Investigating a potential role for irisin as a biomarker in the early detection of Alzheimer’s disease

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Abstract

Dementia, and in particular its most common cause Alzheimer's disease (AD), is predicted to reach pandemic status by mid-century. Individuals with AD typically present with significant clinical manifestations, including a decline in memory and language, with later stages of the disease associated with negative impacts on independent living and normal bodily functions, and ultimately death of the patient. There is currently no effective treatment or cure for AD, despite heightened research efforts worldwide. A number of factors contribute to the ongoing failure of AD clinical trials, including advanced damage in the brain of AD patients and severe side-effects of some compounds. Therefore, it is vital to identify biomarkers that are indicative of AD pathology well before clinical symptoms of AD manifest, so that intervention strategies can be developed before marked neurodegeneration.

In light of limited progress in the development of an AD pharmaceutical, research is shifting toward identifying preventative strategies, such as changes in lifestyle, to delay or prevent the onset of AD. Due to numerous known beneficial impacts of exercise on the brain, such as the increase of a molecular key player in brain health (i.e. brain-derived neurotrophic factor, BDNF), the potential of exercise to change the course of AD has received significant research attention. Irisin is a recently identified hormone, proposed to increase energy expenditure in muscle and white adipose cells, which potentially plays a mediating role in the beneficial effects of physical activity on the body. Interestingly, irisin has shown to activate BDNF in mice and humans following exercise, potentially implicating irisin as an important mechanistic factor underlying the relationship between exercise and brain health. Nevertheless, no research to date has thoroughly investigated the association between circulating irisin levels, physical
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activity and markers of cognitive health in human studies; a gap in the literature that this thesis seeks to address.

The discovery of irisin and the observation that it may be linked to BDNF activation has therefore led to the development of the following research questions for my PhD thesis:

Is irisin associated with

1) Cognitive health, AD risk factors and AD-related biomarkers in older adults over 60 years of age (Chapter 3)?

2) Physical activity and/or exercise in older adults over 60 years of age (Chapter 4)?

3) A neuroprotective effect in a frequently used in vitro model of AD (SH-SY5Y neuroblastoma cells) (Chapter 5)?

Data derived from neuropsychological assessments, blood samples and measures of habitual physical activity were utilised from the Australian Imaging, Biomarker & Lifestyle Study of Ageing (AIBL). Furthermore, neuropsychological, blood biomarker and physical activity data from a physical exercise intervention study in participants over 60 years of age was analysed (Physical Exercise and Cognitive Stimulation Study, PEACS). Finally, blood samples from a small resistance exercise pilot study were analysed to assess irisin in younger adults following an acute bout of resistance exercise (Acute Strength Training-Study, AST).

In this project, I demonstrated that, in cognitively healthy older adults, irisin is associated with age, cognition, habitual physical activity intensity and volume, and lipids; with a number of these associations dependent on gender or Apolipoprotein E.
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(genetic risk factor for AD; APOE ε4 carrier status. I also demonstrated that irisin levels were significantly increased following a 16 week exercise intervention in older adults; however, irisin levels were not significantly altered following acute resistance training in younger adults. In my in vitro work, I observed cell protection from the neurotoxic effects of Aβ, and cell proliferation when SH-SY5Y cells were treated with irisin.

To my knowledge, this thesis is the first investigation of the role of irisin as a biomarker of brain health. This research has the potential to provide new insights into the impact irisin has on cognition. It further shows that physical activity levels can be associated with irisin levels in older adults, when a continuous exercise regimen is followed for four months. The observations made in this project demonstrated that irisin is a worthy candidate to be investigated as a biomarker in brain health. Nevertheless, the results in my thesis also clearly demonstrate that the relationship between irisin and brain health is complex and more work is required to establish solid conclusions regarding irisin’s distribution and function in the human brain.
Dedication

The people that this thesis is truly dedicated for might not be amongst us anymore, but nonetheless, they are the people that have influenced me the most until this day: my parents. They taught me to see the beauty in the contrary, to embrace the calm and the storm, to move forward whilst being pushed backwards, and to keep daring to push limits without the fear of failure. This applies to life as much as it does to science.

“In the middle of difficulty lies opportunity.”

Albert Einstein
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Thesis Declaration

I, Sabine M. Bird, certify that:

This thesis comprises my original work and has been substantially accomplished during enrolment in this PhD degree.

This thesis does not contain material, which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution.

No part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of The University of Western Australia and where applicable, any partner institution responsible for the joint-award of this degree.

This thesis does not contain any material previously published or written by another person, except where due reference has been made in the text. Due acknowledgements are also made in the acknowledgement sections.

The work(s) are not in any way a violation or infringement of any copyright, trademark, patent, or other rights whatsoever of any person.

The research involving human data from the Physical Exercise and Cognitive Stimulation Study (PEACS) reported about in this thesis was assessed and approved by The University of Western Australia Human Research Ethics Committee, Approval #: 3286.

The research involving human data from the Acute Strength Training-Study (AST) reported about in this thesis was assessed and approved by Murdoch University Human Research Ethics Committee. Approval #: 2014076.

The Human Research Ethics Committee at The University of Western Australia approved that data from the established Australian Imaging, Biomarker and Lifestyle Study of Ageing (AIBL) can be collected and included in my project. Reference #: RA/4/1/6503. The following approvals were obtained prior to commencing the relevant work with the AIBL study for my project: The research involving human data was assessed and approved by Human Research Ethics Committees at Edith Cowan University (Project

XXIII
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Written patient consent has been received and archived for the research involving patient data reported in this thesis.

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This thesis contains work prepared for publication.

Signature:

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607 180 / 10 ml  
760 180 / 25 ml |
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### Data analysis

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<td>AST</td>
<td>Acute Strength Training</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATT</td>
<td>Attention (cognitive composite score)</td>
</tr>
<tr>
<td>BACE1</td>
<td>β-secretase</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-Brain-Barrier</td>
</tr>
<tr>
<td>BDNF (p/m)</td>
<td>Brain-derived neurotrophic factor (pro/mature)</td>
</tr>
<tr>
<td>BL</td>
<td>Baseline</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BNT</td>
<td>Boston Naming Testing</td>
</tr>
<tr>
<td>BP</td>
<td>Base pair</td>
</tr>
<tr>
<td>C83</td>
<td>α-cleaved carboxyl-terminal fragment of BACE1</td>
</tr>
<tr>
<td>C99</td>
<td>β-cleaved carboxyl-terminal fragment of BACE1</td>
</tr>
<tr>
<td>CAMKII</td>
<td>Ca²⁺-calmodulin dependent kinase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COWAT</td>
<td>Controlled Oral Word Association Test</td>
</tr>
<tr>
<td>CDR</td>
<td>Clinical dementia rating</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CTRL</td>
<td>Control</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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</tr>
<tr>
<td>CVLT</td>
<td>California Verbal Learning Test</td>
</tr>
<tr>
<td>DIA BP</td>
<td>Diastolic blood pressure</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EF</td>
<td>Executive Functioning (cognitive composite score): defined as decision-making, response inhibition and cognitive flexibility.</td>
</tr>
<tr>
<td>EI</td>
<td>Exercise intervention</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EM</td>
<td>Episodic Memory (cognitive composite score)</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-regulated Kinase</td>
</tr>
<tr>
<td>F12</td>
<td>Nutrient mixture F12 (Ham)</td>
</tr>
<tr>
<td>F18</td>
<td>Florbetapir</td>
</tr>
<tr>
<td>FAD</td>
<td>Familial AD</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FNDC5</td>
<td>Fibronectin type III domain containing protein 5</td>
</tr>
<tr>
<td>FT3</td>
<td>Triiodothyronine</td>
</tr>
<tr>
<td>FT4</td>
<td>Free thyroxine</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma Aminobutyric Acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDS</td>
<td>Geriatric Depression Scale</td>
</tr>
<tr>
<td>H2O</td>
<td>Here: Ultrapure water (milliQ®)</td>
</tr>
<tr>
<td>H10/1</td>
<td>Hypertrophy-based resistance training</td>
</tr>
<tr>
<td>HADS</td>
<td>Hospital Anxiety and Depression Scale</td>
</tr>
<tr>
<td>HB</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks' Balanced Salt solution</td>
</tr>
<tr>
<td>HC</td>
<td>Healthy control</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrogen chloride</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HFIP</td>
<td>Hexafluoroisopropanol</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Homeostasis model assessment for insulin resistance</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
</tbody>
</table>
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL-15</td>
<td>Interleukin-15</td>
</tr>
<tr>
<td>IPAQ</td>
<td>International Physical Activity Questionnaire</td>
</tr>
<tr>
<td>irIRN</td>
<td>Irisin-immunoreactivity</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo-Dalton</td>
</tr>
<tr>
<td>L</td>
<td>Language</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LM</td>
<td>Logical Memory</td>
</tr>
<tr>
<td>LMIC</td>
<td>Low- to middle-income countries</td>
</tr>
<tr>
<td>LOAD</td>
<td>Late onset Alzheimer’s disease</td>
</tr>
<tr>
<td>LRG1</td>
<td>Leucine-rich alpha-2-glycoprotein 1</td>
</tr>
<tr>
<td>LRP 1</td>
<td>Lipoprotein-related receptor protein 1</td>
</tr>
<tr>
<td>L-LTP</td>
<td>Late-phase Long-term potentiation (memory)</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
</tr>
<tr>
<td>MCQ</td>
<td>Memory Complaint Questionnaire</td>
</tr>
<tr>
<td>MET</td>
<td>Metabolic Equivalent of Task</td>
</tr>
<tr>
<td>METS</td>
<td>Metabolic syndrome</td>
</tr>
<tr>
<td>ML</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MMSE</td>
<td>Mini–Mental State Examination</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>MVPA</td>
<td><em>Actigraph</em>-defined moderate-to-vigorous physical activity</td>
</tr>
<tr>
<td>NACL</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>NG/ML</td>
<td>Nanograms per millilitre</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth-factor</td>
</tr>
<tr>
<td>NINCDS-ADRDA</td>
<td>National Institute of Neurological and Related Disorders Association</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>NP-40</td>
<td>NP40 Cell Lysis Buffer</td>
</tr>
</tbody>
</table>
Abbreviations

NRF-1 nuclear respiratory factor 1
NT-4/5/6/7 Neurotrophin factor 4/5/6/7

O.D. Optical density

p75NTR p75 neurotrophin receptor
PA Physical activity
pAb Primary antibody
PBS Phosphate-buffered saline
PC Positive control
PCR Polymerase chain reaction
PEACS Physical Exercise And Cognition Study
PET Positron Emission Tomography
PGC-1α PPAR-gamma co-activator 1 alpha
PGE1 Prostaglandin E1
P-gp P-glycoprotein
PI3K Phosphatidylinositol 3-kinase
PiB Pittsburgh compound B
PLCγ Phospholipase Cγ
PMSF Phenylmethylsulfonyl fluoride
PSEN (also: PS) Presenilin
P-TAU Phosphorylated tau

RA Retinoic acid
Rb Rabbit
RCFT Rey Complex Figure Test and Recognition Trial
RFLP Restriction Fragment Length Polymorphism
RT-PCR Real-Time Polymerase Chain Reaction

S5/3 Strength-based resistance training
SA-HRP Streptavidin-horseradish peroxidase
SD Standard deviation
SDS Sodium dodecyl sulphate
SEM Standard error of mean
SH-SY5Y “[…] thrice cloned (SK-N-SH > SH-SY > SH-SY5 > SH-SY5Y) sub-line of the neuroblastoma cell line SK-N-SH, which was established in 1970 from a metastatic bone tumor”, ATCC online.
SMAD Mothers Against Decapentaplegic Homolog
SNP Single nucleotide polymorphism
SP Senile plaques
SUV Standard uptake value
<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sys BP</td>
<td>Systolic blood pressure</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
</tr>
<tr>
<td>T4</td>
<td>Thyroxine</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Boric acid-EDTA</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline + 0.1% Tween® 20</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TFAM</td>
<td>Mitochondrial transcription factor A</td>
</tr>
<tr>
<td>TIMP4</td>
<td>Metalloproteinase inhibitor 4</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethylbenzidine</td>
</tr>
<tr>
<td>Tricine</td>
<td>N-[Tris(hydroxymethyl)methyl]glycine</td>
</tr>
<tr>
<td>TrkB</td>
<td>Tropomyosin-related kinase B</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid-stimulating hormone</td>
</tr>
<tr>
<td>T-TAU</td>
<td>Total Tau</td>
</tr>
<tr>
<td>UCP-1</td>
<td>Uncoupling protein-1</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Microliter</td>
</tr>
<tr>
<td>UN</td>
<td>Untreated</td>
</tr>
<tr>
<td>Val/Met and Val/Val</td>
<td>BDNF-polymorphisms: Valine and methionine; double-Valine</td>
</tr>
<tr>
<td>VEGF-β</td>
<td>Vascular endothelial growth factor - β</td>
</tr>
<tr>
<td>VST</td>
<td>Victorian Stroop Test</td>
</tr>
<tr>
<td>VO$_2$max</td>
<td>Maximum volume of oxygen</td>
</tr>
<tr>
<td>WAIS</td>
<td>Wechsler Adult Intelligence Scale</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WMS</td>
<td>Wechsler memory scales</td>
</tr>
<tr>
<td>YOE</td>
<td>Years of education</td>
</tr>
</tbody>
</table>
Authorship Declaration

This thesis contains work that has been prepared for publication.

- Serum irisin levels are associated with better performance on attention tasks in \textit{APOE-ε4} non-carriers

Location in thesis: Chapter 3
Student contribution to work: 90%

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Maruff, P., 1%

- Irisin is associated with physical activity levels in older adults

Location in thesis: Chapter 4
Student contribution to work: 90%

Co-author signatures and dates:
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Sohrabi, H. R., 1%
Gupta, V., 1%
Laws, S.M., 1%
Ames, D., 1%
Rowe, C.C., 1%
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Authorship Declaration

I, Gary Hulse, certify that the student statements regarding their contribution and contribution of other authors to each of the works listed above are correct. Signatures were not available due to absence of some authors but are currently being collected for publication purpose.

Coordinating supervisor signature:

Date: 05 June 2018


Chapter One

Introduction

Irisin, Physical Activity and The Ageing Brain
1.1 Background - Alzheimer’s disease

Dr Alois Alzheimer described the first case of an Alzheimer’s disease (AD) patient in 1906 (United Cipriani, Dolciotti, Picchi, & Bonuccelli, 2011b). He observed a range of symptoms in his patient, Auguste Deter, including severe memory loss, language problems and psychological changes, such as unpredictable behaviour. Following Auguste Deter’s death, Alzheimer observed dense deposits (today named “amyloid plaques”) around neurons, and tangled fibers (today identified as “neurofibrillary tangles”; NFT) within neurons (D’Andrea, 2014). Increasing age is the greatest risk factor for AD. Thus, with life expectancy increasing significantly over the last century, AD has become an increasingly prevalent condition (WHO & ADI, 2012). According to the latest reports by the World Health Organization, low- to middle-income countries are anticipated to see the steepest increase in AD cases in the near future, due to improvements in quality of life and access to good medical care. AD is a progressive neurodegenerative disorder, for which no effective treatment or cure currently exists. All recent clinical trials targeting the pathological features of AD have failed (Francesca Mangialasche, Alina Solomon, Bengt Winblad, Patrizia Mecocci, & Miia Kivipelto, 2010). Researchers have therefore shifted their focus to early intervention, both with potential drug therapies, and with targeting modifiable risk factors such as the lifestyle components of diet, sleep and physical activity (PA). Indeed, these lifestyle factors have gained considerable interest, and are being extensively investigated in order to identify and characterize brain health promoting activities in an attempt to delay, or ideally prevent, the onset of AD.
1.1.1 Alzheimer’s disease is a worldwide problem

Dementia is a term for a syndrome that covers a number of different degenerative diseases with overlapping symptoms (e.g. Alzheimer’s disease, Lewy body dementia, vascular dementia, etc.) (Cohen, 2015). All of these diseases are associated with a progressive decline in brain function, which impacts on the ability to live independently. Despite dementia affecting a broad range of age groups, the majority of people developing this condition are over 65 years of age (Association, 2011; Brookmeyer, Johnson, Ziegler-Graham, & Arrighi, 2007). With improved medical care, access to better living conditions, increased life expectancy, and declining mortality rates in low- to middle-income countries (LMIC), the number of individuals over the age of 65 is steadily increasing (Figure 1.1a) (UN, 2015). In fact, the World Health Organization (WHO) predicts that by mid-century older adults (i.e. those aged over 60 years of age) will for the first time in human history outnumber the amount of children (WHO, 2016). In accordance with these population predictions, the latest estimation published by Alzheimer’s Disease International (ADI) states that the number of people with dementia will reach 76 million in 2030 and 135 million by 2050 (ADI, 2016). The global burden of dementia will thereby shift to affect both low- and middle-income countries as well as high-income countries (Figure 1.1b). This demographic shift will lead to an unprecedented financial burden on health care systems around the world, with many governments lacking a strategic dementia plan to tackle this upcoming pandemic. Alzheimer’s disease is the most common form of dementia (accounting for 50-70% of all cases), and is already the second leading cause of death in Australians aged 85 years and above, and the fifth leading cause of death in Australians between 65 and 84 years of age (AIHW, 2016).
Figure 1.1: Estimated global population growth, as published by the United Nations (UN, 2015). a) It is predicted that by 2050, the number of people over the age of 60 (2.1 billion) will outnumber the number of 0-9 year old children (1.4 billion), and individuals 10-24 years of age (2.0 billion); b) Estimated growth of the number of people with dementia within both low- and middle-income, as well as high-income countries (Alzheimer’s Disease International, World Alzheimer Report 2010). A recent systematic review of the prevalence of dementia worldwide showed that the condition is a global problem and numbers of dementia cases are rapidly increasing (Prince et al., 2013).
1.1.2 Characterising Alzheimer's disease

Cognition describes mental performance in gaining knowledge and demonstrating comprehension (Esteban-Cornejo, Tejero-Gonzalez, Sallis, & Veiga, 2015). Age-related cognitive decline is a normal phenomenon which increases in frequency over the age of 50 (M. Angevaren, G. Aufdemkampe, H. Verhaar, A. Aleman, & L. Vanhees, 2008). Over the age of 50, faster loss of brain mass (atrophy) occurs, accompanied by accelerating cerebral ventricle size, and declining hippocampal volume (the brain region intrinsic to learning and memory); all of which contributes to cognitive decline (Raz, 2005; Dennis et al., 2008). Although a degree of cognitive decline is considered ‘normal for a certain age’, some experience cognitive decline at an abnormally fast pace, which can lead to AD.

AD is a debilitating, currently incurable, condition unique to the human species, that affects men and women worldwide (Finch & Austad, 2015). Even though AD has been reported to be more prevalent in women, these statistics were initially thought to be due to women generally living longer than men: gender differences in AD are still a matter of debate amongst researchers (Mielke, Vemuri, & Rocca, 2014). AD diagnosis occurs following a long progressive decline of brain health resulting from multiple factors. Continuous clinical and neurophysiological research has led to recent adjustments in the definition and diagnosis of AD; resulting in the designation of a pre-clinical stage, a stage of Mild Cognitive Impairment (MCI), and a full symptomatic disease stage (Sperling et al., 2011). In the pre-clinical stage, individuals do not show any measurable sign of cognitive decline: however, AD-related pathophysiology (e.g. amyloid-beta build-up) is already present in the brain. As this pathophysiology worsens evidence of
cognitive decline becomes apparent, resulting in diagnosis of MCI, and ultimately AD. MCI refers to the stage during which cognitive decline is apparent (in the form of memory and/or thinking problems), yet an individual is still able to live independently and could be well engaged in daily activities (Sperling et al., 2011). Individuals with the amnestic form of MCI often ultimately convert to the AD-stage. In the early AD-stage, cognition has declined markedly; however, it is possible that individuals with mild AD may be able to conduct usual activities of daily living. Nevertheless, due to the swift decline in cognitive abilities in AD, individuals often require constant care shortly after diagnosis.

1.1.2 Clinical symptoms

An individual presenting with AD will often exhibit word-finding problems, disorientation to time and place, issues with misplacing items around the house, and decreased rationality, amongst other clinical symptoms (Adler, 2014; Dubois et al., 2014; Vij, Prashar, Gill, & Jain, 2014). Often, the person with AD starts withdrawing from family and friends or other social activities as the disease progresses (Farrell et al., 2014). The rate of progression from mild to moderate and advanced AD varies considerably from person to person. As the disease progresses, a patient’s cognitive ability declines to such a degree that they become fully dependent on a carer as they struggle to dress and groom themselves, take care of their household, and prepare and remember to eat food. Ultimately, a person with advanced AD fails to recognise close family members and to communicate. Further, in the final stages of the disease, reflexes such as swallowing and walking cease to work, and an individual’s immune system is
compromised, leaving them vulnerable to infection (e.g. pneumonia), from which they frequently die (Morrison & Siu, 2000).

1.1.2.2 Pathophysiological changes

The pathophysiological cascade associated with AD commences well before clinical symptoms are observable (Harari et al., 2014; Solomon et al., 2014; Zhao et al., 2016). Microscopically, AD is characterised by the accumulation of extracellular deposits of the protein amyloid-beta (Aβ) in the brain, as well as by hyperphosphorylation of the microtubule-associated protein tau, resulting in intracellular neurofibrillary tangles (NFT). Ultimately, death of neurons eventuates which is apparent macroscopically as extensive loss of brain mass (atrophy; Figure 1.2) (Braak, Vos, Jansen, Bratzke, & Braak, 1998).

1.1.2.2.1 Cellular hallmarks of AD – Neurofibrillary tangles (NFT)

Microtubules form part of the cytoskeleton, and their filaments reach from the centrosome near the nucleus to the plasma membrane, keeping organelles in place and maintaining the general structure of the cell. The integrity of the microtubule-structure is maintained by a small glycoprotein called tau (Brion, 1998). Tau mostly occurs in neurons but also astrocytes and oligodendrocytes (Alzheimer's Association, 2016). Phosphorylation of tau usually promotes binding activity to microtubules; however, in AD, tau filaments occur in an irreversible hyperphosphorylated state, causing tau molecules to polymerize and form long fibrils tangled around each other (Benzing, Brady, Mufson, & Armstrong, 1993). As a result, tau cannot assist in maintaining the microtubule integrity and the microtubules lose their structure and disintegrate causing
detrimental interruptions to the cytoskeleton, and ultimately, cell death (Brion, 1998). Of note, the presence of NFTs correlate much more strongly with clinical symptoms of AD than deposits of Aβ in the brain, and this strong correlation is maintained with the severity of symptoms (United Nations, 2015b).

1.1.2.2 Cellular hallmarks of AD – Amyloid plaques

In AD, soluble Aβ peptides aggregate abnormally into insoluble and neurotoxic extracellular senile plaques (Masters et al., 1985), resulting in dysfunctional neuronal signalling and ultimately, neuronal loss (Figure 1.3). There is however, some debate as to whether plaque-formation is indeed the underlying cause of neuronal loss and cerebral atrophy in AD; nevertheless plaques are postulated to lead to shrinkage of certain brain areas, particularly the hippocampus and cortex (M. P. Mattson, 2004; Sperling et al., 2011). Some senile plaques contain neurites and are therefore referred to as neuritic plaques. These plaques have been observed most often following neuronal injury and correlate with increased synapse loss and glial activation (United Nations, 2015a).

In AD, significant cerebral Aβ burden is evident, with levels highest in the frontal cortex, cingulate gyrus, precuneus, striatum, parietal cortex, and lateral temporal cortex. By contrast, the occipital cortex, sensorimotor cortex, and mesial temporal cortex are usually less affected (Rowe & Villemagne, 2013). Unlike NFT presence, global brain Aβ load does not correlate with severity of AD symptoms (Rowe & Villemagne, 2013). Importantly, Aβ deposition commences well before detectable cognitive decline, and ~15-20 years before clinical diagnosis of AD (Victor L Villemagne et al., 2013). This time course of Aβ deposition presents a potential window of opportunity to change
disease trajectory. Presently, the leading hypothesis suggests that in AD, Aβ deposition is the primary trigger, which leads to tau hyperphosphorylation resulting in dementia (J. Gotz, Chen, van Dorpe, & Nitsch, 2001). Support for Aβ playing a primary role comes from environmental (e.g. head trauma), and genetic risk factors for AD, including the apolipoprotein E (APOE) gene, and mutations in the presenilin 1 and 2 (PSEN 1 and 2), and the amyloid precursor protein (APP) genes; all of which have been demonstrated to be associated with senile plaque formation (Alzheimer'sAustralia, 2016).
Figure 1.2: Comparison between the size of the ventricles, sulci, and gyri in either healthy ageing or preclinical Alzheimer’s disease (AD) brains, and advanced AD brains (Fox & Schott, 2004; Raz et al., 2005). Picture credit: University of California San Francisco, Memory and Ageing Centre (UCSF), 2017. Source: http://memory.ucsf.edu.

Figure 1.3: Pathological hallmarks of Alzheimer’s disease.

a) Senile plaques (Bielschowski silver stain) are often diffuse but can also occur as neuritic; b) The neuritic plaque includes neurofibrillary tangles (NFT) and microglia. Source: http://neuropathologyweb.org/chapter9/chapter9bAD.html.
1.1.3 The Aβ peptide

The Aβ peptide is a multifunctional protein, best known for its role in a number of neurological diseases due to its various isoforms (Aβ1-39 to Aβ1-43), as well as its ubiquitous appearance in the brain (Garcia-Osta & Alberini, 2009; Mark P Mattson, 1997). Hartmann and colleagues (1997) identified neurons as the major cell type that produces Aβ; with the endoplasmic reticulum (ER) the site of Aβ1-42/43 production and the Trans-Golgi-Network as the site of preferred Aβ1-39/40 production (Chen et al., 2009). Nevertheless, the mechanism of Aβ production and toxicity is yet to be fully elucidated. However, it is known that amyloid precursor protein (APP) cleavage (discussed in detail below) and Aβ oligomerization can occur intra- as well as extra-cellularly (Sakono & Zako, 2010).

Aβ is a cleavage product of its parent molecule, APP, which predominantly exists in axons (Priller et al., 2006). APP is an important and highly conserved transmembrane protein in neurons and other cells that has been shown to be involved in synaptic plasticity (Priller et al., 2006). Figure 1.4 describes the proteolysis of APP to form Aβ isoforms: depending on whether α-secretase or β-secretase (both proteolytic enzymes) cleaves APP, either the non-amyloidogenic or the amyloidogenic pathway is initiated. In the non-amyloidogenic pathway, α-secretase (or A Disintegrin and metalloproteina domain-containing protein 10; ADAM10) cleaves APP to create the C83 fragment, from which γ-secretase can only cleave a smaller P3 fragment, instead of Aβ (Krishnaswamy, Verdile, Groth, Kanyenda, & Martins, 2009). In the amyloidogenic pathway, β-secretase (or Beta-secretase 1; BACE1) cleaves APP to release 2 fragments: a 99 amino acid long component (C99), which stays in the membrane, and a soluble β-APP component which is released into the extracellular space. Following further
proteolytic cleavage of the C99 fragment by γ-secretase, soluble Aβ of 39 to 43 amino acids in length is produced. Soluble Aβ can be rapidly cleared via the CSF into the blood, from where it reaches the liver and the kidney for degradation (Lichtlen & Mohajeri, 2008). Traditionally, research findings most often refer to Aβ1-40 and Aβ1-42 when distinguishing between outcomes of the two cleavage pathways, indicating shorter and longer protein structures of Aβ. Compared to soluble Aβ1-40, the Aβ1-42 isoform is most prone to aggregation and therefore avoid efficient clearance. Soluble Aβ oligomers vary in size (as measured by molecular weight), ranging from < 10 kDa to > 100 kDa depending on assembly-size and structure (Sakono & Zako, 2010). It has been well established that Aβ oligomers impose severe toxicity to neurons, impairing synaptic transmission and causing neuronal death (Dahlgren et al., 2002; Deshpande, Mina, Glabe, & Busciglio, 2006; Shankar et al., 2008). Haughey and colleagues observed that Aβ also inhibits neuronal cell differentiation in vitro (Haughey et al., 2002).

The amyloidogenic, or β-pathway, appears to be dominant following certain environmental triggers, or in the presence of certain autosomal genetic mutations. Once the amyloidogenic pathway is initiated, soluble Aβ monomers begin to interlink, eventually forming insoluble fibrils (oligomers). These fibrils accumulate into dense extracellular (senile) plaques in the brain parenchyma (Figure 1.5). These plaques are very dense, rigid deposits, which gain their strength from a β-sheet interlinked protein structure. As a result, intrinsic Aβ clearing mechanisms become ineffective, eventually leading to neuronal dysfunction and subsequent cell death.

It is still not well understood which factors influence the initiation of the β-secretase cleavage pathway as opposed to the α-secretase pathway. However, autosomal
dominant mutations of genes such as APP (located on chromosome 21) and PSEN-1 and -2 (encoding two of the four individual proteins comprising the γ-secretase complex) cause an increase in the production of Aβ1-42 which leads to the development of familial AD (FAD) at an early age.

**Figure 1.4:** Proteolysis of APP via the non-amyloidogenic and amyloidogenic pathways. Source: (LaFerla, Green, & Oddo, 2007). Abbreviations: Aβ, amyloid-β; ADAM, A Disintegrin and metalloproteinase domain-containing protein; AICD, amyloid precursor protein intracellular domain; APH, anterior pharynx defective; APP, amyloid precursor protein; BACE1, β-secretase 1; C83, α-cleaved carboxyl-terminal fragment of BACE1; C99, β-cleaved carboxyl-terminal fragment of BACE1; PEN2, presenilin enhancer-2; sAPP, soluble APP.
Figure 1.5: The amyloid cascade hypothesis. Amyloidogenic processing of APP causes an overproduction of Aβ$_{1-42}$ and senile plaque formation. The cascade ends with tau aggregation, oxidative stress and neuronal cell death – all of which are the precursor steps of developing dementia. Taken from Verdile, 2004 (Verdile et al., 2004). Abbreviations: α-APPs, alpha-secretase cleaved soluble fragment of the amyloid precursor protein; Aβ, amyloid-β; Aβ$_{40}$, amyloid-β (40 amino acid long isoform); Aβ$_{42}$, amyloid-β (42 amino acid long isoform); APP, amyloid precursor protein; BACE, β-secretase; C, C-terminus; N, N-terminus; PS1, Presenilin-1; PS2, Presenilin-2.
1.1.4 The significance of Apolipoprotein E genotypes (APOE)

Several mechanisms have been identified that help clear excess Aβ from the brain. One protein hypothesized to be involved in Aβ clearance is Apolipoprotein E (ApoE) (Verghese et al., 2013). After the liver, the brain is the second major site of Apolipoprotein E (APOE) gene expression, which yields a cerebral apolipoprotein that has a demonstrated key role in the development of late-onset AD (LOAD). Within the brain, ApoE is mainly synthesised and released by astrocytes, but it is also expressed in neurons immediately upon neuronal injury (Mahley & Huang, 2012; Xu et al., 2006). In humans there are six possible APOE genotypes, coded by two of three possible alleles (ε2, ε3, and ε4), located on the same gene locus of chromosome 19. The resulting genotype variations include; ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, ε4/ε4 (Mahley, Weisgraber, & Huang, 2009). The ApoE hypothesis states that the effect of APOE alleles on the aggregation of senile plaques is as follows: ε4 > ε3 > ε2; indicating an increased risk of developing neurological disorders such as AD in those individuals carrying one or more ε4 alleles.

Several binding affinity studies have confirmed that ApoE ε4 demonstrates a reduced capacity for Aβ binding compared to the other two isoforms (Hone, Martins, Fonte, & Martins, 2003; D. S. Yang, Smith, Zhou, Gandy, & Martins, 1997). The stronger binding affinity to Aβ of the non-ε4 isoforms is associated with more efficient clearance of the neurotoxic peptide from the brain via the blood-brain barrier (BBB). An investigation of ApoE isomer-dependent clearance of Aβ from the brain showed that ApoE ε4 is most disruptive of rapid Aβ40/42-clearance by utilizing the slower very low density lipoprotein-receptor (VLDLR) instead of the faster low density lipoprotein (LDL) receptor–related protein 1 (LRP1) (Verghese et al., 2013). In addition, Verghese
and colleagues reported that ApoE ε2 and ε3 also utilize VLDLR, yet the internalization process of these ApoE isoforms and their complexes with Aβ occurs at a faster rate. Collectively, these studies have resulted in multiple investigations of the APOE gene in relation to AD.

Numerous studies support the hypothesis of abnormal Aβ processing and clearance, as well as malfunctioning signalling by ApoE receptors, in carriers of the APOE ε4 allele (Herz, 2009). An estimation of worldwide APOE allele distribution suggests that approximately 15% of all individuals carry the ε4 allele, compared to 78% carrying the ε3 allele, and about 6% the ε2 allele (Serrano-Pozo, Frosch, Masliah, & Hyman, 2011). Interestingly, it has been found that gender differences are an important factor in analysing the risk associated with ε4 allele carriage for developing late-onset AD. The presence of a single APOE ε4 allele has a much stronger impact on women (over 60 years of age) compared to men (Fagundo et al., 2016) - an observation that has been supported and replicated numerous times, as summarised by Ungar and colleagues (Ungar, Altmann, & Greicius, 2014). Animal and human studies alike continuously report more cognitive problems even in healthy females heterozygous for APOE ε4, compared to their male counterparts (Caselli et al., 2004; F. Liu et al., 2010; Reinvang, Winjevoll, Rootwelt, & Espeseth, 2010; Schiepers et al., 2012; J. A. Siegel, Haley, & Raber, 2012).

Of note, another study has demonstrated the occurrence of neurotoxic fragments in mice, which are derived from the proteolytic cleavage of apoE in neurons, especially from the apoE ε4 isoform (Mahley et al., 2009). Interestingly, these toxic fragments can only be found in neurons, and not in any other apoE-synthesizing cells (Brecht et al.,
2004; Harris et al., 2003; Y. Huang et al., 2001). These fragments are known to cause mitochondrial dysfunction and cytoskeletal degradation, further contributing to axonal swelling and neuronal death (Chang et al., 2005).

### 1.1.5 Metabolic diseases in AD

In addition to APOE genotype and age, metabolic diseases are also established risk factors for AD. Indeed, the relationship between impaired glucose metabolism, insulin sensitivity, diabetes and AD has received considerable research attention in recent years (Candeias et al., 2012; Duron et al., 2012; Haley, Knight-Scott, Simnad, & Manning, 2006; Steen et al., 2005). In brief, this literature as a whole highlights similar pathological features of type 2 diabetes mellitus (T2DM) and AD: including neuronal degeneration, Aβ aggregation, increased oxidative stress and inflammatory response, an association with the APOE ε4 allele, and a number of other shared abnormalities in cellular signalling cascades (Y. Yang & Song, 2013). Due to the similarities of both diseases the term “Type 3 Diabetes” as an alternative description of AD was proposed (Steen et al., 2005). Not only have similar molecular pathways been identified, but in addition, a systematic review conducted by Biessels et al. concluded that a 50-100% risk of developing AD was present among individuals with diagnosed T2DM (Biessels, Staekenborg, Brunner, Brayne, & Scheltens, 2006). Another systematic review, conducted three years later, further confirmed that a diagnosis of T2DM is a risk factor for AD (Kopf & Frolich, 2009).

Obesity, defined by a body mass index (BMI) of 30 or over, is one of the main components of metabolic syndrome. The influence of BMI on AD was recently summarised in a meta-analysis (Okamura et al., 2014), where it was reported that age,
together with an elevated BMI in mid-life (i.e., a mid-life BMI indicative of being overweight or obese, particularly in women), or by contrast a low BMI later in life, play a combined role in increasing AD risk (Victor L Villemagne, Fodero-Tavoletti, Masters, & Rowe, 2015). Weight loss is often reported in AD patients and in the immediate stages preceding an AD diagnosis (Tamura, Masaki, & Blanchette, 2007). How elevated BMI in mid-life can influence AD-risk however, is not yet fully elucidated: some research has associated a change of brain structure and BBB with a higher BMI, hinting towards changes in the microenvironment of the brain as a possible mechanism (Nordestgaard, Tybjærg-Hansen, Nordestgaard, & Frikke-Schmidt, 2017). Indeed, hypercholesterolemia which is frequently associated with elevated BMI, and is itself another widely accepted metabolic syndrome-linked risk factor for AD, has been suggested as a contributor to increased permeability of the BBB (Xue-shan et al., 2016), and thus to altered CNS microenvironment and function. In addition, higher cholesterol levels promote an elevated synthesis of Aβ by increasing APP-decomposition to Aβ by BACE1.

All of the aforementioned metabolic disturbances have been of major interest in the search for causative underlying mechanisms in the development of AD, and have therefore been of interest in the cohort analyses conducted in this thesis.

1.1.6 Neuropathology detection methods in AD

Neuroimaging techniques have become a valuable tool with respect to visualizing and predicting the progress of AD pathology in both diagnosed patients and older adults without clinical symptoms of cognitive decline (who may be at the preclinical disease stage). Magnetic resonance imaging (MRI) provides detailed information on the
structure of the brain. Shrinkage of brain areas associated with memory (e.g. hippocampus, frontal lobe, temporal lobe), as well as enlargement of ventricles and sulci, all assist in quantifying the neuropathology of the disease, and providing informing regarding the rate of atrophy when serial measures are obtained. Previously however, confirmation of the presence of cerebral AD pathology was only possible post-mortem.

In recent times, the functional imaging modality of positron emission tomography (PET) coupled with radioactively labelled amyloid ligands, has been extensively used to assess cerebral Aβ load, and rates of Aβ accumulation in the living brain. To measure Aβ in the living brain, a radioactive tracer is injected into the bloodstream, which after crossing the BBB, binds to cerebral fibrillar Aβ deposits, thereby enabling quantification. One of the most widely used tracers is Pittsburgh Compound B (PiB), a C11 labelled radiotracer that has been validated over the last 12 years by comparing imaging outcomes to both post-mortem histological detection of senile plaques in adult brains (Ikonomovic et al., 2008), and to cognitive performance in older adults pre-mortem (V. L. Villemagne et al., 2008). More recently, the Florbetapir (F18) radiotracer (developed by Amyvid, Eli Lilly) has also been frequently and successfully used to detect Aβ plaques via PET imaging (Morris et al., 2016; Sevigny et al., 2016). The advantage of Florbetapir over the PiB tracer is its longer half-life, thereby facilitating successful transport of the ligand from its production site to the imaging site, and allowing more doses to be obtained from a single tracer batch. However, both tracers demonstrate similar sensitivity and specificity (Morris et al., 2016). More recently, several tau PET tracers have been synthesized (e.g. THK5117, PBB3, and T807) to enable visualization of NFTs in the living brain, which develop in the early stages of
AD, and could serve as early markers for the disease. However, further work is needed to understand the contribution of tau deposits to the temporal-causal development of AD (Okamura et al., 2014; Victor L Villemagne et al., 2015). The detection of protein markers in CSF is still recognized as one of the most definitive methods for identifying the presence of AD pathology in older adults in vivo (ADDF, 2016). Typically, high amounts of total tau (t-tau) and in particular phosphorylated tau (p-tau), as well as low amounts of Aβ₄₂ in CSF are associated with early signs of AD (Anoop, Singh, Jacob, & Maji, 2010; Zetterberg et al., 2014): this ratio is proposed to reflect a dearth of Aβ₄₂ in CSF following sequestration into senile plaques in the brain, and an increase in p- and t-tau released into the CSF following the death and subsequent fragmentation of NFT-containing neurons (Anoop et al., 2010). In comparison, Aβ₄₀ levels are largely unchanged in CSF as AD pathology progresses.

1.1.7 Blood biomarker panel for AD

Biomarkers are crucial tools in the early detection and observation of the progression of AD, whereby confirmed diagnosis has only previously been possible during post mortem examination. Although the use of CSF biomarker quantification and PET neuroimaging have proved to be highly sensitive and specific to measures of AD neuropathology, development of a more cost effective and accessible method, such as a blood test, is vital. Nevertheless, the objective of identifying a single blood-based biomarker indicative of AD neuropathology has been superseded by the search for and identification of a multi-protein biomarker panel, likely as a component of a multistep diagnostic process (Schneider, Hampel, & Buerger, 2009). Several study groups have had some success at identifying various biomarker panels with sensitivity and specificity > 80% (O'Bryant et al., 2015). However, the reproducibility of these results
is generally hampered by a number of variables that are often difficult to control for, including; subjective reporting of medication consumption, and complex dietary and lifestyle habits. Furthermore, methodological approaches are also often subject to discrepancies between laboratories, and the detection of markers in the blood often suffers from a lack of standardized methodology. The biggest challenge to-date has been to identify blood-based biomarkers, which exhibit strong enough signals such that one or two markers would be sufficient for diagnosis, akin to the measurement of tau and Aβ in CSF. Therefore, further research is needed to find strong reliable blood-based biomarkers and reproducible testing methods for the early diagnosis and prognosis of AD.

1.1.8 Current research focus

To-date, studies aiming to identify treatment strategies for AD have produced relatively disappointing results in clinical settings. These potential treatments have been unable to alter the course of the disease, with some even contributing to severe side effects (Golde, Schneider, & Koo, 2011; F. Mangialasche, A. Solomon, B. Winblad, P. Mecocci, & M. Kivipelto, 2010). Worldwide research activities have therefore shifted towards identifying early markers of changing brain health, with the notion that a potential treatment strategy administered at the pre-clinical or even pre-symptomatic stage of AD (before the brain is irreversibly damaged), will likely yield the greatest chance of success (Gandy & DeKosky, 2013). Indeed, the early detection of AD pathology (i.e. well before the onset of clinical symptoms) could trigger a number of potentially personalized preventative strategies, delaying the onset of the predicted disease by as long as possible. In Australia, a delay of AD onset of just five years has the potential to save billions of dollars a year due to a reduced requirement for medical
and residential care. Therefore, there is an urgent need for a sensitive and reliable detection method for pre-clinical or even pre-symptomatic AD, that is easily accessible, affordable and quick to perform, and that can be combined with disease-modifying strategies. Such strategies include modifiable lifestyle factors, such as diet, exposure to stress, and social engagement, or cognitive stimulation (Gandy & DeKosky, 2013). An additional promising lifestyle factor that has gained wide acceptance over recent years, and has been shown to demonstrate multiple beneficial effects with respect to brain health, is physical activity.
1.2 Biomarkers associated with memory and brain health

Over the past decades, a group of powerful cell signalling molecules, termed neurotrophins, has been described, which is heavily involved in the development and function of the brain (Tapia-Arancibia, Aliaga, Silhol, & Arancibia, 2008), including in ‘synaptic plasticity’, a mechanism implicit in memory formation (E. J. Huang & Reichardt, 2001; Y. Lu, Christian, & Lu, 2008; Schjetnan & Escobar, 2012; Yamada & Nabeshima, 2003). Memory is a complex function of the central nervous system (CNS), involving the development, maintenance and function of neurons, as well as the strengthening of their synapses and connections (Bekinschtein et al., 2008; Y. Lu et al., 2008; Tapia-Arancibia et al., 2008).

Neurotrophins belong to a group of mediators that play an important role in neuronal maintenance and survival, as well as activating receptor tyrosine kinases. These receptor-activation steps lead to crucial signalling cascades in neurons, influencing their proliferation, growth, cytoskeleton assembly, and membrane trafficking (E. J. Huang & Reichardt, 2001). In mammals, the neurotrophins are a family of four similarly encoded and structured proteins, namely: Brain-derived neurotrophic factor (BDNF), nerve growth-factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4 and NT-5) (R. Gotz et al., 1994; Greenberg, Xu, Lu, & Hempstead, 2009; Hallbook, 1999; E. J. Huang & Reichardt, 2001; Nilsson, Fainzilber, Falck, & Ibanez, 1998).
1.2.1 Role of brain-derived neurotrophic factor (BDNF)

BDNF is the most widely expressed neurotrophin and is understood to play a key role in the development of long-term memory (Cunha, Brambilla, & Thomas, 2010). Various stimuli regulate the expression of BDNF, such as physical activity and learning tasks. BDNF is strongly associated with hippocampal-dependent cognition and volume (Harrisberger et al., 2015; Pezawas et al., 2004; Szeszko et al., 2005). The mature form of BDNF (mBDNF) represents a dimer, which is derived from the cleavage of its precursor protein, precursor BDNF (proBDNF). ProBDNF can be cleaved intra- and extracellularly by an enzyme, plasmin, to release mBDNF (B. Lu, Pang, & Woo, 2005); however, proBDNF also acts as a signalling molecule on its own (Mowla et al., 2001).

Due to the role of BDNF in maintaining memory and neuronal health, recent research has investigated the link between this neurotrophin and the development of AD. Mounting evidence has demonstrated dysfunctional BDNF signalling in various brain regions in AD patients, as well as a down-regulation of neurotrophins and their receptors in the pre-clinical stages (Fahnestock, Garzon, Holsinger, & Michalski, 2002; Minichiello et al., 1999; Reichardt, 2006; G. J. Siegel & Chauhan, 2000). Significantly, within the field of AD research, evidence pointed towards a neuroprotective function of BDNF against the toxic effects of Aβ1-42 (Arancibia et al., 2008; Kitiyanant, Kitiyanant, Svendsen, & Thangnpon, 2012).

It was suggested in the late 1990’s that BDNF could cross the BBB (Pan, Banks, Fasold, Bluth, & Kastin, 1998; Poduslo & Curran, 1996), a notion that sparked hope for the role of BDNF as a biomarker of AD that could be easily measured in the periphery. However, comparisons of circulating BDNF levels amongst individuals with and
without cognitive decline have been investigated on numerous occasions, with inconclusive outcomes. Serum BDNF was significantly decreased in a Turkish population of early-onset and late-onset AD patients compared to controls; while Nettiksimmons et al. could not confirm these results in a longitudinal study (Gezen-Ak et al., 2013; Nettiksimmons et al., 2014). However, lower baseline levels of CSF-BDNF were strongly associated with an annual cognitive decline in otherwise cognitively normal older adults (Li et al., 2009). Among animal studies, only a few have shown a correlation between blood and brain BDNF levels (rats and pigs); such correlations have not been reported in studies of mice. In a 2011 animal study, Klein et al. showed that plasma and whole blood BDNF levels were positively correlated with hippocampal BDNF in the brains of pigs and rats, respectively (Klein et al., 2011). Yet, this relationship was not found when BDNF levels in the frontal cortex were examined. Thus, debate remains regarding whether BDNF does in fact cross the BBB, and its suitability as a reliable indicator of brain health (Nettiksimmons et al., 2014; Pardridge, 2012). Consideration should however be given to the complexity of measuring the various forms of BDNF (total, mature and pro-), whether each form was measured in the aforementioned studies, and how such measurements might vary between species, as well as how levels of each of these forms reflects brain health, both in humans and animals: the story is highly likely to be more complicated than some of the literature suggests.

A recent meta-analysis attempted to clarify the association between BDNF levels and cognition, confirming the current perspective that lower peripheral BDNF levels are observable in individuals with AD (Qin et al., 2016). Furthermore, higher levels of proBDNF relative to mature BDNF have been found in participants with AD (J. Y. Lim,
Reighard, & Crowther, 2015). However, of note, in addition to the complexities touched upon in the previous paragraph, the effect of BDNF on neurons is likely to vary from person to person, depending on a polymorphism that can be found in the BDNF gene: the Val66Met polymorphism (Gomar et al., 2016; Y. Y. Lim et al., 2014; Y. Y. Lim et al., 2015). This single nucleotide polymorphism is located in the pro-domain of the human BDNF gene, and affects the trafficking and secretion of BDNF. Humans with the amino acid Methionine (Met) instead of Valine (Val) in codon 66, are proposed to demonstrate impairment in hippocampal-dependent episodic memory and abnormal hippocampal function, compared to Val/Val homozygotes. This notion is however, yet to be confirmed and is a topic of much ongoing research and debate (Dodds, Henson, Miller, & Nathan, 2013; Kambeitz et al., 2012). Mechanistically, the Val66Met mutation, is purported to hinder the sorting of BDNF, leading to decreased regulated secretion of this neurotrophin (Anastasia et al., 2013). It is also plausible that other, as yet unknown, genetic variations modulate the effect of BDNF on neurons.

A plethora of stimuli, from light, osmotic and electric stimuli to physical activity, stress and epigenetic factors have been reported to influence BDNF regulation (Binder & Scharfman, 2004; Boulle et al., 2012). One of the important molecular triggers of BDNF activation was found to be the fibronectin type III domain containing 5 (FNDC5) protein; a pro-hormone that was first discovered over a decade ago and which recently has received increased attention as a putative precursor protein for a recently discovered hormone associated with physical activity, namely irisin (Bostrom et al., 2012; Wrann et al., 2013).
1.2.2 Fibronectin type III domain containing 5 (FNDC5) protein

During embryonic development, fibronectin type III domain containing 5 (FNDC5) protein has been found to be highly expressed in the human brain, and has recently been linked to neural differentiation of embryonic stem cells (Ahmadi Ghahrizjani et al., 2015). FNDC5 is a ubiquitous and highly conserved type I membrane protein that is mainly expressed in tissues with high energy demand, specifically, heart, skeletal muscle and brain (H. P. Erickson, 2013; Ferrer-Martinez, Ruiz-Lozano, & Chien, 2002; Teufel, Malik, Mukhopadhyay, & Westphal, 2002). Much FNDC5 research has focussed on managing high-energy demands as well as investigation of a potential role in neural differentiation (Ahmadi Ghahrizjani et al., 2015). In recent years, studies have found that FNDC5 is a dimer with an extracellular domain, which is cleaved, thereby releasing a small fragment, called irisin, into circulation. Irisin was initially described as a newly discovered hormone which could potentially enhance understanding of the molecular pathways underlying cellular energy production. Following its discovery in 2012, several research groups have investigated the importance, abundance and role of irisin in a range of *in vivo* and *in vitro* studies.

1.2.3 The discovery of irisin

Mammals store energy in the form of triglycerides, which mainly occur in white adipose tissue (WAT). Two other types of fatty tissue, namely brown adipose tissue (BAT) and beige adipose tissue (intermediate phenotype), oppose WAT and utilise energy to release heat (Tiano, Springer, & Rane, 2015a). Irisin was identified when factors from skeletal muscle responsible for the browning of white adipose tissues (browning response) were investigated. Browning describes the process in which
adipocytes of WAT gain the properties of adipocytes of BAT, and is an area of great interest in metabolic research. Adipocytes of BAT are hypothesised to aid in heat generation in hibernating animals and infants, without a need for active shivering (Castillo-Quan, 2012).

In early 2012, Boström and colleagues published a report describing the newly identified hormone, irisin (Bostrom et al., 2012). These investigators observed a rise in FNDC5 and Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) mRNA in mice and in middle-aged humans after undertaking exercise (10 weeks of endurance training). Following the exercise regime, these investigators also detected increased circulating levels of a molecule that they would later refer to as irisin. The group then undertook an in vivo experiment whereby FNDC5 was overexpressed in the liver of mice, which resulted in a significant increase of BAT, improved glucose tolerance and increased energy expenditure (Bostrom et al., 2012). The researchers went on to propose the existence of a hormone (irisin) cleaved from FNDC5 that is able to fulfil signalling tasks in WAT and with it, induce associated metabolic benefits. In their model, PGC1-α stimulates the expression of the FNDC5 gene (an observation that has been described elsewhere (Kelly, 2012)); which in turn yields a type I transmembrane protein, that is proteolytically cleaved to release a smaller (112 amino acid long), soluble fragment into the blood stream; later named irisin after the Greek messenger goddess Iris (Bostrom et al., 2012). During their investigation of human plasma and muscle biopsy samples, the group used samples collected from eight non-diabetic male participants who took part in a 10-week aerobic exercise program. Using Western blotting, with antibodies directed against wild-type FNDC5, several bands were apparent which were determined to be a consequence of a glycosylation step.
of the secreted protein. Mass spectroscopy was then employed to identify the cleavage area of FNDC5 and its sequence. The conversion from WAT to BAT is not well understood, however the movement of a shivering body is proposed to be responsible for the initiation of irisin expression and thus, the activation of metabolic, intracellular pathways during the WAT to BAT conversion (Lee et al., 2014). In subsequent years, several studies concluded that irisin could be released by a number of organs, including the brain and heart, since organs with a high-energy expenditure express the FNDC5 gene. Further, because adipose cells were shown to also release irisin, this hormone was described simultaneously as both a myokine and an adipokine (Moreno-Navarrete et al., 2013a; Roca-Rivada et al., 2013). Figure 1.7 (page 51) briefly summarises the proposed pathway that this thesis is based upon; specifically, the PGC-1alpha, irisin, and BDNF-signalling cascade.

The reported discovery of irisin sparked heavy debate, from approximately 2013 to 2015. Following a critical review of commercially available irisin-measuring ELISA kits, this debate surrounded both the molecule’s existence and function (Albrecht et al., 2015; Elsen, Raschke, & Eckel, 2014). The authors reported discrepancies in the specificity of antibodies and therefore, in the definite detection of irisin in plasma and serum samples. These discrepancies appeared to explain wide variability in the detection range of irisin; sometimes reported as low as 25 pg/ml and sometimes as high as over 2,000 ng/ml (Y.-K. Choi et al., 2013; Moreno-Navarrete et al., 2013b). Whilst some companies withdrew their irisin ELISA kits from the market during this time period, Phoenix Pharmaceuticals developed a second, and later a third, generation of irisin-detection kits, which have now been validated, and are the most commonly used detection kits (e.g. Reinehr, Elfers, Lass, & Roth, 2015). Nevertheless, the earlier
human studies are still likely to have provided some insight into the relationship between irisin and varying demographics (e.g. age, gender, BMI). Study limitations are further discussed in Chapter 6 of this thesis.

1.2.3.1 Effects of age and gender on irisin levels

To-date, conflicting data exists linking age and gender with irisin levels. Although some studies report age-dependent irisin levels in young-to-middle aged cohorts (Ates, Altay, Topcuoglu, & Yilmaz, 2015; Huh, Panagiotou, Mougios, Brinkoetter, Vamvini, Schneider, et al., 2012), other studies have reported no relationship between age and irisin in either young adults or children (Oelman et al., 2016) (Huh, Panagiotou, Mougios, Brinkoetter, Vamvini, Schneider, et al., 2012; Reinehr et al., 2015). In older adults, irisin levels have been reported, by some, to decrease with increasing age (A. D. Anastasilakis, S. A. Polyzos, Z. G. Saridakis, et al., 2014; Bluher et al., 2014; Huh, Mougios, Kabasakalis, et al., 2014; Huh, Panagiotou, Mougios, Brinkoetter, Vamvini, Schneider, et al., 2012). Levels of this hormone are known to be associated with skeletal muscle mass, thus, this association between age and irisin in older adults could be due to the differing body compositions between ‘young-old’ and ‘old-old’ individuals; a notion which is supported by evidence of a clear relationship between irisin and lean body mass in some studies (Huh, Panagiotou, Mougios, Brinkoetter, Vamvini, Schneider, et al., 2012; Staiger et al., 2013; Stengel et al., 2013). Reports of differing irisin levels between genders (sexes) have recently started to emerge in the literature, with females often demonstrating higher baseline irisin levels than men (Al-Daghri et al., 2014; Athanasios D. Anastasilakis et al., 2014). Indeed, higher levels of irisin have been reported in healthy young females compared to their male counterparts (A. D. Anastasilakis, S. A. Polyzos, Z. G. Saridakis, et al., 2014), in healthy young Arab girls
compared to young Arab boys (Al-Daghri et al., 2014), and whilst irisin levels were increased in Type I diabetes patients overall compared to healthy controls, this association was particularly prominent in females (Espes, Lau, & Carlsson, 2015): concurrently, these females experienced a reduced requirement for insulin. Importantly, in an exercise study conducted by Zügel and colleagues, no differences in resting serum irisin levels between men and women were observed; however, acute aerobic exercise induced a significant surge in irisin levels among lean women when compared to their male counterparts (Zugel et al., 2016): further supporting the idea that both lean body mass and even obesity may impact this relationship (Crujeiras et al., 2014). However, such gender-related differences in irisin levels are not always observed (Moreno et al., 2015).

Irisin levels have also been positively correlated with levels of the sex hormone oestradiol in healthy middle-aged women in a single study (Huh, Panagiotou, Mougios, Brinkoetter, Vamvini, & Schneider, 2012). Furthermore, higher bone-density and a reduced risk of osteoporotic fractures have been linked with higher irisin levels in postmenopausal women (A. D. Anastasilakis, S. A. Polyzos, P. Makras, et al., 2014; Palermo et al., 2015). These results suggest the need for more sex-specific investigations in regards to irisin’s actions and functionality.

### 1.2.3.2 Irisin, cardiovascular disease and metabolic disorders

A number of recent studies have explored the link between irisin and biomarkers of both cardiovascular disease (CVD) and metabolic disorders such as T2DM: of note, both CVD and T2DM are associated with increased risk of AD (Barnes & Yaffe, 2011; Imtiaz, Tolppanen, Kivipelto, & Soininen, 2014; Profenno, Porsteinsson, & Faraone,
2010). A strong inverse association between irisin and total cholesterol and LDL has been observed in a large male cohort (Oelmann, Nauck, Volzke, Bahls, & Friedrich, 2016). Moreover, in healthy, non-diabetic study participants, Liu and colleagues reported correlations between irisin and total cholesterol (Hecksteden et al., 2013), triglycerides, and fasted blood glucose levels before adjusting for covariates (J. J. Liu et al., 2013). Intriguingly, high density lipoprotein (HDL)-cholesterol has been shown to be positively correlated with irisin levels in patients with Chronic Kidney Disease (CKD), even after adjusting for renal function (Wen, Wang, Lin, & Hung, 2013).

Choi and colleagues reported reduced serum irisin levels in subjects with new-onset T2DM, while higher levels of irisin corresponded with lower risk of developing T2DM (Y. K. Choi et al., 2013): possibly suggesting that decreases in irisin levels could be indicative of insulin resistance, which often precedes the development of T2DM. Zhang and colleagues undertook a case control study of 50 newly diagnosed T2DM patients and 50 controls, and found lower serum irisin levels in the T2DM group (C. Zhang et al., 2016). The outcome of this case study was consistent with the results of a meta-analysis published by the same group, wherein seven other studies reported a similar relationship between irisin concentrations and T2DM (Alis et al., 2014; J. J. Liu et al., 2013; Moreno-Navarrete et al., 2013a; Sanchis-Gomar, Alis, Pareja-Galeano, Romagnoli, & Perez-Quilis, 2014; Sanchis-Gomar, Alis, Pareja-Galeano, Sola, et al., 2014). Contrary to the studies described above, there have been some reports of high irisin levels being associated with T2DM. Indeed, in a study by Park and colleagues (2013), increased serum irisin levels were associated with metabolic syndrome (MetS; a risk factor for T2DM and CVD), BMI, and insulin resistance. Further, a study of pre-diabetic males also showed higher overall plasma irisin levels compared to their control
counterparts (Norheim et al., 2014). Initially, an individual’s BMI was hypothesised to account for the observed between-group differences in irisin levels, with several studies showing a positive correlation between plasma irisin and BMI (Crujeiras et al., 2014; Huh, Panagiotou, Mougiou, Brinkoetter, Vamvini, Schneider, et al., 2012; Moreno-Navarrete et al., 2013a; Park et al., 2013; Stengel et al., 2013). However, Sanchis-Gomar and colleagues found no difference in irisin levels between obese and non-obese T2DM patients (Sanchis-Gomar, Alis, Pareja-Galeano, Sola, et al., 2014). Moreover, BMI is notorious as an unreliable measure of obesity as it does not take into account the body fat/lean mass ratio, and can therefore lead to misleading classifications, particularly in older adults (Burkhauser & Cawley, 2008).

Further, genetic polymorphisms in the FNDC5 gene could contribute to varying estimates when assessing irisin levels and risk of developing MetS or T2DM. Specifically, Staiger and colleagues concluded that the FNDC5 gene determines insulin sensitivity based on four SNPs (Staiger et al., 2013). The authors also showed a positive association between FNDC5 mRNA and insulin in cross-sectional muscle biopsies of younger and elderly subjects.

1.2.3.3 Irisin and the central nervous system

Studies of rat and mouse brains have shown irisin-immunoreactivity in the Purkinje cells of the cerebellum and medulla oblongata (Dun et al., 2013). In an in vivo study of Sprague Dawley rats, central administration (via intra-cerebroventricular injection) of irisin dissolved in CSF, resulted in a marked increase in activity levels of the rats as measured by travel distance, resting time, oxygen consumption and carbon dioxide production (W. Zhang et al., 2015); leading the authors to conclude that irisin likely
signals directly to the CNS. Moreover, exposing mouse H19-7HN hippocampal cells to supraphysiological concentrations of irisin (50-100 nmol/L) was shown to increase cell proliferation, although such effects were not observed at physiological conditions (5-10 nmol/L) (Moon, Dincer, & Mantzoros, 2013). It is perhaps unsurprising that supraphysiological irisin concentrations are needed to modulate cell proliferation in this in vitro model, as treatment concentrations needed to cause an effect in vitro often do not reflect the concentrations required to elicit an equivalent effect in vivo (Laskowski et al., 2016).

As irisin is active within the CNS and regulates metabolic activity with movement, it has been designated as a “cross-organ messenger” acting between the brain and skeletal muscle (W. Zhang et al., 2015). The recent research findings characterising irisin’s actions on the CNS, the activation of irisin signalling upon physical activity, together with the relationship of this hormone with secondary risk factors for AD, such as CVD and T2DM, make irisin an appealing candidate in the quest to identify biomarkers of preclinical AD.

1.2.3.4 Structure and putative function of irisin

The FNDC5 structure, from which irisin is proteolytically cleaved, contains a 29 amino acid (AA) signal peptide at the amino (N)-terminus followed by a fibronectin type III (FNIII) domain, and a hydrophobic domain at the carboxy (C)-terminus (19 AAs), which is inserted in the cell membrane lipid bilayer (Figure 1.6). FNDC5 is a type I transmembrane protein with its FNIII domain positioned extracellularly, similar to some cytokine receptors (Novelle, Contreras, Romero-Pico, Lopez, & Dieguez, 2013). Irisin
production is analogous to the shedding and release of other hormones from transmembrane precursors. Following removal of the N-terminal signal peptide, the irisin peptide is proteolytically cleaved from the C-terminal moiety, glycosylated and released as a hormone of 112 AA that comprises most of the FNIII region.

X-ray Crystallography and biochemical data indicate that irisin may activate receptors via a unique FNIII-like structure, forming a pre-formed “intersubunit β-sheet dimer” (Schumacher, Chinnam, Ohashi, Shah, & Erickson, 2013). This irisin structure is unique because it promotes dimerization, whereas all other known FNIII-structures demonstrate contrary physical features. Overall, irisin’s structural elements are implicated in protein stability, with a putative binding site to a receptor found within the location of the flexible loops and its N-terminus on the hydrophobic face of the dimer (Schumacher et al., 2013). Therefore, irisin has the structural properties to facilitate dimerization and thus, activation of a receptor within a single cell, or even between two cells; leading to this peptide being hypothesised to perform an autocrine and/or paracrine function.
Figure 1.6: The FNDC5 structure, from which irisin is proteolytically cleaved, contains a 29 AA signal peptide at the amino (N)-terminus followed by a fibronectin type III (FNIII) domain extracellularly, and a hydrophobic domain at the carboxy (C)-terminus (19 AAs), which is inserted in the cell membrane lipid bilayer. Following removal of the N-terminal signal peptide, the irisin peptide is proteolytically cleaved from the C-terminal moiety, glycosylated and released as a hormone of 112 AA that comprises most of the FNIII region. Source: (Novelle et al., 2013). Abbreviations: AA, amino acids; FNDC5, Fibronectin type III domain-containing protein 5; kDa, kilo Dalton.
**1.2.3.5 Molecular pathways associated with irisin**

Numerous studies have shown that skeletal muscle produces and secretes endocrine signalling molecules in the form of cytokines or other peptides, collectively referred to as myokines (Schumacher et al., 2013). Exercise does not only release a plethora of myokines, of which irisin is one, it also affects more than 1000 genes. These physiological changes constitute a large variety of potential molecular pathways and markers triggered by exercise or physical activity that can influence overall health of the body, including brain health (Pedersen et al., 2003; Timmons, Baar, Davidsen, & Atherton, 2012).

Recent studies suggest that the browning of WAT (which is stimulated by irisin) occurs via the p38 mitogen-activated protein kinase (MAPK) / extracellular signal-regulated kinases (ERK) pathway (Bostrom et al., 2012; Y. Zhang et al., 2014), or via the PGC-1α estrogen-related receptor-α BDNF pathway (Tiano et al., 2015a; Wrann et al., 2013). The MAPK/ERK pathway is an extensively studied signalling cascade influencing the cell cycle and therefore many human diseases, including AD (Kim & Choi, 2010). As mentioned below in section 1.3, the PGC-1α/BDNF-pathway has recently been investigated in order to understand the signalling cascade that underlies the beneficial effects of physical activity on the brain; wherein, PGC-1α serves as a BDNF-regulating factor.

SMAD3 (mothers against decapentaplegic homolog 3) belongs to a group of regulators involved in the development of obesity (Yadav et al., 2011). Given the link between obesity and irisin described in section 1.2.3.1, it was of interest to investigate a possible relationship of SMAD3 with irisin. Indeed, Tiano and colleagues (2015b) showed that
SMAD3 negatively impacted irisin levels in mice due to active inhibition of FNDC5 and PGC-1α expression. Furthermore, SMAD3 knockout mice showed higher irisin levels following exercise compared to control mice. The same research study confirmed the suppressive effect of SMAD3 on FNDC5 and PGC-1α in C2C12 myotubes. The authors concluded that SMAD3 inhibition could help protect against obesity and diabetes. Further, the group provided evidence that PGC-1α is an upstream regulator of FNDC5, as an increase of SMAD3 activity elicited a decrease of PGC-1α several hours before FNDC5-expression ceased.

1.2.4 Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)

A link between BDNF and another crucial key mediator of normal brain function, namely PGC-1α, was recently established (Cheng et al., 2012). PGC-1α is one of the major factors involved in energy expenditure (i.e. glucose utilisation) (Puigserver and Spiegelman, 2003; Lin et al., 2004; Rodgers et al., 2005), and is a mediator of physical activity (PA)-induced neuronal protection (Qin et al., 2009). The induction of PGC-1α is associated with a number of crucial brain functions, such as oxygen metabolism, neurite growth and mitochondrial biogenesis (Puigserver & Spiegelman, 2003; Wu et al., 1999). Furthermore, Cheng and colleagues (2012) have identified a potential role for PGC1-α in the formation of dendritic spines during neurogenesis.

BAT cells express large amounts of mitochondria. The uncoupling protein 1 (UCP1) in these mitochondria is responsible for the release of energy in the form of heat, instead of storing energy in the form of fat. The expression of UCP-1 is in turn triggered by the transcriptional regulator PGC-1α. PGC-1α is increased upon physical activity and is a
long-known regulator of mitochondrial biogenesis. The more BAT there is in humans or mammals, the more mitochondria are available, the higher the heat production, and the lower the WAT content. Exercise was found to increase the amount of BAT-cells, which results in increased energy utilisation, and improved health. Increasing evidence also suggests that skeletal PGC-1α activity is down-regulated in T2DM patients (Liang & Ward, 2006; J. J. Liu et al., 2013). Further, PGC-1α is involved in insulin sensitivity, the metabolism of fatty acids and glucose (Soyal, Krempler, Oberkofler, & Patsch, 2006), and has also been shown to decrease Aβ generation.

Given that the expression and activity of irisin, neurotrophins, and other molecules in the irisin signalling cascade, have all been linked to exercise and/or physical activity in this section (1.2), it seems logical to describe our current understanding of physical activity in connection to cognition and AD in the following section (1.3).
1.3 Physical activity

The term physical activity (PA) encompasses movements that result in energy expenditure that is greater than a person’s resting state (Blondell, Hammersley-Mather, & Veerman, 2014). Physical activity has been associated with numerous beneficial health outcomes, in particular the prevention and treatment of a plethora of diseases. Evidence from numerous studies demonstrates that physical activity maintains healthy blood pressure and blood cholesterol levels (Dasgupta et al., 2014; Swift, Johannsen, Lavie, Earnest, & Church, 2014), thereby preventing cardiovascular disease (CVD) (Gaesser, 2007), positively affects bone density and therefore, the prevention of osteoporosis (Tan et al., 2014), assists in the prevention and treatment of diabetes (Gaesser, 2007; Swift et al., 2014), and is associated with reduced symptoms of depression and anxiety (Lindwall, Gerber, Jonsdottir, Börjesson, & Ahlborg Jr, 2014; Rosenbaum, Tiedemann, Sherrington, Curtis, & Ward, 2014). Interestingly, more recent studies have shown a strong association between regular physical activity and improved brain health and cognitive skills, particularly in older adults (Blondell et al., 2014).

Worldwide research is increasingly focusing on identifying strategies that may prevent or delay the onset of AD (Reiman et al., 2011). Translational work from preliminary in vitro and in vivo experiments led to a number of clinical studies in the past decade, which sought to investigate the beneficial effects of non-pharmaceutical lifestyle factors, such as physical activity and diet, on overall brain health. Physical activity in particular has the potential to reduce the risk of developing cognitive decline and dementia as shown in a number of studies, e.g. (Cotman, Berchtold, & Christie, 2007; K. I. Erickson, Weinstein, & Lopez, 2012; Hillman, Erickson, & Kramer, 2008; Eric B Larson et al., 2006; N. T. Lautenschlager, Cox, & Cyarto, 2012; Nicola T
Lautenschlager et al., 2008). While physical activity simply refers to any movements a person performs daily that involve the use of skeletal muscle, exercise refers to a planned and structured activity that is being conducted with the purpose of gaining physical fitness (Bherer, Erickson, & Liu-Ambrose, 2013). Within the umbrella term of exercise, either aerobic or resistance exercise can be performed. Aerobic exercise requires large muscle activity over an extended period of time (at least 10 minutes), it increases the heart rate and delivers oxygen more quickly throughout the body (Howley, 2001). Ultimately, a person improves cardiovascular fitness and endurance when regularly conducting aerobic training. Resistance exercise involves muscle contraction against an external weight or resistance. For these exercises, certain muscle groups are targeted and tasks are performed to build muscle strength. Resistance training also counteracts sarcopenia in older adults (Hunter, McCarthy, & Bamman, 2004). Resistance training can be divided into strength training and hypertrophy training. While strength training utilises heavy weights for few repetitions (< 6) at a given time, it is purely for the purpose of increasing muscle strength (i.e. increased contractile force output); hypertrophic resistance training uses moderate weights for more repetitions (6 - 12) and builds muscle endurance and muscle size (Radaelli et al., 2015; Villareal et al., 2017).

The impact of various exercise regimes on brain health has been studied extensively in previous years. However, the mechanisms underpinning precisely how exercise benefits overall health, and in particular brain health, are poorly understood (Neufer et al., 2015). One candidate mechanism involves the myokines. The different types of exercise cause different myokines to be secreted, which are known to initiate cellular signalling cascades that contribute to overall better brain health (Best, Chiu, Hsu, Nagamatsu, &
Liu-Ambrose, 2015); e.g. BDNF is increased following aerobic exercises, while reduced myostatin and increased IL-6 are linked to resistance exercise (Ost, Coleman, Kasch, & Klaus, 2016).

1.3.1 Physical activity and cognition

Throughout evolution the nervous systems and brains of all species adapted according to environmental influences and needs. Humans developed from species that were exposed to long hours of walking, foraging, and exploratory trips. Physical activity therefore always played a crucial role in human development. A link between memory processing and walking or running across vast territory seems obvious; important places needed to be recognised and remembered, cognitive strategies needed to be learnt and applied for increased survival chances. This theory lead to the understanding of a “metabolic brain” (Gomez-Pinilla & Hillman, 2013).

A vast amount of literature exists investigating various types of physical activity in relation to cognitive performance across all human age groups, and in people with diagnosed medical conditions. Reviews are regularly attempting to summarise latest research outcomes with the aim of pinpointing significant methodologies and outcomes which can be successfully applied in the community as a preventative strategy for cognitive decline (Gomez-Pinilla & Hillman, 2013). Physical activity acts as a trigger for improved cellular energy metabolism and synaptic plasticity. A positive association of cardiovascular fitness with cognition is particularly marked in older adults as reported by several groups (M. Angevaren, G. Aufdemkampe, H. J. J. Verhaar, A. Aleman, & L. Vanhees, 2008; Smith et al., 2010). Further, a recent meta-analysis and review confirmed that an association between more physical activity and reduced risk of
cognitive decline exists in healthy older adults (Blondell et al., 2014). Combined aerobic and resistance training have been shown to positively impact executive function, attention and processing speed, as well as memory (Scherder et al., 2014; Smith et al., 2010). Combined activity regimes have shown even greater improvement in this area, when compared to aerobic exercise per se (Smith et al., 2010). Further, the memory of cognitively affected people with MCI, appears to benefit from physical activity the most. Interestingly, a study investigating cognition pre- and post-aerobic intervention confirmed an association with higher fitness level and better neurocognitive performance, but the actual improvements in aerobic fitness during the intervention did not yield the same degree of improved cognitive performance (Etnier, Nowell, Landers, & Sibley, 2006). Interestingly, in the review by Smith and colleagues, no association between aerobic fitness and working memory was observed (Smith et al., 2010). The intensity regime of physical activity may play a significant role in the beneficial impact on cognition here.

Older adults might even benefit from physical activity more than other age groups: low muscle mass in older people has already been linked to cognitive decline (Boyle, Buchman, Wilson, Leurgans, & Bennett, 2009; Burns, Johnson, Watts, Swerdlow, & Brooks, 2010). In a recent literature review, a thorough overview of the protective impact of physical activity from cognitive decline in older adults was described (Bauman, Merom, Bull, Buchner, & Singh, 2016). Indeed, the positive impact of physical activity on individuals appears particularly strong in older subjects. The authors suggested that older (70-75 years old) individuals’ functional status may be preserved with even lower amounts of physical activity than previously recommended. In a 2 year large-scale study conducted by Weuve and colleagues (2004) over 18,700
women reported their activity levels via questionnaires. Cognitive tests over the 2 year period showed that in particular, older women over the age of 70 experienced less cognitive decline, if they kept an active lifestyle that included regular walking (Weuve et al., 2004). A similar result was obtained by Yaffe et al. (2001), who assessed nearly 6,000 women over 65 years of age for their energy expenditure during physical activity in association with their cognitive results (via Mini-Mental State Examination; MMSE) over a 5-8 year period. The results indicated better cognitive performance in active women, during that time period, when compared with sedentary participants. A Cochrane review proposed that intensity of physical activity is a crucial mediator of improved cognitive performance (Maaike Angevaren et al., 2008). Many previous studies have relied on self-report measures of physical activity in the form of questionnaires (Geda et al., 2010; Middleton, Kirkland, & Rockwood, 2008; Weuve et al., 2004; Yaffe et al., 2001). Self-reporting is likely to be biased as our memory is strongly linked to perceived exertion, which in turn depends on the fitness level of an individual (Duron et al., 2012). Nevertheless, 1-2 sessions of resistance training per week has been associated with improvements in selective attention/processing speed (assessed via Stroop test) and working memory (assessed via verbal digital span test) in an older cohort of women between 65 and 75 years of age (Liu-Ambrose & Donaldson, 2009). Moreover, moderate- to high-intensity resistance workouts improved memory and executive function in elderly individuals aged between 67 and 69 years (Cassilhas et al., 2007). Further, strength training leads to stronger muscles and bones, less fragility and more balance, a more positive mind-set, more confidence, community engagement and better quality of life (Liu-Ambrose & Donaldson, 2009; Orr, Raymond, & Singh, 2008).
Although, the notion that physical activity can improve cognitive performance has been frequently documented, it is not yet clear which type of exercise (aerobic or anaerobic), and at what intensity (low, moderate, vigorous) is most beneficial. Moreover, it remains to be investigated if certain cognitive domains improve quicker than others in response to the PA-stimulus, and whether these results are dependent on certain demographic variations within population cohorts. In the largest 24-month PA intervention study to-date, conducted in sedentary older adults over 70 years of age, regular moderate exercise did not lead to an overall cognitive benefit; however, participants over 80 years old and with lower baseline physical ability showed improved scores in executive functioning (Sink et al., 2015). This study was based on independent exercise carried out at home with activities self-reported by participants; elements which are known to be associated with reporting bias. Indeed, it is important to note that the type of exercise, intensity, frequency, study duration and pre-existing fitness levels are all factors that require consideration when assessing the impact of regular exercise in older adults. Thus, much more research is needed in older adults to characterise the true effect of physical activity on brain health. A summary of important study outcomes linking physical activity to AD and dementia follows below.

1.3.2 Physical activity and AD/dementia

A plethora of studies have reported that higher physical activity levels are associated with decreased risk of dementia (Jedrychowski et al., 2015; E. B. Larson et al., 2006; Podewils et al., 2005). The outcome of the Adult Changes in Thought Study found that exercising at least three times per week conferred a protective effect against developing dementia in a cohort over 65 years of age, followed over a nine year period (E. B. Larson et al., 2006). Out of 1740 people who were assessed in regards to self-reported
weekly physical activity levels and incidence of dementia, 158 participants developed
dementia, and 1158 participants remained free of dementia. The researchers calculated a
hazard ratio of dementia (adjusted for age and gender) of 0.62 (CI, 0.44 to 0.86; \( p =
0.004 \)), if people exercised at least three times per week, with a dementia incidence rate
of 13.0 per 1000 person-years (compared with 19.7 per 1000 person-years for
individuals who were less active) (E. B. Larson et al., 2006). Similarly, over a four-year
period, Buchman and colleagues collected objective and self-reported physical activity
data from 716 individuals with a mean age of 81.6 years (Buchman et al., 2012). The
study’s outcome showed a clear association between reduced risk of AD and total daily
physical activity levels. The total physical activity per day also correlated with the
annual rate of cognitive decline.

A lot less information is available from intervention studies conducted on participants
experiencing cognitive impairment. Bherer and colleagues (2013), reviewed the
available results, which included just five intervention studies, and reported that whilst a
beneficial effect of high-intensity aerobic exercise was shown to improve processing
speed and executive function in a mixed-gender cohort of MCI subjects over 55 years of
age (Baker et al., 2010), light-intensity intervention (five days of 30 minutes walking)
yielded no significant result in a cohort with moderate dementia (mean age, 85 years)
(Eggermont, Milberg, Lipsitz, Scherder, & Leveille, 2009). Further, improvements in
balance and visual span as well as cognitive function were observed in a Tai-Chi
intervention group comprised of cognitively impaired older adults (Lam et al., 2011).
Nagamatsu and colleagues were able to confirm this outcome, as they observed
improved spatial memory in a group of 70-80 year old women with probable MCI, who
underwent either aerobic or resistance training twice a week for six months (Nagamatsu
et al., 2013). A modest study outcome was reported by Lautenschlager and colleagues, when assessing 170 subjective memory complainers over an 18-month period. The intervention group took part in a 24-week home-based physical activity program. Neuropsychological assessment showed modest improvements in delayed word list recall (Nicola T Lautenschlager et al., 2008).

1.3.3 Irisin and exercise

As mentioned above, in the original study by Boström, a twofold increase of plasma irisin was measured after a 10-week exercise regime in obese, but otherwise healthy adults (Bostrom et al., 2012). This study immediately led to further investigations involving different exercise programs, varying cohorts, and time frames.

Huh et al. evaluated the relationship between exercise and irisin in varying sample sizes and demographics (Huh, Panagiotou, Mougios, Brinkoetter, Vamvini, Schneider, et al., 2012). In healthy, moderately trained young male subjects (aged 20.5 ± 1.5 years), irisin was acutely increased by approximately 18.4% in serum 30 minutes after exercise, however an effect could barely be seen 1 week following exercise (irisin levels were slightly higher on average, by 3.5%). Observations of acutely elevated irisin levels after bouts of exercise have been frequently confirmed (A. D. Anastasilakis, S. A. Polyzos, Z. G. Saridakis, et al., 2014; Daskalopoulou et al., 2014; Kraemer, Shockett, Webb, Shah, & Castracane, 2014; Norheim et al., 2014; Tsuchiya et al., 2014; Tsuchiya, Ando, Takamatsu, & Goto, 2015). Furthermore, resistance training has been shown to induce a spike in plasma irisin levels one hour after completion of an exercise bout. This was the first study to conclude that resistance training might be more effective than endurance
training in elevating plasma irisin levels in young physically active males (Tsuchiya et al., 2015). Of note, an increase in serum irisin levels was not observed following acute strength training in an early study (Pekkala et al., 2013). In a separate study, a cohort of healthy, physically inactive, middle-aged men was split into two groups; pre-diabetes and healthy controls (Norheim et al., 2014). Both groups underwent a 12-week strength and endurance-training program. Two 45-minute long bicycling tests were performed at 70% of an individual’s VO₂max before and after the 3-month intervention period. Blood samples for irisin detection were taken before, immediately post and 2 hours after the bicycle test. Plasma irisin levels peaked immediately after the exercise (1.2-fold), and returned to baseline levels within 2 hours in both groups (Norheim et al., 2014). Significantly, Daskalopoulou et al. (2014) showed that irisin levels were dependent on the maximal exercise workload of each individual, with fitter individuals, as measured by VO₂max tests, demonstrating higher circulating irisin concentrations. In addition, 10 minutes of sub-maximal exercise also increased circulating irisin in the same study. Similar results were obtained by Kraemer et al., who reported that irisin levels increased by over 20% half way through a 90-minute sub-maximal exercise task (60% VO₂max) (Kraemer et al., 2014). Interestingly, a healthy, but untrained, female cohort underwent a 6-week program in which a whole-body vibration exercise led to acute elevations in blood irisin levels (Huh, Mougios, Skraparlis, Kabasakalis, & Mantzoros, 2014).

Hecksteden and colleagues (2013) exposed healthy untrained men and women to a 6-month strength endurance or aerobic endurance exercise regime (mean age per group 48-50 years), with the authors reporting that serum irisin levels did not change following the intervention. However, Hecksteden et al., did note that the majority of individual irisin levels marked as outliers in the upper range were derived from female
participants, whilst the outliers in irisin levels in the lower range were derived from male subjects. A 12-week strength-training program undertaken on a cohort of untrained women also resulted in no change in serum irisin levels (Ellefsen et al., 2014).

Numerous studies have shown that irisin is indeed involved in the signalling cascades that are triggered in the brain and muscles following PA. Yet, the detection methods have been far from standardized. Moreover, the great variations of physical activity regimes and differing cohort demographics suggest that caution is required in the analysis and interpretation of clinical data.
1.4 Summary

Recent insights into AD development and pathology have provided a range of new ideas in the quest to diagnose the disease early, and ideally prevent or delay onset. Given the complex neuropathology and etiology of AD, our current knowledge about this disease has increased markedly in the last 15-20 years. Researchers are now embracing a more holistic approach in identifying factors and mechanisms that cause small cellular or physiological changes and malfunctions years before clinical symptoms present (Gandy & DeKosky, 2013; Sperling et al., 2011). In order to counteract such pathophysiological changes in the human body, it is paramount to identify these changes early, thereby presenting the best opportunity to positively affect brain health. A blood test which could measure these early changes is highly desirable, as it would provide a cost-effective, rapid and, non-invasive methodological approach. In order to yield a sensitive blood test however, it is important to identify reliable biomarkers of ideally preclinical AD which can either be assessed on their own or as part of a panel of biomarkers: irisin is one such candidate biomarker.

As described in detail earlier, peripheral, circulating irisin levels in serum or plasma have been strongly linked to metabolic diseases (Y. K. Choi et al., 2013; Moreno-Navarrete et al., 2013a; S. Zhang et al., 2016). Since metabolic diseases are a risk factor for developing dementia later in life, irisin needs to be investigated in order to further our understanding of physiological changes that occur in the body years before the clinical symptoms of AD manifest. Adding more weight to the argument that irisin warrants further investigation in the field of AD research, is the fact that levels of this hormone have been shown to fluctuate following physical activity (Huh, Panagiotou, Mougios, Brinkoetter, Vamvini, Schneider, et al., 2012; Kraemer et al., 2014; Tiano et
al., 2015a): a significant discovery given the well-established link between physical activity and enhanced brain health and decreased AD risk in older adults. It is also conceivable that further investigation of irisin levels in response to varying forms of physical activity will provide insight into the mechanisms underlying the association between physical activity and brain health, and potentially assist in the development of strategies aimed at preventing AD. Indeed, if irisin is actually a mediator of the beneficial impact of physical activity on the brain, then levels of this hormone may also have some influence on, or predictive value for, the cognitive status of an individual. Significantly, to-date, only one study has investigated the relationship between irisin and cognition (Belviranli, Okudan, Kabak, Erdogan, & Karanfilci, 2016).
Figure 1.7: Proposed pathway, connecting irisin with its precursor and BDNF. Abbreviations: Aβ, amyloid β; BACE1: β-secretase; BDNF, brain-derived neurotrophic factor; FNDC5, Fibronectin type III domain-containing protein 5; NF-κB, nuclear factor-κB; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPAR-γ, Peroxisome proliferator-activated receptor gamma; ROS, reactive oxygen species.
1.5 Hypotheses and aims of this PhD project

1.5.1 Irisin levels are higher in cognitively normal healthy older adults when compared to individuals with AD.

Aim 1: Measure and examine irisin levels in cognitively normal healthy older adults and those diagnosed with AD.

Aim 2: Evaluate the relationship between serum irisin levels and factors associated with AD risk, including the demographics of age, gender, APOE ε4 allele carriage, as well as brain amyloid (Aβ-load), body mass index (BMI), and a history of diagnosed medical conditions (e.g. diabetes, depression, thyroid dysfunction, hypertension, heart attack).

1.5.2 Higher irisin levels are associated with a better blood biomarker profile of metabolic disease in older adults.

Aim 3: Evaluate the relationship between serum irisin levels and blood biomarkers associated with metabolic diseases (e.g. lipids and thyroid hormones).

1.5.3 Higher levels of irisin are associated with better cognitive functioning in cognitively normal healthy controls.

Aim 4: Examine the association of circulating irisin levels with neuropsychological assessments of cognitively normal healthy older adults.

1.5.4 Higher levels of physical activity, which are known to be associated with better brain health, are associated with increased concentrations of irisin.

Aim 5: Investigate the relationship between irisin and known measures of physical activity in cognitively normal healthy adults in; 1) a longitudinal
observational study cohort of older adults (AIBL), 2) in older participants of a physical activity intervention cohort (PEACS), and 3) in younger to middle-aged participants of a short-term strength-training intervention study.

1.5.5 *Irisin is neuroprotective in vitro against cytotoxic Aβ42.*

Aim 6: Examine the effect of irisin on Aβ-mediated toxicity and BDNF expression in human neuroblastoma cells.
Chapter Two

Materials and Methods
Chapter 2 – Material and Methods

2.1 Introduction

This chapter describes all aspects of data collection from three study cohorts; 1) the Australian Imaging, Biomarkers and Lifestyle Flagship Study of Ageing (AIBL), 2) the Physical Exercise and Cognitive Stimulation (PEACS) Study, and 3) the Acute Strength Training (AST) Study, including ethics processes, selection of participants, neuropsychological assessments, blood sample collection, neuroimaging and physical activity measures. Further, methods of experimental procedures that were utilised to investigate the quantity of the hormone of interest, irisin, and its effect on neuroblastoma cells in vitro, are described in detail. The chapter is concluded with an overview of data cleaning processes and procedures of statistical analyses.
2.2 Data collection for the Australian Imaging, Biomarkers and Lifestyle (AIBL) Flagship Study of Ageing

2.2.1 Ethical approval

AIBL is a collaborative study involving leading Alzheimer’s disease (AD) researchers from Perth and Melbourne, where the recruitment of the cohort was undertaken. Management of the study database is undertaken by Commonwealth Scientific Industrial and Research Organisation (CSIRO) scientists from around Australia. Ethical approval of the study was obtained from the Human Research Ethics Committees of Austin Health, St Vincent’s Health, Hollywood Private Hospital and Edith Cowan University. The University of Western Australia officially recognised the ethical approval granted by Hollywood Private Hospital in order for me to conduct this PhD research project as a student of the university (Appendix 1: Participant information and consent form). Written informed consent was obtained from all participants prior to undertaking any study procedures, in accordance with National Health and Medical Research Council (NHMRC) guidelines.

2.2.2 The AIBL study cohort

The AIBL Study commenced in 2006; a full description of this cohort has been published previously (Ellis et al., 2009). The aim of the AIBL Study was to recruit volunteers over the age of 60, in order to investigate the relationship between neuroimaging and blood-based biomarkers, lifestyle factors, cognitive decline and the development of AD. The cohort is comprised of cognitively healthy controls (HC, approximately 69%) and participants expressing neuropsychological profiles consistent with mild cognitive impairment (MCI, approximately 12%) and AD (approximately 19%) (total inception
cohort: \( n = 1112 \). Data collection occurs every 18 months and involves a thorough neuropsychological assessment, collection of blood for quantification of various blood biomarkers, neuroimaging and collection of information regarding lifestyle.

### 2.2.3 Recruitment of study participants

AIBL participants were recruited from the general public, following advertisements on TV, radio and in newspapers in Perth and Melbourne. Interested volunteers were initially screened via a short telephone interview. Potentially eligible participants were contacted by researchers and provided with a participant information and consent form, which describes all procedures in detail, as well as potential risks and benefits of the study. Written informed consent was obtained from all participants prior to assessment.

### 2.2.4 Eligibility criteria for study participation

An initial screening of interested participants was conducted by telephone or on site, in which contact details, gender, and age was determined. Additionally, a brief questionnaire regarding medical history and alcohol intake was also completed. Exclusion criteria for the study include: history of non-AD dementia, bipolar disorder, schizophrenia, Parkinson’s disease, insulin-dependent diabetes mellitus, current depression, cardiovascular disease, stroke, or recent history (previous 2 years) of cancer (except basal cell carcinoma). The 15-item Geriatric Depression Scale (GDS) questionnaire was also administered during this screening to measure depressive symptoms, with those recording a score of greater than 6 excluded from participation. Further, a history of alcohol or substance abuse also lead to exclusion, specifically; women who reported drinking more than two standard drinks per day, and men who drank more than four standard drinks per
day were excluded from study participation. Participants who met the inclusion criteria were considered eligible for the study and were invited for a full assessment. In this thesis, data from study participants classified as cognitively HC, and participants diagnosed with AD were used. The classification procedure of an individual’s cognitive status will be described in detail below (see sections 2.2.5 and 2.2.8).

2.2.5 Assessment procedures

After a participant was deemed eligible for study inclusion, a baseline assessment date was arranged. At this assessment, vital signs, including blood pressure, pulse, body height, abdominal circumference and weight were measured, before 80 ml of fasted blood was drawn. Participants were then provided with a light breakfast during which they completed a number of questionnaires about their medical history, current mood, self-perceived memory, and physical activity. Shortly after breakfast, participants underwent a thorough cognitive assessment conducted by a trained neuropsychological rater. Separate visits were arranged for the conduct of brain imaging assessments.

Participants were invited to return for follow-up assessments every 18 months. For my PhD project, I collected data at the 54-month (i.e. baseline in this project) and 72-month follow-up time-points (i.e. 18 month follow-up here) of the inception AIBL cohort. A small minority of the study cohort were assessed in their homes. In-home assessments were usually conducted on participants with moderate to advanced AD, as well as participants of all classifications who were physically limited.
2.2.6 Overview of measures

Clinical and laboratory data were collected and analysed for use in my PhD thesis. For the clinical data collection: 1) neuropsychological assessments, 2) demographic cohort characteristics, 3) physical activity measures in the form of objective actigraphy measurements and self-report questionnaires, 4) blood biomarker analyses, and 5) neuroimaging data were utilised. For the laboratory-based component of this PhD project, 1) serum levels of irisin were measured, and experimental results from 2) protein detection via Western blotting, and 3) cell viability assays were collected. These laboratory-based measures are described in detail in section 2.5.4 below.

2.2.7 Neuropsychological assessment

The AIBL neuropsychological assessment battery was compiled by a panel of experts including senior neuropsychologists and psychiatrists of the AIBL Study. All of the tests are well-established and frequently used psychometric measures in ageing research, targeting different cognitive domains. A complete list of tests that comprise the neuropsychological battery is listed in Table 2.1. I administered the full test battery to most of my participants myself. Nevertheless, some participants with AD were unable to complete all tasks. In addition to the neuropsychological battery, participants completed questionnaires regarding their own opinion of their memory (Memory Assessment Clinic-Questionnaire; MAC-Q) and current anxiety and depression levels (Hospital Anxiety and Depression Scale; HADS).
2.2.7.1 Mini-Mental State Examination

The Mini-Mental State Examination (MMSE) is a 30-item assessment established in 1975 by Folstein and colleagues, and is one of the most widely used tools for the primary examination of global cognitive function (Folstein, Folstein, & McHugh, 1975). Questions briefly evaluate language and arithmetic skills, remembering and recalling a short list of words, orientation to time and place, naming items, and copying a figure by drawing.

2.2.7.2 Digit Span

Digit span is a widely used assessment of short-term verbal working memory and forms part of the Wechsler Memory Scale (WMS) and Wechsler Adult Intelligence Scale (WAIS) (Woods et al., 2011). The digit span comprises two components, digit span forward and digit span backward. In both trials, the examiner reads out a list of random digits that the examinee is asked to repeat. Starting with a sequence of two digits, the random sequences gradually increase in length by increments of one digit. Participants undertake two trials per sequence length, whereby the numbers within the sequence are different in both trials. Once the participant fails to correctly repeat back a sequence of the same length twice, the task is discontinued. A nine-digit long sequence is the longest sequence provided, and upon successful completion of this sequence, the task ceases. In the backwards version of the digit span the participant is asked to repeat the sequence back to the examiner in reverse order. As with the forwards task, a participant has to fail two consecutive trials in order for the task to be terminated. Every correct repeat of a sequence is awarded one point. Points from forward and backward digit span are summed to yield the total score for this test.
2.2.7.3 Logical memory

Logical memory is used to assess narrative episodic memory, and is another sub-test of the WMS-Revised (Chapman et al., 2016). A short story is read out loud to the participant at a steady pace, following this, the participant is asked to try and recall as many facts as possible from that story immediately afterwards (LM I). Approximately 25 - 35 minutes later, the participant is asked once more to recall as many details from the story as possible (LM II). Every correct item of information is allocated one point score.

2.2.7.4 California Verbal Learning Test - Second Edition

Episodic verbal learning and memory is measured using the California Verbal Learning Test Second Edition (CVLT II) (Baños & Martin, 2002). A list comprised of 16 words is read out loud to the participant (List A), following which they are asked to recall as many words as they can from the list, in any order. These words belong to one of four unrelated semantic categories, which are made up of four words each. The examiner writes down every word the examinee recalls verbatim. After five trials of List A, the scores of all correct words per trial are added together to create the CVLT learning score. The examiner then reads out a second 16-item interference word list (List B), and the examinee is then asked to repeat back as many words as possible from this second list. After one single trial of List B, the examinee is then asked to recall as many words as possible from List A. Responses are again written down verbatim, and the number of correct responses is summed to yield a CVLT short delay-recall score. After a 20-minute delay, participants are asked to recall words from list A, yielding a CVLT long delay-recall score. The task is concluded with a 48–item forced choice discrimination task, for which the examinee has to make a decision whether the word that was just read out by
the examiner belongs to list A, or was a distractor word. Every correctly identified target word (i.e. word that was in list A) is recorded as a hit and an incorrectly identified target word is recorded as a false positive.

2.2.7.5 Rey Complex Figure Test (RCFT)

Visuospatial functioning and memory are measured using the Rey Complex Figure Test (Meyers, Bayless, & Meyers, 1996). Participants are presented with a copy of the Rey Complex Figure, and asked to draw a copy of this figure as accurately as possible (RCFT copy). After a three-minute (short-delay free recall) and a thirty-minute delay (long-delay free recall), participants are asked to recall and draw the figure again from memory. The scoring of the figure follows a standardized protocol, which allows scores of 0, 0.5, 1 or 2 for each single aspect of the figure; scores are allocated depending on whether the drawing and/or the location of certain shapes is correct.

2.2.7.6 Controlled Oral Word Association Task (COWAT)

Phonemic and categorical verbal fluency are measured by the Controlled Oral Word Association Task (COWAT; Delis, Kaplan, & Kramer, 2001). In the phonemic task, participants are asked to name as many words as possible in one minute starting with a specified letter (F, A, or S). Additionally, participants are asked to follow three rules: 1) not to use proper nouns, i.e. nouns that start with a capital letter, such as names or names of places; 2) not to add endings to previously mentioned words, e.g. if the word “run” was previously stated, then words like “running”, “runner” and “runs” cannot be used; and 3) to avoid repeating words. The numbers of correct words stated for each letter is summed to yield a Letter Fluency Score.
In the categorical fluency task, participants are given a category (animals, boys names) for which they have to list appropriate words for one minute per category. The only rule to be followed in the semantic fluency task is to avoid repetition. The numbers of correct words for each category are summed to yield a *Category Fluency Score*. In a third trial, participants are asked to alternate between two categories, fruit and furniture, for one minute. This third trial yields two scores: *Switching Score* (number of times the participant correctly switches from one category to the other) and *Total Correct Switching Task*.

### 2.2.7.7 Digit Symbol Coding

Digit Symbol Coding is a task from the Wechsler Adult Intelligence Scale (WAIS-III) (Wechsler, 1997). This task measures attention, working memory and speed. Participants are provided with a legend in which nine numbers are each matched to nine different symbols. The examinee is instructed to copy the correct symbol underneath each number that is aligned on the lower part of the sheet. The time limit of this task is two minutes and every correct number/symbol match counts towards the total raw score.

### 2.2.7.8 The Stroop Test

Attention, processing speed and executive function are assessed by the Victorian Stroop test (VST) (Strauss & Sherman and Spreen, 2006). Three Stroop tasks are administered: 1) the ‘dots’ task requires participants to simply name the colour of 24 dots presented, 2) the ‘words’ task requires participants to state the colour of 24 neutral words presented, and 3) the ‘colours’ task requires participants to state the colour of 24 words (which are written in one of four colours). The last task requires the participant to inhibit the
automatic response of reading out the colour word, and instead the participant is required to name the colour of the ink that the word is written in. All three tasks are timed and recorded in seconds.

2.2.8 Classification of participants

Results from the neuropsychological assessment in combination with information regarding medical history, self-perceived memory, and activities of daily living (self-report and Clinical Dementia Rating) were used for the classification of participants. Furthermore, information from a collateral source, such as a close family member or friend was sought, in order to corroborate reports from participants, and provide additional information for the panel of neuropsychologists and geriatricians to classify a participant as either cognitively healthy (HC), MCI or AD. Classification as AD followed the NINCDS-ADRDA (National Institute of Neurological and Related Disorders Association) criteria (McKhann et al., 1984). MCI diagnoses were made according to the protocol based upon the criteria of Winblad et al. (Winblad et al., 2004) which are informed by the criteria of Petersen et al. (Petersen et al., 2001). Consistent with Winblad criteria, all participants classified with MCI had either personally, or through an informant, reported memory difficulties (Winblad et al., 2004). In this thesis, only data from study participants classified as cognitively HC, and participants diagnosed with AD were used.

2.2.8.1 Clinical Dementia Rating

The Clinical Dementia Rating (CDR) is a validated and frequently used tool to assess the severity of dementia symptoms in six different domains. The examiner asks participants
questions about their ability to perform tasks at home, how they feel about their memory, as well as questions assessing difficulties with orientation, judgement and problem-solving, community affairs, hobbies and personal care. In participants diagnosed with AD or MCI, an informant report is necessary to rate these domains. Impairment severity ranges from 0 (no signs of dementia) to 3 (advanced dementia) in increments of 0.5 (Chapman et al., 2016).
Table 2.1: List of neuropsychological tests, and their respective cognitive domains, utilised in this thesis

<table>
<thead>
<tr>
<th>Cognitive Domain</th>
<th>Neuropsychological Test</th>
<th>Outcome Measure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verbal memory</td>
<td>Logical memory II</td>
<td>Number of details correctly recalled</td>
<td>Wechsler, 1945</td>
</tr>
<tr>
<td></td>
<td>CVLT-II delayed recall</td>
<td>Number of words correctly recalled</td>
<td>Delis et al. 2000</td>
</tr>
<tr>
<td></td>
<td>CVLT-II recognition discrimination</td>
<td>Discrimination index for recognition of words</td>
<td>Delis et al. 2000</td>
</tr>
<tr>
<td>Visual memory</td>
<td>RCFT 3 minute delay</td>
<td>Number of elements correctly recalled</td>
<td>Meyers &amp; Meyers, 1995</td>
</tr>
<tr>
<td></td>
<td>RCFT 30 minute delay</td>
<td>Number of elements correctly recalled</td>
<td>Meyers &amp; Meyers, 1995</td>
</tr>
<tr>
<td></td>
<td>RCFT recognition</td>
<td>Number of elements correctly recognised</td>
<td>Meyers &amp; Meyers, 1995</td>
</tr>
<tr>
<td>Executive function</td>
<td>Stroop C/D</td>
<td>Speed of Stroop Colours/Stroop Dots</td>
<td>Strauss, Sherman, &amp; Spreen, 2006</td>
</tr>
<tr>
<td></td>
<td>Controlled Oral Word Association Task</td>
<td>Number of words produced in 1 minute</td>
<td>Delis et al. 2001</td>
</tr>
<tr>
<td></td>
<td>Fruit and furniture switching</td>
<td>Number of words produced in 1 minute</td>
<td>Delis et al. 2001</td>
</tr>
<tr>
<td>Language</td>
<td>Category fluency (Animals/Boys’ Names)</td>
<td>Number of words produced in 1 minute</td>
<td>Delis et al. 2001</td>
</tr>
<tr>
<td>Attention</td>
<td>Digit span</td>
<td>Total correct trials (forwards and backwards)</td>
<td>Wechsler, 1997</td>
</tr>
<tr>
<td></td>
<td>Stroop dots time</td>
<td>Speed of trial</td>
<td>Strauss, Sherman, &amp; Spreen, 2006</td>
</tr>
<tr>
<td></td>
<td>Digit symbol coding</td>
<td>Number of symbols correctly matched</td>
<td>Wechsler, 1997</td>
</tr>
<tr>
<td>Visuospatial functioning</td>
<td>RCFT copy</td>
<td>Number of elements drawn correctly</td>
<td>Meyers &amp; Meyers, 1995</td>
</tr>
</tbody>
</table>

Abbreviations: CVLT-II, California Verbal Learning Test-II; RCFT, Rey Complex Figure Test; Stroop C/D, Stroop colours/dots.
2.2.9 Vitals, medical and demographic history

All participants provided information on their medical history, alcohol and tobacco consumption, and years of education. Weight (in kg) and height (in m; without shoes) was recorded and used to calculate a body mass index (weight/height²; BMI).

2.2.10 Collection of physical activity data

I collected physical activity (PA) data from 151 participants. All 151 participants provided information about their weekly physical activity by completing the International Physical Activity Questionnaire (IPAQ), and 43 of the 151 participants also agreed to wear an actigraphy unit for seven days to enable collection of objective PA data.

2.2.10.1 Actigraphy data

Cognitively healthy AIBL participants, who consented to participate in the actigraphy sub-study, were asked to wear a waist-mounted GT3X actigraphy unit for seven consecutive days, excluding periods of bathing/showering or swimming. These actigraphy monitors were used to obtain an objective measure of physical activity. The pathway of data analysis is briefly depicted in Figure 2.1 below.
Figure 2.1: Actigraphy data processing (cleaning) and analysis (scoring) pathway. Freedson Adult algorithms were used to calculate daily total counts and maximal counts. Only raw data collected between 6.00 AM and 10.30 PM were included. Abbreviations: MET, Metabolic Equivalent of Task; MVPA, *Actigraph*-defined moderate-to-vigorous physical activity.
2.2.10.1.1 Actigraphy data collection and processing

Actigraphy units collect movement information (i.e. changes in acceleration) along the vertical axis (axis 1), which is translated into “counts”. These counts are based on the sum of accelerations (measured 30 times / second) during an epoch (Table 2.2). Only data collected between 6 AM and 10.30 PM were analysed, to reflect the average daily time frame that participants were awake for. The average awakening time used in my thesis is based on a previously published report, as well as on subjectively reported awakening times of participants (Brown et al., 2012).

The maximal count reflects the highest intensity level reached during the day, which was averaged over the 7 day period, whereas the axis counts represent the averaged total counts over seven days. The value of the counts varies based on the frequency and intensity of the raw acceleration. The filtering process by which counts are produced is proprietary to ActiGraph Ltd. (Pensacola, Florida, USA).

The collected data were analysed using ActiLife software, version 6.11.4. Data processing and scoring followed the algorithms listed in Table 2.2 below. Only counts measured between 6 AM and 10.30 PM from axis 1, as well as maximal daily counts were used for data analysis.
Table 2.2: Definition of bouts, cut points and sedentary settings as per Freedson (1998).

<table>
<thead>
<tr>
<th>Bouts</th>
<th>Minimum length: 10 minutes, drop time: 2 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Count levels: Minimum 1952 per minute</td>
</tr>
<tr>
<td>Cut points</td>
<td>5 cut points – counts are based on 60 second epochs (min. 1952):</td>
</tr>
<tr>
<td></td>
<td>Sedentary 0 – 99</td>
</tr>
<tr>
<td></td>
<td>Light 100 – 1951</td>
</tr>
<tr>
<td></td>
<td>Moderate 1952 – 5724</td>
</tr>
<tr>
<td></td>
<td>Vigorous 5725 – 9498</td>
</tr>
<tr>
<td></td>
<td>Very vigorous 9499 +</td>
</tr>
<tr>
<td>Sedentary setting</td>
<td>Minimum of 10 minutes with a maximum of 99 counts per minute</td>
</tr>
</tbody>
</table>

The Freedson algorithms utilised the settings listed here. The definition of a count describes the accelerometer output of the worn actigraphy unit, which depends on the intensity of the movement of a person. All counts measured within a 60 second time frame (i.e. one epoch) allocate the intensity of the epoch into different categories: sedentary, light, moderate, vigorous and very vigorous activity. Ten epochs (equivalent to 10 consecutive minutes) add up to one bout of activity. One bout is successfully registered when every single epoch measured at least 1952 counts. The drop time of 2 minutes in the Freedson calculations states that a bout is still recognised as a bout, even if 2 out of 10 consecutive minutes were spent at less than 1952 counts per epoch.
2.2.10.2 International Physical Activity Questionnaire

The International Physical Activity Questionnaire (IPAQ) collects data on self-reported physical activity undertaken during the previous seven days (Craig et al., 2003). Participants completed the IPAQ on the day of their neuropsychological assessment, referring to the week immediately prior to the assessment date. When a participant agreed to also wear the actigraphy unit, then the IPAQ was completed at the end of the seven-day period of wearing the actigraphy unit, i.e. one week after the assessment date. Participants were asked to report their daily physical activities in five domains, consisting of occupational work (including voluntary positions), transportation via motor vehicles or self-powered (on foot or bicycle), housework (including maintenance work in the garden or around the house), leisure-time and sedentary activity. The sedentary activity (sitting) score was not part of the final calculated physical activity score. To further separate between walking, moderate and vigorous activities, additional questions addressed the perceived intensity by providing examples, such as “carrying light loads”, “washing windows”, “chopping wood” etc. Each item recorded the amount of days in the past week that a particular activity was undertaken, and how many minutes or hours on average this activity was executed for on each of these days.

2.2.10.2.1 IPAQ data collection and data processing

A Metabolic Equivalent of Task (MET) score was calculated for each question with different weightings for the various domains. A mean seven day score was calculated by multiplying the allocated MET score for an activity by the amount of minutes a participant was engaged in that activity for the whole week (MET·min/wk), see Table 2.3 below. The cohort was then stratified into activity tertiles for further analysis, see Table 2.4.
Data cleaning involved the exclusion of questionnaires that were not correctly completed and demonstrated over-reporting, i.e. with MET scores adding to more than two standard deviations (SDs) above the mean; when the same physical activity data was included in two or more different domains of the questionnaire; or when questions were skipped. More details about data cleaning and the determination of outliers are described in section 2.8.1 below.

**Table 2.3: Allocated MET-scores to individual IPAQ-items**

<table>
<thead>
<tr>
<th>Activity</th>
<th>Allocated MET score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Walking</td>
<td>3.3</td>
</tr>
<tr>
<td>(Domains: Occupation, Transport, Leisure-time)</td>
<td></td>
</tr>
<tr>
<td>Moderate activities</td>
<td>4</td>
</tr>
<tr>
<td>(Domains: Occupation, Housework, Leisure-time)</td>
<td></td>
</tr>
<tr>
<td>Vigorous activities</td>
<td>Vigorous work: 8</td>
</tr>
<tr>
<td>(Domains: Occupation, Transport by Bicycle, Gardening/House Maintenance, Leisure-time)</td>
<td>Self-powered transport by bicycle: 6</td>
</tr>
<tr>
<td></td>
<td>Vigorous garden work: 5.5</td>
</tr>
<tr>
<td></td>
<td>Fast bicycling, swimming etc.: 8</td>
</tr>
<tr>
<td>Light housework</td>
<td>3</td>
</tr>
</tbody>
</table>

**MET scores: Walking + Moderate + Vigorous MET-min/week**

MET scores were computed according to the IPAQ user manual (http://www.ipaq.ki.se) and previous literature (Craig et al., 2003). Abbreviations: IPAQ, International Physical Activity Questionnaire; MET, Metabolic Equivalent of Task averaged over 7 days.
Table 2.4: Physical activity Tertiles determined based on physical activity measures for the baseline cohort in the AIBL Study

<table>
<thead>
<tr>
<th></th>
<th>Tertile 1 (T1)</th>
<th>Tertile 2 (T2)</th>
<th>Tertile 3 (T3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPAQ MET·min/wk</td>
<td>0 – 1980</td>
<td>1981 - 5013</td>
<td>5014 – 35865</td>
</tr>
<tr>
<td>Maximal counts (Actigraphy)</td>
<td>3169 – 4435</td>
<td>4436 – 5281</td>
<td>5282 – 7566</td>
</tr>
<tr>
<td>Total counts (Actigraphy)</td>
<td>229610 –</td>
<td>1357011 –</td>
<td>1835629 –</td>
</tr>
<tr>
<td></td>
<td>1357010</td>
<td>1835628</td>
<td>3782492</td>
</tr>
</tbody>
</table>

Abbreviations: AIBL, Australian Imaging, Biomarkers and Lifestyle Flagship Study of Ageing; IPAQ, International Physical Activity Questionnaire (short); MET·min/wk, Metabolic Equivalent of Task averaged in minutes per week.
2.2.11 Pathology data collection

2.2.11.1 Blood collection

Participants were asked to fast for at least 10 hours prior to blood collection. Venous blood was collected and its processing commenced between 8 and 9.30am. Blood was withdrawn from the cephalic vein using a vacutainer and a 21 gauge winged infusion set (Safety Lok Blood Collection Set, BD). Blood was collected into serum-separating tubes (Monovette Serum Gel Z/7.5, Sarstedt) and Ethylenediaminetetraacetic acid (EDTA) tubes containing prostaglandin E1 (PGE1) for plasma isolation. Following fractionation, serum and plasma samples were kept in liquid nitrogen for future analysis.

2.2.11.2 Serum fractionation

Serum-separating tubes containing whole blood were left standing upright for 20 minutes at ambient temperature immediately post-collection. The samples were spun at 1800 x g for 15 min in an Eppendorf 5810R centrifuge with a swing-bucket rotor to remove cells and clotting factors. The serum supernatant was carefully removed and immediately aliquoted into 1 ml Nunc® cryovials, snap-frozen on dry ice and transferred to a liquid nitrogen facility for storage.

2.2.11.3 Plasma fractionation

Plasma was collected to measure selected blood biomarkers. Whole blood in EDTA-PGE1 tubes was mixed thoroughly by incubation at room temperature for 45-60 minutes on a Ratek Tube Roller Mixer (Ratek, Victoria, Australia). The EDTA-PGE1 tubes were then spun at 200 x g for 10 mins (no brake). In order to remove platelets from the plasma, the top phase was transferred into a fresh tube and spun at 1,500 x g for 15 minutes.
(maximum brake). The plasma was then transferred into a fresh tube and spun again at 3,200 x g for a further 30 minutes (maximum brake). This process was concluded by aliquoting the plasma into vials for storage in liquid nitrogen.

2.2.11.4 Pathology test results

Blood-base clinical analytes including lipids, glucose, iron and hormone levels were measured by PathWest Laboratory Medicine, Nedlands, Western Australia and Melbourne Health, Victoria. A list of the analytes of interest and ranges considered normal are provided in Table 2.5 below:

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Fluid</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Plasma</td>
<td>3 – 5.4 mmol/L</td>
</tr>
<tr>
<td>Insulin</td>
<td>Serum</td>
<td>&lt; 12 mU/L</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>Plasma</td>
<td>&lt; 5.5 mmol/L</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>Plasma</td>
<td>&lt; 1.7 mmol/L</td>
</tr>
<tr>
<td>LDL</td>
<td>Plasma</td>
<td>&lt; 3.0 mmol/L</td>
</tr>
<tr>
<td>HDL</td>
<td>Plasma</td>
<td>&gt; 1.0 mmol/L</td>
</tr>
<tr>
<td>Free T4</td>
<td>Serum</td>
<td>9 – 19 pmol/L</td>
</tr>
<tr>
<td>Free T3</td>
<td>Serum</td>
<td>2.6 – 5.7 pmol/L</td>
</tr>
<tr>
<td>TSH</td>
<td>Serum</td>
<td>0.40 – 4.00 mU/L</td>
</tr>
</tbody>
</table>

Clinical blood-based analytes measured by PathWest laboratories in either plasma or serum. Abbreviations: HDL, High-density lipoprotein; LDL, Low-density lipoprotein; mmol/L, Millimoles per litre; mU/L, Millimitis per litre; pmol/L, Picomoles per litre; T3, Triiodothyronine; T4, Thyroxine; TSH, Thyroid-Stimulating Hormone.
2.2.12 *Apolipoprotein E (APOE) genotyping*

Genotyping for all AIBL samples was carried out by A/Prof. Simon Laws and his team at Edith Cowan University, using the following protocol. QIAamp Deoxyribonucleic acid (DNA) Blood Maxi Kits (Qiagen, Hilden, Germany) were utilised to extract DNA from 5 mL of whole blood. The extraction took place at room temperature. In brief, 500 μl of protease K was transferred into a 50 mL Falcon tube before 5 mL whole blood was added. If there was an insufficient volume of whole blood, then the whole blood sample was ‘topped up’ with 1 x phosphate buffered saline (PBS) to yield a 5 mL volume. Six mL of ‘AL buffer’ was then added to the tube and the whole suspension was mixed thoroughly by inverting the tube 15 times and then vigorously shaking it for at least one minute as per the manufacturer’s recommendation. Mixing ensures that the provided (AL) buffer is evenly distributed in the blood-protease mix to ensure adequate cell lysis. The tubes were then incubated at 70°C for 10 minutes. Five mL of absolute ethanol was subsequently thoroughly mixed into the solution by first inverting the tubes 10 times and then vigorously shaking for 30 seconds. The entire contents of the tube was then added to a QIAamp Maxi column and centrifuged at 1,850 x g for 3 minutes. The filtrate was discarded and the column placed back into the 50 mL Falcon tube. Five mL of the first wash buffer (AW1) was added to the column, prior to centrifugation at 2,500 x g for 2 minutes, with 5ml of the second wash buffer (AW2) added immediately afterwards. Centrifuging the whole sample at 2,500 x g for 15 minutes concluded the thorough DNA-purification step. The column was then transferred to another clean 50mL Falcon tube, into which the purified DNA was ultimately eluted by adding 750 μl of ‘AE buffer’ straight onto the membrane and leaving for 5 minutes at room temperature. A final spin of 2,500 x g for 3 minutes ensured that all DNA was collected at the bottom of the tube.
DNA was then transferred into a clean Eppendorf tube and stored at 4°C until sequencing occurred.

TaqMan® genotyping assays were applied to determine the two *APOE* alleles of each participant (rs7412, assay ID: C____904973_10; rs429358, assay ID: C____3084793_20; Life Technologies, Carlsbad, CA). DNA was first amplified via Real-Time Polymerase Chain Reaction (RT-PCR) (QuantStudio 12K Flex™; Applied Biosystems, Foster City, CA). In short, the TaqMan® GTXpress™ Master Mix (Life Technologies, Carlsbad, California, USA) was added to the reaction plate provided by the manufacturer. DNA samples were then added to the mix, and centrifuged for 1 minute at 460 x g. Cycle settings included an initial enzyme activation at 95°C for 20 seconds, followed by 40 cycles of a denaturation step at 95°C for 3 seconds, and an elongation step at 60°C for 30 seconds.

The *APOE* genotypes were determined by identifying 2 single nucleotide polymorphisms (SNPs); rs7412 allele (C) and rs429358 allele (T) as detailed in Table 2.6 below.

### Table 2.6: Allele 1 and 2 configurations yielding each Apolipoprotein E (*APOE*) genotype

<table>
<thead>
<tr>
<th>Allele 1</th>
<th>Allele 2</th>
<th><em>APOE</em> Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>T_T</td>
<td>T_T</td>
<td>ε2/ ε2</td>
</tr>
<tr>
<td>T_C</td>
<td>T_T</td>
<td>ε2/ ε3</td>
</tr>
<tr>
<td>T_C</td>
<td>T_C</td>
<td>ε2/ ε4</td>
</tr>
<tr>
<td>C_C</td>
<td>T_T</td>
<td>ε3/ ε3</td>
</tr>
<tr>
<td>C_C</td>
<td>T_C</td>
<td>ε3/ ε4</td>
</tr>
<tr>
<td>C_C</td>
<td>C_C</td>
<td>ε4/ ε4</td>
</tr>
</tbody>
</table>
2.3 Physical Exercise and Cognitive Stimulation (PEACS) Study

2.3.1 Introduction

The following sections describe the research plan, recruitment of participants, and data collection of the Physical Exercise and Cognitive Stimulation (PEACS) Study. The study was conducted between July 2009 and December 2011 by Dr Tejal Shah and Professor Ralph Martins. The aim was to investigate the impact of an exercise intervention (EI) and/or cognitive stimulation (CS) regime on neuropsychological, brain imaging and blood-based biomarker parameters in an elderly population over the age of 60. For the purpose of my PhD project, I focused only on the EI and control groups. Volunteers undertook a physical activity regimen for 16 weeks. In contrast, the control group only received educational material relating to maintaining a healthy lifestyle. Figure 2.2 below shows the size of each group, whilst the study Participant information and consent form is included in Appendix 2 of this thesis.

2.3.2 Ethical approval

The PEACS Study was conducted by Dr. Tejal Shah and Prof Ralph Martins at the McCusker Alzheimer’s Research Foundation (now the Australian Alzheimer’s Research Foundation), and ethical approval was obtained from the Human Research Ethics Committees of Hollywood Private Hospital, the University of Western Australia and Edith Cowan University.

2.3.3 Recruitment

PEACS Study participants were recruited from an existing database of study participants at the McCusker Alzheimer’s Research Foundation, and via newspaper advertisements, public lectures, and through word of mouth. Eight hundred interested individuals were
contacted and the study outline was described. From this, 660 were screened for eligibility via a 15-minute questionnaire administered over the phone. Detailed information packs were sent to 383 of the eligible participants; of these, two hundred and twenty-four participants consented to take part in the study. The study consisted of a baseline assessment, and two follow-up assessments after 8 and 16 weeks. For the purpose of this thesis, I analysed data from 48 of the 58 available participants who completed the full study program; of whom 26 participants belonged to the EI only group and 22 participants to the control group.

**Figure 2.2:** Flow chart of PEACS Study participants enrolled in the exercise intervention group and the control group. The study duration was 16 weeks, with a 52-week follow-up time point. Abbreviations: EI, exercise intervention; PEACS, Physical Exercise and Cognitive Stimulation Study.
2.3.4 Eligibility and Inclusion criteria

Community-dwelling cognitively healthy men and women aged 60 years of age or older were recruited for this study. To be eligible, participants were required to understand and communicate in English, have no auditory or visual impairment (unless corrected), no history of tremors, and be willing to participate in follow-up assessments. Exclusion criteria included a medical history of cardiovascular diseases, i.e. myocardial infarction, angina or congestive heart failure, as well as schizophrenia, bipolar disorder, anxiety or depression. If arthritis caused restricted movements, then this condition also led to the exclusion of a participant.

An initial screen at baseline investigated a participant’s performance in the Mini-Mental State Examination (MMSE) with a minimum score of 24 required for study eligibility. A physician-issued fitness certificate was also required for individuals assigned to the EI group.

2.3.5 Exercise intervention and control group descriptions

The EI group was asked to walk at least three times per week for 60 minutes, and to perform a home-based resistance training regimen two days per week, for 30-45 minutes at a time, for a total of 16 weeks. The control group received educational material pertaining to maintaining a healthy lifestyle. Participants were allocated to each group based on their preferences.

2.3.6 Research design

All participants attended a baseline assessment which involved the following procedures: provision of written informed consent, collection of a 60 ml fasted venous blood sample
(i.e. after a ten hour overnight fast), collection of demographic information and general practitioner contact details, as well as undertaking a thorough neuropsychological test battery. In addition, participants were provided with an actigraphy unit that collected objective data of daily physical activity for seven subsequent days. Within one week following the baseline assessment, participants allocated to the EI group were invited to a group meeting with an exercise physiologist in which detailed instructions and demonstrations of exercise tasks were provided. Safety instructions, detailed handouts and contact numbers were also provided to ensure proper conduct of exercises at the participants’ homes.

Monthly meetings of the EI group and follow-up phone calls by research assistants ensured regular contact with all participants. The EI group meetings also included contact with an exercise physiologist to check an individual’s technique and performance applied to exercise tasks. Physical fitness assessments were conducted at Baseline, 8 and 16 week follow-ups. Neuropsychological assessments were administered, and blood samples collected, at 8 and 16 weeks of the study in addition to baseline.

2.3.7 Apolipoprotein E genotyping of the PEACS cohort

For DNA-purification QIAamp DNA Blood Maxi Kits (Qiagen, Hilden, Germany) were utilised as described in detail above (2.2.12). The genotyping was conducted by A/Prof Simon Laws and his team at Edith Cowan University. APOE genotyping was conducted using the Restriction fragment length polymorphism (RFLP) method which utilises restriction digest as described by Hixson and Vernier, and primers described by Wenham

The extracted DNA was first amplified via polymerase chain reaction (PCR). For this 10 pmols of each primer (APOE-P1 (TCCAAGGAGCTGCAGGCGGCGCA) and APOE-P2 (ACAGAATTCCGCCGCGCTGTACACTGCA)) were added to the DNA, which then underwent 37 cycles under the following conditions: the initial denaturation was held at 94°C for 5 minutes; at 65°C for 30 seconds and at 70°C for 90 seconds. 35 cycles at 94°C for 30 seconds; 65°C for 30 seconds and 70°C for 90 seconds followed. The final step concluded the amplification at 94°C for 30 seconds, 65°C for 30 seconds, 70°C for 10 minutes and 4°C for 1 minute.

The APOE restriction enzyme HhaI (5.0 U) was incubated with the amplicon at 37°C overnight. HhaI cuts the DNA at various locations according to genotype. When the resultant fragments were separated on an 8% non-denaturing polyacrylamide gel in Tris-Boric acid-EDTA (TBE) buffer, and stained with ethidium bromide, fragments of different lengths were visualised under UV light using a BioRad Gel Doc XR. Fragment sizes and their respective genotypes are outlined in Table 2.7 below.
### Table 2.7: Fragment size in base pairs (bp) determined the Apolipoprotein E (APOE) genotype of a study participant, with six possible outcomes

<table>
<thead>
<tr>
<th>APOE genotype</th>
<th>Fragment lengths in bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>ε2/ ε2</td>
<td>91 bp, 83 bp</td>
</tr>
<tr>
<td>ε3/ ε3</td>
<td>91 bp, 48 bp, 35 bp</td>
</tr>
<tr>
<td>ε4/ ε4</td>
<td>72 bp, 48 bp, 35 bp</td>
</tr>
<tr>
<td>ε2/ ε3</td>
<td>91 bp, 83 bp, 48 bp, 35 bp</td>
</tr>
<tr>
<td>ε2/ ε4</td>
<td>91 bp, 83 bp, 72 bp, 48 bp, 35 bp</td>
</tr>
<tr>
<td>ε3/ ε4</td>
<td>91 bp, 72 bp, 48 bp, 35 bp</td>
</tr>
</tbody>
</table>
2.4 Acute Strength Training (AST) pilot study

The Acute Strength Training (AST) Study, conducted in 2016 by Kieran Marston at Murdoch University, included seventeen ‘young’ and ‘older’ men and women who volunteered to participate in two types of acute resistance training exercises.

2.4.1 Ethical approval

Ethical approval was obtained from Murdoch University’s Human Research Ethics Committee. Participants were informed in detail of the study procedures and associated risks before written informed consent was obtained prior to undertaking study assessments.

2.4.2 The AST study cohort

The study cohort included ‘young’ \((n = 10)\) and ‘older’ \((n = 7)\) subgroups of participants. In order to be included in the ‘young’ subgroup, interested participants needed to be under the age of 30 and at low to moderate risk of medical difficulties that might arise from strenuous exercise. This risk was assessed via the Exercise and Sports Science Australia risk stratification questionnaire. The mean age of the ‘young’ subgroup was \(23.9 \pm 1.1\) years. The ‘older’ subgroup included six middle-aged males and one female with a mean age of \(49.6 \pm 3.6\) years. The study cohort and subgroups of participants are represented diagrammatically in Figure 2.3 below.

2.4.3 Exercise intervention description

Each participant undertook two different training protocols on different days (allowing four to ten days between the two protocols for a wash out period). The training protocols
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included; 1) strength based resistance training, consisting of 5 sets of 5 repetitions, with three-minute recovery time between each set (“S5/3”), and 2) hypertrophy based resistance training, consisting of three sets of ten repetitions, with one minute recovery between sets (“H10/1”). The training protocols were conducted at the same time of day for every participant and every visit.

Before data collection commenced, two familiarisation visits were scheduled for every participant. The first session provided a detailed description and demonstration of strength-exercises, including bench press, latissimus dorsi pull-down, leg press, leg extension, seated row, military press and dumbbell arm curl. A five repetition maximum was established for each participant during the first visit. The second scheduled visit served to establish a ten repetition maximum. On the third and fourth scheduled visits, either the strength or hypertrophy resistance workout was conducted, and the individual’s outcome recorded. A gentle to moderate warm-up on a rowing ergometer for five minutes preceded the workouts.

2.4.4 Blood collection

A fasted venous blood sample was collected from every participant immediately before the warm-up phase, immediately after each workout session, and 30 minutes following completion of each workout. Blood was collected into serum tubes (SST Vacutainer®, Becton-Dickinson, USA), which were centrifuged at 1,800 x g for 15 minutes following clotting. The serum was stored at -80°C until required for analysis.
Figure 2.3: Diagrammatic representation of the Acute Strength Training (AST) cohort which included ‘young’ and ‘older’ subgroups of participants. The cohort undertook strength and hypertrophy based resistance training protocols on two separate occasions. Data including blood samples were collected immediately prior to-, immediately post-, and 30 minutes post- exercise.
2.5 Laboratory-based data collection

2.5.1 Detection of serum irisin by enzyme-linked immunosorbent assay (ELISA)

For the detection of irisin in serum, a commercially available enzyme-linked immunosorbent assay (ELISA) was used (Phoenix Pharmaceuticals, Inc., Burlingame, California, USA – catalogue number EK-067-029), as per the manufacturer’s instructions. In this thesis, two generations of the same kit were used, with the initial so-called ‘second generation kit’ used for irisin detection in the PEACS Study samples, and the ‘third generation kit’ used to detect irisin in the AIBL and AST Study samples.

Before commencing each assay, all solutions of the kit were brought to room temperature. The 20x assay buffer concentrate was diluted with 950 ml distilled water to produce a 1x assay buffer solution. This assay buffer was then used to dilute and dissolve all kit reagents and samples. Initially, the standard peptide was rehydrated with 1 ml assay buffer and mixed thoroughly by vortexing. The resultant 1,000 ng/ml stock solution was left for approximately 10 minutes at room temperature and then used to prepare standard solutions of 100, 10, 1 and 0.1 ng/ml concentrations. Next, the positive control, rabbit anti-IgG primary antibody, and biotinylated peptide, were rehydrated and left for at least 5 minutes to completely dissolve. All serum samples were diluted 1:2 with the assay buffer after fully thawing on ice. A 96-well immunoplate, pre-coated with a secondary antibody with non-specific binding sites blocked, was provided with every kit and used to load controls, standards and a maximum of 40 samples at a time. Every sample, standard and control was loaded onto the plate in duplicate. This step was followed by the addition of the primary antibody and the biotinylated peptide into each well, excluding the blank wells. The plate was then sealed and left on an orbital shaker for 2 hours at room temperature, with an approximate orbital shaking of 300-400 rpm. Following the incubation period, the plate was washed four times by adding 350 μl of assay buffer per
well, emptying the contents into the sink, then inverting and blot drying the plate. 100 μl of Streptavidin-horseradish peroxidase (SA-HRP) was then added to each well, and the plate sealed and incubated again for one hour at room temperature on the same orbital shaker. Afterwards, the plate was again washed four times as before. The next step was conducted under light-reduced conditions. After pipetting 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) into each well, the plate was once more sealed with an acetate plate sealer (APS) and wrapped in aluminium foil before being placed on the orbital shaker for another hour at room temperature. The assay procedure was concluded by stopping the reaction with 100 μl 2M HCl. The colour of the wells ranged from blue to yellow. A gentle tap to the plate ensured proper mixing. The plate was then immediately read on the FLUOstar OPTIMA Plate reader (BMG Technologies, Buckinghamshire, UK). The absorbance O.D. was read at a 450 nm spectrum.

The analysis of irisin levels was conducted using the MasterPlex ReaderFit 2010 software, Version 2.0.0.77 (Hitachi Solutions, Ltd, Tokyo, JP), which provided the option of a five parameter logistical analysis. The standards were plotted on a log-scale x-axis, and the corresponding O.D. reading on the linear y-axis. An inverse relationship between peptide concentration and O.D. readings (absorbance measurements) was shown, and each standard curve from each assay demonstrated a reverse sigmoidal shape: the higher the concentration of the peptide, the weaker the intensity of discoloration in the wells (corresponding to lower absorbance readings). All samples analysed were found on the slope of the curve. Figure 2.4 below illustrates a typical standard curve.

The irisin concentrations in the serum samples were calculated by extrapolation to the standard curve, as described in the manufacturer’s protocol. All serum irisin
concentrations are expressed in ng/ml. Further details regarding evaluation of irisin levels, and the process of winsorizing the data, are given in section 2.8.1.

![Absorbance O.D. (450 nm) vs. [irisin] ng/ml](image)

**Figure 2.4:** A typical standard curve \( r = 0.9998 \) to assess irisin peptide concentration in human serum when conducting an enzyme-linked immunosorbent assay (ELISA).

### 2.5.2 *SH-SY5Y* neuroblastoma cells

Within this PhD project, *in vitro* investigation of irisin and cell protection was undertaken using human *SH-SY5Y* neuroblastoma cells. *SH-SY5Y* neuroblastoma cells were originally acquired from the American Type Culture Collection (ATCC) Global Bioresource Centre (Manassas, Virginia, USA), and detailed information on this cell line was studied prior to use in my experiments. *SH-SY5Y* neuroblastoma cells have been extensively utilised in recent years in the area of neurobiology research (Kovalevich & Langford, 2013). The original cells were derived from a bone-marrow biopsy of a female and thus, contain two X-chromosomes. The *SH-SY5Y* cell line was established in 1978 after the original cells were sub-cloned three times. *SH-SY5Y* cells are considered robust cells that have been demonstrated to float in media, and adhere to the surface of culture vessels. In all experiments described herein, floating cells were discarded during media changes and only adherent cells were used. Throughout the duration of this project, these cells were observed to grow quickly to 80% confluency in conditions described below,
and only showed slower growing rates immediately following thawing. Cells were grown in T-75 flasks in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS), at a pH of 7.4. Flasks were stored in an incubator at 37°C and 5% carbon dioxide (CO₂). At 70-80% confluency, cells were passaged 1:20 by removing the media and washing the flask surface with Hanks buffer, adding 1 ml of 0.25% trypsin-EDTA for 3 minutes at 37°C. All experiments were conducted with cells that had undergone less than or equal to 20 passages to guarantee neuronal characteristics typical for this cell line. Excess cells were recovered and frozen for future experiments. After splitting the cells, the unused cell suspension was centrifuged at 200 x g for 5 minutes to yield a cell pellet thereby separating the cells from the media they were immersed in. After carefully removing the media, the pellet was gently resuspended in a 10% Dimethyl sulfoxide (DMSO) / FCS solution and aliquoted into 1 ml cryovials. Between 1 and 1.5 million cells/ml were frozen in each vial. These cells were then transferred into a freezing unit and stored at -80°C for 2-3 days before transferring them to a liquid nitrogen unit for long-term storage. When needed, these cells were thawed in a 37°C water bath and swiftly transferred to a tube containing standard growing media. The tube was then centrifuged at 200 x g for 5 minutes at room temperature, after which time, the DMSO-suspension was carefully removed, and the pellet dissolved in 12 ml of standard media as described above (DMEM/10% FCS). The cell suspension was gently pipetted into a T-75 flask and left in the incubator for 5-7 days, with a media change after 2 or 3 days. Once the cells reached 80% confluency they were passaged once, before being used in experiments.
Figure 2.5: Light micrograph showing human SH-SY5Y neuroblastoma cells at moderate density.
2.5.3 Testing the ability of treatment-medium to stop cell growth

A number of experiments required Aβ and/or irisin to be administered to SH-SY5Y cells: when required, these peptides were administered to cells in treatment medium (DMEM/F12 + 1% FCS), which differs in composition from normal growth medium (DMEM/F12 + 10% FCS). To establish whether the treatment medium is effective at halting natural cell growth, I counted the cells after two and three days of incubation in either normal growth or treatment medium. As expected, and as illustrated by Figure 2.6, the treatment medium inhibited cell growth, meaning that any observation of increased cell growth during experiments was due to the peptide administered and not to the medium.

![Graph showing cell count comparison between normal medium and treatment medium at 48 hours and 72 hours](image)

**Figure 2.6:** The treatment medium used in experiments when Aβ and/or irisin administration was required is effective at stopping cells from growing and proliferating. Each graph represents one experiment; mean ± standard error of the mean shown. Student’s unpaired t-tests revealed significant differences in the number of viable cells between the two types of medium (normal and treatment) after 48 hours and 72 hours of incubation (*p < 0.05; t = 3.23, p = 0.031 and t = 4.34, p = 0.012, respectively).
2.5.4 Cell viability assays following Aβ and/or irisin treatment

Different treatments and varying well plate layouts were used for a range of cytotoxicity assays. In general, 96-well plates were used, and 10,000 cells per well were seeded and grown in 100 μl standard growth media as described above. Cells were counted using a ViCell™ Cell Viability Analyzer (Beckman Coulter, California, USA), and appropriate volumes to yield necessary concentrations were calculated using the formula $C_1 \times V_1 = C_2 \times V_2$. After two days of normal growth, approximately 40% cell confluency was reached and treatment was performed. Cell media was changed to 1% FCS/phenol-red free DMEM, and Aβ (concentration range 1-20 μM) and/or irisin (ranging from 1-50 nM, depending on the experiment; catalogue number PH-067-16; Phoenix Pharmaceuticals, Burlingame, California, USA) were added. Cells were subsequently incubated for between 3 and 5 days depending on the experiment. On termination of the incubation period, a Lactate dehydrogenase (LDH) assay was performed to determine the toxicity of the treatment. The LDH assay was complemented by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay in order to simultaneously assess cell viability. All steps were conducted under sterile conditions in a cell culture hood, at room temperature, unless otherwise stated.

2.5.4.1 LDH assay

The LDH-assay is widely used to determine cell viability. To measure the release of LDH from damaged membranes of SH-SY5Y cells following treatment with Aβ +/- irisin, the CytoTox-ONE™ Homogeneous Membrane Integrity Assay was used (catalogue number G7890/1/2; Promega, Madison, USA). Two μl of lysis solution (a 9% weight/volume solution of Triton® X-100 in water) was added to the wells and the whole plate was
incubated for 15 minutes at 37°C in 5% CO₂: during which time, LDH from lysed cells was released into the media. Fifty μl from each well was then transferred to a black 96-well plate, and 50 μl of the CytoTox-ONE™ Reagent (comprising the LDH substrate mix provided by the manufacturer) was added to produce a total volume of 100 μl per well. The plate was then wrapped in foil and incubated for a maximum of 10 minutes before the reaction was stopped by adding 100 μl of the provided Stop-solution. The fluorescence of each well was read using the FLUOstar OPTIMA Plate reader (BMG Technologies, Buckinghamshire, UK), with greater fluorescence corresponding to higher numbers of lysed (i.e. dead) cells.

2.5.4.2 MTS-assay

For this assay, 2 μl of the same lysis solution as described in section 2.5.4.1 above was added to each well and the whole plate was incubated for 15 minutes at 37°C in 5% CO₂. Cell media was then carefully removed, and 100 μl of MTS (Promega, Madison, USA) was added to each well. The plate was then wrapped in foil and incubated for four hours at 37°C in 5% CO₂. The absorbance of each well at 450 nm was then read in the same FLUOstar OPTIMA Plate reader (BMG Technologies, Buckinghamshire, UK) as was used for the LDH assays. Raw absorbance values were transferred to an Excel spreadsheet, and mean, SDs and SEMs calculated.

2.5.4.3 Well plate layouts for Aβ +/- irisin treatments

Figures 2.7 and 2.9 illustrate the two well plate layouts used most frequently for the Aβ +/- irisin treatment of SH-SY5Y cells in vitro. Slight variations in irisin concentrations
and Aβ treatments did occur, which are cited in the results section of Chapter 5 as appropriate.

### 2.5.4.4 Confirmation of Aβ-mediated cell toxicity and irisin non-toxicity in SH-SY5Y cells

To confirm the cytotoxicity of Aβ and the non-toxicity of irisin, plates with SH-SY5Y cells grown for two days, were first immersed in 1% FCS/phenol-red free DMEM (treatment media). The well plate layout illustrated in Figure 2.7 demonstrates that each treatment was administered in quadruplicate. ‘Blank’ wells containing treatment media without cells served as a reference to background ‘noise’. ‘Untreated’ wells (Un) contained treatment media with cells, without the addition of Aβ42 or irisin. The toxicity of Aβ with increasing concentrations (1, 5, 10, 15 and 20 μM) was determined after four and five days of treatment. Two ‘controls’ were also employed: a positive control (Ctrl 1) where lysis buffer was added to ensure complete cell death, and a negative control (Ctrl 2) where irisin at a concentration of 10 nM was administered (in the absence of Aβ) to confirm its non-toxic effects on SH-SY5Y cells. Cytotoxicity assays were performed at the end of the four or five day treatment period. To avoid evaporation during the long incubation periods, 100 μl of sterile water was added to the wells at the edges of each plate. An example of the results of the cytotoxicity assays confirming Aβ-mediated toxicity and irisin non-toxicity in SH-SY5Y cells is provided in Figure 2.8.
**Figure 2.7:** Well plate layout for confirmation of Aβ-mediated cell toxicity and irisin non-toxicity in SH-SY5Y cells. Treatments were administered in quadruplicate. ‘Blank’ wells contained treatment media without cells to determine background ‘noise’. ‘Untreated’ wells (Un) contained treatment media with cells, without the addition of Aβ42 or irisin. The toxicity of Aβ with increasing concentrations (1, 5, 10, 15 and 20 μM) was determined after four and five days of treatment. A positive control (Ctrl 1) consisting only of lysis buffer ensured complete cell death, and a negative control (Ctrl 2) comprising of 10 nM irisin in the absence of Aβ confirmed the non-toxic effects of this peptide on SH-SY5Y cells. Cytotoxicity assays were performed at the end of the four or five day treatment period. Sterile water (100 μl) was added to wells at the edges of each plate to avoid evaporation during the long incubation periods. Wells with no labelling were left empty. Abbreviations: Aβ, amyloid-β42; Ctrl, control; H2O, sterile milliQ water; Un, Untreated.
Figures 2.8: Representative LDH and MTS assay results confirming Aβ-mediated cell toxicity and irisin non-toxicity in SH-SY5Y cells (at P11) after 5 days of treatment. Aβ was administered at a concentration range of 1-20 μM. A positive control (Ctrl 1) consisting only of lysis buffer ensured complete cell death, and a negative control (Ctrl 2) comprising of 10 nM irisin in the absence of Aβ confirmed the non-toxic effects of this peptide on SH-SY5Y cells. ‘Untreated’ wells contained treatment media with cells, without the addition of Aβ42 or irisin. Cytotoxicity assays were performed at the end of the five day treatment period. Abbreviations: Aβ, amyloid-β42; Ctrl, control; LDH, Lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; nm, nanometre; P11, passage 11.
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2.5.4.5 Determining the ability of irisin to ameliorate Aβ-mediated toxicity in SH-SY5Y cells

To assess the ability of a physiological concentration of irisin to ameliorate Aβ-mediated toxicity, plates with SH-SY5Y cells grown for two days, were first immersed in 1% FCS/phenol-red free DMEM (treatment media). ‘Blank’ wells containing treatment media without cells served as a reference to background ‘noise’. ‘Untreated’ wells (Un) contained treatment media with cells, without the addition of Aβ42 or irisin. Aβ was administered at concentrations of 1, 5, 10, 15 and 20 μM in the presence or absence of 10 nM irisin for three, four or five days. The well plate layout illustrated in Figure 2.9 demonstrates that each Aβ +/- irisin treatment was administered in triplicate. Two ‘controls’ were also employed: a positive control (Ctrl 1) where lysis buffer was added to ensure complete cell death, and a negative control (Ctrl 2) where irisin alone at a concentration of 10 nM was administered to confirm its non-toxic effects on SH-SY5Y cells. Cytotoxicity assays were performed at the end of the three, four or five day treatment period. To avoid evaporation during the long incubation periods, 100 μl of sterile water was added to the wells at the edges of each plate. Each experiment was conducted at least three times.
**Figure 2.9**: Well plate layout for assessing the ability of a physiological concentration of irisin to ameliorate Aβ-mediated toxicity in SH-SY5Y cells. ‘Blank’ wells contained treatment media without cells to determine background ‘noise’. ‘Untreated’ wells (Un) contained treatment media with cells, without the addition of Aβ$_{42}$ or irisin. Aβ was administered at concentrations of 1, 5, 10, 15 and 20 μM in the presence or absence of 10 nM irisin for three, four or five days. Each Aβ +/- irisin treatment was administered in triplicate. A positive control (Ctrl 1) consisting only of lysis buffer ensured complete cell death, and a negative control (Ctrl 2) comprising of 10 nM irisin in the absence of Aβ confirmed the non-toxic effects of this peptide on SH-SY5Y cells. Cytotoxicity assays were performed at the end of the three, four or five day treatment period. Sterile water (100 μl) was added to wells at the edges of each plate to avoid evaporation during the long incubation periods. Wells with no labelling were left empty. Abbreviations: Aβ, amyloid-β$_{42}$; Ctrl, control; H$_{2}$O, sterile milliQ water; Un, Untreated.
2.6 Preparation of oligomeric Aβ42 for cell treatment

The preparation of hexafluoroisopropanol (HFIP)-treated Aβ peptide stocks was completed as described previously (Stine, Jungbauer, Yu, & LaDu, 2011). Aβ42 has a molecular weight of 4510.6 g/mol. In order to yield a 1 mM solution, 4.5 mg of the lyophilized powder was weighed out and 998 μl of HFIP added, using a glass Hamilton syringe with a Teflon plunger and a sharp needle. The solution was incubated for 30 minutes at room temperature before swiftly aliquoting the solution into Eppendorf tubes at volumes of 100 μl. The tubes were left open in the fume hood so that the liquid could evaporate overnight. On the second day the tubes were transferred into a SpeedVac and samples dried for one hour without heating or pressure, in order to remove all remaining liquid. The Aβ42 peptide was apparent as a clear film on the bottom of the tube. Tubes containing the peptide film were stored in an air-tight container together with desiccant at -30°C until required. Prior to an experiment, a 5 mM Aβ solution, was prepared by adding 20 μl of sterile DMSO to 0.45 mg of the Aβ peptide film. By thoroughly scraping down the walls of the tube and vortexing for at least 30 seconds, the Aβ peptide film was successfully suspended in solution. This Aβ peptide suspension was then sonicated for 10 minutes at room temperature after which time 980 μl of cold phenol-free F12 cell culture media was added. The final working concentration was 100 μM of Aβ. This final solution was vortexed for 15 seconds and left for 24 hours at 4°C before use in cell treatments.
2.7 Western blotting

2.7.1 Cell lysate preparation

Six-well plates were used to seed SH-SY5Y cells at a density of 200,000 cells in a total volume of 3 ml media per well. Once an estimated 40% cell confluency was reached, the media was changed to fresh growth media with the addition of various concentrations of irisin or BDNF, depending on the experiment. Treatment periods lasted for 24, 48 or 72 hours, after which the media was removed and retained, and the cells lysed and the lysates retained. NP40 (Nonidet) lysis buffer was prepared using 50 mM Tris-HCl at pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% NP40, and a protease inhibitor cocktail (Complete®) consisting of 5 μg/ml aprotinin, 5 μg/ml leupeptin and 0.1 mM phenylmethylsulfonyl fluoride (PMSF).

Before cell lysis was commenced, the lysis buffer and washing buffer (PBS) were cooled and kept on ice. The 6-well plates were transferred onto ice, and the media removed and retained. The wells were washed three times with 3 ml of ice-cold PBS. Approximately 150-200 μl of NP-lysis buffer was then added onto the centre of each well. Following an incubation time of 5 minutes on ice, each well was thoroughly scraped and the cell lysates collected into Eppendorf tubes. The tubes were incubated on ice for a further 20-25 minutes: during this time, the lysates were thoroughly mixed every 10 minutes. Following the total incubation time on ice of approximately 30 minutes, the tubes were centrifuged at 200 x g for 10 minutes at 4°C. The supernatant was collected and stored at -80°C for subsequent analysis.
2.7.2 Determination of sample protein concentration

Assays to determine the protein concentration in each cell lysate or media sample were conducted using the Pierce Micro BCA™ Protein Assay Kit (catalogue number 23235, Thermo Scientific, Rockford, USA). An Albumin standard was provided with the kit and used to prepare standard dilutions for a linear standard curve, ranging from 0.5 – 200 μg/ml. A working reagent was prepared by adding 25 parts of reagent A to 24 parts of Reagent B and 1 part of reagent C as per the manufacturer’s instructions. 100 μl of each protein lysate and cell media sample, and of each standard as well as the blank, was added to a clear 96-well plate. 100 μl of the working reagent was then added to each well to yield a final volume of 200 μl per well. After wrapping the plate in foil, it was incubated at 55°C for 15 minutes or until a colour-change occurred from green to purple. The plate was then read by a FLUOstar OPTIMA Plate reader (BMG Technologies, Buckinghamshire, UK) at an absorbance of 550 nm. The protein concentration within each sample was then calculated in μg/ml by reference to the standard curve. A minimum of 25 μg of protein was deemed sufficient in order to continue to the Western blot protein detection stage.

2.7.3 Western blotting

Gels used for stacking and separating proteins included hand-cast Tris-Tricine gels comprising a 12% and 8% Resolving gradient gel and a 4% stacking gel, and Bis-Tris gels which were purchased pre-made (Navix Nu®PAGE 4-12%). The hand-cast gels consisted of acrylamide (49.5% / 3%), gel buffer, deionised water, 10% ammonium persulphate and N,N,N’,N’-tetramethylethylene diamine (TEMED). Once the hand-cast gels were set, they were transferred and assembled into a tank, which was filled with
anode buffer (2M Tris) and cathode buffer (Tris-Tricine) in the appropriate reservoirs, to allow for conduct of an electrical current. In the case of the pre-cast gels, NuPAGE sample buffer (2 μl of 10x reducing agent + 3 μl 4x LDS sample buffer) was added to the protein lysate samples, and a single type of running buffer (1x MES) was added to each tank reservoir. Following the loading of samples into the gel wells, the pre-cast gels were run for 2 hours at 90 Volts.

Once the well-moulding combs were removed from the hand-cast gels and the chambers straightened, a protein standard (Novex Sharp ladder) and the samples were loaded. Ten minutes before loading the samples, a sample buffer made of Bis Tris, Bicine, SDS, sucrose, Bromophenol Blue and 2.5% β-Mercaptoethanol was added to the protein samples before boiling them at 95°C for 5 minutes in a heating block. Once loaded with samples, the hand-cast gels were run at 80 Volts through the stacking phase and at 120 Volts through the resolving phase for approximately 2 hours.

Proteins were subsequently transferred onto nitrocellulose membrane. For this, ice-cold transfer buffer consisting of 20% methanol in deionised water was prepared. The gel(s) were carefully removed from the glass plates, the stacking gel cut off and discarded and the remainder of the gel was placed in a container with transfer buffer to wash off any excess SDS. The washed gel was then placed within a sandwich consisting of two buffer-soaked pieces of filter paper on each side, a wet sponge on each side, and the nitrocellulose membrane on top of the gel facing the anode. The transfer ‘cassette’ was fully emerged in the transfer buffer and was run at a current of 250 mA for approximately 16 hours at 4°C. Once the transfer of proteins from the gel to the nitrocellulose membrane was complete, the membrane was placed into a new container with 5% skim milk in TBS
Chapter 2 – Material and Methods

for one hour at room temperature. This was followed by a two-hour incubation with rabbit primary antibody to BDNF (ab46176, Abcam, Cambridge, UK) in 5% skim milk/TBS at room temperature, at an antibody dilution of 1:1,000. Three 10-minute washing steps with TBS-T followed, before the addition of the secondary antibody; Goat anti-rabbit Immunoglobulin G (IgG)-HRP (ab97051, Abcam, Cambridge, UK) at a 1:10,000 dilution for one hour. Three more washing steps with TBS-T were then undertaken before a quick rinse with TBS to remove the tween. In the final step, an Amersham™ ECL™ Western blotting detection kit (GE Healthcare, Silverwater, New South Wales, Australia) coupled with Amersham Hyperfilm™ (GE Healthcare Life Sciences, Silverwater, New South Wales, Australia) was used to detect protein bands. After which, the membranes were stored in TBS at 4°C until further use.

To detect a different antigen, i.e. GAPDH (catalogue number 2118, Cell Signaling Technology®, Danvers, Massachusetts, USA), as an equal protein loading-reference, membranes were stripped with Restore Plus Stripping Buffer (Thermo Scientific, Rockford, USA). First the membrane was washed with TBS followed by immersion in stripping buffer for 15 minutes at room temperature. Three washes in TBS for 10 minutes each left a clean membrane that was blocked with 5% skim milk/TBS for one hour, before the primary antibody was added, three 10 minute washes in TBS-T followed, after which secondary antibody exposure occurred. Apart from a simple visual inspection of the developed film, quantitative assessments of protein bands were undertaken using the ChemiDoc™ Touch Imaging System and its software Image Lab, Version 5.1 build 8 (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia).
2.8 Statistical Analyses

2.8.1 Data cleaning for statistical analysis: Irisin and MET scores

Irisin levels were detected between 5.9 and 26.2 ng/ml. Winsorization of statistical data determined outliers beyond irisin levels of 18 ng/ml: this level was determined via the Modified Thompson Tau test (δ) (Avbelj, 2012) (Thompson, 1985). Irisin was computed with mean irisin levels of 11.43 ± 3.53 ng/ml, tau = 1.95, and tau*SD = 6.89. The cut-off value was set to 18 ng/ml and thirteen outliers were identified and further visually confirmed as outliers using an SPSS boxplot.

In order to avoid excessive self-reported physical activity levels, a truncation process was put in place, ruling that neither light, moderate nor vigorous activity can exceed 180 min per day per participant. Thirty IPAQ reports were affected by this truncation rule, lowering the total weekly MET scores.

Outliers were visually determined by inspecting the histograms and percentiles for MET scores. One clear outlier was identified and removed (see Figure 2.10). In addition, exact outliers for MET scores were then identified via Hoaglin’s g’ = 2.2 (Lower limit: Q1 - (g*(Q3-Q1)); Upper limit: Q3 + (Q3 - Q1)). A total of nine cases were excluded due to the Hoaglin outlier rule (Hoaglin & Iglewicz, 1987).
Figure 2.10: Frequency distribution of self-reported MET·min/wk from IPAQs, before outliers were determined (a) and after removing an outlier (b). Abbreviations: MET·min/wk, MET, Metabolic Equivalent of Task averaged over 7 days.
2.8.2 Analysis of clinical data

The statistical analyses methods used to evaluate the clinical data (i.e. in Chapters 3 and 4) will be described in detail within each respective methods section of each chapter. Regardless of the statistical tests used, all assumptions for these tests were considered and met before analysis took place. All of the statistical analyses were conducted using IBM® SPSS® Statistics 23.0 for both Mac and Windows 7 (IBM Corp. Released 2015. IBM SPSS Statistics for Mac and Windows, Version 23.0. Armonk, NY: IBM Corp). In all circumstances, a $p$-value of less than 0.05 was always deemed significant. Data is presented as mean ± standard deviation (SD), unless otherwise stated.

2.8.3 Analysis of laboratory data

Protein assays as described above (2.3.5.2) ultimately produced a standard curve against which unknown concentrations were matched in order to compute the protein concentration in a sample. A standard curve with $r = 0.99$ or higher was deemed sufficient to continue to calculate exact protein concentrations (Figure 2.11 below). The standards were retrieved from the Pierce Micro BCA™ Protein Assay Kit. Concentrations were calculated in $\mu g/ml$. 
Figure 2.11: An example standard curve to determine protein concentrations in cell lysates, here $r = 0.9971$.

Mean comparisons between treatment groups in toxicity assays and Western blots were evaluated via an independent samples $t$-test. A $p$-value below 0.05 was deemed significant. Relative quantitative assessments of the density of bands resulting from Western blots was further assessed using the software ImageLab (version 5.1) provided by the ChemiDoc™ Touch Imaging System device (Bio-Rad Laboratories, California, USA).

The mean and standard error of mean (SEM) of raw results were calculated for every triplicate in every single toxicity and viability assay, and is presented for the time-dose Aβ-toxicity experiment.

For the LDH and MTS assays, a minimum of three repeated experiments were conducted and presented as combined results. A comparison between different treatment groups in these assays was conducted via independent samples $t$-test, using IBM® SPSS® Statistics 23.0 for both Mac and Windows 7 (IBM Corp. Released 2015; IBM SPSS Statistics for
Mac and Windows, Version 23.0. Armonk, NY: IBM Corp). A $p$-value below 0.05 was deemed significant.

In the case of Western blot analysis, a simple visual inspection of the developed Amersham Hyperfilm™ (GE Healthcare Life Sciences, Silverwater, New South Wales, Australia) determined the approximate size of the protein bands when compared to a standard protein ladder (Novex Sharp ladder). Further, quantitative assessments of the thickness and density of these bands were conducted using the ChemiDoc™ Touch Imaging System and its software Image Lab, Version 5.1 build 8 (Bio-Rad Laboratories, Gladesville, NSW, Australia).
Chapter Three

The Relationship between Serum Irisin Levels, Cognition and Alzheimer’s disease-Related Blood Biomarkers: Cross-Sectional and Longitudinal Investigations
3.1 Introduction

As described in Chapter 1 (Section 1.2.3.3, page 29), irisin is a recently discovered hormone associated with function of the central nervous system (CNS) (Hashemi et al., 2013; Moon, Dincer, & Mantzoros, 2013; Ostadsharif et al., 2011; Zhang et al., 2015a). Early in vitro studies indicate a role for irisin in neuronal development (Hashemi et al., 2013; Moon et al., 2013; Ostadsharif et al., 2011; Zhang et al., 2015a, 2015b). Nevertheless, to-date, circulating levels (i.e. serum and/or plasma) of irisin have not been investigated in regards to Alzheimer’s disease (AD) diagnosis, nor thorough cognitive testing, in an older adult cohort.

The current gold standard for identifying AD neuropathology in pre-mortem human populations is via neuroimaging and biomarker analysis of cerebrospinal fluid, which are expensive and invasive techniques, respectively. The development of a widely accessible cost-effective, non-invasive, and sensitive method is a vital next step in the identification of AD neuropathology before the onset of clinical symptoms. Although research to-date has made promising progressions toward identifying blood-based biomarkers of AD, further work is required to establish a panel of blood biomarkers that meets the specificity and sensitivity seen with the use of brain imaging and cerebrospinal fluid biomarkers (Lovestone, 2014). Thus, the investigation of newly discovered blood-based molecules that have the potential to enhance such panels, e.g. irisin, is a crucial next step in this field of research.

To my knowledge, there have been two prior investigations into the relationship between circulating irisin levels and cognition, with these studies reporting conflicting results. Recently, one study investigated the relationship between irisin and cognition in
a cohort of young trained athletes (Belviranli, Okudan, Kabak, Erdogan, & Karanfilci, 2016). Belviranli et al. reported an association between higher plasma irisin levels and better performance on the mini-mental state examination (MMSE) (Belviranli et al., 2016). A second study conducted an investigation into executive cognitive profiles of obese and morbidly obese females aged 18-60, who were assessed using the Wisconsin Card Sorting Test, Stroop Colour and Word Test, and Iowa Gambling Task (Fagundo et al., 2016): the authors reported an inverse association between plasma irisin levels and executive functioning. As irisin and its precursor protein, Fibronectin type III domain containing protein 5 (FNDC5), have been found to frequently occur in the mammalian brain, it appears promising to further investigate associations between irisin and cognitive performance in a cohort of older adults.

In this Chapter, several cross-sectional and longitudinal analyses were conducted to investigate the possible role of irisin as a biomarker of AD, cognitive performance and brain health. The study cohort for these analyses was derived from the earlier described Australian Imaging, Biomarkers and Lifestyle flagship study of ageing (AIBL; see Chapter 2, Section 2.2, page 52), and comprised 201 cognitively healthy participants and 92 Alzheimer’s disease (AD) participants, over 60 years of age.

The aims of this Chapter were to:

1) Evaluate differences in serum irisin levels between older adults categorised as cognitively healthy, and those with AD;

2) Evaluate the relationship between serum irisin levels and factors associated with AD risk, including age, gender, apolipoprotein E (APOE) ε4 allele carriage, body mass index (BMI), and education;
3) Evaluate the association between serum irisin levels and cognitive function in cognitively healthy older adults; and

4) Evaluate the relationship between serum irisin and blood biomarkers associated with AD risk.
3.2 Materials and Methods

The materials and methods relevant to this Chapter are briefly described below. For more thorough details of all methods, please refer to Chapter 2.

3.2.1 Study participants

Participants included in these analyses were men and women aged over 60, drawn from the larger AIBL study cohort. 293 participants who were cognitively healthy (HC; \( n = 201 \)) or had a diagnosis of AD (\( n = 92 \)) were selected for inclusion in the current analyses, based on their clinical classification (i.e. individuals with mild cognitive impairment were excluded) and availability of demographic, physical activity, neuropsychological and pathology data. Approximately two thirds of the data utilised in these analyses were from participants of the Perth site (\( n = 191 \)) with the remaining one third of data obtained from participants from the Melbourne site of the AIBL study (\( n = 102 \)); procedures across both sites remained consistent across all time-points. All participants were provided with detailed descriptions of study procedures, and written, informed consent was obtained from all participants at each time-point. Details of recruitment, inclusion criteria, and ethical approval for the AIBL study are documented in Chapter 2, Sections 2.2.1 – 2.2.4 (pages 52-53).

3.2.2 Data collection

The data collection for the two time-points (Baseline and 18-month follow-up) investigated in this project was conducted from 2013 to 2015. At each visit, a fasted blood sample was collected for biomarker analyses, extensive neuropsychological assessment was conducted, and questionnaires were completed for the collection of information
regarding medical history, demographic information, current mood and physical activity level. Height, weight, waist circumference, blood pressure and heart rate were also measured at each visit. Further, blood samples were analysed for a range of blood biomarkers of interest, including cardiovascular disease and metabolic disease biomarkers, hormones, and thyroid function biomarkers.

**3.2.2.1 Neuropsychological data collection**

Neuropsychological assessment was used to evaluate a variety of cognitive domains, with various targeted tests, as thoroughly outlined in Chapter 2 (Section 2.2.7, pages 55-59). Attention, working memory, visuospatial functioning, language, verbal learning, and short-term and delayed memory recall were all assessed by the AIBL neuropsychological battery. Composite scores of cognitive domains of interest were calculated (Lim et al., 2014) and summarised as: Episodic Memory (EM), Executive Functioning (EF), Attention (Att), and Preclinical Alzheimer’s Cognitive Score (ADCS). Composite scores were computed by standardizing results for various cognitive tests against baseline mean and standard deviation (SD) for all HC participants. The Z scores for certain tests (described below) were averaged to gain a single composite score for a specific domain.

The EM composite was calculated by averaging Z-scores of the California Verbal Learning Test (CVLT-II) delayed recall, Logical Memory (LM) delayed recall, and Rey Complex Figure Test delayed recall tasks. The EF composite score comprised Z-scores from Stroop Colours/Dots, Letter Fluency, and Category Fluency Switching (Fruit/Furniture). The Att composite consisted of Z-scores from Digit Symbol, Stroop Dots/Time, and Digit Span (Lim et al., 2014). The ADCS was computed using the average of the Z-scores from CVLT-II total recall and LM (delayed recall) score, attention and
MMSE (Donohue et al., 2014). These composite scores have been shown to be sensitive correlates of AD biomarkers, compared with individual cognitive test scores (Harrington et al., 2013).

### 3.2.2.2 APOE genotyping

Genotyping for all AIBL samples was carried out by A/Prof Simon Laws and his team at Edith Cowan University. The protocol for DNA extraction and APOE genotyping is explained in detail in Chapter 2, Section 2.2.12, pages 71-72. Briefly, QIAamp DNA Blood Maxi Kits (Qiagen, Hilden, Germany) were utilised to extract DNA from 5mL of whole blood. This was followed by TaqMan® genotyping assays to determine the two APOE alleles of each participant (rs7412, assay ID: C____904973_10; rs429358, assay ID: C___3084793_20; Life Technologies, Carlsbad, CA). DNA was first amplified via Real-Time PCR (QuantStudio 12K Flex™; Applied Biosystems, Foster City, CA). APOE genotype was determined by identifying 2 single nucleotide polymorphisms (SNPs) rs7412 allele (C) and rs429358 allele (T).

### 3.2.2.3 Irisin detection

A detailed description of blood collection, serum fractionation and detection assays is contained in Chapter 2, Sections 2.2.11.1 – 2.2.11.3, page 69, and Section 2.5.1, page 81. In brief, for the detection of irisin in human serum, a commercially available enzyme-linked immunosorbsent assay (ELISA) was used (Phoenix Pharmaceuticals, Inc., Burlingame, California, USA – catalogue number EK-067-029), and the manufacturer’s protocol was closely followed. The samples were analysed by measuring optical density
(O.D.) at 450 nm via a FLUOstar OPTIMA plate reader (BMG Technologies, Buckinghamshire, UK).

3.2.3 Data cleaning for statistical analysis

Irisin levels within the HC sub-group ranged between 5.9 and 26.2 ng/ml. Measurements of irisin that were identified as outliers were winsorized at 18 ng/ml: this level was determined via the Modified Thompson Tau test ($\delta$) (Avbelj, 2012) (Thompson, 1985). Irisin was computed with mean irisin levels of $11.43 \pm 3.53$ ng/ml, $tau = 1.95$, and $tau*SD = 6.89$. The cut-off value was set to 18 ng/ml and thirteen outliers were identified and further visually confirmed as outliers on a boxplot.

3.2.4 Statistical analyses: Cross-sectional data

The analysis of irisin levels determined via ELISA was conducted using the MasterPlex ReaderFit 2010 software, Version 2.0.0.77 (Hitachi Solutions, Ltd, Tokyo, JP), which provided the option of a five parameter logistical analysis. The dilution factor was taken into account and concentrations were calculated in ng/ml.

All other statistical analyses were conducted using IBM® SPSS® Statistics 23.0 for both Mac and Windows 7 (IBM Corp. Released 2015. IBM SPSS Statistics for Mac and Windows, Version 23.0. Armonk, NY: IBM Corp). Numerous statistical tests were utilised to address the study aims, as described below. A $p$-value of less than 0.05 was deemed significant. As the current study is the first of its kind, the analyses conducted here are exploratory; thus, adjustments for multiple comparisons would increase the Type 2 error rate (Perneger, 1998), and consequently have not been performed.
To evaluate mean differences in demographic and medical history characteristics between the HC and AD groups, independent t-tests were conducted for continuous data, and chi-square ($\chi^2$) analyses were conducted for categorical data.

When investigating differences in irisin levels within categorical variables (e.g. HC versus AD, Male versus Female, BMI < 25 versus BMI > 25, etc.) and presence or absence of particular medical conditions (i.e. with or without diabetes mellitus), analyses of variance (ANOVA) and analyses of covariance (ANCOVA) were conducted. Further, these analyses were re-run following stratification of the cohort by gender and $APOE \varepsilon 4$ allele carriage. $F$- and $p$-values were reported from the ANOVAs and ANCOVAs. Crude $p$-values were listed and represent unadjusted $p$-values (from ANOVAs). Adjusted $p$-values were calculated, covarying for age, gender and $APOE \varepsilon 4$ carrier status, where appropriate (i.e. gender removed from models when investigating gender-stratified cohort).

In the HC cohort only, I evaluated the relationship between irisin levels and cognitive function (cognitive composite scores) using a series of linear regression models. In these models, the cognitive composite score was entered as the dependent variable, and irisin entered as the independent variable, with the inclusion of age, gender and $APOE \varepsilon 4$ allele carriage as covariates in the model. These models were also re-run following the stratification of the cohort by $APOE \varepsilon 4$ allele carriage (age and gender as covariates) and gender (age and $APOE \varepsilon 4$ allele carriage as covariates).

In the combined HC and AD cohort, partial correlations (adjusting for age, gender and $APOE \varepsilon 4$ allele carriage) were used to explore the relationship between serum irisin levels
and blood biomarkers that have been previously associated with AD risk. These correlational analyses were re-run following stratification of the cohort by clinical classification (HC and AD) and gender.

3.2.5 Statistical Analyses: Longitudinal data

To evaluate changes in irisin from baseline to 18 months, repeated measures ANOVAs and ANCOVAs were conducted. The Greenhouse-Geisser correction was applied to correct for violation of sphericity of the data. In order to investigate whether there was an association between changes in irisin levels over 18 months and changes in cognitive composite scores over the same time period, change (Δ) of each variable was calculated. Linear regression models were conducted to evaluate the relationship between Δ-cognitive variables (entered as dependent variables into separate models), and the Δ-irisin variable (entered as independent variable), with the inclusion of age, gender and APOE ε4 allele carriage as covariates. These linear regressions were re-run following stratification of the cohort by gender.
3.3 Results

3.3.1 Irisin levels vary in human serum of older adults

Irisin concentrations ranged between 6 and 26 ng/ml (please see 3.2.3 for details on outlier windsorizing). A typical result from a single plate is depicted in Figure 3.1 below.

Figure 3.1: Typical results from the ELISA detection of irisin. Irisin concentrations were usually observed at physiological concentrations of approximately 10 ng/ml, with some outliers reaching greater than 20 ng/ml. The error bars provide an indication of the standard deviation observed between duplicates.
3.3.2 Baseline descriptive statistics of the AIBL cohort

The studied cohort was comprised of 201 HC s, and 92 participants clinically classified as AD (Table 3.1). The average age of the cohort was 75.6 ± 7.2 years, and 58% were females. The majority of participants were classified as overweight (62.1% with a BMI > 25), and nearly 40% of all participants were carriers of the APOE ε4 allele.

When stratifying the group by clinical classification (i.e. HC versus AD), the AD participants were on average seven years older than the HC group (80.5 ± 7.7 years versus 73.4 ± 5.8 years; \( t = -7.9, p < 0.001 \)). Additionally, over 70% of ADs carried the APOE ε4 allele, compared to 25.4% of the HC group (\( \chi^2 = 54.1, p < 0.0001 \)). Self-reported depressive symptoms, measured via the GDS, were significantly higher in the AD cohort (\( \chi^2 = 14.2, p < 0.0001 \)). As expected, a significantly higher proportion of individuals had high levels of brain beta-amyloid (Aβ+; indicated by a standardised uptake value ratio of greater than 1.4) in the AD group (88.5%), compared with the HC group (18.2%; \( \chi^2 = 36.12, p < 0.0001 \)).
Chapter 3 – Results I: The Relationship between Serum Irisin Levels, Cognition and Alzheimer’s disease-Related Blood Biomarkers: Cross-Sectional and Longitudinal Investigations

**Table 3.1: Characteristics of the total cohort, and following stratification by cognitively healthy controls and participants with Alzheimer’s disease**

<table>
<thead>
<tr>
<th></th>
<th>Total (n = 293)</th>
<th>HC (n = 201)</th>
<th>AD (n = 92)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean ± SD</td>
<td>75.6 ± 7.2</td>
<td>73.4 ± 5.8</td>
<td>80.5 ± 7.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gender, female, % (n)</td>
<td>58.4 (171)</td>
<td>57.2 (115)</td>
<td>60.9 (56)</td>
<td>0.56</td>
</tr>
<tr>
<td>Years of education, % &gt; 12 years (n)</td>
<td>50.3 (148)</td>
<td>50.2 (101)</td>
<td>51.1 (47)</td>
<td>0.89</td>
</tr>
<tr>
<td>Standard alcohol consumption per day, mean ± SD</td>
<td>0.5 ± 1.0</td>
<td>0.6 ± 1.1</td>
<td>0.3 ± 0.7</td>
<td><strong>0.004</strong></td>
</tr>
<tr>
<td>APOE ε4 carriers, % (n)</td>
<td>39.6 (116)</td>
<td>25.4 (51)</td>
<td>70.6 (65)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI count, n</td>
<td>227</td>
<td>189</td>
<td>38³</td>
<td></td>
</tr>
<tr>
<td>BMI &lt; 25, % (n)</td>
<td>86 (37.9)</td>
<td>72 (38.1)</td>
<td>14 (36.8)</td>
<td>0.76</td>
</tr>
<tr>
<td>PET count, n</td>
<td>81</td>
<td>55</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Aβ+, % (n)</td>
<td>40.7 (33)</td>
<td>18.2 (10)</td>
<td>88.5 (23)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GDS count, n</td>
<td>202</td>
<td>156</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>GDS, mean ± SD</td>
<td>1.6 ± 2.1</td>
<td>1.2 ± 1.7</td>
<td>3.0 ± 2.7</td>
<td></td>
</tr>
</tbody>
</table>

Data presented as mean ± standard deviation, unless otherwise stated. **Count** refers to the number of participants with available data for each variable. p-values determined by independent sample *t*-tests for continuous variables and chi-square for categorical data. ³High level of missing data due to AD patients being assessed at home. Abbreviations: Aβ+, brain amyloid higher than or equal to 1.4 SUVR (standardised uptake value ratio); AD, Alzheimer’s disease; APOE, Apolipoprotein E; BMI, Body Mass Index (kg/height(m)²); GDS, Geriatric Depression Scale; HC, cognitively healthy controls; PET, positron emission tomography to determine beta-amyloid burden; SD, standard deviation.
Medical history and basic physiological measures that may be of relevance to AD risk are summarized in Table 3.2 below. Higher levels of systolic blood pressure ($t = -3.7, p < 0.0001$), and more frequent clinically diagnosed depression ($\chi^2 = 12.2, p < 0.0001$) were observed in AD participants, compared to HCs. Hypertension and clinically diagnosed depression are of particular interest as they have been previously associated with AD (Diniz, Butters, Albert, Dew, & Reynolds, 2013; Hughes & Sink, 2016).
**Table 3.2: Self-reported medical history and basic vital signs of cognitively healthy subjects and participants with Alzheimer’s disease**

<table>
<thead>
<tr>
<th>Medical history / Physiological measures</th>
<th>Total</th>
<th>HC</th>
<th>AD</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension Diagnosed, % (n)</td>
<td>36 (94)</td>
<td>35.9 (71)</td>
<td>36.5 (23)</td>
<td>0.93</td>
</tr>
<tr>
<td>Heart attack Diagnosed, % (n)</td>
<td>3.1 (8)</td>
<td>3.0 (6)</td>
<td>3.1 (2)</td>
<td>0.97</td>
</tr>
<tr>
<td>Serious head injury Diagnosed, % (n)</td>
<td>3.1 (8)</td>
<td>2.0 (4)</td>
<td>6.3 (4)</td>
<td>0.08</td>
</tr>
<tr>
<td>Thyroid dysfunction Diagnosed, % (n)</td>
<td>9.9 (26)</td>
<td>10.6 (21)</td>
<td>7.8 (5)</td>
<td>0.52</td>
</tr>
<tr>
<td>Type 2 Diabetes Diagnosed, % (n)</td>
<td>6.9 (18)</td>
<td>5.6 (11)</td>
<td>11.1 (7)</td>
<td>0.13</td>
</tr>
<tr>
<td>Depression Diagnosed, % (n)</td>
<td>11.6 (30)</td>
<td>7.7 (15)</td>
<td>23.8 (15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sys BP (mmHg) Mean ± SD</td>
<td>130.4 ± 18.6</td>
<td>128.0 ± 18.2</td>
<td>138.3 ± 18.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dia BP (mmHg) Mean ± SD</td>
<td>76.5 ± 10.3</td>
<td>75.8 ± 10.1</td>
<td>79.0 ± 10.4</td>
<td>0.04</td>
</tr>
<tr>
<td>Heart rate Mean ± SD</td>
<td>70.1 ± 12.5</td>
<td>69.64 ± 11.9</td>
<td>71.61 ± 14.0</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Medical history data was not complete for the entire cohort. The \( n \) varied for each medical history measure with maximum missing data of \( n = 34 \). Blood pressure data was missing for \( n = 43 \). \( p \)-values determined by independent samples \( t \)-tests for continuous variables and chi-square analyses for categorical data. A \( p \)-value under 0.05 was deemed significant (bold). Abbreviations: AD, Alzheimer’s disease; Dia BP, Diastolic blood pressure; HC, cognitively healthy controls; SD, standard deviation; Sys BP, Systolic blood pressure.
Chapter 3 – Results I: The Relationship between Serum Irisin Levels, Cognition and Alzheimer’s disease-Related Blood Biomarkers: Cross-Sectional and Longitudinal Investigations

3.3.3 Baseline irisin levels within a cohort of cognitively healthy older adults and people with Alzheimer’s disease

Previous studies have investigated levels of irisin in both serum and plasma samples. However, the majority of these investigations were conducted on small sample sizes of between 6 and 40 participants. Larger sample sizes of 75 to 122 are available, but are mostly restricted to young or middle-aged adults (Anastasilakis et al., 2014; Ates, Altay, Topcuoglu, & Yilmaz, 2015; Daskalopoulou et al., 2014; Hecksteden et al., 2013; Huh et al., 2012; Norheim et al., 2014; Reinehr, Elfers, Lass, & Roth, 2015; Staiger et al., 2013; Stengel et al., 2013; Tsuchiya et al., 2014; Tsuchiya, Ando, Takamatsu, & Goto, 2015), and/or lack the extensive characterization present in the AIBL study cohort; e.g., neuropsychological test battery data, brain imaging, APOE genotyping etc. One published study did draw from a 967 strong participant pool, analyzing samples from individuals in their 50’s for irisin levels. However, the assay used in this study was derived from a commercially available un-validated kit (Oelmann, Nauck, Volzke, Bahls, & Friedrich, 2016).

In this thesis, I investigated serum irisin levels for the first time in a large older adult cohort in relation to extensive demographical and medical characteristics. Moreover, no previous investigation has examined the relationship between irisin and brain Aβ levels.

3.3.3.1 The relationship between irisin levels, demographic characteristics and medical history

Table 3.3 details serum irisin levels with regards to clinical classification (i.e. HC vs AD), age group, gender, APOE ε4 allele carriage, education, BMI and brain Aβ load. Following
adjustment for gender and APOE ε4 allele carriage, a significant difference in irisin levels was observed between those aged 60-75 (11.59 ± 2.92 ng/ml) compared with those aged 75 years and over (10.82 ± 2.82 ng/ml; \( F(1,289) = 4.7, p = 0.031 \)). This categorisation of age was employed for several reasons; many epidemiological studies report incidences of physical activity (a particular focus of this thesis), morbidity and mortality within categorised age groups, frequently with the groupings beginning and ending in mid-decades, e.g. 70-74, 75-79. Moreover, both the Australian Institute of Health and Welfare, and the Australian Department of Health publish reports on physical activity level recommendations and medical conditions in older adults, using the demarcation of “aged 75 years and over” (The Department of Health, 2017; Australian Institute of Health and Welfare, 2017). Furthermore, the mean age of the utilised AIBL cohort is 75.65 years, thereby ensuring a convenient split of the associated data with the implemented age categories.

In addition to the age-related difference in irisin described above, trend level differences in irisin concentrations were observed between the HC and AD groups \( (F(1,291) = 3.70, \ p = 0.06) \) and APOE ε4 allele carriers and non-carriers \( (F(1,291) = 2.92, \ p = 0.09) \); nevertheless, these trend level differences did not remain following adjustment for important covariates.

Interestingly, while I did not observe differences between the HC and AD groups with respect to medical history associated with AD-risk (excepting depression; as shown in Table 3.2), across the total cohort (AD + HC together), serum irisin levels did differ depending on type 2 diabetes mellitus status. Lower mean irisin levels were observed in individuals with type 2 diabetes mellitus \( (9.87 ± 2.79 \text{ ng/ml}) \), compared to those without
the disease (11.36 ± 2.91 ng/ml; \( t = 2.09 \ p = 0.04 \)). The level of significance did not change following adjustment for age, gender and \( APOE \ \varepsilon4 \) allele carriage (Table 3.4; non-significant relationships not shown).
Table 3.3: Irisin levels across various demographic groupings

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Serum irisin, ng/ml</th>
<th>(P_{\text{crude}})</th>
<th>(P_{\text{adjusted}})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HC</td>
<td>201</td>
<td>11.44 ± 3.01</td>
<td>0.06</td>
<td>0.56(^a)</td>
</tr>
<tr>
<td>AD</td>
<td>92</td>
<td>10.75 ± 2.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &lt; 75</td>
<td>153</td>
<td>11.59 ± 2.92</td>
<td>0.02</td>
<td>0.03(^b)</td>
</tr>
<tr>
<td>Age ≥ 75</td>
<td>140</td>
<td>10.82 ± 2.82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>122</td>
<td>11.08 ± 2.85</td>
<td>0.46</td>
<td>0.51(^c)</td>
</tr>
<tr>
<td>Female</td>
<td>171</td>
<td>11.33 ± 2.93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOE ε4-</td>
<td>177</td>
<td>11.46 ± 2.93</td>
<td>0.09</td>
<td>0.15(^d)</td>
</tr>
<tr>
<td>APOE ε4+</td>
<td>116</td>
<td>10.87 ± 2.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YOE &lt; 12</td>
<td>145</td>
<td>11.49 ± 2.91</td>
<td>0.12</td>
<td>0.12(^a)</td>
</tr>
<tr>
<td>YOE ≥ 12</td>
<td>148</td>
<td>10.97 ± 2.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI &lt; 25</td>
<td>86</td>
<td>11.54 ± 3.05</td>
<td>0.28</td>
<td>0.26(^a)</td>
</tr>
<tr>
<td>BMI ≥ 25</td>
<td>141</td>
<td>11.33 ± 2.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aβ⁻</td>
<td>48</td>
<td>11.00 ± 2.72</td>
<td>0.57</td>
<td>0.85(^a)</td>
</tr>
<tr>
<td>Aβ⁺</td>
<td>33</td>
<td>10.62 ± 3.33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(P_{\text{crude}}\), \(p\)-value from analysis of variance without adjustment for covariates; \(P_{\text{adjusted}}\), \(p\)-value from analysis of covariance, adjusting for covariates; \(^a\)\(p\)-value adjusted for age, gender, and APOE ε4 allele carriage; \(^b\)\(p\)-value adjusted for gender and APOE ε4 allele carriage; \(^c\)\(p\)-value adjusted for age and APOE ε4 allele carriage; \(^d\)\(p\)-value adjusted for gender and age. A \(p\)-value < 0.05 was deemed significant (bold). Abbreviations: AD, Alzheimer’s disease; Aβ⁺, high beta-amyloid load, defined by a standardised uptake value ratio ≥ 1.4; Aβ⁻, low beta-amyloid load, defined by a standardised uptake value ratio < 1.4; APOE ε4⁺, carriers of the apolipoprotein E ε4 allele; APOE ε4⁻, non-carriers of the apolipoprotein E ε4 allele; BMI, Body mass index (kg/height(m)²); HC, healthy controls; SD, standard deviation; YOE, Years of Education.
Table 3.4: Irisin levels in those with medical conditions that have been previously associated with AD risk

<table>
<thead>
<tr>
<th></th>
<th>Serum irisin, ng/ml</th>
<th>$P_{\text{crude}}$</th>
<th>$P_{\text{adjusted}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Type 2 DM</td>
<td>242</td>
<td>11.36 ± 2.91</td>
<td>0.037</td>
</tr>
<tr>
<td>Type 2 DM</td>
<td>18</td>
<td>9.87 ± 2.79</td>
<td>0.037*</td>
</tr>
</tbody>
</table>

$P_{\text{crude}}$, $p$-value from analysis of variance without adjustment for covariates; $P_{\text{adjusted}}$, $p$-value from analysis of covariance, adjusting for age, gender, and $APOE$ ε4 allele carriage. A $p$-value < 0.05 was deemed significant. Abbreviations: AD, Alzheimer’s disease; $APOE$, apolipoprotein E; DM, diabetes mellitus; SD, standard deviation.
3.3.3.2 Differences in irisin levels between cognitively healthy controls and Alzheimer’s disease participants, following stratification of the cohort by gender and APOE ε4 allele carriage

As described above, a trend towards higher levels of irisin in HCs (11.44 ± 3.01 ng/ml) compared to AD patients (10.75 ± 2.57 ng/ml; \( t = 1.9; p = 0.055 \)) was observed (Table 3.3, Figure 3.2a). Following adjustment for age, gender, and APOE ε4 allele carriage, this trend was abrogated \((F(1,288) = 0.32, p = 0.57)\). However, following stratification of the cohort by gender, a significant difference in irisin levels was observed between HC and AD females \((F(1,169) = 4.28, p = 0.04; \text{Figure } 3.2b, \text{Table } 3.5)\). Mean irisin levels in HC females were 11.65 ± 3.04 ng/ml, as compared to 10.67 ± 2.59 ng/ml in female AD participants.

Interestingly, the difference in irisin levels between age groupings (< 75 vs. ≥ 75 years) reported above, did not remain when the cohort was stratified by APOE ε4 allele carriage or gender; although trend level associations were seen in APOE ε4 non-carriers and females \((p = 0.07 \text{ and } p = 0.08, \text{ respectively})\).

Following stratification of the cohort by gender, irisin levels in males were also impacted by the presence of the APOE ε4 allele, although this relationship was no longer significant following adjustment for covariates \((p = 0.045 \text{ and } p = 0.06, \text{ respectively})\).
Figure 3.2: Irisin levels in cognitively healthy controls and individuals with Alzheimer’s disease. **a)** Irisin levels were compared between HC and ADs in the whole cohort; **b)** Irisin levels in HC and ADs were compared following stratification of the cohort by gender. \( *p < 0.05 \) (adjusted for age and apolipoprotein E ε4 allele carriage). Abbreviations: AD, Alzheimer’s disease; HC, healthy controls.
Table 3.5: The relationship between serum irisin levels and factors associated with Alzheimer’s disease risk in the entire study cohort, and following stratification of the cohort by gender and APOE ε4 allele carriage

<table>
<thead>
<tr>
<th>Classification (AD / HC)</th>
<th>Total cohort (n = 293)</th>
<th>APOE ε4 carriers (n = 116)</th>
<th>APOE ε4 non-carriers (n = 177)</th>
<th>Male (n = 122)</th>
<th>Female (n = 171)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>p</td>
<td>F</td>
<td>p</td>
<td>F</td>
</tr>
<tr>
<td>Irisin</td>
<td>3.70</td>
<td>0.055</td>
<td>0.03</td>
<td>0.86</td>
<td>4.01</td>
</tr>
<tr>
<td>Irisin¹</td>
<td>0.32</td>
<td>0.57</td>
<td>0.35</td>
<td>0.55</td>
<td>2.08</td>
</tr>
<tr>
<td>Age (&lt; 75 / ≥ 75)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irisin</td>
<td>5.30</td>
<td>0.022</td>
<td>3.27</td>
<td>0.07</td>
<td>1.41</td>
</tr>
<tr>
<td>Irisin¹</td>
<td>4.68</td>
<td>0.031</td>
<td>3.26</td>
<td>0.07</td>
<td>1.58</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irisin</td>
<td>0.54</td>
<td>0.46</td>
<td>1.00</td>
<td>0.76</td>
<td>1.76</td>
</tr>
<tr>
<td>Irisin¹</td>
<td>0.44</td>
<td>0.51</td>
<td>0.08</td>
<td>0.77</td>
<td>2.14</td>
</tr>
<tr>
<td>APOE ε4 allele carriage (+/-)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irisin</td>
<td>2.92</td>
<td>0.09</td>
<td></td>
<td></td>
<td>4.12</td>
</tr>
<tr>
<td>Irisin¹</td>
<td>1.96</td>
<td>0.16</td>
<td></td>
<td></td>
<td>3.53</td>
</tr>
<tr>
<td>Brain beta-amyloid (Aβ+ / Aβ-)²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irisin</td>
<td>0.32</td>
<td>0.57</td>
<td>0.15</td>
<td>0.71</td>
<td>0.11</td>
</tr>
<tr>
<td>Irisin¹</td>
<td>0.04</td>
<td>0.85</td>
<td>0.51</td>
<td>0.48</td>
<td>0.09</td>
</tr>
</tbody>
</table>

One way analysis of variance (ANOVA) was utilised to investigate differences in irisin levels across categorical characteristics. These analyses were repeated following stratification of the cohort by APOE ε4 allele carriage and gender. Rows: ‘Irisin’, unadjusted for covariates; ‘Irisin¹’, model adjusted for age, with or without gender and APOE ε4 carriage depending on cohort stratification; e.g. age and gender where APOE ε4 stratification exists, or age and APOE ε4 where gender stratification exists. ²n for brain imaging = 81. Abbreviations: Aβ+, high beta-amyloid load, defined by a standardised uptake value ratio ≥ 1.4; Aβ-, low beta-amyloid load, defined by a standardised uptake value ratio < 1.4; AD, Alzheimer’s disease; APOE, Apolipoprotein E; HC, healthy controls; SD, standard deviation.
3.3.4 Baseline descriptive characteristics of the cognitively healthy control sub-cohort

Individuals diagnosed with AD often have a number of comorbidities that are likely to influence irisin levels, such as, loss of body mass due to reductions in muscle and fat tissue (Burns, Johnson, Watts, Swerdlow, & Brooks, 2010; Gu et al., 2014), or loss of nerve cells and brain tissue. Depending on diagnosed disease stage (mild, moderate or advance AD), irisin levels may be difficult to interpret in relation to cognitive health. Therefore, the investigation of irisin as a potential biomarker for the early detection of declining brain health (i.e. the preclinical disease stage, before clinical AD diagnosis is possible) should be undertaken in a cognitively healthy cohort. Thus, the AD participants were removed from the dataset and analyses of the relationship between irisin, cognition and additional blood-based analytes were undertaken on only the HC participants ($n = 201$).

The characteristics of the HC sub-cohort are described in Table 3.6, both without and with stratification for $APOE \varepsilon 4$ allele carriage (the greatest genetic risk factor for sporadic AD). The HC sub-cohort had a mean age of $73.1 \pm 7.6$ years, most were female (57%), overweight (BMI ≥ 25; 62%), 50% were highly educated, whilst 18% had significant brain Aβ load. The majority of this HC cohort were $\varepsilon 4$ non-carriers ($n = 150$, 75%). Following stratification of the group on the basis of $APOE \varepsilon 4$ allele carriage, the $\varepsilon 4$ carriers were younger, contained fewer females (45% vs. 61% of non-carriers), and unsurprisingly a higher percentage of Aβ+ individuals (38% vs. 6% of non-carriers).
### Table 3.6: Characteristics of cognitively healthy controls of the AIBL cohort, and following stratification for APOE ε4 allele carriage

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>APOE ε4 non-carriers</th>
<th>APOE ε4 carriers</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 201)</td>
<td>(n = 150)</td>
<td>(n = 51)</td>
<td></td>
</tr>
<tr>
<td>Age (years), mean ± SD</td>
<td>73.1 ± 7.6</td>
<td>73.9 ± 6.0</td>
<td>72.9 ± 4.7</td>
<td>0.02*</td>
</tr>
<tr>
<td>Gender, female, % (n)</td>
<td>57.2 (115)</td>
<td>61.3 (92)</td>
<td>45.1 (23)</td>
<td>0.04*</td>
</tr>
<tr>
<td>Years of education, % &gt; 12 years (n)</td>
<td>50.2 (101)</td>
<td>49.3 (74)</td>
<td>52.9 (27)</td>
<td>0.66</td>
</tr>
<tr>
<td>Standard alcohol consumption per day, mean ± SD</td>
<td>0.6 ± 0.9</td>
<td>0.6 ± 0.9</td>
<td>0.75 ± 1.3</td>
<td>0.37</td>
</tr>
<tr>
<td>BMI count, n</td>
<td>189</td>
<td>142</td>
<td>47</td>
<td>0.08</td>
</tr>
<tr>
<td>BMI &lt; 25, % (n)</td>
<td>38.1 (72)</td>
<td>34.5 (49)</td>
<td>48.9 (23)</td>
<td>0.003*</td>
</tr>
<tr>
<td>PET count, n</td>
<td>55</td>
<td>34</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Aβ+, % (n)</td>
<td>18.2 (10)</td>
<td>5.9 (2)</td>
<td>38.1 (8)</td>
<td></td>
</tr>
<tr>
<td>GDS count, n</td>
<td>163</td>
<td>118</td>
<td>38</td>
<td>0.24</td>
</tr>
<tr>
<td>GDS, mean ± SD</td>
<td>1.2 ± 1.7</td>
<td>1.3 ± 1.7</td>
<td>0.95 ± 1.4</td>
<td></td>
</tr>
</tbody>
</table>

Demographic and descriptive data was not complete for the entire cohort. Data presented as mean ± standard deviation, unless otherwise stated. p-values determined by independent sample t-tests for continuous variables and chi-square analyses for categorical data. A p-value < 0.05 was deemed significant (bold). Abbreviations: Aβ+, beta-amyloid positron emission tomography standardised uptake value ratio ≥ 1.4; AIBL, Australian Imaging, Biomarkers and Lifestyle study of ageing; APOE, Apolipoprotein E; BMI, Body mass index (kg/height (m)^2); GDS, Geriatric Depression Scale; PET, positron emission tomography; SD, standard deviation.
3.3.5 The relationship between irisin levels and demographic characteristics in the cognitively healthy control cohort

A summary of irisin levels across demographic characteristics is reported for the HC group only in Table 3.7. Similar to examination of the total cohort (i.e. including both HCs and ADs; Table 3.3), irisin levels were higher in the younger age group (i.e. < 75 years; 11.82 ± 2.89 ng/ml), compared with those 75 years of age and older (10.74 ± 3.13 ng/ml; t = 2.44, p = 0.02); a relationship which remained significant following adjustment for gender and APOE ε4 allele carriage (p = 0.02). In non-carriers of the APOE ε4 allele, irisin levels were significantly higher in those aged < 75 (11.91 ± 2.83 ng/ml), than those aged 75 and over (10.67 ± 3.11 ng/ml; F(1,147) = 6.19, p = 0.01).

In APOE ε4 allele carriers, females had higher mean irisin levels (12.59 ± 3.24 ng/ml), compared with males (10.52 ± 2.70 ng/ml; F(1,48) = 5.75, p = 0.02), whilst those who were overweight (BMI ≥ 25) had lower levels of irisin (10.35 ± 2.88 ng/ml), compared with those in a healthy weight range (12.73 ± 2.75 ng/ml; F(1,43) = 5.23, p = 0.03). The association between irisin levels and presence of medical conditions associated with AD risk was also evaluated in this HC cohort. No significant differences in irisin levels were observed between those with and without the studied medical conditions (data not shown).
Table 3.7: Irisin levels across demographic characteristics in the total cognitively healthy control group, and following stratification for gender and APOE ε4 allele carriage

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Serum Irisin, ng/ml</th>
<th>Total cognitively healthy control group (n = 201)</th>
<th>Male (n = 86)</th>
<th>Female (n = 115)</th>
<th>APOE ε4 non-carriers (n = 150)</th>
<th>APOE ε4 carriers (n = 51)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td>$P_{\text{crude}}$</td>
<td>$P_{\text{adjusted}}$</td>
<td>$P_{\text{adjusted}}$</td>
<td>$P_{\text{adjusted}}$</td>
<td>$P_{\text{adjusted}}$</td>
</tr>
<tr>
<td>Age &lt; 75</td>
<td>131</td>
<td>11.82 ± 2.89</td>
<td>0.02</td>
<td>0.02</td>
<td>0.22</td>
<td>0.052</td>
<td>0.01</td>
</tr>
<tr>
<td>Age ≥ 75</td>
<td>70</td>
<td>10.74 ± 3.13</td>
<td></td>
<td></td>
<td>0.22</td>
<td>0.052</td>
<td>0.01</td>
</tr>
<tr>
<td>Male</td>
<td>86</td>
<td>11.16 ± 2.97</td>
<td>0.26</td>
<td>0.32</td>
<td>N/A</td>
<td>N/A</td>
<td>0.85</td>
</tr>
<tr>
<td>Female</td>
<td>115</td>
<td>11.65 ± 3.04</td>
<td></td>
<td></td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>YOE &lt; 12</td>
<td>100</td>
<td>11.69 ± 3.03</td>
<td>0.25</td>
<td>0.32</td>
<td>0.23</td>
<td>0.98</td>
<td>0.76</td>
</tr>
<tr>
<td>YOE ≥ 12</td>
<td>101</td>
<td>11.20 ± 2.99</td>
<td></td>
<td></td>
<td>0.23</td>
<td>0.98</td>
<td>0.76</td>
</tr>
<tr>
<td>BMI &lt; 25</td>
<td>72</td>
<td>11.81 ± 3.00</td>
<td>0.25</td>
<td>0.38</td>
<td>0.80</td>
<td>0.23</td>
<td>0.65</td>
</tr>
<tr>
<td>BMI ≥ 25</td>
<td>117</td>
<td>11.29 ± 3.06</td>
<td></td>
<td></td>
<td>0.80</td>
<td>0.23</td>
<td>0.65</td>
</tr>
<tr>
<td>Aβ-</td>
<td>45</td>
<td>10.96 ± 2.79</td>
<td>0.84</td>
<td>0.55</td>
<td>0.94</td>
<td>0.18</td>
<td>0.24</td>
</tr>
<tr>
<td>Aβ+</td>
<td>10</td>
<td>11.25 ± 4.31</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$P_{\text{crude}}$, $p$-value from analysis of variance without adjustment for covariates; $P_{\text{adjusted}}$, $p$-value from analysis of covariance, adjusted for covariates; $^a p$-value adjusted for gender and APOE ε4; $^b p$-value adjusted for age and APOE ε4 allele carriage; $^c p$-value adjusted for age, gender and APOE ε4; $^d p$-value adjusted for APOE ε4 allele carriage; $^e p$-value adjusted for gender; $^f p$-value adjusted for age; $^g p$-value adjusted for age and gender. A $p$-value < 0.05 was deemed significant (bold). Abbreviations: Aβ+, Pittsburgh compound B positron emission tomography standardised uptake value ratio ≥ 1.4; Aβ-, Pittsburgh compound B positron emission tomography standardised uptake value ratio < 1.4; APOE, Apolipoprotein E; BMI, Body mass index (kg/height (m)$^2$); YOE, Years of Education; SD, standard deviation.
### 3.3.6 Cross-sectional analysis of irisin and cognition in the cognitively healthy controls

Linear regression analyses were conducted to evaluate the relationship between serum irisin levels and cognitive composite scores (Table 3.8). A significant association was observed between higher irisin levels and better performance on attention tasks ($\beta = 0.71$, $p = 0.009$), yet this association did not survive adjustment for covariates ($\beta = 0.52$, $p = 0.06$). After stratifying the cohort by APOE $\varepsilon$4 allele carrier status, irisin was significantly associated with attention scores in APOE $\varepsilon$4 non-carriers only (adjusting for age and gender; $\beta = 0.80$, $p = 0.02$; Table 3.8). No other significant associations between irisin and cognition were found in the cognitively healthy control group, either with or without stratification by APOE $\varepsilon$4 allele carriage. Following stratification of the HC cohort by gender, higher levels of irisin were associated with better attention scores in females only (adjusted for age and APOE $\varepsilon$4 allele carriage; $\beta = 0.08$, $p = 0.002$; Figure 3.3).
Table 3.8: Association between irisin and cognitive function in the total cognitively healthy control group, and following stratification by APOE ε4 allele carriage

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Total Cohort</th>
<th>APOE ε4 non-carriers (n = 150)</th>
<th>APOE ε4 carriers (n = 51)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>p-value</td>
<td>β</td>
</tr>
<tr>
<td>Attention</td>
<td>0.04</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>(0.0, 0.07)</td>
<td></td>
<td>(0.01, 0.09)</td>
</tr>
<tr>
<td>Episodic Memory</td>
<td>0</td>
<td>0.60</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(-0.04, 0.02)</td>
<td></td>
<td>(-0.03, 0.04)</td>
</tr>
<tr>
<td>Executive Function</td>
<td>0.01</td>
<td>0.69</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>(-0.03, 0.04)</td>
<td></td>
<td>(-0.02, 0.07)</td>
</tr>
<tr>
<td>ADCS</td>
<td>0.01</td>
<td>0.58</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>(-0.02, 0.04)</td>
<td></td>
<td>(-0.01, 0.05)</td>
</tr>
<tr>
<td>Language</td>
<td>0</td>
<td>0.81</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>(-0.04, 0.03)</td>
<td></td>
<td>(-0.03, 0.05)</td>
</tr>
</tbody>
</table>

A p-value < 0.05 was deemed significant (bold). Evaluated by linear regression with age, gender and APOE ε4 allele carrier status entered as covariates into the model when assessing the complete study cohort; and with age and gender entered as covariates into the model after stratifying the cohort by APOE ε4 carrier status. Abbreviations: ADCS, Preclinical Alzheimer’s Disease Cognitive Score; APOE, Apolipoprotein E.
Figure 3.3: Linear regression analysis showed a cross-sectional association between irisin levels and attention composite score in cognitively healthy females, but not in males. Model adjusted for age and Apolipoprotein E ε4 allele carriage; $\beta = 0.08$, $p = 0.002$. 
3.3.7 Longitudinal analysis of irisin and cognition

3.3.7.1 Description of the longitudinal cohort

Eighteen months following the baseline assessment, blood sampling and cognitive assessments were repeated. Of the initial cohort of 293 HCs and ADs, 45 participants (HC, n = 2; AD, n = 43) did not return. Furthermore, 12 participants converted from HC to mild cognitive impairment (MCI). Of the participants who returned for follow-up, irisin was not measured in the blood samples of 19 of these individuals. Thus, data from a total of 217 participants are used in the subsequent analyses (HC, n = 182; AD, n = 35: Figure 3.4). A brief exploration of the 12 converters to MCI is conducted later in this chapter (3.3.10).

**Figure 3.4:** Diagram detailing participant numbers available for longitudinal analyses of irisin levels. Abbreviations: AD, Alzheimer’s disease; HC, healthy control; MCI, mild cognitive impairment.
3.3.7.2 Changes in irisin levels over 18 months in cognitively healthy subjects and Alzheimer’s disease patients

Data from the HCs and ADs ($n = 217$) was used to evaluate changes in serum irisin levels over an 18 month period, using repeated measures ANOVA and ANCOVA. The time*classification interaction term was a significant predictor of changes in serum irisin ($F(1, 215) = 6.20, p = 0.014$). In the HC group, serum irisin levels increased by 1% (from $11.47 \pm 3.05$ ng/ml to $11.59 \pm 2.92$ ng/ml). However, in the AD group, serum irisin levels increased by 11% ($10.00 \pm 2.53$ ng/ml to $11.18 \pm 2.88$ ng/ml; $F(1,34) = 9.60, p = 0.004$; Table 3.9). A significant effect of a time*gender interaction was also observed on changes in serum irisin ($F(1, 215) = 4.11, p = 0.044$; Table 3.10), whereby serum irisin levels in females rose by 5% over the follow-up period (from $11.31 \pm 3.08$ to $11.88 \pm 2.88$ ng/ml), and declined in males by 0.7% (from $11.10 \pm 2.94$ to $11.02 \pm 2.91$ ng/ml).
### Table 3.9: Changes in serum irisin levels over 18 months when stratifying the cohort by clinical classification

<table>
<thead>
<tr>
<th></th>
<th>Δ BL-18m time*classification (n = 217)</th>
<th>Δ BL-18m HC (n = 182)</th>
<th>Δ BL-18m AD (n = 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>p</td>
<td>F</td>
</tr>
<tr>
<td>Irisin (Δ)</td>
<td>6.2</td>
<td>0.01*</td>
<td>0.65</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>HC (n = 182)</th>
<th>AD (n = 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>18m</td>
</tr>
<tr>
<td>Irisin, ng/ml</td>
<td>11.47 ± 3.05</td>
<td>11.59 ± 2.92</td>
</tr>
</tbody>
</table>

Longitudinal analysis of change in irisin over 18 months in the HC and AD groups. *p*-value < 0.05; **p*-value < 0.01; Δ, change variable. Test includes Greenhouse-Geisser correction. Abbreviations: 18m, 18 month follow-up timepoint; AD, Alzheimer’s disease; BL, Baseline; HC, healthy controls.

### Table 3.10: Changes in serum irisin levels over 18 months when stratifying the cohort by gender

<table>
<thead>
<tr>
<th></th>
<th>Δ BL-18m time*gender (n = 217)</th>
<th>Δ BL-18m Males (n = 89)</th>
<th>Δ BL-18m Females (n = 128)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>p</td>
<td>F</td>
</tr>
<tr>
<td>Irisin (Δ)</td>
<td>4.11</td>
<td>0.04*</td>
<td>0.14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Males (n = 89)</th>
<th>Females (n = 128)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline 18m</td>
<td>Baseline 18m</td>
</tr>
<tr>
<td>Irisin, ng/ml</td>
<td>11.10 ± 2.94</td>
<td>11.02 ± 2.91</td>
</tr>
</tbody>
</table>

Longitudinal analysis of change in irisin over 18 months following stratification of the cohort by gender. *p*-value < 0.05; **p*-value < 0.01; Δ, change variable. Abbreviations: 18m, 18 month follow-up time-point; BL, Baseline.
3.3.7.3 The relationship between changes in irisin levels and changes in cognitive function over the 18 month follow-up period

When examining the HC group only \((n = 182)\), increases in serum irisin levels across the follow-up period were associated with increases in executive functioning composite score \((\beta = 0.05, p < 0.05; \text{Table 3.11})\). After stratifying the cohort by gender, no associations were observed between changes in serum irisin and changes in cognition over the follow-up period (Table 3.12).

**Table 3.11**: Longitudinal analysis of changes in irisin levels and cognitive performance from baseline to 18-month follow-up in cognitively healthy controls

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Δ BL-18m HC ((n = 182))</th>
<th>(\beta)</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attention ((\Delta))</td>
<td>0.00002 (0, 0)</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>Episodic Memory ((\Delta))</td>
<td>0.01 (-0.02, 0.04)</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>Executive Function ((\Delta))</td>
<td>0.05 (0.00, 0.09)</td>
<td><strong>0.031</strong></td>
<td></td>
</tr>
<tr>
<td>ADCS ((\Delta))</td>
<td>0.004 (-0.02, 0.03)</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>Language ((\Delta))</td>
<td>-0.008 (-0.04, 0.02)</td>
<td>0.63</td>
<td></td>
</tr>
</tbody>
</table>

\(\Delta\), change variable. A series of linear regression models were used to evaluate the relationship between \(\Delta\)-irisin and \(\Delta\)-cognitive composite scores over an 18 month period. Abbreviations: 18m, 18 month follow-up time-point; ADCS, Preclinical Alzheimer’s Disease Composite Score; BL, Baseline; HC, Healthy Control.
Table 3.12: Longitudinal analysis of changes in irisin levels and cognitive performance from baseline to 18-month follow-up in the cognitively healthy control cohort following stratification by gender

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Δ BL-18m Males (n = 78)</th>
<th>Δ BL-18m Females (n = 104)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>p</td>
</tr>
<tr>
<td>Attention (Δ)</td>
<td>0.00 (0, 0)</td>
<td>0.89</td>
</tr>
<tr>
<td>Episodic Memory (Δ)</td>
<td>0.01 (-0.04, 0.06)</td>
<td>0.66</td>
</tr>
<tr>
<td>Executive Function (Δ)</td>
<td>0.07 (-0.02, 0.16)</td>
<td>0.17</td>
</tr>
<tr>
<td>ADCS (Δ)</td>
<td>0.01 (-0.03, 0.06)</td>
<td>0.56</td>
</tr>
<tr>
<td>Language (Δ)</td>
<td>-0.03 (-0.09, 0.02)</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Δ, change variable. Following stratification of the healthy control cohort by gender, a series of linear regression models were used to evaluate the relationship between Δ-irisin and Δ-cognitive composite scores over an 18 month period. Available n varied for each cognitive score, with the maximum missing n of 4 males and 10 females. Abbreviations: 18m, 18 month follow-up time-point; ADCS, Preclinical Alzheimer’s Disease Composite Score; BL, Baseline.
3.3.8 Cross-sectional analysis of irisin and blood biomarkers at baseline

Exploratory correlational analyses were conducted in order to evaluate the relationship between serum irisin levels and blood biomarkers that have previously been associated with AD risk (Table 3.13). Results from partial correlations (corrected for age, gender and APOE ε4 allele carriage) demonstrated irisin levels were significantly correlated with cholesterol ($r = 0.18, p < 0.01$), low-density lipoprotein (LDL; $r = 0.19, p < 0.001$), and bilirubin levels ($r = 0.12, p < 0.05$) across the total cohort (HC + AD). In addition, irisin was inversely correlated with calcium ($r = -0.13, p < 0.05$). No significant relationships were observed between irisin levels and thyroid hormones, glucose or insulin (Table 3.13).

Following stratification of the cohort by gender, irisin was significantly associated with levels of cholesterol ($r = 0.22, p < 0.01$), LDL ($r = 0.25, p = 0.001$), bilirubin ($r = 0.15, p = 0.049$), and inversely with calcium ($r = -0.19, p = 0.015$), in females only. Following stratification of the cohort by clinical classification, irisin was associated with cholesterol ($r = 0.18, p < 0.05$), LDL ($r = 0.19, p = 0.008$), and lower levels of calcium ($r = -0.14, p = 0.04$) in HCs only.

Interestingly, when the cohort was stratified by ‘clinically healthy’ and ‘clinically high’ levels of cholesterol, the significant correlation between irisin and cholesterol levels remained in both groups: ‘healthy total cholesterol’, $r = 0.16, p = 0.039$, $n = 165$, and ‘high total cholesterol’ $r = 0.22, p = 0.013$, $n = 126$ (data not shown).
Table 3.13: Cross-sectional correlations between irisin and selected blood-based biomarkers in the entire cohort at baseline, and following stratification by gender and clinical classification

<table>
<thead>
<tr>
<th>Lipids</th>
<th>*Total cohort (n = 287)</th>
<th>bMale (n = 118)</th>
<th>bFemale (n = 166)</th>
<th>*HC (n = 196)</th>
<th>*AD (n = 86)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>0.18**</td>
<td>0.11</td>
<td>0.22**</td>
<td>0.18*</td>
<td>0.19</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.04</td>
<td>-0.01</td>
<td>0.06</td>
<td>0.03</td>
<td>0.07</td>
</tr>
<tr>
<td>HDL</td>
<td>0.03</td>
<td>0.16</td>
<td>-0.05</td>
<td>0.01</td>
<td>0.07</td>
</tr>
<tr>
<td>LDL</td>
<td>0.19***</td>
<td>0.07</td>
<td>0.25**</td>
<td>0.19**</td>
<td>0.19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hormones</th>
<th>*Total cohort (n = 286)</th>
<th>bMale (n = 118)</th>
<th>bFemale (n = 165)</th>
<th>*HC (n = 195)</th>
<th>*AD (n = 86)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>-0.05</td>
<td>0.05</td>
<td>-0.11</td>
<td>-0.01</td>
<td>-0.10</td>
</tr>
<tr>
<td>FT4</td>
<td>&lt; 0.01</td>
<td>0.04</td>
<td>-0.02</td>
<td>-0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>TSH</td>
<td>0.02</td>
<td>0.11</td>
<td>-0.03</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td>FT3</td>
<td>0.04</td>
<td>0.01</td>
<td>0.06</td>
<td>0.07</td>
<td>0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other</th>
<th>*Total cohort (n = 288)</th>
<th>bMale (n = 118)</th>
<th>bFemale (n = 166)</th>
<th>*HC (n = 196)</th>
<th>*AD (n = 86)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>-1.0</td>
<td>-0.09</td>
<td>-0.11</td>
<td>-0.08</td>
<td>-0.10</td>
</tr>
<tr>
<td>Calcium</td>
<td>-0.13*</td>
<td>-0.05</td>
<td>-0.19*</td>
<td>-0.14*</td>
<td>0.02</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>0.12*</td>
<td>0.09</td>
<td>0.15*</td>
<td>0.12</td>
<td>0.15</td>
</tr>
</tbody>
</table>

*r* values shown. *a*Partial correlations were adjusted for age, gender and *APOE ε4* allele carrier status; *b*Partial correlations were adjusted for age and *APOE ε4* allele carrier status.

Abbreviations: AD, Alzheimer’s disease; FT3, Triiodothyronine; FT4, Free thyroxine; HC, healthy controls; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TSH, Thyroid-Stimulating Hormone.
3.3.9 Analysis of participants that converted from HC to MCI within 18 months

Twelve participants converted from being classified as HC at baseline to MCI at 18-month follow-up. Of these 12 participants, irisin was only assessed at both timepoints in \( n = 5 \). Although the sample size was particularly small, I conducted an analysis to examine whether there was any statistically significant change in irisin, within this group of converters. Irisin levels in those that converted from HC to MCI over the 18-month period did not significantly change (Baseline: 11.23 ± 3.01 ng/ml; 18m follow up: 11.67 ± 2.40 ng/ml; \( t = -0.60, p = 0.95 \)).
3.4 Discussion

This Chapter examined the relationship between levels of serum irisin and demographic characteristics, measures associated with cognitive health, and blood biomarkers in a cohort of older men and women. Specifically, the aims of this Chapter were to evaluate: 1) differences in serum irisin levels between those classified as HC and AD, 2) the association between irisin levels and factors associated with AD risk, 3) the relationship between cognitive function and irisin levels in the HC sub-cohort, and 4) the relationship between irisin and a number of blood-based biomarkers that have previously been associated with AD risk. Although the majority of the analyses were cross-sectional, some longitudinal analyses were also performed. The main findings from this Chapter were: 1) an association between increasing age and lower irisin levels, 2) higher irisin levels in older adults without a diagnosis of type 2 diabetes mellitus, 3) no differences in irisin levels between HC and AD groups overall; yet a difference was observed when evaluating females only and in non-carriers of the APOE ε4 allele, 4) higher irisin levels were found in overweight (BMI > 25) APOE ε4 non-carriers and normal weight (BMI < 25) APOE ε4 carriers, 5) a relationship between irisin levels and performance on tasks of attention in non-carriers of the APOE ε4 allele, and 6) irisin correlates with cholesterol, LDL, calcium and bilirubin.

To my knowledge, this is the first study to investigate differences in irisin levels between a group of cognitively healthy older adults and individuals with AD. As discussed in Chapter 1, the need to identify biomarkers of AD is vital, and irisin represents an important target that warrants investigation. Previous research has indicated that lower irisin levels are associated with obesity and the onset of type 2 diabetes (factors which increase AD risk) (Choi et al., 2013; Liu et al., 2013; Moreno-Navarrete et al., 2013).
Consistent with this literature, I observed lower irisin levels in older adults with a diagnosis of type 2 diabetes mellitus (of note, participants developed the condition within five to seven years of the baseline blood draw, and duration of such a medical condition would likely impact results). Due to the strong links between AD, obesity and type 2 diabetes mellitus, I hypothesised that those with AD would have lower levels of irisin, compared with the cognitively healthy group. I observed a trend towards lower irisin levels in the AD group, compared with the HC group; however, this difference was no longer evident following adjustment for age, gender and APOE ε4 allele carriage. The lack of difference between the HC and AD groups in this study may be due to sample size, specifically the AD group was markedly smaller (n = 92), compared to the cognitively healthy control group (n = 201), which may indicate a lack of power to detect significant differences. Alternatively, the results presented here may indeed reflect the notion that circulating irisin levels are not associated with AD neuropathology. Nevertheless, it is important to note that I observed a significant effect of the time*classification interaction on changes in irisin levels across an 18-month period; whereby irisin levels in the AD cohort actually increased, compared with the HC group. This finding was unexpected, as previous work indicates higher levels of irisin reflect a “healthier” outcome. Regardless, these results are preliminary, and further research into this topic is vital, ideally utilising larger sample sizes and a longitudinal study design including follow-up at multiple time-points.

I also observed an effect of age on levels of serum irisin. More specifically, higher levels of irisin were observed in those aged less than 75 years, compared with those aged 75 years or more. This result complements two previous reports, in which lower irisin levels in older adults above 60 years of age were observed when compared to young individuals.
in their 20’s (Huh et al., 2012; Tanisawa et al., 2014). To my knowledge, the current study is the first to report a relationship between age and irisin levels in a cohort of participants over 75 years of age compared to ‘younger older’ participants aged between 60 and 75. While some studies have reported age-dependent irisin levels in young-to-middle aged cohorts (Ates et al., 2015; Huh et al., 2012), no relationship between age and irisin has been reported in younger adults or children (Huh et al., 2012; Reinehr et al., 2015). Irisin levels are known to be associated with skeletal muscle mass; thus, the association between age and irisin could be due to the differing body compositions between the ‘young-old’ and ‘old-old’ groups studied within this Chapter. However, I did not find an association between BMI and irisin levels in this cohort; although, it should be noted that BMI is not an accurate measure of body composition, particularly in older adults (Burkhauser & Cawley, 2008). Future studies should utilise dual-energy X-ray absorptiometry scanning to more effectively evaluate the relationship between irisin and body composition, and the potential role of muscle mass as a mediator in the relationship between circulating irisin levels and age.

To my knowledge, this is the first study to thoroughly evaluate the relationship between serum irisin levels and cognitive function in a cohort of older adults. Here, an association between irisin levels and performance on tasks of attention in cognitively normal non-carriers of the APOE ε4 allele was observed. These findings support and build on those of Belviranli et al., who reported an association between a relatively crude measure of global cognition (Mini Mental State Examination; MMSE) and irisin, in younger male adult athletes (Belviranli et al., 2016). It is somewhat surprising that I only observed a relationship between irisin and attention, and not any other cognitive domain (e.g. episodic memory, executive function). The attention composite is comprised of test
scores from the Digit Symbol, Stroop Dots/Time, and Digit Span. When investigating the relationship between irisin levels and performance on individual tests, it can be seen that processing speed (i.e. digit symbol) appears to be driving the observed relationship. A slowing of processing speed is significantly associated with advancing age (Albinet, Boucard, Bouquet, & Audiffren, 2012; Lu et al., 2013); thus, it is possible that irisin may reflect age-related changes in cognition (i.e. processing speed). Nevertheless, it is important to note that this association remained following adjustment for age; thus, age was unlikely to be the driving factor in this relationship. I also observed that increases in serum irisin levels were associated with better performance on executive function tasks over an 18-month period. It is important to note that although processing speed and executive function are distinct domains, they are related, with processing speed contributing to effective executive functioning (Albinet et al., 2012). The relationship between irisin and multiple cognitive domains cannot be ruled out at this stage, and clearly future work in this field is necessary. Importantly, measures of both irisin and cognition over multiple time-points (i.e. greater than two occasions), and spanning numerous years, would contribute vital evidence towards understanding the relationship between circulating irisin levels, cognitive functioning and risk of cognitive decline.

The relationship between attention and serum irisin levels was observed in non-carriers of the APOE ε4 allele. Although I did not observe a difference in attention scores nor irisin levels between these two genotype groups, these disparate results could possibly be due to the differing action of irisin in APOE ε4 carriers versus non-carriers. As mentioned earlier, it is becoming increasingly apparent that APOE ε4 carriers experience subtle brain changes throughout their lifetime (Michaelson, 2014). Thus, it is likely that pathological changes in older adults need to be addressed and interpreted differently, dependent on
APOE genotype. This would mean that certain changes in blood-based biomarkers might be predictive of AD in non-carriers of the APOE ε4 allele only, and that APOE ε4 carriers need an entirely different biomarker panel to calculate risks of neurological decline. Moreover, an inverse relationship between signs of inflammation and irisin has been demonstrated in several studies (Dong et al., 2015; Tang et al., 2015; Yildiz et al., 2016; Zhang et al., 2016). The exact mechanism of irisin fluctuation in response to inflammation remains unclear. Chronic cerebral or even systemic inflammation is common in older adults; particularly in AD patients with an APOE ε4 allele (Michaelson, 2014). Given the demographic make-up of the studied cohort (i.e. older adults), it is likely a proportion of these subjects were taking anti-inflammatory medications (due to unavailability of medication data this could not be confirmed), which may have had an impact on the level of irisin at a certain point in time. Therefore, irisin levels in APOE ε4 carriers as measured in this study might not represent the actual level comparable to cognitive performance, and could possibly explain my findings being limited to non-carriers of the APOE ε4 allele. Nevertheless, as APOE ε4 carriers represented only a small number of our cognitively healthy cohort (n = 51; ~25%), clearly this association requires investigation in larger study cohorts.

The relationship between irisin and total cholesterol, glucose and insulin has been investigated in numerous study cohorts in recent years. Here, I demonstrated a positive association between irisin levels and cholesterol and LDL. This finding is surprising, as data from this Chapter, and that published by others, suggests that higher irisin levels reflect a “healthier” outcome. However, previous literature examining circulating cholesterol and irisin is equivocal, and has demonstrated both positive and inverse associations. For example, Oelman and colleagues recently observed a strong inverse
association between irisin and total cholesterol and LDL in a large male cohort, but not in females (Oelmann et al., 2016). On the contrary, in healthy, non-diabetic study participants, Liu and colleagues reported significant correlations of irisin with total cholesterol, triglycerides, and fasted blood glucose levels (Liu et al., 2013). It is possible that as yet undetermined use of cholesterol-lowering medication in my cohort (and others previously) may have contributed to the findings reported here.

The results reported within this Chapter should be considered within the context of the study limitations. The nature of our recruitment process for our HC cohort may have selected a larger proportion of high-functioning community-dwelling older adults, and thus the variability in cognitive function may not necessarily reflect that of the wider population. It is also possible that medication use may impact the association between irisin, cognition and blood biomarkers; however, such data was not available at the time of analysis. In addition, as irisin is a relatively newly discovered hormone, factors that are yet to be identified could significantly contribute to basal irisin levels and changes in irisin levels over time. Nevertheless, within this Chapter I rigorously evaluated irisin levels in regards to characteristics associated with cognitive health and AD in older adults; and thus, I believe this information will be vital for informing future studies.

To my knowledge, this is the first study to investigate the relationship between irisin, AD diagnosis and cognitive function. The outcomes here suggest that serum irisin levels do not differ between cognitively healthy older adults and those diagnosed with AD in this well-characterised cohort. However, I report an association between better performance on measures of attention and higher irisin levels in cognitively healthy older adults, which is contingent upon APOE genotype. Clearly, the relationship between serum irisin levels
and cognitive health is complex, and dependent upon consideration of a number of influencing factors. Future studies utilising larger sample sizes, with longer periods of follow-up, are required to validate the findings reported in this Chapter, and to further contribute to the understanding of the role irisin may (or potentially may not) play in reflecting current cognitive health and predicting future cognitive decline.
Chapter Four

The Effect of Physical Activity on Levels of Serum Irisin
4.1 Introduction

There is strong evidence to demonstrate a positive impact of increased physical activity on movability, flexibility and independence in older adults (Bauer, Glenn, Pilhatsch, Pfennig, & Whybrow, 2014; Fukushima et al., 2016; Longhi & Radetti, 2013; Ungar, Altmann, & Greicius, 2014). Thus, increased physical activity is associated with improved quality of life in the elderly (e.g. by maintaining good health, preventing falls and injuries), and reduced risk of numerous medical conditions, including depression, anxiety and cardiovascular disease (Avin et al., 2015; Cadore, Rodriguez-Manas, Sinclair, & Izquierdo, 2013; Gaesser, 2007; Lindwall, Gerber, Jonsdottir, Börjesson, & Ahlborg Jr, 2014; Rosenbaum, Tiedemann, Sherrington, Curtis, & Ward, 2014). In addition, as described in detail in Chapter 1 (Sections 1.3.1 – 1.3.2, pages 35-40), overall higher physical activity levels have been shown to have beneficial effects on brain health, including enhanced cognition and decreased risk of Alzheimer’s disease (AD). Nevertheless, despite continued research into the beneficial effects of physical activity on cognitive health, the exact mechanism(s) by which physical activity maintains a healthy aging brain is relatively poorly understood.

As discussed in Chapter 1 (Section 1.2.1, page 21), previous reports indicate that brain-derived neurotrophic factor (BDNF) plays a vital mediating role in the relationship between physical activity and cognitive health. A large evidence base demonstrates increased BDNF expression following both acute exercise and chronic physical activity/exercise in both younger and older adult cohorts (Huang, Larsen, Ried-Larsen, Møller, & Andersen, 2014). Due to the role of BDNF in maintaining memory and neuronal health, it is of great interest to fully understand triggers of BDNF expression, and importantly, researchers have demonstrated that exercise induces BDNF expression.
via the FNDC5/irisin pathway (P. Bostrom et al., 2012; P. A. Bostrom & Fernandez-Real, 2014; Wrann et al., 2013). The notion that irisin triggers BDNF expression, presents another potential mechanistic factor by which physical activity impacts the brain: could irisin serve as another mechanistic factor linking physical activity to enhanced cognitive health and reduced AD risk? Firstly, an association must be established between circulating irisin levels and physical activity and exercise. Indeed, the relationship between irisin and physical activity has been evaluated in numerous human studies, yet results to-date remain equivocal (Benedini et al., 2017; Daskalopoulou et al., 2014; Huh, Siopi, Mougios, Park, & Mantzoros, 2015; Tsuchiya, Ijichi, & Goto, 2016). Nevertheless, it is important to note that the majority of these previous studies investigating a relationship between irisin and physical activity have been conducted in younger adult cohorts; thus, the evaluation of this association in older adult cohorts is vital.

As described above, there is a lack of consistent evidence linking circulating irisin levels with physical activity and exercise, as the vast majority of studies to-date investigating irisin and physical activity, in humans, only involved young cohorts. Therefore, the purpose of this Chapter is to gain insight into the relationship between serum irisin and habitual physical activity levels, and the effects of exercise interventions on changes in irisin, in various cohorts of older adults (with the additional inclusion of a small subset of younger adults). The cohorts studied in this Chapter each utilised different measures and methods of physical activity and exercise (i.e. habitual physical activity and exercise interventions; aerobic and resistance training). It is important to note the distinction between physical activity and exercise used in this Chapter: ‘physical activity’ refers to any physical movement resulting in increased heart
Chapter 4 – Results II: The Effect of Physical Activity on Levels of Serum Irisin

rate and breathing (can include household activities, exercise, occupational activity etc.),
whilst ‘exercise’ refers to any planned and structured physical activity that is conducted
with the aim of improving or maintaining physical fitness (Caspersen, Powell, &
Christenson, 1985).

The aims of this Chapter were to:

1) Determine whether irisin could serve as a marker for physical activity levels in older
   individuals; and

2) To determine whether irisin levels could be used to assess whether a person is active
   enough to trigger beneficial molecular pathways for healthy well-being and ageing.
4.2 Materials and Methods

4.2.1 Participants of the Australian Imaging, Biomarkers and Lifestyle study cohort

The first cohort investigated in this Chapter is comprised of cognitively healthy older adults (≥ 60 years) of the Australian Imaging, Biomarkers and Lifestyle (AIBL) study. Chapter 2 of this thesis describes the recruitment and inclusion criteria for AIBL study participants (Sections 2.2.1 – 2.2.4, pages 52-53). Only participants with a correctly completed International Physical Activity Questionnaire (IPAQ), for whom irisin levels were available, were included in these analyses (n = 151). A subset of this group also wore an actigraphy unit around their waist for seven days: These individuals comprise the actigraphy sub-cohort (n = 43). A diagram of the study cohorts within AIBL is depicted in Chapter 2, Figure 2.1. All participants were informed about the procedures and potential risks, before consent was obtained (see Chapter 2.2.1, page 52).

4.2.2 Participants of the Physical Exercise And Cognitive Stimulation cohort

Participants from the Physical Exercise And Cognitive Stimulation (PEACS) study also comprised cognitively healthy older adults aged over 60. A detailed description of PEACS methodology, recruitment and eligibility criteria is outlined in Chapter 2 (2.3.2 – 2.3.6, pages 74-75). For the current project, I measured serum irisin levels in the samples of PEACS participants that were allocated to either an educational group only (i.e. control group, no intervention; n = 22), or an exercise intervention group (EI; n = 26; see Chapter 2, Figure 2.3); the intervention is described briefly below. Baseline actigraphy data was collected for the entire PEACS cohort utilising the same methodology as the AIBL cohort, and described in Chapter 2 (Section 2.2.10, pages 63-64).
4.2.3 Participants of the Acute Strength Training study cohort

The Acute Strength Training (AST) study cohort was comprised of seventeen men and women, with the cohort stratified into a “young cohort” (mean age: 23.9 ± 1.1 years; \(n = 10\)) and “older cohort” (mean age: 49.6 ± 3.6 years; \(n = 7\)). Each participant undertook two different resistance exercise training protocols on different days (allowing four to ten days between the two protocols for a wash out period). The resistance training protocols were: a strength based resistance training protocol (“S5/3”) and a hypertrophy based resistance training protocol (“H10/1”), see Chapter 2 (Figure 2.4 and Sections 2.4.2 – 2.4.3, pages 78-80) for full descriptions of the resistance protocols, which are also described briefly below. All individuals were informed about the procedures and potential risks, before consent was obtained from participants to take part in this study (see Chapter 2.4.1, page 78).

4.2.4 Physical activity measurements and exercise interventions

4.2.4.1 The International Physical Activity Questionnaire

Participants of the AIBL study completed the long version of the IPAQ at baseline; this data was used in cross-sectional analyses in this Chapter. As described in detail in Chapter 2 (Section 2.2.10.2, page 65), the IPAQ provides self-reported information on habitual physical activity in five different domains, namely; occupational activity, transportation, household activities, leisure-time and sedentary time during the day. Each question within the IPAQ corresponds to a Metabolic Equivalent of Task (MET) score (i.e. walking is 3.3, moderate-intensity is 4.4, vigorous has a MET of 8.0), which was computed by Ainsworth and colleagues and used as a standard for the last 15 years (Ainsworth et al., 2000; Craig et al., 2003). A mean seven-day score was calculated by
multiplying the allocated MET score for an activity by the amount of minutes a participant engaged in that activity for the week (MET·min/wk). Consistent with published literature in the field, participants were allocated into Tertiles based on their MET·min/wk (Table 4.1). Blood samples collected on the same day as IPAQ completion were assayed for irisin.

**Table 4.1: MET·min/wk cut-offs applied for the categorisation of the AIBL-PA cohort into Tertiles based on IPAQ data**

<table>
<thead>
<tr>
<th>Tertile 1</th>
<th>Tertile 2</th>
<th>Tertile 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 50)</td>
<td>(n = 50)</td>
<td>(n = 51)</td>
</tr>
<tr>
<td>0 - 1980</td>
<td>1981 - 5013</td>
<td>5014 - 18543</td>
</tr>
</tbody>
</table>

Tertile 1 = lowest levels of PA; Tertile 3 = highest levels of PA. Abbreviations: AIBL, Australian Imaging, Biomarkers and Lifestyle study; IPAQ, International Physical Activity Questionnaire; MET·min/wk, Metabolic Equivalent of Task in minutes per week; PA, physical activity.

4.2.4.2 Actigraphy data collection

Cognitively healthy AIBL and PEACS participants who consented to participate in the actigraphy sub-study, were asked to wear a waist-mounted GT3X actigraphy unit for seven consecutive days and nights, except when they were in the shower or participating in water-based activities. The collected data was analysed using ActiLife software, version 6.11.4. The activity units collect movement information (i.e. changes in acceleration) along the vertical axis (axis 1), which is translated into “counts”. Maximal counts (a measure of physical activity intensity; the average from every highest count per
day over the 7 day data collection period), and total counts (all counts recorded for the 7
days of data collection) were used in the current study.

4.2.4.3 Exercise intervention in PEACS

The aim of the PEACS study was to investigate the impact of an exercise intervention
(EI) and cognitive stimulation tasks in an older adult cohort aged over 60 years. For the
purpose of my PhD project I have utilised data from the EI group and a control group.
Briefly, the exercise intervention included aerobic exercise in the form of walking, 3 times
per week for 60 minutes, and home-based resistance training, twice a week for 30-45
minutes. The period of the intervention was 16 weeks. The control group did not
undertake any intervention, but were provided with educational material regarding a
healthy lifestyle. A diagram of the study groups within PEACS is depicted in Chapter 2
(Figure 2.2). Irisin was quantified in blood samples that were collected within the month
prior to the commencement of the EI/control period (pre-intervention) and within a month
of the cessation of the EI/control period (post-intervention).

4.2.4.4 Exercise intervention in Acute Strength Training study

Each participant within the AST study undertook two different resistance training
protocols on different days (with no less than 4 days, and no more than 10 days between
protocols). The resistance training protocols included; 1) strength based resistance
training consisting of 5 sets of 5 repetitions, with three-minute recovery time between
each set (“S5/3”), and 2) hypertrophy based resistance training, consisting of three sets of
ten repetitions, with one minute recovery between sets (“H10/1”). A diagram of the study
groups within AST is depicted in Chapter 2 (Figure 2.3). Blood was taken from each
participant immediately before training commenced, immediately post-training cessation, and 30 minutes post-training cessation. Serum from all three timepoints was assayed for irisin levels.

4.2.5 Measurements of irisin

Irisin was measured in serum samples using a commercially available ELISA kit (EK-067-29) manufactured by Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). The latest version of this kit (3rd generation) has been shown to be the most reliable detector of irisin on the market, and has been validated in several publications (Lee et al., 2014; Polyzos & Mantzoros, 2015; Reinehr, Elfers, Lass, & Roth, 2015). This 3rd generation kit was used when analysing serum samples from the AIBL and AST studies. Measurement of irisin in the PEACS cohort commenced a year prior to the measurements of irisin in the AIBL and AST study cohorts. Consequently, the levels of irisin detected in PEACS serum samples were determined utilising the 2nd generation of the same EK-067-29 kit described above. At the time of PEACS serum sample analysis, the utilised kit was the most reliable on the market. Nevertheless, after completion of irisin quantification in PEACS samples, major improvements were made to this kit, yielding the above-mentioned 3rd generation. The kit adjustments were significant in that the measured range of irisin in the 3rd generation was not directly comparable to the 2nd generation measurements; meaning that originally reported concentrations of approximately 400-600 ng/ml were now only found to be a fraction of the previous results, ranging from approximately 6 – 18 ng/ml on average. Despite these discrepancies, investigations of irisin fluctuations revealed consistent trends between the 2nd and 3rd generation of kits. For the AIBL cohort, I utilised a small number of samples (n = 60) to conduct a reliability
analysis, and confirmed a moderate correlation \( r = 0.51, p < 0.001 \) between irisin levels detected using the 2\textsuperscript{nd} and 3\textsuperscript{rd} generation kits.

### 4.2.6 Statistical analyses

Statistical analyses were conducted using IBM\textsuperscript{®} SPSS\textsuperscript{®} Statistics 23.0 for both Mac and Windows 7 (IBM Corp. Released 2015. IBM SPSS Statistics for Mac and Windows, Version 23.0, Armonk, NY: IBM Corp). Data is presented as mean ± standard deviation, unless otherwise stated. A \( p \)-value of less than 0.05 was deemed significant. For descriptive statistics within each cohort, differences in IPAQ Tertiles, study groups (e.g. actigraphy and non-actigraphy sub-groups), and gender were evaluated by independent sample \( t \)-tests or analysis of variance (ANOVA) for continuous variables, and chi-square (\( \chi^2 \)) analyses for categorical data.

#### 4.2.6.1 IPAQ data cleaning

After the calculation of IPAQ MET\textperiodcentered min/wk scores for the AIBL cohort, these data were searched for outliers by visual inspection of a histogram. One clear outlier was identified and removed, leaving the cohort to be analysed at 151 participants.

#### 4.2.6.2 Cross-sectional statistical analyses: Evaluation of relationship between irisin and physical activity data in AIBL and PEACS study cohorts

For the examination of the relationship between serum irisin and habitual physical activity, a series of linear regression analyses were conducted. More specifically, using data from the AIBL-PA cohort, linear regression analyses were utilised to investigate the association between irisin (independent variable) and MET\textperiodcentered min/wk (dependent variable),
with the inclusion of gender, age, BMI, and APOE ε4 allele carrier status as covariates within the model. The linear regression analyses were repeated after stratifying the AIBL-PA cohort by gender.

One-way ANOVA was utilised to evaluate differences in irisin levels across IPAQ Tertiles, which was followed by analysis of covariance (ANCOVA) adjusting for age, gender, BMI and APOE ε4 allele carrier status; these analyses were then repeated following stratification of the cohort by gender (with gender no longer included as a covariate). Linear regression analyses were also utilised to evaluate the relationship between serum irisin levels (independent variable) and actigraphy measured Maximal Counts and Total Counts (entered into separate models as dependent variables), with the inclusion of age, gender, BMI and APOE ε4 allele carrier status entered into the models as covariates. These linear models were conducted separately for both the AIBL actigraphy sub-cohort and the PEACS cohort (data was not combined due to the different generations of kits used to quantify irisin in the AIBL and PEACS samples).

4.2.6.3 Intervention statistical analyses: Evaluation of irisin levels pre- and post-intervention in the PEACS cohort

A repeated measures ANOVA (with the inclusion of age, gender and BMI as covariates) with a Greenhouse-Geisser correction was performed to evaluate changes in irisin levels pre- and post-intervention between the EI and control groups. The time variable was inspected to assess whether there was a change in irisin levels across both groups from pre- to post-intervention. Furthermore, the time*group interaction was evaluated to assess whether there were differences between the EI and control groups in terms of changes in serum irisin levels from pre- to post-intervention. Estimated marginal means were calculated and reported for each timepoint and group.
4.2.6.4 Intervention statistical analyses: Evaluation of irisin levels pre- and post-intervention in the AST cohort

In order to assess any change of irisin levels over three timepoints (before exercise commencement, T1; immediately post workout, T2; and 30 minutes post workout, T3), repeated measures ANOVA with a Greenhouse-Geisser correction was applied. These statistical analyses were conducted for both resistance training programmes separately (H10/1 and S5/3).
4.3 Results

4.3.1 The relationship between irisin and physical activity in the AIBL cohort

4.3.1.1 Descriptive statistics for the complete AIBL-PA cohort

The average age of the AIBL-PA participants was 73.0 ± 5.5 years, and the group was comprised of 58.6% females (Table 4.2). Over 35% of participants \((n = 54)\) reported a diagnosis of hypertension, and over 11% \((n = 17)\) had a history of thyroid problems (Table 4.3). Depression, diabetes, serious head injury, TIA and heart attack were reported by only a small number of participants (< 10% for each condition).

In order to evaluate whether there was any selection or volunteer bias between those that did and did not wear the actigraph in the AIBL-PA cohort, I conducted an analysis to compare the actigraphy and non-actigraphy groups based on demographic and medical history variables (Tables 4.2 and 4.3). After stratification by actigraphy and non-actigraphy data collection, no significant differences were observed in demographic characteristics, or in self-reported physical activity levels, between the two groups. The BMI amongst participants of the actigraphy and non-actigraphy groups differed at trend level, whereby the actigraphy cohort had a slightly lower BMI than the non-actigraphy cohort \((25.5 ± 3.5 \text{ and } 26.8 ± 4.1 \text{ kg/m}^2, \text{ respectively}; t = 1.89, p = 0.06)\). When comparing self-reported medical history between the two actigraphy-based groups, a history of depression was significantly more prevalent in the actigraphy cohort compared to the non-actigraphy cohort (Table 4.3; 18.6% vs 3.7%, respectively; \(p = 0.002\)).
Table 4.2: Demographic characteristics of the AIBL-PA cohort: The complete AIBL-PA cohort (all of whom had available IPAQ data), the actigraphy sub-group, and the non-actigraphy sub group

<table>
<thead>
<tr>
<th>Descriptive category</th>
<th>AIBL-PA cohort (n = 151)</th>
<th>Actigraphy cohort (n = 43)</th>
<th>Non-actigraphy cohort (n = 108)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean ± SD</td>
<td>73.0 ± 5.5</td>
<td>72.3 ± 4.4</td>
<td>73.3 ± 4.1</td>
<td>0.27</td>
</tr>
<tr>
<td>Gender, female, % (n)</td>
<td>58.3 (88)</td>
<td>55.8 (24)</td>
<td>59.3 (64)</td>
<td>0.70</td>
</tr>
<tr>
<td>Years of education &gt; 12 years, % (n)</td>
<td>48.3 (73)</td>
<td>48.8 (21)</td>
<td>48.1 (52)</td>
<td>0.94</td>
</tr>
<tr>
<td>Alcohol consumption per week, mean ± SD</td>
<td>3.9 ± 5.8</td>
<td>3.6 ± 4.5</td>
<td>4.0 ± 6.2</td>
<td>0.64</td>
</tr>
<tr>
<td>APOE ε4 allele carriers, % (n)</td>
<td>24.5 (37)</td>
<td>32.6 (14)</td>
<td>21.3 (23)</td>
<td>0.15</td>
</tr>
<tr>
<td>BMI (kg/m²), mean ± SD</td>
<td>26.4 ± 4.0</td>
<td>25.5 ± 3.5</td>
<td>26.8 ± 4.1</td>
<td>0.06</td>
</tr>
<tr>
<td>Aβ-SUVR, mean ± SD</td>
<td>1.35 ± 0.44</td>
<td>1.53 ± 0.49</td>
<td>1.30 ± 0.42</td>
<td>0.23</td>
</tr>
<tr>
<td>MET-min/wk, mean ± SD</td>
<td>4381.4 ± 3639.1</td>
<td>4796.1 ± 3889.6</td>
<td>4216.3 ± 3539.7</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Data presented as % (n) or mean ± standard deviation, as appropriate. p-values determined by independent sample t-tests for continuous variables and χ²-analyses for categorical data. Maximum missing data: BMI, n missing = 8 (n = 7 in the non-actigraphy cohort; n = 1 in the actigraphy cohort). A total of 30 participants completed PET-imaging, of whom n = 23 were from the non-actigraphy cohort and the remaining 7 from the actigraphy cohort. Abbreviations: Aβ-SUVR, beta-amyloid positron emission tomography standardised uptake value ratio; AIBL-PA, Australian Imaging, Biomarkers and Lifestyle study-physical activity cohort; APOE ε4, Apolipoprotein ε4 allele; BMI, Body mass index (kg/height (m)²); IPAQ, International Physical Activity Questionnaire; MET-min/wk, Level of self-reported habitual physical activity represented as Metabolic Equivalent of Task in minutes per week; SD, standard deviation.
Table 4.3: Medical history and clinical measurements of the AIBL-PA cohort: The complete AIBL-PA cohort (all of whom had available IPAQ data), the actigraphy subgroup and the non-actigraphy subgroup

<table>
<thead>
<tr>
<th>Medical history or clinical measurement</th>
<th>AIBL-PA cohort (n = 151)</th>
<th>Actigraphy cohort (n = 43)</th>
<th>Non-actigraphy cohort (n = 108)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension, % (n)</td>
<td>35.8 (54)</td>
<td>30.2 (13)</td>
<td>38 (41)</td>
<td>0.35</td>
</tr>
<tr>
<td>Heart attack, % (n)</td>
<td>2.6 (4)</td>
<td>4.8 (2)</td>
<td>1.9 (2)</td>
<td>0.33</td>
</tr>
<tr>
<td>TIA, % (n)</td>
<td>5.3 (8)</td>
<td>9.5 (4)</td>
<td>3.7 (4)</td>
<td>0.15</td>
</tr>
<tr>
<td>Serious head injury, % (n)</td>
<td>2.0 (3)</td>
<td>4.8 (2)</td>
<td>0.9 (1)</td>
<td>0.13</td>
</tr>
<tr>
<td>Thyroid dysfunction, % (n)</td>
<td>11.3 (17)</td>
<td>9.3 (4)</td>
<td>12.0 (13)</td>
<td>0.62</td>
</tr>
<tr>
<td>Type 2 Diabetes Mellitus, % (n)</td>
<td>6.0 (9)</td>
<td>9.5 (4)</td>
<td>4.6 (5)</td>
<td>0.26</td>
</tr>
<tr>
<td>Depression, % (n)</td>
<td>7.9 (12)</td>
<td>18.6 (8)</td>
<td>3.7 (4)</td>
<td><strong>0.002</strong></td>
</tr>
<tr>
<td>Sys BP (mmHg), mean ± SD</td>
<td>125.5 ± 17.6</td>
<td>127.1 ± 19.2</td>
<td>124.9 ± 16.9</td>
<td>0.50</td>
</tr>
<tr>
<td>Dia BP (mmHg), mean ± SD</td>
<td>74.4 ± 9.2</td>
<td>74.4 ± 8.2</td>
<td>74.4 ± 9.6</td>
<td>0.98</td>
</tr>
<tr>
<td>Heart rate, mean ± SD</td>
<td>68.4 ± 11.3</td>
<td>66.83 ± 9.9</td>
<td>69.0 ± 11.8</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Data presented as % (n) or mean ± standard deviation, as appropriate. p-values determined by independent sample t-tests for continuous variables and χ²-analyses for categorical data. Medical information was not available for the entire cohort, therefore n varied in each category. The maximum missing data n was 2 within the complete cohort (for heart attack and type 2 diabetes mellitus). Abbreviations: AIBL-PA, Australian Imaging, Biomarkers and Lifestyle study-physical activity cohort; Dia BP, diastolic blood pressure; IPAQ, International Physical Activity Questionnaire; mmHg, millimetres of mercury; SD, standard deviation; Sys BP, systolic blood pressure; TIA, transient ischaemic attack.
4.3.1.2 Descriptive statistics for the complete AIBL-PA cohort, following stratification by gender

After stratifying the AIBL-PA cohort ($n = 151$) by gender, several significant differences were evident (Table 4.4): 17% of women were $APOE \varepsilon 4$ carriers compared to 34.9% of men ($\chi^2 = 6.34, p = 0.01$). Consistent with this increased presence of $APOE \varepsilon 4$ carriers, men had a higher Aβ burden than women ($1.6 \pm 0.5$ compared to $1.1 \pm 0.1; t = 2.95, p = 0.006$). Furthermore, weekly alcohol intake differed significantly between the genders ($t = 2.38, p = 0.03$), with males consuming nearly twice as many standard drinks per week than females, averaging $5.2 \pm 7.3$ drinks versus $3.0 \pm 4.2$ drinks in females. When investigating gender differences in regards to diagnosed medical conditions, the only significant difference observed was thyroid dysfunction (Table 4.5), which was diagnosed in 15.9% of women, but only in 4.8% of men ($\chi^2 = 4.67, p = 0.03$).
**Table 4.4: Demographic characteristics of the complete baseline AIBL-PA cohort after stratification by gender**

<table>
<thead>
<tr>
<th>Descriptive category</th>
<th>Male (n = 63)</th>
<th>Female (n = 88)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean ± SD</td>
<td>73.7 ± 5.4</td>
<td>72.5 ± 5.6</td>
<td>0.18</td>
</tr>
<tr>
<td>Years of education &gt; 12 years, % (n)</td>
<td>57.1 (36)</td>
<td>42.0 (37)</td>
<td>0.07</td>
</tr>
<tr>
<td>Alcohol consumption per week, mean ± SD</td>
<td>5.2 ± 7.3</td>
<td>3.0 ± 4.2</td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td>APOE ε4 carriers, % (n)</td>
<td>34.9 (22)</td>
<td>17.0 (15)</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td>BMI (kg/m²), mean ± SD</td>
<td>27.1 ± 3.9</td>
<td>25.9 ± 4.0</td>
<td>0.07</td>
</tr>
<tr>
<td>Aβ-SUVR, mean ± SD</td>
<td>1.6 ± 0.5</td>
<td>1.1 ± 0.1</td>
<td><strong>0.006</strong></td>
</tr>
</tbody>
</table>

Data presented as % (n) or mean ± standard deviation, as appropriate. *p*-values determined by independent sample *t*-tests for continuous variables, and χ²-analyses for categorical data. Maximum missing data *n*: BMI = 8 (*n* = 6 in the female cohort and *n* = 2 in the male cohort). A total of 30 participants completed PET imaging, of which *n* = 14 were female. Abbreviations: Aβ-SUVR, beta-amyloid positron emission tomography (PET) standardised uptake value ratio; AIBL-PA, Australian Imaging, Biomarkers and Lifestyle study-physical activity cohort; APOE ε4, Apolipoprotein ε4 allele; BMI, Body mass index (kg/height (m)²); SD, standard deviation.
Table 4.5: Medical history and basic vital signs of the complete AIBL-PA cohort at baseline after stratification by gender

<table>
<thead>
<tr>
<th>Medical history / vital signs</th>
<th>Male (n = 63)</th>
<th>Female (n = 88)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension, % (n)</td>
<td>39.7 (25)</td>
<td>33 (29)</td>
<td>0.43</td>
</tr>
<tr>
<td>Heart attack, % (n)</td>
<td>4.8 (3)</td>
<td>1.9 (2)</td>
<td>0.17</td>
</tr>
<tr>
<td>TIA, % (n)</td>
<td>4.8 (3)</td>
<td>5.7 (5)</td>
<td>0.82</td>
</tr>
<tr>
<td>Serious head injury, % (n)</td>
<td>3.2 (2)</td>
<td>1.1 (1)</td>
<td>0.37</td>
</tr>
<tr>
<td>Thyroid dysfunction, % (n)</td>
<td>4.8 (3)</td>
<td>15.9 (14)</td>
<td>0.03</td>
</tr>
<tr>
<td>Type 2 Diabetes Mellitus, % (n)</td>
<td>4.8 (3)</td>
<td>6.8 (6)</td>
<td>0.60</td>
</tr>
<tr>
<td>Depression, % (n)</td>
<td>9.5 (6)</td>
<td>6.8 (6)</td>
<td>0.54</td>
</tr>
<tr>
<td>Sys BP (mmHg), mean ± SD</td>
<td>127.7 ± 16.8</td>
<td>123.9 ± 18.0</td>
<td>0.20</td>
</tr>
<tr>
<td>Dia BP (mmHg), mean ± SD</td>
<td>74.3 ± 9.4</td>
<td>74.5 ± 9.2</td>
<td>0.94</td>
</tr>
<tr>
<td>Heart rate, mean ± SD</td>
<td>66.4 ± 12.2</td>
<td>69.9 ± 10.4</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Data presented as % (n), or mean ± standard deviation, as appropriate. p-values determined by independent sample t-tests for continuous variables and χ²-analyses for categorical data. Medical information was not available for the entire cohort, therefore n varied in each category. The maximum missing data was n = 7 within the female cohort (heart rate), and n = 2 within the male cohort (heart rate). Abbreviations: AIBL-PA, Australian Imaging, Biomarkers and Lifestyle study-physical activity cohort; Dia BP, diastolic blood pressure; mmHg, millimetres of mercury; SD, standard deviation; Sys BP, systolic blood pressure; TIA, transient ischaemic attack.
4.3.1.3. Descriptive statistics for the complete AIBL-PA cohort stratified by Tertiles

The stratification of the AIBL-PA cohort into Tertiles was conducted based on total MET·min/wk values (see Statistical methodology in this Chapter, 4.2.4.1, pages 165-166). After stratification of the physical activity cohort into Tertiles (Tertile 1 = lowest PA; Tertile 3 = highest PA), demographic characteristics and medical history was re-examined (Tables 4.6 and 4.7 respectively). Women were more likely to report lower levels of physical activity (Tertile 1 = 74% women, Tertile 2 = 58% women, Tertile 3 = 43% women; $\chi^2 = 9.9$, $p = 0.007$). No differences were observed across the Tertiles in terms of self-reported medical history or basic vital signs.
Table 4.6: Demographic characteristics of the complete baseline AIBL-PA cohort after stratification by Tertiles

<table>
<thead>
<tr>
<th>Descriptive category</th>
<th>Tertile 1 (n = 50)</th>
<th>Tertile 2 (n = 50)</th>
<th>Tertile 3 (n = 51)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean ± SD</td>
<td>72.9 ± 5.9</td>
<td>73.1 ± 5.7</td>
<td>73.1 ± 5.1</td>
<td>0.84</td>
</tr>
<tr>
<td>Gender, female, % (n)</td>
<td>74 (37)</td>
<td>58 (29)</td>
<td>43 (22)</td>
<td>0.007</td>
</tr>
<tr>
<td>Years of education &gt; 12 years, % (n)</td>
<td>54 (27)</td>
<td>40 (20)</td>
<td>51 (26)</td>
<td>0.34</td>
</tr>
<tr>
<td>Alcohol consumption per week, mean ± SD</td>
<td>4.0 ± 6.2</td>
<td>4.0 ± 6.3</td>
<td>3.6 ± 4.8</td>
<td>0.93</td>
</tr>
<tr>
<td>APOE ε4 allele carriers, % (n)</td>
<td>24 (12)</td>
<td>26 (13)</td>
<td>23 (12)</td>
<td>0.95</td>
</tr>
<tr>
<td>BMI (kg/m²), mean ± SD</td>
<td>26.9 ± 3.9</td>
<td>26.5 ± 4.4</td>
<td>26.1 ± 3.7</td>
<td>0.30</td>
</tr>
<tr>
<td>Aβ-SUVR, mean ± SD</td>
<td>1.4 ± 0.5</td>
<td>1.3 ± 0.5</td>
<td>1.4 ± 0.4</td>
<td>0.79</td>
</tr>
</tbody>
</table>

Descriptive data for the AIBL-PA cohort after stratification by International Physical Activity Questionnaire Tertiles (1 = lowest PA levels; 3 = highest PA levels). Aβ-SUVR was determined in n = 13 (Tertile 1), n = 9 (Tertile 2), and n = 8 (Tertile 3). The maximum missing data for all other categories was n = 6 within the Tertile 1 cohort, n = 3 in Tertile 2, and n = 2 in Tertile 3. Data presented as mean ± standard deviation, unless otherwise stated. p-values determined by ANOVA for continuous data and χ²-analyses for categorical data. p-value < 0.05 is deemed significant (bold). Abbreviations: Aβ-SUVR, beta-amyloid positron emission tomography standardised uptake value ratio; AIBL-PA, Australian Imaging, Biomarkers and Lifestyle study-physical activity cohort; APOE, Apolipoprotein E; BMI, Body mass index (kg/height (m)²); PA, physical activity; SD, standard deviation.
Table 4.7: Medical history and basic vital signs of the complete baseline AIBL-PA cohort after stratification by Tertiles

<table>
<thead>
<tr>
<th>Medical history</th>
<th>Tertile 1 (n = 50)</th>
<th>Tertile 2 (n = 50)</th>
<th>Tertile 3 (n = 51)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension, % (n)</td>
<td>30 (15)</td>
<td>34 (17)</td>
<td>41.2 (21)</td>
<td>0.58</td>
</tr>
<tr>
<td>Heart attack, % (n)</td>
<td>4 (2)</td>
<td>4 (2)</td>
<td>2 (1)</td>
<td>0.82</td>
</tr>
<tr>
<td>TIA count, % (n)</td>
<td>8 (4)</td>
<td>2 (1)</td>
<td>5.9 (3)</td>
<td>0.37</td>
</tr>
<tr>
<td>Serious head injury, % (n)</td>
<td>0 (0)</td>
<td>2 (1)</td>
<td>3.9 (2)</td>
<td>0.37</td>
</tr>
<tr>
<td>Thyroid dysfunction, % (n)</td>
<td>12 (6)</td>
<td>8 (4)</td>
<td>13.7 (7)</td>
<td>0.61</td>
</tr>
<tr>
<td>Type 2 Diabetes Mellitus, % (n)</td>
<td>8 (4)</td>
<td>2 (1)</td>
<td>11.8 (6)</td>
<td>0.14</td>
</tr>
<tr>
<td>Depression, % (n)</td>
<td>8 (4)</td>
<td>6 (3)</td>
<td>9.8 (5)</td>
<td>0.76</td>
</tr>
<tr>
<td>Sys BP (mmHg), mean ± SD</td>
<td>124.8 ± 16.8</td>
<td>125.3 ± 16.8</td>
<td>126.5 ± 19.1</td>
<td>0.65</td>
</tr>
<tr>
<td>Dia BP (mmHg), mean ± SD</td>
<td>76.1 ± 8.6</td>
<td>74.1 ± 9.0</td>
<td>73.2 ± 9.8</td>
<td>0.12</td>
</tr>
<tr>
<td>Heart rate, mean ± SD</td>
<td>68.4 ± 11.7</td>
<td>68.6 ± 11.1</td>
<td>68.1 ± 11.4</td>
<td>0.90</td>
</tr>
</tbody>
</table>

Descriptive data for the AIBL-PA cohort after stratification by International Physical Activity Questionnaire Tertiles (1 = lowest PA levels; 3 = highest PA levels). Data presented as % of the whole cohort (n), or mean ± standard deviation, as appropriate. p-values determined by Analysis of Variance. No cases of stroke were reported in the physical activity cohort. Medical information was not available for the entire cohort, therefore n varied in each category. The maximum missing n was 7 within the Tertile 1 cohort, n = 5 in Tertile 2, and n = 3 in Tertile 3. Abbreviations: AIBL-PA, Australian Imaging, Biomarkers and Lifestyle study-physical activity cohort; Dia BP, diastolic blood pressure; mmHg, millimetres of mercury; PA, physical activity; SD, standard deviation; Sys BP, systolic blood pressure; TIA, transient ischaemic attack.
Chapter 4 – Results II: The Effect of Physical Activity on Levels of Serum Irisin

4.3.1.4 Cross-sectional analysis of irisin and physical activity in the complete AIBL-PA cohort

Linear regression models were run to evaluate the relationship between self-reported physical activity levels, as represented by IPAQ-determined MET·min/wk, and irisin, while adjusting for age, gender, and BMI (Table 4.8). Irisin was not a significant predictor of the MET·min/wk variable ($\beta = -0.05, p = 0.53$). However, gender and BMI were both significant predictors of MET·min/wk (Gender: $\beta = -0.27, p < 0.01$; BMI: $\beta = -0.18, p = 0.03$).

<table>
<thead>
<tr>
<th>Variables</th>
<th>$\beta$ (CI)</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MET·min/wk Irisin</td>
<td>-0.05 (-274.07, 141.44)</td>
<td>0.53</td>
</tr>
<tr>
<td>MET·min/wk Age</td>
<td>0.06 (-67.46, 154.51)</td>
<td>0.44</td>
</tr>
<tr>
<td>MET·min/wk Gender</td>
<td>-0.27 (-3218.61, -822.91)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MET·min/wk BMI</td>
<td>-0.18 (-319.36, -19.19)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Linear regression models run to evaluate the relationship between self-reported PA levels (n = 151), represented by International Physical Activity Questionnaire-determined MET·min/wk, and irisin, while adjusting for age, gender, and BMI: $\beta$ values shown. Abbreviations: AIBL-PA, Australian Imaging, Biomarkers and Lifestyle study-physical activity cohort; BMI, Body mass index (kg/height (m)$^2$); CI, Confidence Interval; MET·min/wk, Metabolic Equivalent of Task over a mean 7-day score; PA, physical activity.
4.3.1.5 Cross-sectional analysis of irisin and physical activity in the complete AIBL-PA cohort after stratification by gender

Similar to the previous section, linear regression analyses were conducted in order to evaluate the relationship between irisin and MET·min/wk (IPAQ-determined habitual self-reported physical activity levels) in the AIBL-PA cohort, following stratification by gender. There were no observed relationships between irisin and MET·min/wk in either gender (males: $\beta = -0.02$, $p = 0.89$; females: $\beta = -0.09$, $p = 0.45$: data not shown).

4.3.1.6 Irisin in the complete baseline AIBL-PA cohort after stratification by Tertiles

When investigating serum irisin levels across the IPAQ Tertiles, irisin levels were not found to differ ($F(2,148) = 0.40$, $p = 0.67$), with mean irisin levels in Tertile 1 averaging $11.51 \pm 2.93$ ng/ml, Tertile 2 = $11.58 \pm 2.82$ ng/ml, and Tertile 3 = $11.11 \pm 2.79$ ng/ml (Figure 4.1). This lack of difference across Tertiles remained following adjustment for age, gender, BMI and $APOE \varepsilon 4$ allele carriage ($F(2,136) = 0.27$, $p = 0.76$: data not shown).
Figure 4.1: Raw mean serum irisin levels across International Physical Activity Questionnaire Tertiles. Tertile 1 = lowest physical activity levels, Tertile 3 = highest physical activity levels in Metabolic Equivalent of Task over a mean 7-day score (MET·min/wk). Whiskers represent the range of irisin levels, upper horizontal line of the box shows the third quartile above the mean irisin level, middle horizontal line is the mean irisin level, and lower horizontal line shows the first quartile below the mean irisin level.
4.3.1.7 Cross-sectional analysis of irisin and physical activity in the AIBL actigraphy cohort

The actigraphy cohort comprised 43 study participants; thus, this group was not sufficiently powered to stratify into Tertiles. Rather, in the proceeding actigraphy analyses, only continuous data derived from actigraphy units were utilized. The relationship between serum irisin levels and actigraphy-derived physical activity measures was evaluated using linear regression models with the inclusion of age, gender and BMI. A trend-level inverse relationship between max counts (measure of physical activity intensity) and irisin was observed ($\beta = -0.30, p = 0.05$; Table 4.9). BMI was however, significantly inversely associated with max counts ($\beta = -0.33, p = 0.04$). Total counts (measure of volume of physical activity) was not associated with serum irisin levels, or with any of the covariates included in the model.
**Table 4.9: Linear regression analyses of irisin and actigraphy-derived physical activity levels in the AIBL cohort**

<table>
<thead>
<tr>
<th>Variables</th>
<th>β (CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Max counts model</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irisin</td>
<td>-0.30 (-214.78, 1.86)</td>
<td>0.05</td>
</tr>
<tr>
<td>Age</td>
<td>-0.07 (-96.24, 62.30)</td>
<td>0.67</td>
</tr>
<tr>
<td>Gender</td>
<td>-0.29 (-1308.86, 71.61)</td>
<td>0.08</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.33 (-193.16, -4.38)</td>
<td><strong>0.04</strong></td>
</tr>
<tr>
<td><strong>Total counts model</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irisin</td>
<td>0.003 (-78611.62, 80252077)</td>
<td>0.98</td>
</tr>
<tr>
<td>Age</td>
<td>-0.16 (-85225.51, 31039.26)</td>
<td>0.35</td>
</tr>
<tr>
<td>Gender</td>
<td>0.21 (-198173.51, 814177.90)</td>
<td>0.23</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.08 (-85313.0, 53127.37)</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Linear regression models run to evaluate the relationship between actigraphy-determined PA levels in the AIBL cohort (n = 43), and irisin, while adjusting for age, gender, and BMI: β values shown. Maximal (Max) counts (a measure of physical activity intensity; the average from every highest count per day over the 7 day data collection period); Total counts (all counts recorded for the 7 days of data collection). Missing data n = 2 (BMI). Abbreviations: AIBL, Australian Imaging, Biomarkers and Lifestyle study; BMI, Body mass index (kg/height (m)^2); CI, Confidence Interval; PA, physical activity.
4.3.2 The relationship between irisin, physical activity and exercise in the PEACS cohort

4.3.2.1 Descriptive statistics of the control and exercise intervention groups in PEACS

The overall PEACS cohort was comprised of 69% females and had a mean age of 67.9 years (Table 4.10). After stratifying the cohort by the exercise intervention (EI) and control groups, no significant difference was evident when evaluating age, gender, BMI, APOE ε4 allele carrier status (Table 4.10), or medical conditions (Table 4.11) across these groups. Trend level differences were however, observed for APOE ε4 carrier status ($p = 0.05$), and hypertension ($p = 0.07$).

Table 4.10: Demographic characteristics of the PEACS study total cohort, exercise intervention, and control groups at baseline

<table>
<thead>
<tr>
<th>Descriptive category</th>
<th>Overall ($n = 48$)</th>
<th>EI ($n = 26$)</th>
<th>Control ($n = 22$)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean ± SD</td>
<td>67.9 ± 5.6</td>
<td>67.3 ± 5.3</td>
<td>68.7 ± 5.9</td>
<td>0.37</td>
</tr>
<tr>
<td>Gender, female, % ($n$)</td>
<td>69 (33)</td>
<td>65 (17)</td>
<td>73 (16)</td>
<td>0.58</td>
</tr>
<tr>
<td>BMI (kg/m$^2$), mean ± SD</td>
<td>26.1 ± 3.6</td>
<td>25.9 ± 3.9</td>
<td>26.3 ± 3.2</td>
<td>0.69</td>
</tr>
<tr>
<td>APOE ε4 carriers, % ($n$)</td>
<td>27 (13)</td>
<td>38 (10)</td>
<td>14 (3)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Data presented as % ($n$) or mean ± standard deviation, as appropriate. $p$-values determined by independent sample $t$-tests for continuous variables, and $\chi^2$-analyses for categorical data. $p$-value $< 0.05$ is deemed significant. Abbreviations: APOE ε4, Apolipoprotein E ε4 allele; BMI, Body mass index (kg/height (m)$^2$); EI, exercise intervention group; PEACS, Physical Exercise And Cognitive Stimulation; SD, standard deviation.
Table 4.11: Medical history of the PEACS study total cohort, exercise intervention, and control groups at baseline

<table>
<thead>
<tr>
<th>Medical history</th>
<th>Overall (n = 48)</th>
<th>EI (n = 26)</th>
<th>Control (n = 22)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension, % (n)</td>
<td>31.3 (15)</td>
<td>19.2 (5)</td>
<td>45.5 (10)</td>
<td>0.07</td>
</tr>
<tr>
<td>Thyroid dysfunction, % (n)</td>
<td>22.9 (11)</td>
<td>23.1 (6)</td>
<td>22.7 (5)</td>
<td>0.62</td>
</tr>
<tr>
<td>Type 2 Diabetes Mellitus, % (n)</td>
<td>12.5 (6)</td>
<td>15.4 (4)</td>
<td>9.1 (2)</td>
<td>NS*</td>
</tr>
<tr>
<td>Depression, % (n)</td>
<td>14.9 (7)</td>
<td>12.0 (3)</td>
<td>18.2 (4)</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Data presented as % (n). p-values determined by χ²-analyses. p-value < 0.05 is deemed significant. Missing data n = 1 (Depression). *Unable to conduct χ²-analyses due to all cells having a count less than 5. Abbreviations: EI, exercise intervention group; NS, Not significant; PEACS, Physical Exercise And Cognitive Stimulation.
4.3.2.2 Baseline irisin levels in the PEACS cohort

Irisin levels in PEACS samples were measured pre- and post-intervention using the 2\textsuperscript{nd} generation of the Phoenix Pharmaceuticals (Burlingame, California, USA) ELISA kit (catalogue number EK-067-29). Therefore, the ranges of serum irisin levels in this cohort are much higher than the serum samples measured in the AIBL and AST cohorts, which were assessed using the 3\textsuperscript{rd} generation of the same ELISA-kit. Whilst these kits derived from the same company, and whilst I conducted a reliability analysis, which confirmed a moderate correlation of levels determined using the different kits (see section 4.2.5, page 168), it cannot be reliably confirmed that the relationships between irisin and maximal intensity of exercise, measured in the PEACS and AST cohorts, are comparable.

Within the PEACS study cohort, irisin levels did not differ significantly between the EI and control groups at baseline ($F(1, 47) = 0.60, p = 0.44$). Mean levels were measured at 460.7 ± 27.0 ng/ml in the EI group, and 496.6 ± 40 ng/ml in the control group (Figure 4.2). Irisin was not associated with age, gender, or BMI at baseline in this cohort.
Figure 4.2: Raw mean baseline serum irisin levels across study groups in the PEACS cohort. Whiskers represent the range of irisin levels, upper horizontal line of the box shows the third quartile above the mean irisin level, middle horizontal line is the mean irisin level, and lower horizontal line shows the first quartile below the mean irisin level. Abbreviations: EI, exercise intervention; PEACS, Physical Exercise And Cognitive Stimulation.
4.3.2.3 Irisin and physical activity at baseline in PEACS

All PEACS participants had 7-day actigraphy data available from baseline. For cross-sectional analysis, PEACS groups were combined, and levels of irisin were evaluated against actigraphy-measured Maximal and Total counts per week (Table 4.12). Higher Max counts (an indication of physical activity intensity) was associated with higher levels of irisin ($\beta = 0.34$, $p = 0.01$). Similarly, actigraphy-derived Total counts (measure of volume of physical activity) was associated with higher levels of irisin ($\beta = 0.38$, $p = 0.008$). Age was inversely associated with both Max and Total counts ($\beta = -0.43$, $p = 0.002$, and $\beta = -0.28$, $p = 0.04$, respectively).

**Table 4.12: Linear regression analyses of irisin and actigraphy-derived physical activity levels in the PEACS cohort**

<table>
<thead>
<tr>
<th>Variables</th>
<th>$\beta$ (CI)</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Max counts model</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irisin</td>
<td>0.34 (-3.30, 23.74)</td>
<td>0.01</td>
</tr>
<tr>
<td>Age</td>
<td>-0.43 (-805.59, -206.17)</td>
<td>0.002</td>
</tr>
<tr>
<td>Gender</td>
<td>-0.02 (-3905.05, 3397.03)</td>
<td>0.89</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.20 (-813.19, 94.75)</td>
<td>0.12</td>
</tr>
<tr>
<td><strong>Total counts model</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irisin</td>
<td>0.38 (-3.96, 34.03)</td>
<td>0.008</td>
</tr>
<tr>
<td>Age</td>
<td>-0.28 (-61297.69, -1847.44)</td>
<td>0.04</td>
</tr>
<tr>
<td>Gender</td>
<td>0.19 (-101930.89, 622295.70)</td>
<td>0.15</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.16 (-72713.39, 17337.47)</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Linear regression models run to evaluate the relationship between actigraphy-determined PA levels in the PEACS cohort (n = 48), and irisin, while adjusting for age, gender, and BMI: $\beta$ values shown. Maximal (Max) counts (a measure of physical activity intensity; the average from every highest count per day over the 7 day data collection period); Total counts (all counts recorded for the 7 days of data collection). Abbreviations: BMI, Body mass index (kg/height (m)$^2$); CI, Confidence Interval; PA, physical activity; PEACS, Physical Exercise And Cognitive Stimulation.
4.3.2.4 Changes of irisin from pre- to post-intervention in the PEACS cohort groups

Repeated measures ANOVA with a Greenhouse-Geisser correction demonstrated irisin levels changed significantly from pre- (baseline) to post- (16 week follow-up) intervention in the complete PEACS cohort, regardless of group allocation ($Time = F(1, 43) = 8.55, p = 0.005$; Table 4.13). Furthermore, the $Time*Group$ interaction was significant, indicating a significant difference between the EI and control group in terms of change of irisin from pre- to post-intervention ($F(1, 43) = 7.36, p = 0.01$). Serum irisin levels within the EI group increased 16% from pre- to post-intervention; however, the control group experienced a 1% decrease in irisin levels over the same time period. The $Time*BMI$ interaction was also significant, regardless of group allocation ($F(1, 43) = 7.93, p = 0.007$).
**Table 4.13**: Results from repeated measures univariate analysis of variance, examining differences in irisin levels from pre- to post-intervention across study groups of the PEACS cohort

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group and Timepoint</th>
<th>Irisin Adjusted Marginal Means (ng/ml)</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time*Group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control Pre-intervention</td>
<td>497 ± 35</td>
<td>8.55</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Control Post-intervention</td>
<td>491 ± 37</td>
<td>7.36</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>EI Pre-intervention</td>
<td>460 ± 32</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EI Post-intervention</td>
<td>549 ± 34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time*BMI</td>
<td></td>
<td></td>
<td>7.93</td>
<td>0.007</td>
</tr>
<tr>
<td>Time*Age</td>
<td></td>
<td></td>
<td>3.74</td>
<td>0.06</td>
</tr>
<tr>
<td>Time*Gender</td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.90</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, Body mass index (kg/height (m)^2); EI, exercise intervention; PEACS, Physical Exercise And Cognitive Stimulation.
4.3.3 Irisin and physical activity in the AST cohort

4.3.3.1 Description of the AST cohort

The ‘younger’ cohort within the total AST cohort consisted of seven males and three females, with a mean age of 23.90 ± 1.10 years, whilst the ‘older’ cohort consisted of six males and one female with an average age of 49.57 ± 3.55 years. Both age groups underwent two different resistance training protocols (H10/1 and S5/3) and had blood collected immediately before the exercise session commenced (T1), immediately after the session finished (T2), and 30 minutes post-session completion (T3). Irisin levels were measured in all blood samples at all timepoints.

4.3.3.2 Irisin levels following the hypertrophy (H10/1) training protocol

In the total cohort of 17 participants (combining ‘younger’ and ‘older’ participants), following the hypertrophy training protocol, irisin levels rose, from 10.8 ± 3.1 ng/ml at baseline (T1), to 11.9 ± 4.9 ng/ml immediately post-exercise (T2), and returned to 10.7 ± 3.2 ng/ml 30 minutes post-exercise (T3; Table 4.14). Repeated measures ANOVA with a Greenhouse-Geisser correction showed no statistically significant difference in irisin levels between these timepoints within subjects (Time: F(1.65, 26.24) = 1.04, p = 0.36). Following stratification of the cohort into the ‘younger’ and ‘older’ groups (‘younger’: n = 10; ‘older’ n = 7), the Time variable was again not statistically significant (Table 4.14).
Table 4.14: Overview of mean irisin levels in AST cohort participants pre- and post- the H10/1 hypertrophy training protocol

<table>
<thead>
<tr>
<th></th>
<th>T1 (Pre-training)</th>
<th>T2 (Immediately Post-training)</th>
<th>T3 (30 minutes Post-training)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cohort (H10/1)</td>
<td>10.8 ± 3.1</td>
<td>11.9 ± 4.9</td>
<td>10.7 ± 3.2</td>
<td>0.36</td>
</tr>
<tr>
<td>Mean irisin (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Younger’ (H10/1)</td>
<td>9.87 ± 1.59</td>
<td>11.12 ± 4.77</td>
<td>10.85 ± 2.69</td>
<td>0.39</td>
</tr>
<tr>
<td>Mean irisin (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Older’ (H10/1)</td>
<td>12.22 ± 4.22</td>
<td>13.06 ± 5.30</td>
<td>10.54 ± 3.96</td>
<td>0.35</td>
</tr>
<tr>
<td>Mean irisin (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data presented as mean ± standard deviation. p-values determined via repeated measures analysis of variance. Abbreviations: AST, Acute Strength Training; H10/1, hypertrophy-based strength training with 10 repetitions per bout and one minute break between sets; T1, timepoint 1, immediately before warm-up; T2, timepoint 2, immediately following session completion; T3, timepoint 3, 30 minutes after session completion.

4.3.3.3 Irisin levels following the strength (S5/3) training protocol

In the total cohort of 17 participants, following the strength training protocol, irisin was measured at 11.32 ± 3.30 ng/ml at T1, 10.59 ± 2.01 ng/ml immediately post-exercise at T2, and at 11.02 ± 2.26 ng/ml 30 minutes post-exercise completion at T3 (Table 4.15).

Repeated measures ANOVA with a Greenhouse-Geisser correction showed no statistically significant difference in irisin levels across these timepoints (Time: F(1.19, 19.07) = 0.97, p = 0.35). Following stratification of the cohort into the ‘younger’ and ‘older’ groups (‘younger’: n = 10; ‘older’ n = 7), the Time variable was again not statistically significant (Table 4.15).
Table 4.15: Overview of mean irisin levels in AST cohort participants pre- and post- the S5/3 strength training protocol

<table>
<thead>
<tr>
<th></th>
<th>T1 (Pre-training)</th>
<th>T2 (Immediately Post-training)</th>
<th>T3 (30 minutes Post-training)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cohort (S5/3)</td>
<td>11.32 ± 3.30</td>
<td>10.59 ± 2.01</td>
<td>11.02 ± 2.26</td>
<td>0.35</td>
</tr>
<tr>
<td>Mean irisin (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Younger’ (S5/3)</td>
<td>11.72 ± 3.76</td>
<td>10.34 ± 1.81</td>
<td>10.63 ± 1.78</td>
<td>0.21</td>
</tr>
<tr>
<td>Mean irisin (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Older’ (S5/3)</td>
<td>10.75 ± 2.66</td>
<td>10.94 ± 2.38</td>
<td>11.57 ± 2.87</td>
<td>0.35</td>
</tr>
<tr>
<td>Mean irisin (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data presented as mean ± standard deviation. p-values determined via repeated measures analysis of variance. Abbreviations: AST, Acute Strength Training; S5/3, strength-based training with 5 repetitions of maximum load and three minute recovery in between sets; T1, timepoint 1, immediately before warm-up; T2, timepoint 2, immediately following session completion; T3, timepoint 3, 30 minutes after session completion.
4.4 Discussion

This Chapter focused on evaluating; a) the relationship between serum irisin levels and habitual physical activity levels in cognitively healthy older adults (AIBL and PEACS studies), b) the effects of a 16-week exercise regimen (including both aerobic and resistance training) in older adults on serum irisin levels (PEACS study), and c) the acute effects of resistance training protocols in ‘younger’ and ‘older’ adults on serum irisin levels (AST study). In this Chapter, I observed: 1) a relationship between irisin and actigraphy-derived maximal, and total counts (objective measures of physical activity intensity, and total physical activity, respectively), 2) increases in serum irisin levels following a 16-week exercise intervention, and 3) no acute changes in irisin following two different resistance training protocols.

A cross-sectional analysis of serum irisin levels and habitual physical activity within the AIBL-PA and PEACS cohorts was conducted based on frequent reports that blood irisin levels are linked to habitual physical activity levels in humans (A. D. Anastasilakis, S. A. Polyzos, P. Makras, et al., 2014; Kurdiova et al., 2014; Lecker et al., 2012; Norheim et al., 2014). In the current study, I evaluated the relationship between serum irisin and levels of both subjectively measured physical activity (measured using the IPAQ) and objectively measured physical activity (determined using actigraphy). No significant association was observed between self-reported physical activity levels and levels of serum irisin. However, in the PEACS cohort, I observed a relationship between irisin levels and objectively measured intensity and volume of physical activity, whereby more intense and higher volume of physical activity were both associated with higher levels of irisin. To-date, only one other study has evaluated the relationship between circulating irisin levels and objectively-measured physical activity (determined via a SenseWear
armband), from which step counts and METs were extracted for analysis (Hofmann et al., 2014); notably, a measure of PA intensity was not reported. This assessment was conducted in patients with anorexia nervosa, and no association between plasma irisin levels and METs per day was observed.

It is important to note that in this Chapter, the observed associations between actigraphy-derived physical activity and irisin levels in the PEACS cohort were not mirrored in the analyses of the AIBL actigraphy cohort, where a trend-level inverse association was observed between more intense physical activity (i.e. maximal counts) and lower serum irisin levels. The reason for this discrepancy could simply be of a methodological nature, as an older version of the irisin kit was used in the PEACS analysis. However, the main demographical difference between the two studied cohorts (AIBL and PEACS) reflects a higher percentage of women in the PEACS cohort compared to the AIBL cohort (69% vs 56%). It is conceivable that the observed difference between the cohorts is due to this demographic variation, although an irisin induction mechanism which is contingent upon gender is yet to be determined. Further, both of these actigraphy cohorts consisted of small sample sizes of 48 (PEACS) and 43 (AIBL) participants; thus, any potential association between habitual physical activity measures and irisin levels requires validation in a larger cohort. Interestingly, when assessing serum irisin levels acutely following high intensity strength training in the AST study, I did not observe any significant spikes in irisin levels. These results should however be interpreted with caution as the sample size was very small (n = 17), and the age range was large (~20-50 years).
Chapter 4 – Results II: The Effect of Physical Activity on Levels of Serum Irisin

As stated above, I did not observe an association between serum irisin levels and self-reported weekly MET scores in the AIBL-PA cohort. To my knowledge, this is the first study to evaluate serum irisin levels with MET scores in older adults. The lack of association reported in this thesis is consistent with the findings of a previous study that compared IPAQ-MET physical activity scores with irisin levels (Athanasios D. Anastasilakis et al., 2014). However, another two studies did report an association between circulating irisin and MET scores: plasma and serum irisin levels were associated with higher MET scores in adults aged under 65 years (Cooke, Gomez, Mutter, Mantzoros, & Daskalopoulou, 2013; Jameel, Thota, Wood, Plunkett, & Garg, 2015). It is possible that the age of the cohorts may explain the conflicting findings; i.e. younger cohorts may be undertaking more intense physical activity (which could be reflected in their total volume). It is also possible that response-related bias may have had an impact on the outcome reported in this thesis; i.e. efficiency of memory recall may be inferior in the older study cohort. Nevertheless, previous analyses from the AIBL study have reported relationships between IPAQ-MET levels and biomarkers of cardiovascular disease and metabolic syndrome (Brown, Peiffer, & Martins, 2013), and thus I am confident that the data collected by the IPAQ is a true reflection of current physical activity levels.

Data from the PEACS study allowed the evaluation of the impact of a 16-week exercise intervention (combining both aerobic and resistance exercises) on levels of serum irisin. In this Chapter, I reported that irisin from pre- to post-intervention increased significantly (16%) in the exercise intervention (EI) group after the 16-week intervention period, compared with the control group (1% decrease). Although nearly all available exercise intervention studies report an increase of irisin acutely following varying exercise
regimes (A. D. Anastasilakis, S. A. Polyzos, Z. G. Saridakis, et al., 2014; Daskalopoulou et al., 2014; Huh, Mougios, Kabasakalis, et al., 2014; Loffler et al., 2015; Norheim et al., 2014; Nygaard et al., 2015; Tsuchiya et al., 2014; Tsuchiya, Ando, Takamatsu, & Goto, 2015), conflicting results of changes in irisin have emerged from chronic exercise studies, as summarised by Dinas and colleagues (Dinas et al., 2017). Whilst the majority of previous studies evaluating the impact of chronic exercise on irisin have included younger to middle-aged study participants, a small number of studies have been conducted on healthy older adults. The results observed in the current study are supported by those reported by Kim and colleagues (2015), who observed an increase in serum irisin levels, when compared to the control group, following a 12-week resistance exercise intervention in a cohort of older adults (65 years and over) (Kim, So, Choi, Kang, & Song, 2015). Further, eight weeks of endurance training (cycling) was associated with increases in serum irisin levels in an older healthy study cohort (mean age 67 years), compared to their younger counterparts (Miyamoto-Mikami et al., 2015). Although I observed an increase in irisin following the PEACS exercise intervention, this research area requires further evaluation. Specifically, future studies should be rigorously designed whereby participants are supervised and monitored during exercise for consistency and reporting-bias purposes. Furthermore, additional blood collection at acute timepoints following an exercise bout would shed light on the acute fluctuations of irisin, within a longer intervention programme. Moreover, a larger cohort is needed in order to reach sufficient statistical power to make definitive conclusions.

As mentioned above, previous studies of acute exercise programmes have consistently demonstrated increases in irisin levels (Dinas et al., 2017). However, irisin levels have been shown to return to normal within a relatively short period of time, often within half
an hour (A. D. Anastasilakis, S. A. Polyzos, P. Makras, et al., 2014; Daskalopoulou et al., 2014; Kraemer, Shockett, Webb, Shah, & Castracane, 2014b; Norheim et al., 2014). I utilised blood samples from the AST cohort to assess levels of serum irisin before a bout of resistance training, immediately after, and 30 minutes post-workout. Irisin levels were not found to be significantly altered by the two different resistance-training regimens that participants were exposed to (H10/1, hypertrophy; S5/3, strength training). Nevertheless, due to the small sample size utilised in this study (n = 17), and thus a likely lack of power to detect a significant time variable, percentage changes from pre- to post- intervention (T1 versus T2 and T3) were also examined. Immediately following H10/1 (hypertrophy) training, mean irisin levels were marginally elevated (T2) in both the ‘younger’ (11.2%) and ‘older’ (6.4%) participants (9.1% overall); however these levels dropped again within 30 minutes of workout completion (T3), to 2.3% above baseline in the ‘younger’ group and below baseline (T1) in the ‘older’ cohort (13.7% below baseline). Following the S5/3 (strength) training, irisin gradually rose over the three timepoints in the ‘older’ cohort, but only marginally: levels rose by 1.7% between T1 and T2, and by 7.1% between T1 and T3. ‘Younger’ participants experienced a marginal drop at both timepoints, by 11.8% between T1 and T2, and by 9.4% between T1 and T3. Thus, the overall elevations observed in the strength protocol were smaller than the hypertrophy protocol. Studies on resistance training have shown that intensity of exercise bouts plays a key role in the increase of basal hormones associated with muscle strength and hypertrophy, including, serum growth hormone, insulin-like growth factor (IGF-1), and testosterone (West et al., 2010); however, this is not a long-term effect. Thus, it could be hypothesised that strength training with fewer repetitions and more recovery time between sets, such as that employed in the S5/3 protocol, is not sufficient to induce release of hormones such as irisin. Further, the release of systemic hormones is proportionate to the size of the muscle
groups being used. In the AST study, the protocol included seven exercises in the following order: bench press, seated row, leg press, military press, latissimus dorsi pull-down (lat pull-down), leg extension, and arm curl. Although the large leg muscles have been targeted in this study and were likely the reason that marginal changes in irisin were observed, the order of exercise types might have influenced the results, particularly in regards to the strength-protocol. The last exercise to be completed was arm curls targeting the relatively small biceps; thus this exercise, combined with the long rest period, means that blood collection would have taken place 15 minutes after completing the last leg exercise in S5/3. Hypothetically, that is enough time to diminish any increases in irisin levels that may have occurred immediately after working out the larger muscle groups.

One study to-date utilised a resistance training programme to evaluate a change of irisin in a human study cohort. Tsuchiya and colleagues reported an increase of irisin following acute resistance training in ten healthy men (Tsuchiya et al., 2014). While the fluctuations in irisin concentrations I observed in my project were not statistically significant, they are consistent with previously reported trends (Gotshalk et al., 1997; Hakkinen & Pakarinen, 1993; Ratamess et al., 2005). Future studies of larger cohorts should also be sufficiently powered to examine if gender differences influence the results. Furthermore, rigorous design of resistance training protocols will be essential, ideally with large muscle groups targeted during the end portions of the program, followed by swift blood collection. Consideration of mechanistic pathways involved in acute induction of irisin will be discussed in detail in Chapter 6 (Section 6.6 page 266).

Although methodological issues may explain the fact that I observed an effect of chronic exercise (PEACS), but not acute exercise (AST) on irisin levels, possible biological factors that may underlie this difference should be considered. Various types of acute
exercised have frequently been associated with elevated levels of irisin in the bloodstream (Daskalopoulou et al., 2014; Huh, Mougios, Kabasakalis, et al., 2014; Huh, Mougios, Skraparlis, Kabasakalis, & Mantzoros, 2014; Huh et al., 2012; Kraemer, Shockett, Webb, Shah, & Castracane, 2014a; Loffler et al., 2015; Nygaard et al., 2015; Tsuchiya et al., 2014). Initially, irisin was thought to be secreted from skeletal muscle cells following exercise, or even as a result of shivering (due to muscle contraction) (Perakakis et al., 2017). The muscle-related molecular mechanism was hypothesized to trigger a Peroxisome proliferator-activated receptor gamma coactivator 1-alpha / Fibronectin type III domain-containing protein 5 (PGC-1α/FNDC5) pathway. Multiple acute aerobic exercise studies have reported a surge in irisin release (and possible expression) that lasts approximately 15 minutes post-exercise cessation (Dinas et al., 2017). Resistance and aerobic exercise studies have both been demonstrated to activate the AMP-activated protein kinase (AMPK) pathway, which has also been linked to irisin production via PGC-1α (Dreyer et al., 2006; Gibala et al., 2009; Huh, Mougios, Kabasakalis, et al., 2014; Lundberg, Fernandez-Gonzalo, & Tesch, 2014). AMPK’s activation is acute and short-lived in muscles, with increased AMPK occurring within minutes (Lee-Young, Koufogiannis, Canny, & McCONELL, 2008). Therefore, it is logical that only acute elevations of irisin were observed in these prior studies. In the AST study described here, the intensity of resistance training may not have been sufficient to activate the pathway leading to irisin production. Thus, explaining why the increases in irisin observed were only marginal. Why I observed a relationship between increased irisin levels and maximal exercise intensity in the PEACS cohort, in which non-supervised, low-to-moderate intensity resistance training was prescribed, but not in a supervised high intensity strength program, remains to be determined. The AST study consisted of a short-term exercise program in young adults, whereas PEACS was conducted in older adults over several
months. There may be an adaptive response by irisin, in which physically fitter and younger people do not show variations in irisin following exercise as acutely as older adults. Additionally, age, gender and BMI were confounding factors in the PEACS cohort analysis, whilst such data was only partially available for the AST cohort. Moreover, irisin levels of the PEACS and AST cohorts were analysed using two different generations of ELISA kits, which showed marked differences in the concentrations of the hormone (range of 400 – 600 ng/ml in PEACS, versus 6 – 18 ng/ml in AST, as detailed earlier). To what degree an unknown factor impacted the measurements in the earlier kit generation, and how comparable the relationships of irisin fluctuations between the kit generations are, is not known.

All three study cohorts in this Chapter had some limitations that need to be considered: 1) all three studies would greatly benefit from larger cohort numbers to validate the findings reported here; 2) the use of BMI as a major covariate is limited in its interpretation as it does not provide an exact ratio and distribution of body fat and lean mass (DXA body composition scans would shed more light on the actual relationship between the presence of adipose tissue and irisin levels); 3) as discussed earlier, any form of self-report questionnaire can introduce bias (nevertheless, the International Physical Activity Questionnaire is a validated tool used in various studies across the world (Faulkner, Cohn, & Remington; Hagströmer et al., 2008; Mannocci et al., 2012; Oyeyemi et al., 2011; Shenoy, Chawla, & Sandhu, 2014)); and 4) the comparison of irisin levels across cohorts is severely limited by the different ELISA kits used for PEACS and AST, respectively.
To summarise, in this Chapter, I observed objectively-measured physical activity intensity and volume to be associated with higher serum irisin levels. Furthermore, a 16-week aerobic and strength training intervention increased irisin levels, compared with the control group. Although these results are promising, the biological pathways linking exercise-induced irisin to brain health require further validation. Additionally, to gain reliable insight into the actual effect of physical activity or exercise on irisin, a larger highly characterised study cohort is needed that records the history of a participant’s fitness level before exposing the individual to bouts of resistance or aerobic exercise.
Chapter Five

*In vitro* investigation of irisin and cell protection in human SH-SY5Y neuroblastoma cells
5.1 Introduction: The effect of irisin on cells in vitro

A hallmark of Alzheimer’s disease is Aβ-accumulation in the brain, which induces neuronal cell death. Considerable research effort has focussed on finding ways to protect neurons against this Aβ-induced toxicity and even promote cell growth and functionality (Hölscher, 2014; Nagahara et al., 2009; Reddy et al., 2017). One important molecule that has been thoroughly characterised with respect to its neuroprotective properties is brain-derived neurotrophic factor (BDNF) (Burns, Johnson, Watts, Swerdlow, & Brooks, 2010). This neurotrophin is crucial for the development of the central nervous system, and regulates neuronal maintenance throughout life (Binley, Ng, Barde, Song, & Morgan, 2016; Faigle & Song, 2013). A plethora of older and newer research studies conducted in humans have shown that lower levels of serum, plasma, and (post-mortem) brain BDNF are present in individuals with preclinical and clinical AD, compared to healthy controls (Budni, Bellettini-Santos, Mina, Garcez, & Zugno, 2015; Tanila, 2017), leading some to postulate that BDNF administration could be an effective treatment for AD. Indeed, cognitive stimulation (Marosi & Mattson, 2014), and physical activity have been shown to increase peripheral BDNF levels in humans as well as in animal models (Huang, Larsen, Ried-Larsen, Møller, & Andersen, 2014). Moreover, synthetically designed BDNF-mimetics have been developed and tested in a small number of animal studies in an attempt to increase BDNF levels or its effects; see (Tanila, 2017) for a recent review. However, further work is required to demonstrate efficient transport of peripheral BDNF (or its mimetic) across the BBB, and thereby optimise its effect on the CNS.

Whilst the neuroprotective effects of BDNF are well-established, the molecular mechanisms triggering BDNF expression are yet to be fully elucidated. Interestingly,
recent studies suggest that irisin is directly linked to BDNF expression (Wrann et al., 2013; Zsuga, Tajti, Papp, Juhasz, & Gesztelyi, 2016). In this Chapter, I therefore sought to determine whether irisin protects human neuroblastoma cells in vitro against Aβ toxicity, and whether this postulated protective effect was mediated via altered BDNF expression. Whilst some studies have shown that irisin is able to stimulate cell proliferation in osteoblasts, pancreatic β cells, endothelial cells and adipocytes (Kerstholt et al., 2015; Qiao et al., 2016; Song et al., 2014; Zhang et al., 2014; Zugel et al., 2016), only one publication to-date has utilised neurons, demonstrating stimulation of hippocampal neuronal cell proliferation following administration of irisin at supra-physiological concentrations (Moon, Dincer, & Mantzoros, 2013). The human neuroblastoma cell line, SH-SY5Y, was selected for the current study as it is frequently used as an in vitro model in AD research (Nordin, 2013). I hypothesized that irisin would protect SH-SY5Y cells against Aβ42-induced cytotoxicity, and that this protective effect would be mediated by increased BDNF expression.
Chapter 5 – Results III: In vitro investigation of irisin and cell protection in human SH-SY5Y neuroblastoma cells

5.2 Methods

5.2.1 Preparation of SH-SY5Y cells for experiments

SH-SY5Y neuroblastoma cells were originally acquired from the American Type Culture Collection (ATCC) Global Bioresource Centre (Manassas, Virginia, USA), and detailed information on this cell line was studied before using them in my experiments. A thorough description of methodologies concerning cell culture technique is also described in Chapter 2 (Sections 2.5.2 – 2.5.3, pages 84-85, 87).

In brief, viable SH-SY5Y neuroblastoma cells grow by adhering to the flask in which they are cultured, as well as by floating in the medium. As per previously published studies, I used the adherent SH-SY5Y cells only (Kovalevich & Langford, 2013). The results presented in this Chapter utilised cells from passages 13 to 19. In brief, cells were grown in T-75 flasks with standard growth medium (DMEM + 10% FCS) until approximately 80% confluent. Culture media was changed every 2-3 days, with the cells inspected daily to confirm healthy growth. Once the cells reached desired confluency, the culture media was aspirated and the cells washed twice with Hanks buffer (pre-warmed to 37°C). Following these washing steps, cell-detachment was induced with 0.25% Trypsin/EDTA before cells were prepared for counting (ViCell™ Cell Viability Analyzer (Beckman Coulter, California, USA).

5.2.1.1 Cell seeding

The cell-medium mix was spun down for 5 minutes at 200 x g, after which the cell pellet was resuspended in a medium with phenol-red free DMEM + 10% FCS, as the activity of Aβ was shown to be inhibited by phenol-red (B. K. Pedersen, 2013). 10,000
cells in a volume of 100 µl were seeded per well in a 96-well plate (Nunc®). Cell numbers were calculated using the formula, \( C_1 \times V_1 = C_2 \times V_2 \).

### 5.2.1.2 Plate preparation for cell viability assays

The cells in the 96-well plate were grown in normal growth medium (without phenol-red) for either 2 days, or when approximately 40-50% confluency was reached. Following this period, the cell culture media was removed, the cells were washed in Hanks buffer, and treatment medium (DMEM + 1% FCS) was added to each well. The treatment medium was tested before commencing experiments to ensure a halt of cell growth, whilst the cells remained in a healthy, viable state. Aβ and irisin were then added to each well according to the plate layout in Figure 2.8 (Chapter 2, Section 2.5.3.3, p. 91), yielding a total volume of 100 µl per well. Each treatment was conducted at least in triplicate, and every experiment was conducted at least three times. Blank and control wells were also prepared. The treatment periods varied from three to five days.

### 5.2.2 Preparation of peptides for treatment of SH-SY5Y cells

#### 5.2.2.1 Aβ\(_{42}\) oligomer preparation

Oligomeric Aβ\(_{42}\) was prepared as per the protocol of Stine and colleagues (Stine, Jungbauer, Yu, & LaDu, 2011). 1 mM Aβ solution was prepared from lyophilized powder by adding Hexafluoro-2-propanol (HFIP). Several incubation and drying steps followed, ultimately resulting in a very fine peptide film adhering to the bottom and sides of the tubes. The films were stored at -20°C, or immediately prepared for use a day later. In order to prepare the oligomeric Aβ\(_{42}\) immediately prior to each experiment,
the peptide was resuspended thoroughly in Dimethyl sulfoxide (DMSO). A 10-minute sonication step in a water bath followed, which was then interrupted by adding cold phenol-free F12 media prior to thorough mixing. The resulting Aβ42 peptide was used no longer than one hour following reconstitution. Before commencement of the experiments, the toxicity of the Aβ42 peptide was thoroughly tested in a time-dose dependent experiment.

5.2.2.2 Irisin peptide preparation

The irisin peptide was purchased from Phoenix Pharmaceuticals (catalogue number EK-067-16, Phoenix Pharmaceuticals, Burlingame, California, USA), and prepared as per the manufacturer’s protocol, which resulted in a solution comprising 20% acetonitrile and water. The peptide solution was aliquoted and stored at -80°C until used for experiments.

5.2.3 Cell viability assays

5.2.3.1 Lactate dehydrogenase (LDH) assay

A CytoTox-ONE™ Homogeneous Membrane Integrity Assay was used (cat.no G7890/1/2; Promega, Madison, USA) to assess cell viability after treatment with cytotoxic Aβ. Lysing occurred by incubating cells with the manufacturer’s lysis buffer for 15 minute at 37°C. During that time, LDH was released into the medium, which was then transferred into a black 96-well plate and mixed with the CytoTox-ONE™ Reagent (made up of the LDH substrate mix provided by the manufacturer), before reading the fluorescence in each well with a FLUOstar OPTIMA Plate reader (BMG Technologies,
Buckinghamshire, UK). The more fluorescence measured per well, the higher the amount of lysed (i.e. non-viable) cells in that well.

5.2.3.2 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay

Following LDH assay cell lysis (described in section 5.2.3.1), the solution was completely aspirated and replaced with MTS. The wells were incubated in MTS for approximately four hours at 37°C, before the absorbance of each well was read using the FLUOstar OPTIMA Plate reader (BMG Technologies, Buckinghamshire, UK), to enable quantification of viable cells.

5.2.4 Western blotting

5.2.4.1 Cell lysate preparation

6-well plates were used to seed 200,000 SH-SY5Y cells in a total volume of 3 ml media per well. Once the cells reached an estimated 40% confluency, the media was changed to fresh growth media with the addition of 5 or 10 nM irisin. Treatment periods lasted for 48 and 72 hours, after which protein from both the media and the cells was collected. In order to retrieve cell lysates, NP40 lysis buffer (50 mM Tris-HCl at pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% NP40-nonidet) was used in conjunction with a protease inhibitor cocktail (5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.1 mM PMSF (phenylmethylsulfonyl fluoride); Complete®). All cell lysis steps were conducted on ice, with ice-cold lysis and washing buffers used. The cells were incubated in NP-lysis buffer for a total of 25-30 minutes, during which time the wells were scraped thoroughly and the lysate transferred
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into Eppendorf tubes and vortexed regularly. This incubation time was followed by a spin at 200 g for 10 mins at 4°C. The supernatant was collected and stored at -80°C for subsequent analysis.

5.2.4.2 Protein assay

Protein assays to determine the protein concentration in each cell lysate sample were conducted, prior to each Western blot experiment, using the Pierce Micro BCA™ Protein Assay Kit (catalogue number 23235, Thermo Scientific, Rockford, USA). A standard curve was produced using standards provided within the Assay Kit to determine the protein concentration in every cell lysate sample. Plates were read using a FLUOstar OPTIMA Plate reader (BMG Technologies, Buckinghamshire, UK) at an absorbance of 550 nm. A standard curve with $r = 0.99$ or higher was deemed sufficient to proceed and calculate exact protein concentrations per sample (Example, Figure, 5.1). Protein concentrations were calculated in μg/ml. A minimum of 25 μg of protein per sample was needed to conduct a Western blot experiment.

![Graph showing a standard curve for protein assay.](image)

**Figure 5.1:** An example standard curve to determine protein concentrations in cell lysates; here, $r = 0.9971$. 

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5.2.4.3 Western blotting

Tris-Tricine gels were either made of a partly 12% and partly 8% resolving gel and a 4% stacking gel, or, pre-made Navix Nu®PAGE 4-12% Bis-Tris gels were used. Anode buffer (2 M Tris) and cathode buffer (Tris-Tricine) were used to allow for conduction of an electrical current. Novex Sharp ladder was used as a molecular weight marker, indicating different sized proteins. The protein samples were mixed with a sample buffer made of Bis-Tris, Bicine, SDS, Sucrose, Bromophenol Blue and 2.5% β-Mercaptoethanol and heated at 95°C for 5 minutes prior to loading into gel wells. The gel dock was run at 80 Volts through the stacking gel and at 120 Volts through the resolving gel for 2 hours. For the pre-cast gels, protein lysate samples were prepared with Nu®PAGE sample buffer. The dock was run for 2 hours at 90 Volts, after which time resolved proteins were transferred onto nitrocellulose membrane. The transfer occurred at 250 mA for approximately 16 hours at 4°C. The membrane was then blocked in 5% skim milk in TBS for one hour at room temperature, followed by a two-hour incubation with the primary antibody (i.e. Rb pAb to BDNF, ab46176, Abcam, Cambridge, UK) in 5% skim milk/TBS at room temperature, at an antibody dilution of 1:1,000. Three 10-minute washing steps with TBS-T followed, before the addition of the secondary antibody, Goat anti-rabbit IgG (HRP) (ab97051, Abcam, Cambridge, UK), at a 1:10,000 dilution for one hour at room temperature. Three more washing steps with TBS-T, and a quick rinse with TBS to remove the tween, preceded the detection of protein bands, using an Amersham™ ECL™ Western blotting detection kit (GE Healthcare, Parramatta, NSW, Australia), coupled with Amersham Hyperfilm™. The membrane was subsequently stored in TBS at 4°C until further use. Each membrane was later stripped with Restore Plus Stripping Buffer (Thermo Scientific, Rockford, USA), prior to incubation with a GAPDH antibody (catalogue number 2118, Cell
Signaling Technology®, Danvers, Massachusetts, USA), which following secondary antibody incubation and protein band detection, was used to ensure loading of equal protein concentrations into each well.

5.2.5 Statistical analyses

The mean and SEM of raw results were calculated for every triplicate in each cell viability assay. For the LDH and MTS assays, a minimum of three individual experiments were conducted. Normalised results were computed for every experiment in order to present data from all the experiments combined. A comparison between different treatment groups in these assays was conducted via independent t-test, using IBM® SPSS® Statistics 23.0 for both Mac and Windows 7 (IBM Corp. Released 2015; IBM SPSS Statistics for Mac and Windows, Version 23.0. Armonk, NY: IBM Corp). A p-value below 0.05 was deemed significant. For the Western blot analysis, semi-quantitative assessments of the thickness and density of protein bands was conducted using the ChemiDoc™ Touch Imaging System and its software Image Lab, Version 5.1 build 8 (Bio-Rad Laboratories, Gladesville, NSW, Australia).
5.3 Results

5.3.1 Irisin and SH-SY5Y cell viability

In order to first investigate whether irisin affected SH-SY5Y cell proliferation or viability, I administered physiological concentrations of irisin (10 nM) to SH-SY5Y cells for three or four days. Neither cell proliferation nor cell death was observed during this time period (Figure 5.2; MTS (3 days): $t = 0.21, p = 0.84$; MTS (4 days): $t = 0.26, p = 0.91$). By contrast, supraphysiological concentrations of irisin (50 nM) resulted in increased cell viability after three days of treatment, compared to untreated cells, as determined by MTS and LDH assays respectively (Figure 5.3; MTS: $t = -2.19, p = 0.04$; LDH: $t = 2.47, p = 0.025$). Since I demonstrated that irisin is not cytotoxic, I consequently used this peptide within the concentration range tested, for further experiments (Figures 5.4 – 5.6).
Figure 5.2: Physiological concentrations of irisin (10 nM) have no effect on SH-SY5Y cell viability after a) three, or b) four days. Mean and SEM are summarised from n = 3 experiments. Abbreviations: MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl) -2-(4-sulfophenyl)-2H-tetrazolium; nM, Nanomolar.
Figure 5.3: Supraphysiological concentrations of irisin (50 nM) increased cell viability in SH-SY5Y cells after three days, as determined by a) MTS, and b) LDH assays. Mean and SEM are summarised from n = 3 experiments. *p-values were determined by independent t-test. Abbreviations: LDH, Lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; nM, Nanomolar.
5.3.2 Aβ-induced toxicity is reduced by irisin in SH-SY5Y cells

Following demonstration that irisin is non-toxic to SH-SY5Y cells, and actually potentially plays a protective role in this cell line, I sought to investigate whether irisin maintains cell viability and protects against cell toxicity induced by Aβ42. Concentrations of 1-20 μM of Aβ42 were administered over a three to five day period to treat healthy, viable SH-SY5Y cells. Simultaneously, some wells treated with Aβ42 were also exposed to 10 nM irisin for the same amount of time. Following treatment with Aβ42 +/- irisin, MTS and LDH-assays were performed on the same day with the same sample plate at each time point, as described in section 5.2.3.

As expected, overall decreased cell viability was observed with both the MTS assay, and the LDH assay, with increasing concentrations of Aβ42 (Figures 5.4 - 5.6). Specifically, after three days of treatment with Aβ, I saw a gradual decline of cell viability in the 10–20 μM treatment groups, but not in the 1–5 μM treatment groups, in both, irisin-positive and irisin-negative wells. Overall, wells with or without 10 nM irisin showed no significant differences in cell viability patterns (Figure 5.4). In wells only treated with Aβ42, reduction of viable cells at 10 μM was 9.5%, at 15 μM it was 47%, and at 20 μM, 81.7% reduction was observed. Similarly, when additionally treated with 10 nM irisin, the 10 μM Aβ42 + irisin treatment group still demonstrated a 9.5% reduction in viable cells, while 15 μM Aβ42 + irisin induced a 41.5% reduction in viable cells, and 20 μM Aβ42 + irisin caused a nearly 70% reduction in viable cells, compared to the untreated group, in both irisin-positive and negative wells (Figure 5.4a). These results were matched by corresponding LDH-assays that showed increased cell death by a factor of 1.3, 2 and 2.6 in wells exposed to 10, 15 and 20 μM Aβ42, respectively, when no irisin was added. Adding 10 nM irisin did not ameliorate Aβ42-induced cell toxicity,
with increased cell death, compared to untreated cells, occurring at a factor of 1.5, 2.2 and 2.9 in wells treated with 10, 15 and 20 μM Aβ42, respectively.

After four days of treatment with 10 μM Aβ42, a 53-55% reduction in MTS assay-determined cell viability, compared to untreated cells, was observed, irrespective of the presence or absence of irisin (Figure 5.5). Significantly greater cell death was observed in the 15 μM Aβ42 treatment group, and in the 20 μM Aβ42 treatment group (84-86% and 90-97% respectively), both in the presence or absence of irisin: there were no significant differences in MTS assay-determined cell viability between the irisin – and + treatment groups (independent t-test; p > 0.05). As mentioned previously, LDH-assays generally supported the MTS results by showing increased Aβ42-toxicity, compared to untreated wells, at a factor of 2.2, 2.9, and 2.8, respectively, in wells treated with 10, 15, or 20 μM Aβ42 alone. However, in the wells to which irisin was added, this Aβ-induced toxicity was lessened to factors of 1.6, 2.4 and 2.4, respectively, for 10, 15, or 20 μM Aβ42, compared to untreated cells. Student’s unpaired t-tests showed significant differences in this LDH assay-determined Aβ-induced cell toxicity between the irisin-positive and irisin-negative treatment groups at 1, 5 and 10 μM Aβ42: t(1 μM Aβ42) = -3.4, p = 0.004; t (5 μM Aβ42) = -4.1, p = 0.001; and t (10 μM Aβ42) = -2.9, p = 0.01, see Table 5.1.

Five days of treatment with Aβ42 induced 77, 87 and 92.7% reduced MTS assay-determined cell viability in the absence of irisin, in wells treated with 10, 15, or 20 μM Aβ42 respectively (Figure 5.6). The addition of irisin ameliorated this Aβ42–induced toxicity, with 61, 66 and 84% reduced viability observed among the 10, 15, and 20 μM Aβ42 treatment groups. However, these inter-group differences were not significant at this
5-day time-point. Again, LDH-assays supported the MTS-assay determined cell viability results, with increased cell death observed at increasing Aβ42 concentrations.

Overall, concentrations of 10-20 μM Aβ42 caused significant cell death after only three days of treatment, whilst Aβ42 at concentrations of 1 and 5 μM demonstrated cytotoxic effects after four days. Three day incubations were not sufficient to observe significant differences between irisin-treated and untreated cells exposed to Aβ42. However, five day incubations appeared excessive, meaning that a maximum Aβ-induced toxicity was reached, which was no longer distinguishable between the wells treated with 15, and 20 μM Aβ42. Consequently, four days of treatment with 1-20 μM Aβ42 appeared to be the most appropriate duration of incubation to assess dose-dependent effects of Aβ42, on cell viability / toxicity, as well as to assess the effect of irisin co-treatment on this relationship. Furthermore, despite some apparent protection induced by irisin against Aβ-toxicity, 10 nM irisin did not appear to directly affect cell proliferation, as shown by the control groups (Ctrl 2) in every experiment.
Figure 5.4  

**a)** MTS, and **b)** LDH assays showing SH-SY5Y (P14-16) cell viability, after 3 days of treatment with Aβ (1 μM to 20 μM) +/- 10 nM irisin. Graphs represent mean +/- SEM of 3 separate experiments. Abbreviations: Aβ, amyloid-β; Ctrl 1, control 1 (positive control, lysis buffer); Ctrl 2, control 2 (negative control, 10 nM irisin only); LDH, Lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.
Figure 5.5 a) MTS, and b) LDH assays showing SH-SY5Y (P14-16) cell viability, after 4 days of treatment with Aβ (1 μM to 20 μM) +/- 10 nM irisin. Graphs represents mean +/- SEM of 3 separate experiments. Abbreviations: Aβ, amyloid-β42; Ctrl 1, control 1 (positive control, lysis buffer); Ctrl 2, control 2 (negative control, 10 nM irisin only); LDH, Lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.
**Figure 5.6** a) MTS, and b) LDH assays showing SH-SY5Y (P14-16) cell viability, after 5 days of treatment with Aβ (1 μM to 20 μM) +/- 10 nM irisin. Graphs represent mean +/- SEM of 3 separate experiments. Abbreviations: Aβ, amyloid-β; Ctrl 1, control 1 (positive control, lysis buffer); Ctrl 2, control 2 (negative control, 10 nM irisin only); LDH, Lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.
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**Table 5.1:** Irisin (10 nM) ameliorated Aβ-induced cell toxicity after 4 days of treatment

<table>
<thead>
<tr>
<th>[Aβ] / μM</th>
<th>- Irisin</th>
<th>+ Irisin</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25.2</td>
<td>13.7</td>
<td>-3.41</td>
<td>0.004</td>
</tr>
<tr>
<td>5</td>
<td>23.0</td>
<td>16.0</td>
<td>-4.14</td>
<td>0.003</td>
</tr>
<tr>
<td>10</td>
<td>65.7</td>
<td>52.8</td>
<td>-2.93</td>
<td>0.036</td>
</tr>
<tr>
<td>15</td>
<td>87.9</td>
<td>83.8</td>
<td>-1.12</td>
<td>0.28</td>
</tr>
<tr>
<td>20</td>
<td>95.0</td>
<td>90.2</td>
<td>-0.79</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Four days of treatment of SH-SY5Y cells with either Aβ only (1-20 μM), or Aβ and 10 nM irisin, resulted in significant differences in cell toxicity versus untreated wells, as assessed by LDH assay. Between group differences were determined using student’s unpaired t-tests. A p-value < 0.05 was deemed significant (bold).
5.3.3 Irisin stimulates BDNF expression in SH-SY5Y cells

Having determined, in previous sections, that the addition of 10 nM irisin can yield higher cell viability without increasing the cell number in each well, I sought to investigate a possible mechanism responsible for these observations. I therefore conducted Western blot analysis to examine changes in BDNF levels after SH-SY5Y cells were exposed to 5-10 nM irisin for two or three days. I examined both cell lysates and the medium in which cells were incubated, and detected protein bands after 48 and 72 hours following treatment with irisin, using an α-BDNF antibody (Ab46176, Abcam®); see Figure 5.7. Band sizes of approximately 28 kDa and ~56 kDa were detected as early as 48 hours in lysate samples, consistent with the detection of proBDNF, and a dimerized version of the proBDNF protein respectively (Figure 5.7a). Levels of proBDNF increased significantly by a factor of 1.6 following 5 nM irisin treatment, and by a factor of 2 following 10 nM irisin treatment, compared to untreated cells; relative quantitative assessment, student’s unpaired t-test, $t = 15.84$, $p = 0.004$ (Table 5.2). Whilst an increase in dimerised proBDNF was also observed in cell lysates, this increase was only at trend level ($t = 3.37$, $p = 0.08$). Interestingly, the mature form of BDNF (mBDNF; 14 kDa) was only detected at very low levels in the cell lysates. Further, additional bands were clearly detected at ~37 and ~42 kDa, likely representing glycosylated proBDNF or even pre-proBDNF-forms, thereby indicating a highly activated BDNF-expression machinery in the cells. However, in the cell media, no BDNF was detected after 48 hours.

After 72 hours, intracellular BDNF-levels were no longer significantly different between untreated cells and those cells treated with 10 nM irisin (Figure 5.7b).
However, in the media, bands equivalent to the size of dimerised BDNF (~56 kDa) were increased 2.7-fold in density after 72 hours of exposure to 5-10 nM irisin; student’s unpaired t-test, $t = 5.65, p = 0.03$ (Figure 5.7c). No other bands were detected in the media after 72 hours of irisin treatment. Re-probing of nitrocellulose membranes using an antibody to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), demonstrated that observed differences in levels of BDNF species between treatment groups were not due to loading of unequal quantities of protein (right hand panel of Figures 5.7 a and b).
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a)

Irisin treatment (48 hours): lysate

Dimerised BDNF

proBDNF

mBDNF

GAPDH

b)

Irisin treatment (72 hours): lysate

Dimerised BDNF

proBDNF

GAPDH
Figure 5.7: a) Western blot analysis revealed bands at the expected sizes of proBDNF and mature BDNF in the cell lysate. Administration of 5 nM or 10 nM irisin for 48 hours increased BDNF levels in SH-SY5Y cells compared to untreated cells. b) Western blot analysis revealed bands at the expected sizes of proBDNF and dimerised BDNF in the cell lysate after 72 hours of incubation with and without irisin (10 nM). c) Western blot analysis revealed bands only at the 56 kDa mark in cell medium, which is likely to represent dimerised BDNF. Levels of this dimerised BDNF were increased after 72 hours of incubation with 5 nM or 10 nM irisin compared to untreated cells. In summary, 5 nM and 10 nM irisin treatments increased BDNF species levels in SH-SY5Y cells as early as 48 hours in cell lysates, and within 72 hours in cell medium. Images captured using the ChemiDoc™ Touch Imaging System device. GAPDH image obtained by scanning the biofilm with a Bio-Rad GS-800 scanner (Software: Quantity One). Representative Western blots shown. Abbreviations: BDNF, brain-derived neurotrophic factor; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; kDa, kilo-Dalton; mBDNF, mature form of BDNF; nM, nanomolar; PC, positive control (BDNF peptide); proBDNF, BDNF-precursor protein; UN, untreated.
Table 5.2: Relative quantitative assessment of BDNF bands, from Western blots, in cell lysate and medium after 48 and 72 hours of irisin treatment

<table>
<thead>
<tr>
<th>Intracellular (cell lysate)</th>
<th>Untreated (48 h)</th>
<th>5 nM irisin (48 h)</th>
<th>10 nM irisin (48 h)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>proBDNF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative quantitative assessment (average)</td>
<td>1.07</td>
<td>1.69</td>
<td>2.15</td>
<td>0.004</td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>158</td>
<td>202</td>
<td></td>
</tr>
<tr>
<td>Dimerised BDNF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative quantitative assessment (average)</td>
<td>0.94</td>
<td>1.37</td>
<td>1.72</td>
<td>0.08</td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>146</td>
<td>183</td>
<td></td>
</tr>
<tr>
<td>Extracellular space (medium)</td>
<td>Untreated (72 h)</td>
<td>5 nM irisin (72 h)</td>
<td>10 nM irisin (72 h)</td>
<td>p-value</td>
</tr>
<tr>
<td>Dimerised BDNF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative quantitative assessment (average)</td>
<td>1.33</td>
<td>1.73</td>
<td>3.64</td>
<td>0.03</td>
</tr>
<tr>
<td>%</td>
<td>100</td>
<td>130</td>
<td>274.3</td>
<td></td>
</tr>
</tbody>
</table>

A relative quantitative assessment of BDNF-bands, from Western blots, revealed a significant increase in BDNF in the cell lysate following 48 hours of treatment with 5 or 10 nM irisin, in SH-SY5Y cells. BDNF was also released into the extracellular space (medium) before cells were lysed following 72 hours of treatment with 5 or 10 nM irisin. Relative quantity was assessed using ChemiDoc™ Touch Imaging System. Abbreviations: BDNF, brain-derived neurotrophic factor; h, hours; nM, nanomolar; proBDNF, BDNF-precursor protein.
5.4 Discussion

In this Chapter, I sought to investigate whether irisin could confer cell protection in human SH-SY5Y neuroblastoma cells; a well-used model in AD research. I was specifically interested to determine, whether irisin could confer protection against Aβ42-mediated cell toxicity, and whether irisin could modulate cell proliferation. I observed that; 1) physiological concentrations of irisin could indeed confer a degree of protection to SH-SY5Y cells exposed to Aβ42, 2) that supra-physiological concentrations of irisin can stimulate growth of SH-SY5Y cells, and that 3) irisin promotes BDNF expression in these cells, thereby supporting the current notion that irisin is directly linked to levels of BDNF and is a crucial preceding step in the BDNF expression pathway.

It has been previously reported that irisin can promote cell proliferation in osteoblasts, endothelial cells and even in mouse hippocampal cells (Moon et al., 2013; Qiao et al., 2016; Song et al., 2014). However, these studies used irisin concentrations that were not physiological; specifically 20 nM irisin. The treatment medium (1% FCS/DMEM) used in my experiments was shown to halt cell proliferation, as tested prior to commencing the experiments reported in this Chapter. Nevertheless, exposure of my neuroblastoma cells to supra-physiological concentrations of irisin (50 nM), induced cell growth regardless of the treatment medium used. Physiological irisin concentrations did not however, induce cell proliferation. These results should be interpreted with caution. It is possible that my results are skewed by the type of treatment medium utilised. Nonetheless, it is also conceivable that an ‘acute trigger’ of irisin expression or activation would be needed to induce high enough levels of irisin to mediate cell proliferation.
Particularly interesting, however, is the fact that I observed a degree of cell protection against the cytotoxic effects of Aβ, mediated by co-incubation with irisin (10 nM). When translating these *in vitro* results to a hypothetical scenario in humans, if irisin can 1) trigger cell proliferation, and 2) protect cells against Aβ-toxicity, then regular bursts of irisin induced for example by physical activity, could be beneficial for maintaining brain health, particularly in older adults when age-related accumulation of cerebral Aβ can manifest. Taking this a step further, it is conceivable that irisin therapy could induce BDNF levels and protect against neuronal damage and thus cognitive decline.

Notably, the protective effect of irisin against Aβ-toxicity was only observed at 4 days, and not at 3, or 5 days of co-incubation. The exact reason for this timeframe of effect is unknown, however, it seems likely that it relates to the Aβ concentration used in the experiments. Our lab regularly observes the first signs of Aβ-induced toxicity after three days of incubation, at the concentrations utilised in this study, with major effects of toxicity, without excessive cell death, usually observed after four days. At five days, Aβ-induced toxicity is usually so far advanced that most cells are dead, and measurements are difficult to interpret. Thus, at 3 days, the cells are possibly too healthy for irisin to have a significant effect, whilst at 5 days, the cell death is likely too advanced for irisin to counter; hence, 4 days appears to be optimal for the protective effect of irisin against Aβ-toxicity to be observed. Future experiments utilising different concentrations of irisin will determine whether this protective effect could be further enhanced.

In this Chapter, I also showed for the first time, that irisin, at low to moderate concentrations, promotes the expression of BDNF in human neuroblastoma cells. Irisin at 10 nM was sufficient to increase BDNF expression significantly within two days, and
may therefore be linked to the irisin-mediated protective effect observed in the cell viability assessments. The detection of multiple bands during the Western blot analysis is likely to be explained by the presence of several forms of BDNF, i.e. the BDNF precursor protein proBDNF, as well as dimers of BDNF. This could be an indicator that irisin has a perpetual role in the body, promoting induction of the BDNF signalling pathway, and therefore, may be beneficial in the signalling network of ongoing cell maintenance and cell-cell communication. BDNF-mediated protection against exposure to Aβ has previously been reported (Arancibia et al., 2008); the results described in this Chapter suggest that activation of irisin may be a necessary preceding step in this process. However, the exact concentration of irisin required to initiate sufficient BDNF-expression, to mediate full protection against Aβ, remains to be determined. Of note, only large bands of approximately 56 kDa, likely representing dimerised BDNF, were detected in cell media following irisin treatment. This is potentially an important observation, as it was shown by Pang and colleagues, that whilst proBDNF can be processed intra- and extracellularly, extracellular processing is a crucial mechanistic pathway in the induction of long-term potentiation (L-LTP) (Pang, Nagappan, Guo, & Lu, 2016): a process intrinsic to learning and memory formation.

I observed increased intracellular BDNF within the first 48 hours following administration of irisin. Yet, at 72 hours post-irisin treatment, levels of intracellular BDNF were no longer significantly different from untreated cells. However, increasing amounts of extracellular BDNF (56 kDa), at 72 hours, suggests dimerization of proBDNF, and an efflux into the extracellular space. Extracellular proBDNF-processing could possibly be due to exhaustion of intracellular processing capacities, leading to a change in processing pathways. Whilst the impact of the different processing pathways is
not yet fully understood (Park & Poo, 2013), it is clear that differences in intracellular and extracellular processing of BDNF might be more important than we currently understand. Here and elsewhere, irisin served as a trigger of BDNF-expression, and the observation that proBDNF can be detected intra- and extracellularly, might be further indication of irisin’s potential involvement in the initiation and maintenance of L-LTP.

Within the scope of this Chapter, technical difficulties with in vitro experiments were addressed and minimized before results were included. Most importantly, the toxicity of oligomeric Aβ42, and the effectiveness of treatment medium, was verified before commencing the experiments presented here. I chose an upper concentration of 20 µM of Aβ because it has shown the most consistent results in exhibiting cytotoxicity in our lab, and it is a concentration that has also frequently been used in the literature (L. Pedersen & Hojman, 2012; Porter et al., 2016). However, it is not clear how comparable the toxicity of 20 µM in vitro is to the toxicity of Aβ in the human brain. A recent study reported that extracellular Aβ can induce cell death via p75 neurotrophic receptor activity (p75NTR) (Yamamoto et al., 2007). A possible scenario is that irisin is taken up by the cells to initiate BDNF expression, resulting in cell-promoting pathways at the same time as p75NTR is active via Aβ-triggers. These simultaneously occurring opposing events could potentially form the basis of the degree of cell protection against Aβ-toxicity observed in my experiments following irisin treatment.

One notion is that irisin could help neurons to neutralize the effects of Aβ via the BDNF-pathway, e.g. by increasing enzymatic activity to reduce Aβ production (Nigam et al., 2017). Alternatively, it could be speculated that irisin directly protects neurons by inhibiting extracellular Aβ-binding to the cell surface (e.g. Rage), by binding to the same
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receptors as Aβ, and thus, inhibiting Aβ-influx into the intracellular space. The binding activity of irisin has not been investigated to-date, leaving room for speculation, but a structural analysis hints towards receptor-binding activity of irisin (Schumacher, Chinnam, Ohashi, Shah, & Erickson, 2013). If irisin is able to bind to more than one receptor, it is possible that in humans, an influx of irisin from the bloodstream into the brain, via the blood-brain barrier (BBB) occurs, which would leave higher levels of extracellular irisin in the brain upon stimulatory triggers like exercise. Surges of higher amounts of extracellular irisin in the brain following bursts of stimuli, and crossing of the BBB, in combination with intracellular irisin in neurons, could lead to ongoing brain protection via regular increases of BDNF-production and / or alterations in receptor activity relating to neurotoxicity.

Whilst this Chapter provides valuable insight into irisin’s potential for protecting human neuroblastoma cells from Aβ toxicity, and thereby, potentially the AD brain, a great deal more work remains in order to establish an exact mechanistic model. The signalling cascade that stimulates irisin cleavage, and its subsequent effects, is yet to be fully elucidated. The identification of polymorphisms of the FNDC5 and BDNF genes will likely assist in understanding how ‘effective’ irisin is expressed, cleaved, and functions. The results of this Chapter, as well as Chapters 3 and 4, support the idea that investigating irisin is an exciting novel approach for the prevention and treatment of AD. This idea also dovetails with our current understanding of lifestyle factors, particularly exercise, which demonstrate considerable promise for maintaining brain health. One crucial step will be to identify the receptor that irisin binds to, and if multiple receptors are involved in yielding multiple effects of irisin. In this regard, it will be of considerable importance to determine when and how irisin crosses the BBB (if indeed it does). Furthermore,
establishment of the levels of irisin required to trigger brain health promoting activity in humans will be another important subject of future investigations.
Chapter Six

General Discussion and Future Directions
6.1 Introduction

Since the initial discovery of irisin (Bostrom et al., 2012), subsequent research has reported that irisin plays a vital role in the browning of white adipose tissue, promoting heat and energy production, and ultimately protecting the body from insulin-resistance and obesity (Panati, Suneetha, & Narala, 2016). These first observations led to the notion that irisin plays a role in mediating the beneficial effects of physical activity on the human body (Polyzos, Kountouras, Shields, & Mantzoros, 2013). The fact that physical activity is health promoting on numerous levels is widely recognised by health experts, and therefore, regular physical activity is implemented in healthcare policies by governments worldwide (Colaianni, Colucci, Cinti, & Grano, 2016; Kraus et al., 2015; Reis et al., 2016). Of particular interest in the field of Alzheimer’s disease (AD) research, is the beneficial impact of physical activity on brain health (Benedict et al., 2013). However, a lack of understanding still persists as to how these effects on the brain are mediated at a molecular level (Brown, Peiffer, & Martins, 2013; Erickson, Leckie, & Weinstein, 2014). In order to further investigate and characterise potential key mechanistic molecular players in exercise-induced brain changes, it appeared logical to investigate the role of irisin within the context of a neurological disorder, such as AD. Thus, in this PhD project, I focussed on investigating the role of irisin in relation to cognitive abilities, physical activity levels and biomarker profiles associated with AD, in a high-risk group, namely older adults over 60 years of age.

Investigations into the relationship between irisin and cognition, demographic characteristics, and blood-based biomarkers, revealed an Apolipoprotein E (APOE) ε4 - and gender-dependent effect of irisin on 1) attention, 2) body mass index (BMI), 3) total cholesterol, and 4) Low-density lipoprotein (LDL)-cholesterol. Following on from this,
I investigated the relationship between irisin and physical activity levels, with varying results, depending on the cohort examined. Basal serum irisin levels were associated with more intense and greater volume of objectively measured habitual physical activity in the PEACS cohort. Furthermore, in this cohort, irisin levels were shown to be increased following a 16-week exercise intervention, incorporating a regular aerobic and resistance exercise program undertaken on several days per week. Nevertheless, I did not observe any acute changes in irisin levels following resistance exercise in a pilot study of ‘younger’ adults (AST cohort). Moreover, in the AIBL cohort, I saw a trend towards a negative association between irisin levels and objectively measured PA intensity. As discussed later, in section 6.5, irisin has been reported to act acutely, providing a potential explanation for the disparate results observed. However, maximal PA intensity was inversely associated with BMI in the AIBL cohort; a result that is likely to have influenced the negative trend in the linear regression model for irisin and maximal count, particularly given that adipose cells also release irisin (discussed in Section 1.2.3, page 28).

In the quest to identify and characterise key mechanistic molecular players that contribute to the beneficial effects of exercise on the brain, past research has focussed on the actions of the most important neurotrophic factor in the brain; brain-derived neurotrophic factor, or BDNF (Bherer et al., 2013). BDNF is ubiquitously expressed, and plays many key roles intrinsic to developing and maintaining neuronal tissue (Baydyuk & Xu, 2014; Marosi & Mattson, 2014; Van Kanegan et al., 2014). Interestingly, in my project, irisin was shown to trigger the expression of BDNF in human neuroblastoma cells, and to mediate a protective effect against Amyloid-beta (Aβ)-induced toxicity.
To my knowledge, this thesis is the first investigation into the role of irisin as a biomarker of brain health, and provides a first glimpse into a seemingly complex relationship between irisin, cognition and physical activity levels, in a large observational cohort of older adults, as well as following exercise interventions in two studies of ‘older’ and ‘younger’ adults. In addition to emphasising this complex relationship, the results presented in this thesis will likely inform the design of future studies aimed at further investigating the role of irisin in maintaining brain health.
6.2 Does irisin demonstrate potential as a blood biomarker of Alzheimer’s disease?

In Chapter 3, I examined the relationship between levels of serum irisin and particular demographic characteristics, and medical information (i.e., age, gender, BMI, years of education, standard alcohol consumption, APOE ε4 carrier status, as well as geriatric depression scores, medical history, and brain Aβ-burden as assessed by positron emission tomography (PET)-brain imaging), in order to characterise the relationship between irisin levels and these variables in an older study cohort for the first time. This investigation provides a platform for the identification of potential confounding variables for future investigations of irisin as a candidate biomarker of brain health and AD. Identifying new biomarkers of AD is crucial for early intervention strategies (Hampel et al., 2010). Not only must a biomarker have the ability to accurately distinguish people with AD from those with other brain conditions, and also from cognitively healthy subjects, an ideal biomarker should also be able to indicate changes in brain health before the onset of clinical symptoms (Frank & Hargreaves, 2003). Although sensitive and specific biomarkers of AD, such as Aβ_{42} and tau, have been identified, the applicability of the associated tests present challenges, as these biomarkers are measured in cerebrospinal fluid (CSF) retrieved invasively via lumbar puncture, or are quantified from brain scans, which involve the administration of costly, short-lived radioactive tracers (Naylor et al., 2012). Thus, research into AD biomarkers places considerable emphasis on the development of widely applicable and cost-effective detection methods: For this reason, development of a blood test is favoured (Henriksen et al., 2014; Snyder et al., 2014). While many blood-based biomarkers have been examined, an adequately sensitive and specific AD blood test has not been developed, highlighting the need for the investigation of novel blood biomarker candidates. Indeed, irisin to-date has not been investigated as a potential biomarker of brain health. Therefore, for the first time, I investigated serum
irisin levels in a cohort of older adults aged over 60 years, with and without a diagnosis of AD, in conjunction with neuropsychological and medical assessments.

Previous research has indicated that lower irisin levels are associated with obesity and the onset of Type 2 diabetes mellitus (T2DM) (Choi et al., 2013; Liu et al., 2013; Moreno-Navarrete et al., 2013). Due to the strong links between AD, obesity and T2DM, I hypothesised that individuals with AD would have lower levels of irisin compared with the cognitively healthy cohort. However, I only observed a difference between these two participant groups (within the AIBL cohort) at trend level, whereby the healthy control (HC) group had higher mean irisin levels compared with the AD group. Although not statistically significant, the difference does reflect reports in the diabetes literature, where higher irisin levels are observed in “healthy” non-diabetics (de Alencar et al., 2017). When stratifying the AIBL cohort by gender, cognitively HC females had higher mean irisin levels than AD females; however, no difference was observed between the AD and HC males. Furthermore, I also observed an effect of age on serum irisin levels, in which higher levels were observed in those aged less than 75 years, compared with those aged 75 years or more; again supporting the notion that higher irisin levels may reflect a “healthier” outcome.

The AIBL study cohort is a highly characterised longitudinal study of ageing, indicating that incorrect diagnoses were unlikely a mitigating factor in the results reported here. It is conceivable that the lack of significant difference in serum irisin levels between the HC and AD groups, in the total cohort, may be due to sample size, specifically the AD group was relatively small ($n = 92$), compared with the HC group ($n = 201$), which may indicate a lack of adequate statistical power; although, a gender-contingent relationship between
irisin levels and clinical classification was still able to be observed. Furthermore, irisin levels in individuals with AD may be influenced by the effect of AD pathology on normal physiology, e.g. in this instance, a decline in the integrity of the blood-brain-barrier (BBB) may be relevant (Henriksen et al., 2014). The level of functionality of the BBB would play a vital role in levels of most potential biomarkers measured in the blood that are thought to originate in the brain. To-date, assessing the degree of BBB-degradation in an individual throughout the progression of AD, or even before clinical diagnosis, remains difficult; however, this concept should be considered relative to the ability of the investigated molecule to cross the BBB (the ability of irisin to cross the BBB is discussed further on page 256; Henriksen et al., 2014). Alternatively, the results presented in this thesis may indicate that circulating irisin levels are not directly associated with AD neuropathology, and that irisin may not serve as a useful biomarker when neurodegeneration is at an advanced stage. Nevertheless, the association between irisin and AD clearly warrants further investigation in well-characterised cohorts, and in individuals in the pre-clinical stages of AD (i.e. individuals who are cognitively ‘normal’ yet have evidence of AD pathology on brain scans), in order to assess its suitability as a predictive biomarker. It would also be beneficial to analyse irisin in a physical activity cohort of PiB-PET imaged study participants: the small subset of participants with available PiB-PET results in this study limited my ability to draw conclusions.
6.3 The relationship between serum irisin and cognition in cognitively healthy older adults

In order to investigate the applicability of irisin as a biomarker of brain health, I thoroughly investigated the relationship between serum levels of irisin and cognitive performance in cognitively healthy older adults. This work describes, for the first time, that higher levels of irisin are associated with better performance on tasks of attention in cognitively healthy APOE ε4 non-carriers. To-date, few other studies have assessed the relationship between irisin and cognition. Belviranli et al. reported an association between better performance on a relatively simple measure of global cognition (Mini Mental State Examination; MMSE) and higher levels of plasma irisin in younger male adult athletes (Belviranli, Okudan, Kabak, Erdogan, & Karanfilci, 2016). Nevertheless, the low variability in MMSE scores (i.e. there is a strong ceiling effect within this test), particularly in a young adult cohort, makes the interpretation of these reported results difficult. A more recent study utilised an online cognitive assessment tool (Lumosity™) in a cohort of young adults, and found a positive relationship between plasma irisin levels and better performance on a customised, shortened version of the Lumosity™ NeuroCognitive Performance Test (Boland, Dolezal, Garfinkel, & Cooper, 2017). This short cognitive test battery includes measures of processing speed, memory, attention, mental flexibility, and problem solving.

A slowing of processing speed is significantly associated with advancing age (Albinet, Boucard, Bouquet, & Audiffren, 2012; Lu et al., 2013). Thus, it is possible that due to the age of the cohort in my cross-sectional study, subtle changes in serum irisin levels were sufficient to be indicative of changes in the attention composite score, compared with the remaining cognitive composite scores investigated. Given that ageing and APOE ε4 allele
carriage impacts cognitive performance and general physiological changes (i.e. likely to be associated with a greater degree of variability), it is possible that pathological changes in older adults need to be interpreted differently compared to younger cohorts. Nevertheless, it is unclear as to why the reported association between attention and irisin was only observed in non-carriers of the APOE ε4 allele, particularly given that both groups (carriers and non-carriers) were performing within the “normal range”. Future studies are required to further understand the moderating role of APOE ε4 on the relationship between irisin levels and cognitive performance, particularly in the context of Aβ load in the brain. In this group of 201 HC participants, I was unable to find associations between irisin levels and performance in the other cognitive domains investigated, namely; episodic memory, ADCS, executive function (EF), and language. If irisin only works acutely, then the time at which blood was drawn would be important, as irisin levels could drop rapidly, thereby masking a potential relationship with cognition.

Similar to the above results, in a longitudinal analysis of the same cohort of cognitively healthy participants, I found that an increase in irisin levels over 18 months was associated with an increase in EF. EF scores were computed from three different timed tests assessing; processing speed via Stroop C/D, the ability to find words in the Controlled Oral Word Association Task, and the ability to switch back and forth between two categories in the Category Switching Task. EF is dysfunctional in AD, which is reflected by a lack of attention and focus (Kirova, Bays, & Lagalwar, 2015). In both the cross-sectional and longitudinal analyses, better cognitive performance (particularly relating to attention) was associated with higher irisin levels, indicating that serum irisin levels may potentially serve as a biomarker of cognition and/or cognitive decline in cognitively
healthy older adults. Nevertheless, the observations reported here require rigorous validation in larger longitudinal studies, utilising batteries of sensitive and validated neuropsychological tests, and serial, acutely timed blood draws following cognitive tasks or exercise. The notion that higher irisin levels are associated with better cognition in older adults is in agreement with my in vitro findings in human neuroblastoma cells (SH-SY5Y), which are an accepted in vitro model of AD. If irisin is able to confer a degree of protection against cytotoxicity, and even stimulate cell growth, in an in vitro model of AD, then it is conceivable that similar events could occur in the living brain, thereby helping to maintain neuronal connections and maybe even strengthen them.

My observation that irisin increases BDNF levels in neuroblastoma cells is novel. I also demonstrated that physiological concentrations of irisin stimulated growth of these cells. These results collectively suggest a protective role of irisin in the brain. Cell growth and maintenance of neuronal functionality are crucial steps in maintaining a healthy brain and cognitive function. However, some cell types might be influenced differently within the human brain which could explain why the promotional effects of irisin are localised to the domains of attention and executive function. In fact, a “frontal lobe hypothesis of ageing” was established over two decades ago, proposing that age-related changes in cognition are due to a declining white matter morphology primarily in the frontal lobe (Phillips & Henry, 2005). If the frontal lobe is indeed vulnerable to ageing as previously reported, my results imply that irisin could indeed be an indicator of structural integrity in the frontal lobe. Higher irisin levels may indicate a higher level of protection against neuronal loss in the frontal lobe, ensuring that normal attention function is maintained. In addition, similar to BDNF, it is possible that irisin not only protects existing cells and inter-cell connections, but also supports cell proliferation. Osteoblasts, endothelial cells,
and even mouse hippocampal cells have all been shown to be stimulated to proliferate following treatment with irisin (Moon, Dincer, & Mantzoros, 2013; Qiao et al., 2016; Song et al., 2014). All of these studies, including my own work, observed cell proliferation following an exposure to high levels of irisin. Furthermore, I reported that irisin protected cells from Aβ-induced neurotoxicity. Since Aβ is present in human brains and likely detrimental to cells, higher irisin levels would potentially have a protective role against such mediators of cell damage, in the frontal lobe as we age.
6.4 The relationship between irisin and dysfunctional lipid metabolism blood biomarkers

As discussed throughout this thesis, dysfunctional lipid metabolism is related to heightened risk of AD (further: Zarrouk et al., 2017). Therefore, I investigated a possible relationship between irisin and blood lipids. Interestingly, I observed a positive correlation between irisin levels and lipids (total cholesterol, and low-density lipoprotein; LDL) in cognitively healthy older adults. These results are consistent with previous studies that have also reported associations between circulating irisin levels and LDL, though in varying middle-aged cohorts. Liu et al. and Tang et al. utilised a cohort of individuals with T2DM, while Huh and colleagues studied this association in overweight women (Huh, Panagiotou, Mougios, Brinkoetter, Vamvini, & Schneider, 2012; Liu et al., 2013; Tang et al., 2015). However, inverse relationships between irisin and LDL or total cholesterol have also been previously reported. Oelman and colleagues observed an inverse association between irisin and total cholesterol and LDL in a large male cohort (Oelmann, Nauck, Volzke, Bahls, & Friedrich, 2016); nevertheless, it is important to note that in this study, no distinction was made between those taking lipid-lowering medication versus those who were not. In a study conducted by Choi and colleagues, no relationship between irisin and LDL, high-density lipoprotein (HDL) or total cholesterol was observed (Choi et al., 2013).

My findings of a positive association between irisin and lipids may seem contradictory to other findings in this thesis, whereby higher irisin levels reflect a “healthy” status; however, there are numerous possible explanations for the reported results. LDL transports cholesterol, through arteries to cells, where it is needed for producing molecules such as hormones (Jeon & Blacklow, 2005; Zhang, Xue, Ong, & Chen, 2009).
Hence, higher irisin levels may also result in higher activity of cellular pathways, including increased cholesterol transport via LDL. Although, higher levels of LDL are known to be associated with an increased risk of cardiovascular disease (Nordestgaard & Varbo, 2014), the LDL levels reported in this thesis were within normal reference ranges. Furthermore, it is possible that low LDL levels may be more prevalent in individuals currently taking lipid-lowering drugs, even though these individuals may overall have increased risk for poorer health outcomes. It is also important to note that lipid-lowering medications have previously been reported to influence irisin levels (Gouni-Berthold et al., 2013); unfortunately, medications data was not available in my study cohort, thereby precluding investigation of this relationship. High lipid profiles can contribute to slow, long-term damage to the body and brain, including inflammation, by forming oxidised LDL particles, which mediate an inflammatory response (Tintut & Demer, 2014). It could be hypothesised that an individual with a long history of untreated hyperlipidemia could accumulate more cellular damage in the brain and body compared with someone who has been treated with lipid-lowering drugs for numerous years. Therefore, it is possible that long-term exposure to increased pro-inflammatory pathways could hamper long-term efficiency of protein expression machinery (Hotamisligil, 2006) associated with irisin, and thus, lower irisin levels might be found in adults who have started taking lipid-lowering drugs later in life. This notion is supported by previously reported inverse relationships between markers of inflammation and irisin (Dong et al., 2015; Tang et al., 2015; Yildiz et al., 2016; Zhang et al., 2016).

My results support the idea that irisin is related to LDL and total cholesterol. Nevertheless, careful consideration must be given to the interpretation of the reported
results, in order to understand the circumstances in which a certain lipid profile reflects a decline in brain health. All future studies measuring levels of circulating irisin should take the use of lipid-lowering medications into consideration, during both the design and analyses stages, in order to account for this likely confounding variable.
6.5 The relationship between circulating irisin levels and physical activity: The importance of studying acute changes

The notion that irisin is associated with enhanced cognitive abilities is supported by previous reports that irisin is induced following physical activity, and is linked to BDNF, the main neurotrophic factor in the human body (Chen, Li, Liu, & Jia, 2016). With respect to the relationship between irisin and physical activity, I observed the following in Chapter 4: 1) a relationship between habitual exercise intensity (measured by actigraphy) and irisin in the PEACS study cohort, 2) no relationship between self-reported physical activity and irisin in the AIBL cohort, 3) increases in serum irisin following a 16-week exercise intervention, and 4) no acute changes in irisin following resistance training in a small pilot study of ‘younger’ adult participants, assessing the impact of acute strength training (AST).

Following a 16-week exercise intervention (which included aerobic and resistance exercise), participants experienced a 16% increase in irisin levels, compared to the control group, in whom a 1% decrease in irisin levels was observed. This result supports a previous report of an eight week endurance training (cycling) intervention which showed increased serum irisin levels in an older, healthy cohort, post-intervention (J. Q. Chen et al., 2016). In addition, Kim et al. (2015) reported an increase in irisin levels following a 12-week resistance exercise intervention in older adults over 65 years of age (mean age 67 years; Miyamoto-Mikami et al., 2015). However, given an ongoing debate about the differences in physiological responses following strength and aerobic exercises, further research is required to determine whether one type of exercise is more effective than the other with respect to increasing irisin levels.
Other previous research, investigating the effect of an exercise intervention on circulating irisin levels, has predominantly utilised younger to middle-aged cohorts, with varying effects observed across these studies (Kim, So, Choi, Kang, & Song, 2015). The leading explanation for observing differing physiological responses in study cohorts, including elevations or maintenance of irisin levels, centres around the differing intensity, duration, or type of exercise completed in the various studies. It is also likely that differing individual characteristics will contribute to the optimum required exercise for maintaining brain health (e.g., gender, age and genetic make-up). Thus, it is vital that future research focusses on determining the optimum individualised prescription of exercise for maintaining a healthy brain throughout ageing.

In regards to irisin release and timeframe of action, a number of studies have concluded that an acute induction of irisin occurs immediately post-exercise (as summarised by Dinas et al., 2017). In order to investigate this hypothesis, I analysed data from an acute resistance exercise pilot study, which was conducted in young to middle-aged adults. Using data from this study, I did not observe any changes in irisin following acute resistance training bouts. My results are in contrast to Tsuchiya and colleagues, who reported an increase in irisin following acute resistance training in ten healthy men (Y. Tsuchiya, Ijichi, & Goto, 2016). These disparate results could be due to different methodologies in detecting irisin: Tsuchiya and colleagues measured irisin in plasma instead of serum, and reported marked increases in irisin one hour after the cessation of resistance training, while in the current project we only investigated acute changes in serum irisin immediately post-exercise, and 30 minutes post-exercise. Future research studies would benefit from the collection of multiple post-exercise blood samples in order to effectively track the trajectory of irisin levels.
Discrepancies in acute inductions of irisin following similar exercise programmes in similar study cohorts could potentially be attributed to the existing fitness levels of participants. Adaptive changes (e.g. cell- and tissue-dependent modified gene transcription, to yield better use of energy sources, and modified regulation of protein expression) to the human body following regular physical activity have received a great amount of research attention (Neufer et al., 2015). It is plausible that a surge in irisin levels following exercise could vary greatly amongst individuals, leading to different adaptive responses: Future studies should consider the inclusion of participants with similar, or alternatively grouped, fitness levels, following assessment of maximal oxygen consumption (VO$_{2\text{max}}$), to evaluate whether this is indeed the case. Alternatively, the inconsistency in results in this field could be due to the hypothesis that irisin goes through an annual rhythm, peaking in summer (due to heightened activity levels), and winter (due to increases in adipose tissue), while gradually dropping between these seasons (Y. Tsuchiya et al., 2014; Yoshifumi Tsuchiya, Ando, Takamatsu, & Goto, 2015). While this hypothesis, if correct, would not explain differences between individual study participants, it could explain differences in group outcomes depending on when studies were conducted.

Although much of the previous literature suggests acute and transient inductions of irisin, an effect of physical activity on basal irisin levels cannot yet be discounted. In this thesis, I observed a relationship between more intense and greater volume of habitual physical activity and higher irisin levels. This result may indicate that higher basal irisin levels reflect adaptation to regular higher physical activity levels, which clearly would be of great importance in the study of older adults. For this reason, accurate basal
concentrations need to be determined, and standardized detection methods established, before commencement of future studies.

Results from this thesis partially support the notion that exercise is involved in the induction of irisin; nevertheless this research area needs further investigation. Future studies would benefit from high quality, randomized controlled trials, investigating large highly characterised study cohorts of older adults, comparing different types of exercise intervention programs (i.e. aerobic, resistance, and a combination of both), in which participants are monitored, and blood samples collected immediately pre-, post- and at multiples time-points post-exercise. Further, basal fitness levels need to be established and accounted for in each participant, and changes of fitness throughout the intervention must be monitored.
6.6 Proposing a novel interpretation of irisin results

Although varying methodological issues may contribute to discrepancies in the irisin literature, it is important to further evaluate the molecular pathways involved in irisin regulation. Following on from the brief description, in Chapter 4, of the signalling cascade preceding irisin release, and based on the results presented both in this thesis and in other landmark research publications, here I propose a novel interpretation of circulating irisin levels.

Mice and human studies have shown that irisin is highly expressed in the heart, skeletal muscle and brain, and is at much lower concentrations in other tissues such as the kidney and pancreas (Athanasios D. Anastasilakis et al., 2014). Bouts of exercise are known to increase Amp-activated protein kinase (AMPK) and Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), as shown in multiple publications (e.g. Apro, Moberg, Ekblom, Holmberg, & Blomstrand, 2016; Gibala et al., 2009; Taylor et al., 2016). As discussed in Chapter 1, PGC-1α stimulates Fibronectin type III domain-containing protein 5 (FNDC5), the precursor protein for irisin. Further, I earlier discussed that the stimulation of irisin following exercise is reported to be acute and transient. In addition, it was shown that irisin stimulation is dependent on a functional AMPK/PGC-1α/FNDC5 pathway (Shan, Liang, Bi, & Kuang, 2013). Acute changes in irisin levels measured from pre- to post-exercise are likely to represent secreted irisin levels from muscle tissue, as muscle contractions activate the AMPK pathway, and muscles cover the largest area of all tissues expressing FNDC5/irisin. Nevertheless, in addition, activation of this pathway also occurs in the brain, and is likely to have a stimulatory effect on BDNF and brain health overall. However, I propose that baseline irisin levels, measured in participants who were not exposed to exercise at the time, could represent predominantly
brain-derived irisin levels, as ongoing AMPK-stimulation is triggered by ongoing neuronal activity due to glucose demands of neurons when in use, yet at lower levels than when exposed to exercise. This hypothesis would explain recently observed day-night fluctuations (Huh, Panagiotou, Mougiós, Brinkoetter, Vamvini, & Schneider, 2012; Rabiee et al., 2014), in which higher irisin levels were found during the day. It could also account for inter-study variability of irisin levels depending on the time of day blood-draws occurred. Another important point of consideration would be levels of activity immediately before the blood-draw (i.e. walking to appointment) that could influence blood irisin levels. The hypothesis presented here suggests that future studies should adhere to strict scheduling for blood sampling (i.e. a tight time-frame in which blood samples should be collected), and consider a significant “rest” time before a blood sample is collected (data from post-exercise blood sampling could be used to inform the establishment of a sufficient rest time).

In order to support the above-described hypothesis, irisin would need to cross the BBB, a point of interest that is still under intense investigation. Crossing the BBB is theoretically possible given that irisin is a small peptide (12 kDa), considerably smaller than the Interleukin-1 (17.5 kDa) and leptin (16 kDa) peptides, which are well known to cross the BBB (William A Banks, 2015; Chen, Li, Liu, & Jia, 2015; Polyzos, Mathew, & Mantzoros, 2015). The structure of irisin was characterised recently, with a dimer described, that links two interacting four-stranded β-sheets, resulting in protein stability (A. D. Anastasilakis et al., 2014). Schumacher and colleagues suggested that the structure of irisin is unique, as its fibronectin type III (FNIII) domains are optimized for dimerization, and its structure indicates a link to a receptor, which is yet to be identified (Schumacher, Chinnam, Ohashi, Shah, & Erickson, 2013). Passive crossing of the BBB
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is tied to a number of prerequisites for a peptide such as irisin, which currently remain to be determined. Nevertheless, numerous examples demonstrate that crossing of larger molecules still occurs frequently via receptors (W. A. Banks, 2015; Schumacher et al., 2013).

An additional consideration, with respect to interpreting my results, revolves around the fact that the expression and secretion of FNDC5/irisin has also been shown to originate from visceral adipose tissue (VAT), and white adipose tissue (WAT), in an animal study utilising rats (Roca-Rivada et al., 2013). This secretion was hampered as soon as the rats were made to fast for 36 hours. Whether lower irisin levels were found in my study cohort because we also collected fasted blood samples remains to be determined. Once adipose tissue-secretion of irisin is halted, circulating irisin levels would derive from surrounding muscle tissue (mostly skeletal), yet in theory, at a lower level, than when a person is exposed to exercise (Huh, Panagiotou, Mougios, Brinkoetter, Vamvini, Schneider, et al., 2012). This hypothesis certainly warrants further exploration in the context of characterising the biology underlying circulating irisin levels.
6.7 Study limitations

Although this thesis reports a number of novel contributions to the understanding of irisin levels in older adults, some limitations must be considered. Despite access to a large study cohort within the AIBL network, cohort numbers were limited to the availability of physical activity data, and other covariates: In cases of numerous AD participants, vital signs, BMI etc. had not been measured. Furthermore, the collection of objective physical activity data was not undertaken in those with AD.

The characteristics of the studied cohorts may not directly be applicable to all populations. For example, the majority of the cognitively healthy AIBL and PEACS participants were high-functioning community-dwelling older adults. Thus, the results reported here may not reflect the wider community. Furthermore, the degree of disease progression in the AIBL AD patients varies greatly, with some AD participants able to complete nearly the entire neuropsychological testing battery, while others were immobile and unable to communicate. Thus, characteristics allocated to the AD group as a whole are unlikely to reflect all stages of the disease.

The International Physical Activity Questionnaire (IPAQ) was created in order to provide a snapshot of a person’s weekly physical activity levels, and is a well-validated and highly-utilised questionnaire. Despite these positive features, over-reporting in physical activity questionnaires, in general, is a commonly reported phenomenon (Abbott, Patabendige, Dolman, Yusof, & Begley, 2010). In contrast, actigraphy units are subject to under-reporting; whilst they record every movement of the body, they cannot record time spent swimming (not a waterproof unit), and recordings for cycling activities are underestimated due to the relatively stable core position during this activity.
Nevertheless, our actigraphy participants also kept a 7-day diary to ensure that high-levels of cycling or swimming were considered; however, in this cohort these two activities were only reported at low levels (i.e. less than once a week).

In a recent study by Hecksteden and colleagues, it was found that long-term storage of serum samples could potentially result in degradation of the irisin peptide (Heesch, Van Uffelen, Hill, & Brown, 2010; Kurtze, Rangul, & Hustvedt, 2008; Lewis, Hernon, Clark, & Saxton, 2017). This observation is yet to be independently confirmed, or supported by other groups. However, this notion raises the possibility that when irisin concentrations are analysed immediately after a blood-draw, i.e. before sample-freezing for storage, measured irisin levels may differ compared with frozen samples. Additionally, the samples utilised in this thesis were stored either in liquid nitrogen (AIBL), or in a -80°C freezer (PEACS and AST), a factor which may have further impacted the results. Across published studies, irisin concentrations range from 6-26 ng/ml in human serum, whilst other studies have published with concentration ranges of 300-500 ng/ml, or even as low as pg/ml. There are several possible reasons for these differences. Whilst varying laboratory techniques and storage conditions of blood samples could have contributed to these discrepancies, these differing levels are most likely due to the use of older and/or non-validated detection kits that were routinely used in the first two to three years after the discovery of irisin. Indeed, a critical review of commercially available irisin ELISA kits found discrepancies in the specificity of antibodies in the early kits, and therefore, in the inaccurate measurement of the peptide (Hecksteden et al., 2013). Within this thesis, a third generation irisin detection kit, provided by Phoenix Pharmaceuticals (EK-067-29), was utilised to measure samples from the AIBL and AST studies. Studies conducted by Reinehr and colleagues report that this kit is successful at detecting the correct irisin.
peptide (Albrecht et al., 2015; Elsen, Raschke, & Eckel, 2014); a view that was further supported by Park and colleagues (Reinehr, Elfers, Lass, & Roth, 2015). The irisin levels presented for the AIBL and AST cohorts in this thesis display a similar concentration range to irisin levels published most recently (Park et al., 2013). However, the ELISA kit used for PEACS sample assessments in the current study, belongs to the second generation of the same kit (EK-067-29), and therefore the measured irisin concentrations are very high. For this reason, I primarily focussed my analyses on the relationship amongst study subgroups, comparing relative differences, rather than actual measured concentrations.

One important issue that requires further elaboration, is the past discussion of the actual existence and functionality of irisin. In the last 2 years, the most recent irisin detection kit gained support from the scientific community for the accurate detection of this peptide. In addition, the structure of irisin, and its genetic code have been established, suggesting that this peptide could play an important role in mammals. Nevertheless, within this thesis, I have drawn all of my conclusions with caution, understanding that the outcomes of this project require validation, and repetition, for firm conclusions to be reached. Moreover, irisin is likely to be easily influenced by other variables, which further complicates the interpretation of results. There is still a great need to investigate irisin further as too many unanswered questions remain.
6.8 Future directions

As previously described in detail within this discussion, the disparity in previous studies evaluating relationships between blood irisin levels and health measures is likely attributable to numerous methodological factors. With the recent major improvements in the development of more accurate immunoassays, future research should focus on understanding irisin and its correlates in order to provide more insight into the role of this peptide in maintaining brain health throughout aging.

One important aspect to consider in future studies of irisin is the use of genetic data, including information on methylation status and polymorphisms of important genes purported to be linked to irisin, such as BDNF and FNDC5. The results presented in my thesis demonstrate that irisin is likely to play a major role in the body to promote BDNF activity and therefore, be beneficial in the signalling network of ongoing cell maintenance and cell-cell communication. These results are consistent with the observation that both irisin and BDNF are protective against Aβ (Perakakis et al., 2017). To-date, there are few studies that have evaluated circulating BDNF and irisin levels simultaneously in humans (Belviranli et al., 2016; Murawska-Cialowicz, Wojna, & Zuwala-Jagiello, 2015), whilst other studies discussed in this thesis, describe expression levels of BDNF in vitro (reviewed in Chapter 1). While Belviranli and colleagues found a correlation between irisin and BDNF levels in plasma in endurance athletes, Murawska-Cialowicz et al. found a sex dimorphism in serum irisin levels but not an association with BDNF levels following an exercise regimen in young men and women. However, in the latter study, the cohort was very small (n = 12), and the irisin detection kit is no longer used. Since, the majority of publications were able to find a link between irisin and BDNF, the question now is to identify the exact concentration of irisin required to initiate enough
BDNF-expression to confer full protection against Aβ. Further, identifying a relationship between irisin and BDNF or FNDC5 polymorphisms has the potential to elucidate discrepancies in measuring differing levels of irisin in the elderly, and also following bouts of exercise. One recent study has shown that a single nucleotide polymorphism in the FNDC5 gene correlated with obesity and lipid metabolism (Arancibia et al., 2008). Further, simultaneous measurement of levels of circulating BDNF and irisin could assist in providing insight into mechanisms of both irisin and BDNF in combination with cognition and response to exercise.

Longitudinal studies covering a period of many years and including a variety of biomarker detection methods (e.g. blood tests and saliva samples) are crucial in order to observe and interpret true changes of potential biomarkers such as irisin. A recent study suggested naturally fluctuating irisin levels in a day-night rhythm (Al-Daghri et al., 2016): Exercise intervention programmes could therefore have different effects on irisin depending on what time of the day exercise is conducted. Moreover, poor sleep quality or even full sleep deprivation could be responsible for decreased levels of irisin; suggesting another factor which may influence the effectiveness of exercise bouts for irisin induction and brain health. Further research is vital to fully understand such factors that likely play a significant role in modulating irisin levels, and future studies should take these factors into consideration, when establishing new study cohorts and undertaking data analyses. Moreover, in order to gain reliable insight into the effect of physical activity levels on markers of cognitive health in older adults, more exercise intervention studies are needed to explore the type, length and intensity of exercise required for irisin induction and healthier ageing.
Brain health slowly starts to decline between the ages of 40 and 77 (Athanasios D. Anastasilakis et al., 2014); the same time frame during which detectable changes in the brain occur that ultimately lead to clinical symptoms of AD approximately 20 years later (Gram, Holtermann, Sogaard, & Sjøgaard, 2012). Irisin during these early years could be of crucial importance when assessing molecular pathways that are involved in strengthening and forming cells in the brains of middle-aged adults, and when identifying physiological changes in the body that could ultimately lead to diseases identified as risk factors for AD, such as cardiovascular disease. Further, investigation of irisin levels across the full spectrum of AD (preclinical (HC with significant brain Aβ), MCI, mild, moderate and advanced AD) would likely provide considerable insight into the prognostic utility of irisin as a biomarker of AD.

On an in vitro level, the full function of irisin, its signalling mechanism, and pathways require further investigation. A plethora of questions regarding the potential of irisin in neuronal maintenance and protection remain, and would best be answered through studies of primary hippocampal cells in culture animal models of AD expressing human irisin. Further, the identification of the receptor(s) that irisin binds to is of utmost importance in order to fully understand its signalling pathways and biological function.
6.9 Conclusions

The work undertaken as part of this thesis demonstrated that irisin; 1) negatively correlates in older individuals, depending on age, diabetes disease status, BMI, gender and APOE ε4 carrier status; 2) is lower in AD females compared to HC females; 3) positively correlates with tasks of attention in APOE ε4 non-carriers; 4) correlates positively with LDL and total cholesterol; 5) is positively related to intense habitual physical activity; 6) increases following 16-weeks of exercise intervention; 7) does not acutely change following resistance training in ‘younger’ adults; 8) increases BDNF-expression in SH-SY5Y human neuroblastoma cells; 8) demonstrates a protective effect on SH-SY5Y cells against Aβ; and 9) increases cell proliferation in vitro.

In order to identify people at risk of developing cognitive decline later in life, we need to ascertain reliable biomarkers that are able to detect changes in the brains of at-risk individuals. The findings presented in this thesis suggest that irisin may be of interest in this biomarker search, likely when added to a panel of markers. The time/condition of blood draw, a possible interaction with medications, and an even more developed detection kit for irisin should be investigated for this purpose. However, the exact role of irisin in the body also requires elucidation. If irisin proves to be reliable in predicting, 1) brain health, or 2) physiological changes associated with an AD-risk factor, this peptide will contribute to a cost-effective and widely accessible blood test. If early identification of individuals at-risk can be established, we can then continue to develop vital preventative strategies for these people, which will likely be customised to individuals based on their genetic profile. However, we are yet to pinpoint the optimal approach to implementing individualised treatment strategies, and lifestyle choices, which will result in successful and healthy ageing.
Indeed, understanding all aspects of the underlying mechanisms of the relationship between physical activity, brain ageing and AD pathology is a necessity, in order to develop useful strategies for maintaining a healthy brain throughout aging. Thus far, only relatively few researchers have attempted to investigate the function of irisin, particularly in relation to the brain, and much more work is needed to establish firm conclusions regarding irisin’s distribution and function within the human body. Whilst this thesis provides many novel insights into serum irisin in older adults, the investigation of the full potential of irisin as a new biomarker needs the inclusion of many different variables, involving clinical and laboratory work, old and new irisin detection methods, and improved in vitro models to explore its functionality. The new discovery of a peptide or protein provides an exciting platform for vital research, and in the case of irisin, preliminary work suggests this molecule is worth exploring and investigating as a potential candidate for the identification of individuals at-risk of developing AD. Furthermore, if future results confirm the notion that irisin is an essential player in important signalling cascades in the CNS, it could be employed as a therapeutic target; delivering the peptide to organs, and especially the brain, to initiate increased energy expenditure, muscle or brain activity.
References


either teriparatide or denosumab treatment for 3 months. *Osteoporos Int*, 25(5), 1633-1642. doi:10.1007/s00198-014-2673-x


Binley, K. E., Ng, W. S., Barde, Y. A., Song, B., & Morgan, J. E. (2016). Brain-derived neurotrophic factor prevents dendritic retraction of adult mouse retinal ganglion cells. European Journal of Neuroscience, 44(3), 2028-2039.


References


References


References


References

between apolipoprotein E genotype and Alzheimer disease: a meta-analysis. JAMA, 278(16), 1349-1356.


Huh, J. Y., Mougios, V., Kabasakalis, A., Fatouros, I., Siopi, A., Douroudos, I., . . . Mantzoros, C. S. (2014). Exercise-induced irisin secretion is independent of age or fitness level and increased irisin may directly modulate muscle metabolism
References

through AMPK activation. *J Clin Endocrinol Metab*, 99(11), E2154-2161. doi:10.1210/jc.2014-1437


with aerobic exercise performance in patients with heart failure. *Circ Heart Fail*, 5(6), 812-818. doi:10.1161/circheartfailure.112.969543


References


levels is associated with reduction of abdominal visceral fat in middle-aged and older adults. *PLoS One*, 10(3), e0120354. doi:10.1371/journal.pone.0120354


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References


Pang, P. T., Nagappan, G., Guo, W., & Lu, B. (2016). Extracellular and intracellular cleavages of proBDNF required at two distinct stages of late-phase LTP. *npj Science of Learning, 1,* 16003.

References


References


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References


Zhang, Y., Li, R., Meng, Y., Li, S., Donelan, W., Zhao, Y., . . . Cui, T. (2014). Irisin stimulates browning of white adipocytes through mitogen-activated protein kinase p38 MAP kinase and ERK MAP kinase signaling. Diabetes, 63(2), 514-525.

Zhang, Y., Li, R., Meng, Y., Li, S., Donelan, W., Zhao, Y., . . . Tang, D. (2014). Irisin stimulates browning of white adipocytes through mitogen-activated protein kinase p38 MAP kinase and ERK MAP kinase signaling. Diabetes, 63(2), 514-525. doi:10.2337/db13-1106


Appendix 1

AIBL Participant Information and Consent Form Document
# PARTICIPANT INFORMATION AND CONSENT FORM

## Version 16: 1st September 2012

### STUDY EXTENSION

<table>
<thead>
<tr>
<th>Title of Study:</th>
<th>The Australian Imaging Biomarkers and Lifestyle (AIBL) Flagship Study of Ageing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Principal Researcher:</td>
<td>Professor Ralph N. Martins</td>
</tr>
<tr>
<td>Associate Researchers:</td>
<td>Dr. Hamid Sohrabi, Professor David Ames, Dr. Kathryn Ellis, Professor Colin Masters, Dr. Trevor Lockett, Professor Edmond Chiu, Dr. Olga Yastrubetskaya, Dr. Mathew Samuel, Ms. Kristy Draper, Mr. Kevin Taddei, Mr. Mark Rodrigues, Ms. Belinda Brown, Ms. Tania Taddei, Ms. Samantha Gardener, Ms. Rebecca Lachovitzki, Dr. Tejal Shah, Ms. Georgia Martins, Dr. Simon Laws, Dr. Veer Gupta, Dr. Stephanie Rainey-Smith, Dr. Andrea Wilson, Ms Kim Lucy Do, Ms Sabine Matthaes, Ms Rachel Buckley.</td>
</tr>
</tbody>
</table>

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This Participant Information and Consent Form is 16 pages long. Please make sure you have all the pages.

### 1. YOUR CONSENT

You are invited to continue your participation in an extension to this research study of Alzheimer's disease.

This Participant Information document contains detailed information about the research project. Its purpose is to explain to you as openly and clearly as possible all the procedures involved in this project before you decide whether or not to take part in it.

Please read this Participant Information carefully. Feel free to ask questions about any information in the document. Should you have questions about the study you may contact:

**Professor Ralph Martins (Principal Researcher):**
**Phone No.** [Private Number]

**Dr Hamid Sohrabi (Registered Psychologist):**
Appendix 1 – AIBL Participant Information Document and Consent Form

Phone No. [Redacted]
Dr Stephanie Rainey-Smith (Study Co-ordinator)
Phone No. [Redacted]
Office Phone No. for this study: 9347 4200

You may also wish to discuss the project with a relative or friend or your local health worker. Feel free to do this.

Once you understand what the project is about and if you agree to take part in it, you will be asked to sign the Consent Form. By signing the Consent Form, you indicate that you understand the information and that you give your consent to participate in the research project.

You will be given a copy of the Participant Information and Consent Form to keep as a record.

2. PURPOSE OF THE STUDY

Approximately 4 years ago you joined this study and underwent clinical assessments at commencement (baseline) and at an 18 month and 36 month follow-up. The study has generated much valuable data on the genetic and lifestyle risk factors for Alzheimer’s disease (AD) and biochemical profiles that may lead to an early diagnostic test for the condition.

From 2010, the study has approval to continue for a further 3 years. This will enable us to follow study participants over a longer period of time, increasing the quality and amount of valuable information to further develop risk profiles and diagnostic tests. In the extension, you will be invited back for 3 additional assessments at 18 month intervals (i.e. at 36, 54 and 72 months from commencement of the original study). The assessments will be similar to those you underwent at commencement and at 18 months, in that you will give a blood sample, perform memory tasks and complete questionnaires about your diet (one paper and pencil food frequency questionnaire (FFQ) i.e. CCV FFQ, and one computerised FFQ i.e. CSIRO FFQ) and exercise habits.

In addition, as part of the extension (i.e. 36, 54 and 72 month follow-ups), you will be asked to also provide a sample of your buccal (cheek) cells. The procedure for obtaining cheek cells is simply to wipe a disposable toothbrush over the cheek from inside your mouth. The collection will take under 5 minutes to conduct. We will analyse your buccal cells to identify a decrease in frequency of particular cells beyond the normal aging process that could detect Alzheimer’s patients. This may reflect changes in the AD and Mild Cognitive Impairment (MCI) brain.

Memory loss is common among the general adult population. It may be nothing of clinical significance, a symptom of another condition such as depression, or an early sign of dementia such as the most common form of dementia, Alzheimer’s disease (AD).
There is currently no method of clearly detecting whether a person is likely to develop AD or has AD, until the disease is well advanced. As treatment is likely to be most effective if given early, it is critical that researchers establish ways of diagnosing AD at an early stage.

This study aims to examine whether brain images, scores on psychometric tasks, neuropsychological (measurement of memory loss and thinking ability) tests, and/or blood biomarkers can help to diagnose AD at an early stage, or predict individuals who are at high risk for developing AD. This study will also examine lifestyle factors that may be related to AD, which may lead to the development of new treatments in the future.

This study is being carried out at research centres in Melbourne and Perth, with approximately 1000 participants in total. The following individuals over the age of 60 years will be eligible to participate in this study:

1. People with Alzheimer’s disease (AD)
2. People with Mild Cognitive Impairment (MCI)
3. Healthy people, which can include carers of people with AD

This study has been reviewed and granted ethics approval by the Hollywood Private Hospital Research Ethics Committee and Edith Cowan University Human Research Ethics Committee, which oversee the ethical conduct of all research undertaken at the Sir James McCusker Alzheimer’s Disease Research Laboratory.

3. PROCEDURES

We will perform the following tests and procedures at your 36, 54 and 72 month assessments:

Tests of Cognition (Thinking Ability), Memory Loss, Mood and Behaviour
You will be asked about your mood and behaviour, as this helps the study doctor understand if you may be at risk of depression. We will also test your memory and related thinking skills. These tests will take approximately one hour and 10 minutes to complete. The questions cover areas such as general knowledge, calculation, language, ability to recognise familiar objects and the ability to recall word lists.

Blood Sample
A nurse or other qualified person will take from you a blood sample using a sterile, disposable needle. The amount of blood to be taken will be 80 ml, which is equal to about 4 tablespoons. Researchers will then investigate the cells in your blood, along with the genetic information, known as DNA, contained in your genes.

We may also wish to use your blood and DNA in the future for related research and tests that are currently unknown or for other purposes. If this occurs then we have to obtain approval from the Hollywood Private Hospital and Edith Cowan University Human Research Ethics Committees.

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Sometimes blood and/or DNA is sent to other research institutions within Australia and/or overseas. If this occurs, the researchers involved are required to demonstrate to a local Human Research Ethics Committee that the study meets the appropriate Australian ethical and privacy standards. If collaborative research is done with other research institutions, please be assured that your identity will not be disclosed to individuals working in these other institutions.

Your blood and DNA samples will be stored at the Sir James McCusker Alzheimer’s Disease Research Laboratory, or at the premises of our research partners, until it is used up or until you contact us to request it be destroyed. Your samples will be labelled with a unique study code. This code protects your identity from technicians at the laboratory where the samples are analysed but allows your study doctor and members of the research team to identify your results. Procedures for secure and confidential storage of blood and DNA samples are those approved by the Hollywood Private Hospital and Edith Cowan University Human Research Ethics Committees.

**Buccal (Mouth) Sample**

You will need to provide a sample of your buccal (cheek) cells. The procedure for obtaining cheek cells is simply to wipe a disposable toothbrush over the cheek from inside your mouth. The collection will take under 5 minutes to conduct.

**Consent for access to your Medical Records**

In addition to asking your permission to utilise some of your blood for medical research, we also need to ask your permission for access to identified health information kept about you that is relevant to medical research. Such medical records may originate from hospitals, General Practice records, diagnoses by private specialists you have seen in the past, and information that is held on you by the Department of Health at the Health Information Centre.

Members of the research team may examine your health records. Any information obtained during the study is coded so that you cannot be identified. By signing the attached Consent Form, you authorise release of, or access to, this confidential coded information to the relevant study personnel as noted above. The review of these records may be in respect to this study and any further research that may be conducted in relation to it. These records will be made available, as described above, even if you withdraw.

**OPTIONAL PROCEDURES**

**Physical Activity Monitoring – Actigraph Uni-axial Accelerometer**

You may be invited to wear a small device called an actigraph to measure your physical activity levels. The actigraph is a small device (size of matchbox) that is not bulky, and is worn (at waist level) for the entire day excepting bathing and swimming. You will be asked to wear the actigraph for a period of 7 days, on three separate occasions. Each occasion will be up to 4 months apart. This will allow us to measure seasonal variation in your physical activity over a 12-month period. On each occasion, you will be required to come in 7 days later to return the actigraph, at which time a ‘fasting’ blood sample will be taken from
you. The amount of blood to be taken on each occasion will be 80 ml, which is equal to about 4 tablespoons. We will measure levels of your blood constituents, such as lipids (cholesterol and triglyceride) and biomarkers related to AD and the results of these tests will be related to your activity level. If you would like some feedback on your blood test results and your activity level, please notify one of the researchers who will discuss these results with you. The Physical Activity Monitoring sub-study is NOT compulsory. You may continue to participate in the main study even if you decide not to participate in the Physical Activity Monitoring sub-study.

**Body composition and bone density (DXA) scan**

You may be invited to undergo a body composition and bone density (DXA) scan. This will take place on-site at Western Medicine, located within the Hollywood Private Hospital Specialist Centre, by trained professionals. This information will be used to investigate the association between body fat and Alzheimer’s disease related proteins. The results of the scan will be discussed with you at the time of scanning with a qualified person from Western Medicine. You will also be given a copy of these results and an information sheet identifying any lifestyle changes that can be made to improve your bone density. The procedure takes approximately 30-45 minutes and at no cost to you. As this is an **optional part** of the study you therefore do not have to participate in this component of the study.

Please be aware that the DXA scan may reveal you have a low bone mineral density and are at a high risk of bone fracture (osteoporosis). Should the scan reveal that you have osteoporosis, we will forward a specialist report to your GP. A copy of this report will be given to you. We will follow-up with you to ensure you have consulted your GP for further investigation and possible treatment for the condition. In addition, should you and your GP require assistance in managing osteoporosis arrangements can be made with a bone density specialist.

**Neuroimaging**

At each of the clinical assessments you will be invited to receive a magnetic resonance imaging (MRI) scan of your brain and either one or two positron emission tomography (PET) scans of your brain. You may also be invited to receive a PET scan of your pancreas. As these are **optional parts** of the study you therefore do not have to participate in these components. Please discuss any concerns that you may have regarding these imaging procedures with the research staff. For the PET and MRI scans, an additional visit for each scan will be required at each clinical assessment. If you agree to have your pancreas scanned this will take place on the same day as the PET scan of your brain.

**PET Scans**

The PET scans will be conducted at either the WA PET Cyclotron Services Department at Sir Charles Gairdner Hospital and/or the Oceanic Medical Imaging PET-CT Centre, Hollywood Private Hospital Medical Centre. You will be asked to fast for 6 hours prior to undergoing the scan. A small plastic needle will be inserted into a vein in your arm which will be used to inject a small dose of radioactive tracer (C11-PIB, F18-Flutemetamol, F18-Florbetaben, F18-Florbetapir, or F18-FDG) which will mix with your blood to travel to your brain and pancreas. You will then rest for 30 to 40 minutes, after which time you will lie in
the PET scanner, holding your head and body as still as possible for about 30-40 minutes while pictures of your brain and pancreas where appropriate, are taken.

If you are invited to receive one PET scan of your brain, you will be injected with one of three new F18 radioactive tracers; either F18-Flutemetamol, F18-Florbetaben, or F18-Florbetapir. If you are invited to receive two PET scans of your brain you will be injected with C11-PiB and F18-FDG on separate occasions. The radiation dose from the injected tracers is relatively small, and is not considered to be a threat to your health. To put it into perspective, the radiation dose from the tracers is less than the dose you would receive from a conventional CT (X-ray) scan of the chest. Because your radiation exposure is low, you won't feel any effects and should be able to go home soon after your scan is complete. Most of the tracer will be flushed from your body within 6 to 8 hours. Other than the radiation exposure, there are no documented side effects associated with administration of PET tracers. In addition to the radiation risk, the insertion of the intravenous line may cause bruising and very infrequently infection. Should a reaction or complication occur, you will be given appropriate treatment at the hospital.

If you are invited to receive an FDG-PET scan, on the day of your scan, upon arrival at the imaging centre, you will be asked to confirm that you have fasted and your blood glucose level will then be checked. Your blood glucose will be measured via a finger prick, in the same way that your pharmacist or GP may check your glucose levels. Your blood glucose level should be < 140 mg/dL (7.8 mmol/L). If your blood glucose level is 140 mg/dL or above, the FDG-PET scan may be re-scheduled. It is not necessary to check your blood glucose level on the day of any of the other PET scans, it is however, still essential that you fast for 6 hours prior to your scan.

MRI scans
The MRI scans will be conducted at SKG Radiology. It will require you to lie still in another scanner for 45 minutes. MRI is a non-invasive technique that enables images of internal parts of the human body to be generated without the use of ionising radiation such as X-rays. There are no injections involved in this procedure, nor will anything be removed from your body. However, MRI involves the use of a strong magnetic field to generate these images and as such, people with pacemakers, aneurysm clips, artificial heart valves, ear implants or metal/foreign objects in their eyes or elsewhere in their bodies face serious risk of injury. Thus if you think you may have any such implants or objects you should not participate in this part of the study. Furthermore, the MRI may cause anxiety and effects of claustrophobia in some people due to the loud noises generated by the machine and confined space in which the scan is performed.

**Donation of a cerebrospinal fluid (CSF) sample**
The brain and spinal cord are bathed in a clear fluid termed “cerebrospinal fluid” (CSF). CSF can be used to provide an insight into the functioning of the brain. This is in contrast to the blood, as the brain is protected by specialised blood vessels that prevent the passage of certain molecules into the brain. CSF is often sampled for diagnostic purposes, for example, the determination of bacterial meningitis, and is now becoming a substance of great interest in the Alzheimer’s field.
Appendix 1 – AIBL Participant Information Document and Consent Form

We would like to collect CSF (up to 20 ml, approximately 3 teaspoons of fluid) by a procedure termed a “lumbar puncture” (otherwise known as a “spinal tap”). The procedure involves the insertion of a fine needle between the vertebrae (bones that constitute the spine) in the lower back region and is performed under local anaesthesia and under strictly sterile conditions by an experienced and highly trained anaesthetist. As this is an optional part of the study you therefore do not have to participate in this component of the study. Please discuss any concerns that you may have regarding having a lumbar puncture procedure with the research staff.

As this is an invasive medical procedure, you will be required to attend the Hollywood Private Hospital Day Surgery Unit for a period of one to two hours, where the lumbar puncture will be performed and you will be monitored for any side effects (see Risks associated with participation in the optional sub-studies on pages 9, 10 and 11 of this information and consent form). As a further measure to ensure you do understand the nature and risks of a lumbar puncture, we ask that you attend the day surgery clinic with a trusted friend or relative. The anaesthetist will speak to the person accompanying you to confirm your capacity to fully understand what is involved in undergoing a lumbar puncture.

You will be asked to lie on your side in a curled-up position on a mattress. The skin will be cleaned with a disinfectant solution. A local anaesthetic will then be injected into the area to make it numb. A specialised needle will then be inserted between the vertebrae to collect the CSF. You will be asked to remain lying down for one hour while your condition is monitored. You will then be free to leave. The following day, you will receive a follow-up call from the anaesthetist to ensure that you are not experiencing any side effects.

We may wish to use the CSF that we collect from you in the future for related research and tests that are currently unknown or for other purposes. If this occurs then we have to obtain approval from the Hollywood Private Hospital and Edith Cowan University Human Research Ethics Committees.

Sometimes CSF is sent to other research institutions within Australia and/or overseas. If this occurs, the researchers involved are required to demonstrate to a local Human Research Ethics Committee that the study meets the appropriate Australian ethical and privacy standards. If collaborative research is done with other research institutions, please be assured that your identity will not be disclosed to individuals working in these other institutions.

Your CSF samples will be stored at the Sir James McCusker Alzheimer’s Disease Research Laboratory, or at the premises of our research partners, until it is used up or until you contact us to request it be destroyed. Your samples will be labelled with a unique study code. This code protects your identity from technicians at the laboratory where the samples are analysed but allows your study doctor and members of the research team to identify your results. Procedures for secure and confidential storage of CSF samples are those approved by the Hollywood Private Hospital and Edith Cowan University Human Research Ethics Committees.
Appendix 1 – AIBL Participant Information Document and Consent Form

Donation of a urine sample
If you agree to donate a cerebrospinal fluid (CSF) sample, on the day that your CSF is collected, you will also be asked to provide a urine sample. If you agree to provide a urine sample, you will be given a collection cup and directed to a bathroom where you will collect 25 ml (approximately 4 teaspoons of fluid) of your own urine in private. Once collection is complete, you will return the collection cup to a nurse or researcher who will be located nearby. If you do not wish to donate a CSF sample you may still be asked to donate 25 ml of urine on the day that your blood sample is collected. As this is an optional part of the study you therefore do not have to participate in this component of the study.

Cogstate Study
You may be invited to participate in an additional study that will investigate the rate of change in cognitive function (i.e. thinking, reasoning and memory) in older adults. This study will commence from the time of your 36-month follow up visit. It will involve 10 sessions of up to 30 minutes each over the following 18 months.

You will be assessed with the Cogstate test, which is a computerised series of tasks which use playing cards. We can arrange for these sessions to take place at your home if you prefer. In addition, at four time points you will complete four brief neuropsychological measures, which will be familiar to you.

Subjective Memory Complaint Interview
If you are a healthy or Mild Cognitive Impairment participant, you may be asked to participate in an interview focusing on (a) your perception of your memory performance in hypothetical scenarios, and (b) details regarding your recent memories of personal events (for instance how you relive these memories and your reaction to situational demand on your memory). This study will also involve a short computerised task. This assessment will last approximately 55 minutes in total and will be audio recorded with your permission. In order to keep to a minimum the number of visits that you are asked to make to the research unit, if you agree to participate in this optional study component, a member of our research team will, with your consent, visit your home and conduct the interview there.

Study of Care-givers
If you are a participant with Alzheimer’s disease or Mild Cognitive Impairment your next of kin or care-giver may be given the opportunity to complete a phone survey and paper questionnaires about care options and utilization of facilities and about the benefits of physical activity and how a physical activity program would be best implemented.

4. BENEFITS
You will not receive any direct benefit from participating in this study. If you are a patient with Alzheimer’s disease or mild cognitive impairment, you may gain more information about your condition through education during the study. You will receive a report on your current nutritional intake, which will inform you of any excesses or deficiencies and allow you to adjust your diet if needed. As a research participant, you will also be contributing to the overall understanding and knowledge in the area of AD.
5. RISKS AND DISCOMFORTS
There are a number of potential risks associated with this study.

Risks associated with blood tests
There is a small risk of discomfort, bruising, and in extremely rare cases, infection at the site of the needle puncture, as a result of taking blood for laboratory testing. Some people feel dizzy or faint after they give blood. If you must come in for blood tests before a meal i.e. a fasted blood sample, you will be provided with an opportunity to eat before further testing is done. Food will be provided by the Study Group.

Risks associated with cognitive tasks
You may experience anxiety or psychological discomfort while completing the memory assessment.

Risks associated with providing personal information
As with the collection of any personal (private) information, there is a very slight risk of accidental disclosure of information or breach of computer security. Extensive safeguards are in place to minimize this potential risk, with hard copies of your information stored in locked cabinets within the principal researcher’s office and electronic copies stored on file with password restricted access.

If you participate in the Body composition & bone density (DXA) scan sub-study
The DXA scan is a painless, non-invasive procedure and involves the use of X-rays to determine regional body fat composition and bone mineral density (bone strength). The radiation dose from one DXA scan is low, approximately 1/50th of a chest X-ray. Your radiation exposure due to the DXA scan is the same amount you would receive during a 2 hour airplane flight and is less than normal background radiation you are exposed to on a yearly basis.

If you participate in the Actigraph Accelerometer sub-study
There is a very slight risk of increasing injury severity if the participant were to fall on the activity monitor but this is no greater than should they fall with anything else in their pocket or wearing a mobile phone or other tool on their belt.

If you participate in the optional MRI and PET brain imaging component:
Risks associated with MRI scans
The MRI scan does not cause any pain and does not expose you to X-ray radiation. However, MRIs use a magnetic field that can interact with medical devices or metal in your body. It is important that you inform the study team if you have any metal or metal devices or electrical parts in your body. The study doctor will go through a checklist with you. You do not need to tell the study team if you have fillings in your teeth, as they will have no effect. However, it is important that you tell your study team if you have tattoos, piercings, or permanent eye makeup. In addition, you will have to lie still on your back in the MRI scanner, which is a tight space. This may be difficult if you are claustrophobic. Some MRI machines are noisy and you may find this discomforting.

If you have previously had an MRI and experienced any unusual side effects, symptoms, or discomforts please tell the study team. If you experience any unusual side effects,
Appendix 1 – AIBL Participant Information Document and Consent Form

symptoms, or discomforts during the MRI please tell the staff at your MRI appointment and tell the study team either at your next appointment or via a telephone call.

If you experience any disturbing symptoms at any time during the study or directly following your participation, you should contact the study doctor immediately.

**Risks associated with PET scans**

If you take part in the optional PET imaging, you will have a cannula (a small flexible tube with a needle end) inserted into a vein in your arm. The risks of having a cannula inserted are the same as those associated with blood tests, which are a small risk of discomfort, bruising, and on extremely rare occasions infection at the site of needle puncture.

This research study involves exposure to a small amount of radiation at each 18 month scan. As part of everyday living, everyone is exposed to naturally occurring background radiation and receives a dose of about 2 millisieverts (mSv) each year. The effective dose from this research study will vary between 5.4 mSv and 7 mSv at each review, depending on whether you consent to undergo either one or two PET scans of your brain. If you consent to undergo one PET scan of your brain, you will be exposed to up to 7 mSv of radiation through the injection of one of three new F18 radioactive tracers; either F18-Flutemetamol, F18-Florbetaben, or F18-Florbetapir. If you consent to undergo two PET scans of your brain, you will be exposed to a total of 5.4 mSv of radiation through the injection of C11-PiB and F18-FDG on separate occasions. If you are also invited and consent to receive a PET scan of your pancreas, on the day of your C11-PiB brain scan you will additionally undergo a brief ‘scouting’ CT scan of your upper abdomen to locate the pancreas. This is accomplished easily with the combined CT-PET scanner but the ‘scouting’ CT scan entails extra radiation exposure of 0.5 mSv, thereby increasing the combined radiation dose to 5.9 mSv (C11-PiB and F18-FDG brain scans 5.4 mSv, + pancreas 0.5 mSv).

To put these radiation doses into perspective, 5.4 mSv is equivalent to two and a half years of natural background radiation that you would be exposed to through normal daily activities. 5.9 mSv and 7 mSv are equivalent to approximately three and three and a half years of natural background radiation respectively. This can be compared to, up to 10 mSv, which is the radiation dose you would be exposed to during a CT scan of the chest. Furthermore, the Australian Radiation Protection and Nuclear Safety Agency guidelines stipulate that it is acceptable for research participants over the age of 60 years to be exposed to up to 8 mSv per year in addition to radiation received as part of normal clinical management. The acceptable research dose increases to up to 12 mSv per year for research participants who are 70 years or more.

You should however, drink plenty of fluids afterwards to flush the radioactive drugs from your body. Most of the tracer will be flushed from your body within 6 to 24 hours. Other than the radiation exposure, there are no documented side effects associated with administration of PET tracers. It should be noted however, that C11-PiB, F18-Flutemetamol, F18-Florbetaben, and F18-Florbetapir are relatively new radiation markers and are currently only used for research purposes.
It is important to note that there are safety precautions for lactating women that must be adhered to. The F18-FDG tracer does not get into breast milk, but the person who receives the tracer emits a small amount of radiation for about 4 hours after the F18-FDG injection. Therefore, women should not have prolonged skin contact with a baby to breastfeed for at least 4 hours after the injection of the F18-FDG tracer. Breast milk can be safely pumped and fed to a baby by another person anytime after receiving the F18-FDG tracer.

**Risks associated with lumbar puncture**
The risks associated with lumbar puncture are greatly minimized when the procedure is performed by an experienced and highly trained medical professional under sterile conditions. You will also be monitored for a period of one hour in the Day Surgery unit to observe and treat any side effects, should they occur. In addition, you will receive a follow-up phone call the day after the procedure to ensure you are not suffering any side effects. However, there are some risks that you should carefully consider before agreeing to undergo a lumbar puncture. A lumbar puncture can occasionally be uncomfortable or even painful, but the likelihood of this is minimized by the fact it is being performed by an experienced doctor and by the use of local anaesthetics. The most common side effect is headache, and, occasionally temporary sensations (such as tingling or numbness) can occur in the lower limbs. More rarely, bleeding and bruising at the lumbar puncture site and trauma to the spinal cord can occur, which can result in paralysis. Paralysis is however, extremely rare as the site of lumbar puncture is below that where nerves leave the spinal cord to enter the lower limbs. Infection can also occur, however this is reduced by using sterile instruments and cleaning the area with surgical disinfectants.

**Additional possible risks**
The possibility exists for a rare reaction to any of the procedures to which the participant will be exposed.

6. **ALTERNATIVES**
You do not have to participate in this study. Choosing not to participate will in no way affect your current or future medical care at any of the participating health services involved in this study. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage. This action will in no way affect your current or future medical care at any of the participating health services involved in this study.

7. **PRIVACY, CONFIDENTIALITY AND DISCLOSURE OF INFORMATION**
Any information obtained in connection with this research study that can identify you will remain confidential and will not be disclosed to any third party, except as required by law. The results of this research study may be presented at meetings or in publications. However, your identity will not be disclosed in those presentations. No patient will be identifiable by name in any publications/presentations arising from the study.

During the study, research information will be stored within the locked offices of Professor Ralph Martins at the Sir James McCusker Alzheimer’s Disease Research Unit. The researchers named at the beginning of this consent form will have access to this.
information. Electronic data will be stored on computer with restricted access using password protection, with access limited to researchers within the study group.

It is desirable that your family GP be advised of your decision to participate in this research study. By signing the Consent Form, you agree to your family GP being notified of your decision to participate in this research study, and being kept informed of your progress in the study.

Other research centres may assist us with our research in exchange for our study findings and data. If this is the case, neither your name nor other information that can identify you personally will be given to these other research centres. Instead they will only be given data with an ID code, which only the researchers of this project at the Sir James McCusker Alzheimer’s Disease Research Laboratory will be able to trace back to you. Therefore, please be assured that all personal information collected about you as part of this study will remain strictly confidential throughout the conduct of this study.

8. NEW INFORMATION ARISING DURING THE PROJECT

During the research project, new information about the risks and benefits of the project may become known to the researchers. If this occurs, you will be told about this new information. This new information may mean that you can no longer participate in this research. If this occurs, the persons supervising the research will stop your participation. In all cases, you will be offered all available care to suit your needs and medical condition, if applicable.

9. RESULTS OF PROJECT

At the completion of the study, the results will be made available through either publication in a peer-reviewed journal, clinical meetings, and/or study reports. If you would like to be personally informed of the results, the researchers will provide you or your carer (if applicable) a verbal summary of the overall results of the study upon request. However, we wish to advise that information gained at early stages of any research will be preliminary in nature and is unlikely to have any relevance for you or your relative’s health. You will not be informed of the results of the investigations other than if unexpected incidental pathology is detected such as a brain tumour or unknown stroke.

10. FURTHER INFORMATION OR ANY PROBLEMS

If you require further information or if you have any problems concerning this project you can contact the Principal researcher, Professor Ralph Martins, Ph: (08)9347 4201 or 0404-839-305; Associate researcher, Dr. Hamid Sohrabi, Ph: 0404-418-270; the Sir James McCusker Alzheimer’s Disease Research Unit on (08)9347 4200; or Dr Mathew Samuel, Fremantle Hospital and the Fremantle Older Adult Mental Health Service (FOAMHS), Ph: (08)9347 4201.

If after agreeing to participate you have further questions or experience an adverse event, you are encouraged to contact us on the above numbers. Once having agreed to
participate, you are free to withdraw from this study at any time without giving a reason and this will not affect your current or future medical care.

11. OTHER ISSUES

If you have any complaints about any aspect of the project, the way it is being conducted, or any questions about your rights as a research participant, then you may contact:

Dr. Terry Bayliss, Chairperson of the Hollywood Private Hospital Research Ethics Committee on (08)9346 6345, or Ms Kim Gifkins, Research Ethics Officer at Edith Cowan University on (08)6304 2170, or Secretary of the Sir Charles Gairdner Hospital Human Research Ethics Committee on (08)9346 2999, or Chair of the South Metropolitan Adult Health Service (SMAHS) Human Research Ethics Committee on (08)9431 2929, or Professor Frank Van Boekxmeer, Chairman of the Royal Perth Hospital Ethics Committee on (08) 9224 2244.

12. PARTICIPATION IS VOLUNTARY

Participation in any research project is voluntary. If you do not wish to take part you are not obliged to. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage.

Your decision whether to take part or not to take part, or to take part and then withdraw, will not affect your routine treatment, your relationship with those treating you or your relationship with the participating health services involved in this study.

Before you make your decision, a member of the research team will be available to answer any questions you have about the research project. Please feel free to ask for any information you want. Sign the Consent Form only after you have had a chance to ask your questions and have received satisfactory answers.

If you decide to withdraw from this project, please notify a member of the research team of your intent.

13. ETHICAL GUIDELINES

This project will be carried out according to the National Statement on Ethical Conduct in Research Involving Humans (2007) produced by the National Health and Medical Research Council of Australia. This statement has been developed to protect the interests of people who agree to participate in human research studies.

The ethical aspects of this research project have been approved by the Hollywood Private Hospital, Edith Cowan University, Sir Charles Gairdner Hospital and South Metropolitan Adult Health Human Research Ethics Committees and the Ethics Committee of the Royal Perth Hospital.
The Australian Imaging Biomarkers and Lifestyle (AIBL) Flagship Study of Ageing

Participant Revocation of Consent Form
Version 16: 1st September 2012

I hereby wish to WITHDRAW my consent to participate in the research proposal named above and understand that such withdrawal WILL NOT jeopardize any treatment or my relationship with any of the participating Health Services.

Participant’s Name (printed) ____________________________________________

Signature: ___________________________ Date ___________________________
The Australian Imaging Biomarkers and Lifestyle (AIBL) Flagship Study of Ageing

PARTICIPANT CONSENT FORM Version 16: 1st September 2012

Consent for participation in an extension to the research project

I, ________________________________

agree to continue my participation in an extension to "The Australian Imaging Biomarkers and Lifestyle (AIBL) Flagship Study of Ageing". (Doctor or health professional) ___________________________ has explained to me and I understand the consequences involved in my participation in the AIBL Flagship Study of Ageing. I have had an opportunity to ask questions and am satisfied with the answers given.

I understand my participation in the extension to this study will involve follow-up assessments at 36, 54 and 72 months, and that assessments will be similar to those I underwent at commencement and at 18 months.

I understand that participation in this project will involve:
1. Completion of questionnaires relating to my diet (one paper/pencil and one computerised) and exercise levels,
2. Measurement of mood, behaviour, cognition (thinking ability) and memory loss,
3. Voluntary Buccal (cheek) cell donation,
4. Voluntary donation of blood:

In making my donation, I freely agree to participate in this project according to the conditions in the Participant Information Form. I understand and agree that:

- the blood (which in this consent form, includes its constituents, such as DNA, and any cell lines derived from the blood) and buccal cells will be stored and used in relation to The Australian Imaging Biomarkers and Lifestyle (AIBL) Flagship Study of Ageing
- the AIBL Flagship Study of Ageing will not be liable for any loss of, or damage to my blood, or derived cell constituents (including DNA) used in accordance with this form,
- access to my blood or derived cell constituents (including DNA) for future unspecified research will only be released where the research proposal has been approved by a Human Research Ethics Committee,
- access to my health information relevant to this study and any further research that may be conducted in relation to it, only where the research proposal has been approved by a Human Research Ethics Committee,

I also understand that:

- I will be given a copy of the Participant Information and Consent Form to keep,
- the researcher has agreed not to reveal my identity and personal details if information about this project is published or presented in any public form,
- I am free to withdraw from this study at any time without giving a reason and without affecting my current or future medical care,
- I will not benefit financially if this research leads to development of a new treatment or medical test,
- I agree to my family GP being notified of my decision to participate in this research study, and being kept informed of my progress in the study.

Optional parts of the research project, please CIRCLE your response:

- I give my consent to participate in the DXA scan sub-study YES NO
- I give my consent to participate in the Actigraph Accelerometer sub-study YES NO
- I give my consent to be contacted about PET and MRI imaging (including imaging of the pancreas) YES NO
- I may be contacted between visits to provide additional information or receive study updates YES NO
- I give my consent to be contacted about participation in future studies YES NO
- I give my consent to a lumbar puncture YES NO
- I give my consent to provide a urine sample YES NO
- I give my consent to participate in the Cogstate study YES NO
- I give my consent to participate in the Subjective Memory Complaint Interview YES NO

Signature ___________________________ Date ___________________________

Declaration by researcher: I have given a verbal explanation of the research project, its procedures and risks and I believe that the participant has understood that explanation.

Researcher’s Name (printed) ___________________________ Signature ___________________________ Date ___________________________

*Note: All parties signing the Consent Form must date their own signature.*
Appendix 2

PEACS Participant Information and Consent Form Document
Study Title: Effects of Physical Activity and Cognitive Stimulation on Plasma Beta Amyloid and on Cognitive Functioning In the Elderly

Chief Investigator: Professor Ralph Martins

Participant Information Sheet

This participant information and consent form is 12 pages long. The document contains detailed information about the research project. Its purpose is to assist you with as openly and clearly as possible all the procedures involved in this study before you decide whether or not to take part in it.

Please feel free to ask questions about any part of this study that needs more clarification by contacting our staff via the contact details provided at the end of this letter. Once you understand what the study is about and if you agree to take part in it, you will need to sign the consent form. By signing the consent form, you indicate that you understand the information and that you give your consent to participate in the study.

The information sheet contains,

1. Information about the study
2. Consent form to take part in the study

Purpose of the Study

Memory loss is common among the general adult population. It may be nothing of clinical significance, a symptom of another condition such as depression or an early sign of dementia.

The most common form of dementia is Alzheimer’s Disease (AD). Unfortunately, there is no cure for this type of dementia. Currently available medications only treat the symptoms of dementia but do not halt the progression of the disease. However, researchers worldwide are working towards the development of medications that stop the progressive decline of individuals with dementia.

There is currently no definite diagnostic tool for AD and no treatment available. Previous research indicates that lifestyle factors may provide assistance in the prevention and management of AD. Lifestyle factors which have proved beneficial in the influence of AD include staying mentally active, regular exercise and a balanced diet. This research study is investigating whether lifestyle factors may impact upon brain functioning, specifically memory. It is hoped that the outcome of this study may generate future preventive strategies against AD. This study will run from the year 2009 to 2011.

Aims

This study will test the effect of lifestyle directed interventions on brain functioning. The aims of this study are:

1) To evaluate the effects of physical activity and cognitive stimulation on your memory
2) To evaluate the effects of physical activity and cognitive stimulation on various blood biomarkers related to AD
Appendix 2 – PEACS Participant Information Sheet and Consent Form

We hope that this study will help in understanding further the possible effects on memory by staying physically and mentally active. We will specifically identify whether these lifestyle factors can improve learning and memory ability, and determine whether these positive benefits are related with biochemical and other functional parameters. The results of this study could one day assist in designing a strategy to prevent or delay the onset of AD.

What will I be asked to do if I agree to participate in this study?

Firstly, you will be asked to have a ‘baseline’ assessment, which includes the activities listed below. Then you will be asked to have 3 follow-up assessments. One will be conducted during the study period (8 weeks), another immediately after the study period (16 weeks) and the last follow-up will occur 52 weeks (12 months) after the completion of the 16 weeks study.

Procedures

Blood Sample

You will be required to fast for 10 hours prior to your baseline appointment (commence fasting at 10:30pm the night before your appointment and arrive at the clinic by 8.30am). A research officer will then take a 60 ml fasting blood sample, following which, you will be provided with a light breakfast of tea/coffee/toast/muesli. We will then conduct some tests to assess your memory. A fasting blood sample will be collected at your first visit (baseline) and at the 8 and 16 week follow-up assessments.

Tests of Cognition and Memory Loss

At baseline and at all subsequent follow-up visits (8, 16 and 52 weeks), you will undergo memory testing. The tests will be conducted by the researcher or a research assistant who has gained an expertise in this area. These tests are used to assess various memory functions, and will take approximately one hour thirty minutes to complete. The questions cover areas such as general knowledge, calculation, language, ability to recognise familiar objects and the ability to recall word lists.

If the results of these tests suggest that you have a problem with your memory, then we will refer you to your local doctor for an opinion, if this is your wish.

Questionnaires

You will be asked to complete some questionnaires. One of the questionnaires contains questions about you and your family’s medical history. It should take you 15-20 minutes to complete all the questionnaires.

Lifestyle Program

Approximately 15 days after your baseline assessment, you will commence a lifestyle-based program, designed by the research team. The program is home based and the materials required for the program will be provided by the research team. During this time you will be required to allocate 60-120 minutes, five times per day to the program activities, as well as attending a group meeting once a month. These group meetings will be conducted with other participants taking part in the
study, and will allow you to discuss your progress with the program and clarify any problems you may be having. These meetings will also allow you the opportunity to share your experiences with the other participants in the study. The meetings will be chaired by a qualified member of the research team who will be available to answer any questions you might have and provide any support that you require. In addition, your pulse rate, blood pressure and respiration rate will be recorded at these meetings.

Assessment of Physical Activity Levels

Following your initial blood and memory testing you will be provided with a physical activity monitoring device called an ActiGraph for recording daily activities over 7 days. At baseline, you will be called in for assessing your current physical activity levels. This will be done by an exercise physiologist in the fitness centre at Hollywood Private Hospital, Nedlands and will take 1 hour. This will be repeated at 8 weeks and 16 weeks after the baseline assessment.

Monthly Group Meetings

During the entire study period you will be closely monitored. For this, you will be asked to come once a month (once every 4 weeks) along with a group of people undergoing similar activities. An exercise physiologist or a research officer will be conducting these meetings whereby you can interact and exchange your thoughts and ideas with other participants and resolve any queries arising regarding the activities you are doing.

OPTIONAL PARTS OF THE STUDY

You are also invited to participate in optional parts of the study that will be conducted in conjunction with the main lifestyle study. The optional parts of the study will further investigate the effect of lifestyle directed programs. The aims of the optional parts of the study are:

1) To evaluate the effects of physical activity and cognitive stimulation on brain activity by 18F-fluorodeoxyglucose positron emission tomography (FDG PET) scan
2) To evaluate the relationship of physical activity and cognitive stimulation with body composition by dual-energy x-ray absorptiometry (DXA) scan
3) To evaluate the effects of physical activity and cognitive stimulation by analysis of AD related biomarkers in the cerebrospinal fluid (CSF)

What will I be asked to do if I agree to participate in the optional parts of the study?

If you agree to take part in the optional parts of the study you will be required to have a FDG-PET scan OR a DXA scan OR a lumbar puncture (to collect CSF for analysis) as per your wish OR a combination of any one / two / three procedures. The details of the each, including the location and timing of the procedures are mentioned below:

Neuroimaging – FDG PET Scan

If you consent to receive a PET scan of your brain, this will occur at baseline and after 52 weeks (12 months). Not every participant involved in this study will receive a PET scan. The brain scans will be conducted at the WA PET Cyclotron Service located at Sir Charles Gairdner Hospital. You may be
Appendix 2 – PEACS Participant Information Sheet and Consent Form

asked to fast for 6 hours prior to undergoing the scan. A small plastic needle will be inserted into a
vein in your arm. The needle will be used to inject a small dose of radioactive tracer which will mix
with your blood to travel to your brain. Thirty minutes later you will be asked to lie in the PET scanner
for up to 45 minutes while a scan of your brain is taken. The radiation dose from the injected tracer
is very small, and is not considered a threat to your health. The dose is equivalent to the dose you
would receive from a conventional CT (x-ray) scan. To put this into perspective, this is about two and
a half years of natural background radiation you would be exposed to through normal daily activities.
Because your radiation exposure is low, you won’t feel any affects and should be able to go home
after your scan is completed. Most of the tracer will be flushed from your body within 6-8 hours. Other
than the radiation exposure, there are no documented side effects associated with the administration
of PET tracers, however the insertion of the intravenous line may cause bruising and very
infrequently infection. Should a reaction or complication occur, you will be given appropriate
treatment at the hospital. Please discuss any concerns that you may have with these imaging
procedures with the research staff. The procedure takes 45 minutes to complete and will be occur at
no cost to you.

DXA Scan

If you consent to undergo the DXA scan, this will occur at baseline and after 16 weeks. Not every
participant will receive a DXA scan. This will take place on-site at the Varlo Health Institute, located at
Edith Cowan University, Joondalup. The DXA scan will be used to investigate the association
between body fat and AD related proteins. The scan is a non-invasive procedure and involves the
use of X-rays to determine regional body fat composition by trained professionals. The results of the
scan will be discussed with you at the time of scanning with a qualified person from the institute.
You will also be given a copy of these results and an information sheet identifying any lifestyle changes
that can be made to improve your body composition. The procedure takes approximately 40-45
minutes and will be at no cost to you. Please discuss any concerns that you may have about this
procedure with the research staff.

Lumbar Puncture to Collect Your CSF Sample:

The brain and spinal cord are bathed in a clear fluid termed ‘cerebrospinal fluid’ (CSF). CSF can be
used to provide an insight into the functioning of the brain. This is in contrast to the blood, as the
brain is protected by specialised blood vessels that prevent the passage of certain molecules into the
brain. CSF is often sampled for diagnostic purposes, for example, the determination of bacterial
meningitis, and is now becoming a substance of great interest in the Alzheimer’s field.

We would like to collect a fasting sample of CSF (20-30 ml i.e. approximately 3-4 teaspoons) by a
procedure termed a ‘lumbar puncture’ (otherwise known as a ‘spinal tap’). The procedure involves the
insertion of a fine needle between the vertebrae (bones that constitute the spine) in the lower back
region and is performed under local anaesthesia and under strictly sterile conditions by an
experienced and highly trained anaesthetist.

If you consent to undergo a lumbar puncture, you will be required to attend the Hollywood Private
Hospital Day Surgery Unit for a period of two hours, where the lumbar puncture will be performed and
you will be monitored for any side effects (see Risks associated with participation in the optional parts
of the study on page 6 of this information and consent form). As a further measure to ensure you do understand the nature and risks of a lumbar puncture, we ask that you attend the day surgery clinic with a trusted friend or relative. The anaesthetist will speak to the person accompanying you to confirm your capacity to fully understand what is involved in undergoing a lumbar puncture.

You will be asked to lie on your side in a curied-up position on a mattress. The skin will be cleaned with a disinfectant solution. A local anaesthetic will then be injected into the area to make it numb. A specialised needle will then be inserted between the vertebrae to collect the CSF. You will then be asked to remain lying down for one hour while your condition is monitored. You will then be free to leave. The following day, you will receive a follow-up call from the anaesthetist to ensure that you are not experiencing any side effects. A research officer will discuss all the details of the procedure and its complications personally if you need further information. After your ‘baseline’ CSF collection you will be asked to have a ‘follow-up’ lumbar puncture at 16 weeks.

What are the risks if I agree to participate in the main study?

1. Risks associated with blood tests

   There is a small risk of discomfort, bruising and in extremely rare cases, infection at the site of the needle puncture, as a result of taking blood for laboratory testing. There is also the rare chance of more extreme side effects such as infection or fainting. As you would be providing us with a fasted sample, you will be provided with an opportunity to eat before further testing is done. Breakfast will be provided by the study group.

2. Risks associated with the cognitive tasks

   There are no actual risks recognized for doing cognitive tests. You may experience anxiety or psychological discomfort while completing the memory assessment.

3. Risks associated with physical activity assessments

   Physical activity assessment at the gym may be associated with self injury due to incorrect technique, however, the chances of this are negligible as you will be closely supervised and monitored by an exercise physiologist.

4. Risks Associated With Monthly Group Meetings

   You may experience difficulty in adjusting your time as per the date you are asked to come. However, this will be minimized by notifying you of the particular dates for the monthly group meeting well in advance so that you have sufficient time to adjust your other schedules as per your convenience.

5. Physical Exercise Risk:

   Some participants will be required to perform regular exercise. These exercises will not be strenuous; however, there is the risk of acquiring an exercise-related injury. Every precaution will be made to ensure that you are taught the correct way to perform the exercises in order to reduce the risk of injury. Research officers will be available via the
telephone for support and monthly meetings with an exercise physiologist will be arranged to review exercise performance. You will be required to provide a letter from your GP outlining your fitness ability to ensure that the fitness regime meets your abilities.

6. Mental Exercise Risk:

Some participants will be required to perform regular mental activities by using their computers. These activities will not be strenuous and will be within your capability, thereby avoiding frustration and a sense of failure.

What are the risks if I agree to participate in the optional parts of the study?

1. Risks associated with the FDG PET Scan (if you agree to participate)

If you take part in the PET scan you will have a cannula (a small flexible tube with a needle end) inserted into a vein in your arm. The risks of having a cannula inserted are the same as those associated with the blood tests, which are a small risk of discomfort, bruising and in very rare occasions infection at the site of needle puncture.

The procedure involves a small amount of radiation from the scan. As part of everyday living, everyone is exposed to naturally occurring background radiation and receives a dose of about 2 milliSv each year. At this dose level, no harmful effects of radiation have been demonstrated as any effect is too small to measure. To put this dose into perspective, this is about two and a half years of natural background radiation you would be exposed to through normal daily activities.

You should however drink plenty of fluids afterwards to flush the radioactive drugs from your body. Most of the tracer will be flushed from your body within 6 – 24 hours. Other than the radiation exposure, there are no documented side effects associated with administration of PET tracers.

2. Risks associated with the DEXA Scan (if you agree to participate)

The DXA scan is a painless, non invasive procedure and involves the use of x-rays to determine regional body fat composition. The radiation dose from one DXA scan is low, approximately 1/50th of a chest x-ray. Your radiation exposure due to the DXA scan is the same amount you would receive during a 2 hour airplane flight and is less than normal background radiation you are exposed to on a yearly basis.

3. Lumbar Puncture Risk (if you agree to participate)

A lumbar puncture is the sampling of cerebrospinal fluid from the base of the spine. Risks associated with this procedure include minor pain, bruising or swelling and in rare cases infection at the needle puncture site. A small number of participants (10%) experience a headache after the procedure. In most cases the pain is mild and lasts from 0-2 days. In rare cases a more severe headache may develop. In the unlikely event of this occurring there is a treatment available which can alleviate the headache within a few hours. There is also a risk that participants may feel light headed or faint following the Lumbar puncture.
This is similar to the experience of fainting after providing a blood sample. The incidence of this is rare and we will take all the possible precautions to prevent such incidences.

**Privacy and Confidentiality**

Please be assured that any information obtained about you as part of this study will be treated with the strictest confidentiality. Wherever possible, your data is allocated a code or identification (ID) number. Your personal information will only be known to the members of this research team at McCusker Foundation. Other research centres may assist us with our research in exchange for our study findings and data. If this is the case, neither your name nor other information that can identify you personally will be given to these other research groups. Instead, they will only be given data with an ID code, which only the investigators of this project at McCusker Foundation will be able to trace back to you. Therefore, please be assured that all personal information collected about you as part of this study will remain strictly confidential throughout the conduct of this study.

Information collected will be used for the purposes of a PhD research project, scientific papers in conjunction with a number of presentations and possible publications. No identifying information will be presented about you.

**Withdrawal from the Study**

If at any time you wish to withdraw from the study, you are free to do so without prejudice or affecting your current or future medical care, or participation in future studies at the McCusker Foundation.

**Further Information**

Before you agree to participate in this study, we want you to be sure and clear about all aspects of this study. Therefore, we encourage you to ask any questions that you may have before providing written informed consent. Please contact the researcher Tejal Shah on (08)9347 4200 or (08)9347 4206, the chief investigator Professor Ralph Martins on (08)9347 4201 or ask the researcher enrolling you into the study any questions or queries that you may have. If after agreeing to participate you have further questions or experience an adverse event, you are encouraged to contact us on the above numbers.

**Ethical Approval**

The Hollywood Private Hospital Research Ethics Committee has given approval for this study. If you have any concerns about this study, now or in the future, please do not hesitate to contact Dr Terry Bayliss, Chairperson, Research Ethics Hollywood Private Hospital, Monash Avenue, Nedlands WA 6009. Telephone (08) 9345 6249.

**DNA (GENETIC) TESTING & STORINGs**

The purpose of this part of the information sheet is to give you more information about how your DNA (Genetic) material will be analysed for this study and stored at the McCusker Foundation.
WHAT IS DNA?

DNA is an abbreviation for deoxyribonucleic acid, which are chemical compounds that make up your genetic material, or genes.

Your genes are inherited from your (biological) parents. The genes you inherit from your parents may lead to a medical problem in early or later life. A gene mutation is an alteration to your DNA and may also be associated with a particular disease.

Testing of your genes or genetic material can provide us with information on what may happen to your general health, or perhaps that of your family, either now or in the future.

WHY IS DNA TESTED?

DNA testing is done in medical research. It helps us to learn more about diseases and what causes them. In doing so, it may assist in the clinical management of patients with such diseases.

INFORMED CONSENT

DNA testing will only be carried out if you have given your consent in writing. We recommend that you give careful consideration to the important information set out in this Information Sheet. If you have any questions, we encourage you to ask the person enrolling you. Before you give your consent, we want you to be sure and clear about all aspects of the testing and storage of your genetic material.

HOW WILL MY DNA BE OBTAINED IN THIS STUDY?

In this study, your DNA will be extracted from cells in the blood sample that you provide as part of the study.

Once extracted from your blood cells, DNA appears as a clear fluid (like water). For storage purposes, it is kept in a small plastic tube and labelled with only an ID number. This sample is stored in a freezer at McCusker Foundation. Your sample will become part of the McCusker Foundation DNA Bank. There is no cost to you for storing your sample.

DNA can be stored for an indefinite period of time. Therefore, if your sample remains in storage, it may be used in future tests and research that are currently unknown.

Given this, we give you the option of instructing us on how your DNA is to be used and stored. If you agree to participate in this study and when you sign the consent form, you will be asked to select one of the three options for using and storing your DNA:

1. Discard my DNA sample after it has been tested for the specific purpose of this study.
2. Test and then store my DNA sample indefinitely for future research in the field of AD.

3. Test and then store my DNA sample indefinitely for future unspecified research.

It is important that you understand that if you do consent to donate your DNA sample, you will not receive or be entitled to any financial reward or remuneration.

**McCusker Foundation’s DNA Bank**

If you consent to having your DNA sample stored at McCusker for future research purposes, you should consider the possibility that future DNA testing may result in new information about diseases or tendencies toward diseases that you may have. Some of this information will have no importance as far as your health is concerned but may have health implications for yourself and your family including your (biological) children. We suggest that you consider whether or not any significant health information obtained as a result of DNA testing should be disclosed to family members after your death. Before then, the information will be given to you. When making the decision about releasing information to your family, you should consider the impact and value that the results may have on your family. Given this, we give you the option of whether or not you wish to disclose your genetic information to family members in the event of your death.

There may be the rare circumstance when the Chief Investigator is placed in a position where disclosure of your genetic material may be required by law. This may be as a result of a court order, for example. Wherever possible, you will be informed if this should occur.

Sometimes DNA is sent to other research institutions within Australia and/or overseas. If this occurs, your DNA sample will only be labelled with an identified code or number, which only the members of this Research study will be able to trace back to you. If collaborative research is done with other research institution, please be assured that your identity will not be disclosed to individuals working in other research institutions.

**The Chief Investigator’s Commitment to You**

DNA testing is a complex and changing area. The Chief Investigator will endeavour to protect your interest at all times. Your instructions in relation to your DNA sample will be carried out to the best of the Chief Investigator’s ability, that being according to the options you will select in regard to the storage of your DNA sample and disclosure of your genetic material in the event of your death. Your sample will be stored carefully, but a guarantee cannot be given against accidental loss or damage. Your DNA sample will not be used for any other purpose other than what has been detailed in this Information Sheet. If we wished to do further testing on your DNA sample, we must first get consent from you in writing, and this would only occur if we get approval from the Hollywood Private Hospital Research Ethics Committee.
Even after you have given consent for testing and/or storage of your DNA sample, you have the over-riding right to request, at any time in the future, that your DNA sample be discarded.

We highly recommended:

If you agree to participate in this study, the Chief Investigator asks that you:

1. Advise your family members, or the Executor of your will, that you have a DNA sample in storage at the McCusker Foundation. We suggest that you give them, or place with your will, a copy of this Information Sheet, signed Consent Form and Options Sheet.

2. Advise your family members of the purpose, or your wishes, for which you have given your DNA sample.

3. Notify the Chief Investigator of any change of address or contact details for you or your next of kin, preferably in writing.

WHAT IF I CHANGE MY MIND?

You have the right to withdraw your consent and DNA sample at any time.

If you wish to have your DNA sample withdrawn, please notify the Chief Investigator. Be assured that we will promptly discard your DNA sample in an appropriate manner. You may wish that your DNA sample to be discarded upon your death. In which case, we suggest that you make such provisions, either by informing you next-of-kin or advising us in writing either at the commencement of or during the conduct of this study.

PARTICIPANT CONSENT FORM - Effects of Physical Activity and Cognitive Stimulation on Plasma Beta Amyloid and on Cognitive Functioning In the Elderly

To be completed by the Participant of the study:

1. Have you read the Information Sheet about this study?       Yes☐       No☐
Appendix 2 – PEACS Participant Information Sheet and Consent Form

2. Have you had an opportunity to ask questions and discuss this study? Yes □ No □

3. Have you received satisfactory answers to all your questions? Yes □ No □

4. Have you received enough information about this study? Yes □ No □

5. Which Doctor or Researcher has spoken to you about this study? ____________________________

6. Do you understand that you are free to withdraw from this study at any time without giving a reason and without affecting your current or future medical care? Yes □ No □

7. Do you agree to take part in this study? Yes □ No □

8. Have you completed the ‘Options of Duration for Storage of my DNA Sample’ question on the attached Options Sheet? Yes □ No □

9. Have you completed the ‘Options of Disclosure of my Genetic Information to Family Members’ question on the attached DNA Options Sheet? Yes □ No □

10. Have you received a copy of the information sheet and consent form? Yes □ No □

11. OPTIONAL PARTS OF THE STUDY:
    a) I give my consent for a DXA scan Yes □ No □
    b) I give my consent for the FDG PET neuroimaging study Yes □ No □
    c) I give my consent for a lumbar puncture Yes □ No □

If YES TO C: acknowledgement from a second person (trusted friend or relative) is required. Please provide their NAME and CONTACT DETAILS below. This person will be asked to verify your capacity to provide informed consent on the day of your lumbar puncture procedure.

NAME (RELATIVE / FRIEND) ______________________ PHONE NUMBER: ______________________

POSTAL ADDRESS

Participant’s Name ____________________________ Participant’s Signature ____________________________ Date ____________________________

Person Obtaining Consent ____________________________ Signature of Person Obtaining Consent ____________________________ Date ____________________________

Witness’s Name ____________________________ Witness’s Signature ____________________________ Date ____________________________

YOU WILL BE GIVEN A COPY OF THIS CONSENT FORM
Appendix 2 – PEACS Participant Information Sheet and Consent Form

DNA OPTIONS SHEET

A. Options for Duration of Storage of my DNA Sample

In regard to my DNA sample (tick ONLY ONE box):

☐ I consent to the testing and then storage of my DNA sample for research in the field of Alzheimer’s Disease.

☐ I consent to the testing and then storage of my DNA sample for future unspecified research.

☐ I consent to the testing of my DNA sample for the specific purpose of this study on the condition that my DNA sample is discarded immediately thereafter, as detail in the Information Sheet.

B. Options for Disclosure of my Genetic Information to Family Members

In the event of my death (tick ONLY ONE box):

☐ I DO consent to my genetic information, which is obtained from my DNA sample as a result of this study, being revealed to my family members, upon their written request to the Chief Investigator.

☐ I DO NOT consent to my genetic information, which is obtained from my DNA sample as a result of this study, being revealed to my family members.
Appendix 3

International Physical Activity Questionnaire (IPAQ)
Appendix 3 – International Physical Activity Questionnaire (IPAQ)

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the last 7 days. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the vigorous and moderate activities that you did in the last 7 days. Vigorous physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Moderate activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?
   □ Yes
   □ No ➡️ Skip to PART 2: TRANSPORTATION

The next questions are about all the physical activity you did in the last 7 days as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, digging, heavy construction, or climbing up stairs as part of your work? Think about only those physical activities that you did for at least 10 minutes at a time.
   ___ days per week
   □ No vigorous job-related physical activity ➡️ Skip to question 4

3. How much time did you usually spend on one of those days doing vigorous physical activities as part of your work?
   ___ hours per day
   ___ minutes per day

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate physical activities like carrying light loads as part of your work? Please do not include walking.
   ___ days per week
   □ No moderate job-related physical activity ➡️ Skip to question 6

LONG LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised October 2002.
Appendix 3 – International Physical Activity Questionnaire (IPAQ)

5. How much time did you usually spend on one of those days doing moderate physical activities as part of your work?
   ____ hours per day
   ____ minutes per day

6. During the last 7 days, on how many days did you walk for at least 10 minutes at a time as part of your work? Please do not count any walking you did to travel to or from work.
   ____ days per week
   [ ] No job-related walking → Skip to PART 2: TRANSPORTATION

7. How much time did you usually spend on one of those days walking as part of your work?
   ____ hours per day
   ____ minutes per day

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the last 7 days, on how many days did you travel in a motor vehicle like a train, bus, car, or tram?
   ____ days per week
   [ ] No traveling in a motor vehicle → Skip to question 10

9. How much time did you usually spend on one of those days traveling in a train, bus, car, tram, or other kind of motor vehicle?
   ____ hours per day
   ____ minutes per day

Now think only about the bicycling and walking you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the last 7 days, on how many days did you bicycle for at least 10 minutes at a time to go from place to place?
    ____ days per week
    [ ] No bicycling from place to place → Skip to question 12

LONG LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ, Revised October 2002.
Appendix 3 – International Physical Activity Questionnaire (IPAQ)

11. How much time did you usually spend on one of those days to bicycle from place to place?
   ___ hours per day  
   ___ minutes per day

12. During the last 7 days, on how many days did you walk for at least 10 minutes at a time to go from place to place?
   ___ days per week

   [ ] No walking from place to place  
   ___ days per week

   Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

13. How much time did you usually spend on one of those days walking from place to place?
   ___ hours per day  
   ___ minutes per day

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the last 7 days in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, chopping wood, shoveling snow, or digging in the garden or yard?
   ___ days per week

   [ ] No vigorous activity in garden or yard  
   ___ days per week

   Skip to question 16

15. How much time did you usually spend on one of those days doing vigorous physical activities in the garden or yard?
   ___ hours per day  
   ___ minutes per day

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, sweeping, washing windows, and raking in the garden or yard?
   ___ days per week

   [ ] No moderate activity in garden or yard  
   ___ days per week

   Skip to question 18

LONG LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised October 2002.
17. How much time did you usually spend on one of those days doing moderate physical activities in the garden or yard?

____ hours per day
____ minutes per day

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, washing windows, scrubbing floors and sweeping inside your home?

____ days per week

☐ No moderate activity inside home → Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY

19. How much time did you usually spend on one of those days doing moderate physical activities inside your home?

____ hours per day
____ minutes per day

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the last 7 days solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the last 7 days, on how many days did you walk for at least 10 minutes at a time in your leisure time?

____ days per week

☐ No walking in leisure time → Skip to question 22

21. How much time did you usually spend on one of those days walking in your leisure time?

____ hours per day
____ minutes per day

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do vigorous physical activities like aerobics, running, fast bicycling, or fast swimming in your leisure time?

____ days per week

☐ No vigorous activity in leisure time → Skip to question 24

LONG LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised October 2002.
Appendix 3 – International Physical Activity Questionnaire (IPAQ)

23. How much time did you usually spend on one of those days doing vigorous physical activities in your leisure time?
   
   _____ hours per day
   _____ minutes per day

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis in your leisure time?
   
   _____ days per week
   No moderate activity in leisure time → Skip to PART 5: TIME SPENT SITTING

25. How much time did you usually spend on one of those days doing moderate physical activities in your leisure time?
   
   _____ hours per day
   _____ minutes per day

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the last 7 days, how much time did you usually spend sitting on a weekday?
   
   _____ hours per day
   _____ minutes per day

27. During the last 7 days, how much time did you usually spend sitting on a weekend day?
   
   _____ hours per day
   _____ minutes per day

This is the end of the questionnaire, thank you for participating.

LONG LAST 7 DAYS SELF-ADMINISTERED version of the IPAQ. Revised October 2002.