et al.*, 2000; Muller, *et al.*, 2007; Carantoni, *et al.*, 2000; Fujisawa, *et al.*, 1991). This association has been demonstrated in non-diabetic individuals too, suggesting that this feature of DM2 may be more relevant to AD risk than just DM2 itself (Luchsinger, *et al.*, 2004; Stolk, *et al.*, 1997). In support of this, other evidence suggests that AD severity also correlates with plasma insulin levels (Craft, *et al.*, 1998).

Two studies have shown that hyperinsulinemia is a significant risk factor in AD patients who do not possess the *APOE* $\epsilon 4$ allele, indicating that these factors may affect risk independently (Kuusisto, *et al.*, 1997; Craft, *et al.*, 1999). Moreover, Craft *et al.* (1998) found that AD subjects with only one *APOE* $\epsilon 4$ allele or no $\epsilon 4$ alleles had higher plasma insulin levels than $\epsilon 4/\epsilon 4$ homozygotes. Craft *et al.* (2003) later found that memory facilitation occurred with peripheral insulin infusions at a similar dose for normal adults and *APOE* $\epsilon 4/\epsilon 4$ homozygotes with AD, however AD patients with one or less $\epsilon 4$ alleles required much higher doses for the same effect. These results may indicate that those who are not $\epsilon 4/\epsilon 4$ homozygotes may have reduced sensitivity to insulin. Another large study found no association between diabetes and AD risk overall, however when stratified for *APOE* $\epsilon 4$ genotype, this study found an association only in *APOE* $\epsilon 4$ negative individuals (Akomolafe, *et al.*, 2006). These results indicate that insulin metabolism dysfunction may be a relevant pathological mechanism for the approximate 50% of AD patients who do not possess the $\epsilon 4$ allele, in respect to AD risk. It may indicate that for those individuals with *APOE* $\epsilon 4$ alleles, the supposed metabolic deficiencies caused by the ApoE4 protein are sufficient to increase AD risk, whereas in non-*APOE* $\epsilon 4$ individuals, AD may develop after long-term insulin resistance and associated effects.

1.7.1.2 Insulin and Cognition

Accumulating evidence suggests that insulin is involved in a number of key roles in cognition and memory. In support of this, insulin has been shown to play a part in

regulating cerebral levels of neurotransmitters which are involved in memory and cognition, such as dopamine, acetylcholine and norepinephrine (Craft & Watson, 2004). *In vivo* studies have shown memory facilitation in rats after insulin was directly applied to the brain (Park, *et al.*, 2000) and after intravenous insulin infusion in humans (Craft, *et al.*, 2003). The administration of insulin via nasal pathways has the effect of elevating levels in the CNS without raising peripheral levels, and several studies have also demonstrated improvements in memory and cognitive function after such treatment both in diabetic mice, and in early AD and MCI patients (Craft, *et al.*, 2012; Francis, *et al.*, 2008; Reger, *et al.*, 2006; Reger, *et al.*, 2008(a); Reger, *et al.*, 2008 (b)). Interestingly, in the study by Reger *et al.* (2008(a)), memory facilitation only occurred in *APOE* $\epsilon 4$ negative subjects while memory was impaired in $\epsilon 4$ carriers. It is possible that this outcome was dose-related as it is in contrast to previous reports (Craft, *et al.*, 2003).

Conversely, DM2 and elevated plasma insulin has also been associated with impaired cognition and memory (Stolk, *et al.*, 1997; Vanhanen, *et al.*, 1998; Marfaing-Jallat, *et al.*, 1995). The discrepancy in these results may be due to the fact that insulin-induced memory facilitation is attenuated at higher (and lower) doses, indicating an optimum level is required for cognitive improvement (Craft, *et al.*, 2003; Reger, *et al.*, 2008 (a)). In addition, *APOE* genotype-related differences in insulin sensitivity may have affected this result. The study by Stolk *et al.* (1997) excluded diabetics and dementia patients from the analysis, which may have skewed the results, and significant results were only obtained for females. Similarly, Vanhanen's study (1998) was only on elderly glucose intolerant individuals. Much more likely, however, is that acute hyperinsulinemia may facilitate memory and cognition, while chronic hyperinsulinemia appears to produce deleterious effects.

1.7.1.3 Insulin and A β

Insulin has been demonstrated to regulate A β levels, both in the CNS and peripherally, although the mechanisms that control A β levels are unclear. An *in vivo* study on healthy adults found that A β levels in the CSF were elevated after intravenous insulin infusion (Watson, *et al.*, 2003). *In vitro* studies have found that insulin stimulates A β production via upregulation of APP trafficking and increased secretion of A β into the

extracellular milieu (Gasparini, *et al.*, 2001). The same study showed that insulin also increased extracellular levels of A β by impairing its degradation by IDE. This has been supported by other studies that have found that hyperinsulinemia has an inhibitory effect on A β degradation (Qiu, *et al.*, 1998; Kulstad, *et al.*, 2006). Moreover, it has been recently demonstrated that AD patients respond differently to insulin when compared to healthy controls (Kulstad, *et al.*, 2006). While levels of plasma A β dropped in normal adults after receiving a peripheral insulin infusion at optimal levels, the AD patients consistently showed an increase in A β levels in a dose-dependent manner. This indicates that AD patients may have impaired insulin clearance which could be attributable to IDE dysfunction or a reduction in levels of IDE, which then leads to accumulation of A β .

1.7.1.4 Hypercholesterolemia, Insulin and IDE

There are other sources of evidence which link insulin and IDE with A β regulation. Hypercholesterolemia has been associated with AD, MCI and increased levels of A β in some studies (Pappolla, *et al.*, 2003; Kivipelto, *et al.*, 2001), and research has shown that a high-fat diet in AD-model transgenic Tg2576 mice induces insulin resistance and subsequently impairs insulin signalling in the brain (Ho, *et al.*, 2004). Concomitantly, there is a decrease in IDE activity, and an increase in both A β ₄₀ and A β ₄₂ levels and brain plaque load. The suggested role of insulin signalling in IDE regulation is supported by a similar study by Zhao *et al.* (2004). Obesity and elevated free fatty acids (FFA), features related to hyperinsulinemia, have also been shown to impair insulin degradation via direct non-competitive inhibition of IDE (Hamel, *et al.*, 2003; Eckel, *et al.*, 2005).

Insulin potentially affects other aspects of AD pathology. It has been demonstrated that both insulin and insulin-like growth factor-1 (IGF-1) regulate the phosphorylating protein kinase, glycogen synthase kinase-3 β (GSK-3 β) to stimulate transient tau phosphorylation *in vitro* and *in vivo*, which may be involved in the formation of NFT (Engel, *et al.*, 2006; Lesort, *et al.*, 1999; Lesort & Johnson, 2000; Hong & Lee, 1997; Ho, *et al.*, 2004). IGF-1 not only has neurotrophic properties and a role in apoptosis, but also regulates levels of IDE and therefore may modulate A β levels, and as mentioned above may be involved in tau phosphorylation through regulation of GSK-3 β (Sharma,

et al., 2008; Costantini, *et al.*, 2006). Interestingly, IGF-1 is also a substrate for IDE degradation, and low levels have been found in AD brains (Steen, *et al.*, 2005). In addition, IGF-1 signalling has an effect on GSK-3 α , which has been shown to regulate APP processing (Phiel, *et al.*, 2003). Another study has shown that hypercholesterolemia increases production of A β via GSK-3 α , and decreases clearance of A β due to a reduction in levels of IDE, through alterations in the IGF-1 signalling pathway (Sharma, *et al.*, 2008). This association between hypercholesterolemia and low IDE is supported in another study (Prasanthi, *et al.*, 2008). Therefore, hypercholesterolemia may also play an important part in the link between DM2 and AD. In addition, hyperinsulinemia has been shown to promote inflammatory responses in the CNS, a feature associated with AD (Craft, 2005; Aisen, 1997; Fishel, *et al.*, 2005). Research has found that the degree of chronic inflammation correlates with both cognitive decline (Parachikova, *et al.*, 2007) and brain atrophy (Cagnin, *et al.*, 2002) and therefore may exacerbate neuronal degeneration. Increased peripheral insulin levels have been found to lead to increased cerebral levels of pro-inflammatory cytokines such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumour necrosis factor- α (TNF- α), all of which have been reported to be elevated in AD and have been located in SP and their related glial cells in Alzheimer's patients (Craft, 2005; Sokolova, *et al.*, 2009; Griffin, *et al.*, 1989; Bauer, *et al.*, 1991; Fishel, *et al.*, 2005; Dickson, *et al.*, 1993). Interestingly, anti-inflammatory cytokines have been shown to increase both IDE expression as well as A β degradation (Shimizu, *et al.*, 2008). Inflammation is a known characteristic of DM2, and there is mounting evidence that chronic inflammation is an important early stage of AD pathogenesis (reviewed by Bhamra & Ashton, 2012). In fact, inflammation-linked proteins such as alpha-2 macroglobulin and clusterin (amongst others) are considered to be good potential biomarkers of early stages of AD (Thambisetty, *et al.*, 2010; reviewed by Bhamra & Ashton, 2012). Chronic activation of microglia in neurodegenerative disorders such as AD, Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) as well as inflammatory conditions of CNS such as multiple sclerosis (MS) results in overall upregulation of pro-inflammatory cytokines and chemokines in the brain. As a result, many therapeutic strategies aim to alleviate neurodegenerative disease progression by reducing inflammation (Reinhart, *et al.*, 2011; reviewed by Holmes, 2013; Cappellano, *et al.*, 2013; Rizzo, *et al.*, 2013).

1.8 Hyperinsulinemia, IDE and AD

Insulin resistance and hyperinsulinemia are related conditions, such that each condition induces the other (Craft, 2006). Therefore it is plausible that low levels of plasma IDE may lead to hyperinsulinemia, which leads to insulin resistance and possibly the development of DM2. In addition, IDE deficiency-induced hyperinsulinemia could reasonably lead to the accumulation of A β in the periphery, due to competitive inhibition. This could potentially instigate or exacerbate AD pathology. In accordance with this, it has in fact been demonstrated that hyperinsulinemia can increase A β levels in the periphery, and also in the CSF and brain (Fishel, *et al.*, 2005). Certain diets may exacerbate this problem too, if they lead to high peripheral lipid levels and/or chronic insulin release.

In other studies, including studies by our group in the AIBL study, it has been shown that greater levels of exercise have a protective effect against AD, and also induce positive effects on AD-related pathological biomarkers (Brown, *et al.*, 2012(a); Brown, *et al.*, 2012(b); Liang, *et al.*, 2010). This may be, in part at least, associated with exercise-induced elevation of IDE levels, as a recent study in rats confirmed that IDE levels increase, causing a decrease in insulin levels, as a result of even short-term exercise (Kim, *et al.*, 2011).

It has been demonstrated that levels of IDE decline as a result of ageing, in areas of the brain susceptible to AD pathology (Caccamo, *et al.*, 2005); for example IDE levels were found to decline in hippocampal regions, an area commonly affected by AD pathology, but not in the cerebellum, a region of the brain where A β deposits are rare. This ageing-related, regional decline in IDE levels supports the concept that insulin and IDE levels are relevant in AD pathogenesis. Insulin resistance and chronic hyperinsulinemia, as previously mentioned, can also have other effects relevant to AD, such as reducing insulin transport across the BBB into the CNS (Baura, *et al.*, 1996). This may lead to reduced levels of insulin in the brain, attenuating the benefits of insulin on cognition and memory.

If hyperinsulinemia forms the basis for the putative relationship between AD and DM2, IDE could be a good candidate as a plasma biomarker for AD. Leissring and colleagues

(2003) examined the effect of genetic over-expression of IDE in *APP* transgenic mice. The results showed a significant decrease in both soluble and insoluble cerebral A β levels, with a concomitant prevention or reduction in formation of SP when compared with *APP* transgenic controls. In addition, premature death was attenuated in the mice over-expressing IDE. This result paralleled a previous *in vitro* IDE over-expression study, where extracellular levels of both A β_{40} and A β_{42} were also found to be significantly reduced (Vekrellis, *et al.*, 2000). In this cell culture study, the reduction of A β was inhibited by insulin incubation, highlighting the competition between insulin and A β for IDE, and its preference for insulin. Another over-expression study on Chinese hamster ovary cells demonstrated a significant reduction in both extracellular and intracellular A β levels (Sudoh, *et al.*, 2002). Interestingly, IDE has been shown to degrade monomeric but not oligomeric or fibrillar A β , indicating that a reduction in monomeric A β may be sufficient to prevent plaque formation, and that IDE may not recognise/bind aggregated A β (Leissring, *et al.*, 2003; Vekrellis, *et al.*, 2000; Mukherjee, *et al.*, 2000). However, in a more recent study, it was shown that IDE is responsible for a significant portion of the degradation of fibrillar A β in monocyte-derived macrophages (Yamamoto, *et al.*, 2008). It is likely that protein chaperone-mediated disassembly of the fibrils may occur before the degradation by IDE. It has also been suggested that monomeric and polymeric A β may exist in equilibrium, where reduction of monomers may instigate the disassembly of pre-formed fibrillar aggregates (Leissring, *et al.*, 2003). A study by Lazarov *et al.* (2002) supports this equilibrium hypothesis by demonstrating that SP formation is reversible. If this is the case, then IDE may be ideal as a therapeutic target against AD.

Further support for the concept that IDE is important in A β breakdown comes from *in vivo* studies which have found that levels of A β in the brain elevate as a response to deletion of the *IDE* gene, in transgenic knockout mouse models (Farris, *et al.*, 2003; Miller, *et al.*, 2003). While Miller *et al.* (2003) found an increase of both A β_{40} and A β_{42} , Farris and colleagues (2003) demonstrated that murine IDE knockouts not only display chronic elevation of A β levels, but also develop hyperinsulinemia and impaired glucose tolerance, characteristics of DM2. In these experiments, levels of AICD were also found to be elevated, signifying impaired clearance. The function of AICD is not precisely known, however it has been suggested that it may have a role in nuclear

signalling and apoptosis (Edbauer, *et al.*, 2002; Kinoshita, *et al.*, 2002). In addition, AICD is thought to be neurotoxic and it appears to regulate GSK-3 β (Kim, *et al.*, 2003; Ryan & Pimplikar, 2005). This suggests a mechanism that is separate from A β accumulation which could be involved in AD pathogenesis.

Farris *et al.* (2004) demonstrated further evidence for IDE's possible involvement in the development of LOAD, in a study on Goto-Kakizaki (GK) rats. The GK rat is a well-characterised rat model of DM2, with typical features such as hyperinsulinemia and postprandial hyperglycaemia, and mutations within the *IDE* gene (Fakhrai-Rad, *et al.*, 2000). This study showed that partial IDE hypofunction was sufficient to induce elevations in neuronal A β levels; however increases in steady-state levels of A β did not occur until the age of 14 months, indicating a possible compensatory effect in the early stages of life. This may reflect possible mechanisms for the development of LOAD. In addition, the levels of A β in this study compared along with knockout and over-expression studies appear to indicate a dose-dependent effect of *IDE* on A β , further supporting the importance of expression levels on A β homeostasis (Farris, *et al.*, 2003). Overall the results indicate that impaired expression and/or activity of *IDE* are sufficient to generate symptoms of AD pathology.

Clinical evidence supports the premise that IDE may be integral to the development and progression of AD. A study by Perez *et al.* (2000) found that the activity level of IDE was significantly lower in AD brains compared with controls. While this study did not measure the cerebral levels of the full length IDE protein due to detection difficulties, levels of a 44 kDa fragment of IDE in the cytosolic fraction were shown to be decreased in AD brains compared with controls. These findings were supported by a larger-scale study by Cook *et al.* (2003), who found decreased levels of IDE protein and mRNA in hippocampal regions of AD brains compared with control brains. Interestingly, this reduction was only observed in *APOE* $\epsilon 4$ carriers, which suggests that the $\epsilon 4$ allele either directly affects *IDE* expression, or interacts with an independent process of AD pathology which leads to reduced *IDE* expression. This finding of decreased levels of IDE in hippocampal regions was supported in a murine study, where homozygous triple-transgenic models of AD (Oddo, *et al.*, 2003) showed significantly lower levels of IDE in the hippocampus compared with wild-type and hemizygous transgenic mice (Caccamo, *et al.*, 2005). In accordance with these

findings, a new transgenic mouse model of AD has been reported to demonstrate reduced cortical expression of IDE prior to the appearance of SP (Ferretti, *et al.*, 2011).

Conversely, studies have also shown that levels of IDE are increased in certain regions of the AD brain in comparison with controls, the enzyme can be localised to neurons and has also been associated with SP (Bernstein, *et al.*, 1999). It is possible that small sample size, lack of *APOE* $\epsilon 4$ stratification, and/or region-specific alterations in IDE levels may be causing these conflicting results. Miners *et al.* (2008a) demonstrated that hippocampal IDE expression differs depending on the region examined; with CA3/4 neurons showing the highest expression and CA1 the lowest, and significantly lower levels being found in AD patients compared with controls in the CA2 and CA3/4 regions. In this study, the lowest levels were also associated with the most advanced AD cases. Furthermore, slight differences were found between *APOE* $\epsilon 4$ carriers and non-carriers with respect to IDE expression levels; however they failed to reach significance.

1.8.1 IDE Catalytic Activity

It is plausible that it is a combination of decreased activity and expression levels, or simply impaired activity alone that leads to decreased A β degradation. Although there have been reports of increased IDE activity in AD individuals compared with healthy controls in some studies (Miners, *et al.*, 2009), numerous studies have reported reduced activity levels compared with healthy controls (Kim, *et al.*, 2007; Perez, *et al.*, 2000; Qin & Jia, 2008; Zhao, *et al.*, 2007; Morelli, *et al.*, 2004). The relatively recent study by Zhao and colleagues (2007) showed impairment in both IDE levels and activity in membrane fractions of the hippocampus in MCI patients compared with normal adults. Moreover, both activity and concentration levels continued to decrease during the progression from MCI to AD, and were inversely correlated with cerebral A β_{42} levels. This supports the notion that IDE dysfunction and impairment of expression are present and apparent in the prodromal stages of AD. The importance of IDE activity is further highlighted in the study by Morelli *et al.* (2004). This study examined the levels and activity of IDE in cerebral microvessel fractions, in AD patients with concomitant CAA, compared with non-AD controls. The results found that although expression

levels of *IDE* were markedly comparatively increased in AD/CAA patients, the activity levels were significantly decreased.

One recent study on familial EOAD patients with a novel *PS1* mutation found that accumulation of extracellular and intracellular $A\beta_{42}$ was always associated with decreased activity levels of *IDE*, both in the cytosol and on the plasma membrane (Qin & Jia, 2008). Moreover they discovered that elevated intracellular, but not extracellular $A\beta_{42}$, was also associated with decreased expression levels of *IDE*. This indicates that intra- and extracellular *IDE* may function in different ways. It has recently been shown that cytosolic *IDE* has a longer half-life than membrane-bound *IDE*, which has a considerably faster turnover (Bulloj, *et al.*, 2008). This localisation, and *IDE* activity can be modulated by changes in membrane lipid composition, and has been shown to result in alterations in $A\beta$ degradation. This may explain a mechanism for an *IDE*-lipid interaction which impacts on *IDE* activity levels. As previously mentioned, the novel 15b-*IDE* isoform (Farris, *et al.*, 2005), could also suggest a mechanism for the reduced activity levels observed in AD, whether it exists physiologically as a homodimer or with 15a-*IDE* as a heterodimer.

There are a number of other factors that may contribute to the impairment of *IDE* activity. It has been shown that ubiquitin, a regulatory protein which has been suggested as having a possible role in AD and other neurodegenerative diseases, binds to and inhibits the activity of *IDE*, and this interaction was found to be modulated by insulin (Grasso, *et al.*, 2008; Saric, *et al.*, 2003) indicating it is affected via a competitive mechanism. This would be likely to affect the degradation of $A\beta$ in the same manner. Ubiquitin has also recently been found to be a substrate of *IDE* (Ralat, *et al.*, 2011). Pathological mechanisms of AD such as oxidative stress or intracellular accumulation of $A\beta$ could increase the levels of ubiquitin and therefore reduce *IDE* activity.

IDE activity has also been found to be reduced through impaired insulin signalling due to diet-induced insulin resistance (Ho, *et al.*, 2004), inhibition through elevation of FFA's (Hamel, *et al.*, 2003) and elevated glucocorticoids (Kulstad, *et al.*, 2005; Harada, *et al.*, 1996). Interestingly, the glucocorticoid cortisol has been found to be elevated in the periphery of AD patients (Umegaki, *et al.*, 2000; Hartmann, *et al.*, 1997; Davis, 1986). A recent study showed that different types of fatty acids can differentially

affect the expression of IDE as well, with saturated palmitic acid causing down-regulation and polyunsaturated docosahexaenoic acid (DHA) causing the opposite effect (Du, *et al.*, 2010). Interestingly, saturated dietary fatty acids such as palmitic acid have been shown to exacerbate AD pathology while unsaturated fatty acids such as DHA are considered to be protective (Morris, *et al.*, 2003; Patil, *et al.*, 2007; Cole & Frautschy, 2006). It is possible that this may be due in part to their effects on IDE expression and activity.

As previously mentioned, the activity of IDE has also been shown to be inhibited by cysteine-directed modifications such as alkylation, S-nitrosylation by nitric oxide (NO), and oxidation or glutathionylation (Neant-Fery, *et al.*, 2008; Cordes, *et al.*, 2009; Malito, *et al.*, 2008; Shinall, *et al.*, 2005). IDE activity has also been reported to be positively regulated by the peptide hormone somatostatin, which is known to be reduced in AD (Ciaccio, *et al.*, 2009). In addition, the important discovery that fibrillar A β binds to, and inhibits the activity of IDE, is also a very significant observation (Chander, *et al.*, 2007). One or more of these factors may contribute to the impairment of IDE activity and subsequent development of AD pathology. Overall the results suggest a need to examine both expression levels and activity in conjunction with each other to gain a more complete picture of IDE-related AD pathology.

1.8.2 IDE and A β Neurotoxicity

Another positive effect of IDE cleavage of A β is the subsequent neutralization of the neurotoxic effects of the fragments produced. An *in vitro* study by Mukherjee, *et al.* (2000) found that IDE reduced the cytotoxicity of both A β_{40} and A β_{42} in a dose-dependent manner. However, while only 50 ng of IDE was sufficient to almost completely eliminate the toxicity of A β_{40} , a ten-fold increase in IDE was required to neutralize A β_{42} to a similar degree. The significance of this difference is not clear, however may reflect the differing degrees of neurotoxicity and/or propensity to oligomerise, of each of the species. In addition, this study also showed that these hydrolysed fragments of A β produced by IDE did not deposit on synthetic amyloid *in vitro*. This further highlights a possible role for IDE in a therapeutic capacity.

1.8.3 ApoE and IDE

While it is not known how the ApoE protein contributes to AD pathogenesis, it has been reported that ApoE is essential for, and may assist with cerebral clearance of A β , via degradation extracellularly by IDE and within microglia by IDE and its related protease, neprilysin (NEP) (Jiang, *et al.*, 2008). The capacity of ApoE in this degradative facilitation is determined partly by isoform, with ApoE4 demonstrating a reduced capability to assist this process, and ApoE2 being most efficient. In addition, this study demonstrated the importance of ApoE lipidation status in the proteolytic clearance of A β , with unlipidated ApoE also demonstrating a reduced capacity for facilitation compared with the lipidated protein. This finding supports similar previous reports that found that lipidation of ApoE is essential for effective A β clearance (Cole & Ard, 2000). Defects in the ATP-binding cassette transporter A1 (ABCA1) have been shown to result in abnormally lipidated ApoE, and lead to a reduction of ApoE levels in the CNS and plasma (Wahrle, *et al.*, 2004; Hirsch-Reinshagen, *et al.*, 2005). Studies on ABCA1 deficiency have shown an increase in the levels of both soluble and insoluble A β in murine brains (Wahrle, *et al.*, 2005; Koldamova, *et al.*, 2005), that cannot be attributed to an increase in A β production (Hirsch-Reinshagen, *et al.*, 2005). Therefore, another possible mechanism for the development of AD pathology may be related to the interaction between the ApoE and IDE proteins in the clearance of A β .

Another recent study by Du and colleagues (2009) demonstrated that IDE mRNA and protein expression was down-regulated by ApoE4 in comparison to ApoE2 or ApoE3 in rat primary hippocampal neurons and PC12 cells. The same study showed that ApoE is required for normal IDE expression levels as *APOE* knockout mice had significantly lower IDE levels than wild-type mice. By culturing hippocampal primary neurons from these *APOE* knockouts and treating them with ApoE3-containing conditioned medium, they showed that this IDE deficit could be rescued, however treating with ApoE4 containing medium had no effect. These effects could be attenuated by blocking the N-methyl-D-aspartic acid receptor (NMDAR); in fact, by inhibiting this receptor they showed an increase in IDE levels and a corresponding decrease in IDE levels when this receptor was activated. This indicates that the regulatory effect of ApoE isoforms on IDE levels is dependent on the NMDA receptor pathway.

Other interactions appear to occur between *IDE* and *APOE* genotype to alter *IDE* expression levels. Studies have shown that elderly individuals carrying the $\epsilon 4$ allele have higher peripheral levels of the glucocorticoid cortisol than non- $\epsilon 4$ individuals (Peskind, *et al.*, 2001), as do AD patients (Umegaki, *et al.*, 2000; Hartmann, *et al.*, 1997; Davis, 1986), and in a study of aged macaque monkeys, oral cortisol treatment was found to cause a decrease in *IDE* mRNA and protein levels in both the frontal cortex and the hippocampus (Kulstad, *et al.*, 2005). It has also been reported that other glucocorticoids interact with *IDE* to inhibit its capacity for insulin degradation (Harada, *et al.*, 1996). Overall this could suggest that the increased risk conferred by the $\epsilon 4$ allele may be partly due to an increase in glucocorticoid levels, affecting *IDE* expression and activity.

1.9 IDE Levels in Plasma

IDE levels have been studied in regions of the brain, both in cytosolic and membrane fractions, as well as in brain microvessels. It has been investigated in liver, kidneys, erythrocytes, skeletal muscle, testes and ovaries, among other tissues, in both rodents and humans (Miller, *et al.*, 2003; Duckworth, *et al.*, 1988; Morelli, *et al.*, 2004; Vekrellis, *et al.*, 2000; Farris, *et al.*, 2003; Duckworth, *et al.*, 1990). *IDE* has also been detected in extracellular biological fluids such as CSF and wound fluid (Qiu, *et al.*, 1998; Shearer, *et al.*, 1997). It has been shown that the expression of *IDE* is ubiquitous (Kuo, *et al.*, 1993; Weirich, *et al.*, 2008), and evidence also exists for the presence of an enzyme with insulin-degrading capacity in human serum (Misbin, *et al.*, 1981). However, although the activity of *IDE* has been examined in blood in rudimentary studies with respect to insulin degradation (Ionushas, *et al.*, 1987; Ardy & Pontremoli, 1953), and more recently *IDE* expression has been detected in blood and all other tissues examined (Su, *et al.*, 2002), surprisingly it appears that no study at the commencement of this project, had examined *IDE* protein or activity in blood, and certainly not with respect to AD. However as mentioned previously, one very recent study has been published on serum *IDE* activity but had low sample numbers in the AD and MCI groups (Liu, *et al.*, 2012(a)).

Given the evidence associating hyperinsulinemia, elevated plasma A β levels and AD, it would be highly beneficial to examine the blood concentration and catalytic activity of this protease in a large cohort.

1.10 Genetic Linkage and Association of IDE

The gene for *IDE* is located on chromosome 10q23-q25 (Espinosa, *et al.*, 1991; Affholter, *et al.*, 1990), and in addition to functional evidence supporting IDE as a putative biomarker for AD, there is accumulating evidence that the region in and/or around *IDE* may be genetically associated with both AD (Kehoe, *et al.*, 1999; Myers, *et al.*, 2000; Bertram, *et al.*, 2000; Ait-Ghezala, *et al.*, 2002; Bian, *et al.*, 2004; Liu, *et al.*, 2007; Hamshire, *et al.*, 2007; Vepsäläinen, *et al.*, 2007; Mueller, *et al.*, 2007; Bjork, *et al.*, 2006; Zuo & Jia, 2009; Carrasquillo, *et al.*, 2010) and DM2 (Duggirala, *et al.*, 1999; Wiltshire, *et al.*, 2001; Karamohamed, *et al.*, 2003; Furukawa, *et al.*, 2008; Zeggini, *et al.*, 2007). Moreover, as with many conditions, various studies on both dizygotic and monozygotic twins have shown that genetic factors may account for a significant proportion of the risk of LOAD (Petersen, *et al.*, 2004; Bergem, *et al.*, 1997; Gatz, *et al.*, 1997). A recent large study demonstrated an association between a novel *IDE* variant and lower plasma A β_{40} levels and a reduced risk of LOAD. It was found that this variation resulted in an increase in IDE mRNA expression (Carrasquillo, *et al.*, 2010).

In addition, *IDE* has been linked to other quantitative indices (*QTL*) of AD, such as age of onset (Li, *et al.*, 2002; Blacker, *et al.*, 2003; Prince, *et al.*, 2003; Dickson, *et al.*, 2008), plasma A β levels (Ertekin-Taner, *et al.*, 2000, Ertekin-Taner, *et al.*, 2004), MMSE score, memory and cognitive function, CSF levels of tau protein, cerebral A β and NFT load (Prince, *et al.*, 2003; McQueen, *et al.*, 2007; Liu, *et al.*, 2007; Blomqvist, *et al.*, 2005; Mueller, *et al.*, 2007). Quantitative indices of DM2 such as elevated fasting plasma glucose levels (Meigs, *et al.*, 2002; Karamohamed, *et al.*, 2003), plasma insulin levels (Marlowe, *et al.*, 2006; Gu, *et al.*, 2004), and age of onset (Duggirala, *et al.*, 1999) have also been associated with the region in and/or around the *IDE* gene. A recent study by Hong and colleagues (2008) also discovered an association between human life span and *IDE*.

Interestingly, some studies showed genetic association with *IDE* only in *APOE* $\epsilon 4$ negative individuals (Feuk, *et al.*, 2005; Edland, *et al.*, 2003; Zuo & Jia, 2009), supporting findings of insulin resistance and hyperinsulinemia in non- $\epsilon 4$ carriers with AD (Kuusisto, *et al.*, 1997; Craft, *et al.*, 1999), and an association between AD and DM2 only in non- $\epsilon 4$ carriers (Akomolafe, *et al.*, 2006). This suggests that the effect of the *APOE* genotype and *IDE* might affect risk independently, and may be of significance for a subset of AD and DM2 sufferers. In addition, this also highlights the importance of stratifying results by *APOE* genotype.

Although many studies have reported an association between the *IDE* gene and AD, there are also studies that refute this association (Marlowe, *et al.*, 2006; Ozturk, *et al.*, 2006; Nowotny, *et al.*, 2005). It was suggested that these discrepancies could be due to a number of factors including allele frequency, linkage disequilibrium and locus variance in different populations, subject selection bias and genotyping errors. Another common theme which occurs in some of these genetic analyses is that often the association is only seen in males (Gu, *et al.*, 2004; Hong, *et al.*, 2008) or the study only used men in their cohort (Marlowe, *et al.*, 2006). Of course, in addition, it is not possible to analyse every single nucleotide polymorphism within the limitations of a single study. This may be another factor to consider when translating the data.

Although disease-causing mutations have not yet been discovered within the coding region of the *IDE* gene, it is possible that mutations exist within the non-coding region. These non-coding region mutations may affect the cap, tail, polyadenylation signal, promoter or other regulatory sequences which may alter the gene product and/or its expression or function (Knight, 2003). In accordance with this, a recent meta-analysis of the literature found a significant positive association between the *IDE* polymorphism rs1832196 and AD risk (Zhang, *et al.*, 2013). This polymorphism is located within an intronic sequence of *IDE*. Collectively, there is considerable evidence supporting the genetic involvement of *IDE* in both AD and DM2 pathology.

1.11 Summary and Project Hypothesis

For the moment, the only way to ascertain a definite diagnosis of AD is via post-mortem analysis, and current diagnostic methods are not sufficient in the early or prodromal stages of the disease for accurate diagnosis. With the increasing prevalence and incidence of AD in the ageing population, the identification of a suitable biomarker, or set of biomarkers, for prognostic, diagnostic and therapeutic monitoring purposes is becoming all the more imperative. In particular, a blood biomarker would be highly beneficial due to the invasive nature, difficulties and expense associated with current diagnostic procedures. A β is widely believed to be central to the pathogenesis of AD, with increased accumulation as a product of an imbalance between production and clearance of this peptide. The vast majority of AD cases are of the non-familial and/or late-onset variety, and increasing evidence suggests that impairment of A β clearance mechanisms may be the more important factor in LOAD pathogenesis, rather than increased A β production. Enzymes, receptors and any other proteins involved in the clearance and/or degradation of A β are therefore viable biomarker candidates.

IDE is a protease of interest, due to increasing evidence supporting its mechanistic involvement in the regulation of A β and AD pathology, and genetic studies supporting the existence of an AD risk locus, and AD *QTL* loci in the vicinity of the *IDE* gene on chromosome 10q. In addition, IDE is associated with hyperinsulinemia and DM2, which have both been suggested as potential risk factors for AD. IDE has also been shown to interact with the ApoE protein and can be affected by *APOE* genotype. Some gaps in current knowledge include the quantification and activity of IDE in the blood, and the possible association of *IDE* expression and/or IDE activity levels with AD risk.

While numerous studies have reported associations between IDE and AD, replication of these results has been inconsistent, and a number of factors may have led to these discrepancies. An analysis of these variables in a large population would assist in determining the associations between IDE and AD pathogenesis, as well as evaluate the potential of IDE as a functional AD biomarker.

Therefore, the project hypotheses are as follows:

1. The levels and catalytic activities of IDE in human plasma differ between clinical classifications of AD, MCI and healthy controls.
2. The levels and catalytic activities of IDE in human plasma are affected by APOE genotype.

To test these hypotheses, the particular aims of this project are:

1. To measure the levels of plasma IDE protein in a large cohort (n = 1000+).
2. To measure the catalytic activity of plasma IDE in a large cohort.
3. To determine if there is any relationship between IDE levels, catalytic activity, and other biochemical markers including A β , ApoE and insulin in plasma between AD/MCI affected individuals and healthy controls.
4. To determine if *APOE* $\epsilon 4$ genotype impacts upon the levels or catalytic activity of plasma IDE.

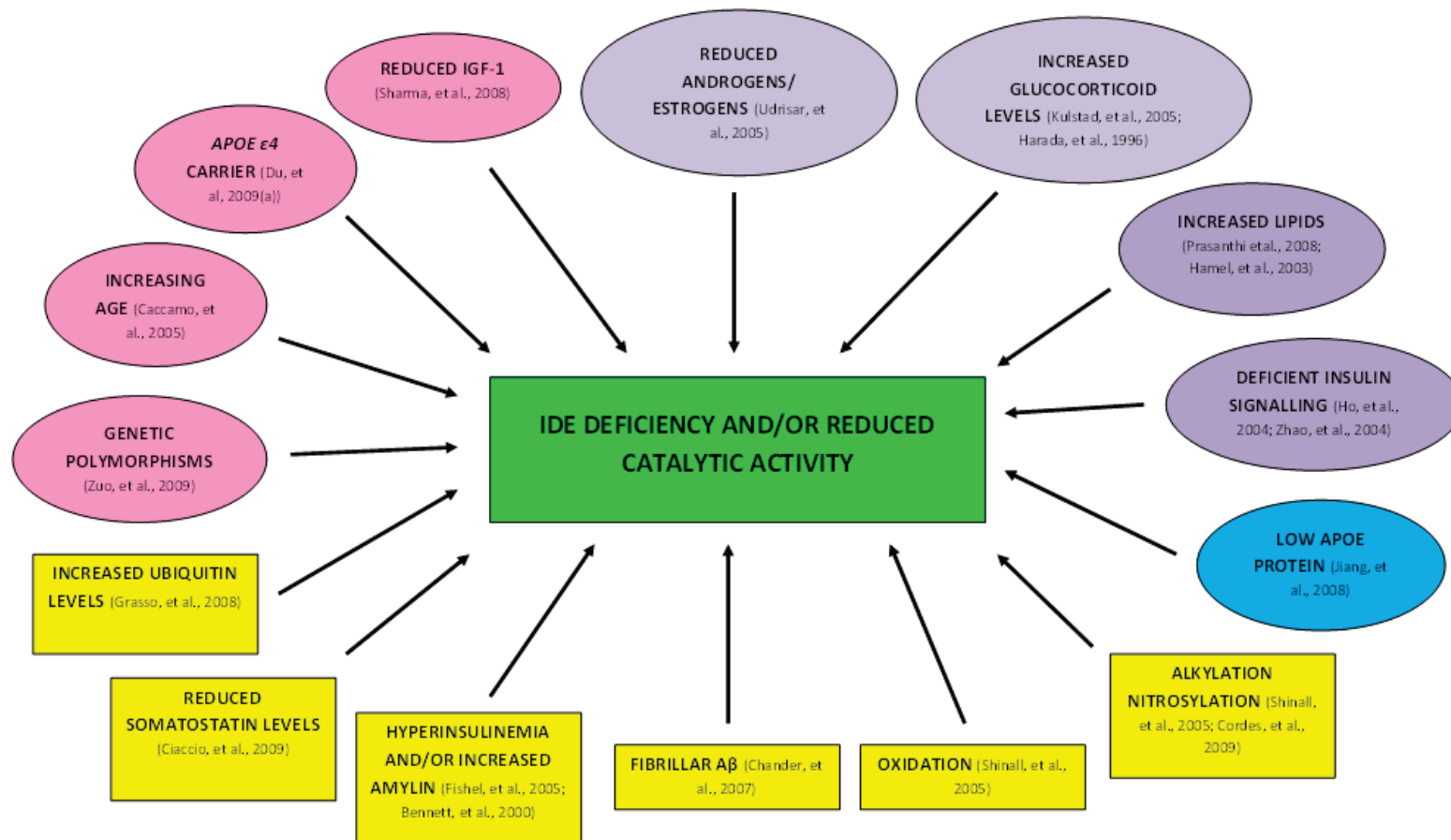


Figure 1.3 Representation of various factors associated with AD or DM2 which may affect IDE function. A number of physiological conditions may negatively regulate the catalytic activity (yellow), expression levels (pink), or both levels and activity (purple) of IDE. Low ApoE protein, indicated in blue, regulates the ability of IDE to degrade A β independently of IDE levels or catalytic activity, and this has been shown to be modulated by the lipidation status of ApoE. Individual factors or a combination of these may lead to an insufficiency of IDE to efficiently degrade A β and lead to its accumulation in the periphery and/or the brain.

(THIS PAGE HAS BEEN INTENTIONALLY LEFT BLANK)

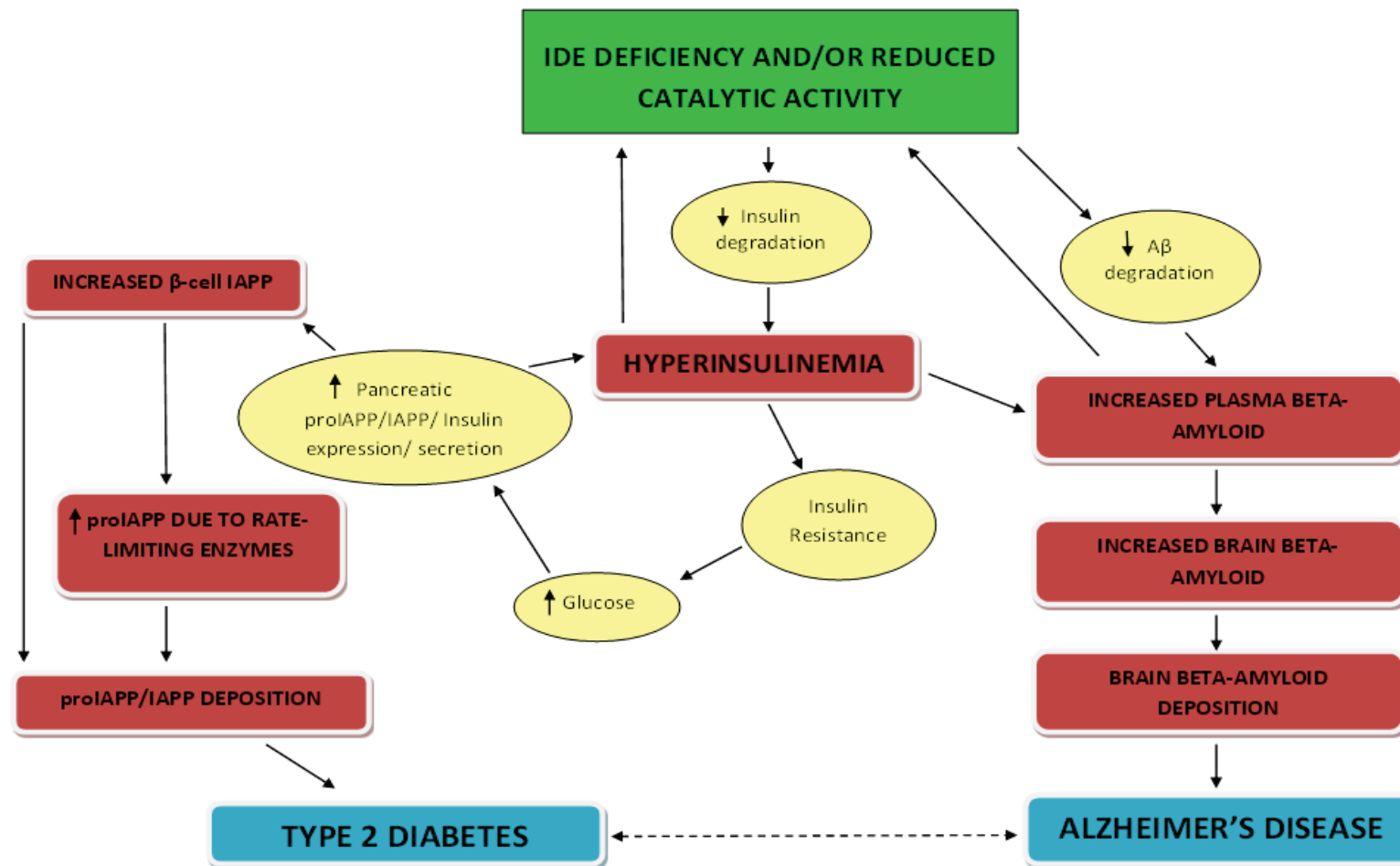


Figure 1.4 Flowchart of how a reduction in IDE levels or activity may lead to the development of both AD and DM2. Reduction of IDE levels or activity cause an insufficiency in both Aβ and insulin degradation, leading to their accumulation in plasma. The increased proteins/peptides then compete further for IDE, causing a positive feedback mechanism. The hyperinsulinemic state is also central to both conditions. This leads to an increase in amyloid deposition in the brain (Aβ) and pancreas (ProIAPP/IAPP) which subsequently develops into DM2 and/or AD.

(THIS PAGE HAS BEEN INTENTIONALLY LEFT BLANK)

CHAPTER 2 – Materials and Methods.

2.1 Materials

2.1.1 Antibodies

The antibodies used in this project are summarised in Tables 2.1 and 2.2. Figure 2.1 illustrates the epitopes on IDE to which each antibody binds.

Table 2.1 Commercial primary IDE antibodies.

PRIMARY IDE ANTIBODIES						
Antibody	Catalogue Number	Supplier	Host	Immunogen Source	Immunogen Sequence	Formulation
Ab 1(a) – Batch 1 Ab 1(b) – Batch 2	AB9210	Chemicon Aust. P/L (now Merck/Millipore, VIC, Australia)	Rabbit	Synthetic rat	AA200-250	Serum
Ab 2	LS-C55007	LifeSpan Biosciences, Inc. (Seattle, WA, USA)	Goat	Synthetic Human	AA914-924	Purified
Ab 3	ab28561	Abcam (Cambridge, UK)	Rabbit	Synthetic Human	AA238-416	Purified
Ab 4	250706	Abbiotec™ (San Diego, CA, USA)	Mouse	Synthetic Human	N-terminus (Not defined)	Purified
Ab 5	AF2496	R&D Systems Inc. (Minneapolis, MN, USA)	Goat	Recombinant Human	AA42-1019	Purified
Ab 6	H00003416- A01	Abnova (Taipei, Taiwan)	Mouse	Synthetic Human	AA 920-1019	Serum
(M)Ab 7	MMS-282R	Covance (MA, USA)	Mouse	Human	AA 1-1019	Ascites
Ab 8	GTX111664	GeneTex Inc. (California, USA)	Rabbit	Synthetic Human	AA 349-585	Purified
(M)Ab 9	5282-1	Epitomics (CA, USA)	Rabbit	Synthetic Human	AA 50-75	Tissue culture supernatant

- (M) denotes monoclonal antibody

Table 2.2 Additional commercial non-IDE and secondary antibodies.

NON-IDE ANTIBODIES					
Antibody	Supplier	Host	Immunogen	Epitope	Formulation
GTX27793 (AL-01) (M)	GeneTex Inc. (California, USA)	Mouse	Human serum albumin	Full-length	Purified
SECONDARY ANTIBODIES					
Antibody	Supplier	Host	Classes	Conjugate	Formulation
P0449	Dako (VIC, Australia)	Goat	All	HRP	Purified
AP322P	Chemicon Australia Pty. Ltd. (VIC, Australia)	Rabbit	IgG	HRP	Purified
AP326P	Chemicon Australia Pty. Ltd. (VIC, Australia)	Mouse	IgG	HRP	Purified

- (M) denotes monoclonal antibody

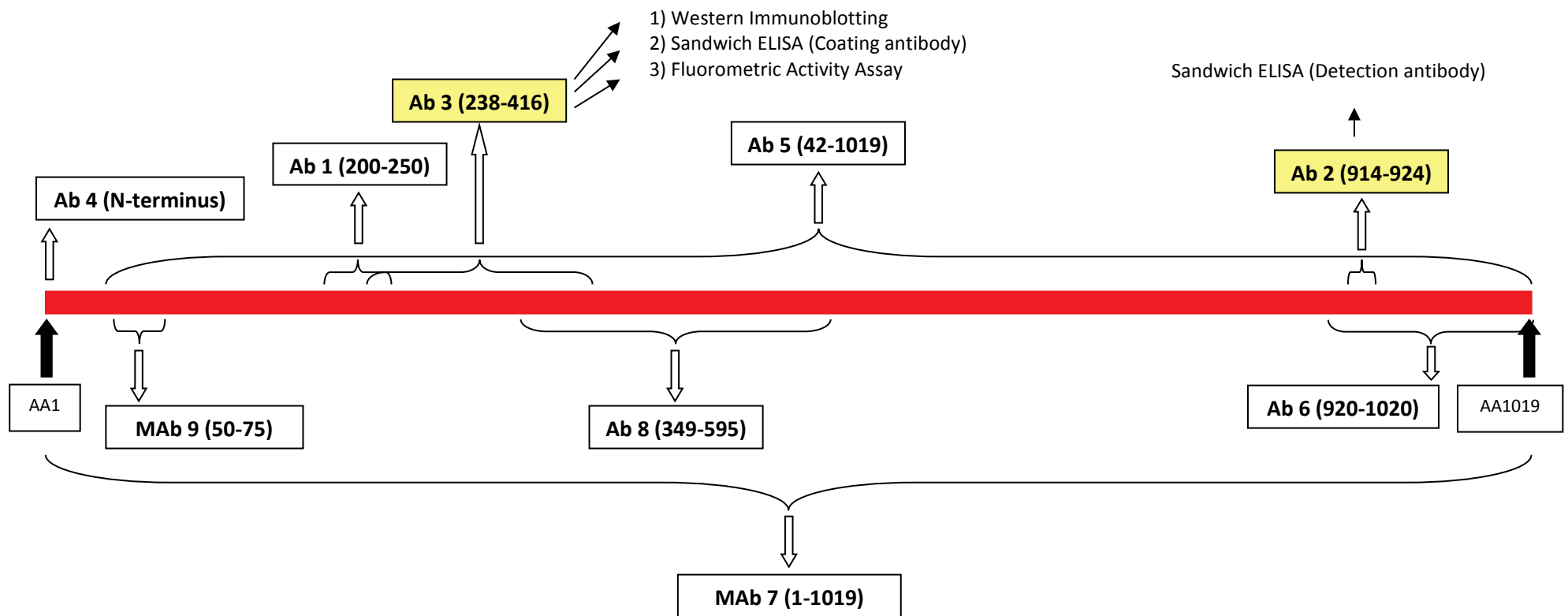


Figure 2.1 Diagram of IDE epitopes to which commercially-available antibodies bind. Monoclonal antibodies are indicated by (M).

2.1.2 Suppliers of Reagents

2.1.2.1 Proteins

The fluorogenic peptide substrate (Mca-R-P-P-G-F-S-A-F-K(Dnp)-OH, also called Substrate V), ES005, and human recombinant IDE, 2496-ZN were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Recombinant His-tagged rat IDE, 407241, was purchased from Calbiochem/EMD Biosciences, Inc. (Merck KGaA, Darmstadt, Germany). Lyophilized recombinant A β ₄₀ and A β ₄₂ was synthesized and purchased from W.M. Keck Foundation Biotechnology Research Laboratory (Yale University, New Haven, CT, USA). The custom-synthesized blocking peptide against LS-C55007 primary antibody was purchased from Mimotopes Pty. Ltd. (Clayton, VIC, Australia). The commercial blocking peptide (and immunogen) against antibody ab28561 was purchased from Abcam (Cambridge, UK).

2.1.2.2 Other reagents

All de-ionized ultra-pure water (MQ H₂O) was obtained from the usual laboratory distilled water system, and filtered through the Sartorius Arium® (611DI) filtration system (Sartorius AG, Göttingen, Germany).

Polyoxyethylene (20) sorbitan monolaurate (Tween-20), bovine serum albumin (BSA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Trizma Hydrochloride (Tris-HCl) (T-3253) and Ponceau S powder (78376) were all purchased from Sigma-Aldrich Pty. Ltd. (Castle Hill, NSW, Australia).

NuPage® Novex 10% (NP0316BOX) and 4-12% (NP0336BOX) Bis-Tris 1.5mm x 15-well pre-cast gels, NuPage® 4x lithium dodecyl sulfate (LDS) sample buffer (NP0007), NuPage® 10x Reducing Agent containing 500 mM dithiothreitol (DTT) (NP0009), NuPage® Antioxidant (NP0005), Novex® Sharp Pre-stained Protein Standard (LC5800), NuPage® 20x MES SDS Running Buffer (NP0002-02) and NuPage® Transfer Stacks – Regular Nitrocellulose (IB3010-01) were all purchased from Invitrogen™ Australia Pty. Ltd. (Mulgrave, VIC, Australia).

Sodium chloride (NaCl), ethylenediaminetetraacetic acid tetra-sodium salt (EDTA), ethanol absolute was purchased from Merck Pty. Ltd. (Merck KGaA, Darmstadt, Germany). Fraction V bovine serum albumin (10 735 086 001) was purchased from Roche Diagnostics Australia Pty. Ltd. (Castle Hill, NSW, Australia). AnalaR sodium carbonate (Na_2CO_3) (10240.4H), AnalaR sodium hydrogen carbonate (NaHCO_3) (10247.4V), and sodium dodecylsulfate (SDS) were all purchased from BDH Chemicals Australia Ltd. (VIC, Australia). The Thermo Scientific Micro BCA™ Protein Assay Kit (23235), Pierce Direct IP Kit (26148), Restore™ Western Blot Stripping Buffer (21059) and Gelcode Blue Stain Reagent (Coomassie stain) (24592) were all purchased from Thermo Fisher Scientific (Scoresby, Vic, Australia). Glycerol (193996), 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) (151245) and Tris-[hydroxymethyl]aminomethane (Tris) (152176) was purchased from MP Biomedicals Australia (Formerly ICN Biomedicals Inc., Seven Hills, NSW, Australia). Amersham ECL™ Western Blotting Detection Reagents (RPN2106), Amersham ECL™ Advance Western Blotting Detection Kit (RPN2135) and Amersham ECL™ Prime Western Blotting Detection Reagents (RPN2232) were all purchased from GE Healthcare Australia Pty. Ltd. (Rydalmere, NSW, Australia). Phosphate-buffered saline (PBS) was purchased from Fisher Biotech International Ltd. (Wembley, WA, Australia). Complete Mini Protease Inhibitor Cocktail (11836153001) was purchased from Roche Australia (Osborne Park, WA, Australia). 3,3',5,5'-Tetramethylbenzidine (TMB) Microwell Peroxidase Substrate System (50-76-00) was purchased from Kirkegaard and Perry Laboratories Inc. (Maryland, USA). Block Ace (BUF029) was purchased from AbD Serotec (Oxford, UK).

2.1.3 Suppliers of Equipment

A β ₄₂ solution concentration was determined using an Implen Nanophotometer (Version 7122, V2.0.0).

2.1.3.1 Western Immunoblotting

Proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Invitrogen™ NuPage® Electrophoresis system, using the XCell SureLock™ Mini-Cell Electrophoresis Blot Module and the Invitrogen™ iBlot® Gel Transfer Device, both purchased from Invitrogen™ Australia Pty. Ltd. (Mulgrave, VIC, Australia). Bands were visualised by densitometric analysis using the Bio-Rad GS-800 Densitometer (Bio-Rad Laboratories Pty. Ltd., CA, USA).

2.1.3.2 ELISA/Fluorescent and BCA Protein Assay

IDE ELISA kit was purchased from Kamiya Biomedical Company (Seattle, USA). 96-well microplates 655900 were purchased from Greiner Bio-One (Frickenhäusen, Germany), U16 MaxiSorp™ Modules 469264 and black F16 MaxiSorp™ Modules 475515 were purchased from Nunc (Rochester, NY, USA). Microplates were washed using either the Bio-Rad Immunowash Microplate Washer (Model 1575) or the BioTek® Select Deep Well Microplate Washer (ELx405™, VT, USA). Proteins were quantified using the Fluostar Optima Microplate Reader (BMG Labtech GmbH, Ortenberg, Germany).

2.2 Methods

2.2.1 The Australian Imaging, Biomarker and Lifestyle Flagship Study of Ageing (AIBL) Cohort

Plasma samples to be analysed were sourced from the Australian Imaging, Biomarker and Lifestyle Flagship Study of Ageing (AIBL) study (<http://www.aibl.csiro.au/>) which has been well characterised (Ellis, *et al.*, 2009). Launched in November 2006, this longitudinal study includes participants recruited from Perth, Western Australia (40%) and Melbourne, Victoria (60%). The primary aim of this study has a focus on early detection, and prevention or delay of Alzheimer's disease, by research into a variety of diagnostic markers, biomarkers and neuroimaging techniques. AIBL is also assessing the role of diet and exercise in relation to the prevention of AD.

The AIBL cohort consists of 1112 individuals, of which 211 have AD, 133 have MCI and 768 are healthy controls (HC) (Figure 2.2). Of the 768 HC participants, 396 reported having memory complaints that did not meet the criteria for MCI or dementia. Using a multidisciplinary approach, all participants underwent extensive clinical (including blood collection for biomarker analysis), neuropsychological and lifestyle assessments at baseline, and neuroimaging with PiB-PET and MRI was performed on approximately one quarter of the cohort. Consenting participants are subsequently re-assessed at 18 month intervals until death. The exclusion criteria included comorbid conditions that could affect cognitive function, including non-AD dementias, uncontrolled diabetes, past serious head injury, current clinical depression, history of stroke, and alcohol consumption exceeding two standard drinks per day for women, and four standard drinks per day for men.

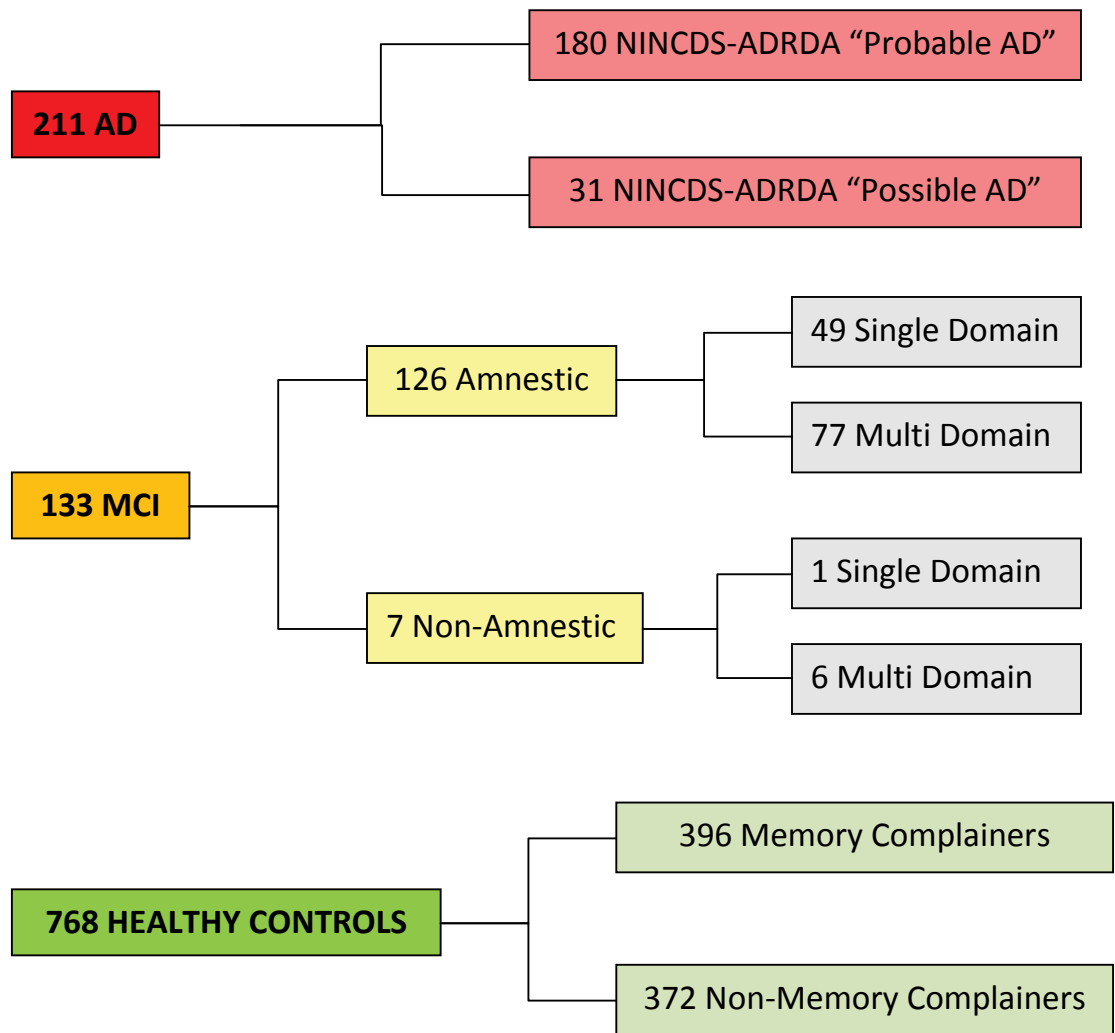


Figure 2.2 Classification of AIBL cohort subgroups at baseline by diagnosis (n=1112).

2.2.2 Sample preparation

Blood collection details can be found in Ellis, *et al.*, 2009. In summary, whole blood from the participants was collected into three types of tubes; lithium-heparin, serum and EDTA (with prostaglandin E1 added at a concentration of 33.3ng/mL to EDTA tubes only). The tubes were divided into aliquots and stored in liquid nitrogen, and after removal samples were subsequently stored at -80°C until use. Immediately prior to assaying, plasma samples were thawed on ice, and then centrifuged at 12,000 RPM for 15 minutes, and the supernatant collected. All plasma samples used in this project were sourced from EDTA tubes unless otherwise specified. It should be noted that separate subsets of the AIBL cohort were used to measure full-length IDE levels by Western immunoblotting, total IDE levels by ELISA, and IDE activity with very few samples in common between each assay.

2.2.2.1 Methods used for the measurement of biomarkers other than IDE

Testosterone, estradiol, total cholesterol, LDL/HDL cholesterol and insulin measurements were collected by clinical pathology services PathWest Laboratory Medicine WA (Perth) or Melbourne Health (Melbourne). With the exception of insulin for which measurements were taken using serum samples, the remainder of the test measurements from the clinical pathology services were taken from blood plasma collected in Lithium-Heparin tubes or serum samples. Total testosterone was measured using a chemiluminescent microparticle immunoassay. Free testosterone was calculated from total testosterone levels and sex-hormone binding globulin (SHBG) levels. Estradiol was measured using a chemiluminescent microparticle immunoassay. Total cholesterol was measured using an enzymatic assay based on the formulation of Allain, *et al.*, 1974 with modifications of this procedure by Roeschlau, *et al.*, 1974. HDL cholesterol was measured with an Accelerator Selective Detergent method. LDL cholesterol was calculated using the Friedewald formula (Friedewald, *et al.*, 1972) from HDL cholesterol, total cholesterol and triglyceride measurements (if triglycerides were < 4.5 mmol/L)

otherwise if triglycerides were $\geq 4.5\text{mmol/L}$, direct measurements were taken with a Measured, Liquid Selective Detergent method. Insulin levels were measured using a chemiluminescent microparticle immunoassay. An in-house sandwich ELISA was used to measure the A β isoforms using EDTA-collected plasma as previously documented (Mehta, *et al.*, 2000). A commercial sandwich ELISA kit (ApoE4/Pan ApoE ELISA, MBL International, Woburn, USA) was used to measure the levels of plasma ApoE and ApoE4 using EDTA-collected plasma as previously documented (Gupta, *et al.*, 2011). DNA for the *APOE* genotyping was isolated from whole blood using a Qiaamp DNA blood Midikit (Qiagen) as per manufacturer instructions. *APOE* genotyping was performed using either *APOE* TaqMan[®] SNP Genotyping Assays (Life Technologies) for rs7412 (Assay ID: C____904973_10) and rs429358 (Assay ID: C____3084793_20), or by amplification by polymerase chain reaction with restriction enzyme digestion as previously detailed (Hixson & Vernier, 1990). Brain A β load was determined by PET imaging using a radioactive imaging agent, Pittsburgh Compound B (PiB) as previously detailed (Pike, *et al.*, 2007).

2.2.3 Determination of plasma total protein content

The total protein content of human plasma was obtained with the Micro BCA[™] Protein Assay kit (Pierce, 23235). To summarise, bovine serum albumin (BSA) standards were prepared with MQ H₂O, in the range of 0 – 80 $\mu\text{g/mL}$ from a stock concentration of 2.0 mg/mL. 150 μL per well of each standard dilution was loaded in duplicate onto a standard 96-well microplate (Greiner Bio-One). Plasma was diluted to 1:100, 1:1000 and 1:2000 with MQ H₂O, and 150 μL of the plasma dilutions were loaded in quadruplicate. A working reagent was prepared with 25 parts Pierce reagent MA, 24 parts reagent MB, and one (1) part reagent MC as per manufacturer instructions, and 150 μL was added to the wells containing the standards and plasma dilutions. The plate was agitated for 30 seconds, then covered and incubated at 37°C for two hours. The plate was then allowed to cool to room temperature and absorbance was read on a Fluostar Optima microplate reader (BMG Labtech GmbH, Ortenburg, Germany) at 562 nm. A standard curve

was generated from the BSA standard dilutions and the plasma total protein concentration was interpolated from this curve.

2.2.4 IDE levels in human plasma by Western immunoblotting

2.2.4.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation of proteins

All samples were prepared using NuPage[®] LDS Sample Buffer and Reducing Agent (Invitrogen) on 10% Bis-Tris 1.5mm 15 well pre-cast gels with MES SDS running buffer, unless otherwise specified. Proteins were resolved by SDS-PAGE under reducing conditions. Total protein concentration of undiluted pooled control plasma (PCP) was determined to be 59.503 µg/ul. IDE as a positive control was prepared from purified recombinant rat IDE (rrIDE) diluted in MQ H₂O to a concentration of 20 ng/µL using a 1 mg/mL stock solution, with the final content per well being 100 ng. Plasma samples were diluted 1:7 with MQ H₂O (1µL per well), including an internal standard of pooled human plasma (PCP) which was loaded onto each gel. Total protein content after dilution was calculated to be 59.503 µg per load for PCP. LDS Sample Buffer and reducing agent (Invitrogen™) were added in the ratio of 8:3:1 (IDE standards or diluted plasma: LDS Sample buffer: reducing agent). The standards and plasma samples were heated at 70°C for 10 minutes. Where rat liver, human frontal cortex or zebrafish brain were used in optimization steps, the samples were heated for 5 mins at 95°C. The samples, PCP and positive control were loaded onto NuPage[®] 10% Bis-Tris mini pre-cast gels, using the XCell SureLock™ Mini-Cell Electrophoresis Blot Module (Invitrogen™), and NuPage[®] 1x MES SDS Running Buffer (Invitrogen™) for inner and outer chambers. 500 µL of NuPage[®] Antioxidant was added to the inner chamber immediately prior to loading samples. Proteins were separated according to their molecular weight along with pre-stained molecular weight markers (in the range of 3.5 kDa to 260 kDa; Invitrogen™) by electrophoresis, at 100 V for 1.5 to 3 hours. The semi-dry transfer of proteins to nitrocellulose membranes by electrophoresis was performed using the iBlot[®] Dry Blotting System (Invitrogen™) on programme P3 for 8.5 mins.

2.2.4.2 LS-C55007 immunoblotting protocol

Following electrophoretic transfer of the proteins, the nitrocellulose membranes were incubated with tris-buffered saline (TBS, pH 7.4) containing 3% non-fat dairy milk powder (NFDM) for one hour at room temperature with rocking, to block any non-specific binding sites. No Tween-20 was included in the blocking buffer at this stage to avoid the detergent loosely binding to the membrane and displacing the NFDM, as this may lead to the Tween-20 being removed by subsequent washes in TBST and leaving spaces for non-specific binding of the antibodies. After washing twice for 2 mins in TBS containing 0.05% Tween-20 (TBST), the membranes were then incubated with primary antibody LS-C55007 (LifeSpan Biosciences Inc.) diluted 1:5000 in TBST at 4°C overnight (approximately 18 hours) with rocking. Following the overnight incubation, the membranes were washed twice for 2 mins, then 4 times for 3 mins each in TBST to remove unbound antibody. The anti-goat HRP-conjugated secondary antibody (P0449) was then diluted 1:7500 in TBST and the membranes were incubated for 1 hour at room temperature (RT) with rocking. This was followed by washing twice for 2 mins and four times for 3 mins each in TBST to remove any unbound secondary antibody, then two 3 minute washes in TBS to remove any traces of Tween-20. The membranes were then incubated with ECL™ detection reagent (for 2 mins) for optimization only, or ECL Advance/Prime detection reagents (GE Healthcare Australia P/L) prepared as per manufacturer's instructions for 5 mins and exposed to film for up to 1.5 hours to obtain the desired signal intensity.

2.2.4.3 AB9210 immunoblotting protocol

Following electrophoresis, the membranes were incubated in TBS containing 5% NFDM. Membranes were then incubated overnight with primary antibody AB9210 diluted 1:8000 in TBST containing 0.5% NFDM at 4°C as per the LS-C55007 protocol. Following the overnight incubation, blots were washed three times for 10 min in TBST, and then incubated with HRP-conjugated anti-rabbit secondary antibody (AP322P) for 1 hour at RT as above. A further three 10 min washes in TBST and two 3 min washes in TBS followed. Secondary antibodies were detected by incubation

in ECL™ detection reagent for 2 mins and exposure to film for up to 20 minutes to achieve desired signal intensity.

2.2.4.4 ab28561 immunoblotting protocol

After electrophoretic transfer or stripping (stripping protocol detailed below in section 2.2.4.5), the membranes were incubated in TBS containing 3% NFDM at 4°C overnight with rocking. After washing twice in TBS, primary antibody ab28561 was diluted 1:2000 (or 1:1000 for some optimization steps) in TBST containing 0.5% NFDM and incubated for 2 hours at RT (rocking). Following primary antibody incubation, membranes were washed as per LS-C55007 protocol and then incubated in HRP-conjugated anti-rabbit secondary antibody, diluted 1:5000 in TBST with 0.5% NFDM, for 1 hour at RT. The membranes were then washed and incubated in ECL™ or ECL™ Prime detection reagent as per the LS-C55007 protocol, and exposed to film for up to 30 mins to achieve desired signal intensity.

2.2.4.5 Stripping of antibodies from membrane

Membranes were stored between cellulose acetate sheets at RT prior to stripping. After removing from acetate, blots were re-hydrated in TBS for 15 mins (no rocking), and then washed in TBS for 7 mins (with rocking for all steps from here on). Blots were then washed in 20 µL of Restore™ stripping buffer for 10 minutes in the fumehood to remove all bound antibodies, before washing in TBS for 2 mins. Following this, blots were washed in TBST four times for 5 mins, and in TBS twice for 2 mins. For AIBL samples, after stripping, the membranes were stained with Ponceau S, then destained and immunoblotted using ab28561 antibody. Alternatively, blots were re-stripped following immunoblotting and Coomassie stained.

2.2.4.6 Ponceau S and Coomassie staining as a loading control

The freshly stripped membranes were subsequently stained using a 0.1% (W/V) solution of Ponceau S in 5% acetic acid, for 5 mins, rocking. They then were rinsed in MQ H₂O twice for 30 seconds to remove excess background staining.

After scanning, blots were then destained using one 2 min wash in hot TBS, then a further two washes for 2 mins in cold TBS prior to immunoblotting.

After immunoblotting, membranes were stripped of antibodies as previously described in Section 2.2.4.5. The freshly stripped membranes were then Coomassie stained using Pierce GelCode Blue Stain Reagent as per manufacturer instructions.

2.2.4.7 Test of EDTA spiking on IDE levels in lithium-heparin collected plasma

The approximate concentration of EDTA in the EDTA-collected tubes is 1.8 mg/mL, and may vary per sample, therefore non-EDTA containing plasma was assessed to determine if varying amounts of EDTA would affect the detectable levels of IDE via Western immunoblotting. EDTA was diluted in MQ H₂O to a concentration of 10 mg/mL, then spiked into diluted lithium-heparin collected plasma to a final concentration of 2.0, 1.5 and 1.0 mg of EDTA per mL of (non-diluted) plasma. A control sample of lithium-heparin plasma containing 0 mg of EDTA was included for comparison, as well as two positive controls of 50ng and 100ng of rrIDE. Samples were subsequently analysed by Western blot using the LS-C55007 protocol as detailed above, with the exception of the washing steps after the antibodies. The wash steps were twice for 2 mins, one 15 min wash and then three 5min washes. Two three minute TBS washes were included after the secondary antibody to remove the Tween-20.

The same membrane was subsequently stripped of antibodies and re-probed using antibody ab28561 using the protocols described previously.

2.2.4.8 Test of cross-reactivity of LS-C55007 (and ab28561) to purified BSA and HSA

To test if antibody LS-C55007 or ab28561 detects purified BSA or HSA within the region of interest in the Western blot, purified BSA and HSA was diluted in MQ H₂O to a final concentration of 30 and 60 µg per well. This represents the lower and upper limits of albumin levels found in human plasma in normal physiology. A

separate HSA sample equivalent to 60 µg per well was also spiked with 175.2 ng of purified human recombinant IDE (rhIDE) and a separate sample spiked with 8 ng rrIDE to determine if IDE was sequestered by albumin under denaturing and reducing conditions. The spiked sample was pre-incubated for 1.5 hours at RT to allow time for binding. Samples were then resolved by SDS-PAGE and immunoblotted using the LS-C55007 or ab28561 protocols as previously described.

2.2.4.9 Test of cross-reactivity of ab28561 to purified BSA and albumin-free blocking agent

To test if antibody ab28561 detects purified BSA or an albumin-free blocking agent (ECL™ Advance Blocking Agent) within the region of interest in the Western blot, purified BSA and ECL™ Advance were both diluted in MQ H₂O to a final concentration of 15, 30 and 60 µg per well. A single lane of rrIDE (7.5 ng/well) was added as a positive control. All samples were resolved on a single gel and immunoblotted as per the previously detailed protocols with antibody ab28561.

2.2.4.10 Pre-incubation with recombinant human IDE blocking test for AB9210 for specificity

PCP was diluted in MQ H₂O (59.503 µg/well), LDS Sample Buffer and Reducing Agent to a ratio of 6 parts diluted PCP/3 parts Sample Buffer/1 part Reducing Agent and four lanes of each were loaded onto a single gel (two duplicates). Proteins were resolved by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane as previously described in Section 2.2.4.1. The single membrane was then cut into two identical pieces, designated membrane A (control) and membrane B. The separate membranes were then immunoblotted with IDE antibody AB9210 as previously described in Section 2.2.4.3, with the exception of a brief pre-incubation step prior to the primary antibody incubation. This step involved spiking and pre-incubating the antibody solution with either 0 (membrane A) or 1.32 µg (membrane B) of rhIDE for 10 minutes prior to adding the respective membranes before incubating overnight. The remainder of the protocol was

identical to previously described, using separate containers to prevent cross-contamination.

2.2.4.11 Pre-incubation with immunizing peptide blocking test for LS-C55007 for specificity

PCP was diluted in MQ H₂O as previously described (59.503 µg/well) as well as rrIDE (200 ng/well) and five lanes of each were loaded onto a single gel. Proteins were resolved by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane as previously described in Section 2.2.4.1. The single membrane was then cut to result in 5 identical pieces; each containing one lane of molecular weight marker (MWM), one lane of PCP, and one lane of rrIDE. The separate pieces were designated membrane A (control), B, C, D and E. All membranes were then immunoblotted with IDE antibody LS-C55007 as previously described in Section 2.2.4.2, with the exception of a pre-incubation step prior to the primary antibody incubation. This step involved spiking the antibody solution with 0 µg (A), 5 µg (B), 10 µg (C), 15 µg (D) and 20 µg (E) of a custom-synthesized peptide matching the immunogen to antibody LS-C55007. The five tubes were then incubated at RT for 30 mins prior to incubating the membranes. The remainder of the protocol was identical to previously described, using separate containers to prevent cross-contamination.

The test was repeated twice more using increasing amounts of blocking peptide up to 500 µg, to ensure validity of results.

2.2.4.12 Pre-incubation with immunizing peptide blocking test for ab28561 for specificity

PCP (59.503 µg/well) and rrIDE (25 ng/well) were diluted in MQ H₂O, prepared with sample buffer and reducing agent prior to SDS-PAGE and stored at -80°C until use. Rat liver (1 µL/well) was pre-homogenized, centrifuged for 10 mins at 12,000 RPM and the supernatant pre-prepared in tris-tricine sample buffer (1M Tris-HCl containing 2% (W/V) SDS, 4% (W/V) glycine, 0.05% (W/V) phenol red). Liver was then stored at -80°C until use. Zebrafish brains (25 ng/well) were pre-homogenized

in PBS containing a protease inhibitor cocktail (PI), and centrifuged at 6,000 RPM for 10 mins at 4°C. The supernatant was collected and the total protein content determined by a BCA Protein Assay.

Samples were all heated for 70°C for 10 mins, with the exception of rat liver which was heated at 95°C. Three lanes of each sample were loaded onto a single gel (NuPage® 4-12% Bis-Tris 1.5mm 15 well pre-cast) and resolved by SDS-PAGE for 2 hours at 100V. After semi-dry transfer as previously described, the membrane was cut into three identical pieces, each with one lane of molecular weight marker and one lane of each sample. The separate pieces were designated membrane A (control), B and C. All membranes were then immunoblotted with ab28561 as previously described, with the exception of a pre-incubation step prior to the primary antibody incubation. This step involved spiking the antibody solution (diluted 1:1000) with 0 µg (A), 5 µg (B) and 40 µg (C) of a commercially available peptide matching the immunogen to antibody ab28561. The three tubes were then incubated at RT for 30 mins prior to incubating the membranes. The remainder of the protocol was identical to previously, using separate containers to prevent cross-contamination.

2.2.4.13 Pre-clearing IgG from plasma

PCP was diluted 1:3 in PBS containing PI, and pre-cleared using the Pierce Direct IP kit, as per manufacturer instructions, to remove any immunoglobulins which may bind non-specifically to the beads.

2.2.4.14 Pre-clearing albumin from plasma

To clear some of the albumin from the PCP, the Pierce Direct IP kit was used as per manufacturer instructions. Briefly, 20 µL of a 50% agarose bead slurry was added to a spin column and equilibrated with a coupling buffer. 10 µg of purified anti-albumin antibody GTX27793 was then covalently bound to the beads for 2 hours. After washing with tris buffer, the pre-cleared (see above) PCP sample was diluted to a volume of 300 µL in PBS containing PI and added to the column. The column

was then sealed and incubated overnight on a rotating mixer at 4°C. The column was centrifuged and the flow-through collected.

2.2.4.15 Preparation of A β ₄₂

To prepare a stock solution of purified A β ₄₂ from lyophilized stock, 100 μ L of hexafluoroisopropanol (HFIP) was added per 1mg of lyophilized peptide into a tube, and allowed to sit open for 15 mins in the fumehood to assist with solubilization. After removing the HFIP by vacuum, and sitting for a further 10 mins, 1 mL of MQ H₂O was added to the tube, vortexed and allowed to sit a further 5 mins. The solution was then centrifuged for 10 mins at 10,000 RPM and the supernatant transferred into a low protein-binding tube. The concentration was subsequently determined using an Implen Nanophotometer at a wavelength of 214nm.

Purified A β was subsequently incubated with rhIDE (9ug A β to 100ng rhIDE) overnight at 4°C with end-over-end mixing, then resolved by SDS-PAGE and probed with an IDE antibody (LS-C55007 or ab28561) as previously described. This was to determine if IDE and A β denaturant-resistant binding occurs, leading to a shift in molecular weight. Purified A β was also used as an IDE inhibitor for the fluorometric activity assay as detailed in Section 2.2.6.3.

2.2.4.16 Immunoprecipitation of IDE from plasma

To assist with determining the band/s of interest on Western blot, PCP and pre-cleared/albumin-depleted PCP (AD-PCP), as well as rrIDE and rhIDE were used in an immunoprecipitation (IP) procedure for enrichment of the target protein (IDE). rhIDE was also spiked with A β ₄₂ and included in the immunoprecipitation experiment, to determine if IDE-A β binding was resistant to the immunoprecipitation method and subsequently denaturing and reducing conditions, leading to an increased molecular weight determined by Western blotting. All dilutions and washes were done using TBS containing a protease inhibitor cocktail (TBS/PI). IDE antibody 250706 or AF2496 was diluted 1:120 in TBS/PI (per tube) and bound to Protein G-coupled Magnetic Sepharose beads (20% slurry) as per manufacturer instructions, using the Classic Protocol. 4-8 μ L each of

PCP and AD-PCP, and 100 ng of rrIDE was diluted to 500 μ L with TBS/PI, and incubated in separate tubes with the antibody-bound beads for 1 hour with end-over-end mixing. The supernatant was then discarded and after washing the beads, the proteins were eluted from the beads by adding NuPage[®] LDS sample buffer and reducing agent, and then heating the tubes for 5 mins at 95°C. The supernatant was then separated from the beads and the samples stored at -80°C until resolved by SDS-PAGE.

2.2.4.17 Western Blotting image analysis and quantitation

Films were scanned using a Bio-Rad GS-800 Calibrated Densitometer, and Quantity One software (version 4.5.1). Images were acquired using high-resolution settings of 36.3 x 36.3 microns. Quantity One software was used to measure the density of the scanned IDE bands, and the data was then exported to Microsoft[®] Excel 2007 for normalization to adjust for gel to gel variation using the internal control sample included on every gel. Background density for each band was determined separately by measuring an area close to the band that did not contain any protein; and was then removed from the density of the band. Duplicates having a coefficient of variation of < 15% were omitted from the analysis. The data was analysed using IBM[®] Statistical Package for the Social Sciences (SPSS[®]) Version 19.0.0 (Section 2.2.6).

2.2.5 IDE levels in human plasma by enzyme-linked immunosorbent assay (ELISA)

All loading volumes are 100 μ L per well and all wells were loaded in duplicate unless otherwise stated.

2.2.5.1 Kamiya IDE ELISA commercial kit

To determine if the Kamiya IDE ELISA commercial kit would be suitable for measuring IDE in plasma samples, IDE standards were prepared as per manufacturer instructions to concentrations of 0.78 to 50 ng/mL, and human plasma samples were applied neat and diluted from 1:1 up to 1:1000 using the buffer supplied with the kit. The remainder of the protocol was performed as per manufacturer instructions and absorbance was measured using a Bio-Rad Fluostar Optima microplate reader at a wavelength of 450nm.

2.2.5.2 Indirect In-house ELISA for IDE

To test if various antibodies would be suitable for use in an indirect in-house ELISA, a screening test was performed using various commercially-available monoclonal (MMS-282R and 5282-1) and polyclonal (GTX111664, AB9210, 250706, H00003416-A01, LS-C55007 and ab28561) IDE antibodies (see Section 2.1.1 for antibody details). 96-well Nunc Maxisorp microplates (modules) were coated with purified rrIDE standards at concentrations of 78.12 to 5000 ng/mL, diluted in 50 mM carbonate/bicarbonate buffer (pH 8.6). PCP was coated neat (undiluted) and at several dilutions of 1:1 up to 1:20 in the same buffer. All samples were applied in duplicate. The plates were incubated O/N at 4°C with shaking. Following the O/N incubation, plates were washed with a microplate washer four times in PBS (pH 7.4) and then incubated in a PBS solution containing 2% bovine serum albumin (BSA) to block any unbound sites on the plate (200 μ L per well), for 1.5 hours at 4°C with shaking. No Tween-20 was included in the blocking buffer at this stage to avoid the detergent loosely binding to the plate and displacing the BSA, as this may lead to the Tween-20 being removed by subsequent washes with PBS containing 0.05% Tween-20 (PBST) and leaving spaces for non-specific binding of the antibodies. The

plates were then loaded with the primary antibody diluted 1:1000 in PBST containing 1% BSA (1% BSA/PBST), and incubated for either 2 hours at RT or 4°C O/N with shaking. Washing was then performed four times with PBST to remove any unbound antibodies. The HRP-conjugated anti-rabbit (AP322P), anti-goat (P0449) or anti-mouse (AP326P) secondary antibodies were then diluted 1:10000 in 1% BSA/PBST, loaded onto the respective plates, and incubated for 2 hours at 4°C with shaking. After washing another four times with PBST, 3,3',5,5'-tetramethylbenzidine (TMB) was prepared as per manufacturer instructions and applied to each well while protecting from light. The substrate was incubated for 30 minutes in darkness and the reaction stopped with the addition of 1M phosphoric acid (H₃PO₄). The absorbance was then quantified using a microplate reader at a wavelength of 450nm to determine optical density.

2.2.5.3 Sandwich ELISA for IDE

96-well Nunc Maxisorp microplates (modules) were coated with an inactive domain IDE antibody (ab28561) at 2.5 µg/mL diluted in 50 mM carbonate/bicarbonate buffer (pH 9.6, or 8.6 for some optimisations). The plates were then incubated O/N (18 hours) at 4°C with shaking. Following the O/N incubation, the plates were washed four times with PBS (pH 7.4) to remove unbound antibodies, on a microplate washer. After washing, wells were incubated (200 µL per well) with 3% BSA/PBS for 1 hour at RT with shaking, to block any unbound sites on the plate. No Tween-20 was included in the washing or blocking buffer prior to this stage to avoid the detergent loosely binding to the plate and displacing the BSA, as this may lead to the Tween-20 being removed by subsequent washes with PBST and leaving spaces for non-specific binding of the antibodies. IDE protein standards were then prepared from rrIDE, serially diluted to concentrations of 50 to 16000 ng/mL, and plasma samples were each diluted 1:2 in PBST (pH 7.4). For some optimisations 1% BSA was added to the diluent. An internal standard of human plasma (PCP) was also included (1:2). IDE standard and plasma samples were loaded in each well in duplicate, and the plates were incubated for 1 hour at RT with shaking. Following this incubation, the plates were further incubated O/N (18 hours) at 4°C with shaking. After the O/N incubation, any unbound protein was removed by washing

four times with a microplate washer. The detection antibody LS-C55007 (or H00003416-A01 for optimisation) was then diluted 1:750 in PBST, loaded into each well and the plates were incubated O/N at 4°C with shaking. Following the O/N incubation, plates were washed four times with PBST. The anti-goat (or anti-mouse for A01 antibody, AP326P) HRP-conjugated secondary antibody (P0449) was then diluted 1:7500 in PBST, loaded into each well, and plates were incubated for two hours at RT with shaking. The plates were then washed again four times in PBST, and TMB was loaded to each well, protected from light. The plates were then incubated in darkness for 15 – 25 minutes. The reaction was stopped by applying 1M H₃PO₄ to each well and the absorbance was measured at a wavelength of 450 nm on a microplate reader to determine optical density.

2.2.5.3.1 Preparation of human (HSA) and bovine (BSA) serum albumin

Lyophilized preparations of purified BSA, Fraction V BSA, and HSA were diluted to 60 and 30 mg/mL in PBST. Solutions were then diluted 1:2 as per plasma dilutions and assayed using the IDE sandwich ELISA protocol as detailed previously in section 2.2.5.3. The diluted solutions of purified BSA and HSA were also diluted as detailed in Section 2.2.6.7 and assayed using the IDE fluorometric activity assay.

2.2.5.4 Low-level data analysis

Blank (background) optical density values were subtracted from the sample optical density values obtained, and were normalised to the internal standard sample value included on each plate to control for plate-to-plate variation. Values less than the mean plus three times the standard deviation of the blanks were considered below the detection limit. Duplicates with a coefficient of variation (CV) >15% were omitted from the analysis; if PCP (internal standard) values exceeded this CV the whole plate was omitted.

2.2.6 Catalytic activity of plasma IDE by immunocapture-based IDE-specific fluorometric assay

The fluorometric assay used was adapted from Miners, *et al.*, 2008(b) and Wang, *et al.*, 2010. All well volumes are 100 μ L per well unless otherwise specified. All assays used PBST for dilutions and washing with the exception of the test between PBST and TBST where TBST was included as a sample buffer.

2.2.6.1 Immunocapture-based IDE specific fluorometric assay

96-well Nunc MaxiSorp™ Black Microplates (modules) were coated with an inactive domain IDE antibody ab28561 diluted at 2.5 μ g/mL in 50 mM carbonate buffer (pH 8.6), and incubated O/N at 4°C, with shaking. Following the O/N incubation, the plate was washed 4 times in PBST using a plate washer, then blocked for 30 mins at RT (with shaking) with a 2% BSA/PBST solution (or 3% ECL Advance Blocking Agent in PBST for one optimisation step). The blocking solution was not removed from the wells after incubation. IDE protein standards were prepared from rrIDE diluted in PBST, at concentrations from 312.5 to 7500 ng/mL (for the AIBL samples, otherwise as specified) and 50 μ L per well was added to the plates in duplicate. Plasma samples including an internal standard of PCP, were diluted in PBST to a concentration of approximately 19 μ g/ μ L total protein (for the AIBL samples, otherwise as specified), and 50 μ L per well was added to the plates in duplicate. The plates were then incubated O/N at 4°C with shaking. After the O/N incubation, plates were washed 4 times with PBST, and the internally-quenched fluorogenic peptide substrate (Mca-R-P-P-G-F-S-A-F-K(Dnp)-OH) was diluted in 50 mM HEPES to a concentration of 2.5 μ L/mL (10 μ M) and applied to each well while protecting from direct light. The fluorescence generated by the cleavage of the fluorogenic substrate was incubated and measured in a microplate reader (in darkness) with excitation at 320 nm and emission at 405 nm (at 31°C), immediately (baseline) and then hourly for 6 hours (for AIBL samples, otherwise as specified). The cut off point of 6 hours was used as maximum activity was reached after 5 hours in the original study (Miners, *et al.*, 2008(b)) and it was considered important to measure the highest reading possible within the linear range to maximise the chances of

detecting any differences between the groups. The gain adjustment setting for early optimisation experiments using Ab 3 (Batch 1) was variable (as specified), however for Batch 2 the gain adjustment was set to 1487 for all assays. The data values were interpolated from the standard curve of rrIDE as a measure of the equivalent amount of recombinant rat IDE (ng/mL).

2.2.6.2 Inhibition of catalytic activity of IDE by IDE-specific antibodies and A β ₄₀

To determine the specificity of the assay, four tubes containing PCP (1180 μ g total protein each) were diluted in PBST to a volume of 125 μ L, including no inhibitor (control), or either 20 μ L of IDE antibodies AB9210 (A), LS-C55007 (B), or 20.5 μ g of A β ₄₀ (C). Antibody AB9210 binds to residues 200-250 of IDE which contains part of the catalytic site, while LS-C55007 binds close to the C-terminus of IDE in a domain which is distinct from the catalytic site. A β ₄₀ was prepared as per A β ₄₂ as previously described in section 2.2.4.15. The four tubes were then pre-incubated for 2 hours at RT on a rolling mixer, prior to adding these samples to the plates. The assay was otherwise conducted as described above in section 2.2.6.1. Specificity was determined by comparing the fluorescence generated by PCP in the presence or absence of each inhibitor.

2.2.6.3 Inhibition of catalytic activity of IDE by A β ₄₂

To assist with the determination of the assay specificity, 50 μ L per well of 50 mM HEPES containing 0, 10 and 100 μ M A β ₄₂ (prepared as detailed in Section 2.2.4.15) was added to the immunocaptured IDE (from PCP), and incubated for 2 hours at RT, with shaking, prior to the addition of 50 μ L per well of 20 μ M fluorogenic substrate. The assay was otherwise conducted as described in Section 2.2.6.1. Specificity was determined by comparing the fluorescence generated by PCP in the presence or absence of each concentration of A β ₄₂.

2.2.6.4 Preparation of insulin

Lyophilized insulin was dissolved in 0.01 M hydrochloric acid (HCl, pH 2.0) to a concentration of 10 mg/mL and then sterile-filtered through a 0.2 µm pore size filter. The insulin solution was then diluted with HEPES (pH of approximately 8.0 to counteract low pH of HCl) to a concentration of 1 mM. Serial dilutions of insulin were then made to 0.5 and 0.25 mM in HEPES (pH 7.5).

2.2.6.5 Inhibition of catalytic activity of IDE by insulin

50 µL per well of 50 mM HEPES containing 0, 0.25, 0.5 and 1.0 mM of insulin was added to the immunocaptured IDE (from PCP), and incubated for 2 hours at RT, with shaking. 50 µL per well of 10 µM fluorogenic substrate diluted in HEPES was then added to each well, and the assay was otherwise conducted as described in section 2.2.6.1. Specificity was determined by comparing the fluorescence generated by PCP in the presence or absence of each concentration of insulin.

2.2.6.6 Determination of fluorometric activity assay cross-reactivity with human (HSA) and bovine (BSA) serum albumin

HSA and BSA (Sigma) was prepared as described in section 2.2.5.3.1 to concentrations of 60 and 30 mg/mL each, diluted in PBST. Each dilution was further diluted as per plasma samples (40 µl + 85 µL PBST), and then assayed in duplicate using the fluorometric activity assay protocol as detailed in Section 2.2.6.1. Cross-reactivity was determined to be fluorescence generated above the value of the (PBST only) blank. Results are shown in the Appendix.

2.2.6.7 Comparison of IDE activity from EDTA collected plasma and Lithium-Heparin (Li-Hep) collected plasma

EDTA plasma and Li-Hep plasma were diluted in PBST to contain 960 ug total protein per well (50 µL) and IDE activity was measured with the activity assay as detailed in Section 2.2.6.1.

2.2.6.8 Low-level data analysis

Duplicate values with a coefficient of variation (CV) of >15%, and assays where the standard curve values were > 2x standard deviation of the mean of each concentration were removed from the analysis. Values were then interpolated from the standard curve included on each plate.

2.2.7 Statistical Analysis

All data was analysed using IBM® Statistical Package for the Social Sciences (SPSS®) Version 19.0.0 (for Windows). Normality of the frequency distribution was determined using z-scores for skewness and kurtosis, the Kolmogorov-Smirnoff and Shapiro-Wilk tests, histograms, boxplots and normal Q-Q plots. Levene's test was used to determine homogeneity of variance.

Data was determined to have a non-normal distribution if z-scores for skewness and kurtosis were outside the range of ± 1.96 , and Kolmogorov-Smirnoff/Shapiro-Wilk tests produced a significance value of $p \leq 0.05$. Histograms, boxplots and normal Q-Q plots were also visualised for normal shape of the distribution and outliers. A significance of $p \geq 0.05$ for Levene's test was used to determine homogeneity of variances. Data found to be not normally distributed was subsequently transformed if possible.

Data that satisfied the normality criteria following transformation was then analysed with the parametric Student's T-test, one-way analysis of variance (ANOVA) or analysis of covariance (ANCOVA) using Tukey Post-Hoc analyses (independent samples, two-tailed analysis). Correlations were determined using Spearman's Rho, with Bonferroni-Holm correction applied to correct for multiple testing, which is a slightly less stringent correction method than the Bonferroni method (Holm, 1979).

Data that still displayed non-normal distribution after transformation was analysed with the non-parametric Mann-Whitney and Kruskal-Wallis tests. Mann-Whitney tests were used as Post-Hoc tests for the Kruskal-Wallis test with Bonferroni correction applied to correct for multiple testing. The Monte Carlo method was used to determine the estimate of significance which is more accurate than the Asymptotic Method when data is poorly distributed. Significance was considered to be $p \leq 0.05$. Correlations were determined using Spearman's Rho, with Bonferroni-Holm correction applied to correct for multiple testing.

(THIS PAGE HAS BEEN INTENTIONALLY LEFT BLANK)

CHAPTER 3 – Full-length plasma IDE Levels are higher in MCI in comparison to healthy controls or AD, as measured by Western Immunoblotting.

3.1 Introduction

Beta-amyloid (A β) is believed to be a key peptide involved in the pathogenesis of Alzheimer's disease (AD), and higher than normal levels of A β peptides in the brain lead to A β aggregation and neurotoxicity, and appear to instigate the development of extracellular senile plaques (SP) and subsequently the formation of intracellular neurofibrillary tangles (NFT) which are the primary lesions found in the brains of AD patients. It has been demonstrated that A β is produced within brain cells as well as within the cerebrovasculature (Reviewed by Wilson, *et al.*, 1999; Kalaria, *et al.*, 1996), and can be transported out into the periphery. However free A β can also be transported into the brain, across the blood-brain barrier from the peripheral circulation (Deane, *et al.*, 2003). It has been shown that plasma levels of A β increase in AD patients prior to the formation of SP, but decrease considerably as levels of brain A β increase and begin forming insoluble fibrillar deposits of amyloid (Kawarabayashi, *et al.*, 2001; Schupf, *et al.*, 2008). This suggests that peripheral A β may be a source for the A β that accumulates in the brain, and it is likely that regulation of A β levels in the periphery may be of importance in the pathogenesis of AD.

Insulin-degrading enzyme (IDE) has been demonstrated to be a primary metalloprotease responsible for the regulation of monomeric A β levels in the brain, but not oligomeric or fibrillar forms (Leissring, *et al.*, 2003; Farris, *et al.*, 2003). It has also been shown that overexpression of IDE not only reduces the soluble levels of brain A β , but also attenuates the formation of SP and prevents early death in transgenic mouse models. These results suggest that the reduction of monomeric A β levels is sufficient to prevent SP formation. In accordance with this, recent work has shown that the formation of A β fibrils is critically influenced by the oligomeric

state of soluble A β , as well as the balance of monomeric to aggregated forms of A β_{42} , revealing more about the role of A β_{42} in the amyloidogenic pathway (Jeong, *et al.*, 2013). It has also been shown that amyloid formation is reversible (Lazerov, *et al.*, 2002). It is possible then that reducing monomeric A β in the periphery by way of IDE clearance may have some impact on preventing cerebral accumulation of the peptide, as well as its oligomerisation and fibrillisation, and hence the development of AD. Therefore, the levels of IDE in human plasma could be integral in regulating not only plasma A β , but also A β in the brain. Furthermore, plasma IDE levels may also reflect cerebral levels of A β , and thus provide a useful biomarker for the assessment and monitoring of brain A β levels and/or early pathological processes that lead to neurodegeneration. Plasma IDE levels could also potentially be utilised as a biomarker in clinical trials for determining the efficacy of drugs, preventative interventions and other relevant treatments for AD.

It has been shown that truncated isoforms of IDE have little or no activity (Li, *et al.*, 2006), therefore the first aim of this project was to determine if full-length IDE was present in human plasma. Western immunoblotting was employed for this purpose, and a number of IDE antibodies were screened for suitability. Once the presence of full-length IDE was confirmed, the levels of plasma IDE in AD patients and MCI individuals were measured against the levels in healthy controls (HC), to see if they were altered in the prodromal period of AD (MCI) or in the disease state.

3.1.1 Aims

1. To use Western immunoblotting techniques to characterise commercial IDE antibodies and to determine whether full-length IDE is present in human plasma.
2. To measure levels of plasma IDE using a well-characterised Western immunoblotting technique to determine if plasma IDE levels can differentiate between classifications of AD, MCI and HC.
3. To determine if the presence of the *APOE4* allele has any impact on the levels of full-length plasma IDE.
4. To identify any relationships between full-length plasma IDE and other biochemical data obtained from the AIBL study.

3.2 Experimental Summary

3.2.1 Antibody characterisation: Pre-incubation of polyclonal IDE antibody Ab 1 with recombinant human IDE to check specificity for human plasma IDE.

Experiments were carried out to test if IDE antibody AB9210 (Ab 1(a) (Batch 1) – refer to Table 2.1 for details) detects full-length IDE in human plasma, and to determine if the bands seen are specific. SDS-PAGE and Western immunoblotting were performed as previously outlined in Sections 2.2.4.1 and 2.2.4.3, including a pre-incubation blocking step prior to the primary antibody incubation as outlined in Section 2.2.4.10.

3.2.2 Testing the batch-to-batch variability of IDE antibody Ab 1 on human plasma and rrIDE.

Experiments were carried out to test if a second batch of IDE antibody AB9210 (Ab 1(b)- refer to Table 2.1 for details) could detect full-length IDE in human plasma as efficiently as the previous batch (Ab 1(a)). Recombinant rat IDE (rrIDE) was used as a positive control, and SDS-PAGE and Western immunoblotting were performed as outlined in Sections 2.2.4.1 and 2.2.4.3.

3.2.3 Test of alternate polyclonal IDE antibody Ab 2: comparison with IDE antibody Ab 1.

SDS-PAGE and Western immunoblotting were performed as outlined in Sections 2.2.4.1, 2.2.4.2 and 2.2.4.3, to determine if an alternate IDE antibody LS-C55007 (Ab 2 – refer to table 2.1 for details) could detect full-length IDE in human plasma and rrIDE as efficiently as IDE antibody AB9210 (Ab 1(a) (Batch 1)). Subsequent experiments showed that an extended development time of one hour was required

to adequately visualise the band of interest in the region of 115-120 kDa, however background was also amplified. Consequently ECL Advance/Prime detection agents were used along with additional washing steps in order to increase the sensitivity of the method for the detection of full-length IDE in human plasma while minimizing the background generated.

3.2.4 Determination of the effect of EDTA in blood collection tubes on levels of human plasma IDE detected with IDE antibody Ab 2.

As EDTA is a chelating agent and IDE is a metalloprotease, an experiment was conducted to analyse whether the EDTA in the blood collection tubes has an effect on the amounts of full-length plasma IDE that are detected using IDE antibody LS-C55007 (Ab2). SDS-PAGE and Western immunoblotting were performed as outlined in Sections 2.2.4.1, 2.2.4.2 and 2.2.4.7.

3.2.5 Investigating potential cross-reactivity of IDE antibody Ab 2 with purified human and bovine albumin.

Human plasma contains diverse components including many proteins, and the most abundant of these is albumin. Cross-reactivity occurring between plasma albumin and a specific antibody of interest could therefore potentially mask the detection of other less abundant proteins by the antibody in question. In addition, bovine serum albumin is often used as a blocking agent in various immune-based protein detection methods, and sometimes cross-reacts with antibodies raised in goats due to species similarity. Therefore purified human (HSA) and bovine (BSA) serum albumin was analysed by SDS-PAGE and Western immunoblotting (as described in Sections 2.2.4.1, 2.2.4.2 and 2.2.4.8 with IDE antibody LS-C55007 (Ab 2) to determine if either of these albumins could be detected in the region of interest. The concentrations of albumin used reflected physiological levels found in plasma in normal human physiology. In addition, HSA was spiked with recombinant human

IDE to determine if any interactions between IDE and HSA occur under denaturing and reducing conditions.

3.2.6 Pre-incubation of IDE antibody Ab 2 with a synthesised immunogen peptide to assess specificity of antibody to IDE in human plasma.

To determine if the bands detected by IDE antibody LS-C55007 (Ab 2) are specific for IDE in human plasma and specific for rrIDE, SDS-PAGE and Western immunoblotting were performed as outlined in Sections 2.2.4.1 and 2.2.4.2. The experiment included a pre-incubation blocking step using a custom-synthesised peptide identical to the Ab 2 immunogen. This step was carried out prior to the primary antibody incubation, as outlined in Section 2.2.4.11.

3.2.7 Test of alternate polyclonal IDE antibody Ab 3: assessment of its detection of rrIDE as well as full-length IDE in human plasma.

To determine if an alternative polyclonal IDE antibody ab28561 (Ab 3 - refer to Table 2.1 for details) can detect full-length IDE in human plasma, as well as purified rrIDE used as a positive control, SDS-PAGE and Western immunoblotting were performed as outlined in Sections 2.2.4.1 and 2.2.4.4. Homogenised rat liver, human frontal cortex and purified recombinant human IDE were also included as further positive controls.

3.2.8 Determination of the effect of EDTA in blood collection tubes on the detection of human plasma IDE using IDE antibody Ab 3.

To determine the effect, if any, of EDTA's presence in human blood samples on the detection of full-length IDE by IDE antibody ab28561 (Ab 3), membranes previously

analysed by IDE antibody LS-C55007 were stripped of antibodies and re-probed (as described in Sections 2.2.4.7 and 2.2.4.5).

3.2.9 Testing IDE antibody Ab 3 for cross-reactivity with BSA, HSA and a non-albumin containing blocking agent.

As IDE antibody LS-C55007 was found to cross-react with albumin, an experiment was conducted to identify any cross-reactivity between polyclonal IDE antibody ab28561 (Ab 3) and purified BSA or an alternative, commonly used blocking agent which contains no albumin (ECL™ Advance Blocking Reagent). SDS-PAGE and Western immunoblotting were performed as described in Sections 2.2.4.1 and 2.2.4.4. The concentrations of albumin (or non-albumin blocking agent) used reflected physiological levels of albumin found in plasma in normal human physiology. The same experiment was subsequently repeated to include HSA and BSA, as well as rrIDE/rhIDE-spiked HSA as outlined in Sections 2.2.4.8 and 3.2.5.

3.2.10 Pre-clearing and albumin-removal from human plasma.

As immunoglobulins and albumin may be interfering factors in antibody-based detection and immunoprecipitation methods, it was considered that it would be beneficial to reduce the immunoglobulin and albumin content of human plasma. A pre-clearing step was performed as described in Section 2.2.4.13 to remove immunoglobulins that may non-specifically bind to the beads used in the immunoprecipitation protocols. The pre-cleared plasma was then also depleted of albumin content using the immunoaffinity-based protocol described in Section 2.2.4.14, using a monoclonal antibody to human albumin GTX27793 (AL-01).

3.2.11 Immunoprecipitation (IP) of IDE from human plasma with polyclonal IDE antibodies Ab 4 and Ab 5.

IDE immunoprecipitation experiments were carried out on untreated human plasma, and to investigate whether there are any non-specific contaminating factors, immunoprecipitation was also carried out on human plasma which had been pre-cleared of immunoglobulins and depleted of albumin prior to IP. RrIDE and rhIDE (AF2496 only) were immunoprecipitated to be included as positive controls. A sample of rhIDE pre-incubated with A β ₄₂ (A β ₄₂ preparation is detailed in Section 2.2.4.15) was also included in the IP experiment, to determine if IDE-A β interactions were resistant to the IP method as well as to the denaturing and reducing conditions of the subsequent Western immunoblotting step, leading to an increased molecular weight. The IP experiment was performed as described in Section 2.2.4.16, using polyclonal IDE antibodies 250706 (Ab 4) or AF2496 (Ab 5)(refer to Table 2.1 for details). The immunoprecipitated samples were then resolved by SDS-PAGE and Western immunoblotting with IDE antibody ab28561 (Ab 3) as described in Sections 2.2.4.1 and 2.2.4.4.

3.2.12 Testing the specificity of IDE antibody Ab 3 for IDE in human plasma by pre-incubation with a commercial blocking peptide.

To determine if the bands detected from human plasma samples by IDE antibody ab28561 (Ab 3) were specific, SDS-PAGE and Western immunoblotting were performed as outlined in Sections 2.2.4.1 and 2.2.4.4, including a pre-incubation blocking step with peptide, that was used as the immunogen, prior to the primary antibody incubation, as outlined in Section 2.2.4.12. rrIDE, rat liver and zebrafish brain homogenates were included as positive controls.

3.2.13 Determination of full-length IDE levels in the plasma samples of the AIBL cohort by SDS-PAGE and Western immunoblotting, using IDE antibody Ab 3.

Samples which had been collected using the methods described in Section 2.2.2 from the AIBL cohort were tested to measure full-length IDE levels. These investigations used SDS-PAGE and Western immunoblotting, and IDE antibody ab28561 (Ab 3), as outlined in Sections 2.2.4.1 and 2.2.4.4. An internal control sample of pooled control plasma (PCP) and a positive control of rrIDE were included on each gel. Ponceau S staining of the antibody stripped membrane prior to immunoblotting, was performed as previously described in Section 2.2.4.6, to ensure complete transfer of proteins as well as providing a gel loading control. Alternatively, blots were re-stripped following immunoblotting and Coomassie stained. Following ECL detection of bands, levels of full-length plasma IDE were quantified by scanning on a Bio-Rad densitometer GS-800 and images were analysed using Quantity-One software (V4.5.1).

3.3 Results

3.3.1 Bands detected by IDE antibody Ab 1(a) (Batch 1) in human plasma are IDE.

When using immunoaffinity methods such as Western immunoblotting, it is common to detect several bands, particularly when using polyclonal antibody sera which may detect multiple epitopes on the protein of interest, and which may also contain antibodies to other proteins. Although the bands detected may be specific and represent multiple isoforms of the protein, it is also common to have non-specific binding occur. Therefore it is important to identify which of the detected bands (if any) is the target protein.

One commonly used technique is to absorb antibody activity by pre-incubation with the immunising peptide, thus blocking the antibody binding sites (also called peptide competition assay); the blocked antibody is then used in immunoblotting. Briefly, the antibody is pre-incubated with an excess of the immunising peptide used to raise the antibody, or a peptide or protein that contains the matching epitope of interest, prior to adding to the membrane. The peptide binds to the antibody, effectively blocking it from binding to the target epitope. The resulting blot will show that the specific bands are significantly reduced in intensity or absent, while non-specific bands remain. While this technique is generally more effective on monoclonal antibodies, which usually recognise just one epitope on the protein of interest, it can also be useful for determining the specificity of polyclonal antibodies.

An immunising peptide blocking experiment was performed on Ab 1(a) (AB9210) to determine if the single band detected at 105 kDa in human plasma is IDE (Figure 3.1). This antibody was raised against a synthetic peptide of rat IDE spanning from amino acids 200 to 250. As this sequence shows 96.1% homology with the human IDE peptide it would be expected to detect IDE in human plasma. Results show that the band density was reduced by more than 50% after using full-length human IDE to neutralise the antibody. This indicates that the band seen is likely to be IDE,

despite a slightly lower estimated molecular weight when compared to most published studies. A limitation of this test was that it was only performed once and should have been repeated twice to ensure validity of the results. In addition, it would have been useful to include increasing doses of IDE to observe a dose-curve effect. However, as the subsequent batches of this antibody performed differently, it was decided not to continue with this antibody (discussed in Section 3.3.2) so further testing was considered unnecessary.

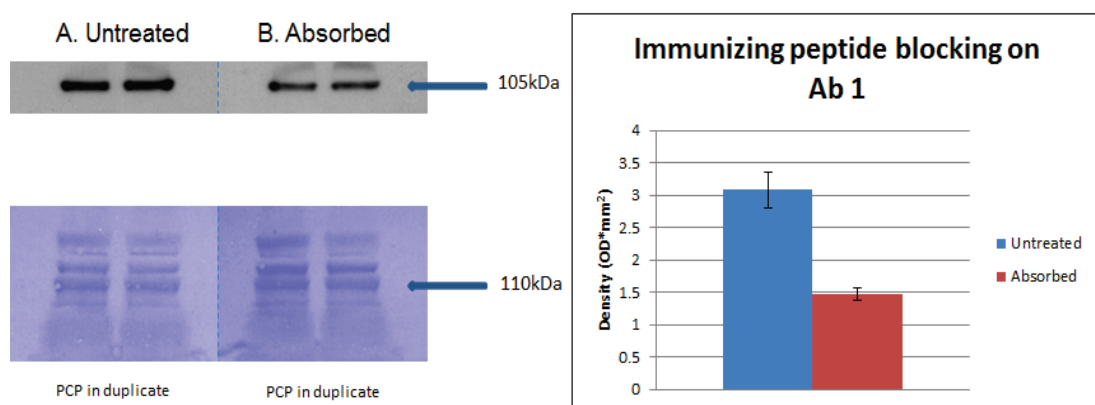


Figure 3.1 Immunising peptide (protein) blocking experiment on IDE antibody AB9210 (Ab 1(a)). Western immunoblot probed with polyclonal IDE antibody AB9210 (1:8000) after pre-incubating primary antibody solution without human IDE protein (membrane A.) or with 1.32 μ g recombinant human IDE as detailed in Sections 2.2.4.1 and 2.2.4.3 and 2.2.4.10. Sample is 1 μ L pooled control plasma (PCP) per lane in duplicate. Results show signal is reduced by approximately 50% in B. as compared with the control. Coomassie stain of membrane was included as a loading control. Error bars indicate \pm SD.

3.3.2 Subsequent batches of polyclonal IDE antibody Ab 1 are unable to detect full-length IDE in human plasma.

While monoclonal antibodies are known to be more specific than polyclonal antibodies, there are nevertheless certain advantages to using polyclonal antibodies. These include reduced cost, and they can also be more sensitive than monoclonals in detecting the protein. One drawback, however, is the possibility of batch-to-batch variation in polyclonal antibodies. It is important to compare each

new batch of polyclonal antibody with the previous batch to ensure consistency, and it is essential to use the same batch when conducting studies on a cohort where samples need to be compared to each other.

The initial batch of IDE antibody AB9210 (Ab 1(a) (Batch 1)) was compared with a subsequent batch (Ab 1(b) (Batch 2)) to determine if results were comparable (Figure 3.2). The results show that Batch 2 was unable to detect IDE in human plasma, even at double the original concentration used for Batch 1. It was concluded that Batch 2 was unsuitable for measuring IDE in human plasma. As supplies of Batch 1 were no longer available, an alternative antibody needed to be screened.

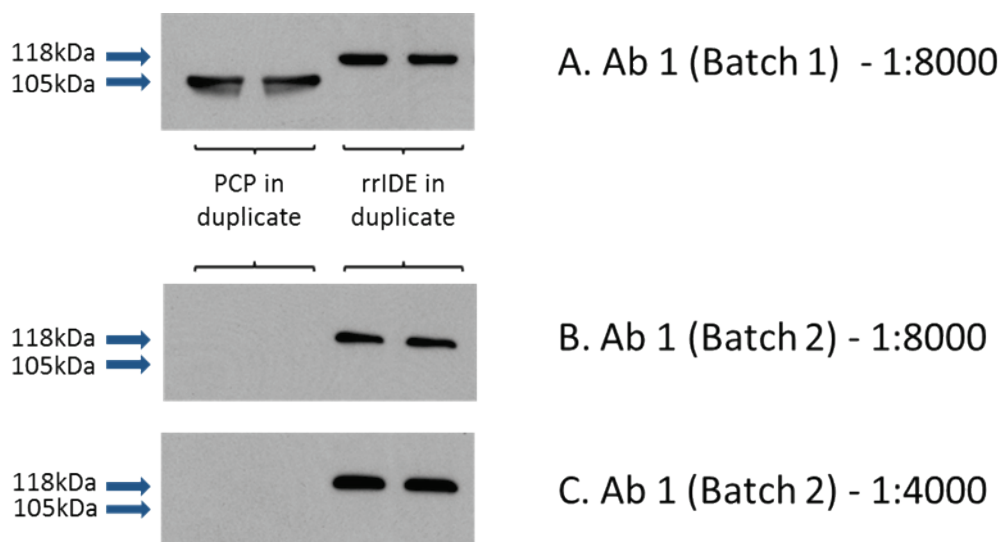


Figure 3.2 Comparison of original batch of IDE antibody AB9210 (Ab 1(a) (Batch 1)) with a subsequent batch of AB9210 (Ab 1(b) (Batch 2)) on human plasma and rrIDE. Western immunoblot of human plasma and rrIDE probed with IDE antibody Ab 1(a) (Batch 1) (membrane A.) compared with Ab 1(b) (Batch 2) (membranes B. and C.) as detailed in Sections 2.2.4.1 and 2.2.4.3. Samples are 1 μ L/lane of pooled control plasma (PCP) and 50 ng/lane rrIDE in duplicate. Results show that Ab 1(b) (Batch 2) is unable to detect IDE in human plasma at concentrations of 1:8000 or 1:4000. Ab 1(b) (Batch 2) is capable of detecting purified rrIDE, however appears to be less sensitive than Ab 1(a) (Batch 1). Once past the primary antibody step, the 3 blots were processed together.

3.3.3 Comparison of IDE antibody Ab 1 (AB9210, Batch 1) and IDE antibody Ab 2 (LS-C55007).

A comparison was made between Ab 1(a) (Batch 1) (AB9210 original batch) and another polyclonal IDE antibody Ab 2 (LS-C55007) (Figure 3.3(A) and (B)). Ab 2 was raised against a synthetic peptide of human IDE, spanning amino acids 914-924, targeting the C-terminal end of the IDE protein. The reported truncated isoform of IDE contains this region, therefore Ab 2 would be expected to detect both the truncated plus the full-length isoforms of IDE. The initial results show that at a concentration of 1:8000 for Ab 1(a) (Batch 1), and 1:5000 for Ab 2, both are able to detect rrIDE at 200-400 ng/well, however Ab 2 appears to have less affinity for recombinant rat IDE. This may be because Ab 2 was raised against a human IDE sequence, whereas Ab 1 was raised against a rat IDE sequence. However these antibodies are difficult to compare in this test. Firstly, Ab 1 is in the form of antiserum, therefore the exact concentration of IDE-specific immunoglobulins cannot be determined (total IgG is estimated to be between 1-2 mg/mL), whereas Ab 2 is purified and the concentration determined to be 0.5 mg/mL. In addition, these antibodies require different secondary antibodies (anti-Rabbit versus anti-Goat), and different concentrations were used [Ab 2 (1:7500), Ab 1 (1:5000)], these issues would also have contributed to the observed differences in signal. In human plasma, no bands were seen under the conditions used with Ab 2 in the range of 100-120 kDa, which is the expected molecular weight for full-length IDE. However a signal was detected at approximately 45 kDa which may correspond to the truncated form that has been reported.

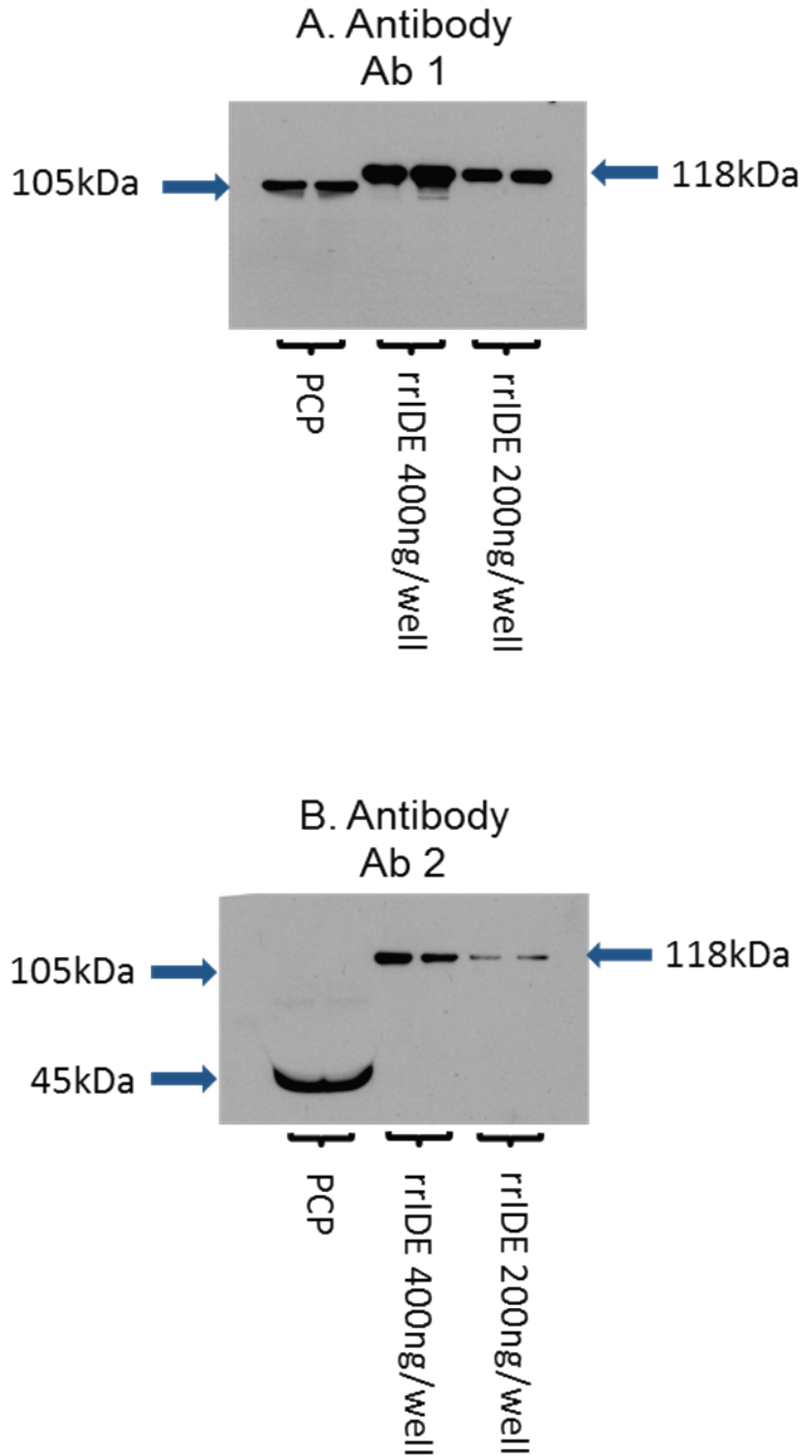


Figure 3.3 (A) and (B) Comparison of polyclonal IDE antibody AB9210 (Ab 1(a) (Batch 1)) with LS-C55007 (Ab 2) on human plasma and rrIDE. Western immunoblot of human plasma and rrIDE probed with IDE antibodies Ab 1(a) (Batch 1) (membrane A) compared with Ab 2 (membrane B) as detailed in Sections 2.2.4.1, 2.2.4.2 and 2.2.4.3. Samples are 1 μ L/lane of pooled control plasma (PCP) and 400 and 200 ng/lane of rrIDE in duplicate. Results show Ab 2 detects a band at approximately 45 kDa, but not within the range of full-length IDE (100-120 kDa) under these conditions.

The Western blotting methods were modified slightly to try to improve the detection of full-length IDE in human plasma by Ab 2. Three individual plasma samples (including PCP) and rrIDE were resolved by SDS-PAGE and probed with Ab 2, using an extended exposure time when developing the film (Figure 3.4). Results show that after a one hour exposure, additional bands to the 45 kDa bands previously demonstrated appeared at approximately 160 (ghost bands), 115-120, 80 and 60 kDa. The band at 115-120 kDa corresponded to various previous reports of the molecular weight of IDE, however differed from the molecular weight of the band seen using Ab 1 (105 kDa). Cross-reactivity with the molecular weight marker (MWM) proteins at 80 and 60 kDa demonstrates some non-specific binding.

To reduce the exposure time required and to retain sufficient visualisation of the bands within the region of interest (specifically the 115-120 kDa band), ECL™ Advance Detection Reagent was tested in this application (Figure 3.5) with five individual plasma samples including PCP, and rrIDE (previously ECL™ Detection Reagent was used). ECL™ Advance is reported to be a more sensitive chemiluminescent reagent than ECL™, increasing the sensitivity of detection by up to ten times compared to standard ECL™. Results show that ECL™ Advance did improve the detection of bands at 115-120, 80, 70 and 45 kDa, although the background was also significantly enhanced. The previously observed “ghost” bands at 160 kDa (Figure 3.4) were not apparent.

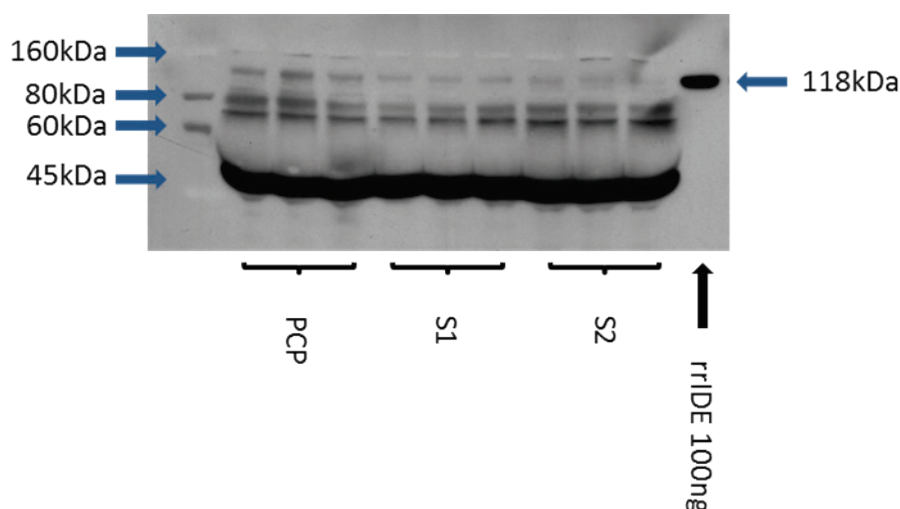


Figure 3.4 Investigating the effect of extended ECL development time on the detection of full-length human plasma IDE with Ab 2. Pooled control plasma (PCP), and two individual plasma samples (S1 and S2) were run in triplicate (1 μ l/lane) as detailed in Sections 2.2.4.1 and 2.2.4.2. The rrIDE sample contained 100 ng. After transfer, the immunoblot was probed with primary antibody Ab 2 (LS-C55007) and developed for 1 hr. Results show that multiple bands are detected in plasma after exposing for one hr, at approximate molecular weights of 160, 115-120 (close to expected size), 80, and 70 kDa, and strong bands at approximately 45 kDa. Cross-reactivity is apparent with the MWM at 80 and 60 kDa. Differences in intensity of the plasma bands between individual samples at 115-120 kDa are apparent, although intra-sample variation is also apparent.

Differences in intensity of the 115-120 kDa band were apparent between individual samples. The development time was reduced from 60 min with standard ECL™ to 1 min using ECL™ Advance. It should be noted that the last lane was markedly lighter than its duplicate; this was unlikely to be due to imprecise pipetting, as this phenomenon was observed consistently. As it was only a positive control loaded in this lane in all gels, and not used for quantification, it was not considered to be an issue in this instance.

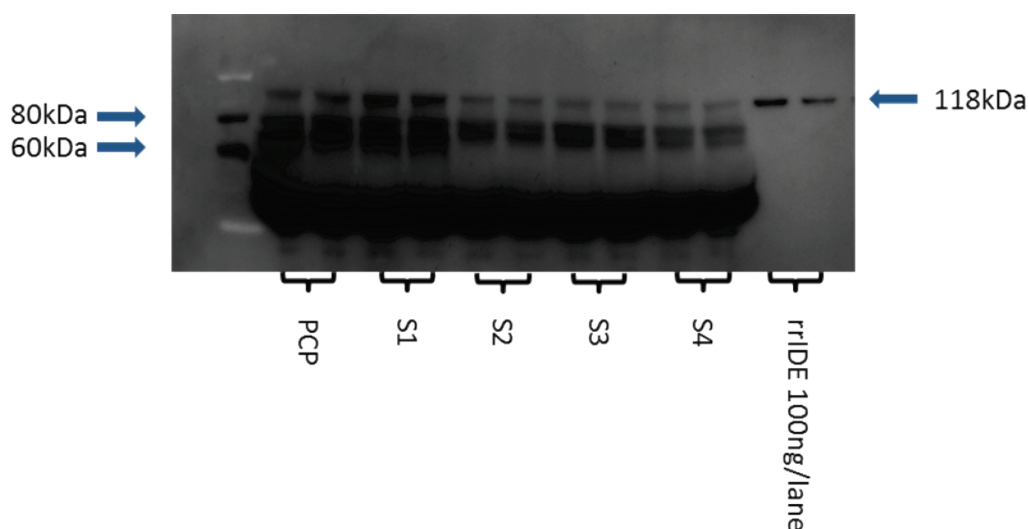


Figure 3.5 Plasma samples probed with Ab 2 (LS-C55007), using rrIDE as positive control, and developed using ECL™ Advance. Pooled plasma (PCP), and four individual plasma samples (S1, S2, S3 and S4) all at 1 μ L plasma per lane, were run in duplicate as detailed in Sections 2.2.4.1 and 2.2.4.2. The rrIDE sample contains 100 ng. Results show that multiple bands are detected in the plasma samples after exposing for 1 min, at approximate molecular weights of 115-120 (close to expected), 80, and 70 kDa, and strong bands at approximately 45kDa. Cross-reactivity is apparent with the MWM at 80 and 60kDa. Differences in intensity of the plasma bands between individual samples at 115-120 kDa are apparent. The background is considerably darker with this developer.

3.3.4 EDTA has no effect on the intensity of the 115-120 kDa band detected with IDE Ab 2.

EDTA is a common anticoagulant used in blood collection tubes to prevent the clotting cascade, when separating plasma out of whole blood. Final concentrations of EDTA in blood after collection range from approximately 1.2 to 2.0 mg/mL, and as EDTA is water-soluble, it is most likely that a majority of this would remain in the plasma following red blood cell removal. EDTA functions as a chelating agent, sequestering metal ions from proteins, and this may have an effect on the antibody-antigen interaction in immunoblotting techniques when detecting metal-

dependent enzymes such as IDE. To determine whether IDE detection is influenced by EDTA in the blood samples, plasma which was collected in non-EDTA collection tubes (using Lithium-heparin as anticoagulant) was spiked with EDTA to give final concentrations from 0 to 2.0 mg/mL. SDS-PAGE and Western immunoblotting were then performed on these samples with IDE Ab 2 (Figure 3.6). Results show there was no difference in the levels of IDE detected when comparing the control plasma (0 mg/mL of EDTA) with the plasma samples containing concentrations of EDTA up to 2.0 mg/mL.

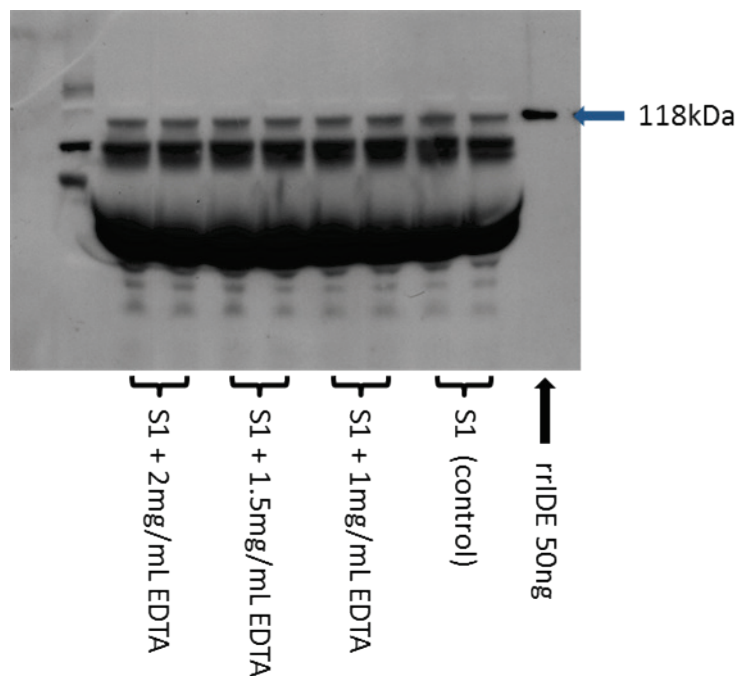


Figure 3.6: Investigating the effect of EDTA on IDE detection. Western blot of an individual plasma sample collected in lithium-heparin collection tube subsequently spiked with EDTA to final concentrations of 1.0, 1.5 and 2.0 mg/mL as detailed in Sections 2.2.4.7, 2.2.4.1 and 2.2.4.2. The blot contained rrIDE as a positive control, and was probed with IDE Ab 2 (LS-C55007). Duplicate aliquots of plasma sample S1 (1 μ L/lane) were loaded. The rrIDE lane contained 50 ng protein. Results show no significant differences between IDE band intensities for EDTA concentrations from 0 to 2.0 mg/mL.

3.3.5 IDE Ab 2 cross-reacts with HSA strongly, and BSA weakly, in Western immunoblotting.

Albumin is the most abundant plasma protein and therefore cross-reactivity between albumin and a detecting antibody may potentially mask true levels of a less abundant protein. The plasma levels of albumin in the AIBL cohort range from approximately 30 to 60 mg/mL (which would equate to 30-60 μ g/lane in the 1 μ l plasma aliquots/lane). Therefore Western immunoblotting experiments were carried out to determine whether IDE Ab 2 (LS-C55007) cross-reacts with purified bovine (BSA) and/or human (HSA) serum albumin at concentrations within the physiological range in AIBL participants (Figure 3.7). HSA was also spiked with purified recombinant human IDE (rhIDE) to determine if any interaction occurs between the proteins that would produce additional bands in Western immunoblotting. The results show that Ab 2 cross-reacted with purified HSA strongly at both 30 and 60 μ g/lane, with two close bands detected at approximately 45 kDa. Very slight cross-reactivity was also observed between Ab 2 and BSA at both concentrations. The bands observed did not appear to be dose-responsive for either HSA or BSA, although a lower exposure time may have shown band intensity differences. No other bands were observed when HSA was spiked with rhIDE. It was concluded that the cross-reactivity between Ab 2 and albumin was of little significance in the Western immunoblotting method as no bands were seen in the region of interest (100-120 kDa). Interestingly the molecular weight of human albumin is 66 kDa and no bands were observed in this region.

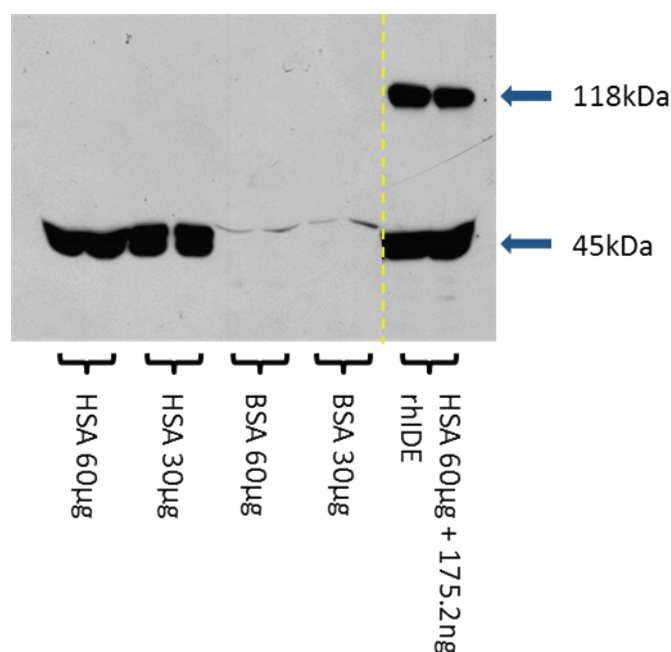


Figure 3.7 Test of cross-reactivity between Ab 2 (LS-C55007) and HSA or BSA. Purified human serum albumin (HSA), bovine serum albumin (BSA), and HSA spiked with recombinant human IDE (rhIDE) were probed with IDE Ab 2 as detailed in Sections 2.2.4.8, 2.2.4.1 and 2.2.4.2. HSA and BSA lanes contain 60 and 30 µg protein per lane in duplicate. HSA + rhIDE contains 30 µg HSA and 175.2 ng rhIDE in duplicate. Results show that the antibody cross-reacts with HSA, with strong bands appearing at approximately 45-50 kDa. Detection appears to be non-dose responsive. BSA also appeared to be detected weakly at approximately 50kDa, also in a non-dose responsive fashion.

3.3.6 Testing the specificity of IDE Ab 2 by pre-incubation blocking of the antibody with the immunising peptide.

To determine which of the bands (if any) observed in human plasma by Western immunoblotting with Ab 2 are IDE, a pre-incubation step with the immunising peptide was performed on Ab 2 (refer to Section 3.3.1 for overview). Such a pre-incubation should block the antibody binding sites. The peptide was custom-synthesised to be identical to the immunogen used to raise Ab 2. The test was carried out in 3 slightly different ways, to ensure accuracy of the results (Figures 3.8(A)(B)(C)). Results showed that the 118 kDa bands observed in rrIDE and rhIDE

lanes were absent after pre-incubating Ab 2 with the blocking peptide, however no differences were seen between any of the bands from human plasma in the molecular weight region of interest. The results indicate that Ab 2 is specifically detecting the purified recombinant IDE, however also indicate that the bands seen in human plasma are most likely non-specific.

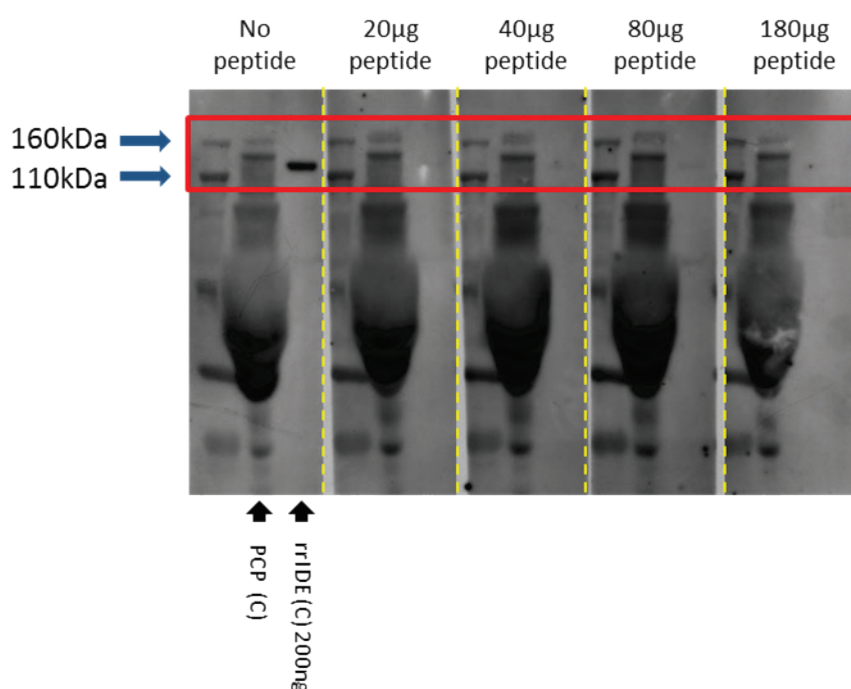


Figure 3.8(A) Pre-incubation of IDE Ab 2(LS-C55007) with the immunising peptide to test antibody specificity (Test 1). Pooled control plasma (PCP) and recombinant rat IDE (rrIDE - positive control) were probed with IDE Ab 2, some antibody aliquots of Ab 2 were blocked (prior to adding to immunoblots) with a custom-made peptide identical to the immunogen (20, 40, 80 and 180 µg peptide/blot) as detailed in Sections 2.2.4.11, 2.2.4.1 and 2.2.4.2. PCP lanes contain 1 µL plasma per lane. rrIDE lanes contain 200 ng per lane. Results show rrIDE bands (118 kDa) absent at all concentrations of blocking peptide, however no differences can be seen in PCP lanes in the region of interest.

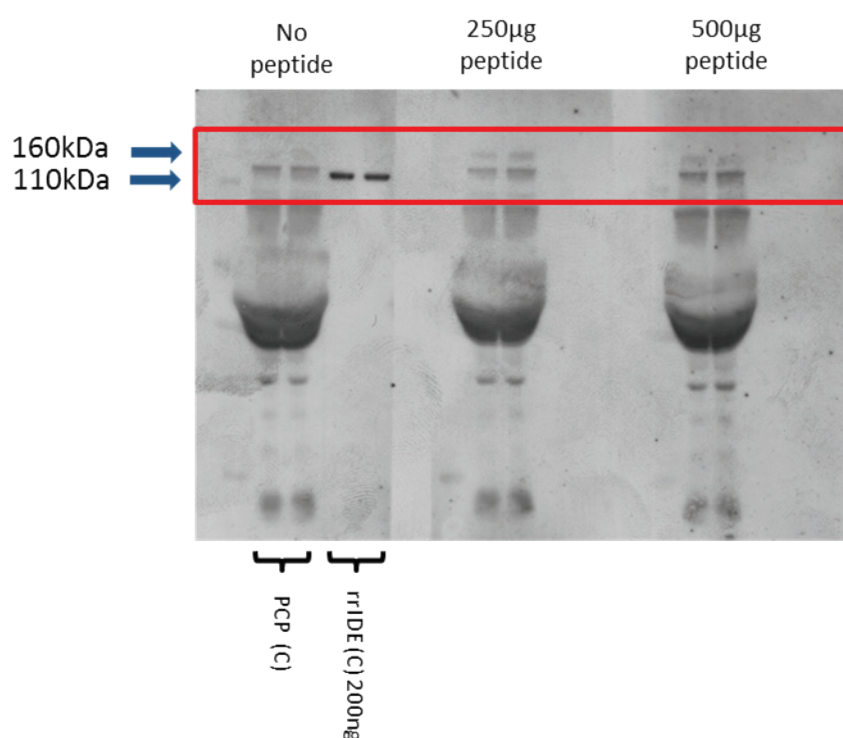


Figure 3.8(B) Pre-incubation of IDE Ab 2(LS-C55007) with the immunising peptide to test antibody specificity (Test 2). Pooled control plasma (PCP) and recombinant rat IDE (rrIDE - positive control) were probed with IDE Ab 2, which had been neutralised/blocked prior to incubation with the custom peptide identical to the immunogen (250 and 500 µg peptide/blot - higher amount/sample than in previous experiment) as detailed in Sections 2.2.4.11, 2.2.4.1 and 2.2.4.2. PCP lanes contain 1 µL plasma per lane in duplicate. rrIDE lanes contain 200 ng per lane in duplicate. Results show that the rrIDE bands (118 kDa) are absent at all concentrations of blocking peptide, however no difference can be seen in PCP bands.

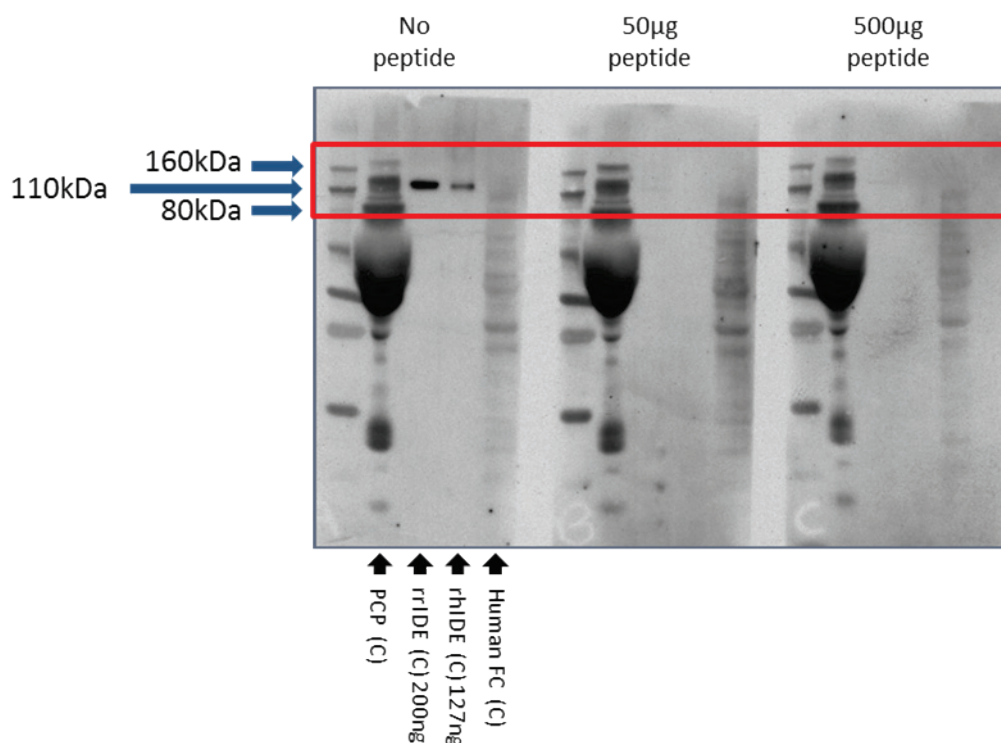


Figure 3.8(C) Pre-incubation of IDE Ab 2 (LS-C55007) with the immunising peptide to test antibody specificity (Test 3). Pooled control plasma (PCP), recombinant rat IDE (rrIDE), recombinant human IDE (rhIDE) and human brain frontal cortex (FC) were probed with IDE Ab 2, with some antibody samples having been blocked prior to incubation with a custom peptide identical to the immunogen (50 and 500 µg peptide/blot) as detailed in Sections 2.2.4.11, 2.2.4.1 and 2.2.4.2. PCP lanes contain 1 µL plasma/lane, rrIDE lanes contain 200 ng/lane, rhIDE lanes contain 127 ng/lane and FC lanes contain 1µL brain homogenate/lane (15.2 µg total protein). Results show rrIDE and rhIDE bands (118 kDa) are absent at all concentrations of blocking peptide, however no difference can be seen in the PCP or FC bands. The immunoblotting results had been transformed with Quantity One software to aid visualisation of bands.

3.3.7 Test of IDE antibody ab28561 (Ab 3).

An alternate IDE antibody (ab28561), which binds to an epitope encompassing an area that is not part of the active site of IDE, was tested for its ability to detect plasma IDE by Western immunoblotting (Figure 3.9). The antibody (designated Ab 3) was tested using both ECL™ (standard) and ECL™ Prime Detection Reagents. ECL™ Prime is a replacement product for ECL™ Advance (now discontinued),

therefore has greater sensitivity than standard ECL™. Samples of rrIDE, rhIDE, rat liver homogenate and human frontal cortex (FC) were also included on the Western blots. The results showed that Ab 3 can detect rrIDE and rhIDE as a single band at 118 kDa, similar to Ab 1 and 2. Using standard ECL™, several bands were detected at approximately 50, 70, 80 and 120-130 kDa in human plasma, as well as a single band at 110 kDa in rat liver and single bands for recombinant IDE (rat and human – rrIDE/rhIDE, respectively). No bands were detected in the FC sample using standard ECL™ under these conditions. When ECL™ Prime was used as the detection reagent, multiple other bands became visible in all samples, including the rrIDE, rhIDE and FC. In plasma, an additional band at approximately 160 kDa was observed. These results indicated that Ab 3 may be suitable for detecting IDE in human plasma, however specificity needed to be checked.

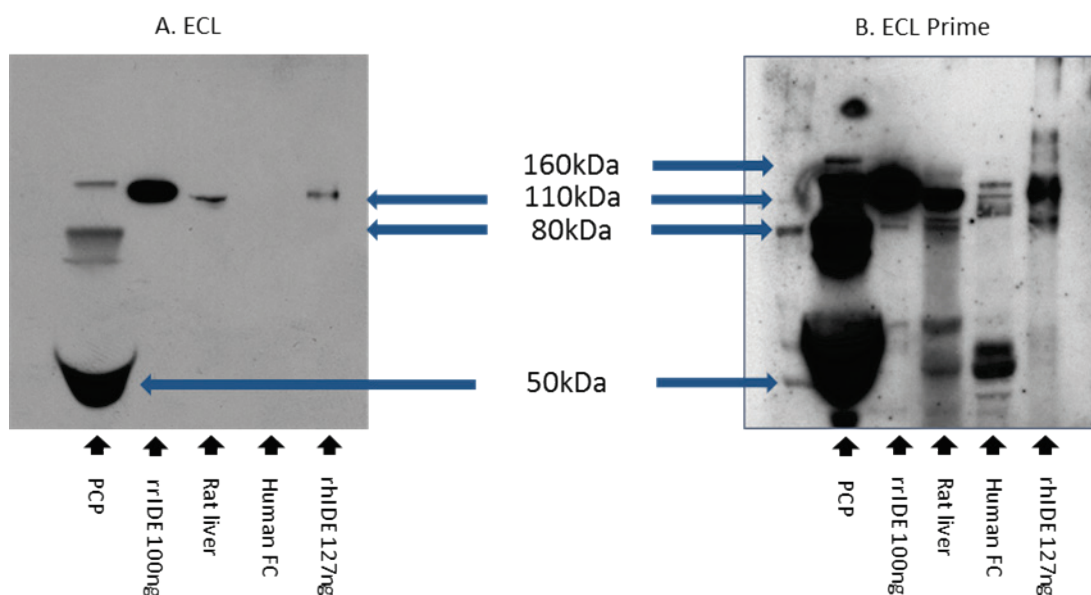


Figure 3.9 Test of Ab 3 (ab28561) on human plasma, rat liver, human frontal cortex and recombinant IDE (rat and human). Pooled plasma (PCP), recombinant rat IDE (rrIDE), rat liver homogenate, human brain frontal cortex (FC and recombinant human IDE (rhIDE) were probed with IDE Ab 3 (ab28561), and developed with either ECL (membrane A) or ECL Prime (membrane B) as detailed in Sections 2.2.4.1 and 2.2.4.4. PCP lanes contain 1 μ L plasma per lane. The rrIDE samples contained 100 ng per lane, rat liver samples: 47.4 μ g total protein per lane, human FC samples: 15.2 μ g total protein per lane and rhIDE samples: 127 ng per lane. Single bands were observed for rrIDE, rat liver and rhIDE at 110-118 kDa using ECL Normal. For PCP, bands appear at approximately 120-130, 80, 70 and 50 kDa. No band was apparent for human FC (Membrane A). With ECL Prime, an extra band was seen in the PCP at just over 160 kDa and several extra bands in rat liver (two at around 80 kDa, one at 55-60 kDa and one at 50 kDa). For human FC, three bands were detected using ECL Prime in the region of interest (approximately 125, 115 and 100 kDa) as well as two strong bands at approximately 55 and 50 kDa. Membrane B has been transformed with Quantity One software to aid visualisation of bands.

3.3.8 EDTA has no effect on the intensity of the 120-130 kDa band detected with IDE Ab 3 (ab28561).

As previously described in Section 3.3.4, Ab 3 was tested to determine if the EDTA in the plasma samples influences the binding of Ab 3 to IDE. The immunoblot which had been used for this purpose to test IDE Ab 2 was stripped of antibodies, and re-probed using Ab 3 (Figure 3.10). Results showed that there was no difference in protein band intensities between the control plasma sample and the samples containing EDTA up to 2.0 mg/mL.

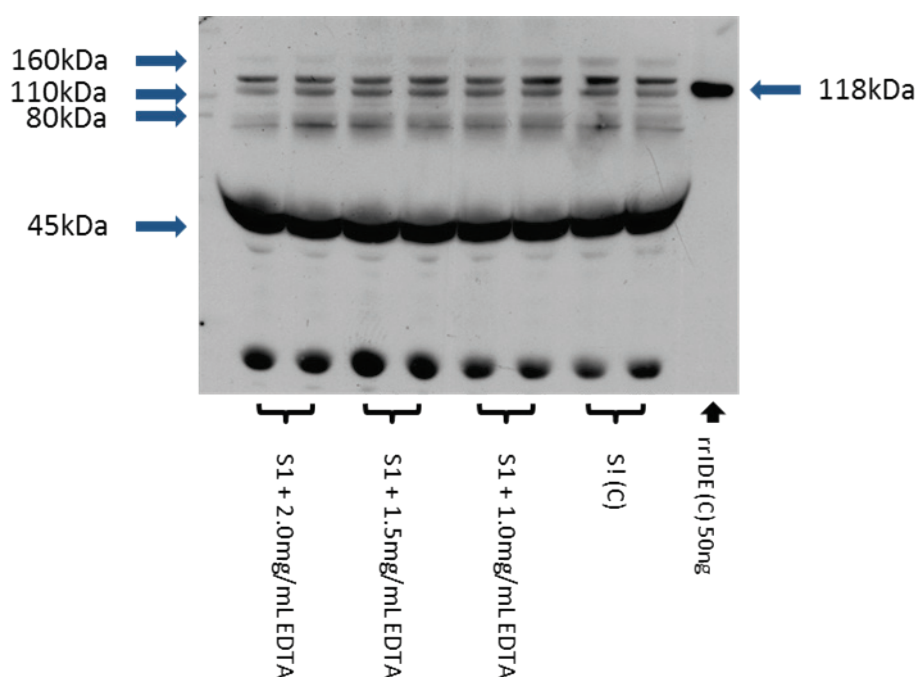


Figure 3.10 Investigating whether EDTA in blood samples interferes with Ab 3 (Ab28561) detection of putative IDE protein bands. The Western blot run for the purpose of investigating Ab 2 in the same manner (see Fig. 3.6) was stripped and re-probed with IDE antibody Ab 3 (ab28561) as detailed in Sections 2.2.4.5 and 2.2.4.4. Plasma sample (S1) contains 1 μ L plasma/lane in duplicate. The rrIDE lane contains 50ng protein. The plasma samples had been spiked with EDTA to 1.0, 1.5 and 2.0mg/mL. Results show no significant difference between band intensities of control plasma samples and samples spiked with EDTA, when detected with Ab 3.

3.3.9 Ab 3 (ab28561) cross-reacts with BSA, HSA and a non-albumin containing blocking agent.

As both monoclonal and polyclonal IDE antibodies have previously been reported to cross-react with other proteins (Yfanti, *et al.*, 2008), and results on Ab 2 (LS-C55007) from the current project show cross-reactivity with HSA and BSA (refer to Section 3.3.5), Ab 3 (ab28561) was also tested (Figure 3.11(A)). The amounts loaded onto the gel were 30 µg and 60 µg which reflected physiological albumin levels found in the plasma volumes normally loaded onto the gels (1 µL/lane). The results showed that Ab 3 strongly cross-reacts with BSA in a dose-dependent manner, with bands visible at 40, 38 and 20 kDa in the 60 µg lane, and only the 40 and 20 kDa bands visible in the 30 µg BSA lane. With HSA, weak signals were detected at ~ 50 and 36 kDa at 60 µg HSA, but not at 36 kDa in the 30 µg HSA lane. In addition, no extra bands were observed when 60 µg HSA was spiked with either rrIDE or rhIDE. It should be noted that the quantity of rhIDE used was approximately 20-fold the rrIDE quantity; as the rhIDE was past its expiry date, it was used in excess in case there had been significant protein degradation. As this was not a test requiring quantification of the IDE, this excessive use of rhIDE was determined to be of no consequence.

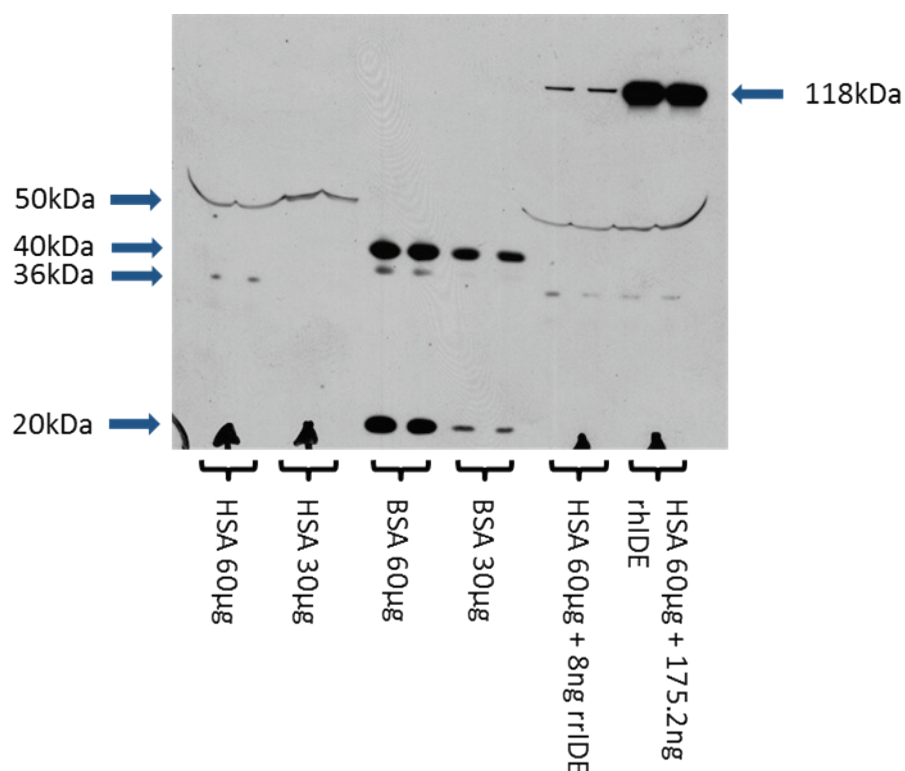


Figure 3.11(A) Test of cross-reactivity of IDE Ab 3 with HSA and BSA. Purified HSA, BSA, and HSA spiked with recombinant rat (rrIDE) or human IDE (rhIDE) were resolved by SDS-PAGE and probed with primary antibody (Ab 3) ab28561 as detailed in Sections 2.2.4.8, 2.2.4.1 and 2.2.4.4. HSA and BSA lanes contained 30 or 60 µg protein, in duplicate. HSA + rrIDE, or rhIDE, contained 60 µg HSA + 8 ng rrIDE, or 175.2ng rhIDE, respectively, in duplicate. Results show that the antibody cross-reacts with BSA at levels that would be found in plasma volumes frequently loaded onto gels, with strong bands appearing at ~ 40 and 20kDa and light bands at ~ 38kDa only in 60µg lanes. Signals appear to be dose responsive. HSA also appeared to be detected weakly at ~ 50 and 36kDa.

To investigate further whether IDE Ab 3 may cross-react with other blocking agents which did not contain albumin, ECL™ Advance Blocking Reagent was tested. Due to proprietary restrictions, it was not possible to determine the components present in the ECL™ blocking agent or their respective physiological concentrations; however it was stated by the manufacturer that the preparation should not contain albumin or globulin. The preparation was primarily produced from bovine milk powder and therefore would be expected to contain milk proteins such as casein

and other whey proteins. The ECL™ blocking agent was tested for Ab 3 cross-reactivity at concentrations up to 60 mg/mL (Figure 3.11(B)). The results show that Ab 3 can cross-react with ECL™ blocking agent at 30-60 mg/mL, with bands at approximately 55 kDa, however no bands were visible at 15 mg/mL. The signal detected was dose-responsive.

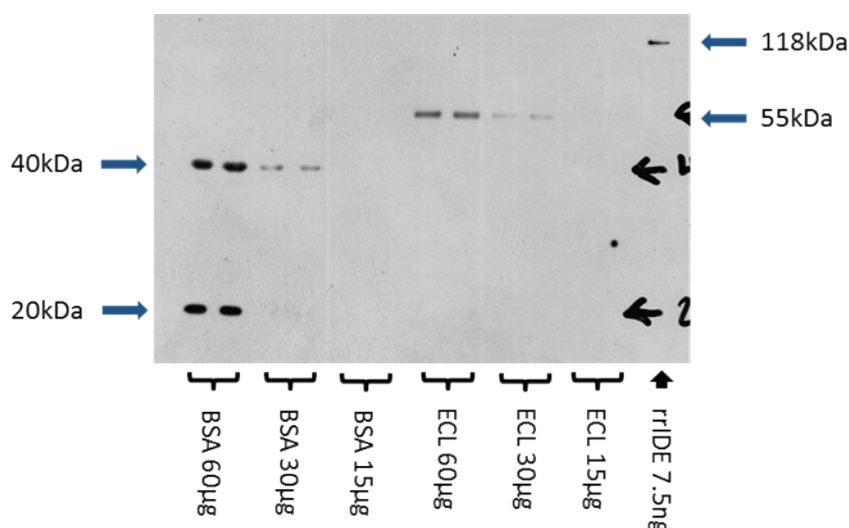


Figure 3.11(B) Test of cross-reactivity of IDE antibody Ab 3 with BSA and ECL™ Advance Blocking Reagent. BSA and ECL lanes contain 15, 30 and 60 µg protein each in duplicate. The recombinant rat IDE (rrIDE) lane contains 7.5 ng. Experiment was performed as detailed in Sections 2.2.4.9, 2.2.4.1 and 2.2.4.4. Results show that the antibody cross-reacts with BSA at levels equivalent to albumin in the physiological range of human plasma, with bands at 40kDa (30-60 µg) and 20kDa (60 µg). The signal appears to be dose-responsive. Similarly, the ECL block is also cross-reactive with bands appearing at 55kDa (for 30-60 µg). No signal was detected for either BSA or ECL at 15 µg at this exposure.

As there was no signal present in the expected 100-130 kDa range for HSA, BSA or ECL™ Blocking Reagent, it was concluded that Ab 3 may still be suitable for the measurement of plasma IDE by Western immunoblotting. However, the cross-reactivity observed may have implications for the detection of IDE in other immunoaffinity-based applications.

3.3.10 Immunoprecipitation of IDE from human plasma.

As IDE Ab 3 (ab28561) binds non-specifically with other proteins in human plasma, it is likely that one or more of the bands seen in Western immunoblotting are non-specific. To reduce the number of confounding factors in the sample, and to improve determination of the bands of interest, an immunoprecipitation (IP) method was trialled using Protein G-coupled magnetic sepharose beads, with yet another polyclonal IDE antibody (Figure 3.12(A)). The IP antibody was raised in mouse against a synthetic peptide derived from the N-terminus of human IDE, and designated Ab 4 (250706). As such, Ab 4 would not be expected to capture the truncated isoform of IDE as it does not contain the N-terminal half. The immunoprecipitated samples were resolved by SDS-PAGE and detected on Western blots using Ab 3 (ab28561). Results showed that the recovery of purified rrIDE was poor (under the conditions employed) with only approximately 14% of the protein recovered after immunoprecipitation with this protocol and antibody. Numerous bands were still observed after both albumin depletion (including pre-clearing of immunoglobulins) and immunoprecipitation of plasma. However, in the region of interest (approximately 100-130 kDa) only one band was clearly visible after either albumin depletion and/or immunoprecipitation, although this band was less intense than in the plasma control which had only 25% of the plasma content used in the IP. This band was determined to be approximately 120-130 kDa. In addition, the band at 160 kDa in plasma was not observed after IP, and the 70 kDa band was significantly reduced. The 80 kDa band observed in plasma however, was still present after albumin depletion and/or IP. It was concluded that the 160 and 70 kDa bands were non-specific, whereas the 120-130 kDa band appears to be specific. Interestingly, the 80 kDa band also appears to be specific, however analysis of this band is outside the scope of this project. Nevertheless, it may be worth characterising this 80 kDa band in future studies.

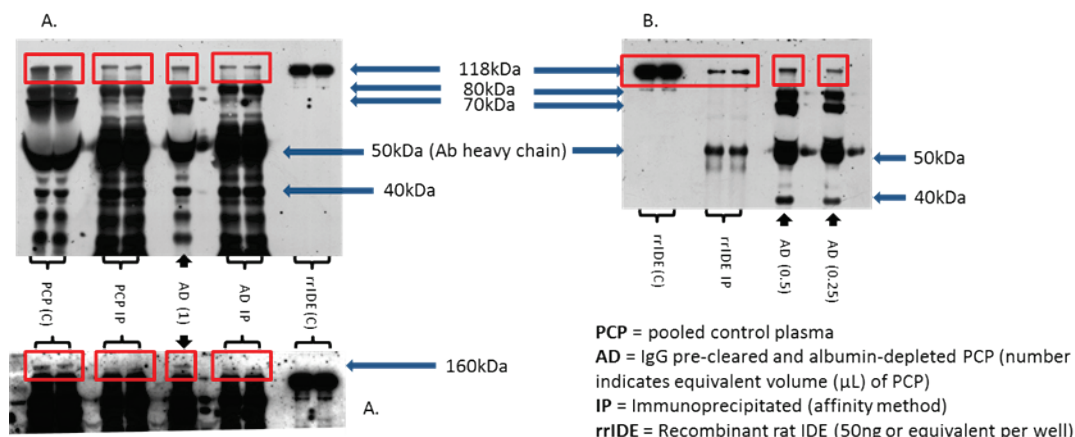


Figure 3.12(A) Immunoprecipitation of IDE from human plasma using IDE mouse polyclonal Ab 4 (250706). IDE was immunoprecipitated from pooled plasma (PCP), albumin-depleted PCP (AD) and recombinant rat IDE (rrIDE) samples, using IDE Ab 4 (250706) and probed with IDE Ab 3 (ab28561) as detailed in Sections 2.2.4.16, 2.2.4.1 and 2.2.4.4. PCP was pre-cleared of IgG, and albumin-depleted with antibody AL-01, prior to IP (see Sections 2.2.4.13 and 2.2.4.14). PCP lanes contain 1 μ L/lane in duplicate. IP from PCP used 4 μ L of plasma/sample (per lane), run in duplicate, and rrIDE lanes contain 50ng or equivalent/lane in duplicate. AD lanes contain 1, 0.5, and 0.25 μ L plasma/lane as indicated. An extra band appears at just over 50kDa in the rrIDE IP, suggesting cross-reactivity of Ab 3 or the secondary antibody to the heavy chain of the eluted IP antibody. Blot A. (lower image) has been transformed with Quantity One software to aid visualisation of upper band.

A further IP experiment was performed using the same protocol, to test a second polyclonal IDE antibody designated Ab 5 (AF2496) (Figure 3.12(B)). Ab 5 was raised in goat against full-length human recombinant IDE. Using a full-length protein as an immunogen for generating an antibody is likely to result in conformational epitopes, thus the antibody is more likely to recognise the native protein, and may be more sensitive in an IP in which IDE has not been denatured or reduced. One rrIDE sample was pre-incubated with A β ₄₂, and immunoprecipitated, to determine if binding between these proteins was resistant to the IP procedure (as well as the denaturing and reducing conditions) which would lead to an upward shift in molecular weight. Immunoprecipitated samples were subsequently resolved by SDS-PAGE and immunoblotted using Ab 3 (ab28561). Results showed that Ab 5

produced similar results to those obtained using Ab 4, with clear bands apparent at 120-130 and 80 kDa. No bands were observed at 160 kDa, and the 70 kDa band previously observed with Ab 4 was very faint and only seen in PCP, and not in the AD PCP. Ab 5 detected very weak bands in the rrIDE lanes compared with rhIDE lanes. No shift in molecular weight was observed in the A β ₄₂-spiked rhIDE compared with rhIDE alone, however the bands were fainter. AD PCP IP bands at 120-130 kDa were also fainter than PCP IP bands. Overall it appears that the 120-130 kDa and the 80 kDa bands in human plasma are specific, while it is unlikely that the 160 kDa or 70 kDa bands are IDE.

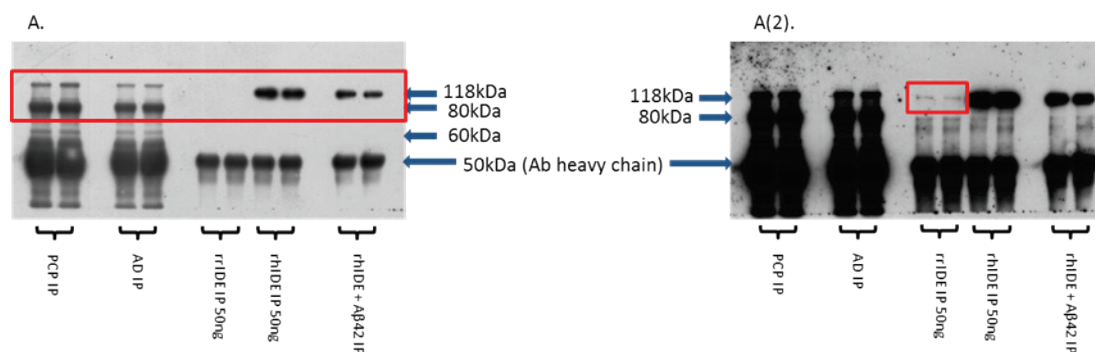


Figure 3.12(B) Immunoprecipitation of IDE from human plasma using IDE antibody Ab 5 (AF2496). IDE immunoprecipitation was carried out from pooled plasma (PCP), albumin-depleted PCP (AD), recombinant rat IDE (rrIDE) and recombinant human IDE (rhIDE) using polyclonal IDE AF2496 as detailed in Sections 2.2.4.16, 2.2.4.1 and 2.2.4.4. Following SDS-PAGE and Western blotting, blots were probed with IDE ab28561 (Ab 3). Image A(2) is a longer exposure of Blot A. The two right lanes contain 50ng rhIDE + 9 μ g beta-amyloid 42 (A β ₄₂). For AD, PCP samples were pre-cleared of IgG and albumin-depleted with antibody AL-01, prior to IP (see Sections 2.2.4.13 and 2.2.4.14). IP PCP and AD samples originally used 2 μ L of plasma per lane in duplicate, and rrIDE/rhIDE and rhIDE+A β ₄₂ lanes contain 50ng or equivalent of IDE per lane in duplicate. Bands lower than 60kDa in all lanes are contaminated with the immunoprecipitating antibody fragments which were eluted with the sample. At 3 min exposure, rhIDE is detected at 118kDa, no visible bands for rrIDE (Blot A). At 10 min exposure, the rrIDE bands are faintly visible at 118kDa (Blot A(2)).

3.3.11 Pre-incubation immunising peptide blocking test on IDE antibody Ab 3 (ab28561) for specificity.

To determine the specificity of the bands detected by Western immunoblotting with Ab 3, a blocking test involving pre-incubation with the immunising peptide was performed (similar to the test performed with Ab 1 and 2, in Sections 3.3.1 and 3.3.6) using a commercial peptide which was the Ab 3 immunogen (Figure 3.13). The results were difficult to interpret as the immunising peptide increased the background of the immunoblot and this caused difficulties in visualisation of the bands. However, despite this issue, it was still possible to observe a reduction in band intensity for rrIDE, the major band in rat liver at 115 kDa and an 80 kDa band in zebrafish brain. It also appeared that the upper two bands in plasma disappeared with the peptide pre-immunisation (120-30 and 160 kDa), as did the 95 and 50 kDa bands in zebrafish brain. Figure 3.13 was transformed by Quantity One software to maximise the signal to noise ratio and shows no bands visible in these regions. Densitometric analysis of the 120-130 kDa band shows a reduction of 97% in the absorbed sample (antibody neutralised with 5 µg peptide). The band in plasma at 115 kDa conversely, appears to be stronger after peptide pre-immunisation. These results support the results obtained by the IP experiments which suggest that the band observed at 120-130 kDa after immunoblotting with Ab 3 is IDE. In addition, IDE has been demonstrated to be 110 kDa in rat liver in previous studies, and Ab 3 specifically detects a band at a similar molecular weight, lending further support to the likelihood that this antibody detects full-length IDE in human plasma. Interestingly, Ab 3 also appeared to detect IDE at different molecular weights in zebrafish brain (50, 80 and 95 kDa) indicating different isoforms may be present in this species, however further characterisation of these bands is outside the scope of this project.

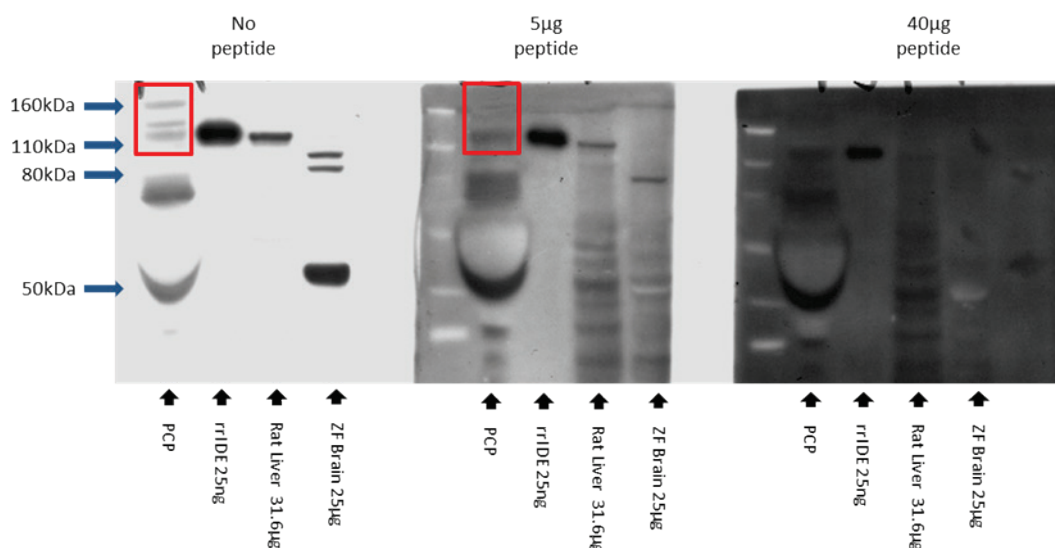


Figure 3.13 Testing IDE Ab 3 (ab28561) specificity by blocking with the immunising peptide prior to adding to Western blots. Pooled plasma (PCP), recombinant rat IDE (rrIDE), rat liver and zebrafish brain (ZF) samples were run on SDS-PAGE and transferred to Western prior to being probed with IDE ab28561, some of which had been blocked prior to incubation with a commercial peptide identical to the immunogen (0, 5 and 40µg peptide/blot). The test was performed as detailed in Sections 2.2.4.12, 2.2.4.1 and 2.2.4.4. PCP: 1 µL plasma per lane. rrIDE: 25 ng per lane. Rat liver: 31.6 µg total protein/lane and ZF brain: 25 µg total protein/lane. Due to high background, the images were enhanced to optimize the signal to noise ratio, to assist in visualising bands.

3.3.12 Measurement of the 120-130 kDa band in plasma samples obtained from the AIBL cohort at baseline

The samples were resolved by SDS-PAGE (under reducing conditions), transferred to Western blots, and immunoblotting was performed using Ab 2 (LS-C55007). However, as this antibody subsequently failed the immunising peptide blocking test, it could not be determined if the bands seen with this IDE antibody were specific (Section 3.3.6). Therefore, the immunoblots were stripped and re-probed using Ab 3 (ab28561) (example Figure 3.14). The band observed at 120-130 kDa was determined to be IDE and quantified using Quantity One software. Pooled control plasma (PCP) was used as an internal standard on each gel to control for gel-to-gel and blot-to-blot variation. The blots were Ponceau S stained after re-

stripping and prior to immunoblotting (or Coomassie stained following immunoblotting and subsequent re-stripping) to ensure equal loading.

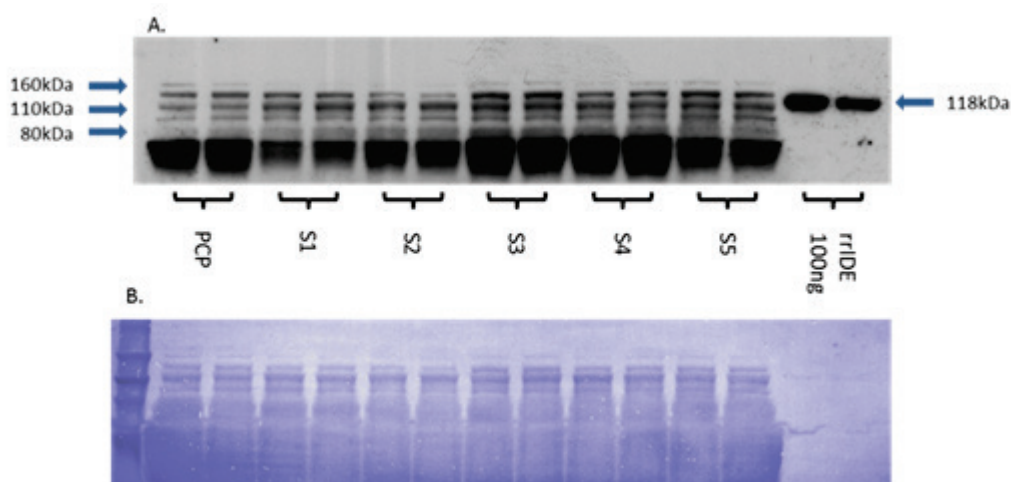


Figure 3.14 120-130 kDa band measured in human plasma samples obtained from the AIBL cohort (Ab 3). Example Western blot of 5 individual AIBL samples, including pooled control plasma as an internal standard (PCP) and recombinant rat IDE (rrIDE) as a positive control (Figure A.). SDS-PAGE and immunoblotting were performed as detailed in Sections 2.2.4.1 and 2.2.4.4. Plasma samples including PCP (1 μ L/lane) were loaded in duplicate, rrIDE (100 ng/lane) was also loaded in duplicate as a positive control. Antibody used here was Ab 3, (ab 28561). Results show differences in band intensities between individual samples. Figure B. Coomassie stain of membrane as loading control.

3.3.13 Low-level data analysis.

Results were considered to be semi-quantitative. Raw data were normalised for inter-gel variation by adjusting to the PCP internal control included on each gel. Coefficient of variation (CV) values of duplicates which were >15% were excluded from the analysis. If the CV of the PCP duplicates was >15%, the entire gel was excluded from the analysis. Data points determined to be outliers were removed from the analysis (n = 2).

The overall data were observed to have a non-normal distribution (Figure 3.15(A), Table 3.1), therefore it was transformed by square root (Figure 3.15(B), Table 3.1). The transformation improved the normality issues, determined by inspecting the

frequency histogram, and with the z-score for skewness being <1.96 . There were still some normality issues after transformation determined by the z-score for kurtosis of 2.189 (absolute value), and significant p-values for the Kolmogorov-Smirnoff (K-S) and Shapiro-Wilk (S-W) tests. However the z-score was very close to the cut-off point of 1.96 indicating that the normality issues were minimal. In addition, the K-S and S-W tests are very sensitive to sample size, and as it was reasonably large, it would be expected to show significance even with minimal normality issues. Furthermore, when the data was stratified by classification, the healthy control group particularly, demonstrated non-normal distribution prior to transformation (Figures 3.16(A)(B)(C), Table 3.2), while after transformation the z-scores for both skewness and kurtosis were below the cut-off point of 1.96 for all classifications, indicating normality (Figures 3.17(A)(B)(C), Table 3.3). While there still seemed to be minor normality issues for healthy controls only, as evidenced by significant K-S and S-W tests, the frequency histogram was a normal shape after transformation. Therefore the normality issues after transformation were determined to be within acceptable limits and parametric tests were used to analyse the data for differences between classification and *APOE4* genotype status (ANOVA (with Tukey HSD post-hoc tests) and Student's t-tests). However, as existing biochemical data had non-normal distribution also, the non-parametric Spearman's Rho test was used to analyse correlations.

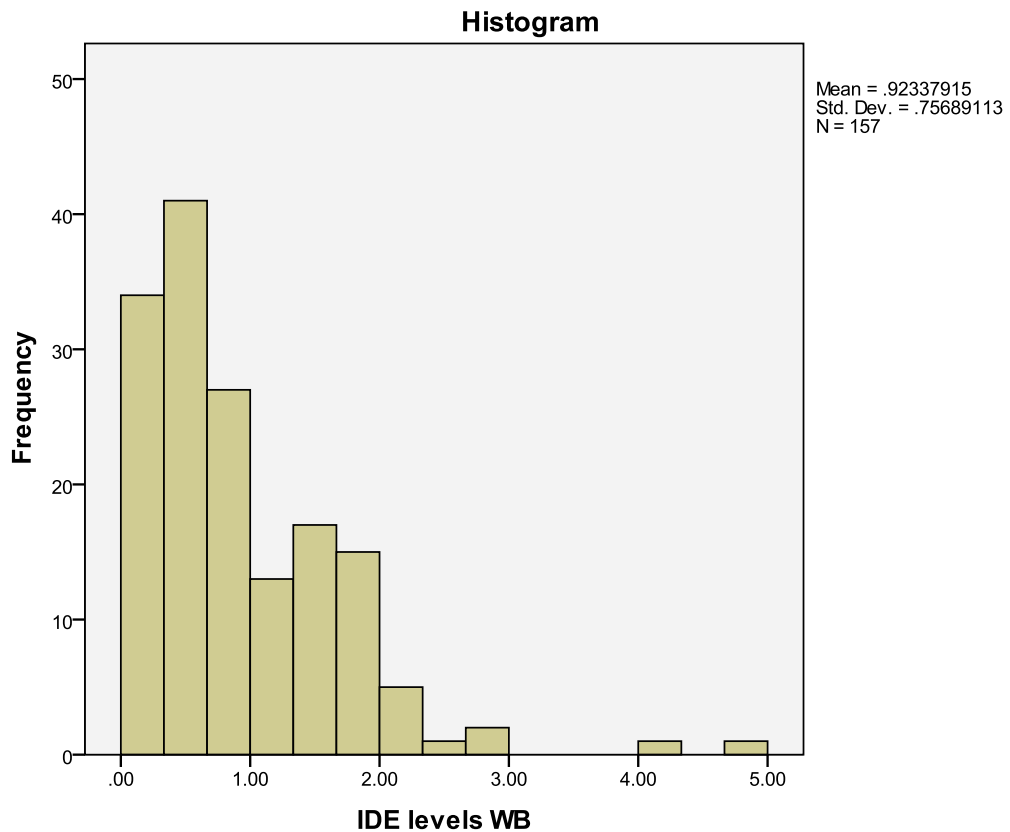


Figure 3.15(A) Frequency histogram of overall data (IDE levels measured by Western immunoblotting) before transformation. Band intensity results for the IDE Western blot (WB) results (untransformed). Histogram shows a positive skew of the data, indicating non-normal distribution (n = 157).

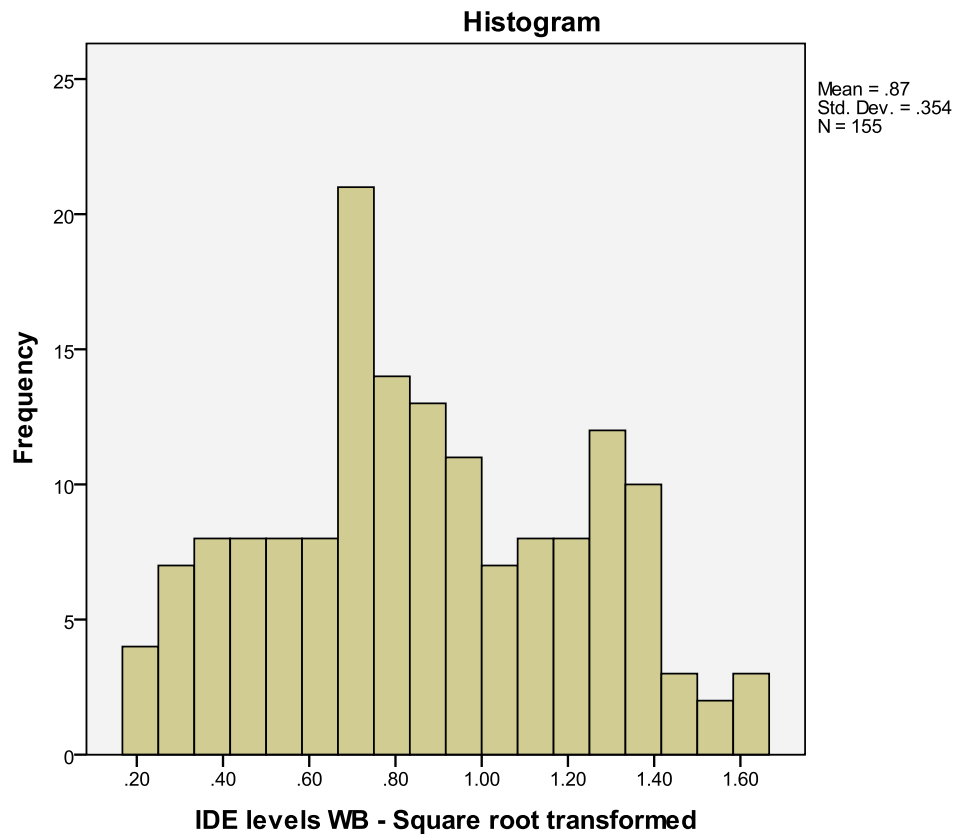
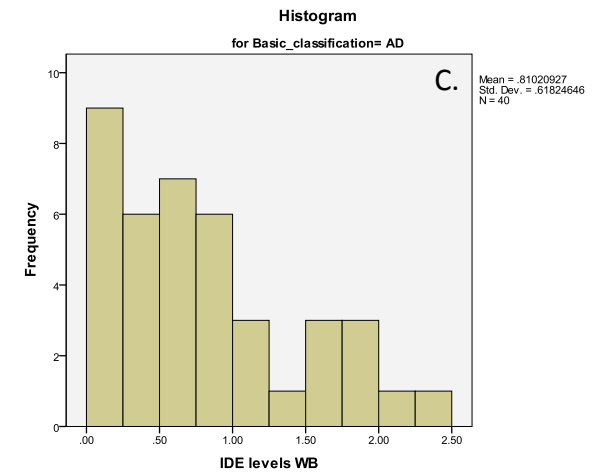
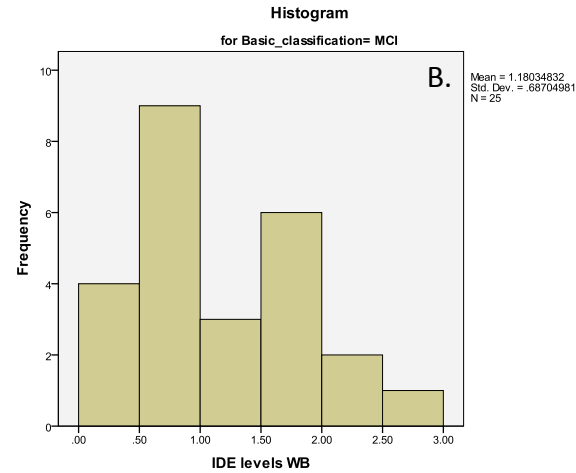
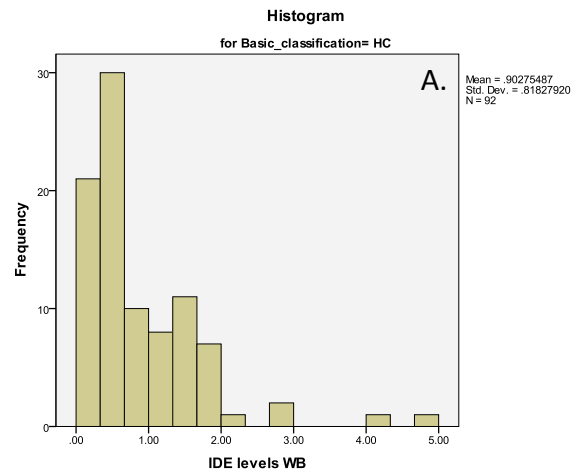


Figure 3.15(B) Frequency histogram of overall data (IDE levels measured by Western immunoblotting) after square root transformation. Band intensity results for the IDE Western blot (WB) results transformed to square root, outliers removed ($n = 2$). Histogram shows the data is improved after transformation by square root and following the removal of two outliers ($n = 155$), indicating a more normal distribution.

Table 3.1 Values of skewness, kurtosis, Kolmogorov-Smirnoff (K-S) and Shapiro-Wilk (S-W) tests of distribution normality in the overall data before and after square root transformation. Values prior to transformation indicate non-normal distribution; after transformation skewness and kurtosis values are within acceptable limits regardless of significant p-values for the K-S and S-W tests (n = 157/155).

	Untransformed	Transformed
Skewness (\pm S.E.)	1.712 \pm .194	.120 \pm .195
Skewness (z score)	8.825	.615
Kurtosis (\pm S.E.)	5.097 \pm .385	-.847 \pm .387
Kurtosis (z score)	13.239	-2.189
Kolmogorov-Smirnoff	$D(157)=.142, p=.000$	$D(155)=.073, p=.041$
Shapiro-Wilk	$W(157)=0.862, p=.000$	$W(155)=.975, p=.007$

(THIS PAGE HAS BEEN INTENTIONALLY LEFT BLANK)



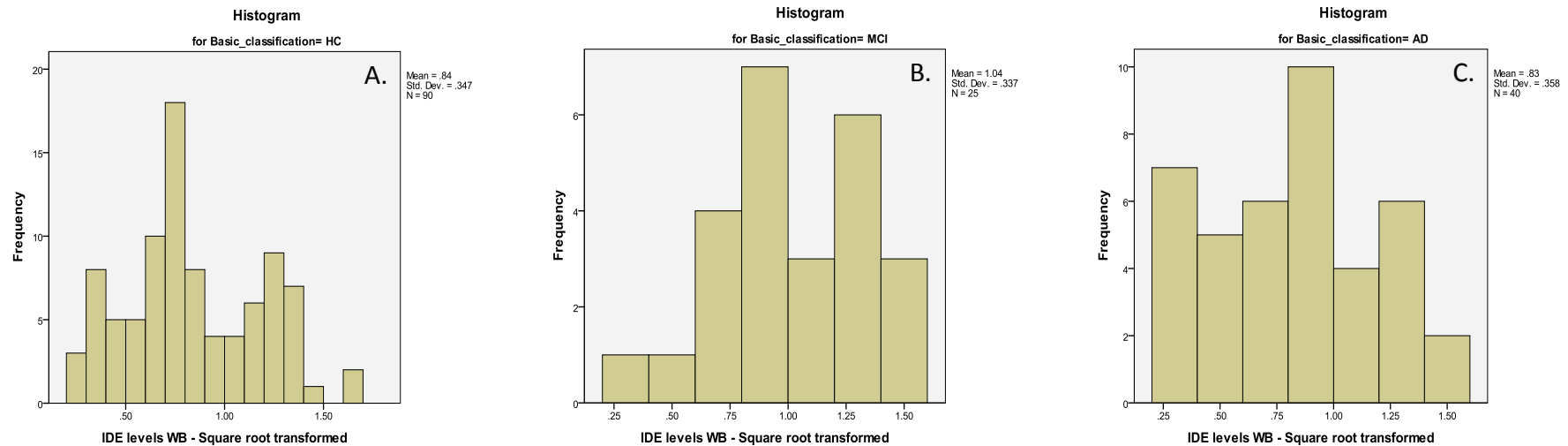
Figures 3.16 (A)(B)(C) Frequency histograms of data (IDE levels measured by Western immunoblotting (WB)) stratified by classification (HC, MCI, AD, respectively) before transformation. Histograms show the data is positively skewed in all classifications, indicating a deviation from normal distribution.

Table 3.2 Values of skewness, kurtosis, Kolmogorov-Smirnoff (K-S) and Shapiro-Wilk (S-W) tests of distribution normality in the data before transformation. Values prior to transformation indicate non-normal distribution in the HC and AD data, MCI data is within acceptable limits.

	HC	MCI	AD
Skewness (\pm S.E.)	2.156 \pm .251	.343 \pm .464	.739 \pm .374
Skewness (z score)	8.5	.74	1.98
Kurtosis (\pm S.E.)	6.868 \pm .498	-.974 \pm .902	-.374 \pm .733
Kurtosis (z score)	13.79	-1.08	-.51
Kolmogorov-Smirnoff	$D(92)=.18$, $p=.000$	$D(25)=.163$, $p=.084$	$D(40)=.112$, $p=.200$
Shapiro-Wilk	$W(92)=0.803$, $p=.000$	$W(25)=.950$, $p=.250$	$W(40)=.921$, $p=.008$

Table 3.3 Values of skewness, kurtosis, Kolmogorov-Smirnoff (K-S) and Shapiro-Wilk (S-W) tests of distribution normality in the data after transformation by square root. Values after transformation by square root and removal of 2 outliers indicate normal distribution in the MCI and AD data. Values for skewness and kurtosis for HC data indicate normality, however the K-S and S-W tests still indicate some deviation from normality.

	HC	MCI	AD
Skewness (\pm S.E.)	.232 \pm .254	-.197 \pm .464	.103 \pm .374
Skewness (z score)	.913	-.425	.275
Kurtosis (\pm S.E.)	-.705 \pm .503	-.744 \pm .902	-.958 \pm .733
Kurtosis (z score)	-1.4	-.825	-1.31
Kolmogorov-Smirnoff	$D(90)=.103$, $p=.020$	$D(25)=.129$, $p=.200$	$D(40)=.076$, $p=.200$
Shapiro-Wilk	$W(90)=0.969$, $p=.031$	$W(25)=0.969$, $p=.612$	$W(40)=0.966$, $p=.276$



Figures 3.17 (A)(B)(C) Frequency histograms of data (IDE levels measured by Western immunoblotting) stratified by classification (HC, MCI, AD, respectively) after transformation by square root. Histograms show the data is improved after transformation (and removal of 2 outliers) in all classifications, particularly in the HC data, indicating a more normal distribution.

3.3.14 Full-length plasma IDE levels are increased in MCI compared with HC in the total cohort.

To determine IDE levels according to cognitive status, the cohort was divided into the clinically classified groups, healthy controls (HC - including subjective memory complainers), mild cognitive impairment (MCI) and probable AD (AD). As only a subset of the cohort was used, the HC and SMC groups were combined to form the healthy control group. Mean age was significantly different between the groups. It should be noted that the subset of samples used to measure IDE levels by Western immunoblotting was different from the subsets subsequently used to measure IDE levels by ELISA and IDE activity (detailed in Chapters 4 and 5, respectively). This is because all samples in the cohort were initially going to be assayed, however due to the time-frame required for optimisation of the procedures, the whole cohort was not able to be completed.

The demographics for this subset of the AIBL cohort are shown in Table 3.4. AD and MCI patients were significantly older than the HC group. As expected, AD patients had a significantly higher percentage of *APOE* $\epsilon 4$ carriers and brain $A\beta$ load than the control or MCI groups. Plasma $A\beta_{1-42}$ was significantly decreased in the AD group compared with MCI and HC groups. For $A\beta_{x-40}$, the MCI group had significantly higher levels compared with controls, with the AD group having levels in between MCI and HC groups. Levels of ApoE4 were significantly decreased in the MCI group compared with HC and AD, and estradiol was significantly reduced in MCI and AD groups (HC>MCI>AD). All other variables were not significantly different between classifications.

(THIS PAGE HAS BEEN INTENTIONALLY LEFT BLANK)

Table 3.4 Demographics of the AIBL cohort subset used for the Western immunoblotting IDE analysis. Plasma samples (1.0 μ L per well in duplicate) from the AIBL cohort were measured by Western immunoblotting as detailed in Sections 2.2.4.1 and 2.2.4.4. Other variables were measured as outlined in Section 2.2.2.1. Full-length plasma IDE level data underwent square root transformation; the remainder of the variables are not transformed. Continuous variables are expressed as mean (median) \pm standard deviation. P values determined by Kruskal-Wallis tests using the Monte Carlo method (untransformed) or one-way analysis of variance (transformed). As only a subset of the cohort was tested, the HC and subjective memory (SMC) groups were combined to provide the healthy controls for this assay. As ApoE4 is only expressed in individuals carrying the *APOE* ϵ 4 allele, the n-values are reduced for this variable. As only a subset of the cohort were measured with Pib-PET (for Neocortical SUVR – Brain A β load), n-values are reduced for this variable.

	HC	MCI	AD	p-value
Full-length Plasma IDE Levels (OD*mm ²)	0.839 (0.770) ± 0.347	1.04 (0.963) ± 0.337	0.828 (0.838) ± 0.358	0.034
n (Full-length IDE Levels)	90	25	40	-
Age (years)	71.1 (70) ± 7.14	75.48 (75) ± 8.08	75.53 (76) ± 10.63	0.013
APOE ε4 +ve (%)	28.1	40	50	0.046
Male (%)	42.2	52	27.5	0.103 (NS)
Plasma insulin (mU/L)	7.22 (7) ± 4.61 n = 89	8.58 (6.5) ± 8.29 n = 25	7.81 (5.2) ± 6.84 n = 39	0.889 (NS)
Plasma Aβ ₁₋₄₀ (pg/mL)	153.2 (152.3) ± 40.18 n = 90	153.9 (157.75) ± 47.19 n = 24	146.06 (141.17) ± 40.76 n = 40	0.390 (NS)
Plasma Aβ ₁₋₄₂ (pg/mL)	32.69 (32.8) ± 9.7 n = 90	33.94 (30.17) ± 14.4 n = 24	28.8 (27.95) ± 9.37 n = 40	<0.001
Plasma Aβ _{1-42/1-40} ratio	0.226 (0.213) ± 0.115 n = 90	0.235 (0.215) ± 0.105 n = 24	0.205 (0.206) ± 0.064 n = 40	0.513 (NS)
Plasma Aβ _{x-40} (pg/mL)	141.35 (149.89) ± 52.68 n = 90	166.14 (168.92) ± 45.41 n = 24	152.16 (156.64) ± 45.89 n = 40	0.013
Plasma Aβ _{x-42} (pg/mL)	31.87 (31.81) ± 12.56 n = 90	35.54 (32.93) ± 18.77 n = 24	28.48 (27.12) ± 13.15 n = 40	0.117 (NS)
Plasma Aβ _{x-42/x-40} ratio	0.286 (0.221) ± 0.287 n = 90	0.227 (0.182) ± 0.129 n = 24	0.216 (0.185) ± 0.18 n = 40	0.052 (NS)
Plasma ApoE – Total (mg/dL)	15.49 (15.33) ± 2.73 n = 89	15.23 (15.27) ± 3.26 n = 24	14.59 (14.78) ± 2.6 n = 38	0.278 (NS)
Plasma ApoE4 (mg/dL)	6.64 (6.3) ± 2.08 n = 25	5.48 (4.77) ± 2.99 n = 9	6.84 (5.18) ± 4.21 n = 19	<0.001
Plasma Testosterone - Total (nmol/L)	7.61 (1.9) ± 8.19 n = 87	10.79 (14.3) ± 9.97 n = 24	5.61 (1.4) ± 7.74 n = 39	0.087 (NS)
Plasma Testosterone – Free (nmol/L)	0.146 (0.022) ± 0.183 n = 90	0.198 (0.242) ± 0.194 n = 25	0.097 (0.017) ± 0.147 n = 40	0.052 (NS)
Plasma Estradiol (pmol/L)	114.6 (88.5) ± 90.19 n = 68	107.45 (96.5) ± 51.81 n = 20	82.18 (69.5) ± 51.94 n = 38	0.032
Plasma Cholesterol – total (mmol/L)	5.55 (5.65) ± 1.11 n = 90	5.42 (5.1) ± 0.984 n = 25	5.69 (5.75) ± 1.42 n = 40	0.626 (NS)
Plasma LDL Cholesterol (mmol/L)	3.27 (3.3) ± 1.002 n = 90	3.14 (2.9) ± 0.918 n = 25	3.29 (3.15) ± 1.22 n = 40	0.710 (NS)
Plasma HDL Cholesterol (mmol/L)	1.70 (1.63) ± 0.493 n = 90	1.72 (1.77) ± 0.344 n = 25	1.75 (1.69) ± 0.413 n = 40	0.652 (NS)
Brain Aβ load (Neocortical SUVR)	1.57 (1.34) ± 0.505 n = 35	1.75 (1.78) ± 0.584 n = 14	2.29 (2.18) ± 0.334 n = 11	<0.001

Full-length IDE levels as determined by Western immunoblotting (Figure 3.18) were found to be significantly higher in MCI individuals when compared with HC subjects ($p = 0.037$). IDE levels in MCI individuals were also higher than in AD patients, however this did not reach significance ($p = 0.054$ – a significant difference for all results requires $p \leq 0.05$). There was no significant difference between the HC and AD groups.

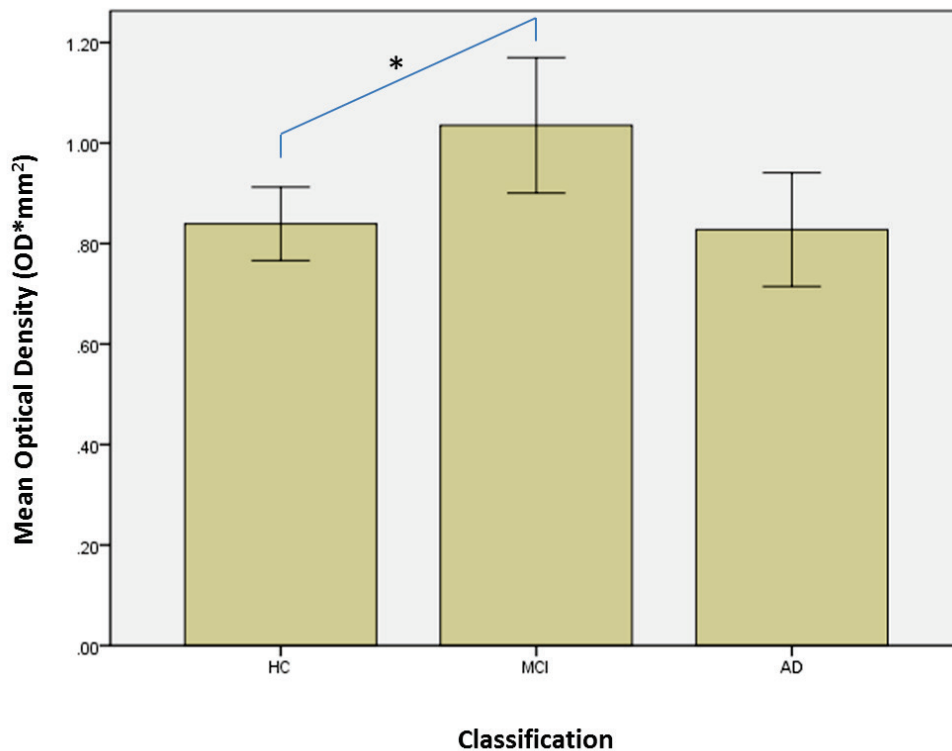


Figure 3.18 Levels of full-length plasma IDE are increased in MCI compared with HC and AD. Plasma samples (60 μ g/ lane total protein in duplicate) from the AIBL cohort were resolved by 10% SDS-PAGE, transferred to Western blots and probed for full-length IDE as described in Sections 2.2.4.1 and 2.2.4.4, the antibody used was Ab 3 (Ab 28561). The average optical density of the duplicate bands was obtained for each sample, and the mean of these values was compared by ANOVA with Tukey post-hoc analysis. Data represents mean \pm SEM, $n = 155$ (90 HC/25 MCI/40 AD). Results show IDE levels in MCI are significantly increased compared with HC ($p = 0.037$) and trended towards an increase in levels compared with AD ($p = 0.054$).

3.3.15 Full-length plasma IDE levels are increased in females with MCI compared with HC and AD

Further analysis revealed a significant difference in IDE levels between males and females ($p = 0.000$), with females having higher levels of full-length IDE than males (Figure 3.19). Therefore, the data was subsequently analysed by classification separately for males and females (Figure 3.20). The results show that the significant difference seen in the MCI group compared with the HC group is only demonstrated in females ($p = 0.014$). In addition, the difference between the female MCI and AD groups was also highly significant ($p = 0.008$). When analysing the data for the male participants only, no significant difference between classifications was observed.

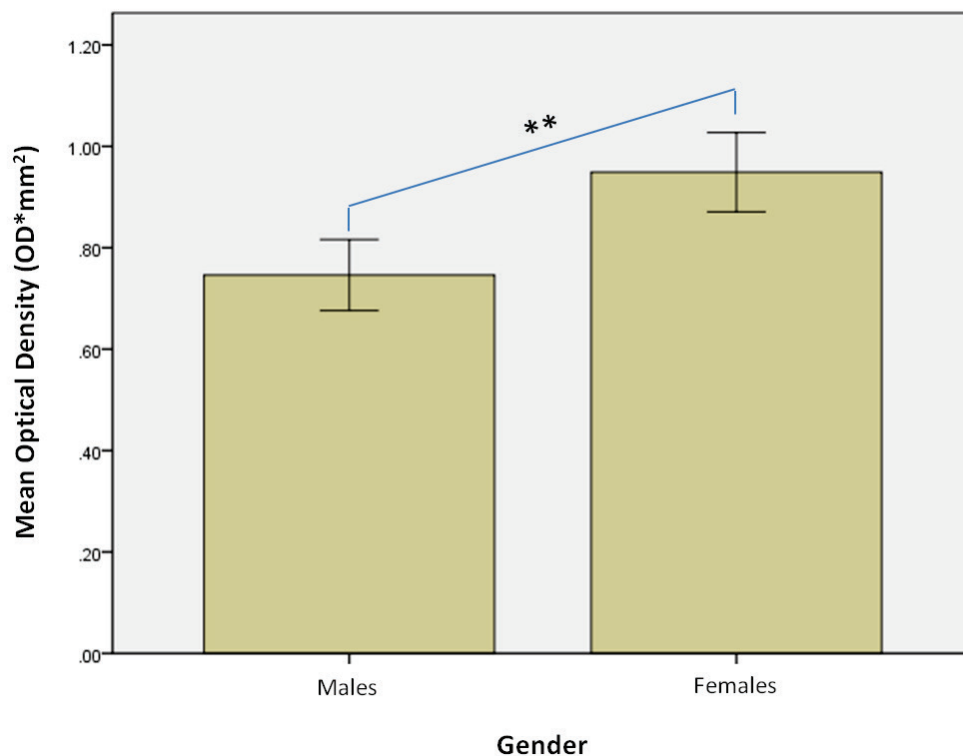


Figure 3.19 Full-length plasma IDE is higher in females compared with males. Plasma samples (60 $\mu\text{g}/\text{lane}$ total protein in duplicate) were resolved by 10% SDS-PAGE, transferred to Western blots and probed for IDE as described in Sections 2.2.4.1 and 2.2.4.4, the antibody used was Ab 3, (Ab 28561). The average optical density of duplicate bands was obtained for each sample, and the mean of these values was compared by Students T-test. Data represents mean \pm SEM, $n = 155$ (62 M/93 F). Results show that full-length plasma IDE is significantly higher in females compared with males ($p = 0.000$).

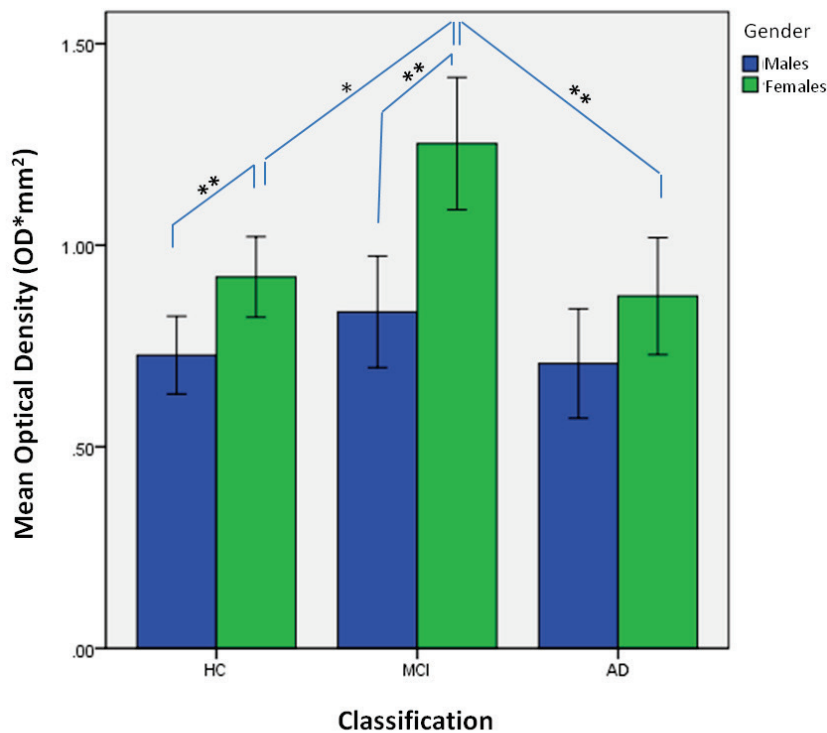


Figure 3.20 Full-length plasma IDE levels are increased in MCI compared with HC and AD in females only. Plasma samples (60 µg/lane total protein in duplicate) were resolved by 10% SDS-PAGE, transferred to Western blots and probed for full-length IDE as described in Sections 2.2.4.1 and 2.2.4.4, using Ab 3. The average optical density of the duplicate bands was obtained for each sample, and the mean of these values was compared by ANOVA with Tukey post-hoc analysis. Data represents mean ± SEM, n = 155 (HC-38 M/52 F, MCI-13 M/12 F, AD-11 M/29 F). Results show that full-length plasma IDE is significantly increased in female MCI individuals compared with both female HC (p = 0.014) and female AD (p = 0.008) participants. In addition, IDE is significantly higher in females compared with males in both the HC (p = 0.008) and the MCI (p = 0.001) groups but not in the AD group (p = 0.190).

3.3.16 Levels of full-length plasma IDE do not differ in *APOE ε4* carriers compared with non-carriers.

As the *APOE ε4* genotype is considered a risk factor for AD, and it has been reported that levels of IDE are reduced in neurons exposed to the ApoE4 protein *in vitro* (Du, *et al.*, 2009), the data was analysed to see if there was any difference between full-length plasma IDE in individuals carrying the *ε4* allele compared to non-carriers (Figure 3.21

(A)). The results show that there was no significant difference in plasma full-length IDE levels between $\epsilon 4$ carriers and non-carriers, for the data overall ($p = 0.183$) When stratified by $\epsilon 4$ status, a similar pattern was seen by classification as in the overall subset, however the increase in IDE levels in the MCI group was no longer significant for either $\epsilon 4$ carriers ($p = 0.128$) or non-carriers ($p = 0.271$)(Figure 3.21(B) and (C), respectively).

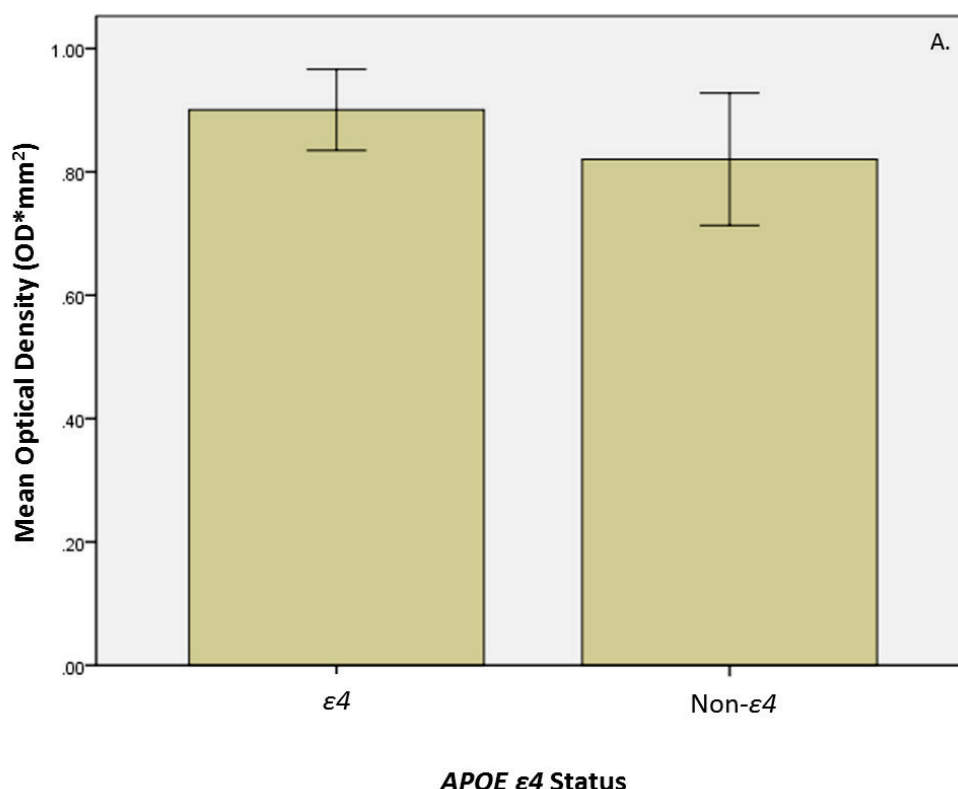
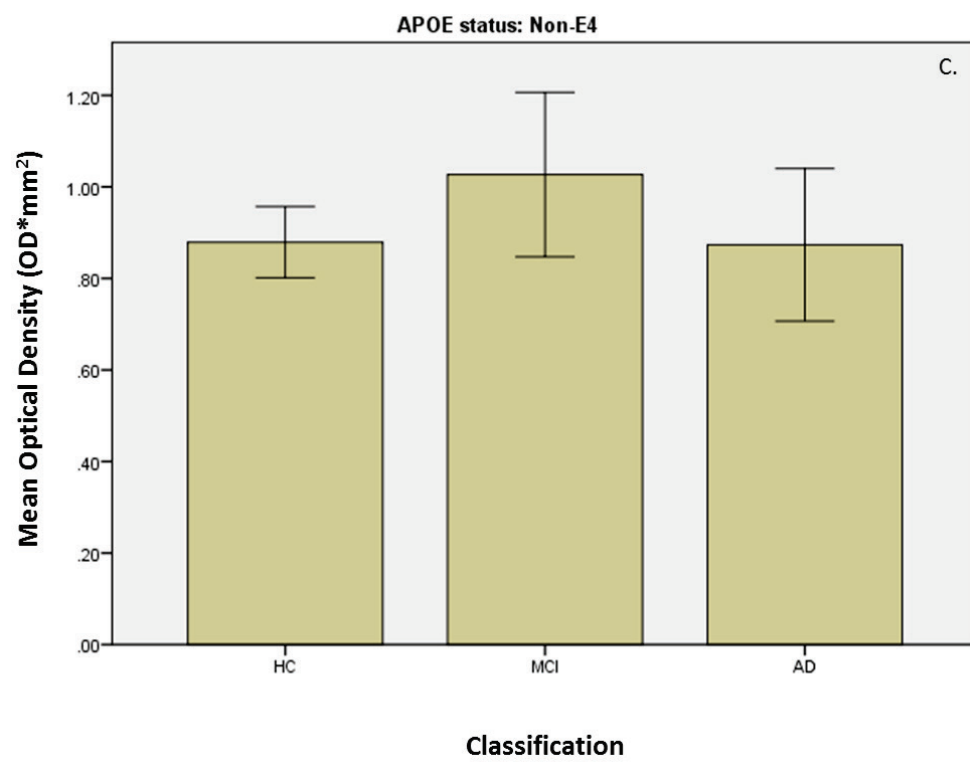
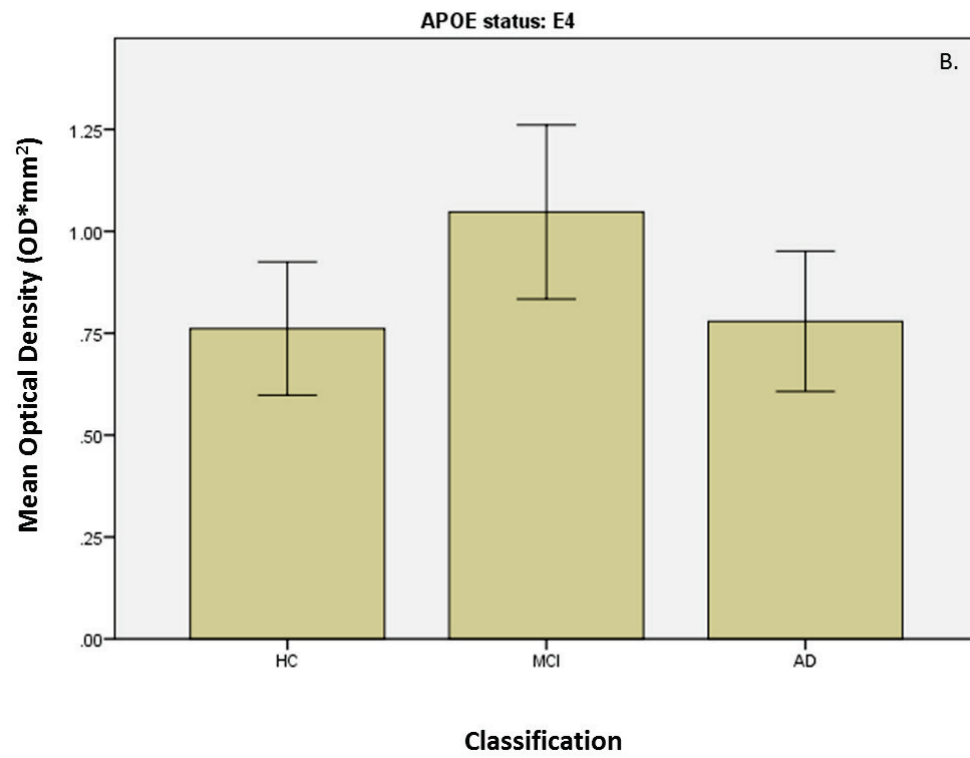


Figure 3.21(A) Full-length plasma IDE levels are no different in $\epsilon 4$ non-carriers compared with carriers. Plasma samples (60 $\mu\text{g}/\text{lane}$ total protein in duplicate) were resolved by 10% SDS-PAGE, transferred to Western blots and probed for full-length IDE as described in Sections 2.2.4.1 and 2.2.4.4, using Ab 3. The average optical density of the duplicate bands was obtained for each sample, and the mean of these values was compared by Students T-test. Data represents mean \pm SEM, $n = 152$ (54 $\epsilon 4/98$ non- $\epsilon 4$). Results show that there is no significant difference in full-length plasma IDE levels for $\epsilon 4$ non-carriers compared with carriers ($p = 0.183$).

Figure 3.21(B) and (C) Full-length plasma IDE levels are not significantly different by clinical classification for $\epsilon 4$ non-carriers or carriers. Plasma samples (60 μ g/lane total protein in duplicate) were resolved by 10% SDS-PAGE, transferred to Western blots and probed for full-length IDE as described in Sections 2.2.4.1 and 2.2.4.4, using Ab 3. The average optical density of the duplicate bands was obtained for each sample, and the mean of these values was compared by Students T-test. Data represents mean \pm SEM, ($\epsilon 4$ carriers) n = 54 (25 HC/10 MCI/19AD), (non-carriers) n = 98 (64 HC/15 MCI/19AD). Results show that there is no significant difference in full-length plasma IDE levels by clinical classification for either $\epsilon 4$ carriers (p = 0.128) or non-carriers (p = 0.271).



When the overall data is controlled for gender, similar results are observed between classifications, with the MCI group having significantly higher full-length IDE levels than controls ($p = 0.026$), but with levels in the MCI group being significantly higher than the AD group as well ($p = 0.040$). As age has also been shown to be significantly different between the groups in this subset ($p = 0.013$), this was also controlled for along with gender, and results showed the same trend: MCI subjects have significantly higher IDE levels than both HC ($p = 0.010$) and AD ($p = 0.014$). Even though there were no significant differences in IDE levels in $\epsilon 4$ carriers versus non-carriers, the $\epsilon 4$ status has the potential to affect the levels of IDE in plasma, and controlling for this as well as gender and age strengthened this trend with the MCI group again having significantly higher levels than both HC ($p = 0.004$) and AD ($p = 0.006$) (Figure 3.22). Conversely when analysing full-length IDE levels by $\epsilon 4$ status, no effect of covariates was observed demonstrating no significant differences between carriers and non-carriers (data not shown).

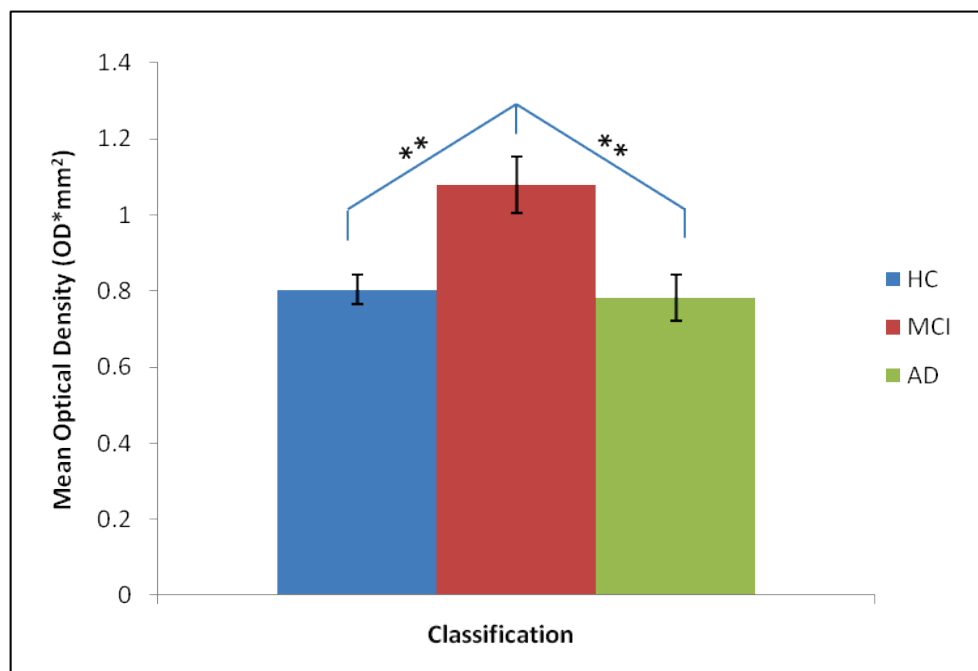


Figure 3.22 Full-length plasma IDE levels are significantly increased in MCI compared with HC and AD. Plasma samples (60 µg/lane total protein in duplicate) were resolved by 10% SDS-PAGE, transferred to Western blots and probed for full-length IDE as described in Sections 2.2.4.1 and 2.2.4.4, using Ab 3. The average optical density of the duplicate bands was obtained for each sample, and the mean of these values was compared by ANCOVA. Data represents mean \pm SEM, $n = 152$ (89 HC/25 MCI/38 AD). Data is controlled for gender, age and *APOE* $\epsilon 4$ status. Results show that full-length plasma IDE levels are significantly higher in the MCI group compared with the HC ($p = 0.004$) and AD ($p = 0.006$) groups.

3.3.17 Relationships between total plasma IDE levels and other biochemical data collected in the AIBL study.

To determine if there were any relationships between plasma levels of full-length IDE and other biochemical data collected as part of the AIBL study, correlation analyses were performed. Details of the methods used to measure the variables other than IDE are outlined in Section 2.2.2.1. As full-length IDE was shown to differ by gender in the current study, it was included in the correlation analyses. Plasma IDE was also analysed for any correlation with age as it has been reported that expression of IDE shows an age-dependent reduction in the brain (Caccamo, *et al.*, 2005). Plasma insulin, $A\beta_{40}$ and $A\beta_{42}$ (and N-terminally truncated fragments), and total ApoE and

ApoE4 were analysed for correlations with IDE, as both insulin and A β are substrates of IDE and ApoE has been associated with reduced IDE expression (Cook, *et al.*, 2003; Du, *et al.*, 2009). The levels of testosterone, estradiol and cholesterol (plus LDL/HDL subfractions) were also examined for correlation with full-length IDE levels, as these have also been associated with changes in IDE expression (Udrisar, *et al.*, 2005; Sharma, *et al.*, 2008). Furthermore, full-length plasma IDE levels were also compared with brain A β load to see if any correlation exists between these two variables.

Results showed that in the whole subset (Table 3.2), and in addition to a positive correlation with gender ($r_s = .282$, $p = 0.000$), only total and free testosterone negatively correlated with full-length plasma IDE, however these associations were weak [$(r_s = -.235$, $p = 0.004$) and $(r_s = -.255$, $p = 0.001$), respectively]. Oestradiol, the predominant female sex hormone, almost reached significance ($r_s = -.173$, $p = 0.053$), however the association was again weak. No other variables correlated with plasma full-length IDE. After Bonferroni-Holm correction for multiple testing (Holm, 1979), only gender ($p = 0.000$) and free testosterone ($p = 0.017$) were still significant although total testosterone also showed a trend towards significance ($p = 0.064$).

As both testosterone and oestradiol both significantly correlate with gender and with each other, males and females were analysed separately to see if the relationship between full-length plasma IDE with these variables was due to the effect of gender (Tables 3.3(A) and (B)). The results showed that these correlations were not significant when males and females were analysed separately. No correlations between plasma IDE and the remaining biochemical variables were observed when the genders were analysed separately, however in males insulin approached significance for a negative association ($r_s = -.239$, $p = 0.064$).

Table 3.2 Correlation matrix of full-length plasma IDE levels with other variables.

Plasma levels of full-length IDE were analysed for relationships with sex, age, and plasma insulin, A β species, ApoE species, testosterone, oestradiol, lipids and brain A β load (two tailed) (methods of data collection are outlined in Section 2.2.2.1). n values vary due to missing values for some data or in the case of brain A β load only a subset of the cohort underwent PiB-PET scans. For ApoE4, n value is lower as only the *APOE* ϵ 4 carriers express this isoform. Results show a significant but weak correlation exists between IDE levels and both sex ($r_s = .282$, $p = .000$), total testosterone ($r_s = -.235$, $p = .004$) and free testosterone ($r_s = -.282$, $p = 0.001$). Oestradiol approached a weak correlation however did not reach significance ($r_s = -.173$, $p = 0.053$). Only sex (gender) and free testosterone remained significant following Bonferroni-Holm correction for multiple testing. * indicates significance at the 0.05 level, ** indicates significance at the 0.01 level. ("unadj." = unadjusted, prior to Bonferroni-Holm correction, "adj." = adjusted by Bonferroni-Holm correction).

Overall	Correlation Coefficient (r_s)	N	Significance (2-tailed)
Sex	.282	155	<0.001 ** (unadj.) <0.001 ** (adj.)
Age	-.030	155	0.709 (NS)
Insulin	-.038	153	0.645 (NS)
A β_{1-40}	-.069	154	0.398 (NS)
A β_{1-42}	.022	154	0.790 (NS)
A $\beta_{42/40}$ ratio	.048	154	0.553 (NS)
A β_{x-40}	-.050	154	0.541 (NS)
A β_{x-42}	-.027	154	0.737 (NS)
A $\beta_{x-42/x-40}$ ratio	.032	154	0.690 (NS)
Total ApoE	.133	151	0.104 (NS)
ApoE4	-.061	53	0.665 (NS)
Total Testosterone	-.235	150	0.004 ** (unadj.) 0.064 (adj.)(NS)
Free Testosterone	-.255	155	0.001 ** (unadj.) 0.017 * (adj.)
Oestradiol	-.173	126	0.053 (NS)
Total Cholesterol	.010	155	0.904 (NS)
LDL Cholesterol	-.036	155	0.655 (NS)
HDL Cholesterol	.034	155	0.671 (NS)
Brain A β Load	-.138	60	0.294 (NS)

Tables 3.3 (A) Correlation matrix of full-length plasma IDE levels with other biochemical variables (males only). Table 3.3 (A) shows correlation analysis of full-length plasma IDE with age, and plasma insulin, A β species, ApoE species, testosterone, oestradiol, lipids and brain A β load (two tailed). Methods of data collection are outlined in Section 2.2.2.1. n values vary due to missing values for some data or in the case of brain A β load only a subset of the cohort underwent PiB-PET scans. For ApoE4, n value is lower as only the *APOE* ϵ 4 carriers express this isoform. Results show that insulin demonstrates a trend toward a negative correlation ($r_s = -.239$, $p = 0.064$), however there were no other significant correlations or trends in males.

Males (A)	Correlation Coefficient (r_s)	N	Significance (2-tailed)
Sex	-	-	-
Age	.095	62	0.460 (NS)
Insulin	-.239	61	0.064 (NS)
A β_{1-40}	-.099	62	0.445 (NS)
A β_{1-42}	-.086	62	0.508 (NS)
A $\beta_{42/40}$ ratio	-.044	62	0.735 (NS)
A β_{x-40}	-.054	62	0.678 (NS)
A β_{x-42}	.078	62	0.547 (NS)
A $\beta_{x-42/x-40}$ ratio	.076	62	0.558 (NS)
Total ApoE	.187	61	0.149 (NS)
ApoE4	-.258	26	0.203 (NS)
Total Testosterone	.103	62	0.427 (NS)
Free Testosterone	.073	62	0.574 (NS)
Oestradiol	-.192	60	0.142 (NS)
Total Cholesterol	.081	62	0.533 (NS)
LDL Cholesterol	.071	62	0.582 (NS)
HDL Cholesterol	.023	62	0.862 (NS)
Brain A β Load	-.086	28	0.664 (NS)

Tables 3.3 (B) Correlation matrix of full-length plasma IDE levels with other biochemical variables (females only). Table 3.3 (B) shows correlation analysis of full-length plasma IDE with age, and plasma insulin, A β species, ApoE species, testosterone, oestradiol, lipids and brain A β load (two tailed). Methods of data collection are outlined in Section 2.2.2.1. n values vary due to missing values for some data or in the case of brain A β load only a subset of the cohort underwent PiB-PET scans. For ApoE4, n value is lower as only the *APOE* ϵ 4 carriers express this isoform. Results show no significant correlations or trends in females.

Females (B)	Correlation Coefficient (r_s)	N	Significance (2-tailed)
Age	-.064	93	0.540 (NS)
Insulin	.060	92	0.573 (NS)
A β ₁₋₄₀	-.084	92	0.426 (NS)
A β ₁₋₄₂	.118	92	0.262 (NS)
A β _{42/40} ratio	.152	92	0.147 (NS)
A β _{x-40}	-.061	92	0.566 (NS)
A β _{x-42}	-.056	92	0.596 (NS)
A β _{x-42/x-40} ratio	-.005	92	0.959 (NS)
Total ApoE	.072	90	0.503 (NS)
ApoE4	.035	27	0.863 (NS)
Total Testosterone	-.082	88	0.449 (NS)
Free Testosterone	-.079	93	0.454 (NS)
Oestradiol	-.036	66	0.774 (NS)
Total Cholesterol	-.087	93	0.408 (NS)
LDL Cholesterol	-.087	93	0.405 (NS)
HDL Cholesterol	-.143	93	0.172 (NS)
Brain A β Load	-.169	32	0.355 (NS)

When stratified by *APOE* $\epsilon 4$ status (Tables 3.4 (A) and (B)), for the non- $\epsilon 4$ carriers there were positive correlations observed between full-length IDE levels and sex ($r_s = .314$, $p = 0.002$) and total ApoE ($r_s = .232$, $p = 0.021$), and inverse correlations with $A\beta_{1-40}$ ($r_s = -.230$, $p = 0.023$), total testosterone ($r_s = -.282$, $p = 0.006$), free testosterone ($r_s = -.306$, $p = 0.002$), oestradiol ($r_s = -.242$, $p = 0.034$) and brain $A\beta$ load ($r_s = -.401$, $p = 0.019$). Following Bonferroni-Holm correction however, only gender ($p = 0.036$) and free testosterone ($p = 0.034$) remained significant. For the $\epsilon 4$ carriers, no significant correlations were seen between full-length IDE and any of the variables tested.

Table 3.4 (A) Correlation matrix of full-length plasma IDE levels with other variables for *APOE ε4* non-carriers only. Plasma levels of full-length IDE were analysed for relationships with sex, age, and plasma insulin, Aβ species, ApoE species, testosterone, oestradiol, lipids and brain Aβ load (two tailed) after stratifying data by *APOE ε4* status (methods of data collection are outlined in Section 2.2.2.1). n values vary due to missing values for some data or in the case of brain Aβ load only a subset of the cohort underwent PiB-PET scans. For ApoE4, no values are shown as only the *APOE ε4* carriers express this isoform. Results show significant positive correlations between IDE levels and sex ($r_s = .314$, $p = 0.002$) and total ApoE ($r_s = .232$, $p = 0.021$), and inverse correlations with Aβ₁₋₄₀ ($r_s = -.230$, $p = 0.023$), total testosterone ($r_s = -.282$, $p = 0.006$), free testosterone ($r_s = -.306$, $p = 0.002$), oestradiol ($r_s = -.242$, $p = 0.034$) and brain Aβ load ($r_s = -.401$, $p = 0.019$). Only gender ($p = 0.036$) and free testosterone (0.034) remained significant following Bonferroni-Holm correction for multiple testing. * indicates significance at the 0.05 level, ** indicates significance at the 0.01 level.

<i>APOE ε4</i> non-carriers only (A)	Correlation Coefficient (r_s)	N	Significance (2-tailed)
Sex	.314	98	0.002 ** (unadj.) 0.036 * (adj.)
Age	-.038	98	0.708 (NS)
Insulin	-.050	96	0.628 (NS)
Aβ ₁₋₄₀	-.230	98	0.023 * (unadj.) 0.299 (adj.)(NS)
Aβ ₁₋₄₂	.022	98	0.828 (NS)
Aβ _{42/40} ratio	.168	98	0.099 (NS)
Aβ _{x-40}	-.151	98	0.138 (NS)
Aβ _{x-42}	-.017	98	0.865 (NS)
Aβ _{x-42/x-40} ratio	.093	98	0.364 (NS)
Total ApoE	.232	98	0.021 * (unadj.) 0.294 (adj.)(NS)
ApoE4	-	-	-
Total Testosterone	-.282	94	0.006 ** (unadj.) 0.096 (adj.)(NS)
Free Testosterone	-.306	98	0.002 ** (unadj.) 0.034 * (adj.)
Oestradiol	-.242	77	0.034 * (unadj.) 0.408 (adj.)(NS)
Total Cholesterol	.003	98	0.977 (NS)
LDL Cholesterol	-.030	98	0.772 (NS)
HDL Cholesterol	-.024	98	0.811 (NS)
Brain Aβ Load	-.401	34	0.019 * (unadj.) 0.285 (adj.)(NS)

Table 3.4 (B) Correlation matrix of full-length plasma IDE levels with other variables for *APOE ε4* carriers only. Plasma levels of full-length IDE were analysed for relationships with sex, age, and plasma insulin, Aβ species, ApoE species, testosterone, oestradiol, lipids and brain Aβ load (two tailed) after stratifying data by *APOE ε4* status (methods of data collection are outlined in Section 2.2.2.1). n values vary due to missing values for some data or in the case of brain Aβ load only a subset of the cohort underwent PiB-PET scans. Results show no significant correlations between full-length plasma IDE and any other variables tested.

<i>APOE ε4</i> carriers only (B)	Correlation Coefficient (r_s)	N	Significance (2-tailed)
Sex	.197	54	0.153 (NS)
Age	-.037	54	0.791 (NS)
Insulin	.001	54	0.997 (NS)
Aβ ₁₋₄₀	.115	53	0.411 (NS)
Aβ ₁₋₄₂	-.039	53	0.783 (NS)
Aβ _{42/40} ratio	-.129	53	0.359 (NS)
Aβ _{x-40}	.069	53	0.622 (NS)
Aβ _{x-42}	-.099	53	0.481 (NS)
Aβ _{x-42/x-40} ratio	-.091	53	0.519 (NS)
Total ApoE	-.145	53	0.301 (NS)
ApoE4	-.061	53	0.665 (NS)
Total Testosterone	-.123	53	0.381 (NS)
Free Testosterone	-.140	54	0.314 (NS)
Oestradiol	-.116	46	0.444 (NS)
Total Cholesterol	.021	54	0.878 (NS)
LDL Cholesterol	.007	54	0.960 (NS)
HDL Cholesterol	.115	54	0.409 (NS)
Brain Aβ Load	-.005	25	0.983 (NS)

3.3.17.1 The ratio of full-length IDE to insulin is not significantly different between classifications.

IDE has a high affinity for both insulin and A β and as a result they may compete for the enzyme in the circulation. This does depend on a plethora of other factors such as binding affinities of these peptides (and other IDE substrates) for IDE, plasma levels of the free peptides (as opposed to those bound to other proteins), levels of other IDE substrates, levels of other enzymes that degrade one or both of these peptides (for example neprilysin, amongst others, can also degrade A β), binding/clearance by other tissues, and conformation of the peptides. For many reasons, it is possible that the ratio of IDE to insulin in plasma may help to differentiate between HC and MCI and/or AD. Therefore a ratio was calculated between the untransformed measurements of full-length plasma IDE and plasma insulin levels and compared between clinical classifications (Figure 3.23(A)). The results showed no significant differences in the ratio of full-length IDE to insulin ($p = 0.092$), although the ratio tended to be a little higher in MCI compared with HC group. It was concluded that there was no overall improvement when considering full-length IDE levels relative to insulin for differentiating between clinical classifications, compared with IDE alone.

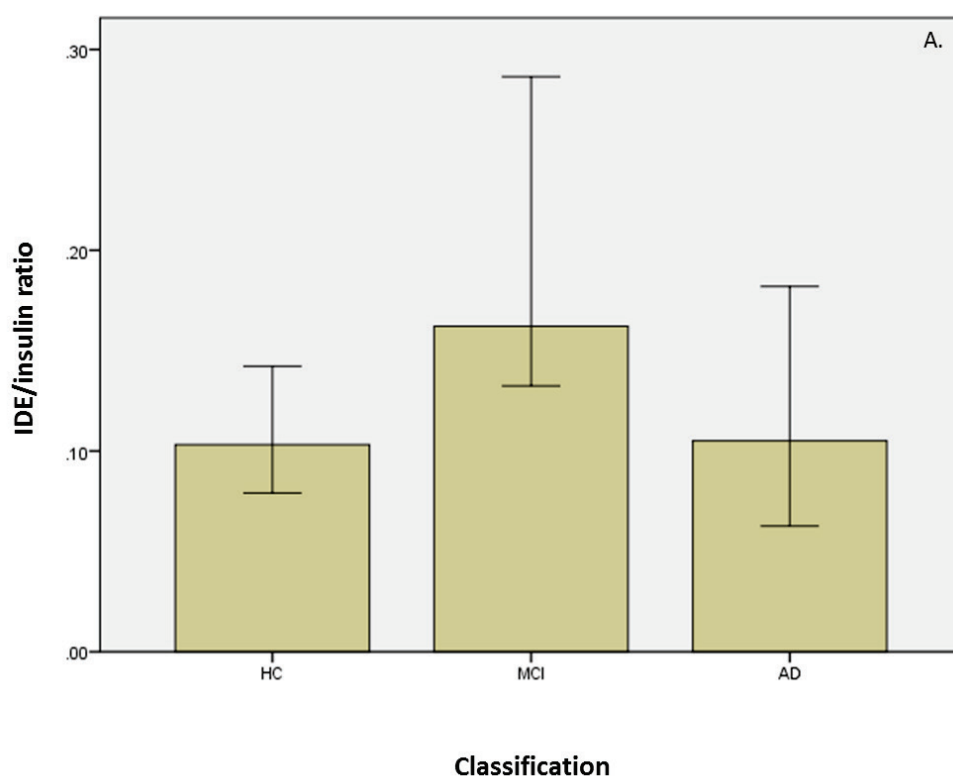


Figure 3.23(A) The ratio of full-length IDE to insulin is not significantly different between classifications. Insulin levels were measured as outlined in Section 2.2.2.1. Plasma samples (60 µg/lane total protein in duplicate) were resolved by 10% SDS-PAGE, transferred to Western blots and probed for full-length IDE as described in Sections 2.2.4.1 and 2.2.4.4, using Ab 3. The average optical density of the duplicate bands was obtained for each sample, and a ratio was calculated of (untransformed) full-length plasma IDE to plasma insulin (IDE/Insulin ratio). The median of these values were compared between classifications by a Kruskal-Wallis test. Data represents medians with 95% confidence interval (CI), n = 153 (89 HC/25 MCI/39 AD). Results show no significant differences in the IDE/insulin ratio between classifications (p = 0.092).

3.3.17.2 The ratio of full-length IDE to total A β (42+40) tends to be higher in MCI compared with HC.

Similarly to the IDE/insulin ratio, the levels of full-length IDE relative to the levels of A β_{1-42} and A β_{1-40} may be more informative with respect to differentiating between clinical classifications of AD and MCI with controls, than levels of IDE alone. It has been demonstrated that plasma A β is generally elevated in early AD compared with controls and reduces as A β is deposited into SP in the brain (Kawarabayashi, *et al.*, 2001; Schupf, *et al.*, 2008), therefore the ratio of plasma IDE/A β levels may be pertinent in the prodromal stages of AD with respect to the regulation of plasma A β levels. This may be reflected in the MCI group, which represents the pre-symptomatic period prior to full-blown AD symptoms becoming apparent. Therefore, a ratio of plasma full-length IDE with total levels of A β_{1-42} + A β_{1-40} were calculated and compared between clinical classifications (Figure 3.23(B)). Results showed that the ratio of full-length IDE/A β_{42+40} demonstrated a trend toward an increase in MCI compared with HC, however did not quite reach significance ($p = 0.052$). Similar to the IDE/insulin ratio, it was concluded that there were no benefits in considering IDE relative to A β_{42+40} compared with IDE alone, for differentiating between clinical classifications.

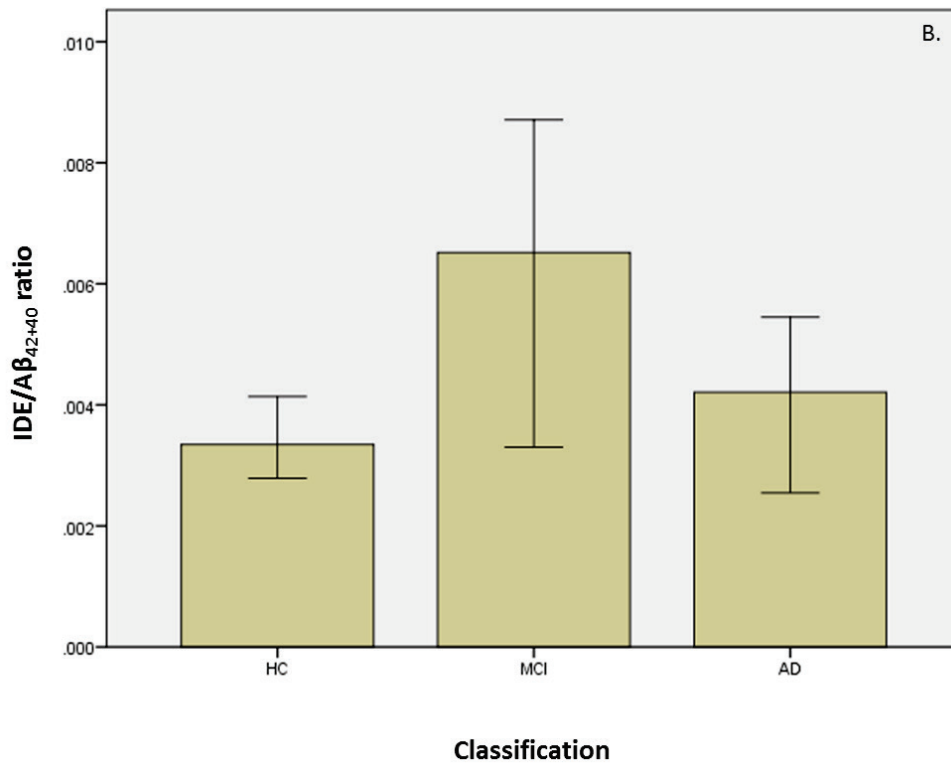


Figure 3.23(B) The ratio of full-length IDE to Aβ₄₂₊₄₀ is not significantly different between classifications. Aβ₁₋₄₂ and Aβ₁₋₄₀ levels were measured as outlined in Section 2.2.2.1. Plasma samples (60 μg/lane total protein in duplicate) were resolved by 10% SDS-PAGE, transferred to Western blots and probed for full-length IDE as described in Sections 2.2.4.1 and 2.2.4.4, using Ab 3. The average optical density of the duplicate bands was obtained for each sample, and a ratio was calculated of (untransformed) full-length plasma IDE to plasma Aβ₁₋₄₂ + Aβ₁₋₄₀ (IDE/Aβ₄₂₊₄₀ ratio). The median of these values were compared between classifications by a Kruskal-Wallis test. Data represents medians with 95% confidence interval (CI), n = 154 (90 HC/24 MCI/40 AD). Results show a trend towards MCI having higher full-length IDE/Aβ₄₂₊₄₀ ratios than HC, however this did not reach significance (p = 0.052).

3.3.17.3 The ratio of full-length IDE to insulin + total A β_{42+40} is not different between clinical classifications.

As both insulin and A β are competing substrates of IDE, it is possible that there is a combined effect of these which may affect their relationship with levels of full-length IDE. The total levels of these substrates relative to IDE may therefore be able to differentiate between HC, MCI and AD better than the IDE/insulin or IDE/A β_{42+40} ratios alone. Thus, to investigate this possibility, the values of plasma insulin (in mU/L) were converted to pg/mL and added to the values of A β_{1-42} + A β_{1-40} , to give a total value of insulin+A β_{42+40} in pg/mL. The ratio of full-length IDE to insulin+ A β_{42+40} was then analysed by clinical classification to determine if they could differentiate between MCI, AD and controls better than either IDE alone, the IDE/insulin ratio or the IDE/ A β_{42+40} ratio (Figure 3.23(C)). Results showed no significant differences in IDE/insulin+A β_{42+40} ratio between clinical classifications ($p = 0.171$). Hence, it was concluded that the ratio of full-length IDE with the combination of insulin and A β_{42+40} provided no additional benefits for differentiating between clinical classifications than levels of full-length IDE alone, the IDE/insulin ratio or the IDE/A β_{42+40} ratio.

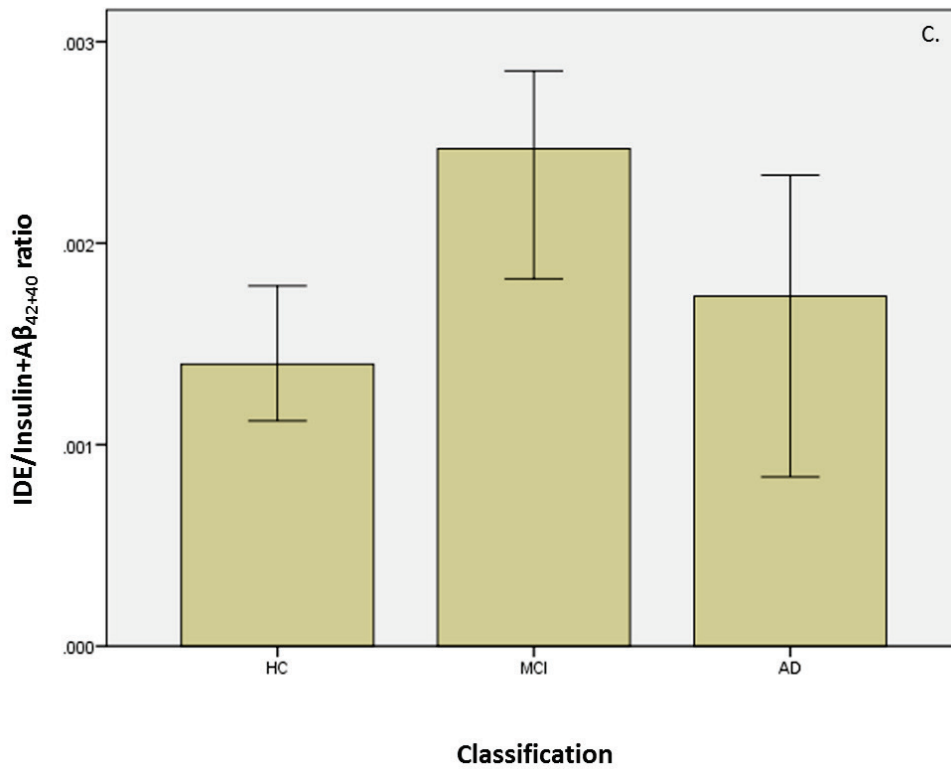


Figure 3.23(C) The ratio of full-length IDE to insulin + Aβ₄₂₊₄₀ is not significantly different between classifications. Aβ₁₋₄₂ and Aβ₁₋₄₀ levels were measured as outlined in Section 2.2.2.1. Plasma samples (60 μg/lane total protein in duplicate) were resolved by 10% SDS-PAGE, transferred to Western blots and probed for full-length IDE as described in Sections 2.2.4.1 and 2.2.4.4, using Ab 3. The average optical density of the duplicate bands was obtained for each sample, and a ratio was calculated of (untransformed) full-length plasma IDE to the combined values of plasma insulin + Aβ₁₋₄₂ + Aβ₁₋₄₀ (IDE/insulin + Aβ₄₂₊₄₀ ratio). The median of these values were compared between classifications by a Kruskal-Wallis test. Data represents medians with 95% confidence interval (CI), n = 154 (89 HC/24 MCI/39 AD). Results show no difference between clinical classifications for the full-length IDE/insulin + Aβ₄₂₊₄₀ ratio (p = 0.171).

3.4 Discussion

The aim of this chapter was to optimise a Western immunoblotting protocol to be able to quantitate levels of full-length IDE in human plasma. Using blood samples obtained from AIBL study participants, the test was used to determine if IDE levels differed in AD patients and MCI individuals compared with HC individuals, and to determine whether IDE levels were related to other AD-related genetic and biochemical factors.

While most studies of IDE involve generating in-house antibodies for use, there are a number of commercially-available antibodies which have been used in the published literature. At the commencement of this project, no other study had been undertaken on determining the levels of IDE in human plasma, therefore a number of commercially-available antibodies were screened for their suitability for this purpose. It was also important that the antibody used was able to measure levels of full-length IDE specifically, as truncated forms of IDE are not catalytically active.

Human blood plasma is a complex mixture with many constituents, and as such can present difficulties when attempting to detect low-abundance proteins. Non-specific cross-reactions may occur between the antibodies used and other proteins in the plasma, leading to false-positive results, or banding which is inconsistent with the published molecular weight of the protein of interest. Non-specific binding of the antibody to other proteins may also reduce the availability of the antibody to bind to the target protein, therefore leading to reduced detection. As such, it is necessary to determine specificity of the immune reaction when using methods such as Western immunoblotting to detect proteins in complex samples. Data was collected in the current study on three polyclonal IDE antibodies (Ab 1, Ab 2 and Ab 3) to determine if bands observed in the region of interest are in fact IDE. At the start of this study, there were no publications which had used these antibodies specifically for Western immunoblotting. It was demonstrated that for the single band detected with Ab 1(a), approximately 50% of the immunoreactivity could be blocked by pre-incubation of the antibody with full-length recombinant IDE (not the immunizing peptide), suggesting that some of the signal observed was specific, and the single band observed at 105 kDa was of a molecular weight similar to that previously reported in numerous publications. Therefore, it was likely that this band was indeed IDE. However, only a

small quantity of full-length recombinant IDE was used as the blocking protein, rather than a peptide specific to the epitope to which the antibody binds, and this may be why the band was not completely absent. In addition, 1.32 µg of full-length protein would have considerably fewer epitopes to block the binding sites of the IDE antibody than 1.32 µg of peptide specific to the paratope of the antibody. It may be that prohibitively large quantities of full-length IDE are required to block all the paratopes to prevent binding to the band on the immunoblot. However, it cannot be ruled out that another protein can be detected non-specifically by Ab 1 at the same molecular weight.

Subsequent batches of Ab 1 were unable to detect the same band in the plasma, and this may have been due to the antibody being sourced from a different bleed of the immunised animal, or even a different animal altogether. Polyclonal antibodies are essentially a mixture of antibodies which bind to different epitopes within the region of the immunogen, and sometimes to other proteins altogether. Batch to batch as well as bleed to bleed variation is not uncommon. As the immunogen for Ab 1 was derived from rat, the antibody would be expected to detect the recombinant rat IDE. However, it is possible that Ab 1(b) (Batch 2) did not contain one or more of the antibodies that were present in Ab 1(a) (Batch 1) and part of the epitope in human IDE was altered enough to resist binding of the antibodies from Batch 2, either from an alteration in the sequence or some post-translational modification that may occur in the plasma (refer to Figure 3.20 - diagram of hypothetical mechanism). Regardless, it was concluded that this antibody was unsuitable.

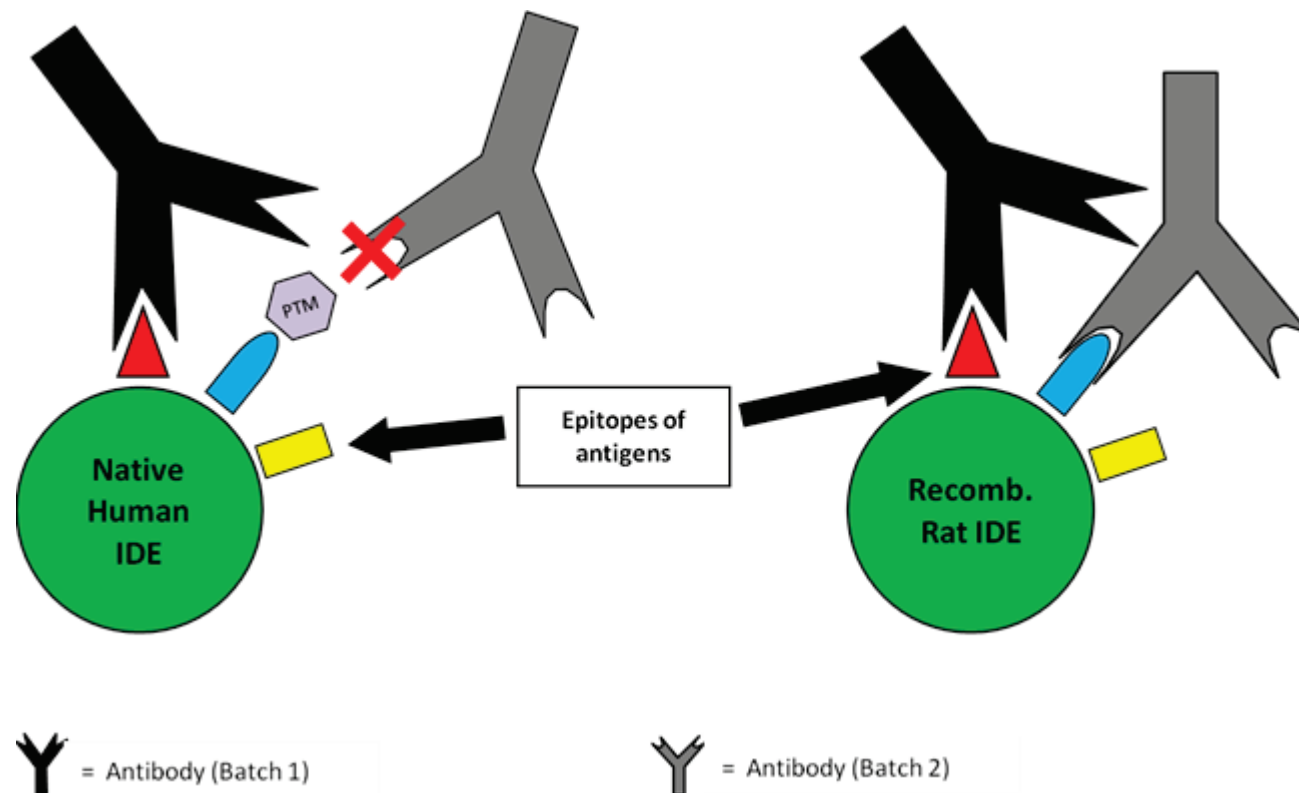


Figure 3.24 Hypothetical representation of potential mechanism of batch-to-batch variability in polyclonal antibodies. Antibodies in different batches may recognise different epitopes within the same region of the antigenic protein. Due to batch-to-batch variation in antibody generation, the presence of post-translational modifications can cause non-recognition of the protein leading to loss of binding by the antibody.

Ab 2 initially did not show immunoreactivity in the region of interest for IDE in plasma, but detected a large, band at approximately 45 kDa. This band was excised and sent for mass-spectrometry analysis, however was not able to be identified due to albumin contamination (data not shown). When testing purified rat IDE, the antibody detected a band at 118 kDa, which corresponds to the published molecular weight of full-length IDE in the UniProtKB database of 117.968 kDa. This is slightly higher than most published studies which report IDE at 110 kDa. After using extended development times or a more sensitive detection system, a band at 115-120 kDa was observed as well as other bands at lower molecular weights of approximately 80, 70 and 45 kDa. It was difficult to determine the exact molecular weight of the 115-120 kDa band as the difference between the 110 kDa and the 160 kDa marker was only several millimetres wide. Ideally, it would have been useful to run the gel for considerably longer to allow better resolution of the bands to improve the estimation of the molecular weight, or to use lower acrylamide content (8.5% gels, for example). Alternatively, a different molecular weight marker could have been used which was more specific to the region of interest. Using the enhanced detection system (ECL™ Prime) with Ab 2 dramatically enhanced the background of the blots, although not enough to impair visualisation of the bands. The background was subsequently minimised to an acceptable level by increasing the number of washing steps performed after the primary and secondary antibody incubations. Although the 115-120 kDa band was slightly higher than the published molecular weight for IDE, it is possible that some post-translational modifications may occur in plasma. Another possibility considered was that IDE-A β binding was resistant to the denaturing and reducing conditions used in the SDS-PAGE (as suggested by Llovera, *et al.*, 2008), increasing the molecular weight of the band by 4 kDa. However, pre-incubation of purified A β and purified rat IDE did not produce an upward shift in molecular weight for Ab 3, suggesting that the denaturing and reducing conditions of SDS-PAGE was sufficient to dissociate any binding. It should be noted that while this result was observed with purified protein, it cannot be ruled out that in a complex matrix such as plasma, other factors may affect the binding affinity of IDE to A β and the results

may not reflect what occurs in human plasma. Plasma IDE may also bind to another, as yet unknown, peptide or protein.

Strong cross-reactivity was noted with human serum albumin (HSA) and weakly with bovine serum albumin (BSA) using Ab 2, as well as with some of the molecular weight marker proteins. However, none of these proteins were in the molecular weight region of interest, suggesting that Ab 2 may still be a valid antibody for the measurement of IDE in human plasma. A blocking test using the immunising peptide was therefore performed to check specificity. The results showed that Ab 2 can detect purified IDE, as the purified IDE band disappeared from the Western blot when the primary antibody Ab 2 was blocked. However this was not the case with the bands seen in the plasma samples: the non-specific binding of Ab 2 to other bands in the molecular weight region of interest indicated this antibody was not going to be suitable for the assay.

A third IDE antibody was therefore screened for its suitability for measuring plasma IDE (Ab 3). This purified rabbit polyclonal detected a band at approximately 110 kDa in rat liver and human frontal cortex, as well as a band at 118 kDa for both purified rat and human IDE. In human plasma, Ab 3 detected several bands of similar molecular weight to those detected by Ab 2; 120-130, 80 and 70 kDa, as well as the characteristic wide band at 50 kDa. Using the enhanced detection system, other bands also became apparent in the plasma around the region of interest, at 160 and 115 kDa, as well as several others in rat liver and human frontal cortex. While it is possible that these extra bands are simply due to non-specific binding, it is also possible that multiple isoforms of human IDE exist in plasma.

Further analysis of Ab 3 showed strong cross-reactivity with BSA, and weak cross-reactivity with HSA, however the bands were not in the region of interest and not likely to impair visualisation of full-length IDE. With BSA, the bands were visible at 40 and 20 kDa, thus smaller than the reported molecular weight of BSA (approximately 66-67 kDa). It is possible that these bands represent degraded fragments of BSA, as a BLAST (NCBI) protein search showed three regions of BSA which share sequence similarity with regions of the immunogen for Ab 3

(<http://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed 18th February, 2014).

Interestingly, a study on BSA degradation demonstrated bands at 40 and 20 kDa (as well as some others) which correspond to the molecular weights observed in the current study (Estey, *et al.*, 2006), however it should be noted that this degradation occurred as a result of acidic conditions and may not be relevant to our study. Two regions in HSA also share sequence similarity with regions of the immunogen for Ab 3, which may explain the cross-reactivity with HSA. Alternatively, the bands seen may also represent contaminants of the BSA/HSA preparations. Furthermore, when testing a commercial blocking agent composed primarily of bovine milk powder, Ab 3 again showed some cross-reactivity. The company which produced this blocking agent stated that the blocking agent was unlikely to contain BSA or globulin, but instead contained milk proteins. Interestingly, two other unrelated IDE antibodies have been reported to cross-react with fibrinogen, casein and thrombin, all of which are found in bovine milk (Yfanti, *et al.*, 2008). A BLAST comparison of sequences revealed one region of sequence similarity (to the immunogen used to produce Ab 3) with bovine fibrinogen (gamma chain), three regions in the alpha chain, and two in the beta chain (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed 18th February, 2014). Furthermore, three regions of bovine beta-casein, and one region of bovine prothrombin also showed sequence similarity. However, human fibrinogen and beta-casein had no sequence similarity with the immunogen for Ab 3 but there were still two regions of similarity with human prothrombin. Regardless, the bands observed were not in the region of interest and therefore would not interfere with the detection of full-length plasma IDE.

As mentioned, co-incubation of purified recombinant A β with both purified recombinant rat and human IDE showed no shift in molecular weight of IDE detected with Ab 3 indicating that any binding between recombinant IDE and A β was dissociated by the denaturing and reducing conditions of the SDS-PAGE. However, it cannot be excluded that such binding observed with purified proteins and peptides may be quite different to that which occurs when the proteins are in

native form, and in their normal more complex environment, in this case – in plasma (Cavalcante Braga, *et al.*, 2013).

To remove some of the components of plasma which may be causing non-specific cross-reactivity with the antibody, and to clarify the band of interest, an immunoprecipitation (IP) procedure was performed with two separate IDE antibodies (Ab 4 and Ab 5). The IP was performed with both unprocessed plasma and also plasma which had been pre-cleared of immunoglobulins and depleted of albumin. The two antibodies produced a similar reaction pattern, with only one band remaining in the region of interest at 120-130 kDa. Interestingly, the 80 kDa band persisted following IP, and it would be of value to investigate this band in the future. However, within the scope of this project only the full-length isoform of IDE being the 120-130 kDa band was determined to be relevant.

To investigate further the specificity of the 120-130 kDa band, an antibody blocking test using the immunising peptide was performed. However, the blocking peptide significantly increased the background of the immunoblot; nevertheless, it could be seen that the band at 118 kDa from recombinant rat IDE was reduced dose-dependently, as was the 115 kDa band in rat liver, and the 95 kDa band observed in zebrafish disappeared. This indicated that the bands observed close to the region of interest in several different species and tissues were most likely IDE. In plasma, the 160 and 120-30 kDa bands were not visible after peptide treatment, however the band at 115 kDa appeared to be stronger. This would suggest that the band at 120-130 was specifically IDE, in accordance with this band also persisting after IP with a different IDE antibody. However, as the 115 kDa persisted after peptide blocking it could suggest that this band is not specific. Interestingly, the observation that the 160 kDa band also was not visible after peptide blocking was not what was expected, as this band seemed to be absent following immunoprecipitation. This would indicate that the band seen is specifically IDE. As this band was considerably higher than the expected molecular weight of IDE, it may be part of a complex of proteins including IDE, which is resistant to denaturing and reducing conditions. Unfortunately, investigating this band was outside of the scope of this project.

Our studies and other studies have found that different IDE antibodies detect different bands by Western blotting (Yfanti, *et al.*, 2008). There are a number of possible explanations for this. Firstly, each of the three antibodies used were raised against a different size epitope of IDE, for example Ab 2 was raised to an 11 amino acid peptide, whereas Ab 3 was raised to an IDE sequence of over 150 amino acids, and secondly, the regions of the IDE sequence used were all different. Thirdly, the immunogens were all synthetic, and it is not known how much of the immunogen sequences were in a secondary structure that would be similar to native or denatured IDE. Fourthly, synthetic proteins do not contain post-translational modifications which may be present on the native protein.

Yet another factor which may have an effect on the reaction patterns seen in Western immunoblotting is the species from which the immunogen was sourced: the Ab 1 immunogen was a rat IDE sequence, whereas Ab 2 and 3 were raised against human IDE sequences. The host in which the antibodies are raised can also be an issue; Ab 1 and 3 were raised in rabbits, whereas Ab 2 was raised in a goat. Regardless of this, however, even antibodies raised in the same type of host can vary from animal to animal and even from bleed to bleed, which is one major drawback of using polyclonal antibodies. A recent study demonstrated that even the method of immunisation can produce highly variable results when it comes to antibody production (Brown, *et al.*, 2011).

Following preliminary testing, Ab 3 was chosen for the measurement of full-length IDE in human plasma: the antibody has a high affinity for recombinant human IDE, and it showed specificity on a number of different tissues from different species, including the 120-130 kDa band in human plasma. Importantly, immunoprecipitation methods using two alternative IDE antibodies (Ab 4 and Ab 5) showed almost identical reaction patterns with the band at 120-130 kDa being the only remaining band to persist after IP in the region of interest. Although results are not shown in this chapter, Ab 3 also showed specificity in an IDE activity assay using several inhibitors which will be discussed in Chapter 5. Results from our group also previously demonstrated a reduction of the 110 and 80 kDa bands in an

IDE knock-down experiment in zebrafish, using Ab 3 for the immunoblotting (Chen, *et al.*, 2012).

IDE is widely reported as being 110 kDa in a number of tissues and species, therefore the band at 120-130 kDa seen with Ab 3 is larger than expected. A number of factors could be responsible for this difference. It is possible that IDE undergoes post-translational modifications in plasma such as phosphorylation, acetylation, ubiquitination and/or glycosylation. Sequence analysis of IDE using prediction programs shows that the protein contains numerous motifs and sites that may be modified by such processes (Prasad, *et al.*, 2009; Li, *et al.*, 2006; Hastie, *et al.*, 2001; Gupta & Brunak, 2002). Therefore it is possible that the increased molecular weight may be due to post-translational modifications. Another possibility is that a larger isoform may be found in human blood, however there have been no reports to date of a larger isoform existing in any other tissues, with the exception of the 115 kDa membrane-bound form of IDE and the mitochondrial isoform of IDE (Vekrellis, *et al.*, 2000; Leissring, *et al.*, 2004). However, these isoforms are unlikely to be in the peripheral circulation. It may be simply that our estimation of IDE molecular weight is different due to the lack of protein separation on the 10% acrylamide gels – the 110 kDa and 160 kDa weight markers were very close. However, the band for recombinant rat IDE was consistently observed to be higher than the 110 kDa marker, and the plasma band (estimated to be approximately 120-130 kDa) was consistently observed to be higher than that. In addition and as mentioned previously, the predicted molecular weight of the full-length isoform as determined from the UniProtKB database is 117.968 kDa, and this is similar to the molecular weight observed in the current study. In addition, this antibody detected a band in rat liver of 110 kDa which is consistent with previous reports, suggesting that it is unlikely that this is an estimation error.

It has been reported in the literature that IDE levels in the brain tend to be lower in AD patients and MCI individuals (Zhao, *et al.*, 2007; Qin & Jia, 2008; Cook, *et al.*, 2003; Perez, *et al.*, 2000), and that this drop in IDE levels has been associated with higher levels of soluble cerebral A β . A large body of evidence indicates that the levels of soluble A β in the brain are integral to the process of AD pathogenesis. It is

also known that brain A β can originate from the periphery, such that levels of soluble A β in the blood may influence brain levels of the peptide. Furthermore, it is highly likely that levels of A β -degrading enzymes in the blood play an important part in regulating the peripheral A β content. As IDE has been found to be one of the primary A β -degrading enzymes, its blood concentration may be related to the accumulation of both peripheral, and subsequently brain A β .

The levels of full-length IDE measured by Western immunoblotting in this project were found to be similar in healthy controls and AD patients. However, a significant increase in full-length IDE was observed in the MCI group compared with both controls and AD patients. Upon further analysis this observation was determined to be significant only in females, however a similar pattern existed in males. This increase in IDE levels in the MCI group is contrary to what would be expected; genetic polymorphisms in *IDE* have been associated with increased levels of plasma A β in individuals at risk of LOAD (Ertekin-Taner, *et al.*, 2004; Ertekin-Taner, *et al.*, 2008) and if IDE is primarily responsible for regulating the levels of plasma A β , as is seen in the CNS, then it might be expected that early deficits in the levels of plasma IDE could feasibly be a contributing factor to increased circulating A β . Based on the results of the current study, it is possible then that any potential reductions in levels of plasma IDE occur well before the onset of cognitive deficits which are observable in the MCI participants. Furthermore, it is therefore possible that the increase in IDE levels in MCI individuals may be indicative of a compensatory mechanism comprising the upregulation of full-length (active isoform) IDE, perhaps in response to increased levels of IDE substrates such as A β and insulin in plasma. It may be that increased A β species and/or an increase in insulin may be a very early event in AD pathogenesis which saturates all available IDE in the plasma and thus triggers its upregulation. However, in this subset of the AIBL cohort there was no significant increase observed in either A β or insulin for the MCI participants overall, and there was no correlation observed between any of the A β species or insulin and levels of full-length IDE overall. In addition, there were no significant differences between clinical classifications for any of the calculated ratios of IDE with insulin or A β (or the combination), although a trend toward a higher IDE/A β_{42+40} ratio was observed

in MCI participants compared with controls (but not in AD), which would be primarily due to the increase seen in full-length IDE levels. However, as pathogenic processes have already commenced by the MCI stage, an increase in IDE substrates may occur much earlier than this prodromal period which may return to more normal levels following the upregulation of IDE.

Alternatively, any potential deficit in IDE function may not be necessarily related to a reduction in IDE levels but possibly a reduction in IDE catalytic activity. A previous study reported increased levels of IDE concomitantly with reduced IDE activity in brain microvessels of AD/CAA patients compared with controls, and this may be related to the increase in full-length IDE levels found in the circulation in the current study (Morelli, *et al.*, 2004). The high variation evident in the MCI group also highlights heterogeneity in the group itself; not all MCI individuals will convert to AD and some will even revert to normal, therefore this is likely to be a confounding factor. It is not clear why the increase in MCI seems to be more pronounced in females than in males, however may be reflective of hormonal differences.

It is possible that levels of both A β and IDE in the plasma negatively reflect the levels in the brain. Thus high levels of IDE in the plasma may indicate low levels of IDE in the brain, and concomitantly low levels of A β in the plasma may reflect high cerebral A β levels. One group has shown this to be the case for A β_{42} after glucocorticoid treatment, with increasing levels of A β_{42} observed in the brain and a corresponding decrease observed in plasma A β_{42} (Kulstad, *et al.*, 2005). This was also accompanied by a reduction of IDE in the brain, and did not appear to affect A β_{40} levels. This would be consistent with A β_{42} being the primary species implicated in AD. Unfortunately plasma IDE was not measured in the same study so it is unknown if a corresponding increase occurs in the plasma. Another study supporting the hypothesis that IDE is primarily responsible for regulating the levels of A β showed that increases in cerebral A β_{42} (both intracellular and extracellular) were consistently associated with a reduction in IDE levels (both membrane-bound and cytosolic) in patients with a PS1 mutation (Qin & Jia, 2008). It must be noted though that early-onset familial AD may not have the same pathogenic processes as

sporadic AD. However, as previously mentioned, genetic studies have demonstrated association between IDE variants and plasma A β ₄₂ levels in LOAD families (Ertekin-Taner, *et al.*, 2004; Ertekin-Taner, *et al.*, 2008).

Accumulating evidence appears to support the notion that during the development of AD pathology, plasma A β ₄₂ levels decline as plaques develop in the brain (Kwarabayashi, *et al.*, 2001; Kuo, *et al.*, 2000; Schupf, *et al.*, 2008). One of these investigations was a large study examining both A β ₄₂ and A β ₄₀, and demonstrated that only A β ₄₂ levels in plasma were affected during the conversion to AD, with high initial levels followed by a decline during the onset of AD (Schupf, *et al.*, 2008), reflecting the deposition of A β in the brain. Another study showed that intraperitoneal inoculation of A β can also trigger amyloid formation in the brains of transgenic mice (Eisele, *et al.*, 2010). This is supportive of the theory that the elevated A β levels in the brain originate, at least in part, from the peripheral blood supply. However, it should be noted that previous work by Eisele and colleagues (2009) showed no initiation of A β deposition when injected intravenously, although soluble levels of brain A β were not monitored in that study.

A recent study using AD transgenic mice demonstrated that an increase in plasma A β levels occur concurrently with a reduction in intracellular A β in the brain, after treatment with a γ -secretase inhibitor which also acts on microglial activation (Sivilia, *et al.*, 2013). This study also reported cognitive improvement and reduced amyloid deposition in the mice. It is believed that a potential therapeutic pathway involves reducing levels of soluble A β in the brain by increasing its efflux across the BBB into the plasma, where it may be degraded by IDE and other A β -degrading pathways (Deane, *et al.*, 2009). Recent studies support this concept, as it has been demonstrated that a reduction in plasma A β leads to a reduction in cerebral A β in AD transgenic mice (Guan, *et al.*, 2009; Liu, *et al.*, 2010; Sutcliffe, *et al.*, 2011), although another recent study refutes these findings (Walker, *et al.*, 2013). However, as IDE is the primary enzyme responsible for insulin degradation in the periphery, the challenge would be to increase the degradation of A β selectively without disturbing the degradation of insulin by IDE (and other substrates). As it

has been established that IDE may be regulated selectively in this fashion by small peptide activators, and other regulatory factors, this may prove possible.

It is important to note that IDE is too large to cross the blood-brain barrier (BBB), therefore separate pools are likely to exist in the periphery and the brain. It would be unlikely that IDE would move from the brain to the periphery. Therefore it is more likely that IDE would be independently upregulated in the periphery, possibly in response to increased plasma A β or insulin. Upregulation of IDE has been reported previously, in response to A β , insulin and also various cellular stressors (Vepsäläinen, *et al.*, 2008; Zhao, *et al.*, 2004; Tundo, *et al.*, 2013).

Furthermore, it can be speculated that reduced brain IDE may initiate the increase of cerebral A β that many studies have documented, and this excess may increase transfer of A β across the BBB into the periphery. This could subsequently instigate the upregulation of plasma IDE also, however if the enzyme is unable to control the levels of peripheral A β sufficiently, the peptide may reach a critical level, disruptions to insulin metabolism may occur, both of which may then cause alterations to BBB permeability. This would probably lead to increased A β influx back into the brain and a reduction in efflux to the periphery. In support of this theory, it has been demonstrated that increased levels of brain A β are capable of upregulating IDE levels and therefore it is possible that this also occurs in the periphery (Vepsäläinen, *et al.*, 2008). The increased transport of peripheral A β to the brain, as well as the reduction in efflux (partly due to greater aggregation and deposition of A β in A β fibrillar deposits and plaques), in conjunction with the increased plasma A β degradation via elevated levels of plasma IDE may then lead to the characteristic reduction in plasma A β during A β deposition which is seen later in AD patients. The drop in A β may then lead to the subsequent down-regulation of plasma IDE as less would be required, and this is reflected in the results of the current study.

When the cohort was split into carriers and non-carriers of *APOE* $\epsilon 4$ alleles, no significant difference in full-length plasma IDE levels was observed. This is an interesting finding as it has been reported that carriers of the $\epsilon 4$ allele have lower

levels of brain IDE compared with non-carriers or controls (Cook, *et al.*, 2003). This does not support the hypothesis that plasma levels of IDE negatively reflect the levels in the brain, and instead supports the notion that brain and plasma IDE function independently. However, it would be of value to examine the results more thoroughly by way of specific *APOE* genotypes rather than just $\epsilon 4$ carriers versus non-carriers, as it has been shown that the different genotypes confer different risks for developing AD (Farrer, *et al.*, 1997). To characterise properly the effect of *APOE* genotype on IDE levels, the results should ideally be analysed after dividing according to the 6 possible *APOE* genotypes. Unfortunately the small sample size precluded this analysis due to a lack of power.

One limitation of this study is that it was not possible to analyse potential effects of medications on plasma IDE levels, due to the size of the cohort, and the fact that only a small number of participants were not taking medication. A number of compounds and medications have been implicated in the regulation of IDE (this will be discussed in more detail in Chapter 6). Therefore, as a substantial proportion of the AIBL cohort were taking at least one, or a combination of these medications at the time blood samples were taken, it is likely that these will have influenced the measured levels of plasma IDE. A significant correlation was observed between testosterone and IDE in the whole cohort, although this disappeared when analysing male and female participants separately. Oestradiol showed a trend toward association with IDE however this also disappeared when data was split by gender. This suggests that the potential relationship between IDE and these sex hormones is not necessarily gender specific. Larger cohorts and additional analysis will be required to determine if such hormones and medications influence plasma IDE levels in humans.

Another limitation which should be mentioned is the method of Western immunoblotting itself. As there were a large number of samples, and only five samples could be run on each gel in duplicate, a large number of gels were needed. An internal standard of pooled control plasma was included in each gel, to which sample results were normalised to control for inter-gel variation. However, the large number of gels would have increased the chance that errors were going to

occur when normalising to correct for inter-gel variation. Another source of variation in the experiment was due to the step in which the blots were stripped of antibodies following the initial immunoblotting procedure, and re-probed with a different primary antibody; although this is generally considered an acceptable practice.

Western immunoblotting is considered to be a semi-quantitative procedure, and subject to variation even through such things as the method of quantification (Gassmann, *et al.*, 2009). Therefore, even a small degree of imprecision may affect the means or medians, and as such it cannot be excluded that during normalisation procedures the trend of this data may have been affected by these factors.

3.4.1 Summary

The results in this chapter demonstrate that levels of plasma full-length IDE are elevated in MCI individuals in comparison to healthy controls as well as AD patients, and that no difference is observed between healthy controls and AD. In addition, no difference in plasma IDE levels was seen in *APOE* $\epsilon 4$ carriers versus non-carriers. These results for full-length plasma IDE were contrary to what was expected, as it conflicts with the hypothesis that low plasma IDE levels are an initiating factor in the development of AD. However, one could argue that AD pathogenesis starts well before MCI stages, as AD-related early memory loss often indicated by MCI indicates that synaptic and neuronal loss have already occurred to an extent.

Based on these results and others from the AIBL study, an alternative chain of events may be occurring. Firstly, it is possible that a trigger such as high plasma A β and/or insulin initiate an upregulation of plasma IDE. Although within this subset of the AIBL cohort there was no significant increase of either A β_{1-40} , A β_{1-42} or insulin observed in MCI, potentially this may have occurred well prior to the MCI stage. However, recent results from the AIBL study have shown that individuals who convert from healthy to MCI have a reduction in plasma A β_{42} , while an increase is seen with A β_{40} in MCI and AD participants after controlling for various factors such

as gender and *APOE* status, among others (Rembach, *et al.*, 2014). As $A\beta_{40}$ encompasses the majority of the $A\beta$ species, this alone may be adequate for the saturation of IDE, which may potentially lead to its upregulation in the plasma. Furthermore, even though the full-length $A\beta_{40}$ and $A\beta_{42}$ peptides were not significantly increased, a significant increase in $A\beta_{x-40}$ (but not $A\beta_{x-42}$) was observed within this subset. This also highlights the need to consider $A\beta_{42}$, $A\beta_{40}$ and $A\beta$ fragments separately in addition to as a single species of peptide. BBB damage may then contribute to increased influx and decreased efflux of excess $A\beta$ to/from the brain, respectively, and lead to the characteristic accumulation of cerebral $A\beta$ seen in AD. The subsequent decrease in plasma $A\beta$ that has been documented may then be a result of both $A\beta$ trafficking to the brain and increased plasma degradation of $A\beta$ due to elevated plasma IDE. Reports from another recent study have shown that alterations of plasma insulin can occur at the MCI stage, but again appear to be influenced by gender and *APOE* status (Cholerton, *et al.*, 2012). Collectively, these results outline the complexity of the mechanisms surrounding AD pathology and highlight the need to take into account various factors such as gender, *APOE* genotype and differentiating between $A\beta$ species when translating the data. It would be useful in future studies to examine blood IDE at regular intervals in AD transgenic animals from a very early age to see if the decrease in plasma $A\beta$ that has been documented occurs prior to or concurrently with an increase in plasma IDE, or alternatively if the decrease occurs following an increase in plasma IDE, as well as monitoring plasma insulin levels.

The overall results though suggest that the differences observed in plasma full-length IDE levels measured with Western immunoblotting could be reflective of biochemical changes in the prodromal period of AD, and that IDE may be a suitable biomarker candidate to include in a panel of biomarkers for early detection. Various limitations, however, such as the effects of medications, sex hormones, gender and *APOE* status among others, on plasma IDE must be acknowledged and need further investigation before any conclusions may be formed from these results. In addition, studies that progressively monitor levels of IDE from a non-pathological state through the conversion to AD would provide more information to

the comparison of MCI and AD with controls and would be of considerable value as a future direction.

(THIS PAGE HAS BEEN INTENTIONALLY LEFT BLANK)

CHAPTER 4 – Total plasma IDE Levels measured by enzyme-linked immunosorbent assay (ELISA) are decreased in MCI in comparison to healthy controls or AD.

4.1 Introduction

Western blotting techniques are widely used in research and have several advantages over other immunoaffinity applications for the detection of proteins; one being good sensitivity to detect low-abundance proteins in a complex sample such as plasma, and also the specificity to differentiate between proteins based on molecular weight. However, there are some limitations to Western immunoblotting; firstly it is considered a semi-quantitative application, and secondly it is not ideal for use when measuring a large number of samples due to the inter-assay variation. In addition, the proteins in Western blotting are usually in a denatured and reduced conformation compared to the native protein. Hence some antibodies may not detect specific proteins as they bind to conformational epitopes and this generally occurs when using antibodies that are raised against the full-length protein. There are other immunoaffinity applications which are more suited to protein quantification and also more suited to processing large numbers of samples, such as the enzyme-linked immunosorbent assay, or ELISA. Using a 96-well plate, this application permits the simultaneous measurement of up to 40 samples in duplicate per assay, including an 8-point standard curve (in duplicate).

One common limitation of ELISA-based detection of proteins is the inability to differentiate between different isoforms of a protein. However, several publications have reported total levels of IDE (primarily in brain), and it would be useful to characterise IDE in human plasma by ELISA and compare with the results from these other studies. The measurement of total plasma IDE using an ELISA may also uncover differences compared to measuring with Western immunoblotting: ELISAs may prove more suitable for measuring IDE as a clinical biomarker. There is

one commercially available ELISA kit for the measurement of IDE, however it was found to be unsuitable for our purposes. Therefore an in-house ELISA was developed for the purpose of measuring total IDE in human plasma from the AIBL cohort.

4.1.1 Aims

1. To develop an in-house ELISA for the purpose of measuring total IDE levels in human plasma.
2. To determine if levels of total IDE differ between HC, MCI and AD participants.
3. To determine if the presence of the *APOE4* allele has any impact on levels of total plasma IDE.
4. To identify any relationships between total plasma IDE, full-length IDE and other biochemical data obtained from the AIBL study.

4.2 Experimental Summary

4.2.1 Test of commercial IDE ELISA kit for suitability in testing human plasma.

To screen a commercial ELISA kit to determine its suitability for the measurement of IDE in human plasma, the assay was performed twice (Run 1 and Run 2) as per manufacturer instructions, as described in Section 2.2.5.1.

4.2.2 Screening of eight commercial IDE antibodies to determine suitability for an in-house, indirect IDE ELISA for use on human plasma samples.

Antibodies that were screened for their suitability to be used in an indirect ELISA for plasma IDE are listed in Section 2.1.1 and in Table 2.1. An indirect ELISA was performed using each of the antibodies as described in Section 2.2.5.2 using purified rat IDE as a standard, and plasma, both neat and diluted.

4.2.3 Test of the three most suitable IDE antibodies for an IDE sandwich ELISA.

Using IDE antibody ab28561 (Ab 3) as the coating antibody, a sandwich ELISA was performed using either H00003416-A01 (Ab 6) or LS-C55007 (Ab 2) as detection antibody, as described in Section 2.2.5.3. Purified rat IDE was used as a standard, and both neat and diluted plasma samples were tested.

4.2.4 Assay control tests.

Using IDE antibody ab28561 (Ab 3) as coating antibody, and LS-C55007 (Ab 2) as detection antibody, a sandwich ELISA was performed as described in Section

2.2.5.3, with minor changes. The coating buffer was at a pH of 8.6 rather than 9.6; and no Tween detergent was present in the PBS used for the washing steps, with the exception of the wash following the secondary antibody incubation. The blocking agent used in this protocol was Fraction V BSA. Omission of the capture and detection antibodies, and the blocking agent was tested to ensure there was no interference by any of these components in the assay, and these controls used recombinant rat IDE at a concentration of 625 ng/mL as the control sample.

4.2.5 Optimisation of coating conditions for the IDE sandwich ELISA.

Using IDE antibody ab28561 (Ab 3) as coating antibody, and LS-C55007 (Ab 2) as detection antibody, a sandwich ELISA was performed as described in Section 2.2.5.3. The conditions of coating overnight at 4°C was compared with coating for 2 hr at room temperature, as well as the effect of the buffer pH of 9.6 compared with 8.6. Purified rat IDE was used for the standard, and plasma was tested, both neat and diluted.

4.2.6 Optimisation of sample and antibody diluents in the IDE ELISA.

For this purpose, the assay was performed as described in Section 2.2.5.3, using PBST alone for diluting the samples, detection antibody and secondary antibody, compared with adding 1% bovine serum albumin (BSA) to the sample diluents, or adding 2% polyethylene glycol (6000) to the detection antibody diluent. Purified rat IDE was used for the standard, and plasma was tested, diluted 1:1 and 1:2.

4.2.7 Optimisation of detection antibody incubation in the IDE ELISA.

To determine if overnight incubations of the detection antibody at 4°C can improve the signal obtained in the sandwich ELISA, the ELISA was performed as described in Section 2.2.5.3 (Ab 3 and 2), using a 2 hr incubation at room temperature for the

detection antibody, compared with an overnight incubation at 4°C. Purified rat IDE was used for the standard, and five individual plasma samples were used diluted 1:2.

4.2.8 Investigation of the upper detection limit of the IDE sandwich ELISA.

For this purpose, the experiment was performed as described in Section 2.2.5.3. An 11-point standard curve of recombinant rat IDE ranging from 50 to 64000 ng/mL and five individual plasma samples (1:2) were included in the assay.

4.2.9 Optimisation of coating antibody concentration and extension of standard curve for upper detection limit analysis in the IDE sandwich ELISA.

The ELISA was performed as previously described in Section 2.2.5.3. Three concentrations of coating antibody (Ab 3) were used on a standard curve of recombinant rat IDE to determine the optimal concentration (5, 1 and 0.1 µg/mL). The IDE concentrations ranged from 50 to 160000 ng/mL.

4.2.10 Test of non-albumin containing blocking agent to reduce background in the IDE sandwich ELISA.

To try and decrease the elevated background absorbance, the ELISA was performed as described in Section 2.2.5.3, substituting 3% ECL™ Blocking agent in the blocking buffer. Recombinant rat IDE was used for the standard curve ranging from 500 to 16000 ng/mL, and PCP was diluted 1:2.

4.2.11 Measurement of total plasma IDE levels in a subset of the AIBL cohort.

Total IDE levels of 177 samples from the AIBL cohort (57 HC/50 MCI/70 AD) were measured with the in-house sandwich ELISA as detailed in Section 2.2.5.3. Recombinant rat IDE was used for the standard curve ranging from 50 to 16000 ng/mL and an internal standard of pooled control plasma (PCP) was included on each plate for normalisation of inter-assay variance (all in duplicate). AIBL samples were all assayed in duplicate.

4.2.12 Testing for potential cross-reactivity of BSA (standard and Fraction V) and HSA in the IDE sandwich ELISA.

The IDE sandwich ELISA was performed as described in Section 2.2.5.3. BSA (standard and Fraction V) and HSA were prepared as outlined in Section 2.2.5.3.1 at concentrations equivalent to the lowest and highest levels of albumin likely to be found in human serum, then diluted as per PCP. PCP was diluted 1:2. Fraction V BSA was used for the blocking step.

4.2.13 Spike and recovery assessment of IDE sandwich ELISA.

To assess the effect of plasma proteins and other plasma constituents on the detection of IDE, recombinant rat IDE was diluted 1) in PBST (control), and 2) in PBST-diluted plasma (1:2) to provide matching standard curves ranging from 1250 to 10000 ng/mL IDE. The IDE ELISA was performed as outlined in Section 2.2.5.3. Absorbance value of the diluted plasma blank (no spike) was subtracted from the plasma diluted standard curve to account for the signal from the plasma itself.

4.3 Results

4.3.1 The commercial IDE ELISA kit is unsuitable for the testing of human plasma samples.

When using commercial kits to measure proteins in a complex mixture, it is important to test the kit to determine if it produces expected results. Therefore, the commercial IDE ELISA kit that was available at the time was trialled using two human plasma samples at various dilutions. The test was carried out twice to ensure the results were accurate. The results showed that goodness of fit of the standard curve was $r^2 = 0.8674$ (Run 1) and $r^2 = 0.9858$ (Run 2) (Figure 4.1(A)). There was considerable difference in the absorbance values of the standard curve for each Run; the maximum absorbance (50 ng/mL) for Run 1 was 1.77 compared with Run 2 which was only 1.04. In addition, there was little or no correlation between plasma sample dilution levels and measured levels of IDE for either run, with high inconsistency between Run 1 and Run 2 (Figure 4.1(B)).

To address the possibility that the poor standard curve in Run 1 may have affected the interpolated plasma values and therefore contributed to the variation observed, raw absorbance values of the plasma dilutions were normalised to the mean of the blanks and compared between Run 1 and Run 2 (Figure 4.1 (C)). Results demonstrated that the plasma dilutions showed considerable variation independently of the standard curve. Inter-assay variation was up to 56%, while intra-assay variation was up to 30%. It was considered to be particularly important to have consistency between assays due to the fact that the sample size was large, requiring more than one plate, and therefore plates needed to be compared. Although some assay-to-assay and within-assay variation is expected (preferably <15%), these results demonstrated that there was excessive inter- and intra-assay variation with this commercial ELISA kit.

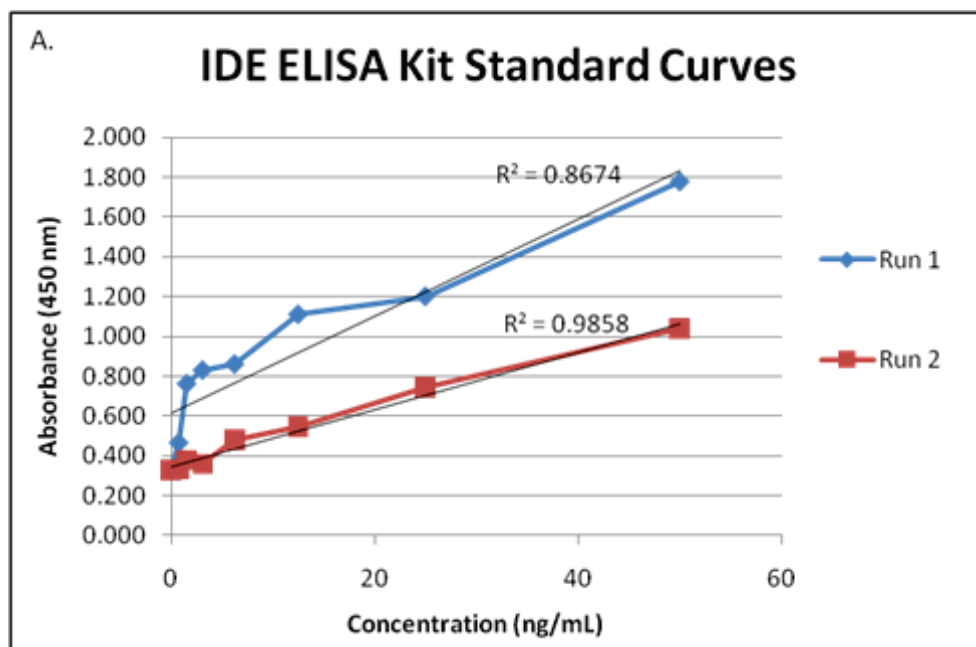


Figure 4.1 (A) Comparison of standard curves from Run 1 and Run 2, using the commercial IDE ELISA kit. Assay was performed as detailed in Section 2.2.5.1. Standards were prepared in a range of 0.78 ng/mL to 50 ng/mL as per manufacturer instructions. Results showed considerable differences in the standard curves for each Run; goodness of fit was $r^2 = 0.8674$ with a maximum absorbance value of 1.77 for Run 1 compared with $r^2 = 0.9858$ and a maximum absorbance value of 1.04 for Run 2.

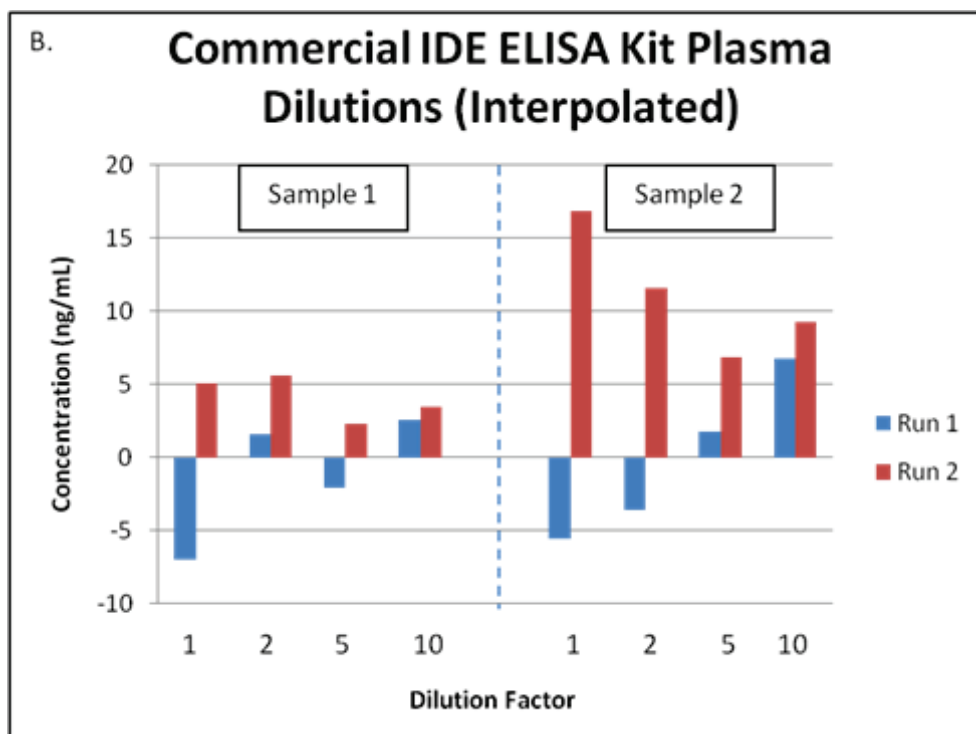


Figure 4.1 (B) Test of commercial IDE ELISA kit with human plasma (Run 1 versus Run 2). Assay was conducted as detailed in Section 2.2.5.1. Individual human plasma samples (2) were prepared as per manufacturer instructions and diluted up to 1:10 (4 dilutions). Results show that the interpolated concentrations from the plasma dilutions were not dose-responsive for the plasma in either run. In addition, concentrations for the plasma samples were inconsistent between Run 1 and Run 2. The interpolated concentration of undiluted plasma for sample 1 in Run 1 was below the limit of detection compared with 5.06 ng/mL in Run 2. The interpolated concentration for undiluted sample 2 was below the limit of detection in Run 1 and 16.85 ng/mL in Run 2. The results indicate that this kit has high inter-assay variation and poor linearity of dilution.

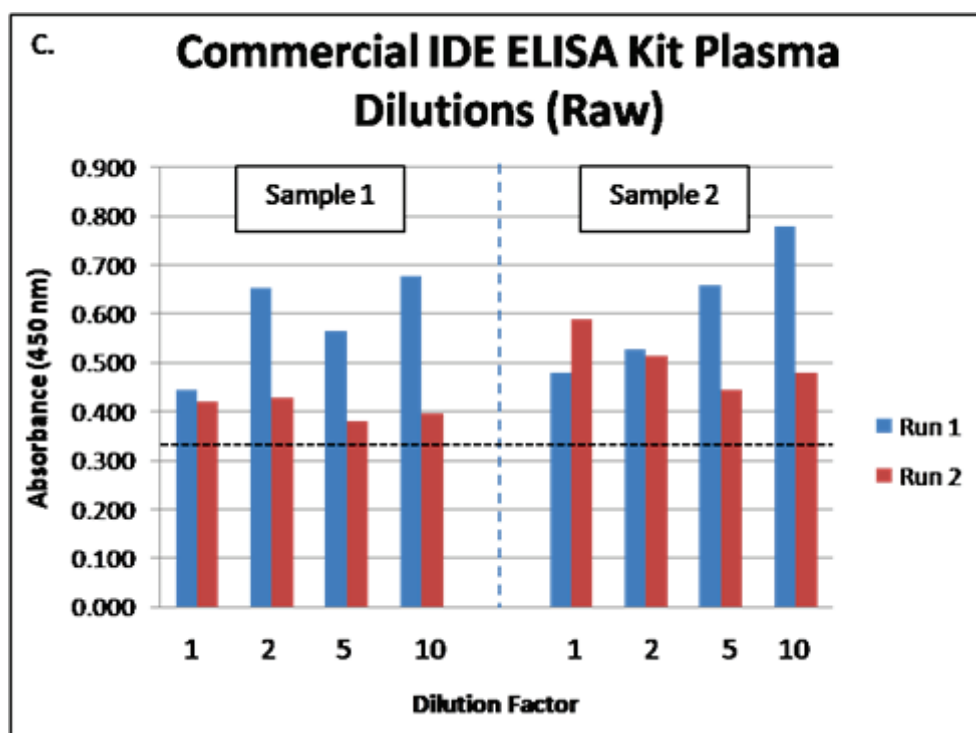


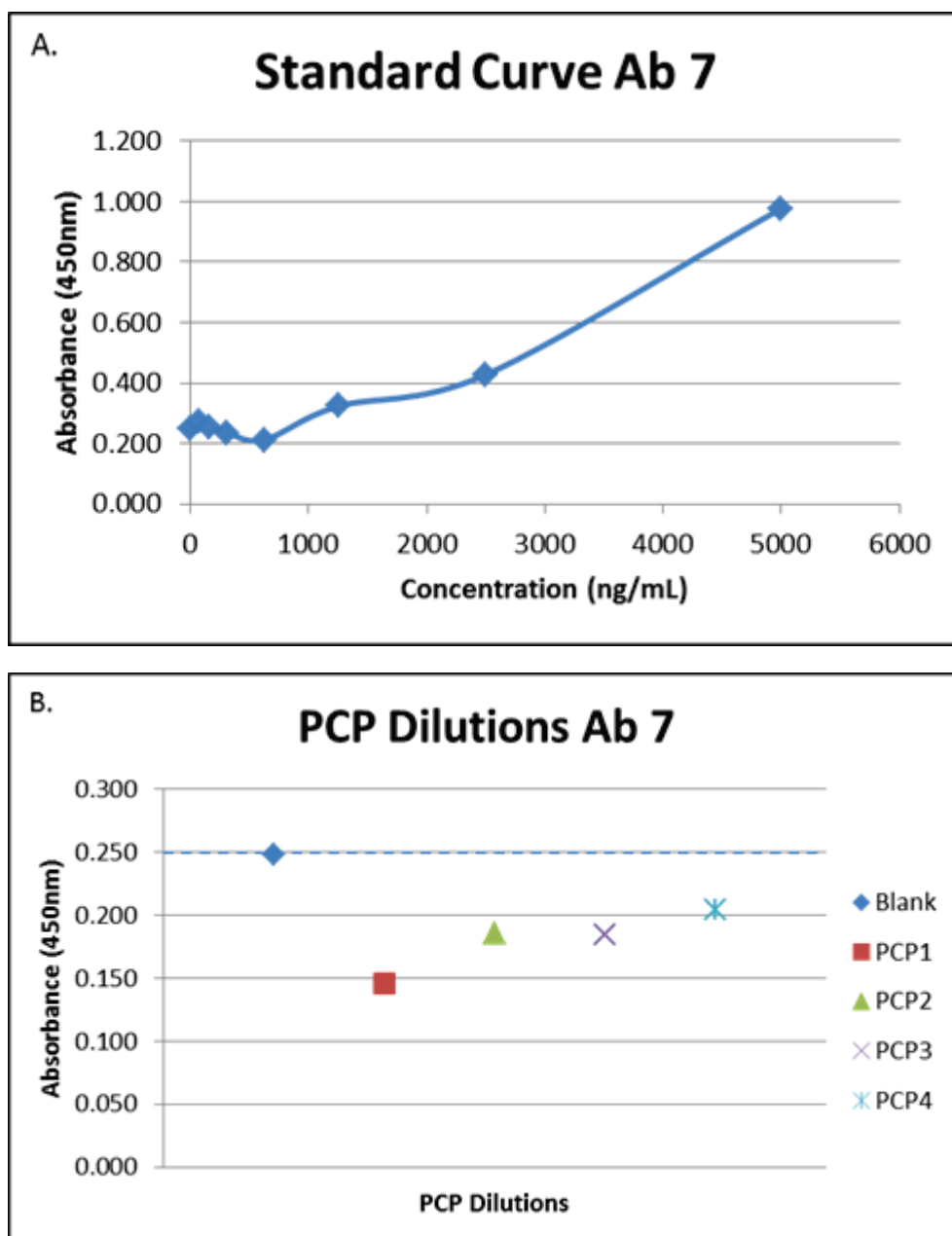
Figure 4.1 (C) Test of commercial IDE ELISA kit with human plasma – raw values (Run 1 versus Run 2). Individual human plasma samples (2) were prepared as per manufacturer instructions and diluted up to 1:10 (4 dilutions). The dashed (black) line indicates background absorbance (blank). Results showed no linearity of dilution and poor concordance between runs.

4.3.2 Indirect ELISA detection is effective for purified rat IDE, but not for IDE in human plasma.

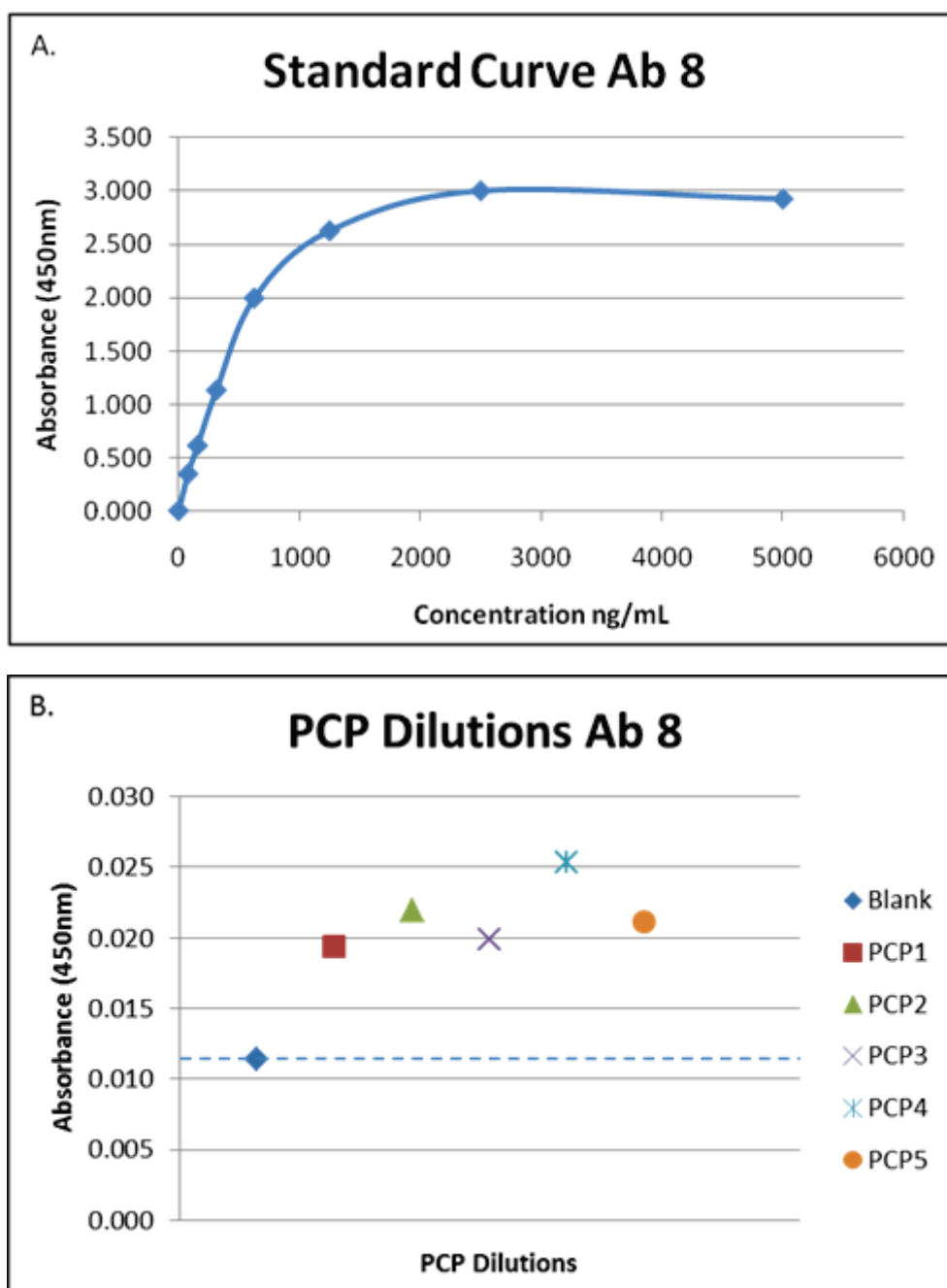
As the commercial IDE ELISA kit was determined to be unsuitable for measurement of plasma IDE in the AIBL study, development of an in-house ELISA assay was required. As a result, there were a number of important factors to consider. Two options were available – an indirect ELISA and a sandwich ELISA. As an indirect ELISA is simpler, requiring only one detection antibody, this was trialled first. With an indirect ELISA, the antigen or sample is directly adsorbed to a surface (microplate well) and the protein of interest is detected by the addition of a specific antibody to which a labelled secondary antibody is used to quantify the protein present. An ideal antibody would have low background, produce a high dynamic

range of the standard curve, and demonstrate good linearity of dilution. Not all antibodies are effective for use in ELISA, especially if the antibody has been raised against a small peptide. This may produce antibodies which prefer a linear conformation of the antigen, which may be buried in the structure of the natively-folded protein. However, this is not always the case, as some linear epitopes are presented on the surface of the native enzyme. In addition, it is important to determine if there is any cross-reactivity of the antibodies (primary and secondary) to non-specific components of the sample matrix.

Eight commercially-available antibodies were selected for screening for the development of an in-house indirect IDE ELISA; A-MMS-282R (monoclonal), B-GTX111664, C-AB9210, D-250706, E-5282-1 (monoclonal), F-H00003416-A01, G-LS-C55007 and H-ab28561; designated Abs 1-4 and Abs 6-9 as summarised in Table 2.1 (Figure 4.2, A-H). The screening tests used purified rat IDE for the standard curve and pooled control plasma (PCP) both undiluted and using a range of dilutions. Results showed that all antibodies detected the purified IDE dose-dependently, although the dynamic range varied considerably. The three antibodies with the highest dynamic range were MAb 9 (Graph set E), Ab 8 (Graph set B) and Ab 3 (Graph set H), respectively. However, the results for the plasma samples varied considerably, with a majority of the antibodies demonstrating a dose-dependent signal in the reverse direction. For monoclonal MAb 7, the plasma signals fell below the background signal (Graph set A). With antibodies Ab 8, Ab 1 (Graph set C) and Ab 4 (Graph set D) the plasma signals observed were not dose-dependent and were situated toward the lower end of the standard curve. When testing MAb 9 (Graph set E), plasma samples showed very little difference in signal between the undiluted samples and those diluted 1:5, and OD readings were almost as low as the background signal. Of the three remaining antibodies; Ab 6, Ab 2 and Ab 3 (Graph sets F-H, respectively), all showed good dynamic range of the standard curve with the highest being for Ab 3. For the plasma (neat and diluted) samples, all three antibodies showed a similar reverse trend, however for Ab 6 and Ab 2, the dynamic range of the plasma dilutions was much lower than Ab 3.



Figures 4.2 A (A) and (B) Screening of monoclonal IDE antibody MMS-282R (MAb 7). Test of indirect ELISA using mouse antibody MAb 7 (Clone 9B12) at a dilution of 1:1000, with a recombinant rat IDE (rrIDE) standard curve (Graph A.) and pooled control plasma (PCP) dilutions (Graph B.). Assay was performed as detailed in Section 2.2.5.2. The dashed line indicates the OD of the blank. The standard curve ranged from 78ng/mL to 5000ng/mL with each concentration in duplicate. PCP was neat (PCP1), diluted 1:1 (PCP2), 1:2 (PCP3) and 1:3 (PCP4) in duplicate. Results show that while the assay works for rrIDE with the highest OD reading of almost 1.000, the background is very high (~0.250) and all PCP OD readings fell below the OD of the blank.



Figures 4.2 B (A) and (B) Screening of polyclonal IDE antibody GTX111664 (Ab 8). Test of indirect ELISA using polyclonal rabbit Ab 8 at a dilution of 1:1000, with a recombinant rat IDE (rrIDE) standard curve (Graph A.) and pooled control plasma (PCP) dilutions (Graph B.). Assay was performed as detailed in Section 2.2.5.2. The dashed line indicates the OD of the blank. The standard curve ranged from 78ng/mL to 5000ng/mL with each concentration in duplicate. PCP was neat (PCP1), and diluted 1:1 (PCP2), 1:2 (PCP3), 1:3 (PCP4) and 1:4 (PCP5) in duplicate. Results show that the assay works for rrIDE with the highest reading of approximately 3.000, and the background is very low (0.011). However, while all PCP dilutions fall above the OD of the blank, values were very low (all ≤ 0.025), inconsistent, and did not demonstrate a dose-response.

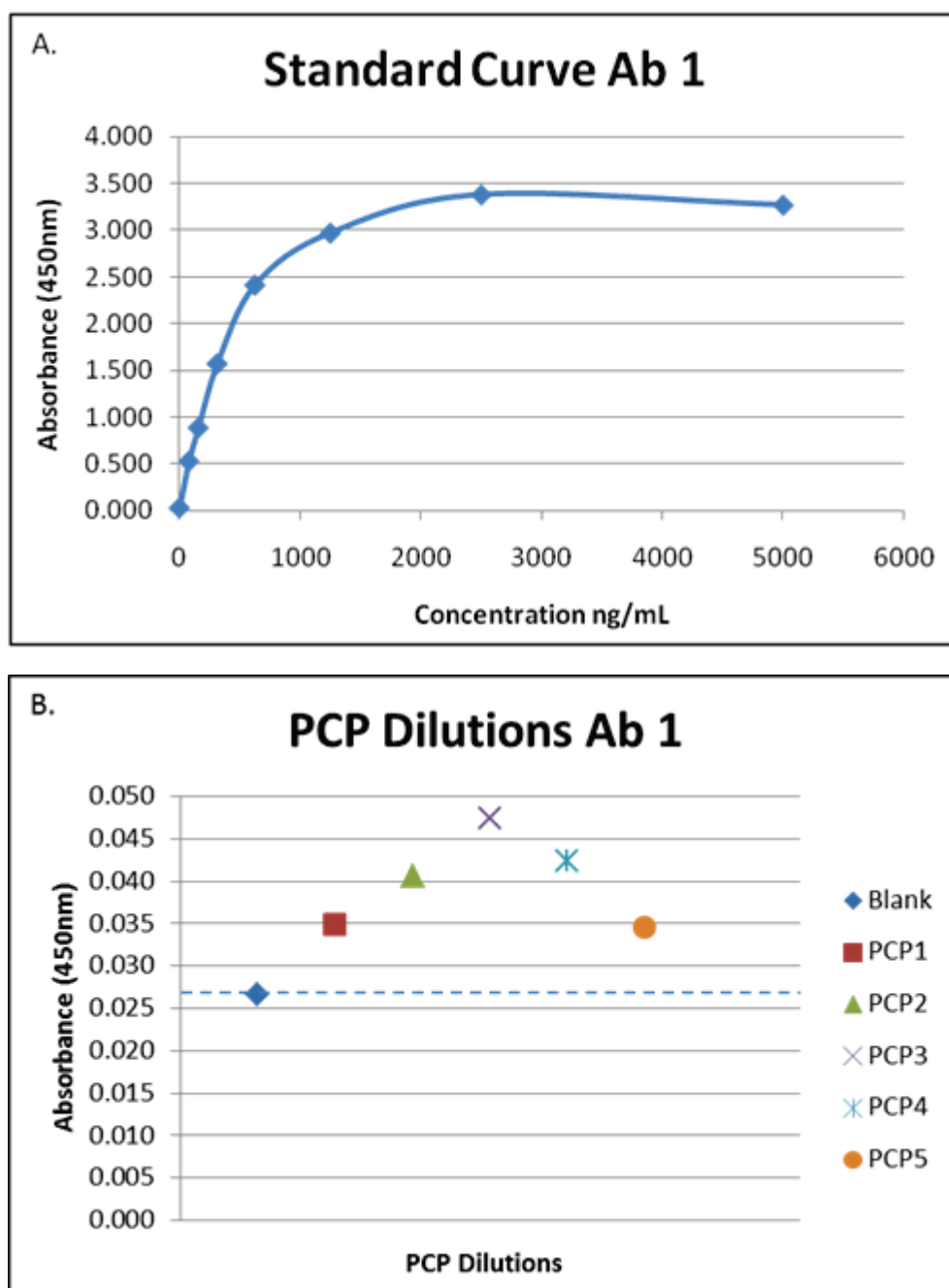


Figure 4.2 C (A) and (B) Screening of polyclonal IDE antibody AB9210 (Ab 1). Test of indirect ELISA using polyclonal rabbit Ab 1 at a dilution of 1:1000, with a recombinant rat IDE (rrIDE) standard curve (Graph A.) and pooled control plasma (PCP) dilutions (Graph B.). The dashed line indicates the OD of the blank. Assay was performed as detailed in Section 2.2.5.2. The standard curve ranged from 78ng/mL to 5000ng/mL with each concentration in duplicate. PCP was neat (PCP1), and diluted 1:1 (PCP2), 1:2 (PCP3), 1:3 (PCP4) and 1:4 (PCP5) in duplicate. Results show that the assay works for rrIDE with the highest reading of approximately 3.400, and the background is quite low (0.027). However, while all PCP dilutions fall above the OD of the blank, values were very low (all ≤ 0.048) and not dose-responsive.

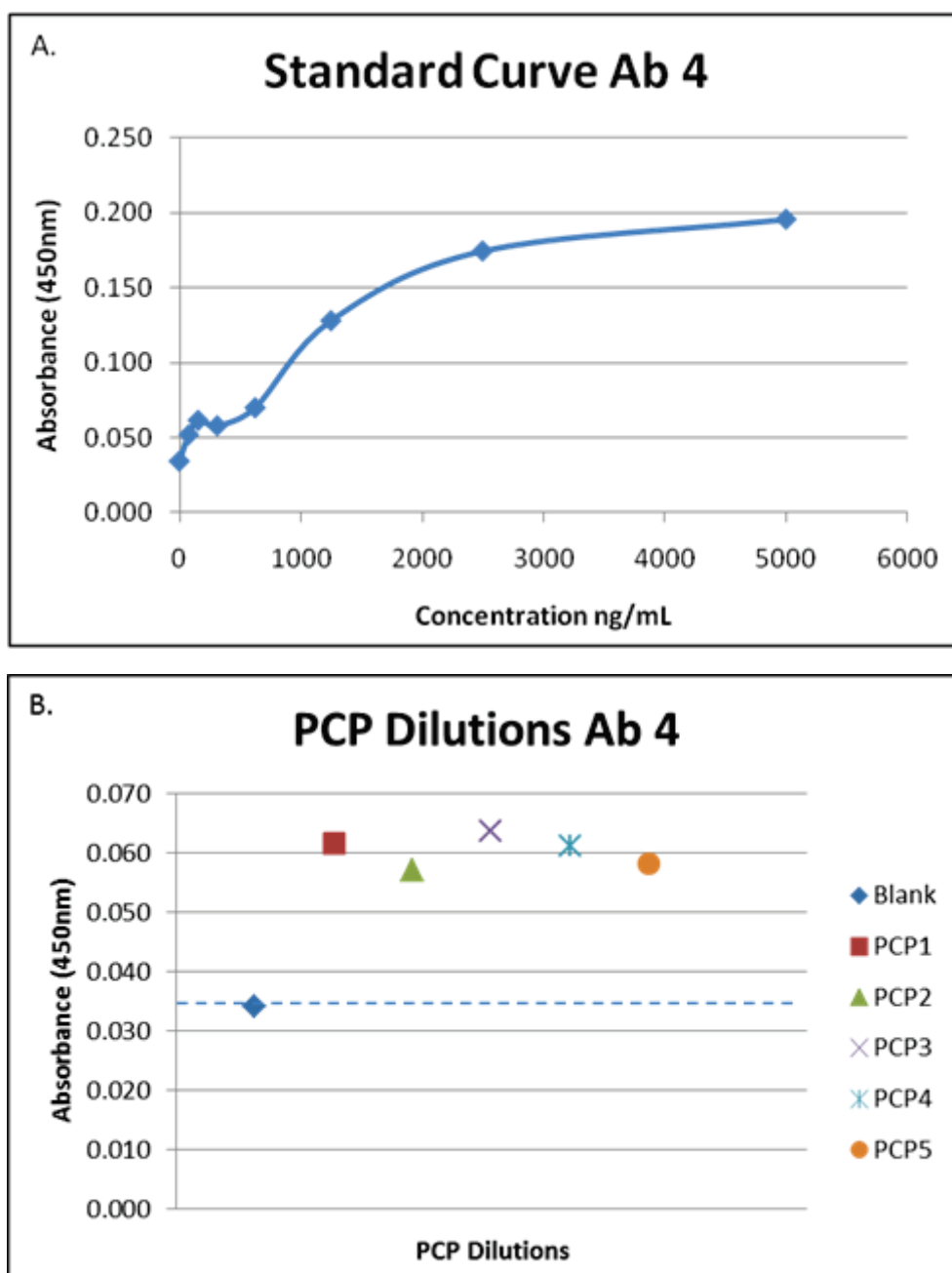


Figure 4.2 D (A) and (B) Screening of polyclonal IDE antibody 250706 (Ab 4). Test of indirect ELISA using polyclonal mouse Ab 4 at a dilution of 1:1000, with a recombinant rat IDE (rrIDE) standard curve (Graph A.) and pooled control plasma (PCP) dilutions (Graph B.). The dashed line indicates the OD of the blank. Assay was performed as detailed in Section 2.2.5.2. The standard curve ranged from 78ng/mL to 5000ng/mL with each concentration in duplicate. PCP was neat (PCP1), and diluted 1:1 (PCP2), 1:2 (PCP3), 1:3 (PCP4) and 1:4 (PCP5) in duplicate. Results show that while the assay seems dose-responsive for rrIDE, the highest reading of 0.195 is very low. The background is reasonably low (0.034). However, readings for the PCP dilutions were only just above the blank OD (all ≤ 0.064) and did not demonstrate a dose-response.

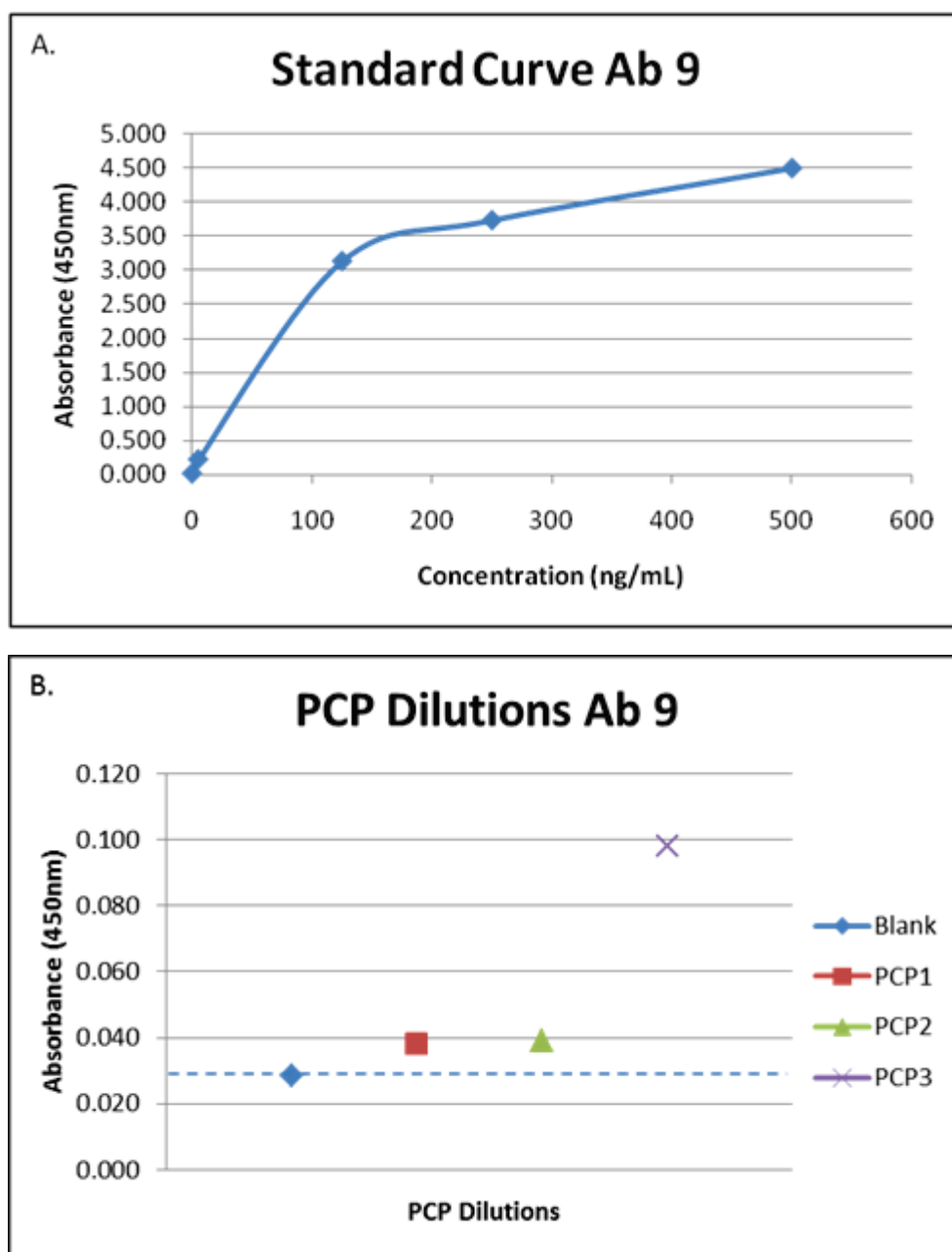


Figure 4.2 E (A) and (B) Screening of monoclonal IDE antibody 5282-1 (MAb 9). Test of indirect ELISA using rabbit MAb 9 as detection antibody, at a dilution of 1:1000, with a recombinant rat IDE (rrIDE) standard curve (Graph A.) and pooled control plasma (PCP) dilutions (Graph B.). Dashed line shows OD of blank. Assay was performed as detailed in Section 2.2.5.2. The standard curve ranged from 5ng/mL to 500ng/mL. PCP was diluted 1:1 (PCP1), 1:5 (PCP2) and 1:20 (PCP3). Results show that the assay works for rrIDE with the highest reading of approximately 4.500, and the background is very low (0.014). However, while all PCP dilutions fall above the OD of the blank, values are very low for 1:1 and 1:5 (<0.04), while diluting to 1:20 yields the highest signal of almost 0.100.

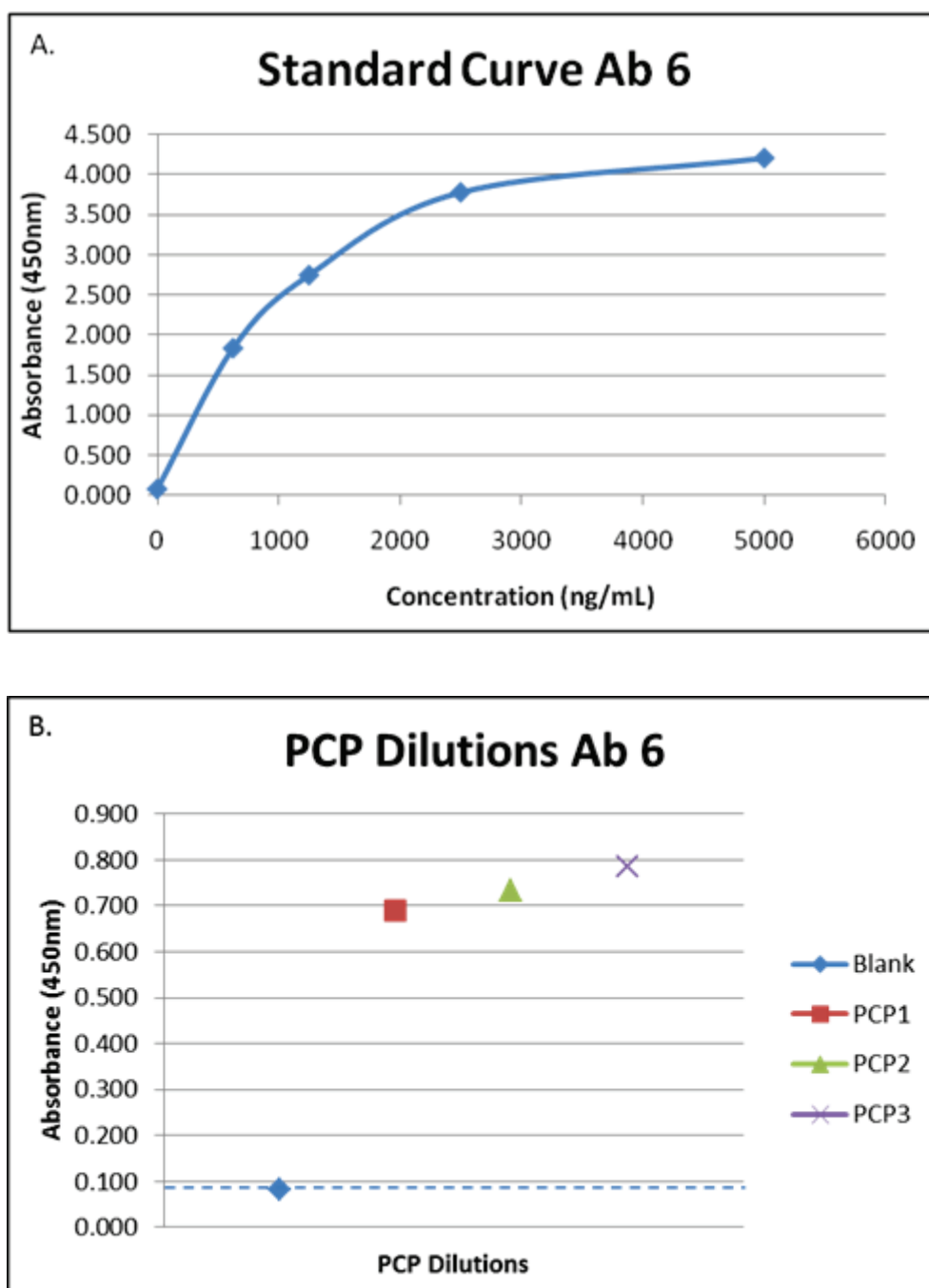


Figure 4.2 F (A) and (B) Screening of polyclonal IDE antibody H00003416-A01 (Ab 6). Test of indirect ELISA using polyclonal mouse Ab 6 at a dilution of 1:1000, with a recombinant rat IDE (rrIDE) standard curve (Graph A.) and pooled control plasma (PCP) dilutions (Graph B.). The dashed line indicates the OD of the blank. Assay was performed as detailed in Section 2.2.5.2. The standard curve ranged from 625ng/mL to 5000ng/mL. PCP was diluted 1:1 (PCP1), 1:5 (PCP2) and 1:20 (PCP3). Results show that the assay works for rrIDE with the highest reading of approximately 4.200, the background is negligible (0.082) and all PCP dilutions fall well above the OD of the blank. However, PCP dilutions appear to be dose-responsive in the reverse direction.

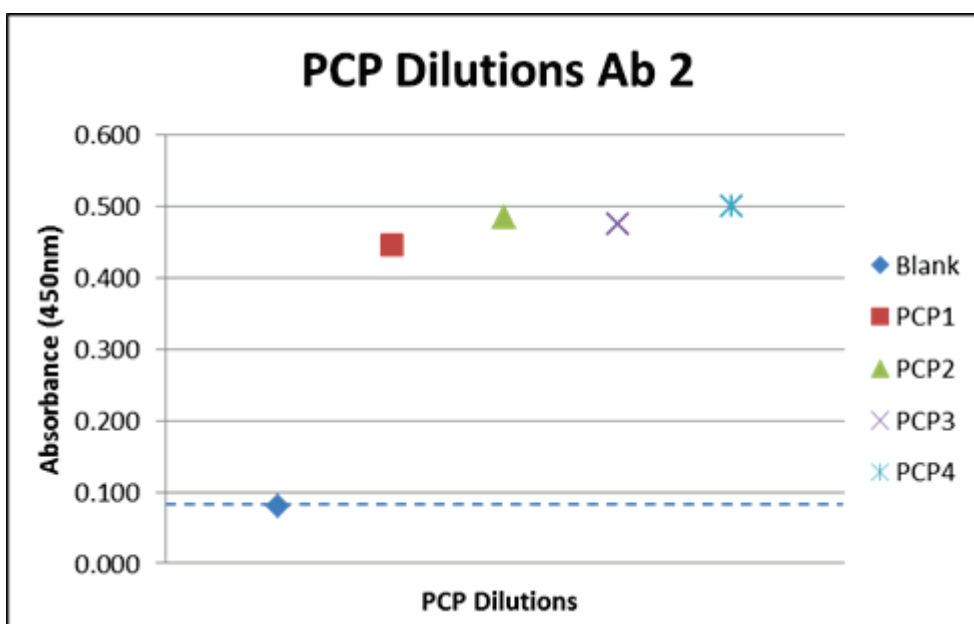
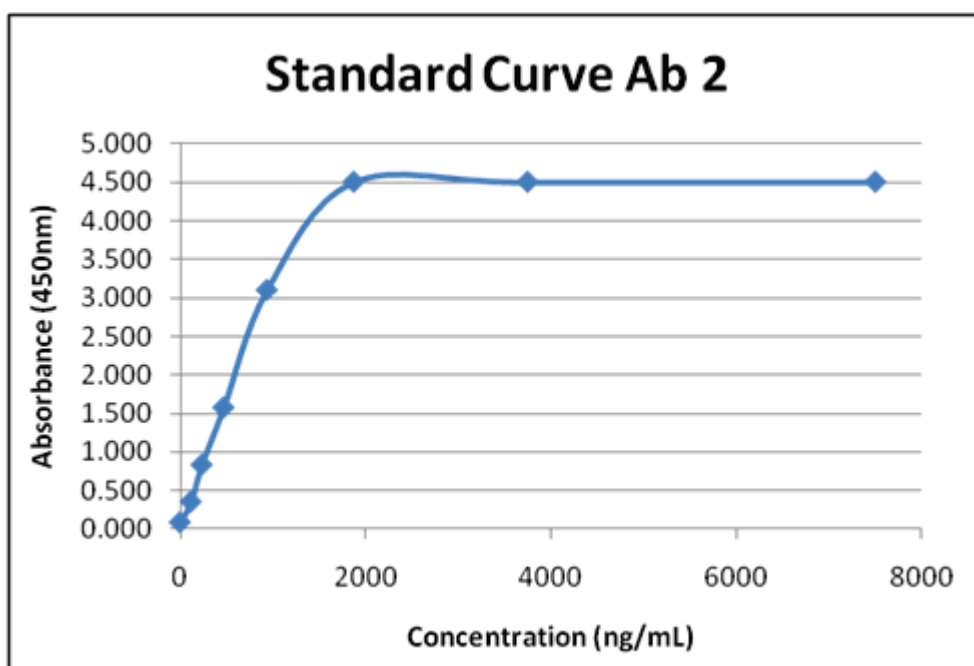


Figure 4.2 G (A) and (B) Screening of polyclonal IDE antibody LS-C55007 (Ab 2). Test of indirect ELISA using polyclonal goat Ab 2 at a dilution of 1:1000, with a recombinant rat IDE (rrIDE) standard curve (Graph A.) and pooled control plasma (PCP) diluted samples (Graph B.). The dashed line indicates the OD of the blank. Assay was performed as detailed in Section 2.2.5.2. The standard curve ranged from 117ng/mL to 7500ng/mL, with each concentration in duplicate. PCP was diluted 1:1 (PCP1), 1:2 (PCP2), 1:5 (PCP3) and 1:10 (PCP4) in duplicate. Results show that the assay works for rrIDE, however the top three concentrations have exceeded the maximum absorbance of 4.500. The background is reasonable (0.081) and all PCP dilutions fall well above the OD of the blank. However, PCP dilutions have a very small dynamic range, with less than 0.054 OD units between the 1:2 dilution and 1:10 dilution. In addition, PCP appears to be dose-responsive in the reverse direction.

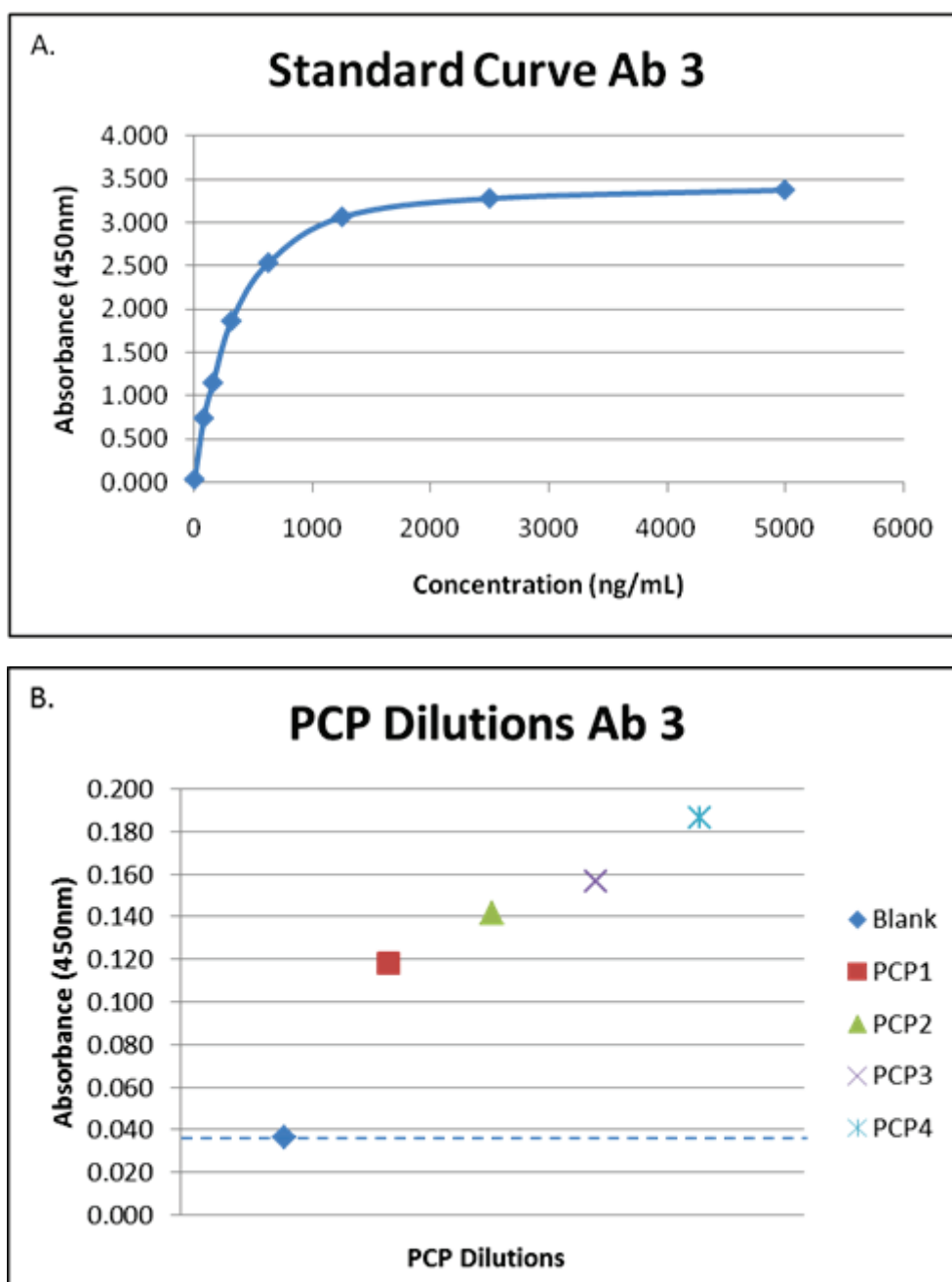
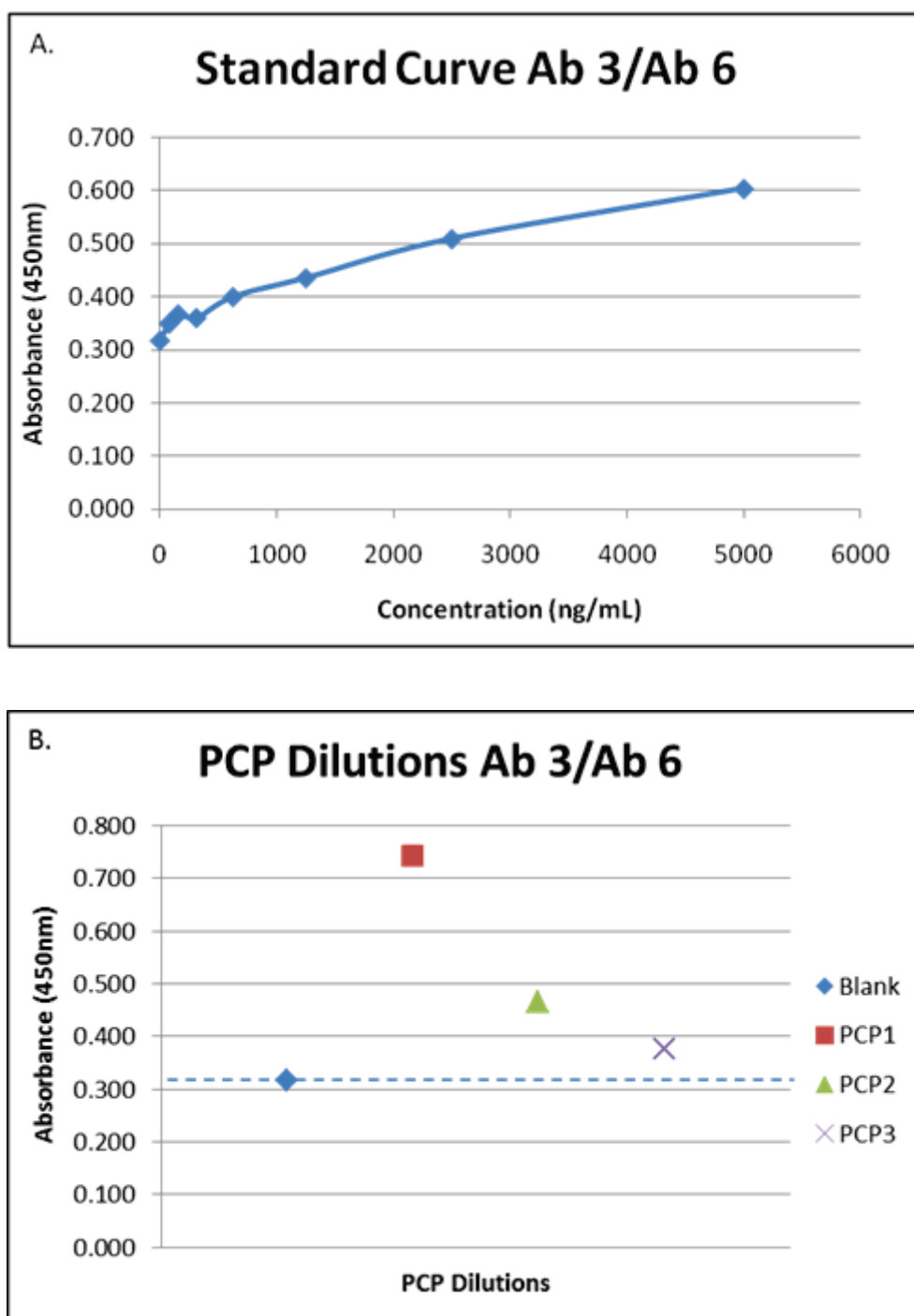


Figure 4.2 H (A) and (B) Screening of polyclonal IDE antibody ab28561 (Ab 3). Test of indirect ELISA using polyclonal rabbit Ab 3 at a dilution of 1:1000, with a recombinant rat IDE (rrIDE) standard curve (Graph A.) and pooled control plasma (PCP) dilutions (Graph B.). The dashed line indicates the OD of the blank. Assay was performed as detailed in Section 2.2.5.2. The standard curve ranged from 78ng/mL to 5000ng/mL with each concentration in duplicate. PCP was neat (PCP1), and diluted 1:1 (PCP2), 1:2 (PCP3) and 1:3 (PCP4) in duplicate. Results show that the assay works for rrIDE, with the highest reading of almost 3.400, the background being negligible (0.036) and all PCP dilutions falling well above the OD of the blank. However, PCP dilutions again appear to be dose-responsive in the reverse direction.

4.3.3 Optimisation of a sandwich ELISA for measuring plasma IDE in the AIBL cohort.

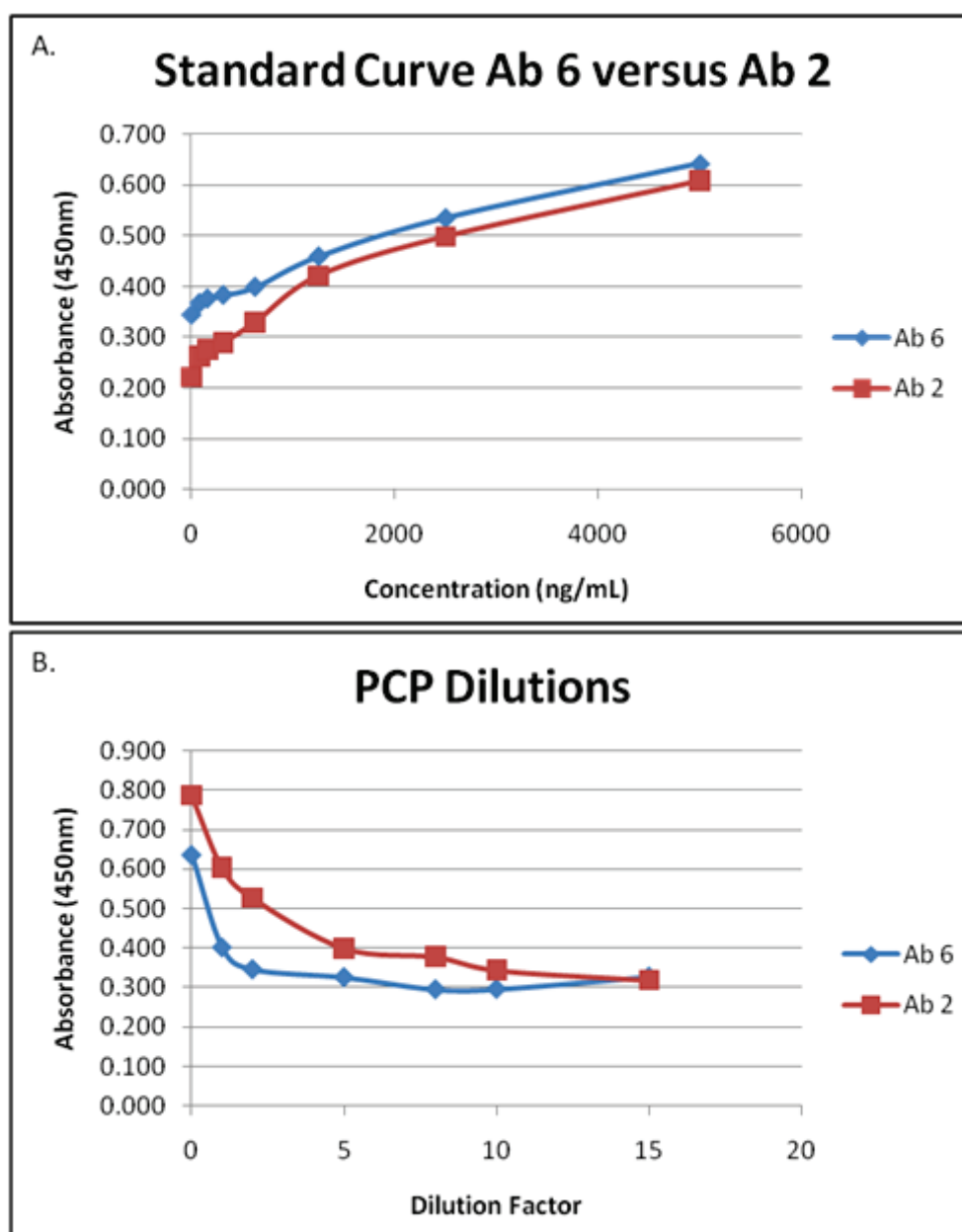
The trials above of the indirect ELISA method demonstrate that this does not appear to be an appropriate method to detect IDE from complex mixtures like plasma. Due to the high content of other proteins, it may be that the levels of IDE binding to the plate were not reflective of the levels in plasma, due to non-specific binding of other proteins, both to the plate and to IDE itself, for example. Therefore, the use of an indirect ELISA was determined to be unsuitable for the measurement of plasma IDE.

As there was a selection of IDE antibodies available, raised to a variety of regions of the IDE protein and from different sources (rabbit, goat, mouse), a sandwich ELISA was then trialled. The sandwich ELISA was developed using polyclonal antibody ab28561 (Ab 3) as the coating antibody and H00003416-A01 (Ab 6) as the detection antibody (Figures 4.3 (A) and (B)). Ab 3 was used as a coating antibody for two reasons; firstly, it was determined to be the antibody with the highest affinity for plasma IDE. Secondly, it was affinity purified, which is essential for a coating antibody, as impurities in the antibody will compete for binding sites on the ELISA plate. Ab 6 is an unpurified antibody, it therefore was not appropriate as a coating antibody, but could function well as a detection antibody. Ab 3 binds to a location of IDE that spans the interface between Domains 1 and 2, between residues 238-416, while Ab 6 binds to an epitope in Domain 4 between residues 920-1019. Therefore these two antibodies bind to distinct sites and should not interfere with each other as a result of steric hindrance. Results showed that the background was considerably higher in the sandwich ELISA than using the antibodies separately in an indirect ELISA. In addition, the standard curve values were considerably reduced compared with the values seen in the indirect ELISA. However, the PCP sample values were much higher than seen in the indirect ELISA and were appropriately dose-responsive up to a dilution of 1:10.



Figures 4.3 (A) and (B) Optimisation of IDE sandwich ELISA using Ab 3 and Ab 6. Sandwich ELISA trial using ab28561 (Ab 3) as coating antibody (1:400) and H00003416-A01 (Ab 6) as detection antibody (1:750), using a recombinant rat IDE (rrIDE) standard curve (Graph A.) and pooled control plasma (PCP) dilutions (Graph B.) The dashed line indicates the OD of the blank. The assay was performed as detailed in Section 2.2.5.3. The standard curve ranged from 78ng/mL to 5000ng/mL with each concentration in duplicate. PCP was neat (PCP1), diluted 1:1 (PCP2) or 1:10 (PCP3) in duplicate. Results show that the assay has a low dynamic range for rrIDE and background is high (0.318). PCP readings are above the blank OD, and appear dose-responsive up to a 1:10 dilution.

To determine if antibody LS-C55007 (Ab 2) would be a better detection antibody than Ab 6, the sandwich ELISA was performed again, trialling both Ab 2 and Ab 6 (Figures 4.4 (A) and (B)). As Ab 2 bound to a similar region to Ab 6 (close to the C-terminus), it was expected that Ab 3 and Ab 2 would also not interfere with each other when bound to IDE. As Ab 2 is affinity purified, it would also allow greater control over the assay, as it would be possible to determine exact concentrations of the antibody. In addition, there would be no other contaminants which could potentially affect the function of the assay. The results were similar for both antibodies, however Ab 2 demonstrated a slightly better dynamic range for both the standard curve and plasma dilutions. In addition, the background was approximately 35% lower with Ab 2 than with Ab 6. It was concluded that Ab 2 was the superior detection antibody.



Figures 4.4 (A) and (B) Comparison of the potential detection antibodies Ab 6 and Ab 2. Comparison of H00003416-A01 (Ab 6) and LS-C55007 (Ab 2) as detection antibodies using ab28561 (Ab 3) as coating antibody, a recombinant rat IDE (rrIDE) standard curve (Graph A.) and pooled control plasma (PCP) dilutions (Graph B.) The assay was performed as detailed in Section 2.2.5.3. Data is shown without blank-adjustment. Both primary antibodies were diluted to 1:1000. The standard curve ranged from 78ng/mL to 5000ng/mL with each concentration in duplicate. PCP was neat (0), and diluted 1:1, 1:2, 1:5, 1:8, 1:10 and 1:15 in duplicate. Results show that Ab 2 demonstrated a slightly higher dynamic range for both the standard curve and PCP dilutions, and lower background compared with Ab 6 (Background of 0.222 and 0.344, respectively). PCP dilutions for both antibodies fall above the OD of the blanks and appear dose-responsive. Ab 2 shows linearity of dilution for PCP up to approximately 1:5.

4.3.4 Tests for non-specific binding of IDE sandwich ELISA reagents.

To determine if there was any signal being generated which was not specific to the antigen being measured, the ELISA was tested by separate omission of the capture antibody, detection antibody and blocking agent (Figure 4.5). Results showed that omission of either the capture and detection antibody resulted in a low background signal, indicating negligible cross-reactivity of the antibodies, with either the secondary antibody or the blocking agent. However, it should be noted that there were some differences in the protocol used to measure the AIBL samples from this protocol: 1) The coating pH was 8.6 in this protocol instead of 9.6; 2) Tween-20 was only present in the PBS wash following the secondary antibody, and 3) Fraction V BSA was used instead of cell-culture grade BSA. There was negligible cross-reactivity observed between assay reagents using this protocol.

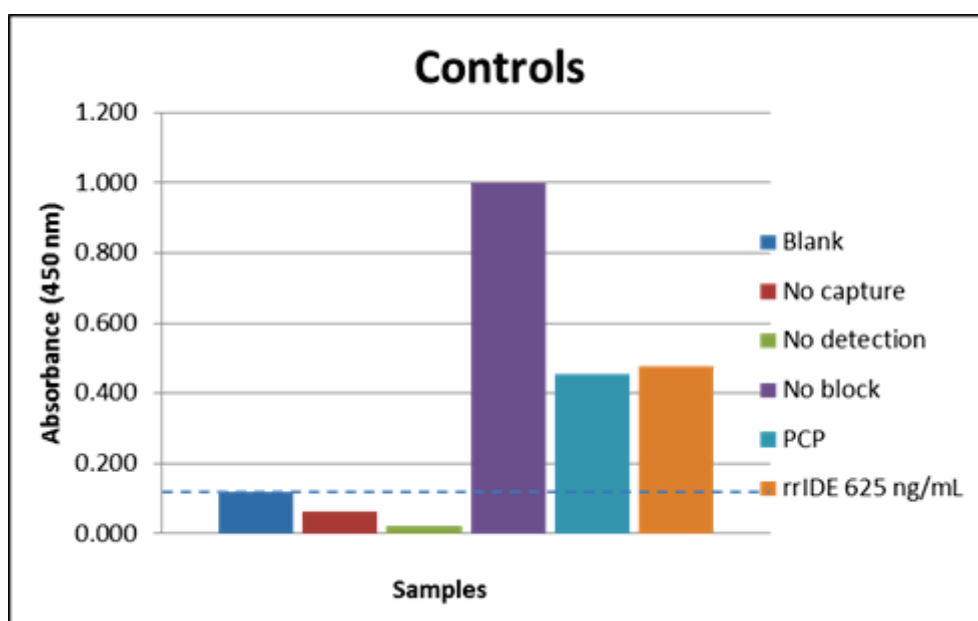
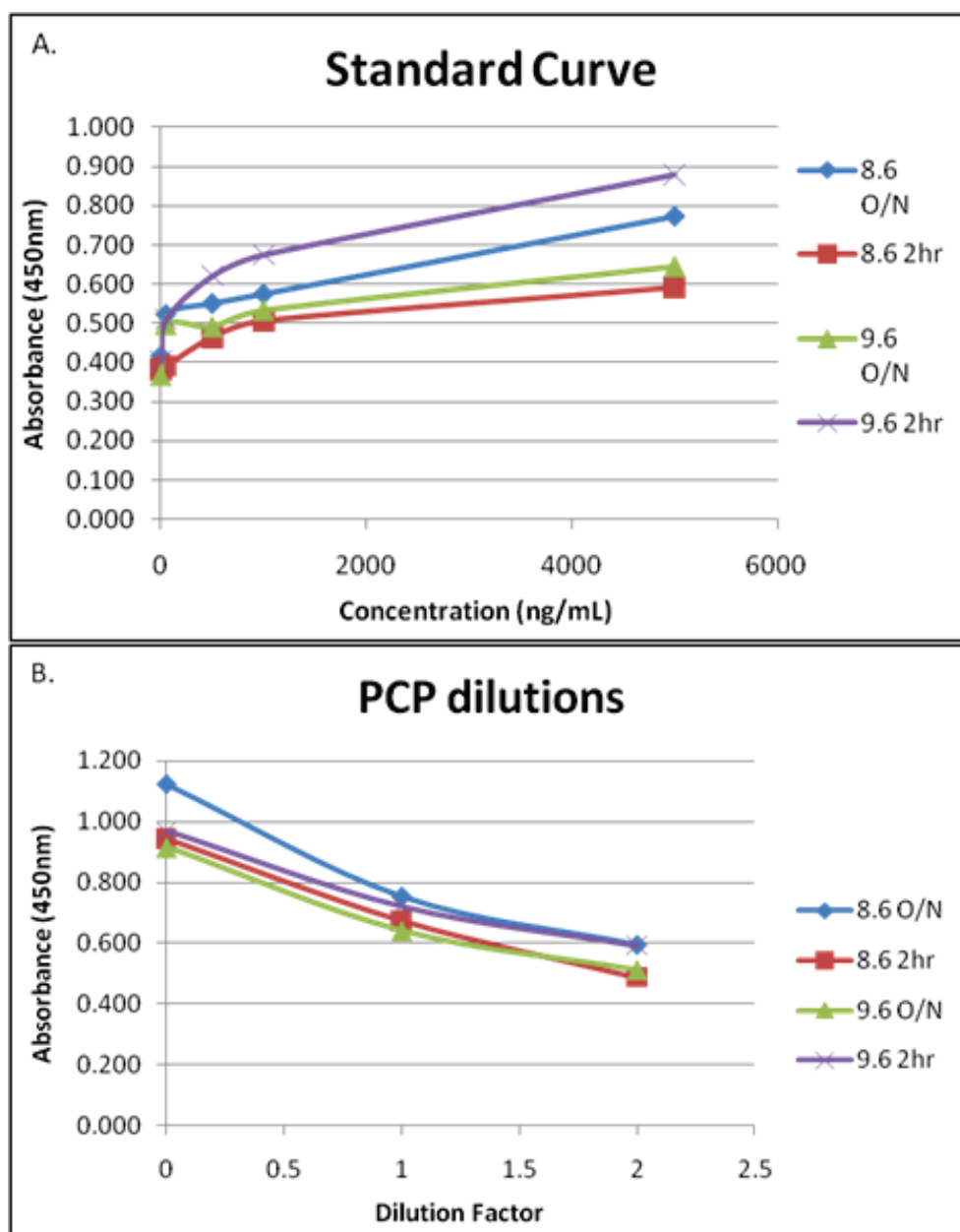


Figure 4.5 Testing for non-specific binding/cross-reactivity in the IDE sandwich ELISA using ab28561 as coating antibody (1:400) and LS-C55007 as detection antibody (1:750). Pooled control plasma (PCP) was run diluted at 1:2 and all control wells contained 625 ng/mL of purified recombinant rat IDE (rrIDE). Plates were coated at pH 8.6 O/N at 4°C. Otherwise, the assay was performed as detailed in Section 2.2.5.3. Data is not blank adjusted – dashed line indicates blank. Results show there is negligible signal generated when either coating or detection antibody was omitted, whereas the signal was increased 2-fold when blocking agent was omitted.

4.3.5 Optimising the coating conditions for the IDE sandwich ELISA

For this purpose, a comparison was made between coating overnight at 4°C and coating for 2 hr at room temperature (RT ~ 25°C). The pH of the coating buffer was also compared (original conditions of pH 8.6 were compared to pH 9.6), as the recommended pH for the coating buffer was pH 9.6. The results show that coating at a pH of 9.6 for 2hr at RT was superior to other conditions for the rrIDE standard curve (Figure 4.6 (A)), with a larger dynamic range, however background was similar for all conditions (difference of 0.049 between lowest and highest value).

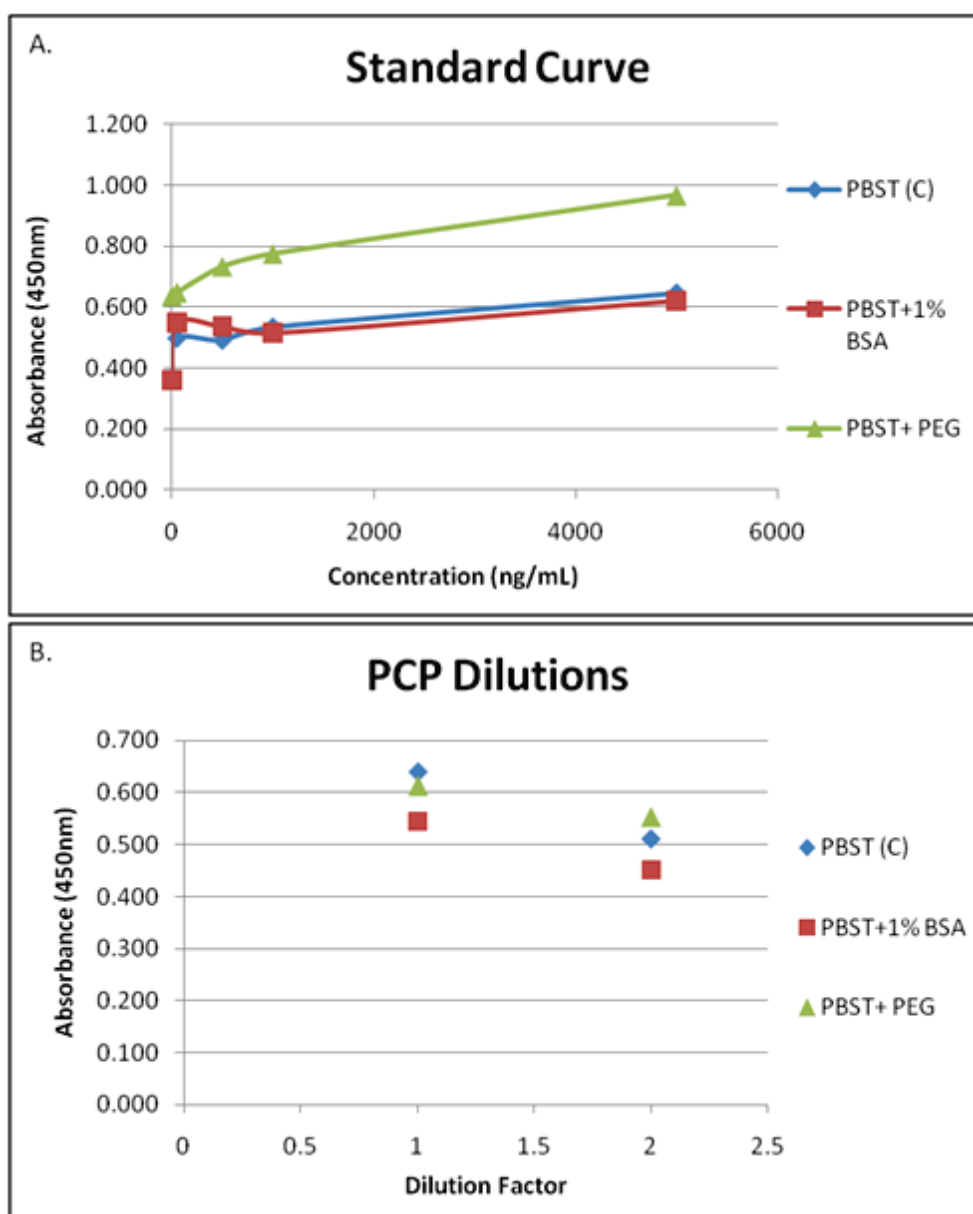
For the plasma dilutions (Figure 4.6 (B)), the coating condition of overnight at 4°C with the coating buffer at pH 8.6 was optimal. Coating for 2 hr at room temperature (pH 9.6) provided the next best result, however for the 1:2 dilution there was negligible difference in signal compared with coating overnight at 4°C (pH 8.6) (difference of 0.032 OD units for 1:1 dilution and 0.002 units for 1:2). As the plasma would need to be diluted to fall within the range of the standard curve, it was concluded that overall the optimal coating condition was 2 hr at room temperature (pH 9.6).



Figures 4.6 (A) and (B) Optimisation of coating conditions for the IDE sandwich ELISA. The sandwich ELISA used ab28561 (Ab 3) as coating antibody (1:400) and LS-C55007 (Ab 2) as detection antibody (1:750). The assay was performed as detailed in Section 2.2.5.3. Data is shown without blank-adjustment. Recombinant rat IDE (rrIDE) used for the standard curve (Graph A.) at 50 to 5000ng/mL. Pooled control plasma (PCP) was run neat (0) and diluted 1:1 and 1:2 (Graph B.). Plates were coated at pH 8.6 or 9.6 O/N at 4°C, and pH 8.6 or 9.6 for 2hr at room temperature (RT - approximately 25°C).

4.3.6 Optimising the sample and detection antibody diluent in the IDE sandwich ELISA.

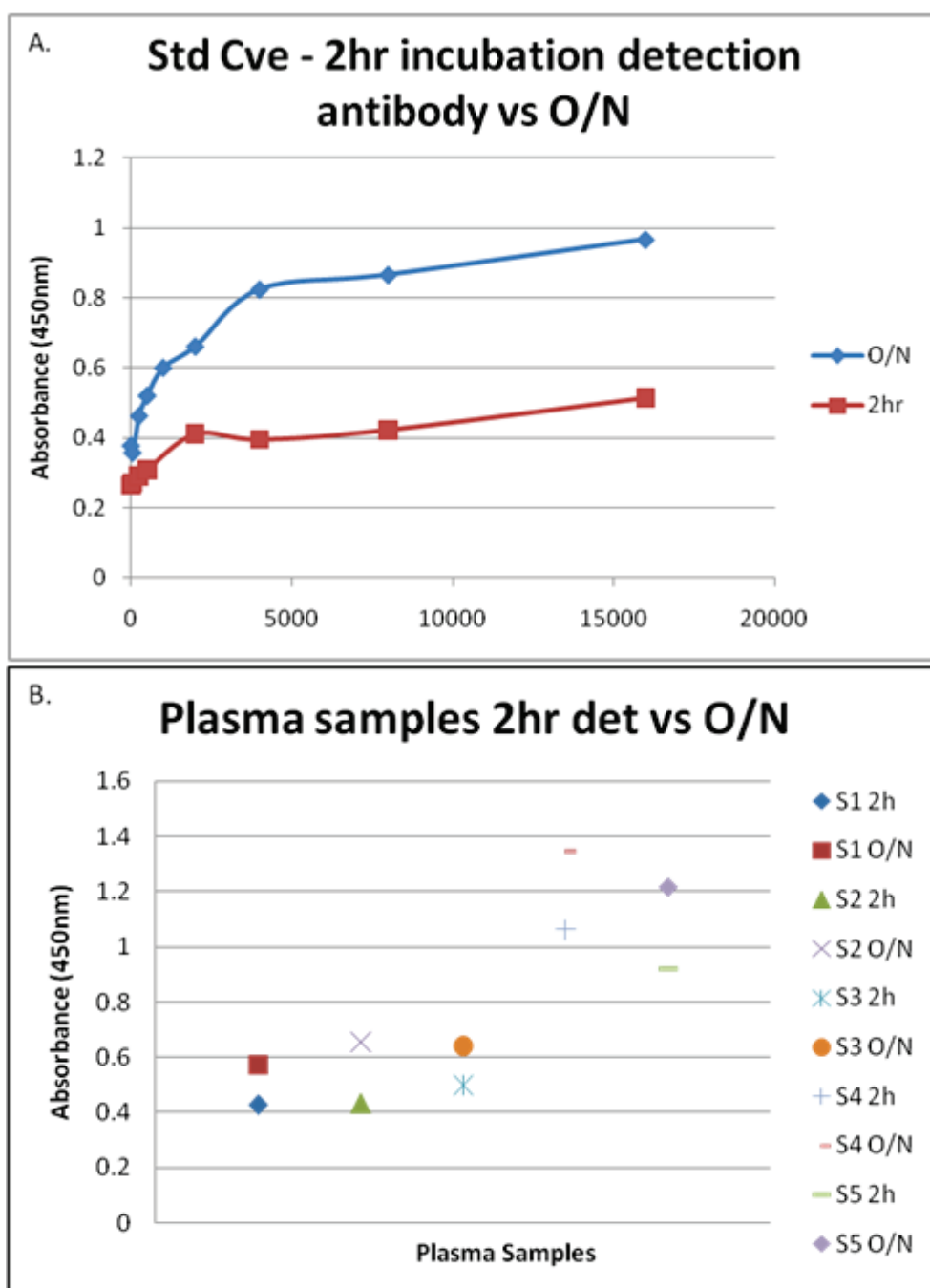
The composition of the sample and antibody diluents in a sandwich ELISA can have a dramatic impact on the binding behaviour of the antibodies to the antigen. Phosphate-buffered saline at a pH of 7.4 is one of the most commonly used diluents in ELISAs due to its stability at physiological pH. Often low concentrations of BSA are added to buffers to stabilise proteins in solution. Therefore the addition of 1% BSA to PBST as the sample diluent was tested compared with PBST alone (Figures 4.7 (A) and (B)). In addition, it has been shown that the addition of polyethylene glycol (PEG) can increase the speed of an immune reaction and enhance the signal in an ELISA (Morissette, *et al.*, 1991). Consequently, PEG (6000) was also added to the detection antibody buffer (PBST) and compared with using PBST alone (Figures 4.7 (A) and (B)). The buffer conditions were tested on purified rat IDE as well as two plasma dilutions (1:1 and 1:2). The results showed that the addition of 1% BSA to the sample diluents had virtually no effect on the standard curve (Graph A.), however caused a reduction of signal in the plasma (Graph B.). The addition of PEG to the detection antibody buffer (PBST) increased the signal for the standard curve, however also increased the background in a similar fashion, resulting in no overall benefit (Graph A.). Results for plasma were similar with or without the addition of PEG. Overall, it was concluded that no benefit in signal amplification or dynamic range was seen with either the addition of BSA or PEG to the buffers. PBST alone was therefore considered to be the optimal buffer for all dilution steps in the sandwich ELISA.



Figures 4.7 (A) and (B) Optimising buffer constituents for the dilution of samples and detection antibody in sandwich ELISA. Optimisation of sample and antibody diluents for sandwich ELISA using ab28561 (Ab 3) as coating antibody (1:400) and LS-C55007 (Ab 2) as detection antibody (1:750). The assay was performed as detailed in Section 2.2.5.3. Recombinant rat IDE (rrIDE) was used for the standard curve (Graph A.) at 50 to 5000ng/mL. Pooled control plasma (PCP) was diluted at 1:1 and 1:2 (Graph B.). Data is shown without blank-adjustment. Standard diluent (PBST alone for sample and antibody) was compared with adding 1% BSA to the sample diluent, and also compared to adding 2% PEG 6000 to the detection antibody diluent.

4.3.7 Improvements in detected signals in the IDE sandwich ELISA following changes to the detection antibody incubation time and temperature.

Overnight incubations of antibodies at 4°C rather than 2 hr at room temperature have frequently been found to improve the signals obtained (Dako, Abcam, Cell-signalling technology (CST)). Therefore a 2 hr incubation (at room temperature) of the detection antibody was compared with incubating overnight at 4°C (Figures 4.8 (A) and (B)). The results demonstrated a considerably increased signal and improved dynamic range when incubating overnight at 4°C for both the standard curve and all of the individual plasma samples. Although the background was also increased (background OD of 0.263 (2hr) compared with 0.377 (overnight)), this increase was negligible compared to the increase in signal. Therefore, it was concluded that overnight incubation of the detection antibody at 4°C was superior to 2 hr at room temperature.



Figures 4.8 (A) and (B) Optimisation of detection antibody incubation time and temperature. Optimisation of detection antibody (Ab 2 diluted to 1:750) incubation conditions for sandwich ELISA using Ab 3 as coating antibody (1:400). The assay was performed as detailed in Section 2.2.5.3. Recombinant rat IDE (rrIDE) was used for the standard curve (Graph A.) at 50 to 16000ng/mL. Individual plasma samples were diluted at 1:2 (S1-S5 - Graph B.). Detection antibody was applied and incubated for 2 hr (2hr or 2h) at room temperature (RT), compared with overnight (O/N) incubation at 4°C. All other conditions were identical for both plates. Data is shown without blank-adjustment.

4.3.8 A trial IDE sandwich ELISA was linear to approximately 4000 ng/mL and upper detection limit was not reached at 64000 ng/mL.

The standard curve in an ELISA is generally sigmoidal in shape, therefore discrimination between data points is reduced at the extreme ends of the curve. As the concentration values of the samples are interpolated from the standard curve it is necessary for the absorbance readings of the samples fall within the linear range. The lower detection limit can be determined by calculating the mean of the optical density of the blanks and adding three standard deviations. Values that fall below this detection limit should be (and were) considered to be too low to differentiate from background noise.

An ELISA was run to determine the upper detection limit; this involved setting up an extended standard curve. On the same ELISA plate, five individual plasma samples were diluted and measured to see if their readings fell within the linear portion of the standard curve (Figures 4.9 (A) and (B)). The results showed that the assay was linear to 4000 ng/mL, the curve flattened out somewhat above this concentration, yet the signal continued to rise up to 64000 ng/mL. This observation suggests an upper detection limit was not reached. Considerable variation can be seen in the plasma samples; three of the five plasma samples assayed had values within the standard curve range, yet two of the samples fell above the highest standard curve value. It was concluded that the standard curve would need to be extended further to establish the upper limit of detection.

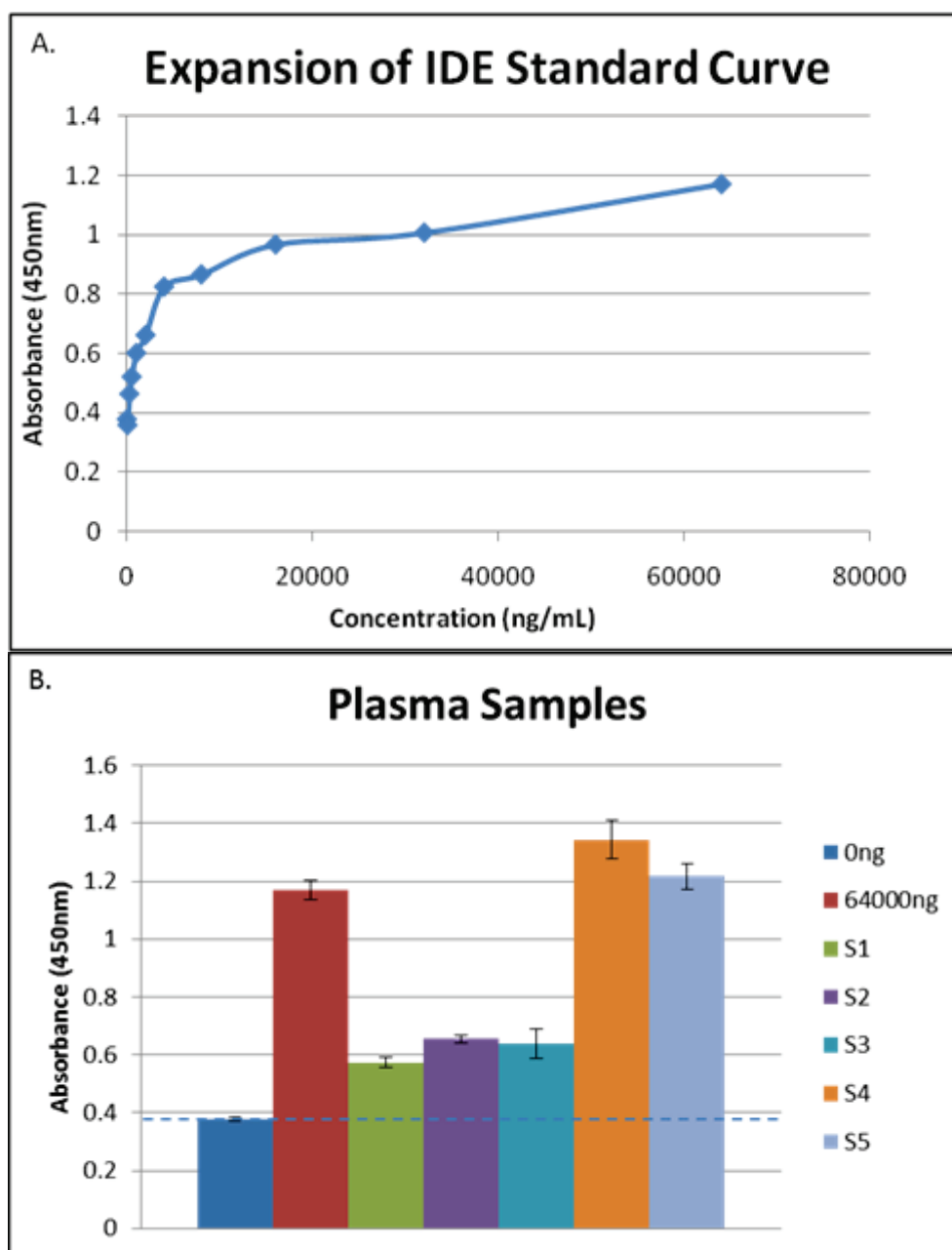


Figure 4.9 (A) and (B) Trial IDE sandwich ELISA to give an extended 11-point standard curve and measure five individual plasma samples. Investigation of the upper detection limit in the sandwich ELISA using Ab 3 as coating antibody (1:400) and Ab 2 as detection antibody at 1:750. The assay was performed as detailed in Section 2.2.5.3. Recombinant rat IDE (rrIDE) was used for the 11-point standard curve (Graph A.) at 50 to 64000ng/mL. Individual plasma samples were diluted at 1:2 (S1-S5 - Graph B.) and shown on the graph, so can be compared with the lowest and highest data point of the standard curve (0 ng (blank) and 64000 ng). Data is shown without blank-adjustment, dashed line indicates blank.

4.3.9 Coating concentration of Ab 3 in IDE sandwich ELISA produces the highest signal at 5 µg/mL and upper detection limit is not reached at 160000 ng/mL IDE.

To optimise the coating concentration for the ELISA, and to investigate further the potential upper detection limit of the assay, three concentrations of Ab 3 were trialled with a standard curve range of rat IDE from 50 to 160000 ng/mL (Figure 4.10). The three concentrations of Ab 3 used were 5, 1 and 0.1 µg/mL. It should be noted that only 50 µL/well of diluted coating antibody was used in this particular test therefore the concentration of 5 µg/mL would contain the same amount of antibody as 100 µL/well of 2.5 µg/mL, which was the coating concentration used in previous optimisation steps. The results demonstrated that a reduction of the concentration of coating antibody caused a reduction in background, signal and dynamic range. With all three concentrations the signal was linear to 10000 ng/mL but continued to rise and a plateau was not reached, even at 160000 ng/mL. However, the slope of the curve above 10000 ng/mL was flatter, and past the linear range. Based on these observations it was determined that the 5 µg/mL was the most suitable coating concentration, as regardless of the increased background, it produced the highest signal to noise ratio and largest dynamic range.

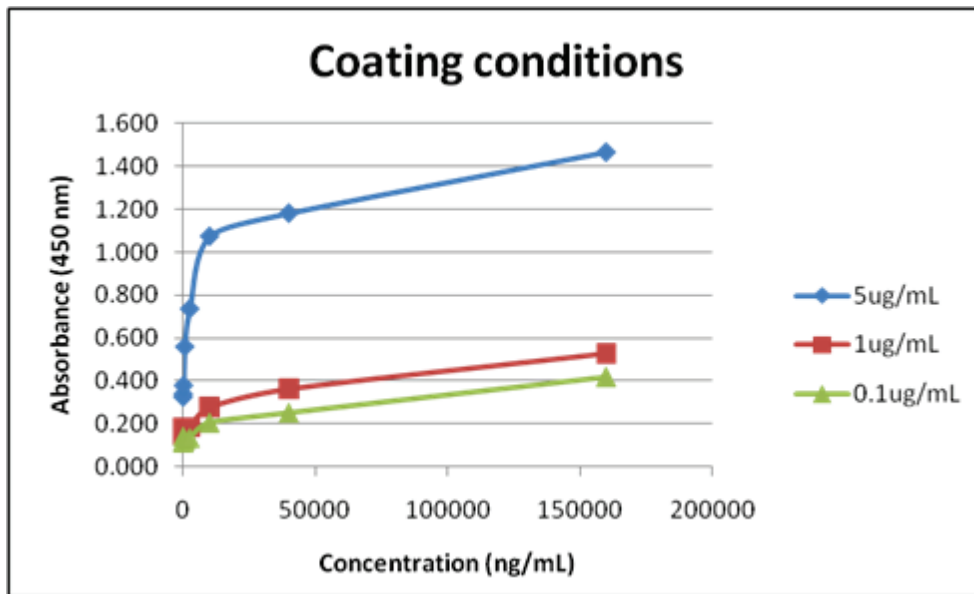


Figure 4.10 Optimisation of coating conditions with Ab 3 and extension of standard curve to 160000 ng/mL. Comparison of coating antibody (Ab 3) concentration for sandwich ELISA using Ab 2 as detection antibody (1:750). The assay was performed as detailed in Section 2.2.5.3. Recombinant rat IDE (rrIDE) was used for the standard curve at concentrations of 50 to 160000 ng/mL. Coating antibody is diluted to 5 µg/mL, 1.0 µg/mL and 0.1 µg/mL (50 µl volume). Data is shown without blank-adjustment. Results show that standard curve is linear to approximately 10000 ng/mL for all three coating concentrations, however continues to increase with increasing concentration of rrIDE. Increasing coating antibody concentration also increases background, yet the signal to noise ratio is optimal at 5 µg/mL.

4.3.10 Testing a non-albumin containing blocking agent in the IDE sandwich ELISA.

Due to the high background absorbance of the ELISA, and the observation that both Ab 2 and Ab 3 cross-reacted with albumin in Western immunoblotting, the blocking agent (BSA) was replaced by a 3% solution of a non-albumin containing blocking agent (ECL™ Advance Blocking agent) to see if the background would decrease. Results showed that the non-albumin blocking agent increased the background compared with previous assays which used BSA (Figure 4.11). It should be noted that the assays were not performed simultaneously and therefore a direct comparison could not be made. However, when previously using BSA as the blocking agent, the background absorbance was consistently approximately 0.35; with the non-albumin block it was over 0.40. As BSA produced a lower background, it was used as the blocking agent in subsequent experiments.

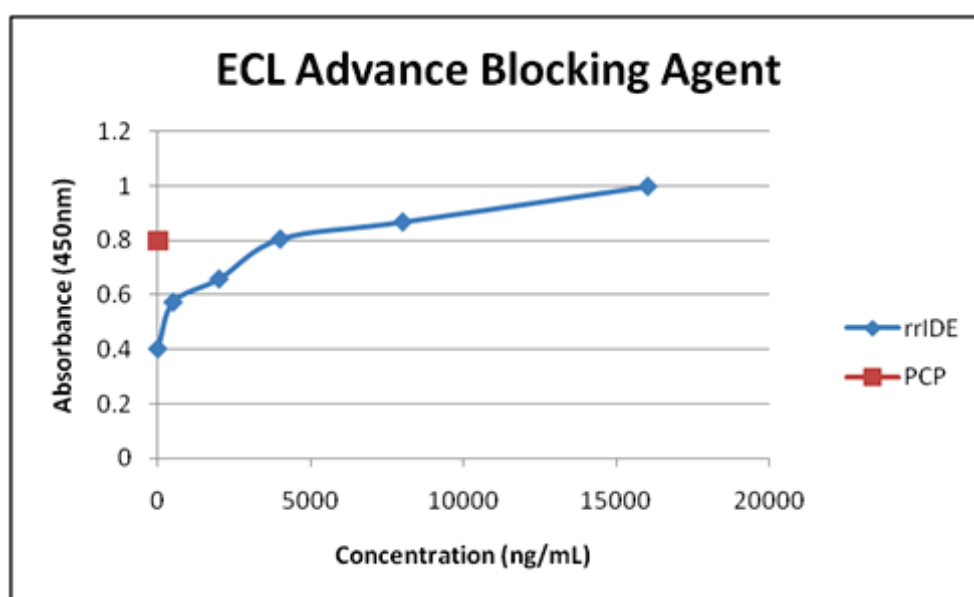


Figure 4.11 Test of non-albumin containing blocking agent in the IDE sandwich ELISA. Test of ECL™ Advance Blocking Agent to improve background absorbance for IDE sandwich ELISA using Ab 3 as coating antibody (1:400) and Ab 2 as detection antibody (1:750). The assay was performed as detailed in Section 2.2.5.3. Recombinant rat IDE (rrIDE) was used for the standard curve at 500 to 16000ng/mL. Pooled control plasma (PCP) diluted to 1:2. Data is shown without blank-adjustment. Blocking step was using 3% ECL Advance Blocking Agent in PBS. Results show that using the non-albumin containing blocking reagent produces a high background absorbance of >0.4 in the sandwich ELISA.

4.3.11 Low-level data analysis.

Raw data was normalised for inter-plate variation by adjusting to a PCP internal control which was included on each plate. Intra-assay variation ($n = 215$, based on the 6 plates used in the analysis) was determined to be $\sim 11\%$ (including samples with a CV of $>15\%$ that were subsequently removed from the analysis). Following the removal of these samples, average CV was 5% . Inter-assay variation was determined to be $\sim 16\%$ ($n = 6$). Coefficient of variation (CV) values of duplicates which were $>15\%$ were excluded from the analysis. If the CV of the PCP duplicates was $>15\%$, the entire plate was excluded from the analysis. Values $< \text{mean} + 3 \times \text{standard deviation}$ of the blanks were considered as below the detection limit. Sample values for each plate were normalised to the PCP sample. As some of the sample values fell outside the range of the standard curve, the concentrations could not be interpolated and therefore only relative differences in absorbance (minus blank absorbance) were compared for all samples.

The data was noted to have a non-normal distribution as determined by visually inspecting histograms, box-plots and normal Q-Q plots, as well as calculating z-scores of skewness and kurtosis. In addition, normality tests such as the Kolmogorov-Smirnoff and Shapiro-Wilks tests were highly significant. A variety of transformations were used to correct the normality issues (square root, natural log, natural log +1, reciprocal), however these were not effective. Therefore non-parametric tests were used to analyse the data (Kruskall-Wallis, Mann-Whitney and Spearman's Rho).

4.3.12 Total plasma IDE levels are reduced in MCI and AD compared with HC.

A sample standard curve and set of 10 AIBL samples is shown in Figures 4.12(A) and (B). To determine IDE levels according to cognitive status, the cohort was divided into the clinically classified groups, healthy controls (HC - including subjective memory complainers), mild cognitive impairment (MCI) and probable AD (AD). As

only a subset of the cohort was used, the HC and SMC groups were combined to form the healthy control group. Mean age was significantly different between the groups. It should be noted that the subset used to measure IDE levels by ELISA was different from the subsets used to measure IDE levels by Western immunoblotting and IDE activity (detailed in Chapters 3 and 5, respectively).

The demographics for this subset of the AIBL cohort are shown in Table 4.1. AD patients were significantly older, and as expected had a significantly higher percentage of *APOE* $\epsilon 4$ carriers than the control or MCI groups. The plasma $A\beta_{1-42/1-40}$ ratio was significantly decreased in the MCI and AD groups, while $A\beta_{x-40}$ was significantly increased in both MCI and AD groups as compared with HC. Plasma ApoE was significantly lower in AD patients, while ApoE4 levels were higher in the MCI group compared with HC and AD groups. P values were not shown for brain $A\beta$ load as in this subset there was only one individual in the HC group who underwent the PiB-PET scan. All other variables were not significantly different between groups.

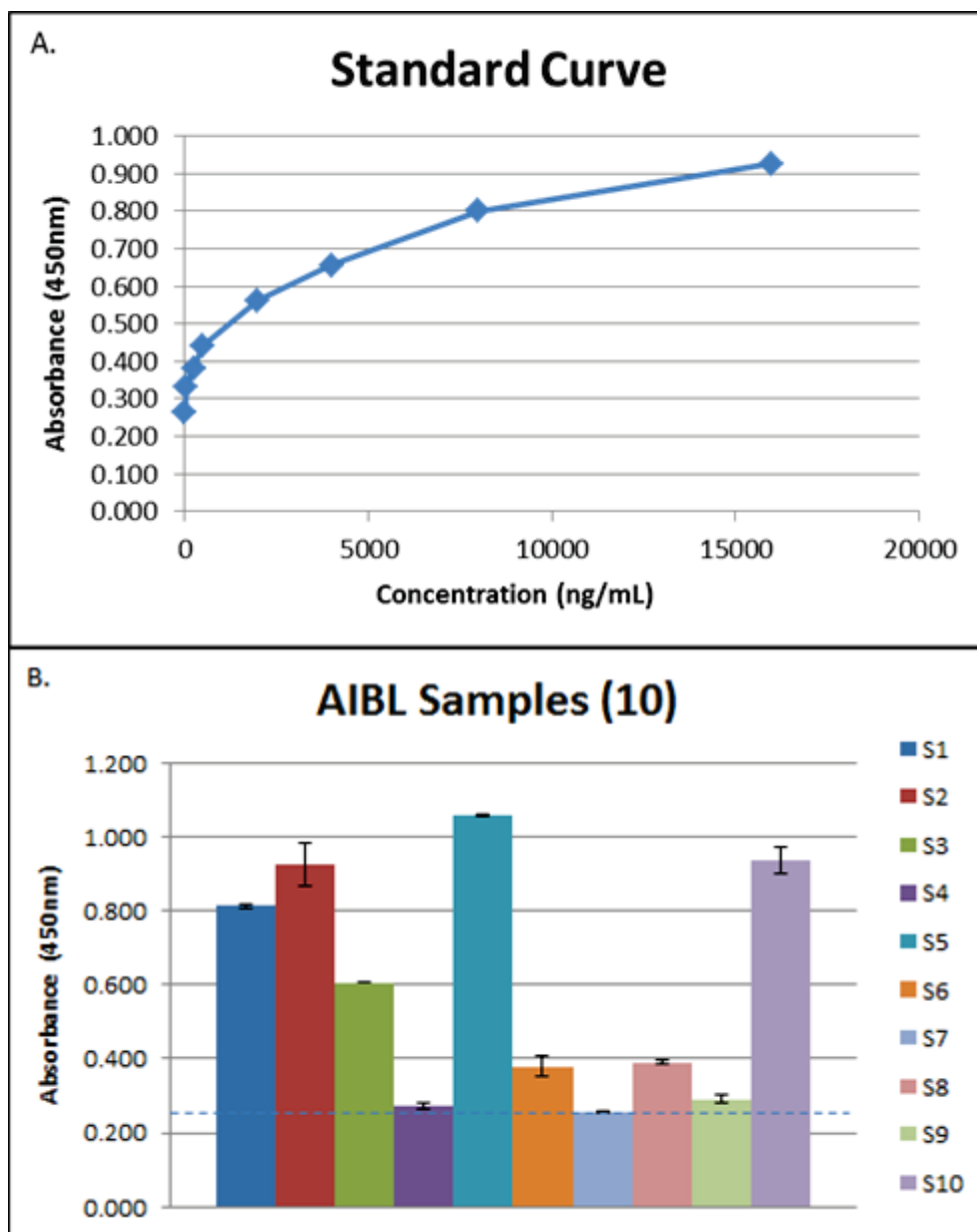


Figure 4.12 (A) and (B) Sample standard curve and 10 sets of samples. The assay was conducted as detailed in Section 2.2.5.3. Recombinant rat IDE (rrIDE) was diluted to concentrations of 50 to 16000 ng/mL (Graph A.) and plasma samples (Graph B) including PCP were loaded at a dilution of 1:2 (33.3 μ L plasma per well) in duplicate. Error bars indicate \pm SD. Data is not blank-adjusted, dashed line indicates blank (Graph B). Some samples had absorbances outside of the range of the standard curve; as a result only relative differences could be compared.

(THIS PAGE HAS BEEN INTENTIONALLY LEFT BLANK)

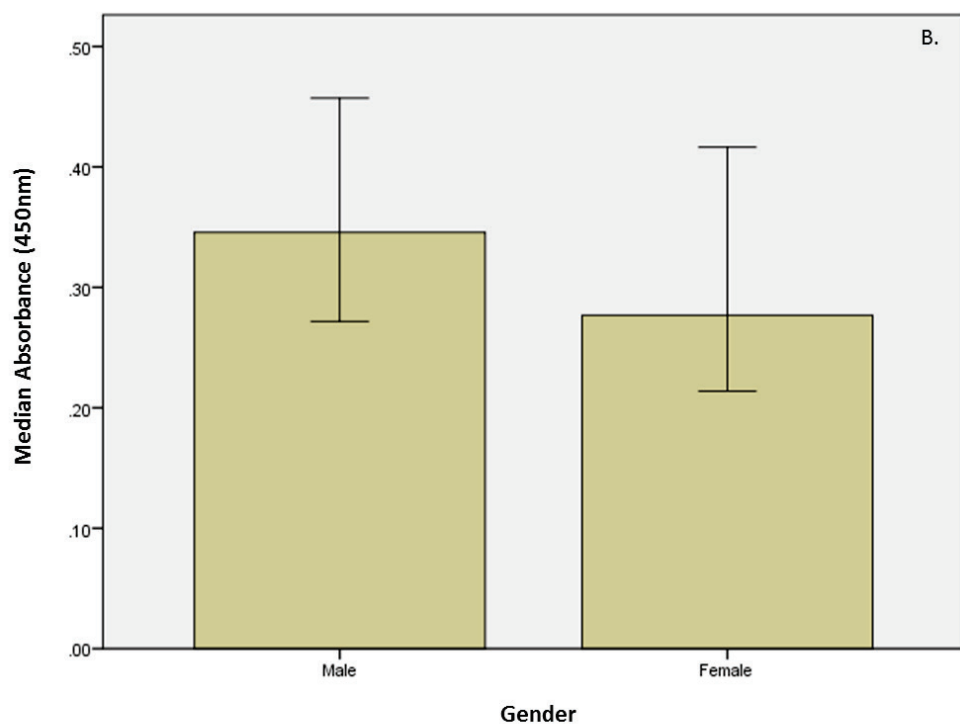
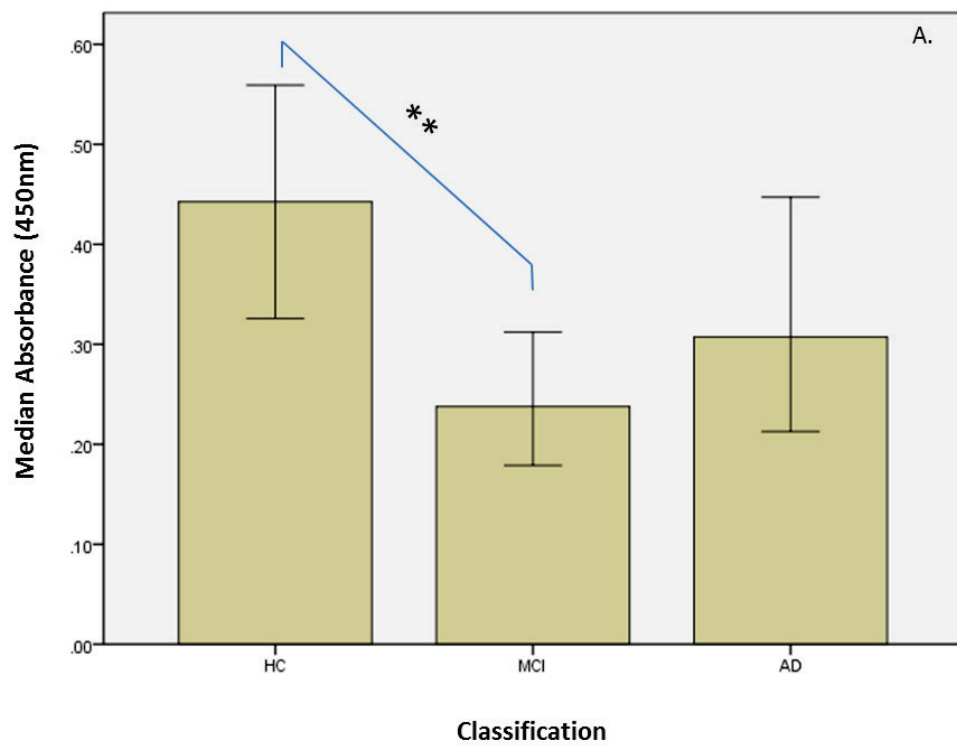
Table 4.1 Basic demographics of the AIBL cohort subset of participants for whom plasma IDE levels were measured by ELISA. Plasma samples (33.3 μ L per well in duplicate) from the AIBL cohort were assayed with an in-house sandwich ELISA as detailed in Section 2.2.5.3. Other variables were measured as outlined in Section 2.2.2.1. Continuous variables are expressed as mean (median) \pm standard deviation. P values were determined by Kruskal-Wallis tests using the Monte Carlo method. As only a subset of the cohort was tested, the HC and subjective memory (SMC) groups were combined to provide the healthy controls for this assay. n values vary due to missing values for some data or in the case of brain A β load, only a subset of the cohort underwent Pib-PET scans. P value was not given for brain A β load as there was only one healthy control in this subgroup. As ApoE4 is only expressed in individuals carrying the *APOE* ϵ 4 allele, the n-values are reduced for this variable.

	HC	MCI	AD	p-value
Total IDE levels (Absorbance 450nm)	0.553 (0.443) ± 0.413	0.391 (0.238) ± 0.413	0.419 (0.307) ± 0.318	0.006
n (Total IDE Levels)	57	50	70	-
Age (years)	68.67 (68) ± 6.12	74.96 (75.5) ± 6.85	79.26 (81) ± 7.85	<0.001
APOE ε4 +ve (%)	33.3	46.9	67.7	<0.001
Male (%)	40.4	36	35.7	0.819 (NS)
Plasma insulin (mU/L)	6.74 (4.9) ± 6.53 n = 57	7.8 (5) ± 9.04 n = 50	6.73 (5.1) ± 7.16 n = 69	0.910 (NS)
Plasma Aβ ₁₋₄₀ (pg/mL)	133.5 (134.4) ± 33.35 n = 57	146.34 (152.39) ± 37.04 n = 48	147.48 (144.96) ± 40.38 n = 70	0.103 (NS)
Plasma Aβ ₁₋₄₂ (pg/mL)	29.28 (27.8) ± 10.46 n = 57	27.8 (27.9) ± 7.56 n = 48	29.86 (29.09) ± 8.82 n = 70	0.463 (NS)
Plasma Aβ _{1-42/1-40} ratio	0.226 (0.224) ± 0.088 n = 57	0.2004 (0.19) ± 0.074 n = 48	0.207 (0.21) ± 0.046 n = 70	0.034
Plasma Aβ _{x-40} (pg/mL)	139.6 (142.6) ± 39.86 n = 56	152.38 (161.43) ± 57.23 n = 48	164.39 (168.34) ± 45.36 n = 70	<0.001
Plasma Aβ _{x-42} (pg/mL)	34.69 (32.98) ± 14.27 n = 55	32.10 (30.91) ± 12.88 n = 48	37.69 (36.86) ± 12.88 n = 69	0.081 (NS)
Plasma Aβ _{x-42/x-40} ratio	0.296 (0.251) ± 0.27 n = 55	0.294 (0.197) ± 0.31 n = 48	0.242 (0.231) ± 0.14 n = 69	0.110 (NS)
Plasma ApoE – Total (mg/dL)	15.4 (15.22) ± 2.8 n = 57	15.2 (15.25) ± 2.72 n = 47	13.85 (14.18) ± 2.77 n = 68	<0.001
Plasma ApoE4 (mg/dL)	6.61 (6.48) ± 1.34 n = 19	8.44 (6.4) ± 4.26 n = 22	7.06 (4.96) ± 4.49 n = 46	0.023
Plasma Testosterone - Total (nmol/L)	8.07 (1.00) ± 9.77 n = 57	5.86 (1.00) ± 7.63 n = 49	6.49 (1.00) ± 8.08 n = 70	0.869 (NS)
Plasma Testosterone – Free (nmol/L)	0.161 (0.016) ± 0.195 n = 57	0.116 (0.013) ± 0.163 n = 50	0.119 (0.016) ± 0.152 n = 70	0.356 (NS)
Plasma Estradiol (pmol/L)	101.37 (88) ± 49.1 n = 57	90.49 (76) ± 52.69 n = 47	86.57 (79.5) ± 40.9 n = 70	0.339 (NS)
Plasma Cholesterol – total (mmol/L)	5.74 (5.8) ± 1.19 n = 57	5.4 (5.25) ± 1.05 n = 50	5.64 (5.6) ± 1.41 n = 70	0.322 (NS)
Plasma LDL Cholesterol (mmol/L)	3.58 (3.6) ± 1.02 n = 57	3.13 (3.1) ± 0.874 n = 50	3.35 (3.2) ± 1.22 n = 70	0.090 (NS)
Plasma HDL Cholesterol (mmol/L)	1.58 (1.5) ± 0.371 n = 57	1.64 (1.69) ± 0.365 n = 50	1.68 (1.59) ± 0.452 n = 70	0.373 (NS)
Brain Aβ load (Neocortical SUVR)	** n = 1	2.06 (2.25) ± 0.712 n = 23	2.51 (2.35) ± 0.631 n = 6	n/a

Total IDE levels as determined by the developed sandwich ELISA (Figure 4.13(A)) were found to be significantly lower in the MCI ($p = 0.000$) group compared with the HC group. IDE levels were also lower in the AD group compared with the controls, however failed to reach significance ($p = 0.062$, after Bonferroni correction for multiple testing $p = 0.186$). There was no significant difference between IDE levels in the MCI and AD groups ($p = 0.260$, after Bonferroni correction $p = 0.780$). No differences were observed in total IDE levels between males and females (Figure 4.13(B), $p = 0.136$).

(THIS PAGE HAS BEEN INTENTIONALLY LEFT BLANK)

Figure 4.13(A) and (B) Levels of total plasma IDE are decreased in MCI and AD compared with HC, and no different in males versus females. Plasma samples (diluted 1:2 - 33.3 μ L per well in duplicate) from the AIBL cohort were assayed with an in-house sandwich ELISA to measure total IDE as detailed in Section 2.2.5.3. The average absorbance of the duplicates was obtained for each sample, and the median of these values by classification were compared by a Kruskal-Wallis test, using Mann-Whitney tests for post-hoc analysis with Bonferroni correction applied to adjust for multiple testing for significant comparisons only. Data represents medians with 95% confidence interval (CI), $n = 177$ (57 HC/50 MCI/70 AD). Values were blank adjusted and normalised to the internal standard sample value included on each plate to control for plate-to-plate variation. Results showed a significant reduction in total IDE for MCI compared with HC ($p = 0.000$), however the reduction seen in AD compared with HC lost significance after Bonferroni correction (Graph A., $p = 0.186$). No differences in total IDE levels were seen between males and females (Graph B., $p = 0.136$).



4.3.13 Total plasma IDE levels reduce (not significantly) with age.

As IDE levels in the hippocampus have previously been reported to decline with ageing (Caccamo, *et al.*, 2005), total levels measured with ELISA were analysed by age quartiles to determine if this phenomenon occurs in the periphery (Figure 4.14). Results showed that similar to the hippocampus, IDE levels in the blood also tend to decrease with age, particularly in age groups over 78 years, however the reduction was not statistically significant.

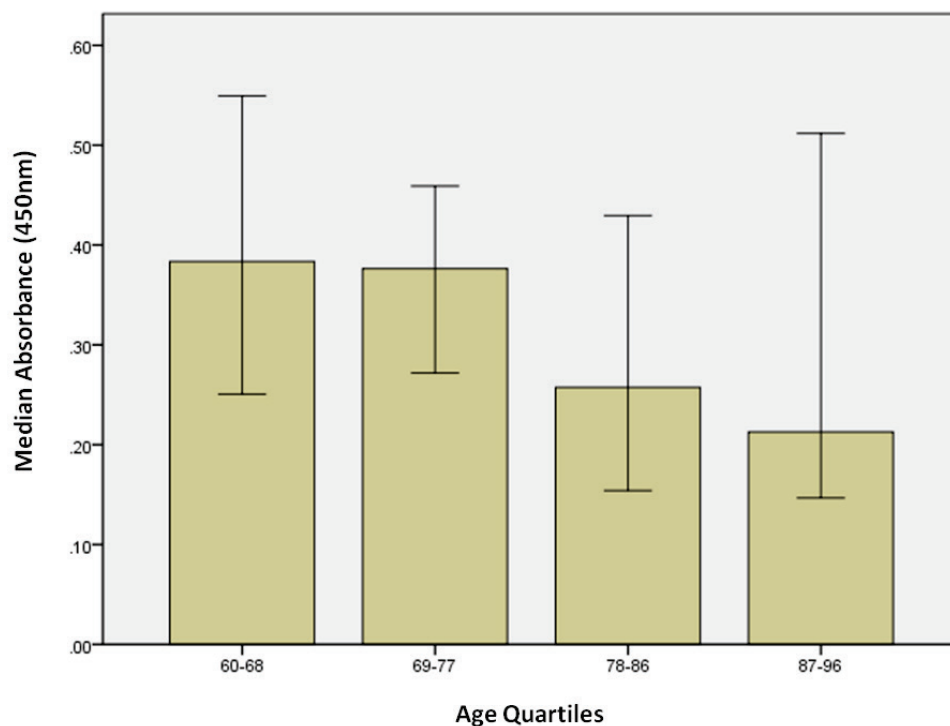


Figure 4.14 Total plasma IDE levels reduce (not significantly) with age. Plasma samples (diluted 1:2 - 33.3 μ L per well in duplicate) from the AIBL cohort were assayed with an in-house sandwich ELISA to measure total IDE as detailed in Section 2.2.5.3. The average absorbance of the duplicates was obtained for each sample, and the median of the groups were compared by a Kruskal-Wallis test. Data represents medians with 95% confidence interval (CI). $n = 177$ (Quartile 1 (60-68) $n = 52$ /Quartile 2 (69-77) $n = 59$ /Quartile 3 (78-86) $n = 50$ /Quartile 4 (87-96) $n = 15$). Results show that IDE levels tend to reduce with age, albeit not significantly ($p = 0.119$).

4.3.14 *APOE* $\epsilon 4$ carriers do not have significantly different total plasma IDE levels compared with non-carriers.

Several studies have demonstrated a difference in cerebral IDE expression between *APOE* $\epsilon 4$ carriers and non-carriers, with $\epsilon 4$ carriers shown to have reduced expression of IDE. To ascertain if this effect also occurs in the plasma, total IDE levels were compared between carriers and non-carriers (Figure 4.15(A)). Results showed that $\epsilon 4$ carriers tended to have lower total IDE levels than non-carriers, however this was not statistically significant ($p = 0.109$). When stratified by $\epsilon 4$ status and analysed by classification (Figures 4.15(B) and (C)), there was a non-significant trend toward lower IDE levels in MCI and AD compared with controls still in $\epsilon 4$ carriers ($p = 0.068$), but not in non-carriers ($p = 0.114$).

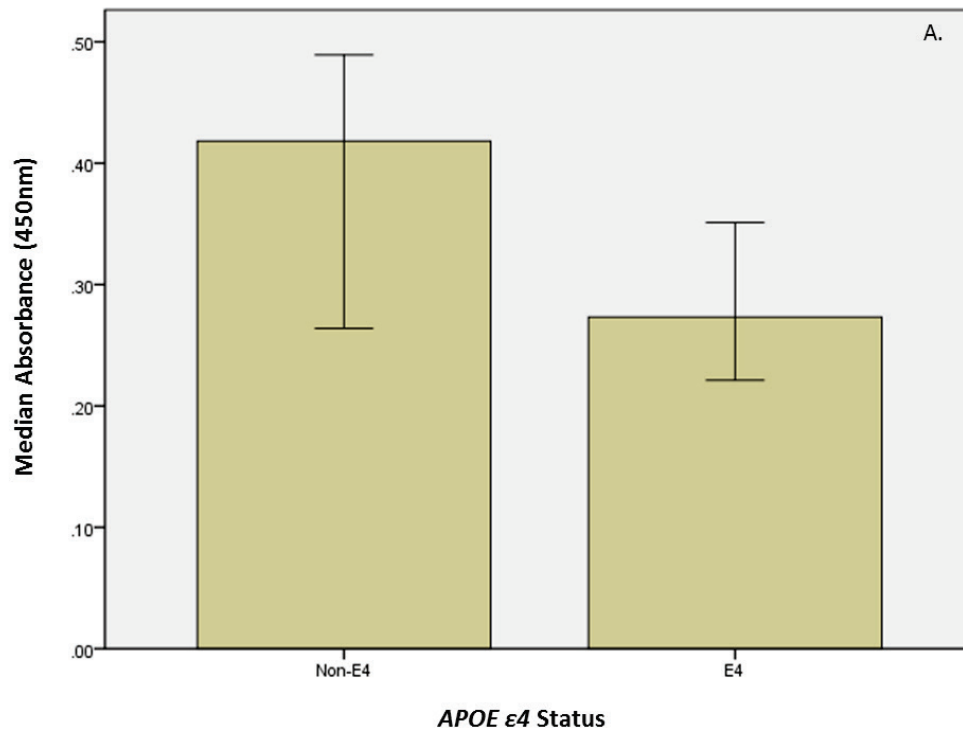
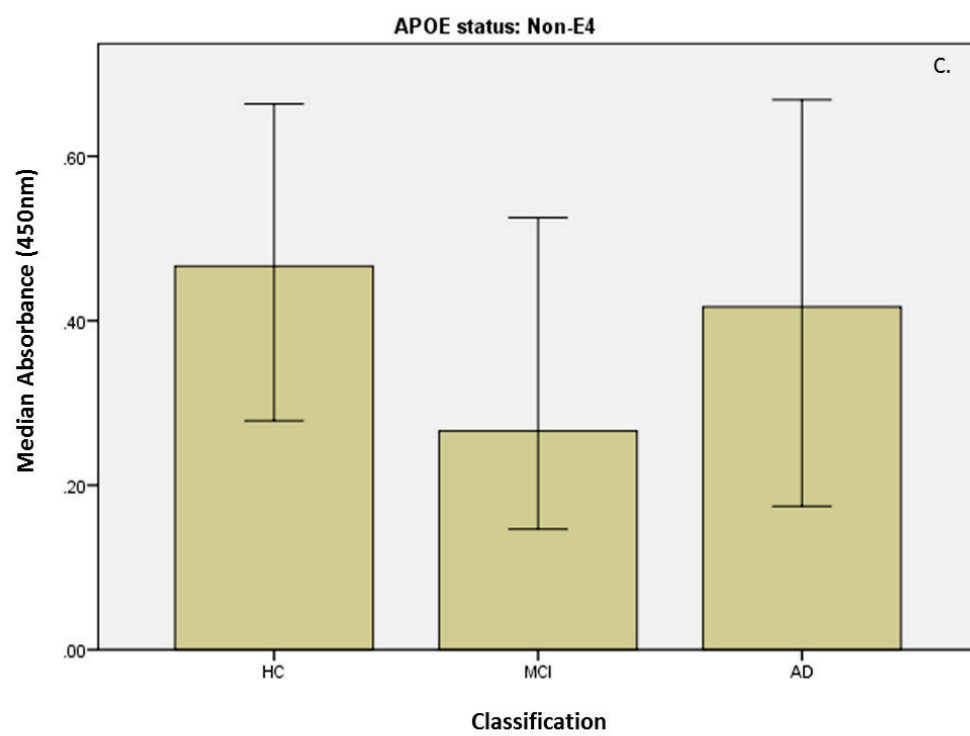
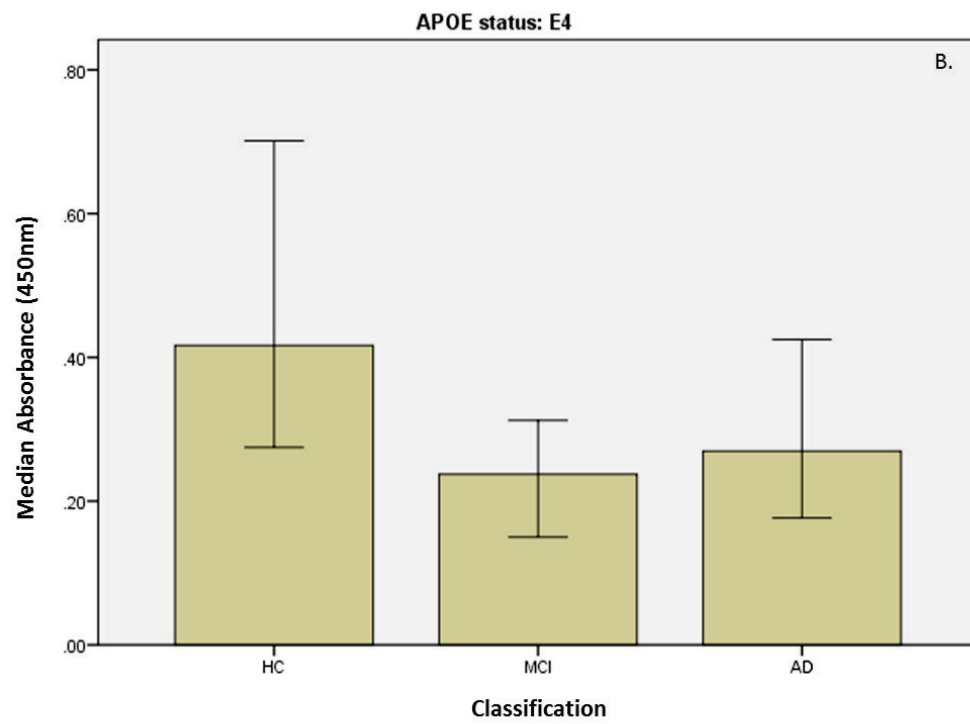


Figure 4.15(A) Total IDE levels are not significantly reduced, in *APOE* ε4 carriers compared with non-carriers. Plasma samples (diluted 1:2 - 33.3 μL per well in duplicate) from the AIBL cohort were assayed with an in-house sandwich ELISA to measure total IDE as detailed in Section 2.2.5.3. The average absorbance of the duplicates was obtained for each sample, and the median of these values by *APOE* ε4 genotype status were compared by a Kruskal-Wallis test. Data represents medians with 95% confidence interval (CI), n = 174 (89 ε4/85 non-ε4). Results show no significant reduction in IDE levels between ε4 carriers and non-carriers (p = 0.109).

(THIS PAGE HAS BEEN INTENTIONALLY LEFT BLANK)

Figure 4.15(B) and (C) Total IDE levels are reduced in MCI and AD (but not significantly), in *APOE* $\epsilon 4$ carriers but not in non-carriers. Plasma samples (diluted 1:2 - 33.3 μ L per well in duplicate) from the AIBL cohort were assayed with an in-house sandwich ELISA to measure total IDE as detailed in Section 2.2.5.3. The average absorbance of the duplicates was obtained for each sample, and the median of these values (after stratification by *APOE* $\epsilon 4$ genotype status) by clinical classification were compared by a Kruskal-Wallis test. Data represents medians with 95% confidence interval (CI), ($\epsilon 4$ carriers) n = 88 (19 HC/23 MCI/46 AD), (non-carriers) n = 86 (38 HC/26 MCI/22 AD). Results show a trend toward lower IDE in MCI and AD for $\epsilon 4$ carriers (Graph B., p = 0.068) but not for non-carriers (Graph C., p = 0.114).



4.3.15 Relationships between total plasma IDE levels and other biochemical data collected in the AIBL study

4.3.15.1 Total IDE levels correlate with age, A β , testosterone, and HDL cholesterol.

To determine if there were any relationships between total plasma IDE levels and other biochemical data collected in AIBL study, correlation analyses were performed (Table 4.2). Details of the methods used to measure the variables other than IDE are outlined in Section 2.2.2.1. Plasma IDE was analysed for any correlation with age as it has been reported that IDE expression shows an age-dependent reduction in the brain. Plasma insulin, A β_{40} and A β_{42} (and fragments), and total ApoE and ApoE4 were analysed for correlations with IDE, as both insulin and A β are substrates of IDE, and ApoE has been associated with reduced IDE expression. Testosterone (total and calculated free), estradiol and cholesterol were also examined for correlation with total IDE levels, as these have been associated with changes in IDE expression. Total plasma IDE was also analysed against brain A β load to see if any correlation existed. Results demonstrated a significant inverse correlation between levels of total plasma IDE and both plasma A β_{1-42} ($r_s = -.236$, $p = 0.002$) and A β_{1-40} ($r_s = -.225$, $p = 0.003$). IDE also tended towards an inverse correlation with A β_{x-42} ($r_s = -.144$, $p = 0.060$), however this did not reach significance. Total IDE in plasma also showed an inverse correlation with age ($r_s = -.182$, $p = 0.015$) and HDL cholesterol ($r_s = -.158$, $p = 0.036$), and a positive correlation with total testosterone ($r_s = .173$, $p = 0.022$) and free testosterone ($r_s = .172$, $p = 0.022$). No other variables correlated with plasma total IDE. Because using Bonferroni correction to adjust the rejection criteria for multiple testing would be too stringent in this instance (because of 18 tests in total), Bonferroni-Holm correction was instead used to reduce the chances of Type II errors (Holm, 1979). After the correction was made, only A β_{1-42} ($p = 0.036$) was still significant, however A β_{1-40} almost reached significance ($p = 0.051$).

(THIS PAGE HAS BEEN INTENTIONALLY LEFT BLANK)

Table 4.2 Correlation matrix of total plasma IDE levels with other variables.

Plasma levels of total IDE were analysed for relationships with sex (gender), age, and plasma insulin, A β species, ApoE species, testosterone, oestradiol, lipids and brain A β load (two tailed) (methods of data collection are outlined in Section 2.2.2.1). n values vary due to missing values for some data or in the case of brain A β load only a subset of the cohort underwent PiB-PET scans. For ApoE4, n value is lower as only the *APOE* ϵ 4 carriers express this isoform. Results show a significant but weak negative correlation exists between IDE levels and age ($r_s = -.182$, $p = .015$), A β_{1-42} ($r_s = -.236$, $p = .002$), A β_{1-40} ($r_s = -.225$, $p = .003$), and HDL cholesterol ($r_s = -.158$, $p = .036$). Significant positive correlations were observed with total testosterone ($r_s = .173$, $p = .022$) and free testosterone ($r_s = .172$, $p = .022$). A β_{x-42} approached a weak correlation however did not reach significance ($r_s = -.144$, $p = .060$). Following Bonferroni-Holm correction for multiple testing, only A β_{1-42} ($p = 0.036$) was still significant, although A β_{1-40} almost reached significance ($p = 0.051$). * indicates significance at the 0.05 level, ** indicates significance at the 0.01 level. (“unadj.” = unadjusted, prior to Bonferroni-Holm correction, “adj.” = adjusted by Bonferroni-Holm correction).

Overall	Correlation Coefficient (r_s)	N	Significance (2-tailed)
Sex	-.122	177	0.107 (NS)
Age	-.182	176	0.015 * (unadj.) 0.240 (adj.)(NS)
Insulin	.105	176	0.167 (NS)
A β_{1-40}	-.225	175	0.003 ** (unadj.) 0.051 (NS) (adj.)
A β_{1-42}	-.236	175	0.002 ** (unadj.) 0.036 * (adj.)
A $\beta_{42/40}$ ratio	-.030	175	0.693 (NS)
A β_{x-40}	-.094	174	0.218 (NS)
A β_{x-42}	-.144	172	0.060 (NS)
A $\beta_{x-42/x-40}$ ratio	-.049	172	0.519 (NS)
Total ApoE	.043	172	0.578 (NS)
ApoE4	-.111	87	0.307 (NS)
Total Testosterone	.173	176	0.022 * (unadj.) 0.330 (adj.)(NS)
Free Testosterone	.172	177	0.022 * (unadj.) 0.308 (adj.)(NS)
Oestradiol	.101	174	0.185 (NS)
Total Cholesterol	.056	177	0.458 (NS)
LDL Cholesterol	.104	177	0.169 (NS)
HDL Cholesterol	-.158	177	0.036 * (unadj.) 0.468 (adj.)(NS)
Brain A β Load	.045	30	0.814 (NS)

To exclude the possibility of an effect of gender on the results, particularly regarding testosterone and oestradiol which differ greatly between males and females, the overall subset was stratified by gender and correlation analyses performed again (Tables 4.3(A) and (B)). Results showed that the unadjusted correlation between IDE and $A\beta_{1-42}$ was still significant in both males ($r_s = -.265$, $p = 0.031$) and females ($r_s = -.225$, $p = 0.019$). In males the correlation with $A\beta_{1-40}$ was also still significant ($r_s = -.287$, $p = 0.019$), but in females was reduced to a non-significant trend ($r_s = -.180$, $p = 0.061$). Similarly, the correlation with age was reduced to a non-significant trend for both males ($r_s = -.224$, $p = 0.070$) and females ($r_s = -.160$, $p = 0.095$). Associations between IDE and HDL cholesterol disappeared when stratified by gender. The positive correlations of IDE with total and free testosterone, however, were strengthened in males when stratified [$(r_s = .314$, $p = 0.010)$ and $(r_s = .334$, $p = 0.006)$, respectively] but in females this correlation disappeared ($r_s = .039$, $p = 0.689$). When adjusted for multiple testing by Bonferroni-Holm correction, all associations became non-significant.

When stratified by *APOE* $\epsilon 4$ status (Tables 4.4 (A) and (B)), for the non- $\epsilon 4$ carriers significant inverse correlations were observed between total plasma IDE and $A\beta_{1-40}$ ($r_s = -.255$, $p = 0.015$), $A\beta_{1-42}$ ($r_s = -.226$, $p = 0.038$), and HDL cholesterol ($r_s = -.247$, $p = 0.022$). For $\epsilon 4$ carriers, an inverse correlation was seen with $A\beta_{1-42}$ ($r_s = -.245$, $p = 0.022$) and a positive correlation with total testosterone ($r_s = .213$, $p = 0.048$) and free testosterone ($r_s = .221$, $p = 0.038$). Following Bonferroni-Holm correction however, associations for both $\epsilon 4$ carriers and non-carriers were no longer significant.

After stratification by clinical classification, the correlations seen between total IDE and $A\beta_{1-42}$ and $A\beta_{1-40}$ were found to be restricted to AD patients only ($r_s = -.361$, $p = 0.002$, $n = 70$) and ($r_s = -.268$, $p = 0.025$, $n = 70$), respectively (not shown in tables). Stratifying further by $\epsilon 4$ status showed that the association in AD was only in non- $\epsilon 4$ carriers for $A\beta_{1-42}$ ($r_s = -.634$, $p = 0.002$, $n = 22$), and $A\beta_{1-40}$ ($r_s = -.527$, $p = 0.012$, $n = 22$). This stratification strengthened the correlations by approximately two-fold.

Tables 4.3 (A) Correlation matrix of total plasma IDE levels with other biochemical variables separately by gender (males only). Table 4.3 (A) shows correlation analysis of total plasma IDE with age, and plasma insulin, A β species, ApoE species, testosterone, oestradiol, lipids and brain A β load (two tailed). Methods of data collection are outlined in Section 2.2.2.1. n values vary due to missing values for some data or in the case of brain A β load only a subset of the cohort underwent PiB-PET scans. For ApoE4, n value is lower as only the *APOE* ϵ 4 carriers express this isoform. Results show that significant negative correlations are retained in males between IDE and A β ₁₋₄₂ ($r_s = -.265$, $p = 0.031$), A β ₁₋₄₀ ($r_s = -.287$, $p = .019$), and the positive correlation in males was stronger with total testosterone ($r_s = .314$, $p = .010$) and free testosterone ($r_s = .334$, $p = .006$). However, following Bonferroni-Holm correction, all associations were no longer significant. ** indicates significance at the 0.01 level, * indicates significance at the 0.05 level.

Males (A)	Correlation Coefficient (r_s)	N	Significance (2-tailed)
Age	-.224	66	0.070 (NS)
Insulin	.192	66	0.122 (NS)
A β ₁₋₄₀	-.287	66	0.019 * (unadj.) 0.304 (adj.)(NS)
A β ₁₋₄₂	-.265	66	0.031 * (unadj.) 0.465 (adj.)(NS)
A β _{42/40} ratio	.012	66	0.921 (NS)
A β _{x-40}	-.013	65	0.919 (NS)
A β _{x-42}	-.097	65	0.441 (NS)
A β _{x-42/x-40} ratio	-.086	65	0.497 (NS)
Total ApoE	.159	65	0.207 (NS)
ApoE4	.038	34	0.831 (NS)
Total Testosterone	.314	66	0.010 ** (unadj.) 0.170 (adj.)(NS)
Free Testosterone	.334	66	0.006 ** (unadj.) 0.108 (adj.)(NS)
Oestradiol	.130	66	0.299 (NS)
Total Cholesterol	.040	66	0.751 (NS)
LDL Cholesterol	.060	66	0.634 (NS)
HDL Cholesterol	-.157	66	0.209 (NS)
Brain A β Load	-.105	12	0.746 (NS)

Tables 4.3 (B) Correlation matrix of total plasma IDE levels with other biochemical variables separately by gender (females only). Table 4.3 (B) shows correlation analysis of total plasma IDE with age, and plasma insulin, A β species, ApoE species, testosterone, oestradiol, lipids and brain A β load (two tailed). Methods of data collection are outlined in Section 2.2.2.1. n values vary due to missing values for some data or in the case of brain A β load only a subset of the cohort underwent PiB-PET scans. For ApoE4, n value is lower as only the *APOE* ϵ 4 carriers express this isoform. Results show that significant negative correlations are retained in females only between total IDE and A β ₁₋₄₂ ($r_s = -.225$, $p = 0.019$),. However, following Bonferroni-Holm correction, the association was no longer significant. * indicates significance at the 0.05 level.

Females (B)	Correlation Coefficient (r_s)	N	Significance (2-tailed)
Age	-.160	110	0.095 (NS)
Insulin	.066	110	0.490 (NS)
A β ₁₋₄₀	-.180	109	0.061 (NS)
A β ₁₋₄₂	-.225	109	0.019 * (unadj.) 0.323 (adj.)(NS)
A β _{42/40} ratio	-.063	109	0.516 (NS)
A β _{x-40}	-.089	109	0.356 (NS)
A β _{x-42}	-.153	107	0.115 (NS)
A β _{x-42/x-40} ratio	-.046	107	0.641 (NS)
Total ApoE	-.019	107	0.847 (NS)
ApoE4	-.194	53	0.165 (NS)
Total Testosterone	.039	110	0.689 (NS)
Free Testosterone	.027	111	0.777 (NS)
Oestradiol	-.020	108	0.835 (NS)
Total Cholesterol	.118	111	0.219 (NS)
LDL Cholesterol	.143	111	0.134 (NS)
HDL Cholesterol	-.122	111	0.200 (NS)
Brain A β Load	.236	18	0.345 (NS)

Table 4.4 (A) Correlation matrix of total plasma IDE levels with other variables for APOE ϵ 4 non-carriers only. Plasma levels of full-length IDE were analysed for relationships with sex, age, and plasma insulin, A β species, ApoE species, testosterone, oestradiol, lipids and brain A β load (two tailed) after stratifying data by APOE ϵ 4 status (methods of data collection are outlined in Section 2.2.2.1). n values vary due to missing values for some data or in the case of brain A β load only a subset of the cohort underwent PiB-PET scans. For ApoE4, no values are shown as only the APOE ϵ 4 carriers express this isoform. Results show significant inverse correlations between IDE levels and A β ₁₋₄₀ ($r_s = -.262$, $p = 0.015$), A β ₁₋₄₂ ($r_s = -.226$, $p = 0.038$) and HDL cholesterol ($r_s = -.247$, $p = 0.022$). Following Bonferroni-Holm correction for multiple testing, no associations remained significant. * indicates significance at the 0.05 level.

APOE ϵ4 non-carriers only (A)	Correlation Coefficient (r_s)	N	Significance (2-tailed)
Sex	-.125	86	0.250 (NS)
Age	-.179	86	0.099 (NS)
Insulin	.167	85	0.126 (NS)
A β ₁₋₄₀	-.262	85	0.015 * (unadj.) 0.255 (adj.)(NS)
A β ₁₋₄₂	-.226	85	0.038 * (unadj.) 0.570 (adj.)(NS)
A β _{42/40} ratio	.021	85	0.847 (NS)
A β _{x-40}	-.053	85	0.630 (NS)
A β _{x-42}	-.152	84	0.168 (NS)
A β _{x-42/x-40} ratio	-.064	84	0.565 (NS)
Total ApoE	.017	85	0.878 (NS)
ApoE4	-	-	-
Total Testosterone	.138	86	0.206 (NS)
Free Testosterone	.142	86	0.194 (NS)
Oestradiol	.085	85	0.437 (NS)
Total Cholesterol	-.069	86	0.528 (NS)
LDL Cholesterol	-.019	86	0.864 (NS)
HDL Cholesterol	-.247	86	0.022 * (unadj.) 0.352 (adj.)(NS)
Brain A β Load	-.015	14	0.958 (NS)

Table 4.4 (B) Correlation matrix of total plasma IDE levels with other variables for APOE ϵ 4 carriers only. Plasma levels of full-length IDE were analysed for relationships with sex, age, and plasma insulin, A β species, ApoE species, testosterone, oestradiol, lipids and brain A β load (two tailed) after stratifying data by APOE ϵ 4 status (methods of data collection are outlined in Section 2.2.2.1). n values vary due to missing values for some data or in the case of brain A β load only a subset of the cohort underwent PiB-PET scans. Results show significant inverse correlations between IDE levels A β ₁₋₄₂ (r_s = -.245, p = 0.022) and a positive correlation with total testosterone (r_s = .213, p = 0.048) and free testosterone (r_s = .221, p = 0.038). Following Bonferroni-Holm correction for multiple testing, no associations remained significant. * indicates significance at the 0.05 level.

APOE ϵ4 carriers only (B)	Correlation Coefficient (r_s)	N	Significance (2-tailed)
Sex	-.147	88	0.172 (NS)
Age	-.149	88	0.166 (NS)
Insulin	.015	88	0.889 (NS)
A β ₁₋₄₀	-.172	87	0.110 (NS)
A β ₁₋₄₂	-.245	87	0.022 * (unadj.) 0.396 (adj.)(NS)
A β _{42/40} ratio	-.119	87	0.272 (NS)
A β _{x-40}	-.180	86	0.097 (NS)
A β _{x-42}	-.143	85	0.193 (NS)
A β _{x-42/x-40} ratio	-.007	85	0.949 (NS)
Total ApoE	.053	87	0.624 (NS)
ApoE4	-.111	87	0.307 (NS)
Total Testosterone	.213	87	0.048 * (unadj.) 0.768 (adj.)(NS)
Free Testosterone	.221	88	0.038 * (unadj.) 0.646 (adj.)(NS)
Oestradiol	.122	86	0.262 (NS)
Total Cholesterol	.141	88	0.191 (NS)
LDL Cholesterol	.185	88	0.084 (NS)
HDL Cholesterol	-.071	88	0.512 (NS)
Brain A β Load	-.118	15	0.676 (NS)

4.3.15.2 The ratio of total IDE to insulin is lower in MCI and AD individuals.

As previously mentioned, IDE has a high affinity for both insulin and A β , and as a result the levels of these substrates in relation to IDE may provide better discrimination between clinical classifications than the levels of IDE alone. Therefore the ratio of total IDE to insulin was calculated and compared between classifications (Figure 4.16(A)). An increase in insulin relative to total IDE levels or a decrease in IDE relative to insulin levels would lead to a reduction of the IDE/Insulin ratio. Either of these changes could influence the degradation of A β (both directly and indirectly via other effects of insulin, for example), and may lead to an increase in plasma A β due to decreased degradation. The results showed that the IDE/insulin ratio was significantly lower in MCI subjects compared with HC subjects ($p = 0.009$). After applying Bonferroni correction for multiple testing the difference was still significant ($p = 0.027$). The IDE/insulin ratio was also lower in the AD group compared with controls ($p = 0.032$) however after Bonferroni correction for multiple testing was no longer significant ($p = 0.096$).

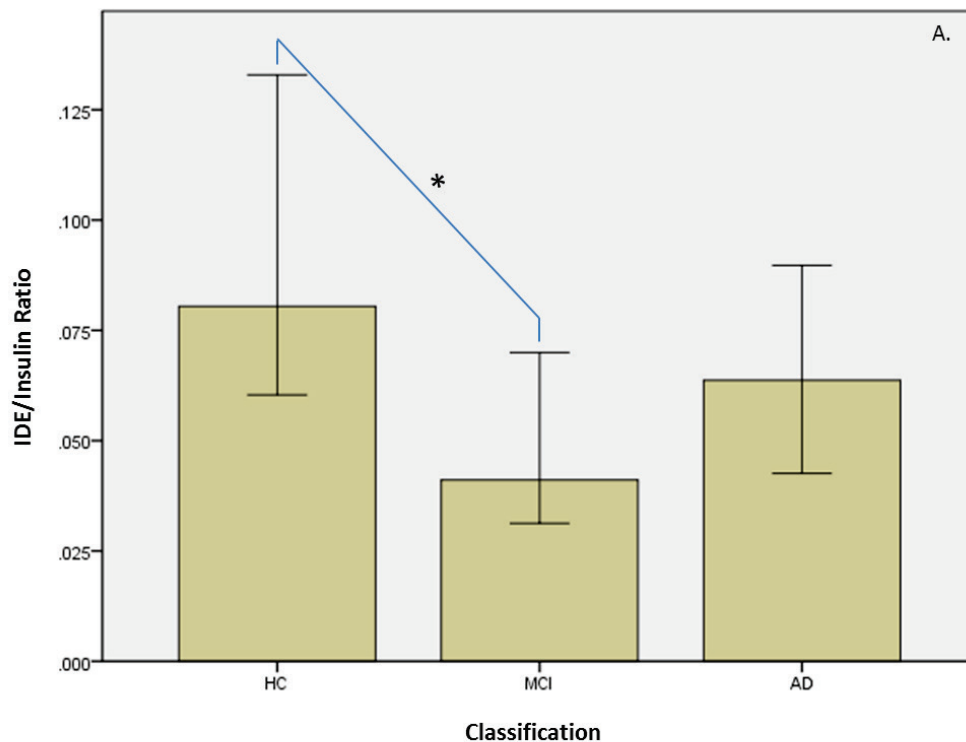
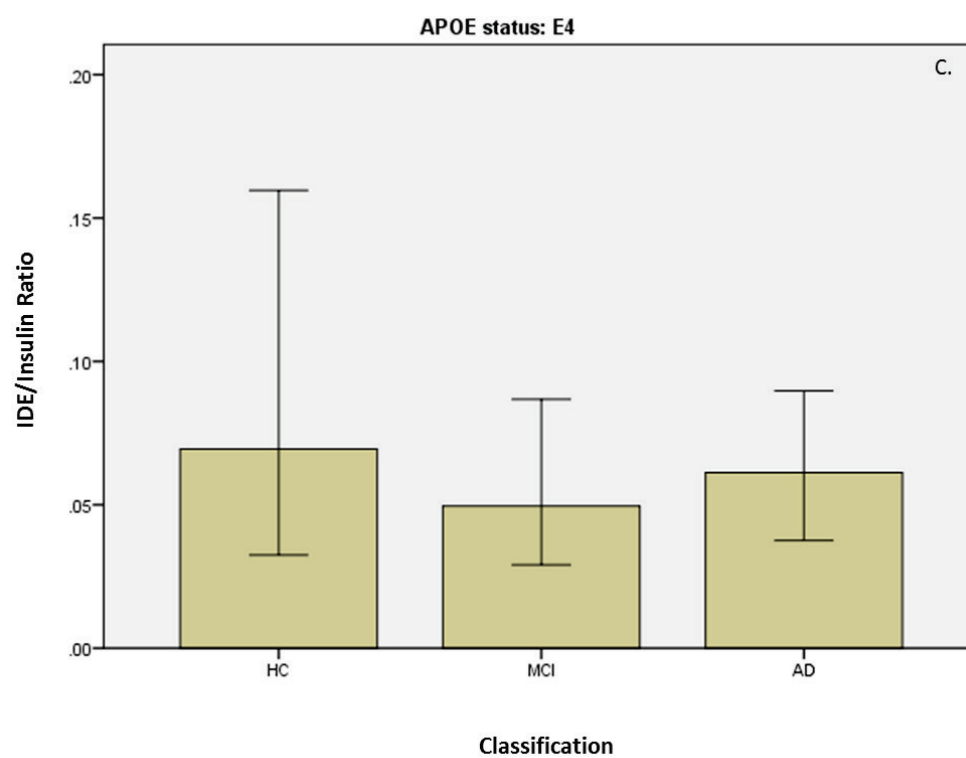
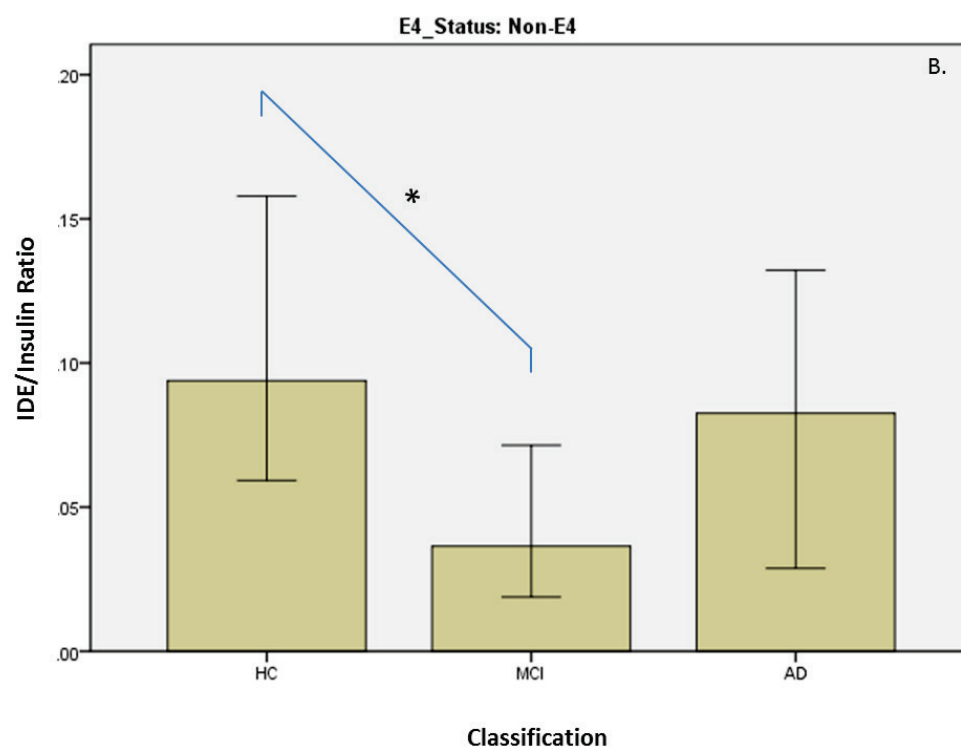


Figure 4.16(A) AD and MCI individuals have a lower IDE/Insulin ratio than controls (overall). Insulin levels were measured as outlined in Section 2.2.2.1. Plasma samples (diluted 1:2 - 33.3 μ L per well in duplicate) from the AIBL cohort were assayed with an in-house sandwich ELISA to measure total IDE as detailed in Section 2.2.5.3. The average absorbance of the duplicates was obtained for each sample, and a ratio was calculated of total plasma IDE to plasma insulin (IDE/Insulin ratio). The median of these values were compared between classifications by a Kruskal-Wallis test with Mann-Whitney post-hoc tests (Bonferroni correction applied for multiple testing). Data represents medians with 95% confidence interval (CI), $n = 176$ (57 HC/50 MCI/69 AD). Results show the MCI group has a lower IDE/insulin ratio than HC ($p = 0.027$ after Bonferroni correction).

When the groups were stratified for *APOE* $\epsilon 4$ status, only the non- $\epsilon 4$ carriers had significantly different IDE/Insulin ratios by classification (Figure 4.16(B) and (C)). Results for this group showed a significant reduction of the IDE/Insulin ratio in the MCI group compared with HC group ($p = 0.012$, after Bonferroni correction $p = 0.036$). There was no significant differences observed between the AD group and either MCI ($p = 0.247$), or HC group ($p = 0.235$). For the $\epsilon 4$ carriers there were no significant differences between classifications ($p = 0.386$).

(THIS PAGE HAS BEEN INTENTIONALLY LEFT BLANK)

Figure 4.16(B) and (C) MCI individuals that do not carry an *APOE* $\epsilon 4$ allele have a lower IDE/Insulin ratio than non- $\epsilon 4$ controls. Insulin levels were measured as outlined in Section 2.2.2.1. Plasma samples (diluted 1:2 - 33.3 μ L per well in duplicate) from the AIBL cohort were assayed with an in-house sandwich ELISA to measure total IDE as detailed in Section 2.2.5.3. The average absorbance of the duplicates was obtained for each sample, and a ratio was calculated of total plasma IDE to plasma insulin (IDE/Insulin ratio). Data was stratified by *APOE* $\epsilon 4$ status and the median of these values were compared between HC/MCI/AD classifications by a Kruskal-Wallis test with Mann-Whitney post-hoc tests, and found to be significantly different in the non- $\epsilon 4$ carriers only ($p = 0.036$ after Bonferroni correction applied for multiple testing) but no differences were observed between classifications for $\epsilon 4$ carriers ($p = 0.386$). Data represents medians with 95% confidence interval (CI), (non- $\epsilon 4$) $n = 85$ (38 HC/26 MCI/21 AD), ($\epsilon 4$ carriers) $n = 88$ (19 HC/23 MCI/46 AD).



4.3.15.3 The ratio of total IDE to $A\beta_{42+40}$ is significantly lower in MCI and AD compared with HC.

Similar to the IDE/insulin ratio, the ratio of IDE to total levels of both $A\beta_{42}$ and $A\beta_{40}$ may offer some information about AD, and in fact may be more relevant to Alzheimer's pathology than IDE/Insulin ratios. It has been observed that plasma $A\beta$ is generally elevated in early AD compared with controls and reduces as $A\beta$ is deposited into SP in the brain (Kawarabayashi, *et al.*, 2001; Schupf, *et al.*, 2008), therefore the ratio of plasma IDE/ $A\beta$ levels may be pertinent in the prodromal stages of AD with respect to the regulation of plasma $A\beta$ levels. This may be reflected in the MCI group, which represents the pre-symptomatic period prior to full-blown AD symptoms becoming apparent. Hence, the IDE/ $A\beta_{42+40}$ ratios were calculated (using $A\beta_{1-42}$ and $A\beta_{1-42}$) and analysed by classification (Figure 4.17(A)).

The results show a significant reduction in the IDE/ $A\beta_{42+40}$ ratio in MCI ($p = 0.000$) and AD ($p = 0.024$) compared with HC after Bonferroni correction for multiple testing (MCI<AD<HC). When stratified for *APOE* status (Figures 4.17(B) and (C)), no significant differences were observed between clinical classifications for the IDE/ $A\beta_{42+40}$ ratio, however a trend toward lower ratios were seen in the MCI group compared with controls and AD ($p = 0.071$) but only in the non- $\epsilon 4$ carriers.

The decrease in IDE levels (as shown in the current study) relative to $A\beta$, together with an increase in $A\beta$ accounts for the reduction in IDE/ $A\beta_{42+40}$ ratios observed. Only a slight increase was observed in $A\beta_{40}$ species indicating that the reduction of IDE was the major factor contributing to the differences observed. It was concluded that the IDE/ $A\beta_{42+40}$ ratio may be of benefit for differentiating between healthy controls and pre-symptomatic or symptomatic AD patients.

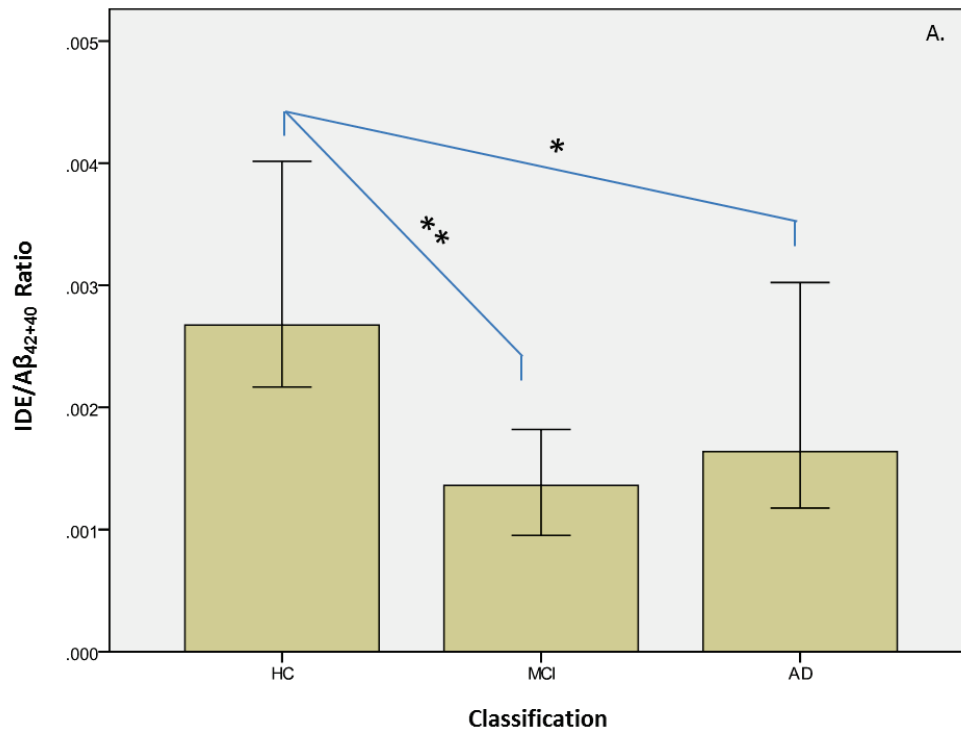
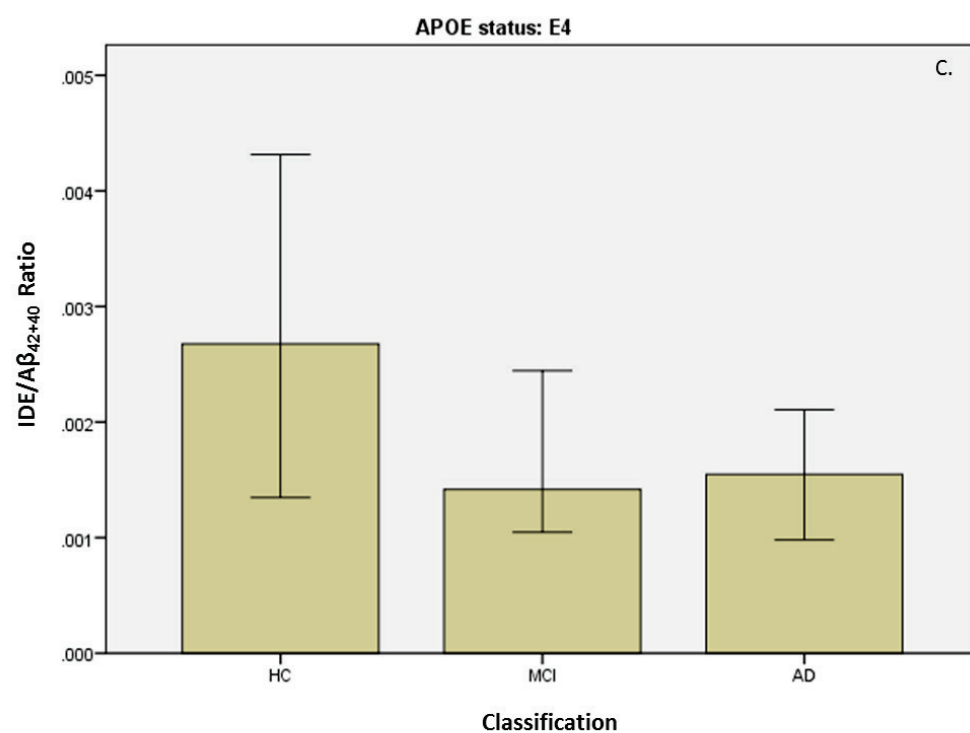
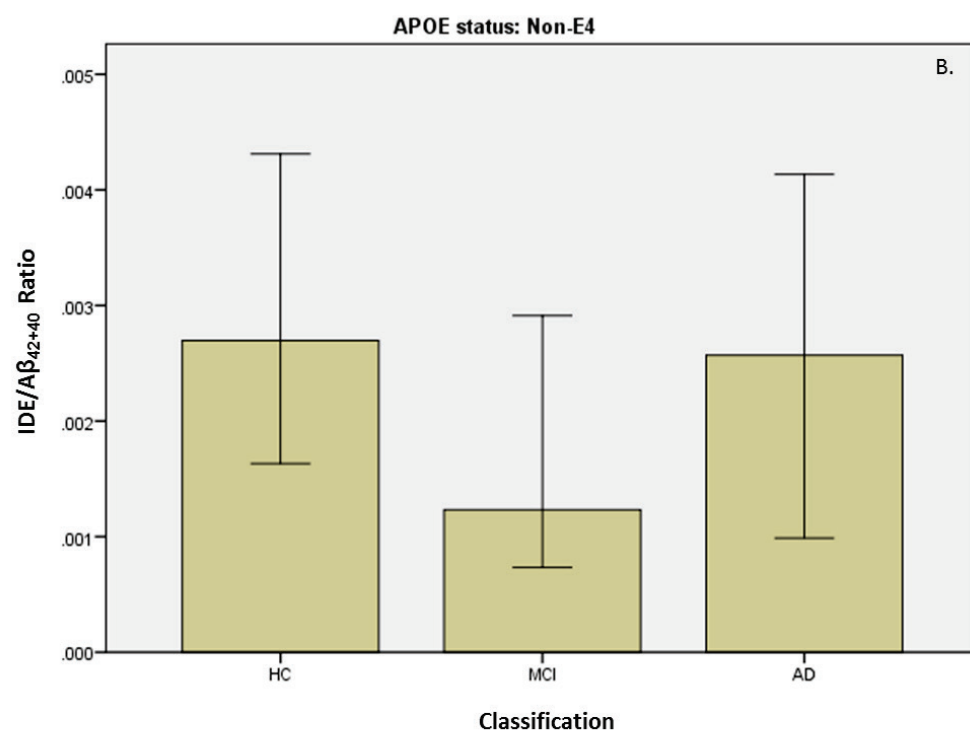


Figure 4.17(A) The plasma IDE/Aβ₄₂₊₄₀ ratio is significantly reduced in MCI and AD in comparison to HC. Aβ₁₋₄₂ and Aβ₁₋₄₀ were measured as outlined in Section 2.2.2.1. Plasma samples (diluted 1:2 - 33.3 μL per well in duplicate) from the AIBL cohort were assayed with an in-house sandwich ELISA to measure total IDE as detailed in Section 2.2.5.3. The average absorbance of the duplicates was obtained for each sample, and a ratio of total plasma IDE to plasma Aβ₄₂₊₄₀ (IDE/Aβ₄₂₊₄₀ ratio) was calculated. The median of these values were compared between classifications by a Kruskal-Wallis test with Mann-Whitney post-hoc tests (with Bonferroni correction applied for multiple testing). Data represents medians with 95% confidence interval (CI), n = 175 (57 HC/48 MCI/70 AD). Results show that after Bonferroni correction the IDE/Aβ₄₂₊₄₀ ratio is significantly lower in MCI (p = 0.000) and AD (p = 0.024) compared with HC.

Figure 4.17(B) and (C) The plasma IDE/A β_{42+40} ratio is not significantly reduced in MCI and AD in comparison to HC when stratified by *APOE* status. A β_{1-42} and A β_{1-40} were measured as outlined in Section 2.2.2.1. Plasma samples (diluted 1:2 - 33.3 μ L per well in duplicate) from the AIBL cohort were assayed with an in-house sandwich ELISA to measure total IDE as detailed in Section 2.2.5.3. The average absorbance of the duplicates was obtained for each sample, and a ratio of total plasma IDE to plasma A β_{42+40} (IDE/A β_{42+40} ratio) was calculated. The median of these values were compared between classifications by a Kruskal-Wallis test with Mann-Whitney post-hoc tests (with Bonferroni correction applied for multiple testing). Data represents medians with 95% confidence interval (CI). (Non- $\epsilon 4$ carriers) n = 85 (38 HC/25 MCI/22 AD), ($\epsilon 4$ carriers) n = 87 (19 HC/22 MCI/46 AD). Results show no significant differences between classifications for $\epsilon 4$ carriers (p = 0.092), however a trend toward lower IDE/A β_{42+40} ratio was seen in MCI for the $\epsilon 4$ non-carriers only (p = 0.071).



4.3.15.4 The ratio of total IDE to Insulin + A β ₄₂₊₄₀ is significantly lower in MCI and AD compared with HC.

Because significant differences were observed between the classifications for both the IDE/Insulin ratio and also the IDE/A β ₄₂₊₄₀ ratio, there may be a combined effect of increased insulin and increased A β with reduced IDE in plasma, thus the ratio of IDE to combined levels of insulin and A β could potentially differentiate MCI and AD compared to control subjects better than the IDE/insulin or IDE/A β ₄₂₊₄₀ ratios. To address this possibility, insulin values (in mU/L) were converted into pg/mL and added to the A β ₄₂₊₄₀ values to give a total value of Insulin + A β ₄₂₊₄₀ in pg/mL. The ratio of IDE to insulin + A β ₄₂₊₄₀ was then analysed by classification to see if it could differentiate between MCI and/or AD with controls more effectively than insulin or A β ₄₂₊₄₀ alone (Figure 4.18(A)).

The results show that the IDE/Insulin + A β ₄₂₊₄₀ ratio was similar to the IDE/A β ₄₂₊₄₀ ratio in that there was a highly significant decrease in MCI compared with controls ($p = 0.000$) and a significant reduction in AD compared with controls ($p = 0.024$) after Bonferroni correction for multiple testing (MCI<AD<HC). Using the combination of insulin and A β levels in the ratio rather than just A β alone did not produce any additional benefits to using A β levels alone in this subgroup. However, when stratified by APOE status (Figures 4.18(B) and (C)), the non- $\epsilon 4$ carriers showed a similar result to the overall results with a significantly lower ratio in MCI compared with HC ($p = 0.048$ after Bonferroni correction), while the $\epsilon 4$ carriers were not significantly different between classifications ($p = 0.172$). It was concluded that similar to the IDE/A β ₄₂₊₄₀ ratio, the IDE/Insulin + A β ₄₂₊₄₀ ratio may be useful for differentiating between healthy controls and pre-symptomatic or symptomatic AD patients.

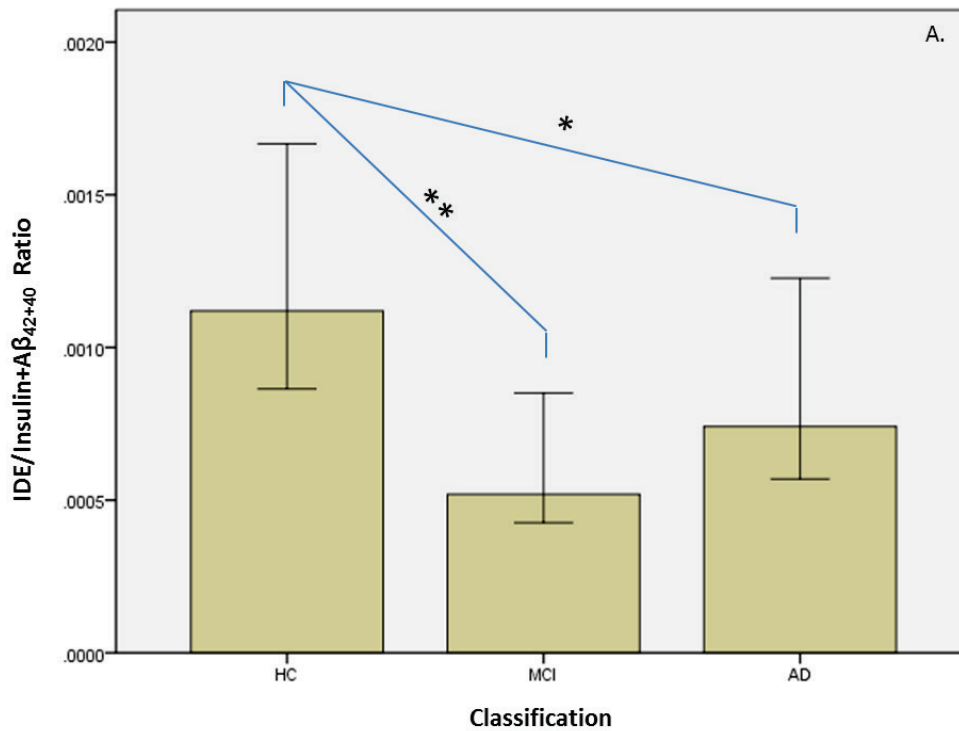
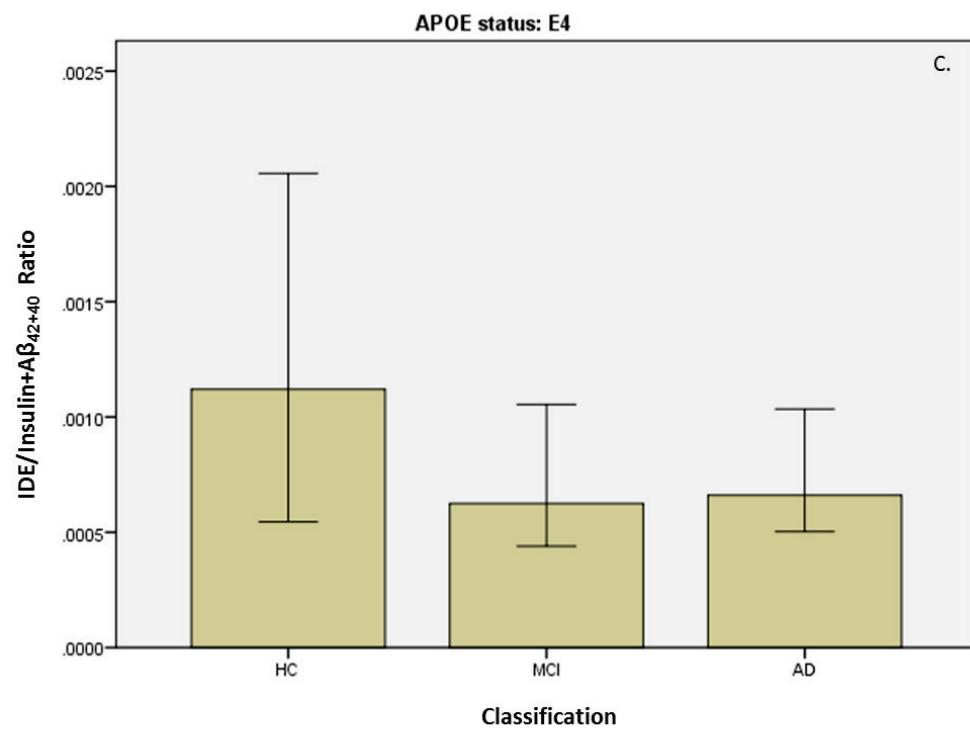
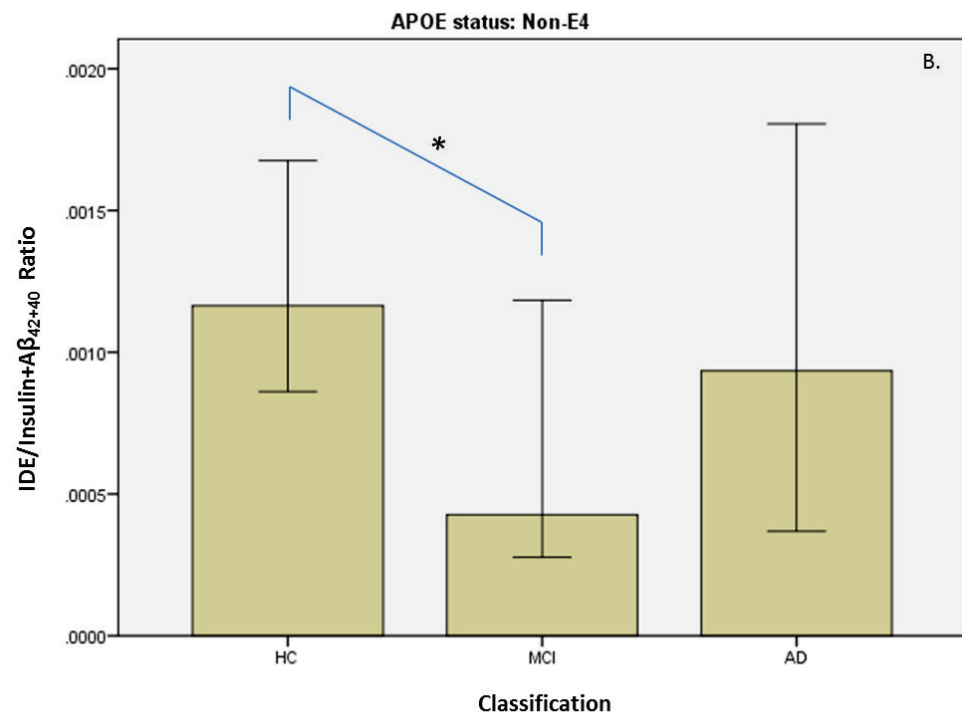


Figure 4.18(A) The plasma IDE/Insulin+A β_{42+40} ratio is significantly reduced in MCI and AD in comparison to HC. Insulin, A β_{1-42} and A β_{1-40} were measured as outlined in Section 2.2.2.1. Plasma samples (diluted 1:2 - 33.3 μ L per well in duplicate) from the AIBL cohort were assayed with an in-house sandwich ELISA to measure total IDE as detailed in Section 2.2.5.3. The average absorbance of the duplicates was obtained for each sample, and a ratio of total plasma IDE to plasma Insulin + A β_{42+40} (IDE/Insulin+A β_{42+40} ratio) was calculated. The median of these values were compared between classifications by a Kruskal-Wallis test with Mann-Whitney post-hoc tests (with Bonferroni correction applied for multiple testing for significant results only). Data represents medians with 95% confidence interval (CI), n = 174 (57 HC/48 MCI/69 AD). Results show that after Bonferroni correction MCI (p = 0.000) and AD (p = 0.024) were significantly lower than HC.

Figure 4.18(B) and (C) The plasma IDE/Insulin+A β_{42+40} ratio is significantly reduced in MCI in comparison to HC in APOE $\epsilon 4$ non-carriers only. Insulin, A β_{1-42} and A β_{1-40} were measured as outlined in Section 2.2.2.1. Plasma samples (diluted 1:2 - 33.3 μ L per well in duplicate) from the AIBL cohort were assayed with an in-house sandwich ELISA to measure total IDE as detailed in Section 2.2.5.3. The average absorbance of the duplicates was obtained for each sample, and a ratio of total plasma IDE to plasma Insulin + A β_{42+40} (IDE/Insulin+A β_{42+40} ratio) was calculated. The median of these values were compared between classifications by a Kruskal-Wallis test with Mann-Whitney post-hoc tests (with Bonferroni correction applied for multiple testing for significant results only). Data represents medians with 95% confidence interval (CI), (non- $\epsilon 4$ carriers) n = 84 (38 HC/25 MCI/21 AD), ($\epsilon 4$ carriers) n = 87 (19 HC/22 MCI/46 AD). Results show that after Bonferroni correction MCI (p = 0.000) and AD (p = 0.024) were significantly lower than HC.



4.3.16 Follow-up optimisation (1): Cell-culture grade BSA produces a dose-dependent signal in IDE sandwich ELISA but Fraction V BSA and HSA do not.

After the AIBL samples were assayed in the IDE sandwich ELISA assays mentioned above (using cell-culture grade BSA), some experiments were performed to find out if a purer form of BSA could reduce the high background observed in the ELISA. Cross-reactivity had been seen with both Ab 2 and Ab 3 with cell-culture grade BSA and HSA at an equivalent range of concentrations of albumin found in human serum with Western immunoblotting, therefore it was deemed worthwhile to determine if there was still cross-reactivity under non-denaturing and reducing conditions. Fraction V derived BSA is a purer formulation of BSA than cell-culture grade, and hence may result in less background in the sandwich ELISA due to reduced levels of contaminants. Therefore, cell-culture grade BSA was compared with Fraction V BSA and human serum albumin (HSA) to determine if these latter two could contribute to any part of the signal observed in the ELISA (Figure 4.19). Fraction V BSA was used as the blocking agent. The results demonstrate that there is no signal derived above the absorbance of the blank, from the Fraction V BSA or from HSA at any of the concentrations tested. However, a dose-dependent signal was observed with the cell-culture grade BSA. Furthermore, the background absorbance was considerably reduced by using the Fraction V BSA as a blocking agent instead of the cell-culture grade BSA, compared with previous experiments. Nevertheless, the cell-culture grade BSA was used in the experiments in this chapter, and the background absorbance was removed prior to analysing the values, therefore it was concluded that it was not a confounding issue in the assay. In addition, since the readings of HSA in this assay were negligible, it is likely that the human albumin in the plasma samples was not interfering with the detection of IDE in the sandwich ELISA. Finally, Fraction V BSA was determined to be the superior grade of BSA as a blocking agent in this assay as compared with cell-culture grade BSA. This highlights the importance of choosing the correct blocking agent for the assay.

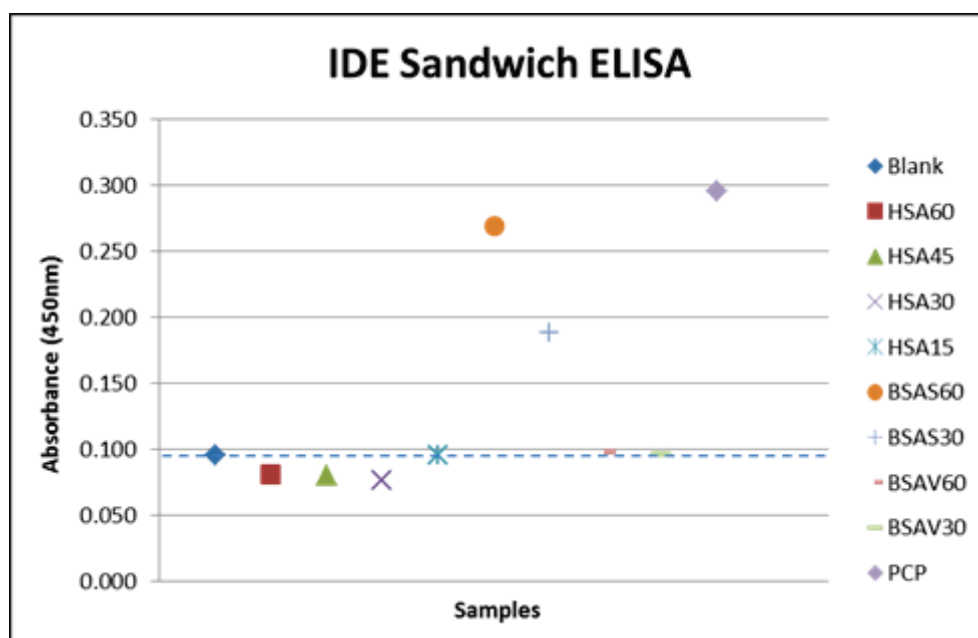


Figure 4.19 Determination and comparison of potential cross-reactivity of different albumins in the IDE sandwich ELISA. A sandwich ELISA was carried out using HSA and two types of BSA at different dilutions in place of plasma samples. HSA was diluted to physiological concentrations (60, 45, 30 and 15mg/mL) and then diluted as per plasma samples (1:2). The two types of BSA were also diluted to concentrations equivalent to the highest and lowest physiological plasma levels of HSA (60 and 30 mg/mL, respectively) and then diluted as per plasma samples (1:2). The two types of BSA tested were Cell Culture Grade BSA (BSAS) and Fraction V BSA (BSAV). Fraction V BSA was also used for the blocking step. The OD readings obtained for the blank and a pooled plasma control sample are included in graph (PCP diluted to 1:2). Data is shown without blank-adjustment, the blank reading is indicated by the dashed line. The results show that the background absorbance can be reduced considerably using Fraction V BSA or HSA as blocking agents, compared with previous experiments which used cell-culture grade BSA.

4.3.17 Follow-up optimisation (2): Spike and recovery assessment of IDE sandwich ELISA.

As plasma contains a plethora of constituents which potentially may interfere with the detection of IDE in the ELISA, a spike and recovery assessment was performed using recombinant rat IDE (Figure 4.20). The assay was performed as detailed in Section 2.2.5.3 with the following exceptions. Rat IDE was used to prepare two identical standard curves; one diluted in PBST alone (control) and the other diluted in a 1:2 solution of PCP. Ideally, the two standard curves should be the same; however values that fall within 80-120% of the control curve are considered to be an acceptable recovery. Results demonstrated significant interference by the plasma matrix with 100% of the standard curve signal suppressed, even at the highest concentrations of IDE.

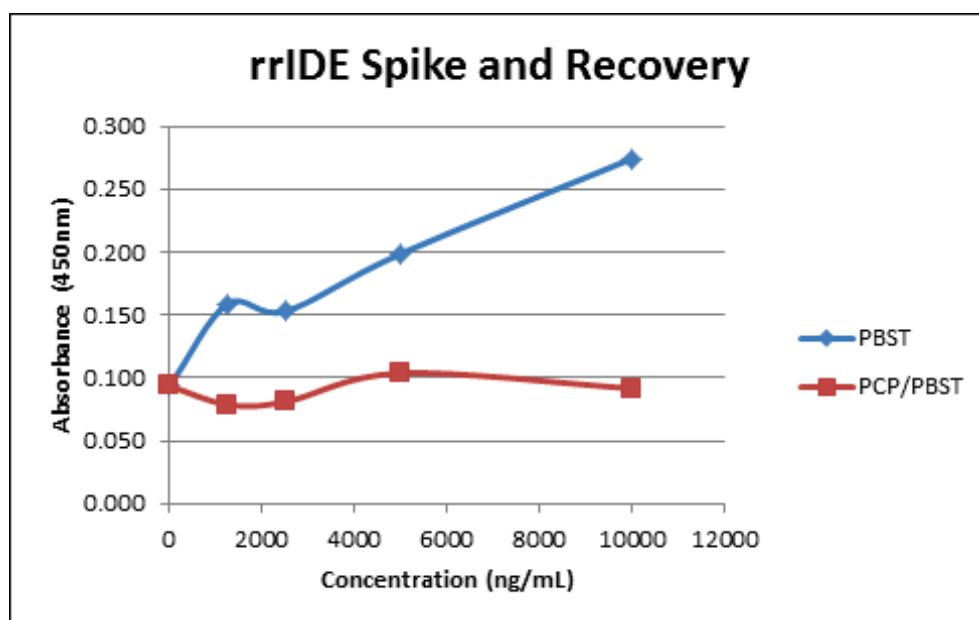


Figure 4.20 Assessment of the potential interference of plasma components in the IDE sandwich ELISA. Spike and recovery assessment ELISA was carried out on plasma using Ab 3 as coating antibody (1:400) and Ab 2 as detection antibody (1:750) as detailed in Section 2.2.5.3. Recombinant rat IDE (rrIDE) used for the standard curve at concentrations of 1250 to 10000ng/mL in PBST (PBST - Control). Data is shown without blank-adjustment. Pooled control plasma (PCP) was diluted 1:2 in PBST, and used as the diluent for an identical standard curve to the control (PCP/PBST). Values for the PCP/PBST samples were obtained by subtracting control blank from the PCP/PBST blank to obtain the signal generated from the plasma-containing diluent, then removing this value from the spiked values (without blank adjustment).

4.4 Discussion

The aim of this chapter was to determine IDE levels in human plasma, in samples which had been obtained from AIBL study participants, using an ELISA method for the enzyme measurement. The test was developed to ascertain if there were any differences in total plasma IDE levels in AD or MCI patients, when compared with healthy controls. Correlations between the IDE levels and levels of other AD-related genetic or biochemical parameters which had been analysed in the AIBL study were also investigated, to determine if there were any significant relationships.

At the time of starting this project, there was only one available commercial IDE ELISA kit recommended for the detection of IDE in human plasma. This kit was screened for its suitability for measuring plasma IDE and reproducibility between runs. From two runs, the kit showed a high coefficient of variation (CV) within duplicates (intra-assay variation) and between runs (inter-assay variation); ideally the variation should not exceed 15%. The reasons for the high coefficients of variation were not clear; however one possibility may be related to the storage of the plasma samples. The company states that plasma samples should be stored for less than 2 months at -80°C. Although stored correctly, the samples used to test the kit were in excess of 6 months old, as were the AIBL samples which were to be assayed. Another possible reason for the variation may be related to the assay procedure: during the first 37°C incubation on Run 1, the plate sealer provided with the kit had warped and partially peeled off the plate. This may have introduced some variation due to uneven evaporation of the wells and temperature differences. An alternate brand of plate sealers were therefore used for subsequent incubations. However, this would not have accounted for the high CV observed in the second test. While it would have been of value to screen the kit further, this was not viable due to the high cost of the kit and therefore this kit was deemed unsuitable for this project.

As a result, the development of an in-house ELISA was necessary. The indirect (antigen coating) method was chosen initially due to its simpler design; only one

primary antibody was required, negating the need to screen for matching pairs of antibodies. There were considerable differences in assay results, dependent on the primary antibody used. Eight commercially-available IDE-specific primary antibodies were screened for this purpose, and these were rated according to a number of criteria. These included the amount of background signal produced, the strength of the signal above the blank, dynamic range of the standard curve, and linearity of dilution. MAb 7, one of two monoclonal antibodies that were screened, was also the only antibody that produced a high background, with plasma signal values being less than the background signal. This antibody had been reported (Yfanti, *et al.*, 2008) to cross-react with several proteins which are present in human plasma; it is possible that it also cross-reacted with the BSA used as the blocking agent, although this was not tested. Cross-reactivity with BSA may also have accounted for the plasma signal being lower than the background as the HSA in plasma would have bound to the plate, acting as a blocking agent itself and preventing the BSA from binding. This would be feasible if the antibody cross-reacted with BSA and reacted weakly or not at all with HSA. However, again this was not tested. The other monoclonal MAb 9 produced an adequate standard curve dynamic range, however the values for the plasma dilutions did not demonstrate a dose-response. These antibodies were therefore considered unsuitable for the indirect ELISA.

The polyclonal antibodies screened showed considerable variation. While Ab 8 and Ab 1 had good dynamic range on the standard curve, plasma values were very low and there was no linearity of dilution. Similarly Ab 4 had low plasma values but also a poor range on the standard curve. In addition there was no linearity of dilution apparent for all three of these antibodies. Therefore Ab 8, 1 and 4 were excluded as candidates for the indirect assay. The three remaining antibodies (Ab 6, Ab 2 and Ab 3) showed an acceptable dynamic range on the standard curve, but plasma dilutions demonstrated signal in the reverse direction: showing increasing absorbance as the dilution increased. One reason for this observation that is likely is that plasma proteins may have acted like a blocking agent, occupying binding sites on the plate and essentially competing for space with IDE, therefore more

dilution would result in less non-specific protein present, leading to less of a blocking effect and a more specific signal. Therefore, it was concluded that the indirect method would not be appropriate for the measurement of IDE in a complex matrix such as plasma, and that a sandwich ELISA would be more suitable for this purpose.

Three antibodies (Ab 6, 2 and 3) from the indirect ELISA tests were selected to develop a sandwich ELISA based on a good dynamic range on the standard curve, and positive signal above the blank in the plasma. Of these, Ab 6 was in an unpurified form therefore was unsuitable to use as a coating antibody, as contaminants in the antibody preparation would compete for binding sites on the plate. In addition, as Ab 2 demonstrated a strong cross-reaction with HSA, which is present in human plasma, it was also considered not to be suitable for a coating antibody as it would be capturing the HSA from the plasma as well as the IDE. Hence Ab 3 was tested as a coating antibody with both Ab 6 and Ab 2 tested as potential detection antibodies. As both bind to similar regions which are distinct from the Ab 3 IDE epitope, this reduced the likelihood of steric hindrance between the capture and detection antibodies. Both Ab 6 and Ab 2 worked well as detection antibodies, producing similar results and plasma dilutions being correctly reflected dose-dependently in absorbances observed. Ab 2, however, had a marginally higher dynamic range for both standard curve and plasma dilutions, a lower background, and as Ab 2 was affinity purified it would not contain contaminants which could potentially interfere with the assay. Therefore Ab 2 was chosen as detection antibody.

The reagents for the sandwich ELISA were tested for cross-reactivity and generation of any non-specific signal by omitting each from the assay separately. Omission of the capture or detection antibodies resulted in low background signals, indicating negligible cross-reactivity. When the blocking agent was omitted, signal was increased approximately two-fold. This would be expected as unbound sites on the plate surface would therefore be available for the sample or detection antibody to bind to, leading to a higher overall signal. However, there were two limitations of this experiment; firstly the controls were performed using purified IDE and may not

be reflective of potential cross-reactivity which may occur with the assay reagents and other components of plasma. Secondly, there were three differences in protocol for this experiment compared with the protocol used to measure IDE in the AIBL samples; the coating step was at pH 8.6 instead of 9.6, there was no Tween-20 in the PBS buffer washes until the final wash step after the secondary antibody, and Fraction V BSA was used instead of cell-culture grade BSA. The change in pH would only be likely to affect the strength of binding of the antibody to the microplate minimally, and potentially lead to a slightly lower overall signal. However, it was demonstrated that coating at a pH of 8.6 produced a slightly higher signal in rrIDE compared with pH 9.6. Similarly, the presence of Tween-20 in the wash steps would probably only slightly reduce the likelihood of non-specific binding, and its absence might lead to a higher overall signal. As there was only a very low background observed by omission of both the coating and detection antibodies, it is unlikely that these two factors would significantly alter the results observed in a negative direction (ie. increase the background). At the time of this particular experiment, the concept that assay results could be influenced by different formulations of BSA had not been considered, and the results obtained using Fraction V BSA possibly did not reflect potential cross-reactivity with cell-culture grade BSA which was used in the protocol when testing the AIBL samples. Therefore in hindsight, the control tests should have been done using the cell-culture grade BSA, and this would need to be investigated in future. In addition, it would have been of value to test the assay using plasma from an animal which had the expression of IDE knocked out to ensure that other contaminants of the plasma sample were not cross-reacting with the assay components. Unfortunately, no IDE-knockout plasma samples were available at that time. The cross-reactivity of BSA will be discussed in subsequent paragraphs. Nonetheless, while there were some limitations, these control tests confirmed that negligible cross-reactivity exists between the secondary antibody and both the capture and detection antibodies.

A number of conditions for the sandwich ELISA were subsequently optimised. For the coating step; the antibody concentration, the buffer type and pH, and the time and temperature of the coating incubation were tested. A shorter incubation time

of two hours at room temperature in carbonate/bicarbonate buffer (pH 9.6) was demonstrated to be superior for results. Regarding blocking agents, BSA (cell-culture grade) versus a non-albumin blocking agent were also compared (discussed below). PBST was chosen as sample diluent due to its superiority in maintaining its pH at different temperatures (discussed further in Chapter 5). Additionally, as the plasma sample dilutions were observed to be linear to 1:5, a dilution of 1:2 was chosen as the dilution for the AIBL samples as a midpoint between undiluted plasma and 1:5. Furthermore, the addition of BSA to the sample and detection antibody diluents (common in ELISA assays) led to the slight reduction in signal. However, this test used cell-culture grade BSA which was observed to be cross-reactive with the antibodies, and this may have caused inhibition of the signal through interfering with the antibody-antigen binding. The addition of polyethylene glycol (PEG) to incubation buffers has been reported to increase the speed of the antigen-antibody reaction (Kątnik, *et al.*, 1987), however in the present study there was no overall benefit observed by its addition to the detection antibody diluent.

The IDE sandwich ELISA, although not completely optimised due to time limitations, was subsequently used to measure the total IDE in the plasma of a subset of AIBL participants ($n = 177$, 57 HC/50 MCI/70 AD). The results showed that there was a reduction of IDE levels for both MCI and AD groups, with only the MCI group being statistically significant. The trend was observed in both male and female participants. The effect of the *APOE* $\epsilon 4$ allele was seen to reduce the level of IDE but was not statistically significant. Similarly, the levels of plasma IDE were observed to reduce with age, similar to previous reports of cerebral IDE (Caccamo, *et al.*, 2005) but results were again not statistically significant; however there was a significant inverse correlation between IDE levels and age, albeit very weak. The high variation in IDE levels was evident, and may be related to diet, exercise or medications which alter the expression of IDE. It would be of value to investigate the potential effect of these factors on IDE expression.

Total plasma IDE levels also correlated inversely with levels of plasma $A\beta_{40}$ and $A\beta_{42}$, and this result is supported by genetic evidence of a link between IDE

polymorphisms and levels of plasma A β (Carrasquillo, *et al.*, 2010; Ertekin-Taner, *et al.*, 2000). This may highlight the importance of IDE in the regulation of circulating A β . This result would support the hypothesis that a reduction in plasma IDE may contribute to an accumulation of A β species in the periphery. Statistically this association was highly significant, yet the correlation was weak, which suggests that there are other A β -degrading enzymes which contribute to the regulation of plasma A β . However when stratified by classification and *APOE* $\epsilon 4$ status, the correlation strengthened by two-fold only in AD non-carriers, while the association disappeared in MCI, HC, and $\epsilon 4$ carriers. This suggests a major contribution of IDE toward A β regulation in AD patients who do not carry an $\epsilon 4$ allele.

A positive correlation was also observed between plasma IDE levels and both total and free testosterone levels, but when split by *APOE* $\epsilon 4$ status only the $\epsilon 4$ carriers showed significant correlation. This is not entirely unexpected due to reports in the literature which show that testosterone upregulates IDE expression (Udrisar, *et al.*, 2005; César Vieira, *et al.*, 2011) but this has not been investigated by *APOE* genotype. Statins have been found to lower levels of testosterone, and as there was a high level of statin use amongst AIBL participants, this may have confounded the results (Schooling, *et al.*, 2013). While the literature reports that oestrogen and progesterone also upregulate IDE (Udrisar, *et al.*, 2005; Jayaraman, *et al.*, 2012; Zhao, *et al.*, 2010), no association was found between ELISA-measured levels of plasma IDE and oestrogen in the current study, and progesterone levels were not measured. It would be useful to investigate the potential interaction between progesterone and IDE, and perhaps the cumulative effect of sex hormones on the regulation of IDE in future studies.

The negative correlation between total IDE levels and plasma levels of HDL cholesterol is also puzzling. When split by *APOE* $\epsilon 4$ status, only the $\epsilon 4$ non-carriers showed significance. This result appears counter-intuitive as HDL cholesterol is considered to be beneficial, and as lower levels of IDE were found in the MCI and AD groups compared with controls it would suggest that higher levels of HDL should be found in the pathological state, which is contrary to what the literature has reported. In fact, high levels of plasma HDL have been associated with a reduced

risk of AD while low levels have been found in AD patients (Reitz, *et al.*, 2010; Kuo, *et al.*, 1998) although the levels of HDL may be different prior to the MCI stage of pathogenesis. No other studies have reported a potential interaction between IDE and HDL cholesterol, however it has been shown that both IDE and HDL can be regulated by dietary factors (Du, *et al.*, 2010; Mensink, *et al.*, 2003) and exercise (Kim, *et al.*, 2011; Kodama, *et al.*, 2007). In particular, saturated fatty acids have been shown to decrease expression of IDE while other studies have demonstrated that they increase levels of HDL. This perhaps could be a chance association, however while outside the scope of this project it would be of interest to investigate this potential association further.

As the levels of insulin and A β were likely to be influenced by IDE levels, ratios of IDE to these peptides were calculated (both individually and in combination). The ratios of IDE/A β_{40+42} , and IDE/insulin+A β_{40+42} showed better sensitivity for differentiating between the HC/MCI/AD classifications than IDE levels alone. Conversely, no improvement of sensitivity was seen with the ratio of IDE/insulin compared to IDE alone. All ratios of IDE:peptides were consistently lower in MCI than HC, and the IDE/A β_{40+42} , and IDE/insulin+A β_{40+42} ratios were significantly lower in both MCI and AD, when compared with HC. When stratified by APOE $\epsilon 4$ status, all IDE:peptide ratios were considerably lower in MCI when compared to HC, yet for non- $\epsilon 4$ carriers the ratios were higher in AD patients, almost to the same level as the controls. Only in case of the IDE/A β_{40+42} , and IDE/insulin+A β_{40+42} ratios did the $\epsilon 4$ carriers remain significantly lower in the AD group. In the case of the non- $\epsilon 4$ carriers, only the IDE/insulin ratio and the IDE/insulin+A β_{40+42} ratio reached statistical significance between MCI and HC. The differences observed in ratios between $\epsilon 4$ carriers and non-carriers in the AD group may be related to insulin resistance that has been previously observed to be characteristic of non-carriers (Craft, *et al.*, 2000). However, no significant differences in insulin levels were found between classifications in the AIBL cohort either overall, or stratified by APOE $\epsilon 4$ status.

When A β_{1-42} and A β_{1-40} levels were analysed separately, there was no difference in plasma A β_{42} between classifications in this AIBL subgroup. However, there was an

increase in plasma $A\beta_{1-40}$ for MCI and AD groups compared with HC, albeit not significant, as well as a significant increase in $A\beta_{x-40}$. Although the results for $A\beta_{1-40}$ were not significantly different between classifications in this subgroup, it was shown that after controlling for a number of covariates the differences became significant (Rembach, *et al.*, 2014). It is possible that the combination of reduced IDE and increased $A\beta_{1-40}$ with $A\beta_{x-40}$ could lead to the saturation of, or at least a major reduction in available IDE in the plasma. Impairment of $A\beta$ or insulin degradation would then lead to an accumulation of these substrates in the peripheral circulation, contributing to the characteristic hyperinsulinemia and elevated plasma $A\beta$ which has been linked with AD pathogenesis and pathology (Ertekin-Taner, *et al.*, 2008; Kuusisto, *et al.*, 1997; Muller, *et al.*, 2007).

Analysing ELISA background problems

One noteworthy observation was the considerably higher background of the sandwich ELISA compared with the background of the indirect assays. This background was shown not to be related to the secondary antibody cross-reacting with either the detection or coating antibodies. Therefore, following the measurement of the AIBL samples above, further optimisation tests were performed. The use of Fraction V BSA as the blocking agent reduced the background OD reading to about 1/3 of that observed with cell-culture grade BSA (used as a blocking agent in the AIBL sample assays). However, the background signal observed with Fraction V BSA was still considerably higher than the background observed when either the coating or detection antibody was omitted. This result suggests that there may be some small degree of cross-reactivity between the capture and detection antibody. This is not an unusual phenomenon in sandwich ELISA assays, and as the concentrations and volumes were constant for both antibodies the background generated was consistent across wells, and therefore easily removed from the analysis. However, the potential interaction between the antibodies may have reduced the overall specific signal due to this non-specific binding which may have rendered a portion of the antibodies inaccessible for binding to IDE. It is unfortunate that the cell-culture grade BSA was

used in the protocol for the AIBL samples, however, a constant concentration and amount was added to all the wells in the blocking step and the background OD reading was subtracted from all samples, thus this may not be an issue. Similarly though, the use of cell-culture grade BSA might lead to a reduced amount of Ab 3 available for specific binding to IDE. These factors may account for the decreased values seen in the standard curve in the sandwich assay as compared with each of the antibodies separately in the indirect assay. However, the low background observed in the indirect assays for both of the antibodies suggests there was negligible cross-reaction with BSA, as the antibody was applied subsequent to the blocking agent. Regardless, the sandwich assay could not be accurately quantitative because of this interference and only relative differences could be compared. Future assays should use BSA preparations such as Fraction V to minimise the background.

The use of antibodies raised in goat can be problematic, for example, BSA may contain contaminating bovine IgG which may cross react with the anti-goat secondary antibody, causing problems with detection. In addition, human samples may contain anti-bovine IgG as well, which may cross-react with one or more of the three antibodies used in the assay (Kragstrup, *et al.*, 2013; Henning & Nielsen, 1992). As a goat raised antibody is used for detection in this assay, it was important to make sure this was not occurring to ensure that there were not false positives. However, a mouse raised antibody was initially used for detection, and was compared against the goat antibody. Although the mouse antibody produced slightly overall lower signal in the plasma, it demonstrated a very similar dilution curve to the goat antibody. This suggests that there was unlikely to be interference by anti-bovine IgG in the assay.

Although Ab 2 was shown to cross react with HSA in the Western immunoblotting method, plasma-derived human albumin would have been washed away prior to the detection antibody being applied so therefore it was determined not to be an issue. Even though Ab 3 demonstrated a weak cross-reactivity with HSA on Western immunoblotting tests, activity experiments on degradation of a fluorescent peptide substrate in plasma following immunocapture with Ab 3 show

that 100% of activity can be inhibited with two known IDE inhibitors (to be discussed further in Chapter 5), providing further evidence that IDE Ab 3 shows negligible binding to anything else in the plate-based assays. In addition, when testing purified HSA at levels found physiologically in plasma, the OD signal produced in the sandwich ELISA was not above background, therefore it was thought that HSA was unlikely to be a confounding factor in the plasma samples tested in the assay. A major reason HSA was detected by Ab 3 in the Western Blot but not in the sandwich ELISA may be related to the denatured conformation of the protein under the denaturing and reducing conditions in the former method, while the proteins in ELISA are in native conformation.

One limitation of this assay was related to the particularly high background observed when using a non-albumin containing blocking agent (that is derived mainly from bovine milk). One previous study reported that two IDE antibodies (one monoclonal, one polyclonal) used in their project were observed to cross-react with fibrinogen, casein and thrombin; clotting elements of plasma which are found in bovine milk (Yfanti, *et al.*, 2008). Even though the antibodies used in the current study were not the same ones, it is possible therefore that the higher background seen with the use of the non-albumin blocking agent was due to the IDE antibodies similarly cross-reacting with these or other proteins in this preparation. If this is the case, these elements being present in human plasma as well may be cross-reacting with the assay. However, contrary to this there was little or no background observed when using this antibody in Western immunoblotting experiments, which used milk powder as a block. This may have been due to the fact that the milk powder was in a less purified form, therefore having lower levels of potential cross-reactants. Although not addressed within the time-frame of this project, it would be of benefit to further investigate these potentially confounding factors. It may be of value to assess circulating IDE levels in serum rather than plasma if this is the case.

Perhaps the most concerning limitation of this IDE ELISA assay is the poor spike and recovery results. Generally, recovery should be approximately 80-120% of the control value (Shah, *et al.*, 1992). In the current study 100% of the signal was

suppressed by the plasma matrix, indicating major interference. The reason for this result is unclear; however there are a number of possibilities to consider. It is possible that the recombinant IDE is sequestered by plasma proteins and therefore its detection is masked in the ELISA. This would not have been an issue in the denaturing and reducing conditions of the Western blot as the conditions cause most proteins to disassociate. It would be of value in the future to perform a spike and recovery experiment using Western immunoblotting as the method of measurement. Co-immunoprecipitation experiments in plasma would also help determine if this is the case. As mentioned earlier, IDE has been shown to bind to numerous other proteins that are present in human plasma (Bennett, *et al.*, 2000(a); Kurochkin & Goto, 1994; Roth, *et al.*, 1984). It is also known that IDE can be complexed with the proteasome, and that the proteasome is present in human plasma (Bennett, *et al.*, 2000(b); Ichihara, 1993; Egerer, *et al.*, 2002).

Furthermore, heterophilic antibodies, human anti-animal antibodies and rheumatoid factors, which are present in human plasma, can also be responsible for analyte masking (Urbonaviciute, *et al.*, 2007; reviewed in Tate & Ward, 2004). Cross-reactants (including BSA) can weakly link the antigen to the antibodies which are then removed by the washing steps along with the analyte or antibodies of interest, leading to falsely negative results (Valdes & Jortani, 2002). Recent studies have sought to address such issues by outlining some validation steps designed to identify these confounding variables (Kragstrup, *et al.*, 2013).

Another possibility for apparently low plasma readings of IDE is that IDE itself is degraded by other proteases present in the plasma. It would be of value to repeat the experiment with a protease inhibitor cocktail present in the plasma diluent.

An increase in IDE concentration may cause what is known as the “hook effect” (reviewed in Tate & Ward, 2004) leading to a false negative result. This occurs due to competition for the capture antibody. However, the likelihood of this is low considering the observation that the highest concentration of standard tested in this assay was 160000 ng/mL and did not show a reduction of signal at the higher end of the curve. The lowest concentration used in the spike and recovery test was

1250 ng/mL. Unless the concentration of IDE in the plasma was higher than 160000 ng/mL, it would be unlikely that this phenomenon would be the cause of the lack of signal. Alternatively, the increase in IDE concentration in the plasma in association with components of the plasma matrix may cause IDE to self-aggregate. This would lead to the epitopes being masked and inaccessible for antibody binding.

Another potential factor may be that rat IDE and human IDE behave differently in plasma. Although rat IDE has 95% sequence similarity to human IDE, the differences may still lead to structural or functional differences as observed with other enzymes (Pearce, *et al.*, 1992; Glatt and Oesch, 1987). It is also possible that the recombinant protein behaves differently in plasma to the native protein, for example altering conformation to an extent that would reduce or prevent antibodies from binding to the rrIDE (Cavalcante Braga, *et al.*, 2013).

However, failure of the spike and recovery test does not necessarily mean that the signal observed from human plasma samples in the AIBL cohort is not IDE. It may be that the recombinant rat IDE changes conformation in human plasma, and/or binds to plasma proteins that do not influence human IDE. Alternatively, it may be that only a portion of IDE present in the plasma is free protein, with a proportion of it bound to other proteins, or present in the form of dimers and tetramers which may not be detected by ELISA. As mentioned previously, increasing the concentration of IDE may also change the binding properties toward other plasma constituents and/or may also induce self-aggregation. Regardless, in this project there is good evidence that the capture antibody Ab 3 specifically and exclusively binds to IDE (to be discussed further in Chapter 5) and therefore it is likely that this in-house IDE sandwich ELISA is specifically measuring only IDE. However, if aggregation does occur, it is likely that the true level of IDE in plasma samples is much higher than the values measured with this assay.

4.4.1 Summary

The aim of this chapter was to develop and optimise an in-house ELISA which could accurately and specifically measure IDE in human plasma from the AIBL cohort. The development of the assay was complicated by a number of cross-reactivity issues associated with the antibodies and blocking buffer chosen, and the finding that factors in plasma appeared to interfere significantly with the detection of IDE. As such, a fully-quantitative assay could not be developed, and only relative differences could be compared. Promising results were observed, with altered levels of plasma IDE in the prodromal and symptomatic stages of AD, particularly when considered together with circulating insulin and A β levels. However, more work needs to be done to ascertain the source/s of the confounders and to determine if this has affected the accuracy of the IDE levels measured. The ELISA protocol needs to be improved, and followed by validation studies and tests of other cohorts before the results can be confirmed, and therefore, caution should be exercised when considering the current results. Indeed, the IDE levels measured by ELISA contradict the results obtained via Western immunoblotting. If the results reflected by the sandwich ELISA are subsequently confirmed to be accurate, then plasma IDE may be useful as part of a biomarker panel for very early stages of AD. Studies of a much larger cohort would allow for the testing of a larger number of classifications, for example HC under 65, HC over 65, memory complainers, MCI and AD. This would then reveal how IDE changes in early AD pathogenesis, and also help to determine IDE's role in the development of AD.

CHAPTER 5 –IDE enzyme activity in plasma is higher in MCI compared with HC and AD.

5.1 Introduction

Changes in enzyme protein levels are often indicative of biochemical changes which may cause or reflect pathological changes in human physiology. Often however, these measurements are not truly representative of the functional abilities of the protein in question. In the case of an enzyme, its catalytic activity may differ due to a number of factors which are unrelated to enzyme protein levels themselves. Some of these factors include different concentrations of enzyme substrates or co-factors such as metals, inhibitors or activators of the enzyme, or post translational modifications such as phosphorylation, alkylation, oxidation, s-nitrosylation, s-glutathionylation and ubiquitination. While there are many other types of post-translational modifications that can occur, the types mentioned here have been associated specifically with the inhibition of IDE. Other factors such as medications, or even alternate isoforms or the aggregation status of IDE can also have an effect on the function of the protein.

A number of studies suggest that the catalytic activity of IDE does not correlate with IDE expression levels (Kim, *et al.*, 2007; Miners, *et al.*, 2009; Behl, *et al.*, 2009). Therefore, assessing IDE catalytic activity is an important consideration when assessing IDE's influence on A β and insulin levels. An immunocapture-based fluorometric activity assay has been previously developed to assess the catalytic activity of IDE in brain homogenates (Miners, *et al.*, 2008(b); Wang, *et al.*, 2010). The assay uses a specific IDE antibody to capture IDE from the sample onto a solid-phase (96-well microplate). A fluorogenic substrate of IDE (derived from the sequence of bradykinin), fluoresces when cleaved and directly represents activity levels. In this chapter, the suitability of this assay for measuring IDE activity in plasma is investigated, prior to assessing activity in samples from the AIBL cohort.

5.1.1 Aims

1. To use a fluorometric activity assay to measure the catalytic activity of IDE in human plasma, and determine whether the catalytic activity can differentiate between HC, MCI and AD.
2. To determine if the presence of the *APOE4* allele has any impact on the catalytic activity of plasma IDE.
3. To identify any relationships between plasma IDE catalytic activity, expression levels of plasma IDE and other biochemical data obtained from the AIBL study.

5.2 Experimental summary

The catalytic activity assay was adapted from the original assay developed by Miners and colleagues (2008(b), using amendments to the protocol as published (Wang, *et al.*, 2009). All of the catalytic activity assays performed were based on this method. Gain adjustment was set at 1487 for all assays unless otherwise specified. Assays were incubated with the fluorogenic peptide for 6 hours unless otherwise specified.

5.2.1 Test of immunocapture-based fluorometric assay for the measurement of the catalytic activity of plasma IDE.

The catalytic activity of plasma IDE was tested as detailed in Section 2.2.6.1 using pooled control plasma (PCP), following a previously published method (Miners, *et al.*, 2008(b); Wang, *et al.*, 2009).

5.2.2 Test of fluorometric IDE activity assay specificity

To test the specificity of the IDE catalytic activity assay further, the IDE substrates/inhibitors insulin and A β were trialled as IDE enzyme inhibitors in the assay, as detailed in Sections 2.2.6.2, 2.2.6.3, 2.2.6.4 and 2.2.6.5.

5.2.3 Comparison of EDTA-collected blood and lithium-heparin- (LiHep) collected blood: determining which is better in the IDE activity assay.

To ascertain if the presence of EDTA as an anticoagulant in the plasma samples was inhibitory in the IDE activity assay, plasma isolated from EDTA-collected blood was compared with plasma isolated from LiHep-collected blood in an assay performed as described in Section 2.2.6.1.

5.2.4 Determination of plasma IDE catalytic activity in the AIBL cohort using the IDE immunocapture-based fluorometric activity assay.

IDE catalytic activity was measured in the sub-set of samples obtained from the AIBL cohort as detailed in Section 2.2.6.1. An internal control of PCP was included on each plate as a reference sample. IDE activity was interpolated from the standard curve and expressed as the Equivalent Concentration of rrIDE (ng/mL).

5.3 Results

5.3.1 PCP and rrIDE generate a time and dose-dependent signal in the fluorometric activity assay.

The immunocapture-based fluorometric activity assay developed by Miners and colleagues (2008(b)) was originally used to measure IDE activity in brain tissue homogenates. The assay has not previously been used to assess IDE activity in plasma, therefore it was important to carefully characterise the assay prior to adapting it to be used for plasma. The capture antibody used for development of the assay in plasma was Ab 3 (see Section 2.1.1 for details), since it was the most suitable antibody to detect IDE protein in plasma by Western immunoblotting and to capture IDE in ELISAs (see Sections 3.3.7 and 4.3.3, respectively). Miners, *et al.*, (2008(b)) also found that, of the four IDE-specific antibodies tested, Ab 3 showed a consistent dose-response in activity with purified human and rat IDE and produced the highest signal in brain homogenates.

To initially assess the efficacy for Ab 3 to capture IDE, the activity of diluted rrIDE or pooled control plasma (PCP) was measured. Purified recombinant rat IDE (rrIDE) was chosen as it has similar activity to recombinant human IDE (rhIDE) (Miners, *et al.*, 2008(b)). A concentration range of rrIDE and or PCP was assessed, however 1250 to 12500 ng/mL rrIDE and 480-1680 µg total protein PCP were found to show a signal above that observed for the blank. It is noted that the blank generated a signal of an average of ~ 8000 fluorescence units, which may have impacted on the sensitivity of the assay particularly detecting lower concentrations of the rrIDE or PCP. Nevertheless, the results [Figure 5.1(A) and (B)] showed a strong linear relationship between the amount of fluorescence and increasing concentrations of rrIDE (A) or PCP (B). This association was more prominent with increasing incubation periods (up to the 6 hours), consistent with that shown for brain homogenates by Miners and colleagues where a maximum signal level was observed at 5 hours. There was little difference between the 1440 µg TP/well samples and 1680 µg TP/well samples; the reason for this was not pursued, but it was decided that the loading of plasma TP/well for the measurement of IDE

catalytic activity needed to be restricted to between 480 and 1440 μg TP. Overall these results indicate that a standard curve could be generated with Ab 3, and a dose and time dependent fluorescence signal was observed for plasma. The specificity of the observed activity in plasma was subsequently further investigated.

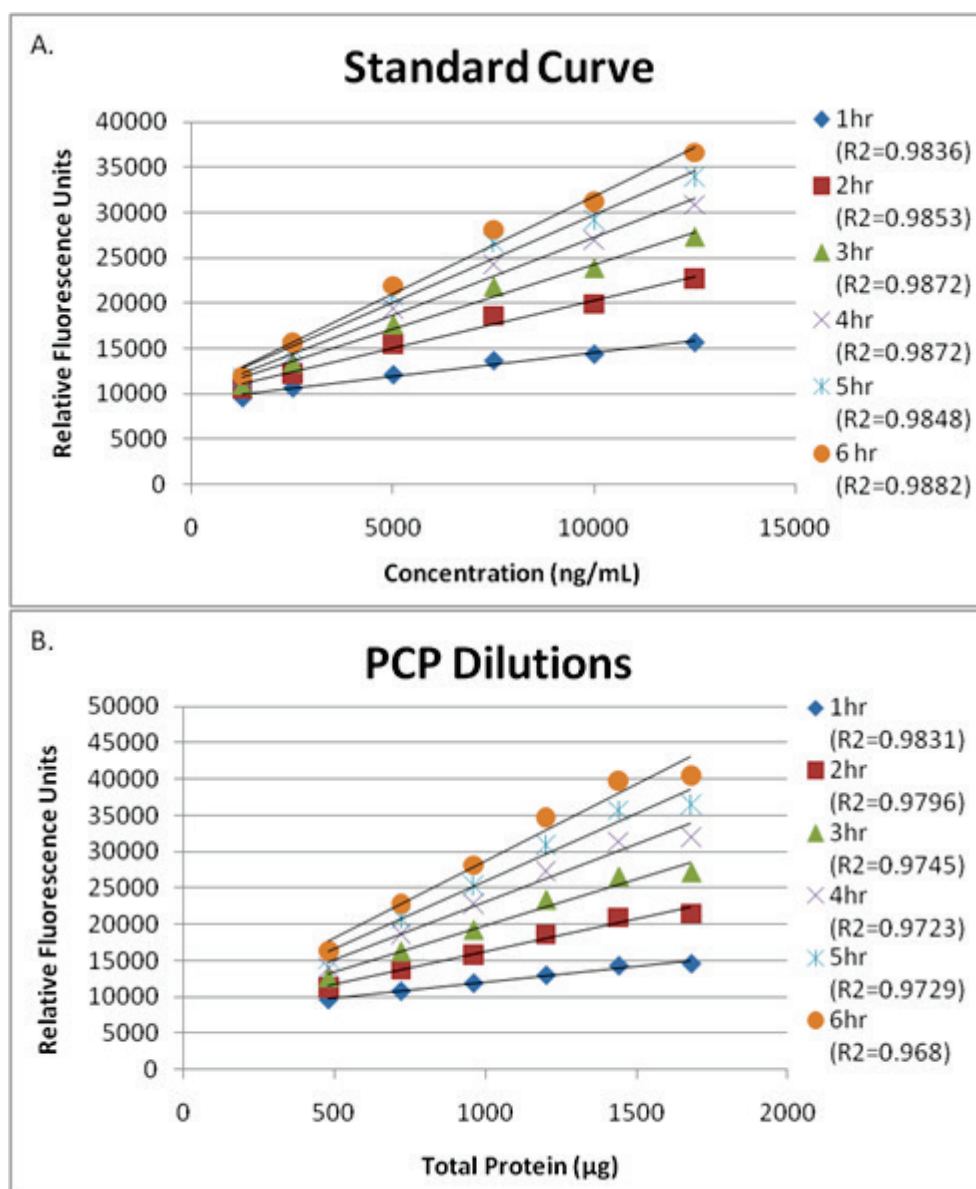
The assay was performed with PCP (480 μg TP) under several conditions, including: without the use of blocking agent, no substrate and no sample. The results are shown in Figure 5.1(C). As before, the PCP sample generated a signal that increased with time. Omission of the blocking agent (BSA) led to a negligible reduction in signal, therefore it was concluded that there was no significant effect of the blocking agent in the activity assay. Omission of the sample (vehicle buffer only) did result in a moderate fluorogenic signal however this did not increase with time, indicating that cleavage of the peptide was not occurring. This was considered to be the background signal of the assay. The majority of the background fluorescence appeared to be related to the fluorogenic peptide itself, with omission of the peptide reducing the fluorescence by $\sim 85\%$. In addition, it was observed that the gain adjustment setting was related to the background reading (as well as the samples); for a single test plate the background signal changed from 8736 RFU with a gain of 1712, 7435 RFU with a gain of 1679 and 3147 RFU with a gain of 1487. At the higher gain settings the signal exceeded the maximum RFU of the plate reader, thus it was decided that subsequent assays would be gain adjusted to 1487.

The specificity for the Ab 3 antibody to detect IDE in plasma samples was assessed through incubating PCP (480 μg TP) with IDE antibodies Ab 1 (final amount was 8 μL anti-serum/well), Ab 2 (4 μg /well, purified), or with $\text{A}\beta_{40}$ (8.2 μg /well). Ab 1 binds to an epitope which is located within the catalytic domain of IDE (Domain 1), whereas Ab 2 binds to an epitope distal to the catalytic domain in Domain 4 (see section 2.1.1 for antibody details). As the $\text{A}\beta_{40}$ peptide is a substrate for IDE it was used as a competitive inhibitor thought to occupy the catalytic site of IDE, resulting in reduced cleavage of the fluorogenic peptide and thus a reduced signal.

Results (Figure 5.1(D)) showed that pre-incubating the PCP sample with either Ab 1 or $\text{A}\beta_{40}$ resulted in an approximately 50% reduction in cleavage of the fluorogenic

peptide. Pre-incubating PCP with Ab 2 had no effect in reducing IDE activity. It is possible that this reflects that Ab 2 binds too distal to the catalytic domain to have an inhibitory effect on enzyme activity. The inhibition seen by Ab 1 and A β ₄₀ suggests however, that at least 50% of the fluorescence observed in the assay is specifically due to the cleavage of the fluorogenic peptide by IDE.

The stocks of Ab 3 were exhausted at this stage and a new batch was obtained. As batch to batch variations do occur amongst antibodies, the new batch required similar validation as the old antibody, prior to any further analysis and assaying the AIBL samples.



Figures 5.1(A) and (B) Test of fluorometric IDE enzyme activity assay, using ab28561 (Ab 3) as coating antibody. The activity assay was performed as detailed in Section 2.2.6.1 for 6 hours. The recombinant rat IDE (rrIDE) standard curve is represented in Graph A, and the assay results of the pooled control plasma (PCP) dilutions are shown in Graph B. The standard curve ranged from 1250ng/mL to 12500ng/mL (50 µl/well) with each concentration in duplicate. PCP was diluted to provide from 480 to 1680 µg of total protein per well in duplicate. Fluorescence was generated by incubation with the fluorogenic substrate (Mca-RPPGFSAFK(Dnp)-OH) and fluorescence measurements reflect the amount of cleavage of the substrate at pH 7.5 by the immunocaptured IDE. Gain adjustment was set to 1712. Data is not blank-adjusted.

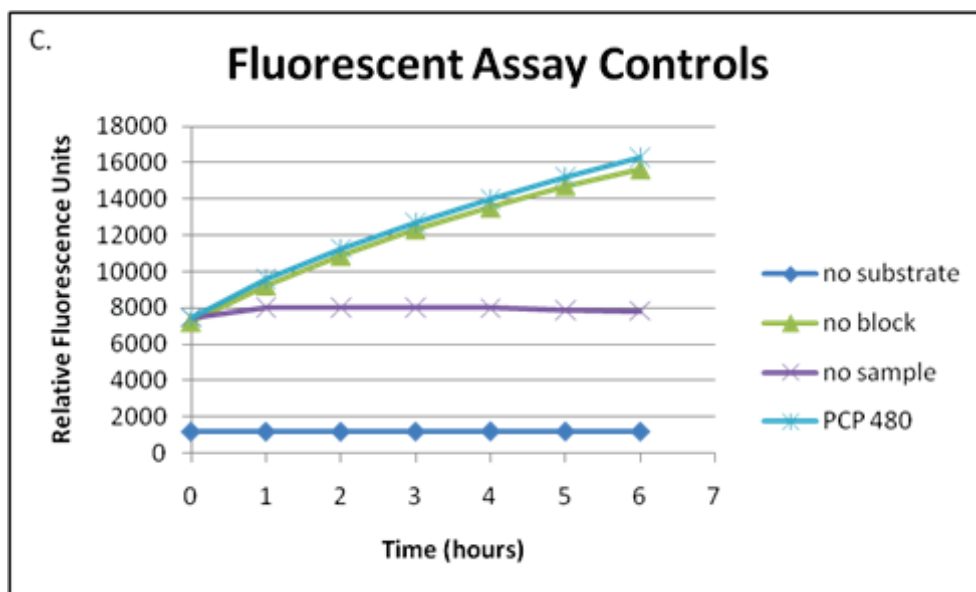


Figure 5.1(C). Test of immunocapture-based IDE fluorometric activity assay (with Ab 3) to analyse components of the assay. The activity assay was performed as detailed in Section 2.2.6.1 and 2.2.6.2. Controls include the addition of PCP at 480 μg TP for all wells with the exception of the “no sample” wells, and all samples were run in duplicate. “No substrate” indicates all assay components as usual without the addition of the fluorogenic peptide (HEPES only). Results show a low reading without the addition of fluorogenic peptide. With all components added except the sample (PBST only) there is a high level of background fluorescence with this gain adjustment setting (1712). Negligible difference occurs with or without the blocking step.

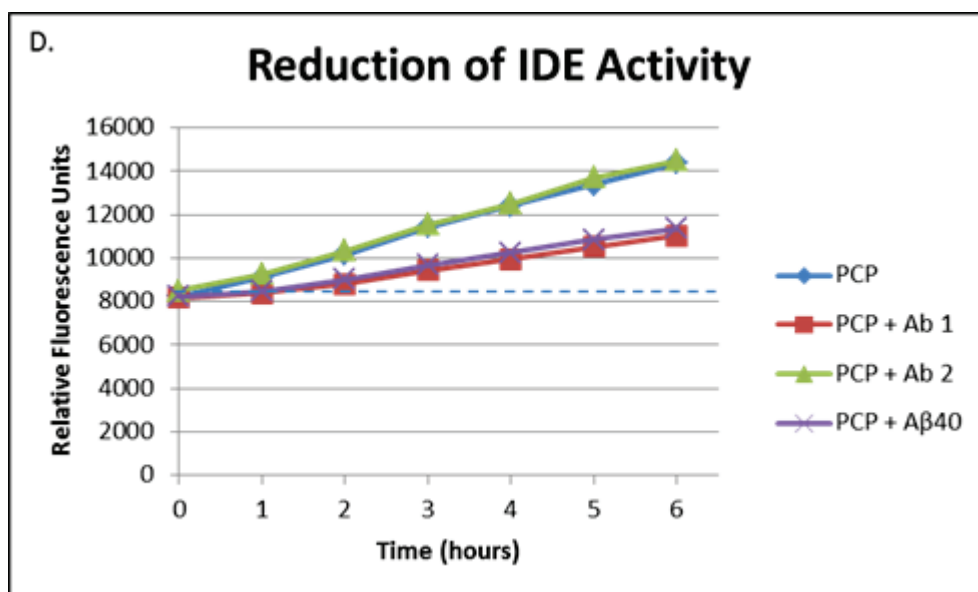


Figure 5.1(D). Further validation test of fluorometric activity assay using specific IDE antibodies and Aβ₄₀ as inhibitors of IDE-based cleavage of the fluorogenic peptide. Prior to being added to the microplate, PCP dilutions (480 μg TP/well) were pre-incubated with 20 μL each of IDE antibodies Ab 1 or Ab 2, or with 20.5 μg of Aβ₄₀. IDE antibody Ab 1 was raised against an epitope of IDE which is within the catalytic domain, while Ab 2 was raised against an epitope close to the C-terminus of IDE distinct from the catalytic site. The assay was carried out as described in section 2.2.6.1 and 2.2.6.2 over 6 hours, and all samples were run in duplicate. Blank (background) is indicated by the dashed line. Gain adjustment was set to 1712. Results show that pre-incubation of PCP with Ab 2 had no effect on the catalytic activity of plasma IDE, however Ab 1 and Aβ₄₀ partially reduced the activity of IDE by approximately 50% for the course of the experiment.

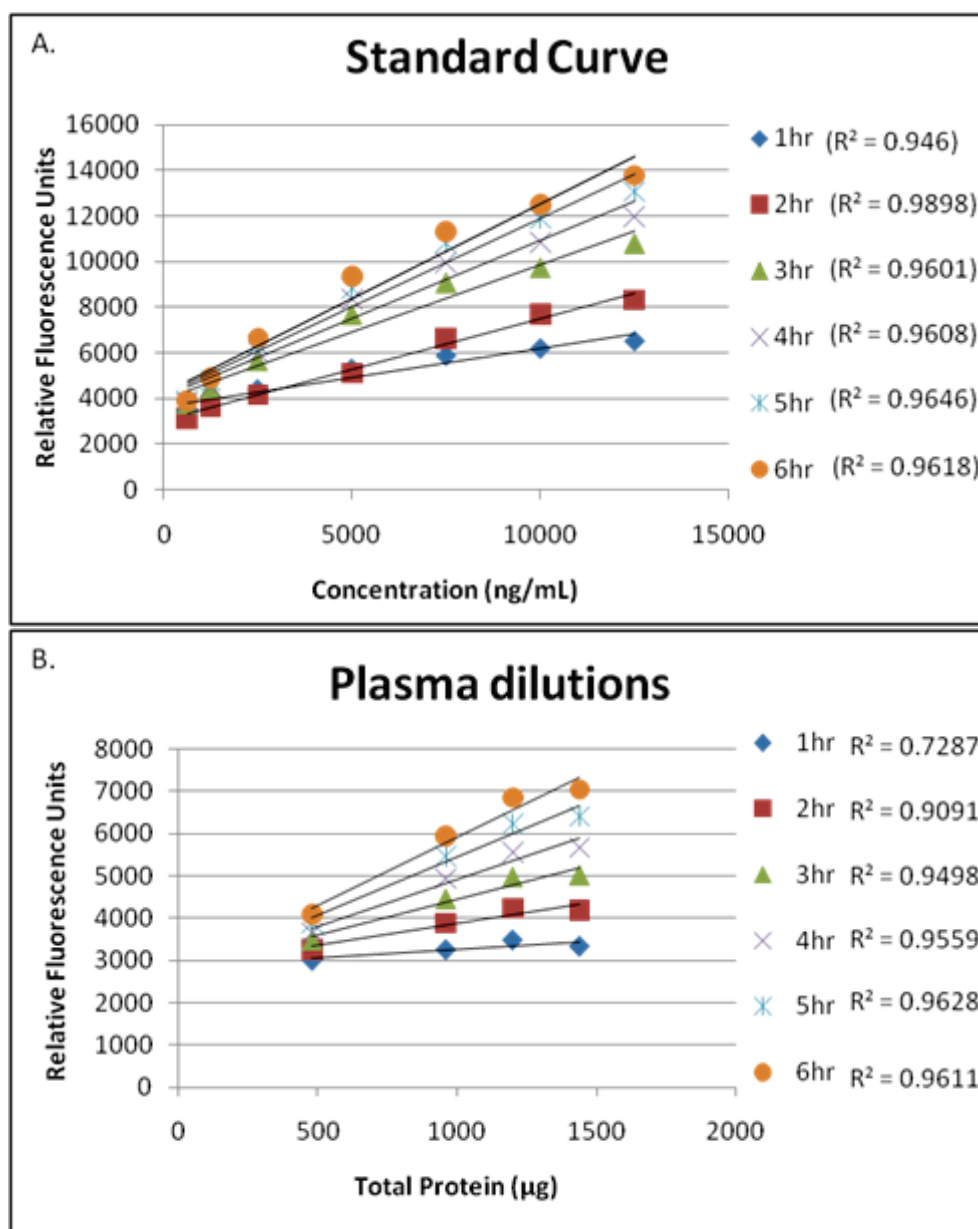
5.3.2 Validating the new batch of coating antibody Ab 3 (ab28561).

Polyclonal antibodies may vary considerably between batches due to a number of factors. Therefore, it is essential to thoroughly screen each batch prior to use, and if comparisons need to be made between runs of an assay, it is important to use the same batch of antibody throughout. Hence, as stocks of the original batch of Ab 3 were exhausted, a new batch needed to be validated under the same conditions as the previous batch.

Results (Figure 5.2(A) and (B)) showed the new batch of Ab 3 generated a similar standard curve correlation to that of the original batch (see Figure 5.1(A)), with R²

values of 0.9882 for Batch 1 and 0.9618 for Batch 2 at the 6 hour timepoint. However the maximum signal achieved was ~4-fold lower than that observed for the original batch. Compared with the previous batch (using the same gain adjustment setting) the background was similar, with values of 3090.5 for Batch 1 and 3114 for Batch 2. Signal was also reduced in the PCP for the new batch, although not to the same extent. The lower fluorescence signals most likely indicate lower IDE levels captured, possibly due to a lower affinity of IDE-specific antibodies in the newest batch of Ab 3, however the original and new batches were not tested on the same plate so could not be directly compared.

Similarly to the previous batch of Ab 3 (Figures 5.1(A) and (B)), using the new batch in the fluorogenic assay resulted in standard curves that increased linearly in slope with increasing IDE concentration, with apparently proportional increases in signal up to at least 7500 ng/ml (375 ng/well IDE). For the PCP dilutions, the assay demonstrated a good linear time- and protein loading-dependent response for all dilutions tested up to 6 hr, and up to the concentration of 1200 µg TP/well. Similar to the results from the previous batch of Ab 3, little difference between the 1200 µg TP/well and the 1440 µg TP/well was observed. With this batch of antibody the optimal protein loading of PCP appeared to be 960 µg TP/well, as this was the highest concentration where the signal could be differentiated from the higher concentrations of PCP. It was concluded that although the overall signal was reduced with the new batch of Ab 3 compared to the old batch, it was still suitable to use in the assay.



Figures 5.2(A) and (B) Test of fluorometric IDE activity assay using new batch of IDE Ab 3 as capture antibody. Recombinant rat IDE (rrIDE) was used to produce a standard curve (Graph A) with wells containing concentrations from 625 to 12500 ng/mL. Pooled control plasma (PCP) was diluted to aliquot 480 to 1440 µg total protein/well (Graph B). All samples were run in duplicate. The fluorogenic catalytic assay was run according to the method described in section 2.2.6.1. Data is not blank-adjusted. Results show that the new lot of Ab 3 produced fluorescence that was, overall, considerably lower than the previous lot of antibody. However, the fluorescent signal was consistently reflective of the amount of protein present up to 7500 ng/mL for the standard curve and 1200 µg total protein/well for PCP, as well as the time incubated.

Similar to the old batch, control tests were subsequently performed using the new batch of Ab 3 to ascertain specificity. The first assessment was whether the background observed was related to the capture antibody or its components, or BSA which was used as the blocking agent (Figure 5.3). The plate was coated with Ab 3 diluted to 2.5, 1.25 and 0.625 $\mu\text{g/mL}$, washed, and then assayed with the fluorogenic peptide (diluted in HEPES as previously detailed in Section 2.2.6.1). As 50% glycerol was a component of Ab 3, glycerol alone was also tested (1.25 $\mu\text{L/mL}$) with the peptide, with and without the blocking agent. Finally, the blocking agent was tested alone with the peptide. These control tests were run alongside two samples, containing all components of the assay with and without PCP. Results showed that the signal was at the same intensity as the blank for all wells not containing PCP, while the wells containing PCP increased in signal for the duration of the assay (2 hours). The assay was incubated for 2 hours as increases in fluorescent signal (above the blank) occurred within the first hour and reflected the pattern of results at 6 hours. As samples wells that did not contain PCP did not change over time, a true signal was observed at this time point. In addition, (on separate plates) the peptide alone diluted in HEPES (no other assay components) was tested as well as a no peptide control. The results showed that the diluted peptide alone generated a similar signal to the background, with an average reading of 3544.25 RFU (± 83.7551 SD, $n=4$) for the diluted peptide, while omission of the peptide reduced the signal by $\sim 64\%$. This indicates that the background observed is a product of the fluorogenic peptide itself, and not related to either the capture antibody, glycerol contained in the antibody, or blocking agent reacting with the fluorogenic peptide, in agreement with previous optimisation experiments (see Graph 5.1 (C)). However, even though the extent of the reduction in signal after omission of the peptide was not as pronounced in this experiment, this may be related to the different gain adjustment settings. The assay was also tested to see if different buffers may have any effect on the background or signal generated; and results showed that there was little difference between using PBST or TBST as the sample diluent and for washes (see Appendix). It was also demonstrated that the highest physiological levels of HSA or BSA produced no signal above the blank,

confirming no cross-reactivity was occurring with albumin in the plasma or in the blocking agent (See Appendix).

Together, these results suggest that the background was a product of the peptide alone, and not because of any cross-reactivity between components of the assay. Therefore the background was not likely to be an interfering factor in the measurement of IDE activity with this assay. Therefore, for subsequent experiments, data was subtracted from the background signal (from Section 5.3.3 onward).

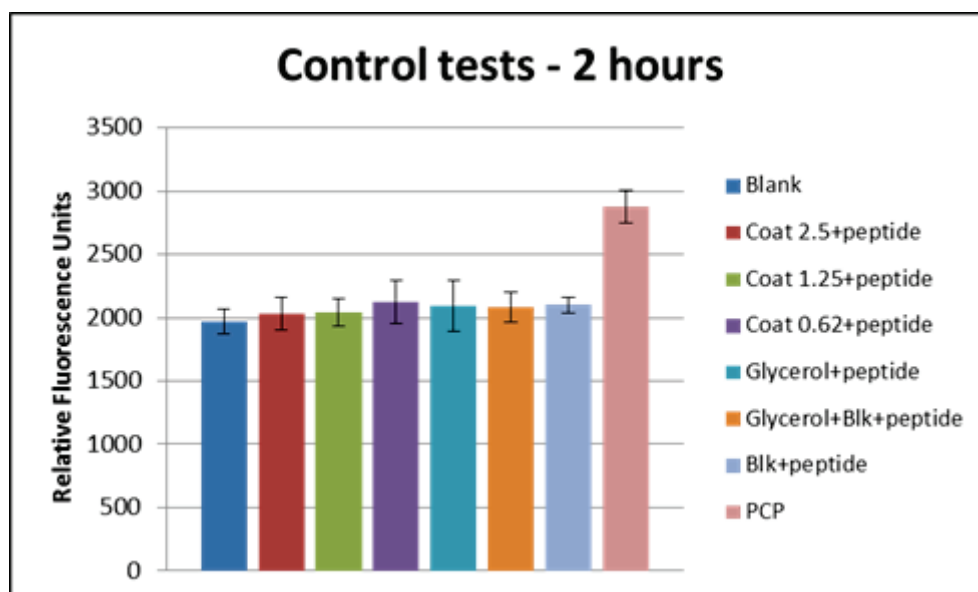


Figure 5.3 Test of immunocapture-based IDE fluorometric activity assay (with new batch of Ab 3) to analyse components of the assay. The activity assay was performed as detailed in Section 2.2.6.1 for 2 hours. The test includes blank wells (all components except sample), and PCP at 480 μg TP/well (including all other assay components), as well as Ab 3 coated at 2.5, 1.25 and 0.625 $\mu\text{g}/\text{mL}$ with the fluorogenic peptide (diluted in HEPES) only. Glycerol (1.25uL/mL) was also tested with the peptide with and without the blocking agent (BSA – “Blk”), and BSA was also tested alone with the peptide. All tests were done in duplicate. Negligible difference occurs for the background signal obtained for all control wells including the blank, while the PCP showed an increasing signal over the three readings. Error bars indicate $\pm\text{SD}$.

5.3.3 EDTA collected plasma produces a higher signal than Lithium-heparin collected plasma in the IDE fluorometric activity assay.

IDE is a metalloprotease which is dependent on the presence of a zinc atom for its catalytic activity (Ansorge, *et al.*, 1984; Ebrahim, *et al.*, 1991), and therefore, chelating agents such as EDTA or phenanthroline are likely to affect the measurable activity of IDE. The collection protocol of plasma from blood requires the inclusion of an anticoagulant such as EDTA or Lithium-Heparin (LiHep) so that the cellular components of blood may be removed while retaining the other factors and proteins, without activating the clotting cascade. Much of the anticoagulant is also retained in the plasma, which may interfere with certain down-stream applications. Plasma collected from the AIBL cohort is collected in the presence of either EDTA or

Heparin, dependent on the assay that is performed. Thus, to determine which collection method is suitable for the fluorescent assay, IDE activity was assayed in either EDTA-collected plasma or LiHep-collected plasma.

The results showed that whilst LiHep-collected plasma generated a similar signal to the blank (ie background fluorescence), EDTA collected plasma generated a signal above the blank (Figure 5.4). The enzymatic activity measured in the LiHep plasma was only approximately 15% of the signal generated from EDTA plasma. Although the assay was incubated for 4 hours rather than the full 6 hours, it is not likely that the signal would increase to a level comparable to that seen for EDTA plasma. While the reasons for this difference in signal was not pursued, the findings indicate that EDTA collected plasma was more suitable for measurements of IDE activity than plasma collected in LiHep tubes.

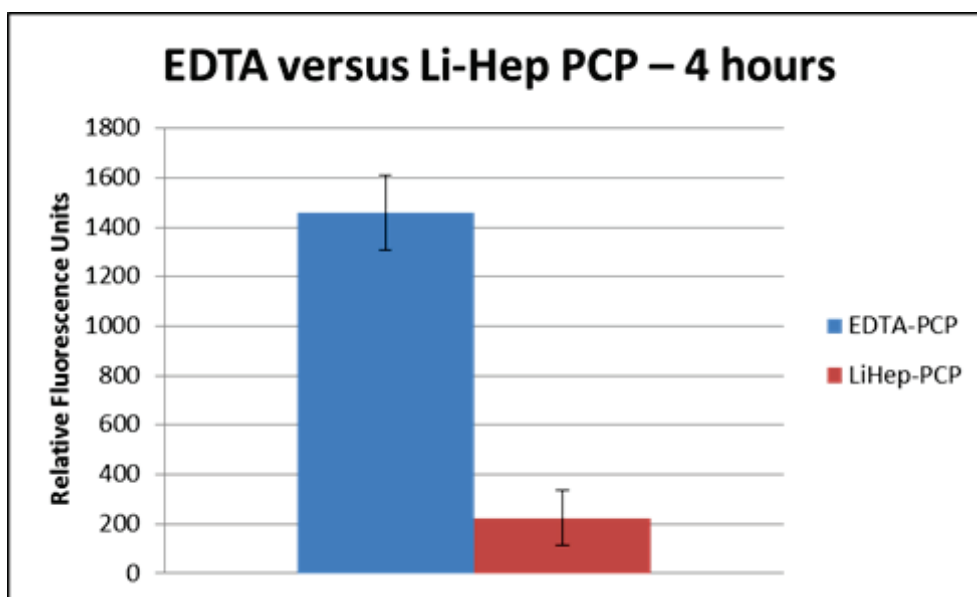


Figure 5.4 Comparison of IDE activity in PCP originating from blood collected in EDTA tubes versus LiHep tubes. Pooled control plasma (PCP) isolated from blood collection tubes originally containing EDTA or Lithium-Heparin (LiHep), were loaded at a concentration of 960 µg total protein per well with 8 replicates of each. The IDE activity assay was conducted over 4 hr, otherwise was carried out as described in section 2.2.6.1 and 2.2.6.9. Data has been blank-adjusted. The results show that PCP obtained from LiHep-collected blood had very little fluorescence compared with PCP obtained from EDTA-collected blood, implying less active enzyme in the LiHep-collected blood. Error bars indicate \pm SD.

5.3.4 Investigating the specificity of the IDE catalytic activity assay using the IDE inhibitors A β ₄₂ and insulin.

According to the company product datasheet, the fluorogenic peptide Mca-R-P-P-G-F-S-A-F-K(Dnp)-OH (Substrate V) is able to be cleaved by a number of different enzymes such as neprilysin (NEP), endothelin converting enzyme-1 (ECE-1), angiotensin-converting enzyme (ACE), cathepsin A and cathepsin X (also called cathepsin Z or P). The peptide may also theoretically be cleaved by any protease capable of cleaving the amide bond between the amide group and quencher group. While the signal derived in the assay is likely to be from IDE due to the initial immunocapture step by a specific IDE antibody, specificity can also be assessed

with competing substrates. For IDE the major substrates are A β and insulin which binds to the catalytic site, rendering it unavailable for the fluorogenic peptide. Plasma samples were therefore incubated with either A β_{42} or insulin prior to assaying.

The results (Figures 5.5(A) and (B)) show that the signal generated from the fluorogenic activity assay in plasma was reduced dose-dependently, by either A β_{42} or insulin. At concentrations of 100 μ M A β_{42} and 1mM insulin, IDE activity was inhibited completely. These results indicate that the activity observed in plasma is most likely attributed to IDE.

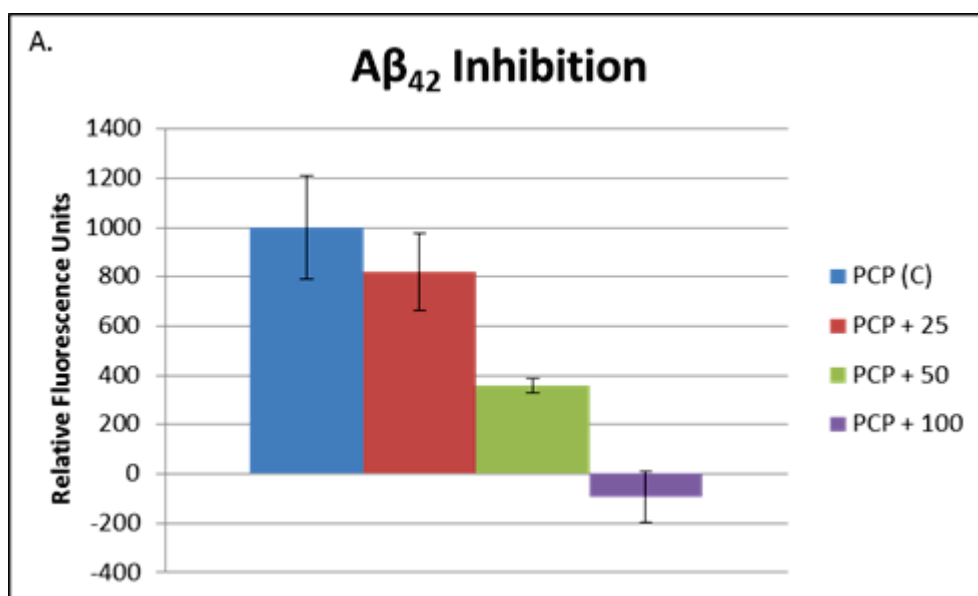


Figure 5.5(A). Test of fluorometric activity assay specificity by inhibition with beta-amyloid isoform 42 (A β_{42}). The assay was conducted as detailed in Sections 2.2.4.15, 2.2.6.3 and 2.2.6.1 over 6 hours. Pooled control plasma (PCP) was loaded at a dilution of 960 μ g total protein per well in duplicate and subsequently incubated with concentrations of 0, 25, 50 and 100 μ M A β_{42} . Data has been blank-adjusted. Results show that A β_{42} inhibited the activity of immunocaptured IDE in a dose-responsive fashion, and total inhibition was seen at a concentration of 100 μ M. Error bars indicate \pm SD.

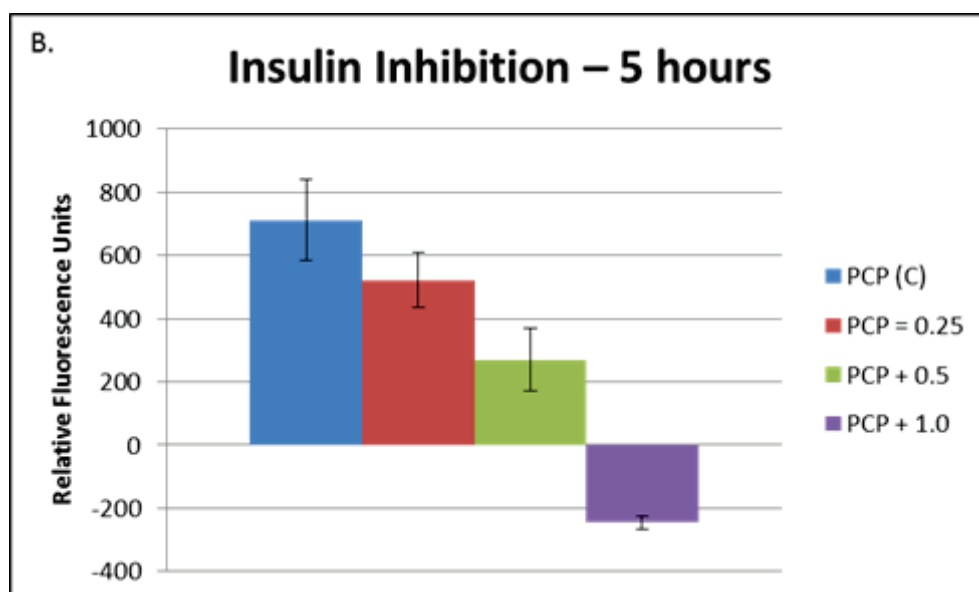


Figure 5.5(B). Test of fluorometric activity assay specificity by inhibition with insulin. The assay was conducted as detailed in Sections 2.2.6.4, 2.2.6.5 and 2.2.6.1 over 5 hours. Pooled control plasma (PCP) was loaded at a dilution of 960 μg total protein per well in duplicate and subsequently incubated with 0, 0.25, 0.5, and 1 mM concentrations of insulin. Data has been blank-adjusted. The results show that insulin inhibited the activity of immunocaptured IDE in a dose-responsive fashion, and total inhibition was seen at a concentration of 1.0 mM insulin. Error bars indicate $\pm\text{SD}$.

5.3.5 Optimisation Summary

The IDE fluorometric activity assay that had previously been used for assaying IDE activity in brain homogenates was adapted for use in human plasma. It was demonstrated that the fluorescent signal obtained for the standard curve and plasma sample increased in a time- and concentration-dependent manner, and no cross-reactivity occurred between the fluorogenic peptide and the capture antibody, glycerol, BSA or HSA. Although a moderate background signal was observed, it was similar to that observed for the diluted peptide alone and did not increase over time, indicating no cleavage of the peptide was occurring. Omission of the fluorogenic peptide significantly reduced the signal by at least two-thirds, indicating that the background was primarily due to the peptide alone. Fluorescence signal obtained for the sample was above the blank and could be completely inhibited by two known IDE substrates (insulin and A β). This is

consistent with the original study (Miners, *et al.*, 2008(b)) demonstrating minimal cross-reactivity with other related A β -degrading enzymes which cleave the fluorogenic peptide. In addition, it was demonstrated that plasma collected with Lithium-Heparin collection tubes had very little activity compared with samples collected in EDTA-containing tubes, presumably due to differences in enzyme stability. Therefore the assay was subsequently used to measure IDE catalytic activity in EDTA-collected plasma samples from a subset of the AIBL cohort.

5.3.6 Low-level data analysis.

A total of 384 plasma samples from a subset of the AIBL cohort were assayed for plasma IDE activity. Coefficient of variation (CV) values of duplicates which were >15% were excluded from the analysis. Plates with standard curves that did not fall within 2 times the standard deviation \pm the mean of each concentration of rrIDE were also excluded ($n = 2$). Values that fell below the mean of the blank (background) + 2 times the standard deviation were considered to be below the lowest level of detection. Values were interpolated from the standard curve (rrIDE) of each microplate, multiplied by the dilution factor (3.125) and expressed as Equivalent amount of rrIDE (ng/mL). Intra-assay variation ($n = 351$, based on the 9 plates used in the analysis) was determined to be ~17% (including samples with a CV of >15% that were subsequently removed from the analysis). Following the removal of these samples, average CV was 6%. Inter-assay variation was determined to be ~15% based on low, medium and high concentrations of the standard curve ($n = 9$).

The data was noted to have a non-normal distribution as determined by visually inspecting histograms, box-plots and normal Q-Q plots, as well as calculating z-scores of skewness and kurtosis. In addition, normality tests such as the Kolmogorov-Smirnoff and Shapiro-Wilks tests were highly significant. A variety of transformations were used to correct the normality issues (square root, natural log, natural log +1, reciprocal), however were not effective. Therefore non-parametric

tests were used to analyse the data (Kruskall-Wallis, Mann-Whitney and Spearman's Rho).

5.3.7 Plasma IDE catalytic activity is higher in MCI compared with HC and AD.

To determine IDE activity according to cognitive status, the cohort was divided into the clinically classified groups, healthy controls (HC - including subjective memory complainers), mild cognitive impairment (MCI) and probable AD (AD). As only a subset of the cohort was used, the HC and SMC groups were combined to form the healthy control group. It should be noted that the subset used to measure IDE activity was different from the subsets used to measure IDE levels by Western immunoblotting and ELISA (detailed in Chapters 3 and 4, respectively).

The demographics for this subset of the AIBL cohort are shown in Table 5.1. As expected, AD patients were significantly older, and had a significantly higher percentage of *APOE* $\epsilon 4$ carriers and brain $A\beta$ load than the control or MCI groups. Plasma $A\beta_{1-42}$ was significantly decreased in the MCI and AD groups compared with controls (HC>MCI>AD) while $A\beta_{x-42}$ was significantly reduced in both MCI and AD groups as compared with HC. For $A\beta_{1-40}$, AD patients also had a trend toward lower plasma levels, however this did not quite reach significance. The $A\beta_{x-42/x-40}$ ratio also showed significant alterations with AD and MCI being lower than controls (HC>MCI>AD), along with levels of ApoE and ApoE4. All other variables were not significantly different between groups.

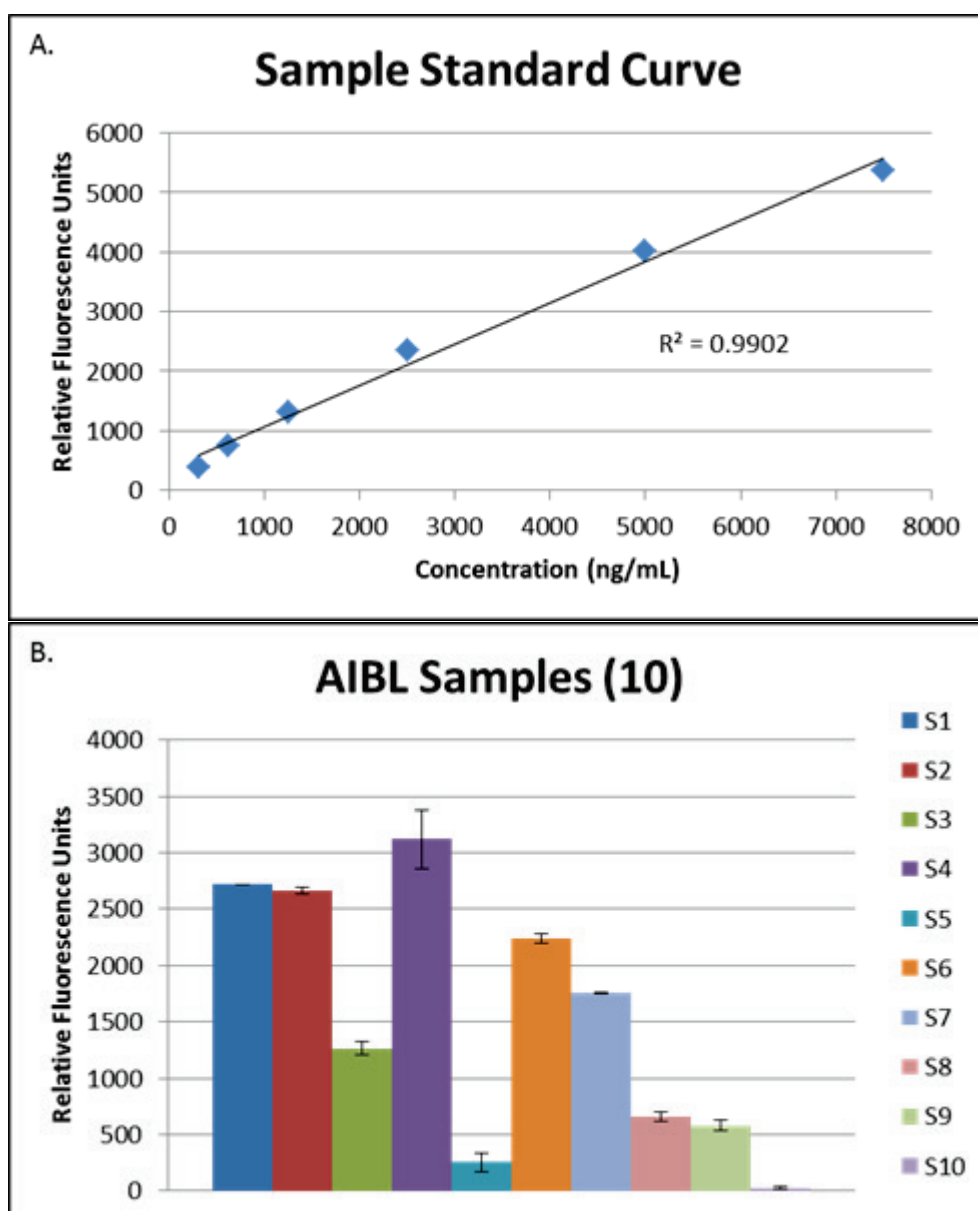


Figure 5.6(A) and (B) Sample standard curve and 10 sets of samples. The assay was conducted as detailed in Sections 2.2.6.1 over 6 hours. Recombinant rat IDE (rrIDE) was diluted to concentrations of 312.5 to 7500 ng/mL (Graph A.) and plasma samples (Graph B) including PCP were loaded at a dilution of 960 µg total protein per well (16 µL plasma per well) in duplicate. Error bars indicate \pm SD. Graphs show raw values of final readings at 6 hours. Data is blank-adjusted.

(THIS PAGE HAS BEEN INTENTIONALLY LEFT BLANK)

Table 5.1 Basic demographics of the AIBL cohort subset of participants for whom plasma IDE catalytic activity was measured by fluorometric assay. Plasma samples (16 μ L per well in duplicate) from the AIBL cohort were assayed in the IDE fluorometric activity assay for 6 hours as detailed in Section 2.2.6.1. Other variables were measured as outlined in Section 2.2.2.1. Continuous variables are expressed as mean (median) \pm standard deviation. P values were determined by Kruskal-Wallis tests using the Monte Carlo method. As only a subset of the cohort was tested, the HC and subjective memory (SMC) groups were combined to provide the healthy controls for this assay. As ApoE4 is only expressed in individuals carrying the *APOE* ϵ 4 allele, the n-values are reduced for this variable. As only a subset of the cohort were measured with Pib-PET (for Neocortical SUVR – Brain A β load), n-values are reduced for this variable.

	HC	MCI	AD	p-value
Catalytic activity plasma IDE (Equivalent rrlIDE (ng/mL))	1909.32 (1275.91) ± 2190.73	3340.9 (1807.09) ± 3652.1	2889.03 (1642.73) ± 3369.29	0.032
n (IDE Activity)	84	56	50	-
Age (years)	71.1 (69) ± 7.6	75.4 (76) ± 7.9	76.8 (79) ± 7.9	<0.001
APOE ε4 +ve (%)	32.1	46.4	71.4	<0.001
Male (%)	38.1	35.7	38	0.958 (NS)
Plasma insulin (mU/L)	7.53 (5.00) ± 13.36 n = 79	9.24 (6.00) ± 13.93 n = 55	6.86 (5.20) ± 6.14 n = 49	0.825 (NS)
Plasma Aβ ₁₋₄₀ (pg/mL)	151.23 (150.80) ± 35.17 n = 82	154.87 (160.09) ± 51.26 n = 54	137.36 (141.91) ± 42.1 n = 50	0.054 (NS)
Plasma Aβ ₁₋₄₂ (pg/mL)	32.11 (32.06) ± 9.28 n = 82	30.37 (29.72) ± 11.17 n = 54	27.08 (24.39) ± 11.38 n = 50	<0.001
Plasma Aβ _{1-42/1-40} ratio	0.217 (0.222) ± 0.059 n = 82	0.214 (0.193) ± 0.099 n = 54	0.204 (0.205) ± 0.068 n = 50	0.102 (NS)
Plasma Aβ _{x-40} (pg/mL)	145.14 (153.17) ± 54.87 n = 82	153.31 (158.39) ± 58.73 n = 54	144.77 (147.97) ± 55.32 n = 50	0.392 (NS)
Plasma Aβ _{x-42} (pg/mL)	35.72 (35.45) ± 12.18 n = 81	29.32 (26.79) ± 12.48 n = 54	29.5 (26.35) ± 13.51 n = 49	0.005
Plasma Aβ _{x-42/x-40} ratio	0.327 (0.242) ± 0.335 n = 81	0.270 (0.174) ± 0.306 n = 54	0.241 (0.209) ± 0.183 n = 49	<0.001
Plasma ApoE – Total (mg/dL)	15.5 (15.43) ± 2.68 n = 82	15.29 (15.30) ± 2.82 n = 54	14.21 (14.32) ± 2.53 n = 49	0.016
Plasma ApoE4 (mg/dL)	7.7 (6.46) ± 3.01 n = 28	6.85 (5.25) ± 4.22 n = 25	6.69 (5.07) ± 4.003 n = 36	<0.001
Plasma Testosterone - Total (nmol/L)	6.62 (1.10) ± 8.58 n = 82	7.77 (1.55) ± 9.65 n = 52	6.63 (1.00) ± 7.91 n = 50	0.625 (NS)
Plasma Testosterone – Free (nmol/L)	0.136 (0.017) ± 0.183 n = 79	0.122 (0.022) ± 0.164 n = 56	0.126 (0.014) ± 0.151 n = 49	0.848 (NS)
Plasma Estradiol (pmol/L)	97.01 (79.5) ± 57.81 n = 74	106.41 (81.00) ± 73.21 n = 44	94.43 (81.00) ± 45.81 n = 47	0.945 (NS)
Plasma Cholesterol – total (mmol/L)	5.78 (5.80) ± 1.19 n = 82	5.49 (5.30) ± 0.99 n = 56	5.79 (5.70) ± 1.28 n = 50	0.303 (NS)
Plasma LDL Cholesterol (mmol/L)	3.46 (3.30) ± 1.05 n = 82	3.15 (3.00) ± 0.93 n = 56	3.55 (3.45) ± 1.15 n = 50	0.181 (NS)
Plasma HDL Cholesterol (mmol/L)	1.76 (1.73) ± 0.45 n = 82	1.67 (1.70) ± 0.43 n = 56	1.65 (1.60) ± 0.44 n = 50	0.309 (NS)
Brain Aβ load (Neocortical SUVR)	1.45 (1.30) ± 0.42 n = 33	1.77 (1.63) ± 0.56 n = 23	2.32 (2.32) ± 0.53 n = 19	<0.001

The catalytic activity of plasma IDE determined by the fluorometric activity assay (Figure 5.7) was found to be significantly higher in the MCI group ($p = 0.007$) compared with the HC group. There was a trend towards higher IDE activity in the AD group compared with the controls ($p = 0.082$). No significant difference was observed in the catalytic activity of MCI compared with AD groups ($p = 0.642$). After Bonferroni correction for multiple testing, the difference between HC and MCI groups remained significant ($p = 0.021$). No differences were observed in total IDE activity between males and females overall [$p = 0.726$](Figure 5.8(A)), however when stratified by gender [Figures 5.8(B) and (C)] there was a trend toward higher IDE activity in MCI compared with controls for males only. No difference was observed between AD and HC in males, or between classifications in females. Large standard deviation was observed within the each classification, particularly for the MCI and AD groups.

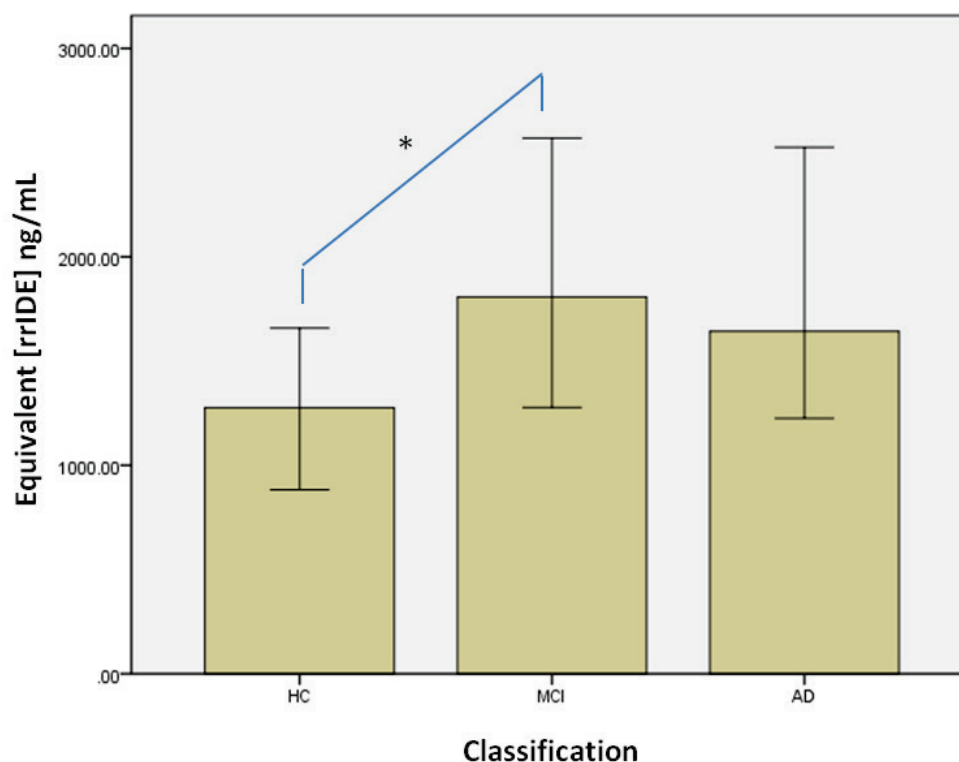


Figure 5.7 Catalytic activity of plasma IDE is increased in MCI compared with HC and AD. Plasma samples (16 μ L per well in duplicate) from the AIBL cohort were assayed in the IDE fluorometric activity assay as detailed in Section 2.2.6.1. The average value of the duplicates was obtained for each sample, and the median of these values by classification were compared by a Kruskal-Wallis test, using Mann-Whitney tests for post-hoc analysis with Bonferroni correction applied to adjust for multiple testing only with significant comparisons. The data represent medians with 95% confidence interval (CI), $n = 190$ (84 HC/56 MCI/50 AD). The results show a significantly higher level of plasma IDE catalytic activity in the MCI group when compared with the HC group ($p = 0.007$, after Bonferroni correction $p = 0.021$). No significant difference was observed in IDE activity in the MCI group compared with the AD group ($p = 0.642$), however there was a trend toward the AD group being higher than HC group ($p = 0.082$).

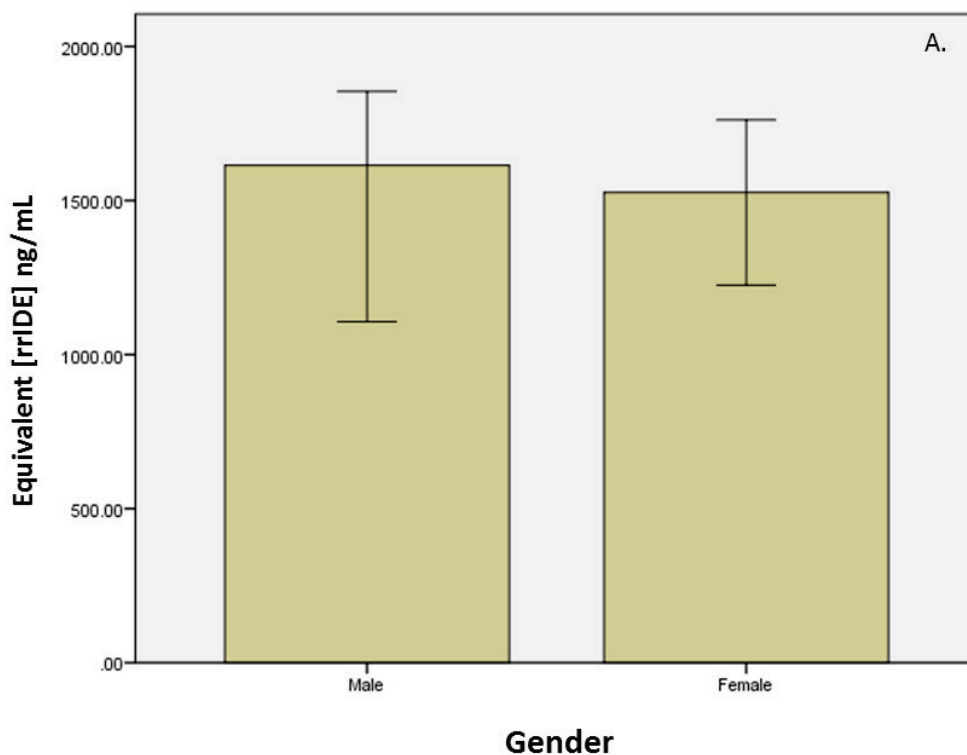
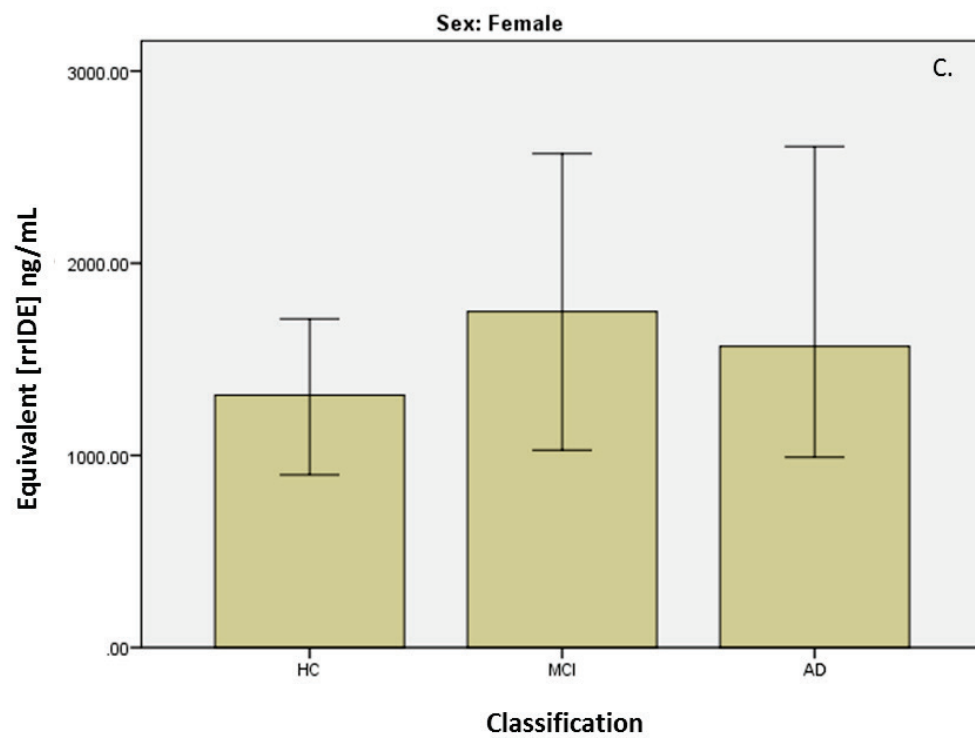
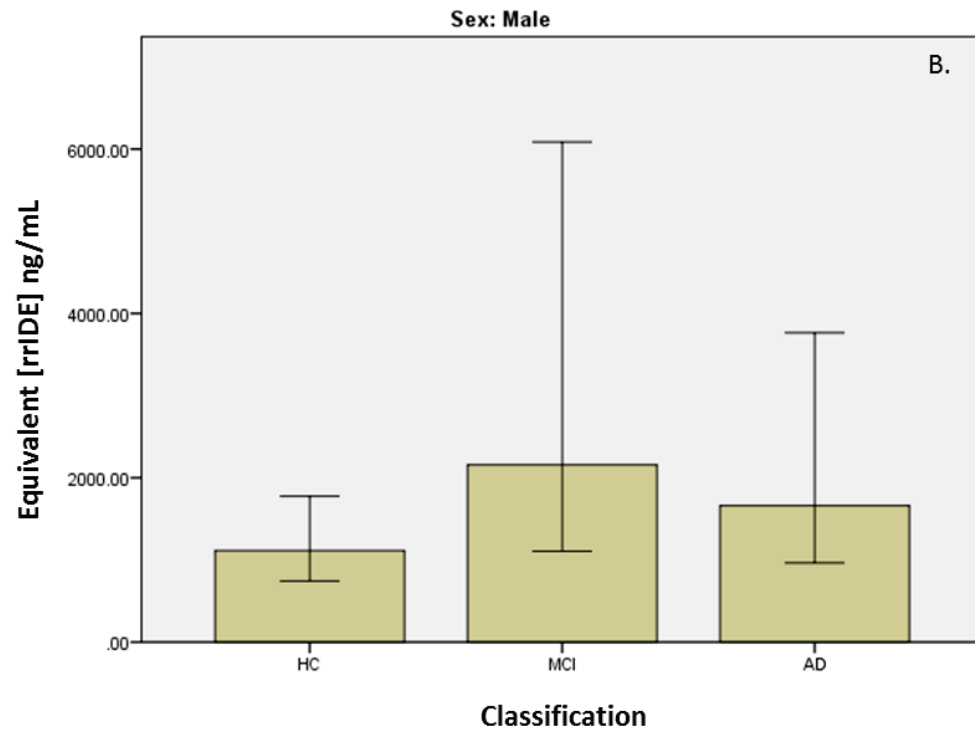


Figure 5.8(A) Gender has no effect on the catalytic activity of plasma IDE overall. Plasma samples (16 μ L per well in duplicate) from the AIBL cohort were assayed in the IDE fluorometric activity assay as detailed in Section 2.2.6.1. The average value of the duplicates was obtained for each sample, and the median of these values by classification were compared by a Kruskal-Wallis test. The data represent medians with 95% confidence interval (CI), $n = 190$ (71 Males/119 Females). The results show no difference in IDE activity between males and females ($p = 0.726$).

(THIS PAGE HAS BEEN INTENTIONALLY LEFT BLANK)

Figure 5.8(B) and (C) Plasma IDE activity is increased (not significantly) in male MCI compared with HC. Plasma samples (16 μ L per well in duplicate) from the AIBL cohort were assayed in the IDE fluorometric activity assay as detailed in Section 2.2.6.1. The average value of the duplicates was obtained for each sample, and the median of these values by classification were compared by a Kruskal-Wallis test. The data represent medians with 95% confidence interval (CI), (Males) n = 71 (32 HC/20 MCI/19 AD), (Females) n = 119 (52 HC/36 MCI/31 AD). The results show a trend toward higher IDE activity in male MCI compared with HC and AD ($p = 0.085$), while no differences were seen between classifications in females ($p = 0.336$).



5.3.8 Plasma IDE catalytic activity does not increase with age.

As levels of IDE have been reported to reduce with age (Caccamo, *et al.*, 2005), the activity of plasma IDE was analysed according to age quartiles (Figure 5.9). No significant effect of age was seen on IDE activity ($p = 0.395$). There was considerable variation in IDE activity in the oldest group, however it should be noted that this group had a small sample size ($n = 13$).

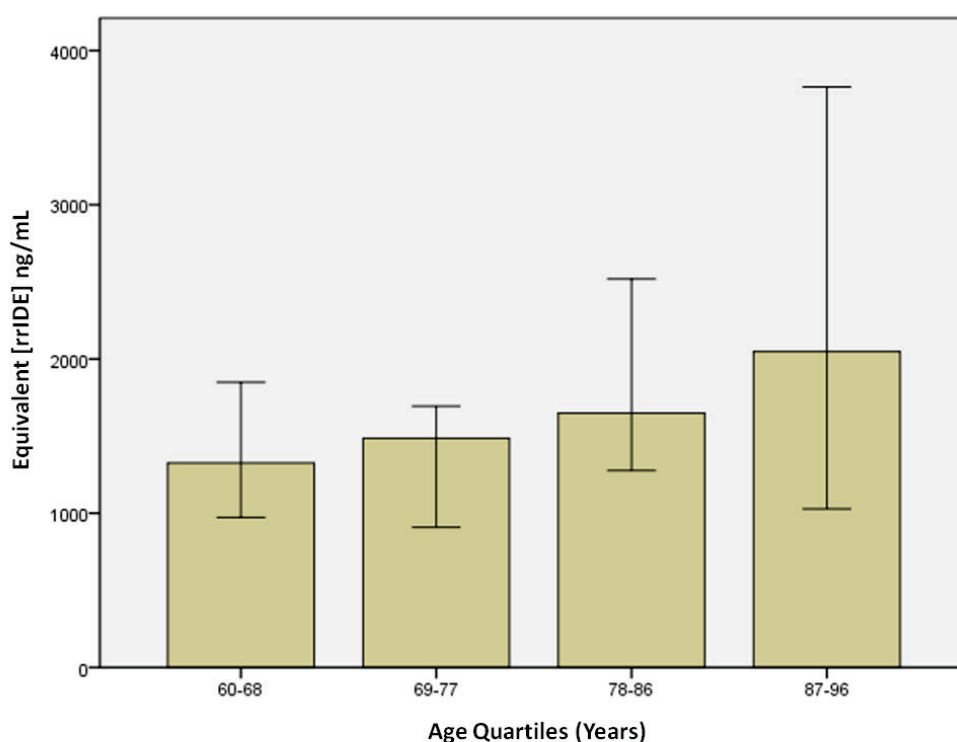


Figure 5.9 Plasma IDE activity does not increase with age. Plasma samples (16 μ L per well in duplicate) from the AIBL cohort were assayed with a fluorometric activity assay to measure the catalytic activity of IDE as detailed in Section 2.2.6.1. The average value of the duplicates was obtained for each sample, and the median of these values by age quartiles were compared by a Kruskal-Wallis test (Q1=60-68/Q2=69-77/Q3=78-86/Q4=87-96). The data represent medians with 95% confidence interval (CI), $n = 189$ (61=Q1/63=Q2/52=Q3/13=Q4). The results show that there was no significant effect of age on IDE activity ($p = 0.395$). .

5.3.9 APOE $\epsilon 4$ genotype does not impact on plasma IDE catalytic activity.

Some studies have reported a reduction in levels of IDE in APOE $\epsilon 4$ carriers compared with non-carriers in AD brains (Cook, *et al.*, 2003) and other studies have observed an increase in soluble and insoluble A β accumulation associated with the $\epsilon 4$ allele and/or ApoE4 isoform in both transgenic mice and humans (Youmans, *et al.*, 2012; Reiman, *et al.*, 2009; Bales, *et al.*, 2009; Castellano, *et al.*, 2011). Furthermore, this observation has been associated with A β clearance, and it has been shown that clearance of A β by IDE is facilitated by ApoE in an isoform-specific manner ($E4 < E3 < E2$) (Jiang, *et al.*, 2008). Hence there is a possibility that the impairment of A β degradation in $\epsilon 4$ carriers may be associated with a reduction in the catalytic activity of IDE. It should be noted that no APOE $\epsilon 4$ –related differences in IDE levels were observed in the current study using either Western immunoblotting or ELISA methods (see Sections 3.3.16 and 4.3.14). It is possible, however, that catalytic activity may be altered without a change in protein levels. To assess if this may be reflected in the IDE activity data in this project, the catalytic activity of $\epsilon 4$ carriers was compared with non-carriers (Figure 5.10(A)).

The results indicate that there is no difference in the catalytic activity of plasma IDE between $\epsilon 4$ carriers and non-carriers ($p = 0.529$). When the data was assessed in relation to classification when stratified by $\epsilon 4$ status, the MCI and AD $\epsilon 4$ carriers were observed to have a trend towards slightly higher IDE catalytic activity than the HC ($HC < AD < MCI$) ($p = 0.091$), while the $\epsilon 4$ non-carriers had similar activity between classifications ($p = 0.277$) (Figure 5.10(B) and (C)).

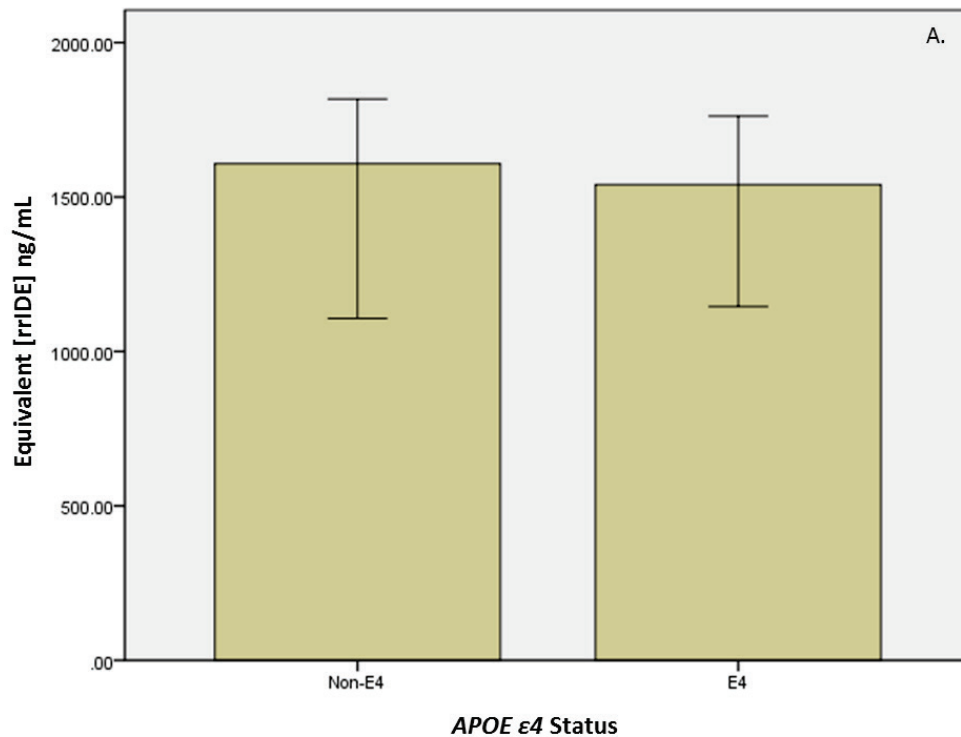
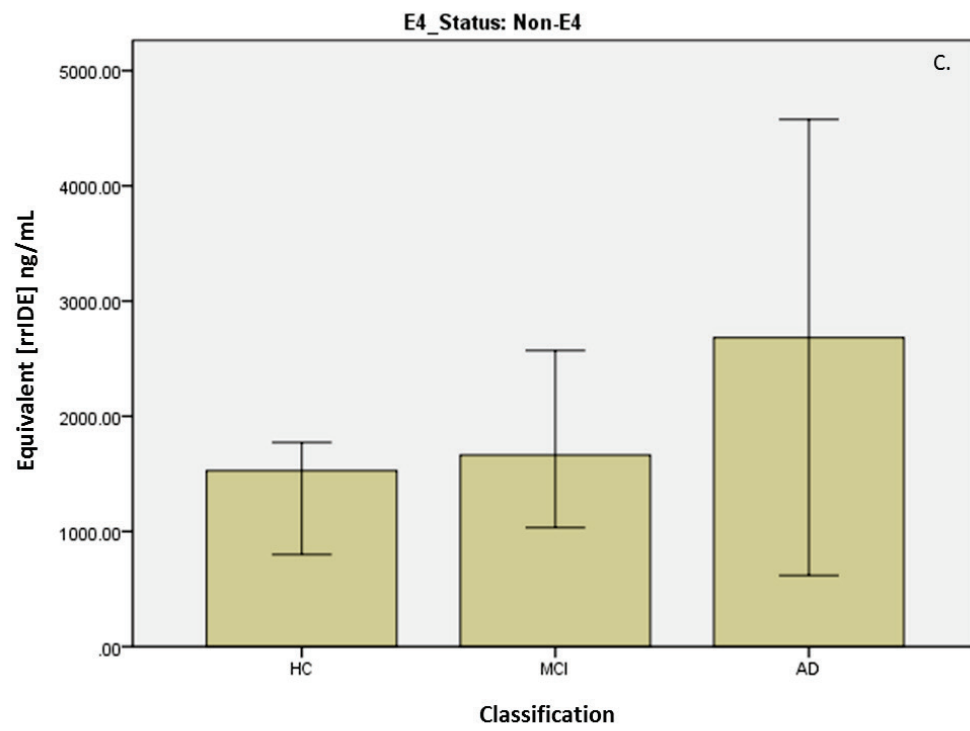
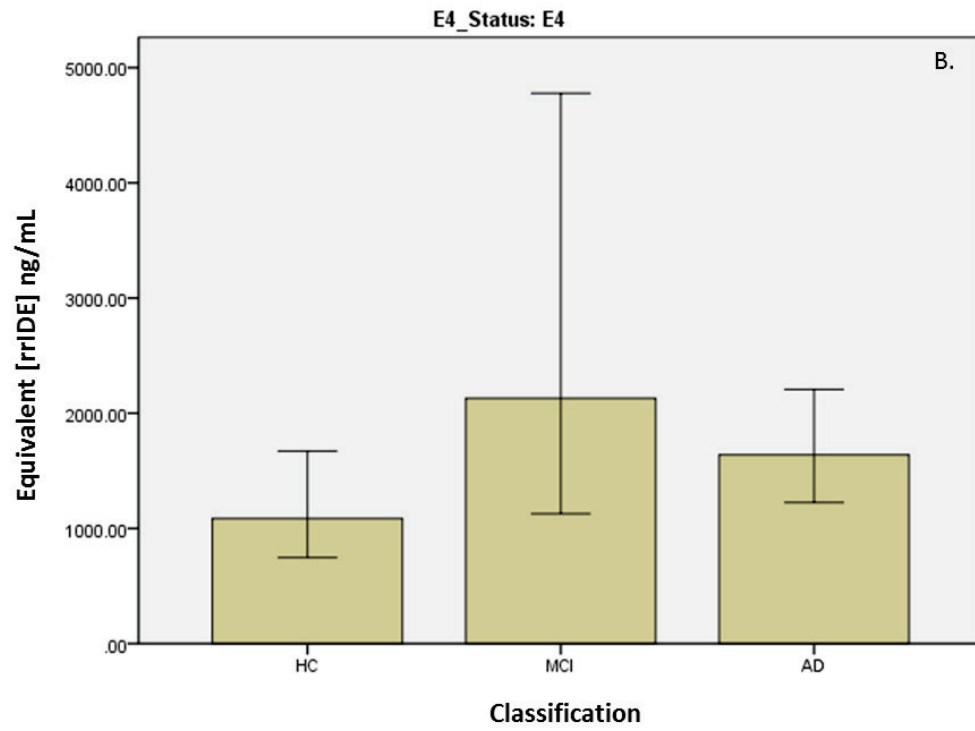


Figure 5.10(A) Plasma IDE catalytic activity is not significantly different in *APOE* ε4 carriers compared with non-carriers. Plasma samples (16 μL per well in duplicate) from the AIBL cohort were assayed with a fluorometric activity assay to measure the catalytic activity of IDE as detailed in Section 2.2.6.1. The average value of the duplicates was obtained for each sample, and the median of these values by *APOE* ε4 status was compared by a Kruskal-Wallis test. Data represents medians with 95% confidence interval (CI), n = 189 (88 ε4/101 non-ε4). Results show no difference in the catalytic activity of plasma IDE in the ε4 carriers as compared with the non-carriers (p = 0.529).

(THIS PAGE HAS BEEN INTENTIONALLY LEFT BLANK)

Figure 5.10(B) and (C) Plasma IDE catalytic activity is not significantly different between HC/MCI/AD classifications after dividing results into *APOE* $\epsilon 4$ carriers and non-carriers. Plasma samples (16 μ L per well in duplicate) from the AIBL cohort were assayed with a fluorometric activity assay to measure the catalytic activity of IDE as detailed in Section 2.2.6.1. The average value of the duplicates was obtained for each sample, and the median of these values by classification separately by *APOE* $\epsilon 4$ status were compared by a Kruskal-Wallis test. Data represents medians with 95% confidence interval (CI). ($\epsilon 4$ carriers) $n = 88$ (27 HC/26 MCI/35 AD), (non-carriers) $n = 101$ (57 HC/30 MCI/14 AD). The results show no significant difference in the catalytic activity of plasma IDE between HC/MCI/AD classifications for either $\epsilon 4$ carriers ($p = 0.091$, Graph B.) or non-carriers ($p = 0.277$, Graph C.).



5.3.10 The ratio of IDE activity to insulin levels in plasma is not significantly different between classifications.

The ELISA results from this study demonstrated that there is a significant reduction in the IDE levels/insulin levels ratio for MCI individuals who do not carry the *APOE* $\epsilon 4$ allele compared with controls (see Section 4.3.14.1). Similar to the IDE/insulin ratio (for levels), an increase in insulin level or a decrease in IDE activity would lead to a reduction of the IDE activity/insulin level ratio. To characterise further this potential relationship and to determine if the relationship of IDE activity to insulin levels may be able to differentiate between classifications in this subgroup, the ratio of plasma IDE catalytic activity and insulin levels was analysed both overall, as well as after stratification by *APOE* $\epsilon 4$ genotype.

The results indicate no significant difference between classifications overall ($p = 0.294$, Figure 5.11(A)) or between males and females ($p = 0.596$, Figure 5.11(B)). When stratified by *APOE* $\epsilon 4$ genotype (Figures 5.11(C) and (D)), no significant differences in IDE activity/insulin level ratio were seen between classifications for the $\epsilon 4$ carriers ($p = 0.141$) or the non-carriers ($p = 0.577$), although in $\epsilon 4$ carriers the MCI group was slightly higher when compared with the HC group.

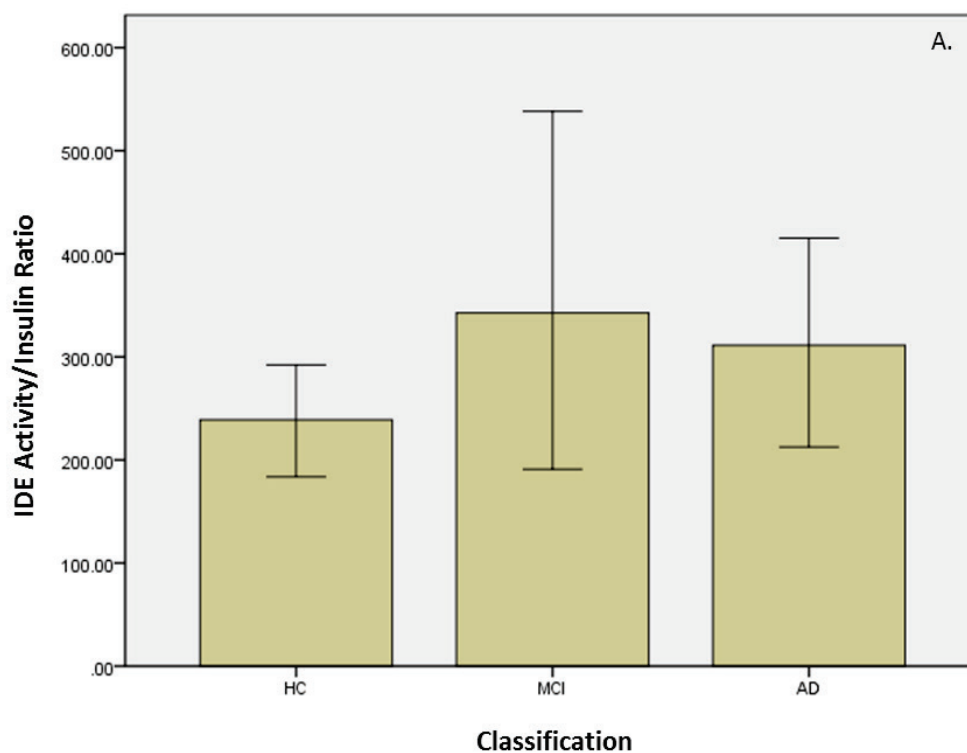


Figure 5.11(A) The IDE activity/insulin level ratio is not significantly different between classifications in the overall subgroup. Plasma samples (16 μ L per well in duplicate) from the AIBL cohort were assayed with a fluorometric activity assay to measure the catalytic activity of IDE as detailed in Section 2.2.6.1. The average value of the duplicates was obtained for each sample, and the ratios of these values to the level of plasma insulin were compared by classification using a Kruskal-Wallis test. Data represents medians with 95% confidence interval (CI), $n = 183$ (79 HC/55 MCI/ 49 AD). Results show that the IDE activity/insulin ratio is not significantly different between classifications ($p = 0.294$).

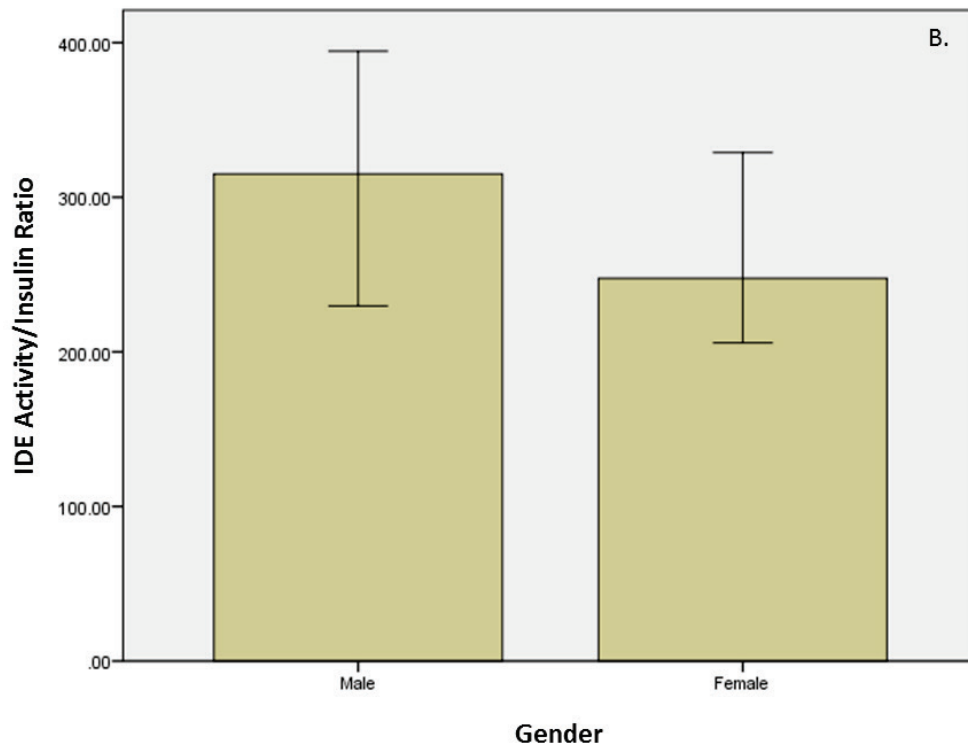
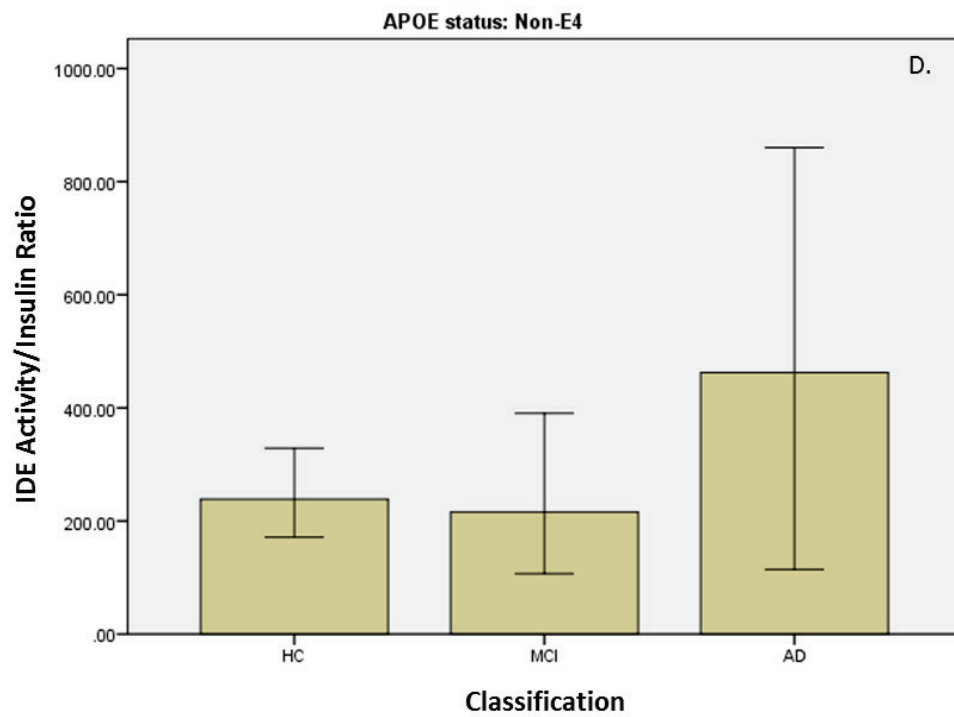
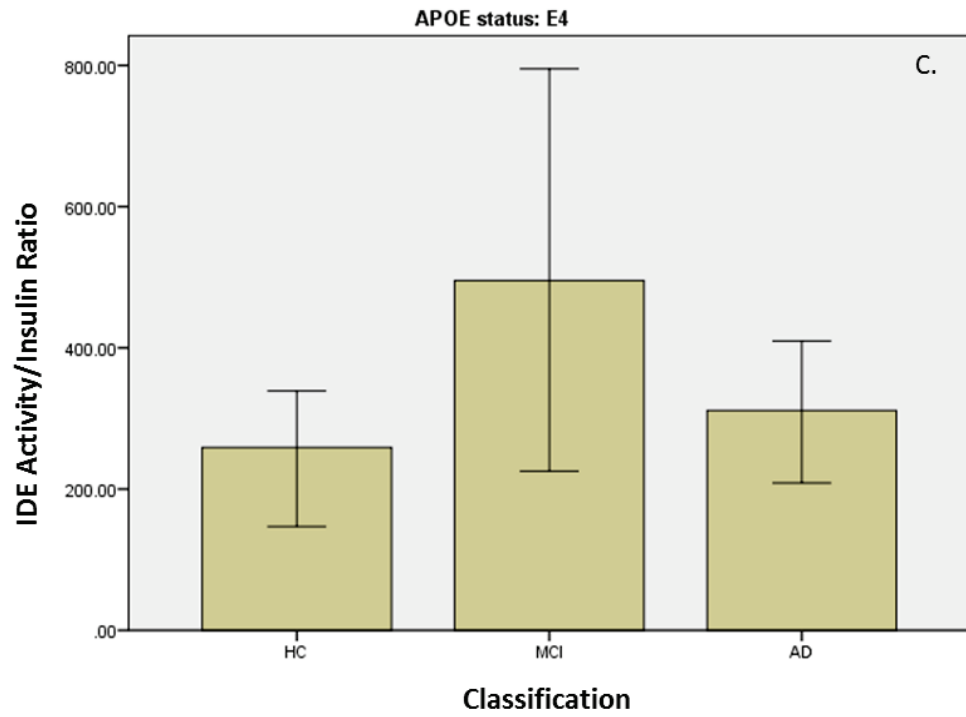


Figure 5.11(B) The IDE activity/insulin level ratio is not significantly different between males and females in the overall subgroup. Plasma samples (16 μ L per well in duplicate) from the AIBL cohort were assayed with a fluorometric activity assay to measure the catalytic activity of IDE as detailed in Section 2.2.6.1. The average value of the duplicates was obtained for each sample, and the ratios of these values to the level of plasma insulin were compared by classification using a Kruskal-Wallis test. Data represents medians with 95% confidence interval (CI), $n = 183$ (67 males/116 females). Results show that the IDE activity/insulin ratio is not significantly different between males and females ($p = 0.596$).

(THIS PAGE HAS BEEN INTENTIONALLY LEFT BLANK)

Figure 5.11(C) and (D) The IDE activity/insulin level ratio is not significantly different between classification when stratified by APOE $\epsilon 4$ status. Plasma samples (16 μ L per well in duplicate) from the AIBL cohort were assayed with a fluorometric activity assay to measure the catalytic activity of IDE as detailed in Section 2.2.6.1. The average value of the duplicates was obtained for each sample, and the ratios of these values to the level of plasma insulin were compared by classification using a Kruskal-Wallis test. Data represents medians with 95% confidence interval (CI), ($\epsilon 4$ carriers – Graph A.) $n = 85$ (25 HC/25 MCI/35 AD), (non-carriers – Graph B.) $n = 97$ (54 HC/30 MCI/13 AD). Results show that the IDE activity/insulin ratio is not significantly different between classifications for $\epsilon 4$ carriers ($p = 0.141$) or non-carriers ($p = 0.577$).



5.3.11 The ratio of IDE Activity: $A\beta_{42} + A\beta_{40}$ levels increases in MCI and AD compared with HC.

In the current study, it was shown that the ratio of plasma IDE levels to $A\beta_{42} + A\beta_{40}$ was significantly lower in the MCI group when compared with the HC group, and there was also a significant reduction in the ratio in AD compared with HC. To determine if the ratio of plasma IDE activity to $A\beta_{42} + A\beta_{40}$ was also significantly different between classifications, the ratio was calculated and compared between MCI, AD and controls (Figure 5.12(A)). The data was also stratified for *APOE* $\epsilon 4$ genotype and then compared by classification (Figure 5.12(B) and (C)).

Results showed that the IDE Activity/ $A\beta_{42+40}$ ratio was increased in MCI and AD groups compared with HC, with HC having the lowest ratio values and AD having the highest ratio values (HC<MCI<AD). Although these differences showed significance between HC vs MCI ($p = 0.022$) and HC vs AD ($p = 0.008$), only the AD group compared with the HC group remained significant following Bonferroni correction for multiple testing ($p = 0.024$). When stratified for *APOE* $\epsilon 4$ genotype, no significant differences or trends were observed between classifications in the $\epsilon 4$ non-carriers ($p = 0.182$), however, similar to the overall results an increasing trend was observed for the $\epsilon 4$ carriers (HC<MCI<AD, $p = 0.058$), however this did not reach significance.

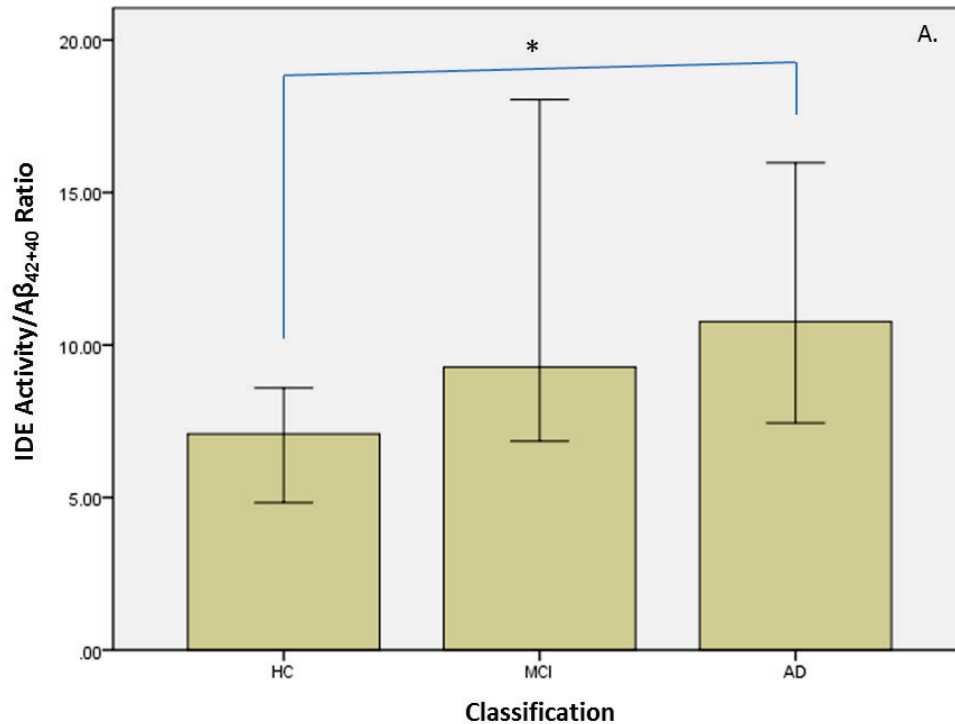
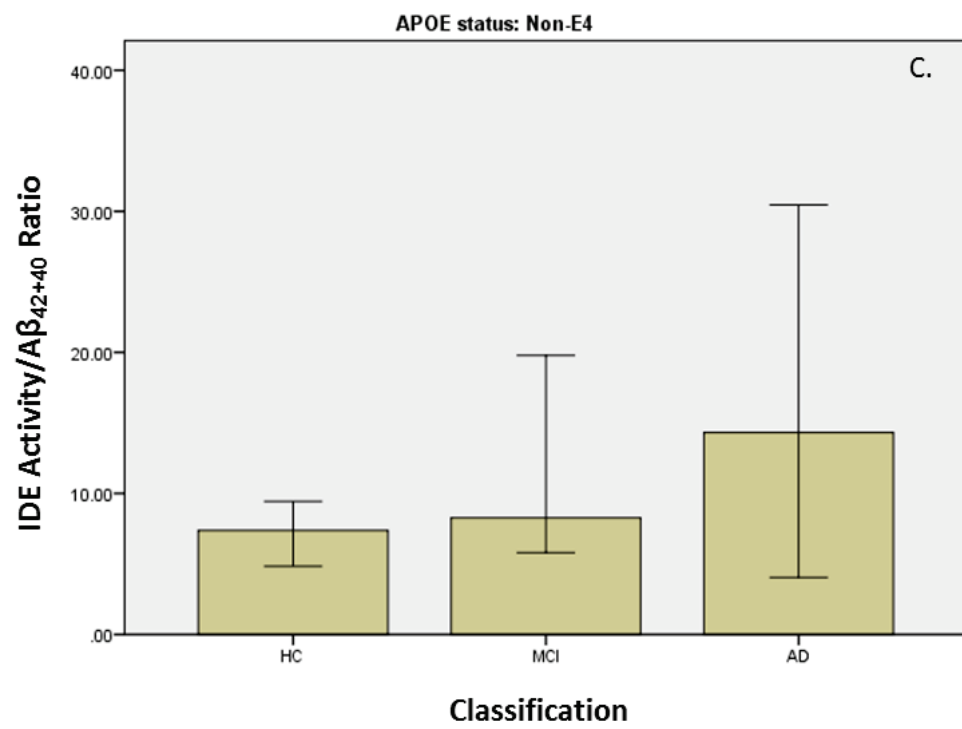
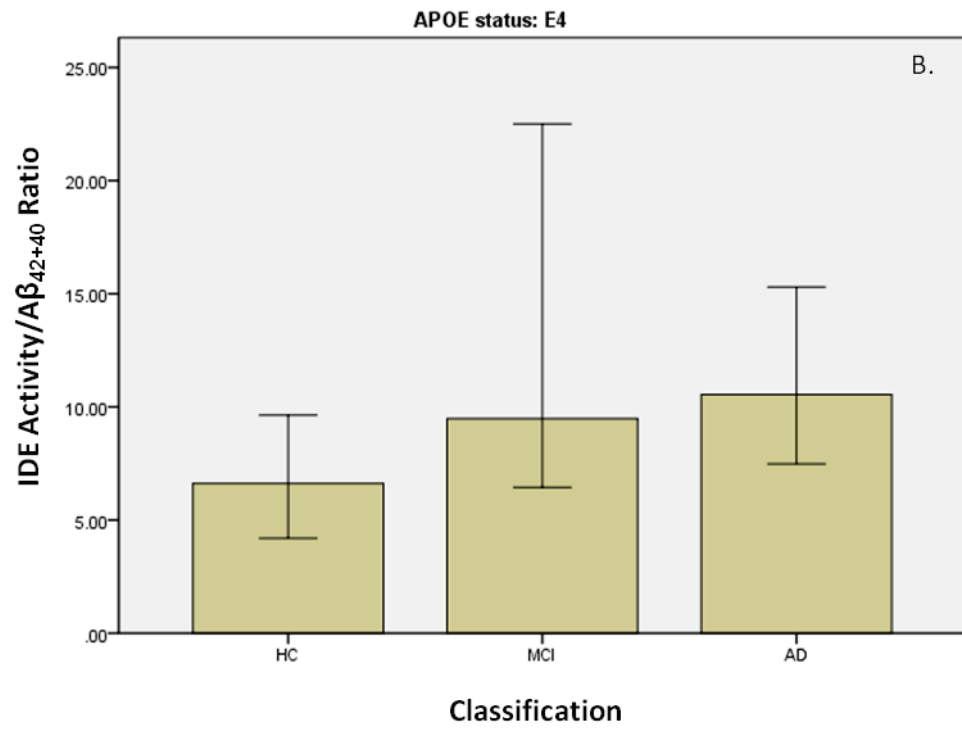


Figure 5.12 (A) The IDE Activity/ $A\beta_{42+40}$ ratio is increased in MCI (not significantly) and AD (significantly) when compared with HC. Plasma samples (16 μ L per well in duplicate) from the AIBL cohort were assayed with a fluorometric activity assay to measure the catalytic activity of IDE as detailed in Section 2.2.6.1. The average value of the duplicates was obtained for each sample, and the ratios of these values to the combined levels of plasma $A\beta_{42}$ and $A\beta_{40}$ were compared by classification using a Kruskal-Wallis test, using Mann-Whitney tests for post-hoc analysis with Bonferroni correction applied to adjust for multiple testing only with significant comparisons. Data represents medians with 95% confidence interval (CI), $n = 186$ (82 HC/54 MCI/50 AD). Results show that the IDE activity/ $A\beta_{42+40}$ ratio is significantly different in the AD group compared with HC following Bonferroni correction ($p = 0.024$), while the difference between MCI and HC is no longer significant, however a trend towards an increase is observed ($p = 0.066$). No significant difference was seen between the MCI and AD groups ($p = 0.981$).

Figure 5.12 (B) and (C) The IDE Activity/ $A\beta_{42+40}$ ratio is increased in MCI and AD (not significantly) when compared with HC in APOE $\epsilon 4$ carriers only. Plasma samples (16 μ L per well in duplicate) from the AIBL cohort were assayed with a fluorometric activity assay to measure the catalytic activity of IDE as detailed in Section 2.2.6.1. The average value of the duplicates was obtained for each sample, and the ratios of these values to the combined levels of plasma $A\beta_{42}$ and $A\beta_{40}$ were compared by classification after dividing by *APOE $\epsilon 4$* status, using a Kruskal-Wallis test. Data represents medians with 95% confidence interval (CI), $n = 86$ (26 HC/35 MCI/25 AD) for $\epsilon 4$ carriers and $n = 99$ (56 HC/29 MCI/14 AD) for $\epsilon 4$ non-carriers. Results show that an increasing trend is observed in the IDE activity/ $A\beta_{42+40}$ ratio for $\epsilon 4$ carriers (Graph B) however does not quite reach significance ($p = 0.058$), and no significant differences were seen in non $\epsilon 4$ carriers (Graph C, $p = 0.182$).



5.3.12 IDE Activity/Insulin + $A\beta_{42+40}$ ratio increases in MCI compared with HC and AD.

To determine if there was any additive effect of $A\beta_{42+40}$ combined with insulin in relation to the catalytic activity of IDE, a ratio of IDE Activity and insulin + $A\beta_{42+40}$ was calculated and compared between classifications in the subset of the AIBL cohort (Figure 5.13(A)). The results indicated that the MCI group was significantly higher than the HC group ($p = 0.045$ after Bonferroni correction) for the IDE Activity/insulin + $A\beta_{42+40}$ ratio, while the increase in the AD group compared with the HC was no longer significant after Bonferroni correction, but demonstrated a trend ($p = 0.072$). When the data was stratified by *APOE* $\epsilon 4$ status, no significant difference between classifications for $\epsilon 4$ carriers ($p = 0.108$, Figure 5.13(B)), or non-carriers ($p = 0.168$, Figure 5.13(C)) was observed.

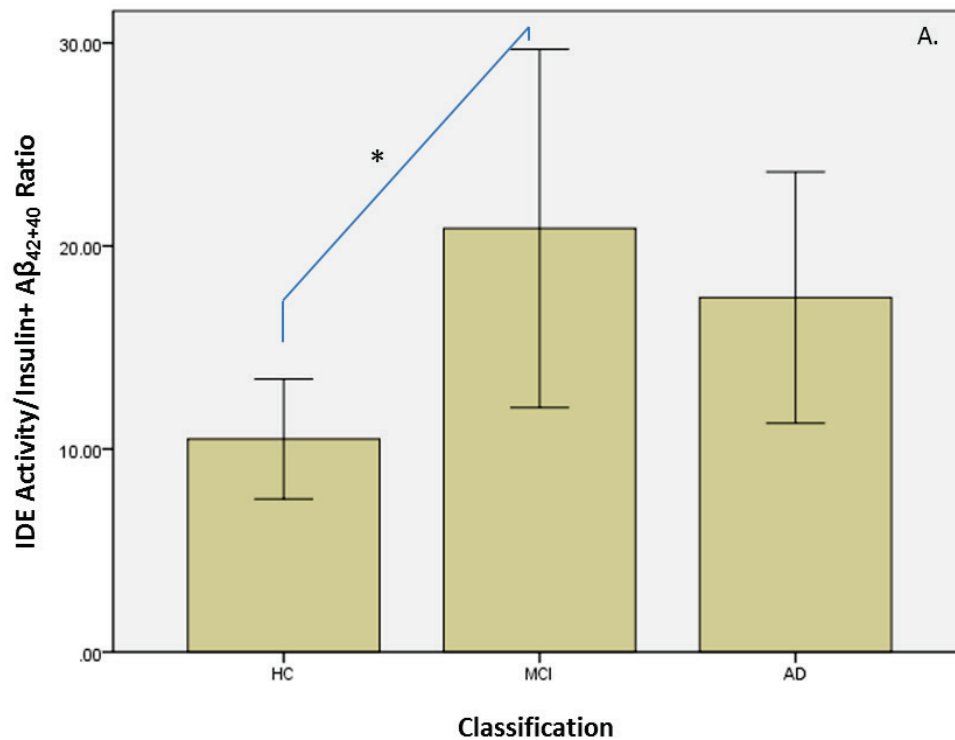
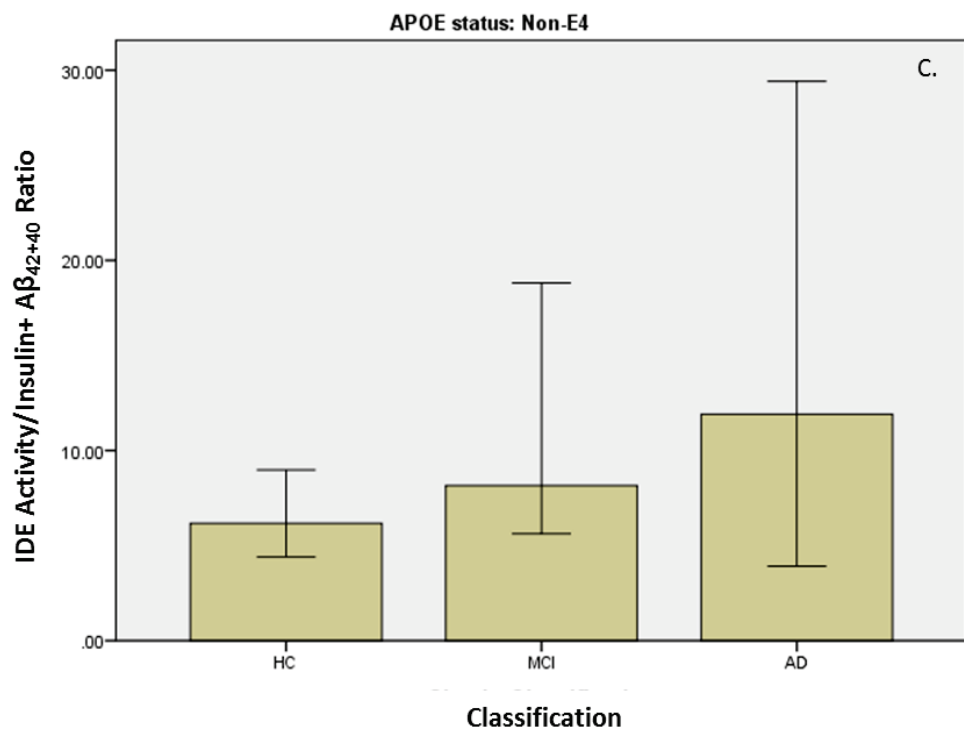
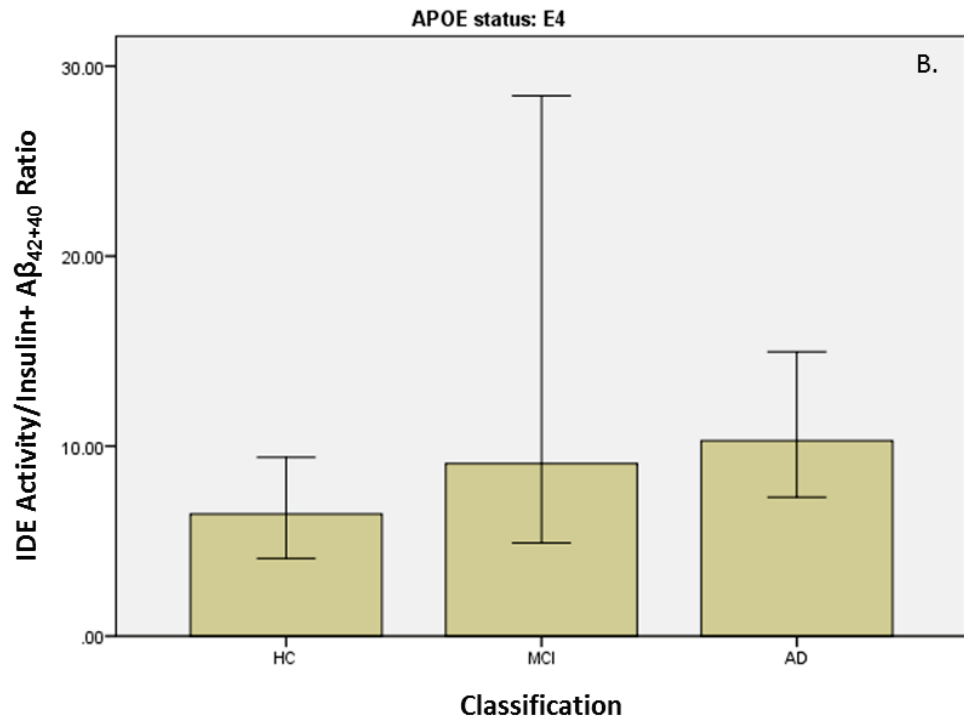


Figure 5.13(A) IDE Activity/Insulin + $A\beta_{42+40}$ ratios are increased in MCI (significantly) and AD (not significantly) compared with HC. Plasma samples (16 μ L per well in duplicate) from the AIBL cohort were assayed with a fluorometric activity assay to measure the catalytic activity of IDE as detailed in Section 2.2.6.1. The average value of the duplicates was obtained for each sample, and the ratios of these values to the combined levels of plasma insulin, $A\beta_{42}$ and $A\beta_{40}$ were calculated. The ratios were compared by classification using a Kruskal-Wallis test, using Mann-Whitney tests for post-hoc analysis with Bonferroni correction applied to adjust for multiple testing only with significant comparisons. Data represents medians with 95% confidence interval (CI), $n = 179$ (77 HC/53 MCI/49 AD). Results show that the IDE activity/Insulin + $A\beta_{42+40}$ ratio is significantly increased in MCI compared with HC ($p = 0.045$ after Bonferroni correction), while AD demonstrates a trend towards an increase compared with HC ($p = 0.072$, after Bonferroni correction). No significant difference was seen between MCI and AD groups ($p = 0.951$).

Figure 5.13(B) and (C) IDE Activity/Insulin + $A\beta_{42+40}$ ratios are no different by classification when stratified by *APOE* $\epsilon 4$ status. Plasma samples (16 μ L per well in duplicate) from the AIBL cohort were assayed with a fluorometric activity assay to measure the catalytic activity of IDE as detailed in Section 2.2.6.1. The average value of the duplicates was obtained for each sample, and the ratios of these values to the combined levels of plasma insulin, $A\beta_{42}$ and $A\beta_{40}$ were calculated. The ratios were compared by classification using a Kruskal-Wallis test. Data was stratified by *APOE* $\epsilon 4$ status. Data represents medians with 95% confidence interval (CI), ($\epsilon 4$ carriers) $n = 83$ (24 HC/24 MCI/35 AD), (non-carriers) $n = 95$ (53 HC/29 MCI/13 AD). Results show no significant differences in IDE Activity/Insulin + $A\beta_{42+40}$ ratio between classifications for $\epsilon 4$ carriers ($p = 0.108$, Graph B.) or non-carriers ($p = 0.168$, Graph C.).



5.3.13 Correlation analyses of plasma IDE catalytic activity with multiple biochemical measures in the AIBL cohort.

To determine if there were any significant correlations between IDE activity and other parameters for which biochemical data was collected as part of the AIBL study, correlation analyses were performed. As with the IDE levels measured in the ELISA, IDE activity was analysed for any correlations with sex, age, plasma insulin levels, A β species/ratios, total ApoE/ApoE4 levels, levels of testosterone/estradiol, plasma lipids (total cholesterol, LDL/HDL subfractions) and brain A β load. IDE activity was also stratified by *APOE* ϵ 4 status and analysed again for any correlations. The biochemical variables and their data collection details are outlined in Section 2.2.2.1.

Results overall (Table 5.2) showed a weak positive correlation with oestradiol ($r_s = .155$, $p = 0.047$). A weak inverse correlation was also seen between IDE activity and the A $\beta_{x42/x40}$ ratio ($r_s = -.156$, $p = .035$). Following Bonferroni-Holm correction for multiple testing, these correlations were no longer significant. No other significant correlations or trends were observed with the remaining variables.

When stratified by gender (Tables 5.3(A) and (B)), IDE activity positively correlated with age in males only ($r_s = .320$, $p = 0.065$), however after correction was no longer significant. In addition, the A $\beta_{x-42/x-40}$ ratio was observed to have a trend toward significance, while no other variables showed any significant relationships or trends with IDE activity. For females, only estradiol was observed to be positively associated with IDE activity, but again, after correction was no longer significant.

When stratified by ϵ 4 status (Tables 5.4 (A) and (B)), IDE activity showed no significant correlations in the ϵ 4 non-carriers, however a trend toward an inverse correlation with testosterone was observed ($r_s = -.176$, $p = 0.082$). For the ϵ 4 carriers, a significant positive correlation was shown between IDE activity and total testosterone ($r_s = .250$, $p = 0.022$), but not for calculated levels of free testosterone which are considered a better measure of bioavailable testosterone ($r_s = .175$, $p = 0.102$). Inverse correlations were observed between IDE activity and A β ratios

($A\beta_{42/40} - r_s = -.235, p = 0.030$, $A\beta_{x-42/x-40} - r_s = -.305, p = 0.004$), and total cholesterol ($r_s = -.214, p = 0.047$). No other significant correlations were observed with the remaining variables for $\epsilon 4$ carriers, however there were trends toward a negative correlation with HDL cholesterol ($r_s = -.196, p = 0.070$) and a positive correlation with brain $A\beta$ load ($r_s = .320, p = 0.065$). Following Bonferroni-Holm correction, however, none of the observed correlations were still significant, although there was a trend toward significance for the $A\beta_{x-42/x-40}$ ratio ($p = 0.072$).

Table 5.2 Correlation matrix of plasma IDE catalytic activity with other biochemical variables measured as part of the AIBL study. The catalytic activity of plasma IDE was analysed for relationships with sex, age, and plasma insulin, A β species, ApoE species, testosterone, oestradiol, lipids and brain A β load (two tailed). n values vary due to missing values for some data or in the case of brain A β load only a subset of the cohort underwent PiB-PET scans. For ApoE4, n value is lower as only the *APOE* ϵ 4 carriers express this isoform. Results show a weak positive correlation with oestradiol ($r_s = .155$, $p = .047$) and a weak inverse correlation between IDE activity and the A $\beta_{x42/x40}$ ratio ($r_s = -.156$, $p = .035$) (“unadj.” = unadjusted, prior to Bonferroni-Holm correction). No other variables showed correlations with IDE activity. After Bonferroni-Holm correction these correlations were no longer significant (“adj.” = Bonferroni-Holm adjusted).

Overall	Correlation Coefficient (r_s)	N	Significance (2-tailed)
Sex	-.030	190	0.681 (NS)
Age	.114	190	0.118 (NS)
Insulin	-.006	183	0.936 (NS)
A β_{1-40}	.116	186	0.114 (NS)
A β_{1-42}	.048	186	0.512 (NS)
A $\beta_{42/40}$ ratio	-.063	186	0.390 (NS)
A β_{x-40}	.098	186	0.183 (NS)
A β_{x-42}	-.076	184	0.303 (NS)
A $\beta_{x-42/x-40}$ ratio	-.156	184	0.035 * (unadj.) 0.630 (adj.)(NS)
Total ApoE	.019	185	0.794 (NS)
ApoE4	-.123	86	0.259 (NS)
Total Testosterone	.020	184	0.785 (NS)
Free Testosterone	-.005	184	0.944 (NS)
Oestradiol	.155	165	0.047 * (unadj.) 0.799 (adj.)(NS)
Total Cholesterol	-.054	188	0.460 (NS)
LDL Cholesterol	-.064	188	0.384 (NS)
HDL Cholesterol	-.042	188	0.565 (NS)
Brain A β Load	.109	75	0.353 (NS)

Table 5.3(A) Correlation matrix of plasma IDE catalytic activity with other biochemical variables measured as part of the AIBL study after stratification for gender – males only. The catalytic activity of plasma IDE was split by *APOE* $\epsilon 4$ status and analysed for relationships with sex, age, and plasma insulin, A β species, ApoE species, testosterone, oestradiol, lipids and brain A β load (two tailed). n values vary due to missing values for some data or in the case of brain A β load only a subset of the cohort underwent PiB-PET scans. For ApoE4, only the *APOE* $\epsilon 4$ carriers express this isoform therefore is not included for the $\epsilon 4$ non-carriers. Results show no correlations of IDE activity with these variables.

Males (A)	Correlation Coefficient (r_s)	N	Significance (2-tailed)
Age	.300	71	0.011 * (unadj.) 0.198 (adj.)(NS)
Insulin	-.154	67	0.214 (NS)
A β_{1-40}	.179	71	0.134 (NS)
A β_{1-42}	.084	71	0.484 (NS)
A $\beta_{42/40}$ ratio	-.068	71	0.574 (NS)
A β_{x-40}	.202	71	0.092 (NS)
A β_{x-42}	-.047	70	0.700 (NS)
A $\beta_{x-42/x-40}$ ratio	-.216	70	0.072 (NS)
Total ApoE	-.115	71	0.338 (NS)
ApoE4	-.083	38	0.618 (NS)
Total Testosterone	-.039	69	0.751 (NS)
Free Testosterone	-.177	68	0.149 (NS)
Oestradiol	.043	67	0.728 (NS)
Total Cholesterol	-.128	69	0.293 (NS)
LDL Cholesterol	-.140	69	0.251 (NS)
HDL Cholesterol	.001	69	0.994 (NS)
Brain A β Load	.250	33	0.161 (NS)

Table 5.3(B) Correlation matrix of plasma IDE catalytic activity with other biochemical variables measured as part of the AIBL study after stratification for gender – females only. The catalytic activity of plasma IDE was split by *APOE* $\epsilon 4$ status and analysed for relationships with sex, age, and plasma insulin, A β species, ApoE species, testosterone, oestradiol, lipids and brain A β load (two tailed). n values vary due to missing values for some data or in the case of brain A β load only a subset of the cohort underwent PiB-PET scans. For ApoE4, only the *APOE* $\epsilon 4$ carriers express this isoform therefore is not included for the $\epsilon 4$ non-carriers. Results show no correlations of IDE activity with these variables.

Females (A)	Correlation Coefficient (r_s)	N	Significance (2-tailed)
Age	.003	119	0.970 (NS)
Insulin	.095	116	0.311 (NS)
A β_{1-40}	.085	115	0.365 (NS)
A β_{1-42}	.023	115	0.806 (NS)
A $\beta_{42/40}$ ratio	-.068	115	0.469 (NS)
A β_{x-40}	.036	115	0.700 (NS)
A β_{x-42}	-.086	114	0.361 (NS)
A $\beta_{x-42/x-40}$ ratio	-.114	114	0.227 (NS)
Total ApoE	.117	114	0.217 (NS)
ApoE4	-.169	48	0.250 (NS)
Total Testosterone	-.033	115	0.725 (NS)
Free Testosterone	-.029	116	0.761 (NS)
Oestradiol	.222	98	0.028 * (unadj.) 0.504 (adj.)(NS)
Total Cholesterol	-.006	119	0.949 (NS)
LDL Cholesterol	-.017	119	0.856 (NS)
HDL Cholesterol	-.035	119	0.709 (NS)
Brain A β Load	-.017	42	0.917 (NS)

Table 5.4(A) Correlation matrix of plasma IDE catalytic activity with other biochemical variables measured as part of the AIBL study after stratification for *APOE* $\epsilon 4$ status - $\epsilon 4$ non-carriers only (unadjusted). The catalytic activity of plasma IDE was split by *APOE* $\epsilon 4$ status and analysed for relationships with sex, age, and plasma insulin, A β species, ApoE species, testosterone, oestradiol, lipids and brain A β load (two tailed). n values vary due to missing values for some data or in the case of brain A β load only a subset of the cohort underwent PiB-PET scans. For ApoE4, only the *APOE* $\epsilon 4$ carriers express this isoform therefore is not included for the $\epsilon 4$ non-carriers. Results show no correlations of IDE activity with these variables.

<i>APOE</i> $\epsilon 4$ non-carriers only (A)	Correlation Coefficient (r_s)	N	Significance (2-tailed)
Sex	.120	101	0.231 (NS)
Age	.104	101	0.300 (NS)
Insulin	-.088	97	0.393 (NS)
A β_{1-40}	.135	99	0.183 (NS)
A β_{1-42}	.133	99	0.190 (NS)
A $\beta_{42/40}$ ratio	.084	99	0.410 (NS)
A β_{x-40}	.123	99	0.225 (NS)
A β_{x-42}	-.029	98	0.777 (NS)
A $\beta_{x-42/x-40}$ ratio	-.040	98	0.697 (NS)
Total ApoE	.049	99	0.633 (NS)
ApoE4	n/a	n/a	n/a
Total Testosterone	-.176	99	0.082 (NS)
Free Testosterone	-.166	96	0.107 (NS)
Oestradiol	.126	89	0.239 (NS)
Total Cholesterol	.077	100	0.444 (NS)
LDL Cholesterol	.046	100	0.646 (NS)
HDL Cholesterol	.074	100	0.464 (NS)
Brain A β Load	-.147	40	0.365 (NS)

Table 5.4(B) Correlation matrix of plasma IDE catalytic activity with other biochemical variables measured as part of the AIBL study after stratification for *APOE* $\epsilon 4$ status - $\epsilon 4$ carriers only (unadjusted). The catalytic activity of plasma IDE was split by *APOE* $\epsilon 4$ status and analysed for relationships with sex, age, and plasma insulin, A β species, ApoE species, testosterone, oestradiol, lipids and brain A β load (two tailed). n values vary due to missing values for some data or in the case of brain A β load only a subset of the cohort underwent PiB-PET scans. Results show a significant but weak positive correlation with testosterone ($r_s = -.250$, $p = .022$) but not calculated free testosterone, and IDE activity was also significantly correlated inversely with cholesterol ($r_s = -.214$, $p = .047$), A $\beta_{42/40}$ ratio ($r_s = -.235$, $p = .030$) and A $\beta_{x42/x40}$ ratio ($r_s = -.305$, $p = .004$) ("unadj." = unadjusted, prior to Bonferroni-Holm correction). Following Bonferroni-Holm correction for multiple testing ("adj." = Bonferroni-Holm adjusted), these correlations were no longer significant, however a trend toward significance was seen for the A $\beta_{x42/x40}$ ratio ($p = 0.072$). * indicates significance at the < 0.05 level. ** indicates significance at the < 0.01 level.

<i>APOE</i> $\epsilon 4$ carriers only (B)	Correlation Coefficient (r_s)	N	Significance (2-tailed)
Sex	-.184	88	0.086 (NS)
Age	.097	88	0.370 (NS)
Insulin	.090	85	0.413 (NS)
A β_{1-40}	.082	86	0.453 (NS)
A β_{1-42}	-.043	86	0.694 (NS)
A $\beta_{42/40}$ ratio	-.235	86	0.030 * (unadj.) 0.480 (adj.)(NS)
A β_{x-40}	.049	86	0.653 (NS)
A β_{x-42}	-.133	85	0.226 (NS)
A $\beta_{x-42/x-40}$ ratio	-.305	85	0.004 ** (unadj.) 0.072 (adj.)(NS)
Total ApoE	-.017	86	0.877 (NS)
ApoE4	-.123	86	0.259 (NS)
Total Testosterone	.250	84	0.022 * (unadj.) 0.374 (adj.)(NS)
Free Testosterone	.175	88	0.102 (NS)
Oestradiol	.186	75	0.111 (NS)
Total Cholesterol	-.214	87	0.047 * (unadj.) 0.705 (adj.)(NS)
LDL Cholesterol	-.175	87	0.106 (NS)
HDL Cholesterol	-.196	87	0.070 (NS)
Brain A β Load	.320	34	0.065 (NS)

5.4 Discussion

The aim of this chapter was to optimise a previously developed immunocapture-based IDE-specific fluorometric activity assay (Miners, *et al.*, 2008(b)) in order to measure the catalytic activity of IDE in plasma from individuals from the AIBL cohort. The assay was used to determine if there were any differences in IDE catalytic activity in AD and MCI individuals compared with healthy controls, or if any differences in IDE catalytic activity were related to various AD-related genetic and biochemical factors.

The assay was shown to reflect the concentration of protein over time both for purified recombinant rat IDE (rrIDE) and human plasma using two different batches of Ab 3. Using the new batch of antibody, no significant difference was observed when using phosphate-based buffers for the blocking step, sample incubation and washes as compared with using tris-based buffers. The signal had good linearity up to a concentration of 7500 ng/mL purified rrIDE and also for plasma (up to approximately 1200 µg total protein per well). Over the 6 hour incubation time period the signal for rrIDE was linear for the first few hours but then appeared to plateau slowly at all concentrations assayed, while the signal in plasma remained linear for the duration of the assay. It is possible that the plasma IDE is more stable than the purified enzyme due to the presence of endogenous proteins, while the rrIDE began degrading early in the incubation period. Although the non-bound components of the plasma were washed away prior to the fluorogenic peptide being added, it is possible that the immunocaptured IDE is bound to other proteins which do not interfere with the catalytic site.

It should be noted that Miners also found that the signal was maximal after 5 hours for brain homogenates, however the current study demonstrated that the signal in plasma continued to increase in a linear fashion up to at least 6 hours incubation. The signal may have increased further after extending the incubation beyond 6 hours but this was not pursued; however it may be of value to investigate this in the future. It may be that IDE levels are higher in the brain than in the plasma

which may have saturated the fluorogenic peptide earlier, leading to the plateau in signal observed in the Miners study.

The finding that the assay was linear for rrIDE up to 7500 ng/mL is contrary to the results of Miners who found that the assay was linear only between 156 – 2500 ng/mL for rrIDE. Furthermore, the fluorescent values were more than double in the Miners results compared to the values observed in this study. There were a number of differences in the Miners protocol by comparison which may account for these differences. Firstly, the coating antibody was diluted in PBS (pH 7.4) in Miners study, while our study used a carbonate/bicarbonate coating buffer (pH 8.6). The microplate manufacturer advised that a coating buffer pH of 9.6 was optimal for antibody adsorption. The lower pH though, would be more likely to result in weaker binding of the capture antibody to the microplate, which may have been displaced by the subsequent washes and led to a reduced signal, which is opposite to what was observed. However, as well as the pH, the different components of the buffers may also have had an effect on the binding capabilities of the antibody. Another difference was the use of BSA as a blocking agent in the current study. This step was not included in the Miners assay and could potentially have led to exposed binding sites on the plate which may have allowed additional IDE or other enzymes to bind to the plate, leading to a higher signal. Although omission of the blocking step did not alter the signal observed in plasma, it may be that the presence of BSA in the incubation step provided some extra stability to the purified IDE during the overnight incubation. The Miners protocol also included an additional two washes in each washing step, although it is unclear how this would have contributed to an increased signal; it is possible though that the extra washes may have decreased the background leading to an improved signal to noise ratio. Similarly, the samples in our study were incubated overnight at 4°C rather than 1.5 hours at RT; again it is not clear why this may contribute to a lower overall signal or to the differences observed in the range of linearity of the rrIDE. Other differences include the presence of Tween-20 in the wash buffers and sample buffer (PBS) in the current study, which may have reduced the background reading and led to a better signal to noise ratio. Perhaps though, most notable differences may be the

dilution buffer composition and incubation temperature of the fluorogenic substrate, which in the current study was 31°C compared with 37°C in the Miners protocol. This was likely to have led to a reduction of enzyme activity and account for the lower signal observed. In addition to this, the dilution buffer used for the fluorogenic peptide in the current study was HEPES, while Miners used a Tris-based buffer containing zinc. The addition of zinc in the incubation buffer may have stimulated the activity of IDE, and this effect has been demonstrated in a recent study (Grasso, *et al.*, 2012). The combination of the activation of IDE by zinc, as well as the increased incubation temperature in the Miners study would have resulted in an increase in IDE activity by comparison with the current study, and would have saturated the peptide earlier, perhaps providing an explanation for why the linear range was lower in the Miners study. One other factor which was not mentioned in the Miners study is the gain adjustment setting, which in our study was found to have a large impact on the values obtained. This alone may have accounted for the higher values observed in the Miners study.

The activity assay was seen to have a high level of background signal, and while the source of this background noise has not been confirmed, there are a few possible reasons. It was determined that the background was not attributable to an interaction of the fluorogenic peptide with BSA (or contaminating proteases within the BSA preparation) used as the blocking agent, as no difference was seen when it was omitted from the assay. Similarly, no interaction was seen between the fluorogenic peptide and the capture antibody or its component glycerol. It was shown that a similar background signal occurred with just the diluted peptide alone, and omission of the peptide with all other assay components led to a significantly reduced signal, suggesting that the background was primarily due to the fluorescent peptide itself. Regardless, the background fluorescence for both batches of antibody were similar, and also did not increase over time and this indicated that ongoing cleavage of the peptide was not occurring. Although the increased background may have reduced the sensitivity of the assay at the lower limit of detection, the background was therefore not considered to be a significant factor and was subtracted from the values observed. Furthermore, the reduced

signal observed with the second batch of Ab 3 compared with the original batch is likely due to a reduced affinity of the new batch of antibody to IDE, leading to reduced amounts of the enzyme captured. This is not unusual for polyclonal antibodies, and highlights the importance of using a single batch of antibody for immunoassays which require comparisons between different experiments.

The activity assay was also tested using samples collected in tubes containing different anticoagulants; namely EDTA and Lithium-Heparin (LiHep). It was expected that the samples containing EDTA might have a lower activity by comparison due to the chelating effect of the EDTA on the essential zinc ion bound to the catalytic site of IDE. Surprisingly, the opposite result was observed with LiHep samples having only approximately 15% of the activity of EDTA samples. It is not clear exactly why this difference would occur, however may be potentially related to the tendency of EDTA collected samples to be vulnerable to haemolysis during blood processing, releasing intracellular IDE into the plasma and leading to an increased signal. Alternatively, the differences may be related to the reduced stability of plasma proteins in LiHep collected blood, resulting in more degradation of IDE over time in LiHep tubes. Other studies have shown that levels of certain analytes measured in EDTA-collected plasma are higher than samples collected in LiHep tubes or sodium-heparin tubes (Kragstrup, *et al.*, 2013; de Jager, *et al.*, 2009), and while the levels of IDE were not compared for differences between the two different anticoagulants in the current study, it would be of value to investigate in future whether the activity differences were related to IDE protein level, and/or due to haemolysis.

The internally-quenched fluorogenic peptide which was used for this assay is not specific to IDE, and other enzymes such as neprilysin (NEP), endothelin converting enzyme (ECE-1), angiotensin converting enzyme (ACE), cathepsin A and cathepsin X (also called cathepsin Z or P) are also able to cleave this substrate according to the company datasheet. Therefore it was essential to ensure that the capture antibody (Ab 3) was able to specifically bind to IDE but not bind to any other enzyme capable of degrading the peptide. When testing activity assays for specificity, a common approach is to use specific inhibitors to block the activity generated by the assay.

Unfortunately most inhibitors are not specific for just one enzyme; for example A β is a substrate of IDE which inhibits its catalytic activity toward the fluorogenic substrate by competitive inhibition. As some other proteases also cleave A β , it is likely that it would also inhibit other A β -degrading enzymes. Therefore more than one inhibitor may need to be used to confirm the specificity of an assay in measuring a particular enzyme. In the current study, two commonly used IDE inhibitors were employed; insulin and A β (both A β_{40} and A β_{42}). A β and insulin are both competitive inhibitors of IDE, by binding to the active site and preventing binding or cleavage of the fluorogenic peptide. However, A β is also a substrate for NEP, ECE-1 and ACE as well as IDE and therefore not specific. Insulin though, is primarily degraded by, and has a high affinity for insulin-degrading enzyme and has been previously used in IDE activity assays to determine specificity (Liu, *et al.*, 2012(a); Alper & Schmidt, 2009). However, it should be noted that there are other enzymes present in the plasma that bind to insulin, and have been reported to be inhibited by insulin, although it is unknown if these enzymes are capable of cleaving the fluorogenic substrate (Lokhov, *et al.*, 2004; Kashyap, *et al.*, 2008). Miners and colleagues (2008(b)) had previously showed that there was negligible signal from recombinant NEP, ACE and ECE-1 using the same IDE-specific capture antibody as the one in the current study (Ab 3), indicating that the signal observed is unlikely to be contributed to substantially by these particular proteases. The signal observed in this activity assay derived from human plasma could be completely inhibited by both insulin and A β_{42} , in a dose-dependent fashion. Although the reduction in signal was only around 50% for A β_{40} , this initial experiment used a lower concentration of A β and was the reason that larger amounts of A β_{42} were subsequently used. These results indicate that it is likely that the signal is attributable to cleavage of the substrate by IDE, although it would be of interest to test other IDE-specific inhibitors in this assay. Recently this has been addressed with the development of promising novel and specific IDE inhibitors (Abdul-Hay, *et al.*, 2013; Leissring, *et al.*, 2010).

In addition, IDE-specific antibodies were also used in an attempt to neutralize the activity of IDE in plasma by interference of the fluorogenic substrate cleavage. One

antibody (Ab 1) was targeted to a region within Domain 1 of IDE which is in close proximity to the catalytic site, while the other (Ab 2) targets a region near the C-terminus of IDE which is far away from the catalytic site. There was around 50% reduction of signal when the sample was pre-incubated with Ab 1, while no such decrease was observed with Ab 2. This may suggest that Ab 1, by binding close to the catalytic site, is somehow interfering with the cleavage of the fluorogenic substrate, possibly by physically blocking binding to the catalytic site, or by steric hindrance. It is also possible that binding of the antibody to IDE causes a conformational change that negatively affects binding or cleavage of the substrate. However, it should be noted that Ab 1 was in the form of anti-serum (as opposed to Ab 2 which was purified), which would contain other components which may have inhibited the signal also, and therefore this may not necessarily confirm specificity. Nevertheless, in Western immunoblotting experiments (see Section 3.3.1), Ab 1 only detected a single band at the expected molecular weight, which could be considerably reduced by pre-incubation of the antibody with purified IDE, suggesting that the antibody is specific. The observation that Ab 2 does not interfere with substrate cleavage indicates that the epitope location may be too distal from the catalytic region to interfere with binding or cleavage. While it cannot be completely ruled out that other components of the antiserum (Ab 1) may have contributed to the reduction in signal in the activity assay, these results support that the signal observed is derived from IDE.

The specificity of the assay was demonstrated by the complete reduction of signal by IDE substrates, A β and insulin, and no cross-reactivity was observed between assay reagents or with plasma albumin. Therefore the assay was used to measure IDE activity in plasma samples obtained from the well-characterised AIBL cohort. The results for the AIBL samples showed that IDE activity in plasma was significantly higher in MCI individuals compared with healthy controls. AD patients also had elevated IDE activity however this did not reach significance. These results for IDE activity were contrary to what was expected; it was expected that MCI and AD might have decreased activity compared with HC. A recent study by Liu, *et al.*, 2012(a) showed that MCI and AD individuals had lower serum IDE activity than

healthy controls, and the subjects with the lowest serum IDE catalytic activity also had correspondingly lower IDE levels compared with subjects with the highest catalytic activity (who had higher IDE levels) as determined by Western immunoblotting. In the current study, plasma IDE activity was increased in MCI (and a trend toward an increase in AD) compared with HC, which was reflected by an increase in IDE levels for MCI (but not AD) in Western immunoblotting results. However, this was opposite to what was observed with IDE levels measured by ELISA, where MCI levels were significantly reduced compared with HC. This will be discussed further in Chapter 6. It should be noted that the study by Liu and colleagues used serum samples as opposed to plasma; one consideration related to this is that unlike serum samples, the plasma samples contained EDTA. As mentioned above, EDTA has been shown to increase the likelihood of haemolysis, and as red blood cells contain IDE, this may have affected the results in this assay. It was demonstrated that EDTA containing plasma had significantly higher IDE activity than plasma sourced from Lithium-Heparin (LiHep) tubes and it is possible this may be due to haemolysis. Conversely, it may be that IDE in the LiHep plasma samples was less stable than IDE in the EDTA plasma, and this may be the case for serum IDE also. Until the implications of these differences are investigated, caution should be exercised when translating the results of this study. In addition, the study by Liu and colleagues used a different capture antibody than the one used in the current study. Although they demonstrated a reduction in activity with several substances which are known to inhibit IDE activity (insulin, A β , amylin and EDTA), the inhibition seen in their study was not complete (unlike in the current study where complete inhibition was seen with A β and insulin) and therefore it cannot be ruled out that proteases other than IDE were implicated in generating the measurable activity observed in the Liu study.

As the Western immunoblotting data indicated that IDE levels differed between males and females, the IDE catalytic activity was also analysed by gender. Although no significant differences were seen in IDE activity overall between males and females, when stratified by gender the males demonstrated a trend toward increased activity in MCI compared with HC, while no differences in IDE activity was

seen between classifications for females. While it was noted that the increase did not reach significance, this may be due to reduced numbers as a result of stratifying the subset. This indicates that the observed increase in IDE activity in MCI is primarily due to the effects in males. The reason for the increase in the activity in male MCI participants is not clear; there was no increase in IDE substrates or hormones in the plasma that have been shown to upregulate IDE such as insulin, A β , testosterone (free or total) or oestrogen levels in this subset of the AIBL cohort, indicating other as yet unknown or non-assessed factors are involved. It would be of value to investigate this finding in future studies.

Based on the results of the current study, it is possible that the activity of IDE is upregulated in early AD (MCI) in an attempt to compensate for a reduction in total IDE levels, as reflected in the IDE ELISA data (although the Western immunoblotting results did not concur with the ELISA results; the different assays may be measuring distinct forms of IDE and this will be discussed more in Chapter 6). This finding is consistent with previous reports on IDE activity in the brain using the same fluorometric activity assay; Miners and colleagues (2009) found an increase in brain IDE activity in AD patients compared with controls, which was not influenced by *APOE* $\epsilon 4$ genotype and independent of IDE levels. Although not significant, differences in activity patterns between classifications in the current study observed between genders may be related to other factors such as intrinsic hormonal differences, effects of sex hormone-based or other medications, or lifestyle factors such as diet and exercise. Indeed the high variation seen in the MCI and AD groups may indicate the influence of other variables. Due to time-restraints it was not possible to investigate these possibilities within the time-frame of this project; however it would be of value to explore these theories in the future.

The plasma catalytic activity of IDE was shown to be similar for *APOE* $\epsilon 4$ carriers and non-carriers overall. When stratifying the classifications by $\epsilon 4$ status, although not statistically significant MCI and AD $\epsilon 4$ carriers tended to have increased IDE activity compared with control group, while no differences were seen in the non-carriers. The difference in IDE activity between classifications in carriers versus non-carriers may potentially reflect the pathological changes that may be occurring faster in $\epsilon 4$

carriers; as mentioned in Chapter 1 it has been shown that the onset of AD tends to be earlier, and the progression of degeneration faster for individuals carrying the $\epsilon 4$ allele (Corder, *et al.*, 2003; Chen, *et al.*, 2007).

To ascertain if there were any associations between IDE activity and other biochemical data collected as part of the AIBL study, correlation analyses were performed. The variables were chosen based on two criteria; variables which have been associated with AD pathology such as age, plasma levels of $A\beta$ (and N-terminally truncated species), ApoE/ApoE4, cholesterol (and LDL/HDL subtypes), and brain $A\beta$ load, and those which have been associated with alterations in IDE levels (sex, insulin, testosterone, and estradiol). The correlations that were observed between IDE activity and other variables (only overall and in *APOE* $\epsilon 4$ carriers) were not significant following Bonferroni-Holm correction, however this type of correction for multiple testing is very conservative and increases the chances of Type II errors occurring, albeit not quite as stringent as the original Bonferroni method. However, as a large number of correlates were analysed, Bonferroni/Bonferroni-Holm is the best correction method to avoid the occurrence of Type I errors. For the *APOE* $\epsilon 4$ carriers in the current study, four variables out of seventeen tested were significantly correlated with IDE activity prior to correction, which is more than would be expected by chance alone if none of these variables had a true relationship with IDE activity. Therefore, one or more of these correlations may actually be true associations despite the corrected results. In particular, the observation that both the $A\beta_{42/40}$ ratio and $A\beta_{x-42/x-40}$ ratio showed a negative correlation with IDE activity prior to Bonferroni correction, and the correlation with $A\beta_{x-42/x-40}$ ratio was still close to significance following Bonferroni correction, is probably worth investigating further in the future.

In the case of testosterone data, total levels were used in the correlation analyses as well as calculated levels of free testosterone, which is regarded as a better measure of bioavailable testosterone. Analysing males and females together may be a limitation, as males have considerably higher levels of testosterone than females, although there were no associations overall or after stratification by gender in males or females between total or free testosterone and IDE activity.

However, in females a positive correlation was observed between estradiol and IDE activity; therefore the overall correlation seen with estradiol was due primarily to the association in females. Although not significant after correction, this relationship is consistent with the published literature which suggests that estradiol positively regulates IDE activity (Udrisar, *et al.*, 2005).

As the subset of AIBL samples used in the IDE activity assay was different to the subsets used to measure IDE levels by Western immunoblotting and ELISA, it was not possible to assess any correlations between activity and levels due to the small number of samples in common. However, a small sample set which were in common with the activity and levels (by Western immunoblotting) subsets showed a borderline correlation (data not shown).

5.4.1 Summary

In this project, a previously described fluorometric assay that had been developed for the purpose of measuring the catalytic activity of IDE in brain homogenates was adapted for measuring IDE activity in human plasma. Although factors such as background signal may have impacted on the sensitivity of the assay at the lower limit of detection, specificity of the assay was shown by demonstrating no cross-reactivity between assay reagents, as well as a complete reduction of signal by the use of IDE substrates that competitively and dose-dependently inhibited the activity generated from plasma. Further development of the assay such as identifying ways to reduce the background signal, as well as investigating the reason for the differences in plasma collected with different anticoagulants would be of value. The use of additional IDE inhibition studies as well as genetic knock-down or knock-out studies to further characterise and validate this assay for use in plasma should also be considered.

This initial pilot study on the catalytic activity of plasma IDE was promising, and showed alterations in the plasma IDE activity which differed by clinical classification. While gender did not have an effect on IDE activity overall, when

stratified by gender the differences seen between classifications were only found in the male subgroup. IDE activity overall did not differ by *APOE* status, however a non-significant increasing trend was seen in IDE activity for MCI and AD compared with controls in $\epsilon 4$ carriers only, as well as an association of IDE activity with the $A\beta$ ratios in $\epsilon 4$ carriers which requires further investigation. It is possible that with a larger cohort these trends may become significant. Notably, the ratio of IDE activity to $A\beta$ levels was higher in MCI and AD participants compared with controls, as was the ratio of IDE activity to the collective levels of insulin and $A\beta$. While only full-length $A\beta_{40}$ and $A\beta_{42}$ was included in these ratios, it would be of interest to investigate if the N-terminally truncated species of $A\beta$ were also implicated. However, there was high variation within each classification group, and this may be related to hormone levels, lipid composition and/or other factors which may inhibit or enhance the activity of IDE.

The results suggest that IDE activity in plasma is upregulated in both the prodromal (represented by the MCI group) and symptomatic periods of AD, however whether IDE activity might be clinically useful as an early diagnostic biomarker is not clear at this stage. It may be that other factors which regulate IDE may prove to be more relevant than IDE itself. Indeed the observed associations with sex hormones requires further investigation. Further work towards understanding the complex regulatory mechanisms of IDE catalytic activity in the peripheral circulation, and specifically in relation to its role in $A\beta$ degradation or other potential roles in AD would therefore be of importance in future studies. It is notable that the results are contrary to the only other published study on blood IDE activity who found lower IDE activity in MCI and AD individuals (Liu, *et al.*, 2012(a)), however in their study they used serum rather than plasma, and a number of covariates were controlled for. As non-parametric analyses were used in the current study this was not possible. While the results from the current study show potential, and particularly in respect to plasma $A\beta$ and insulin levels, they would require validation following further assay characterisation in a larger cohort before any conclusions could be made regarding the suitability of IDE activity as a plasma biomarker for the early detection of Alzheimer's disease.

(THIS PAGE HAS BEEN INTENTIONALLY LEFT BLANK)

Chapter 6 – Final Discussion

6.1 Introduction

The number of AD sufferers is predicted to increase significantly in the coming years, therefore the development of a blood-based diagnostic test is crucial. There are no effective treatments for AD, therefore prevention and/or delaying the onset of disease symptoms is the next best goal for anti-AD therapy. AD has a long pre-symptomatic phase, and thus diagnosis at the earliest stage possible will maximise the effect of disease-delaying treatments. Therefore, the ideal panel of blood biomarkers will identify AD patients in early pre-symptomatic stages with high specificity and sensitivity. To this end, potential blood biomarkers need to be characterised extensively, and reliable biomarker assays need to be developed. The aim of the current study was to investigate insulin-degrading enzyme (IDE) in the plasma of MCI individuals, AD patients and healthy controls to ascertain if it is a suitable biomarker to include in such a biomarker panel.

The specific aims of this project were:

- To characterise commercial IDE antibodies for the measurement of plasma IDE levels using two distinct methods,
- To measure the catalytic activity of plasma IDE using a previously developed activity assay to determine if the levels are altered in the prodromal period of AD (MCI) or in symptomatic AD,
- To investigate any association between IDE (levels or activity) and other AD-related biomarkers and genetic influences.

The potential implications of these results will be discussed in the context of the current literature, and the limitations of the study will be addressed. Prospective future directions will also be discussed.

6.2 Assay development and validation

The only available commercial IDE ELISA kit on the market at the commencement of the project did not perform within the acceptable limits of inter-assay and intra-assay variation, therefore it was not used for the measurement of plasma IDE levels in this project. Although only two tests were performed using this kit, the prohibitively high cost prevented further characterisation. Similarly, an in-house indirect ELISA was not suitable for measuring levels of IDE in plasma, as other proteins present in the matrix competed with IDE for binding to the ELISA plate. Therefore, an in-house sandwich ELISA assay was developed for the measurement of IDE levels.

An ELISA is an antibody-based assay, and this project has demonstrated some of the numerous factors that should be considered when characterising antibodies for use in such an assay, as the choice of antibody is central to the integrity of the results. These are discussed in detail in Chapters 3 and 4, but highlighted below are some of the factors that were found to have the greatest impact. Variable results were obtained in this project when using different antibodies in all the applications tested. The likely reasons for these variations or inconsistencies include differences in IDE epitopes to which the antibodies bind, differences in the immunogens used to initiate the immune response to generate the antibodies, or differences in host species in which the antibodies were generated. Another important consideration when trying to set up a reliable immunoassay is the batch-to-batch variability of the antibodies. It was demonstrated in this project that there can be substantial differences in assay results caused by differences in batches of polyclonal antibodies (both from commercial as well as private sources), highlighting one of the advantages of monoclonal antibodies, or if using polyclonals, the importance of sourcing antibodies from a single batch. This is necessary to ensure consistency when measuring an analyte which will be compared between subjects in a cohort.

In addition, at least two of the commercially-available IDE antibodies that were tested cross-reacted significantly with components of blocking solutions such as BSA, as well as plasma components such as HSA, under the denaturing and reducing

conditions of Western immunoblotting (WB), while not cross-reacting under the native conditions of the ELISA or fluorometric activity assay. This outlines the importance of testing the specificity and potential cross-reactivity of the antibody within each application for which it is intended. It was also demonstrated that some common reagents such as BSA that are used routinely in these applications can vary in purity, and this can also have a major impact on the outcome of the assay (Chapter 4, Section 4.3.16). The finding that Fraction V grade BSA did not generate a signal in the ELISA while cell-culture grade BSA produced a dose-dependent signal may have possibly been due to contaminants in the latter preparation. Therefore it is critical that antibodies are tested thoroughly for cross-reactivity with blocking reagents and with non-specific components of the sample matrix. Although the ELISA assay cross-reacted with cell-culture grade BSA, no cross-reactivity issues were demonstrated with the fluorometric activity assay, which suggests that the cross-reactivity issues were related to the detection antibody in the ELISA. While it is probably not feasible to test for cross-reactivity with every component in a complex fluid such as plasma, at least the major proteins or proteins known to be highly antigenic should be assessed, and ideally plasma from an IDE knock-out animal model could be used to validate the assay.

The elimination of signal observed with the spike and recovery assessment in the sandwich ELISA (Chapter 4, Section 4.3.17) indicates a significant matrix effect of the plasma components on the assay. It is important to identify the reason for this signal reduction, and the effect minimised, as part of assay validation. Regardless, peptide blocking and immunoprecipitation experiments indicated that the 120-130 kDa protein band detected by Ab 3 in plasma samples was IDE (discussed further in Chapter 3, Section 3.4).

As Ab 3 detected multiple other bands which appeared to be non-specific in the Western immunoblotting results, it was also important to determine this antibody's specificity in the ELISA and the fluorometric activity assay. It was shown for the sandwich ELISA that there was negligible cross-reactivity between the secondary antibody and either the detection or capture antibodies. The slightly elevated signal for the blank may indicate some cross-reactivity between the capture and

detection antibodies; however, the concentrations of these were consistent between wells and the background reading subtracted from results to account for this. Nevertheless this high blank signal may have impacted on the sensitivity of the assay, leading to a reduced overall signal. In addition, cell-culture grade BSA was used in the ELISA for the AIBL samples and was responsible for some cross-reactivity, which may also have blocked some of the available antibody, leading to a reduced signal.

Recent research is beginning to address potential interfering factors and cross-reactivity issues in immunoassays. Emerging studies on assay validation and cross-reactivity show that interference can occur from other endogenous factors such as heterophilic, anti-animal or auto-antibodies and rheumatoid factors (reviewed in Tate & Ward, 2004). Some exogenous potentially interfering factors have also been identified such as some plasticisers and organosilicone surfactants in plastic tubes (Bowen, *et al.*, 2005 and reviewed in Bowen, *et al.*, 2010). While these factors were not addressed in the current study, variables such as these need to be considered carefully when developing and optimising an immunoassay, and therefore would be important for further refinement of the ELISA.

The most robust specificity tests for Ab 3 were performed using the IDE activity assay; firstly at least half of the signal generated could be removed by pre-incubation with A β ₄₀ and an IDE-specific antibody (Ab 1) which binds within the catalytic domain of IDE. However, it should be noted that Ab 1 was in the form of antiserum which potentially could contain other inhibitory components as well as the specific IDE antibody. Conversely, another IDE-specific antibody (Ab 2) had no effect on the catalytic activity observed, probably as it binds to an epitope of IDE too distal from the catalytic site to block the activity. However it cannot be ruled out that because Ab 2 binds IDE within the C-terminal region it may also be binding to the inactive truncated isoform which is comprised of the C-terminal half of IDE. This may have bound a large amount of available antibody leading to insufficient antibody amounts left to neutralise active IDE. However, such an effect would depend on the concentration of IDE fragments and the concentration of IDE-specific antibody in the sample, neither of which were determined. Secondly, it was

demonstrated that the catalytic activity obtained from plasma after immunocapture with Ab 3 could be completely eliminated when the immunocaptured sample was pre-incubated with either of the known IDE substrates, A β ₄₂ or insulin. These results indicate strongly that the signal derived from the immunocapture of protein using Ab 3 is specifically due to IDE. However, it cannot be completely ruled out that part or all of the activity observed may be due to an unknown peptidase with similar properties to IDE. Despite this possibility, Miners, *et al.*, 2008(b) reported negligible cross-reactivity of the activity assay with other known A β -degrading enzymes such as neprilysin (NEP), angiotensin-converting enzyme (ACE) and endothelin-converting enzyme-1 (ECE-1), further supporting the theory that IDE cleavage of the peptide was responsible for the signal observed. Overall, these results strongly suggest that the antibody is specifically binding IDE in the activity assay, and as the ELISA works on similar immunocapture principles, it is likely that the ELISA is also specific, regardless of any potential cross-reactivity of the detection antibody. Conversely, this does not necessarily mean that Ab 3 is exclusively detecting IDE following the denaturing and reducing conditions of WB. In fact, the observation of many additional bands in this application may be a result of these denaturing and reducing conditions, exposing different epitopes which were then detected by the primary antibody. However, it cannot be ruled out that Ab 3 is detecting other fragments or forms of IDE.

6.3 Potential significance of plasma IDE in AD pathology

According to the “peripheral sink” hypothesis, the levels of brain A β may be controlled by peripheral clearance mechanisms (reviewed by Kurz & Perneczky, 2011), and several studies have shown that this is possible. For example, recent studies have demonstrated that peripherally-applied therapeutic compounds such as γ -secretase inhibitors which reduce plasma A β , can also deplete levels of brain A β (Sutcliffe, *et al.*, 2011; Sivilia, *et al.*, 2013). Immunotherapy studies (despite autoimmune reactions) and studies of other compounds such as an extract of *Withania somnifera* suggest strongly that peripheral clearance of A β will reduce

brain A β levels (Sehgal, *et al.*, 2012; Liu, *et al.*, 2012(b)). Similarly, reducing levels of peripheral A β by the upregulation of circulating proteases may also have some therapeutic benefits. In accordance with this concept, it has been demonstrated that the peripheral expression of the metalloproteinase neprilysin (NEP) in transgenic mice reduces the levels of blood A β , and subsequently reduces levels of soluble brain A β as well as oligomeric and insoluble A β (Liu, *et al.*, 2009; Liu, *et al.*, 2010; Guan, *et al.*, 2009). Importantly, other studies have shown that an increase in NEP in the brain is associated with a reduction in cognitive deficits (Poirier, *et al.*, 2006; Spencer, *et al.*, 2008). However, two recent studies have contradicted the hypothesis (Walker, *et al.*, 2013; Henderson, *et al.*, 2013). These studies showed that while NEP peripheral administration did in fact reduce the levels of soluble A β in the plasma, it had no effect on cerebral A β accumulation in both wild-type and AD mouse models, or in monkeys. However, these studies had a number of limitations including small sample sizes. In both studies the degraded fragments of A β in the periphery were not measured and it is possible that the presence of these fragments may have had an effect on the A β efflux and/or influx across the BBB. In the Walker study, they were also not able to measure circulating levels of A β_{42} , and this may be more relevant to AD pathology. Conversely, in the Henderson study, the soluble levels of A β_{40} in the primate brain were not measured. It is likely that both isoforms of A β need to be considered in conjunction, to conduct a more thorough investigation between reduction of plasma A β and its potential effects on brain A β . Furthermore, the transgenic mouse models used in the studies were different, making comparisons difficult, and some (or all) of these transgenic models may not be truly representative of the mechanisms of AD which occur in humans. For example, a recent study has shown that some commonly used transgenic mouse models do not display the classic reduction in IDE levels and corresponding reduction in A β degradation that is seen in very early AD pathology in humans (Stargardt, *et al.*, 2013). These transgenic mice have mutations in the *PS1* and/or *APP* genes which would be more relevant for EOFAD rather than LOAD. In addition, while NEP and IDE are considered to be two of the major A β -degrading enzymes, the two enzymes may function differently and perhaps have effects on AD pathology which are independent of their catalytic function. Although the

results from some of these studies might suggest that the efflux of brain A β may not be able to be stimulated simply by reducing peripheral A β via increasing or upregulating plasma metalloproteinases, there is evidence that the pools of peripheral and cerebral A β exist in some kind of equilibrium and that the regulation of plasma A β -degrading enzymes offers a promising treatment option for AD. Further work which addresses these issues would need to be undertaken in order to confirm or negate this hypothesis.

6.3.1 Findings of IDE levels/activity measurements by classification

The results from the current study are difficult to interpret. However, with all methods used in this study, alterations were detected in IDE levels and activity in the prodromal period of AD, represented by the MCI group. The Western blot (WB) results show increased levels of full-length IDE in the MCI group, while for total IDE levels as measured by ELISA the opposite was observed, with decreased levels of IDE in MCI and also in the AD group compared with controls. This discrepancy is likely to be related to the methods used for these measurements, but may also offer some further information in respect to this. While there were some limitations in these applications, it is possible that these methods are reflecting particular characteristics or mechanisms of IDE. It is probable that the denaturing and reducing conditions of the WB dissociated IDE which may have been bound to other proteins in the ELISA, and increased the overall detectable levels of IDE, whereas the ELISA may not have detected a portion of the total IDE due to the enzyme binding to other proteins. Alternatively, the ELISA may have detected both full-length IDE as well as forms of IDE which were not full-length, which may have been missed on the WB, or may have been present at a different molecular weight. Supporting this idea, it cannot be discounted that some of the other bands seen in the WB experiments might also be IDE, particularly the 80kDa band, which was shown to be reduced in the peptide blocking experiments, and also was retained following immunoprecipitation. These factors may at least partially account for the discrepancies seen between the WB and ELISA data.

Full-length IDE may not necessarily be catalytically active, as a portion of the measured IDE may represent the inactive 15b isoform, although the increase in protein levels in MCI individuals is reflected in the fluorometric activity assay, with a corresponding increase in activity in the MCI group compared with controls. However, it was observed that this trend of increased protein levels in MCI was seen only in females, while for the activity data only the males showed a trend toward increased activity in the MCI group. However, the loss of significance may be because of smaller numbers due to stratification. Thus, MCI levels of full-length IDE were higher than healthy control levels, yet interestingly, IDE levels in the AD group were similar to the healthy controls, and differences between MCI and AD almost reached significance. This may suggest a compensatory upregulation of full-length IDE as the disease progresses from HC to MCI which is then reduced during the progression from MCI to AD, possibly due to increasing severity of AD pathology. However, when looking at the total IDE levels and activity data, the AD group is not statistically different from the MCI group, indicating no significant changes in IDE expression or activity occur during the progression from MCI to AD. Changes in IDE levels and activity may occur at early stages in the disease process, which would have been missed here as some of the healthy controls who will eventually develop AD may have already experienced these undetected changes as part of very early AD pathogenesis. Longitudinal studies of larger healthy control cohorts may reveal earlier and greater changes in IDE expression and activity; therefore this enzyme warrants further investigation.

In this project, the increase in full-length IDE as well as catalytic activity that was detected in the MCI group compared with controls was somewhat unexpected, as it was hypothesised that a reduction of IDE levels or activity might be seen in the MCI and AD groups. However this may be a reflection of some compensatory mechanism in response to an earlier pathological event; since by the time MCI is apparent, AD pathogenesis has already started. Studies have reported an upregulation in IDE levels and/or activity in the liver in response to a diet high in sugar, salt and fat (Castell-Auvi, *et al.*, 2012) and as a response to a number of cellular stressors (Tundo, *et al.*, 2013), both of which are related to AD pathology.

This may be regulated by the induction of heat-shock proteins (HSP), particularly HSP70 which has been demonstrated to induce expression of IDE (Hoshino, *et al.*, 2011). More importantly, several studies in transgenic mice have reported an upregulation in IDE expression that may be a compensatory response to A β deposition in the brain (Leal, *et al.*, 2006; Vepsäläinen, *et al.*, 2008; Zhao, *et al.*, 2011). One of these studies found that 17 β -estradiol could upregulate IDE expression, correlating with an attenuation of A β deposition in the brains of ovariectomised AD-transgenic mice, also suggesting such a mechanism for the oestrogen-mediated preventative effect against AD, when hormone treatment is started at the onset of menopause (Zhao, *et al.*, 2011). While it would appear that the increase in IDE levels and/or activity in these studies may be in response to the formation of insoluble A β plaques rather than soluble A β , many other metabolic changes are likely to be occurring concurrently, and the changes that may be occurring in the plasma were not measured in these studies. In another brain study carried out by Miners and colleagues (2010), an increase in IDE activity was reported with age which was not due to an increase in expression. This study measured 4 enzymes involved in A β production and clearance in normal control brains (beta-secretase (BACE-1), NEP, IDE and angiotensin-converting enzyme (ACE)), and found that both A β -synthesising and -degrading enzyme activities increase with age, coinciding with declining soluble A β and increasing insoluble A β levels. Furthermore, IDE activity correlated directly with insoluble A β levels, NEP activity was inversely related to soluble A β levels, whereas ACE level correlated directly with insoluble and inversely with soluble A β , in controls but not AD (Miners, *et al.*, 2010). These enzymes may have quite different profiles in plasma, and all should be characterised further to understand their involvement in AD.

One factor to consider was the observation of the plasma IDE band being higher than the previously reported molecular weight of 110 kDa. Although it is possible that the difference in molecular weight in this instance is due to incorrect estimation because of the lack of resolution of the protein bands, it was shown that the band was consistently higher than the 110 kDa molecular weight marker. In addition, the single band observed for rrIDE was estimated to be approximately 118

kDa, which is consistent with the UniProtKB database estimation of 117.968 kDa, and the plasma band in question was consistently higher than that. Furthermore, Ab 3 detected a band at 110 kDa for rat liver which is consistent with published studies (Kurochkin, *et al.*, 1994; Authier, *et al.*, 1996(b)). Hence, it is perhaps more likely that the higher estimated molecular weight was due to some post-translational modification of IDE that may occur in the plasma. Sequence analysis of IDE shows multiple sites that could be subject to processes such as phosphorylation, acetylation, ubiquitination, and glycosylation, all of which would increase molecular weight to some extent. In addition, IDE has been shown to be modified by glutathionylation (Cordes, *et al.*, 2011), nitrosylation (Cordes, *et al.*, 2009) and alkylation (Shinall, *et al.*, 2005). Alternatively, there may be other isoforms of IDE in the plasma which have yet to be identified. Thus, there may be structural and functional differences associated with such potential modifications of IDE that may also be relevant to A β clearance or to other AD-related pathological processes, and as a future direction, these would be of interest to explore.

It is possible that total IDE levels (which potentially include truncated and inactive forms as measured by ELISA) are low in the early stages of AD pathology, and the full-length isoform is upregulated as a response to some event such as A β or insulin accumulation in the plasma. The low IDE levels detected by ELISA may also have more relevance in AD in respect to other reported functions of IDE that are independent of its catalytic function. These roles could include as a chaperone molecule to inhibit the fibrillisation of and toxicity of A β (de Tullio, *et al.*, 2013), to regulate the efflux of A β through the blood-brain barrier (Ito, *et al.*, 2014), or through regulation of IGF-1 signalling which may affect AD pathology via GSK-3 α -related effects on APP processing and GSK-3 β -related effects on tau (Sharma, *et al.*, 2008; Hong & Lee, 1997). Another non-catalytic IDE-mediated mechanism may involve the regulation of proteasomal degradation of AD-related proteins (Bennett, *et al.*, 2003). As the roles of IDE are diverse and probably relevant to many different aspects of AD pathology, it would be of value to investigate these potential functions further.

6.3.2 Impact of *APOE* $\epsilon 4$ genotype on IDE levels and activity

There were no significant differences in full-length IDE protein levels or activity levels when comparing *APOE* $\epsilon 4$ carriers with non-carriers in these studies. This was unexpected, although total IDE levels did tend to be lower in the $\epsilon 4$ carriers than the non-carriers (non-significantly). This result is consistent with a study by Miners, *et al.* (2009), that showed no overall effect of the $\epsilon 4$ genotype on IDE levels or activity in the frontal cortex. The only study of IDE levels in blood did not investigate the effects of $\epsilon 4$ genotype (Liu, *et al.*, 2012(a)). When stratified by $\epsilon 4$ status, there were still no significant differences in IDE levels (full-length or total) between classifications, although for total IDE only the $\epsilon 4$ carriers (but not non-carriers) showed a trend toward reduced IDE levels in MCI and AD compared with controls. This indicates that the overall results were primarily due to the differences in the proportions of $\epsilon 4$ carriers in the 3 groups, with the healthy control, MCI and AD groups consisting of approximately 33%, 47% and 68% $\epsilon 4$ carriers, respectively. After stratification of the IDE activity data by $\epsilon 4$ status, the differences seen between classifications overall were once again only reflected in the $\epsilon 4$ carriers, with a trend toward higher IDE in the MCI and AD groups. In fact, correlation analyses showed differences between $\epsilon 4$ carriers and non-carriers for both IDE levels (full-length and total) and enzyme activity. This indicates that the presence of the $\epsilon 4$ allele may indeed have some effect on the levels and activity of IDE. It is possible that these potential effects of the *APOE* $\epsilon 4$ allele on IDE are indirect, as several factors reported to affect IDE expression and activity have been observed to be affected by $\epsilon 4$ status; these include levels of glucocorticoids, plasma lipids, insulin levels and even the ApoE4 protein as well as the lipidation status of the ApoE protein (Peskind, *et al.*, 2001; Knouff, *et al.*, 1999; Dallongeville, *et al.*, 1992; Craft, *et al.*, 1999; Jiang, *et al.*, 2008; Du, *et al.*, 2009). Hence the potential effects of $\epsilon 4$ status on levels and activity of IDE may be mediated by any combination of these factors, and this list is not exhaustive. In the current study, no significant correlations were observed between IDE and plasma levels of the ApoE or ApoE4 protein. It has been previously reported that ApoE protein regulates the expression of IDE in brain (Cook, *et al.*, 2003; Du, *et al.*, 2009); however, this

association may not be reflected in the blood. In addition, it may not simply be the presence or absence of the $\epsilon 4$ allele that has an effect on IDE expression or activity, but perhaps there are differential effects resulting from specific *APOE* genotypes. It was not possible to stratify the subsets by genotype due to loss of power in our study, but it would be of value to investigate this further with a larger cohort.

6.3.3 Associations between IDE levels and activity with biochemical variables measured in the AIBL cohort

6.3.3.1 Testosterone

After Bonferroni-Holm correction, the results for full-length IDE levels in the overall AIBL subset correlated negatively with free testosterone levels, and trended toward a negative correlation with total testosterone levels. This appears counter-intuitive, as testosterone has been reported to increase IDE levels (César Vieira, *et al.*, 2011; Udrisar, *et al.*, 2005). The associations with testosterone were absent after stratifying the subset by gender, indicating that the association was not restricted to males or females. This is interesting considering the much higher levels of testosterone found in males. The association was again seen after stratification for *APOE* status, but only in non- $\epsilon 4$ carriers. However, after Bonferroni-Holm adjustment it was no longer significant for total testosterone, though was still significant for free testosterone. This may be due to the fact that free testosterone is a better measure of bioactive testosterone than total testosterone. Conversely, when looking only at total IDE levels, positive correlations (unadjusted) were seen between IDE levels and total as well as free testosterone, which after stratification appeared to be only in males and in $\epsilon 4$ carriers. It may be that females do not have a high enough level of testosterone to be able to regulate IDE levels in this way. Although not significant following correction for multiple testing, this would be consistent with previously published reports. No overall association was seen in IDE activity with testosterone (total or free), but a positive correlation was observed with total testosterone only in $\epsilon 4$ carriers, although again this was not significant

following correction. Changes in testosterone have been implicated in the pathogenesis of AD and differences related to *APOE* genotype have been reported (Verdile, *et al.*, 2012; Moffat, *et al.*, 2004; Hogervorst, *et al.*, 2004). Although the association observed between testosterone and IDE levels by WB was negative rather than positive which is a puzzling result, the results for the ELISA and activity data are consistent with the published literature and hence the association of IDE with testosterone in all three datasets is worth investigating further.

6.3.3.2 IDE levels and plasma A β

Numerous associations were observed between measurements of plasma A β and IDE levels/activity in the current study. Negative correlations were observed between plasma A β_{1-40} and IDE levels (full-length and total), but not with the activity data; for the full-length levels the association was only seen in non-*APOE* $\epsilon 4$ carriers, while with total IDE the association was seen in the overall subset and in non-*APOE* $\epsilon 4$ carriers (but not in $\epsilon 4$ carriers). When stratified by gender, the association with total IDE was only observed for males. Following correction for multiple testing, these associations were no longer significant although the correlation with total levels was close to significance. The observation that the associations with A β_{1-40} and IDE levels were seen mainly in non- $\epsilon 4$ carriers is interesting, as this was not seen with A β_{1-42} , and may indicate that regulation of the individual A β species by IDE is differentially affected by $\epsilon 4$ status; this raises the possibility that the presence of the $\epsilon 4$ allele disrupts the normal interaction between IDE and A β_{1-40} . In the AIBL study, A β_{1-40} was shown to increase in MCI and AD individuals compared to controls (Rembach, *et al.*, 2014) and this would be consistent with the reduction of total IDE in MCI and AD that is seen in the current study. Furthermore, the observation that the reduction of total IDE was primarily seen in the $\epsilon 4$ carriers supports the hypothesis that the presence of the $\epsilon 4$ allele may reduce the normal expression of total IDE, hence disturbing the balance of total IDE to A β_{1-40} . Indeed in the brain and *in vitro*, the presence of the $\epsilon 4$ allele has been reported to decrease the expression of IDE (Cook, *et al.*, 2003; Du, *et al.*, 2009). Additionally, in non- $\epsilon 4$ carriers, an inverse relationship was seen between levels of full-length IDE and brain A β load, although this lost significance after

correction, while in $\epsilon 4$ carriers a trend toward a positive correlation was seen between IDE activity and brain A β load. Although the levels of full-length IDE were seen to increase in MCI compared to controls, this may be part of a compensatory mechanism to try and control the rising levels of A β_{1-40} during early AD pathology by upregulating the active form of IDE. However, as a decrease was seen in the ELISA data, this may indicate that the levels of active (full-length) IDE contribute to only a small proportion of total IDE protein.

Negative correlations were also seen between A β_{1-42} and total IDE levels (not with full-length IDE or activity) and unlike A β_{1-40} , the association was not affected by $\epsilon 4$ status or gender. After correction for multiple testing these associations were still significant in the overall subset, but not after stratification for gender and $\epsilon 4$ status, probably due to reduced numbers. Further analysis after stratifying by clinical classification showed that the relationship between total IDE and A β_{1-42} was restricted to AD patients only and this correlation was stronger than in the overall subset. In accordance with these results, the relationship between total IDE and A β_{1-40} also was only seen in AD patients.

It is probably not surprising that no relationship was seen between total IDE and A β in the MCI group. Although the majority of MCI individuals will convert to AD at some stage, some will remain stable and others revert to healthy status. In addition, the progression and stage of pathology may differ between MCI participants, therefore the heterogeneity in the MCI group may confound the results.

Further investigation revealed that the correlation between total IDE and A β in AD patients was only seen in non- $\epsilon 4$ carriers. This supports the idea that the presence of the $\epsilon 4$ allele contributes to a dysregulation of the IDE/A β interaction, possibly by reducing the efficiency of IDE to degrade A β (Jiang, *et al.*, 2008). Alternatively, it may also be related to differences in insulin sensitivity between $\epsilon 4$ carriers and non-carriers (Kuusisto, *et al.*, 1997; Craft, *et al.*, 2003).

Regarding IDE activity, no correlations were seen with levels of A β_{1-40} or A β_{1-42} species, however inverse relationships between activity and A β ratios were

observed but only in $\epsilon 4$ carriers; following multiple testing correction these were no longer significant, although a trend remained for the $A\beta_{x-42/x-40}$ ratio. The significance of this is uncertain, although as higher IDE activity was seen in MCI and AD compared with controls, this would suggest that $A\beta$ ratios were lower with AD pathology, which is consistent with results from a meta-analysis of the published literature (reviewed by Koyama, *et al.*, 2012). The potential mechanisms behind this association are not clear, however as a trend still remained with the $A\beta_{x-42/x-40}$ ratio following correction, it would be interesting to explore this further, in a larger cohort.

As IDE was associated with levels of $A\beta$ in plasma, the levels (both full-length and total) and activity of plasma IDE relative to levels of $A\beta$ (42+40) were analysed as an IDE/ $A\beta_{42+40}$ ratio. As insulin is also a substrate for IDE, similar to $A\beta$, its plasma concentration relative to IDE concentration may be more capable of differentiating between clinical classifications than IDE alone. Therefore an IDE/insulin ratio as well as an IDE/insulin+ $A\beta_{42+40}$ ratio were calculated. For full-length IDE, the results showed no significant differences between classifications for all three ratios, however, the IDE/ $A\beta_{42+40}$ ratio almost reached significance, with MCI having a higher ratio than controls. When looking at total IDE on the other hand, the IDE/insulin ratio for the MCI group was significantly lower than for the control group, while for the IDE/ $A\beta_{42+40}$ and IDE/insulin+ $A\beta_{42+40}$ ratio, both MCI and AD groups were significantly lower than the controls. Therefore, there may be some value in using total levels of IDE as a ratio with insulin and $A\beta$ in a biomarker panel. Regarding IDE activity, the IDE activity/ $A\beta_{42+40}$ ratio was significantly higher in AD compared with controls, and MCI individuals showed a trend toward being significantly higher than controls after Bonferroni correction for multiple testing. In addition, the IDE activity/insulin+ $A\beta_{42+40}$ ratio was significantly higher in MCI than controls, while the AD group showed a trend toward being higher than controls following correction. While the consideration of insulin and $A\beta$ levels in conjunction with IDE activity may show differences between clinical classifications which may be useful as a diagnostic biomarker, similar to full-length IDE there was no additional benefit of including them in a ratio.

As IDE is an A β -degrading enzyme, these observations support the notion that IDE may have a role in regulating the plasma levels of A β , and in particular in AD patients who do not carry an $\epsilon 4$ allele. However as the correlations were only moderate/strong it indicates that other proteases are implicated. Plasma contains other known A β -degrading enzymes such as neprilysin (NEP) and angiotensin-converting enzyme (ACE) which would contribute to the clearance of A β . Nevertheless, these results do support the concept that IDE has a major role in the regulation of plasma A β .

6.3.3.3 Associations with gender.

IDE protein and activity levels were also seen to have relationships with other biochemical measures in the current study. For example, it was shown that females have a higher level of full-length IDE than males. This association was not affected by $\epsilon 4$ status, and was still significant after correction for multiple testing. Females are considered to be at greater risk of AD than males generally (Bachman, *et al.*, 1992), and it was shown in the current study that full-length IDE levels are higher in MCI, which may be indicative of upregulation of the active form of IDE due to pathological mechanisms such as hyperinsulinemia or increased plasma A β levels. As a greater proportion of women develop AD than men, it may be argued that the higher IDE levels in women measured here reflect the greater level of AD pathology that is developing in the MCI females of the cohort. However this gender difference was not seen with total (ELISA-measured) levels of IDE or activity.

There are several potential reasons for this gender difference, with differences in sex hormones being the most likely. Although the hormones oestrogen (estradiol), progesterone, testosterone, and luteinising hormone are best known for their roles in reproduction, many studies show these hormones have other roles, including neuroprotection. Changes in the levels of these hormones that occur in reproductive senescence are hypothesised to increase risk of AD, as a result of reduced protection against oxidative insults (Barron, *et al.*, 2006). Both oestrogen and testosterone levels were lower in the females of this subset compared to the males (progesterone was not measured), and both of these hormones have been

shown to influence A β production and levels (Amtul, *et al.*, 2010; Barron & Pike, 2012). Oestrogen has also been shown to influence lipid raft signalling complexes, which may modulate susceptibility to A β toxicity (Marin, *et al.*, 2011). Oestrogen effects on IDE expression and protein levels may play an important role in the oestrogen-mediated protection, as studies have shown that IDE expression is significantly increased by both oestrogen and progesterone in cultured neurons as well as *in vivo*, and is inversely associated with the soluble A β levels *in vivo* (Jayaraman, *et al.*, 2012). Others have shown in AD-model transgenic mice that 17 β -estradiol increases IDE expression in a brain region-specific manner; for example, in the hippocampus (but not the cerebellum) 17 β -estradiol increased IDE expression and attenuated A β accumulation/plaque formation. Furthermore, this IDE regulation was shown to be mediated by an ER β /PI3-K pathway (Zhao, *et al.*, 2011). It is interesting to note that in many AD-transgenic mouse models, the females show earlier and more prominent A β accumulation and deposition, as well as poorer behavioural performance, than the corresponding age-matched males (Vest & Pike, 2013), supporting the concept that the differences in AD risk seen in humans may be hormone-related, as diet and environmental factors are identical in the studies of male and female mice.

In our studies, estradiol was observed to correlate negatively with levels of full-length IDE (although only in non- ϵ 4 carriers), and to correlate positively with IDE activity levels, a finding which appeared to be primarily due to the females in the cohort. These associations were not significant after correction, and are contrary to the published reports which demonstrate that IDE expression is upregulated by estradiol. However, other studies investigated effects of oestrogen in the brain or *in vitro* (Jayaraman, *et al.*, 2012; Zhao, *et al.*, 2011). It is possible that estradiol may have a different effect on plasma IDE, and future studies of oestrogen effects on IDE and A β levels in animal models should expand to investigate A β levels and IDE enzyme protein and activity level measurements in both brain and plasma.

6.4 Limitations

Western immunoblotting is a commonly used method for the measurement of proteins, however it has a number of limitations. Firstly, the technique is considered to be semi-quantitative; meaning only relative differences between samples can be obtained without a standard curve; even then it is generally only quantitative over a very narrow range. In addition, the molecular weight can only be estimated rather than confirmed exactly. In the current study, the molecular weight of full-length IDE was higher than reported previously, and while this may be due to inaccurate estimation, it may also be indicative of post-translational modifications. As there were a large number of samples, many gels needed to be run, as only five samples could be measured in duplicate on any one gel. Therefore errors may have been introduced when normalising for gel-to-gel variation, although a single (pooled control plasma) sample was run on each gel to adjust for this. Furthermore, the membranes were stripped of the original primary antibody and re-probed with another antibody which may also have introduced variation. Even the method of quantification can produce variable results (Gassmann, *et al.*, 2009). Therefore Western immunoblotting is not ideal for measuring and comparing analytes in large cohorts, and it cannot be excluded that potential variations related to this method may have rendered the results less accurate.

A limitation of the sandwich ELISA assay is the poor spike and recovery results, with the signal being completely suppressed in plasma. This indicates significant matrix effects, which may be affecting the accuracy of the IDE measurements. However it has been shown that NEP, a similar peptidase to IDE, is unstable when added to plasma and quickly loses its catalytic activity, potentially by its rapid clearance (Liu, *et al.*, 2007). It is possible that the addition of recombinant IDE has the same effect; it may be degraded by endogenous proteases. This could be overcome by the use of a protease inhibitor cocktail, and/or by further dilution of the plasma sample; these possibilities require further investigation.

It should be noted that because some of the samples had values which fell above the standard curve range in the ELISA, and as cross-reactivity with the blocking

agent was possibly reducing sensitivity, only relative differences could be obtained and analysed. However, as the standard curve began to plateau at higher concentrations, it is possible that this occurs in plasma also, and hence the overall results could have been affected because of reduced sensitivity at higher concentrations. Ideally, a standard curve should only be used in the linear range, with plasma samples diluted to fall within this linear region, from which plasma concentrations of the protein in question are interpolated. It would be of value to refine this assay to ensure this, possibly by screening alternative antibodies or recombinant IDE.

Furthermore, cell-culture grade BSA was used to measure the AIBL samples, which was later found to cross-react with the ELISA assay; this was more than likely the reason for the high background. Ideally the samples should be re-assayed using Fraction V BSA. In addition, there may have been some small degree of cross-reactivity between the capture and detection antibody which may have also contributed to the background. Although the background reading was deducted from sample readings to account for this, both factors may have affected the sensitivity of the assay. Moreover, cross-reactivity tests in the work-up of the assay were performed using Fraction V BSA instead of cell-culture grade BSA, which was used for the AIBL samples. This is a limitation which requires further investigation; the control tests should be repeated using the cell-culture grade BSA.

Similar to the sandwich ELISA assay, the background readings in the IDE activity assay were rather high. Although it was confirmed that this was not due to cross-reactivity between reagents, and that most likely it was a product of the fluorogenic peptide alone, the high background may have affected the sensitivity of the assay, particularly at the lower limit of detection. In future studies it would be of interest to investigate ways to reduce the level of background noise seen, to improve assay sensitivity.

Enzyme catalytic activity appeared to be significantly influenced by the type of anticoagulant used (EDTA or LiHep) in the plasma collection tubes, and clearly this needs to be taken into consideration. It was shown that EDTA did not affect the

measurement of IDE protein levels. However, in the activity assay, EDTA-collected plasma samples produced a significantly higher signal than the very low signal observed in the Lithium-heparin (LiHep)-collected plasma. This was contrary to the expected result, despite the fact that there are some studies which have reported similar findings; IDE is a thiolmetalloendopeptidase that requires zinc for activity, thus EDTA would be expected to inhibit activity (depending on EDTA, enzyme and metal ion concentrations, as well as a host of other factors). It is possible that the increased activity in EDTA plasma may be related to haemolysis of erythrocytes; it has been shown that in ostrich plasma, EDTA increases the likelihood of erythrocyte haemolysis when compared with the LiHep anticoagulant (Mafuvadze & Erlwanger, 2007). As erythrocytes are a rich source of IDE, haemolysis may release IDE into the plasma, thus increasing the levels measured. However, great care was taken during plasma processing, and samples which had obvious haemolysis were not used. Despite this, there may have been some level of haemolysis in the EDTA samples, and the blood collection and processing methods would need to be investigated and possibly improved to minimise this problem in the future. Another possibility is that the stability of plasma proteins is affected differently by the two anti-clotting agents, as it has been shown that plasma proteins in EDTA are less likely to be subject to degradation than proteins in LiHep plasma (Evans, *et al.*, 2001; Käkönen, *et al.*, 2000). Therefore it may be that active IDE was degraded during storage at a faster rate in LiHep plasma compared with EDTA plasma. Until the reasons for the differences have been identified, caution should be exercised when interpreting the data. It would be of value to carry out further assay optimisation experiments, comparing serum with plasma samples, and comparing anticoagulants in the activity assay.

Another limitation of this study worth noting is that the samples used in each of the experimental methods were from separate subsets, and very few individual samples were run on all three assays. Therefore, it is difficult to compare the results from all three methods, as analysing only the common samples would lead to a loss of power to detect any differences due to small numbers. Ideally, it would be of value to complete the analysis of all samples in the AIBL cohort as was the

intention at the commencement of the study. However, due to the extended time-period required for optimisation of the assays, it was not possible to complete these within the time-frame of the project.

Another general limitation for all assays is that the AIBL samples should have been run at least twice to ensure accuracy of the results, however time and sample availability were factors. Regardless, the samples were run in duplicate, and duplicates with a coefficient of variation greater than 15% were removed from the analysis. In addition, assays were omitted from the analysis if any of the standard curve values were more than 2 standard deviations from the mean of each concentration.

A potentially confounding factor of this study is that it was not possible to analyse potential effects of medications on plasma IDE levels due to the size of the cohort, and the fact that only a small number of participants were not taking medication. The AIBL cohort is comprised of elderly participants, the majority of whom take at least one type of medication: at baseline for example, 79% of HC, 87% of MCI and 97% of AD participants were taking at least one medication (Ellis, *et al.*, 2009). A number of medications have been reported to increase levels of IDE; statins, for example, have recently been shown to increase levels of IDE in the plasma of mice via increased secretion (Tamboli, *et al.*, 2010). Similarly, another recent study has shown that propranolol (an antihypertensive drug) increases the blood (and hippocampal) levels of IDE in rats (Solas, *et al.*, 2009). Other medications such as oestrogen, progesterone, testosterone, *N*-methyl-D-aspartate receptor (NMDAR) antagonists and glucocorticoids have also all recently been reported to increase expression of IDE *in vitro* or *in vivo* (Zhao, *et al.*, 2010; Jayaraman, *et al.*, 2012; Vieira, *et al.*, 2011; Du, *et al.*, 2009). Therefore, as a substantial proportion of the AIBL cohort were taking at least one, or a combination of these medications at the time blood samples were taken, it is likely that these will have influenced the results of this project.

6.5 Future Directions

Greater assay characterisation is needed, as has been detailed in the sections above, before any conclusions may be made about the results. For example, additional cross-reactivity tests should be performed to identify potentially confounding plasma components. In the Western immunoblotting assay, it would be useful to increase the resolution to improve estimation of the molecular weight of the bands, perhaps by performing SDS-PAGE on a lower percentage gel and running the gels more slowly. It would also be of interest to characterise the 80 kDa band which was observed in plasma that appeared to be specific. It is possible that this band is indeed IDE and has some function in plasma that might be relevant to AD. Testing other IDE antibodies, for example using immunoprecipitation followed by Western blotting with a different antibody, may help determine whether the 80 kDa band is IDE and whether it is relevant when determining enzyme activity.

Further refinement of the IDE sandwich ELISA is also required to develop a quantitative assay, by ensuring firstly that the standard curve provides a reasonable linear range, and secondly that the plasma samples are diluted to fall within that range. It would be of interest to screen additional antibodies to determine if there are others more suitable (less cross-reactive), perhaps by the use of monoclonal antibodies rather than polyclonal. While there are few commercially-available monoclonal IDE antibodies currently, it may be of value to develop in-house antibodies for this purpose. In addition, investigation of the potential effects of interfering substances in blood such as heterophilic and anti-animal antibodies should be carried out to ensure they are not affecting the assay.

Similarly, although the results from the current study indicate that the activity assay is specifically detecting IDE, it requires further validation, perhaps by the use of other known IDE inhibitors such as bacitracin, phenanthroline and N-ethylmaleimide. It would also be of interest to try other non-IDE protease inhibitors to eliminate the possibility that other proteases are contributing to the signal observed. The screening of alternate IDE antibodies may also be of value to

compare the results obtained, particularly with monoclonal antibodies which are generally considered to be more specific.

As the IDE was immunocaptured prior to adding the fluorogenic substrate, and the remaining plasma washed away, it would not have been subject to regulatory elements which may alter the activity when in the plasma matrix. It is quite possible that the regulatory elements governing IDE activity may be more relevant to its function than expression levels. Alternatively, the assay may have caused IDE to bind to other plasma constituents that it would normally not bind to, which were captured as a complex, and this may have led to a loss of detectable catalytic activity due to interference with the catalytic site. If possible, an activity assay which could specifically measure IDE activity within the plasma, while in the presence of normal regulatory factors, would give a better representation of its true physiological activity.

The fluorogenic peptide substrate that was used in the activity assay was derived from a bradykinin sequence, and this may not necessarily reflect the contribution of IDE toward the cleavage of A β or insulin. It would be of interest to use a fluorescently-labelled peptide substrate derived from the sequence of A β to analyse IDE more accurately toward A β cleavage activity. In fact, an A β -derived fluorescent substrate has recently been generated by Stargardt and colleagues (2013) which would be more relevant in future A β -degrading protease activity studies.

The many bands observed by Western immunoblotting in plasma suggest that some post-translational modifications occur to IDE in human plasma. In particular, the finding in this study that the molecular weight of IDE was higher than expected supports this possibility. Therefore, it would be of interest to investigate potential modifications, and identify how they affect the catalytic or non-catalytic functions of IDE. For example, initial experiments could include cell culture IDE transfection experiments followed by treatment with N- and O-glycosylation inhibitors, then Western immunoblotting to investigate changes in IDE molecular weight. In addition, co-purification experiments might assist with determining which other

plasma proteins interact with IDE, and this could help not only with assay development, but also may uncover functions of IDE which may directly or indirectly impact on aspects of AD pathology. As a band of 160 kDa was retained in some of the immunoprecipitation experiments, it may indicate that IDE is found as part of a complex of proteins which are resistant to the denaturing and reducing conditions of Western immunoblotting. Specifically, it would be of value to identify and characterise any regulatory components in the blood which affect the ability of IDE to degrade A β . Such components may also alter in some way in the earliest stages of AD, and may be useful to identify, for the purposes of developing therapeutic treatments.

Given the heterogeneity of the MCI group, it would also be of use to investigate potential differences in IDE expression and activity in separate subsets of MCI, including amnesic versus non-amnesic and MCI individuals who convert to AD compared with those who remain stable, or revert back to healthy. Such longitudinal studies would reveal differences in those who are AD converters compared with those who are not, and would be of great benefit for the development of future diagnostic tests. The same argument may apply to the control group, as AD pathology may already have started in some members of this group.

Other factors may also affect the levels and activity of IDE, such as the presence or absence of hyperinsulinemia, the levels of plasma glucose, the interaction of IDE with the proteasome and effects of specific *APOE* genotypes. It is also not known if IDE is subject to circadian fluctuations, although in the current study the samples tested were all from overnight fasted participants. It would be of interest to investigate these factors in relation to circulating levels and activity of IDE.

It is not currently known how IDE is cleared from the peripheral circulation (or the brain for that matter), therefore alterations in levels or activity could be related to the balance between production and clearance, similar to A β . Therefore it would be interesting to explore this in a future study; it is probable that IDE could be

regulated through such clearance mechanisms rather than the upregulation of IDE expression, and this may have value in a therapeutic capacity.

Nutritional status and nutraceutical intake are likely to influence the regulation of IDE, particularly sugar and carbohydrate intake, and the presence of, and/or treatments for diabetes and insulin resistance. There are a number of dietary supplements and nutraceutical treatments which have recently been shown to offer some potential benefits in regulating IDE, such as docosahexaenoic acid (Du, *et al.*, 2010), withanolide A (Patil, *et al.*, 2010), Geranylgeranylacetone (Hoshino, *et al.*, 2013), asiaticoside (Lin, *et al.*, 2013), ginseng (Quan, *et al.*, 2013), Tong Luo Jiu Nao (Liu, *et al.*, 2011) and curcumin (Wang, *et al.*, 2014). A number of these have been investigated for their effects on AD pathology, for example, Withanolide A has been shown in primary neuronal cultures to upregulate IDE and downregulate BACE-1, one of the enzymes necessary for A β production from APP, and to upregulate ADAM10 which is necessary for non-amyloidogenic processing of APP (Patil, *et al.*, 2010). Even spirulina may have some benefits; there is no evidence as yet that it regulates IDE but it has been shown to improve insulin sensitivity more effectively than Pioglitazone and therefore may have indirect effects either by suppressing insulin secretion which would reduce the competition for IDE, or by increasing insulin signalling due to a reduction in insulin resistance (Ou, *et al.*, 2013). It may have further benefits in preventing or improving AD pathology through its antioxidant properties and also its positive effects on dyslipidemia and inflammation (Romay, *et al.*, 2003; Devesh, *et al.*, 2012; Fujimoto, *et al.*, 2012; reviewed by Deng & Chow, 2010).

6.6 Conclusions

Despite the potential limitations with the assays developed in this project, the main findings of the present study have demonstrated that plasma IDE levels and activity were significantly different in MCI compared with controls. This may be of value eventually as part of an AD biomarker panel, and is an interesting finding

considering the heterogeneity of the MCI group. The strength of these differences were improved by considering levels of plasma A β in conjunction with IDE (for total levels), which then demonstrated a similar significant finding in AD as seen in MCI. The significance of plasma A β in AD is still under investigation, however may prove to be a contributing factor in the initiation or progression of AD pathology. There was still some overlap between groups, and as such this may limit the usefulness of IDE as a clinically relevant biomarker. However, identifying the effects of potentially confounding variables may help address these issues. It appears from these results and others that the regulation and functions of IDE are particularly complex, and require ongoing work to unravel the potential significance these may have in the pathology of AD. Overall, however, based on the results from the current study it does appear that there are changes in both the levels and activity of plasma IDE in the early stages of AD, and while this pilot study suggests that IDE may have potential as an AD biomarker, further investigation is needed to confirm or negate the putative benefits of using IDE in a biomarker panel.

REFERENCES

- Alzheimer Research Forum - Diagnosis. (2009). Retrieved May 3, 2009, from <http://www.alzforum.org/dis/dia/default.asp>
- 2012 Alzheimer's disease facts and figures. (2012). *Alzheimer's & Dementia*, 8(2), 131-168.
- Abdul-Hay, S. O., Lane, A. L., Caulfield, T. R., Claussin, C., Bertrand, J., Masson, A., *et al.* (2013). Optimization of peptide hydroxamate inhibitors of insulin-degrading enzyme reveals marked substrate-selectivity. *Journal of medicinal chemistry*, 56(6), 2246-2255.
- Abdullah, L., Paris, D., Luis, C., Quadros, A., Parrish, J., Valdes, L., *et al.* (2007). The influence of diagnosis, intra- and inter-person variability on serum and plasma A β levels. *Neuroscience Letters*, 428(2-3), 53-58.
- Abraham, R., Myers, A., Wavrant-De Vriesé, F., Hamshere, M. L., Thomas, H. V., Marshall, H., *et al.* (2001). Substantial linkage disequilibrium across the insulin-degrading enzyme locus but no association with late-onset alzheimer's disease. *Human Genetics*, 109(6), 646-652.
- Affholter, J. A., Fried, V. A., & Roth, R. A. (1988). Human insulin-degrading enzyme shares structural and functional homologies with *E. coli* Protease III. *Science*, 242(4884), 1415-1418.
- Affholter, J. A., Hsieh, C. L., Francke, U., & Roth, R. A. (1990). Insulin-degrading enzyme: stable expression of the human complementary DNA, characterization of its protein product, and chromosomal mapping of the human and mouse genes. *Molecular Endocrinology*, 4(8), 1125-1135.
- Agnosta, F., Vessel, K. A., Miller, B. L., Migliaccio, R., Bonasera, S. J., Filippi, M., *et al.* (2009). Apolipoprotein E ϵ 4 is associated with disease-specific effects on brain atrophy in Alzheimer's disease and frontotemporal dementia. . *Proceedings of the National Academy of Sciences*, 106(6), 2018-2022.
- Aisen, P. S. (1997). Inflammation and Alzheimer's disease: Mechanisms and therapeutic strategies. *Gerontology*, 43(1-2), 143-149.

- Ait-Ghezala, G., Abdullah, L., Crescentini, R., Crawford, F., Town, T., Singh, S., *et al.* (2002). Confirmation of association between D10S583 and Alzheimer's disease in a case-control sample. *Neuroscience Letters*, 325(2), 87-90.
- Akiyama, H., Shii, K., Yokono, K., Yonezawa, K., Sato, S., Watanabe, K., *et al.* (1988). Cellular localization of insulin-degrading enzyme in rat liver using monoclonal antibodies specific for this enzyme. *Biochemical and Biophysical Research Communications*, 155(2), 914-922.
- Akomolafe, A., Beiser, A., Meigs, J. B., Au, R., Green, R. C., Farrer, L. A., *et al.* (2006). Diabetes mellitus and risk of developing Alzheimer disease. Results from the Framingham Study. *Archives of Neurology*, 63(11), 1551-1555.
- Ali, M., & Plas, C. (1989). Glucocorticoid regulation of chloroquine nonsensitive insulin degradation in cultured fetal rat hepatocytes. *The Journal of Biological Chemistry*, 264(35), 20992-20997.
- Allain, C. C., Poon, L. S., Chan, C. S., Richmond, W., & Fu, P. C. (1974). Enzymatic determination of total serum cholesterol. *Clinical chemistry*, 20(4), 470-475.
- Alper, B. J., & Schmidt, W. K. (2009). A capillary electrophoresis method for evaluation of A β proteolysis in vitro. *Journal of Neuroscience Methods*, 178(1), 40-45.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215(3), 403-410.
- Amata, O., Marino, T., Russo, N., & Toscano, M. (2009). Human insulin-degrading enzyme working mechanism. *Journal of the American Chemical Society*, 131(41), 14804-14811.
- American Psychiatric Association. (2000). *Diagnostic and Statistical Manual of Mental disorders, 4th Edition Text Revision (DSM-IV-TR)*. Washington DC.
- Amtul, Z., Wang, L., Westaway, D., & Rozmahel, R. (2010). Neuroprotective mechanism conferred by 17 β -estradiol on the biochemical basis of Alzheimer's disease. *Neuroscience*, 169(2), 781-786.
- Anastasio, T. J. (2013). Exploring the contribution of estrogen to amyloid-Beta regulation: a novel multifactorial computational modeling approach. *Frontiers in pharmacology*, 4.

- Ansorge, S., Bohley, P., Kirschke, H., Langner, J., & Wiederanders, B. (1983). The insulin and glucagon degrading proteinase of rat liver: a metal-dependent enzyme. *Biomedica biochimica acta*, 43(1), 39-46.
- Ardy, C., & Pontremoli, S. (1953). Blood insulinase activity in diabetics abnormally sensitive to insulin. *Archivio 'E. Maragliano' Di Patologia E Clinica*, 8(4), 821-830.
- Arrigada, P. V., Marzloff, K., & Hyman, B. T. (1992). Distribution of Alzheimer-type pathologic changes in nondemented elderly individuals matches the pattern in Alzheimer's disease. *Neurology*, 42(9), 1681-1688.
- Arvanitakis, Z., Wilson, R. S., Bienias, J. L., Evans, D. A., & Bennett, D. A. (2004). Diabetes mellitus and risk of Alzheimer disease and decline in cognitive function. *Archives of Neurology*, 61(5), 661-666.
- Association, A. s. (2012). 2012 Alzheimer's disease facts and figures. *Alzheimer's & dementia: the journal of the Alzheimer's Association*, 8(2), 131.
- Authier, F., Cameron, P. H., & Taupin, V. (1996(b)). Association of insulin-degrading enzyme with a 70 kDa cytosolic protein in hepatoma cells. *Biochemical Journal*, 319(Pt 1), 149-158.
- Authier, F., Posner, B. I., & Bergeron, J. J. M. (1996(a)). Insulin-degrading enzyme. *Clinical and Investigative Medicine*, 19(3), 149-160.
- Bachman, D., Wolf, P. A., Linn, R., Knoefel, J., CobbS, J., Belanger, A., *et al.* (1992). Prevalence of dementia and probable senile dementia of the Alzheimer type in the Framingham Study. *Neurology*, 42(1), 115-115.
- Bales, K. R., Liu, F., Wu, S., Lin, S., Koger, D., DeLong, C., *et al.* (2009). Human APOE isoform-dependent effects on brain β -amyloid levels in PDAPP transgenic mice. *The Journal of Neuroscience*, 29(21), 6771-6779.
- Banks, W. A., Jaspan, J. B., & Kastin, A. J. (1997). Selective, physiological transport of insulin across the blood-brain barrier: novel demonstration by species-specific radioimmunoassays. *Peptides*, 18(8), 1257-1262.

- Barger, S. W., DeWall, K. M., Liu, L., Mrak, R. E., & Griffin, W. S. T. (2008). Relationships between expression of apolipoprotein E and β -amyloid precursor protein are altered in proximity to Alzheimer β -amyloid plaques: Potential explanations from cell culture studies. *Journal of Neuropathology and Experimental Neurology*, 67(8), 773-783.
- Barron, A. M., Fuller, S. J., Verdile, G., & Martins, R. N. (2006). Reproductive hormones modulate oxidative stress in Alzheimer's disease. *Antioxidants & redox signaling*, 8(11-12), 2047-2059.
- Barron, A. M., & Pike, C. J. (2012). Sex hormones, aging, and Alzheimer's disease. *Frontiers in bioscience (Elite edition)*, 4, 976.
- Barter, P. J., Brandrup-Wognsen, G., Palmer, M. K., & Nicholls, S. J. (2010). Effect of statins on HDL-C: a complex process unrelated to changes in LDL-C: analysis of the VOYAGER Database. *Journal of lipid research*, 51(6), 1546-1553.
- Bates, K. A., Sohrabi, H. R., Rodrigues, M., Beilby, J., Dhaliwal, S. S., Taddei, K., *et al.* (2009). Association of Cardiovascular Factors and Alzheimer's Disease Plasma Amyloid- β Protein in Subjective Memory Complainers. *Journal of Alzheimer's Disease*, 17(2), 305-318.
- Bauer, J., Strauss, S., Schreitergasser, U., Ganter, U., Schlegel, P., Witt, I., *et al.* (1991). Interleukin-6 and alpha-2-macroglobulin indicate an acute-phase state in Alzheimer's-disease cortices. *FEBS Letters*, 285(1), 111-114.
- Baura, G. D., Foster, D. M., Kaiyala, K., Porte (Jnr), D., Kahn, S. E., & Schwartz, M. W. (1996). Insulin transport from plasma into the central nervous system is inhibited by dexamethasone in dogs. *Diabetes*, 45(1), 86-90.
- Behl, M., Zhang, Y., & Zheng, W. (2009). Involvement of insulin-degrading enzyme in the clearance of beta-amyloid at the blood-CSF barrier: Consequences of lead exposure. *Cerebrospinal Fluid Res*, 6(11).
- Bell, R. D., Sagare, A. P., Friedman, A. E., Bedi, G. S., Holtzman, D. M., Deane, R., *et al.* (2007). Transport pathways for clearance of human Alzheimer's amyloid β -peptide and apolipoproteins E and J in the mouse central nervous system. *Journal of Cerebral Blood Flow and Metabolism*, 27(5), 909-918.

- Bennett, R., Fawcett, J., Kruer, M., Duckworth, W., & Hamel, F. (2003). Insulin inhibition of the proteasome is dependent on degradation of insulin by insulin-degrading enzyme. *Journal of endocrinology*, 177(3), 399-405.
- Bennett, R. G., Duckworth, W. C., & Hamel, F. G. (2000(a)). Degradation of amylin by insulin-degrading enzyme. *The Journal of Biological Chemistry*, 275(47), 36621-36625.
- Bennett, R. G., Hamel, F. G., & Duckworth, W. C. (2000(b)). Insulin inhibits the ubiquitin-dependent degrading activity of the 26S proteasome. *Endocrinology*, 141(7), 2508-2517.
- Bentley, N. M., LaDu, M. J., Rajan, C., Getz, G. S., & Reardon, C. A. (2002). Apolipoprotein E structural requirements for the formation of SDS-stable complexes with β -amyloid-(1–40): the role of salt bridges. *Biochemical Journal*, 366, 273-279.
- Bergem, A. L., Engedal, K., & Kringlen, E. (1997). The role of heredity in late-onset alzheimer disease and vascular dementia. A twin study. *Archives of General Psychiatry*, 54(3), 264-270.
- Bernstein, H. G., Ansorge, S., Riederer, P., Reiser, M., Frolich, L., & Bogerts, B. (1999). Insulin-degrading enzyme in the Alzheimer's disease brain: prominent localization in neurons and senile plaques. . *Neuroscience Letters*, 263(2-3), 161-164.
- Bernstein, H.-G., Stricker, R., Lendeckel, U., Bertram, I., Dobrowolny, H., Steiner, J., et al. (2009). Reduced neuronal co-localisation of nardilysin and the putative α -secretases ADAM10 and ADAM17 in Alzheimer's disease and Down syndrome brains. *Age*, 31(1), 11-25.
- Bertram, L., Blacker, D., Mullin, K., Keeney, D., Jones, J., Basu, S., et al. (2000). Evidence for genetic linkage of Alzheimer's disease to chromosome 10q. *Science*, 290(5500), 2302-2303.
- Bhamra, M. S., & Ashton, N. J. (2012). Finding a pathological diagnosis for Alzheimer's disease: Are inflammatory molecules the answer? *Electrophoresis*, 33(24), 3598-3607.

- Bian, L., Yang, J. D., Guo, T. W., Sun, Y., Dian, S. W., Chen, W. Y., *et al.* (2004). Insulin-degrading enzyme and Alzheimer disease: a genetic association study in the Han Chinese. *Neurology*, 63(2), 241-245.
- Björk, B. F., Katzov, H., Kehoe, P., Fratiglioni, L., Winblad, B., Prince, J. A., *et al.* (2007). Positive association between risk for late-onset *alz*heimer disease and genetic variation in *IDE*. *Neurobiology of Aging*, 28(9), 1374-1380.
- Blacker, D., Bertram, L., Saunders, A. J., Moscarillo, T. J., Albert, M. S., Wiener, H., *et al.* (2003). Results of a high-resolution genome screen of 437 Alzheimer's disease families. *Human Molecular Genetics*, 12(1), 23-32.
- Blennow, K. (2004). Cerebrospinal fluid protein biomarkers for Alzheimer's disease. *NeuroRx: The Journal of the American Society for Experimental Neurotherapeutics*, 1(2), 213-225.
- Blennow, K., De Meyer, G., Hansson, O., Minthon, L., Wallin, A., Zetterberg, H., *et al.* (2009). Evolution of A β 42 and A β 40 levels and A β 42/A β 40 ratio in plasma during progression of Alzheimer's disease: A multicenter assessment. *The Journal of Nutrition, Health and Aging*, 13(3), 205-208.
- Blomqvist, M. E. L., Chalmers, K., Andreasen, N., Bogdanovic, N., Wilcock, G. K., Cairns, N. J., *et al.* (2005). Sequence variants of *IDE* are associated with the extent of β -amyloid deposition in the Alzheimer's disease brain. *Neurobiology of Aging*, 26(6), 795-802.
- Blomqvist, M. E. L., Silburn, P. A., Buchanan, D. D., Andreasen, N., Blennow, K., Pedersen, N. L., *et al.* (2004). Sequence variation in the proximity of *IDE* may impact age at onset of both Parkinson disease and Alzheimer disease. *Neurogenetics*, 5(2), 115-119.
- Borroni, B., Di Luca, M., & Padovani, A. (2006). Predicting Alzheimer dementia in mild cognitive impairment patients. Are biomarkers useful? *European Journal of Pharmacology*, 545(1), 73-80.
- Bouras, C., Hof, P. R., Giannakopoulos, P., Michel, J. P., & Morrison, J. H. (1994). Regional distribution of neurofibrillary tangles and senile plaques in the cerebral cortex of elderly patients: a quantitative evaluation of a one-year autopsy population from a geriatric hospital. *Cerebral Cortex*, 4(2), 138-150.

- Boussaha, M., Hannequin, D., Verpillat, P., Brice, A., Frebourg, T., & Campion, D. (2002). Polymorphisms of insulin degrading enzyme gene are not associated with Alzheimer's disease. *Neuroscience Letters*, 329(1), 121-123.
- Brookmeyer, R., Gray, S., & Kawas, C. (1998). Projections of Alzheimer's disease in the United States and the public health impact of delaying disease onset. *American Journal of Public Health*, 88(9), 1337-1342.
- Brown, B., Peiffer, J., Sohrabi, H., Mondal, A., Gupta, V., Rainey-Smith, S., *et al.* (2012(b)). Intense physical activity is associated with cognitive performance in the elderly. *Translational psychiatry*, 2(11), e191.
- Brown, B., Peiffer, J., Taddei, K., Lui, J., Laws, S., Gupta, V., *et al.* (2012(a)). Physical activity and amyloid- β plasma and brain levels: results from the Australian Imaging, Biomarkers and Lifestyle Study of Ageing. *Molecular Psychiatry*.
- Brown, M. C., Joaquim, T. R., Chambers, R., Onisk, D. V., Yin, F., Moriango, J. M., *et al.* (2011). Impact of immunization technology and assay application on antibody performance—a systematic comparative evaluation. *PLoS ONE*, 6(12), e28718.
- Bulloj, A., Leal, M. C., Surace, E. I., Zhang, X., Xu, H., Ledesma, M. D., *et al.* (2008). Detergent resistant membrane-associated IDE in brain tissue and cultured cells: Relevance to A β and insulin degradation. *Molecular Neurodegeneration*, 3(1), 22-34.
- Burdick, D., Soreghan, B., Kwon, M., Kosmoski, J., Knauer, M., Henschen, A., *et al.* (1991). Assembly and aggregation properties of synthetic Alzheimer's A4/ β amyloid peptide analogs. *The Journal of Biological Chemistry*, 267(1), 546-554.
- Burghen, G. A., Kitabchi, A. E., & Brush, J. S. (1972). Characterization of a rat liver protease with specificity for insulin. *Endocrinology*, 91(3), 633-642.
- Cabrejo, L., Guyant-Maréchal, L., Laquerrière, A., Vercelletto, M., De La Fournière, F., Thomas-Antérion, C., *et al.* (2006). Phenotype associated with APP duplication in five families. *Brain*, 129(11), 2966-2976.
- Cabrol, C., Huzarska, M. A., Dinolfo, C., Rodrigues, M. C., Reinstatler, L., Ni, J., *et al.* (2009). Small-molecule activators of insulin-degrading enzyme discovered through high-throughput compound screening. *PLoS ONE*, 4(4), e5274.

- Caccamo, A., Oddo, S., Sugarman, M. C., Akbari, Y., & LaFerla, F. M. (2005). Age- and region-dependent alterations in A β -degrading enzymes: implications for A β -induced disorders. *Neurobiology of Aging*, 26(5), 645-654.
- Cagnin, A., Gerhard, A., & Banati, R. B. (2002). In vivo imaging of neuroinflammation. *European Neuropsychopharmacology: The Journal of the European College of Neuropsychopharmacology*, 12(6), 581-586.
- Camberos, M. C., & Cresto, J. C. (2007). Insulin-degrading enzyme hydrolyzes ATP. *Experimental Biology and Medicine*, 232(2), 281-292.
- Camberos, M. C., Perez, A. A., Udrișar, D. P., Wanderley, M. I., & Cresto, J. C. (2001). ATP inhibits insulin-degrading enzyme activity. *Experimental Biology and Medicine*, 226(4), 334-341.
- Cappellano, G., Carecchio, M., Fleetwood, T., Magistrelli, L., Cantello, R., Dianzani, U., *et al.* (2013). Immunity and inflammation in neurodegenerative diseases. *American journal of neurodegenerative disease*, 2(2), 89.
- Carantoni, M., Zuliani, G., Munari, M. R., D'Elia, K., Palmieri, E., & Fellin, R. (2000). Alzheimer disease and vascular dementia: Relationships with fasting glucose and insulin levels. *Dementia and Geriatric Cognitive Disorders*, 11(3), 176-180.
- Cardon, L. R., Smith, S. D., Fulker, D. W., Kimberling, W. J., Pennington, B. F., & DeFries, J. C. (1994). Quantitative trait locus for reading disability on chromosome 6. *Science*, 266(5183), 276-279.
- Carpenter, J., Jackson, W., De Souza, G., Haarr, L., & Grose, C. (2010). Insulin-Degrading Enzyme Binds to the Nonglycosylated Precursor of Varicella-Zoster Virus gE Protein Found in the Endoplasmic Reticulum. *Journal of virology*, 84(2), 847-855.
- Carrasquillo, M. M., Belbin, O., Zou, F., Allen, M., Ertekin-Taner, N., Ansari, M., *et al.* (2010). Concordant Association of Insulin Degrading Enzyme Gene (IDE) Variants with IDE mRNA, A β , and Alzheimer's Disease. *PLoS ONE*, 5(1), e8764.

- Caspersen, C., Wang, N., Yao, J., Sosunov, A., Chen, X., Lustbader, J. W., *et al.* (2005). Mitochondrial Abeta: a potential focal point for neuronal metabolic dysfunction in Alzheimer's disease. *The FASEB journal: official publication of the Federation of American Societies for Experimental Biology*, 19(14), 2040.
- Castellano, J. M., Kim, J., Stewart, F. R., Jiang, H., DeMattos, R. B., Patterson, B. W., *et al.* (2011). Human apoE isoforms differentially regulate brain amyloid- β peptide clearance. *Science translational medicine*, 3(89), 89ra57.
- Castell-Auvi, A., Cedó, L., Pallares, V., Blay, M., Ardévol, A., & Pinent, M. (2012). The effects of a cafeteria diet on insulin production and clearance in rats. *The British journal of nutrition*, 108(7), 1155-1162.
- Cavalcante Braga, A. R., Manera, A. P., da Costa Ores, J., Sala, L., Maugeri, F., & Juliano Kalil, S. (2013). Kinetics and Thermal Properties of Crude and Purified β -Galactosidase with Potential for the Production of Galactooligosaccharides. *Food Technology and Biotechnology*, 51(1), 45-52.
- Cedazo-Mínguez, A. (2007). Apolipoprotein E and Alzheimer's disease: molecular mechanisms and therapeutic opportunities. *Journal of Cellular and Molecular Medicine*, 11(6), 1227-1238.
- Cedazo-Mínguez, A., Huttinger, M., & Cowburn, R. F. (2001). Beta-VLDL protects against A beta(1-42) and apoE toxicity in human SH-SY5Y neuroblastoma cells. *Neuroreport*, 12(2), 201-206.
- Cellini, E., Bagnoli, S., Tedde, A., Nacmias, B., Piacentini, S., & Sorbi, S. (2005). Insulin degrading enzyme and alpha-3 catenin polymorphisms in Italian patients with Alzheimer disease. *Alzheimer Disease and Associated Disorders*, 19(4), 246-247.
- César Vieira, J. S., Saraiva, K. L., Barbosa, M. C., Porto, R. C., Cresto, J. C., Peixoto, C. A., *et al.* (2011). Effect of dexamethasone and testosterone treatment on the regulation of insulin-degrading enzyme and cellular changes in ventral rat prostate after castration. *International Journal of Experimental Pathology*, 92(4), 272-280.
- Chander, H., Chauhan, A., & Chauhan, V. (2007). Binding of proteases to fibrillar amyloid-beta protein and its inhibition by Congo red. *Journal of Alzheimer's Disease*, 12(3), 261-269.

- Chang, C. L. (2008). Obesity and Alzheimer's disease. *Hong Kong Journal of Psychiatry*, 18(1), 28-35.
- Chen, J. X., & Yan, S. S. (2010). Role of mitochondrial amyloid- β in Alzheimer's disease. *Journal of Alzheimer's Disease*, 20, 569-578.
- Chen, K., Reiman, E. M., Alexander, G. E., Caselli, R. J., Gerkin, R., Bandy, D., *et al.* (2007). Correlations between apolipoprotein E ϵ 4 gene dose and whole brain atrophy rates. *The American Journal of Psychiatry*, 164(6), 916-921.
- Chen, M., Tegg, M., Avdesh, A., Mondal, A., Lardelli, M., Verdile, G., *et al.* (2012). Analysis of insulin-degrading enzyme (IDE) expression and function in zebrafish embryos. *Alzheimer's & Dementia: The Journal of the Alzheimer's Association*, 8(4), P674-P674.
- Chesneau, V., Pierotti, A. R., Barré, N., Creminon, C., Tougard, C., & Cohen, P. (1994). Isolation and characterization of a dibasic selective metalloendopeptidase from rat testes that cleaves at the amino terminus of arginine residues. *Journal of Biological Chemistry*, 269(3), 2056-2061.
- Chesneau, V., Vekrellis, K., Rosner, M. R., & Selkoe, D. J. (2000). Purified recombinant insulin-degrading enzyme degrades amyloid β -protein but does not promote its oligomerization. *Biochemical Journal*, 351(Pt 2), 509-516.
- Cholerton, B., Baker, L. D., Trittschuh, E. H., Crane, P. K., Larson, E. B., Arbuckle, M., *et al.* (2012). Insulin and sex interactions in older adults with mild cognitive impairment. *Journal of Alzheimer's Disease*, 31(2), 401-410.
- Chou, Y.-H., Kuo, W.-L., Rosner, M. R., Tang, W.-J., & Goldman, R. D. (2009). Structural changes in intermediate filament networks alter the activity of insulin-degrading enzyme. *The FASEB Journal*, 23(11), 3734-3742.
- Chow, K. M., Oakley, O., Goodman, J., Ma, Z., Juliano, M. A., Juliano, L., *et al.* (2003). Nardilysin cleaves peptides at monobasic sites. *Biochemistry*, 42(7), 2239-2244.
- Ciaccio, C., Tundo, G. R., Grasso, G., Spoto, G., Marasco, D., Ruvo, M., *et al.* (2009). Somatostatin: a novel substrate and a modulator of insulin-degrading enzyme activity. *Journal of Molecular Biology*, 385(5), 1556-1567.

- Clement, K., Garner, C., Hager, J., Philippi, A., LeDuc, C., Carey, A., *et al.* (1996). Indication for linkage of the human OB gene region with extreme obesity. *Diabetes*, 45(5), 687-690.
- Clifford, P. M., Zarrabi, S., Siu, G., Kinsler, K. J., Kosciuk, M. C., Venkataraman, V., *et al.* (2007). Abeta peptides can enter the brain through a defective blood-brain barrier and bind selectively to neurons. . *Brain Research*, 1142, 223-236.
- Cohen, J. (1988). *Statistical power analysis for the behavioural sciences*. (2nd ed.). Hillsdale, New Jersey: Lawrence Erlbaum Associates.
- Cole, G. M., & Ard, M. D. (2000). Influence of lipoproteins on microglial degradation of Alzheimer's amyloid beta-protein. *Microscopy Research and Technique*, 50(4), 316-324.
- Cole, G. M., & Frautschy, S. A. (2006). Docosahexaenoic acid protects from amyloid and dendritic pathology in an Alzheimer's disease mouse model. *Nutrition and health*, 18(3), 249-259.
- Constantini, C., Scrable, H., & Puglielli, L. (2006). An aging pathway controls the TrkA to p75NTR receptor switch and amyloid β -peptide generation. *The EMBO Journal*, 25(9), 1997-2006.
- Cook, D. G., Leverenz, J. B., McMillan, P. J., Kulstad, J. J., Ericksen, S., Roth, R. A., *et al.* (2003). Reduced hippocampal insulin-degrading enzyme in late-onset alzheimer's disease is associated with the apolipoprotein E- ϵ 4 allele. *American Journal of Pathology*, 162(1), 313-319.
- Cooper, G. J. S., Willis, A. C., Clark, A., Turner, R. C., Sim, R. B., & Reid, K. B. M. (1987). Purification and characterization of a peptide from amyloid-rich pancreases of type 2 diabetic patients. *Proceedings of the National Academy of Sciences*, 84(23), 8628-8632.
- Corder, E. H., Saunders, A. M., Strittmatter, W. J., Schmechel, D. J., Gaskell, P. C., Small, G. W., *et al.* (1993). Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science*, 261(5123), 921-923.

- Cordes, C. M., Bennett, R. G., Siford, G. L., & Hamel, F. G. (2009). Nitric oxide inhibits insulin-degrading enzyme activity and function through S-nitrosylation. *Biochemical Pharmacology*, 77(6), 1064-1073.
- Cordes, C. M., Bennett, R. G., Siford, G. L., & Hamel, F. G. (2011). Redox regulation of insulin degradation by insulin-degrading enzyme. *PloS one*, 6(3), e18138.
- Craft, S. (2005). Insulin resistance syndrome and Alzheimer's disease: Age- and obesity-related effects on memory, amyloid, and inflammation. *Neurobiology of Aging*, 26S, S65-S69.
- Craft, S. (2006). Insulin resistance syndrome and Alzheimer disease: Pathophysiologic mechanisms and therapeutic implications. *Alzheimer Disease and Associated Disorders*, 20(4), 298-301.
- Craft, S., Asthana, S., Cook, D. G., Baker, L. D., Cherrier, M., Purganan, K., et al. (2003). Insulin dose-response effects on memory and plasma amyloid precursor protein in Alzheimer's disease: interactions with apolipoprotein E genotype. *Psychoneuroendocrinology*, 28(6), 809-822.
- Craft, S., Asthana, S., Schellenberg, G., Baker, L., Cherrier, M., Boyt, A. A., et al. (2000). Insulin Effects on Glucose Metabolism, Memory, and Plasma Amyloid Precursor Protein in Alzheimer's Disease Differ According to Apolipoprotein-E Genotype. *Annals of the New York Academy of Sciences*, 903(1), 222-228.
- Craft, S., Asthana, S., Schellenberg, G., Cherrier, M., Baker, L. D., Newcomer, J., et al. (1999). Insulin metabolism in Alzheimer's disease differs according to apolipoprotein E genotype and gender. *Neuroendocrinology*, 70(2), 146-152.
- Craft, S., Baker, L. D., Montine, T. J., Minoshima, S., Watson, G., Claxton, A., et al. (2012). Intranasal insulin therapy for Alzheimer disease and amnesic mild cognitive impairment: a pilot clinical trial. *Archives of Neurology*, 69(1), 29.
- Craft, S., Peskind, E., Schwartz, M. W., Schellenberg, G. D., Raskind, M., & Porte, D. (1998). Cerebrospinal fluid and plasma insulin levels in Alzheimer's disease - Relationship to severity of dementia and apolipoprotein E genotype. *Neurology*, 50(1), 164-168.

- Craft, S., & Watson, G. S. (2004). Insulin and neurodegenerative disease: shared and specific mechanisms. *Insulin and Neurodegenerative disease*, 3(3), 169-178.
- Cummings, J. L. (2001). Treatment of Alzheimer's disease. *Clinical Cornerstone*, 3(4), 27-39.
- Cummings, J. L., & Cole, G. (2002). Alzheimer Disease. *Journal of the American Medical Association*, 287(18), 2335-2338.
- Dahlgren, K. N., Manelli, A. M., Stine, W. B., Baker, L. K., Krafft, G. A., & LaDu, M. J. (2002). Oligomeric and fibrillar species of amyloid- β peptides differentially affect neuronal viability. *The Journal of Biological Chemistry*, 277(35), 32046-32053.
- Dallongeville, J., Lussier-Cacan, S., & Davignon, J. (1992). Modulation of plasma triglyceride levels by apoE phenotype: a meta-analysis. *Journal of lipid research*, 33(4), 447-454.
- Davidson, M. B. (1979). The effect of aging on carbohydrate metabolism: a review of the English literature and a practical approach to the diagnosis of diabetes mellitus in the elderly. *Metabolism: Clinical and Experimental*, 28(6), 688-705.
- Davies, L., Wolska, B., Hilbich, C., Multhaup, G., Martins, R., Simms, G., *et al.* (1988). A4 amyloid protein deposition and the diagnosis of Alzheimer's disease: prevalence in aged brains determined by immunocytochemistry compared with conventional neuropathologic techniques. *Neurology*, 38(11), 1688-1693.
- Davis, J., & Van Nostrand, W. E. (1996). Enhanced pathologic properties of Dutch-type mutant amyloid β -protein. *Proceedings of the National Academy of Sciences*, 93(2), 2996-3000.
- Davis, K. L. (1986). Cortisol and Alzheimer's disease: I. Basal studies. *American Journal of Psychiatry*, 143(3), 300-305.
- Daw, E. W., Payami, H., Nemens, E. J., Nochlin, D., Bird, T. D., Schellenberg, G. D., *et al.* (2000). The number of trait loci in late-onset *alzheimer* disease. *American Journal of Human Genetics*, 66(1), 196-204.

- de Jager, W., Bourcier, K., Rijkers, G. T., Prakken, B. J., & Seyfert-Margolis, V. (2009). Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. *BMC immunology*, 10(1), 52.
- De Santi, S., de Leon, M. J., Rusinek, H., Convit, A., Tarshish, C. Y., Roche, A., *et al.* (2001). Hippocampal formation glucose metabolism and volume losses in MCI and AD. *Neurobiology of Aging*, 22(4), 529-539.
- De Santi, S., de Leon, M. J., Rusinek, H., Convit, A., Tarshish, C. Y., Roche, A., *et al.* (2001). Hippocampal formation glucose metabolism and volume losses in MCI and AD. *Neurobiology of Aging*, 22(4), 529-539.
- de Tullio, M. B., Castelletto, V., Hamley, I. W., Adami, P. V. M., Morelli, L., & Castaño, E. M. (2013). Proteolytically Inactive Insulin-Degrading Enzyme Inhibits Amyloid Formation Yielding Non-Neurotoxic A β Peptide Aggregates. *PLoS ONE*, 8(4), e59113.
- Deane, R., Bell, R., Sagare, A., & Zlokovic, B. (2009). Clearance of amyloid- β peptide across the blood-brain barrier: implication for therapies in Alzheimer's disease. *CNS & neurological disorders drug targets*, 8(1), 16.
- Deane, R., Yan, S. D., Subramanyam, R. K., LaRue, B., Jovanovic, S., Hogg, E., *et al.* (2003). RAGE mediates amyloid- β peptide transport across the blood-brain barrier and accumulation in brain. *Nature Medicine*, 9(7), 907-914.
- DeMattos, R. B., Bales, K. R., Cummins, D. J., Paul, S. M., & Holtzman, D. M. (2002). Brain to plasma amyloid- β efflux: a measure of brain amyloid burden in a mouse model of Alzheimer's disease. *Science*, 295(5563), 2264-2267.
- Deng, R., & Chow, T. J. (2010). Hypolipidemic, antioxidant, and antiinflammatory activities of microalgae *Spirulina*. *Cardiovascular therapeutics*, 28(4), e33-e45.
- Desmarais, W. T., Bienvenue, D. L., Bzymek, K. P., Holz, R. C., Petsko, G. A., & Ringe, D. (2002). The 1.20 Å Resolution Crystal Structure of the Aminopeptidase from *Aeromonas proteolytica* Complexed with Tris: A Tale of Buffer Inhibition. *Structure*, 10(8), 1063-1072.

- Devaskar, S. U., Giddings, S. J., Rajakumar, P. A., Carnaghi, L. R., Menon, R. K., & Zahm, D. S. (1994). Insulin gene expression and insulin synthesis in mammalian neuronal cells. *The Journal of Biological Chemistry*, 269(11), 8445-8454.
- Devesh, C., Kritika, M., Anroop, N., Kumar, S., & Gupta, S. (2012). Spirulina Reverses Histomorphological Changes in Diabetic Osteoporosis in Pioglitazone Treated Rats. *Journal of Diabetes & Metabolism*.
- Dickson, D. W., Lee, S. C., Mattiace, L. A., Yen, S. H., & Brosnan, C. (1993). Microglia and cytokines in neurological disease, with special reference to AIDS and Alzheimer's disease. *Glia*, 7(1), 75-83.
- Dickson, M. R., Li, J., Wiener, H. W., Perry, R. T., Blacker, D., Bassett, S. S., *et al.* (2008). A genomic scan for age at onset of Alzheimer's disease in 437 families from the NIMH Genetic Initiative. *American Journal of Medical Genetics Part B (Neuropsychiatric Genetics)*, 147B(6), 784-792.
- Diniz, B. S. O., Pinto(Jnr), J. A., & Forlenza, O. V. (2008). Do CSF total tau, phosphorylated tau, and β -amyloid 42 help to predict progression of mild cognitive impairment to Alzheimer's disease? A systematic review and meta-analysis of the literature. *The World Journal of Biological Psychiatry*, 9(3), 172-182.
- Dobarro, M., Gerenu, G., & Ramírez, M. J. (2013). Propranolol reduces cognitive deficits, amyloid and tau pathology in Alzheimer's transgenic mice. *The International Journal of Neuropsychopharmacology*, 1-13.
- Dobarro, M., Orejana, L., Aguirre, N., & Ramírez, M. J. (2012). Propranolol reduces cognitive deficits, amyloid β levels, tau phosphorylation and insulin resistance in response to chronic corticosterone administration. *The International Journal of Neuropsychopharmacology*, 1(1), 1-10.
- Dragicevic, N., Mamcarz, M., Zhu, Y., Buzzeo, R., Tan, J., Arendash, G. W., *et al.* (2010). Mitochondrial amyloid- β levels are associated with the extent of mitochondrial dysfunction in different brain regions and the degree of cognitive impairment in Alzheimer's transgenic mice. *Journal of Alzheimer's Disease*, 20, 535-550.

- Du, J., Chang, J., Guo, S., Zhang, Q., & Wang, Z. (2009). ApoE 4 reduces the expression of A β degrading enzyme IDE by activating the NMDA receptor in hippocampal neurons. *Neuroscience Letters*, 464(2), 140-145.
- Du, J., Zhang, L., Liu, S., & Wang, Z. (2010). Palmitic acid and docosahexaenoic acid opposingly regulate the expression of insulin-degrading enzyme in neurons. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, 65(3), 231-232.
- Dubois, B., Feldman, H. H., Jacova, C., DeKosky, S., Barberger-Gateau, P., Cummings, J., *et al.* (2007). Research criteria for the diagnosis of Alzheimer's disease: revising the NINCDS-ADRDA criteria. *The Lancet Neurology*, 6(8), 734-746.
- Duckworth, W. C. (1988). Insulin degradation: mechanisms, products and significance. *Endocrine Reviews*, 9(3), 319-345.
- Duckworth, W. C., Bennett, R. G., & Hamel, F. G. (1998). Insulin degradation: Progress and potential. *Endocrine Reviews*, 19(5), 608-624.
- Duckworth, W. C., Hamel, F. G., Bennett, R., Ryan, M. P., & Roth, R. A. (1990). Human red blood cell insulin-degrading enzyme and rat skeletal muscle insulin protease share antigenic sites and generate identical products from insulin. *The Journal of Biological Chemistry*, 265(5), 2984-2987.
- Duckworth, W. C., & Kitabchi, A. E. (1974). Insulin and glucagon degradation by the same enzyme. *Diabetes*, 23(6), 536-543.
- Duggirala, R., Blangero, J., Almasy, L., Dyer, T. D., Williams, K. L., Leach, R. J., *et al.* (1999). Linkage of type 2 diabetes mellitus and of age at onset to a genetic location on chromosome 10q in Mexican Americans. *American Journal of Human Genetics*, 64(4), 1127-1140.
- Duyckaerts, C., Delatour, B., & Potier, M.-C. (2009). Classification and basic pathology of Alzheimer disease. *Acta Neuropathologica*, 118(1), 5-36.
- Ebrahim, A., Hamel, F. G., Bennett, R. G., & Duckworth, W. C. (1991). Identification of the metal associated with the insulin degrading enzyme. *Biochemical And Biophysical Research Communications*, 181(3), 1398-1406.
- Eckel, R. H., Grundy, S. M., & Zimmet, P. Z. (2005). The metabolic syndrome. *The Lancet*, 365(9468), 1415-1428.

- Economics, D. A. (2011). DEMENTIA ACROSS AUSTRALIA: 2011-2050. *Canberra: Delloite Access Economics Pty Ltd.*
- Edbauer, D., Willem, M., Lammich, S., Steiner, H., & Haass, C. (2002). Insulin-degrading enzyme rapidly removes the β -amyloid precursor protein intracellular domain (AICD). *The Journal of Biological Chemistry*, 277(16), 13389-13393.
- Edland, S. D., Wavrant-De Vriesé, F., Compton, D., Smith, G. E., Ivnik, R., Boeve, B. F., *et al.* (2003). Insulin degrading enzyme (IDE) genetic variants and risk of Alzheimer's disease: evidence of effect modification by apolipoprotein E (APOE). *Neuroscience Letters*, 345(1), 21-24.
- Egerer, K., Kuckelkorn, U., Rudolph, P. E., Rückert, J. C., Dörner, T., Burmester, G.-R., *et al.* (2002). Circulating proteasomes are markers of cell damage and immunologic activity in autoimmune diseases. *The Journal of rheumatology*, 29(10), 2045-2052.
- Ehm, M. G., Karnoub, M. C., Sakul, H., Gottschalk, K., Holt, D. C., Weber, J. L., *et al.* (2000). Genomewide search for type 2 diabetes susceptibility genes in four American populations. *American Journal of Human Genetics*, 66(6), 1871-1881.
- Eisele, Y. S., Bolmont, T., Heikenwalder, M., Langer, F., Jacobson, L. H., Yan, Z.-X., *et al.* (2009). Induction of cerebral β -amyloidosis: Intracerebral versus systemic A β inoculation. *Proceedings of the National Academy of Sciences*, 106(31), 12926-12931.
- Eisele, Y. S., Obermüller, U., Heilbronner, G., Baumann, F., Kaeser, S. A., Wolburg, H., *et al.* (2010). Peripherally applied A β -containing inoculates induce cerebral β -amyloidosis. *Science*, 330(6006), 980-982.
- Ellis, K. A., Bush, A. I., Darby, D., De Fazio, D., Foster, J., Hudson, P., *et al.* (2009). The Australian Imaging, Biomarkers and Lifestyle Study of ageing (AIBL): methodology and baseline characteristics of 1112 individuals recruited for a longitudinal study of Alzheimer's disease. *International Psychogeriatrics*, 21(4), 672-687.

- Engel, T., Goñi-Oliver, P., Lucas, J. J., Avila, J., & Hernández, F. (2006). Chronic lithium administration to FTDP-17 tau and GSK-3 β overexpressing mice prevents tau hyperphosphorylation and neurofibrillary tangle formation, but pre-formed neurofibrillary tangles do not revert. *Journal of Neurochemistry*, 99(6), 1445-1455.
- Ertekin-Taner, N., Allen, M., Fadale, D., Scanlin, L., Younkin, L., Petersen, R. C., *et al.* (2004). Genetic variants in a haplotype block spanning IDE are significantly associated with plasma A β 42 levels and risk for Alzheimer disease. *Human Mutation*, 23(4), 334-342.
- Ertekin-Taner, N., Graff-Radford, N., Younkin, L. H., Eckman, C., Adamson, J., Schaid, D. J., *et al.* (2001). Heritability of plasma amyloid β in typical late-onset *alzheimer's* disease pedigrees. *Genetic Epidemiology*, 21(1), 19-30.
- Ertekin-Taner, N., Graff-Radford, N., Younkin, L. H., Eckman, C., Baker, M., Adamson, J., *et al.* (2000). Linkage of plasma A β 42 to a quantitative locus on chromosome 10 in late-onset *alzheimer's* disease pedigrees. *Science*, 290(5500), 2303-2304.
- Ertekin-Taner, N., Younkin, L., Yager, D., Parfitt, F., Baker, M., Asthana, S., *et al.* (2008). Plasma amyloid β protein is elevated in late-onset *alzheimer* disease families. *Neurology*, 70(8), 596-606.
- Espinosa, R., Lemons, R. S., Perlman, R. K., Kuo, W. L., Rosner, M. R., & Lebeau, M. M. (1991). Localization of the gene encoding insulin-degrading enzyme to human chromosome-10, bands q23-q25 *Cytogenetics and Cell Genetics*, 57(4), 184-186.
- Estey, T., Kang, J., Schwendeman, S. P., & Carpenter, J. F. (2006). BSA degradation under acidic conditions: a model for protein instability during release from PLGA delivery systems. *Journal of pharmaceutical sciences*, 95(7), 1626-1639.
- Evans, M. J., Livesey, J. H., Ellis, M. J., & Yandle, T. G. (2001). Effect of anticoagulants and storage temperatures on stability of plasma and serum hormones. *Clinical biochemistry*, 34(2), 107-112.

- Fakhrai-Rad, H., Nikoshkov, A., Kamel, A., Fernström, M., Zierath, J. R., Norgren, S., *et al.* (2000). Insulin-degrading enzyme identified as a candidate diabetes susceptibility gene in GK rats. *Human Molecular Genetics*, 9(14), 2149-2158.
- Farrer, L. A., Cupples, L. A., Haines, J. L., Hyman, B., Kukull, W. A., Mayeux, R., *et al.* (1997). Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. *JAMA: the journal of the American Medical Association*, 278(16), 1349-1356.
- Farris, W., Leissring, M. A., Hemming, M. L., Chang, A. Y., & Selkoe, D. J. (2005). Alternative splicing of human insulin-degrading enzyme yields a novel isoform with a decreased ability to degrade insulin and amyloid β -protein. *Biochemistry*, 44(17), 6513-6525.
- Farris, W., Mansourian, S., Chang, Y., Lindsley, L., Eckman, E. A., Frosch, M. P., *et al.* (2003). Insulin-degrading enzyme regulates the levels of insulin, amyloid β -protein, and the β -amyloid precursor protein intracellular domain in vivo. *Proceedings of the National Academy of Sciences*, 100(7), 4162-4167.
- Farris, W., Mansourian, S., Leissring, M. A., Eckman, E. A., Bertram, L., Eckman, C. B., *et al.* (2004). Partial loss-of-function mutations in insulin-degrading enzyme that induce diabetes also impair degradation of amyloid β -protein. *American Journal of Pathology*, 164(4), 1425-1434.
- Ferretti, M., Partridge, V., C Leon, W., Canneva, F., Allard, S., N Arvanitis, D., *et al.* (2011). Transgenic mice as a model of pre-clinical Alzheimer's disease. *Current Alzheimer Research*, 8(1), 4-23.
- Feuk, L., McCarthy, S., Andersson, B., Prince, J. A., & Brookes, A. J. (2005). Mutation screening of a haplotype block around the insulin degrading enzyme gene and association with Alzheimer's disease. *American Journal of Medical Genetics Part B (Neuropsychiatric Genetics)*, 136B(1), 69-71.
- Fishel, M. A., Watson, G. S., Montine, T. J., Wang, Q., Green, P. S., Kulstad, J. J., *et al.* (2005). Hyperinsulinemia provokes synchronous increases in central inflammation and beta-amyloid in normal adults. *Archives of Neurology*, 62(10), 1539-1544.

- Florez, J. C., Wiltshire, S., Agapakis, C. M., Burt, N. P., de Bakker, P. I. W., Almgren, P., *et al.* (2006). High-density haplotype structure and association testing of the insulin-degrading enzyme (IDE) gene with type 2 diabetes in 4,206 people. *Diabetes*, 55(1), 128-135.
- Francis, G. J., Martinez, J. A., Liu, W. Q., Xu, K., Ayer, A., Fine, J., *et al.* (2008). Intranasal insulin prevents cognitive decline, cerebral atrophy and white matter changes in murine type I diabetic encephalopathy. *Brain*, 131(12), 3311-3334.
- Frank, R. A., Galasko, D., Hampel, H., Hardy, J., de Leon, M. J., Mehta, P. D., *et al.* (2003). Biological markers for therapeutic trials in Alzheimer's disease. Proceedings of the biological markers working group; NIA initiative on neuroimaging in Alzheimer's disease. *Neurobiology of Aging*, 24(4), 521-536.
- Fratiglioni, L. (1996). Epidemiology of Alzheimer's disease and current possibilities for prevention. *Acta Neurologica Scandinavica Supplementum*, 165, 33-40.
- Friedewald, W. T., Levy, R. I., & Fredrickson, D. S. (1972). Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clinical chemistry*, 18(6), 499-502.
- Fujimoto, M., Tsuneyama, K., Fujimoto, T., Selmi, C., Gershwin, M. E., & Shimada, Y. (2012). Spirulina improves non-alcoholic steatohepatitis, visceral fat macrophage aggregation, and serum leptin in a mouse model of metabolic syndrome. *Digestive and Liver Disease*, 44(9), 767-774.
- Fujisawa, Y., Sasaki, K., & Akiyama, K. (1991). Increased insulin levels after OGTT load in peripheral blood and cerebrospinal fluid of patients with dementia of Alzheimer type. *Biological Psychiatry*, 30(12), 1219-1228.
- Fukumoto, H., Tennis, M., Locascio, J. J., Hyman, B. T., Growdon, J. H., & Irizarry, M. C. (2003). Age but not diagnosis is the main predictor of plasma amyloid beta-protein levels. *Archives of Neurology*, 60(7), 958-964.
- Funk, K., Mrak, R., & Kurek, J. (2011). Granulovacuolar degeneration (GVD) bodies of Alzheimer's disease (AD) resemble late-stage autophagic organelles. *Neuropathology and Applied Neurobiology*, 37(3), 295-306.

- Furukawa, Y., Shimada, T., Furuta, H., Matsuno, S., Kusuyama, A., Doi, A., *et al.* (2008). Polymorphisms in the IDE-KIF11-HHEX gene locus are reproducibly associated with type 2 diabetes in a Japanese population. *Journal of Clinical Endocrinology and Metabolism*, 93(1), 310-314.
- Gao, W. W., Eisenhauer, P. B., Conn, K., Lynch, J. A., Wells, J. M., Ullman, M. D., *et al.* (2004). Insulin degrading enzyme is expressed in the human cerebrovascular endothelium and in cultured human cerebrovascular endothelial cells. *Neuroscience Letters*, 371(1), 6-11.
- Garcia, J. V., Gehm, B. D., & Rosner, M. R. (1989). An evolutionarily conserved enzyme degrades transforming growth factor-alpha as well as insulin. *The Journal of Cell Biology*, 109(3), 1301-1307.
- Gascón-Bayarri, J., Reñé, R., Del Barrio, J. L., De Pedro-Cuesta, J., Ramón, J. M., Manubens, J. M., *et al.* (2007). Prevalence of dementia subtypes in El Prat de Llobregat, Catalonia, Spain: The PRATICON Study. *Neuroepidemiology*, 28(4), 224-234.
- Gasparini, L., Gouras, G. K., Wang, R., Gross, R. S., Beal, M. F., Greengard, P., *et al.* (2001). Stimulation of β -amyloid precursor protein trafficking by insulin reduces intraneuronal β -amyloid and requires mitogen-activated protein kinase signaling. *The Journal of Neuroscience*, 21(8), 2561-2570.
- Gassmann, M., Grenacher, B., Rohde, B., & Vogel, J. (2009). Quantifying Western blots: pitfalls of densitometry. *Electrophoresis*, 30(11), 1845-1855.
- Gatz, M., Pedersen, N. L., Berg, S., Johansson, B., Johansson, K., Mortimer, J. A., *et al.* (1997). Heritability for Alzheimer's disease: The study of dementia in Swedish twins. *Journal of Gerontology: Medical Sciences*, 52A(2), M117-M125.
- Gauthier, S., Reisberg, B., Zaudig, M., Petersen, R. C., Ritchie, K., Broich, K., *et al.* (2006). Mild cognitive impairment. *The Lancet*, 367(9518), 1262-1270.
- Geerlings, M. I., Jonker, C., Bouter, L. M., Adèr, H. J., & Schmand, B. (1999). Association between memory complaints and incident Alzheimer's disease in elderly people with normal baseline cognition. *The American Journal of Psychiatry*, 156(4), 531-537.

- Ghalanbor, Z., Ghaemi, N., Marashi, S.-A., Amanlou, M., Habibi-Rezaei, M., Khajeh, K., *et al.* (2008). Binding of Tris to Bacillus licheniformis alpha-Amylase Can Affect Its Starch Hydrolysis Activity. *Protein and peptide letters*, 15(2), 212-214.
- GherssiEgea, J. F., Gorevic, P. D., Ghiso, J., Frangione, B., Patlak, C. S., & Fenstermacher, J. D. (1996). Fate of cerebrospinal fluid-borne amyloid beta-peptide: Rapid clearance into blood and appreciable accumulation by cerebral arteries. *Journal of Neurochemistry*, 67(2), 880-883.
- Ghosh, S., Watanabe, R. M., Valle, T. T., Hauser, E. R., Magnuson, V. L., Langefeld, C. D., *et al.* (2000). The Finland--United States investigation of non--insulin-dependent diabetes mellitus genetics (FUSION) Study. I. An autosomal genome scan for genes that predispose to type 2 diabetes. *American Journal of Human Genetics*, 67(5), 1174-1185.
- Gibson, P. H., & Tomlinson, B. E. (1977). Numbers of Hirano bodies in the hippocampus of normal and demented people with Alzheimer's disease. *Journal of the Neurological Sciences*, 33(1-2), 199-206.
- Glatt, H., & Oesch, F. (1987). Species differences in enzymes controlling reactive epoxides. In *Mouse Liver Tumors* (pp. 111-124): Springer.
- Glebov, K., & Walter, J. (2011). Statins in Unconventional Secretion of Insulin-Degrading Enzyme and Degradation of the Amyloid- β Peptide. *Neurodegenerative Diseases*, 10(1-4), 309-312.
- Glenner, G. G., & Wong, C. W. (1984). Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochemical And Biophysical Research Communications*, 120(3), 885-890.
- Gluschkof, P., Gomez, S., Morel, A., & Cohen, P. (1987). Enzymes that process somatostatin precursors. A novel endoprotease that cleaves before the arginine-lysine doublet is involved in somatostatin-28 convertase activity of rat brain cortex. *Journal of Biological Chemistry*, 262(20), 9615-9620.
- Goate, A. M. (1997). Molecular genetics of Alzheimer's disease. *Geriatrics*, 52(Supplement 2), S9-S12.

- Goate, A. M., Chartier-Harlin, M. C., Mullan, M., Brown, J., Crawford, F., Fidani, L., *et al.* (1991). Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature*, 349(6311), 704-706.
- Goldfine, I. D., Williams, J. A., Bailey, A. C., Wong, K. Y., Iwamoto, Y., Yokono, K., *et al.* (1984). Degradation of insulin by isolated mouse pancreatic acini. Evidence for cell surface protease activity. *Diabetes*, 33(1), 64-72.
- Gouras, G. K., Tsai, J., Naslund, J., Vincent, B., Edgar, M., Checler, F., *et al.* (2000). Intraneuronal A β 42 accumulation in human brain. *American Journal of Pathology*, 156(1), 15-20.
- Graff-Radford, N. R., Crook, J. E., Lucas, J., Boeve, B. F., Knopman, D. S., Ivnik, R. J., *et al.* (2007). Association of low plasma A β 42/A β 40 ratios with increased imminent risk for mild cognitive impairment and Alzheimer disease. *Archives of Neurology*, 64(3), 354-362.
- Grasso, G., Rizzarelli, E., & Spoto, G. (2008). How the binding and degrading capabilities of insulin degrading enzyme are-affected by ubiquitin. *Biochimica et Biophysica Acta - Proteins & Proteomics*, 1784(7-8), 1122-1126.
- Grasso, G., Salomone, F., Tundo, G. R., Pappalardo, G., Ciaccio, C., Spoto, G., *et al.* (2012). Metal ions affect insulin-degrading enzyme activity. *Journal of Inorganic Biochemistry*, 117, 351-358.
- Griffin, W. S. T., Stanley, L. C., Ling, C., White, L., MacLeod, V., Perrot, L. J., *et al.* (1989). Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. *Proceedings of the National Academy of Sciences*, 86(19), 7611-7615.
- Grisel, J. E. (2000). Quantitative trait locus analysis. *Alcohol Research and Health*, 24(3), 169-174.
- Grodstein, F., Wilson, R. S., Chen, J., & Manson, J. E. (2001). Type 2 diabetes and cognitive function in community-dwelling elderly women. *Diabetes Care*, 24(6), 1060-1065.

- Groves, C. J., Wiltshire, S., Smedley, D., Owen, K. R., Frayling, T. M., Walker, M., *et al.* (2003). Association and haplotype analysis of the insulin-degrading enzyme (IDE) gene, a strong positional and biological candidate for type 2 diabetes susceptibility. *Diabetes*, 52(5), 1300-1305.
- Grundke-Iqbal, I., Iqbal, K., Quinlan, M., Tung, Y. C., Zaidi, M. S., & Wisniewski, H. M. (1986). Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. *The Journal of Biological Chemistry*, 261(13), 6084-6089.
- Grupe, A., Abraham, R., Li, Y., Rowland, C., Hollingworth, P., Morgan, A., *et al.* (2007). Evidence for novel susceptibility genes for late-onset *alzheimer's* disease from a genome-wide association study of putative functional variants. *Human Molecular Genetics*, 16(8), 865-873.
- Grupe, A., Li, Y., Rowland, C., Nowotny, P., Hinrichs, A. L., Smemo, S., *et al.* (2006). A scan of chromosome 10 identifies a novel locus showing strong association with late-onset *alzheimer* disease. *American Journal of Human Genetics*, 78(1), 78-88.
- Gu, H. F., Efendic, S., Nordman, S., Östenson, C. G., Brismar, K., Brookes, A. J., *et al.* (2004). Quantitative trait loci near the insulin-degrading enzyme (IDE) gene contribute to variation in plasma insulin levels. *Diabetes*, 53(8), 2137-2142.
- Guan, H., Liu, Y., Daily, A., Police, S., Kim, M. H., Oddo, S., *et al.* (2009). Peripherally expressed neprilysin reduces brain amyloid burden: a novel approach for treating Alzheimer's disease. *Journal of neuroscience research*, 87(6), 1462-1473.
- Guiroy, D. C., Miyazaki, M., Multhaup, G., Fischer, P., Garruto, R. M., Beyreuther, K., *et al.* (1987). Amyloid of neurofibrillary tangles of Guamanian parkinsonism-dementia and Alzheimer disease share identical amino acid sequence. *Proceedings of the National Academy of Sciences*, 84(7), 2073-2077.
- Gupta, R., & Brunak, S. (2002). *Prediction of glycosylation across the human proteome and the correlation to protein function*. Paper presented at the Pacific Symposium on Biocomputing.

- Gupta, V., Laws, S. M., Villemagne, V. L., Ames, D., Bush, A. I., Ellis, K. A., *et al.* (2011). Plasma apolipoprotein E and Alzheimer disease risk The AIBL study of aging. *Neurology*, 76(12), 1091-1098.
- Gyure, K. A., Durham, R., Stewart, W. F., Smialek, J. E., & Troncoso, J. C. (2001). Intraneuronal A β -amyloid precedes development of amyloid plaques in Down syndrome. *Archives of pathology & laboratory medicine*, 125(4), 489-492.
- Haass, C., Schlossmacher, M. G., Hung, A. Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B. L., *et al.* (1992). Amyloid β -peptide is produced by cultured cells during normal metabolism. *Nature*, 359(6393), 322-325.
- Hahs, D. W., McCauley, J. L., Crunk, A. E., McFarland, L. L., Gaskell, P. C., Jiang, L., *et al.* (2006). A genome-wide linkage analysis of dementia in the Amish. *American Journal of Medical Genetics Part B (Neuropsychiatric Genetics)*, 141B(2), 160-166.
- Hamel, F. G., Mahoney, M. J., & Duckworth, W. C. (1991). Degradation of intraendosomal insulin by insulin-degrading enzyme without acidification. *Diabetes*, 40(4), 436-443.
- Hamel, F. G., Upward, J. L., & Bennett, R. G. (2003). In vitro inhibition of insulin-degrading enzyme by long-chain fatty acids and their coenzyme A thioesters. *Endocrinology*, 144(6), 2404-2408.
- Hampel, H., Frank, R., Broich, K., Teipel, S. J., Katz, R. G., Hardy, J., *et al.* (2010). Biomarkers for Alzheimer's disease: academic, industry and regulatory perspectives. *Nature Reviews Drug Discovery*, 9(7), 560-574.
- Hamshere, M. L., Holmans, P. A., Avramopoulos, D., Bassett, S. S., Blacker, D., Bertram, L., *et al.* (2007). Genome-wide linkage analysis of 723 affected relative pairs with late-onset *alzheimer's* disease. *Human Molecular Genetics*, 16(22), 2703-2712.
- Harada, S., Smith, R. M., Hu, D. Q., & Jarett, L. (1996). Dexamethasone inhibits insulin binding to insulin-degrading enzyme and cytosolic insulin-binding protein p82. *Biochemical And Biophysical Research Communications*, 218(1), 154-158.

- Hardy, J. (1997). Amyloid, the presenilins and Alzheimer's disease. *Trends in Neurosciences*, 20(4), 154-159.
- Hardy, J., & Allsop, D. (1991). Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends in pharmacological sciences*, 12(10), 383.
- Hardy, J., & Higgins, G. A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. *Science*, 256(5054), 184-186.
- Hardy, J., & Selkoe, D. J. (2002). The amyloid hypothesis of Alzheimer's Disease: progress and problems on the road to therapeutics. . *Science*, 297(5580), 353-356.
- Hartley, D. M., Walsh, D. M., Ye, C. P., Diehl, T., Vasquez, S., Vassilev, P. M., *et al.* (1999). Protofibrillar intermediates of amyloid β -protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. *The Journal of Neuroscience*, 19(20), 8876-8884.
- Hartmann, A., Veldhuis, J. D., Deuschle, M., Standhardt, H., & Heuser, I. (1997). Twenty-four hour cortisol release profiles in patients with Alzheimer's and Parkinson's disease compared to normal controls: ultradian secretory pulsatility and diurnal variation. *Neurobiology of Aging*, 18(3), 285-289.
- Hassing, L. B., Johansson, B., Nilsson, S. E., Berg, S., Pedersen, N. L., Gatz, M., *et al.* (2002). Diabetes mellitus is a risk factor for vascular dementia, but not for Alzheimer's disease: A population-based study of the oldest old. *International Psychogeriatrics*, 14(3), 239-248.
- Hastie, T., Tibshirani, R., Friedman, J., & Franklin, J. (2005). The elements of statistical learning: data mining, inference and prediction. *The Mathematical Intelligencer*, 27(2), 83-85.
- Henderson, S. J., Andersson, C., Narwal, R., Janson, J., Goldschmidt, T. J., Appelkvist, P., *et al.* (2013). Sustained peripheral depletion of amyloid- β with a novel form of neprilysin does not affect central levels of amyloid- β . *Brain*, awt308.
- Henning, D., & Nielsen, K. (1992). Cross-reactivity of monoclonal antibodies to bovine immunoglobulins with immunoglobulins of other species. *Veterinary immunology and immunopathology*, 34(3), 235-243.

- Herukka, S. K., Helisalmi, S., Hallikainen, M., Tervo, S., Soininen, H., & Pirttilä, T. (2007). CSF A β 42, tau and phosphorylated tau, APOE ϵ 4 allele and MCI type in progressive MCI. *Neurobiology of Aging*, 28(4), 507-514.
- Hirsch-Reinshagen, V., Burgess, B. L., & Wellington, C. L. (2009). Why lipids are important for Alzheimer disease? *Molecular and Cellular Biochemistry*, 326(1-2), 121-129.
- Hirsch-Reinshagen, V., Maia, L. F., Burgess, B. L., Blain, J. F., Naus, K. E., Mclsaac, S. A., *et al.* (2005). The absence of ABCA1 decreases soluble apoE levels but does not diminish amyloid deposition in two murine models of Alzheimer disease. *The Journal of Biological Chemistry*, 280(52), 43243-43256.
- Hixson, J. E., & Vernier, D. T. (1990). Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with HhaI. *Journal of lipid research*, 31(3), 545-548.
- Ho, L., Qin, W., Pompl, P. N., Zhongmin, X., Wang, J., Zhao, Z., *et al.* (2004). Diet-induced insulin resistance promotes amyloidosis in a transgenic mouse model of Alzheimer's disease. *The FASEB Journal*, 18(3), 902-904.
- Hogervorst, E., Bandelow, S., Combrinck, M., & Smith, A. (2004). Low free testosterone is an independent risk factor for Alzheimer's disease. *Experimental gerontology*, 39(11), 1633-1639.
- Holm, S. (1979). A simple sequentially rejective multiple test procedure. *Scandinavian journal of statistics*, 65-70.
- Holmans, P., Hamshere, M., Hollingworth, P., Rice, F., Tunstall, N., Jones, S., *et al.* (2005). Genome screen for loci influencing age at onset and rate of decline in late onset alzheimer's disease. *American Journal of Medical Genetics Part B (Neuropsychiatric Genetics)*, 135B(1), 24-32.
- Holmes, C. (2013). Systemic inflammation and Alzheimer's disease. *Neuropathology and Applied Neurobiology*, 39(1), 51-68.
- Hone, E., Martins, I. J., Fonte, J., & Martins, R. N. (2003). Apolipoprotein E influences amyloid-beta clearance from the murine periphery. *Journal of Alzheimer's Disease*, 5(1), 1-8.

- Hong, M., & Lee, V. M. Y. (1997). Insulin and insulin-like growth factor-1 regulate tau phosphorylation in cultured human neurons. *The Journal of Biological Chemistry*, 272(31), 19547-19553.
- Hopkins, P. C., Huang, Y., McGuire, J. G., & Pitas, R. E. (2002). Evidence for differential effects of apoE3 and apoE4 on HDL metabolism. *Journal of lipid research*, 43(11), 1881-1889.
- Hoshino, T., Murao, N., Namba, T., Takehara, M., Adachi, H., Katsuno, M., *et al.* (2011). Suppression of Alzheimer's disease-related phenotypes by expression of heat shock protein 70 in mice. *The Journal of Neuroscience*, 31(14), 5225-5234.
- Hoshino, T., Suzuki, K., Matsushima, T., Yamakawa, N., Suzuki, T., & Mizushima, T. (2013). Suppression of Alzheimer's Disease-Related Phenotypes by Geranylgeranylacetone in Mice. *Plos one*, 8(10), e76306.
- Ichihara, A. (1993). Serum concentration and localization in tumor cells of proteasomes in patients with hematologic malignancy and their pathophysiologic significance. *J Lab Clin Med*.
- Ignatius, M. J., Gebicke-Härter, P. J., Pate Skene, J. H., Schilling, J. W., Weisgraber, K. H., Mahley, R. W., *et al.* (1986). Expression of apolipoprotein E during nerve degeneration and regeneration. *Proceedings of the National Academy of Sciences*, 83(4), 1125-1129.
- Ikonomovic, M. D., Klunk, W. E., Abrahamson, E. E., Mathis, C. A., Price, J. C., Tsopelas, N. D., *et al.* (2008). Post-mortem correlates of in vivo PiB-PET amyloid imaging in a typical case of Alzheimer's disease. *Brain*, 131(Pt 6), 1630-1645.
- Im, H., Manolopoulou, M., Malito, E., Shen, Y., Zhao, J., Neant-Fery, M., *et al.* (2007). Structure of substrate-free human insulin-degrading enzyme (IDE) and biophysical analysis of ATP-induced conformational switch of IDE. *The Journal of Biological Chemistry*, 282(35), 25453-25463.
- Ionushas, B. S., Matuliavichus, V. A., Ostrauskas, R. V., Brazauskas, A. P., & Vareikis, E. I. (1987). Blood insulinase activity in patients with diabetes mellitus. *Terapevticheskii Arkhiv*, 59(11), 12-15.

- Irizarry, M. C. (2004). Biomarkers of Alzheimer disease in plasma. *NeuroRx*, 1(2), 226-234.
- Ito, S., Ohtsuki, S., Murata, S., Katsukura, Y., Suzuki, H., Funaki, M., *et al.* (2014). Involvement of Insulin-Degrading Enzyme in Insulin-and Atrial Natriuretic Peptide-Sensitive Internalization of Amyloid- β Peptide in Mouse Brain Capillary Endothelial Cells. *Journal of Alzheimer's Disease*, 38(1), 185-200.
- Iwata, N., Tsubuki, S., Takaki, Y., Shirotani, K., Lu, B., Gerard, N. P., *et al.* (2001). Metabolic regulation of brain A β by neprilysin. *Science*, 292(5521), 1550-1552.
- Iwata, N., Tsubuki, S., Takaki, Y., Watanabe, K., Sekiguchi, M., Hosoki, E., *et al.* (2000). Identification of the major A β 1–42-degrading catabolic pathway in brain parenchyma: suppression leads to biochemical and pathological deposition. *Nature Medicine*, 6(2), 143-150.
- Jack, C. R., Albert, M. S., Knopman, D. S., McKhann, G. M., Sperling, R. A., Carrillo, M. C., *et al.* (2011). Introduction to the recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's and Dementia*, 7(3), 257-262.
- James, A. P., & Mamo, J. C. L. (2005). The immunodetection of lipoprotein-bound amyloid- β is attenuated because of the presence of lipids. *Annals of Clinical Biochemistry*, 42(Pt 1), 70-72.
- Janson, J., Laedtke, T., Parisi, J. E., O'Brien, P., Petersen, R. C., & Butler, P. C. (2004). Increased risk of type 2 diabetes in Alzheimer disease. *Diabetes*, 53(2), 474-481.
- Jarrett, J. T., Berger, E. P., & Lansbury, P. T. (1993). The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry*, 32(18), 4693-4697.
- Jayaraman, A., Carroll, J. C., Morgan, T. E., Lin, S., Zhao, L., Arimoto, J. M., *et al.* (2012). 17 β -Estradiol and progesterone regulate expression of β -amyloid clearance factors in primary neuron cultures and female rat brain. *Endocrinology*, 153(11), 5467-5479.

- Jeong, J. S., Ansaloni, A., Mezzenga, R., Lashuel, H. A., & Dietler, G. (2013). Novel mechanistic insight into the molecular basis of amyloid polymorphism and secondary nucleation during amyloid formation. *Journal of Molecular Biology*.
- Jiang, Q., Daniel Lee, C. Y., Mandrekar, S., Wilkinson, B., Cramer, P., Zelcer, N., *et al.* (2008). ApoE promotes the proteolytic degradation of A β . *Neuron*, 58(5), 681-693.
- Jordan, J., Cena, V., & Prehn, J. H. M. (2003). Mitochondrial control of neuron death and its role in neurodegenerative disorders. *Journal of Physiology and Biochemistry*, 59(2), 129-141.
- Kaiyala, K. J., Prigeon, R. L., Kahn, S. E., Woods, S. C., & Schwartz, M. W. (2000). Obesity induced by a high-fat diet is associated with reduced brain insulin transport in dogs. *Diabetes*, 49(9), 1525-1533.
- Käkönen, S.-M., Hellman, J., Karp, M., Laaksonen, P., Obrant, K. J., Väänänen, H. K., *et al.* (2000). Development and evaluation of three immunofluorometric assays that measure different forms of osteocalcin in serum. *Clinical Chemistry*, 46(3), 332-337.
- Kalaria, R. N., Premkumar, D. R., Pax, A. B., Cohen, D. L., & Lieberburg, I. (1996). Production and increased detection of amyloid β protein and amyloidogenic fragments in brain microvessels, meningeal vessels and choroid plexus in Alzheimer's disease. *Molecular brain research*, 35(1), 58-68.
- Karamohamed, S., Demissie, S., Volcjak, J., Liu, C., Heard-Costa, N., Liu, J., *et al.* (2003). Polymorphisms in the insulin-degrading enzyme gene are associated with type 2 diabetes in men from the NHLBI Framingham Heart Study. *Diabetes*, 52(6), 1562-1567.
- Karran, E., Mercken, M., & De Strooper, B. (2011). The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. *Nature*, 10, 698-712.
- Kątnik, I., Podgórska, M., & Dobryszczyka, W. (1987). Polyethylene glycol enzyme immunoassay for screening anti-haptoglobin monoclonal antibodies. *Journal of immunological methods*, 102(2), 279-282.

- Kaur, M., & Balgir, P. P. (2006). APOE polymorphism and susceptibility to dementia of the Alzheimer's type in the Indian population. *Internet Journal of Mental Health*, 3(1).
- Kawarabayashi, T., Younkin, L. H., Saido, T. C., Shoji, M., Ashe, K. H., & Younkin, S. G. (2001). Age-dependent changes in brain, CSF, and plasma amyloid β protein in the Tg2576 transgenic mouse model of Alzheimer's disease. *The Journal of Neuroscience*, 21(2), 372-381.
- Kayalar, C., & Wong, W. T. (1989). Metalloendoprotease inhibitors which block the differentiation of L6 myoblasts inhibit insulin degradation by the endogenous insulin-degrading enzyme. *Journal of Biological Chemistry*, 264(15), 8928-8934.
- Kehoe, P., Wavrant-De Vriesé, F., Crook, R., Wu, W. S., Holmans, P., Fenton, I., *et al.* (1999). A full genome scan for late onset *alzheimer's* disease. *Human Molecular Genetics*, 8(2), 237-245.
- Kim, H. S., Kim, E. M., Lee, J. P., Park, C. H., Kim, S., Seo, J. H., *et al.* (2003). C-terminal fragments of amyloid precursor protein exert neurotoxicity by inducing glycogen synthase kinase-3 β expression. *The FASEB Journal*, 17(13), 1951-1953.
- Kim, J., Basak, J. M., & Holtzman, D. M. (2009). The role of apolipoprotein E in Alzheimer's disease. *Neuron*, 63(3), 287-303.
- Kim, M., Hersh, L. B., Leissring, M. A., Ingelsson, M., Matsui, T., Farris, W., *et al.* (2007). Decreased catalytic activity of the insulin-degrading enzyme in chromosome 10-linked Alzheimer disease families. *The Journal of Biological Chemistry*, 282(11), 7825-7832.
- Kim, M. S., Goo, J. S., Kim, J. E., Nam, S. H., Choi, S. I., Lee, H. R., *et al.* (2011). Overexpression of Insulin Degrading Enzyme could Greatly Contribute to Insulin Down-regulation Induced by Short-Term Swimming Exercise. *Laboratory animal research*, 27(1), 29-36.
- Kinoshita, A., Fukumoto, H., Shah, T., Whelan, C. M., Irizarry, M. C., & Hyman, B. T. (2003). Demonstration by FRET of BACE interaction with the amyloid precursor protein at the cell surface and in early endosomes. *Journal of Cell Science*, 116(16), 3339-3346.

- Kinoshita, A., Whelan, C. M., Berezovska, O., & Hyman, B. T. (2002). The γ Secretase-generated carboxyl-terminal domain of the amyloid precursor protein induces apoptosis via tip60 in H4 cells. *The Journal of Biological Chemistry*, 277(32), 28530-28536.
- Kivipelto, M., Helkala, E. L., Hanninen, T., Laakso, M. P., Hallikainen, M., Alhainen, K., *et al.* (2001). Midlife vascular risk factors and late-life mild cognitive impairment: A population-based study. *Neurology*, 56(12), 1683-1689.
- Klunk, W. E., Engler, H., Nordberg, A., Wang, Y., Blomqvist, G., Holt, D. P., *et al.* (2004). Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B. *Annals of Neurology*, 55(3), 306-319.
- Knight, J. C. (2003). Functional implications of genetic variation in non-coding DNA for disease susceptibility and gene regulation. *Clinical Science*, 104(5), 493-502.
- Knobloch, M., Konietzko, U., Krebs, D. C., & Nitsch, R. M. (2007). Intracellular Abeta and cognitive deficits precede beta-amyloid deposition in transgenic arcAbeta mice. *Neurobiology of Aging*, 28(9), 1297-1306.
- Knopman, D. S., Dekosky, S. T., Cummings, J. L., Chui, H., Corey-Bloom, J., Relkin, N., *et al.* (2001). Practice parameter: Diagnosis of dementia. *American Academy of Neurology*, 56, 1143-1153.
- Knouff, C., Hinsdale, M. E., Mezdour, H., Altenburg, M. K., Watanabe, M., Quarfordt, S. H., *et al.* (1999). Apo E structure determines VLDL clearance and atherosclerosis risk in mice. *Journal of Clinical Investigation*, 103(11), 1579-1586.
- Kodama, S., Tanaka, S., Saito, K., Shu, M., Sone, Y., Onitake, F., *et al.* (2007). Effect of aerobic exercise training on serum levels of high-density lipoprotein cholesterol: a meta-analysis. *Archives of internal medicine*, 167(10), 999-1008.
- Kolb, H. J., & Standl, E. (1980). Purification to homogeneity of an insulin-degrading enzyme from human erythrocytes. *Hoppe-Seyler's Zeitschrift für physiologische Chemie*, 361(2), 1029-1040.

- Koldamova, R., Staufenbiel, M., & Lefterov, I. (2005). Lack of ABCA1 considerably decreases brain apoE level and increases amyloid deposition in APP23 mice. *The Journal of Biological Chemistry*, 280(52), 43224-43235.
- Kong, Y., Ruan, L., Qian, L., Liu, X., & Le, Y. (2010). Norepinephrine promotes microglia to uptake and degrade amyloid β peptide through upregulation of mouse formyl peptide receptor 2 and induction of insulin-degrading enzyme. *The Journal of Neuroscience*, 30(35), 11848-11857.
- Korovkina, V. P., Stamnes, S. J., Brainard, A. M., & England, S. K. (2009). Nardilysin convertase regulates the function of the maxi-K channel isoform mK44 in human myometrium. *American Journal of Physiology-Cell Physiology*, 296(3), C433-C440.
- Kosik, K. S., Joachim, C. L., & Selkoe, D. J. (1986). Microtubule-associated protein tau is a major antigenic component of paired helical filaments in Alzheimer disease. *Proceedings of the National Academy of Sciences*, 83, 4044-4048.
- Koudinov, A., Matsubara, E., Frangione, B., & Ghiso, J. (1994). The Soluble Form of Alzheimer's Amyloid β Protein Is Complexed to High Density Lipoprotein 3 and Very High Density Lipoprotein in Normal Human Plasma. *Biochemical And Biophysical Research Communications*, 205(2), 1164-1171.
- Koyama, A., Okereke, O. I., Yang, T., Blacker, D., Selkoe, D. J., & Grodstein, F. (2012). Plasma Amyloid-beta as a Predictor of Dementia and Cognitive Decline: A Systematic Review and Meta-analysis. *Archives of Neurology*, archneurol. 2011.1841 v2011.
- Kragstrup, T. W., Vorup-Jensen, T., Deleuran, B., & Hvid, M. (2013). A simple set of validation steps identifies and removes false results in a sandwich enzyme-linked immunosorbent assay caused by anti-animal IgG antibodies in plasma from arthritis patients. *SpringerPlus*, 2(1), 263.
- Kril, J. J., Hodges, J., & Halliday, G. (2004). Relationship between hippocampal volume and CA1 neuron loss in brains of humans with and without Alzheimer's disease. *Neuroscience Letters*, 361(1-3), 9-12.
- Kulstad, J. J., Green, P. S., Cook, D. G., Watson, G. S., Reger, M. A., Baker, L. D., *et al.* (2006). Differential modulation of plasma β -amyloid by insulin in patients with Alzheimer disease. *Neurology*, 66(10), 1506-1510.

- Kulstad, J. J., McMillan, P. J., Leverenz, J. B., Cook, D. G., Green, P. S., Peskind, E. R., *et al.* (2005). Effects of chronic glucocorticoid administration on insulin-degrading enzyme and amyloid-beta peptide in the aged macaque. *Journal of Neuropathology and Experimental Neurology*, 64(2), 139-146.
- Kuo, W. L., Gehm, B. D., Rosner, M. R., Li, W., & Keller, G. (1994). Inducible expression and cellular localization of insulin-degrading enzyme in a stably transfected cell line. *The Journal of Biological Chemistry*, 269(36), 22599-22606.
- Kuo, W. L., Montag, A. G., & Rosner, M. R. (1993). Insulin-degrading enzyme is differentially expressed and developmentally regulated in various rat tissues. *Endocrinology*, 132(2), 604-611.
- Kuo, Y. M., Crawford, F., Mullan, M., Kokjohn, T. A., Emmerling, M. R., Weller, R. O., *et al.* (2000). Elevated A β and apolipoprotein E in A β PP transgenic mice and its relationship to amyloid accumulation in Alzheimer's disease. *Molecular Medicine*, 6(5), 430-439.
- Kuo, Y. M., Emmerling, M. R., Bisgaier, C. L., Essenburg, A. D., Lampert, H. C., Drumm, D., *et al.* (1998). Elevated low-density lipoprotein in Alzheimer's disease correlates with brain A beta 1-42 levels. *Biochemical And Biophysical Research Communications*, 252(3), 711-715.
- Kuo, Y. M., Emmerling, M. R., Lampert, H. C., Hempelman, S. R., Kokjohn, T. A., Woods, A. S., *et al.* (1999). High levels of circulating A β 42 are sequestered by plasma proteins in Alzheimer's disease. *Biochemical And Biophysical Research Communications*, 257(3), 787-791.
- Kupfer, S. R., Wilson, E. M., & French, F. S. (1994). Androgen and glucocorticoid receptors interact with insulin degrading enzyme. *Journal of Biological Chemistry*, 269(32), 20622-20628.
- Kurklinsky, S., Abdul-Hay, S., McGuire, M., Howard, E., Knight, J., & Leissring, M. The Blood Glucose-lowering Effect of Racecadotril is not Attributable to Inhibition of Insulin-degrading Enzyme. *Hormone and metabolic research*(EFirst).
- Kurochkin, I. V. (1998). Amyloidogenic determinant as a substrate recognition motif of insulin-degrading enzyme. *FEBS Letters*, 427(2), 153-156.

- Kurochkin, I. V. (2001). Insulin-degrading enzyme: embarking on amyloid destruction. *Trends in Biochemical Sciences*, 26(7), 421-425.
- Kurochkin, I. V., & Goto, S. (1994). Alzheimer's β -amyloid peptide specifically interacts with and is degraded by insulin degrading enzyme. *FEBS Letters*, 345(1), 33-37.
- Kurz, A., & Perneczky, R. (2011). Amyloid clearance as a treatment target against Alzheimer's disease. *Journal of Alzheimer's Disease*, 24, 61-73.
- Kuusisto, J., Koivisto, K., Mykkänen, L., Helkala, E. L., Vanhanen, M., Hänninen, T., et al. (1997). Association between features of the insulin resistance syndrome and Alzheimer's disease independently of apolipoprotein E4 phenotype: cross sectional population based study. *BMJ*, 315, 1045-1049.
- Kuwano, R., Miyashita, A., Arai, H., Asada, T., Imagawa, M., Shoji, M., et al. (2006). Dynamin-binding protein gene on chromosome 10q is associated with late-onset Alzheimer's disease. *Human Molecular Genetics*, 15(13), 2170-2182.
- Kwak, S. H., Cho, Y. M., Moon, M. K., Kim, J. H., Park, B. L., Cheong, H. S., et al. (2008). Association of polymorphisms in the insulin-degrading enzyme gene with type 2 diabetes in the Korean population. *Diabetes Research and Clinical Practice*, 79(2), 284-290.
- Laakso, M. (1993). How good a marker is insulin level for insulin resistance? *American Journal of Epidemiology*, 137(9), 959-965.
- LaDu, M. J., Falduto, M. T., Manelli, A. M., Reardon, C. A., Getz, G. S., & Frail, D. E. (1994). Isoform-specific binding of apolipoprotein E to β -amyloid. *The Journal of Biological Chemistry*, 269(38), 23403-23406.
- LaDu, M. J., Pederson, T. M., Frail, D. E., Reardon, C. A., Getz, G. S., & Falduto, M. T. (1995). Purification of apolipoprotein E attenuates isoform-specific binding to β -amyloid. *The Journal of Biological Chemistry*, 270(16), 9039-9042.
- LaFerla, F. M., Green, K. N., & Oddo, S. (2007). Intracellular amyloid- β in Alzheimer's disease. *Nature Reviews. Neuroscience*, 8(7), 499-509.
- Lan, X., Xu, J., Kiyota, T., Peng, H., Zheng, J. C., & Ikezu, T. (2011). HIV-1 reduces A β -degrading enzymatic activities in primary human mononuclear phagocytes. *The Journal of Immunology*, 186(12), 6925-6932.

- Launer, L. J., Andersen, K., Dewey, M. E., Letenneur, L., Ott, A., Arnaducci, L. A., *et al.* (1999). Rates and risk factors for dementia and Alzheimer's disease - Results from EURODEM pooled analyses. *Neurology*, 52(1), 78-84.
- Laws, S. M., Clarnette, R. M., Taddei, K., Martins, G., Paton, A., Hallmayer, J., *et al.* (2002). APOE- ϵ 4 and APOE -491A polymorphisms in individuals with subjective memory loss. *Molecular Psychiatry*, 7, 768-775.
- Lazarov, O., Lee, M., Peterson, D. A., & Sisodia, S. S. (2002). Evidence that synaptically released β -amyloid accumulates as extracellular deposits in the hippocampus of transgenic mice. *The Journal of Neuroscience*, 22(22), 9785-9793.
- Le, A., Barton, L. D., Sanders, J. T., & Zhang, Q. (2010). Exploration of bovine milk proteome in colostrum and mature whey using an ion-exchange approach. *Journal of proteome research*, 10(2), 692-704.
- Le Bastard, N., Aerts, L., Leurs, J., Blomme, W., De Deyn, P. P., & Engelborghs, S. (2009). No correlation between time-linked plasma and CSF A β levels. *Neurochemistry international*, 55(8), 820-825.
- Leal, M. C., Dorfman, V. B., Gamba, A. F., Frangione, B., Wisniewski, T., Castaño, E. M., *et al.* (2006). Plaque-associated overexpression of insulin-degrading enzyme in the cerebral cortex of aged transgenic tg2576 mice with Alzheimer pathology. *Journal of Neuropathology & Experimental Neurology*, 65(10), 976-987.
- Leal, M. C., Magnani, N., Villordo, S., Buslje, C. M., Evelson, P., Castaño, E. M., *et al.* (2013). Transcriptional regulation of Insulin Degrading Enzyme modulates mitochondrial A [beta] catabolism and functionality. *Journal of Biological Chemistry*.
- Lee, C. D., Tse, W., Smith, J. D., & Landreth, G. E. (2012). Apolipoprotein E promotes β -amyloid trafficking and degradation by modulating microglial cholesterol levels. *Journal of Biological Chemistry*, 287(3), 2032-2044.
- Lee, J.-P., Cheng, K.-C., Chung, H.-H., Wu, H.-T., Chen, C.-T., & Cheng, J.-T. (2011). Inhibition of insulin degrading enzyme by racecadotril in the brain of Wistar rats. *Hormone and metabolic research*, 43(07), 489-493.

- Leibson, C. L., Rocca, W. A., Hanson, V. A., Cha, R., Kokmen, E., O'Brien, P. C., *et al.* (1997). Risk of dementia among persons with diabetes mellitus: A population-based cohort study. *American Journal of Epidemiology*, 145(4), 301-308.
- Leissring, M. A., Farris, W., Chang, A. Y., Walsh, D. M., Wu, X., Sun, X., *et al.* (2003). Enhanced proteolysis of β -Amyloid in APP transgenic mice prevents plaque formation, secondary pathology, and premature death. *Neuron*, 40(6), 1087-1093.
- Leissring, M. A., Farris, W., Wu, X., Christodoulou, D. C., Haigis, M. C., Guarente, L., *et al.* (2004). Alternative translation initiation generates a novel isoform of insulin-degrading enzyme targeted to mitochondria. *Biochemical Journal*, 383(Pt 3), 439-446.
- Lesort, M., & Johnson, G. V. (2000). Insulin-like growth factor-1 and insulin mediate transient site-selective increases in tau phosphorylation in primary cortical neurons. *Neuroscience*, 99(2), 305-316.
- Lesort, M., Jope, R. S., & Johnson, G. V. W. (1999). Insulin transiently increases tau phosphorylation: involvement of glycogen synthase kinase-3 β and fyn tyrosine kinase. *Journal of Neurochemistry*, 72(2), 576-584.
- Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D. M., Oshima, J., Pettingell, W. H., *et al.* (1995). Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science*, 269(5226), 973-975.
- Li, A., Xue, Y., Jin, C., Wang, M., & Yao, X. (2006). Prediction of N-acetylation on internal lysines implemented in Bayesian Discriminant Method. *Biochemical And Biophysical Research Communications*, 350(4), 818-824.
- Li, H., Wetten, S., Li, L., St Jean, P. L., Upmanyu, R., Surh, L., *et al.* (2008). Candidate single-nucleotide polymorphisms from a genomewide association study of Alzheimer disease. *Archives of Neurology*, 65(1), 45-53.
- Li, P., Kuo, W.-L., Yousef, M., Rosner, M. R., & Tang, W.-J. (2006). The C-terminal domain of human insulin degrading enzyme is required for dimerization and substrate recognition. *Biochemical And Biophysical Research Communications*, 343(4), 1032-1037.

- Li, Q., Ali, M. A., & Cohen, J. I. (2006). Insulin degrading enzyme is a cellular receptor mediating varicella-zoster virus infection and cell-to-cell spread. *Cell*, 127(2), 305-316.
- Li, Y. J., Scott, W. K., Hedges, D. J., Zhang, F., Gaskell, P. C., Nance, M. A., *et al.* (2002). Age at onset in two common neurodegenerative diseases is genetically controlled. *American Journal of Human Genetics*, 70(4), 985-993.
- Liang, K. Y., Mintun, M. A., Fagan, A. M., Goate, A. M., Bugg, J. M., Holtzman, D. M., *et al.* (2010). Exercise and Alzheimer's disease biomarkers in cognitively normal older adults. *Annals of Neurology*, 68(3), 311-318.
- Liang, X., Martin, E. R., Schnetz-Boutaud, N., Bartlett, J., Anderson, B., Züchner, S., *et al.* (2007). Effect of heterogeneity on the chromosome 10 risk in late-onset *alzheimer* disease. *Human Mutation*, 28(11), 1065-1073.
- Liao, L., Cheng, D., Wang, J., Duong, D. M., Losik, T. G., Gearing, M., *et al.* (2004). Proteomic characterization of postmortem amyloid plaques isolated by laser capture microdissection. *Journal of Biological Chemistry*, 279(35), 37061-37068.
- Lin, M. T., & Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*, 443(7113), 787-795.
- Lin, X., Huang, R., Zhang, S., Wei, L., Zhuo, L., Wu, X., *et al.* (2013). Beneficial effects of asiaticoside on cognitive deficits in senescence-accelerated mice. *Fitoterapia*.
- Lindgren, C. M., Mahtani, M. M., Widen, E., McCarthy, M. I., Daly, M. J., Kirby, A., *et al.* (2002). Genomewide search for type 2 diabetes mellitus susceptibility loci in Finnish families: The Botnia study. *American Journal of Human Genetics*, 70(2), 509-516.
- Lindsay, J., Laurin, D., Verreault, R., Hébert, R., Helliwell, B., Hill, G. B., *et al.* (2002). Risk factors for Alzheimer's disease: A prospective analysis from the Canadian Study of Health and Aging. *American Journal of Epidemiology*, 156(5), 445-453.

- Liu, F., Arias-Vásquez, A., Sleegers, K., Aulchenko, Y. S., Kayser, M., Sanchez-Juan, P., *et al.* (2007). A genomewide screen for late-onset *alzheimer* disease in a genetically isolated Dutch population. *American Journal of Human Genetics*, 81(1), 17-31.
- Liu, Y., Guan, H., Beckett, T. L., Juliano, M. A., Juliano, L., Song, E. S., *et al.* (2007). *In vitro* and *in vivo* degradation of A β peptide by peptidases coupled to erythrocytes. *Peptides*, 28(12), 2348-2355.
- Liu, Y., Hua, Q., Lei, H., & Li, P. (2011). Effect of Tong Luo Jiu Nao on A β -degrading enzymes in AD rat brains. *Journal of ethnopharmacology*, 137(2), 1035-1046.
- Liu, Y., Studzinski, C., Beckett, T., Murphy, M. P., Klein, R. L., & Hersh, L. B. (2010). Circulating neprilysin clears brain amyloid. *Molecular and Cellular Neuroscience*, 45(2), 101-107.
- Liu, Y.-H., Giunta, B., Zhou, H.-D., Tan, J., & Wang, Y.-J. (2012(b)). Immunotherapy for Alzheimer disease—the challenge of adverse effects. *Nature Reviews Neurology*, 8(8), 465-469.
- Liu, Z., Zhu, H., Fang, G. G., Walsh, K., Mwamburi, M., Wolozin, B., *et al.* (2012(a)). Characterization of Insulin Degrading Enzyme and Other Amyloid- β Degrading Proteases in Human Serum: A Role in Alzheimer's Disease? *Journal of Alzheimer's Disease*, 29(2), 329-340.
- Llovera, R. E., de Tullio, M., Alonso, L. G., Leissring, M. A., Kaufman, S. B., Roher, A. E., *et al.* (2008). The Catalytic Domain of Insulin-degrading Enzyme Forms a Denaturant-resistant Complex with Amyloid β Peptide IMPLICATIONS FOR ALZHEIMER DISEASE PATHOGENESIS. *Journal of Biological Chemistry*, 283(25), 17039-17048.
- Lokhov, P. G., Moshkovskii, S. A., Ipatova, O. M., & Prozorovskii, V. N. (2004). Cytosolic insulin-binding proteins of mouse liver cells. *Protein and peptide letters*, 11(1), 29-33.
- Luchsinger, J. A., Tang, M., Shea, S., & Mayeux, R. (2004). Hyperinsulinemia and risk of Alzheimer disease. *Neurology*, 63(7), 1187-1192.
- Luchsinger, J. A., Tang, M. X., Stern, Y., Shea, S., & Mayeux, R. (2001). Diabetes mellitus and risk of Alzheimer's disease and dementia with stroke in a multiethnic cohort. *American Journal of Epidemiology*, 154(7), 635-641.

- Lue, L. F., Kuo, Y. M., Roher, A. E., Brachova, L., Shen, Y., Sue, L., *et al.* (1999). Soluble amyloid β peptide concentration as a predictor of synaptic change in Alzheimer's disease. *American Journal of Pathology*, 155(3), 853-862.
- Lui, J. K., Laws, S. M., Li, Q.-X., Villemagne, V. L., Ames, D., Brown, B., *et al.* (2010). Plasma amyloid- β as a biomarker in Alzheimer's disease: the AIBL study of aging. *Journal of Alzheimer's Disease*, 20(4), 1233-1242.
- Lundgren, H., Bengtsson, C., Blohme, G., Lapidus, L., & Waldenstrom, J. (1990). Fasting serum insulin concentration and early insulin response as risk determinants for developing diabetes. *Diabetic Medicine: A Journal of the British Diabetic Association*, 7(5), 407-413.
- Luo, D., Hou, X., Hou, L., Wang, M., Xu, S., Dong, C., *et al.* (2011). Effect of pioglitazone on altered expression of A β metabolism-associated molecules in the brain of fructose-drinking rats, a rodent model of insulin resistance. *European Journal of Pharmacology*, 664(1), 14-19.
- MacKnight, C., Rockwood, K., Awalt, E., & McDowell, I. (2002). Diabetes mellitus and the risk of dementia, Alzheimer's disease and vascular cognitive impairment in the Canadian Study of Health and Aging. *Dementia and Geriatric Cognitive Disorders*, 14(2), 77-83.
- Maechler, P. (2002). Mitochondria as the conductor of metabolic signals for insulin exocytosis in pancreatic β -cells. *Cellular and Molecular Life Sciences*, 59(11), 1803-1818.
- Mafuvadze, B., & Erlwanger, K. H. (2007). The effect of EDTA, heparin and storage on the erythrocyte osmotic fragility, plasma osmolality and haematocrit of adult ostriches (*Struthio camelus*). *Veterinarski arhiv*, 77(5), 427-434.
- Mahley, R. W. (1988). Apolipoprotein E: Cholesterol transport protein with expanding role in cell biology. *Science*, 240(4852), 622-630.
- Mahley, R. W., Weisgraber, K. H., & Huang, Y. (2006). Apolipoprotein E4: A causative factor and therapeutic target in neuropathology, including Alzheimer's disease. *Proceedings of the National Academy of Sciences*, 103(15), 5644-5651.

- Mahtani, M. M., Widen, E., Lehto, M., Thomas, J., McCarthy, M., Brayer, J., *et al.* (1996). Mapping of a gene for type 2 diabetes associated with an insulin secretion defect by a genome scan in Finnish families. *Nature Genetics*, 14(1), 90-94.
- Malito, E., Ralat, L. A., Manolopoulou, M., Tsay, J. L., Wadlington, N. L., & Tang, W. J. (2008). Molecular bases for the recognition of short peptide substrates and cysteine-directed modifications of human insulin-degrading enzyme. *Biochemistry*, 47(48), 12822-12834.
- Manczak, M., Anekonda, T. S., Henson, E., Park, B. S., Quinn, J., & Reddy, P. H. (2006). Mitochondria are a direct site of A β accumulation in Alzheimer's disease neurons: implications for free radical generation and oxidative damage in disease progression. *Human Molecular Genetics*, 15(9), 1437-1449.
- Manelli, A. M., Bulfinch, L. C., Sullivan, P. M., & LaDu, M. J. (2007). Abeta42 neurotoxicity in primary co-cultures: effect of apoE isoform and Abeta conformation. *Neurobiology of Aging*, 28(8), 1139-1147.
- Marfaing-Jallat, P., Portha, B., & Penicaud, L. (1995). Altered conditioned taste aversion and glucose utilization in related brain nuclei of diabetic GK rats. *Brain Research Bulletin*, 37(6), 639-643.
- Marin, R., Marrero-Alonso, J., Fernandez, C., Cury, D., & Diaz, M. (2011). Estrogen receptors in lipid raft signalling complexes for neuroprotection. *Frontiers in bioscience (Elite edition)*, 4, 1420-1433.
- Marlowe, L., Peila, R., Benke, K. S., Hardy, J., White, L. R., Launer, L. J., *et al.* (2006). Insulin-degrading enzyme haplotypes affect insulin levels but not dementia risk. *Neurodegenerative Diseases*, 3(6), 320-326.
- Martin, B. L., Schrader-Fischer, G., Busciglio, J., Duke, M., Paganetti, P., & Yankner, B. A. (1995). Intracellular accumulation of β -amyloid in cells expressing the Swedish mutant amyloid precursor protein. *Journal of Biological Chemistry*, 270(45), 26727-26730.
- Martini, F. H., Ober, W. C., Garrison, C. W., Welch, K., & Hutchings, R. T. (1998). *Fundamentals of Anatomy and Physiology* (4th ed.): New Jersey, USA: Prentice Hall, Inc.

- Martins, R. (2009). Exploring the association between plasma apoE levels and Alzheimer's disease: Australian Imaging, Biomarker and Lifestyle (AIBL) flagship study of ageing. *Alzheimer's and Dementia: The Journal of the Alzheimer's Association*, 5(4), P308-P309.
- Masters, C. L., Multhaup, G., Simms, G., Pottgiesser, J., Martins, R. N., & Beyreuther, K. (1985). Neuronal origin of a cerebral amyloid: neurofibrillary tangles of Alzheimer's disease contain the same protein as the amyloid of plaque cores and blood vessels. *The EMBO Journal*, 4(11), 2757-2763.
- Mattsson, N., Zetterberg, H., Hansson, O., Andreasen, N., Parnetti, L., Jonsson, M., *et al.* (2009). CSF biomarkers and incipient Alzheimer disease in patients with mild cognitive impairment. *JAMA: the journal of the American Medical Association*, 302(4), 385-393.
- Mawuenyega, K. G., Sigurdson, W., Ovod, V., Munsell, L., Kasten, T., Morris, J. C., *et al.* (2010). Decreased clearance of CNS β -amyloid in Alzheimer's disease. *Science*, 330(6012), 1774-1774.
- Mayeux, R., Honig, L. S., Tang, M. X., Manly, J., Stern, Y., Schupf, N., *et al.* (2003). Plasma A β 40 and A β 42 and Alzheimer's disease: Relation to age, mortality, and risk. *Neurology*, 61(9), 1185-1190.
- Mayeux, R., Tang, M. X., Jacobs, D. M., Manly, J., Bell, K., Merchant, C., *et al.* (1999). Plasma amyloid β -peptide 1–42 and incipient Alzheimer's disease. *Annals of Neurology*, 46(3), 412-416.
- McDermott, J. R., & Gibson, A. M. (1997). Degradation of Alzheimer's β -amyloid protein by human and rat brain peptidases: involvement of insulin-degrading enzyme. *Neurochemical Research*, 22(1), 49-56.
- McKee, A. C., Kosik, K. S., & Kowall, N. W. (1991). Neuritic pathology and dementia in Alzheimer's disease. *Annals of Neurology*, 30(2), 156-165.
- McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D., & Stadlan, E. M. (1984). Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's disease. *Neurology*, 34, 939-944.

- McLean, C. A., Cherny, R. A., Fraser, F. W., Fuller, S. J., Smith, M. J., Beyreuther, K., *et al.* (1999). Soluble pool of A β amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. *Annals of Neurology*, 46(6), 860-866.
- McQueen, M. B., Bertram, L., Lange, C., Becker, K. D., Albert, M. S., Tanzi, R. E., *et al.* (2007). Exploring candidate gene associations with neuropsychological performance. *American Journal of Medical Genetics Part B (Neuropsychiatric Genetics)*, 144B(8), 987-991.
- Mega, M. S., Masterman, D. M., O'Connor, S. M., Barclay, T. R., & Cummings, J. L. (1999). The spectrum of behavioral responses to cholinesterase inhibitor therapy in Alzheimer disease. *Archives of Neurology*, 56, 1388-1393.
- Mehta, P. D., Pirttilä, T., Mehta, S. P., Sersen, E. A., Aisen, P. S., & Wisniewski, H. M. (2000). Plasma and cerebrospinal fluid levels of amyloid beta proteins 1-40 and 1-42 in Alzheimer disease. . *Archives of Neurology*, 57(1), 100-105.
- Meigs, J. B., Panhuysen, C. I. M., Myers, R. H., Wilson, P. W. F., & Cupples, L. A. (2002). A genome-wide scan for loci linked to plasma levels of glucose and HbA1c in a community-based sample of Caucasian pedigrees. *Diabetes*, 51(3), 833-840.
- Mensink, R. P., Zock, P. L., Kester, A. D., & Katan, M. B. (2003). Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. *The American journal of clinical nutrition*, 77(5), 1146-1155.
- Mielke, M. M., & Lyketsos, C. G. (2006). Lipids and the pathogenesis of Alzheimer's disease: Is there a link? *International Review of Psychiatry*, 18(2), 173-186.
- Miller, B. C., Eckman, E. A., Sambamurti, K., Dobbs, N., Chow, K. M., Eckman, C. B., *et al.* (2003). Amyloid- β peptide levels in brain are inversely correlated with insulin activity levels in vivo. *Proceedings of the National Academy of Sciences*, 100(10), 6221-6226.
- Miller, D. L., Papayannopoulos, I. A., Styles, J., Bobin, S. A., Lin, Y. Y., Biemann, K., *et al.* (1993). Peptide compositions of the cerebrovascular and senile plaque core amyloid deposits of Alzheimer's disease. . *Archives of Biochemistry and Biophysics*, 301(1), 41-52.

- Milord, E., & Gragnoli, C. (2007). Chromosome 10 locus in linkage to type 2 diabetes in Italians. *Obesity and Metabolism - Milan*, 3(1), 44-47.
- Miners, J. S., Baig, S., Palmer, J., Palmer, L. E., Kehoe, P. G., & Love, S. (2008a). A β -degrading enzymes in Alzheimer's disease. *Brain Pathology*, 18(2), 240-252.
- Miners, J. S., Baig, S., Tayler, H., Kehoe, P. G., & Love, S. (2009). Neprilysin and insulin-degrading enzyme levels are increased in Alzheimer disease in relation to disease severity. *Journal of Neuropathology and Experimental Neurology*, 68(8), 902-914.
- Miners, J. S., Kehoe, P. G., & Love, S. (2008b). Immunocapture-based fluorometric assay for the measurement of insulin-degrading enzyme activity in brain tissue homogenates. *Journal of Neuroscience Methods*, 169(1), 177-181.
- Mirsky, I. A., & Broh-Kahn, R. H. (1949). The inactivation of insulin by tissue extracts; the distribution and properties of insulin inactivating extracts. *Archives of biochemistry*, 20(1), 1-9.
- Misbin, R. I., Almira, E. C., & Cleman, M. W. (1981). Insulin degradation in serum of a patient with apparent insulin resistance. *The Journal of Clinical Endocrinology and Metabolism*, 52(2), 177-180.
- Moffat, S. D., Zonderman, A. B., Metter, E. J., Kawas, C., Blackman, M. R., Harman, S. M., *et al.* (2004). Free testosterone and risk for Alzheimer disease in older men. *Neurology*, 62(2), 188-193.
- Mölsä, P. K., Marttila, R. J., & Rinne, U. K. (1986). Survival and cause of death in Alzheimer's disease and multi-infarct dementia. *Acta Neurologica Scandinavica*, 74(2), 103-107.
- Mooradian, A. D. (2009). Dyslipidemia in type 2 diabetes mellitus. *Nature clinical practice endocrinology & metabolism*, 5(3), 150-159.
- Morelli, L., Llovera, R. E., Mathov, I., Lue, L. F., Frangione, B., Ghiso, J., *et al.* (2004). Insulin-degrading enzyme in brain microvessels. *The Journal of Biological Chemistry*, 279(53), 56004-56013.
- Mori, C., Spooner, E. T., Wisniewski, K. E., Wisniewski, T. M., Yamaguchi, H., Saido, T. C., *et al.* (2002). Intraneuronal A β accumulation in Down syndrome brain. *Amyloid*, 9(2), 88-102.

- Morissette, C., Goulet, J., & Lamoureux, G. (1991). Rapid and sensitive sandwich enzyme-linked immunosorbent assay for detection of staphylococcal enterotoxin B in cheese. *Applied and environmental microbiology*, 57(3), 836-842.
- Morris, M. C., Evans, D. A., Bienias, J. L., Tangney, C. C., Bennett, D. A., Aggarwal, N., *et al.* (2003). Dietary fats and the risk of incident Alzheimer disease. *Archives of Neurology*, 60(2), 194.
- Mukherjee, A., Song, E. S., Kihiko-Ehmann, M., Goodman (Jnr), J. P., St Pyrek, J., Estus, S., *et al.* (2000). Insulysin hydrolyzes amyloid β peptides to products that are neither neurotoxic nor deposit on amyloid plaques. *The Journal of Neuroscience*, 20(23), 8745-8749.
- Müller, D., Baumeister, H., Buck, F., & Richter, D. (1991). Atrial natriuretic peptide (ANP) is a high-affinity substrate for rat insulin-degrading enzyme. *European Journal of Biochemistry*, 202(2), 285-292.
- Muller, M., Tang, M. X., Schupf, N., Manly, J. J., Mayeux, R., & Luchsinger, J. A. (2007). Metabolic syndrome and dementia risk in a multiethnic elderly cohort. *Dementia and Geriatric Cognitive Disorders*, 24(3), 185-192.
- Myers, A., Holmans, P., Marshall, H., Kwon, J., Meyer, D., Ramic, D., *et al.* (2000). Susceptibility locus for Alzheimer's disease on chromosome 10. . *Science*, 290(5500), 2304-2305.
- Namba, Y., Tomonaga, M., Kawasaki, H., Otomo, E., & Ikeda, K. (1991). Apolipoprotein E immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in Alzheimer's disease and kuru plaque amyloid in Creutzfeldt-Jakob disease. *Brain Research*, 541(1), 163-166.
- Nathan, B. P., Bellosta, S., Sanan, D. A., Weisgraber, K. H., Mahley, R. W., & Pitas, R. E. (1994). Differential effects of apolipoproteins E3 and E4 on neuronal growth *in vitro*. *Science*, 264(5160), 850-852.
- Neant-Fery, M., Garcia-Ordoñez, R. D., Logan, T. P., Selkoe, D. J., Li, L., Reinstatler, L., *et al.* (2008). Molecular basis for the thiol sensitivity of insulin-degrading enzyme. *Proceedings of the National Academy of Sciences*, 105(28), 9582-9587.

- Nelson, P. T., Alafuzoff, I., Bigio, E. H., Bouras, C., Braak, H., Cairns, N. J., *et al.* (2012). Correlation of Alzheimer disease neuropathologic changes with cognitive status: a review of the literature. *Journal of Neuropathology & Experimental Neurology*, 71(5), 362.
- Noinaj, N., Bhasin, S. K., Song, E. S., Scoggin, K. E., Juliano, M. A., Juliano, L., *et al.* (2011(a)). Identification of the allosteric regulatory site of insulysin. *PLoS ONE*, 6(6), e20864.
- Nordberg, A. (2004). Is amyloid plaque imaging the key to monitoring brain pathology of Alzheimer's disease in vivo? *European Journal of Nuclear Medicine and Molecular Imaging*, 31(11), 1540-1543.
- Notkola, I. L., Sulkava, R., Pekkanen, J., Erkinjuntti, T., Ehnholm, c., Kivinen, P., *et al.* (1998). Serum total cholesterol, apolipoprotein E epsilon 4 allele, and Alzheimer's disease. *Neuroepidemiology*, 17(1), 14-20.
- Nowotny, P., Hinrichs, A. L., Smemo, S., Kauwe, J. S. K., Maxwell, T., Holmans, P., *et al.* (2005). Association studies between risk for late-onset *alzheimer's* disease and variants in insulin degrading enzyme. *American Journal of Medical Genetics Part B (Neuropsychiatric Genetics)*, 136B(1), 62-68.
- O'Brien, J. T., Beats, B., Hill, K., Howard, R., Sahakian, B., & Levy, R. (1992). Do subjective memory complaints precede dementia - a 3-year follow-up of patients with supposed benign senescent forgetfulness. *International Journal of Geriatric Psychiatry*, 7(7), 481-486.
- O'Brien, T. D., Butler, A. E., Roche, P. C., Johnson, K. H., & Butler, P. C. (1994). Islet amyloid polypeptide in human insulinomas: evidence for intracellular amyloidogenesis. *Diabetes*, 43(2), 329-336.
- O'Brien, T. D., Butler, P. C., Kreutter, D. K., Kane, L. A., & Eberhardt, N. L. (1995). Human islet amyloid polypeptide expression in COS-1 cells. A model of intracellular amyloidogenesis. *American Journal of Pathology*, 147(3), 609-616.
- O'Bryant, S. E., Xiao, G., Barber, R., Reisch, J., Doody, R., Fairchild, T., *et al.* (2010). A serum protein-based algorithm for the detection of Alzheimer disease. *Archives of Neurology*, 67(9), 1077.

- Oddo, S., Caccamo, A., Shepherd, J. D., Murphy, M. P., Golde, T. E., Kaye, R., *et al.* (2003). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular A β and synaptic dysfunction. *Neuron*, 39(3), 409-421.
- Okello, A., Koivunen, J., Edison, P., Archer, H. A., Turkheimer, F. E., Nagren, K., *et al.* (2009). Conversion of amyloid positive and negative MCI to AD over 3 years. An C-11-PIB PET study. *Neurology*, 73(10), 754-760.
- Okereke, O. I., Xia, W., Selkoe, D. J., & Grodstein, F. (2009). Ten-Year Change in Plasma Amyloid β Levels and Late-Life Cognitive Decline. *Archives of Neurology*, 66(10), 1247.
- Ott, A., Stolk, R. P., Hofman, A., van Harskamp, F., Grobbee, D. E., & Breteler, M. M. (1996). Association of diabetes mellitus and dementia: the Rotterdam Study. *Diabetologia*, 39(11), 1392-1397.
- Ott, A., Stolk, R. P., van Harskamp, F., Pols, H. A. P., Hofman, A., & Breteler, M. M. B. (1999). Diabetes mellitus and the risk of dementia. *Neurology*, 53(9), 1937-1942.
- Ou, Y., Lin, L., Yang, X., Pan, Q., & Cheng, X. (2013). Antidiabetic potential of phycocyanin: Effects on KKAy mice. *Pharmaceutical biology*, 51(5), 539-544.
- Ozturk, A., DeKosky, S. T., & Kamboh, M. I. (2006). Lack of association of 5 SNPs in the vicinity of the insulin-degrading enzyme (IDE) gene with late-onset Alzheimer's disease. *Neuroscience Letters*, 406(3), 265-269.
- Paganini-Hill, A., & Clark, L. J. (2000). Preliminary assessment of cognitive function in breast cancer patients treated with tamoxifen. *Breast cancer research and treatment*, 64(2), 165-176.
- Pappolla, M. A., Bryant-Thomas, T. K., Herbert, D., Pacheco, J., Fabra Garcia, M., Manjon, M., *et al.* (2003). Mild hypercholesterolemia is an early risk factor for the development of Alzheimer amyloid pathology. *Neurology*, 61(2), 199-205.
- Parachikova, A., Agadjanyan, M. G., Cribbs, D. H., Blurton-Jones, M., Perreau, V., Rogers, J., *et al.* (2007). Inflammatory changes parallel the early stages of Alzheimer disease. *Neurobiology of Aging*, 28(12), 1821-1833.

- Park, C. R., Seeley, R. J., Craft, S., & Woods, S. C. (2000). Intracerebroventricular insulin enhances memory in a passive-avoidance task. *Physiology and Behaviour*, 68(4), 509-514.
- Parmentier, N., Stroobant, V., Colau, D., De Diesbach, P., Morel, S., Chapiro, J., *et al.* (2010). Production of an antigenic peptide by insulin-degrading enzyme. *Nature immunology*, 11(5), 449-454.
- Patil, S., Melrose, J., & Chan, C. (2007). Involvement of astroglial ceramide in palmitic acid induced Alzheimer like changes in primary neurons. *European Journal of Neuroscience*, 26(8), 2131-2141.
- Patil, S. P., Maki, S., Khedkar, S. A., Rigby, A. C., & Chan, C. (2010). Withanolide A and asiatic acid modulate multiple targets associated with amyloid- β precursor protein processing and amyloid- β protein clearance. *Journal of natural products*, 73(7), 1196-1202.
- Pearce, R., Greenway, D., & Parkinson, A. (1992). Species differences and interindividual variation in liver microsomal cytochrome P450 2A enzymes: effects on coumarin, dicumarol, and testosterone oxidation. *Archives of Biochemistry and Biophysics*, 298(1), 211-225.
- Peila, R., Rodriguez, B. L., & Launer, L. J. (2002). Type 2 Diabetes, APOE Gene, and the risk for dementia and related pathologies. The Honolulu-Asia Aging Study. *Diabetes*, 51(2002), 1256-1262.
- Pérez, A., Morelli, L., Cresto, J. C., & Castaño, E. M. (2000). Degradation of soluble amyloid β -peptides 1-40, 1-42, and the Dutch variant 1-40Q by insulin degrading enzyme from Alzheimer disease and control brains. *Neurochemical Research*, 25(2), 247-255.
- Perlman, R. K., & Rosner, M. R. (1994). Identification of zinc ligands of the insulin-degrading enzyme. *The Journal of Biological Chemistry*, 269(52), 33140-33145.
- Peskind, E. R., Wilkinson, C. W., Petrie, E. C., Schellenberg, G. D., & Raskind, M. A. (2001). Increased CSF cortisol in AD is a function of APOE genotype. *Neurology*, 56(8), 1094-1098.

- Petersen, K. F., Dufour, S., Befroy, D., Garcia, R., & Shulman, G. I. (2004). Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *The New England Journal of Medicine*, 350(7), 664-671.
- Petersen, R. C., Smith, G. E., Waring, S. C., Ivnik, R. J., Tangalos, E. G., & Kokmen, E. (1999). Mild cognitive impairment: Clinical characterization and outcome. *Archives of Neurology*, 56, 303-308.
- Phiel, C. J., Wilson, C. A., Lee, V. M. Y., & Klein, P. S. (2003). GSK-3 α regulates production of Alzheimer's disease amyloid- β peptides. *Nature*, 423(6938), 435-439.
- Phillips, K.-A., Aldridge, J., Ribb, K., Sun, Z., Thompson, A., Harvey, V., *et al.* (2011). Cognitive function in postmenopausal breast cancer patients one year after completing adjuvant endocrine therapy with letrozole and/or tamoxifen in the BIG 1-98 trial. *Breast cancer research and treatment*, 126(1), 221-226.
- Phillips, K.-A., Ribb, K., Sun, Z., Stephens, A., Thompson, A., Harvey, V., *et al.* (2010). Cognitive function in postmenopausal women receiving adjuvant letrozole or tamoxifen for breast cancer in the BIG 1-98 randomized trial. *The Breast*, 19(5), 388-395.
- Pike, C. J., Burdick, D., Walencewicz, A. J., Glabe, C. G., & Cotman, C. W. (1993). Neurodegeneration induced by β -amyloid peptides *in vitro*: The role of peptide assembly state. *The Journal of Neuroscience*, 13(4), 1676-1687.
- Pike, C. J., Walencewicz, A. J., Glabe, C. G., & Cotman, C. W. (1991). In vitro aging of beta-amyloid protein causes peptide aggregation and neurotoxicity. *Brain Research*, 563(1-2), 311-314.
- Pike, K. E., Savage, G., Villemagne, V. L., Ng, S., Moss, S. A., Maruff, P., *et al.* (2007). Beta-amyloid imaging and memory in non-demented individuals: evidence for preclinical Alzheimer's disease. . *Brain: A Journal of Neurology*, 130(11), 2837-2844.
- Pivovarov, O., Gögebakan, Ö., Pfeiffer, A., & Rudovich, N. (2009). Glucose inhibits the insulin-induced activation of the insulin-degrading enzyme in HepG2 cells. *Diabetologia*, 52(8), 1656-1664.

- Poirier, R., Wolfer, D. P., Welzl, H., Tracy, J., Galsworthy, M. J., Nitsch, R. M., *et al.* (2006). Neuronal neprilysin overexpression is associated with attenuation of A β -related spatial memory deficit. *Neurobiology of disease*, 24(3), 475-483.
- Portelius, E., Zetterberg, H., Gobom, J., Andreasen, U., & Blennow, K. (2008). Targeted proteomics in Alzheimer's disease: focus on amyloid- β . *Expert Review of Proteomics*, 5(2), 225-237.
- Prasad, T. K., Goel, R., Kandasamy, K., Keerthikumar, S., Kumar, S., Mathivanan, S., *et al.* (2009). Human protein reference database—2009 update. *Nucleic Acids Research*, 37(suppl 1), D767-D772.
- Prasanthi, R. P. J., Schommer, E., Thomasson, S., Thompson, A., Feist, G., & Ghribi, O. (2008). Regulation of beta-amyloid levels in the brain of cholesterol-fed rabbit, a model system for sporadic Alzheimer's disease. *Mechanisms of Ageing and Development*, 129(11), 649-655.
- Preston, S. D., Steart, P. V., Wilkinson, A., Nicoll, J. A. R., & Weller, R. O. (2003). Capillary and arterial cerebral amyloid angiopathy in Alzheimer's disease: defining the perivascular route for the elimination of amyloid β from the human brain. *Neuropathology and Applied Neurobiology*, 29(2), 106-117.
- Prince, J. A., Feuk, L., Gu, H. F., Johansson, B., Gatz, M., Blennow, K., *et al.* (2003). Genetic variation in a haplotype block spanning IDE influences Alzheimer disease. *Human Mutation*, 22(5), 363-371.
- Qin, W., & Jia, J. (2008). Down-regulation of insulin-degrading enzyme by presenilin 1 V97L mutant potentially underlies increased levels of amyloid beta 42. *The European Journal of Neuroscience*, 27(9), 2425-2432.
- Qiu, W. Q., & Folstein, M. F. (2006). Insulin, insulin-degrading enzyme and amyloid- β peptide in Alzheimer's disease: review and hypothesis. *Neurobiology of Aging*, 27(2), 190-198.
- Qiu, W. Q., Walsh, D. M., Ye, Z., Vekrellis, K., Zhang, J., Podlisny, M. B., *et al.* (1998). Insulin-degrading enzyme regulates extracellular levels of amyloid β -protein by degradation. *The Journal of Biological Chemistry*, 273(49), 32730-32738.

- Qiu, W. Q., Ye, Z., Kholodenko, D., Seubert, P., & Selkoe, D. J. (1997). Degradation of amyloid β -protein by a metalloprotease secreted by microglia and other neural and non-neural cells. *The Journal of Biological Chemistry*, 272(10), 6641-6646.
- Quan, Q., Wang, J., Li, X., & Wang, Y. (2013). Ginsenoside Rg1 Decreases A β 1–42 Level by Upregulating PPAR γ and IDE Expression in the Hippocampus of a Rat Model of Alzheimer's Disease. *PLoS ONE*, 8(3), e59155.
- Ralat, L. A., Kalas, V., Zheng, Z., Goldman, R. D., Sosnick, T. R., & Tang, W.-J. (2011). Ubiquitin is a novel substrate for human insulin-degrading enzyme. *Journal of Molecular Biology*, 406(3), 454-466.
- Ray, S., Britschgi, M., Herbert, C., Takeda-Uchimura, Y., Boxer, A., Blennow, K., *et al.* (2007). Classification and prediction of clinical Alzheimer's diagnosis based on plasma signaling proteins. *Nature Medicine*, 13(11), 1359-1362.
- Rebeck, G. W., Reiter, J. S., Strickland, D. K., & Hyman, B. T. (1993). Apolipoprotein E in sporadic Alzheimer's disease: allelic variation and receptor interactions. *Neuron*, 11(4), 575-580.
- Reddy, P. H., & Beal, M. F. (2008). Amyloid beta, mitochondrial dysfunction and synaptic damage: implications for cognitive decline in aging and Alzheimer's disease. *Trends in molecular medicine*, 14(2), 45.
- Reger, M. A., Watson, G. S., Baker, L. D., Cholerton, B., Fishel, M. A., Plymate, S. R., *et al.* (2008(a)). Intranasal insulin administration dose-dependently modulates verbal memory and plasma amyloid-beta in memory-impaired older adults. *Journal of Alzheimer's Disease*, 13(3), 323-331.
- Reger, M. A., Watson, G. S., Frey (2nd), W. H., Baker, L. D., Cholerton, B., Keeling, M. L., *et al.* (2006). Effects of intranasal insulin on cognition in memory-impaired older adults: modulation by APOE genotype. *Neurobiology of Aging*, 27(3), 451-458.
- Reger, M. A., Watson, G. S., Green, P. S., Wilkinson, C. W., Baker, L. D., Cholerton, B., *et al.* (2008(b)). Intranasal insulin improves cognition and modulates beta-amyloid in early AD. *Neurology*, 70(6), 440-448.

- Reiman, E. M., Chen, K., Liu, X., Bandy, D., Yu, M., Lee, W., *et al.* (2009). Fibrillar amyloid- β burden in cognitively normal people at 3 levels of genetic risk for Alzheimer's disease. *Proceedings of the National Academy of Sciences*, 106(16), 6820-6825.
- Reiman, E. M., Webster, J. A., Myers, A. J., Hardy, J., Dunckley, T., Zismann, V. L., *et al.* (2007). GAB2 alleles modify Alzheimer's risk in APOE ϵ 4 carriers. *Neuron*, 54(5), 713-720.
- Reinhart, P. H., Kaltenbach, L. S., Essrich, C., Dunn, D. E., Eudailey, J. A., DeMarco, C. T., *et al.* (2011). Identification of anti-inflammatory targets for Huntington's disease using a brain slice-based screening assay. *Neurobiology of Disease*, 43(1), 248-256.
- Reitz, C., Tang, M.-X., Schupf, N., Manly, J. J., Mayeux, R., & Luchsinger, J. A. (2010). Association of higher levels of high-density lipoprotein cholesterol in elderly individuals and lower risk of late-onset *alzheim*er disease. *Archives of Neurology*, 67(12), 1491.
- Rembach, A., Faux, N. G., Watt, A. D., Pertile, K. K., Rumble, R. L., Trounson, B. O., *et al.* (2013). Changes in plasma amyloid beta in a longitudinal study of aging and Alzheimer's disease. *Alzheimer's & Dementia*.
- Rembach, A., Faux, N. G., Watt, A. D., Pertile, K. K., Rumble, R. L., Trounson, B. O., *et al.* (2014). Changes in plasma amyloid beta in a longitudinal study of aging and Alzheimer's disease. *Alzheimer's & Dementia*, 10(1), 53-61.
- Rissman, R. A., Trojanowski, J. Q., Shaw, L. M., & Aisen, P. S. (2012). Longitudinal plasma amyloid beta as a biomarker of Alzheimer's disease. *Journal of Neural Transmission*, 1-8.
- Rizzo, F., Riboldi, G., Salani, S., Nizzardo, M., Simone, C., Corti, S., *et al.* (2013). Cellular therapy to target neuroinflammation in amyotrophic lateral sclerosis. *Cellular and Molecular Life Sciences*, 1-17.
- Roberts, S. B., MacLean, C. J., Neale, M. C., Eaves, L. J., & Kendler, K. S. (1999). Replication of linkage studies of complex traits: An examination of variation in location estimates. *American Journal of Human Genetics*, 65(3), 876-884.

- Roeschlau, P., Bernt, E., & Gruber, W. (1974). Enzymatic determination of total cholesterol in serum. *Zeitschrift für klinische Chemie und klinische Biochemie*, 12(5), 226.
- Roher, A. E., Chaney, M. O., Kuo, Y. M., Webster, S. D., Stine, W. B., Haverkamp, L. J., *et al.* (1996). Morphology and toxicity of A β -(1–42) dimer derived from neuritic and vascular amyloid deposits of Alzheimer's disease. *The Journal of Biological Chemistry*, 271(34), 20631-20635.
- Roher, A. E., Esh, C. L., Kokjohn, T. A., Castaño, E. M., Van Vickle, G. D., Kalback, W. M., *et al.* (2009). Amyloid beta peptides in human plasma and tissues and their significance for Alzheimer's disease. *Alzheimer's and Dementia*, 5(1), 18-29.
- Roher, A. E., Kuo, Y. M., Kokjohn, K. M., Emmerling, M. R., & Gracon, S. (1999). Amyloid and lipids in the pathology of Alzheimer disease. *Amyloid*, 6(2), 136-145.
- Romay, C., Gonzalez, R., Ledon, N., Ramirez, D., & Rimbau, V. (2003). C-phycocyanin: a biliprotein with antioxidant, anti-inflammatory and neuroprotective effects. *Current protein and peptide science*, 4(3), 207-216.
- Rosvall, L., Rizzuto, D., Wang, H. X., Winblad, B., Graff, C., & Fratiglioni, L. (2009). APOE-related mortality: Effect of dementia, cardiovascular disease and gender. *Neurobiology of Aging*, 30(10), 1545-1552.
- Roth, R. A., Mesirow, M. L., Yokono, K., & Baba, S. (1984). Degradation of insulin-like growth factors I and II by a human insulin degrading enzyme. *Endocrine Research*, 10(2), 101-112.
- Ryan, K. A., & Pimplikar, S. W. (2005). Activation of GSK-3 and phosphorylation of CRMP2 in transgenic mice expressing APP intracellular domain. *The Journal of Cell Biology*, 171(2), 327-335.
- Safavi, A., Miller, B., Cottam, L., & Hersh, L. B. (1996). Identification of γ -endorphin-generating enzyme as insulin-degrading enzyme. *Biochemistry*, 35(45), 14318-14325.
- Sagare, A., Deane, R., Bell, R. D., Johnson, B., Hamm, K., Pendu, R., *et al.* (2007). Clearance of amyloid- β by circulating lipoprotein receptors. *Nature Medicine*, 13(9), 1029-1031.

- Sakai, A., Ujike, H., Nakata, K., Takehisa, Y., Imamura, T., Uchida, N., *et al.* (2004). No association between the insulin degrading enzyme gene and Alzheimer's disease in a Japanese population. *American Journal of Medical Genetics Part B (Neuropsychiatric Genetics)*, 125B(1), 87-91.
- Saric, T., Muller, D., Seitz, H. J., & Pavelic, K. (2003). Non-covalent interaction of ubiquitin with insulin-degrading enzyme. *Molecular and Cellular Endocrinology*, 204(1-2), 11-20.
- Saunders, A. M., Schmader, K., Breitner, J. C. S., Benson, M. D., Brown, W. T., Goldfarb, L., *et al.* (1993). Apolipoprotein E ϵ 4 allele distributions in late-onset Alzheimer's disease and in other amyloid-forming diseases. *The Lancet*, 342(8873), 710-711.
- Savage, M. J., Trusko, S. P., Howland, D. S., Pinsky, L. R., Mistretta, S., Reaume, A. G., *et al.* (1998). Turnover of amyloid β -protein in mouse brain and acute reduction of its level by phorbol ester. *The Journal of Neuroscience*, 18(5), 1743-1752.
- Scacchi, R., Gambina, G., Ruggeri, M., Martini, M. C., Ferrari, G., Silvestri, M., *et al.* (1999). Plasma levels of apolipoprotein E and genetic markers in elderly patients with Alzheimer's disease. *Neuroscience Letters*, 259(1), 33-36.
- Scahill, R. I., Schott, J. M., Stevens, J. M., Rossor, M. N., & Fox, N. C. (2002). Mapping the evolution of regional atrophy in Alzheimer's disease: Unbiased analysis of fluid-registered serial MRI. *Proceedings of the National Academy of Sciences*, 99(7), 4703-4707.
- Schechter, R., Holtzman, L., Sadiq, F., Kahn, A., & Devaskar, S. (1988). Insulin synthesis by isolated rabbit neurons. *Endocrinology*, 123(1), 505-513.
- Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., *et al.* (1996). Secreted amyloid β -protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nature Medicine*, 2(8), 864-870.

- Schiele, F., De Bacquer, D., Vincent-Viry, M., Beisiegel, U., Ehnholm, C., Evans, A., *et al.* (2000). Apolipoprotein E serum concentration and polymorphism in six European countries: the ApoEurope Project. *Atherosclerosis*, 152(2), 475-488.
- Schilder, C. M., Seynaeve, C., Beex, L. V., Boogerd, W., Linn, S. C., Gundy, C. M., *et al.* (2010). Effects of tamoxifen and exemestane on cognitive functioning of postmenopausal patients with breast cancer: results from the neuropsychological side study of the tamoxifen and exemestane adjuvant multinational trial. *Journal of Clinical Oncology*, 28(8), 1294-1300.
- Schmand, B., Jonker, C., Hooijer, C., & Lindeboom, J. (1996). Subjective memory complaints may announce dementia. *Neurology*, 46(1), 121-125.
- Schmidtke, K., & Hermeneit, S. (2008). High rate of conversion to Alzheimer's disease in a cohort of amnesic MCI patients. *International Psychogeriatrics*, 20(1), 96-108.
- Schooling, C. M., Yeung, S. L. A., Freeman, G., & Cowling, B. J. (2013). The effect of statins on testosterone in men and women, a systematic review and meta-analysis of randomized controlled trials. *BMC medicine*, 11(1), 1-9.
- Schupf, N., Tang, M. X., Fukuyama, H., Manly, J., Andrews, H., Mehta, P., *et al.* (2008). Peripheral A β subspecies as risk biomarkers of Alzheimer's disease. *Proceedings of the National Academy of Sciences*, 105(37), 14052-14057.
- Sehgal, N., Gupta, A., Valli, R. K., Joshi, S. D., Mills, J. T., Hamel, E., *et al.* (2012). Withania somnifera reverses Alzheimer's disease pathology by enhancing low-density lipoprotein receptor-related protein in liver. *Proceedings of the National Academy of Sciences*, 109(9), 3510-3515.
- Selkoe, D. J. (1994). Alzheimer's disease: a central role for amyloid. *Journal of Neuropathology and Experimental Neurology*, 53(5), 438-447.
- Selkoe, D. J. (2001). Clearing the brain's amyloid cobwebs. *Neuron*, 32, 177-180.
- Selkoe, D. J. (2011). Resolving controversies on the path to Alzheimer's therapeutics. *Nature*, 17(9), 1060-1065.
- Seta, K. A., & Roth, R. A. (1997). Overexpression of insulin degrading enzyme: cellular localization and effects on insulin signaling. *Biochemical And Biophysical Research Communications*, 231(1), 167-171.

- Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., *et al.* (1992). Isolation and quantification of soluble Alzheimer's β -peptide from biological fluids. *Nature*, 359(6393), 325-327.
- Shah, V. P., Midha, K. K., Dighe, S., McGilveray, I. J., Skelly, J. P., Yacobi, A., *et al.* (1992). Analytical methods validation: bioavailability, bioequivalence, and pharmacokinetic studies. *Journal of pharmaceutical sciences*, 81(3), 309-312.
- Sharma, S., Prasanthi, R. P. J., Schommer, E., Feist, G., & Ghribi, O. (2008). Hypercholesterolemia-induced A β accumulation in rabbit brain is associated with alteration in IGF-1 signaling. *Neurobiology of Disease*, 32(3), 426-432.
- Shearer, J. D., Coulter, C. F., Engeland, W. C., Roth, R. A., & Caldwell, M. D. (1997). Insulin is degraded extracellularly in wounds by insulin-degrading enzyme. *American Journal of Physiology: Endocrinology and Metabolism*, 273(4), E657-E664.
- Shen, Y. (2006). Structures of human insulin-degrading enzyme reveal a new substrate recognition mechanism. *Nature*, 443(7113), 870-874.
- Shen, Y. (2006). University of Chigaco. Retrieved August 13, 2009, from Argonne National Laboratory website: <http://www.aps.anl.gov/Science/Highlights/2006/20061012.htm>
- Sheng, J. G., Mrak, R. E., & Griffin, W. S. T. (1996). Apolipoprotein E distribution among different plaque types in Alzheimer's disease: implications for its role in plaque progression. *Neuropathology and Applied Neurobiology*, 22, 334-341.
- Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E. A., Levesque, G., Ikeda, M., *et al.* (1995). Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature*, 375(6534), 754-760.
- Shibata, M., Yamada, S., Kumar, S. R., Calero, M., Bading, J., Frangione, B., *et al.* (2000). Clearance of Alzheimer's amyloid- β 1-40 peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier. *The Journal of Clinical Investigation*, 106(12), 1489-1499.
- Shimizu, E., Kawahara, K., Kajizono, M., Sawada, M., & Nakayama, H. (2008). IL-4-Induced Selective Clearance of Oligomeric β -Amyloid Peptide1-42 by Rat Primary Type 2 Microglia. *The Journal of Immunology*, 181(9), 6503-6513.

- Shin, J., Lee, S. Y., Kim, S. H., Kim, Y. B., & Cho, S. J. (2008). Multitracer PET imaging of amyloid plaques and neurofibrillary tangles in Alzheimer's disease . *Neuroimage*, 43(2), 236-244.
- Shinall, H., Song, E. S., & Hersh, L. B. (2005). Susceptibility of amyloid β peptide degrading enzymes to oxidative damage: A potential Alzheimer's disease spiral. *Biochemistry*, 44(46), 15345-15350.
- Siest, G., Bertrand, P., Qin, B., Herbeth, B., Serot, J. M., Masana, L., *et al.* (2000). Apolipoprotein E polymorphism and serum concentration in Alzheimer's disease in nine European centres: The ApoEurope Study. *Clinical Chemistry and Laboratory Medicine*, 38(8), 721-730.
- Sinclair, A. J., Girling, A. J., & Bayer, A. J. (2000). Cognitive dysfunction in older subjects with diabetes mellitus: impact on diabetes self-management and use of care services. All Wales Research into Elderly (AWARE) Study. *Diabetes Research and Clinical Practice*, 50(3), 203-212.
- Sivilia, S., Lorenzini, L., Giuliani, A., Gusciglio, M., Fernandez, M., Baldassarro, V. A., *et al.* (2013). Multi-target action of the novel anti-Alzheimer compound CHF5074: in vivo study of long term treatment in Tg2576 mice. *BMC neuroscience*, 14(1), 1-14.
- Sladek, R., Rocheleau, G., Rung, J., Dina, C., Shen, L., Serre, D., *et al.* (2007). A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature*, 445(7130), 881-885.
- Slooter, A. J., de Knijff, P., Hofman, A., Cruts, M., Breteler, M. M., Van Broeckhoven, C., *et al.* (1998). Serum apolipoprotein E level is not increased in Alzheimer's disease: the Rotterdam study. *Neuroscience Letters*, 248(1), 21-24.
- Snyder, S. W., Ladrer, U. S., Wade, W. S., Wang, G. T., Barrett, L. W., Matayoshi, E. D., *et al.* (1994). Amyloid- β aggregation: Selective inhibition of aggregation in mixtures of amyloid with different chain lengths. *Biophysical Journal*, 67(3), 1216-1228.
- Soares, H. D., Potter, W. Z., Pickering, E., Kuhn, M., Immermann, F. W., Shera, D. M., *et al.* (2012). Plasma biomarkers associated with the Apolipoprotein E genotype and Alzheimer's disease. *Archives of Neurology*, 69(10), 1310-1317.

- Sokolova, A., Hill, M. D., Rahimi, F., Warden, L. A., Halliday, G. M., & Shepherd, C. E. (2009). Monocyte chemoattractant protein-1 plays a dominant role in the chronic inflammation observed in Alzheimer's disease. *Brain Pathology*, 19(3), 392-398.
- Song, E. S., Daily, A. D., Fried, M. G., Juliano, M. A., Juliano, L., & Hersh, L. B. (2005). Mutation of active site residues of insulin-degrading enzyme alters allosteric interactions. *The Journal of Biological Chemistry*, 280(18), 17701-17706.
- Song, E. S., & Hersh, L. B. (2005). Insulysin - An allosteric enzyme as a target for Alzheimer's disease. *Journal of Molecular Neuroscience*, 25(3), 201-206.
- Song, E. S., Juliano, M. A., Juliano, L., & Hersh, L. B. (2003). Substrate activation of insulin-degrading enzyme (insulysin). *The Journal of Biological Chemistry*, 278(50), 49789-49794.
- Song, E. S., Rodgers, D. W., & Hersh, L. B. (2010). A monomeric variant of insulin degrading enzyme (IDE) loses its regulatory properties. *PLoS ONE*, 5(3), e9719.
- Song, F., Poljak, A., Valenzuela, M., Mayeux, R., Smythe, G. A., & Sachdev, P. S. (2011). Meta-analysis of plasma amyloid- β levels in Alzheimer's disease. *Journal of Alzheimer's Disease*, 26(2), 365-375.
- Spencer, B., Marr, R. A., Rockenstein, E., Crews, L., Adame, A., Potkar, R., *et al.* (2008). Long-term neprilysin gene transfer is associated with reduced levels of intracellular A β and behavioral improvement in APP transgenic mice. *BMC neuroscience*, 9(1), 109.
- Stargardt, A., Gillis, J., Kamphuis, W., Wiemhoefer, A., Kooijman, L., Raspe, M., *et al.* (2013). Reduced amyloid- β degradation in early Alzheimer's disease but not in the APPswePS1dE9 and 3xTg-AD mouse models. *Aging cell*.
- Steen, E., Terry, B. M., Rivera, E. J., Cannon, J. L., Neely, T. R., Tavares, R., *et al.* (2005). Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes? *Journal of Alzheimer's Disease*, 7(1), 63-80.
- Stelzmann, R. A., Schnitzlein, H. N., & Murtagh, F. R. (1995). An English translation of Alzheimer's 1907 paper, "Über eine eigenartige Erkankung der Hirnrinde". *Clinical Anatomy*, 8, 429-431.

- Stewart, R., & Liolitsa, D. (1999). Type 2 diabetes mellitus, cognitive impairment and dementia. *Diabetic Medicine*, 16(2), 93-112.
- Stine, W. B., Dahlgren, K. N., Krafft, G. A., & LaDu, M. J. (2003). *In vitro* characterization of conditions for amyloid- β peptide oligomerization and fibrillogenesis. *The Journal of Biological Chemistry*, 278(13), 11612-11622.
- Stolk, R. P., Breteler, M. M. B., Ott, A., Pols, H. A. P., Lamberts, S. W. J., Grobbee, D. E., *et al.* (1997). Insulin and cognitive function in an elderly population. The Rotterdam Study. *Diabetes Care*, 20(5), 792-795.
- Strittmatter, W. J., Saunders, A. M., Schmechel, D. J., Pericak-Vance, M. A., Enghild, J., Salvesen, G. S., *et al.* (1993(a)). Apolipoprotein E: High-avidity binding to B-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proceedings of the National Academy of Sciences*, 90(5), 1977-1981.
- Strittmatter, W. J., Weisgraber, K. H., Huang, D. Y., Dong, L. M., Salvesen, G. S., Pericak-Vance, M. A., *et al.* (1993(b)). Binding of human apolipoprotein E to synthetic amyloid B peptide: Isoform-specific effects and implications for late-onset Alzheimer disease. *Proceedings of the National Academy of Sciences*, 90(17), 8098-8102.
- Su, A. I., Cooke, M. P., Ching, K. A., Hakak, Y., Walker, J. R., Wiltshire, T., *et al.* (2002). Large-scale analysis of the human and mouse transcriptomes. *Proceedings of the National Academy of Sciences*, 99(7), 4465-4470.
- Sudoh, S., Frosch, M. P., & Wolf, B. A. (2002). Differential effects of proteases involved in intracellular degradation of amyloid β -protein between detergent-soluble and -insoluble pools in CHO-695 cells. *Biochemistry*, 41(4), 1091-1099.
- Sutcliffe, J. G., Hedlund, P. B., Thomas, E. A., Bloom, F. E., & Hilbush, B. S. (2011). Peripheral reduction of β -amyloid is sufficient to reduce brain β -amyloid: Implications for Alzheimer's disease. *Journal of neuroscience research*, 89(6), 808-814.
- Suzuki, N., Cheung, T. T., Cai, X. D., Odaka, A., Otvos Jr., L., Eckman, C., *et al.* (1994). An increased percentage of long amyloid Beta protein secreted by familial amyloid Beta protein precursor mutants. *Science*, 264(5163), 1336-1340.

- Szoeke, C., Ellis, K. A., Bush, A., Darby, D., De Fazio, D., Foster, J., *et al.* (2009). The AIBL study: Baseline data from a multicenter, prospective longitudinal study of ageing in 1,100 volunteers. *Alzheimer's and Dementia: The Journal of the Alzheimer's Association*, 5(4), P184-P185.
- T Ferretti, M., Partridge, V., C Leon, W., Canneva, F., Allard, S., N Arvanitis, D., *et al.* (2011). Transgenic Mice as a Model of Pre-Clinical Alzheimers Disease. *Current Alzheimer Research*, 8(1), 4-23.
- Taddei, K., Clarnette, R., Gandy, S. E., & Martins, R. N. (1997). Increased plasma apolipoprotein E (apoE) levels in Alzheimer's disease. *Neuroscience Letters*, 223(1), 29-32.
- Takata, K., Hirata-Fukae, C., Becker, A. G., Chishiro, S., Gray, A. J., Nishitomi, K., *et al.* (2007). Deglycosylated anti-amyloid beta antibodies reduce microglial phagocytosis and cytokine production while retaining the capacity to induce amyloid beta sequestration. *European Journal of Neuroscience*, 26(9), 2458-2468.
- Talbot, K., Wang, H.-Y., Kazi, H., Han, L.-Y., Bakshi, K. P., Stucky, A., *et al.* (2012). Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. *The Journal of Clinical Investigation*, 122(4), 1316.
- Tamboli, I. Y., Barth, E., Christian, L., Siepmann, M., Kumar, S., Singh, S., *et al.* (2010). Statins promote the degradation of extracellular amyloid β -peptide by microglia via stimulation of exosome-associated insulin-degrading enzyme (IDE) secretion. *Journal of Biological Chemistry*, 285(48), 37405-37414.
- Tate, J., & Ward, G. (2004). Interferences in immunoassay. *The Clinical Biochemist Reviews*, 25(2), 105.
- Thal, D. R., Griffin, W. S. T., de Vos, R. A. I., & Ghebremedhin, E. (2008). Cerebral amyloid angiopathy and its relationship to Alzheimer's disease. *Acta Neuropathologica*, 115(6), 599-609.

- Thambisetty, M., Simmons, A., Velayudhan, L., Hye, A., Campbell, J., Zhang, Y., *et al.* (2010). Association of plasma clusterin concentration with severity, pathology, and progression in Alzheimer disease. *Archives of General Psychiatry*, 67(7), 739.
- The Ronald and Nancy Reagan Research Institute of the Alzheimer's Association and the National Institute on Aging Working Group. (1998). Consensus report of the working group on molecular and biochemical markers of Alzheimer's disease. *Neurobiology of Aging*, 19(2), 109-116.
- Tiraboschi, P., Hansen, L. A., Thal, L. J., & Corey-Bloom, J. (2004). The importance of neuritic plaques and tangles to the development and evolution of AD. *Neurology*, 62(11), 1984-1989.
- Tokuda, T., Calero, M., Matsubara, E., Vidal, R., Kumar, A., Permanne, B., *et al.* (2000). Lipidation of apolipoprotein E influences its isoform-specific interaction with Alzheimer's amyloid β peptides. *Biochemical Journal*, 348(Pt 2), 359-365.
- Tundo, G., Ciaccio, C., Sbardella, D., Boraso, M., Viviani, B., Coletta, M., *et al.* (2012). Somatostatin modulates insulin-degrading-enzyme metabolism: implications for the regulation of microglia activity in AD. *PLoS ONE*, 7(4), e34376.
- Tundo, G. R., Sbardella, D., Ciaccio, C., Bianculli, A., Orlandi, A., Desimio, M. G., *et al.* (2013). Insulin-degrading Enzyme (IDE) A NOVEL HEAT SHOCK-LIKE PROTEIN. *Journal of Biological Chemistry*, 288(4), 2281-2289.
- Udrisar, D. P., Wanderley, M. I., Porto, R. C., Cardoso, C. L., Barbosa, M. C., Camberos, M. C., *et al.* (2005). Androgen-and estrogen-dependent regulation of insulin-degrading enzyme in subcellular fractions of rat prostate and uterus. *Experimental Biology and Medicine*, 230(7), 479-486.
- Umegaki, H., Ikari, H., Nakahata, H., Endo, H., Suzuki, Y., Ogawa, O., *et al.* (2000). Plasma cortisol levels in elderly female subjects with Alzheimer's disease: a cross-sectional and longitudinal study. *Brain Research*, 881(2), 241-243.
- UniProt Consortium. (2014). Activities at the Universal Protein Resource (UniProt). *Nucleic Acids Research*, 42(D1), D191-D198.

- Urbonaviciute, V., Fürnrohr, B. G., Weber, C., Haslbeck, M., Wilhelm, S., Herrmann, M., *et al.* (2007). Factors masking HMGB1 in human serum and plasma. *Journal of leukocyte biology*, 81(1), 67-74.
- Valdes, R., & Jortani, S. A. (2002). Unexpected suppression of immunoassay results by cross-reactivity: now a demonstrated cause for concern. *Clinical Chemistry*, 48(3), 405-406.
- van den Berg, E., Kessels, R. P. C., de Haan, E. H. F., Kappelle, L. J., & Biessels, G. J. (2005). Mild impairments in cognition in patients with type 2 diabetes mellitus: the use of the concepts MCI and CIND. *Journal of Neurology, Neurosurgery, and Psychiatry*, 76(10), 1466-1467.
- van Duijn, C. M. (1996). Epidemiology of the dementias: recent developments and new approaches. *Journal of Neurology, Neurosurgery, and Psychiatry*, 60(5), 478-488.
- van Duijn, C. M., Clayton, D. G., Chandra, V., Fratiglioni, L., Graves, A. B., Heyman, A., *et al.* (1994). Interaction between genetic and environmental risk factors for Alzheimer's disease: a reanalysis of case-control studies. *Genetic Epidemiology*, 11(6), 539-551.
- van Oijen, M., Hofman, A., Soares, H. D., Koudstaal, P. J., & Breteler, M. M. B. (2006). Plasma A beta(1-40) and A beta(1-42) and the risk of dementia: a prospective case-cohort study. *Lancet Neurology*, 5(8), 655-660.
- Vanderstichele, H., Van Kerschaver, E., Hesse, C., Davidsson, P., Buyse, M. A., Andreasen, N., *et al.* (2000). Standardization of measurement of -amyloid(1-42) in cerebrospinal fluid and plasma. *Amyloid*, 7(4), 245-258.
- Vanhanen, M., Koivisto, K., Kuusisto, J., Mykkänen, L., Helkala, E. L., Hänninen, T., *et al.* (1998). Cognitive function in an elderly population with persistent impaired glucose tolerance. *Diabetes Care*, 21(3), 398-402.
- Vega, C. A., Butler, R. A., Perez, B., & Torres, C. (1985). Thermodynamics of the dissociation of protonated tris (hydroxymethyl) aminomethane in 25 and 50 wt% 2-propanol from 5 to 45 ° C. *Journal of Chemical and Engineering Data*, 30(4), 376-379.

- Vekrellis, K., Ye, Z., Qiu, W. Q., Walsh, D. M., Hartley, D., Chesneau, V., *et al.* (2000). Neurons regulate extracellular levels of amyloid β -protein via proteolysis by insulin-degrading enzyme. *The Journal of Neuroscience*, 20(5), 1657-1665.
- Venugopal, C., Pappolla, M. A., & Sambamurti, K. (2007). Insulysin cleaves the APP cytoplasmic fragment at multiple sites. *Neurochemical Research*, 32(12), 2225-2234.
- Vepsäläinen, S., Hiltunen, M., Helisalmi, S., Wang, J., van Groen, T., Tanila, H., *et al.* (2008). Increased expression of A β degrading enzyme IDE in the cortex of transgenic mice with Alzheimer's disease-like neuropathology. *Neuroscience Letters*, 438(2), 216-220.
- Vepsäläinen, S., Parkinson, M., Helisalmi, S., Mannermaa, A., Soininen, H., Tanzi, R. E., *et al.* (2007). Insulin-degrading enzyme is genetically associated with Alzheimer's disease in the Finnish population. *Journal of Medical Genetics*, 44(9), 606-608.
- Verdile, G., Fuller, S., Atwood, C. S., Laws, S. M., Gandy, S. E., & Martins, R. N. (2004). The role of beta amyloid in Alzheimer's disease: still a cause of everything or the only one who got caught? . *Pharmacological Research: The Official Journal of the Italian Pharmacological Society*, 50(4), 397-409.
- Verdile, G., Laws, S., Henley, D., Ames, D., Bush, A., Ellis, K., *et al.* (2012). Associations between gonadotropins, testosterone and β amyloid in men at risk of Alzheimer's disease. *Molecular psychiatry*.
- Vest, R. S., & Pike, C. J. (2013). Gender, sex steroid hormones, and Alzheimer's disease. *Hormones and behavior*, 63(2), 301-307.
- Vincent-Viry, M., Schiele, F., Gueguen, R., Bohnet, K., Visvikis, S., & Siest, G. (1998). Biological variations and genetic reference values for apolipoprotein E serum concentrations: results from the STANISLAS cohort study. *Clinical Chemistry*, 44(5), 957-965.
- Visser, P. J., Verhey, F., Knol, D. L., Scheltens, P., Wahlund, L. O., Freund-Levi, Y., *et al.* (2009). Prevalence and prognostic value of CSF markers of Alzheimer's disease pathology in patients with subjective cognitive impairment or mild cognitive impairment in the DESCRIPA study: a prospective cohort study. *Lancet Neurology*, 8(7), 619-627.

- von Strauss, E., Viitanen, M., De Ronchi, D., Winblad, B., & Fratiglioni, L. (1999). Aging and the occurrence of dementia: Findings from a population-based cohort with a large sample of nonagenarians. *Archives of Neurology*, 56(5), 587-592.
- Wahrle, S. E., Jiang, H., Parsadanian, M., Hartman, R. E., Bales, K. R., Paul, S. M., *et al.* (2005). Deletion of ABCA1 Increases A β deposition in the PDAPP transgenic mouse model of Alzheimer disease. *The Journal of Biological Chemistry*, 280(52), 43236-43242.
- Wahrle, S. E., Jiang, H., Parsadanian, M., Legleiter, J., Han, X., Fryer, J. D., *et al.* (2004). ABCA1 is required for normal central nervous system apoE levels and for lipidation of astrocyte-secreted apoE. *The Journal of Biological Chemistry*, 279(39), 40987-40993.
- Walker, J. R., Pacoma, R., Watson, J., Ou, W., Alves, J., Mason, D. E., *et al.* (2013). Enhanced Proteolytic Clearance of Plasma A β by Peripherally Administered Neprilysin Does Not Result in Reduced Levels of Brain A β in Mice. *The Journal of Neuroscience*, 33(6), 2457-2464.
- Wallin, A. K., Blennow, K., Andreasen, N., & Minthon, L. (2006). CSF biomarkers for Alzheimer's disease: Levels of β -amyloid, tau, phosphorylated tau relate to clinical symptoms and survival. *Dementia and Geriatric Cognitive Disorders*, 21(3), 131-138.
- Wallum, B. J., Taborsky, G. J., Porte, D., Figlewicz, D. P., Jacobson, L., Beard, J. C., *et al.* (1987). Cerebrospinal fluid insulin levels increase during intravenous insulin infusions in man. *The Journal of Clinical Endocrinology*, 64(1), 190-194.
- Walsh, D. M., Lomakin, A., Benedek, G. B., Condron, M. M., & Teplow, D. B. (1997). Amyloid β -protein fibrillogenesis. Detection of a protofibrillar intermediate. *The Journal of Biological Chemistry*, 272(35), 22364-22372.
- Walsh, D. M., & Selkoe, D. J. (2007). Ab oligomers – a decade of discovery. *Journal of Neurochemistry*, 101(5), 1172-1184.

- Walsh, D. M., Townsend, M., Podlisny, M. B., Shankar, G. M., Fadeeva, J. V., El Agnaf, O., *et al.* (2005). Certain inhibitors of synthetic amyloid β -peptide (A β) fibrillogenesis block oligomerization of natural A β and thereby rescue long-term potentiation. *The Journal of Neuroscience*, 25(10), 2455-2462.
- Wang, D. S., Dickson, D. W., & Malter, J. S. (2006). β -amyloid degradation and Alzheimer's disease. *Journal of Biomedicine and Biotechnology*, 2006(3), 1-12.
- Wang, H. W., Pasternak, J. F., Kuo, H., Ristic, H., Lambert, M. P., Chromy, B., *et al.* (2002). Soluble oligomers of beta amyloid (1-42) inhibit long-term potentiation but not long-term depression in rat dentate gyrus. . *Brain Research*, 924(2), 133-140.
- Wang, L., Yu, C.-j., Liu, W., Cheng, L.-y., & Zhang, Y.-n. (2011). Rosiglitazone protects neuroblastoma cells against advanced glycation end products-induced injury. *Acta Pharmacologica Sinica*, 32(8), 991-998.
- Wang, P., Su, C., Li, R., Wang, H., Ren, Y., Sun, H., *et al.* (2014). Mechanisms and effects of curcumin on spatial learning and memory improvement in APPswe/PS1dE9 mice. *Journal of neuroscience research*, 92(2), 218-231.
- Wang, R., Wang, S., Malter, J. S., & Wang, D.-S. (2009). Effects of 4-Hydroxy-Nonenal and Amyloid- β on Expression and Activity of Endothelin Converting Enzyme and Insulin Degrading Enzyme in SH-SY5Y Cells. *Journal of Alzheimer's Disease*, 17(3), 489-501.
- Wang, S., Wang, R., Chen, L., Bennett, D. A., Dickson, D. W., & Wang, D. S. (2010). Expression and functional profiling of neprilysin, insulin-degrading enzyme, and endothelin-converting enzyme in prospectively studied elderly and Alzheimer's brain. *Journal of Neurochemistry*, 115(1), 47-57.
- Watanabe, R. M., Ghosh, S., Langefeld, C. D., Valle, T. T., Hauser, E. R., Magnuson, V. L., *et al.* (2000). The Finland--United States investigation of non--insulin-dependent diabetes mellitus genetics (FUSION) study. II. An autosomal genome scan for diabetes-related quantitative-trait loci. *American Journal of Human Genetics*, 67(5), 1186-1200.

- Watson, G. S., Peskind, E. R., Asthana, S., Purganan, K., Wait, C., Chapman, D., *et al.* (2003). Insulin increases CSF Abeta42 levels in normal older adults. *Neurology*, 60(12), 1886-1887.
- Wehr, H., Bednarska-Makaruk, M., Lojkowska, W., Graban, A., Hoffman-Zacharska, D., Kuczynska-Zardzewialy, A., *et al.* (2006). Differences in risk factors for dementia with neurodegenerative traits and for vascular dementia. *Dementia and Geriatric Cognitive Disorders*, 22(1), 1-7.
- Weirich, G., Mengele, K., Yfanti, C., Gkazepis, A., Hellmann, D., Welk, A., *et al.* (2008). Immunohistochemical evidence of ubiquitous distribution of the metalloendoprotease insulin-degrading enzyme (IDE; insulysin) in human non-malignant tissues and tumor cell lines. *Biological Chemistry*, 389(11), 1441-1445.
- Weisgraber, K. H., & Mahley, R. W. (1996). Human apolipoprotein E: the Alzheimer's disease connection. *The FASEB Journal*, 10(13), 1485-1494.
- Weller, S. K. (2011). *Alphaherpesviruses: Molecular Virology*: Horizon Scientific Press.
- Wertkin, A. M., Turner, R. S., Pleasure, S. J., Golde, T. E., Younkin, S. G., Trojanowski, J. Q., *et al.* (1993). Human neurons derived from a teratocarcinoma cell line express solely the 695-amino acid amyloid precursor protein and produce intracellular β -amyloid or A4 peptides. *Proceedings of the National Academy of Sciences*, 90(20), 9513-9517.
- Westermarck, P., Wernstedt, C., Wilander, E., Hayden, D. W., O'Brien, T. D., & Johnson, K. H. (1987). Amyloid fibrils in human insulinoma and islets of Langerhans of the diabetic cat are derived from a neuropeptide-like protein also present in normal islet cells. *Proceedings of the National Academy of Sciences*, 84(11), 3881-3885.
- Weyer, C., Hanson, R. L., Tataranni, P. A., Bogardus, C., & Pratley, R. E. (2000). A high fasting plasma insulin concentration predicts type 2 diabetes independent of insulin resistance. Evidence for a pathogenic role of relative hyperinsulinemia. *Diabetes*, 49(12), 2094-2101.
- Williams, J., Lye, D. C. B., & Umapathi, T. (2008). Diagnostic lumbar puncture: minimizing complications. *Internal Medicine Journal*, 38(7), 587-591.

- Wilson, C. A., Doms, R. W., & Lee, V. M. (1999). Intracellular APP Processing and A[beta] Production in Alzheimer Disease. *Journal of Neuropathology & Experimental Neurology*, 58(8), 787-794.
- Wiltshire, S., Hattersley, A. T., Hitman, G. A., Walker, M., Levy, J. C., Sampson, M., *et al.* (2001). A genomewide scan for loci predisposing to type 2 diabetes in a U.K. population (The Diabetes UK Warren 2 Repository): Analysis of 573 pedigrees provides independent replication of a susceptibility locus on chromosome 1q. *American Journal of Human Genetics*, 69(3), 553-569.
- Wisniewski, T., Castaño, E. M., Golabek, A., Vogel, T., & Frangione, B. (1994). Acceleration of Alzheimer's fibril formation by apolipoprotein E *in vitro*. *American Journal of Pathology*, 145(5), 1030-1035.
- Wisniewski, T., & Frangione, B. (1992). Apolipoprotein-E - a pathological chaperone protein in patients with cerebral and systemic amyloid. *Neuroscience Letters*, 135(2), 235-238.
- Wolfe, M. S., Xia, W., Moore, C. L., Leatherwood, D. D., Ostaszewski, B., Rahmati, T., *et al.* (1999). Peptidomimetic probes and molecular modeling suggest that Alzheimer's γ -secretase is an intramembrane-cleaving aspartyl protease. *Biochemistry*, 38(15), 4720-4727.
- Xia, W., Yang, T., Shankar, G., Smith, I. M., Shen, Y., Walsh, D. M., *et al.* (2009). A specific enzyme-linked immunosorbent assay for measuring β -amyloid protein oligomers in human plasma and brain tissue of patients with Alzheimer disease. *Archives of Neurology*, 66(2), 190-199.
- Xu, H., Greengard, P., & Gandy, S. (1995). Regulated formation of Golgi secretory vesicles containing Alzheimer β -amyloid precursor protein. *The Journal of Biological Chemistry*, 270(40), 23243-23245.
- Xu, W. L., Qiu, C. X., Wahlin, A., Winblad, B., & Fratiglioni, L. (2004). Diabetes mellitus and risk of dementia in the Kungsholmen project: a 6-year follow-up study. *Neurology*, 63(7), 1181-1186.
- Yamaguchi, H., Hirai, S., Morimatsu, M., Shoji, M., & Harigaya, Y. (1988). Diffuse type of senile plaques in the brains of Alzheimer-type dementia. *Acta Neuropathologica*, 77(2), 113-119.

- Yamaguchi, H., Sugihara, S., Ogawa, A., Oshima, N., & Ihara, Y. (2001). Alzheimer β amyloid deposition enhanced by apoE ϵ 4 gene precedes neurofibrillary pathology in the frontal association cortex of nondemented senior subjects. *Journal of Neuropathology and Experimental Neurology*, 60(7), 731-739.
- Yamamoto, M., Kiyota, T., Walsh, S. M., Liu, J., Kipnis, J., & Ikezu, T. (2008). Cytokine-mediated inhibition of fibrillar amyloid- β peptide degradation by human mononuclear phagocytes. *The Journal of Immunology*, 181(6), 3877-3886.
- Yan, H., Yuan, W., Velculescu, V. E., Vogelstein, B., & Kinzler, K. W. (2002). Allelic variation in human gene expression. *Science*, 297(5584), 1143.
- Yang, D. S., Small, D. H., Seydel, U., Smith, J. D., Hallmayer, J., Gandy, S. E., *et al.* (1999). Apolipoprotein E promotes the binding and uptake of beta-amyloid into Chinese hamster ovary cells in an isoform-specific manner. *Neuroscience*, 90(4), 1217-1226.
- Yankner, B. A., Duffy, L. K., & Kirschner, D. A. (1990). Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides. *Science*, 250(4978), 279-283.
- Yenki, P., Khodagholi, F., & Shaerzadeh, F. (2013). Inhibition of Phosphorylation of JNK Suppresses A β -Induced ER Stress and Upregulates Prosurvival Mitochondrial Proteins in Rat Hippocampus. *Journal of Molecular Neuroscience*, 49(2), 262-269.
- Yfanti, C., Mengele, K., Gkazepis, A., Weirich, G., Giersig, C., Kuo, W.-L., *et al.* (2008). Expression of metalloprotease insulin-degrading enzyme insulysin in normal and malignant human tissues. *International journal of molecular medicine*, 22(4), 421.
- Yokono, K. (1981(a)). [Studies on insulin metabolism: enzymatic characteristics and biological significance of insulin-degrading activities in rat liver cell fraction (author's transl)]. *Nihon Naibunpi Gakkai Zasshi*, 57(12), 1683-1698.
- Yokono, K., Imamura, Y., Shii, K., Sakai, H., & Baba, S. (1981(b)). Purification and characterization of insulin-degrading enzyme from pig skeletal muscle. *Endocrinology*, 108(4), 1527-1532.

- Youmans, K. L., Tai, L. M., Nwabuisi-Heath, E., Jungbauer, L., Kanekiyo, T., Gan, M., *et al.* (2012). APOE4-specific changes in A β accumulation in a new transgenic mouse model of Alzheimer disease. *Journal of Biological Chemistry*, 287(50), 41774-41786.
- Zanetti, O., Solerte, S., & Cantoni, F. (2009). Life expectancy in Alzheimer's disease (AD). *Archives of Gerontology and Geriatrics*, 49, 237-243.
- Zeggini, E., Weedon, M. N., Lindgren, C. M., Frayling, T. M., Elliott, K. S., Lango, H., *et al.* (2007). Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes. *Science*, 316(5829), 1336-1341.
- Zhang, L., Ding, Q., & Wang, Z. (2012). Nuclear Respiratory Factor 1 Mediates the Transcription Initiation of Insulin-Degrading Enzyme in a TATA Box-Binding Protein-Independent Manner. *PLoS ONE*, 7(8), e42035.
- Zhang, Y., Wang, B., Wan, H., Zhou, Q., & Li, T. (2013). Meta-analysis of the Insulin degrading enzyme polymorphisms and susceptibility to Alzheimer's disease. *Neuroscience Letters*.
- Zhao, L., Teter, B., Morihara, T., Lim, G. P., Ambegaokar, S. S., Ubeda, O. J., *et al.* (2004). Insulin-degrading enzyme as a downstream target of insulin receptor signaling cascade: implications for Alzheimer's disease intervention. *The Journal of Neuroscience*, 24(49), 11120-11126.
- Zhao, L., Yao, J., Mao, Z., Chen, S., Wang, Y., & Brinton, R. D. (2011). 17 β -Estradiol regulates insulin-degrading enzyme expression via an ER β /PI3-K pathway in hippocampus: Relevance to Alzheimer's prevention. *Neurobiology of Aging*, 32(11), 1949-1963.
- Zhao, X., Pan, F., Garcia-Gancedo, L., Flewitt, A. J., Ashley, G. M., Luo, J., *et al.* (2012). Interfacial recognition of human prostate-specific antigen by immobilized monoclonal antibody: effects of solution conditions and surface chemistry. *Journal of The Royal Society Interface*, 9(75), 2457-2467.
- Zhao, Z., Xiang, Z., Haroutunian, V., Buxbaum, J. D., Stetka, B., & Pasinetti, G. M. (2007). Insulin degrading enzyme activity selectively decreases in the hippocampal formation of cases at high risk to develop Alzheimer's disease. *Neurobiology of Aging*, 28(6), 824-830.

- Zlokovic, B. V., Deane, R., Sallstrom, J., Chow, N., & Miano, J. M. (2005). Neurovascular pathways and Alzheimer amyloid β -peptide. *Brain Pathology*, 15(1), 78-83.
- Zubenko, G. S. (1997). Molecular neurobiology of Alzheimer's disease. *Harvard Review of Psychiatry*, 5(4), 177-213.
- Zuo, X., & Jia, J. (2009). Promoter polymorphisms which modulate insulin degrading enzyme expression may increase susceptibility to Alzheimer's disease. *Brain Research*, 1249, 1-8.

APPENDIX – Further optimisation tests for Fluorometric Activity Assay

A1 Determination of fluorometric activity assay cross-reactivity with human (HSA) and bovine (BSA) serum albumin

It was demonstrated in the IDE ELISA assay that cell-culture grade BSA was cross-reactive and produced a dose-responsive signal. As the capture antibody Ab 3 was used for both the IDE ELISA and also the IDE fluorometric activity assay, it was important to test for any interference of both BSA (cell-culture grade) and HSA in the activity assay. PCP as a control was also included to compare the extent of any HSA/BSA signal generated.

HSA and BSA were prepared as described in section 2.2.5.3.1 to a concentration of 60 mg/mL each (representing the highest concentration of human albumin in plasma) diluted in PBST (Figure A1). Each dilution was further diluted as per plasma samples (40 µl + 85 µL PBST), and then assayed in duplicate along with PCP using the fluorometric activity assay protocol as detailed in Section 2.2.6.1. The test was terminated after 2 hours incubation. Cross-reactivity was determined to be fluorescence generated above the value of the (PBST only) blank.

Results showed that neither BSA nor HSA generated a signal above the level of the blank, while the PCP control did generate a signal as expected. It can be concluded by these results that neither HSA nor BSA was an interfering factor in this assay.

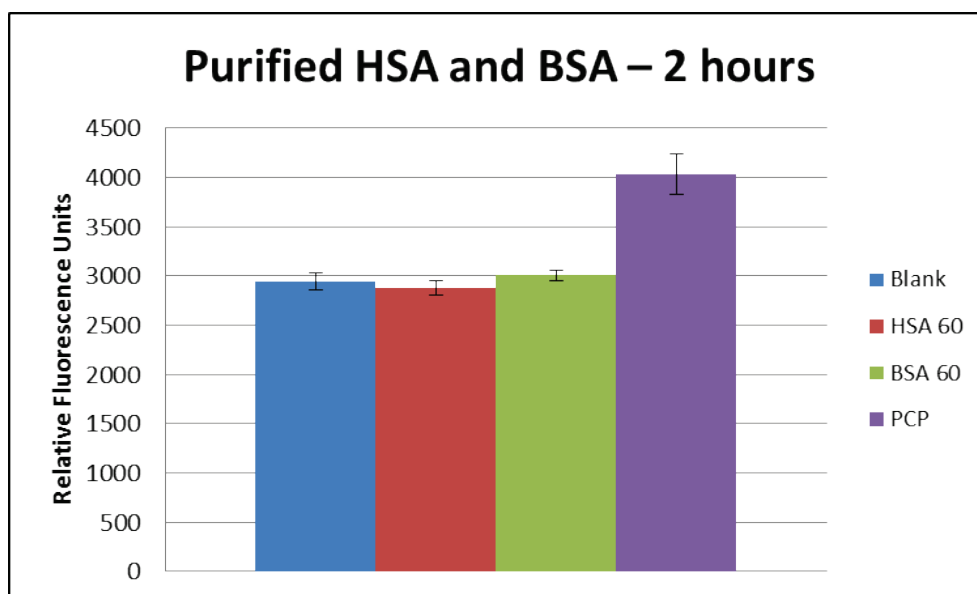


Figure A1 Investigating potential interference in the IDE fluorometric activity assay by purified human (HSA) or bovine serum albumin (BSA). All tests were done in duplicate. Solutions of BSA and HSA were prepared to a concentration of 60mg/mL and diluted as per PCP in PBST. PCP was determined to contain 960 μ g total protein per well. The assay was carried out as described in section 2.2.6.1, although conducted for only 2 hr. The results show that purified HSA and BSA generate no detectable signal above the blank in the IDE fluorometric activity assay at the equivalent of the highest physiological level of albumin found in human plasma. Error bars indicate \pm SD.

A2 Testing phosphate-based buffers against tris-based buffers as sample diluents in the IDE fluorometric activity assay.

Phosphate-based buffers have been known to have a chelating effect on some enzymes, and particularly when using long incubation times. Similar to some other enzymes, IDE catalytic activity has previously been shown to be dependent on metal ions such as zinc. As the samples in the IDE activity assay are diluted in PBST, and incubated overnight, it was important to determine if the chelating effect of the phosphates in PBST were having an effect on the amount of IDE activity measured. Therefore the assay was tested with purified rrIDE and PCP using either PBST or TBST for blocking agents, sample dilutions and washes (Figure A2(A) and (B)).

Results showed minimal differences between TBST and PBST in the fluorometric assay for both the standard curve (Graph A.), and for the plasma (Graph B.). It was concluded that due to the pH instability of tris-based buffers with temperature changes, and the overnight incubation of the samples at 4°C in the protocol, PBST would be used for the IDE fluorometric assay.

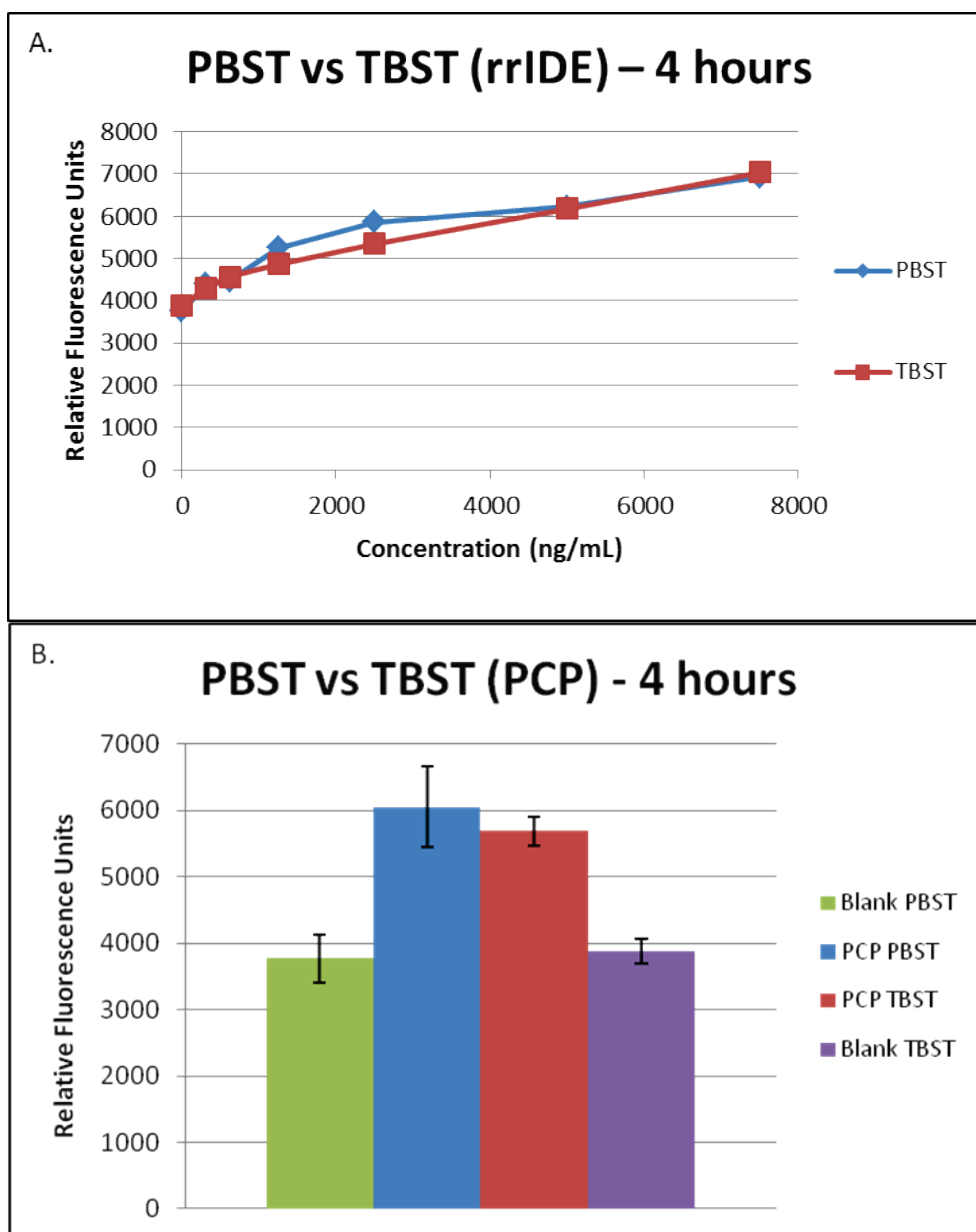


Figure A2(A) and (B). Test of PBST versus TBST as dilution buffers in the fluorescent activity assay for IDE. Assay was performed as detailed in Section 2.2.6.1 over 4 hours. Recombinant rat IDE (rrIDE) was diluted to concentrations of 312.5 to 7500 ng/mL (Graph A.) and pooled control plasma (PCP) was loaded at a dilution of 960 μ g total protein per well (Graph B) in duplicate, using either PBST or TBST as assay diluents. Data is shown at the 4 hour timepoint. Results show that there is negligible difference in the rrIDE standard curve or plasma values when using either PBST or TBST diluents. Error bars in Graph B indicate \pm SD.