The Roles of Sirtuins and Polyphenols in Brain Ageing and Neurodegeneration

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The Roles of Sirtuins and Polyphenols in Brain Ageing and Neurodegeneration

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Alzheimer's disease is a neurodegenerative disease characterised by progressive decline in memory. Sirtuins proteins have been shown to deacetylate numerous proteins that regulate pathways implicated in neurodegeneration. However, relatively little is known about the role of sirtuins in the central nervous system and methods for quantification are limited. In this thesis (chapter 2), I describe a targeted mass spectrometric assay developed and validated for the quantification of all mammalian sirtuins (SIRT1-7). All sirtuins were detected in the human brain, with SIRT2 being the most abundant. In chapter 3, I report a significant drop in SIRT1 levels in AD plasma and an increase in SIRT2 in the occipital AD brain. Interestingly, completion of binding partner work for SIRT2 showed association with a number of energy metabolism associated proteins. Increased protein, lipid and nucleic acid oxidation and reduced activities of antioxidants have been reported in AD plasma. In chapter 4, I investigated the effect of plasma from controls, individuals with mild cognitive impairment and AD, on a microglial cell line. I found that AD plasma significantly decreased cell viability and enhanced glycolytic flux in microglia compared to plasma from healthy controls. Proteomic methods found altered levels of complement and other acute phase proteins, in MCI and AD plasma and an upregulation of glycolytic enzymes in cells exposed to AD plasma. It is well established that amyloid aggregation is enhanced by the presence of metals and various polyphenols are thought to have both antioxidant and anti-aggregation properties. In chapter 5, I investigated the ability of copper and iron to enhance amyloid aggregation and the ability of polyphenols such as resveratrol, curcumin and epigallocatechin gallate to prevent aggregation as well as modulate energy metabolism and attenuate cell toxicity in a neuronal cell culture model. In conclusion, I have successfully applied mass spectrometry for the quantification of sirtuins in the CNS. I have also shown that altered expression of acute phase reactants in AD plasma may alter the energy metabolism of glia. Finally, neurons treated with polyphenols were shown to prevent energy deficits and cell toxicity caused by the Aβ peptide aggregates.

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Abstract

Alzheimer's disease (AD) is a complex multi-factorial neurodegenerative disease characterised by progressive decline in memory and other cognitive abilities. Sirtuins are a family of NAD-dependent histone deacetylase proteins that have been recently associated with longevity. Sirtuins have been shown to deacetylate numerous proteins which regulate multiple biological pathways implicated in neurodegeneration. However, relatively little is known about the role of the seven mammalian sirtuins in the central nervous system (CNS). At present, quantitative western blotting, PCR and ELISA are the main techniques used to quantify sirtuin protein and mRNA levels. However, these methodologies are limited due to low sensitivity and selectivity in a multiplex-format.

In this thesis (chapter 2), I describe a novel, more robust, targeted mass spectrometric assay developed and validated for the quantification of all seven mammalian sirtuins (SIRT1-7). Quantification of all peptides was performed by multiple reaction monitoring (MRM) using three mass transitions per protein-specific peptide, two specific peptides for each sirtuin and a stable isotope labelled internal standard. All sirtuin peptides were detected in the human brain, with SIRT2 being the most abundant. Sirtuins were also detected in human CSF and plasma, and guinea pig and mouse tissues. I applied (chapter 3) the assay to a variety of samples including cultured brain cells, human control and AD *post mortem* brain tissue, CSF and plasma. I found a significant drop in SIRT1 levels in AD plasma and an increase in SIRT2 in the occipital AD brain tissue. Interestingly, completion of binding partner work for SIRT2 using immunoprecipitation and mass spectrometry based techniques showed that SIRT2 is associated with a number of energy metabolism associated proteins such as ATP synthase and enolase.

Increased protein, lipid and nucleic acid oxidation and reduced activities of antioxidant enzymes have also been reported in AD plasma, and are associated with sirtuin function. Amyloid plaques in the AD brain elicit a range of reactive inflammatory responses including complement activation and acute phase reactions, which may also be reflected in plasma. In chapter 4, I investigated the effect of pooled plasma (n=20 each) from healthy controls, individuals with amnestic mild cognitive impairment (aMCI) and AD, on a cultured microglial cell line. I found that AD plasma significantly decreased cell viability and enhanced glycolytic flux in microglial cells compared to plasma from healthy controls. This effect was prevented by heat inactivation of complement protein. Proteomic methods and

isobaric tags (iTRAQ) were used to identify the expression level of complement and other acute phase proteins, which were found to be altered in MCI and AD plasma and an upregulation of key enzymes involved in the glycolysis pathway in cells exposed to AD plasma.

It is well established that amyloid beta (Aβ) aggregation is enhanced by the presence of redox-active metals and various polyphenols are thought to have both antioxidant and anti-aggregation properties. Furthermore, polyphenolic compounds such as resveratrol are known to affect sirtuin expression and activity. In chapter 5, I investigated the ability of transition metals, copper and iron to enhance amyloid aggregation and the ability of polyphenols such as resveratrol, curcumin and epigallocatechin gallate to prevent aggregation as well as modulate energy metabolism and attenuate cell toxicity in a neuronal cell culture model.

In conclusion, I have successfully applied MRM mass spectrometry for the detection and quantification of sirtuin proteins in the CNS, paving the way for more quantitative and functional studies. I have also shown that altered expression levels of acute phase reactants in AD plasma may alter the energy metabolism of glia. Finally, neurons treated with polyphenols were shown to prevent energy deficits and cell toxicity caused by the Aβ peptide aggregates, and with future work investigating these compounds efficacy in activating sirtuins could provide promising results for the development of combination therapies targeting various pathways involved in AD such as oxidative stress, sirtuin activation, mitochondrial dysfunction and energy metabolism for use in transgenic animal models and/or human pilot clinical trials.

ORIGINALITY STATEMENT

'I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at UNSW or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at UNSW or elsewhere, is explicitly acknowledged in the thesis. I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.'

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Publications and Presentations

First Author Peer Reviewed Publications

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Jayasena T, Poljak A, Braidy N, Smythe G, Raftery M, Hill M, Brodaty H, Trollor J, Kochan N, Sachdev P (2015). Upregulation of glycolytic enzymes, mitochondrial dysfunction and increased cytotoxicity in glial cells treated with Alzheimer's disease plasma. PLoS One 10(3):e0116092.

Jayasena T, Poljak A, Smythe G, Braidy N, Münch G, Sachdev P (2013). The role of polyphenols in the modulation of sirtuins and other pathways involved in Alzheimer's disease. Ageing Research Reviews 12(4):867-883

Collaborative Peer Reviewed Publications

Braidy N, Jugder BE, Poljak A, **Jayasena T**, Mansour H, Nabavi SM, Sachdev P, Grant R (2016). Resveratrol as a potential therapeutic candidate for the treatment and management of Alzheimer's disease. Current Topics in Medicinal Chemistry 16(17):1951-60.

Braidy N, Poljak A, Grant R, **Jayasena T**, Mansour H, Chan-Ling T, Smythe G, Sachdev P and Guillemin GJ (2015). Differential expression of sirtuins in the ageing rat brain. Frontiers in Cellular Neuroscience 9:167

Braidy N, Poljak A, **Jayasena T**, Mansour H, Inestrosa NC, Sachdev PS (2015). Accelerating Alzheimer's disease research through 'natural' animal models. Current Opinions in Psychiatry 28(2):155-64.

Braidy N, Poljak A, Marjo C, Rutlidge H, Rich A, **Jayasena T**, Inestrosa NC, Sachdev P (2014). Metal and complementary molecular bioimaging in Alzheimer's disease. Frontiers in Aging Neuroscience 6:138.

Braidy N, Poljak A, Grant R, **Jayasena T**, Mansour H, Chan-Ling T, Guillemin GJ, Smythe G, Sachdev P (2014). Mapping NAD(+) metabolism in the brain of ageing Wistar rats: potential targets for influencing brain senescence. Biogerontology 15(2):177-98

Braidy N, **Jayasena T**, Poljak A, Sachdev P (2012). Sirtuins in cognitive ageing and Alzheimer's disease. Current Opinions in Psychiatry 25(3):226-30.

Book Chapters

Braidy N, Poljak A, **Jayasena T**, Sachdev P (2014). Ionotropic Receptors in the Central Nervous System and Neurodegenerative Disease. R.M. Kostrzewa GJ (ed.), *Handbook of Neurotoxicity*, pg1071-1092; Springer Science. ISBN 978-1-4614-5835-7.

Braidy N, Poljak A, **Jayasena T**, Sachdev P (2014). Drug Treatments for Alzheimer's Disease: Hopes and Challenges. R.M. Kostrzewa GJ (ed.), *Handbook of Neurotoxicity*, pg 1173-1190; Springer Science. ISBN 978-1-4614-5835-7.

Braidy N, Poljak A, **Jayasena T**, Adams S, Sachdev P (2014). Glutamate in the Pathogenesis of Gliomas. R.M. Kostrzewa GJ (ed.), *Handbook of Neurotoxicity*, pg 1287-1299; Springer Science. ISBN 978-1-4614-5835-7.

Conference Presentations

Alzheimer's Association International Conference 2013, Boston, USA

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17th Lorne Proteomics Symposium, Victoria, Australia 2012

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Alzheimer's Australia Dementia Research Foundation Project Grant 2016 ($50,000)

Title: Involvement of SIRT3 and related energy metabolite changes in the AD brain

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List of Abbreviations

The following abbreviations have been used in the text and diagrams of this thesis:

4-HNE	4-hydroxynonenal
αKGDH	α-ketoglutarate dehydrogenase
Aβ	amyloid beta
ABAD	Aβ-binding alcohol dehydrogenase
ABC	avidin-biotin-complex
AD	Alzheimer's disease
ADP	adenosine diphosphate
aMCI	amnestic mild cognitive impairment
APOE	apolipoprotein E
APP	amyloid precursor protein
ATP	adenosine triphosphate
BBB	blood brain barrier
BCA	bicinchoninic acid
BCR	basal control ratio
C4BP	complement 4 binding protein
CNS	central nervous system
CR	calorie restriction
CSF	cerebrospinal fluid
Cu^{2+}	cupric copper
CV	coefficient of variance
DDA	data dependent acquisition
ECAR	extracellular acidification rate
EGCG	epigallocatechin gallate

FBA A/C	fructose bisphosphate aldolase A/C
FCS	fetal calf serum
FDR	false discovery rate
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSEP	grape seed extract polyphenols
H^{+}	proton
H_2O_2	hydrogen peroxide
HA	high abundance fraction
HDTIC	4-Hydroxy-5-hydroxymethyl-[1,3]dioxolan-2,6'-spirane-5',6',7',8'-tetrahydro-indolizine-3'-carbaldehyde
HSP	heat shock protein
IDA	information dependent acquisition
iTRAQ	isobaric tags for relative and absolute quantitation
LA	low abundance fraction
LAP	low abundance plasma
LDH	lactate dehydrogenase
LOD	limit of detection
LOQ	limit of quantification
M-CSF	macrophage colony-stimulating factor
MCI	mild cognitive impairment
MDH	malate dehydrogenase
MnSOD	manganese superoxide dismutase
mPTP	mitochondrial permeability transition pore

MRM	multiple reaction monitoring
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NAD	nicotinamide adenine dinucleotide
NFT	neurofibrillary tangles
NSAIDs	non-steroidal anti-inflammatory drugs
OCR	oxygen consumption rate
$OH^{\bullet}$	hydroxyl radical
OS	oxidative stress
PARP-1	poly (ADP-ribose) polymerase-1
PDH	pyruvate dehydrogenase
PDI	protein disulphide isomerise
PET	positron emission tomography
PGM1	phosphoglucomutase 1
PK	pyruvate kinase
PSEN1	presenilin 1
PSEN2	presenilin 2
RARβ	retinoic acid receptor β
ROS	reactive oxygen species
SIRT	sirtuin
TCEP	tris-(2-carboxyethyl) phosphine
TNFα	tumour necrosis factor-alpha
TPI	triose-phosphate isomerise
UCR	uncoupling ratio
ZnT3	zinc transporter 3

Chapter 1

General Introduction

The majority of this chapter has been published as a review article:

Jayasena T, Poljak A, Smythe G, Braidy N, Münch G, Sachdev P (**2013**). *The role of polyphenols in the modulation of sirtuins and other pathways involved in Alzheimer's disease*. Ageing Research Reviews 12(4):867-883.

Please see Appendix for full publication

1.1 Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disease characterised by decline in cognitive and functional abilities [1]. Pathologically AD presents with progressive and irreversible neuronal and synaptic loss, particularly in the hippocampus [2]. Neurodegenerative diseases are the most common cause of senile dementia with incidence doubling every five years after the age of sixty. The current ageing boom of most developed societies is predicted to translate into an increase in the incidence of AD in coming decades, creating substantial financial and social burdens however there are still no disease modifying therapies available to stop or reverse the decline in patients with AD.

The disease was first defined by psychiatrist Alois Alzheimer in 1907 describing the case of 55 year old Mrs Auguste Deter who presented with memory impairment, aphasia, psychosocial incompetence and disorientation, which progressed to worsening cognitive function [3-5]. From microscopic analysis of her brain, Alzheimer observed and described the presence of 'fibrillary bundles' and 'small miliary foci,' nowadays recognised as neurofibrillary tangles (NFT) and amyloid beta (Aβ) plaques, the two main histological hallmarks of AD [4]. Neurofibrillary tangles are intracellular lesions found within the cytoplasm of neurons and Aβ plaques are extracellular lesions consisting of an Aβ peptide core surrounded by activated microglia (Figure 1.1) [2,6,7]. These plaques also contain high levels of divalent metals such as iron and copper sequestered within them [6-8].

Figure 1.1 Histological hallmarkers of AD – Amyloid Plaques and Neurofibrillary Tangles

(image taken from http://www.brightfocus.org/alzheimers/infographic/amyloid-plaques-and-neurofibrillary-tangles)
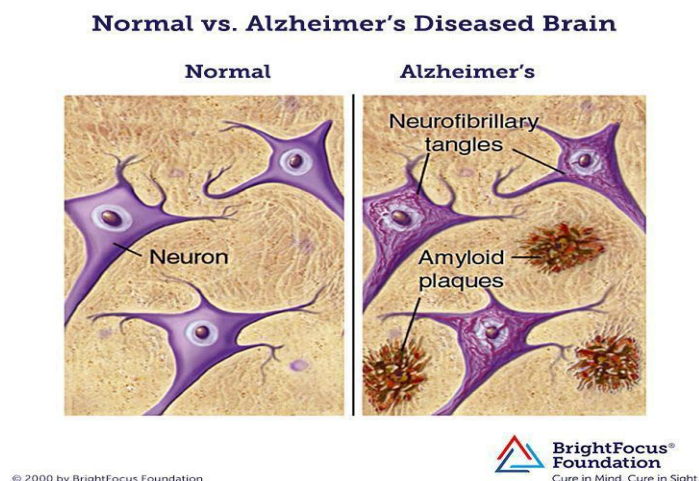
However AD itself wasn't considered a disease distinct from dementia until decades later when research by Blessed et al in 1968 showed that there was a connection between the plaques and tangles and cognitive decline [4,9]. Additionally other researchers also established AD as a condition differing from normal ageing, and genetic mutations involved in the hereditary form of the disease were also discovered [4,10-13].

The most consistent and strongest genetic predisposition for the late onset form of AD (the most prevalent form, affecting about 95% of individuals with AD) to the disease comes from the gene for apolipoprotein E (*APOE*), with an increase in the risk of developing AD reported to be up to 30 times higher for individuals carrying two copies of the *APOE* ε4 allele [14,15]. By contrast, evidence suggests that the *APOE* ε2 allele is protective from AD pathology [16]. Other genes have also been implicated as disease risk factors including those involved with inflammation, cholesterol metabolism, transport/trafficking proteins, neurotransmission and signalling [15,17].

AD remains a difficult disease to study due to its long preclinical phase and the inability to reliably detect the initial stages of the disease. Today, brain scans and improving imaging techniques have provided insight to the aetiopathology of the disease, but reaching agreement on the pathological aspects and causes of AD, in addition to the lack of ways to confirm these, have caused impediments to treatments and cures for the disorder.

Although AD is characterised by the progressive cognitive deterioration of a patient, each individual advances through the course of the disease uniquely. Initial symptoms are often mistaken for age-related problems or stress-induced indicators, including the inability to form new memories and recall recently learnt facts [18]. These subtle problems in the pre-dementia stage usually go unnoticed or may be identified as mild cognitive impairment (MCI) [18]. Early stages of the disease are characterised by behavioural and psychological changes, as well as a gradual inability to handle normal daily activities, including newly learned skills. Usually a patient is able to manage their own affairs, although their vocabulary and language fluency tend to be noticeably affected at this point of the disease [18]. Later phases of AD require full-dependency on a caregiver, leading in some cases to a complete loss of speech and accompanied by a loss in muscle mass due to lack of mobility, ultimately causing the patient to become bedridden [18]. Delusional symptoms and irritability, confusion, aggression and wandering tend to become less common than in the intermediate stages of the disease [18].

Postmortem analyses of AD patients' brains show losses of neurons and synapses in the cerebral cortex, atrophy of the hippocampi, temporal and parietal lobes, parts of the frontal cortex and cingulate gyrus, leading to brain atrophy (Figure 1.2), as well as a presentation of large numbers of plaques (in the cortex) and NFT (beginning in the hippocampal region and extending into the cerebral cortex), providing the basis for a confirmed diagnosis of AD [2,19].

Figure 1.2 Brain Atrophy in AD

(Image taken from: http://memory.ucsf.edu/education/diseases/alzheimer)
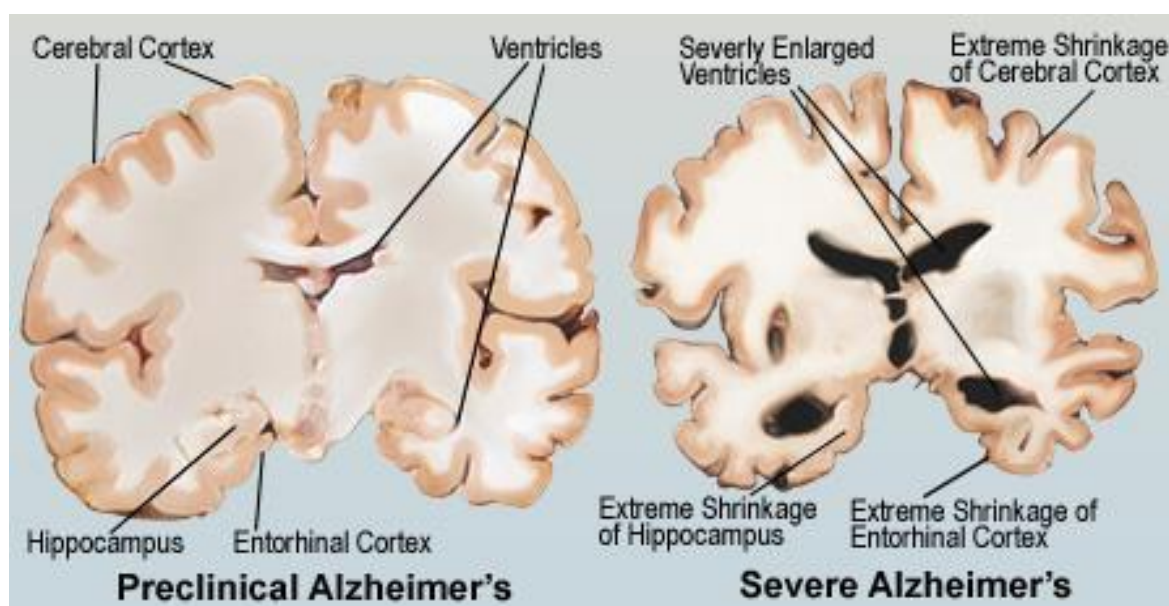
There are two main types of AD: familial and sporadic. The familial form accounts for less than 5% of cases. These are caused by mutations in one of three genes and have an early age onset, before 65 years. Most mutations within the three genes - amyloid presuror protein (*APP*), presenilin 1 (*PSEN*1) and presenilin 2 (*PSEN*2) increase brain and CSF levels of the Aβ peptide. Excess levels of toxic forms of the Aβ peptide (particularly the longer Aβ1-42 version, β-pleated sheet conformers and Aβ oligomers), which over time may aggregate into plaques, disrupt neuronal messaging, and ultimately cause the death of neurons [15,17]. The more common sporadic version of AD has no commonly acknowledged genetic causes (though the *APOE* ε4 gene allele is a recognised risk factor) and is thought to be a multifactorial disease with many risk factors adding up to the dysfunction and disease

pathogenesis. These factors include: acetylcholine decline, increased oxidative stress, amyloid aggregation, mitochondrial dysfunction and divalent metal ion imbalance.

1.2 Cholinergic Hypothesis

The cholinergic hypothesis was theorised because investigations showed that AD brains had lower levels of acetylcholine – a major neurotransmitter in the brain – than non-demented elderly cases [20]. As one of the oldest causal theories of AD, the disturbances in the cholinergic system have also been the focus of most treatments available on the market, although they provide relatively modest postponement of clinical symptoms and fail to alter the course of the pathology[20,21]. Treatments of AD based on the cholinergic hypothesis (donepezil, rivastigmine and tacrine etc.) are cholinesterase inhibitors, which act simply by inhibiting cholinesterase – an enzyme which breaks down the neurotransmitter acetylcholine. By reducing cholinesterase activity, the brain retains higher levels of acetylcholine and therefore neurotransmission [20,21]. However these drugs provide only a temporary reprieve, and do not alter plaque or tangle formation or neuronal cell death.

1.3 Oxidative Stress

The accumulation of oxidative damage from free radical production during normal oxidative metabolism increases with age and is known as the free radical theory of ageing. Originally proposed by Denham Harman, it is thought to be linked to age-related diseases such as AD [22-24]. In fact, one of the earliest pathological events in AD is oxidative damage to the brain cells of affected individuals [1]. Oxidative stress results when pro-oxidant and anti-oxidant activities in the body are not occurring at a balanced rate, resulting in dyshomeostasis (Figure 1.3) and generation of toxic reactive species (ROS), such as hydrogen peroxide, superoxide and the highly reactive hydroxyl radical [25]. The generation of free radicals is catalysed by redox-active metals such as copper and iron. For example, hydrogen peroxide in the presence of copper or iron produces hydroxyl radicals via the Fenton reaction (Figure 1.4). Brain tissue is particularly vulnerable to oxidative damage due to its high oxygen consumption, relatively low antioxidant levels, high content of redox-active metals (copper and iron), and limited regenerative capacity. Thus free radicals have been hypothesised to play an important role in the brain ageing process [26]. Increased oxidative stress due to free radical damage, and the

resulting cellular dysfunction are widely believed to be responsible for the neuronal degeneration that occurs in AD [26].

Oxidative stress results in impaired cellular functions and the formation of toxic species, such as peroxides, alcohols, aldehydes and ketones [27]. The oxidatively modified lipids acrolein and HNE induce toxicity by crosslinking to cysteine, lysine and histidine. Acrolein down regulates the uptake of glutamate and glucose from cell culture. Modifications to proteins result in the impairment of enzymes, whereas ROS interaction with DNA potentially leads to mutations [27]. The excess generation of ROS leads to dysregulation of intracellular calcium signalling. Such dysregulation has been widely observed in neurodegenerative diseases in which aberrant calcium levels stimulate multiple pathways that ultimately induce an apoptotic cascade [1,27].

Figure 1.3 Evidence of Oxidative Stress in AD and its prevention
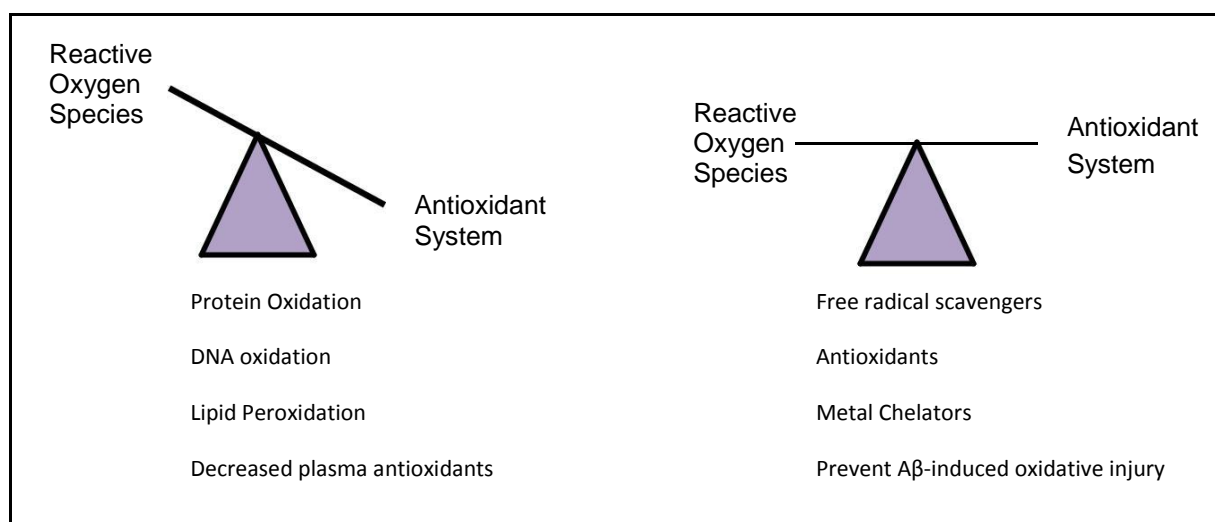
Markers of free-radical damage such as increased protein and DNA oxidation, increased levels of lipid peroxidation, advanced glycation end products, carbonyls, dityrosine, malondialdehyde, and peroxynitrite damage have all been reported to occur in AD [28-30]. Furthermore, decreased levels of plasma antioxidants are reported in AD patients. Peroxidation of cellular membrane lipids generates highly reactive aldehydes such as 4-hydroxynonenal (4-HNE). This product is elevated in multiple AD brain regions compared to nondemented elderly controls [31]. 4-HNE is also increased in the plasma and CSF of AD patients [31]. Elevated isoprostanes have also been observed in the plasma, CSF and urine of

AD individuals [29,32] and protein carbonyls are significantly increased in the AD hippocampus [33]. Increased levels of protein carbonyls are observed in both in NFTs as well as in the cytoplasm of tangle free neurons [23,34]. AD patients have higher dityrosine in plasma [35] and increased nucleic acids oxidation. A significant increase in the oxidised purine 8-OH guanosine (a signal product of hydroxyl radical attack), was found in the cortex and cerebellum of AD brains relative to controls [36]. Elevated levels of this marker were also found in lymphocytes of AD patients [37].

DNA damage by ROS, particularly the hydroxyl radical is a key feature of neuronal cell death in AD patients and in experimental models of this disease [38,39]. Studies demonstrating increased oxidative DNA damage, and helicase activity especially the activity of 8-oxoguanine glycosylase with a concomitant decrease in base excision repair have been reported in the AD brain [40]. DNA damage induced by free radicals or enzymatic modifications can be a trigger that initiates the cell death cascade. These observations have been interpreted to suggest that in addition to the brain being subject to increased oxidative stress in AD, there is a compounding effect of decreased repair of oxidative damage leading to an accumulation of DNA errors, which may be an important factor in the progression of neuronal loss in AD [40].

The increased oxidative modification of cellular and lesion macromolecules seen in AD may originate from multiple sources, including: increased intracellular production of the membrane permeable oxidant, hydrogen peroxide by abnormal or stressed mitochondria, increased extra-neuronal production of hydrogen peroxide from activated microglia, and by Aβ deposits [1,26,41]. These Aβ plaques are another source of oxidative stress in AD as they sequester divalent metals such as copper and iron, which have the potential to catalyse free radical formation [42]. Copper and iron in abnormally high concentrations and markers representing the endproduct of oxidative stress have been found in amyloid plaques, and are elevated in the neocortex of the AD brain [25,43]. Iron has been shown to facilitate the aggregation and deposition of Aβ and to induce aggregation of the major constituent of neurofibrillary tangles, hyperphosphorylated tau [44,45]. Reaction of hydrogen peroxide with available redox active metal ions via Fenton chemistry leads to the generation of the hydroxyl radical (see figure 1.4). This highly reactive and non-selective free radical has the potential to induce many oxidative modifications to a variety of macromolecules.

Figure 1.4 Production of the hydroxyl radical via Fenton chemistry

$$H_2O_2 + Fe^{2+}/Cu^+ \longrightarrow Fe^{3+}/Cu^{2+} + {}^{\bullet}OH + {}^-OH$$

As the body ages, increasing levels of oxidative stress markers are produced and the efficient functioning of molecules to clean up and repair these errors also deteriorates with time [46]. This in turn leads to further oxidation and the destructive spiral continues [46]. These processes themselves are reported to cause inflammation and the hallmarks of AD – amyloid plaques and tangles. How and why are still unanswered questions in this debate, although some researchers suggest that these lesions are the end result of a malfunctioning repair system that ineffectually manages oxidative stress within the brain. The creation of ROS with age results from dyshomeostasis of multiple pathways: – from energy production to the replication of DNA and cellular growth and repair [46]. During ageing, the efficiency of processes that maintain protein integrity and cleanup systems are compromised and numerous errors accumulate. These in turn cause further mistakes to occur and the system suffers with the build-up of dysfunctional proteins and the vicious oxidative stress cycle continues. ROS themselves can directly oxidise and damage DNA, lipids and proteins and induce stress-responses, as well as facilitate apoptosis through mitochondrial pathways [47]. Levels of oxidative stress have been linked with AD, which has lead to clinical trials utilising anti-oxidant therapies [1]. These trails have in general been disappointing. For example trials with vitamin E and curcumin have been inconclusive. However it may be that the window of treatment has been surpassed in patients who have converted to late stages of AD [48,49]. This point has been raised for most failed AD treatments and it is proposed that late stage AD patients have exceeded the threshold for resolving the disease and its causes. It is difficult to determine whether oxidation is causative in AD, since oxidative stress is also a normal part of ageing, and it may just be a matter of degree. It is difficult to determine which individuals will be affected, and also challenging to undo years of cumulative damage. To optimise their efficacy, it is likely that measures to reduce excess levels of ROS and oxidation should be initiated early and ideally at preclinical stages.

1.4 Amyloid Cascade Theory

The main therapeutic approaches for AD are linked to the amyloid cascade hypothesis. This theory highlights the importance of Aβ, the major component of plaques, as an upstream factor in AD pathogenesis, and suggests that Aβ initiates a cascade of toxicity, which recruits tau, the major component of tangles in AD pathology and consequently causes neuronal dysfunction, neurotransmitter deficits, and ultimately neuronal cell death [20,50]. Aβ has normal physiological activity and is present in the brain, cerebrospinal fluid (CSF) and plasma of healthy individuals throughout life. These peptides do, however, have the ability to aggregate and generate the amyloid that is found in the brain parenchyma and around blood vessels [51].

Aβ possesses physiochemical properties that are consistent with both an antioxidant and a prooxidant. Several studies have shown that Aβ exhibits neurotrophic and neuroprotective properties when present at physiological (nM) concentrations. Aβ1-40 and to a lesser extent Aβ1-42 prevents lipoprotein oxidation in CSF and plasma [52]. Evidence that Aβ has antioxidant activity comes from studies showing that increased production of Aβ by mutant PS1 fibroblasts is accompanied by a decrease in the production of ROS, particularly the hydroxyl radical formation [53]. There is a positive correlation between CSF resistance to oxidation and its levels of Aβ [54].

Evidence supporting a neurotoxic role for Aβ in AD comes primarily from experiments on cultures of primary neuronal cells from neonatal rodents or cultures of neuroblastoma cell lines. Such studies typically report neurotoxic effects from the addition of micromolar concentrations of Aβ peptide (particularly the longer Aβ1-42 variant prevalent in AD) to the culture media [55-57]. The role of Aβ in disease is not therefore completely understood. It is possible that it has a dual role in AD pathogenesis. At low nM concentrations it may play a protective role against oxidative stress, however, as the burden of oxidative stress becomes excessive it may become complicit by exacerbating free radical damage [41].

Aβ is generated by cleavage of amyloid precursor protein (APP) which can occur via 2 sequential pathways: the non amyloidogenic and amyloidogenic pathways [58] (see figure 1.5). The first cleavage event initiated by either α-secretase or β-secretase, determines whether Aβ is generated. The next cleavage by γ-secretase determines the species of Aβ generated [58]. Although its function is not completely understood, APP is suggested to be critical for neuronal growth, signalling, and may also function as an antioxidant and a metalloprotein,

modulating copper transport and metabolism [58]. Cleavage of the APP protein can occur at many sites within the cell, including the trans-Golgi network, mitochondrial membrane and plasma membrane, with location and reactants present said to dictate which pathway is followed.

Figure 1.5 APP processing by secretase enzymes. Top showing non amyloidogenic pathway and bottom showing amyloidogenic pathway producing Aβ peptides.
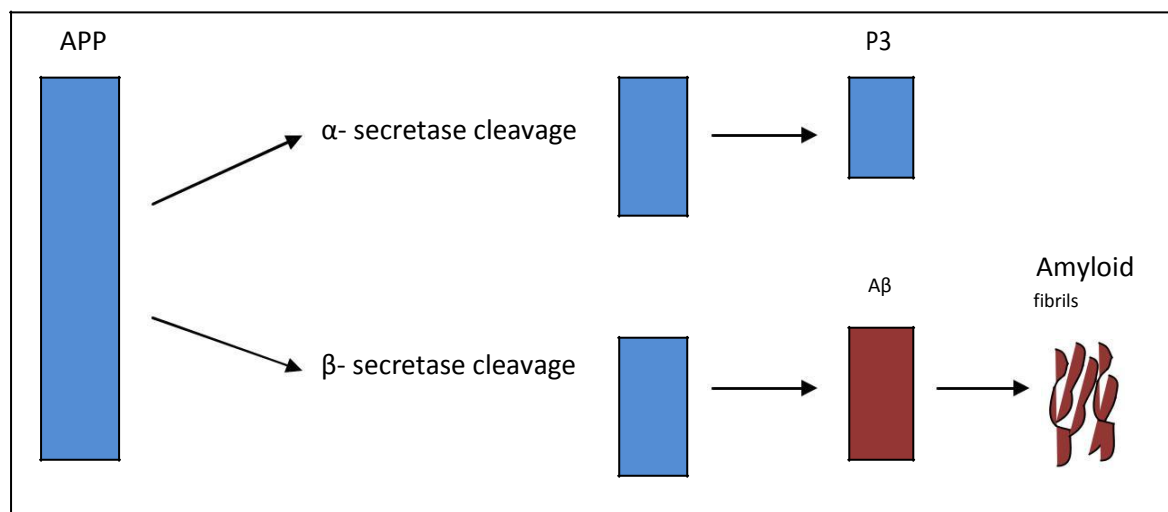
The non-amyloidogenic pathway results in the production of the P3 peptide fragment, which consists of 16 amino acids and involves α-secretase cleavage followed by a γ-secretase cut within the Aβ domain of the APP protein (Figure 1.5). The amyloidogenic version of the pathway involves cleavage by β-secretase followed by the γ-secretase (Figure 1.5), releasing the 40-43 amino acid Aβ peptide, thought to cause the neurodegenerative disease. [58] The enzymatic action of β-secretase leaves a C-terminal fragment known as APP-CTFβ or C99, within the membrane and releases APPsβ into the extracellular space. After Aβ peptide generation by γ-secretase from the C99 fragment, the Aβ peptide is extracellularly secreted [58].

Most of the Aβ peptides produced are 40 amino acids long, however it is thought in the diseased state Aβ42 peptide production increases, causing havoc within the cell and the surrounding environment. This longer form (Aβ42) is more hydrophobic and forms β-pleated sheets and fibrils more easily, and is also thought to be more neurotoxic than the Aβ40 peptide [50]. Various Aβ species exist within the brain, including Aβ1-39, Aβ1-40, Aβ1-42 and

Aβ1-43. These peptides can be found as monomers, oligomers, protofibrils and fibrils and in the latter stages form the amyloid plaques seen in the brains of AD patients [59]. Much debate has surrounded the identification of the actual toxic form of Aβ, ranging from the aggregation of the plaques themselves, to the newer idea that soluble forms, specifically oligomers of the peptide, are the detrimental form [60]. It is believed that Aβ1-42 is predominantly responsible for toxicity observed in AD. Mainly because familial AD mutations in APP elevate the expression of Aβ1-42 and that Aβ1-42 is more prone to aggregation and is more toxic than Aβ1-40 [60]. In AD, Aβ exists in both soluble and insoluble forms, with the later associated with plaques. Plaques were initially thought to be the toxic Aβ source however it is now suggested that soluble Aβ oligomers are more likely to be the source of Aβ toxicity, while fibers are inert [60]. This is supported by studies indicating the neurotoxicity of Aβ oligomers [61]. Research has confirmed the difficulty in identifying the toxic form of Aβ, due to its different assembly states, with this ability in itself being a potential mechanism of toxicity [60].

The implication of the amyloid casade hypothesis is that targeting Aβ should prevent the molecular pathology of AD and alter the course of the disease. Accordingly, Aβ-based therapies have been investigated in preclinical models and clinical trials [62]. However to date these clinical trials have been disappointing [62]. For example the ELAN immunotherapy trial was halted due to development of encephalitis in approximately 6% of patients [63]. However, subsequent studies have also reported some positive effects on plaque clearance [64]. While the amyloid cascade hypothesis of AD has broad support, the failure of drugs that target Aβ indicates possible limitations of this explanation. Furthermore positron emission tomographic (PET) imaging data have shown that while amyloid can be removed from the brain by immunotherapy, but this does not result in improved clinical outcomes [65], possibly due to treatment being applied too late in the course of the disease. This supports the possibility that amyloid plaques reflect the presence of disease but are not directly causative of neurodegeneration. Another possibility is that amyloid plaques are a protective response to underlying disease processes, acting as a storage mechanism to contain neurotoxic oligomers, as the presence of plaque inversely correlates with oxidative burden and synaptic loss [41].

Copper enhances Aβ cytotoxicity to cultured cells, which is ameliorated by co-incubation with zinc. By chelating divalent metals, plaques may confer protection against ROS damage [41].

Apart from a suggested increase in Aβ peptide production, the accumulation of the peptide in older age can be attributed to the malfunction or ineffectiveness of Aβ peptide clearance from

the brain [19,66]. Elimination of Aβ peptide can be instigated by phagocytosis of the peptide by the resident immune cells – microglia and astrocytes – said to be capable of degrading limited amounts and also transporting Aβ peptide from the brain into blood or CSF [67]. Other mechanisms of reducing Aβ peptide include enzymatic degradation such as that by neprilysin and insulin-degrading enzyme. Additionally, removal of the peptide across the blood brain barrier (BBB) can be mediated by proteins such as low-density lipoprotein receptor related protein and P-glycoprotein. Traditional consensus indicates that Aβ peptide accumulations occur extracellularly and that this is the cause of AD degeneration, comprising the basis of the amyloid theory of pathogenesis [26]. While validity of the amyloid hypothesis has been questioned due to the failure of Aβ immunotherapy approaches to improve cognition, further research to identify the actual component or form of the peptide that is neurotoxic may still lead to successful treatments.

1.5 Neuroinflammation

Whilst plaques and tangles are hallmark features of AD, there are other processes which contribute to disruption of brain function and neurodegeneration. Inflammation, seen in many diseases of the elderly, is observed in AD brains and has long been thought to trigger the pathology [68]. In addition, an important AD risk factor is head/brain injury, suggesting that chronic inflammation could initiate or at least contribute to the course of AD [69]. Epidemiological studies in the early 1990's suggested that exposure to anti-inflammatory drugs (more specifically non-steroidal anti-inflammatory drugs – NSAIDs) reduced the risk of developing AD in later life [70]. Whilst contradictory results have stemmed from this, including failed clinical trials of NSAID use in AD patients, it is evident that inflammation plays some part in the disease [70].

Much discussion surrounds the type of effect that inflammation plays and there is evidence for both beneficial and detrimental roles in the disease aetiology. Multiple inflammatory markers are observed in *postmortem* AD brains, including pro-, anti- and post-acting molecules, as well as activation of resident immune cells (glia) in the central nervous system – microglia and astrocytes [71-73]. These have been found in the AD-affected regions of the brain and also localised with plaques and NFT [73]. Throughout the progression of Aβ peptide accumulation, plaques are described as having particular immunoreactive elements. Studies have identified various inflammatory components in AD brains and plaques, including ApoE,

tau protein, ubiquitin, protein kinase C, complement proteins, fibroblast growth factor, advanced glycation end products, prostaglandins, cytokines, proteases, free radicals, redox metal ions, and chemokines [74-77]. These in turn can initiate further inflammatory pathways and indicate that inflammation participates in disease progression, although to what extent and in what pathways is still to be determined.

Microglia make up 12% of brain cells and fully activated microglia are essential for brain development and maintenance, although once activated, they can remain in this state and exacerbate neuronal damage in affected regions [73]. Activated microglia increase in number throughout AD progression and studies suggest that Aβ peptide deposits are capable of stimulating this activation, and microglia cluster around Aβ peptide aggregation sites [73]. Microglia reportedly produce, or signal other cells to produce pro-inflammatory molecules such as interleukin-1, interleukin-6 and tumour necrosis factor-alpha (TNFα), which recruits lymphocytes to inflamed areas [73].

In addition to triggering apoptosis, the excretion of neurotoxins and excitatory neurotransmitters can escalate and spiral out of control in the AD brain. Astroglia are major players in the maintenance of the BBB, including regulating its permeability [78]. Compromised BBB integrity is key to AD progression, as it regulates clearance of waste and metabolic by-products, influx of important nutrients and is a barrier against potential neurotoxic elements. However permeability changes may not be solely the effect of astroglial actions, and factors such as cerebral amyloid angiopathy may have a contributory role [78]. Whilst microglia and astrocytes are the resident immune cells within the brain, the neurons also respond to inflammatory signals, and may participate in pathology [79].

Additional regulatory molecules involved in inflammatory responses such as the COX enzymes, which produce prostaglandins by conversion of arachidonic acid, may link inflammation to increased production of Aβ peptide through increased prostaglandin E2 [80]. Prostaglandin E2 has been shown to increase cleavage of APP, producing Aβ40 and Aβ42 and can be reduced with COX inhibitors [80,81]. The mechanism/s by which inflammation contributes to AD pathology and/or progression is complex and probably includes multiple pathways. Genes or polymorphisms that enhance an individual's inflammatory response or susceptibility to a particular disease(s), in addition to compromised homeostasis of biological systems in old age, potentially give rise to chronic inflammatory activation and increase vulnerability to additional comorbidities. The ageing immune system with enhanced innate

immune responses has been suggested by those backing the 'inflammaging' theory [82,83]. Whilst a substantial body of evidence supports an important role for inflammation in AD, it is still unclear as to whether abnormal immune functioning is a causative, or secondary event in the development of AD pathology.

1.6 Mitochondrial dysfunction and energy metabolism

Mitochondria are the energy production "powerhouses" of the cell and any defects within these usually causes serious disorders, including many neurodegenerative diseases [84,85]. In dealing with large amounts of energy generation, including ATP and NAD^+ production, mitochondria have developed ways of managing the vast amounts of waste that are created. During ageing however, these systems become less efficient and can break down potentially initiating AD through mitochondrial dysfunction [86-88]. The ROS generated by dysfunctional mitochondria as well as other oxidised molecules generated by ROS, such as lipids and glucose, can become taxing to the cellular machinery, and increasingly difficult to clear with advancing age, with adverse implications for both individual cells and the body as a whole[86,87,89,90].

The main portion of ATP consumed by the brain is produced in mitochondria by oxidative phosphorylation. ATP production occurs within the inner mitochondrial membrane, and involves electron transport through a chain of protein complexes (I-IV) [88]. These complexes transfer electrons from electron donors (NADH, FADH) to O_2. During the electron carrying steps, protons (H^+) are transferred through the inner membrane against the chemiosmotic concentration gradient. The potential energy stored in this H^+ gradient is utilized to synthesize ATP from ADP and inorganic phosphate, as the protons are released through the ATP synthase [88]. Neurons require large amounts of energy for continuously operating processes such as: action potentials, resting membrane potential maintenance, active transport, receptor function, vesicle release, and neurotransmitter recycling [91]. Mitochondria can also mediate apoptosis and programmed cell death [86]. Therefore, the number and functionality of mitochondria play a significant role in neuronal function, proliferation and death, and have an impact on health and disease of the central nervous system [88].

Since mitochondria are central to cellular metabolism, findings of metabolic impairment have led to extensive investigation of the role of mitochondria in AD and other neurodegenerative

diseases. Early studies of AD brain tissue demonstrated that bioenergetic deficits occur at early stages of the disease [92-94]. For example, cerebral glucose metabolic rate observed via positron emission tomography scan in AD patients showed region-specific deficits in energy metabolism that preceded clinical impairments in attention, abstract reasoning, and language and visuospatial functions [95]. This was corroborated by evidence of reduced cerebral glucose metabolism prior to onset of clinical AD symptoms in patients carrying familial AD mutant genes and healthy elderly adults who were later diagnosed with AD [95]. Patients with MCI and patients with sporadic AD associated with the apolipoprotein Eε4 allele who subjectively complained of memory loss also exhibited reduced brain metabolism[96]. This coincides with reduced activity of pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (αKGDH) complexes, components of the tricarboxylic acid cycle, in the AD brain [97,98]. While abnormal activity of TCA cycle proteins were sometimes found concurrent with the presence of Aβ plaques, NFTs, and neuronal loss, they also occurred in the absence of these histological markers of AD neurodegeneration [92]. This suggests that altered PDH and αKGDH activities are not simply side effects of neurodegeneration [97,99] and further supports the possibility that metabolic deficiency occurs early and may contribute to AD progression.

Mitochondrial uncoupling occurs in tissue homogenates from patients with AD, frontotemporal dementia and Pick's disease, and may underlie the hypometabolism seen in AD[100]. In addition, the expression and activity of mitochondrial proteins are compromised in tissues and cells derived from AD patients [101-103]. Deregulation of mitochondrial fission/fusion proteins also occurs in AD brain [94], and in *post mortem* studies, fragmented mitochondria are observed in several areas of AD patient brains [93]. It has been suggested that highly functional, mature neurons require elongated highly connected mitochondrial networks to reduce the amount of mitochondrial movement necessary to handle the energy demand of the cell. This may explain why mitochondrial fragmentation coincided with their mislocalisation away from synapses in AD brain [94]. Taken together, this suggests that the energy demands of neurons are not being met in AD, which may contribute to the slow, progressive synaptic dysfunction and neuronal death observed in the disease. Cellular and animal models of AD exhibiting Aβ and tau pathologies replicate the metabolic and mitochondrial impairment observed in human AD patients [104,105].

In addition to generating energy for the cell, mitochondria regulate subcellular calcium signalling and homeostasis as well as subserving neuroplasticity. Distributed throughout the cell, including presynaptic terminals and the axons of neurons, mitochondria also have the ability to undergo fusion and fission, move rapidly between subcellular compartments, function as signalling platforms and respond to electrical, neurotransmitter and growth factor receptor stimuli. Mitochondrial fission and fusion pertains to the regulation of location, number, function and morphology of these organelles. Changes in mitochondrial dynamics have been associated with the processes of learning and memory such as long term potentiation and recovery of synapses in phases of high synaptic activity. When these processes malfunction, oxidised DNA and RNA and structural mitochondrial abnormalities are observed. Additionally, mitochondrial DNA mutations accumulated over the cell's lifecycle may contribute to ageing and disease aetiology as suggested by animal studies. Evidence of the presence of deformed mitochondria, including abnormal shapes and sizes and age related decrease in numbers of mitochondria have all been reported [106-109].

Glucose is essential for healthy brain function and is a primary source of energy in the brain. PET scanning shows a consistent reduction of cerebral glucose consumption in the brain of AD patients [110]. These observations support the notion that AD is linked to metabolic disorders among which diabetes is currently considered one of the highest risk factors for development of cognitive decline and AD [111,112]. These findings have led to preclinical studies aimed at establishing the efficacy of anti-diabetic treatments in animal models of the disease [113].

Redox proteomics studies on AD brain have demonstrated the oxidation of pyruvate kinase (PK), Phosphoglucomutase 1 (PGM1), Eno1, Triose-phosphate isomerase (TPI), fructose bisphosphate aldolase A/C (FBA A/C), ATP synthase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Malate dehydrogenase (MDH) [1]. Similarly, ATP synthase, PK, MDH, Eno1 and FBA C were found increasingly oxidized in amnestic MCI brain [114]. All these proteins are directly or indirectly involved in ATP synthesis in the brain; the inactivation of these proteins induced by oxidation might reduce glucose metabolism. In agreement with this view, CK, Eno1, PGM1, GAPDH, and ATPase activities are reportedly diminished in AD brain [110]. ATP synthase is an essential enzyme in the inner mitochondrial membrane, is the last complex (complexV) of the electron transport chain and is responsible for ATP synthesis. ATP synthase is oxidatively modified at different stages of AD [115] leading to inactivation of the complex. Moreover, it has been shown that ATP synthase activity is

decreased in the cortex of an AD mouse model [115]. Failure of ATP synthase activity significantly contributes to lower ATP levels, and possibly results in electron leakage and increased ROS production, suggesting an alternate rationale for the oxidative stress observed in AD. Thus, the reduced glucose utilization, mitochondrial deficits and decreased production of ATP seen in AD may be contributing factors to the neurodegenerative process and its progression culminating in AD pathology.

1.7 Metal Homeostasis

Metals are required in the body, especially in the brain, in order to perform redox reactions and also as components of some enzymes. An imbalance in metals can cause oxidative stress and neurodegeneration [77]. Metal chelators are capable of disrupting plaques and oxidative stress, although their toxicity and inability to cross the BBB has limited their use as therapeutic treatments for the disease [116,117]. Research on the metal chelator clioquinol has proven to be at least partially successful in both animal and human studies [118]. Metals that are in dyshomeostasis in AD brains include divalent ions of iron, zinc, and copper. These metals instigate aggregation of Aβ peptides, and Aβ peptides, bind the divalent metal ions of copper, iron and zinc [119]. Whether peptide accumulation is a compsenatory response to sequester excess metal or if the presence of metals causes Aβ aggregation in AD is still yet to be determined. Zinc is distributed ubiquitously in the brain, and its level significantly increases in the brain and serum of patients with AD [120,121]. Synaptic zinc levels are regulated by the zinc transporter 3 (ZnT3), which is essential for memory and normal cognitive function [122,123]. In AD, there is a redistribution of zinc into extracellular plaques and surrounding neuropil. Zinc binds Aβ residues 6-28 causing rapid aggregation and precipitation of the protein; thus co-deposition of zinc with Aβ could initiate plaque formation in the disease.

Zinc-induced plaque formation in disease is also supported by the anatomic distribution of plaque and zinc in the brain [124]. Plaque formation only occurs in neocortical regions of AD-affected brains, but Aβ is expressed in all brain regions [124]. The distribution of plaque closely aligns with the expression of ZnT3 prevalent in glutametergic neurons [125]. This protein traffics zinc into synaptic vesicles and zinc is released into the synapse upon exocytosis [125]. APP transgenic mice crossed with mice lacking ZnT3 were shown to have reduced plaque burden. Zinc also interfers with Aβ processing, the α-secretase involved in the processing of

APP is a zinc-dependent enzyme and zinc increases APP proteolysis, while zinc inhibits γ-secretase activity [126].

Copper is thought to interact with Aβ in two ways significant to AD pathology. First copper mediates the aggregation of Aβ under slightly acidic conditions [127]. Second and more importantly, copper and Aβ act synergistically to cause oxidative stress. A study of synthetic Aβ found that submicromolar concentrations of copper induced the aggregation of low nanomolar concentrations of Aβ under slightly acidic conditions *in vitro* [127]. The study also found that the aggregating effects of copper were reversible upon addition of a copper-chelating agent or with an increase in pH [127]. More importantly, another study established that Aβ in the presence of a catalytic quantity of copper produces hydrogen peroxide [128]. Toxicity due to the generation of hydrogen peroxide was confirmed by the addition of catalase which reduced Aβ toxicity by 75% [128]. Copper is also a redox active metal that can catalyse the formation of the hydroxyl radical via the Fenton reaction, and can cause free radical mediated damage and cell death in the same way as iron (Figure 1.4).

In the presence of amyloid, copper undergoes redox cycling, which enhances the potential for copper induced toxicity in AD. This is important because copper is increased in amyloid plaques, however, copper levels are decreased in AD neuronal tissue, which could deprive copper-binding proteins such as superoxide dismutase and ceruloplasmin of their metal cofactor, and consequently impair their function [121,129]. Superoxide dismutase is a major copper binding protein and antioxidant in neurons, which ultilises copper to convert the superoxide free radical into hydrogen peroxide. Ceruloplasmin is another major copper-binding protein, which functions as a ferroxidase to promote iron export. Copper also accelerates Aβ aggregation, but unlike zinc, copper induces oligomer rather than fibril structures [129]. Toxicity of Aβ oligomers can be attenuated in cultures by copper chelation [43,130]. The binding of copper to Aβ is likely to be a factor in AD because copper, too, is enriched in amyloid plaques.

Serum copper levels are elevated in patients with AD. In the brain, copper is concentrated in the extracellular compartment and in the amyloid plaques [129]. In a mouse model of AD, copper accumulates in the brain capillaries and parenchyma, and this accumulation is related to increased Aβ production, neuroninflammation, and reduced Aβ clearance [131]. Furthermore, oxidative stress and neuronal toxicity, induced by hydrogen peroxide and toxic hydroxyl radicals, reduce Cu(II) to Cu(I) through copper and Aβ interactions [7]. Therefore copper

affects Aβ production, clearance, aggregation and fibrillisation through diverse mechanisms, either directly or indirectly.

Iron is the most abundant transition metal in the body and in the brain. It exists in two forms: ferrous (Fe^{2+}) and ferric (Fe^{3+}). Its strong redox potential affords it versatile functions as a cofactor and biocatalyst in many vital as well as potentially damaging reactions in the cell [43]. In AD patients, accumulation of iron in the hippocampus and cerebral cortex colocalises with the lesions, NFTs and Aβ plaques [44]. Abnormal iron deposition in senile plaques can associate with Aβ and may promote increased formation of oxygen radicals [25]. The ability of iron to interact with oxygen is critical for energy production in the cell, but the same property makes it capable of producing damaging free radicals via the Fenton reaction. ROS resulting from this reaction, such as the hydroxyl radical, nonselectively damage intracellular structures via lipid peroxidation, protein cross-linking and carboxylation, and DNA mutation [132]. Aβ itself can also be a substrate for hydroxyl radical production [133]. Deposition of Aβ fragment and APP cleavage and synthesis are promoted by the presence of iron [134]. Several MRI studies have shown a positive correlation between ageing and iron accumulation in the brain [135,136]. As a consequence of iron accumulation, especially if ferritin does not also increase in parallel, the brain becomes increasingly vulnerable to iron-catalysed oxidative stress. Hence the age-related accumulation of iron may underlie or at least contribute to the commonly accepted theory that age is the biggest risk factor for most neurodegenerative diseases.

In vitro studies using synthetic Aβ have shown that iron induces the aggregation and potentiates the neurotoxicity of Aβ. Aβ binds to and reduces Fe^{3+} to Fe^{2+}, which is followed by formation of hydrogen peroxide [41]. The generation of Fe^{2+} and hydrogen peroxide creates conditions ideal for the Fenton reaction, which leads to the formation of the highly reactive hydroxyl radical.

Iron elevation in affected areas of the brain is a feature of a number of neurodegenerative diseases including AD [137]. Neuronal iron deposition causes oxidative stress via the Fenton reaction, which may contribute to elevated oxidative stress observed in AD [77]. Iron-induced oxidative stress has been shown to initiate several apoptotic pathways in neurons and damages proteins [77]. Oxidative damage to proteins and lipids by iron can cause synaptic dysfunction and neuronal cell death. The overload of iron in AD may also lead to increased Aβ production by increasing the expression of APP, and altering its processing. High iron

conditions lead to increased processing of APP, which in turn leads to accelerated degeneration in a mouse model of AD [138].

1.8 Plasma Biomarkers and AD

Although clinical manifestations of cognitive dysfunction and impairment of activities of daily living are the current standard for the diagnosis of AD, neurochemical and neuroimaging biomarker candidates are receiving increasing attention as potential objectively measurable diagnostic tools. Biochemical biomarkers in CSF have been extensively studied and are already a well-established diagnostic markers at least for the last stages of diagnostic validation [139]. Measurement of Aβ42, tau protein, and hyperphosphorylated tau protein (p-tau) in CSF of AD patients, as well as patients with other dementias and normal elderly controls revealed sensitivity and specificity levels between 80% and 90% for detecting AD versus normal elderly [140]. CSF is known to act as a valuable source of biomarkers, as being in direct contact with the brain and spinal cord it provides a complete representation of various biochemical and metabolic profiles of the brain. However, this fluid is obtained by lumbar puncture in patients which is invasive, more painful for patients, and carries a small risk of serious side effects. Thus obtaining CSF, especially repeatedly, on large numbers of elderly individuals in the community is inherently challenging, risky, and is not likely to be accepted world-wide as routine approach for AD diagnosis. Thus, it is essential to identify new biomarkers in other sources such as plasma and urine, which are cheaper, less invasive, easily collectable and allow follow-up and tracking of patients over time. Blood samples are easy to obtain and offer a rich source of disease biomarkers, as approximately 500 mL of CSF are absorbed daily into the circulating blood. Furthermore, damage to the blood brain barrier which occurs in the course of AD may enhance exchange of proteins between CSF and blood in either direction [141].

Previous studies have revealed some potential AD biomarkers in plasma. One such biomarker are miRNAs, which belong to the class of non-coding RNA molecules which regulate more than 60% of all known genes through post-transcriptional gene silencing [142]. The dysregulation of miRNA expression in peripheral blood can serve as a potential source of diagnosis of Alzheimer's disease. Schipper et al identified a number of downregulated miRNAs in sporadic AD patients [143]. The targets of these miRNAs were interestingly found to be part of p53, Notch and Bcl-2 pathways which are already known to be involved in AD

pathogenesis [143]. Other studies have also found decreased levels of miRNA in the brain and blood of AD patients [144,145]. It has been suggested that specific miRNAs can regulate important cellular functions in the brain. Thus the contribution of miRNAs to functions such as redox defences and DNA repair mechanisms highlight the potential of miRNAs as therapeutic biomarkers for AD.

Although the efficiency of amyloid beta as a highly sensitive and specific biomarker from CSF for AD has already been established, studies focussing on evaluating $A\beta$ as a potential biomarker from blood serum have been undertaken as well. A meta-analysis performed by Koyama et al showed a highly statistical and clinically significant decrease in $A\beta_{1-42}$ ratio to predict cognitive impairment [146].

Neuroinflammation is a critical and invariable component of many neurodegenerative diseases. Indeed, the co-occurrence of inflammatory markers such as interleukins, cytokines, chemokines, growth factors and acute phase reactant proteins in plasma, CSF and *post-mortem* brain tissue has become a hallmark feature of AD [72,147-151]. The complement system may also have a role in the clearance of amyloid [152].

Oxidative stress is another area being explored for biomarkers in AD. The parts of brain affected by neurodegeneration show increased ROS levels. In such conditions, proteins undergo post-translation modifications, leading to formation of mixed disulphides, nitration of tyrosine residues, and formation of lipid peroxides. Protein oxidation besides causing toxic cell damage also results in fragmentation and aggregation, leading to proteolysis. The most common known markers for oxidative stress reported in AD include protein DNA oxidation, isoprostane formation, 4-Hydroxy 2 trans nonenal (HNE), lipid peroxidation and advanced glycation end products [37,153,154].

There is also growing evidence of the positive effects of parabiosis experiments, in which circulatory systems of young and aged animals are connected. These studies show that blood from young animals can have rejuvenating and disease modifying effects on older animals [155,156], however the specific components of blood responsible for this effect are not well understood.

Conboy et al hypothesized that there are systemic factors that support the robust regeneration of tissues in young animals and/or inhibit regeneration in old animals, and that these factors act to modulate the key molecular pathways that control regenerative properties [155].

Exposing old mice to factors present in young serum restored the activation of Notch signalling as well as the proliferation and regenerative capacity of aged satellite cells [155]. Villeda et al showed that exposure of an aged animal to young blood can counteract and reverse pre-existing effects of brain ageing at the molecular, structural, functional and cognitive level [156,157]. That blood can have such potent disease attenuating effects in the CNS also indicates that there is important information exchange between blood and the CNS. Synaptic plasticity–related transcriptional changes in the hippocampus of aged mice was found, dendritic spine density of mature neurons increased and synaptic plasticity improved in the hippocampus of aged heterochronic parabionts [156]. At the cognitive level, systemic administration of young blood plasma into aged mice improved age-related cognitive impairments in both contextual fear conditioning and spatial learning and memory [156].

1.9 Polyphenols

Polyphenols may hold potential therapeutic benefits in age-related disorders due to their potent free-radical scavenging and antioxidant effects, thus promoting cell longevity through preventing oxidative damage [158]. Polyphenols are secondary plant metabolites that are involved in plant defence against pathogens and ultraviolet damage [159]. They are found in many fruits, herbs and vegetables and thus serve as important dietary micronutrients. Chemically, polyphenols include a wide variety of biomolecules which contain several hydroxyl groups on one or more aromatic rings. They can be divided into various groups according to chemical structure – including flavonoids, stilbenes and lignans [160,161]. Natural phenolics are compounds which contain hydroxyl groups on only a single aromatic ring and therefore are smaller in size than polyphenols. They include phenolic acids and phenolic alcohols (see Table 1.1 for further details). Recently, the potential role of polyphenols in ageing and neurodegeneration has widened with discoveries that they can modulate various important pathways in the pathogenesis of AD by reducing amyloid aggregation and inflammation and by modulating sirtuins which are involved in longevity and cell survival.

Polyphenols are well known as antioxidants and direct scavengers of free radicals [158,162,163]. In addition, polyphenols can act as metal chelators [164-166], which adds to the antioxidant effects of these compounds through inhibition of transition metal-catalysed free radical formation [77]. Chelation of transition metals such as Fe^{2+} can directly reduce the rate of the Fenton reaction thus preventing oxidation caused by highly reactive hydroxyl radicals [167,168]. Epigallocatechin

gallate (EGCG), a green tea polyphenol, for examples has also been shown to be a potent chelator of transition metals such as copper and iron [169], and inhibits over 90% of iron-mediated DNA damage caused by Fe^{2+} and H_2O_2 [170]. It has also been found that polyphenols are involved in the regeneration of essential vitamins [171,172]. Polyphenols can activate antioxidant enzymes such as gluthathione peroxidase, catalase, superoxide dismutase, as well as hydrogen peroxide and superoxide anions, and inhibit the expression of enzymes such as xanthine oxidase, which is involved in the generation of free radicals [170,173-180]. These multiple antioxidant effects prevent oxidative damage to important cellular components and have lead to investigation of polyphenols as therapeutic agents for the treatment of AD, to combat the increase in oxidative stress seen in this disease.

Several studies have suggested that polyphenols may have anti-amyloidogenic effects. A number of polyphenols including tannic acid, quercetin, kaempferol, curcumin, catechin and epicatechin were shown to dose-dependently inhibit the formation of Aβ fibrils as well as their elongation [181,182]. In addition, polyphenols can bind directly to Aβ or mature aggregates and impair their stability, as all the compounds tested destabilised pre-formed Aβ fibrils [182]. EGCG significantly inhibits Aβ aggregation and has the ability to remodel large Aβ fibrils into smaller aggregates which displayed no toxic effects [183] (Figure 1.5). Fish oil has a synergistic effect in combination with EGCG, leading to reduction in Aβ plaque formation and levels of Aβ(1-40) and Aβ(1-42) in AD transgenic Tg2576 mice [184].

Tannic acid was shown to reduce Aβ deposits as well as Aβ species including oligomers in the transgenic AD mouse brain [182,185]. Tannic acid decreased cleavage of the β-carboxyl-terminal APP fragment, lowered APP-β production, and attenuated neuroinflammation [185]. It is thought to inhibit amyloidogenic APP metabolism by reducing β-site APP cleaving enzyme 1 expression and lowering β-secretase activity [185]. EGCG also down-regulates APP levels and cerebral amyloidosis in Alzheimer transgenic mice [186].

Curcumin, found in the spice turmeric, has been shown to prevent brain lipid peroxidation in rats and increase gluthathione levels and the activity of other detoxifying enzymes [187,188]. Curcumin has also been shown to protect cells against Aβ-induced oxidative insult [189]. When administered to AD transgenic mice, curcumin was found to prevent protein oxidation and inflammation and reduce the formation of soluble and insoluble Aβ by up to 50% [190].

Luteolin, a citrus polyphenol was found to reduce Aβ peptide production in cultured neurons and was also found to decrease amyloidogenic γ-secretase APP processing [191]. In the AD

mouse model Tg2576 mice, this compound along with a structurally similar compound, diosim, were both shown to reduce soluble Aβ levels [191]. In a recent study anthocyanin-enriched fractions of bilberry and blackberry extracts were found to modulate APP processing and significantly alter Aβ40 and Aβ42 levels. Both compounds also alleviated spatial working memory deficits in a mouse model of AD [192].

Abnormal folding of the microtubule-associated protein tau leads to aggregation of tau into neurofibrillary tangles, another major pathological hallmark of AD. It has been shown that polyphenols derived from grape seed extract reduce tau pathology in mouse models of AD [193-195]. These polyphenols also enhanced disaggregation of neurofibrillary filaments, and improved structural stability [193,195]. EGCG also modulates tau pathology in a mouse model of Alzheimer's disease [196]. Tannic acid dose-dependently inhibits tau aggregation and polymerisation through formation of a hairpin binding motif [197].

Table 1.1 Influence of Polyphenols on Sirtuins and Pathways involved in Ageing and AD

Polyphenol	Fruit/Vegetable	Effect on Sirtuins	Influence on AD	Influence in Ageing	Reference
Resveratrol	Red Grapes (1.5-7.8ug/g), Red wine (1.98-7.13mg/L),	Increased SIRT1,SIRT2, SIRT3, SIRT4 and SIRT7	Selectively remodels toxic amyloid aggregates; reduces Aβ plaques in animal models;	CR mimetic; increases longevity in a variety of organisms from yeast to mammals;	198-205
Quercetin	Black and Green Tea (2-2.5ug/g) Apples (0.4ug/g) Red Onion (0.2ug/g)	Increased SIRT1	Reduces Aβ-mediated cyctotoxicity and OS	Extends life span in worms and yeast	206-210
Curcumin	Tumeric (50mg/g)	Increased SIRT1	Reduces Aβ levels in AD mice; enhances memory in AD rat model	Extends life span in flies and mice	211-215
Kaempferol	Tea, Broccoli, Cabbage, Beans, Leek, Tomato, Berries, Grapes, Apple	Increased SIRT3	Reversed Aβ-induced impaired performance of mice in a maze test; inhibit Aβ formation and toxicity	Protective effects against OS-induced cytotoxicity;	182,216-219
Catechin, gallic acid, gentisic acid, chlorogenic acid, epicatechin	Bitter Melon	Increased SIRT1 and SIRT3	Attenuates OS and neuroinflammation		220,221
Procyanidins, phloridzin and other unidentified phenolics	**Apple**	No extension of lifespan in worms lacking Sir2.1; increase Sirt1 activity in yeast	Not known	Extend lifespan of Drosophila flies and in worms; upregulates superoxide dismutase and catalase genes	222-224
Astragaloside, astragalus saponin I	**Astragalus root**	Not known	Prevents amyloid induced memory and neuronal loss in AD mouse model	Reduced DNA damage after OS, increased telomere length in mice fibroblasts	225-229
EGCG, Catechins	**Green tea**	Increase Sir2 activity in yeast	Neuroprotective; Inhibits formation of fibrillar Aβ;	Increases lifespan in worms, flies and mice and	171,186,196,230-235

			metal chelating properties	delays memory regression in mice models of ageing	
Quercetin, kaempferol, catechin, rutin, naringin	**Centella Asiatica**	Not known	Cognitive enhancing effects; reduces brain Aβ levels; protective against Aβ-induced neurotoxicity;	reduced markers of oxidative stress in the brain, increases activity of antioxidant enzymes	[236-242]
Ginsenoside Rbl	**Ginseng**	Not known	Inhibit β-secretase activity; protect against Aβ toxicity, reduce ROS production and apoptosis; reduced tau phosphorylation; anti-inflammatory effect in the brain of AD rat model	Improves cognition in senescence-accelerated mice; improves memory loss in mice; ginseng intake decreased mortality in older males	[243-249]
Proanthocyanidins	**Blueberry**	Not known	Suppress OS; improves learning and memory in mice;	Increase lifespan in worms	[250-255]
Catechins, Proanthocyanidins, Tannins, Resveratrol	**Grape seed**	Not known	Inhibit abnormal protein aggregation and enhance cognition	Increases telomere length in mice; increase lifespan in Drosophilia and mice	[256-260]
Procyanidins	**Cocoa**	Not known	Increased resistence to oxidative stress in yeast and worms; improved cognition	Increase lifespan of worms and rats	[261,262]
α-mangostin	Mangosteen	Not known	Inhibits Aβ aggregation and Aβ-induced cytotoxicity [263] [264]		

1.10 Involvement of Sirtuins in Ageing and Neurodegeneration

Sirtuins are a class of proteins that possess deacetylase or mono-ribosyltransferase activity and play critical roles in cell survival in response to oxidative stress and caloric restriction (CR) regimes [265,266]. The reversible acetylation of proteins controls their activity, and deacetylation leads to their activation or inactivation. Sirtuins are orthologues of yeast Sir2 protein, where SIR stands for silent information regulator, because in yeast, where Sir2 was first discovered, the protein silences certain genes and results in the extension of replicative lifespan. Sirtuins are NAD^+ dependent lysine deacetylases. The sirtuin-mediated deacetylation reaction couples lysine deacetylation to nicotinamide adenine dinucleotide (NAD) hydrolysis. The NAD^+/NADH ratio affects sirtuin activity; at times of low energy availability, this ratio increases, leading to higher sirtuin activity and deacetylation of other proteins.

During the deacetylation catalysed by sirtuins, a cleavage of the chemical bond between nicotinamide and ribose in NAD is coupled with the transfer of an acetyl group from the substrate (acetylated lysine residue) to ribose within the remaining ADP-ribose molecule. The final products of the reaction are: deacetylated lysine residue, O-acetyl-ADP-ribose, and nicotinamide. Thus sirtuin activity may be determined by the quantity of sirtuin molecules, availability of NAD as a co-substrate, and local concentration of nicotinamide which is a product inhibitor of sirtuin activity.

In mammals, seven sirtuins (SIRT1-7) have been identified. All mammalian sirtuins contain a conserved NAD-binding and catalytic domain, but differ in their N and C-terminal domains [265]. They have different specific substrates including histones, transcriptional regulators and enzymes [266]. They also have various biological functions, are localised in specific cell compartments, and play various roles in health and disease including ageing and neurodegeneration [266] (Table 1.2).

Sirtuins can affect ROS production and increase resistance to its damaging effects. Oxidative stress has been shown to decrease SIRT1 expression in the rat hippocampus and cortex [267]. SIRT1 overexpression prevents oxidative stress-induced apoptosis and increases resistance to oxidative stress through regulation of the FOXO family of forkhead transcription factors [268]. The only cytoplasmic sirtuin protein SIRT2, has been shown to increase in response to oxidative stress but promotes cell death through FOXO proteins [269]. SIRT3, a mitochondrial

protein, reduces oxidative stress through activation of superoxidase dismutase activity [270]. In a recent study it was found that SIRT3 mRNA expression is elevated in response to increased mitochondrial ROS production in primary mouse hippocampal cells and the SIRT3 over-expression increases neuronal lifespan [271]. In AD transgenic mice SIRT3 expression was shown to mirror the pattern of Aβ deposition [271]. The study also showed a significant increase in SIRT3 expression in the AD temporal cortex of human *post-mortem* brain tissue [271]. SIRT6, like the founding member of the sirtuin family SIRT1, is a nuclear protein which is involved in oxidative-stress induced DNA repair through its activation of the PARP-1 DNA repair enzyme [272], (for a summary see Table 1.2 and Figure 1.6).

Table 1.2 The Role of the Sirtuin family in Calorie Restriction, Ageing and Neurodegeneration

Name	Location	Involvement in Calorie Restriction, Ageing and Neurodegeneration
SIRT1	**Nucleus**	Suppresses amyloid production by promoting α-secretase cleavage of APP. [273]
		Ameliorates learning impairment and neurodegeneration in mouse models. [274]
		Induces the Notch pathway, involved in repair of neuronal damage in the brain. [273]
		Regulates proteins and genes involved in antioxidant response (FOXO3), apoptosis (p53) and anti-inflammatory response (NFκB) [268,275,276,277,278]
		Modulates memory formation and synaptic plasticity. Activation of SIRT1 in brain enhances, and its deletion impairs, memory function and synaptic plasticity in mice [279]
		Reduction in SIRT1 is associated with accumulation of Aβ and tau in the cerebral cortex of AD patients [280,281]
		Promotes feeding behaviour during dietary limited conditions including calorie restriction or fasting [282]
		Mouse KO: impaired cognitive abilities, decreased dendritic branching, length and complexity, reduced oxidised proteins and lipids in the brain, lower growth hormone levels, shortened lifespan [279,283,284]
SIRT2	**Cytoplasm**	Appears to promote neurodegeneration as SIRT2 inhibition prevents α-synuclein-mediated toxicity in a Parkinson's disease model [285]
		A tubulin deacetylase which regulates cell division and differentiation and accumulates with age [286]
		Impairs neurite outgrowth and oligodendrocyte differentiation and promotes axonal degeneration [287,288,289]
		Small molecule inhibitor of SIRT2 showed neuroprotection via cholesterol-reducing properties in a Huntington's disease model [290,291]
		Upregulates in response to calorie restriction and oxidative stress, and

		promotes cell death under severe stress conditions via interaction with FOXO3a. [269] Involved in myelin formation [292]
SIRT3	**Mitochondria**	Protects against excitotoxic injury in neurons [293] Plays a role in CR-mediated reduction in oxidative stress and decreases reactive oxygen species by increasing activity of superoxide dismutase 2 [294 295 296] Variability of the SIRT3 gene may have a role in human longevity [297 298] SIRT3 upregulates fatty acid oxidation and lowers ATP levels during fasting [299] Involved in the maintenance of mitochondrial function by preventing premature opening of the mitochondrial permeability transition pore [300]
SIRT4	**Mitochondria l Matrix**	Downregulates glutamate dehydrogenase activity, and controls insulin secretion in response to calorie restriction [301] Negatively regulates fatty acid oxidation in combination with SIRT1 [302]
SIRT5	**Mitochondria l Matrix**	SIRT5 promoter polymorphism is associated with a higher brain molecular ageing [303] Involved in regulation of the urea cycle during fasting and calorie restriction [304,305]
SIRT6	**Nucleus**	Neuronal SIRT6 is involved in regulation of somatic growth and obesity prevention in mice [306] Involved in DNA double strand break repair following oxidative stress by inducing PARP1 activity [307] SIRT6 associates with telomeric chromatin and depletion of SIRT6 results in premature cellular senescence and telomere dysfunction [308 309] Prevention of transforming growth factor-β-induced cell senescence in human epithelial cells [310] SIRT6 genes suppress NF-kappaB signalling and thus can delay the ageing process and extend lifespan [311] SIRT6-knockout mice exhibit genomic instability, ageing-like phenotypes and die from hypoglycaemia by 4-5 weeks. Stem cells that are deficient in SIRT6 show genomic instability [312]
SIRT7	**Nucleus**	SIRT7 overexpression in fibroblasts inhibits cell growth and proliferation and promotes cell cycle arrest [313] Positive regulator of RNA polymerase I transcription [314] SIRT7 deficent mice exhibit heart hypertrophy, inflammatory cardiomyopathy and a reduced lifespan [315]

Interest in sirtuins increased greatly after the discovery that the sirtuin gene, Sir2 (SIRT1 being the equivalent in mammals) extends yeast lifespan up to 30 % [316]. A similar effect was found in nematodes and flies and it was thought the effect may occur through a pathway related to caloric restriction [317,318]. Recent work however suggest that the effects on longevity may not be as strong as were observed in the early studies, as controlling for genetic background removed any effects on longevity [319]. The sirtuin system is strongly influenced by caloric restriction (CR), which refers to a reduction in calorie intake without malnutrition [320]. CR has a wide range of benefits in humans [321-323] and life span extension in many species from yeast, fruit flies, nematodes, rodents to primates [324-327]. Furthermore, CR for 3 months was found to improve memory functions in the elderly [328]. CR is also known to modulate neuroinflammation and oxidative stress in various animal models [329-331].

The mechanism by which CR increases life span is still unclear. Some theories include reduced cellular divisions, reduced metabolic rate, reduced production of ROS and hormesis [332-335]. Hormesis is used to describe the beneficial actions resulting from the response of an organism to a low-intensity biological stressor [334]. The hormesis theory of CR proposes that diet imposes a low-intensity biological stress on the organism, which causes a defence response that helps protect it against the causes of ageing [334]. Therefore, it is highly likely that CR places the organism in a defensive state, and enables it to survive adversity, leading to improved health and life span.

Evidence suggests that some of the beneficial effects of CR occur through its influence on sirtuins. The functions of mitochondrial sirtuins suggest that they control activity of several metabolic enzymes and play a crucial role in metabolic adaptation to dietary conditions, such as CR and fasting [336] (Table 1.2). CR does not extend lifespan in SIRT1-deficient mice, suggesting that CR works via activation of SIRT1 [327,337]. Conversely, transgenic mice overexpressing SIRT1 display a number of phenotypic features, such as increased metabolic activity, glucose tolerance, and reduced body weight and cholesterol levels [337]. The ability of SIRT1 to mimic the anti-ageing effects of CR is most likely linked to its regulation of various proteins and genes involved in oxidative stress, inflammation, amyloidogenesis and synaptic plasticity (Table 1.2 and Figure 1.6).

CR prevents Aβ neuropathology in animal models of AD and modulation of SIRT1 expression or activity may be a mechanism by which CR influences AD-type neuropathology [281,327,338]. In one study 30% CR resulted in significantly lower levels of Aβ (1-40) and Aβ (1-

42) in the temporal cortex of Squirrel monkeys relative to control fed monkeys. The cortical Aβ levels were inversely correlated with SIRT1 protein concentrations in the same brain region, and there was also a selective elevation of α- but not β- or γ-secretase activity [327]. As mentioned previously, the cleavage of the membrane-bound APP leads to a range of protein fragments that have a role in AD pathogenesis [339]. Cleavage of APP by β-secretase leads to the formation of APPs-β, and together with γ-secretase leads to the generation of the Aβ peptides (1-40) and (1-42). The latter is thought to be the main toxic component in AD [339]. If APP is processed by α-secretase, then the formation of these pathogenic peptides is prevented, and instead a fragment APPs-α that is associated with a neuroprotective effect is generated [340,341]. SIRT1 is able to modulate APP processing by activating the expression of the α-secretase gene ADAM10, thereby reducing brain levels of Aβ(1-42) as well as Aβ(1-40) [340] (Figure 1.5). Moreover, deacetylation of the retinoic acid receptor β (RARβ) by SIRT1 can induce transcription of the ADAM10 gene and suppress the production of the neurotoxic Aβ(1-42) peptide [340]. There is however, no published literature to date on the effects of CR or sirtuin activation in AD using human clinical trials.

Figure 1.6: Influence of SIRT1 and polyphenols on APP processing and amyloid formation. SIRT1 and the green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) activate ADAM10 and thus enhance α-secretase APP processing leading to the generation of non-amyloidogenic peptides. Resveratrol is able to selectively remodel toxic amyloid aggregates into non-toxic species. Other polyphenols such as curcumin, kaempferol and grape seed extract polyphenols (GSEP) also have the ability to reduce Aβ levels in the brain.
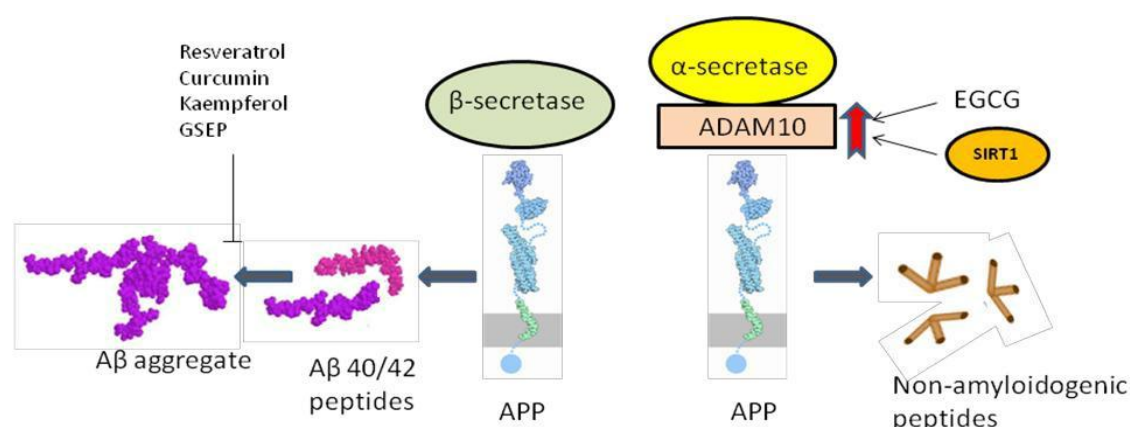
SIRT1 induces a neuroprotective effect through its interaction with FOXO proteins, p53 and NFκB [268,275,277]. SIRT1 is also directly activated by resveratrol. SIRT2 is involved in regulation of the cell cycle as well as neurite outgrowth and axonal degeneration, possibly through its ability to deacteylate tubulin [269,286]. The cellular effects of mammalian sirtuins are broad-ranging since they are found in multiple organelles, can translocate across membranes and between organelles and are likely involved in active responses to changing cellular environments. However the mitochondria have more sirtuins than any other cellular compartment, which may reflect an important link between cellular energy metabolism and longevity since mitochondria are the primary cellular organelles responsible for energy production. SIRT3, 4, and 5 are expressed only in mitochondria. SIRT3 appears to be the predominant mitochondrial deacetylase and to play an important role in several mitochondrial pathways in all tissues [336]. The targets of SIRT3 include proteins involved for example in substrate utilization, electron transfer, and redox homeostasis [342]. SIRT3 reduces oxidative stress by increasing superoxidase dismutase activity. It also deacetylates cyclophilin D (which regulates opening of the mPTP) and thus prevents age-related increase in mitochondrial swelling due to increased opening of the mPTP. In response to caloric restriction or fasting, SIRT3 deacetylates a set of mitochondrial proteins, resulting in

activation, inhibition, and allosteric modification of protein functioning [270]. Unlike other sirtuins, SIRT4 does not possess deacetylase activity, but it is instead an ADP-ribosyltransferase [343]. It is activated in response to amino acids, downregulating insulin secretion by inhibiting mitochondrial glutamate dehydrogenase 1 activity . SIRT4 is also involved in regulation of fatty acid oxidation and mitochondrial gene expression in liver and muscle [344]. SIRT5 is found in the cytosol and mitochondria however, the targets of SIRT5 are not yet well identified. It has been shown to regulate energetic flux through glycolysis and also protein malonylation [345]. The deacetylase activity of SIRT5 is weak, and its main targets are succinyl and malonyl groups. In addition to glycolysis, it is likely involved in many other metabolic pathways, including the urea cycle, where it activates detoxification of excess ammonia that may accumulate during fasting [304]. In a recent study using a SIRT3-/- mouse knockout model relevant for neurological disease, hippocampal SIRT3 expression was found to be enhanced by running wheel exercise [346]. The striatal and hippocampal neurons of mice lacking SIRT3 showed increased vulnerability in pathological conditions. Another study showed that overexpression of SIRT3 protected against age-related hearing loss in mice via enhancing the mitochondrial glutathione antioxidant defense system, suggesting a neuroprotective role for SIRT3 [347]. SIRT6 is involved in DNA repair following oxidative stress through activation of the DNA repair enzyme PARP-1 [307]. SIRT7 is thought to be involved in RNA transcription as it activates RNA polymerase 1 transcription [314].

Figure 1.7 Cellular Roles/Potential Roles and mechanisms of action of Sirt1-7
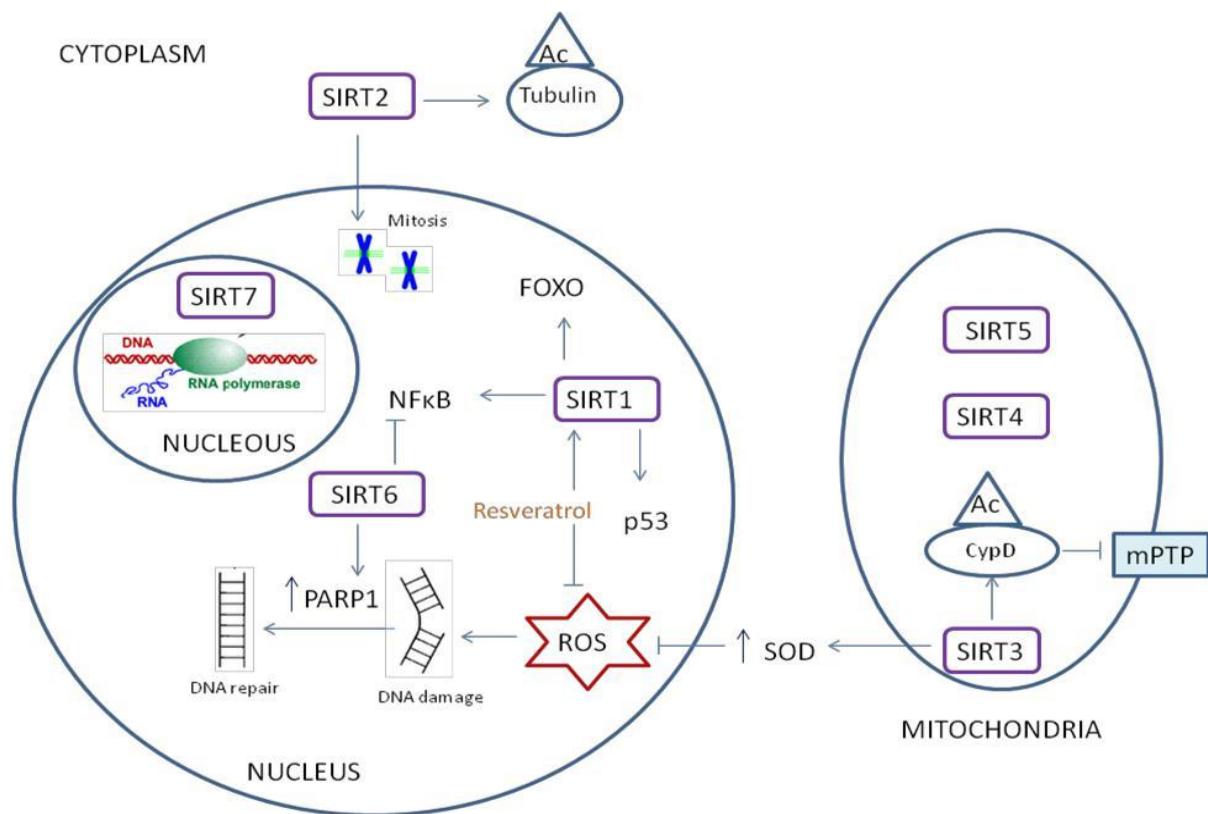
1.11 Polyphenols, sirtuins and ageing

The most studied polyphenol which shows effects on the sirtuin family of proteins is resveratrol which is found in its highest levels in grape seed and skin as well as in red wine (see Table 1.3 for details). Apart from its free radical scavenging and antioxidant properties, resveratrol has many of the same benefits as calorie restriction. Resveratrol is associated with neuroprotective effects [348-350], and other beneficial effects such as: maintenance of mitochondrial integrity, reduction of insulin-like growth factor-1 expression, activation of SIRT1, and increased longevity in some animals models [207,351-358], though life span effects are controversial [359-361]. Epidemiological evidence suggests that moderate red wine consumption, which is rich in resveratrol, reduces the risk of dementia [362].

Resveratrol has also been shown to improve outcomes after ischemic episodes in the heart and brain of mice by a SIRT1-mediated process [363,364]. Resveratrol strongly stimulates SIRT1 deacetylase activity in a dose-dependent manner by increasing its binding affinity to both the acetylated substrate and NAD^{+} [365]. Whether resveratrol is a direct or indirect activator of SIRT1 remains controversial [366,367]. Resveratrol and CR have also been shown to modulate

mitochondrial sirtuins [270,347,367,368]. Resveratrol-fed rats showed increased expression of SIRT3 and SIRT4 in the heart compared to control-fed mice [369], however the opposite effect was seen in the liver of a zebrafish model [370]. Resveratrol also increased expression of SIRT3 in adipocytes [371]. Resveratrol mediated changes to sirtuin expression in the brain have yet to be intensively studied. Investigation of polyphenolic sirtuin-activating compounds are thought to hold promise for enhancing longevity [207,372].

Table 1.3: Influence of Polyphenols on Sirtuins and Pathways involved in Ageing and AD

Polyphenol	Fruit/Vegetable	Effect on Sirtuins	Influence on AD	Influence in Ageing	Reference
Resveratrol	Red Grapes (1.5-7.8ug/g), Red wine (1.98-7.13mg/L),	Increased SIRT1,SIRT2, SIRT3, SIRT4 and SIRT7	Selectively remodels toxic amyloid aggregates; reduces Aβ plaques in animal models;	CR mimetic; increases longevity in a variety of organisms from yeast to mammals;	[198-205]
Quercetin	Black and Green Tea (2-2.5ug/g) Apples (0.4ug/g) Red Onion (0.2ug/g)	Increased SIRT1	Reduces Aβ-mediated cyctotoxicity and OS	Extends life span in worms and yeast	[206-210]
Curcumin	Tumeric (50mg/g)	Increased SIRT1	Reduces Aβ levels in AD mice; enhances memory in AD rat model	Extends life span in flies and mice	[211-215]
Kaempferol	Tea, Broccoli, Cabbage, Beans, Leek, Tomato, Berries, Grapes, Apple	Increased SIRT3	Reversed Aβ-induced impaired performance of mice in a maze test; inhibit Aβ formation and toxicity	Protective effects against OS-induced cytotoxicity;	[182,216-219]
Catechin, gallic acid, gentisic acid, chlorogenic acid,	Bitter Melon	Increased SIRT1 and SIRT3	Attenuates OS and neuroinflammation		[220,221]

epicatechin					
Procyanidins, phloridzin and other unidentified phenolics	**Apple**	No extension of lifespan in worms lacking Sir2.1; increase Sirt1 activity in yeast	Not known	Extend lifespan of Drosophila flies and in worms; upregulates superoxide dismutase and catalase genes	[222-224]
Astragaloside, astragalus saponin I	**Astragalus root**	Not known	Prevents amyloid induced memory and neuronal loss in AD mouse model	Reduced DNA damage after OS, increased telomere length in mice fibroblasts	[225-229]
EGCG, Catechins	**Green tea**	Increase Sir2 activity in yeast	Neuroprotective; Inhibits formation of fibrillar Aβ; metal chelating properties	Increases lifespan in worms, flies and mice and delays memory regression in mice models of ageing	[171,186,196,230-235]
Quercetin, kaempferol, catechin, rutin, naringin	**Centella Asiatica**	Not known	Cognitive enhancing effects; reduces brain Aβ levels; protective against Aβ-induced neurotoxicity;	reduced markers of oxidative stress in the brain, increases activity of antioxidant enzymes	[236-242]
Ginsenoside Rbl	**Ginseng**	Not known	Inhibit β-secretase activity; protect against Aβ toxicity, reduce ROS production and apoptosis; reduced tau phosphorylation; anti-inflammatory effect in the brain of AD rat model	Improves cognition in senescence-accelerated mice; improves memory loss in mice; ginseng intake decreased mortality in older males	[243-249]
Proanthocyanidins	**Blueberry**	Not known	Suppress OS; improves learning and memory in	Increase lifespan in worms	[250-255]

			mice;	
Catechins, Proanthocyanidins, Tannins, Resveratrol	**Grape seed**	Not known	Inhibit abnormal protein aggregation and enhance cognition	Increases telomere length in mice; increase lifespan in Drosophilia and mice [256-260]
Procyanidins	**Cocoa**	Not known	Increased resistence to oxidative stress in yeast and worms; improved cognition	Increase lifespan of worms and rats [261,262]
α-mangostin	Mangosteen	Not known	Inhibits Aβ aggregation and Aβ-induced cytotoxicity [263] [264]	

A variety of polyphenols (or foods rich in polyphenolic compounds) have been investigated for their effects on AD and longevity (Table 1.3). Interestingly, many of these compounds influence sirtuins through direct or indirect pathways. For example, in a recent study, the functional food, bitter melon was found to attenuate oxidative stress and neuroinflammation in mice on a high fat diet [373]. Functional foods are foods or food components which beyond providing basic nutritional needs, also have physiological/pharmacological benefits or reduce the risk of chronic diseases [374]. Bitter melon was also found to regulate sirtuin protein and mRNA expression. A high fat diet significantly reduced SIRT1 mRNA expression by 20%, and brain SIRT1 protein levels were reduced by 25% in C57BL/6 female mice [373]. Feeding of bitter melon normalised both. Interestingly, bitter melon not only normalised SIRT3 levels but also up-regulated SIRT3 mRNA by 20% [373]. Bitter melon also attenuated oxidative stress and neuroinflammatory marker levels caused by the high fat diet. The bitter melon extract contains a variety of polyphenols including gallic acid, gentistic acid, chlorogenic acid and epicatechin, but the most abundant polyphenol isolated was catechin [373]. Catechins are also most abundant in tea, cocoa and berries and daily consumption of catechin increases life span and delays memory regression in animal models of ageing [375,376].

Another traditional food, the herb, *Centella asiatica* has been reported to have a variety of beneficial effects on Aβ-mediated toxicity, and to promote longevity [237]. It contains numerous polyphenols including quercetin, kaempferol, catechin, rutin and naringin, some of which are potent antioxidants and have demonstrated effects on sirtuins (Table 1.3). Therefore *C.asiatica* may have the ability to modulate the effects of oxidative stress in the brain directly, and through regulation of sirtuin activity since several of its polyphenols influence the activity and expression of sirtuins. Most of the work to date has focused on SIRT1 as it is the mammalian homolog of yeast Sir2, which was the first organism in which sirtuin functions were studied. SIRT1 was also the first sirtuin which was shown to be activated by a polyphenol, namely resveratrol. Future investigation into the interaction between sirtuins (especially SIRT2-7) and polyphenols may help determine which polyphenols are specific to which sirtuin and which if any have a general effect on the whole family of proteins.

1.12 Protective effects of polyphenols against inflammation

Excessive inflammation is considered a critical factor in the pathogensis of AD. The AD brain has an increased expression of pro-inflammatory cytokines such as interleukin-1 and tumour necrosis factor and evidence of microglial activation [377]. Several inflammatory genes are associated with an increased risk of AD, and epidemiological studies on AD patients using non-steroidal anti-inflammatory drugs suggest some benefits [378]. Inflammation is also another source of oxidative stress as superoxide and nitric oxide are released.

The anti-inflammatory activity of phenolic compounds has been demonstrated in a number of *in vitro* and *in vivo* studies [379]. *Polyphenols* may affect inflammation not only as antioxidants, but also as modulators of inflammatory redox signalling pathways. Polyphenols can work as modifiers of signal transduction pathways to elicit their beneficial effects [379]. These compounds express anti-inflammatory activity by modulating the expression of pro-inflammatory genes such as cyclooxygenase, lipoxygenase, nitric oxide synthases and several important cytokines, mainly acting through nuclear factor-kappaB and mitogen-activated protein kinase signalling [379-384].

In a transgenic mouse model of AD, treatment with pomegranate extract was found to reduce microgliosis and Aβ deposition. Significant behavioural improvements were also seen and collated with decreased tumor necrosis factor α concentration and lower nuclear factor of activated T-cell activity in the pomegranate-fed mice. Pomegranate polyphenols punicalagin and ellagic acid were also shown to decrease Aβ-induced tumor necrosis factor α in cultured mouse microglia [385].

Polyphenols may also modulate programmed cell death pathways. Resveratrol, EGCG and luteolin have been shown to significantly inhibit the activation of caspase-3 and are able to modulate mitogen-activated protein kinases known to play an important role in neuronal apoptosis [386].

1.13 Protective effects of polyphenols on mitochondrial dysfunction

Changes to the mitochondria seem to be another pathological characteristic of AD. The AD brain shows a reduction in mitochondrial enzyme activities including dehydrogenase enzymes such pyruvate dehydrogenase, and cyclooxygenase enzymes [387]. The cause is

unknown, although OS is thought to play a role. Furthermore the number of intact, normal appearing mitochondria has been shown to decline in the AD brain, particularly in hippocampal neurons [387]. Elevation of intracellular Aβ levels may also potentially alter mitochondrial function and lead to increased reactive oxygen species generation [388].

Polyphenols have also been proposed to protect mitochondrial function, as has been shown for quercetin, rutin and resveratrol [389]. Apple peel polyphenols have similar protective effects by preventing mitochondrial complex 1 inhibition [390]. Grape seed extract has been shown to protect the enzyme activities of the mitochondrial respiratory electron transport chain (complexes I and II) and pyruvate dehydrogenase against oxidative stress [391]. EGCG and luteolin have been found to restore amyloid-induced mitochondrial dysfunction by reducing reactive oxygen species production and normalising mitochondrial membrane potential and cellular ATP levels [392]. Moreover, polyphenols have also been reported to exert their anti-apoptotic action by inhibiting apoptosis-inducing factor release from mitochondria, thus providing a new mechanism of action for polyphenols [393].

1.14 Involvement of polyphenols in telomere maintenance

The role of a number of polyphenols in the maintenance of telomere length has been investigated (Table 1.3). Telomeres are specialised nucleoprotein structures that protect the end of eukaryotic chromosomes from unscheduled DNA repair reactions and degradation. Due to the intrinsic inability of the DNA replication machinery to copy the end of linear molecules, and the endogenous DNA end-degrading activity, telomeres become progressively shorter after every cell division cycle. When a critical number of replications are reached, the cells enter senescence [394] or the end of their replicative ability (Hayflick limit).

In humans, several studies have described an inverse correlation between telomere length and age or age-related disease [395-397]. Telomere length shortens with normal ageing, life stress, inflammation and chronic diseases [394] and telomere dysfunction is linked to the pathogenesis of age-related diseases including AD [396,398]. Various nutrients such as nicotinamide and vitamins A, D, C and E are positively associated with telomere length [399-402] via mechanisms that reflect their role in cellular functions, including DNA repair and chromosome maintenance, DNA methylation, inflammation, oxidative stress modulation and activity of the enzyme telomerase which repairs chromosome ends by adding telomeric repeat subunits.

Healthy lifestyles and diets are positively correlated with telomere length. Changes in diet and lifestyle can modulate telomerase activity in peripheral blood mononuclear cells, although it is not yet clear if this translates to changes in telomere length [403].

Resveratrol has been shown to induce telomere maintenance factor WRN helicase [404]. Another study showed that habitual tea drinkers have longer telomeres in peripheral blood cells than those who drink tea less often [405]. This may possibly be due to the anti-inflammatory effect of tea polyphenols [406]. Similarly, the dietary administration of grape seed polyphenols to mice resulted in a trend for longer telomeres compared with controls [258]. It is clear that diet and lifestyle can together play a pivotal role in influencing inflammation, oxidative stress and psychological stress, all of which cause telomere attrition. A healthy lifestyle with a diet rich in fruits and vegetables combined with exercise and lower body mass index is indeed associated with longer telomeres [407]. EGCG, quercetin and carvediol were shown to prevent telomere shortening and oxidative stress markers in rat cardiomyocytes after cardiac hypertrophy [408]. Two isomers, 4-Hydroxy-5-hydroxymethyl-[1,3]dioxolan-2,6'-spirane-5',6',7',8'-tetrahydro-indolizine-3'-carbaldehyde (HDTIC-1 and HDTIC-2) extracted from *Astragalus membranaceus* root have been shown to extend lifespan of human fetal lung fibroblasts [409]. Cells pre-treated with these compounds show a significant reduction in DNA damage after exposure to oxidative stress. The authors hypothesised that the *Astragalus* root extract related delay in replicative senescence was due to the combined effects of: (1) reduction in telomere shortening rate, (2) reduced DNA damage and (3) improvement in DNA repair [409]. A small-molecule activator of telomerase known as TA-65 has been purified from the root of the *Astragalus,* and is capable of increasing average telomere length and decreasing the percentage of critically short telomeres, and attenuating DNA damage in mouse fibroblasts [410].

More recently, SIRT1 was reported to be a positive regulator of telomere length *in vivo* and to attenuate age related telomere shortening, an effect dependent on telomerase activity [411]. By contrast, another study found that SIRT1 inhibition was associated with increased telomerase activity in human cells [412]. These findings link SIRT1 to telomere biology and global DNA repair. Furthermore this effect does not appear to be specific to SIRT1 alone. SIRT6 is associated with telomeric chromatin, and depletion of SIRT6 has been shown to result in premature cellular senescence and telomere dysfunction [308].

1.15 Therapeutic Potential of Polyphenols and Sirtuin Modulation

As the human population continues to age, interventions that improve the quality of life and delay the onset of age-related diseases are highly desirable. Polyphenols are candidates for such interventions through their diverse biochemical roles including free radical scavenging, protein homeostasis, metal chelation, telomere maintenance and stimulation of sirtuins. Their potent effects in combination with little or no known toxicity may give them a therapeutic advantage. In the past, clinical trials using vitamins and antioxidants have frequently had equivocal outcomes in the treatment of AD. One explanation for the failure of single drugs or natural compounds in AD is that this disorder has multiple aetiologies, involving various pathological and molecular events occurring in parallel or sequentially. This has lead to the assumption that a single drug targeting two or more active neuroprotective moieties or a combination of compounds, targeting multiple pathological aspects of the disease, may offer a superior therapeutic benefit over a drug targeting one pathway alone. For example, a treatment regime using a mixture of polyphenols which together exhibit antioxidant, metal chelating, anti-amyloidogenic and sirtuin activation properties may prove to be superior. Initial studies using polyphenol combination therapy or "cocktails" have shown some promising preliminary results [413,414].

Another important issue is the bioavailability of such compounds. Most polyphenols have poor bioavailability following oral ingestion [415]. Those found in food take the form of glycosides, esters or polymers which cannot be absorbed directly and therefore must be hydrolysed by microflora or enzymes of the small intestine [159]. A further confounder for the central nervous system is that polyphenols cannot pass easily through the blood brain barrier. There are however, various strategies currently under investigation to improve bioactivity. It has been found that some polyphenols can specifically modify metabolic and transport processes that govern bioavailability [415]. Therefore, specific synergistic combinations and interactions could be designed to improve entry into the brain. Another promising method is the use of nanoparticle carriers coupled to polyphenols [416]. Brain permeable nanoparticles may be used to enhance entry of poorly absorbed phenolic compounds into the brain [117,417,418]. Creation and administration of EGCG nanolipidic complexes were found to double the oral bioavailability of EGCG in male Spague-Dawley rats and also improved the EGCG's ability to enhance α-secretase activity *in vitro* [419]. Cocrystallisation is another technique which could increase solubility and thus bioavailability of polyphenols. This procedure used

with quercetin has shown promising results with cocrystals of quercetin showing significant increases in solubility and oral bioavailability [420].

AD is a multifactorial disease involving a variety of different pathways including oxidative stress, inflammation, amyloid aggregation and mitochondrial dysfunction. Furthermore the emerging view that polyphenols may exert a variety of modulatory actions which influence the pathogenesis of AD has brought attention to functions beyond their usual antioxidant activities. Sirtuins are a class of proteins that have been linked to longevity, ageing and neurodegeneration. Investigating the role of sirtuins and other related pathways may lead to the development of effective treatment regimes for AD.

1.16 Aims and Rationale

The overall aim was to gain insight into mechanisms, risk and protective factors for MCI and AD. Sirtuins have been shown to play important roles in ageing and neurodegeneration however the quantification of these proteins in the CNS is limited. Parabiosis experiments show that plasma carries factors which vary with age and disease, and can have have an impact when introduced into other living systems. Here we have explored: (1) the role of mammalian sirtuins in ageing and AD, since these proteins may have a role in cellular health and longevity, (2) the impact of MCI and AD plasma on neuronal cell line health, viability and proteomic expression, (3) how the constituents of MCI and AD plasma vary from controls and (4) the effect of polyphenols on a specific hallmark mechanisms in AD such as Aβ1–42 aggregation and oxidative stress.

The specific foci of each chapter were as follows:

> Mass spectrometry has been used to successfully assay metabolites of SIRT1 activator drugs in plasma and urine, but to date has not been used to quantify protein levels directly. Mass spectrometry has a great advantage over the conventionally used antibody based methods, such as western blotting, in that it offers greater specificity, linearity, reproducibility and typical limits of quantification down to the low fmol range. It also removes some of the antibody specificity issues that are associated with methods such as western blotting and ELISA, as peptides unique to each protein are measured. Targeted mass spectrometry may provide a more specific and sensitive method to detect and quantify sirtuin expression at the protein level, providing a tool

for comprehensive sirtuin protein expression analysis. Therefore one of the aims of this project was to develop a targeted mass spectrometry method using multiple reaction monitoring (MRM) to quantify the seven human sirtuins at the protein level and to apply the assay to CNS biological samples.

The second aim was to apply this mass spectrometry based assay to quantify levels of sirtuins in a variety of biological samples such as primary and cancer brain cells, animal tissues, human *post mortem* brain tissue, CSF and plasma.

The third aim was to investigate changes to sirtuin levels during ageing and neurodegeneration by measuring sirtuins in CSF from control patients over an age range of 30-65yrs and also in control and AD *post mortem* brain tissues.

Since previous studies indicate that AD plasma may contain oxidative stress markers as well as cytotoxic factors, the fourth aim of the project was to investigate the effect of the addition of pooled control, MCI and AD plasma from 20 individuals each on a microglial cell line by measuring cell viability, proliferation and mitochondrial function following 48 hour treatment with non heat-inactivated plasma and plasma in which complement proteins had been deactivated.

The fifth aim was to complete a proteomic analysis using iTRAQ quantitative proteomic analysis of cell extracts exposed to plasma from each group to investigate possible plasma protein alterations unique to MCI or AD, and to detect any protein aberrations within the cells treated with the plasma and to correlate these finding to cell viability and mitochondrial function assays measured *in vitro*.

It has previously been shown that transition metal ions such as iron, zinc and copper have the ability to enhance aggregation of Aβ and polyphenols such as resveratrol, curcumin and EGCG can disrupt this process. Therefore the final aim was to investigate the effect of various polyphenols and metals on amyloid aggregation using electron microscopy, and also to detect any neuroprotective effects of polyphenol treatment on cell viability and mitochondrial energy metabolism using a neuronal cell line treated with aggregated "aged" amyloid.

Chapter 2

Application of Targeted Mass Spectrometry for the Quantification of Sirtuins in the Central Nervous System

2.1 Introduction

Sirtuins are a class of proteins that possess histone deacetylase or mono-ribosyltransferase activity and play critical roles in cell survival in response to oxidative stress and caloric restriction (CR) regimes [421]. In mammals, seven sirtuins (SIRT1-7) have been identified. All mammalian sirtuins contain a conserved NAD-binding and catalytic domain, but differ in their N and C-terminal domains. They have different specific substrates including histones, transcriptional regulators and enzymes. They localise to cell compartments which regulate cellular structure, metabolism and gene expression, including the cytoskeleton (SIRT2), mitochondria (SIRT3, SIRT4 and SIRT5) and nucleus/nucleolus (SIRT1, SIRT6 and SIRT7), and play important roles in health and disease [421,422]. SIRT1 is the best characterized and has the broadest substrate specificity. Sirtuins have emerged as critical modulators of metabolic adaptive responses, and their activities have been linked to ageing and multiple diseases, from metabolic abnormalities to neurodegeneration.

Sirtuins can affect reactive oxygen species (ROS) production and promote resistance to their damaging effects. Oxidative stress has been shown to decrease SIRT1 expression in the hippocampus and cortex, possibly by direct degradation by ROS [423]. SIRT1 overexpression prevents oxidative stress-induced apoptosis and increases resistance to oxidative stress through regulation of the FOXO family of forkhead transcription factors [424]. The cytoplasmic sirtuin protein SIRT2, has been shown to increase in response to oxidative stress but promotes cell death through FOXO proteins [269]. SIRT3, a mitochondrial protein, reduces oxidative stress through activation of superoxidase dismutase [270]. SIRT6 and SIRT7, like the founding member of the sirtuin family SIRT1, are nuclear proteins involved in oxidative-stress induced DNA repair through activation of the PARP-1 DNA repair enzyme.

SIRT1 is expressed in the adult brain, in the cortex, hippocampus, cerebellum, and hypothalamus, and in lower levels in the white matter [425]. Among the brain cell types, SIRT1 is predominantly expressed in neurons and viewed as a nuclear protein [425]. The mRNAs for all seven sirtuins have been identified in mouse brain tissue and also neural stem cells [426]. SIRT1, SIRT2 and SIRT3 have also been detected in human serum, and levels were shown to decline with age and were linked to frailty [427,428]. SIRT3 is elevated at both the mRNA and protein levels in Alzheimer's disease (AD) *post mortem* brain tissue compared to controls [271].

The most common techniques currently utilised for detecting a change in sirtuin levels at the mRNA or protein level are PCR and western blotting, respectively (Table 2.1). Other studies

have used methods such as immunohistochemistry, surface plasmon resonance and ELISA assays (Table 2.1). The majority of these methods are only semi-quantitative with moderate sensitivity, use antibodies which may not have sufficient specificity or detect expression at the mRNA level which may not reflect protein expression. Furthermore, there is no current assay which detects multiple sirtuins simultaneously.

Table 2.1: Expression of sirtuins in the CNS and current methods used for analysis

Name	Areas detected in CNS	Function in CNS	Techniques used
SIRT1	Human hippocampus and cortex [425]. Has also been detected in human serum at approx 8.16ng/µl [427]. Mouse neural stem cells and adult mouse brain [426]. Porcine brain [429].	Modulates memory formation and synaptic plasticity. Reduces with age in mice. Metabolic sensor.	TR-qPCR [425], immunohistochemistry [430], western blotting, surface plasmon resonance and ELISA [427].
SIRT2	Mouse neural stem cells and adult mouse brain [426]. Porcine brain [429]. Human serum [428].	Inhibitor of microglia-mediated inflammation and neurotoxicity [431]. Impairs neurite outgrowth and oligodendrocyte differentiation. Involved in myelin formation.	Mouse knockouts [431], western blotting.
SIRT3	Cortex, Hippocampus and Cerebellum [271]. Mouse neural stem cells and adult mouse brain [426]. Porcine brain [429]. Rat brain. Human serum [428].	Responses to oxidative stress and involved in maintenance of mitochondrial function.	Western blotting and qPCR [271].
SIRT4	Mouse neural stem cells and adult mouse brain [426]. Porcine brain [429]. Rat cortical cells and brain tissue [432].	Regulation of glial development [432]; involved in glutamate transport and protective role against excitotoxicity [433].	qPCR, western blotting and immunofluorescence [429,432].
SIRT5	Mouse neural stem cells and adult mouse brain [426]. Porcine brain [429].	SIRT5 gene polymorphism may promote molecular brain ageing and be a risk factor for mitochondrial dysfunction-related diseases [434].	qPCR [429]
SIRT6	Mainly localised in the nucleus in the cortical layers [435]. Mouse neural stem cells and adult mouse brain [426,436,437]. Rat brain. Porcine brain [429].	Regulator of somatic growth by modulating neural chromatin and gene activity [436]. Modulated DNA repair in the brain [438]. Suppresses proinflammatory gene expression.	Brain specific mouse knockout models and primary brain cell models [436,438,439]; immunohistochemistry [435]; western blotting, immunofluorescence [437].
SIRT7	Mouse neural stem cells and adult mouse brain [426]. Porcine brain [429].	Positive regulator of RNA polymerase I transcription.	Mouse knockout [436].

Quantitative expression analysis of mammalian sirtuin proteins (especially SIRT2-7), across cell and tissue types is limited in the current literature. Previous studies have shown an increase in SIRT3 in AD *post-mortem* brain tissue using western blotting for protein expression and multiplex qPCT to assay SIRT3 mRNA levels [271]. Both SIRT3 protein and mRNA were shown to be significantly elevated in the AD group [271]. Another recent paper detected SIRT1 in human serum samples using western blotting, surface plasmon resonance and ELISA to measure SIRT1 protein levels [427]. SIRT1 declines with age and is more dramatically reduced in MCI and AD patients compared to age matched controls, suggesting that SIRT1 may warrant further investigation as a potential plasma biomarker for AD [427]. SIRT1 and SIRT3 levels in serum were found to be significantly lower in frail subjects as compared to the non-frail [428]. Another study reported a decrease in SIRT1 and SIRT2 mRNA levels using quantitative real time PCR in the primary motor cortex of human *post-mortem* amyotrophic lateral sclerosis brain tissue [440].

Mass spectrometry has been used to successfully assay metabolites of SIRT1 activator drugs in plasma and urine [441], but to date has not been used to quantify protein levels directly. Mass spectrometry has a great advantage over the conventionally used antibody based methods, such as western blotting, in that it offers greater specificity, linearity, reproducibility and typical limits of quantification down to the low fmol range. It also removes some of the antibody specificity issues that are associated with methods such as western blotting and ELISA, as peptides unique to each protein are measured. Targeted MRM based mass spectrometry may provide a more specific and sensitive method to detect and quantify sirtuin expression at the protein level, providing a tool for comprehensive sirtuin protein expression analysis. The aim of this study was to develop a targeted mass spectrometry method using multiple reaction monitoring (MRM) to quantify the seven human sirtuins at the protein level and to apply the assay to CNS biological samples.

Methods

Selection of target sirtuin peptides

Recombinant protein standards were purchased for seven human sirtuins (Cayman Chemical, USA), and 5 µg of each was run by 1D SDS-PAGE gel and colloidal coomassie stained. Sirtuin bands were excised, trypsin digested overnight followed by LC-MS/MS analysis on a

QToF Ultima API hybrid tandem mass spectrometer (Micromass, UK) as previously described [442-444].

Digested peptides were separated by nano-LC using a Cap-LC autosampler system (Waters, USA). Samples (5 μl) were concentrated and desalted onto a micro C18 precolumn (500 μm x 2 mm, Michrom Bioresources, Auburn, CA) with $H_2O:CH_3CN$ (98:2, 0.05% HFBA) at 15 ul/min. After a 4 min wash, the pre-column was automatically switched (Valco 10 port valve, Houston, TX) into line with a fritless nano column (75 μm x ~12 cm) containing Magic C18 (~10cm, 200Å, Michrom). Peptides were eluted using a linear gradient of $H_2O:CH_3CN$ (98:2, 0.1% formic acid) to $H_2O:CH_3CN$ (55:45, 0.1% formic acid) at ~300 nl/min over 30 min. The precolumn was connected via a fused silica capillary (10 cm, 25 μ) to a low volume tee (Upchurch Scientific) where high voltage (2400 V) was applied and the column tip positioned ~1 cm from the Z-spray inlet of an QTof Ultima API hybrid tandem mass spectrometer (Micromass, UK). Positive ions were generated by electrospray and the QTof operated in data dependent acquisition mode (DDA). A Tof MS survey scan was acquired (m/z 350-1700, 1 s) and the two largest multiply charged ions (counts > 20) were sequentially selected by Q1 for MS-MS analysis. Argon was used as collision gas and an optimum collision energy chosen (based on charge state and mass). Tandem mass spectra were accumulated for up to 2 s (*m/z* 50-2000). Peak lists were submitted to the database search program Mascot (Matrix Science, UK) to confirm identification of sirtuin peptides.

The two peptides with the highest signal intensity for each sirtuin were cross-referenced with Skyline software (MacCoss Lab Software, USA). Peptide sequences were checked to ensure no overlap with other sirtuins. Sirtuin standards were run using MRM LC-MS/MS on a 4000 Q TRAQ (SCIEX, USA) mass spectrometer to ensure good signals were detected for all 14 peptides selected for the final list (two unique peptides for each sirtuin).

Table 2.2 List of 14 unique sirtuin peptides selected for sirtuin protein quantification.
Arg and Lys amino acids highlighted in bold used in 'heavy' peptides, labelled with a stable isotope (^{13}C and ^{15}N). Precursor charge state for all peptides was 2^+.

Sirtuin	Peptide	Light Precursor Ion *m/z*	Heavy Precursor Ion *m/z*
SIRT1	DINTIEDAVK	559.2904	563.2975
	TSVAGTVR	395.7245	400.7286
SIRT2	LLDEELTLEGVAR	664.8746	669.8788
	IFSEVTPK	460.758	464.7651
SIRT3	LYTQNIDGLER	661.341	666.3451
	VSGIPASK	379.724	383.7311
SIRT4	RPIQHGDFVR	612.8334	617.8376
	FILTAWEK	504.2817	508.2888
SIRT5	VVVITQNIDELHR	512.6229	515.9589
	NLLEIHGSLFK	635.8613	639.8613
SIRT6	FLVSQNVDGLHVR	742.4044	747.4086
	LVIVNLQPTK	562.8555	566.8626
SIRT7	LLAESADLVTELQGR	807.9385	812.9426
	DTIVHFGER	537.2724	542.2765

2.2.2 Targeted mass spectrometry

MRM analyses were performed on a 4000 Q TRAP hybrid triple quadrupole linear ion trap mass spectrometer (SCIEX, USA) interfaced with a nanospray ion source, operating in positive ion mode and controlled by Analyst 1.5 software. Peptides were concentrated and desalted onto a micro C18 precolumn (500 μm x 2 mm, Michrom Bioresources, USA) with H_2O:CH_3CN (98:2, 0.05% TFA) at 15 μl/min. After a 4 min wash, the pre-column was automatically switched (10 port valve, Valco, USA) into line with a nano column (as described in the previous section). Peptides were eluted using a linear gradient of H_2O:CH_3CN (98:2, 0.1% formic acid) to H_2O:CH_3CN (36:64, 0.1% formic acid) at ~300 nl/min over 30 minutes. Samples were analyzed with an ion spray voltage of 2.4 kV, curtain gas flow of 12 and nebulizing gas flow of 5 L/min. Quadrupoles were operated in the low resolution mode, and the dwell time was 50 ms. For validation runs, the MRM experiment triggered MS/MS spectrum acquisition. MS/MS spectra were acquired in the trap mode (enhanced product ion) with dynamic fill time, Q1 was operated using low resolution. Each sirtuin protein (SIRT1-7, 2 peptides per protein) was run with 12 transition ions per run, per

protein and with a 50ms dwell time. See Table 2.3 for a full list of transitions for each peptide and corresponding collision energies (estimated using Skyline software).

Table 2.3 List of product and transition ions with collision energies used for all 14 selected sirtuin peptides. Each sirtuin protein (SIRT1-7, with 2 peptides per protein) was run with 12 transition ions per run, per protein and with a 50ms dwell time. Precursor charge state for all peptides was 2^+.

Precursor Ion *m/z*	Transition Ion	Peptide	Declustering Potential	Collision Energy
559.2904	889.4625	SIRT1.DINTIEDAVK.y8.light	71.9	27.6
559.2904	775.4196	SIRT1.DINTIEDAVK.y7.light	71.9	27.6
559.2904	674.3719	SIRT1.DINTIEDAVK.y6.light	71.9	27.6
563.2975	897.4767	SIRT1.DINTIEDAVK.y8.heavy	71.9	27.6
563.2975	783.4338	SIRT1.DINTIEDAVK.y7.heavy	71.9	27.6
563.2975	682.3861	SIRT1.DINTIEDAVK.y6.heavy	71.9	27.6
395.7245	602.362	SIRT1.TSVAGTVR.y6.light	60	18.3
395.7245	503.2936	SIRT1.TSVAGTVR.y5.light	60	18.3
395.7245	432.2565	SIRT1.TSVAGTVR.y4.light	60	18.3
400.7286	612.3703	SIRT1.TSVAGTVR.y6.heavy	60	18.3
400.7286	513.3019	SIRT1.TSVAGTVR.y5.heavy	60	18.3
400.7286	442.2648	SIRT1.TSVAGTVR.y4.heavy	60	18.3
664.8746	858.5043	SIRT2.LLDELTLEGVAR.y8.light	79.6	33.6
664.8746	745.4203	SIRT2.LLDELTLEGVAR.y7.light	79.6	33.6
664.8746	644.3726	SIRT2.LLDELTLEGVAR.y6.light	79.6	33.6
669.8788	868.5126	SIRT2.LLDELTLEGVAR.y8.heavy	79.6	33.6
669.8788	755.4285	SIRT2.LLDELTLEGVAR.y7.heavy	79.6	33.6
669.8788	654.3809	SIRT2.LLDELTLEGVAR.y6.heavy	79.6	33.6
460.758	807.4247	SIRT2.IFSEVTPK.y7.light	64.7	22
460.758	660.3563	SIRT2.IFSEVTPK.y6.light	64.7	22
460.758	573.3243	SIRT2.IFSEVTPK.y5.light	64.7	22
464.7651	815.4389	SIRT2.IFSEVTPK.y7.heavy	64.7	22
464.7651	668.3705	SIRT2.IFSEVTPK.y6.heavy	64.7	22
464.7651	581.3385	SIRT2.IFSEVTPK.y5.heavy	64.7	22
661.341	944.4796	SIRT3.LYTQNIDGLER.y8.light	79.3	33.4
661.341	816.421	SIRT3.LYTQNIDGLER.y7.light	79.3	33.4
661.341	702.3781	SIRT3.LYTQNIDGLER.y6.light	79.3	33.4
666.3451	954.4879	SIRT3.LYTQNIDGLER.y8.heavy	79.3	33.4
666.3451	826.4293	SIRT3.LYTQNIDGLER.y7.heavy	79.3	33.4

666.3451	712.3863	SIRT3.LYTQNIDGLER.y6.heavy	79.3	33.4
379.724	659.3723	SIRT3.VSGIPASK.y7.light	58.8	17.4
379.724	572.3402	SIRT3.VSGIPASK.y6.light	58.8	17.4
379.724	515.3188	SIRT3.VSGIPASK.y5.light	58.8	17.4
383.7311	667.3865	SIRT3.VSGIPASK.y7.heavy	58.8	17.4
383.7311	580.3544	SIRT3.VSGIPASK.y6.heavy	58.8	17.4
383.7311	523.333	SIRT3.VSGIPASK.y5.heavy	58.8	17.4
612.8334	1068.559	SIRT4.RPIQHGDFVR.y9.light	75.8	30.7
612.8334	971.5057	SIRT4.RPIQHGDFVR.y8.light	75.8	30.7
612.8334	858.4217	SIRT4.RPIQHGDFVR.y7.light	75.8	30.7
617.8376	1078.567	SIRT4.RPIQHGDFVR.y9.heavy	75.8	30.7
617.8376	981.514	SIRT4.RPIQHGDFVR.y8.heavy	75.8	30.7
617.8376	868.4299	SIRT4.RPIQHGDFVR.y7.heavy	75.8	30.7
504.2817	747.4036	SIRT4.FILTAWEK.y6.light	67.9	24.5
504.2817	634.3195	SIRT4.FILTAWEK.y5.light	67.9	24.5
504.2817	533.2718	SIRT4.FILTAWEK.y4.light	67.9	24.5
508.2888	755.4178	SIRT4.FILTAWEK.y6.heavy	67.9	24.5
508.2888	642.3337	SIRT4.FILTAWEK.y5.heavy	67.9	24.5
508.2888	541.286	SIRT4.FILTAWEK.y4.heavy	67.9	24.5
512.6229	896.4585	SIRT5.VVVITQNIDELHR.y7.light	68.5	23
512.6229	782.4155	SIRT5.VVVITQNIDELHR.y6.light	68.5	23
512.6229	669.3315	SIRT5.VVVITQNIDELHR.y5.light	68.5	23
515.9589	906.4667	SIRT5.VVVITQNIDELHR.y7.heavy	68.5	23
515.9589	792.4238	SIRT5.VVVITQNIDELHR.y6.heavy	68.5	23
515.9589	679.3397	SIRT5.VVVITQNIDELHR.y5.heavy	68.5	23
635.8613	930.5043	SIRT5.NLLEIHGSLFK.y8.light	77.5	32
635.8613	801.4618	SIRT5.NLLEIHGSLFK.y7.light	77.5	32
635.8613	688.3777	SIRT5.NLLEIHGSLFK.y6.light	77.5	32
639.8684	938.5185	SIRT5.NLLEIHGSLFK.y8.heavy	77.5	32
639.8684	809.4759	SIRT5.NLLEIHGSLFK.y7.heavy	77.5	32
639.8684	696.3919	SIRT5.NLLEIHGSLFK.y6.heavy	77.5	32
742.4044	1124.581	SIRT6.FLVSQNVDGLHVR.y10.light	85.2	38.1
742.4044	909.4901	SIRT6.FLVSQNVDGLHVR.y8.light	85.2	38.1
742.4044	696.3787	SIRT6.FLVSQNVDGLHVR.y6.light	85.2	38.1
747.4086	1134.589	SIRT6.FLVSQNVDGLHVR.y10.heavy	85.2	38.1
747.4086	919.4984	SIRT6.FLVSQNVDGLHVR.y8.heavy	85.2	38.1
747.4086	706.387	SIRT6.FLVSQNVDGLHVR.y6.heavy	85.2	38.1
562.8555	912.5513	SIRT6.LVIVNLQPTK.y8.light	72.1	27.8
562.8555	799.4672	SIRT6.LVIVNLQPTK.y7.light	72.1	27.8
562.8555	700.3988	SIRT6.LVIVNLQPTK.y6.light	72.1	27.8

566.8626	920.5655	SIRT6.LVIVNLQPTK.y8.heavy	72.1	27.8
566.8626	807.4814	SIRT6.LVIVNLQPTK.y7.heavy	72.1	27.8
566.8626	708.413	SIRT6.LVIVNLQPTK.y6.heavy	72.1	27.8
807.9385	915.5258	SIRT7.LLAESADLVTELQGR.y8.light	90	41.8
807.9385	802.4417	SIRT7.LLAESADLVTELQGR.y7.light	90	41.8
807.9385	703.3733	SIRT7.LLAESADLVTELQGR.y6.light	90	41.8
812.9426	925.5341	SIRT7.LLAESADLVTELQGR.y8.heavy	90	41.8
812.9426	812.45	SIRT7.LLAESADLVTELQGR.y7.heavy	90	41.8
812.9426	713.3816	SIRT7.LLAESADLVTELQGR.y6.heavy	90	41.8
537.2724	958.5105	SIRT7.DTIVHFGER.y8.light	70.3	26.4
537.2724	857.4628	SIRT7.DTIVHFGER.y7.light	70.3	26.4
537.2724	744.3787	SIRT7.DTIVHFGER.y6.light	70.3	26.4
542.2765	968.5188	SIRT7.DTIVHFGER.y8.heavy	70.3	26.4
542.2765	867.4711	SIRT7.DTIVHFGER.y7.heavy	70.3	26.4
542.2765	754.387	SIRT7.DTIVHFGER.y6.heavy	70.3	26.4

2.2.3 Sirtuin Peptide Standards

Multiple point calibration was used where a series of standard 'light' peptides with known concentrations together with fixed amounts of stable isotope-labelled 'heavy' peptides (100fmol/µl) were used to generate calibration curves for each sirtuin. The curves were expressed as ratios of light/heavy peak area versus concentration of light peptide for each of the 14 peptides selected. A commercially available stable isotope-labelled peptide standard (AQUA peptide, Sigma, USA) was used for absolute quantification of proteins. This heavy surrogate of each of the peptide standards is added at a constant level to all samples and standards and is used for correction of sample losses during workup and normalisation across runs, allowing accurate quantification of the target protein in samples. Peptides labelled with a stable isotope (^{13}C and ^{15}N) are chemically identical to their native counterparts and have identical chromatographic behaviour but can be distinguished from the calibration standards based on a small specific mass difference.

Isotopically labelled internal standards are excellent tools to normalise the variability in sample handling, injection volume and the performance of the mass spectrometer. Synthetic light and heavy peptides were purchased from GL Biochem (China) and Sigma-Aldrich (USA) respectively. Calibration curves in the range of 1-200 fmol/µl (three replicates using

three transitions) were prepared. Heavy peptide (100 fmol/μl) was added to all standards and peptide ratios (light/heavy) were obtained using Skyline MRM analysis software. Average inter- and intra- assay %CV values (n=3) were calculated for each of the seven sirtuins at the 100fmol/ul concentration point. The linear range, limit of detection (LOD) and limit of quantification (LOQ) were determined. A signal-to-noise ratio of >3:1 and >10:1 were used to define LOD and LOQ respectively. Sirtuin standard curves can be seen in Figure 2.1.

2.2.4 Cell Culture

Cells were cultured in medium RPMI 1640 supplemented with 10% foetal bovine serum, 1% 1-glutamax, 1% antibacterial/antifungal, and 0.5% glucose. Cells were maintained at 37°C in a humidified atmosphere containing 95% air/5% CO_2. Cells were seeded into 24-well tissue culture plates to a density of 1×10^5 cells 24 hours prior to experimentation. Neurons were prepared from the same mixed brain cell cultures as previously described [445]. Briefly, cells were plated in 24-well culture plates coated with Matrigel (1/20 in Neurobasal) and maintained in Neurobasal medium supplemented with 1% B-27 supplement, 1% Glutamax, 1% antibiotic/antifungal, 0.5% HEPES buffer, and 0.5% glucose. Microglia were prepared from the mixed brain cell cultures using a published protocol [446]. Briefly, the original mixed brain cell culture was shaken at 220 rpm for 2 h, floating cells were centrifuged, transferred to Permanox chamber slides (NUNC) at a density of 5×10^5 cells/ml and grown in DMEM supplemented with 10% fetal calf serum (FCS), 1% L-glutamax, 0.5% glucose, IL-3 (300 IU/ml), macrophage colony-stimulating factor (M-CSF) (50 IU/ml), and 1% antibiotic/antifungal. Oligodendrocytes were prepared from the mixed brain cell cultures using a published protocol [446]. Briefly, cells were seeded and grown in flasks coated with poly-L-lysine to confluence at 34°C, the permissive temperature, in DMEM/F12 supplemented with 10% FBS, 1% antibiotic/antifungal, 1% of G418 (Invitrogen, Melbourne, Australia) and then shifted to 39°C, the non-permissive temperature that leads to the 'differentiated' state for 72 hours. Cells were cultured in medium RPMI supplemented with 1% myelin binding protein, 1% 1-glutamax, and 1% antibacterial/antifungal. CNS cell lines of neurons (SKNSH), astrocytes (U251), oligodendrocytes (M310) and microglia (CHM5) were cultured in medium RPMI supplemented with 10% foetal bovine serum, 1% 1-glutamax, 1% antibacterial/antifungal, and 0.5% glucose. All cells were maintained at 37°C in a humidified atmosphere containing 95% air/5% CO_2. Cells were seeded into 24-well

tissue culture plates (3 wells per cell type) to a density of 1×10^{5} cells 24 hours prior to experimentation. Wells were pooled prior to cell lysis and probe sonication and all measurements were performed in triplicate.

2.2.5 Sample Preparation of Cell Cultures, Tissues, CSF and Plasma

Cells: Adult human primary microglia, astrocytes, neurons and oligodendrocytes were cultured from resected normal adult brain tissue following removal of brain tumour with informed consent at the Minimally Invasive Cancer Centre, Prince of Wales Hospital, Sydney, Australia. Astrocytes were prepared from the mixed brain cell cultures using a protocol previously described by Guillemin et al [446]. All cells and cell lines were lysed using RIPA buffer followed by probe sonication and cell debris removed by centrifugation at 10,000RPM for 5 min. Total protein concentration was assayed in the supernatant using the Pierce BCA protein assay kit (Life Technologies, Australia), and three 10 µg replicates were run by 1D SDS-PAGE followed by colloidal coomassie staining.

Human Tissues: 20 µg of protein from five individual control *post mortem* brain tissue samples were extracted as described for cells in the previous section, and run on a 1D SDS-PAGE gel and coomassie stained. Brain tissue samples included both gray and white matter and were frozen and stored at -80°C. Detailed patient information including age, sex and *post-mortem* delay of frontal lobe brain tissue collection times are summarised in Table 2.4.

Table 2.4 Human frontal lobe brain tissue sample and subject details

Patient	Sex	Age at death	Post mortem tissue collection time
1	Male	72	24h
2	Female	71	24h
3	Male	72	24h
4	Male	71	29h
5	Male	54	24h

For the fractionated samples, 200 mg of frontal lobe brain tissue from five control subjects were pooled and differential detergent fractionation performed to obtain cytosol, nucleus,

cytoskeletal and membrane protein fractions [447]. Each fraction (20ug) was run on a 1D SDS-PAGE gel and coomassie stained.

Animals: Female guinea pigs (Dunkin–Hartley) and C57Bl6 mice were housed in temperature-controlled rooms (21-22°C; 49-55% humidity) with 12 h light-dark cycle (lights on 7:00-19:00). Food and water was available *ad libitum*. Wild type mice and guinea pig organs were lysed (RIPA buffer) by probe sonication. Protein (20μg/lane) were run by 1D SDS-PAGE and coomassie stained. Protein concentrations were determined using the Pierce BCA protein assay kit (Life Technologies, Australia).

CSF and plasma: CSF samples were collected by standard lumbar puncture from five patients assessed as clinically well after investigation for suspected meningitis, returning normal results for routine CSF pathology markers (white cell count, protein, glucose and bacterial sterility). Aliquots (50 μl) for each patient were obtained for the study. Control human plasma from a healthy individual was immunodepleted of the six most abundant plasma proteins using the Hu6 column (Agilent, USA) according to manufacturer's instructions [448]. For immunodepletion using the Multiple Affinity Removal System Hu6 column and buffer kit (Agilent, Santa Clara, CA, USA) , 24 μl EDTA plasma was diluted into
120 μl Buffer A. Diluted EDTA plasma (100 μL) was injected onto the Hu6 column connected to a HP 1090 HPLC system (Agilent, Santa Clara, CA, USA) and the low abundance protein fraction was collected following the manufacturer's instructions. The low abundance protein fractions from six injections were pooled, buffer exchanged and concentrated into 45 mM NaHCO$_3$ using Amicon 3 kDa centrifugal devices (Millipore, Billerica, MA, USA). CSF protein (5 μg) and depleted plasma protein (20 μg), as determined by a BCA protein assay, were run on a 1D SDS-PAGE gel and colloidal coomassie stained.

For all samples, the band corresponding to the molecular weight for each sirtuin was excised, de-stained and trypsin digested overnight with heavy sirtuin peptides added as internal standards to all samples prior to digestion (the internal standard was constant for all samples and standards and added at a level at about the midrange of the standard curve). The tryptic peptides were dried under vacuum (Savant Speedvac, Thermo Scientific, USA), reconstituted in 0.1% formic acid (5 μl) injected into the mass spectrometer (1uL) and analysed using MRM. Peak area ratios (light/heavy) were calculated for each endogenous peptide (light)

relative to the spiked isotope-labelled (heavy) peptide using Skyline MRM analysis software. Protein concentrations were determined using calibration curves. All samples were fractionated by 1D-SDS-PAGE to reduce sample complexity. A workflow of the sample preparation procedure can be found in Figure 2.6.

2.2.6 Sirtuin mRNA Expression in Human Brain Tissue using PCR

For the gene expression studies RNA was extracted from human brain cells using RNeasy mini kits (Qiagen, Germany). The cDNA was prepared using SuperScript III First-Strand Synthesis System and random hexamers (Invitrogen Corporation, USA) as previously described [449].

Briefly, for each reaction 2 µl of diluted cDNA, 10 µL of SYBR green master mix, 0.15 µL of 10 µM forward and reverse primers and 7.7 µl of nuclease-free water was used making a total volume of 20 µl. Q-PCR was carried out using the Mx3500P Real-Time PCR system (Stratagene, Australia). The primer sequences are shown in Table 2.5. The relative expression levels of sirtuin transcripts were calculated using a mathematical model based on the individual Q-PCR primer efficiencies and the quantified values were normalized against the housekeeping gene 18S. From these values, fold-differences in the levels of transcripts between individual cell cultures were calculated according to the formula $2^{-\Delta\Delta Ct}$

Table 2.5 Real-time primer sequences used for PCR analysis

Primer name	Oligo name	Sequence (5'→3')
SIRT1-Forw	NM_012238-Forw	CAC-CAG-AAA-GAA-CTT-CAC-CAC-CAG
SIRT1-Rev	NM_012238-Rev	ACC-ATC-AAG-CCG-CCT-ACT-AAT-CTG
SIRT2-Forw	AJ505014-Forw	AGG-GAC-AAG-GAG-CAG-GGT-TC
SIRT2-Rev	AJ505014-Rev	GAA-GAG-AGA-CAG-CGG-CAG-GAC
SIRT3-Forw	NM_12239-Forw	GAG-GTT-CTT-GCT-GCA-TGT-GGT-TG
SIRT3-Rev	NM_12239-Rev	AGT-TTC-CCG-CTG-CAC-AAG-GTC
SIRT4-Forw	NM_012240-Forw	TTG-TGC-AGC-AA-GTC-CTC-CTC
SIRT4-Rev	NM_012240-Rev	GTC-TCT-TGG-AAA-GGG-TGA-TGA-AGC
SIRT5-Forw	NM_12241-Forw	TCC-AGC-GTC-CAC-ACG-AAA-CC
SIRT5-Rev	NM_12241-Rev	AAC-ACC-AGC-TCC-TGA-GAT-GAT-GAC
SIRT6-Forw	NM_016539-Forw	GCT-GGA-GCC-CAA-GGA-GGA-ATC
SIRT6-Rev	NM_016539-Forw	AGT-AAC-AAA-GTG-AGA-CCA-CGA-GAG

SIRT7-Forw	NM_016538-Forw	GAG-CCA-ACC-CTC-ACC-CAC-ATG
SIRT7-Rev	NM_016538-Rev	ACG-CAG-GAG-GTA-CAG-ACT-TCA-ATG
GAPDH-Forw	NM_017008-Forw	TGG-AGT-CTA-CTG-GCG-TCT-T
GAPDH –Rev	NM_017008-Rev	TGT-CAT-ATT-TCT-CGT-GGT-TCA

2.2.7 Sirtuin Expression in Human Brain Cells and Tissue using immunohistochemical staining

Post mortem brain tissue from a control male aged 63 years was obtained from the Sydney Brain Bank. Individual had no evidence of neurological disease. Immunohistochemical staining was performed using a previously published procedure [450]. Anti-human sirtuin (1:250) primary antibodies (raised in rabbit) were used.

Formalin fixed blocks from the hippocampal region were embedded in paraffin and cut into 5 μm sections on superfrost plus slides. For sirtuin staining, sections were deparaffinised in xylene for 20 min and rehydrated through graded alcohol treatment. Antigen retrieval was performed through the use of Target Retrieval solution pH 6 (DAKO, Denmark) and autoclaving at 121°C for 20 min. Endogenous peroxidase was blocked using 3% w/v hydrogen peroxide for 5 min and endogenous biotin was blocked as described previously. After incubation with 10% horse serum for 30 min to block non-specific binding, sections were incubated with anti-human sirtuin (1:250) primary antibodies (raised in rabbit), for 2 hours at ambient temperature. After three rinses, sections were incubated with secondary antibodies–biotinylated goat-anti rabbit or mouse (Vector Laboratories, USA, 1:200) for 30 min at ambient temperature followed by 30 min treatment with avidin-biotin-complex (ABC) elite (Vector). Labelling was visualized with liquid DAB and sections were counterstained and mounted. Negative controls for non-specific staining included replacement of the primary antibody by normal rabbit or mouse IgG.

2.2.8 Sirtuin Protein Expression in human control brain tissue using western blotting

Protein from three individual control *post mortem* frontal brain tissue samples were extracted as described in the previous section, and run on a 1D SDS-PAGE gel (20 μg protein per lane), and western blotted with antibodies for SIRT1, SIRT2 and SIRT3.

The gel was electroblotted (20V, 110mA, 90mins) onto a nitrocellulose membrane in transfer buffer of 50mM tris, 40mM glycine, 1.3mM SDS, 20% methanol, pH 9.2. After transfer of proteins, the membrane was incubated in 10% skim milk powder solution containing primary sirtuin antibody. SIRT1, SIRT2 and SIRT3 rabbit polyclonal antibodies were used (1:1000 dilution, Abcam, UK), followed by a secondary antibody (1:100,000 dilution of anti-mouse IgG, Pierce, USA). See Table 2.6 for antibody details and dilutions. Chemiluminescence blots were developed using 1:1 solution of Super Signal West Femto luminol and peroxide buffer (Pierce, USA) according to manufacturer's instructions.

Table 2.6 Antibodies and dilutions used for western blotting

Antibody	Dilution	Manufacturer
Rabbit Polyclonal SIRT1	1:1000	Abcam (Cambridge, UK)
Rabbit Polyclonal SIRT2	1:1000	Abcam (Cambridge, UK)
Rabbit Polyclonal SIRT3	1:1000	Abcam (Cambridge, UK)

2.2.9 Statistics

Sirtuin concentrations are presented as means ± SEM using peak area ratios of light and heavy peptides obtained from Skyline MRM Proteomics software v3.1 (MacCoss Lab, USA). Sirtuins were quantified based on an average of data from both peptides used for each sirtuin where possible. Statistical comparisons were performed using two-tailed student t-tests assuming equal variance. Differences between groups were considered statistically significant at the $p \leq 0.05$ level.

2.2.10 Ethics

All human and animal brain tissue samples were obtained and experiments conducted in accordance with the guidelines of the National Health and Medical Research Council of Australia and were approved by the University of New South Wales Human Research Ethics Committee (human brain tissue reference number HC12563) and the University of New South Wales Animal Care Ethics Committee (guinea pig tissue reference number 14/40B and mice 13/39B). Adult human primary microglia, astrocytes, neurons and oligodendrocytes

were cultured from resected normal adult brain tissue following removal of brain tumour with informed consent at the Minimally Invasive Cancer Centre, Prince of Wales Hospital, Sydney, Australia (reference number X12-0314 and HREC/12/RPAH/481). All control CSF samples were obtained with ethics approval from the Sydney Adventist Hospital, Sydney Australia, with informed consent obtained from all subjects (reference number SAHHREC #13-02).

Results

Peptide standards

Standard curves for sirtuin peptides are shown in Figure 2.1. The LOD and LOQ were determined when the signal to noise ratio of the transition with the highest intensity was approximately 3:1 and 10:1 respectively. All peptides had good linearity in the 1-200 fmol/µl range and intra- and inter- assay coefficients of variance (CV %) were <10% and less than <14% respectively.

Figure 2.1 Sirtuin peptide standard curves, variance and limits of detection and quantification. Sirtuin peptide standard curves (average of two peptides, in triplicate for each sirtuin, with heavy peptide spike of 100fmol/µl). Both inter- and intra-assay CV% were calculated for three replicates and the LOD and LOQ are shown in fmol/ul. CVs calculated using peptide intensity ratios (light/heavy) at the 100fmol/µl peptide concentration level.

SIRT1

$y = 0.0148x + 0.0015$
$R^2 = 0.9987$

Inter-assay CV: 13.81%

Intra-assay CV: 3.1%

LOD: 0.5   LOQ: 2.5

SIRT 2

$y = 0.0138x - 0.0163$
$R^2 = 0.9921$

Inter- assay CV: 9.27%

Intra-assay CV: 4.15%

LOD: 0.25   LOQ: 2.5

SIRT3

$y = 0.0078x - 0.0039$
$R^2 = 0.999$

Inter-assay CV: 12.14%

Intra-assay CV: 7.26%

LOD: 0.25   LOQ: 1.0

SIRT 4

$y = 0.0087x + 0.0658$
$R^2 = 0.9742$

Inter-assay CV: 11.78%

Intra-assay CV: 6.03%

LOD: 0.5   LOQ: 2.5

SIRT5

$y = 0.0226x + 0.1119$
$R^2 = 0.989$

Inter-assay CV: 12.71%

Intra-assay CV: 4.84%

LOD: 0.25   LOQ: 1.0

SIRT6

$y = 0.0087x + 0.1162$
$R^2 = 0.9743$

Inter-assay CV: 13.64%

Intra-assay CV: 6.79%

LOD: 0.25   LOQ: 2.5

SIRT7

$y = 0.0298x - 0.0201$
$R^2 = 0.9908$

Inter-assay CV: 10.67%

Intra-assay CV: 8.64%

LOD: 0.25   LOQ: 1

Figure 2.2 Representative chromatograms for all 14 sirtuin peptides used in the MRM method. Showing transition ions, retention times and signal intensities. The vertical dotted lines either side of the peak clusters indicate the boundaries for peak area integration.

SIRT1 DINTIEDAVK (precursor mass, 2$^+$ *m/z* = 559.2904)
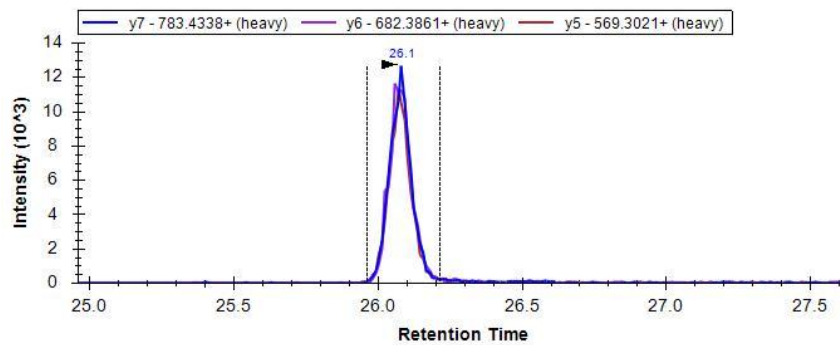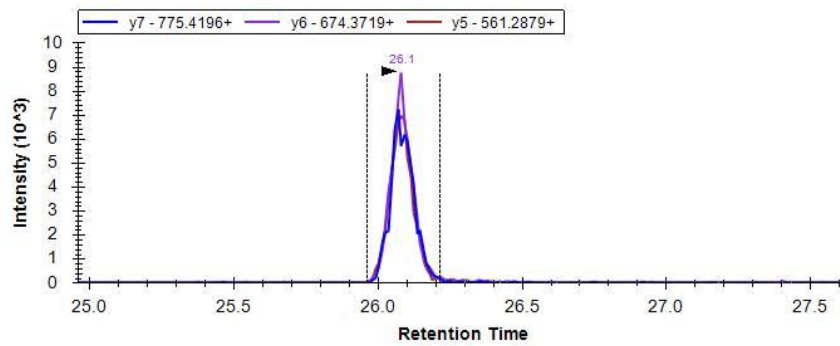
SIRT1 TSVAGTVR (precursor mass, 2$^+$ *m/z* = 395.7245)
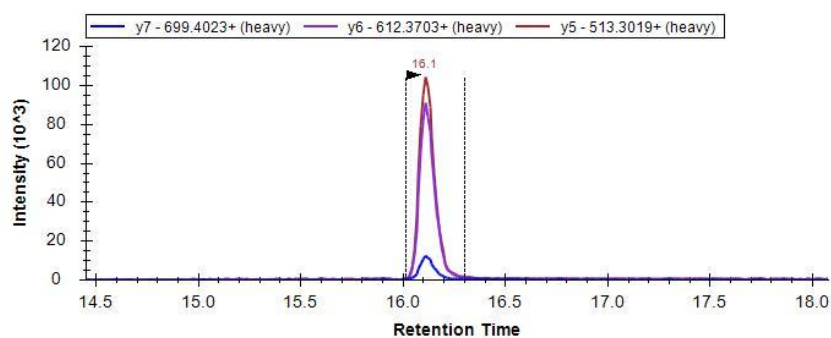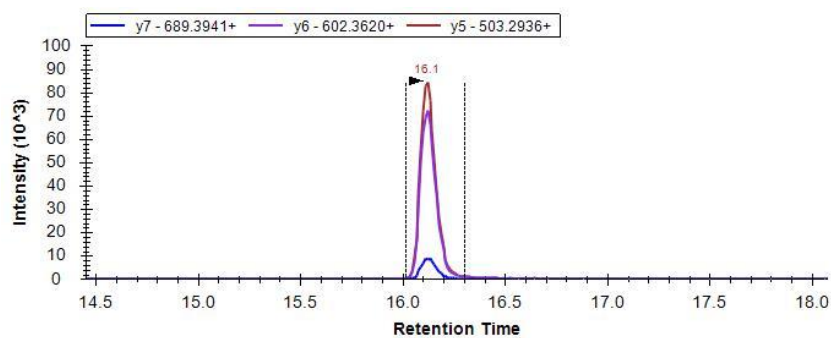
SIRT2 IFSEVTPK (precursor mass, 2$^+$ *m/z* = 460.758)
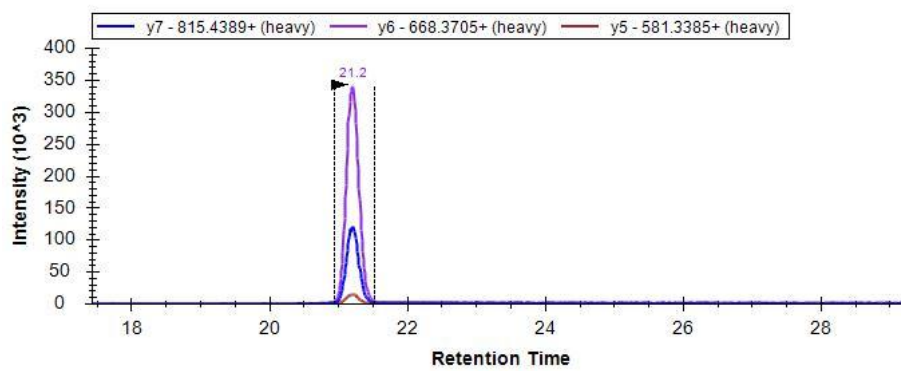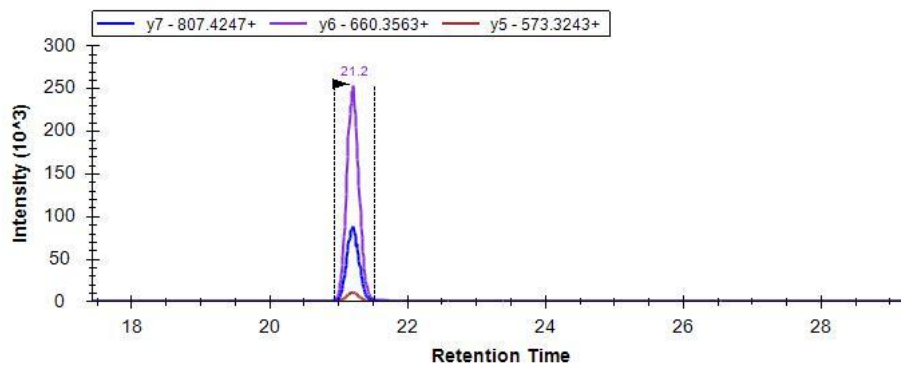
SIRT 2 LLDEELTLEGVAR (precursor mass, 2$^+$ *m/z* = 664.8746)
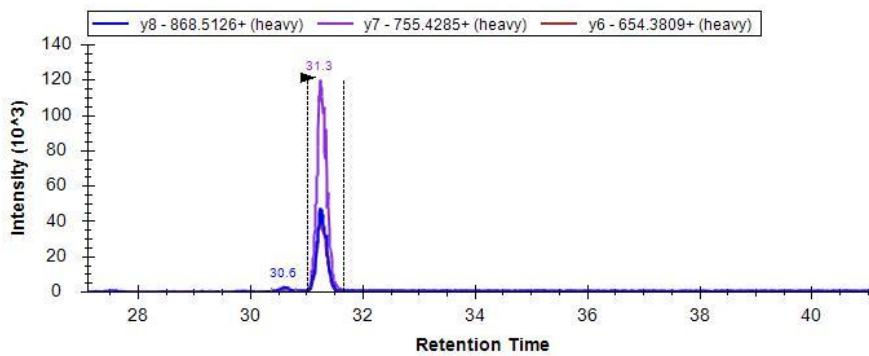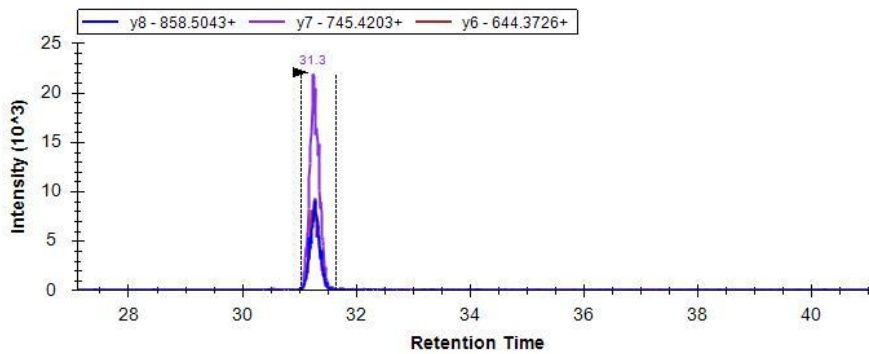
SIRT3 LYTQNIDGLER (precursor mass, 2$^+$ *m/z* = 661.341)
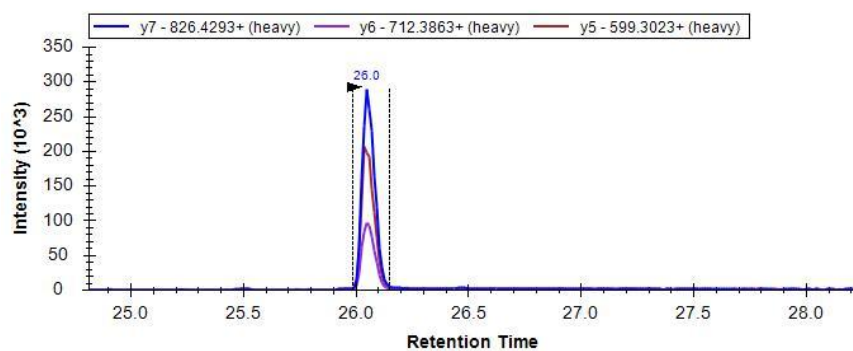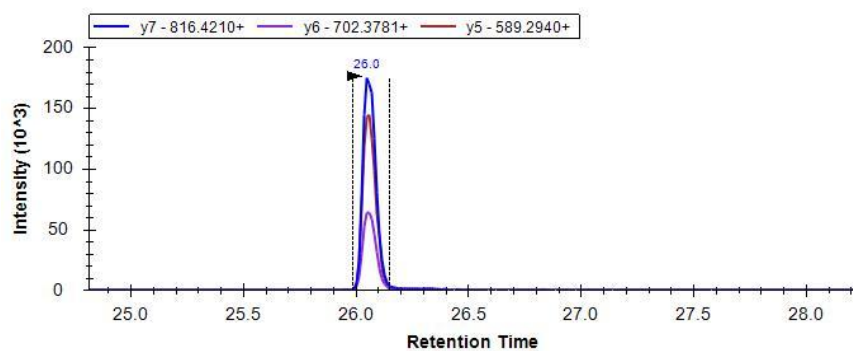
SIRT3 VSGIPASK (precursor mass, 2$^+$ *m/z* = 379.24)
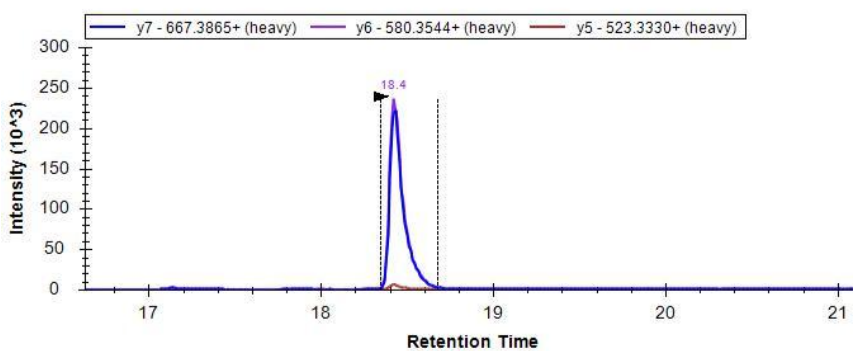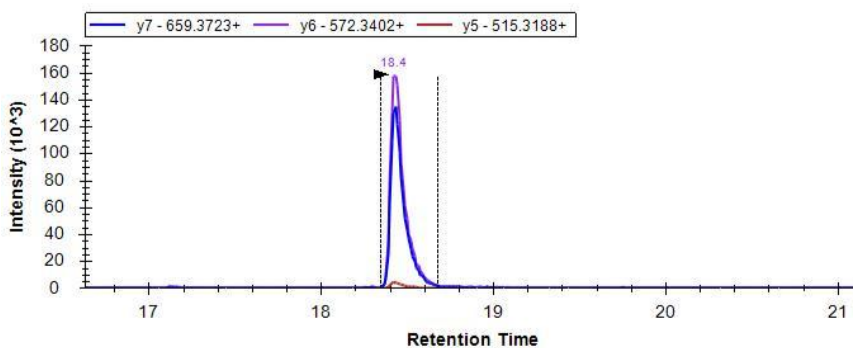
SIRT4 FILTAWEK (precursor mass, 2$^+$ m/z = 612.8334)
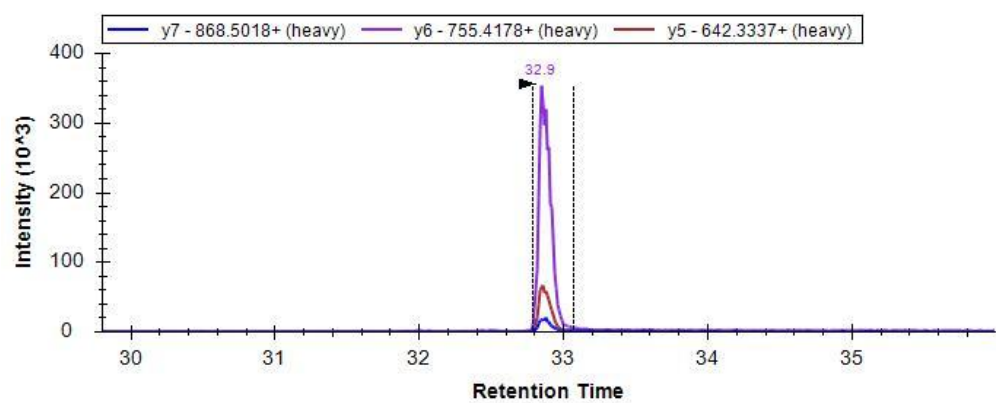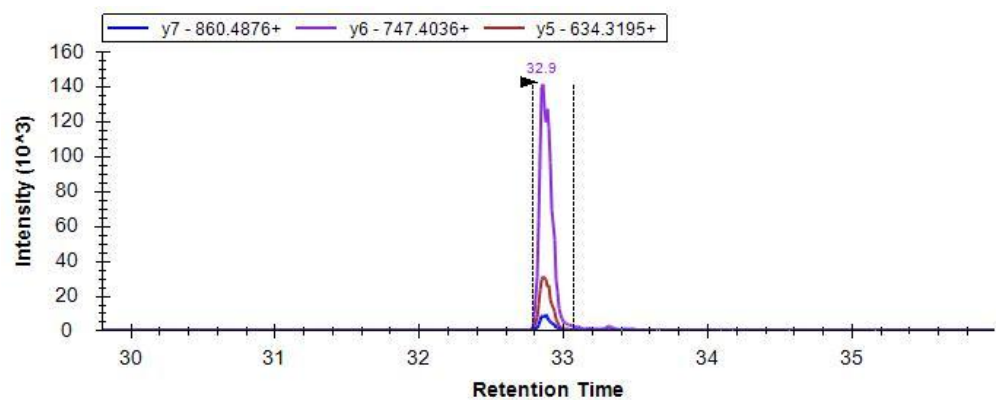
SIRT5 VVVITQNIDELHR (precursor mass, 2$^+$ m/z = 512.6229)
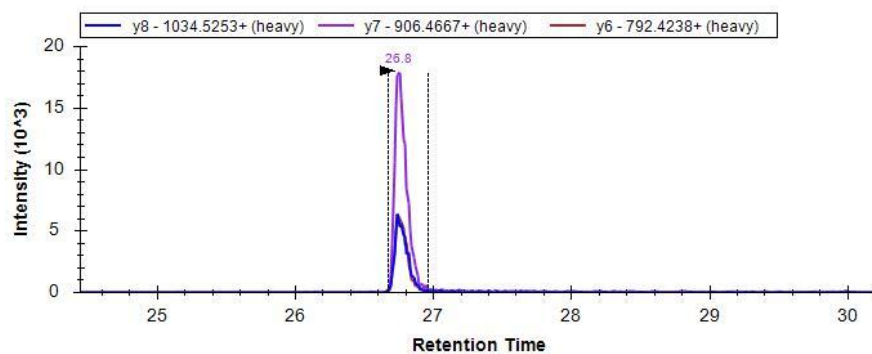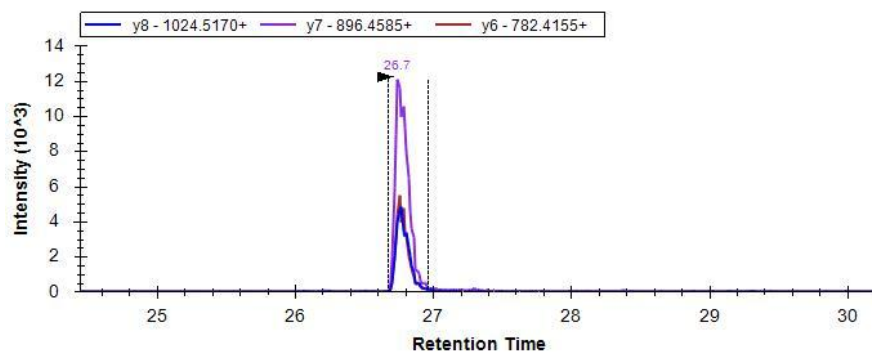
SIRT5 NLLEIHGSLFK (precursor mass, 2^+ *m/z* = 635.8613)
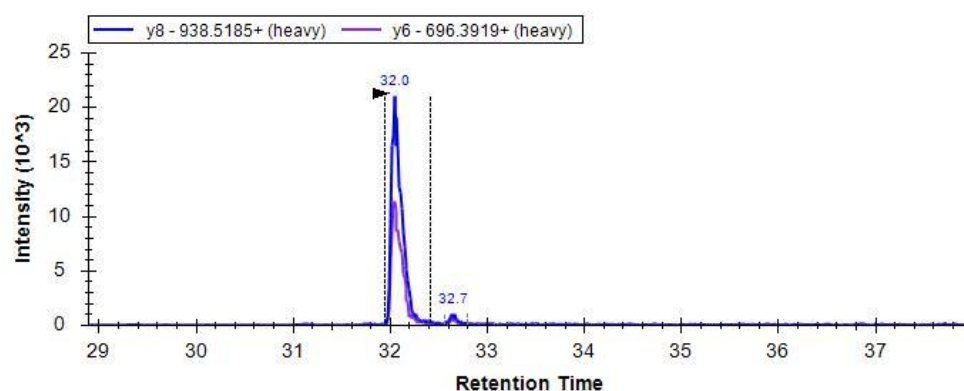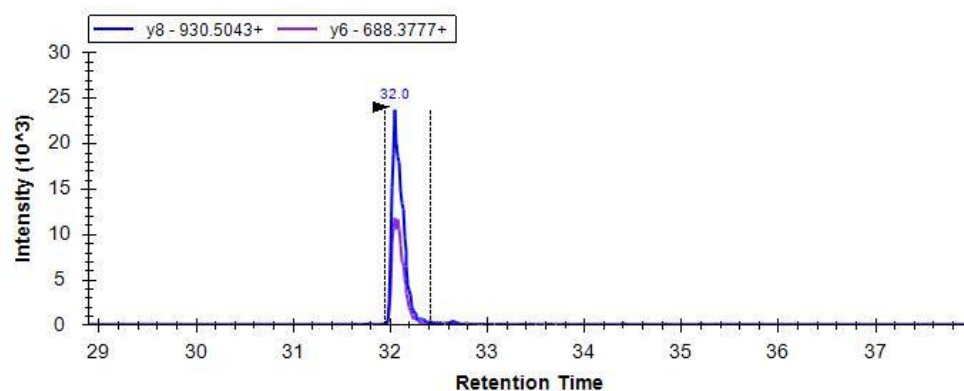
SIRT6 FLVSQNVDGLHVR (precursor mass, 2^+ *m/z* = 742.4044)
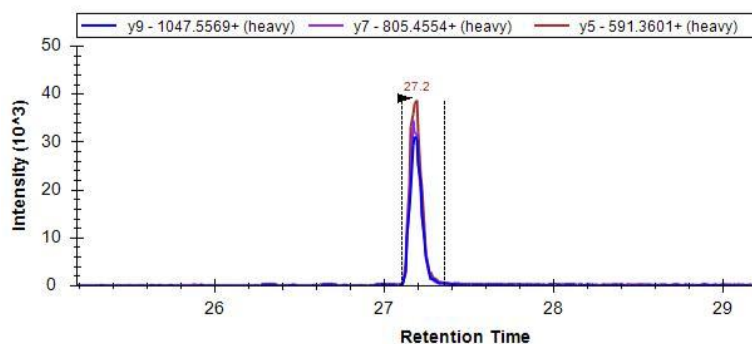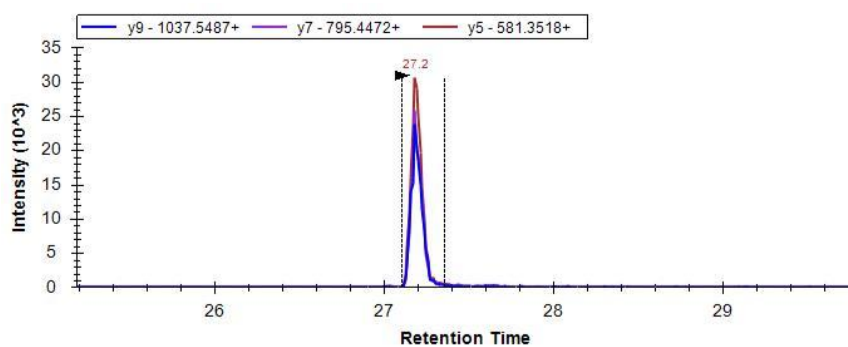
SIRT6 LVIVNLQPTK (precursor mass, 2^+ m/z = 562.8555)
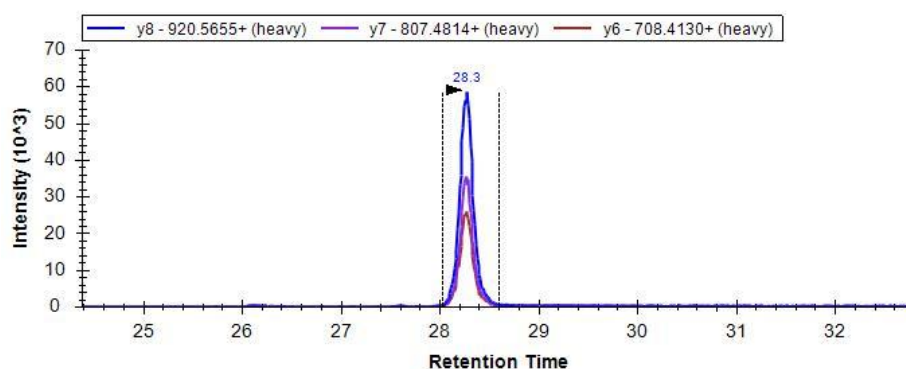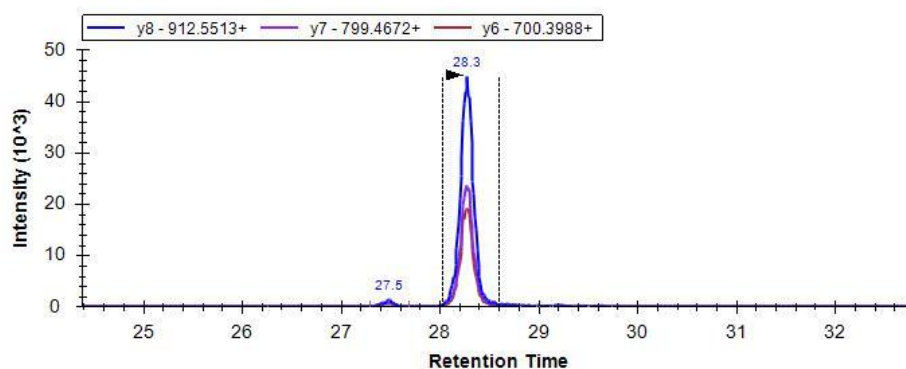
SIRT7 DTIVHFGER (precursor mass, 2^+ m/z = 537.2724)
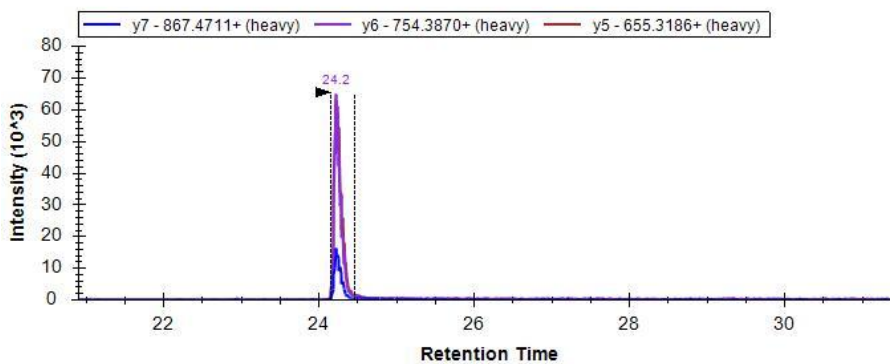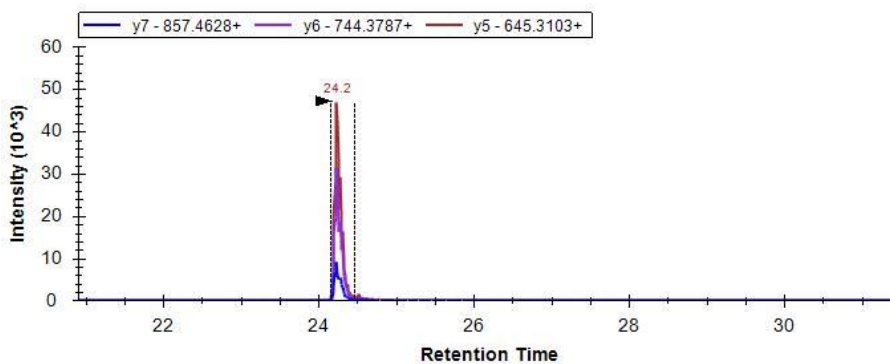
SIRT7 LLAESADLVTELQGR (precursor mass, 2$^+$ *m/z* = 807.9385)
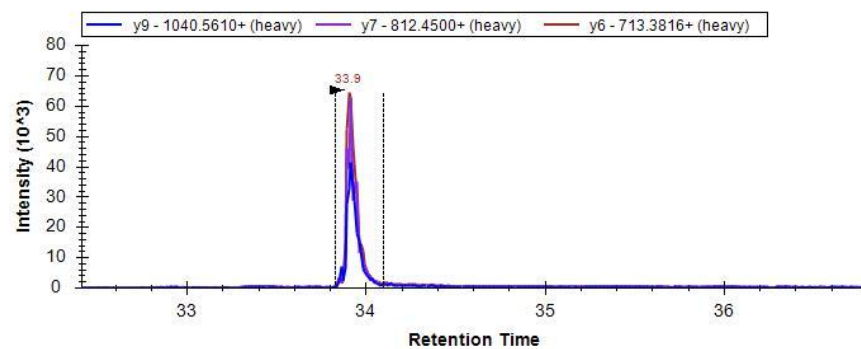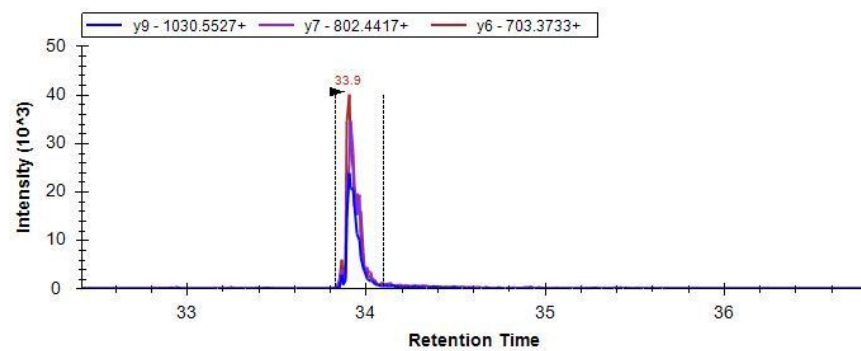
2.3.2 Quantification of sirtuin expression in cells and tissues

In all the human primary brain cell types and human brain cell lines SIRT1, 2, 3, 6 and 7 were detected and quantified (Figure 2.3).

Figure 2.3 Sirtuin expression in primary cultured brain cells and cell lines.

In primary neurons SIRT1 was found to be the most abundant sirtuin (*p<0.05) compared to other neuronal sirtuins (n=3). SIRT2 was found to be abundant in primary oligodendrocytes (*p<0.05) compared to other cell types (n=3). SIRT1 and SIRT2 were the most abundant in all the cell cultures. SIRT4 and SIRT5 were below the detection limits and only small amounts of SIRT6 and SIRT7 were detected.

All seven sirtuins were detected in human control brain tissue, with SIRT2 highly expressed (Figure 2.4, Panel A). Detergent fractionation into subcellular groups improved identification of mitochondrial sirtuins (SIRT3-5) (Figure 2.4, Panel B).

Figure 2.4: Sirtuin expression in human frontal lobe brain tissue. Sirtuin expression in unfractionated (Panel A) and fractionated (Panel B) human frontal lobe brain tissue. All seven sirtuins were detected in unfractionated human frontal lobe brain tissue with SIRT2 the most abundant (*$p < 0.05$) compared to all other sirtuins (Panel A). The mitochondrial sirtuins (SIRT3-5) were found to be close to the LOQ, but their signals improved following fractionation of the tissue into cytosolic, nuclear, cytoskeletal and membrane fractions (Panel B). Fractionation showed SIRT1 and SIRT2 expressed in all fractions, whereas SIRT3-5 were below the LOD in the cytosol and SIRT6 and SIRT7 were below the LOD in the cytoskeletal fraction. SIRT1 was most abundant in the nucleus and SIRT2 in the cytoskeletal fraction (*$p < 0.05$, Panel B). In the cytosol, SIRT2 was the most abundant sirtuin (*$p < 0.05$), and all sirtuins were captured in the membrane fraction.

Quantification of some sirtuins was possible in guinea pig and mouse tissue due to their common sequences with human sirtuin peptides. SIRT1-3 levels in the guinea pig (Figure 2.5 Panel A) and SIRT 1 and 3 levels in mouse organs (Figure 2.5 Panel B) were quantified. Furthermore the method could be adapted for all sirtuins from other species with purchase of the appropriate synthetic peptides and their heavy internal standards.

Figure 2.5 SIRT1-3 protein expression in animal organs. SIRT1-3 protein expression in guinea pig (Panel A) and mouse organs (Panel B). SIRT2 was found to be the most abundant sirtuin in guinea pig, with higher levels expressed in the brain (*p<0.01, n=2) compared to liver and kidney. SIRT2 in mouse was not quantified due to peptide sequence difference with the human peptide standards.
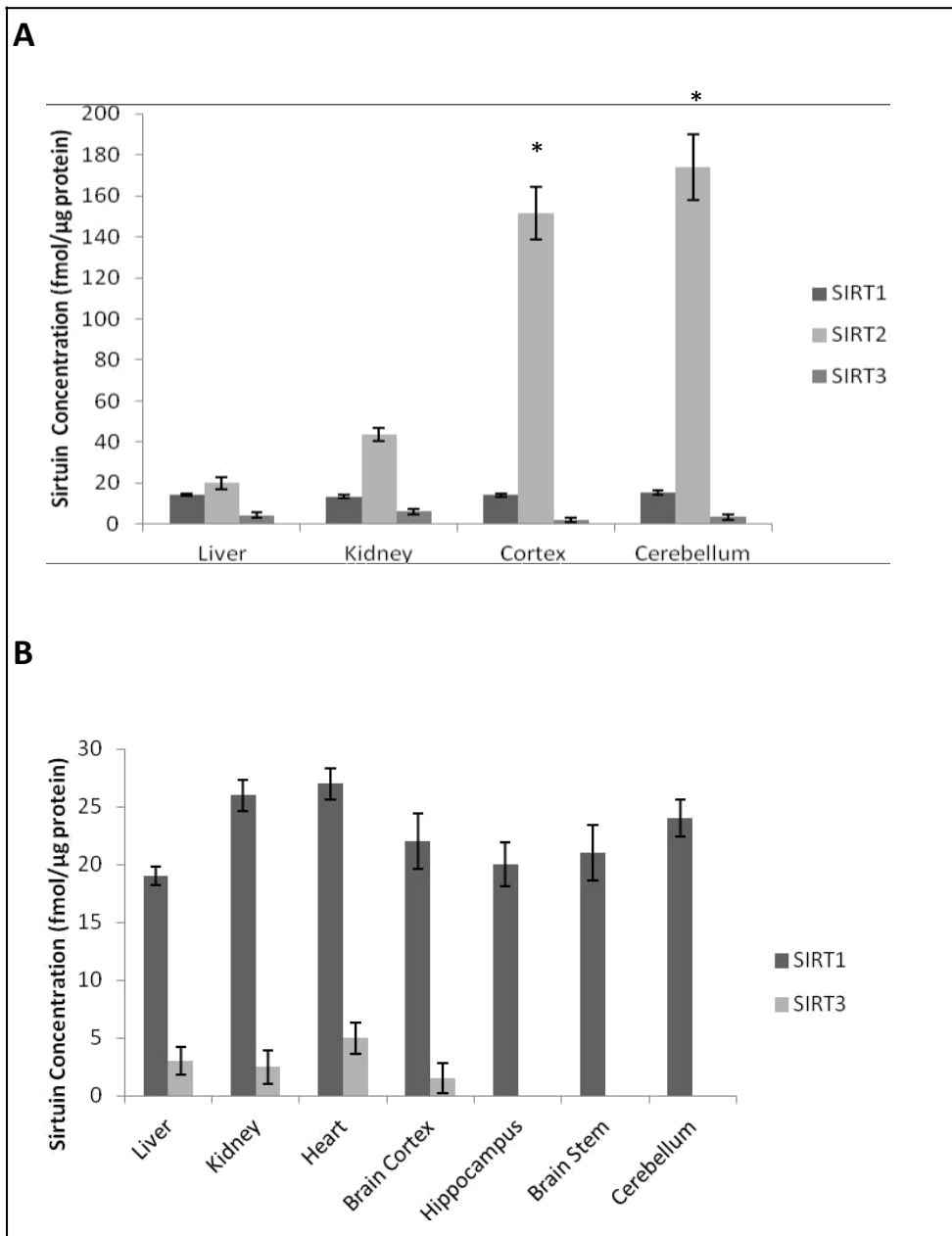
Table 2.7 shows the level of homology (or identity) of human SIRT1-7 peptide sequences with mouse and guinea pig.

Table 2.7 Human sirtuin peptide homology with guinea pig and mouse. Sequence variations from the human are highlighted in red.

Sirtuin	Human Peptide	Homology in Guinea Pig and Mice
SIRT1	DINTIEDAVK	Identical sequence found in both Guinea Pig and Mouse
	TSVAGTVR	In Mice: TSVAETVR In GP: TSVAETVR
SIRT2	LLDELTLEGVAR	Identical sequence found in Guinea Pig In Mice: LLDELTLEGVTR
	IFSEVTPK	In mice: IFSEATPR In GP: LFSDVTPK
SIRT3	LYTQNIDGLER	Identical sequence found in both Guinea Pig and Mouse
	VSGIPASK	In mice: ASGIPASK In GP: ASGIPASK
SIRT4	RPIQHGDFVR	In mice: RPIQHIDFVR In GP: RPIQHSDFVR
	FILTAWEK	In mice: FILTAREQ In GP: FTLTAQDK
SIRT5	VVVITQNIDELHR	Identical sequence found in Mouse In GP: VAVITQNIDELHLR
	NLLEIHGSLFK	In mice: NLLEIHGTLFK In GP: NLVEIHGTIFK
SIRT6	FLVSQNVDGLHVR	Identical sequence found in both Guinea Pig and Mouse
	LVIVNLQPTK	Identical sequence found in both Guinea Pig and Mouse
SIRT7	LLAESADLVTELQGR	In mice: LLAESEDLVTELQGR In GP: LLAESEDLVTELQGR
	DTIVHFGER	Identical sequence found in both Guinea Pig and Mouse

All samples were fractionation on a 1D-SDS-PAGE to reduce sample complexity and a workflow of the sample preparation procedure can be found in Figure 2.6 below.

Figure 2.6 Sample Fractionation and MRM Workflow
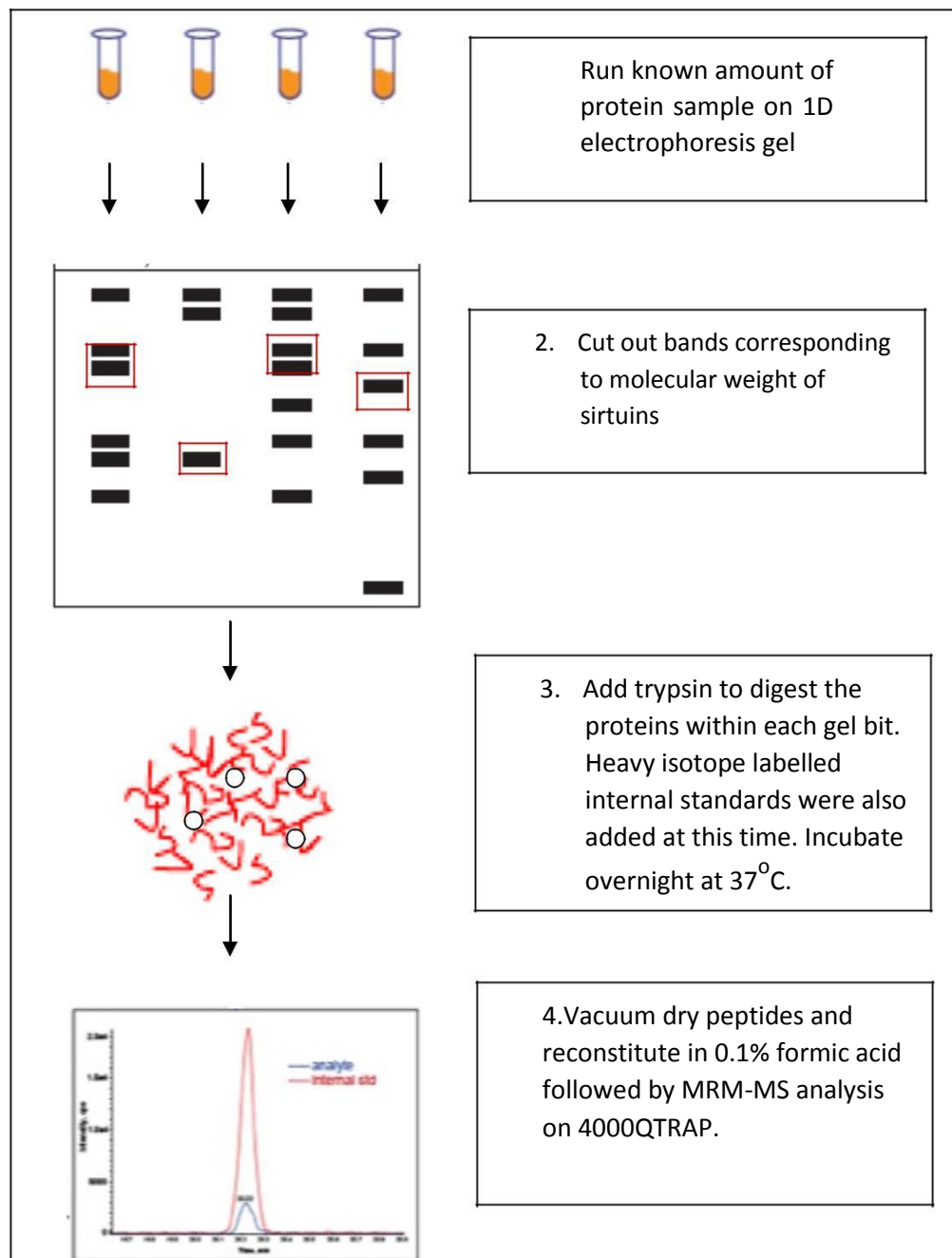
2.3.3 Sirtuin Isoforms

Another important issue is the existence of splice variants or isoforms of the sirtuin proteins. Sequences of all 7 human sirtuins and their isoforms (obtained from UniProt). The peptides used for MRM quantification experiments are highlighted in red and in larger font size in Figure 2.7. All sirtuin isoforms contain at least one of the peptides used for quantification, and both peptides are represented in the majority of isoforms.

Figure 2.7 Sequences for all seven human sirtuins and their isoforms

---

SIRT1 Isoform 1 >sp|Q96EB6|SIR1_HUMAN NAD-dependent protein deacetylase sirtuin-1 OS=Homo sapiens GN=SIRT1 PE=1 SV=2
MADEAALALQPGGSPSAAGADREAASSPAGEPLRKRPRRDGPGLERSPGEPGGAAPER
EVPAAARGCPGAAAAALWREAEAEAAAAGGEQEAQATAAAGEGDNGPGLQGPSREP
PLADNLYDEDDDDEGEEEEAAAAAIGYRDNLLFGDEIITNGFHSCESDEEDRASHASSS
DWTPRPRIGPYTFVQQHLMIGTDPRTILKDLLPETIPPPELDDMTLWQIVINILSEPPKRK
KRK**DINTIEDAVK**LLQECKKIIVLTGAGVSVSCGIPDFRSRDGIYARLAVDFPDLPDPQA
MFDIEYFRKDPRPFFKFAKEIYPGQFQPSLCHKFIALSDKEGKLLRNYTQNIDTLEQVAGIQ
RIIQCHGSFATASCLICKYKVDCEAVRGDIFNQVVPRCPRCPADEPLAIMKPEIVFFGENL
PEQFHRAMKYDKDEVDLLIVIGSSLKVRPVALIPSSIPHEVPQILINREPLPHLHFDVELLGD
CDVIINELCHRLGGEYAKLCCNPVKLSEITEKPPRTQKELAYLSELPPTPLHVSEDSSSPERT
SPPDSSVIVTLLDQAAKSNDDLDVSESKGCMEEKPQEVQTSRNVESIAEQMENPDLKNV
GSSTGEKNER**TSVAGTVR**KCWPNRVAKEQISRRLDGNQYLFLPPNRYIFHGAEVYSDS
EDDVLSSSSCGSNSDSGTCQSPSLEEPMEDESEIEEFYNGLEDEPDVPERAGGAGFGTDG
DDQEAINEAISVKQEVTDMNYPSNKS

SIRT1 Isoform 2 >sp|Q96EB6-2|SIR1_HUMAN Isoform 2 of NAD-dependent protein deacetylase sirtuin-1 OS=Homo sapiens GN=SIRT1
MADEAALALQPGGSPSAAGADREAASSPAGEPLRKRPRRDGPGLERSPGEPGGAAPER
EVPAAARGCPGAAAAALWREAEAEAAAAGGEQEAQATAAAGEGDNGPGLQGPSREP
PLADNLYDEDDDDEGEEEEAAAAAIGYRDNLLFGDEIITNGFHSCESDEEDRASHASSS
DWTPRPRIGPYTFVQQHLMIGTDPRTILKDLLPETIPPPELDDMTLWQIVINILSEPPKRK
KRK**DINTIEDAVK**LLQECKKIIVLTGAGVSVSCGIPDFRSRDGIYARLAVDFPDLPDPQA
MFDIEYFRKDPRPFFKFAKEIYPGQFQPSLCHKFIALSDKEGKLLRNYTQNIDTLEQVAGIQ
RIIQCHGSFATASCLICKYKVDCEAVRGDIFNQVVPRCPRCPADEPLAIMKPEIVFFGENL
PEQFHRAMKYDKDEVDLLIVIGSSLKVRPVALIPSNQYLFLPPNRYIFHGAEVYSDSEDDV
LSSSSCGSNSDSGTCQSPSLEEPMEDESEIEEFYNGLEDEPDVPERAGGAGFGTDGDDQ
EAINEAISVKQEVTDMNYPSNKS

SIRT2 Isoform 1 >sp|Q8IXJ6|SIR2_HUMAN NAD-dependent protein deacetylase sirtuin-2

OS=Homo sapiens GN=SIRT2 PE=1 SV=2

MAEPDPSHPLETQAGKVQEAQDSDSDSEGGAAGGEADMDFLRNLFSQTLSLGSQKER

LLDELTLEGVARYMQSERCRRVICLVGAGISTSAGIPDFRSPSTGLYDNLEKYHLPYPE

AIFEISYFKKHPEPFFALAKELYPGQFKPTICHYFMRLLKDKGLLLRCYTQNIDTLERIAGLE

QEDLVEAHGTFYTSHCVSASCRHEYPLSWMKEK**IFSEVTPK**CEDCQSLVKPDIVFFGES

LPARFFSCMQSDFLKVDLLLVMGTSLQVQPFASLISKAPLSTPRLLINKEKAGQSDPFLG

MIMGLGGGMDFDSKKAYRDVAWLGECDQGCLALAELLGWKKELEDLVRREHASIDAQ

SGAGVPNPSTSASPKKSPPPAKDEARTTEREKPQ

SIRT2 Isoform 2 >sp|Q8IXJ6-2|SIR2_HUMAN Isoform 2 of NAD-dependent protein deacetylase sirtuin-2 OS=Homo sapiens GN=SIRT2

MDFLRNLFSQTLSLGSQKER**LLDELTLEGVAR**YMQSERCRRVICLVGAGISTSAGIPDF

RSPSTGLYDNLEKYHLPYPEAIFEISYFKKHPEPFFALAKELYPGQFKPTICHYFMRLLKDKG

LLLRCYTQNIDTLERIAGLEQEDLVEAHGTFYTSHCVSASCRHEYPLSWMKEK**IFSEVTP**

KCEDCQSLVKPDIVFFGESLPARFFSCMQSDFLKVDLLLVMGTSLQVQPFASLISKAPLST

PRLLINKEKAGQSDPFLGMIMGLGGGMDFDSKKAYRDVAWLGECDQGCLALAELLGW

KKELEDLVRREHASIDAQSGAGVPNPSTSASPKKSPPPAKDEARTTEREKPQ

SIRT2 Isoform 3 >sp|Q8IXJ6-3|SIR2_HUMAN Isoform 3 of NAD-dependent protein deacetylase sirtuin-2 OS=Homo sapiens GN=SIRT2

MPLAECPSCRCLSSFRSVDFLRNLFSQTLSLGSQKER**LLDELTLEGVAR**YMQSERCRR

VICLVGAGISTSAGIPDFRSPSTGLYDNLEKYHLPYPEAIFEISYFKKHPEPFFALAKELYPGQ

FKPTICHYFMRLLKDKGLLLRCYTQNIDTLERIAGLEQEDLVEAHGTFYTSHCVSASCRHE

YPLSWMKEK**IFSEVTPK**CEDCQSLVKPDIVFFGESLPARFFSCMQSDFLKVDLLLVMGT

SLQVQPFASLISKAPLSTPRLLINKEKAGQSDPFLGMIMGLGGGMDFDSKKAYRDVAWL

GECDQGCLALAELLGWKKELEDLVRREHASIDAQSGAGVPNPSTSASPKKSPPPAKDEA

RTTEREKPQ

SIRT2 Isoform 4 >sp|Q8IXJ6-4|SIR2_HUMAN Isoform 4 of NAD-dependent protein deacetylase sirtuin-2 OS=Homo sapiens GN=SIRT2

MAEPDPSHPLETQAGKVQEAQDSDSDSEGGAAGGEADMDFLRNLFSQTLSLGSQKER

LLDELTLEGVARYMQSERCRRVICLVGAGISTSAGIPDFRSPSTGLYDNLEKYHLPYPE

AIFEISYFKKHPEPFFALAKELYPGQFKPTICHYFMRLLKDKGLLLRCYTQNIDTLERIAGLE

QEDLVEAHGTFYTSHCVSASCRHEYPLSWMKEK**IFSEVTPK**CEDCQSLVKPDIVFFGES

LPARFFSCMQSDFLKVDLLLVMGTSLQGRGLAG

SIRT2 Isoform 5 >sp|Q8IXJ6-5|SIR2_HUMAN Isoform 5 of NAD-dependent protein deacetylase sirtuin-2 OS=Homo sapiens GN=SIRT2

MAEPDRRRVICLVGAGISTSAGIPDFRSPSTGLYDNLEKYHLPYPEAIFEISYFKKHPEPFFA

LAKELYPGQFKPTICHYFMRLLKDKGLLLRCYTQNIDTLERIAGLEQEDLVEAHGTFYTSHC

VSASCRHEYPLSWMKEK**IFSEVTPK**CEDCQSLVKPDIVFFGESLPARFFSCMQSDFLKV

DLLLVMGTSLQVQPFASLISKAPLSTPRLLINKEKAGQSDPFLGMIMGLGGGMDFDSKK

AYRDVAWLGECDQGCLALAELLGWKKELEDLVRREHASIDAQSGAGVPNPSTSASPKK

SPPPAKDEARTTEREKPQ

SIRT3 Isoform 1 >sp|Q9NTG7|SIR3_HUMAN NAD-dependent protein deacetylase sirtuin-3, mitochondrial OS=Homo sapiens GN=SIRT3 PE=1 SV=2
MAFWGWRAAAALRLWGRVVERVEAGGGVGPFQACGCRLVLGGRDDVSAGLRGSHGARGEP
LDPARPLQRPPRPEVPRAFRRQPRAAAPSFFFSSIKGGRRSISFSVGASSVVGSGGSSDKG
KLSLQDVAELIRARACQRVVVMVGAGISTPSGIPDFRSPGSGLYSNLQQYDLPYPEAIFEL
PFFFHNPKPFFTLAKELYPGNYKPNVTHYFLRLLHDKGLLLR**LYTQNIDGLERVSGIPA**
SKLVEAHGTFASATCTVCQRPFPGEDIRADVMADRVPRCPVCTGVVKPDIVFFGEPLPQ
RFLLHVVDFPMADLLLILGTSLEVEPFASLTEAVRSSVPRLLINRDLVGPLAWHPRSRDVA
QLGDVVHGVESLVELLGWTEEMRDLVQRETGKLDGPDK

SIRT3 Isoform 2 >sp|Q9NTG7-2|SIR3_HUMAN Isoform 2 of NAD-dependent protein deacetylase sirtuin-3, mitochondrial OS=Homo sapiens GN=SIRT3
MVGAGISTPSGIPDFRSPGSGLYSNLQQYDLPYPEAIFELPFFFHNPKPFFTLAKELYPGNY
KPNVTHYFLRLLHDKGLLLR**LYTQNIDGLERVSGIPASK**LVEAHGTFASATCTVCQR
PFPGEDIRADVMADRVPRCPVCTGVVKPDIVFFGEPLPQRFLLHVVDFPMADLLLILGTS
LEVEPFASLTEAVRSSVPRLLINRDLVGPLAWHPRSRDVAQLGDVVHGVESLVELLGWT
EEMRDLVQRETGKLDGPDK

SIRT4 >sp|Q9Y6E7|SIR4_HUMAN NAD-dependent protein lipoamidase sirtuin-4, mitochondrial OS=Homo sapiens GN=SIRT4 PE=1 SV=1
MKMSFALTFRSAKGRWIANPSQPCSKASIGLFVPASPPLDPEKVKELQRFITLSKRLLVM
TGAGISTESGIPDYRSEKVGLYARTDR**RPIQHGDFVR**SAPIRQRYWARNFVGWPQFS
SHQPNPAHWALSTWEKLGKLYWLVTQNVDALHTKAGSRRLTELHGCMDRVLCLDCGE
QTPRGVLQERFQVLNPTWSAEAHGLAPDGDVFLSEEQVRSFQVPTCVQCGGHLKPDV
VFFGDTVNPDKVDFVHKRVKEADSLLVVGSSLQVYSGYR**FILTAWEK**KLPIAILNIGPTR
SDDLACLKLNSRCGELLPLIDPC

SIRT5 Isoform 1 >sp|Q9NXA8|SIR5_HUMAN NAD-dependent protein deacylase sirtuin-5, mitochondrial OS=Homo sapiens GN=SIRT5 PE=1 SV=2
MRPLQIVPSRLISQLYCGLKPPASTRNQICLKMARPSSSMADFRKFFAKAKHIVIISGAGV
SAESGVPTFRGAGGYWRKWQAQDLATPLAFAHNPSRVWEFYHYRREVMGSKEPNAG
HRAIAECETRLGKQGRR**VVVITQNIDELHR**KAGTK**NLLEIHGSLFK**TRCTSCGVVA
ENYKSPICPALSGKGAPEPGTQDASIPVEKLPRCEEAGCGGLLRPHVVWFGENLDPAILE
EVDRELAHCDLCLVVGTSSVVYPAAMFAPQVAARGVPVAEFNTETTPATNRFRFHFQG
PCGTTLPEALACHENETVS

SIRT5 Isoform 2 >sp|Q9NXA8-2|SIR5_HUMAN Isoform 2 of NAD-dependent protein deacylase sirtuin-5, mitochondrial OS=Homo sapiens GN=SIRT5
MRPLQIVPSRLISQLYCGLKPPASTRNQICLKMARPSSSMADFRKFFAKAKHIVIISGAGV
SAESGVPTFRGAGGYWRKWQAQDLATPLAFAHNPSRVWEFYHYRREVMGSKEPNAG
HRAIAECETRLGKQGRR**VVVITQNIDELHR**KAGTK**NLLEIHGSLFK**TRCTSCGVVA
ENYKSPICPALSGKGAPEPGTQDASIPVEKLPRCEEAGCGGLLRPHVVWFGENLDPAILE

EVDRELAHCDLCLVVGTSSVVYPAAMFAPQVAARGVPVAEFNTETTPATNRFSHLISISS
LIIIKN

SIRT5 Isoform 3 >sp|Q9NXA8-3|SIR5_HUMAN Isoform 3 of NAD-dependent protein deacylase sirtuin-5, mitochondrial OS=Homo sapiens GN=SIRT5

MRPLQIVPSRLISQLYCGLKPPASTRNQICLKMARPSSSMADFRKFFAKAKHIVIISGAGV
SAESGVPTFRGAGGYWRKWQAQDLATPLAFAHNPSRVWEFYHYRREVMGSKEPNAG
HRAIAECETRLGKQGRR**VVVITQNIDELHR**KAGTK**NLLEIHGSLFK**TRCTSCGVVA
ENYKSPICPALSGKGCEEAGCGGLLRPHVVWFGENLDPAILEEVDRELAHCDLCLVVGTS
SVVYPAAMFAPQVAARGVPVAEFNTETTPATNRFRFHFQGPCGTTLPEALACHENETV
S

SIRT5 Isoform 4 >sp|Q9NXA8-4|SIR5_HUMAN Isoform 4 of NAD-dependent protein deacylase sirtuin-5, mitochondrial OS=Homo sapiens GN=SIRT5

MGSKEPNAGHRAIAECETRLGKQGRR**VVVITQNIDELHR**KAGTK**NLLEIHGSLFK**T
RCTSCGVVAENYKSPICPALSGKGAPEPGTQDASIPVEKLPRCEEAGCGGLLRPHVVWF
GENLDPAILEEVDRELAHCDLCLVVGTSSVVYPAAMFAPQVAARGVPVAEFNTETTPAT
NRFRFHFQGPCGTTLPEALACHENETVS

SIRT6 Isoform 1 >sp|Q8N6T7|SIR6_HUMAN NAD-dependent protein deacetylase sirtuin-6 OS=Homo sapiens GN=SIRT6 PE=1 SV=2

MSVNYAAGLSPYADKGKCGLPEIFDPPEELERKVWELARLVWQSSSVVFHTGAGISTAS
GIPDFRGPHGVWTMEERGLAPKFDTTFESARPTQTHMALVQLERVGLLR**FLVSQNV
DGLHVR**SGFPRDKLAELHGNMFVEECAKCKTQYVRDTVVGTMGLKATGRLCTVAKA
RGLRACRGELRDTILDWEDSLPDRDLALADEASRNADLSITLGTSLQIRPSGNLPLATKRR
GGR**LVIVNLQPTK**HDRHADLRIHGYVDEVMTRLMKHLGLEIPAWDGPRVLERALPPL
PRPPTPKLEPKEESPTRINGSIPAGPKQEPCAQHNGSEPASPKRERPTSPAPHRPPKRVKA
KAVPS

SIRT6 Isoform 2 >sp|Q8N6T7-2|SIR6_HUMAN Isoform 2 of NAD-dependent protein deacetylase sirtuin-6 OS=Homo sapiens GN=SIRT6

MSVNYAAGLSPYADKGKCGLPEIFDPPEELERKVWELARLVWQSSSVVFHTGAGISTAS
GIPDFRGPHGVWTMEERGLAPKFDTTFESARPTQTHMALVQLERVGLLR**FLVSQNV
DGLHVR**SGFPRDKLAELHGNMFVEECAKCKTQYVRDTVVGTMGLKATGRLCTVAKA
RGLRACRNADLSITLGTSLQIRPSGNLPLATKRRGGR**LVIVNLQPTK**HDRHADLRIHGY
VDEVMTRLMKHLGLEIPAWDGPRVLERALPPLPRPPTPKLEPKEESPTRINGSIPAGPKQ
EPCAQHNGSEPASPKRERPTSPAPHRPPKRVKAKAVPS

SIRT7 Isoform 1 >sp|Q9NRC8|SIR7_HUMAN NAD-dependent protein deacetylase sirtuin-7 OS=Homo sapiens GN=SIRT7 PE=1 SV=1

MAAGGLSRSERKAAERVRRLREEQQRERLRQVSRILRKAAAERSAEEGR**LLAESADLV
TELQGR**SRRREGLKRRQEEVCDDPEELRGKVRELASAVRNAKYLVVYTGAGISTAASIP
DYRGPNGVWTLLQKGRSVSAADLSEAEPTLTHMSITRLHEQKLVQHVVSQNCDGLHLR
SGLPRTAISELHGNMYIEVCTSCVPNREYVRVFDVTERTALHRHQTGRTCHKCGTQLR**D**

TIVHFGERGTLGQPLNWEAATEAASRADTILCLGSSLKVLKKYPRLWCMTKPPSRRPKL
YIVNLQWTPKDDWAALKLHGKCDDVMRLLMAELGLEIPAYSRWQDPIFSLATPLRAGE
EGSHSRKSLCRSREEAPPGDRGAPLSSAPILGGWFGRGCTKRTKRKKVT

SIRT7 Isoform 2 >sp|Q9NRC8-2|SIR7_HUMAN Isoform 2 of NAD-dependent protein deacetylase sirtuin-7 OS=Homo sapiens GN=SIRT7
MAAGGLSRSERKAAERVRRLREEQQRERLRQVSRILRKAAAERSAEEGR**LLAESADLV
TELQGR**SRRREGLKRRQEEVCDDPEELRGKVRELASAVRNAKYLVVYTGAGISTAASIP
DYRGPNGVWTLLQKGRSVSAADLSEAEPTLTHMSITRLHEQKLVRALGGWYTCQGPGR
APWCPVGN

SIRT7 Isoform 3 >sp|Q9NRC8-3|SIR7_HUMAN Isoform 3 of NAD-dependent protein deacetylase sirtuin-7 OS=Homo sapiens GN=SIRT7
MPGPRRRSPSACPQVSRILRKAAAERSAEEGR**LLAESADLVTELQGR**SRRREGLKRR
QEEVCDDPEELRGKVRELASAVRNAKYLVVYTGAGISTAASIPDYRGPNGVWTLLQKGR
SVSAADLSEAEPTLTHMSITRLHEQKLVQHVVSQNCDGLHLRSGLPRTAISELHGNMYIE
VCTSCVPNREYVRVFDVTERTALHRHQTGRTCHKCGTQLRDTIVHFGERGTLGQPLNW
EAATEAASRADTILCLGSSLKVLKKYPRLWCMTKPPSRRPKLYIVNLQWTPKDDWAALKL
HGKCDDVMRLLMAELGLEIPAYSRVL

2.3.4 Validation of MRM method with established protocols

Human brain cells and tissue samples were compared with established protocols for sirtuin detection such as immunohistochemical staining, PCR and western blotting. Immunohistochemical staining showed that all seven sirtuins are expressed in astrocytes (Figure 2.8, Panel A, rows 1-4) and control human frontal lobe brain tissue (Panel A, row 5). PCR identified SIRT1 and SIRT2 mRNA in control human frontal, occipital and hippocampus brain tissue (Figure 2.8 Panel B). Western blotting confirmed the identification of SIRT1, 2 and 3 in control human frontal lobe brain tissue.

Figure 2.8 Sirtuin expression in human brain cells and tissue. Immunohistochemical staining of astrocytes (Panel A row 1-4) and human frontal brain tissue (Panel A, row 5). Row 1 is blue (DAPI) staining of nuclei, row 2 is green cell marker for GFAP, row 3 is red stain for sirtuin and row 4 is a merge of above three stains. Row 5 shows brown staining (highlighted with arrows) for sirtuin and pruple haemoatoxylin staining. PCR of SIRT1 and SIRT2 mRNA in occipital, frontal and hippocampal human control brain (Panel B) and cropped images of western blotting of SIRT1-3 protein expression in human control frontal brain tissue (n=3) at molecular weights of approximately 40kDa, 50kDa and 30kDa respectively (Panel C).

A
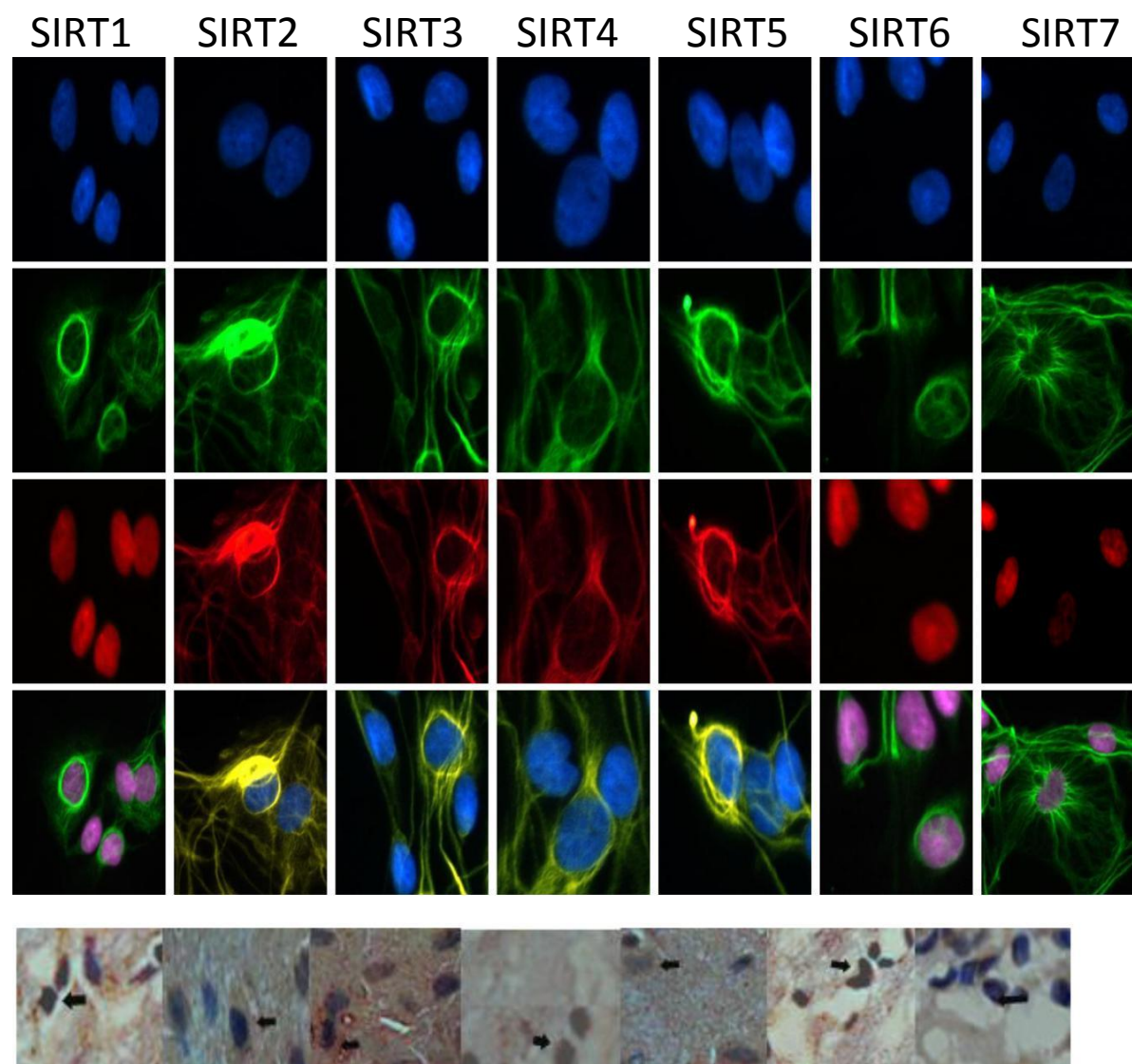
B
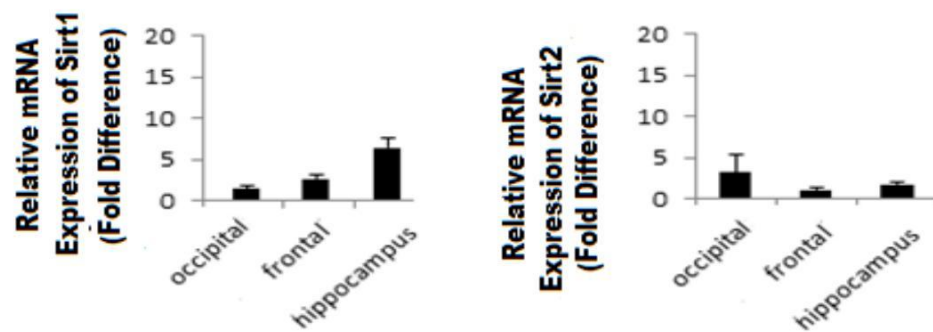
C

2.3.5 Quantification of sirtuins in CSF and immunodepleted plasma

SIRT1 was quantified in five control CSF patients and in control plasma immunodepleted of the six most abundant proteins. SIRT1 was the only sirtuin detected and was found to range from 4.30 ± 0.19 to 5.09 ± 0.53 fmol/μg protein in CSF and 8.68 ± 0.35 fmol/μg protein in plasma (Figure 2.9). SIRT2-7 in CSF and immunodepleted plasma were found to be below the detection limits of the assay. All samples were fractionation by 1D-SDS-PAGE to reduce sample complexity and a workflow of the sample preparation procedure can be found in Figure 2.6.

Figure 2.9 SIRT1 levels in CSF and depleted plasma. SIRT1 levels in CSF (n=5) samples and a plasma sample depleted of the six most abundant plasma proteins (high abundance protein removal was achieved using an Agilent Hu6 affinity column).
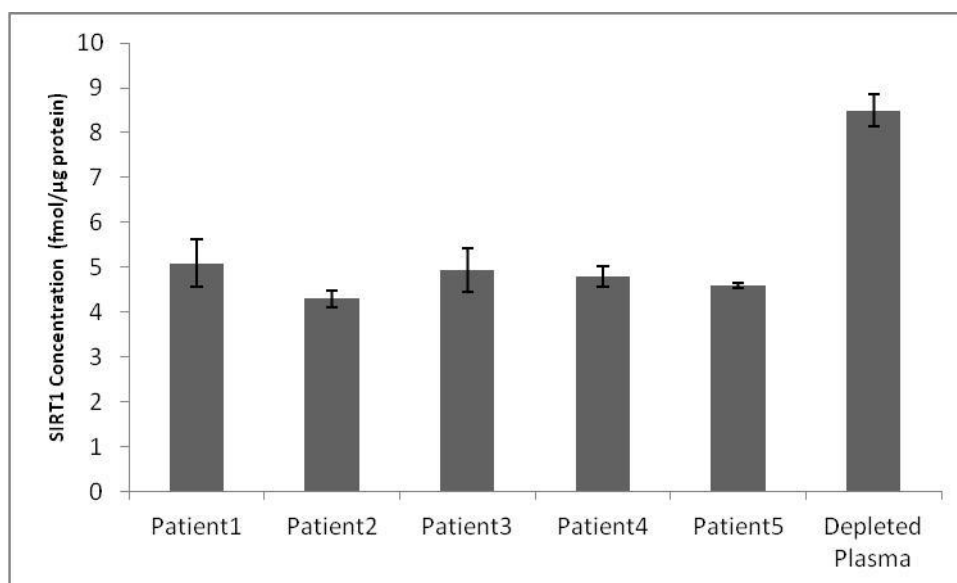
Sirtuin Standard Recoveries and Matrix Effects

The sirtuin levels presented in samples tested are the amounts after the in-gel extraction. Additional experiments to determine the recovery of peptides after this SDS/PAGE step were completed as were experiments to determine matrix effects.

Standard curves were prepared in 3 different matrices and recoveries compared after spiking known amounts of commercially obtained standard sirtuin proteins (Abcam, UK) into depleted human plasma samples.

For the experiments investigating matrix effects, standard curves using buffer only (0.1% formic acid), gel control (blank gel bits) and depleted plasma protein (LAP) were completed using gel bits cut from the appropriate sections of gel lanes containing 20ug of depleted plasma. For the blank and LAP gel matrix work, the various concentrations of light synthetic peptide were added after the gel bits were excised, followed by tryptic digestion and then MRM analysis. Standard curves for SIRT1, 2, 3, 5 and 6 with a seven point standard curve for each were completed. The gel image can be seen in Figure 2.10 (each label representing one point on the standard curve) and the standard curves for each of the individual peptides across the 3 matrices can be seen in Figure 2.11

Figure 2.10 A typical colloidal coomassie stained SDS/PAGE gel used to assess matrix effects on standard curves. Bands were cut from positions at which intact sirtuin proteins would typically be expected, and used to test matrix effects on the synthetic peptide standards. In addition to this, intact commercial recombinant sirtuin preparations were spiked into depleted plasma and run by SDS/PAGE (see Figure 2.11).

Depleted plasma
(20ug per lane)

Blank Gel Lanes
(Gel Control)
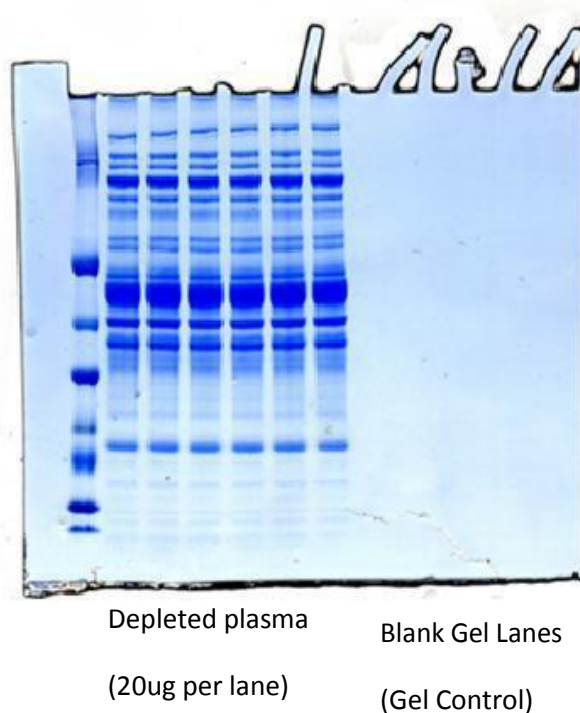
Figure 2.11 Sirtuin peptide standard curves. Prepared in several matrices, including buffer only, a blank gel control, a gel containing low abundance plasma (LAP) proteins (see figure 2.10 for the gel used for the gel matrices).

SIRT2 LLDELTLEGVAR

y = 0.0205x − 0.0342
R² = 0.9942

y = 0.0212x + 0.0045
R² = 0.994

y = 0.0245x − 0.0121
R² = 0.9988

SIRT2 IFSEVTPK

y = 0.0194x − 0.006
R² = 0.998

y = 0.0205x + 0.0112
R² = 0.9981

y = 0.0238x + 0.0046
R² = 0.9981

86

SIRT3 LYTQNIDGLER

Peptide Intensity Ratio (Light/Heavy) vs. Light Peptide Concentration (fmol/µl)

Buffer: $y = 0.0137x - 0.025$, $R^2 = 0.9943$

Gel Control: $y = 0.0126x - 0.0355$, $R^2 = 0.9888$

LAP: $y = 0.0178x - 0.0402$, $R^2 = 0.9911$

SIRT3 VSGIPASK

Peptide Intensity Ratio (Light/Heavy) vs. Light Peptide Concentration (fmol/µl)

Buffer: $y = 0.014x - 0.0225$, $R^2 = 0.9895$

Gel Control: $y = 0.016x - 0.0106$, $R^2 = 0.9996$

LAP: $y = 0.0196x - 0.0403$, $R^2 = 0.9976$

SIRT5 VVVITQNIDELHR

◆ Buffer   y = 0.0107x - 0.0435
            R² = 0.9907

■ Gel Control y = 0.0127x - 0.0604
               R² = 0.9841

▲ LAP   y = 0.0116x - 0.0518
             R² = 0.9864

SIRT5 NLLEIHGSLFK

◆ Buffer   y = 0.0061x + 0.0159
             R² = 0.9926

■ Gel Control y = 0.0067x + 0.0033
               R² = 0.981

▲ LAP   y = 0.0072x + 0.0109
             R² = 0.9805

SIRT6 LVIVNLQPTK

Buffer: $y = 0.0172x - 0.0867$, $R^2 = 0.9853$

Gel Control: $y = 0.0182x - 0.0865$, $R^2 = 0.9861$

LAP: $y = 0.0178x - 0.0943$, $R^2 = 0.9888$

SIRT6 FLVSQNVDGLHVR

Buffer: $y = 0.0169x - 0.0968$, $R^2 = 0.9861$

Gel Control: $y = 0.0148x + 0.0002$, $R^2 = 0.9845$

LAP: $y = 0.017x - 0.0132$, $R^2 = 0.9752$

Our results show that the introduction of the gel bits (blank and LAP) did not affect the linearity of the standard curves produced, and the regression equations are similar.

For further validation of extraction efficiency, intact recombinant sirtuin protein standards were purchased (SIRT1,2,3,5 and 6) and were spiked into depleted control plasma and recoveries calculated after gel fractionation, and in-gel digestion of the recombinant sirtuins.

To determine recoveries of sirtuin proteins, commercial intact sirtuin standards were run by SDS PAGE (5 μg per lane). Hu6 high abundance depleted plasma spiked with 2 ug and 5 μg of the sirtuin standard for SIRT1, 2, 3, 5 and 6 were also run on the same gel (Figure 2.12). Sirtuin 5 μg spike recoveries were calculated in the LAP spike relative to the sirtuin 5 μg standard protein in buffer only samples. The sirtuin 2 μg spike recoveries were calculated in the LAP spike relative to values extrapolated from the results of the sirtuin 5 μg standard protein in buffer only samples (Table 2.8).

 The main issue confronted with this approach was that the highest quality commercial sirtuin standards were not highly pure as can be seen by the multiple bands stained on the gel (Figure 2.12). Consequently it is likely that there is an overestimation of the amount of sirtuin loaded on the gel, and therefore a concomitant underestimation of sirtuin peptide recovery.

However, even with the below ideal standards, the majority of sirtuin peptides had reasonable recovery estimates from the samples after gel bit digestion, relative to buffer only preparations. Sirtuin recoveries (from the gel spike experiment, Figure 2.12), relative to sirtuin run in buffer only achieved 61–96% recovery and 58–131% recovery for the 5 μg and 2 μg sirtuin levels respectively, all recoveries are shown in Table 2.8.

Figure 2.12 Colloidal coomassie stained SDS/PAGE gel used for sirtuin recovery experiments. Lane 1: prestained molecular weight markers; Lanes 2-6: commercial SIRT1, 2, 3, 5 and 6 recombinant sirtuin protein standards (5ug per lane); Lanes 8-12: 20ug Depleted plasma spiked with 5ug of sirtuin standards 1, 2, 3, 5 and 6; Lanes 14-18: Depleted plasma spiked with 2ug of sirtuin standards 1, 2, 3, 5 and 6. Sirtuins 4 and 7 were not used for this experiment as the SIRT4 yield was very low, and SIRT7 was particularly impure.
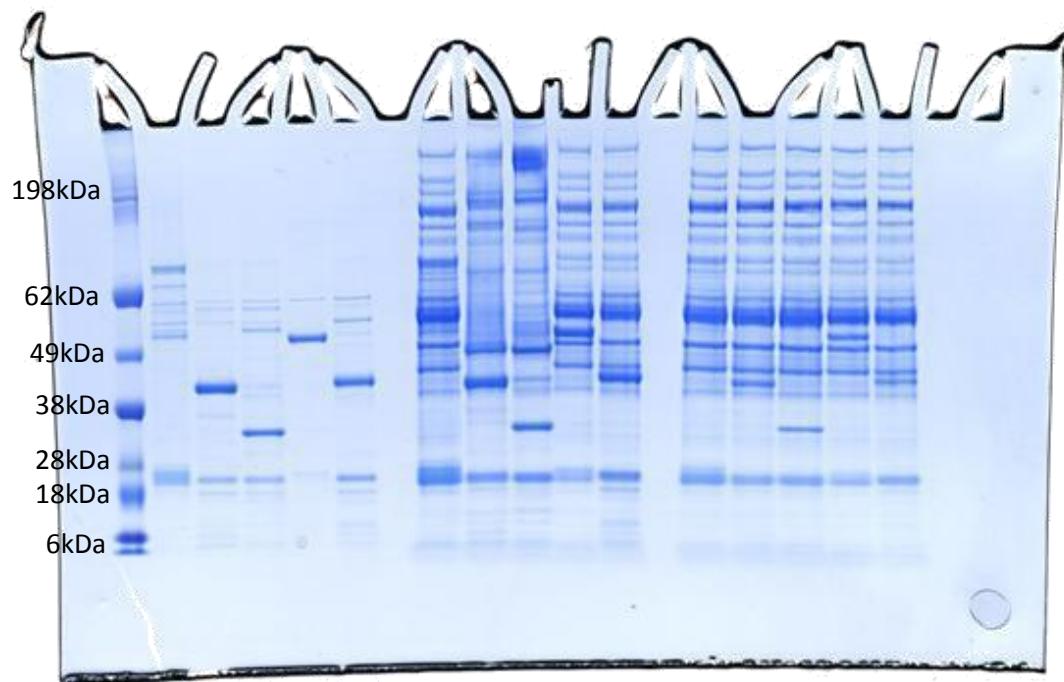
Table 2.8 Sirtuin Recoveries

Sirtuin recovery using the full protocol (sirtuins run by SDS/PAGE) with spiking into matrices consisting of either buffer only (5μg sirtuin) or low abundance plasma (LAP) proteins with either 5μg or 2μg sirtuin spiked. Sirtuin 5μg spike recoveries were calculated in the LAP spike relative to sirtuin 5μg standard protein in buffer only samples. Sirtuin 2μg spike recoveries were calculated in the LAP spike relative to values extrapolated from the results of the sirtuin 5μg standard protein in buffer only samples. The peak ratios and percentage recovery values is from three transitions used for each of the two sirtuin peptides per sirtuin protein and were obtained from one gel lane per sirtuin protein. The gel on which these samples were run is shown in Figure 2.12.

	Avg Peak Intensity Ratio (Light/Heavy)	Avg Peak Intensity Ratio (Light/Heavy)	% Recovery	%Recovery
	DINTIEDAVK	TSVAGTVR	DINTIEDAVK	TSVAGTVR
SIRT1 Std 5μg	0.0106	0.0196		
SIRT1 Std 2μg (extrapolated)	0.0042	0.0078		
SIRT1 Std 5μg + 20μg LAP	0.0080	0.0178	76.10	90.79
SIRT1 Std 2μg + 20μg LAP	0.0046	0.0101	108.50	128.83

	LLDELTLEGVAR	IFSEVTPK	LLDELTLEGVAR	IFSEVTPK
SIRT2 Std 5μg	0.0656	0.0514		
SIRT2 Std 2μg (extrapolated)	0.0262	0.0206		
SIRT2 Std 5μg + 20μg LAP	0.0523	0.0496	79.70	96.34
SIRT2 Std 2μg + 20μg LAP	0.0245	0.0219	93.51	106.31
	LYTQNIDGLER	VSGIPASK	LYTQNIDGLER	VSGIPASK
SIRT3 Std 5μg	0.1015	0.0085		
SIRT3 Std 2μg (extrapolated)	0.0406	0.0034		
SIRT3 Std 5μg + 20μg LAP	0.0620	0.0067	61.08	78.80
SIRT3 Std 2μg + 20μg LAP	0.0236	0.0035	58.13	102.9
	VVVITQNIDELHR	NLLEIHGSLFK	VVVITQNIDELHR	NLLEIHGSLFK
SIRT5 Std 5μg	0.0224	0.0271		
SIRT5 Std 2μg (extrapolated)	0.00896	0.01084		
SIRT5 Std 5μg + 20μg LAP	0.01812	0.0198	80.89	73.33
SIRT5 Std 2μg + 20μg LAP	0.0093	0.0104	103.80	95.94
	FLVSQNVDGLHVR	LVIVNLQPTK	FLVSQNVDGLHVR	LVIVNLQPTK
SIRT6 Std 5μg	0.0789	0.0837		
SIRT6 Std 2μg (extrapolated)	0.0316	0.0335		
SIRT6 Std 5μg + 20μg LAP	0.0571	0.0743	72.37	88.76
SIRT6 Std 2μg + 20μg LAP	0.0326	0.0459	103.16	131.14

2.4 Discussion

The rapid expansion of instruments and software in the field of targeted protein quantification by MRM is expected to have vast applications in quantitative protein biochemistry [451,452]. Most of the currently used protocols to measure sirtuin changes in the CNS have ultilised methods such as ELISA, surface Plasmon resonance, western blotting and fluorescent staining. Mass spectrometry provides a platform that overcomes some of the limitations of antibody based approaches for protein quantification. Isotopically labelled peptide standards allow for the quantification of protein levels and also provide the ability to monitor stability

of analytes throughout the sample processing steps. This greatly simplifies the development of assays from standard immunological formats such as ELISA where well-characterised antibodies are needed. Furthermore MRM facilitates multiplexed analysis, identifying several proteins in a single run, thereby maximising information obtained per sample, and minimising assay time. It measures several transitions per quantified protein, thus generating several independent measurements, and with the use of heavy peptide standards allows for generation of standard curves and accurate quantification, thus having advantages over methods such as western blotting and staining techniques which are semi-quantitative at best and sometimes lack specificity. The MRM approach is also complementary to PCR which targets mRNA levels only.

Perhaps the major limitation to mass spectrometry based protein quantification is throughput and cost. Immunological assays can be performed in 96 well formats and plate readers allow measurement of an entire plate in a single run. Mass spectrometry requires each sample to be run individually with longer run times. Future developments in mass spectrometer detectors may help address this limitation. However one of the main advantages of mass spectrometry is its ability to accurately distinguish different isoforms or modified forms of proteins, even in complex samples. The sensitivity of mass spectrometry is very high and limitations to sensitivity of quantitative assays are often caused by the dynamic range of the proteins in the sample. Thus improved sample purification procedures are often needed for better sensitivity. For example in this study all samples were fractionation on a 1D-SDS-PAGE gel and gel bands excised and tryptic digested prior to MRM analysis to reduce sample complexity. A workflow of the sample preparation procedure can be found in Figure 2.6.

MRM mass spectrometry was successfully utilised in this study to quantify seven mammalian sirtuins in human brain cells, tissues and fluid. Furthermore the method can be used to measure sirtuin expression in animal tissues, such as mouse and guinea pig, in cases where peptide sequences are identical to the human standards. In line with previous data, our results affirm that there is significant divergence in abundance amongst members of the sirtuin family of proteins in the brain. We report SIRT1 and SIRT2 to be the most abundant sirtuins in cultured brain cells with SIRT1 highest in neurons and SIRT2 highest in oligodendrocytes, validating previous studies showing SIRT2 to be highly expressed in oligodendrocytes [453]. Our study and others have also found SIRT2 expressed in neurons and glial cells [454,455]. Further validation with higher sample numbers and across a wider range of cell lines may help elucidate differences between cell types for the lower abundant sirtuins.

We found SIRT2 to be the most abundant sirtuin in the adult human frontal lobe and cortex and cerebellum homogenates from the guinea pig. SIRT2 is known to co-localise with microtubules and functions as a tubulin-deacetylase. It is a suppressor of microglial activation and brain inflammation, with reduced levels of SIRT2 leading to increased production of free radicals and neurotoxicity, while its overexpression inhibits brain inflammation [431]. In other studies, however, it has been shown to increase in cells with oxidative stress and promote death cell [269]. In addition, SIRT2 inhibition has been shown to protect against Parkinson's disease and Huntington's disease [285,456]. Previously published results have shown SIRT2 to be abundant in the brain and serum [428,429,440,457] and our study provides further evidence for this and extends its abundance to the guinea pig brain.

SIRT1 was the second-most abundant sirtuin in the brain and the only sirtuin detected in the CSF and plasma using our method. SIRT1 is a neuroprotective factor in a variety of models for neurodegenerative diseases, including Huntington's disease, Multiple Sclerosis and AD [458,459]. It increases following exposure to cellular stressors, including energy/nutrient depletion and has been linked to increased lifespan in animal models [460]. We were only able to detect SIRT1 in both CSF and plasma while Kumar *et al* have recently identified SIRT1, SIRT2 and SIRT3 in human serum using surface plasmon resonance [428]. The use of more sensitive mass spectrometry instrumentation, and/or fractionation or enrichment of plasma proteins, may improve sensitivity for the other sirtuins in plasma.

The mitochondrial sirtuins SIRT3-5 were found at lower levels in human brain but detection was improved after detergent fractionation. Further fractionation of samples or purification of mitochondria may facilitate improved mitochondrial sirtuin quantification. Our data also show that SIRT6 and SIRT7 are the third and fourth most abundant members of the sirtuins family in the adult human frontal lobe. Both SIRT6 and SIRT7 have been associated with the maintenance of DNA stability and promotion of DNA repair following exposure to cellular stressors [461,462].

We also confirm that mammalian sirtuins are localised to several subcellular compartments. While SIRT1, SIRT6 and SIRT7 are predominantly found in nuclear fractions, they are also detected in cytosol, cytoskeleton and membrane fractions, albeit at much lower levels. Similarly, although SIRT2 is the only human sirtuin primarily localised in the cytoplasm, it may also be found at lower levels in the nucleus and cellular membrane. Our data confirm that SIRT1 and SIRT2 may interact with both the nuclear and cytoplasmic subcellular

compartments. Similarly, while the mitochondrial sirtuins (SIRT3-5) have been previously reported to be exclusively localised to the mitochondria, other studies have reported the localisation of several variants in the nucleus as well as the cytoplasm, in line with our study [463]. SIRT5 has recently been found to have both desuccinylase and de-malonylase activities in both the mitochondria and cytosol [345,464]. The expression patterns of sirtuins in brain cells remains controversial and it is unclear whether certain sirtuins are specific to cell types. Our MRM study indicates that the majority of sirtuins (SIRT1, 2, 6 and 7) are present across all the main brain cell types.

For validation, analyses of the samples by immunohistochemical staining confirmed that all seven sirtuins are found in human primary astrocytes (Figure 2.8, Panel A row 1-5). PCR detected SIRT1 and SIRT2 mRNA in frontal, occipital and hippocampal brain tissue. Western blotting identified protein levels in frontal brain tissue with SIRT2 showing the strongest bands, corresponding well with the results from our MRM assay (Figure 2.8, Panel C).

Various studies have demonstrated that abnormal changes to sirtuin proteins occur during disease states as well as ageing. SIRT2 accumulates with age in the mouse brain and spinal cord [457]. Other recent studies have shown that SIRT1 decreases with age in the rat brain [465] and SIRT2 is upregulated in brain tumours [466]. Hence, there is great value in developing robust quantitative assays such as MRM mass spectrometry to accurately quantify these proteins in control vs disease states. Another important aspect of sirtuin biology is that their deacetylase activity is nicotinamide and NAD^+ dependent, establishing a direct link between their function and energy metabolism. To improve sensitivity, we found our fractionation method of running the samples on SDS PAGE and excising gel bands corresponding to the molecular weights of the intact sirtuin proteins to improve detection. The use of more sensitive mass spectrometers with high resolution MRM capability may improve detection of low abundant sirtuins and/or allow removal of the gel fractionation step. This would allow for sample lysates to be digested and analysed directly and increase sample throughput. Using hybrid mass spectrometers capable of high resolution and accurate mass measurements such as the quadrupole-orbitrap may overcome these issues and yield better sensitivity for high-complexity samples. This will help future validation of levels in large cohorts which may be useful for the identification of sirtuins as potential biomarkers.

Collectively, our results add further insights to the limited data which currently exist regarding the expression of sirtuins in the human CNS. This technique provides a powerful tool and helps improve upon the limitations of current protocols. While mass spectrometry based assays for protein quantification may still have some barriers to overcome before they can be used in a clinical setting due to low throughput and expense compared to methods such as ELISA, it has great value in investigating lower abundance proteins such as sirtuins in complex samples such as the brain with great sensitivity and specificity. MRM mass spectrometry can also be utilised for the quantification of sirtuin related metabolites and thus targeted mass spectrometry methodologies have the potential to not only validate and complement currently established methods for investigation of sirtuin protein expression changes but can also help build a larger view of sirtuin biology in the normal brain and in disease conditions by linking sirtuin protein expression with metabolism.

Chapter 3

Sirtuin Changes During Ageing and Alzheimer's Disease

Introduction

Sirtuins have generated considerable interest as important players in ageing biology. It is generally agreed that sirtuins have a role in extending lifespan, in laboratory model organisms, by mediating the anti-ageing effects of a low-calorie diet (calorie restriction) [421]. In mammals, there are seven sirtuin homologues (SIRT1 to SIRT7), each having diverse locations and multiple targets, and affecting a broad range of cellular processes. Of these seven, the biological activities of SIRT1 has been studied the most extensively. SIRT1 is a nuclear protein in most cell types. It deacetylates transcription factors and cofactors that regulate several metabolic pathways in energy metabolism, lipid metabolism and glucose homeostasis and also targets proteins related to DNA repair and inflammation [288,421,467].

SIRT2 is predominantly a cytoplasmic protein and can deacetylate several cytoskeletal proteins, including α-tubulin, histones, and forkhead proteins and suppresses inflammation [431,468]. SIRT2 is protective against neurodegenerative pathology in mouse models of Alzheimer's disease, with overactivation of SIRT2 protecting against axonopathy and neurodegeneration in a mouse model of Wallerian degeneration [469]. Small-molecule inhibitors targeting SIRT2 have been shown to attenuate several models of neurodegeneration [456,470,471].

Mitochondria represent the primary site for the production of ROS through one-electron carriers in the respiratory chain and this organelle is highly vulnerable to the cytotoxic effect of oxidative stress. Interestingly, three mammalian sirtuins localize to mitochondria: SIRT3, SIRT4 and SIRT5. SIRT3 responds to changes in mitochondrial redox status by altering the enzymatic activity of specific downstream targets, including manganese superoxide dismutase (MnSOD) [472]. MnSOD is the primary mitochondrial ROS scavenging enzyme which modulates ROS levels as well as metabolic homeostatic poise [472].

SIRT4 may be involved in brain ageing, and controls glutamate metabolism through glutamate dehydrogenase, an enzyme which converts glutamate to α-ketoglutarate in the mitochondria in an NAD^+ dependent manner [301]. Over expression of SIRT4 has been shown to alter synaptic activity [473]. In addition, serotonin1b (5-HT1b) receptor knockout mice, up-regulate SIRT5 in adult male mice, causing early onset of brain ageing [474]. SIRT4 has also been shown to regulate fatty acid oxidation and mitochondrial gene expression in liver and muscle cells [344]. SIRT4 knockdown in hepatocytes increases SIRT1 mRNA and protein levels both *in vitro* and *in vivo*, suggesting that the effect of SIRT4 on fatty acid oxidation may be

SIRT1 dependent [344]. A significant increase in fatty acid oxidation in the ageing rat brain has been reported with a reduction in SIRT4 expression [465].

SIRT6, is another nuclear sirtuin protein which promotes longevity [308,475,476]. SIRT6 knockout mice display premature ageing, including excessive loss of subcutaneous fat, a significant reduction in bone density, and die within 4 weeks of birth [312]. Another less characterized sirtuin, SIRT7, is localized to the nucleolus of mammalian cells. SIRT7 protein expression levels correlate with tissue proliferation and its expression is reduced in non-proliferating tissue, such as the heart, brain and muscle [314]. SIRT7 has been associated with rDNA and interacts with RNA polymerase I (Pol I), suggesting a role in NAD-dependent regulation [314]. Recently SIRT7 has been shown to be a histone desuccinylase which is recruited to DNA double-strand breaks in a PARP1-dependent manner and promotes chromatin condensation and DNA repair [477].

Several reports have been published on the association between sirtuins and diseases, such as diabetes, metabolic diseases, cardiovascular diseases, cancer and neurodegenerative diseases [265,421,478]. Of the seven mammalian sirtuins, SIRT1 is the best characterized and is considered to be responsible for delaying ageing in animal models [421]. SIRT1 has also been credited with neuro-protective action in response to stress, in cell culture models. It is also believed to mediate the lifespan extending effects of calorie restriction, which protects experimental animals from neurodegenerative diseases such as AD [281,327,479]. In AD transgenic mouse models SIRT1 overexpression is neuroprotective against amyloid and tau pathologies, and more recently has been shown to improve cognitive performance [340,480]. Since SIRT-1 is a deacetylase that regulates several neuroprotective cellular pathways promoting neuron survival, by deacetylating a broad spectrum of molecules it has been hypothesized that the loss of SIRT-1 protein in the brain contributes to the pathogenesis of AD.

Although a number of sirtuins have promising effects in cellular and animal models of AD, mammalian distribution and functional roles in the central nervous system are still not well understood. In the present study we aimed to investigate changes to sirtuin protein expression during normal human ageing and also changes occurring in AD. We examined sirtuin levels in control, MCI and AD plasma, in the CSF of both a younger and elderly age group, in control and AD *post-mortem* brain tissue and finally in young and older samples of a potential new natural AD animal model – the *Octogon Degus*. To further investigate the functional role of SIRT2, which we found to be the most abundant in the human brain (see

Chapter 2), we completed an immunoprecipitation pulldown followed by mass spectrometry detection of potential binding partners. Owing to the importance of mammalian sirtuins in neurodegeneration and ageing, we hypothesized that they may be differentially expressed in the plasma, CSF and brain and may regulate various targets involved in metabolic processes and neurodegenerative diseases.

3.2 Methods

3.2.1 Plasma, CSF and brain tissue samples

CSF and plasma: CSF samples were collected by standard lumbar puncture from 13 patients during epidural and 13 patients assessed as clinically well after investigation for suspected meningitis, returning normal results for routine CSF pathology markers (white cell count, protein, glucose and bacterial sterility). Aliquots (50 µl) from each patient were obtained for our study. Pooled plasma from MCI, AD, and cognitively normal control subjects, balanced for age, sex and level of education, were obtained from the Sydney Memory and Ageing Study (261 MCI subjects, 24 AD subjects, 411 controls). Demographics of the subjects have previously been published by our group. Plasma was immunodepleted of the six most abundant plasma proteins using the Hu6 column (Agilent,

USA) according to manufacturer's instructions [448]. For immunodepletion using the Multiple Affinity Removal System Hu6 column and buffer kit (Agilent, Santa Clara, CA, USA) , 24 µl EDTA plasma was diluted into 120 µl Buffer A. Diluted EDTA plasma (100µL) was injected onto the Hu6 column connected to a HP 1090 HPLC system (Agilent, Santa Clara, CA, USA) and the low abundance protein fraction was collected, following manufacturer's instructions. The low abundance protein fractions from six injections were pooled, and buffer exchanged and concentrated into 45 mM $NaHCO_3$ using Amicon 3 kDa centrifugal devices (Millipore, Billerica, MA, USA). CSF protein (5 µg) and depleted plasma protein (20 µg), as determined by a BCA protein assay, were run on a 1D SDS-PAGE gel and colloidal coomassie stained.

Human Tissues: Protein (20 µg) from 5 individual control and AD *post mortem* brain tissue samples across 3 brain regions (occipital, frontal and temporal lobes) were extracted. Tissue was lysed using RIPA buffer followed by probe sonication and cell debris removed by centrifugation at 10,000RPM for 5 min. Total protein concentration was assayed in the supernatant using the Pierce BCA protein assay kit (Life Technologies, Australia), and three

10 µg replicates were run by 1D SDS-PAGE followed by colloidal coomassie staining. Detailed patient information can be found in Chapter 2, Table 2.4.

Animal Tissues: Brain tissue from the *O. degus* were from the laboratory of Professor Nibaldo C. Inestrosa, to whom we are greatly appreciative for his provision of these samples. The *O. degus* were bred at the animal facility of the University of Valparaiso, Chile and maintained in a controlled temperature room (23 ± 1 °C), under a 12:12 light/dark cycle, with water and food provided *ad libitum.* At the time of this study, 16 Male *O. degus* were grouped by age, from 12 to 36 months of age (n = 8 per group). Ages were selected to represent the development of AD-like pathology (36 months). All efforts were made to minimize animal discomfort and stress while also limiting the number of animals used. Aged animals were anesthetized with Equitesin (2.5 ml/kg, i.p.) and injected with heparin (4 USP/kg, i.p.). Afterwards, brains were surgically removed from their skulls and frozen in isopentane at -78.5°C. All procedures were conducted according to animal protocols approved by the Institutional Animal Care and Use Committee at the University of Valparaiso and Pontificial Catholic University of Chile. Tissue was lysed using RIPA buffer followed by probe sonication and cell debris removed by centrifugation at 10,000RPM for 5 min. Total protein concentration was assayed in the supernatant using the Pierce BCA protein assay kit (Life Technologies, Australia), and three 10 µg replicates were run by 1D SDS-PAGE followed by colloidal coomassie staining.

For all samples, the band corresponding to the molecular weight for each sirtuin was excised, de-stained and trypsin digested overnight with heavy sirtuin peptides added as internal standards to all samples prior to digestion (the internal standard was constant for all samples and standards and added at a level at about the midrange of the standard curve). The tryptic peptides were dried under vacuum (Savant Speedvac, Thermo Scientific, USA), reconstituted in 0.1% formic acid (5 µl) injected into the mass spectrometer (1uL) and analysed using the MRM approach described in Chapter 2. Peak area ratios (light/heavy) were calculated for each endogenous peptide (light) relative to the spiked isotope-labelled (heavy) peptide using Skyline MRM analysis software. Protein concentrations were determined using calibration curves. All samples were fractionated by 1D-SDS-PAGE to reduce sample complexity. Full details of the SIRT MRM targeted mass spectrometry quantification protocol is provided in Chapter 2 (section 2.2.2) and has been published [481].

3.2.2 Western Blotting and PCR validation

Protein from five individual control and AD *post mortem* occipital brain tissue samples were extracted as described in section 2.1, and run on a 1D SDS-PAGE gel (20 μg protein per lane), and western blotted with antibody against SIRT2. The gel was electroblotted (20V, 110mA, 90mins) onto a nitrocellulose membrane in transfer buffer of 50mM tris, 40mM glycine, 1.3mM SDS, 20% methanol, pH 9.2. After transfer of proteins, the membrane was incubated in 10% skim milk powder solution containing primary SIRT2 antibody. SIRT2 rabbit polyclonal antibody was used (1:1000 dilution, Abcam, UK), followed by a secondary antibody (1:100,000 dilution of anti-mouse IgG, Pierce, USA). Chemiluminescence blots were developed using 1:1 solution of Super Signal West Femto luminol and peroxide buffer (Pierce, USA) according to manufacturer's instructions.

For the PCR studies RNA was extracted from control and AD *post mortem* tissue using RNeasy mini kits (Qiagen, Germany). The cDNA was prepared using SuperScript III First-Strand Synthesis System and random hexamers (Invitrogen Corporation, USA) as previously described [449].

Briefly, for each reaction 2 μl of diluted cDNA, 10 μL of SYBR green master mix, 0.15 μL of 10 μM forward and reverse primers and 7.7 μl of nuclease-free water was used, making a total volume of 20 μl. Q-PCR was carried out using the Mx3500P Real-Time PCR system (Stratagene, Australia). The SIRT2 primer sequences are shown in Chapter 2, Table 2.5. The relative expression levels of SIRT2 transcripts were calculated using a mathematical model based on the individual Q-PCR primer efficiencies and the quantified values were normalized against the housekeeping gene 18S. From these values, fold-differences in the levels of transcripts between individual subjects were calculated according to the formula $2^{-\Delta\Delta Ct}$

3.2.3 Immunoprecipitation of SIRT2 interacting proteins

Immunoprecipitation is a method that enables the purification of a protein, together with any binding partners that it may have. An antibody for the protein of interest is incubated with a cell extract enabling the antibody to bind to the protein in solution. The antibody/antigen/binding partner complex is then pulled out of the sample using protein A/G-coupled agarose beads. This isolates the protein of interest (and its specific binding partners)

from the rest of the sample. The sample can then be separated by SDS-PAGE followed by trypic digestion and identification of bound proteins using mass spectrometry.

Occipital lobe tissue (mix of white and grey matter) from five control subjects (1mg each) were pooled and homogenised in 1ml of lysis buffer containing 20 mM Tris HCl pH 8, 137mM NaCl, 1% Nonidet P-40 and 2mM EDTA and homogenised using a probe sonicator. The sample was then centrifuged for 20min at 10,000RPM at 4°C. The supernatant was removed and placed in a fresh polypropylene tube. Proteins were immunoprecipitated with SIRT2 antibody in solution according to manufacturer's instructions. Briefly, 50 μg of occipital tissue lysate plus SIRT2 antibody were mixed and incubated overnight at 4°C with gentle rotation. Protein A-coupled human IgG1 Sepharose beads were prepared, 1mL PBS, 0.1% BSA added and the solution mixed for 1hr with rotation. The supernatant was removed and 400 μL lysis buffer added. This slurry was mixed well and then 100 μL of beads were added to the lysate sample. The sample was incubated at 4°C with rotation for 4hrs. Tubes were centrifuged, supernatant removed and beads washed three times to remove non-specifically bound proteins. The specific binding-partner proteins were then eluted from the beads and samples run by 1D SDS PAGE. The procedural workflow and representative gel is shown in Figure 3.9.

3.2.4 Mass Spectrometry for Identification of SIRT2 Interacting Proteins

A one dimensional SDS 4-12% NuPage (Thermo Fisher Scientific Inc, MA, USA) gel was run using the elute, unbound and wash fractions from the immunoprecipitation experiments. The gel was colloidal coomassie stained [482], and the lanes uniformly cut into 7-8 bits using a gridcutter and mount from The Gel Company, CA, USA. The gel bits were trypsin digested and then analysed using mass spectrometry as outlined below.

Digested peptides were separated by nano-LC using a Cap-LC autosampler system (Waters, Milford MA). Samples (5 μl) were concentrated and desalted onto a micro C18 precolumn (500 μm x 2 mm, Michrom Bioresources, Auburn, CA) with $H_2O:CH_3CN$ (98:2, 0.05 % HFBA) at 15 ul/min. After a 4 min wash the precolumn was automatically switched (Valco 10 port valve, Houston, TX) into line with a fritless nano column (75 μm x ~12 cm) containing Magic C18 (~10cm, 200Å, Michrom) manufactured according to Gatlin [35]. Peptides were eluted using a linear gradient of $H_2O:CH_3CN$ (98:2, 0.1 % formic acid) to $H_2O:CH_3CN$ (55:45, 0.1 % formic acid) at ~300 nl/min over 30 min. The precolumn was connected via a fused silica capillary (10 cm, 25 μ) to a low volume tee (Upchurch Scientific)

where HV (2400 V) was applied and the column tip positioned ~ 1 cm from the Z-spray inlet of a QTof Ultima API hybrid tandem mass spectrometer (Micromass, Manchester, UK). Positive ions were generated by electrospray and the QTof operated in data dependent acquisition mode (DDA). A Tof MS survey scan was acquired (*m/z* 350-1700, 1 s) and the 2 largest multiply charged ions (counts > 20) were sequentially selected by Q1 for MS-MS analysis. Argon was used as collision gas and an optimum collision energy chosen (based on charge state and mass). Tandem mass spectra were accumulated for up to 2 s (*m/z* 50-2000).

Peak lists were generated by MassLynx (version 4.0 SP1, Micromass) using the Mass Measure program and submitted to the database search program Mascot (version 2.2, Matrix Science, London, England). Search parameters were: Precursor and product ion tolerances ± 0.25 and 0.2 Da respectively; Met(O) and Cys-carboxyamidomethyl were specified as variable modification, enzyme specificity was trypsin, one missed cleavage was allowed on the UniProtKB/Swiss-Prot database.

3.3 Results

3.3.1 Sirtuin expression in pooled Control, MCI and AD immunodepleted plasma

SIRT1-3 and SIRT6 were all detected in pooled human control, MCI and AD plasma. A significant drop in SIRT1 levels was found in the AD plasma samples compared to controls.

Figure 3.1 Sirtuin expression in pooled control, MCI and AD plasma. *p<0.01 vs control plasma.
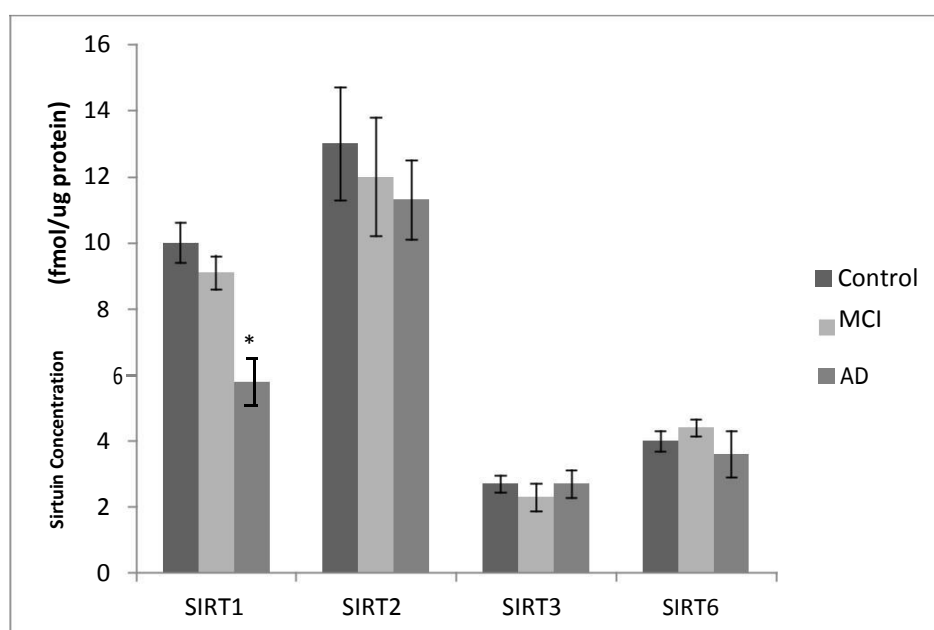
Figure 3.2 Representative chromatograms of SIRT1 peptides in control and AD plasma samples for SIRT1. Showing analyte peptide transitions (top) and the internal standards (bottom) for each sample.
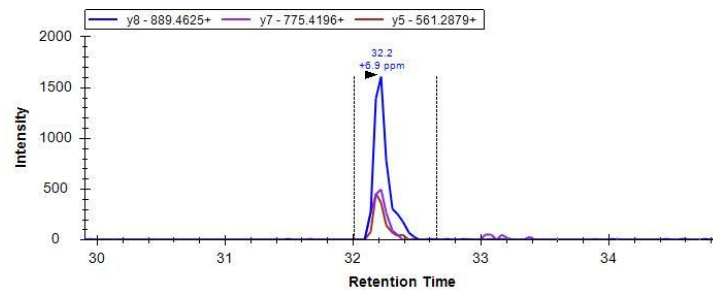
SIRT1 in Control plasma
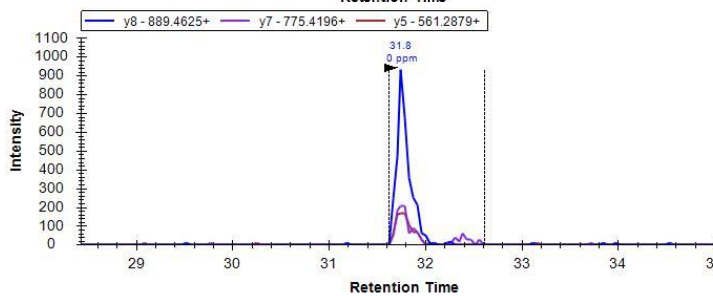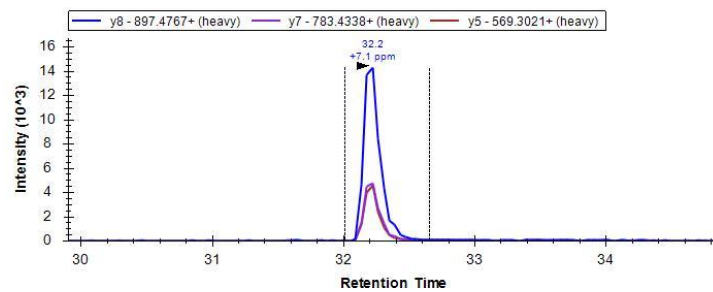
SIRT1 in AD plasma
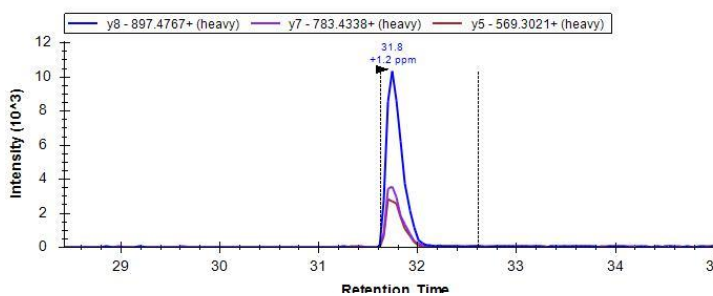
3.3.2 Sirtuin Expression in control and AD *post mortem* brain tissues

SIRT1-3, 6 and 7 were quantified in various brain regions of control and AD *post mortem* brain tissues. A significant increase in SIRT2 was found in AD brain tissues of the occipital lobe (Figure 3.3). SIRT1 and SIRT3 were elevated in the temporal lobe (Figure 3.4). There was also a trend upwards in SIRT2 in the temporal lobe. No significant differences were found in frontal lobe tissue (Figure 3.5).

Figure 3.3 Sirtuin expression in occipital *post mortem* control and AD brain tissue, n=5; *p<0.05 vs control.
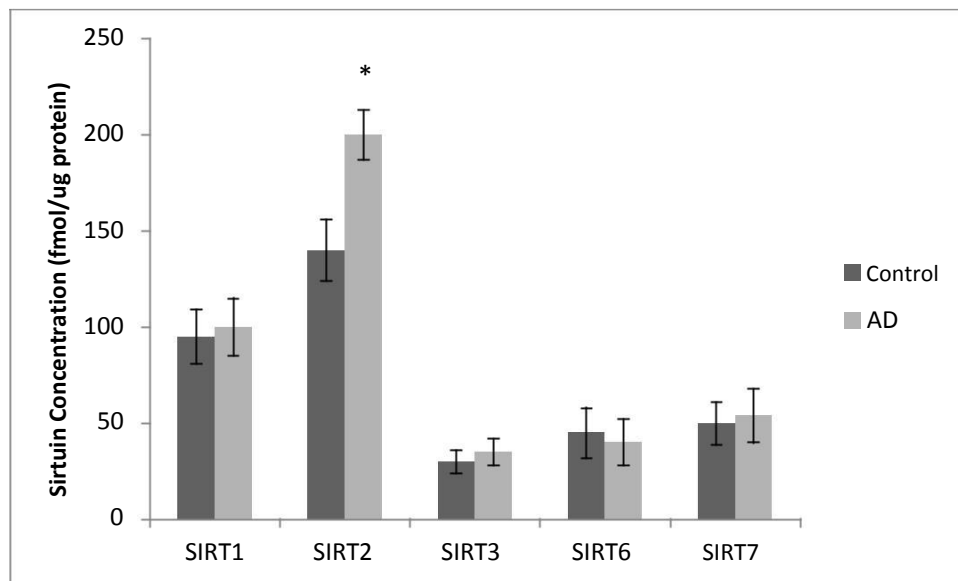
Figure 3.4 Sirtuin expression in temporal *post mortem* control and AD brain tissue, n=5; *p<0.05 vs control.
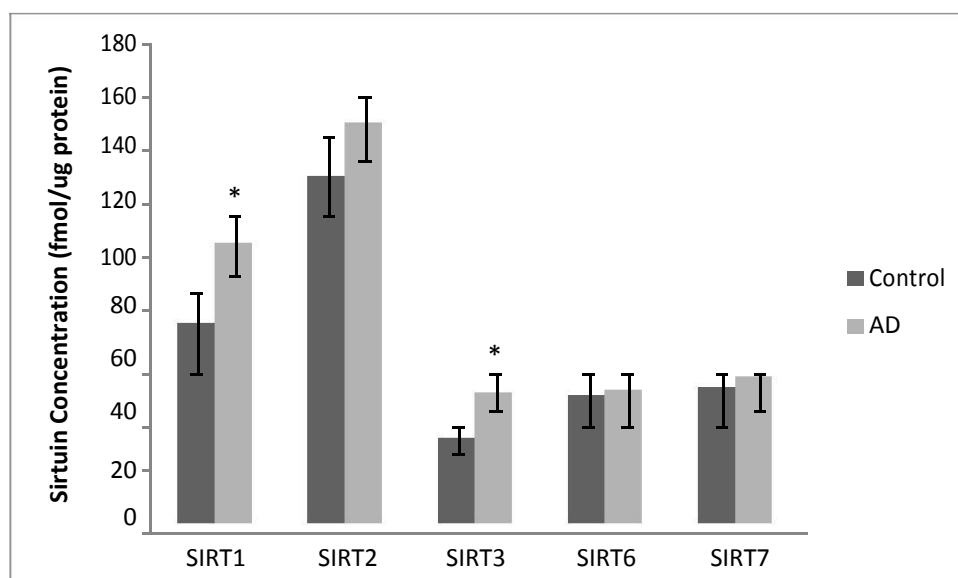
Figure 3.5 Sirtuin expression in frontal *post mortem* control and AD brain tissue, n=5.
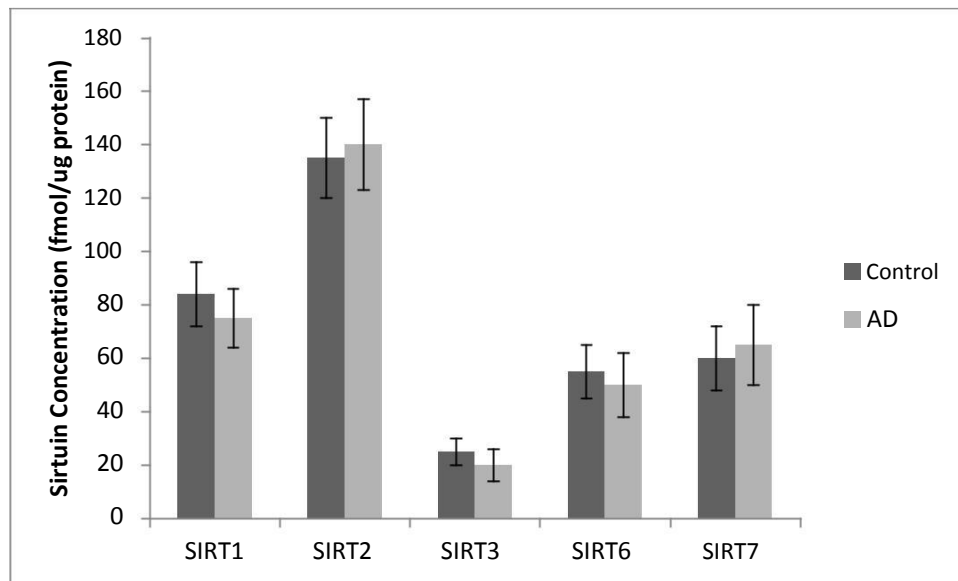
3.3.3 Sirtuin expression in control CSF in younger and elderly age groups

SIRT1 and SIRT2 levels were compared between a younger age group (27-45yrs) and a older age group (62-88yrs). SIRT1 and SIRT2 were found in control CSF and though there was a downward trend in SIRT1 in the older age group, there were no statistically significant differences between the two groups.

Figure 3.6 SIRT1 and SIRT2 levels in younger and older age groups (n=12 in each group).
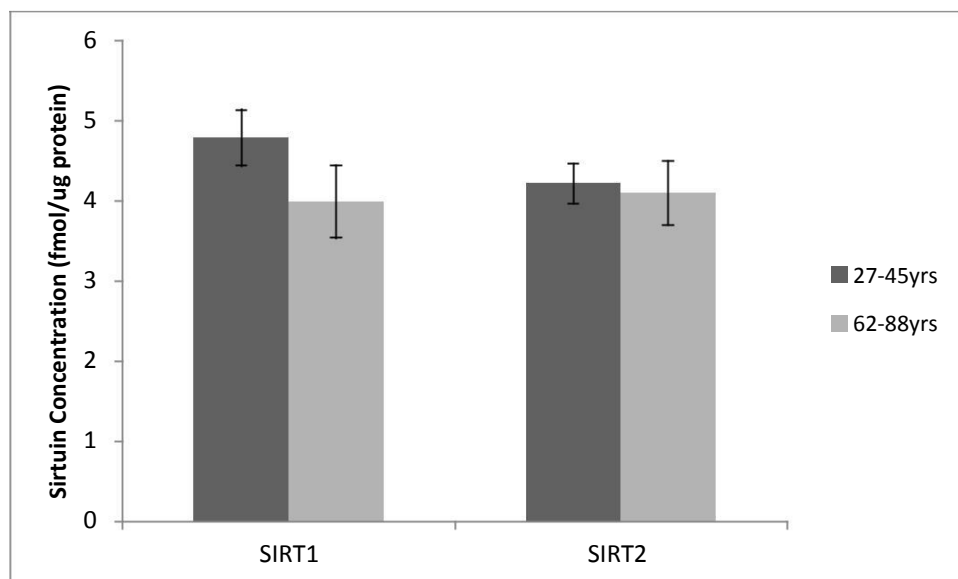
3.3.4 Sirtuin expression in the ageing *Octogon Degus* rodent brain

SIRT1-3 and SIRT5-7 were detected in the *O. Degus* rodent brain. No marked age related changes within the cortex of the ageing brain were observed in this small sample set. However SIRT5 was found to be the most abundant brain sirtuin.

Figure 3.7 Brain sirtuin levels in *Octogon Degus* cortex, n=2.
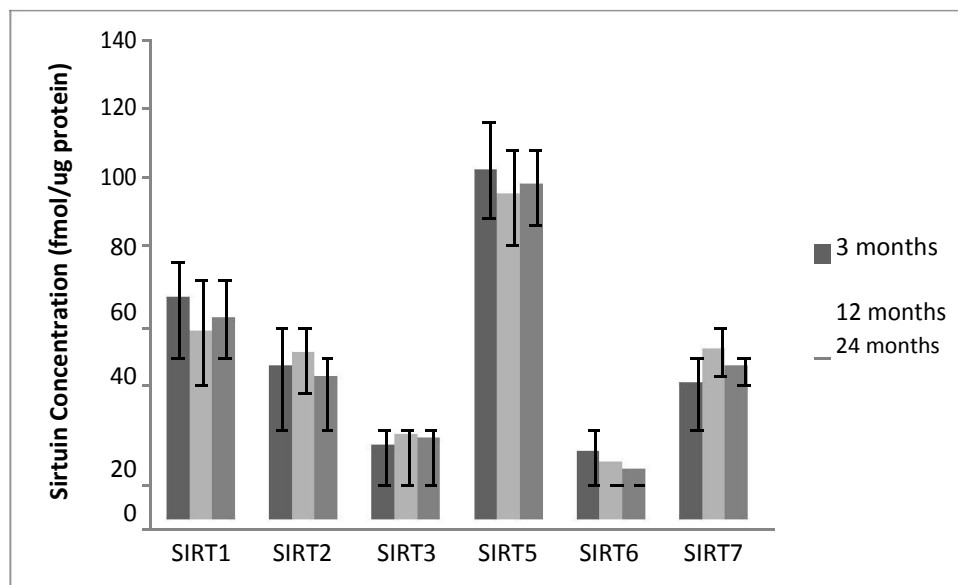
3.3.5 Validation of SIRT2 changes in AD occipital lobe tissue using western blotting and PCR

The significant elevation seen in SIRT2 in the AD occipital lobe using the MRM method was validated using orthogonal methods. In this case western blotting was used to validate SIRT2 protein expression and PCR to measure SIRT2 mRNA changes. These methods identified both upregulation of SIRT2 protein and mRNA expression in AD occipital lobe, validating the MRM quantification.

Figure 3.8 SIRT2 protein levels in control and AD occipital lobe. Using western blotting (panel A) and SIRT2 mRNA in control and AD *post mortem* brain tissue of various brain regions (panel B) both showing a significant elevation of SIRT2 protein and mRNA in the occipital lobe of AD brain, n=5, *p<0.05 vs control.

A

Control   AD   Control   AD   Control   AD   Control   AD
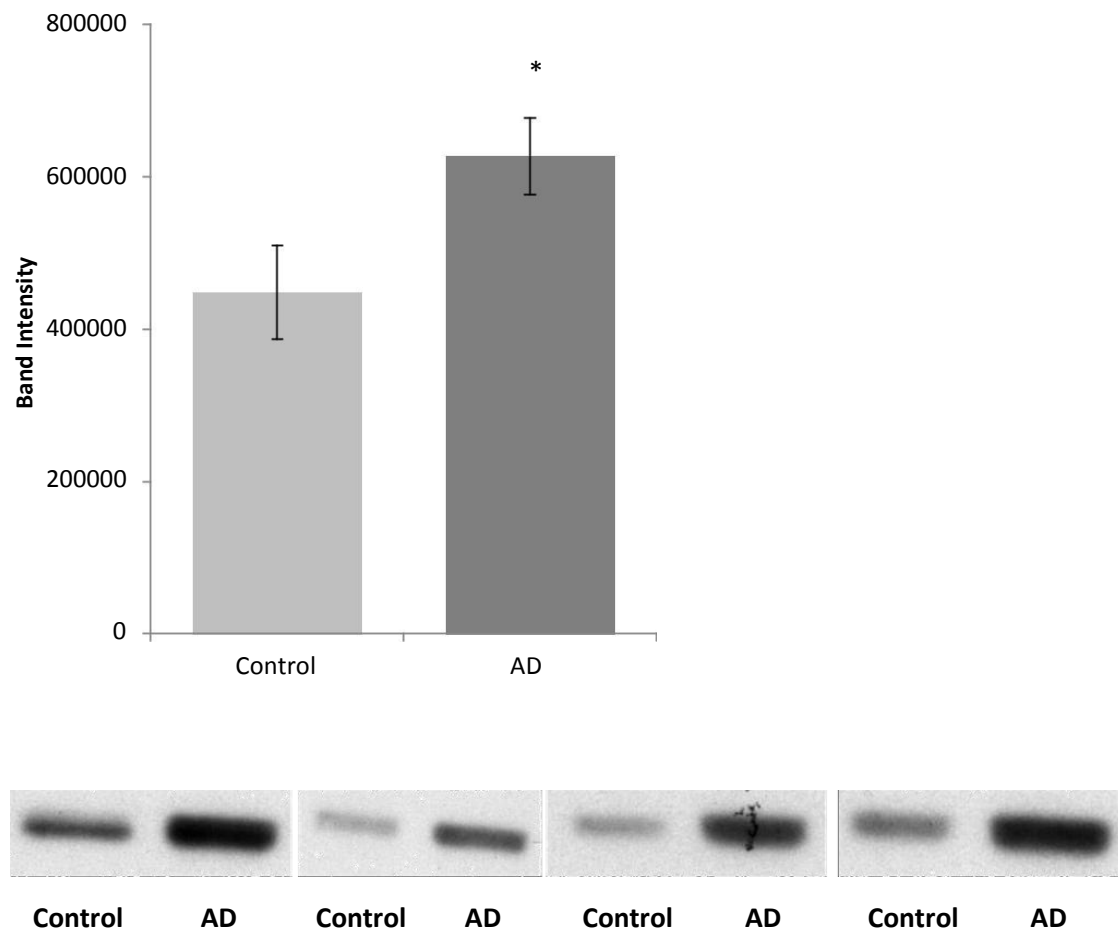
B
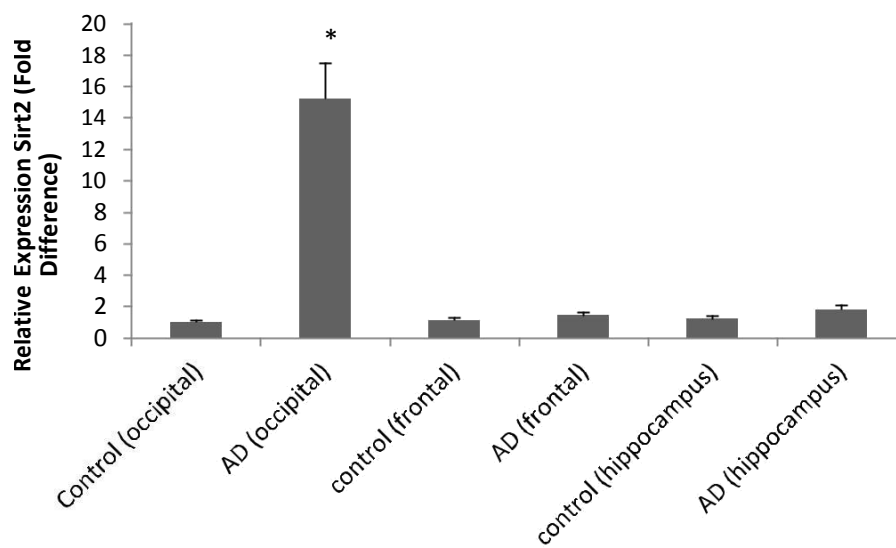
Figure 3.9 Workflow of immunoprecipitation experiment to target SIRT2 binding partner proteins. Using in gel digestion followed by mass spectrometry analysis showing fractions collected after pulldown. Gel lanes 2-4: Elute fraction triplicates, lanes 6-8: Unbound fraction triplicates, lanes 9-11: Wash fraction triplicates.
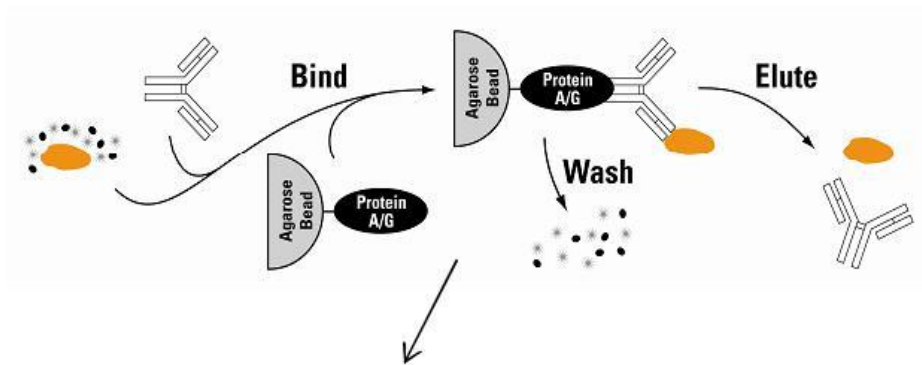
1D SDS-PAGE Gel of fractions collected

In-gel tryptic digest

LC-MS/MS

Mascot
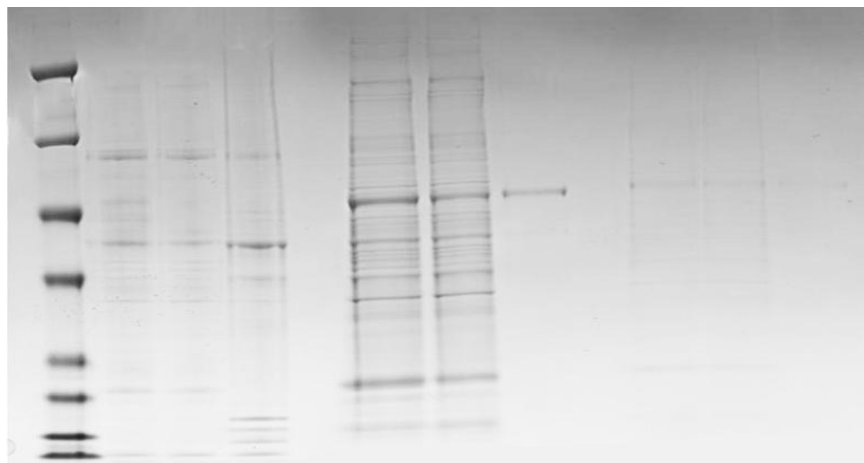
Elute fractions
(potential SIRT2
binding partners)

Unbound fractions

Wash fractions
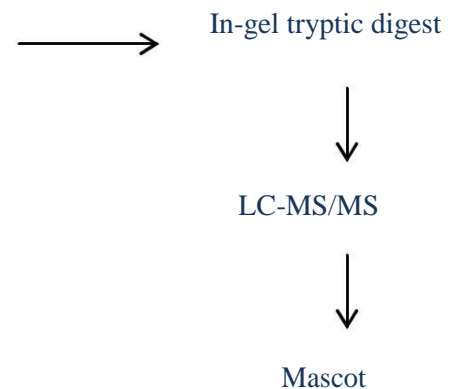
Table 3.1 List of SIRT2 putative binding protein partners identified by immunoprecipation pulldown and mass spectrometry and their functions.

Accession #	Protein Name	emPAI	Protein Sequence Coverage	Peptide Sequences	Function
Q13885	Tubulin beta-2A	5.38	64	26	polymerize into dynamic microtubules; one of the major components of the cytoskeleton [485]
P60709	Actin, cytoplasmic 1	4.64	55	24	form microfilaments, one of the three major components of the cytoskeleton [484]
O60814	Histone H2B	2.71	55	10	one of the main histone proteins involved in chromatin structure [485]
P04075	Fructose-bisphosphate aldolase A	2.07	46	16	enzyme catalyzing a reversible reaction that splits the aldol, fructose 1,6-bisphosphate, into the triose phosphates, dihydroxyacetone phosphate and glyceraldehydes 3-phosphate in gluconeogenesis and glycolysis [486]
P02686	Myelin basic protein	1.57	44	20	provide structural integrity to the myelin sheath of nerves [487]
P00441	Superoxide dismutase 1	1.56	68	5	an important constituent in apoptotic signaling and oxidative stress, most notably as part of the mitochondrial death pathway [488]
P62805	Histone H4	0.87	24	2	proteins involved in the structure of chromatin in eukaryotic cells [489]
P40926	Malate dehydrogenase, mitochondrial	0.77	39	9	is an enzyme that reversibly catalyzes the oxidation of malate to oxaloacetate using the reduction of NAD^+ to NADH. This reaction is part of many metabolic pathways and is involved in the citric acid cycle and gluconeogenesis [490]
P04406	Glyceraldehyde-3-phosphate dehydrogenase	0.67	39	12	catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules and can also initiate apoptosis [491]
P06576	ATP synthase, subunit beta,	0.62	26	11	important enzyme that creates the energy storage molecule adenosine triphosphate (ATP). ATP is the most commonly

	mitochondrial				used "energy currency" of cells for most organisms [492]
P09936	Ubiquitin	0.52	25	5	functions through covalent attachment to other cellular proteins, thereby changing the stability, localization, or activity of the target protein [493]
Q06830	Peroxiredoxin 1	0.51	25	5	ubiquitous family of antioxidant enzymes that also control cytokine-induced peroxide levels and thereby mediate signal transduction in mammalian cells [494]
P09104	Gamma-enolase	0.45	24	7	responsible for the catalysis of the ninth and penultimate step of glycolysis [495]
P07900	Heat shock protein 90-alpha	0.34	18	14	a chaperone protein that assists other proteins to fold properly, stabilizes proteins against heat stress, and aids in protein degradation [496]
P14618	Pyruvate kinase	0.25	20	10	catalyzes the final step of glycolysis. It catalyzes the transfer of a phosphate group from phosphoenolpyruvate to adenosine diphosphate, yielding one molecule of pyruvate and one molecule of ATP [497]
P09211	Glutathione s-transferase	0.14	14	2	catalyses the conjugation of the reduced form of glutathione to xenobiotic substrates for the purpose of detoxification [498]

3.4 Discussion

Ageing is the strongest risk factor for developing MCI and AD in epidemiological studies. There is a progressive increase in the prevalence of AD with increasing age (doubling every 5 to 6 years after the age of 60 up to the 9th decade of life), therefore it is worth considering whether anti-ageing interventions could be of preventive value in dealing with AD in an ageing population. The sirtuin family of proteins has been associated with lifespan extension in lower organisms. Since mammals contain multiple sirtuins, we explored the expression of these in ageing and AD, using a variety of sample types, including brain tissue, CSF and plasma.

3.4.1 Sirtuin levels in control, MCI and AD plasma and young vs old plasma

We investigated sirtuin expression in immunodepleted plasma from pooled control, MCI and AD subjects and found a significant drop in SIRT1 levels in the plasma of AD patients (figure 3.1 and figure 3.2). We also investigated sirtuin CSF concentrations in younger and elderly controls. We found a downward trend in SIRT1 levels in the elderly group (62-88yrs) compared to the younger group (27-45yrs) but statistical significance was not reached (Figure 3.6). In general changes to most sirtiun levels with age appear to be quite modest in the mammalian tissues we assayed.

Recently the first study investigating sirtuin levels in serum have been reported. The study investigated serum SIRT1 concentration in patients with AD and MCI as well as in young and elderly controls. A significant decline in SIRT1 with age in serum and a larger drop in patients with MCI and AD was found. Thus the authors suggest that SIRT1 may be a promising biomarker for AD. This data also suggests that sirtuin activators may potentially have a preventive role in AD. Another study by the same group examined the association of sirtuins to a major geriatric syndrome, frailty. Serum levels of SIRT1, 2 and 3 were found to be significantly lower in frail patients compared to nonfrail controls.

3.4.2 Changes in sirtuin levels in control vs AD brain tissue

Interestingly when control and AD *post mortem* brain tissue were compared, we found a significant elevation of a number of sirtuins. In the occipital lobe we found an elevation of SIRT2 (Figure 3.3), this result was further confirmed using both western blotting and PCR (Figure 3.8) as both these techniques also showed a significant increase in SIRT2 both at the protein and mRNA level in the AD occipital lobe. In the temporal lobe we found an increase in both SIRT1 and SIRT3 in the AD brain (Figure 3.4). No significant differences in sirtuin expression was found in the frontal lobe (Figure 3.5).

Previous studies from our group have identified the *Octodon degus (O. degus*), a South American rodent endemic to Central Chile, as a "natural" rodent model for AD The *O. degus* is a diurnal, visual, and highly social rodent that "naturally" develops several features liinked to the neuropathology of AD. This caviomorph rodent lives up to an average of 7 years in captivity, making it a useful model for use in longitudinal studies, including those related to the neurobiology of AD . The amino acid sequence of wild-type *O. degus* Aβ shares a high degree (97.5%) of genetic homology with humans and it has been reported that age-relate changes in Aβ oligomers and *tau* phosphorylation in *O. Degus* correlates with a decrease in both spatial and object recognition memory, and impaired postsynaptic and neural plasticity. In the *octodon degus* model of AD we found no changes to sirtuins during normal ageing (3 months to 24 months). However interestingly we found the largest amounts of SIRT5 in the brain of this rodent model (Figure 3.7), which was quite a different pattern of expression to the other mammalian tissues we examined (this chapter and in Chapter 2).

Sirtuin expression levels have not been very well investigated in AD, a few recent studies have reported significant changes. One of the first papers to report change to sirtuin in AD was published by Weir et al showing a significant increase in SIRT3 in the temporal cortex of AD *post mortem* brain tissue using western blotting [271]. Progressive mitochondrial dysfunction contributes to neuronal degeneration in age-mediated disease and SIRT3 is an essential regulator of mitochondrial function [499]. Pharmacological augmentation of mitochondrial ROS was shown to increase SIRT3 expression in hippocampal cells with SIRT3 over-expression being neuroprotective. SIRT3 expression was also found to correlate with the deposition of β-amyloid in an AD mouse model and SIRT3 was also upregulated in AD patient temporal neocortex [271]. Our results are consistent with these previous findings, confirming an elevation of SIRT3 in the AD temporal lobe. This suggests a possible role for

SIRT3 in mechanisms sensing and protecting against ROS and AD-mediated mitochondrial stress.

Another recent study has reported changes to SIRT1, 3 and 5 expression in the entorhinal cortex and hippocampal subregions and white matter of 45 cases grouped according to Braak and Braak stages of neurofibrillary degeneration using western blotting and immunohistochemistry analysis [500]. They report a decline in SIRT1 and SIRT3, and an elevation of SIRT5 with AD progression in these brain regions [500]. SIRT3 decline has also been reported in an AD transgenic mouse model. The expression of SIRT3 was significantly lower in the cortex of APP/PS1 double transgenic mice than in wild types at both the

protein and mRNA level [501]. These results suggest that mitochondrial SIRT3 might participate in the development of AD via mitochondrial dysfunction. The elevation we see in the temporal lobe may therefore be a compensatory mechanism by the brain to induce these neuroprotective proteins to boost neuronal longevity. SIRT1 and SIRT3 are also upregulated in response to fasting and calorie restriction and have been shown to reduce ROS levels and reduce oxidative damage. SIRT3 also enhances the mitochondrial glutathione antioxidant defense system in cochlear neurons [347]. The decline reported by others in the hippocampus where the highest level of AD pathology is seen may indicate loss of these neuroprotective activities and metabolic dysfunction reported in this vulnerable region may thus, in part be due to reduction of SIRT1 and SIRT3 levels. Kim et al have reported that mitochondrial SIRT3 protects neurons against excitotoxic injury [502]. Excitotoxic injury was induced by exposure of primary cultured mouse cortical neurons to NMDA resulting in a rapid decrease of cytoplasmic NAD^+ in neurons through poly (ADP-ribose) polymerase-1 (PARP-1) activation [502]. Mitochondrial SIRT3 was increased following PARP-1 mediated NAD^+ depletion, which was reversed by either inhibition of PARP-1 or exogenous NAD^+ 502. The large amount of ROS produced under this NAD+ depleted condition mediated the increase in mitochondrial SIRT3 [502]. SIRT3 overexpression confirmed that SIRT3 is required for neuroprotection against excitotoxicity [502]. Thus SIRT3 seems to play an essential role in protecting neurons against excitotoxic injury. As AD is associated with significant increases in neuronal ROS production and previous studies have shown that SIRT3 mRNA expression is regulated by mitochondrial ROS levels, it seems likely that the SIRT3 upregulation in AD we see in the temporal lobe may be a consequence of Aβ-related oxidative stress. Given that SIRT3 upregulation has been shown to increase neuronal lifespan, it is tempting to speculate that upregulation of SIRT3 in response to Aβ-induced oxidative stress might prolong neuronal function by decreasing ROS, maintaining ATP-levels and sustaining synaptic activity. However this possibility will need further investigation. Furthermore, due to inevitable inter-individual variations in protein expression, as well as potentially variable tissue quality, human AD *post mortem* studies will need to be expanded to include a larger cohort of control and AD samples to validate these findings.

SIRT1 increases the expression of the ADAM10 gene encoding α secretase. In AD the down regulation of SIRT1 reduces the expression of α-secretase and as a result increases the accumulation of pathogenic Aβ peptide formed by β and γ secretase [340]. Serum Aβ1–40 levels were shown to be higher in the AD group than both controls and MCI [340]. Transgenic

over-expression of SIRT1 in the brain markedly reduced amyloid plaque formation, gliosis and decline in learning and memory capabilities, while brain SIRT1 specific knock-out greatly attenuated lifespan likely due to juvenile onset of an AD-like disease [340]. Resveratrol, a SIRT1 activator, has proved to be beneficial in models of AD (*in vitro* and *in vivo*), reducing amyloid-β protein accumulation and also a variety of other neuroprotective effects [355,389,479,503].

A significant decrease in SIRT1 concentration in parietal cortex of AD brain has been reported and a strong correlation was established between tissue SIRT1 concentration, duration of symptoms and tau accumulation [280], but the exact relationship and its role in the sequence of events leading to development of AD remains unclear.

3.4.3 Binding partners of SIRT2

This work has mainly highlighted SIRT2 as the most abundant sirtuin in the brain and we also see a substantial elevation of this protein in the AD occipital lobe. This may be a homeostatic response to pathology, and as the most significant change relative to other sirtuins, it was of interest to explore potential mechanisms of action, by identifying some putative SIRT2 binding partners. We therefore aimed to gain further insights into its biological functions through investigating its binding partners. Using immunoprecipitation pulldown and protein identification using mass spectrometry we identified a list of potential binding partners for SIRT2 (Table 3.1). Interestingly our results reveal a novel potential role of SIRT2 as a regulator of mitochondrial function and metabolism. To date SIRT1, 3, 4 and 5 are the only sirtuins directly shown to be involved in mitochondrial function. However interestly, many of the binding partners identified in our SIRT2 binding partner study are mitochondrial and involved in energy metabolism (Table 3.1). These include superoxide dismutase, which is also a known target of the mitochondrial sirtuin SIRT3, aldolase, ATP synthase, enolase, pyruvate kinase and glucose-6-phosphate isomerise which are all involved in energy metabolism and glycolysis. Our pulldown also highlights other binding partners which have already been validated as being associated with SIRT2 function such as myelin, histones, tubulin and actin. A recently published study has also suggested SIRT2 may regulate the activity of mitochondria. Liu et al found that SIRT2 can localise to mitochondria and directly interact with mitochondrial proteins [504]. In addition, the depletion of SIRT2 was

shown to increase markers of oxidative stress and reduce ATP production in the mouse brain [504]. In SIRT2 knockout mice 20 mitochondrial proteins were found to have changes in acetylation including protein targets also found in our study such as ATP synthase [504]. ATP synthase has also been identified as a SIRT3 target in a previous study which also used mass spectrometry to identify SIRT1 and SIRT3 interacting proteins [505]. Taken together our data and previous studies suggest that SIRT2 is not only a cytosolic protein but can interact with mitochondrial proteins and appears to have multiple mitochondrial targets and may have an emerging role in regulating mitochondrial function, oxidative stress and energy metabolism, all of which has been well established as important factors in the pathogenesis of AD.

Sirtuins represent a unique class of enzymes that not only regulate protein acetylation and metabolism, but also play prominent roles in promoting longevity, preventing neurodegeneration and improving cell survival. Our present study helps add further insight into the changes and distribution of mammalian sirtuins in the CNS during ageing and AD and some potential targets of SIRT2 within the brain, revealing evidence of novel roles for these proteins in the brain and revealing new pathways for future research and validation.

Chapter 4

Effect of AD Plasma on Cultured Glia: Metabolic and Cytotoxic Effects

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See Appendix for full publication

Introduction

A variety of inflammatory markers are increased with the onset of AD pathology, including cytokines and chemokines, coagulation factors and acute-phase reactive proteins as well as reactive astrocytes and activated microglial cells, the main cells involved in the neuroinflammation process [506,507]. Whether accumulation of cytokines and acute phase reactants within the brain is reflected in plasma has not been elucidated as many of these proteins do not easily cross the blood brain barrier. Alternatively, AD may be associated with a more widespread immune dysregulation, detectable in plasma.

Evidence from parabiosis experiments has shown that blood from young, old or diseased animals can have phenotypic effects when introduced into test animals [155,156]. Previous studies investigating the effects of human AD plasma on cells in culture have found differential effects on protein expression and cell biology. One study aimed to determine if exposure to serum from AD patients would affect markers for AD brain lesions [508], and found that 24 hour exposure to AD serum increased four molecular markers characteristic of AD senile plaques and neurofibrillary tangles (NFTs) in rat hippocampal neurons [508]. These markers were Alz-50, beta-amyloid, MAP2 and ubiquitin [508]. This stimulation of AD markers by human serum suggests that the genesis of both neuronal plaques and tangles may arise from exposure of susceptible neurons to toxic serum factors and/or failure to detoxify these factors. Another study found that antibodies in serum of patients with AD caused immunolysis of cholinergic nerve terminals from the rat cerebral cortex, supporting the hypothesis that autoimmune mechanisms may operate in the pathogenesis of AD [509].

Other studies have shown that serum of multiple sclerosis patients causes demyelination in rat CNS explant cultures and also induces cytotoxicity in rat oligodendrocytes in culture [510,511]. Demyelination was present not only in multiple sclerosis sera but was also found in sera from patients with other neurological diseases and complement was shown to be a factor involved in the effect [512,513]. In yet another study, human serum from patients with septic shock was shown to induce apoptosis in human cardiac myocytes [514]. This work demonstrates the utility of examining effects of disease plasma on cell culture systems, to facilitate the study of both disease markers and disease mechanisms. These potent biological effects of blood highlight the likelihood that disease and ageing biomarkers and risk factors are present and could potentially be identified in blood.

Previous studies indicate that AD plasma may contain oxidative stress markers as well as cytotoxic factors, and because there is growing evidence from parabiosis experiments of the impact of blood factors , we aimed to investigate the effect of pooled control, MCI and AD plasma from 20 individuals each on a microglial cell line. Cell viability, proliferation and mitochondrial function were investigated following 48 hour treatment with non heat-inactivated plasma and plasma in which complement proteins had been deactivated. We also tested the effect of commercially purchased complement factors alone and in combination on cultured glia. We then undertook proteomic analysis of the plasma from each group and iTRAQ quantitative proteomic analysis of cell extracts exposed to plasma from each group to investigate possible plasma protein alterations unique to MCI or AD, to detect any protein aberrations within the cells treated with the plasma and to correlate these findings with cell viability and mitochondrial function assays measured *invitro*.

4.2   Methods

4.2.1 Subjects

Age matched healthy control (n = 20), amnestic mild cognitive impairment (aMCI, n = 20), and probable AD (n = 20) fasting EDTA plasmas were aliquoted with a minimum of freeze-thaw cycles (generally ≤2) and stored at −80°C. Plasmas were pooled and used in both the cell culture and plasma proteomics experiments. AD patients were recruited from the Memory Clinic of the Department of Old Age Psychiatry of the Prince of Wales Hospital and participants in a clinical drug trial of donepezil (Aricept®). All met the NINCDS-ADRDA criteria [515] for probable AD. The aMCI subjects were recruited from the Memory and Ageing Study, a longitudinal study of community dwelling individuals aged 70-90 [516]. The diagnosis of aMCI was determined using the Petersen Criteria as follows [517]: (i) subjective complaint of memory impairment, (ii) objective impairment in memory (performance >1.5 SD below normal for age on a standardised memory test) (iii) essentially preserved general cognitive function (MMSE ≥ 24) (iv) intact functional activities as indicated by instrumental activities of daily living; and (v) not meeting DSM-IV criteria for dementia. Healthy control subjects had a normal performance for age on a range of neuropsychological tests and intact functional activities. Ethics committee approval was obtained from the University of New South Wales (UNSW) and the South Eastern Sydney Illawarra Area Health Service (SESIAHS) ethics committees and written informed consent was obtained from all participants.

4.2.2 Cell Culture

CHME-5 cells are a human microglial cell line obtained from embryonic fetal human microglia through transformation with SV-40 virus [518,519] and were a generous gift of Prof Gilles Guillemin (Macquarie University, Sydney, Australia). These cells express antigens present on adult human microglia, secrete pro-inflammatory cytokines upon activation, exhibit properties of primary human microglia and have been successfully used as a model of microglial activation by others [518,520]. Cells were maintained in RPMI1640 cell culture medium, supplemented with 10% heat inactivated foetal bovine serum, 2 mM l-glutamine, and 1% penicillin/streptomycin, at $37^{o}C$ in a humidified atmosphere containing 95% air/5% CO_2. Before experimentation, cells were seeded into 24 or 96 well culture plates to a density of approximately $1x10^{4}$ or $2x10^{3}$ cells respectively. Cells were left overnight and then supplemented with up to 20% (by volume) heat-inactivated and non heat-inactivated control, MCI or AD plasma for 48 hours. For the cell viability and iTRAQ proteomic analyses cells were washed to remove all plasma, and lysed in RIPA buffer (Thermo Fisher Scientific, IL, USA) followed by sonication. For the complement factor experiments cells were seeded into plates and treated with 1, 5 or 10 μg of each complement factor or the complement standard (in a total volume of 200μL cell culture media) and then incubated for 48hrs. These concentrations are within the physiological range of these proteins in plasma [521].

4.2.3 MTT Cell Proliferation Assay

In actively proliferating cells, an increase in 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) conversion in cells relative to controls represents an increase in cellular proliferative activity. Conversely, in cells that are undergoing apoptosis, MTT conversion, and thus biological activity, decreases. Cell proliferation was analysed using established protocols [522,523].

4.2.4 NAD(H) Assay

Damaged cells show mitochondrial dysfunction, which results in decreased cellular nicotinamide adenine dinucleotide (NAD) levels. Intracellular NAD(H) concentration was quantified using the thiazolyl blue microcycling assay established by Bernofsky and Swan

and adapted here for the 24 well plate format [524].

4.2.5 Lactate Dehydrogenase (LDH) Leakage

Cytoplasmic enzyme leakage has been shown to be a useful tool for measuring early cellular damage or impairment [525], and has also been used as a sign of cytotoxicity [526,527]. LDH is released from cells due to loss of membrane integrity. Therefore LDH was measured in the cell culture medium as well as in cell homogenates as another measure of cell viability.

4.2.6 XF24 Microplate-Based Respirometry as a measure of mitochondrial function

To determine the effect of human control, MCI and AD plasma on oxygen consumption rates (OCRs; an indicator of mitochondrial respiration) and extracellular acidification rates (ECARs; a measure of glycolytic flux) in the microglial cell line, the Seahorse XF24, extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA, USA) was employed and assays performed as previously described [528-530]. Briefly, culture plates were incubated in a CO_2-free incubator at 37°C for 1 hr to equilibrate temperature and pH. The microplate was then loaded into the XF24 and further incubated for 15 min with 3 min mix, 2 min wait cycles before commencement of the assay. After determination of the basal respiration in the cell culture, oligomycin (2 µM), carbonylcyanide-p-trifluoromethoxy-phenylhydrazone (FCCP, 500 nM), and antimycin (3 µM) were sequentially added and the OCRs and ECARs for each culture well were quantified for 2 minutes. The basal control ratio (BCR) and the uncoupling ratio (UCR) were determined as previously described [531]. Essentially, the BCR is a measure of how close the basal level of respiration is to the maximum level of respiration. The closer this ratio is to 1, the greater the mitochondrial malfunction. The UCR is a measurement of mitochondrial functional integrity and measures the ratio of uncoupled to physiologically normal respiration levels. The greater the maximum level of respiration, the greater the mitochondrial functional integrity.

4.2.7 Fractionation of Plasma

Control, MCI and AD plasma from 20 subjects was pooled and fractionated by two methods. For analysis of effects of plasma protein vs metabolite fractions on cultured glia, a PD10

column separation method was used. The PD10 column was washed with MilliQ water before the addition of 500 µL of plasma. The flow-through was collected as 750 µL fractions topping the column with MilliQ water. In total 20 fractions were collected and the absorbances read at 280nm.

For the proteomics analysis of plasma proteins, fractionation into low and high abundant protein fractions was undertaken using an MARS-Hu6 column (Agilent Technologies, CA, USA) according to the manufacturer's instructions. The MARS-Hu6 column depletes the top 6 highest abundance proteins (albumin, IgG, IgA, transferrin, haptoglobin and antitrypsin) from plasma. This eliminates the masking effect of highly abundant proteins so that lower-abundant proteins can be more easily detected by the mass spectrometer. The low abundance fraction was used for the proteomic analysis experiments.

4.2.8 Proteomics Analysis of Human Control, MCI and AD plasma

A one dimensional SDS 4-12% NuPage (Thermo Fisher Scientific Inc, MA, USA) gel was run using the low abundance fraction from each of the three groups. The gel was colloidal coomassie stained [482], and the lanes uniformly cut into 7-8 bits using a gridcutter and mount from The Gel Company, CA, USA. The gel bits were trypsin digested and then analysed using mass spectrometry as outlined in Chapter 3, under section 3.2.4.

Peak lists were generated by MassLynx (version 4.0 SP1, Micromass) using the Mass Measure program and submitted to the database search program Mascot (version 2.2, Matrix Science, London, England). Search parameters were: Precursor and product ion tolerances ± 0.25 and 0.2 Da respectively; Met(O) and Cys-carboxyamidomethyl were specified as variable modification, enzyme specificity was trypsin, one missed cleavage was possible on the NCBInr database.

Scaffold Q+ (version 4.3.4), Proteome Software, Portland, OR, USA) was used to identify any altered proteins between the groups. The Scaffold programme uses mass spectrometric data to identify protein changes in biological samples by collating Mascot data and using the ProteinProphet algorithm [532]. We compared the normalised total spectral count values from Scaffold [533] with the emPAI values from Mascot [534], which uses a different algorithm for spectral counting.

4.2.9 iTRAQ Proteomics of Cell Lysates Treated with Human Control, MCI and AD Plasma

Two biological replicates of cells treated with 10% (by volume) non-heat inactivated fetal bovine serum, human control, MCI or AD plasma were washed to remove all plasma/serum and then lysed using RIPA buffer and probe sonicated. Total protein concentrations were determined using the Bicinchoninic acid (BCA) protein assay kit (Pierce, IL, USA). The total protein (120 µg) from each sample was reduced with 2 µl of 5mM tris-(2-carboxyethyl) phosphine (TCEP) for 60 min at 60°C followed by alkylation with 200 mM iodoacetamide (1 µL) for 10 min at room temperature. To remove any buffer components incompatible with mass spectrometry a buffer exchange was performed with 50 mM $NaHCO_3$ using Microcon centrifugal filter devices with a 3,000 Da nominal molecular weight limit membrane (Millipore, MA, USA) to give a final protein concentration of 1 µg/µl.

For tryptic digestion 100 µg total protein from each sample was incubated overnight at 37°C with 4ug of trypsin. Samples were labelled using the 8-plex iTRAQ kit (Applied Biosystems, CA, USA). Each iTRAQ reporter label was mixed with a biological replicate of cell lysate sample, pH adjusted to basic (*ca* pH = 9) with 2 µl of 50 mM of Na_2CO_3 and incubated for 2 hours. The reporter masses for the samples were labelled as follows: fetal bovine serum; 113 and 117, human control plasma; 114 and 118, human MCI plasma; 115 and 119, human AD plasma; 116 and 121.

Sample clean-up was performed using a strong cation exchange cartridge (Applied Biosystems, CA, USA) and a syringe pump at a flow rate of 9.5 ml/hr, and using the manufacturer's protocol. Sample was then passed through a C18 Peptide Macrotrap (Michrom Bioresources, CA, USA). The flow-through from the C18 step was passed through an Oasis cartridge (Waters, MA, USA) to maximise peptide recovery and the two eluants pooled and dried under vacuum. Dried peptide pellets were resuspended in 0.2% heptafluorobutyric acid and then analysed using mass spectrometry as described below.

4.2.10 Proteomic Analysis of Cell Lysates Treated with Human Control, MCI and AD Plasma

Chromatography was performed using an LC Packings capillary HPLC system (Dionex, Amsterdam, Netherlands), comprised of an Ultimate pump system, Switchos valve unit and

Famos autosampler. Samples (iTRAQ labelled peptide mixtures) were captured onto a C18 pre-column cartridge (500 μm x 2 mm, Michrom Bioresources, Auburn, CA). After a 10 min wash to remove any residual buffers or salts, the pre-column was automatically switched into line with a capillary column (75 μm x ~12 cm) containing C18 reverse phase packing material (ReproSil-Pur 120 C18-AQ, 1.9 μM, Dr Maisch, Ammerbuch-Entringen, Germany). Peptides were eluted using a 240 min linear gradient of $H_2O:CH_3CN$ (98:2, 0.1 % formic acid) to $H_2O:CH_3CN$ (20:80, 0.1 % formic acid) at ~300 nl/min. The precolumn was connected via a fused silica capillary (10 cm, 25 μ) to a low volume tee (Upchurch Scientific, WA, USA) where high voltage (2300 V) was applied and the column tip positioned ~ 1 cm from the spray inlet of a TripleTOF 5600$^+$ hybrid tandem mass spectrometer (ABSciex, CA, USA). Positive ions were generated by electrospray and the instrument operated in information dependent acquisition (IDA) mode. A TOF MS survey scan was acquired (*m/z* 350-1700, 0.75 s) and the three largest multiply charged ions (counts > 20, charge state ≥ 2 and ≤ 4) were sequentially selected by Q1 for MS-MS analysis. Nitrogen was used as collision gas and an optimum collision energy automatically chosen (based on charge state and mass). Tandem mass spectra were accumulated for up to 2.5 s (*m/z* 65-2000).

Protein identification and quantification was performed using the MS/MS data (WIFF files) and the Paragon algorithm as implemented in Protein Pilot v4.0 software (Applied Biosystems, CA, USA) using the NCBInr database. Only proteins identified with a ProteinPilot unused score of ≥ 1.3 (greater than 95% confidence in sequence identity) were accepted, as previously described [535,536]. The unused score is a ProteinPilot generated value for the level of confidence in protein sequence identification. As an approximate guide, ProteinPilot unused scores give the following percentage levels of confidence: score ≥1.3 (≥95% confidence), score ≥2 (≥99% confidence), score ≥3 (≥99.9% confidence) [535]. The only fixed modification used was iodoacetamide alkylation of cysteine residues. Mass tolerances were set to 50ppm for the precursor and 0.2 Da for the fragment ions. Autobias correction was applied to correct for any systematic bias in total protein concentration during sample pooling. Both biological replicates for the three human plasma types (control, MCI and AD) were compared to the fetal bovine serum control (iTRAQ reporter 117) and data exported to Microsoft Excel software (Microscoft, WA, USA).

Protein interactions between dysregulated proteins were determined using the WEB-based bioinformatics tool STRING v9.1 (http://string-db.org). STRING has a database that collates information on protein-protein interactions and associations. It scores and weights connections and provides predicted interaction network maps from literature mining searches. The 27 proteins which were significantly dysregulated in glia treated with AD plasma, but not dysregulated in either control or MCI plasma treated glia (shown in Table 4.4) were analysed in STRING v9.1. MCL clustering was used with the 2 clusters option picked and with the confidence view selected to display the strength of evidence for protein associations. Analysis of enrichment was also performed.

Figure 4.1 Schematic workflow of methodological procedures
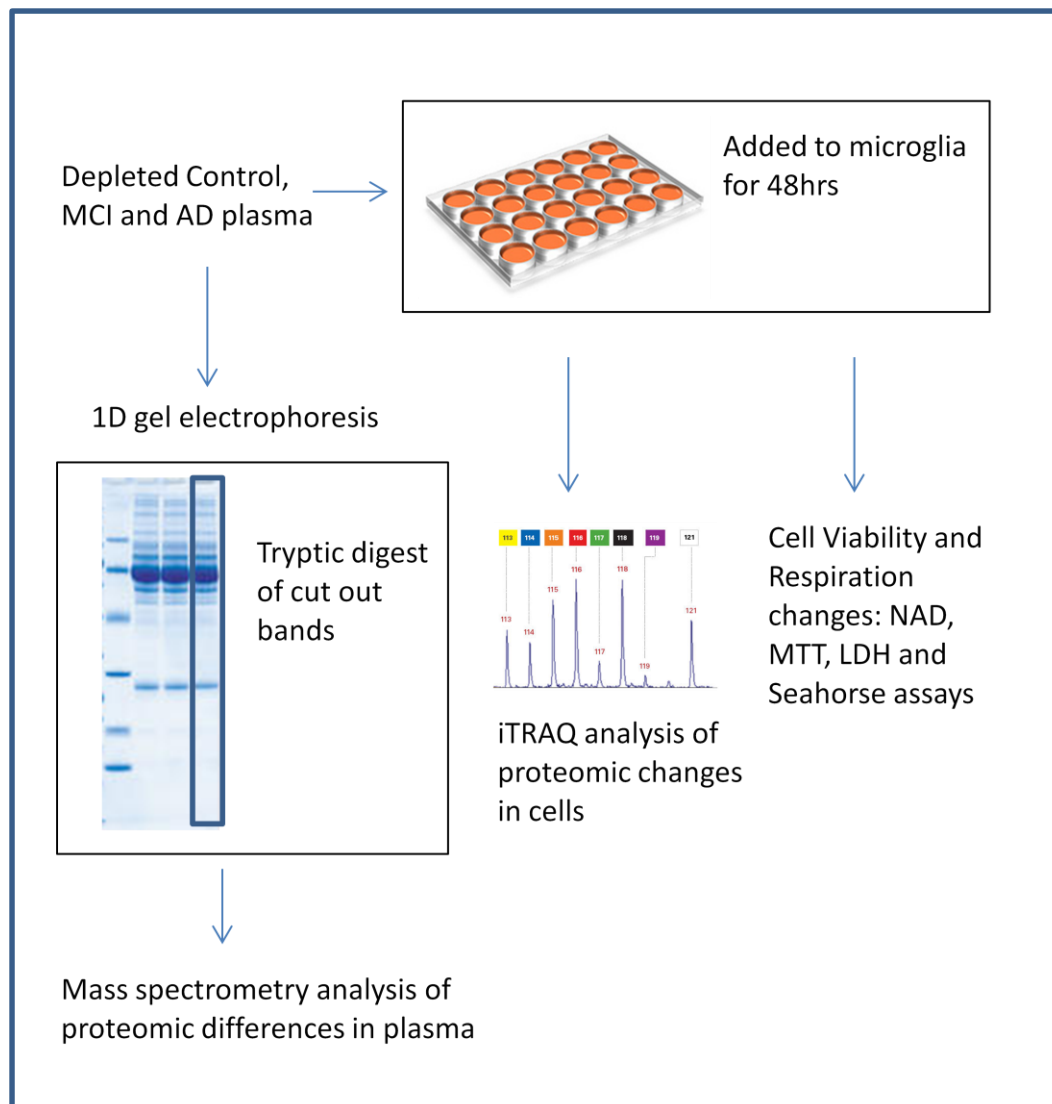
4.2.11 Statistics

All cell viability values are presented as means ± SEM. Statistical comparisons were performed using two-tailed student *t*-tests assuming equal variance. Differences between treatment groups were considered statistically significant at the $p \leq 0.05$ level. Scaffold values are represented as normalised total spectral counts and p-values for significantly altered proteins were obtained using the ProteinProphet algorithm of the Scaffold Q^+ software (Proteome Software, OR, USA). All iTRAQ quantitative data are presented as iTRAQ tag peak area ratios of cells treated with human plasma to cells treated with fetal bovine serum control. Ratios and p-values for significantly altered proteins were obtained through the Paragon algorithm of the Protein Pilot v4.0 software (Applied Biosystems, CA, USA).

Results

4.3.1 Cell Proliferation

Cells treated with non heat-inactivated AD plasma for 48 hours showed a significant decrease in cell proliferation compared to controls (Table 4.1). The addition of MCI plasma to the cells also caused a similar drop in cell proliferation, though not reaching statistical significance (Table 4.1). Mild heat treatment of the plasma at 56°C for 30 minutes is an established approach for inactivating complement proteins [537,538]. Such heat treatment eliminated the

effects of MCI and AD plasma on cell proliferation (Table 4.1). Even with heat treatment there was a slight, though not statistically significant downward trend in cell proliferation.

4.3.2 NAD levels and LDH Leakage

Incubation with non heat inactivated plasma caused a significant drop in cell viability as reflected in lower NAD levels, for cells treated with both MCI and AD plasma, compared to controls (Table 4.1). This result was again prevented by plasma heat inactivation (Table 4.1). A significant increase in LDH leakage into the cell culture medium was seen in cells incubated with non heat inactivated AD plasma (Table 4.1). A concurrent decrease was seen in the amount of intracellular LDH in these same cells (Table 4.1).

Table 4.1: Cell viability of microglial cells exposed to MCI and AD plasma. Measured by MTT absorbance, LDH release and intracellular NAD levels of microglial cells after 48 hour incubation in pooled, non heat inactivated and heat inactivated control, MCI and AD plasma. Cell viability was determined by measurement of cell proliferation, intracellular NAD levels and LDH activity in cell culture media and cell homogenates. n=9 (nine replicates) for cell proliferation measurements, n=6 (six replicates) for NAD concentration and LDH activity. Plasma used for the measurements were obtained from the pooled plasma of 20 patients from each of the three groups (Control, MCI and AD).

		Non Heat Inactivated Plasma									Heat Inactivated Plasma					
		Control			MCI			AD			Control		MCI		AD	
		MTT assay Cell Proliferation (abs at 570nm)	NAD (ng)	LDH Activity (U/L)	MTT assay Cell Proliferation (abs at 570nm)	NAD (ng)	LDH Activity (U/L)	MTT assay Cell Proliferation (abs at 570nm)	NAD (ng)	LDH Activity (U/L)	MTT assay Cell Proliferation (abs at 570nm)	NAD (ng)	MTT assay Cell Proliferation (abs at 570nm)	NAD (ng)	MTT assay Cell Proliferation (abs at 570nm)	NAD (ng)
plasma conc/Vol media	Mean	0.21	10.71		0.23	10.84		0.14	10.3							
volume	S.E.M	0.05	3.84		0.05	1.26		0.03*	1.82							
10 % of media-derived plasma volume	Mean	0.13	3.61	3.35 (media) 11.4 (homogenate)	0.09	2.35	2.7 (media) 13.06 (homogenate)	0.04	0.89	6.09 (media) 7.76 (homogenate)	0.13		0.11		0.11	
	S.E.M	0.03	1.25	0.36 (media) 1.19 (homogenate)	0.02	0.85	0.3 (media) 1.22 (homogenate)	0.01**	1.3*	1.05* (media) 1.0 (homogenate)	0.03		0.04		0.02	

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plasma represents 20% of media	volume	**Mean**	0.06	0.79		0.06	0.56		0.03	0			14.02	12.3	12.1
		S.E.M	0.01	0.14		0.004	0.07		0.004**	0**			1.15	0.60	0.65

* p ≤ 0.05 vs Control, ** p ≤ 0.01 vs Control

4.3.3 The effect of complement proteins on cellular proliferation and viability

Since heat inactivation seemed to ameliorate the effect of plasma, we hypothesised that the complement protein family may be responsible. Treatment of cells with complement factors C1q, C1 inhibitor, C4, C5 and C9 effected a downward trend in cell proliferation with increasing concentration, but did not reach significance (Table 4.2). In combination, the factors were found to reduce cell proliferation at the highest concentration tested. The human complement standard which contains the factors C1q, C2, C3, C4, C5, C6, C7, C8, C9 and factor B was found to be the most potent at minimising cell proliferation (Table 4.2). The addition of human complement standard containing C1q, C2, C3, C4, C5, C6, C7, C8, C9 and factor B was found to significantly reduce NAD levels in the microglia (Table 4.2).

Table 4.2: Cell viability of microglial cells after 48 hour incubation with human complement components (C1q, C1 inhibitor, C4, C5 and C9), both individually and in combination with each other; and a human complement standard containing complement components C1q, C2, C3, C4, C5, C6, C7, C8, C9 and factor B. Cell viability was determined by MTT assay of cell proliferation and intracellular NAD levels (for complement standard samples). Replicates included n=6 for cell proliferation measurements and, n=3 for NAD concentrations. *** p ≤ 0.05 vs Control, ** p ≤ 0.01 vs Control**

	Cell Proliferat-ion (abs at 570nm)	Complement C1q	Complement C1 inhibitor	Complement C4	Complement C5	Complement C9	C1q + C4	C5 + C9	Complement C1q, C1inhib, C4, C5, + C9	Complement Standard	Complement Standard (NAD, ng)
Control (0µg/µl)	Avg	0.317	0.609	0.454	0.375	0.324	0.384	0.356	0.378	0.511	0.114
	SEM	0.019	0.07	0.025	0.031	0.014	0.025	0.018	0.013	0.004	0.003
0.005µg/µl	Avg	0.3	0.593	0.458	0.382	0.333	0.377	0.371	0.377	0.426	0.104
	SEM	0.042	0.02	0.019	0.028	0.0098	0.019	0.025	0.019	0.031	0.010
0.025µg/µl	Avg	0.31	0.571	0.461	0.364	0.336	0.366	0.359	**0.331***	**0.210****	0.094
	SEM	0.018	0.03	0.02	0.025	0.0096	0.025	0.021	0.015	0.036	0.063
0.05µg/µl	Avg	0.267	0.49	0.435	0.355	0.344	**0.289***	**0.265***	**0.292***	**0.129****	**0.063***
	SEM	0.0075	0.05	0.019	0.03	0.021	0.027	0.013	0.024	0.018	0.0024

4.3.4 Effects of plasma metabolites vs proteins on cell proliferation and viability

To determine whether the effect on cell proliferation was due to the protein or metabolite component of plasma (or both), the protein and low molecular weight components of plasma were separated. Plasma was fractionated using a PD10 column (GE Healthcare Life Science, UK) into its protein and metabolite portions to determine which portion of the plasma was causing the cytotoxic effect (Figure 4.1). Addition of these two fractions to the cells showed that it was exclusively the protein portion which was initiating the reduction in cell proliferation (Figure 4.1). Protein fractions of both MCI and AD plasma were found to significantly reduce cell proliferation (Figure 4.1). The metabolite fraction of the plasma had no significant effect on microglial proliferation (Figure 4.1).

Figure 4.2: Fractionation of non heat inactivated control plasma into protein and metabolite fractions using PD10 column, and the effect of these fractions on cell proliferation. Three replicates were performed. Plasma used for the measurements were obtained from the pooled plasma of 20 patients from each of the three groups (Control, MCI and AD). Effect on cell proliferation at a plasma concentration of 10% and 20% of total media volume are shown. * $p \leq 0.01$ vs Control, ** $p \leq 0.001$ vs Control. **Panel A**: Fractionation of non heat inactivated control plasma into protein and metabolite fractions using PD10 column **Panel B**: Effect of these fractions on cell proliferation. **Panel C**: Images of microglia after 48 hour incubation with non heat inactivated 20% control plasma (left) and non heat inactivated 20% AD plasma (right), showing increased toxicity and reduced cell proliferation in the AD plasma treated cells.

A
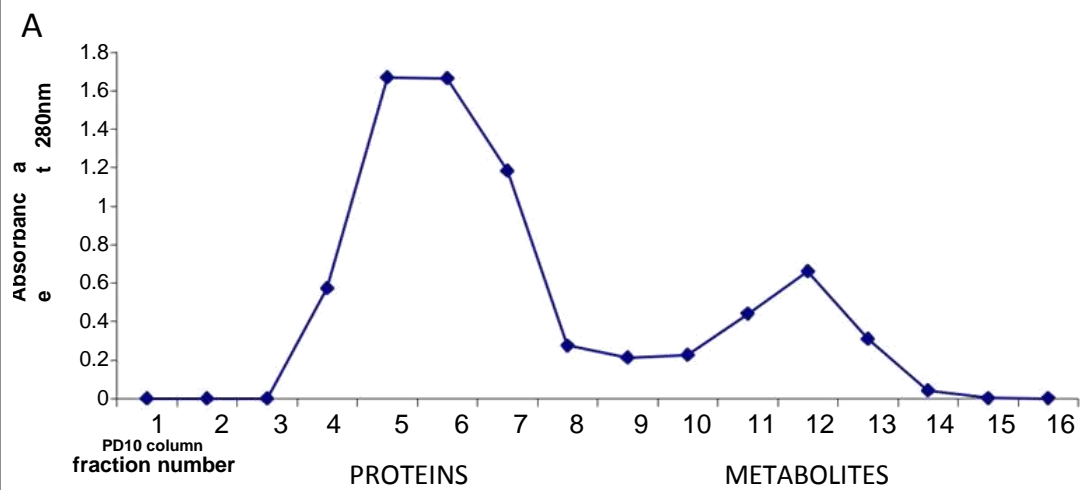
B

	Protein Fraction				Metabolite Fraction			
	MCI		AD		MCI		AD	
Fraction Volume added (% of total media volume)	10%	20%	10%	20%	10%	20%	10%	20%
Average Cell Proliferation (% of Control)	90.77	**75.96***	**53.37****	**37.39****	111.57	103.78	99.19	101.18
S.E.M	3.65	0.92	1.88	1.84	9.36	10.02	3.37	7.59

C
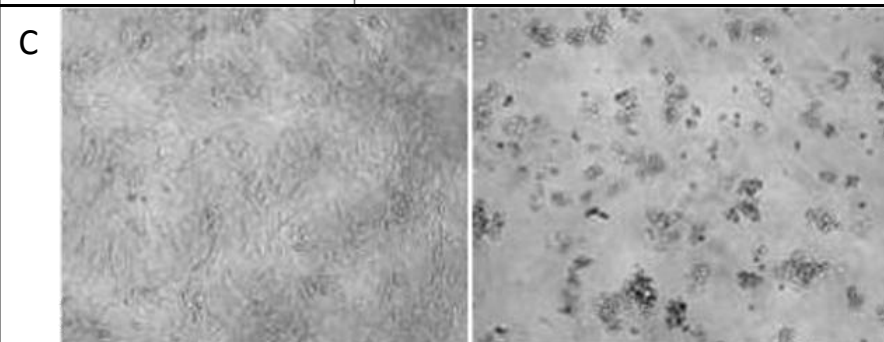
4.3.5 Plasma fractionation and 1D gel electrophoresis

Chromatogram using the MARS-Hu6 column provided a baseline separation of low and high abundant proteins (Figure 4.2). Low abundant proteins are eluted first (first peak on chromatogram) and high abundant proteins are eluted after (second peak, containing the six highest abundance plasma proteins including: albumin, IgG, IgA, transferrin, haptoglobin and antitrypsin). These fractions were run on a 1D SDS NuPAGE gel and proteins were shown to be effectively separated with a substantial depletion of high abundant proteins, revealing many lower abundant protein bands in the low abundant fraction (Figure 4.2). Loading was 50 µg/lane. First and last lanes contained molecular weight markers. Each fraction was run in duplicate.

Figure 4.3 Representative chromatogram of separation of plasma into low and high abundant fractions and 1D SDS NuPage gel of these protein fractions.
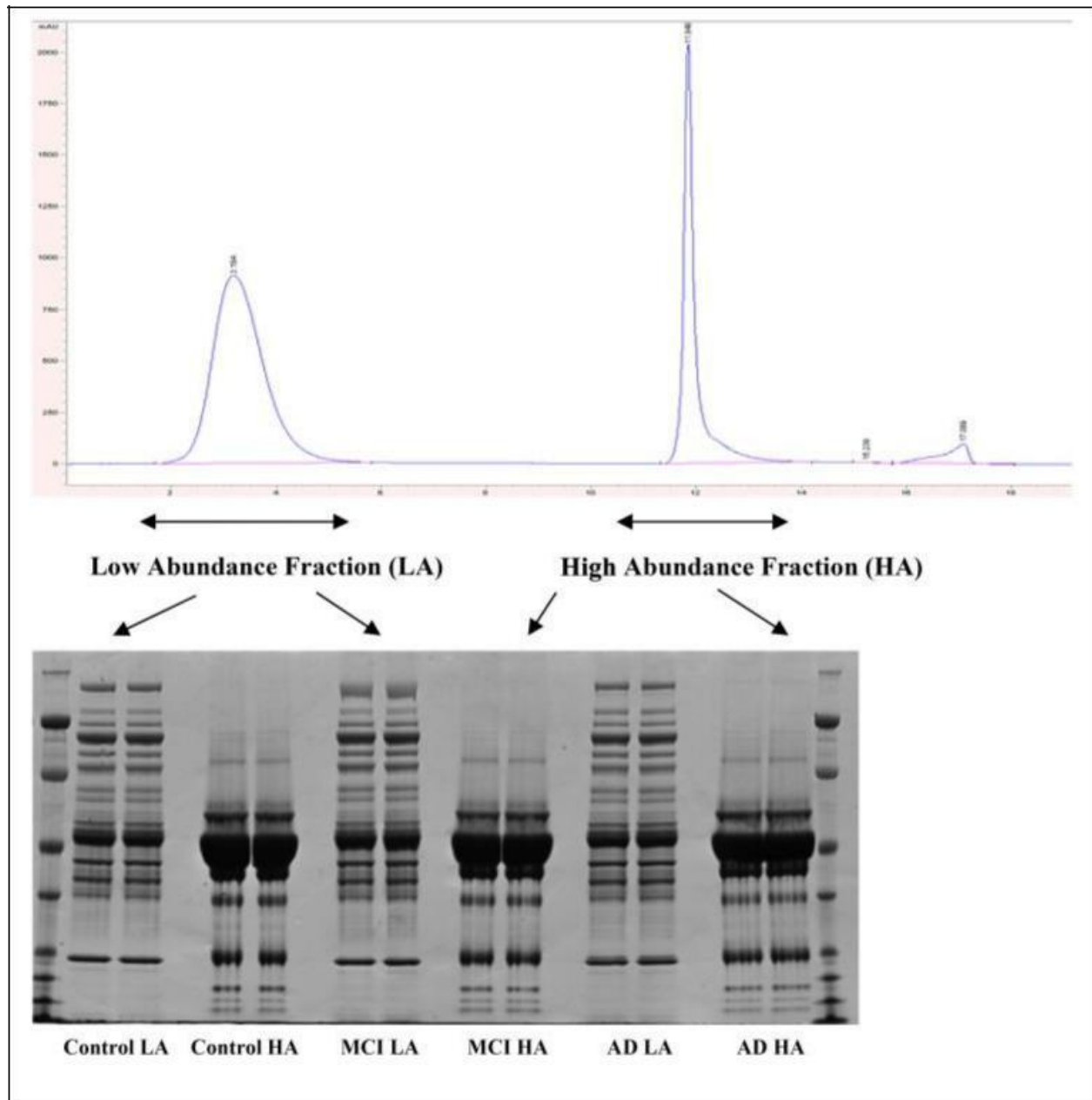
4.3.6 Proteomics of MCI and AD plasma

We used an unbiased label free proteomics approach to explore the MCI and AD plasma proteomes, to identify any dysregulated proteins . Following proteomic analysis of pooled control, MCI and AD plasma, normalised total spectral counts using Scaffold software and emPAI values from Mascot showed complement component 2, fibronectin and fibrinogen to be significantly increased in the AD group compared to the control group (Table 4.3). Thrombin was decreased in the MCI and AD patients compared to controls (Table 4.3).

Table 4.3: Average normalised spectral counts (obtained from Scaffold) and emPAI values (obtained from Mascot) of significantly deregulated proteins identified in pooled plasma samples between the Control, MCI and AD groups. Values are averages from 4 replicates. Plasma used was obtained from the pooled plasma of 20 patients from each of the three groups (Control, MCI and AD) with depletion of high abundance proteins.

		Complement component 2 (gi|14550407)			Complement component 1 inhibitor (gi|114642584)			Complement component 4 binding protein (gi|4502503)			Fibronectin (gi|109658664)			Thrombin (gi|119588383)			Fibrinogen (gi|70906435)			Alpha-1B-glycoprotein (gi|46577680)		
		C	MCI	AD	C	MCI	AD	C	MCI	AD	C	MCI	AD	C	MCI	AD	C	MCI	AD	C	MCI	AD
Normalised Spectral Count	Average	0	0.82	2.77	9.98	7.45	11.30	4.18	3.38	4.51	18.9	15.1	32.2	10.34	5.57	6.29	39.78	35.93	43.19	6.08	8.32	7.64
	S.E.M	0	0.50	0.29	1.0	0.46	2.10	0.51	0.46	0.83	0.18	2.55	2.29	1.18	0.50	0.88	5.58	2.42	7.25	0.49	0.70	1.01
	p-value		0.015#	0.000074**		p = 0.06 vs AD						0.0025##	0.0012**		0.098*	0.033*					0.040*	
emPAI value	Average	0.08	0.06	0.145	0.74	0.62	0.95	0.35	0.18	0.26	0.39	0.30	0.64	0.83	0.46	0.48	5.40	4.74	7.87	0.78	0.86	0.75
	S.E.M	0.010	0.020	0.014	0.090	0.06	0.13	0.027	0.037	0.029	0.068	0.065	0.033	0.068	0.055	0.033	0.48	0.62	0.71	0.10	0.032	0.029
	p-value		0.014#	0.025*		p = 0.06 vs AD				0.013*		0.0071##	0.0018**		0.0057*	0.0034*		0.016#	0.036*		0.050#	

* p ≤ 0.05 vs Control, ** p ≤ 0.01 vs Control

p ≤ 0.05 vs AD, # #p ≤ 0.01 vs AD

4.3.7 Mitochondrial Function and Cellular Bioenergetics

Since changes in NAD and LDH were seen in response to cells exposed to AD plasma, we explored the possibility that cellular energy generating pathways were affected more broadly. To determine whether mitochondrial bioenergetic mechanisms are associated with AD pathogenesis, we assessed mitochondrial function in glial cells treated with human plasma using the Seahorse XF24 bioanalyzer (Seahorse Bioscience, MA, USA). We observed a significant decrease in oxygen consumption rates (OCRs) and an increase in extracellular acidification rates (ECARs) for cells treated with AD plasma. MCI plasma effected similar trends though did not achieve statistical significance (Figure 4.3). We also observed a significant increase in the basal control ratio (BCR) and a decline in the uncoupling ratio (UCR) in microglial cells after 48 hr incubation with AD plasma (Figure 4.3), consistent with impaired mitochondrial function and increased shift towards glycolysis.

Figure 4.4: Effects of human plasma on cellular bioenergetics in a microglial cell line**.**

Effect of human plasma on oxygen consumption rates (OCR) in a microglial cell line for 48 hours. *p<0.05 compared to non-treated cells (control); (n=4 for each treatment group).

Effect of human plasma on extracellular acidification rates (ECAR) in a microglial cell line for 48 hours. *p<0.05 compared to non-treated cells (control); (n=4 for each treatment group). **(C)** Effect of human plasma on the basal control ratio (BCR) in a microglial cell line for 48 hours. *p<0.05 compared to non-treated cells (control); (n=4 for each treatment group). **(D)** Effect of human plasma on the uncoupling ratio (UCR) in a microglial cell line for 48 hours. *p<0.05 compared to non-treated cells (control); (n=4 for each treatment group).
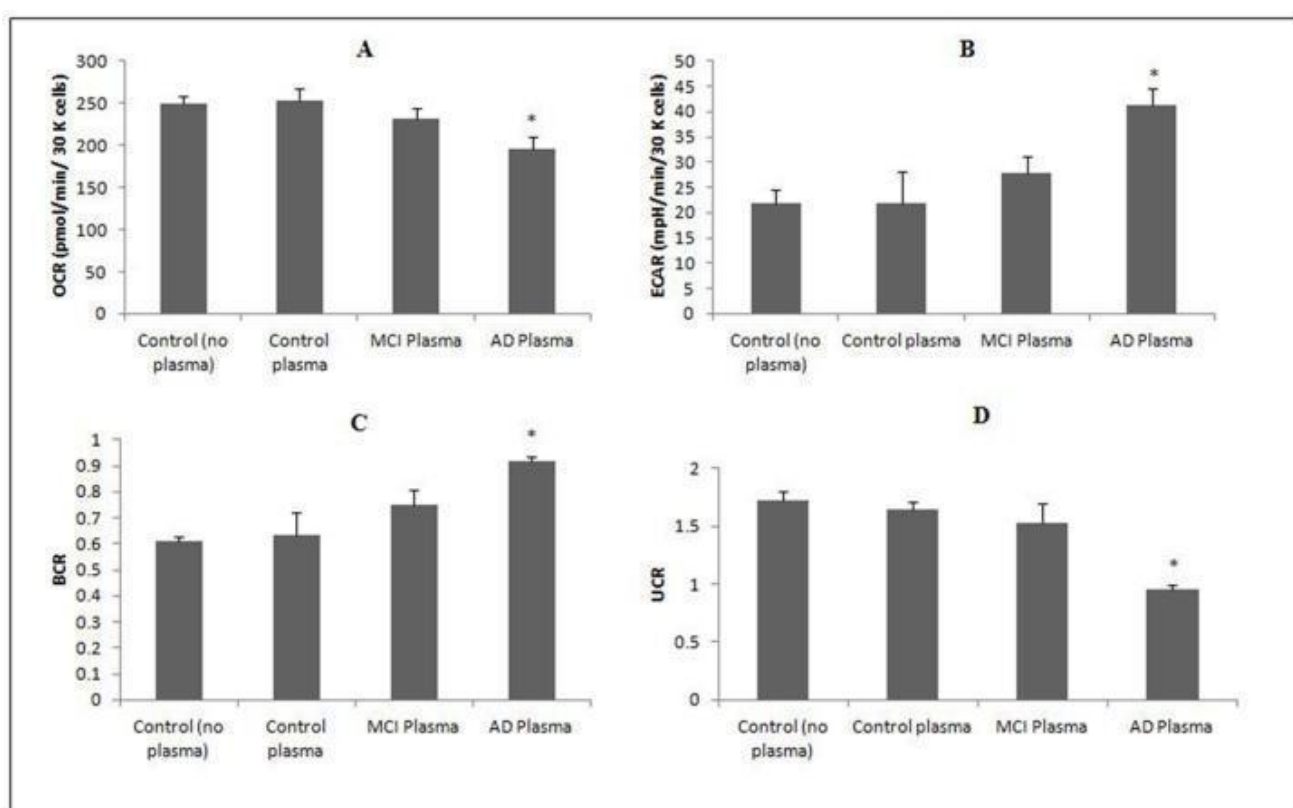
4.3.8 iTRAQ proteomic analysis of cell lysates treated with Control, MCI and AD plasma

Differential protein expression in glial cells treated with fetal bovine serum (control) or human plasma from control, MCI and AD subjects were analysed with two biological replicates performed using an 8-plex iTRAQ experimental design. In total, 791 proteins were identified with 95% or greater confidence in correct protein sequence identification and 750 proteins with a false discovery rate of 5%. Fourty-nine proteins were found significantly altered between the three groups of cellular lysates (Table 4.4). The highest number of dysregulated proteins were found in the cells treated with AD plasma (Table 4.4, 27 proteins highlighted in bold).

Interestingly quite a few proteins involved in the glycolysis cycle were shown to be upregulated in this group, namely glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, enolase, aldolase and pyruvate kinase. These enzymes catalyse five of the ten enzyme reactions of the pathway and their functions are shown in Figure 4.4.

Transketolase was also shown to be significantly elevated in both the AD plasma treated cell sample replicates. This enzyme is part of the pentose phosphate pathway and connects this pathway to glycolysis. Analysis of protein interactions of the 27 dysregulated glial proteins treated with AD plasma using the online STRING v9.1 tool confirmed a significant enrichment of proteins involved in glucose metabolism (Figure 4.5 and Table 4.5).

Table 4.4: Dysregulated proteins in glial cells treated with human control, MCI and AD plasma compared to FBS (non human serum control) following iTRAQ analysis. Cells were incubated with plasma in two 24 well plates, 3 wells for each of the plasma types were pooled from each plate to obtain two biological replicates for the 8-plex iTRAQ experiment. iTRAQ reporter ratios and p-values for altered proteins are shown for both replicates. Proteins found to be dysregulated in MCI and AD treated cells are shown in the table in bold: upregulation in red and downregulation in blue.

Protein Function	Accession #	Name	Replicate 1 Ctrl:FBS	PVal	Replicate 2 Ctrl:FBS	PVal	Replicate 1 MCI:FBS	PVal	Replicate 2 MCI:FBS	PVal	Replicate 1 AD:FBS	PVal	Replicate 2 AD:FBS	PVal
Glycolysis	gi|7669492	glyceraldehyde-3-phosphate dehydrogenase	0.97	0.524	1.02	0.603	0.98	0.676	0.98	0.655	**1.27**	**3.4E-5***	**1.32**	**1.0E-4***
	gi|4505763	phosphoglycerate kinase 1	1.07	0.175	1.05	0.322	1.05	0.279	1.06	0.207	**1.2**	**7.0E-5***	**1.31**	**1.1E-4***
	gi|4503571	alpha-enolase isoform 1	0.93	0.12	0.98	0.753	0.99	0.719	0.98	0.553	**1.19**	**2.7E-4***	**1.22**	**1.0E-4***
	gi|342187211	fructose-bisphosphate aldolase A	0.99	0.829	1	0.989	1.02	0.836	1	0.991	**1.2**	**0.004***	**1.22**	**0.001***
	gi|33286418	pyruvate kinase isozymes M1/M2	0.98	0.746	1.02	0.726	1.01	0.869	1.03	0.6	**1.22**	**0.008***	**1.22**	**0.002***
	gi|4507521	transketolase isoform 1	0.98	0.686	1.01	0.881	1.04	0.323	0.99	0.749	**1.13**	**0.022***	**1.16**	**0.002***
Chaperone	gi|20070125	protein disulfide-isomerase precursor	0.98	0.566	0.96	0.352	0.93	0.057	1.01	0.821	**0.93**	**0.026***	0.98	0.478
	gi|153792590	heat shock protein HSP 90-alpha	1.03	0.679	1.05	0.601	1.06	0.512	1.06	0.473	**1.3**	**0.036***	1.2	0.059

Category	gi	Protein	Ratio	p	Ratio	p	Ratio	p	Ratio	p	Ratio	p	Ratio	p
	gi|66933005	calnexin precursor	1	0.942	0.98	0.7	0.94	0.315	0.93	0.149	0.92	0.266	**0.88**	**0.010***
	gi|5031973	protein disulfide-isomerase A6 precursor	0.97	0.429	0.98	0.662	0.94	0.134	1	0.952	0.96	0.546	**0.91**	**0.019***
	gi|21361657	protein disulfide-isomerase A3 precursor	0.96	0.427	0.98	0.653	**0.9**	**0.013***	1	0.871	0.92	0.117	0.89	0.06
	gi|16507237	78 kDa glucose-regulated protein precursor	0.99	0.806	0.97	0.729	**0.92**	**0.038***	0.98	0.643	0.91	0.101	0.91	0.108
Cytoskeletal	gi|4505257	moesin	1.08	0.265	1.14	0.243	1.12	0.106	1.03	0.721	**1.22**	**0.021***	**1.2**	**0.028***
	gi|21614499	ezrin	1.06	0.498	0.95	0.718	1.05	0.555	1.13	0.446	**1.31**	**0.033***	0.9	0.712
	gi|38176300	nestin	0.99	0.773	1	0.984	0.97	0.518	1.04	0.354	0.94	0.173	**0.85**	**0.004***
	gi|44680105	caldesmon isoform 1	0.91	0.154	0.97	0.773	0.9	0.174	0.94	0.478	0.87	0.085	**0.86**	**0.027***
	gi|19920317	cytoskeleton-associated protein 4	**0.85**	**0.049***	**0.77**	**0.012***	0.92	0.229	**0.79**	**0.032***	**0.74**	**1.0E-4***	**0.77**	**0.019***
	gi|62414289	vimentin	0.97	0.23	1	0.965	**0.9**	**2.4E-4***	0.99	0.778	0.97	0.261	0.96	0.277
Proteolysis	gi|4506713	ubiquitin-40S ribosomal protein S27a	0.98	0.738	0.82	0.159	1.06	0.286	1.04	0.594	0.84	0.066	**0.77**	**0.022***
	gi|66346681	plasminogen activator inhibitor 1	1.04	0.587	0.99	0.922	1	0.999	0.93	0.476	0.97	0.612	**0.79**	**0.023***
	gi|54792069	small ubiquitin-related modifier 2	1.15	0.291	1.02	0.948	1.46	0.083	1.41	0.052	1.23	0.26	**1.29**	**0.043***
	gi|109637759	calpastatin isoform f	0.86	0.228	0.75	0.138	0.81	0.24	0.88	0.46	0.82	0.206	**0.74**	**0.045***
Translation	gi|4503471	elongation factor 1-alpha 1	0.97	0.493	1.02	0.76	0.93	0.225	0.97	0.563	**1.13**	**0.024***	**1.24**	**0.014***
	gi|17158044	40S ribosomal protein S6	0.95	0.62	0.54	0.063	0.83	0.091	0.7	0.172	**0.74**	**0.041***	0.72	0.178

Category	gi	Protein												
	gi|15431288	60S ribosomal protein L10a	1.14	0.206	1.31	0.037*	1.19	0.098	1.27	0.048*	1.41	0.079	1.06	0.732
	gi|214010226	40S ribosomal protein S24 isoform d	0.68	0.062	0.66	0.015*	0.75	0.011*	0.66	0.085	0.6	0.063	0.64	0.022*
	gi|124494254	proliferation-associated protein 2G4	0.97	0.897	0.99	0.974	1.36	0.040*	1.22	0.194	1.24	0.276	1.15	0.146
Transcription	gi|4885379	histone H1.4	0.86	0.158	0.78	0.053	0.79	0.063	0.96	0.61	0.58	0.006*	0.79	0.349
	gi|4885377	histone H1.3	1.04	0.538	1.21	0.386	0.94	0.438	1.18	0.369	0.75	0.012*	0.96	0.613
	gi|4885381	histone H1.5	0.83	0.011*	0.77	0.057	0.79	0.006*	0.9	0.164	0.65	3.4E-4*	0.85	0.205
	gi|4885375	histone H1.2	0.87	0.059	0.73	0.002*	0.74	0.002*	0.95	0.468	0.54	0.001*	0.76	0.102
Immune response	gi|4502101	annexin A1	1	0.999	0.99	0.848	0.93	0.161	1.02	0.794	0.87	0.023*	0.83	0.042*
	gi|50845388	annexin A2 isoform 1	1.04	0.339	1.1	0.021*	0.89	8.0E-4*	0.99	0.647	0.95	0.192	0.93	0.1
	gi|48255891	glucosidase 2 subunit beta	1.04	0.679	1.13	0.292	1.14	0.081	1.16	0.012*	1.01	0.914	1.05	0.584
Antioxidant	gi|32189392	peroxiredoxin-2 isoform a	1.34	0.096	1.2	0.268	1.28	0.15	1.16	0.454	1.19	0.026*	1.28	0.146
	gi|4505591	peroxiredoxin-1	1.01	0.871	0.95	0.379	1.01	0.871	0.96	0.479	1.07	0.223	1.17	0.021*
Cell Growth Regulation	gi|4503057	alpha-crystallin B chain	1.04	0.429	1.02	0.718	1.04	0.45	0.99	0.931	1.24	0.002*	1.26	0.043*
	gi|19743823	integrin beta-1 isoform 1A precursor	1.32	0.102	1.05	0.81	1.14	0.259	1.52	0.038*	1.35	0.069	1.08	0.829
Fatty Acid Metabolism	gi|4557585	fatty acid-binding protein, brain	0.85	0.22	0.84	0.176	0.8	0.228	0.87	0.353	0.9	0.247	0.83	0.049*
	gi|4758504	3-hydroxyacyl-CoA dehydrogenase	1.17	0.445	1.09	0.795	1.33	0.097	1.57	0.015*	1.18	0.49	1.14	0.381

Energy Metabolism	gi|19923437	GTP:AMP phosphotransferase	1.4	0.083	1.27	0.371	**1.46**	**0.047***	1.14	0.44	1.43	0.052	1.43	0.149

* p ≤ 0.05 vs Fetal Bovine Serum Control

Figure 4.5 Glycolysis Pathway highlighting enzymes which were shown to be upregulated in cells treated with AD plasma.
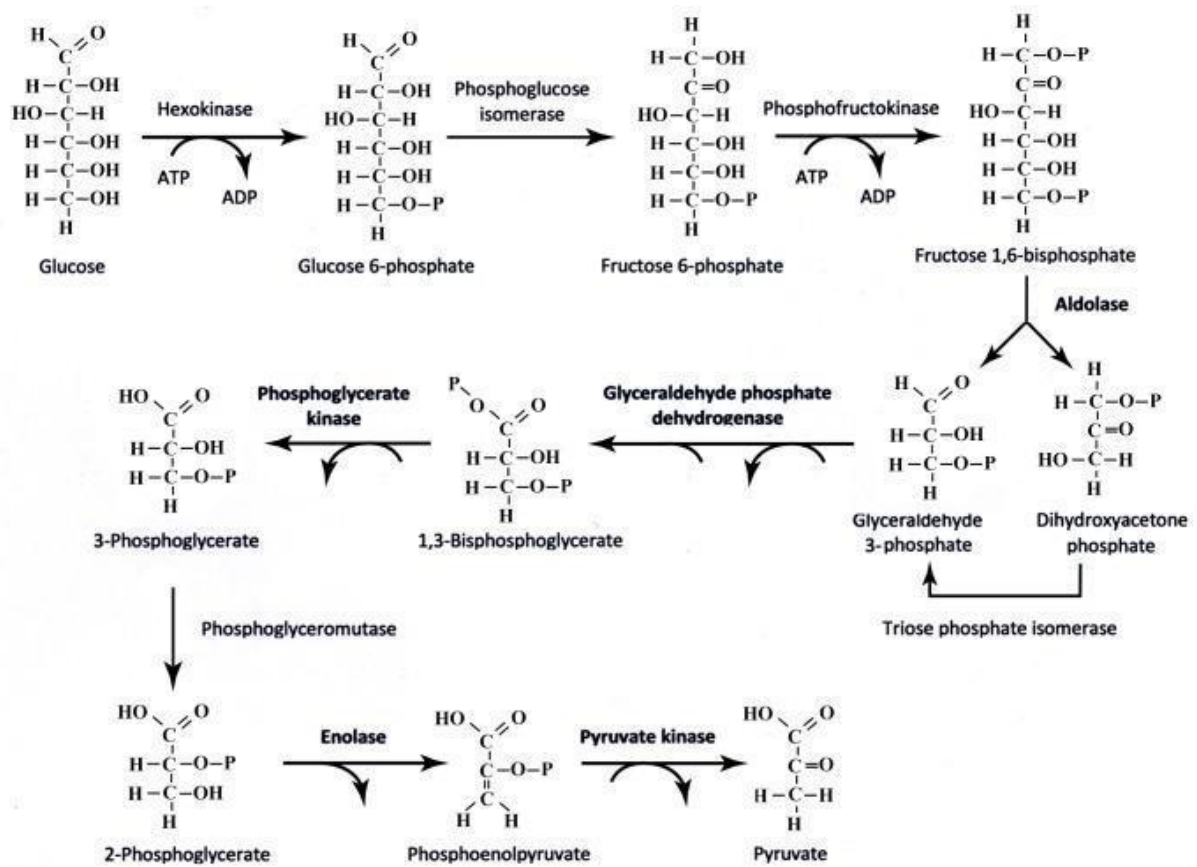
Figure 4.6: Protein associations of proteins dysregulated in AD plasma treated glia. The 27 proteins which were significantly dysregulated in glia treated with AD plasma, but not dysregulated in either control or MCI plasma treated glia (shown in Table 4.4) were analysed in STRING v9.1. MCL clustering was used with the 2 clusters option picked and with the confidence view selected to display the strength of evidence for protein associations (panel A). Analysis of enrichment was also performed and the most significantly enriched biological process was glucose metabolic process (FDR p-value = $1.759 \times 10E-8$, with the 9 proteins involved in this process shown in panel B). Other distinct biological processes which were also significantly enriched included response to hydrogen peroxide (FDR p-value = $3.559 \times 10E-2$ with 4 proteins involved; ANXA1, PRDX1, PRDX2, CRYAB) and membrane to membrane docking (FDR p-value = $4.299 \times 10E-2$ with 2 proteins involved; MSN, EZR). Several molecular functions were also enriched, the most significant being RNA binding (FDR p-value = $5.340 \times 10E-9$ with 17 proteins involved shown in panel C). Another distinct and significantly enriched molecular function is thioredoxin peroxidase activity (FDR p-value = $9.220 \times 10E-3$ with 4 proteins involved; PRDX1, PRDX2). Several cellular components were also enriched, the most significant of these being extracellular vesicle exosome (FDR p-value = $5.019 \times 10E-9$ with 17 proteins involved as shown in panel D). Multiple other significantly enriched cellular components were also observed, and all enriched protein groups are shown in Table 4.5.

Table 4.5 STRING v9.1 analysis of the 27 proteins dysregulated only in glia exposed to AD plasma (shown in Table 4.4) for enrichment in gene ontology biological processes. Glucose metabolism was found to be the most significant biological process, and is also highlighted in the STRING network map (Figure 4.5).

molecular function enrichment

GO_id	Term	Number of Proteins	p-value	p-value_fdr	p-value_bonferroni
GO:0003723	RNA binding	17	1.94E-12	5.34E-09	7.51E-09
GO:0044822	poly(A) RNA binding	16	2.75E-12	5.34E-09	1.07E-08
GO:0008379	thioredoxin peroxidase activity	2	8.88E-06	9.22E-03	3.45E-02
GO:0003676	nucleic acid binding	16	9.50E-06	9.22E-03	3.69E-02
GO:0051920	peroxiredoxin activity	2	2.95E-05	2.29E-02	1.15E-01

biological process enrichment

GO_id	Term	Number of Proteins	p-value	p-value_fdr	p-value_bonferroni
GO:0006006	glucose metabolic process	9	1.42E-12	1.76E-08	1.76E-08
GO:0019318	hexose metabolic process	9	7.28E-12	4.51E-08	9.02E-08
GO:0005996	monosaccharide metabolic process	9	3.63E-11	1.50E-07	4.49E-07
GO:0046364	monosaccharide biosynthetic process	6	2.23E-10	6.89E-07	2.75E-06
GO:0016051	carbohydrate biosynthetic process	7	7.56E-10	1.87E-06	9.35E-06
GO:0006094	gluconeogenesis	5	5.43E-09	1.12E-05	6.73E-05
GO:0019319	hexose biosynthetic process	5	9.29E-09	1.64E-05	1.15E-04
GO:0042542	response to hydrogen peroxide	4	2.30E-05	3.56E-02	2.85E-01
GO:0022614	membrane to membrane docking	2	3.13E-05	4.30E-02	3.87E-01
GO:0016584	nucleosome positioning	2	6.56E-05	8.12E-02	8.12E-01

Cellular component enrichment

GO_id	Term	Number of Proteins	p-value	p-value_fdr	p-value_bonferroni
GO:0070062	extracellular vesicular exosome	17	1.04E-11	5.02E-09	1.51E-08
GO:0044421	extracellular region part	18	1.23E-09	4.44E-07	1.77E-06
GO:0005576	extracellular region	18	1.08E-07	3.06E-05	1.57E-04
GO:0031988	membrane-bounded vesicle	15	1.27E-07	3.06E-05	1.84E-04
GO:0031982	vesicle	15	1.83E-07	3.77E-05	2.64E-04
GO:0043233	organelle lumen	16	2.50E-07	4.32E-05	3.62E-04
GO:0042470	melanosome	5	2.98E-07	4.32E-05	4.32E-04
GO:0005829	cytosol	15	1.22E-06	1.47E-04	1.77E-03
GO:0070013	intracellular organelle lumen	13	5.33E-05	5.93E-03	7.71E-02
GO:0031254	cell trailing edge	2	7.00E-05	6.76E-03	1.01E-01
GO:0001931	uropod	2	7.00E-05	6.76E-03	1.01E-01
GO:0030016	myofibril	4	1.41E-04	1.28E-02	2.05E-01
GO:0016323	basolateral plasma membrane	4	1.51E-04	1.29E-02	2.19E-01
GO:0043292	contractile fiber	4	1.66E-04	1.33E-02	2.40E-01
GO:0060205	cytoplasmic membrane-bounded vesicle lumen	3	2.64E-04	1.91E-02	3.82E-01
GO:0031983	vesicle lumen	3	2.64E-04	1.91E-02	3.82E-01
GO:0031528	microvillus membrane	2	2.98E-04	2.05E-02	4.31E-01
GO:0016023	cytoplasmic membrane-bounded vesicle	7	3.68E-04	2.42E-02	5.32E-01
GO:0005719	nuclear euchromatin	2	5.70E-04	3.43E-02	8.24E-01
GO:0000791	euchromatin	2	7.98E-04	4.62E-02	1.00E+00

4.4 Discussion

There is growing evidence from parabiosis experiments on the positive effects of young blood in ageing, in which circulatory systems of young and aged animals are connected. These studies show that blood from young animals can rejuvenate older animals and ameliorate disease [155,156]. A variety of studies have looked at the effects of plasma on cell cultures in different diseases. For example, Brewer *et al* found that 24 hr exposure of human serum from AD patients to rat hippocampal neurons increased four molecular markers characteristic of Alzheimer senile plaques and neurofibrillary tangles [508]. Another study has shown that Parkinsonian serum has complement-dependent toxicity to rat dopaminergic neurons [539]. A study using a differentiated neuronal cell line investigated the susceptibility of neuronal cells to human complement. It was found that human serum caused lysis of the neurons by complement, as tested by cell viability. The effect was lost when cells were treated with complement-depleted serum by heat inactivation [540].

Our cell culture results also showed that the loss of cell viability and reduction in cell proliferation caused by AD plasma can be prevented by heat inactivation, which inhibits the activity of plasma complement proteins. Alterations to the expression of peripheral proteins may reflect changes in the brain, possibly through a compromised blood brain barrier. Damage to the blood-brain-barrier resulting in increased permeability has been reported in MCI and AD [141,541]. Together these data suggests that complement may have the capacity to contribute to cell loss seen in AD. Complement factors may work synergistically to cause loss in cell viability. We observed reduced cell viability when cells were exposed to the complement standard mixture, as compared to addition of single complement factors (Table 4.2). However in all cases we observed a downward trend in cell viability as complement concentration levels increased regardless of the number of complement proteins present. The data achieved statistical significance with exposure to as few as two complement factors (Table 4.2), indicating that the full spectrum of complement proteins are not necessary for cytotoxicity. Indeed it has been found that treatment of a transgenic mouse model with an agonist to a single complement receptor, C5aR, decreased pathology and improved behavioural performance [542].

There is significant evidence for the involvement of inflammation in the pathogenesis of Alzheimer's disease. In the AD brain, damaged neurons and highly insoluble Aβ peptide deposits and NFTs provide stimuli for inflammation [506,507]. Various neuroinflammatory mediators including complement activators and inhibitors, chemokines, cytokines, radical oxygen species and inflammatory enzymes have been shown to be altered in AD [506,507]. Another prominent feature of AD neuropathology is the association of activated proteins of the classical complement pathway with the lesions. The full-range of classical pathway complement proteins from C1q to C5b-9, known as the membrane attack complex, have been found localised with Aβ deposits in neuritic plaques [543,544]. Complement proteins are also present in dystrophic neurites in AD. The fact that complement activation has progressed until the final membrane attack complex stage and the observation that complement regulators are associated with AD lesions suggests a disturbance in the regulatory mechanisms controlling complement activation in this disease [545-548].

Aβ itself can induce complement-mediated toxicity against neurons in culture, suggesting that Aβ-induced complement activation may contribute to the neuropathogenesis of AD [544,549]. Hyperphosphorylated tau protein, the main component of NFTs, is also a potent stimulator of the complement cascade. Purified NFTs have been shown to activate the complement system in plasma, resulting in a significant increase in the level of membrane attack complex [550]. Tau and Aβ are both able to increase inflammatory responses and cytokine production. Since the complement system is strongly activated in AD, it could possibly participate either in the exacerbation or amelioration of the pathology. Because Aβ deposits and extracellular NFTs are present during early preclinical until terminal stages of AD, their ability to activate complement provides a mechanism for initiating and sustaining chronic, low-level inflammatory responses that may have cumulative effects over the disease course. This supports the idea that complement system cascade intervention might be a useful pharmacological approach to treat early stages of AD.

Proteomic analysis of MCI and AD plasma in this study revealed a number of proteins which were significantly altered between the three groups. The majority of these proteins were acute phase reactants, including proteins which were related to the complement system (Table 4.3). This data is consistent with a number of other published proteomics studies, which have

shown changes in complement protein levels and other acute phase proteins in AD plasma
[536,551-553]. A summary of proteins found altered in such studies is provided in Table 4.6 and some of the proteins found in our study overlap with those found by other groups using larger cohorts of patients. Furthermore, one study has also shown a correlation between brain hippocampal volume changes and plasma levels of acute phase proteins, including complement [551], suggesting a mechanistic link.

Table 4.6 Summary of previous studies showing changes in acute phase proteins in Alzheimer's disease.

Name	Site of effect	Function	Modification	Reference
Alpha-2-macroglobulin	plasma	Inhibitor of coagulation; inhibitor of fibrinolysis	Increased in MCI and AD	[536,552]
Complement C3	plasma	Most abundant protein of the complement system, enhances response	Increased in AD	[554-557]
Complement C4	plasma	Protein involved in the complement system and undergoes cleavage	Increased in AD	[552,558]
C4b-binding protein	plasma	Inhibits C4 and binds necrotic cells	Decreased in MCI and AD	[536]
Complement C5	plasma	Fifth component of the complement pathway	Increased in MCI	[536]
Complement C9	mRNA and protein levels, vascular amyloid deposits	Involved in MAC formation	Increased in AD brain areas, increased deposition in vascular plaques	[554,555,559]

Complement factor H	plasma	Regulation of alternative pathway of the complement system, ensuring no damage to host tissue	Increased in AD	552,560
Fibrinogen	plasma	Involved in blood clotting	Decreased in AD	536,557,561,562
Haptogloblin	Plasma, CSF	Binds free haemoglobin thereby reducing its oxidative activity	Increased in AD plasma, decreased in CSF of MCI and AD patients Other studies show increase in CSF of AD	563,564
Hemopexin	plasma and CSF	Binds heme, preserves iron levels in the body	Increased in AD	558,565,566
Thrombin	Brain tissue, amyloid plaques, neurofibrillary tangles	Coagulation protein that converts fibrinogen into fibrin, also catalyses other coagulation related reactions	Increased in AD	567-569
Transthyretin	Plasma, CSF	Carrier of the thyroid hormone thyroxine	Decreased in AD	536,565,570,571

Interestingly one of the complement proteins that was downregulated in the AD plasma, complement 4 binding protein (C4BP), is a complement inhibitor which is detected in Aβ plaques and on apoptotic cells in the AD brain [572]. *In vitro*, C4BP binds apoptotic and necrotic but not viable brain cells. It also binds to Aβ(1-42) peptide directly and limits the extent of complement activation by Aβ [572]. C4BP levels in CSF of dementia patients and controls were low compared to levels in plasma and correlated with CSF levels of other

inflammation-related factors [572]. Therefore it possibly protects against excessive complement activation in AD brains.

Fibronectin is present in plaques of AD brains and may modify biosynthesis of APP in microglia [573]. Addition of Aβ to cultured astrocytes has been shown to induce a marked increase in the production of fibronectin [574]. This suggests that *in vivo* fibronectin accumulation in senile plaques may be the result, at least in part, of the response of reactive astrocytes to the presence of Aβ. Fibrinogen is associated with an increased risk of AD and vascular dementia [575]. Our study found fibronectin and fibrinogen to be significantly increased in the AD group compared to controls (Table 4.3).

Furthermore, we have also shown that treatment with AD plasma can affect cellular bioenergetics in a microglial cell line, by increasing glycolysis to compensate for declining oxygen consumption and mitochondrial respiration (Figure 4.3). The reduction in cerebral glucose metabolism, as measured by FDG-PET, is a common diagnostic tool for AD. Positron emission tomography (PET) imaging has identified a strong correlation between the spatial distribution of increased glycolysis, and Aβ plaques in the AD brain [576]. It is estimated that aerobic glycolysis accounts for up to 90% of glucose consumed [577]. By contrast, a recent neuroimaging study which correlated multimodal neuronal parameters including glucose metabolism and hippocampal volume with Aβ deposition in cognitively normal older individuals, did not find any association between the multimodal neurodegenerative biomarkers [578]. However, diminished neuronal integrity and cognitive function correlated with an increased Aβ burden in brain regions that are most affected by AD pathology [578]. Increased glycolysis was associated with better verbal episodic memory in individuals with elevated amyloid levels in another study [579]. The increased shift towards glycolysis may occur in regions of the brain most vulnerable to insult, or may occur in response to Aβ accumulation during ageing. Loss of this protective mechanism may increase the vulnerability of certain brain regions to Aβ-induced neurotoxicity.

Quantitative iTRAQ analysis of glial cells treated with human AD plasma showed the highest number of dysregulated proteins (Table 4.4). The most significantly enriched biological

process was glucose metabolism (Figure 4.5 and Table 4.5), and a significant number of upregulated glycolytic proteins were found (highlighted in bold in Figure 4.4), which is in agreement with the ECAR effect we found in cellular biogenetics using mitochondrial function assays (Figure 4.3). Increased expression of glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, enolase, aldolase and pyruvate kinase may increase glycolytic flux leading to the accumulation of pyruvate and thus stimulating anaerobic metabolism to lactic acid. We found an increased level of LDH activity in the cell culture media and an increase in extracellular acidification, as indicated by the increase in ECAR in the microglial cells exposed to human AD plasma (Figure 4.3). These findings suggest a role for mitochondrial bioenergetic deficits in AD pathogenesis. Our study is consistent with

previous PET metabolic analyses in individuals with AD, MCI, or incipient to late AD[580]. Our findings are also consistent with microarray analyses and activity assays of ageing, incipient AD, and AD human samples and rodent models which indicate that genes and the catalytic activity of several glycolytic enzymes are altered in AD or MCI patients[581,582]. Similarly, increased amyloid production and nerve cell atrophy, have been shown to induce mitochondrial dysfunction[583]. Overexpression of pyruvate dehydrogenase kinase and lactate dehydrogenase in neurons has been shown to provide resistance to Aβ toxicity and reduces mitochondrial respiration and oxidative stress[584]. Previous proteomic studies have also revealed that enzymes involved in energy metabolism show altered oxidative modification in the AD brain[585]. A recent study has also shown a number of proteins significantly oxidised in the Down syndrome brain with and without AD pathology[586]. A significant number of proteins involved in energy metabolism were identified including some of the glycolysis enzymes which we found altered in this study.

We also report an increase in the protein expression of the enzyme transketolase in microglial cells in response to human AD plasma. Transketolase is a thiamine-dependent enzyme which catalyses the first reaction in the pentose phosphate pathway. Transketolase alterations have been previously identified in (i) several probable AD patients regardless of age-of-onset and severity of disease; (ii) all early-onset AD patients and APOE ε4/4 carriers; and (iii) nearly half of asymptomatic AD relatives[587]. Increased transketolase activity has been correlated with increased levels of BACE1, the key rate-limiting enzyme for the production of the Aβ peptide[587].

Increased oxidative stress, mitochondrial dysfunction and alterations to energy metabolism have all been identified as early events in the pathogenesis of AD. Cellular models for AD display functional impairment of the mitochondrial respiratory chain, and a decrease in oxygen respiration and ATP production [588]. All functional measures highlight biogenetic impairment in AD cells and correlation with amyloid peptide accumulation [588].

Factors which induce the upregulation of glycolysis in glial cells treated with AD plasma remain unclear. However, aerobic glycolysis has been correlated spatially with amyloid deposition in AD brains [576]. Furthermore, elevated levels of the enzymes pyruvate dehydrogenase kinase and lactate dehydrogenase provide resistance to amyloid and other neurotoxins [584]. The ability of the brain to maintain expression of these enzymes involved in mitochondrial energy metabolism may explain why some individuals show high levels of amyloid deposition without neurodegeneration [584,589-591]. In our study, the indicators of increased glycolysis in microglia may be a compensatory response to the loss of cell viability and mitochondrial function following exposure to AD plasma, very possibly triggered by complement and/or other inflammatory factors. Altered activities of key glycolytic enzymes have been found in hippocampal, frontal and temporal cortex of AD brains and thought to possibly be due to the astrocytosis that occurs in AD [581].

A number of chaperone proteins such as protein disulfide isomerases were found to be downregulated in cells treated with MCI and AD plasma. Protein disulfide isomerases can inhibit the aggregation of misfolded proteins and are also involved in modulating apoptosis and endoplasmic reticulum redox balance [592]. It has been shown that the proinflammatory activation of microglia suppresses mitochondrial function and increases glycolysis and overexpression of the mitochondrial chaperone mortalin can attenuate this effect [593]. Heat shock proteins are chaperone proteins which have an important impact on the proteotoxic effects of tau and Aβ accumulation. Immunohistochemical studies and expression analyses in AD brain tissue have shown that expression levels of a number of heat shock proteins are upregulated and it has been hypothesised that this effect may be due to a hybridisation of activated glia and dysregulated/stressed neurons [594,595]. Dysregulated chaperone proteins in

the cells in our study may reflect a homeostatic attempt to clear toxic plasma proteins and protect mitochondrial function. Some chaperone proteins such as heat shock proteins were upregulated wheras other chaperones, such as several protein disulphide isomerise (PDI) isoforms were found to be downregulated. It is known that HSP90alpha expression is induced when a cell experiences proteotoxic stress. Since it, and several other HSPs, are upregulated, in the iTRAQ results, it could be inferred that it is a homeostatic response to such stress induced by the addition of AD/MCI plasma. So perhaps it is not just complement, but also proteotoxicity of the AD plasma which is inducing the proteomic changes in the glia. It has been shown that PDIs can be anti-apoptotic, but they can also have toxic effects, particularly when nitrosylated, in which case they are associated with protein misfolding [596-598]. Therefore, in a high oxidative stress environment it may be advantageous to downregulate PDI expression to minimise the potential unwanted side effects of exacerbated proteotoxicity. There is evidence from the proteomic data that oxidative stress is indeed at play, because several peroxidases are upregulated. Therefore the dual oxidoreductase and chaperone roles of PDI may not always act synergistically.

Cytoskeletal proteins are another group proteins with altered expression level identified in our iTRAQ proteomics data (Table 4.5). Nestin expression is seen during pathological situations and is a marker of cell proliferation and is reduced in the cells treated with AD plasma, supporting our MTT data (Table 4.1 and Figure 4.1). Ezrin and moesin are involved in crosslinking actin filaments with plasma membranes and stabilising microtubules respectively [599,600] and both were found to be upregulated in cells treated with AD plasma. The antioxidant proteins peroxiredoxins were also found to be elevated in cells exposed to AD plasma which may be another indication of a compensatory mechanism triggered to attenuate the toxic effects of AD plasma.

In conclusion, this study shows that plasma expression levels of acute phase proteins are altered in AD and MCI, supporting a role for increased inflammatory activity in this disease which is detectable in the plasma. Cells exposed to AD plasma show an upregulation of glycolysis possibly as a compensatory mechanism in response to compromised mitochondrial function. Together our observations support an emerging body of evidence that inflammation and metabolism are closely linked processes, which are regulated by transcriptional and

protein translation events [601,602]. In the CNS, complement proteins are synthesised by a variety of cells including neurons, microglia, astrocytes, oliogendrocytes and endothelial cells [603]. Since disruptions in the blood-brain-barrier have been reported in AD there is a possible source of increased complement levels in the AD brain from plasma. It is however likely that there may be other thermolabile factors in disease plasma which facilitate the cytotoxic and glycolytic effects in microglia, one example may be micro RNAs as they are emerging as important factors in neurogenesis, synaptic plasticity and AD [604,605]. Other yet to be characterised substances may also make a significant contribution. This study shows that the use of biological assays in combination with proteomic analysis may help uncover possible mechanisms of disease and may be complementary techniques to validate cellular changes and effects in a range of biological samples.

Chapter 5

Effects of Polyphenols on Cell Viability, Mitochondrial Dysfunction, and Amyloid Aggregation

Introduction

Alzheimer's disease is a multifactorial neurodegenerative condition with a complex pathology including oxidative stress, metal deposition, formation of aggregates of amyloid and tau and enhanced inflammatory responses[606]. Drugs targeted toward reduction of amyloidal load have been identified, and some have been trialed [607,608], but there is no effective pharmacological treatment for combating the disease so far. Early intervention, or preventative measures targeting risk factors may be a better approach than treatment once the disease and neuronal death have substantially progressed. Attempts at disease reversal are likely to be both more challenging and have less certain outcomes than bypassing or at least decelerating disease progression. However this approach requires early intervention, well before substantial neuronal damage, and ideally at preclinical stages. Such intervention is likely to be most efficacious if sustained over a long period of time, and should therefore carry no or low risk of side effects. Consequently natural products may be an important potential avenue for disease intervention. Many are components of foods or indigenous medicines which have been used in human communities for aeons, and therefore substantial knowledge is available about safety or potential side effects (in a sense a natural clinical trial). Furthermore such natural substances can be selected to target multiple risk factors and pathways dysregulated in AD, such as oxidative stress, metal ion accumulation, and most importantly the hallmark protein aggregates which define the disease. AD is characterized by the progressive loss of cholinergic neurons in the cortex and hippocampus along with global cognitive decline and by the accumulation $A\beta$ deposits in the form of extracellular senile plaques[609]. $A\beta$ is produced by the aberrant cleavage of APP, by the sequential action of β- and γ-secretases, that gives rise to a 39–42 amino acid residue polypeptide which is part of the α-helical transmembrane segment of APP [609]. $A\beta$ released extracellularly after the catalytic activity of secretases, undergoes conformational modifications and becomes prone to aggregation [609]. The mechanisms by which the self-assembly of $A\beta1-42$ leads to toxicity, are not well understood. This is because all transient intermediates and oligomers preceding fibrils comprise a very large number of conformations. $A\beta$ aggregates can exist in multiple forms, from soluble oligomers or protofibrils to insoluble large fibrils, which are responsible for various pathological effects [610]. Amyloid fibrils range in size from nanometers to microns and are composed of aggregated peptide β-sheets formed from parallel or anti-parallel alignments of peptide β-strands [610]. The fibrillogenesis process proceeds with the deposition of oligomers into

protofibrils, characterized by a 2–5 nm diameter observed by electron microscopy, and then in protofilaments or fibrillar aggregates larger in size (7–13 nm wide up to 30 nm) [610]. At present, the biological role of different aggregation states of Aβ protein remains unclear. Indeed, whether the accumulation of these large fibrils is the cause or rather the consequence of the disease remains to be elucidated. It is noteworthy that numerous inherited forms of AD display mutations of the APP gene that increases susceptibility to β- and γ-secretase cleavage and the aggregation properties of Aβ, unequivocally linking the production of Aβ to the disease [611]. Moreover, several studies using a variety of cellular and animal models have shown that Aβ neuronal toxicity may depend on soluble oligomeric fractions rather than on mature fibrils [612-614]. Therefore, preventing oligomer formation is an attractive approach as a potential treatment for preventing or delaying the onset of sporadic and familial AD.

Mitochondria, which are abundant in synapses and neurons, are a target site for amyloid oligomer complexes. Mitochondria are critical regulators of neuronal survival and death, and there is strong evidence that mitochondrial dysfunction might represent an important initiator of the pathophysiological cascade in AD [85]. Mitochondria are important targets of the Aβ peptide, and disturbances of membranes and bioenergetics may be key triggers of neuritic damage leading to dementia [615]. Recognised mitochondrial targets of Aβ include the translocase import machinery, enzymes involved in the Krebs cycle, respiratory chain enzymes, the mitochondrial permeability transition pore (mPTP), the mitochondrial matrix protein Aβ-binding alcohol dehydrogenase (ABAD), and mitochondrial DNA [615-617].

Polyphenols are the most abundant micronutrients of our daily food, occupying the major portion of naturally occurring phytochemicals in plants [618]. They are produced in plants as a protective response to various stress stimuli, including bacterial and viral infections, and UV-light [618]. They are well known for their antioxidant capacity and the ability to scavenge the free radicals formed during pathological processes such as cancer, cardiovascular diseases and neurodegenerative disorders [618]. Dietary intake of polyphenols is known to attenuate the progression of AD by showing strong potential to tackle the alterations and reduce the risk of AD [619]. A number of polyphenolic compounds showing promising results against AD pathologies have been identified [619]. Recent research is unravelling novel molecular mechanisms and elucidating specific interactions of polyphenols with their targets in a variety of pathways involved in neurodegeneration.

Numerous polyphenols from natural products such as turmeric, grapes, red wine and green tea, have been reported to inhibit Aβ aggregation and toxicity and show promise *in vivo*, including epigallocatechin gallate (EGCG), curcumin and resveratrol [183,190,620-626]. The major tea polyphenol, EGCG, shows neuroprotective activity against neurological disorders, and inhibits toxicity induced by Aβ and α-synuclein oligomers [183]. Moreover, using isothermal titration calorimetry at different EGCG and salt concentrations, it has been shown that the interactions between Aβ1-42 and EGCG are mainly hydrogen bonds in the region 1–16 and hydrophobic in the region 17–42 [621,627]. Experimentally, EGCG has been reported to generate unstructured, off-pathway Aβ1-42 oligomers, and by this means offsets formation of the structured toxic forms [620].

Figure 5.1: Chemical structures of the polyphenols EGCG, Curcumin and Resveratrol
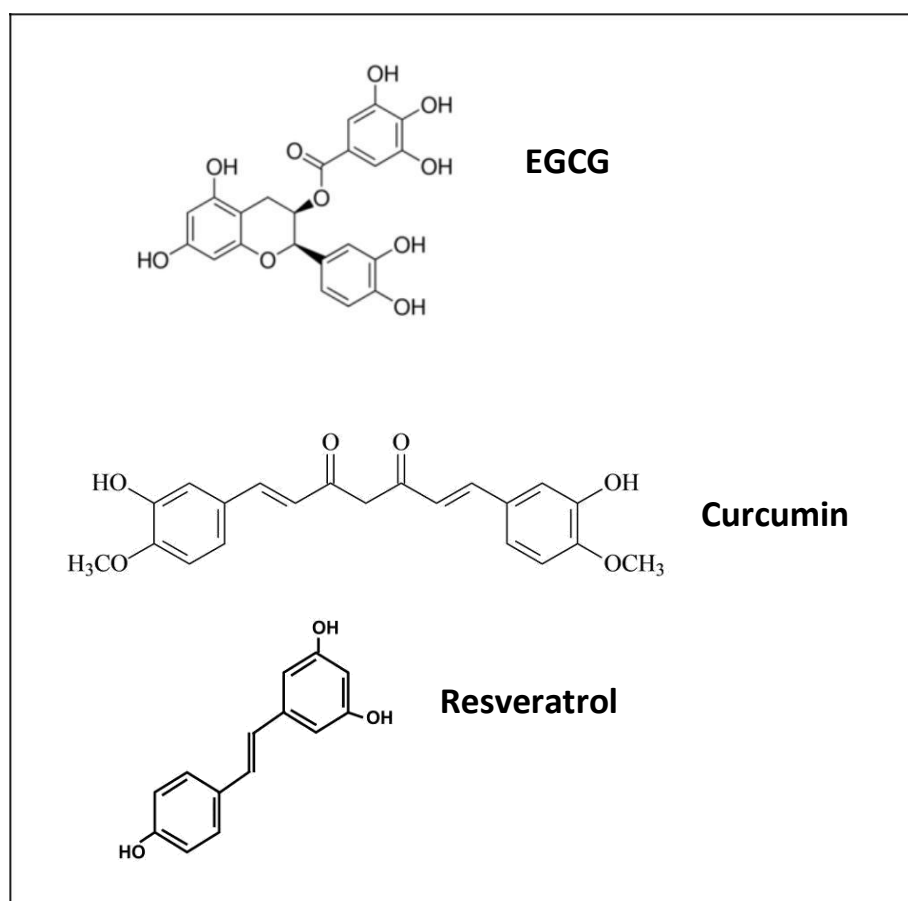
Polyphenols exhibit remarkable antioxidant and anti-inflammatory activities that may exert a leading role in reducing age-related oxidative stress and inflammation, thereby hampering the

onset and progression of degenerative diseases. Oxidative stress is a key hallmark in AD. It occurs early in disease pathogenesis and can exacerbate its progression [26]. Several causes of oxidative stress have been determined, which point to both mitochondrial dysfunction and neuroinflammation [50]. Based on these observations, various neuroprotective strategies against reactive oxygen species-mediated damage have been proposed for the treatment of AD. Epidemiological studies have suggested associations between lack of consumption of phenolic-rich foods or beverages and various diseases, such as stroke, cardiovascular diseases, cancer, and neurodegenerative diseases [618,628]. Conversely polyphenolic and flavenoid rich diets (such as the Mediterranean diet) have documented health benefits [618,628]. For example, cognitive performance in elderly subjects is associated with the consumption of polyphenol-rich foods such as chocolate, red wine, and tea [628-632]. As a modifiable lifestyle factor, diet is an important target, since (1) the benefits of calorie restriction on longevity have been well documented [322,325,633], and (2) the disease preventive benefits of specific dietary nutrients, such as the multitude of polyphenolic constituents, are emerging. Therefore a diet with a high polyphenolic/calorie ratio is likely to be both disease preventative and longevity promoting.

A very important feature of polyphenols is their multiple modes of action. Firstly, their well known antioxidant capacity may counteract oxidative stress induced by $A\beta$, thus hampering neurodegeneration and blunting the self-propagating effect of the vicious cycle between $A\beta$ generation and oxidative stress [634,635]. Secondly, these compounds may competitively interact with aromatic residues in amyloidogenic proteins and block the self-assembly process [635]. Furthermore, they may modulate $A\beta$ production by stimulating the α-secretase pathway and inhibiting the β-and γ-secretase pathways [635]. Some phenolics show a strong enzymatic inhibition of the cascade of $A\beta$ generation and other phenolics show a much pronounced physico-chemical effect against amyloid aggregation and fibrillogenesis. Polyphenols also have metal chelating properties [636]. Copper, iron or zinc are present in $A\beta$ deposits and induce the production of hydrogen peroxide, which may mediate oxidative damage in AD [77,128]. Therefore another therapeutic approach for preventing AD pathogenesis may be linked to metal chelation. These important chemical characteristics of polyphenols might be harnessed to ameliorate multiple pathological processes and pathways and prevent or postpone the onset of neurodegeneration.

EGCG is a member of a family of plant-derived flavan-3-ols, and is the major polyphenolic component of green tea [183]. EGCG directly binds to natively unfolded Aβ, reduces thioflavin T fluorescence, and promotes the assembly of large, spherical oligomers which seem to be off-pathway species (non-toxic or low toxicity variants), as they are unable to seed fibrillogenesis [620]. Importantly, EGCG-stabilized oligomers are not toxic to cells, and are not recognized by the A11 antibody, which binds to pathologically relevant high-molecular weight oligomers formed by numerous amyloid peptides [620]. It has also been demonstrated that EGCG is able to remodel Aβ mature fibrils into smaller, amorphous protein aggregates that are nontoxic to mammalian cells, by direct binding to the β-sheet-rich aggregates and mediating a conformational change without generating potentially toxic monomers or oligomers [606,637]. Notably, *in vivo* administration of EGCG improves spatial cognition in rats and reduces Aβ levels and plaque by as much as 50% in Tg2576 transgenic mice [186,196,638]. This effect may be due to its ability to counteract Aβ fibrillogenesis.

Curcumin is the main constituent of the spice turmeric, whose extensive use is thought to account for the significantly lower prevalence of AD in the Indian population [639]. *In vitro* studies have shown that curcumin can inhibit fibril formation and destabilizes preformed fibrils [640]. Curcumin also binds to plaques and reduces amyloid levels *in vivo* [624]. The ability of curcumin to bind Aβ and to inhibit its aggregation is due to its biphenolic structure that mimics congo red. Curcumin comprises three structural features: a hydroxyl substitution on the aromatic endgroup, a rigid linker region between 8 and 16 Å in length, and a second terminal phenyl group [606]. *In vitro* studies have revealed that curcumin can only bind to the fibrillar conformer of Aβ, and not to shorter, aggregation-incompetent Aβ fragments [606]. Tg2576 transgenic mice fed with curcumin for six months showed decreased levels of Aβ and inflammatory cytokines in the brain [624]. The ability of curcumin to cross the blood–brain barrier, to bind to Aβ and to induce rapid dissolution of plaques was verified using multi-photon microscopy in APPsw/PS1dE9 mice [641]. Furthermore, curcumin was found to reduce the levels of interleukin-1β and isoprostanes (an index of oxidative stress) in the central nervous system and ameliorated amyloid plaque burden in transgenic mice [190].

Resveratrol is a stilbene polyphenol which holds great promise as a therapeutic agent. Many studies have demonstrated its beneficial effects, though some doubts still remain regarding its clinical efficacy [202,642,643]. Epidemiological studies have suggested that moderate red wine consumption may protect against the development of AD and other studies have revealed that

certain components of red wine, including resveratrol, reduce Aβ production and toxicity by a number of different mechanisms [202,626,644]. Recent studies on the effects of grape seed polyphenolic extracts revealed that these compounds prevent β-structural transitions, thereby reducing formation of oligomers and protofibrils, and attenuate Aβ-induced cellular toxicity [606].

In this chapter, we explored the ability of three common polyphenols to prevent the neurotoxicity caused by amyloid and Fenton chemistry on astrocytes using a brain cell model for oxidative stress and amyloid toxicity. We also investigated the effects of polyphenol treatment on amyloid aggregation and the metal chelating properties of these compounds using electron microscopy and mass spectrometry respectively.

5.2 Methods

5.2.1 Preparation of Aβ peptides

Recombinant Aβ(1-42) peptides were reconstituted in HFIP, vortexed, aliquoted and the HFIP evaporated in a fume hood. The resulting peptide films were dried under vacuum in a Speed Vac for 2 hours and the desiccated peptides stored at -20 degrees until use. 5µM concentration of peptides were resuspended in neat DMSO followed by bath sonication for 10 minutes and stored at -20°C freezer until required. 10mM HCL was added to peptides and samples incubated at 37^oC for 48 hours to inititate fibril formation for TEM experiments.

For cell culture experiments soluble aliquots were added to cells and incubated for at 37^oC for 48 hours to inititate fibril formation.

5.2.2 Cell Culture

U251 cells are a human astroglial cell line obtained from embryonic fetal human astrocytes following viral transformation [518,519] and were a generous gift of Prof Gilles Guillemin (Macquarie University, Sydney, Australia). These cells express antigens present on adult human astrocytes, secrete pro-inflammatory cytokines upon activation, exhibit properties of primary human astrocytes and have been successfully used as a model of microglial activation by others [518,520]. Cells were maintained in RPMI1640 cell culture medium, supplemented with 10% heat inactivated foetal bovine serum, 2 mM l-glutamine, and 1%

penicillin/streptomycin, at $37^{o}C$ in a humidified atmosphere containing 95% air/5% CO_2. Before experimentation, cells were seeded into 24 or 96 well culture plates to a density of approximately $1x10^{4}$ or $2x10^{3}$ cells respectively.

5.2.3 MTT Cell Proliferation Assay

In actively proliferating cells, an increase in 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) conversion in cells relative to controls represents an increase in cellular proliferative activity. Conversely, in cells that are undergoing apoptosis, MTT conversion, and thus biological activity, decreases. Cell proliferation was analysed using established protocols in a 96 well plate format [645]. Briefly MTT substrate was prepared at a final concentration of 0.5mg/ml, added to cells in culture and incubated for 2 hours. The quantity of formazan (proportional to the number of viable cells) is measured by recording changes in absorbance at 570 nm using a Amersham Biosciences Biotrak II plate reading spectrophotometer (Buckinghamshire, UK). When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells. The formazan product of the MTT tetrazolium accumulates as an insoluble precipitate inside cells as well as being deposited near the cell surface and in the culture medium. Acidified isopropanol was added to all wells prior to obtaining absorbance readings to solubilize the formazan product, stabilize the color, avoid evaporation, and reduce interference by phenol red and other culture medium components.

5.2.4 XF24 Microplate-Based Respirometry as a Measure of Mitochondrial Function
A mitochondrial function assay was used to determine the effect of selected polyphenols on oxygen consumption rates (OCRs; an indicator of mitochondrial respiration) and extracellular acidification rates (ECARs; a measure of glycolytic flux) following co-treatment with redox active metals and/or aggregated Aβ in the microglial cell line. The Seahorse XF24, extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA, USA) was employed and assays performed as previously described [528-530]. Briefly, culture plates were incubated in a CO_2-free incubator at 37°C for 1 hr to equilibrate temperature and pH. The microplate was then loaded into the XF24 and further incubated for 15 min with 3 min and 2 min wait cycles before commencement of the assay. After determination of the basal respiration in the cell

culture, oligomycin (2 μM), carbonylcyanide-p-trifluoromethoxy-phenylhydrazone (FCCP, 500 nM), and antimycin (3 μM) were sequentially added and the OCRs and ECARs for each culture well were quantified for 2 minutes. This allowed the basal control ratio (BCR) and the uncoupling ratio (UCR) to be determined as previously described [531]. Essentially, the BCR is a measure of how close the basal level of respiration is to the maximum level of respiration. The closer this ratio is to 1, the greater the mitochondrial malfunction. The UCR is a measurement of mitochondrial functional integrity and measures the ratio of uncoupled to physiologically normal respiration levels. The greater the maximum level of respiration, the greater the mitochondrial functional integrity.

5.2.5 Transmission Electron Microscopy

An aliquot (5μl) of Aβ and polyphenol samples were spotted onto copper mesh electron microscopy grids (Sigma-Aldrich, USA) for 1min and blot dried. The grids were subsequently stained using 5μl of 2% uranyl acetate for 1min followed by blot drying. Samples were imaged using a Hitachi JEOL 1400 transmission electron microscope, which operates at accelerating voltages up to 120kV. The microscope is equipped with a high contrast lens configuration especially suited to imaging biological, medical or polymer specimens that lack electron density and inherent contrast. Specimens undergo preparation procedures to ensure they are 100 nm or thinner in order to transmit the electron beam. Dense regions within the specimen cause electrons to scatter and an aperture, positioned near the specimen in the electron column, prevents the scattered electrons from reaching the imaging plane. This results in light and dark regions or 'contrast' within the TEM image. The microscope is equipped with a Gatan digital camera and Digital Micrograph software to facilitate acquisition of digital images.

5.2.6 Polyphenol Metal Complexes using Mass Spectrometry

Polyphenol:metal samples (0.5mg/ml:1mg/ml ratio) using copper (II) sulphate and iron (II) sulphate were prepared in methanol and run on an Orbitrap LTQ XL (Thermo Fisher Scientific, San Jose Ca, USA) ion trap mass spectrometer using a nanospray (nano-electrospray) ionization source to generate ions from the analyte in solution. The instrument was calibrated with the manufacturer's standard calibration solution on the day of analysis

using direct infusion into the ESI source. The instrument conditions were optimized for sensitivity of the compounds using the XCalibur LC tune software. The analysis was carried out in positive ion mode using the orbitrap FTMS analyser at a resolution of 60000. Samples (5μL of 1mg/ml polyphenol in methanol) were transferred into a conductive glass needle and inserted into the nanospray source. Ions generated were measured over a 150 to 2000 Da mass range. Data was acquired in full scan mode over 30 seconds and visualised using the Qual Browser feature in Xcaliber 2.1 (Thermo Fisher Scientific, San Jose, Ca, USA).

5.3 Results

5.3.1 Effect of Aggregation on Cell Viability in Astrocytes

Treatment of astroglial cultures with 5μM of fresh soluble amyloid followed by 48hr incubation at $37^{o}C$ (to initiate aggregation) caused a significant drop in cell proliferation. This was prevented by co-incubation with EGCG (Figure 5.2). A further drop was seen with the addition of transition metals iron and copper in which a ~50% drop in cell viability was observed relative to controls. Again EGCG was shown to prevent the toxicity caused by the addition of these metals and amyloid (Figure 5.2). Curcumin and resveratrol did not significantly prevent the toxicity caused by amyloid alone but were shown to significantly prevent the toxic effects of metal addition to amyloid in the same astroglial model (Figures 5.3 and 5.4).

Figure 5.2: Effect of amyloid, metal ion and EGCG treatment on astrocyte viability. 5μM Aβ42 and/or 5μM metals were used for all treatments *p<0.05 vs Control, **p<0.05 vs Aβ42 + 5 μM Fe/Cu, n=6.
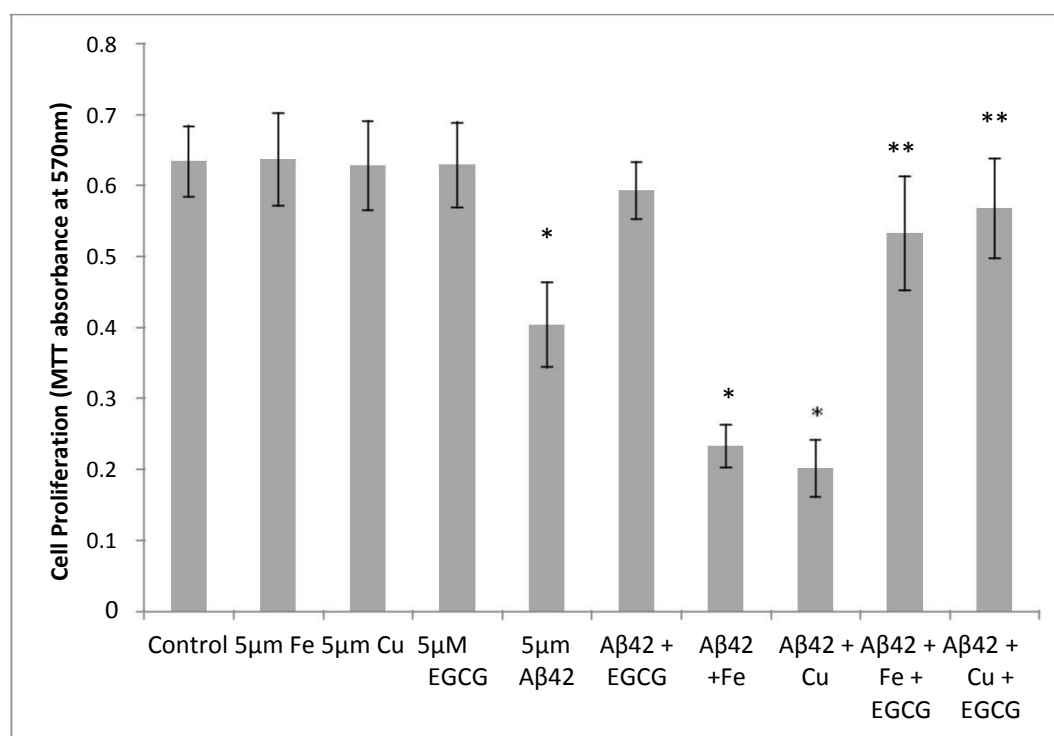
Figure 5.3: Effect of amyloid, metal ion and curcumin treatment on astrocyte viability. 5μM Aβ42 and/or 5 μM metals were used for all treatments *p<0.05 vs Control, **p<0.05 vs 5μM Aβ42 + 5μM Fe/Cu, n=6.
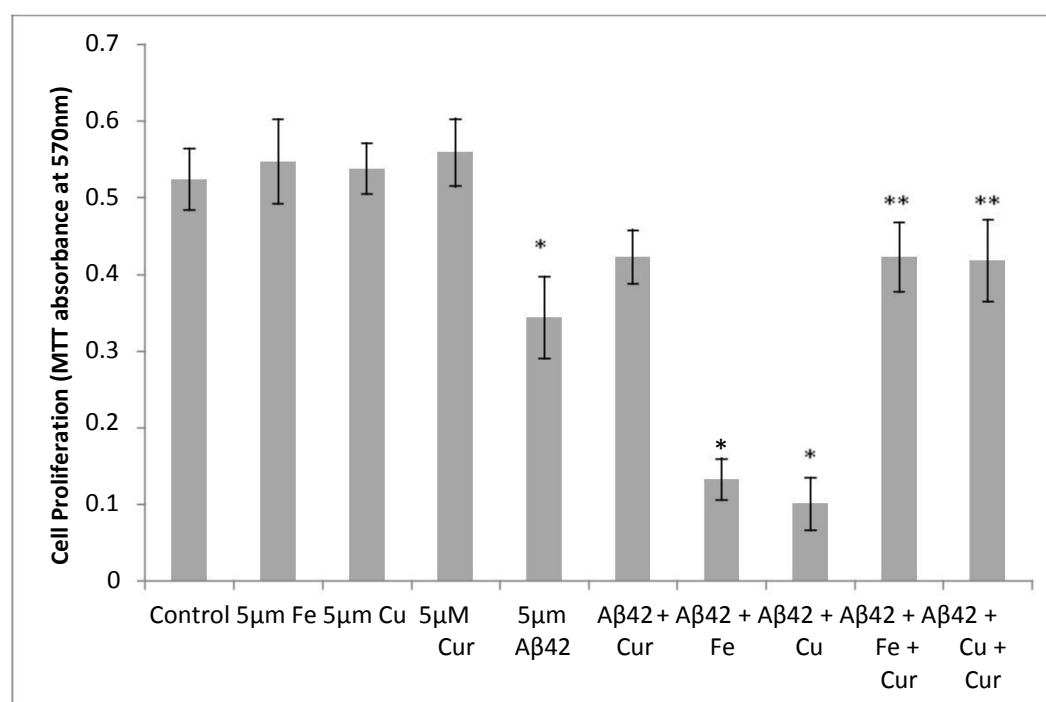
Figure 5.4: Effect of amyloid, metal ion and resveratrol treatment on astrocyte viability. 5μM Aβ42 and/or 5 μM metals were used for all treatments *p<0.05 vs Control, **p<0.05 vs 5μM Aβ42 + 5μM Fe/Cu, n=6.
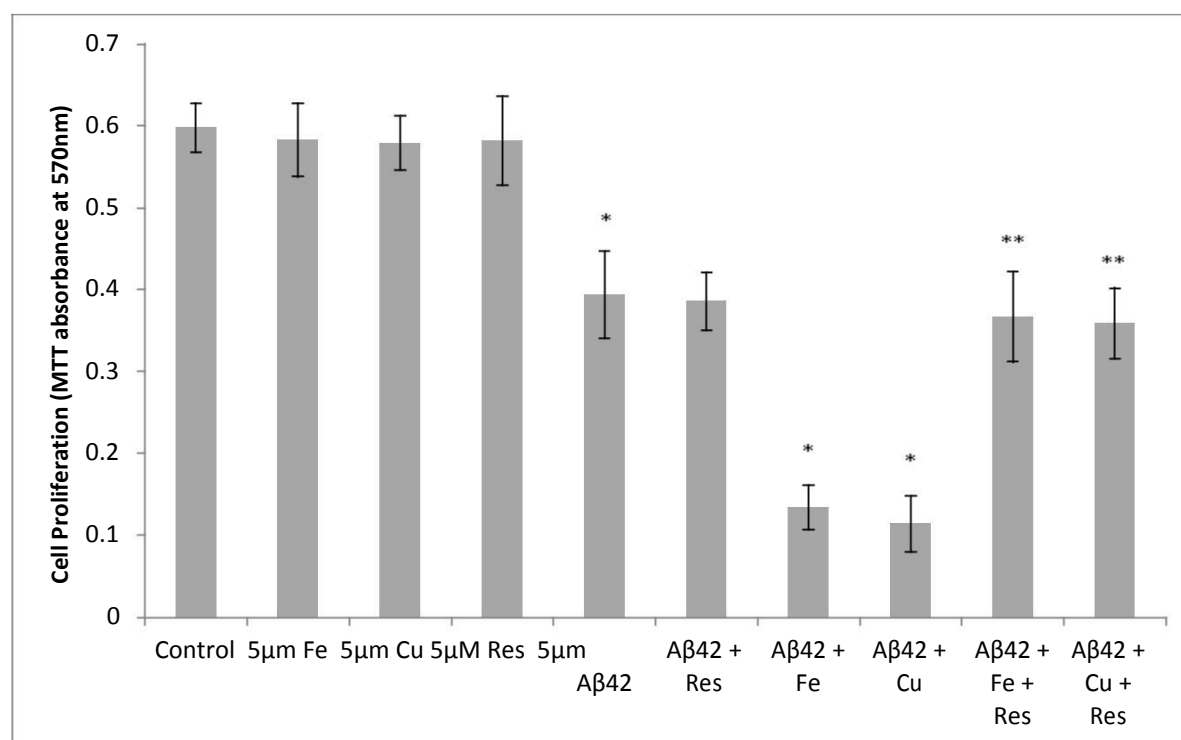
Effect of Polyphenols on Oxidative Stress in Astrocytes

Oxidative stress was modelled in an astrocyte cell line by the addition of hydrogen peroxide (H_2O_2), iron and copper, followed by 24hr incubation at 37°C. The resulting initiation of Fenton chemistry caused a drop in cell proliferation by approximately 75%. All three polyphenols were found to be effective in preventing this toxicity in a dose dependent fashion with 50uM concentrations restoring neurons to control levels (Figures 5.5, 5.6 and 5.7).

Figure 5.5: Effect of EGCG to prevent cell toxicity caused by oxidative stressed initiated by Fenton chemistry (addition of H_2O_2 and Fe^{2+}/Cu^{2+} for 24hrs) to astroglial cells. *$p<0.05$ vs Control, **$p<0.05$ vs 5μM Aβ42 + 5μMFe/Cu, n=6.
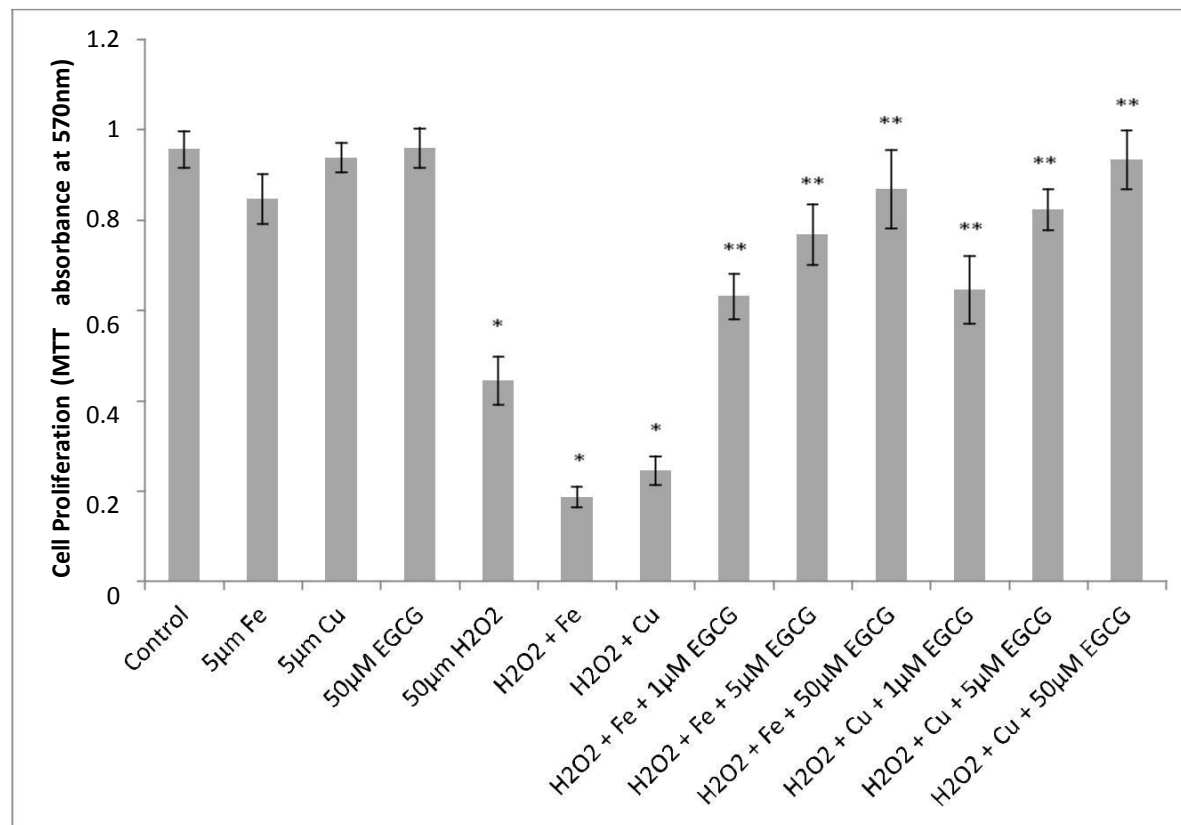
Figure 5.6: Effect of curcumin to prevent cell toxicity caused by oxidative stressed initiated by Fenton chemistry (addition of H_2O_2 and Fe^{2+}/Cu^{2+} for 24hrs) to astroglial cells. *$p<0.05$ vs Control, **$p<0.05$ vs Aβ42 + Fe/Cu, n=6.
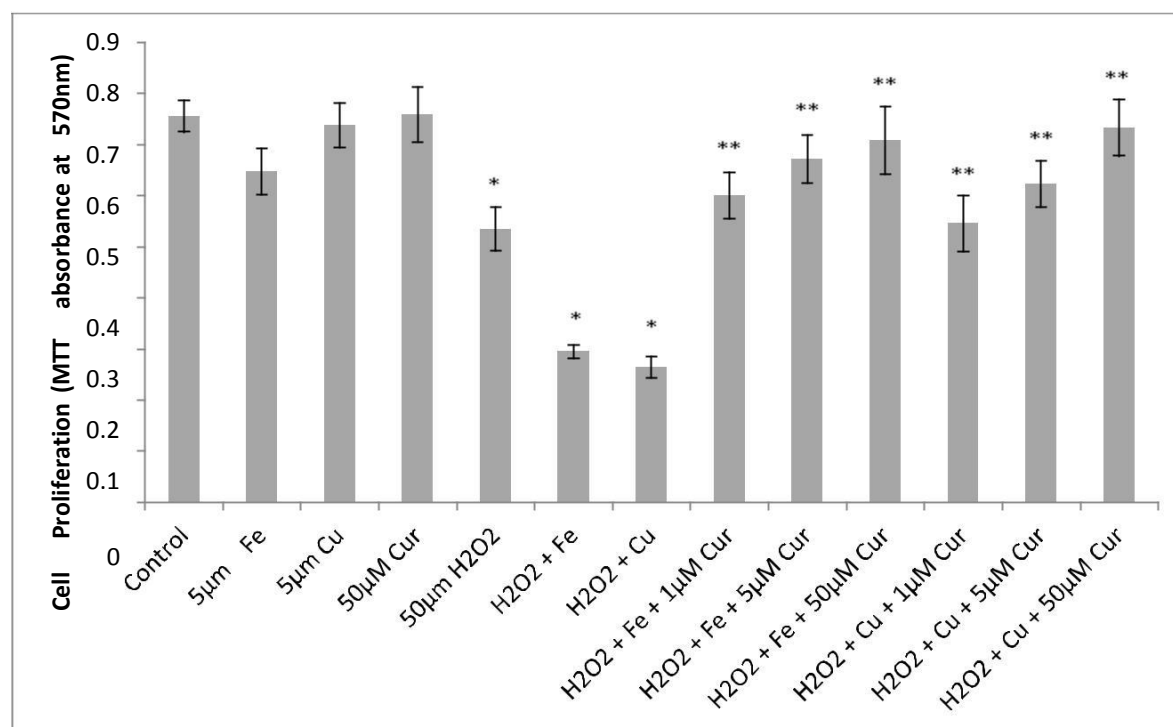
Figure 5.7: Effect of resveratrol to prevent cell toxicity caused by oxidative stressed initiated by Fenton chemistry (addition of H_2O_2 and Fe^{2+}/Cu^{2+} for 24hrs) to astroglial cells. *$p<0.05$ vs Control, **$p<0.05$ vs Aβ42 + Fe/Cu, n=6.
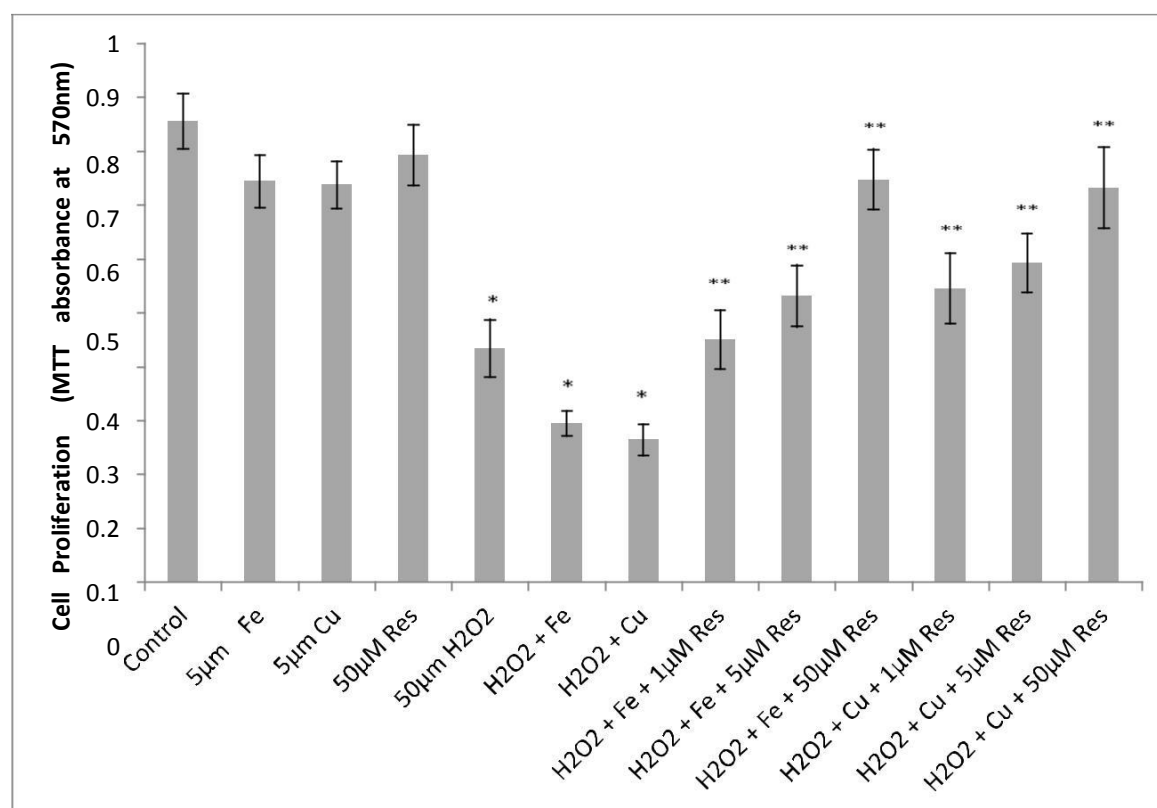
Effect of Polyphenols on Mitochondrial Function in Astrocytes

Using the Seahorse XF24 assay, we observed a significant increase in basal control ratio (BCR), Figure 5.8 and a decline in uncoupling ratio (UCR), Figure 5.9, in astrocytes after 48 hr incubation with soluble Aβ42 (resulting in aggregated Aβ42, and in combination with iron, and copper, consistent with impaired mitochondrial function. Co-treatment with selected polyphenols attenuated mitochondrial function and restored the BCR and UCR to control levels.

Figure 5.8: Effect of amyloid, metal and polyphenol treatment on mitochondrial basal control ratio in cultured astrocytes. *p<0.05 vs Ctrl, **p<0.05 vs Aβ42+Fe/Cu, n=3.
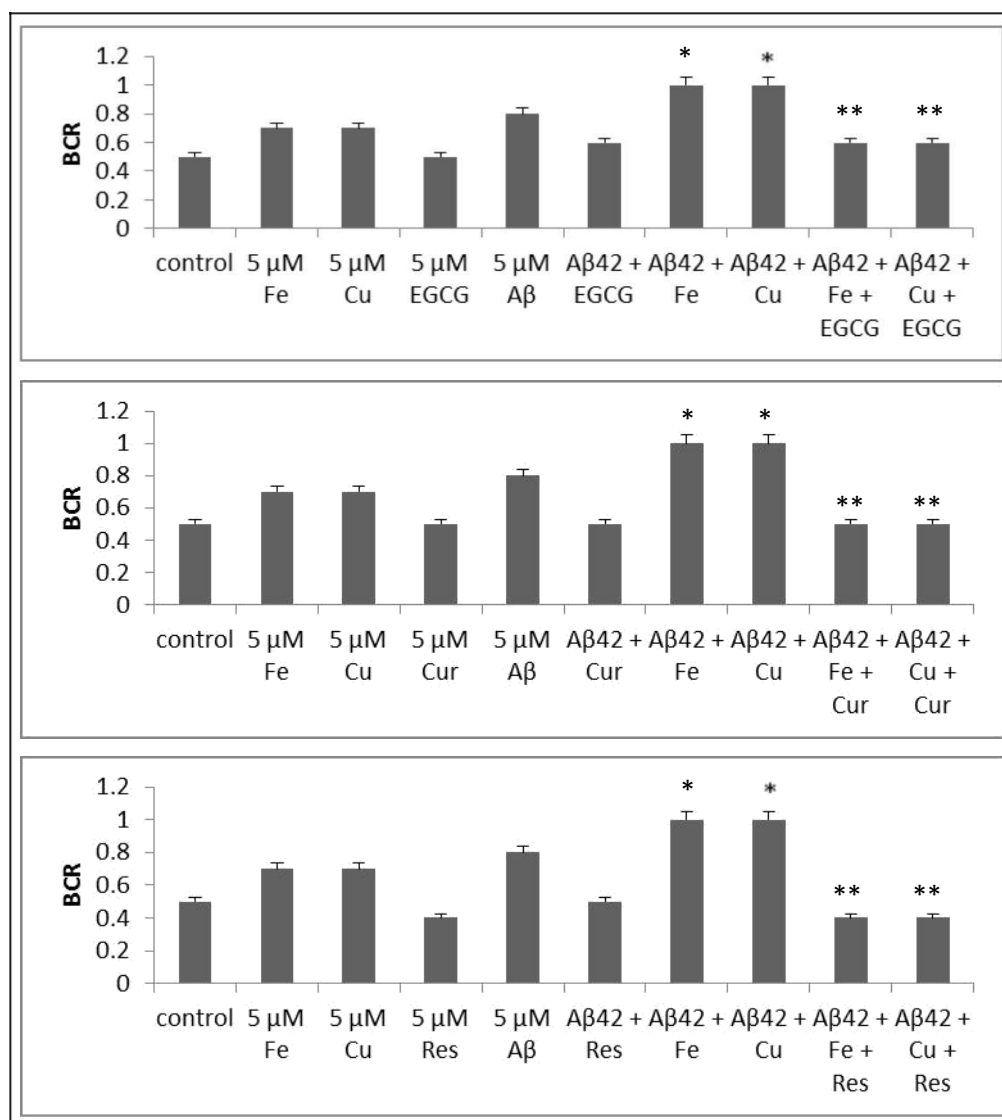
Figure 5.9: Effect of amyloid, metal and polyphenol treatment on mitochondrial uncoupling ratio in cultured astrocytes. *$p<0.05$ vs Control, **$p<0.05$ vs Aβ42+Fe/Cu, n=3.
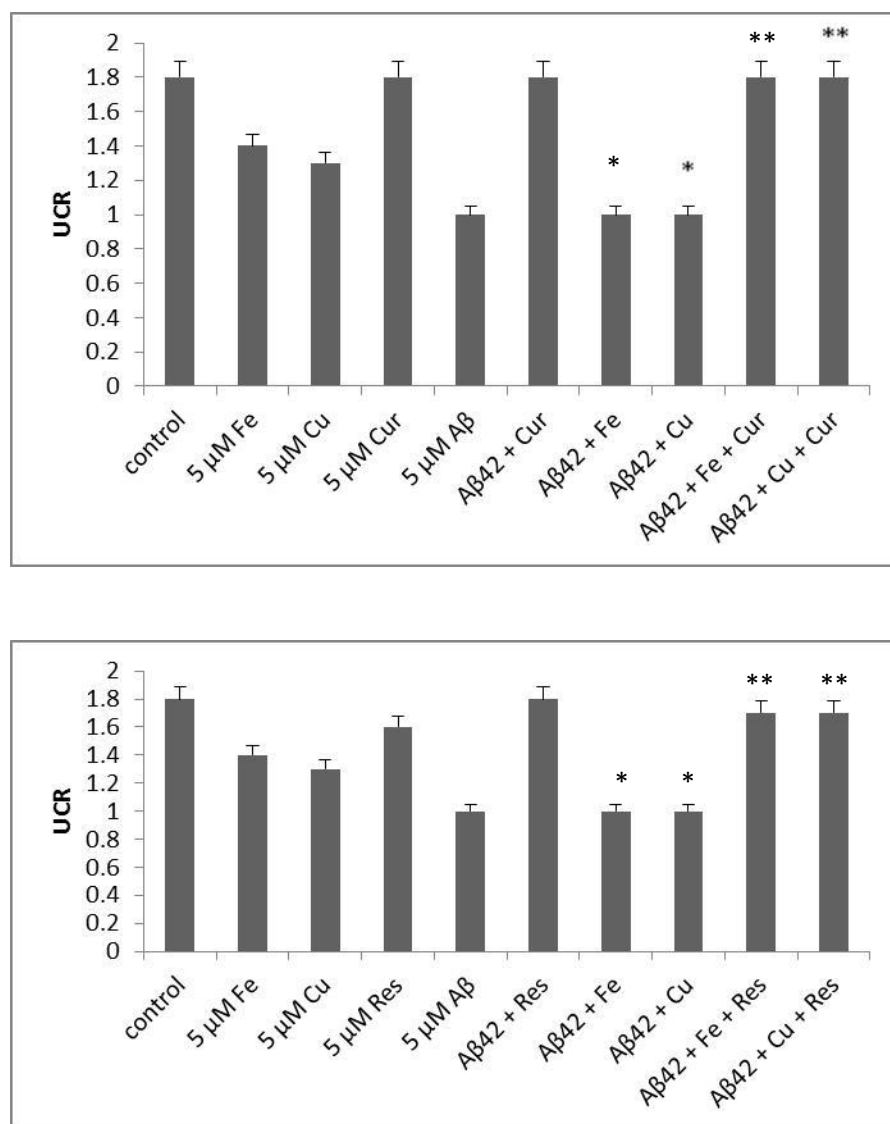
5.3.4 Amyloid Aggregation in the Presence of Polyhpenols

Electron microscopy of amyloid samples co-incubated metals showed that both iron and copper enhanced aggregation of the peptide with iron seeming to be the more potent of the two (Figure 5.10). Addition of EGCG, curcumin and resveratrol created structures distinct from those formed in the presence of metal ions (Figure 5.10). Structures formed in the presence of amyloid peptide and of the polyphenolics with the addition of iron and copper to enhance aggregation, resembled those in amyloid peptide and polyphenolics alone more so

than those with amyloid peptide and metal ion alone (Figure 5.10). This was particularly true for the ECGC and curcumin samples.

Figure 5.10: Representative transmission electron microscopy images of soluble Aβ42 after co-incubation with metals (Panel A) and polyphenols, EGCG (Panel B), Curcumin (Panel C) and Resveratrol (Panel D) for 48hrs at 37°C. Aβ1-42 alone was shown to form some fibril clusters (Panel A), however in the presence of metal, more complex, larger fibril networks were observed (Panel A). Polpyhenols both with and without metals were found to produce more pronounced dense amorphous aggregates with not much fibril structures found (Panel B-D).

B

Aβ + EGCG

Aβ + Fe + EGCG

Aβ + Cu + EGCG

C

Aβ + Cur

Aβ + Fe + Cur

Aβ + Cu + Cur

D

Aβ + Res

Aβ + Fe + Res
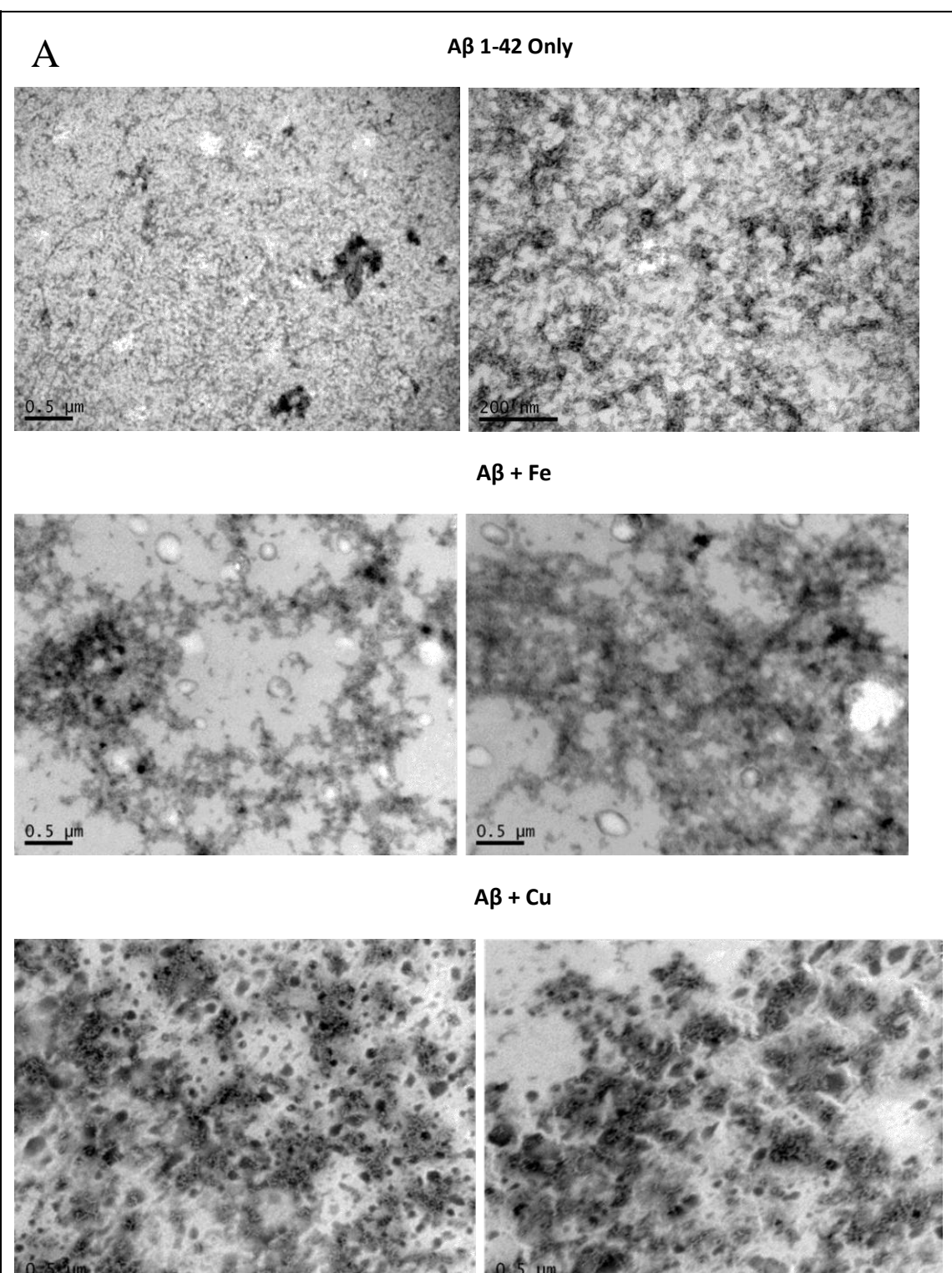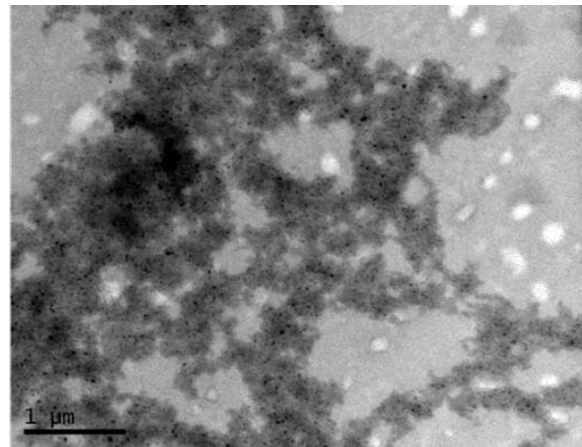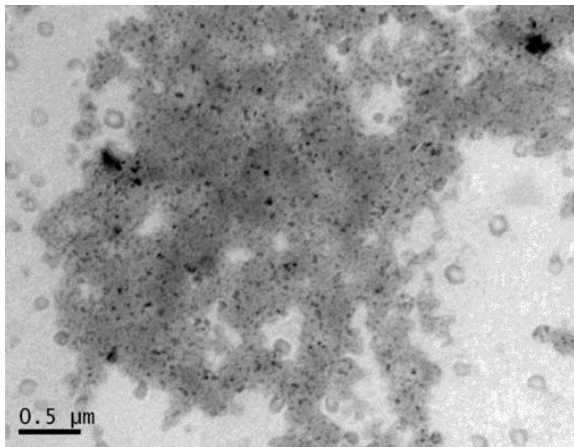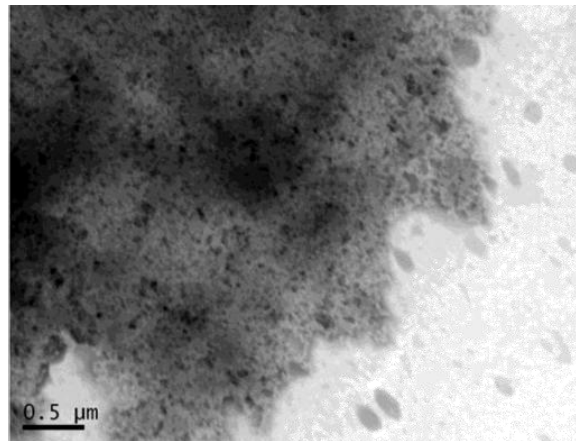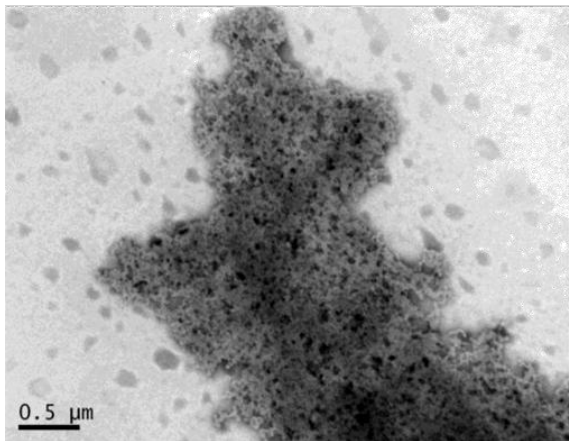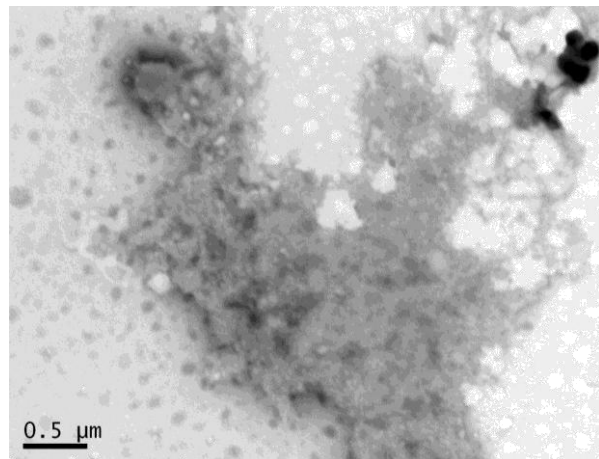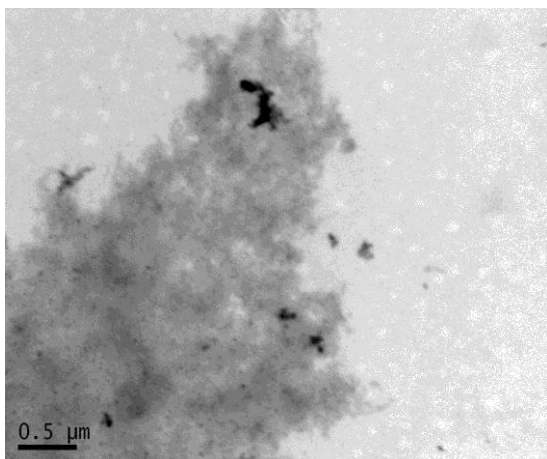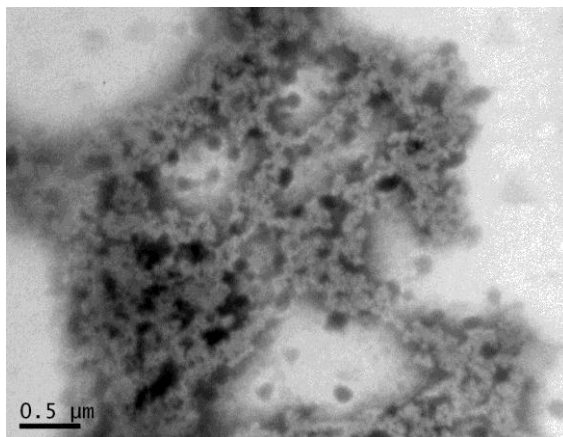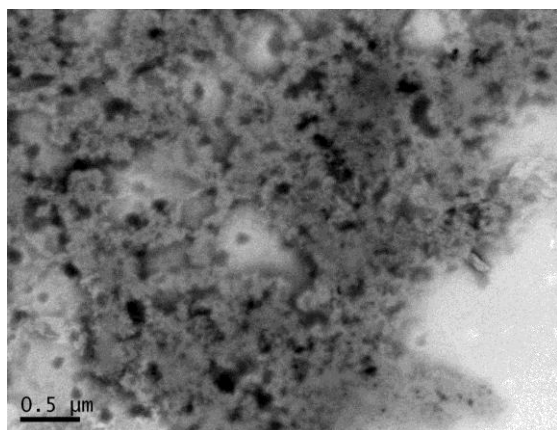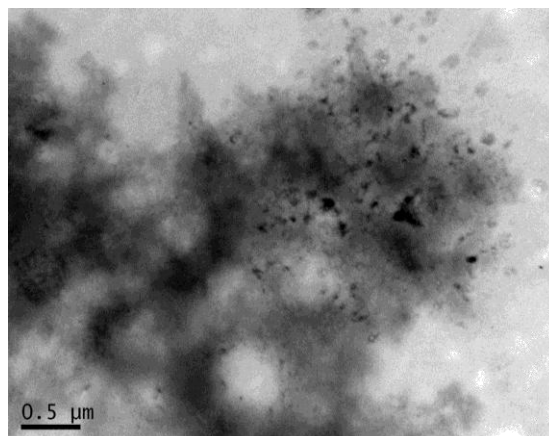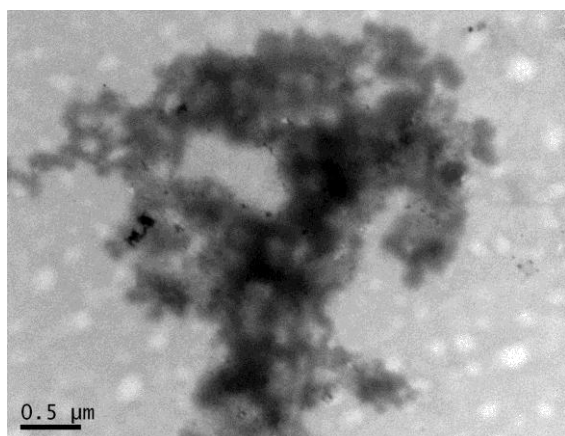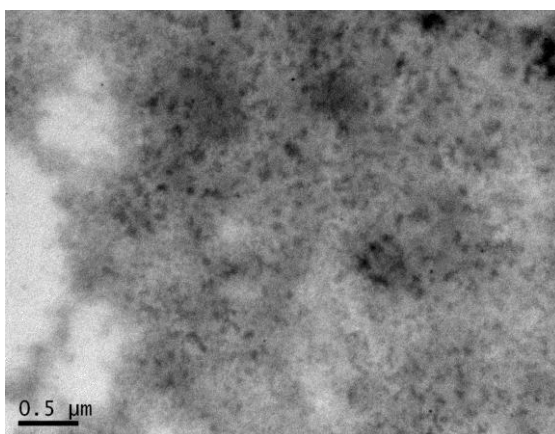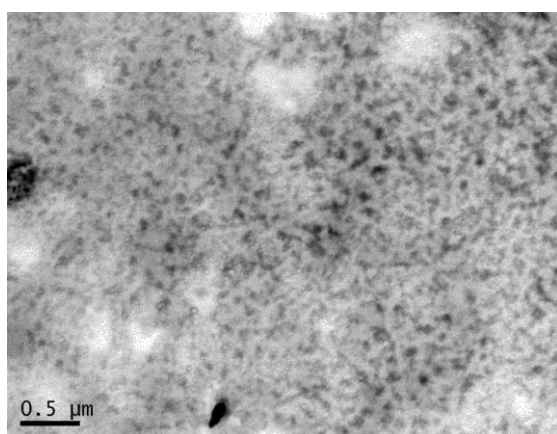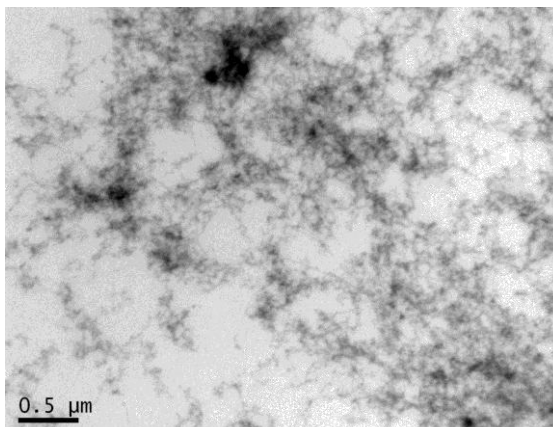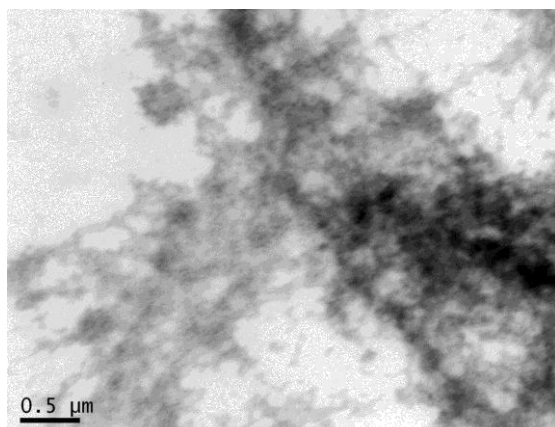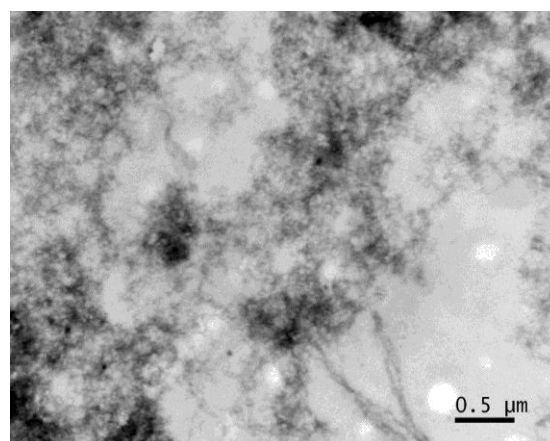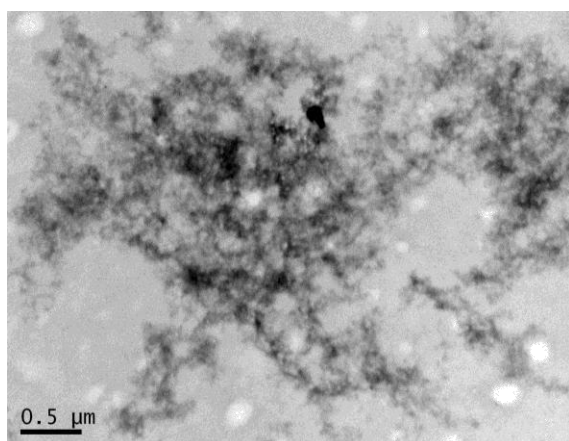
Aβ + Cu + Res
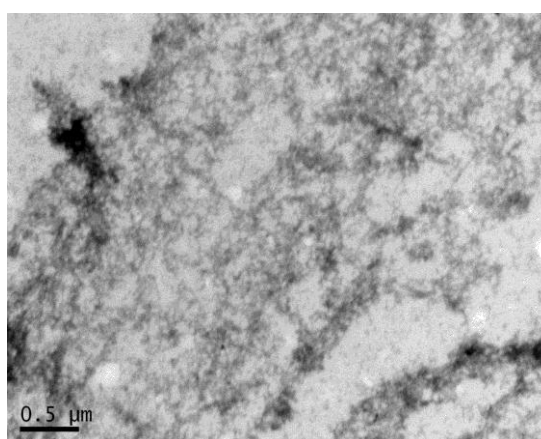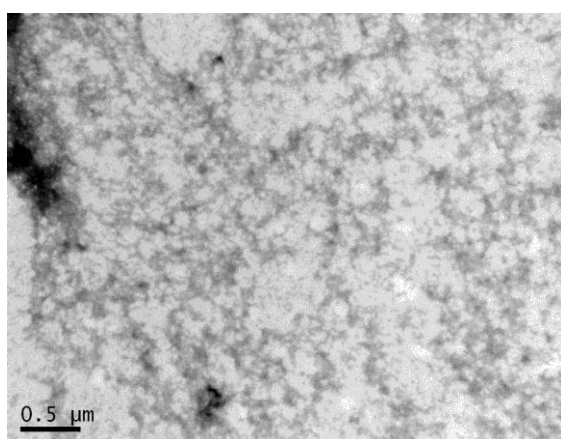
Chelating Properties of Polyphenols with Iron

Mass spectrometry was used to look at the ability of EGCG and Resveratrol to bind to iron. Both polyphenols were found to form metal chelate complexes with iron (see Figures 5.11 and 5.12). These data are not conclusive regarding solution phase chemistry, since gas phase adducts with metals such as sodium and potassium are known to form readily in the gas phase and are common in mass spectra. However it should be noted that the metal ion adducts with EGCG and resveratrol, both $[M+metal]^+$ and the $[2M+metal]^+$ species, are quite intense relative to the protonated species. This extent of adduct formation, is much stronger than the more common 10-20% relative to the protonated ion, which is typically observed in MALDI-TOF. This outcome may therefore reflect an inherently stronger association with metals which may well be recapitulated in solution phase properties and would be worth further investigation.

Figure 5.11: Mass spectrometry chromatogram of EGCG and Fe (1:2 ratio). Top chromatrogram showing sodium adduct of EGCG and bottom spectrum showing iron chelate.
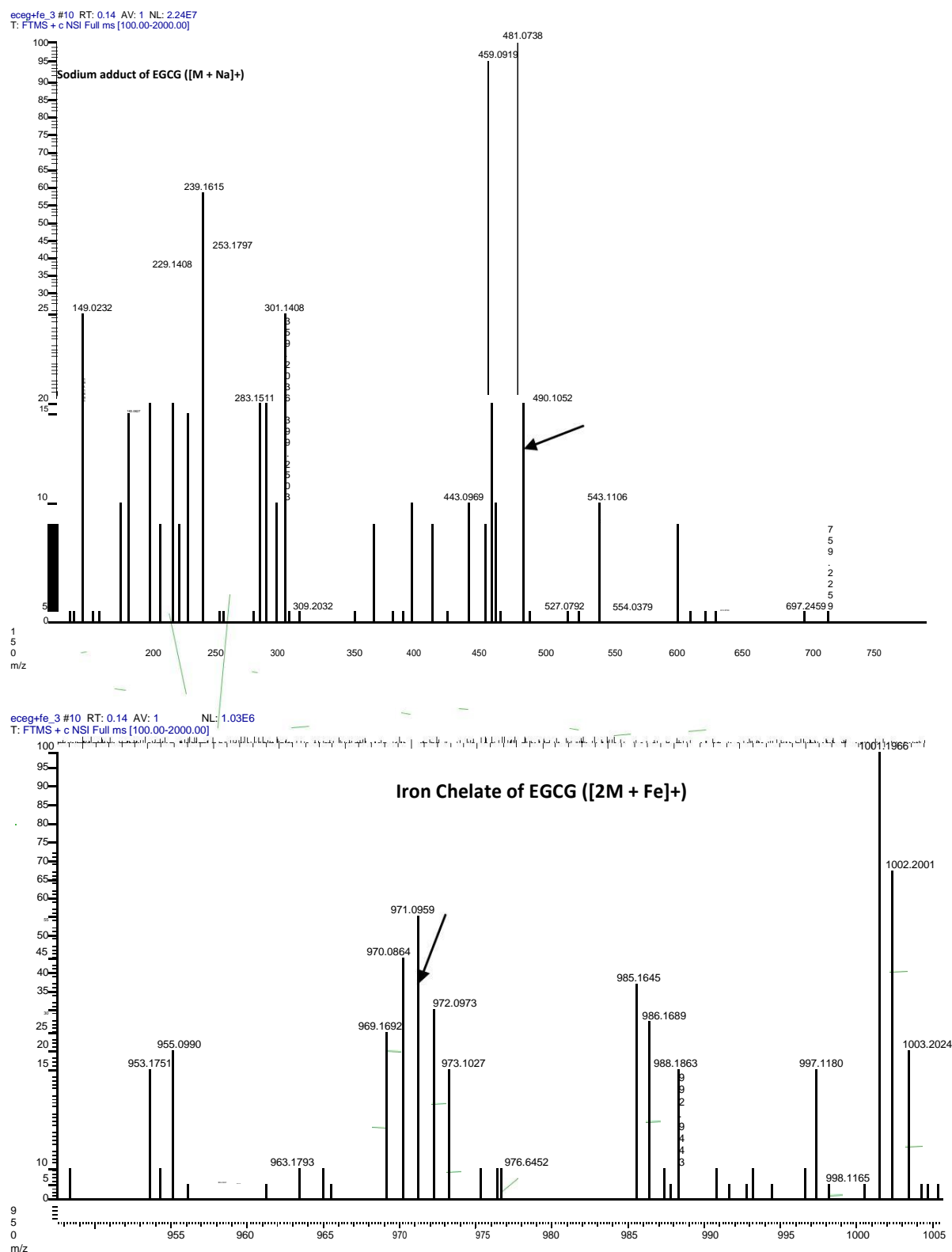
Figure 5.12: Mass spectrometry chromatogram of Resveratrol and Fe (1:2 ratio). Top chromatrogram showing resveratrol only, middle and bottom showing formation of iron chelates with resveratrol.
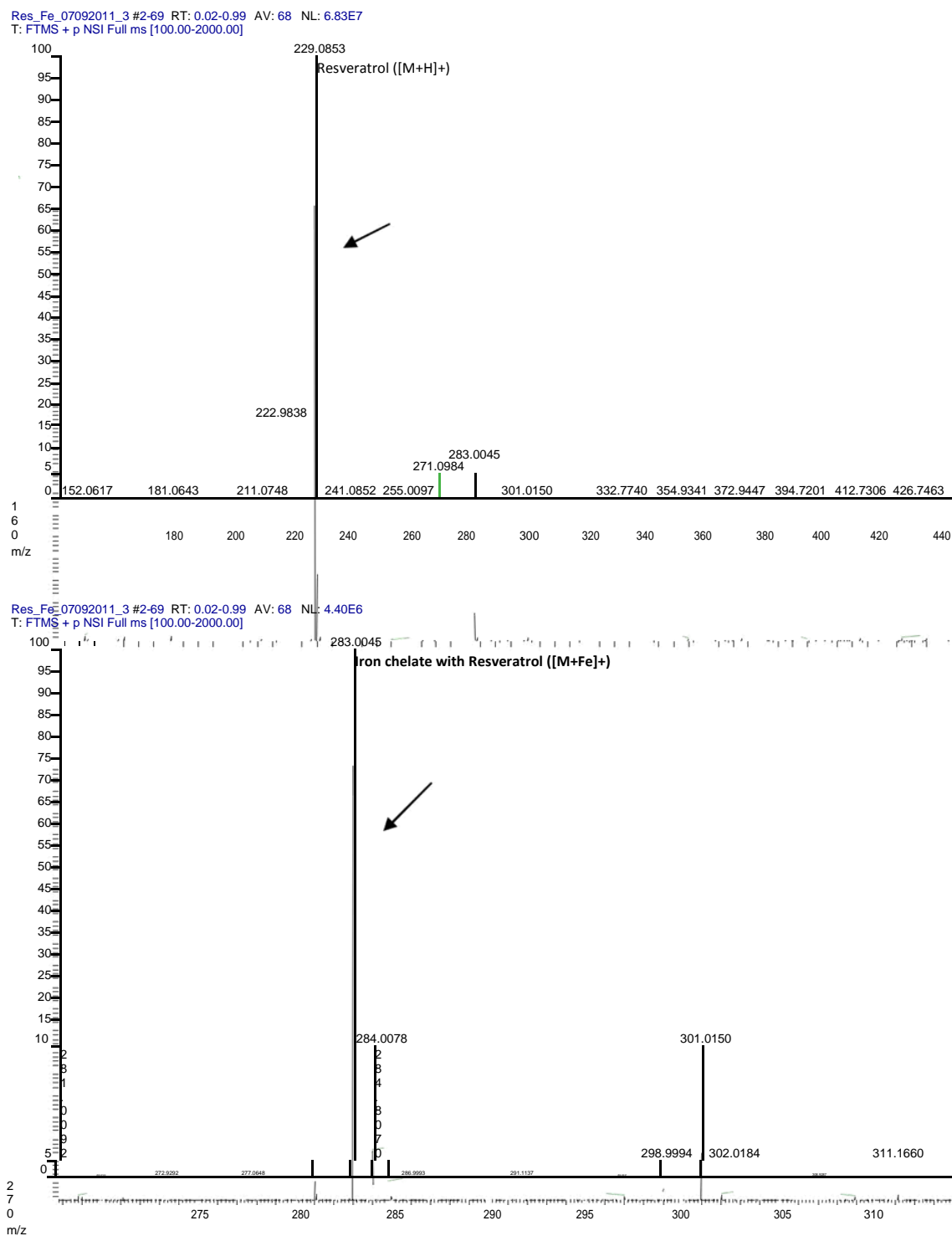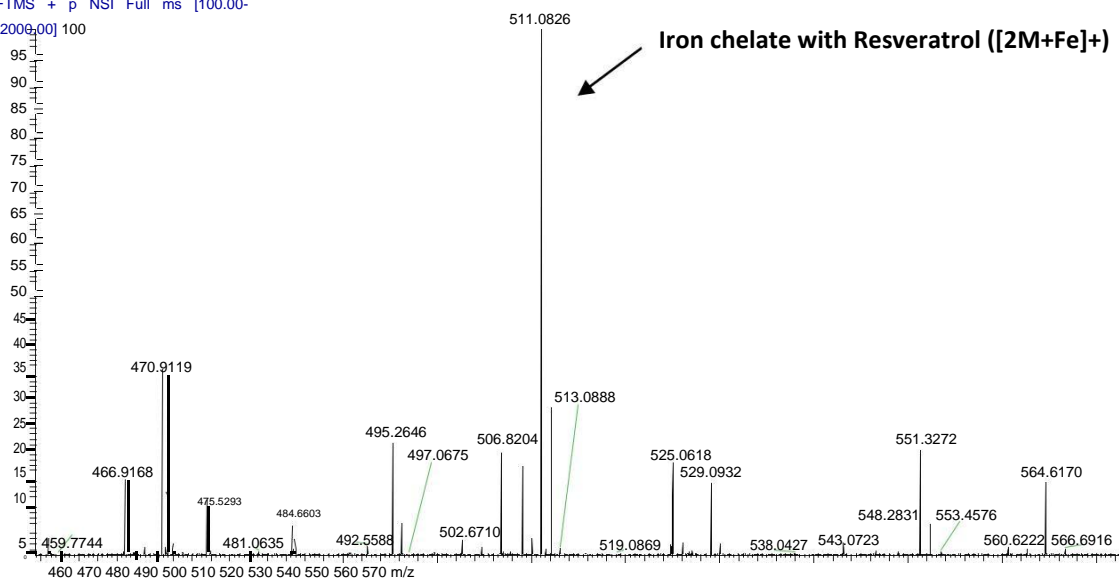
5.4 Discussion

AD is the most prevalent neurodegenerative disorder of ageing. It is a multifactorial disease with several pathology related pathways. Therefore, therapy or preventative strategies may be more efficaceous if they target multiple pathological processes simultaneously. In this regard, cumulative data have demonstrated that polyphenols can display neuroprotective effects through a variety of mechanisms [619].

Protein aggregation is a feature of a number of human diseases including many age related neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. The underlying mechanism/s explaining how amyloidoses cause cell death in neurodegenerative diseases still evades our knowledge. Even the process of amyloid formation, it's endogenous triggers and it's mechanism/s of proliferation and propagation are incompletely understood. Amyloid fibrils exhibit a common molecular architecture in which arrays of β-strands are connected by hydrogen bonds oriented parallel to the fiber long axis, into an array known as a cross-β structure [621]. The substructure of mature fibrils consists of one or more protofilament units, which can assemble laterally or intertwine in various ways as rope-like or ribbon-like modifications to the common fibrillar framework [621]. The finding that amyloid fibrils are stabilized primarily by hydrogen bonds involving the polypeptide main chain explains why fibrils formed from polypeptides of different sequence are morphologically and structurally similar. The toxic effects of amyloid aggregates to exposed cells, includes nonspecific membrane permeabilization, oxidative stress, mitochondrial impairment and eventually apoptosis [26,610,621]. The use of polyphenols is promising, as agents that are capable to protect cells against oxidative stress or inhibit amyloid formation at its earliest stages and disrupt the toxic fibrillar structures.

Studies of the interaction of polyphenols such as EGCG and resveratrol with Aβ lead to the proposal, that polyphenols function as amyloid aggregation inhibitors by diverting polypeptides from their normal amyloid formation pathway into nonproductive off-pathway states [620]. Polyphenols are characterized by the presence of several phenolic hydroxyl groups with acidic properties and with their planar structures form hydrogen bonds with peptides [646].

The various types of damage seen in AD including glycation, protein oxidation, lipid peroxidation and nucleic acid oxidation, almost all result directly or indirectly from metal catalysed hydroxyl radical production [43,128]. Redox active metals therefore play a primary role in the oxidative damage associated with AD [43,128]. Importantly redox active metals such as

copper and iron also accumulate in the neuropil of the AD brain at concentrations 3-5 fold higher than age-matched controls [129,130]. An increase in the concentration of CSF copper and an accompanying increase in ceruloplasmin, iron and iron-binding proteins have been observed in AD [130,647].

Evidence for the link between this metal dyshomeostasis, free radical production and Aβ plaques is abundant. Accumulation of iron in the hippocampus and cerebral cortex of AD patients colocalises with the lesions, NFTs and Aβ and may promote increased formation of oxygen radicals [25]. Several MRI studies have found positive correlations between ageing and iron accumulation in the brain [135,648]. As a consequence, with increased free iron accumulation the brain becomes increasingly vulnerable to the iron-catalysed oxidative stress. It is possible that age-related accumulation of iron may underlie or at least contribute to age being the most significant risk factor for most neurodegenerative diseases.

In vitro studies using synthetic Aβ have shown that iron induces the aggregation and potentiates the neurotoxicity of Aβ [649]. Aβ binds to and reduces Fe^{3+} to Fe^{2+}, which is followed by formation of H_2O_2 [649]. The generation of Fe^{2+} and H_2O_2 creates conditions ideal for the Fenton reaction, leading to the formation of the highly reactive hydroxyl radical [649]. The hydroxyl radical can then initiate lipid peroxidation, protein modifications and damage to DNA. Copper in the presence of Aβ, has been observed to facilitate the generation of oxidative stress. Synthetic Aβ in the presence of catalytic quantities of copper has been shown to produce significant quantities of H_2O_2 via similar mechanisms to iron and suggests a possible mechanism for Aβ-mediated neurotoxicity [649].

Since metals such as iron and copper induce aggregation of Aβ and facilitate free radical-mediated oxidative stress reactions, metal chelation has gained increasing popularity as a means of reducing cell damage in AD. Metal chelation involves introducing compounds to bind metal ions, making them nonbioavailable and facilitating their elimination [650]. Consequently the redox active metals are no longer available to bind other larger ligands such as Aβ, promoting its deposition, nor to promote oxidative stress reactions, through participation in Fenton type reactions [650]. Thus, chelating agents have the potential to inhibit the copper and iron-dependent neurotoxicity in the AD brain.

We have found that the polyphenols, EGCG, resveratrol and curcumin displayed neuroprotective properties against amyloid and metal ion toxicity in cultured astrocytes by protecting cell viability (Figures 5.2 – 5.4) and preventing the effects of oxidative stress via

Fenton chemistry (Figures 5.5 – 5.7). Electron microscopy images of the amyloid:polyphenol complexes showed that they have the ability to bind directly to amyloid, and look similar regardless of whether metal ions are present (Figure 5.10, Panels B-D), and differ in appearance to the aggregates where there are no polyphenolics present (Figure 5.10, Panel A). This suggests that the different structures formed in the presence of polyphenols may be a mechanism of preventing the neurotoxic properties of Aβ42 aggregates. This may also explain the protective effects of the polyphenolics toward glial cells and mitochondrial function (Figures 5.2 – 5.9). Our data are consistent with studies which have shown that EGCG binds to amyloid, forming highly stable structures which are not in the β-sheet conformation [651]. TEM images showed that curcumin had a similar effect to EGCG. Resveratrol however displayed slightly different structure to EGCG and curcumin in the TEM images but was none-the -less neuroprotective as shown via the cell viability effects in the amyloid and oxidative stress cellular model.

EGCG and resveratrol may also chelate iron as suggested from the mass spectrometry results (Figures 5.11 and 5.12). Further investigation using methods such as iTC will help further validate the binding stoichiometry and kinetics of these polyphenolic compounds. Thus these compounds may have the dual ability to provide neuroprotection by binding to amyloid directly and also preventing the effects of metal ions to promote oxidative stress via chelation.

The soluble aggregates of amyloid seen in Alzheimer's disease have been shown to disrupt mitochondrial function, initiating a pathophysiological cascade leading to synaptic and neuronal degeneration [652]. Treatment with EGCG, resveratrol and curcumin mitigated the mitochondrial dysfunction caused by incubation of cells with amyloid and metals (Figures 5.8 and 5.9). Polyphenols have been shown to affect the mitochondria-related redox parameters by acting either directly or indirectly upon the organelle. Santos et al. [653] showed that quercetin or its metabolite 3'-O-methyl-quercetin (50 μM each for 30 min) did suppress Fe^{2+}/ADP-induced lipid peroxidation in mitochondria isolated from rat liver, demonstrating a direct antioxidant action in that experimental model. Furthermore, quercetin at 50 μM (but not its metabolite) prevented potassium phosphate or mefenamic acid-induced protein thiol group oxidation in isolated mitochondria (incubation for 15 min). Quercetin was the most potent compound tested, inhibiting mitochondrial permeability transition pore (MPTP) and, consequently, swelling of the organelle, when compared to other flavonoids. Some authors stated that the enolic 3-OH group in the quercetin structure (C-ring) is responsible for

enhancing the antioxidant activity of quercetin. Additionally, this 3-OH group would exhibit an ability to chelate Fe^{2+}, decreasing its availability to react with H_2O_2 through Fenton chemistry reaction [654]. Devienne et al. [655] found that quercetin (1-50 μM) scavenged superoxide anion radicals ($O_2^{-\bullet}$) (and strongly inhibited hydrogen peroxide generation) in mitochondrial suspensions, leading to decreased rates of lipid peroxidation through a mechanism involving a Fe^{2+}-chelating activity. Dorta et al. [656] reported that quercetin (5-50 μM for 5 min) was the most potent agent in scavenging $O_2^{-\bullet}$ and preventing Fe^{2+}/citrate-induced lipid peroxidation in mitochondria isolated from rat liver when compared to taxifolin, galangin, and catechin. In spite of this, there are controversial data regarding the chelating capacity of quercetin and other flavonoids [657,658].

Thus, the protective ability of quercetin upon isolated mitochondria is apparent, but the exact mechanism by which this flavonoid elicits beneficial effects on the organelle still remains a matter of debate. Polyphenols have been shown to elicit antioxidant effects upon mitochondria by mechanisms that involve its chemical structure and/or its direct action on components of the mitochondrial electron transport chain, the main source of free radicals in this organelle. Moreover, polyphenols interact with other proteins located in the mitochondrial matrix, causing alterations in redox-related parameters. On the other hand the mode of action of polyphenolic compounds is not without contradictory findings. Consequently this area needs further research to determine the mechanisms by which polyphenols modulate mitochondria-related redox parameters.

As with many other drugs developed to counteract AD, the role of these compounds in ameliorating disease progression is still to be clinically verified. First of all, bioavailability of polyphenols is in general low, and concerns still remain regarding their capacity to bypass the blood brain barrier. Studies conducted on cell cultures demonstrate that in most cases the concentration able to exert a protective effect range from 5 to 50 μM, levels that are seldom if ever reached *in vivo* after oral ingestion [606]. To date, clinical trials conducted with these compounds have failed to demonstrate the efficacy of this family of molecules to slow disease progression in patients affected by AD [659,660]. Although many attempts have been made to elucidate the molecular mechanism of natural polyphenols against amyloidogenesis, the structure-activity relationship is still obscure and remains to be further explored. Considerable work remains before the potential clinical benefits of polyphenolic compounds can be fully realised. Nonetheless the advantages of polyphenol rich diets warrants further research.

[661-663].

Chapter 6

General Discussion and Future Directions

6.1 Introduction

AD is a multifactorial disease on several levels: (a) it has a wide variety of risk factors, (b) multiple cell types are affected (neurons, glia, astrocytes, oligodendrocytes, endothelial cells), (c) both intracellular and extracellular changes are observed, and (d) there are both genetic and environmental factors in the aetiology of the disease (autosomal dominant AD, Down's syndrome, late onset AD). Ageing is the strongest risk factor for AD, yet most researchers would agree that ageing and AD are not synonymous. This thesis has explored some common pathways which affect both ageing and AD, to better understand what ageing and AD have in common, and also how they are different, a summary of findings is shown in Figure 6.1.

Figure 6.1 Summary of main findings from thesis.
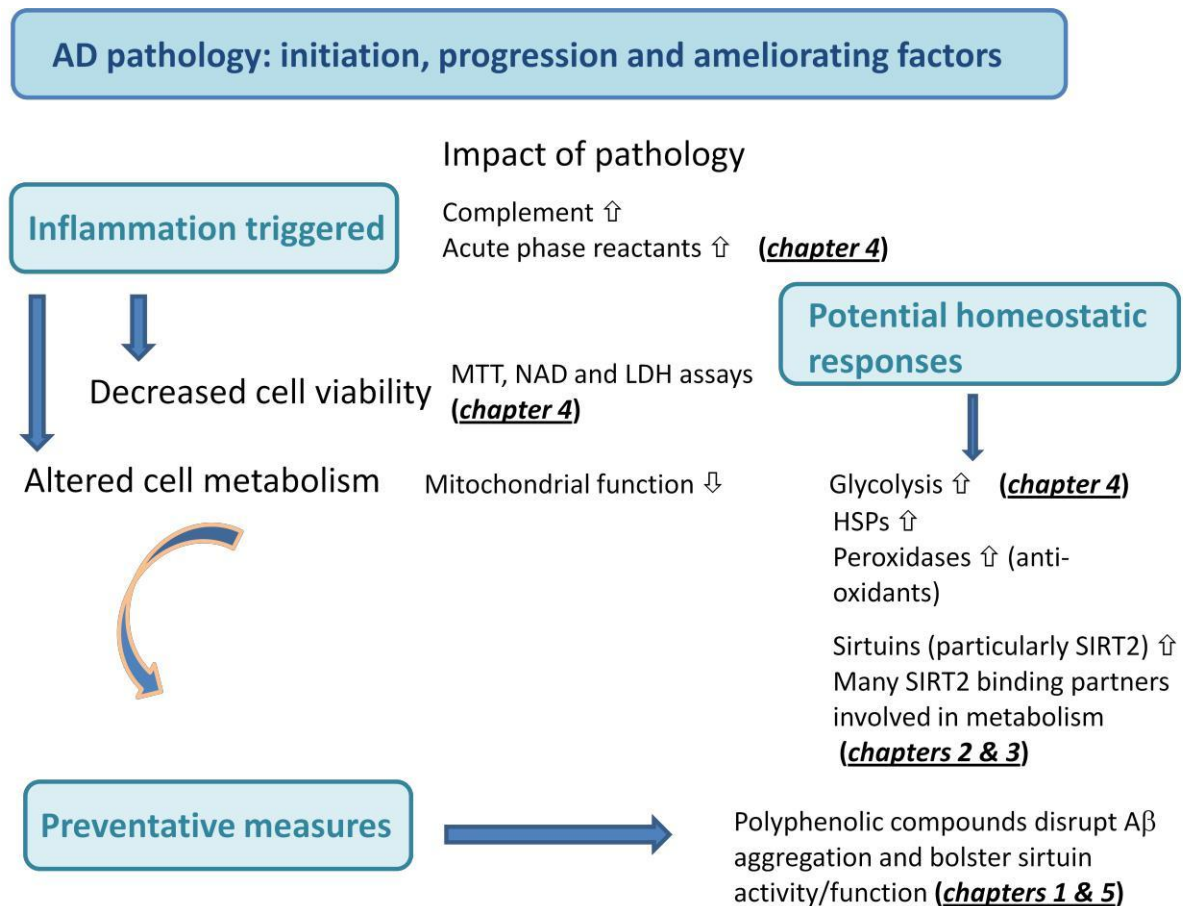
In chapter 3 the role of the longevity associated sirtuins family was explored in ageing and AD, showing that while the sirtuins in general are on a downward trajectory with age, in AD

there appears to be a homeostatic upregulation of specific sirtuins, particularly SIRT1 and SIRT2. In exploring potential mechanisms by which SIRT2 might mediate its effect/s, a variety of binding partners were identified. About 50% were metabolism related, particularly several glycolysis pathway enzymes. The remainder were from diverse cellular locations known to be affected in AD, including histones, cytoskeletal proteins, antioxidant proteins and regulators of protein structure and folding. In chapter 4, the effect of AD vs age matched control plasma on glial cells was explored, with effects on the complement pathway and metabolism (particularly glycolysis) differentiating ageing from AD. In chapter 5, the question of how AD pathology might be modulated was explored. For a multifactorial disease, perhaps disease modifying approaches also need to be multifactorial. Polyphenolic compounds can potentially impact multiple aspects of AD pathology, including: (a) ROS and oxidative stress, (b) function of sirtuins (e.g., resveratrol), (c) protein structure and activity, and (d) metabolism/mitochondrial function.

6.2 Distribution of Sirtuins across CNS cell types and tissues

Sirtuin proteins have a variety of intracellular targets, regulating multiple biological pathways, and could be considered pleiotropic mediators of cellular homeostasis. Both ageing and neurodegenerative conditions such as AD impact multiple cellular pathways. Consequently, proteins with pleiotropic effects such as the sirtuin family may be ideal targets for modulating some of the deleterious effects of both ageing and AD. Relatively little is currently known about the role or expression of the 7 mammalian sirtuins in the central nervous system. Quantitative expression analysis of mammalian sirtuin proteins (especially SIRT2-7), in the CNS is limited in the current literature. SIRT1 has been reported to be expressed in the cortex, hippocampus, cerebellum, and hypothalamus, and in lower levels in the white matter [425]. Among the brain cell types, SIRT1 is predominantly expressed in neurons and viewed as a nuclear protein [425]. The mRNAs for all seven sirtuins have been identified in mouse brain tissue and also neural stem cells [426]. SIRT1, SIRT2 and SIRT3 have also been detected in human serum, and levels were shown to decline with age and were linked to frailty [427,428]. SIRT3 has been shown to be elevated at both the mRNA and protein levels in Alzheimer's disease (AD) *post mortem* brain tissue compared to controls [271].

The most common techniques currently utilised for detecting a change in sirtuin levels at the mRNA or protein level are PCR and western blotting, respectively. Other studies have used

methods such as immunohistochemistry, surface plasmon resonance and ELISA assays. The majority of these methods are only semi-quantitative with moderate sensitivity, use antibodies which may not have sufficient specificity or detect expression at the mRNA level which may not reflect protein expression. Furthermore, there is no current assay which detects multiple sirtuins simultaneously.

Mass spectrometry has a great advantage over the conventionally used antibody based methods, such as western blotting, in that it offers greater specificity, linearity, reproducibility and typical limits of quantification down to the low fmol range. It also removes some of the antibody specificity issues that are associated with methods such as western blotting and ELISA, as peptides unique to each protein are measured. Thus in Chapter 2, I focus on the development of a targeted mass spectrometry method using multiple reaction monitoring (MRM) to quantify the seven human sirtuins at the protein level. To achieve sufficient sensitivity and selectivity in a multiplex-format, a targeted mass spectrometric assay was developed and validated for the quantification of all seven mammalian sirtuins (SIRT1-7). The assay was applied to a variety of samples including cultured brain cells, mammalian brain tissue, CSF and plasma. In line with previous data, my results affirm that there is significant divergence in abundance amongst members of the sirtuin family of proteins in the brain. SIRT1 and SIRT2 are the most abundant sirtuins in cultured brain cells with SIRT1 highest in neurons and SIRT2 highest in oligodendrocytes, validating previous studies showing SIRT2 to be highly expressed in oligodendrocytes[453]. My study and others have also found SIRT2 expressed in neurons and glial cells[454,455]. Further validation with higher sample numbers and across a wider range of cell lines may help elucidate differences between cell types for the lower abundant sirtuins.

 SIRT2 was found to be the most abundant sirtuin in the adult human frontal lobe and cortex and cerebellum homogenates from the guinea pig. Previously published results have shown SIRT2 to be abundant in the brain and serum[428,429,440,457] and my study provides further evidence for this and extends its abundance to the guinea pig brain. The mitochondrial sirtuins SIRT3-5 were found at lower levels in human brain but detection was improved after detergent fractionation. Further fractionation of samples or purification of mitochondria may facilitate improved mitochondrial sirtuin quantification. I also confirmed that mammalian sirtuins are localised to several subcellular compartments. While SIRT1, SIRT6 and SIRT7 are predominantly found in nuclear fractions, they are also detected in cytosol, cytoskeleton and membrane fractions, albeit at much lower levels. Similarly, although SIRT2 is the only

human sirtuin primarily localised in the cytoplasm, it may also be found at lower levels in the nucleus and cellular membrane. My data confirm that SIRT1 and SIRT2 may interact with both the nuclear and cytoplasmic subcellular compartments. Similarly, while the mitochondrial sirtuins (SIRT3-5) have been previously reported to be exclusively localised to the mitochondria, other studies have reported the localisation of several variants in the nucleus as well as the cytoplasm, in line with my study [463]. The expression patterns of sirtuins in brain cells remains controversial and it is unclear whether certain sirtuins are specific to cell types. My MRM study indicates that the majority of sirtuins (SIRT1, 2, 6 and 7) are present across all the main brain cell types. Sirtuins were also detected in human CSF and plasma, and guinea pig and mouse tissues.

6.3 Sirtuins in Ageing and Alzheimer's disease

Although a number of sirtuins have been found to be promising in boosting longevity and neuroprotection in cellular and animal models of AD, mammalian distribution and functional roles in the central nervous system are still not well studied. There is a paucity of information about the role of CNS sirtuins in ageing brain and neurodegenerative diseases. Previous studies have shown an increase in SIRT3 in AD *post-mortem* brain tissue using western blotting for protein expression and multiplex qPCT to assay SIRT3 mRNA levels [271]. Both SIRT3 protein and mRNA were shown to be significantly elevated in the AD group [271]. Another recent paper detected SIRT1 in human serum samples using western blotting, surface plasmon resonance and ELISA to measure SIRT1 protein levels [427]. SIRT1 declines with age and is more dramatically reduced in MCI and AD patients compared to age matched controls, suggesting that SIRT1 may warrant further investigation as a potential plasma biomarker for AD [427]. SIRT1 and SIRT3 levels in serum were found to be significantly lower in frail subjects as compared to the non-frail [428]. Another study reported a decrease in SIRT1 and SIRT2 mRNA levels using quantitative real time PCR in the primary motor cortex of human *post-mortem* amyotrophic lateral sclerosis brain tissue [440].

In AD transgenic mouse models SIRT1 overexpression has been reported to be neuroprotective against amyloid and tau pathologies as well as more recently also shown to induce cognitive enhancement [340,480]. Since SIRT-1 is a deacetylase that regulates several neuroprotective cellular pathways promoting neuron survival by deacetylating a broad spectrum of molecules, it has been hypothesized that the loss of SIRT-1 protein in the brain

contributes to the pathogenesis of AD. A significant decrease in SIRT1 concentration in parietal cortex of AD brain has been reported and a strong correlation was established between tissue SIRT1 concentration, duration of symptoms and tau accumulation [280], but the exact relationship and its role in the sequence of events leading to development of AD remains unclear.

Recently the first study investigating sirtuin levels in serum has been reported. The study investigated serum SIRT1 concentration in patients with AD and MCI as well as in young and elderly controls [427]. A significant decline in SIRT1 with age in serum and a larger drop in patients with MCI and AD was found [427]. The authors suggest that SIRT1 may be a promising biomarker for AD. These data also suggest that sirtuin activators may potentially have a preventive role in AD. Another study by the same group examined the association of sirtuins to a major geriatric syndrome, frailty [428]. Serum levels of SIRT1, 2 and 3 were found to be significantly lower in frail patients compared to nonfrail controls [428].

Thus, in Chapter 3, I investigated changes to sirtuin protein expression during normal human ageing and also changes occurring in AD. I examined sirtuin levels in control, MCI and AD plasma, in the CSF of both a younger and elderly age group, in control and AD *post-mortem* brain tissue and finally in young and older samples of a potential new natural AD animal model – the *Octodon Degus*. To further investigate the functional role of SIRT2, which I found to be the most abundant in the human brain, I completed an immunoprecipitation pulldown followed by mass spectrometry detection of potential binding partners. Owing to the potential importance of mammalian sirtuins in neurodegeneration and ageing, I hypothesized that they may be differentially expressed in the plasma, CSF and brain and may regulate various targets involved in metabolic processes and neurodegenerative diseases. I investigated sirtuin expression in immunodepleted plasma from pooled control, MCI and AD subjects and found a significant drop in SIRT1 levels in the plasma of AD patients, in line with other studies in the literature [427,428]. My data and the work of others suggest the possibility that SIRT1 may be a useful clinical biomarker of AD. However, further validation in larger cohorts is needed with longitudinal samples and non-AD dementia controls.

I also investigated sirtuin CSF concentrations in younger and elderly controls and found a downward trend in SIRT1 in the elderly group but not reaching significance. It would be interesting to further valid these results by looking at sirtuin expression in matched plasma samples and to also investigate levels in larger cohorts of subjects. Evaluation of sirtuin

levels in the CSF, plasma and *post mortem* brain tissue of centenarians would also provide greater insights into how sirtuins may change in those who have "super aged" brains and may provide clues as to how these individuals stay healthy during exceptional longevity.

 Interestingly, when comparing control and AD *post mortem* brain tissue, I found a significant elevation of a number of sirtuins. In the occipital lobe, I found a higher level of SIRT2 and in the temporal lobe an increase in both SIRT1 and SIRT3 in the AD brain. Published reports on sirtuin expression in the AD brain are limited in the current literature, though a few recent studies have reported significant changes. One study showed a significant increase in SIRT3 in the temporal cortex of AD *post mortem* brain [271]. SIRT3 expression was found to correlate with the deposition of β-amyloid in an AD mouse model[271]. My results also confirmed an elevation of SIRT3 in the AD temporal lobe. This suggests a possible role for SIRT3 in mechanisms of sensing and protecting against ROS and AD-mediated mitochondrial stress. Another recent study has reported changes to SIRT1, 3 and 5 expression, reporting a decline in SIRT1 and SIRT3, and an elevation of SIRT5 in the cortex and hippocampus, with AD progression [500]. SIRT3 decline has also been reported in an AD transgenic mouse model[501]. These results suggest that mitochondrial SIRT3 might participate in the development of AD via mitochondrial dysfunction. The elevation we see in the temporal lobe may therefore a compensatory mechanism by the brain to induce these neuroprotective proteins and bolster neuronal longevity. SIRT1 and SIRT3 are also upregulated in response to fasting and calorie restriction and have been shown to reduce ROS levels and reduce oxidative damage. The decline reported by others in the hippocampus where the highest level of AD pathology is seen may indicate loss of these neuroprotective activities and metabolic dysfunction reported in this vulnerable region may be thus, in part due to reduction of SIRT1 and SIRT3 levels. As AD is associated with significant increases in neuronal ROS production and previous studies have shown that SIRT3 mRNA expression is regulated by mitochondrial ROS levels, it seems likely that the SIRT3 upregulation in AD we see in the temporal lobe may be a consequence of Aβ-related oxidative stress. Given that others have shown that SIRT3 upregulation subsequently increases neuronal lifespan, it is tempting to speculate that upregulation of

SIRT3 in response to Aβ-induced oxidative stress might prolong neuronal function by decreasing ROS, maintaining ATP-levels and sustaining synaptic activity. These possibilities will need further investigation, and will no doubt yield important information about mechanistic links between metabolism, longevity and defence against the impacts of pathology.

This work has identified SIRT2 as the most abundant sirtuin in the brain and we also see an elevation of SIRT2 in the AD occipital lobe, one of the least AD pathology susceptible brain regions. I therefore aimed to gain further insights into its biological functions by identifying its binding partners. Using immunoprecipitation pulldown and protein identification using mass spectrometry, I identified a list of potential binding partners for SIRT2. Interestingly, our results reveal a novel potential role of SIRT2 as a regulator of mitochondrial function and metabolism with many of the binding partners identified being involved in energy metabolism. These include aldolase, ATP synthase, enolase, pyruvate kinase and glucose-6-phosphate isomerise which are all involved in energy metabolism and glycolysis. A recently published study has also suggested SIRT2 may regulate the activity of mitochondria. Liu et al found that SIRT2 can localise to mitochondria and directly interact with mitochondrial proteins [504]. Furthermore, the depletion of SIRT2 was shown to increase markers of oxidative stress and reduce ATP production in the mouse brain [504]. Thus taken together my data shown in Chapter 3 and previous studies suggest that SIRT2 is not only a cytosolic protein but may translocate to mitochondria, appears to have many mitochondrial targets and a new role for SIRT2 in mitochondrial function, oxidative stress and energy metabolism may be an emerging. All of these processes are established as important factors in the pathogenesis of AD, and as a modulator, SIRT2 may represent a potential target for disease prevention and/or amelioration.

6.4 Inflammation and Metabolism in AD Pathology

Markers of increased protein, lipid and nucleic acid oxidation and reduced activities of antioxidant enzymes have been reported in AD plasma [664-666]. Amyloid plaques in the AD brain elicit a range of reactive inflammatory responses including complement activation and acute phase reactions, which may also be reflected in plasma. Previous studies have shown that human AD plasma may be cytotoxic to cultured cells [508,509,511]. In Chapter 4, I investigated the effect of pooled plasma from healthy controls and individuals with MCI and AD, on cultured microglial cells. Since previous studies indicate that AD plasma may contain oxidative stress markers as well as cytotoxic factors, in Chapter 4, I investigated the effect of the addition of pooled control, MCI and AD plasma from 20 age matched older individuals on a microglial cell line. Cell viability, proliferation and mitochondrial function were investigated following 48 hour treatment with non-heat-inactivated plasma and plasma in

which complement proteins had been deactivated. I also tested the effect of commercially purchased complement factors alone and in combination on cultured glia. I then undertook proteomic analysis of the plasma from each group and iTRAQ quantitative proteomic analysis of cell extracts exposed to plasma from each group to investigate possible plasma protein alterations unique to MCI or AD, to detect any protein aberrations within the cells treated with the plasma and to correlate these finding to cell viability and mitochondrial function assays measured *in vitro*.

AD plasma was found to significantly decrease cell viability and increase glycolytic flux in microglia compared to plasma from healthy controls. This effect was prevented by heat inactivation. Proteomic methods and isobaric tags showed expression levels of complement and other acute phase proteins to be altered in MCI and AD plasma and an upregulation of key enzymes involved in the glycolysis pathway in cells exposed to AD plasma. The increased expression of glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, enolase, aldolase and pyruvate kinase may increase glycolytic flux leading to the accumulation of pyruvate and thus stimulating anaerobic metabolism to lactic acid. I found an increased level of LDH activity in the cell culture media and an increase in extracellular acidification, as indicated by the increase in ECAR in the microglial cells exposed to human AD plasma. These findings suggest a role for mitochondrial bioenergetic deficits in AD pathogenesis, and a possible homeostatic response through upregulation of glycolysis. Thus my data suggested that altered expression levels of acute phase reactants in AD plasma may alter the energy metabolism of glia.

Damage to the blood-brain barrier is thought to occur in AD, and this may increase movement of proteins between the brain and the vasculature [552]. A variety of inflammatory markers are increased with the onset of AD pathology, including cytokines and chemokines, coagulation factors and acute-phase reactive proteins as well as reactive astrocytes and activated microglial cells, the main cells involved in the neuroinflammation process [506,507]. Whether accumulation of cytokines and acute phase reactants within the brain is reflected in plasma has not been elucidated as many of these proteins do not easily cross the blood brain barrier. Alternatively, AD may be associated with a more widespread immune dysregulation, detectable in plasma.

Previous studies investigating the effects of human AD plasma on cells in culture have found differential effects on protein expression and cell biology. One study aimed to determine if exposure to serum from AD patients would affect markers for AD brain lesions [508], and found

that 24 hour exposure to AD serum increased four molecular markers characteristic of AD senile plaques and neurofibrillary tangles in rat hippocampal neurons [508]. This stimulation of AD markers by human serum suggests that the genesis of both neuronal plaques and tangles may arise from exposure of susceptible neurons to toxic serum factors and/or failure to detoxify these factors. Another study found that antibodies in serum of patients with AD caused immunolysis of cholinergic nerve terminals from the rat cerebral cortex, supporting the hypothesis that autoimmune mechanisms may operate in the pathogenesis of AD [509]. This work demonstrated the utility of examining effects of disease plasma on cell culture systems, to facilitate the study of both disease markers and disease mechanisms. The role of blood factors in ageing and disease is underscored by recent experiments using the parabiosis technique and showing the rejuvenating and pathology ameliorating effects of young blood in older animals [667,668].

Factors which induce the upregulation of glycolysis in glial cells treated with AD plasma remain unclear. However, aerobic glycolysis has been correlated spatially with amyloid deposition in AD brains [576]. It has also been shown that elevated levels of the enzymes pyruvate dehydrogenase kinase and lactate dehydrogenase provide resistance to amyloid and other neurotoxins [584]. The ability of the brain to maintain expression of these enzymes involved in mitochondrial energy metabolism may explain why some individuals could show high levels of amyloid deposition without neurodegeneration [584,589-591]. Interestingly I found some of these glycolytic proteins overlapped with the SIRT2 binding partner proteins we found in Chapter 3. In my study, the indicators of increased glycolysis in microglia may be a compensatory action caused by the loss of cell viability and mitochondrial function following exposure to AD plasma. Altered activities of key glycolytic enzymes have also been found in hippocampal, frontal and temporal cortex of AD brains and thought to possibly be related to the astrocytosis that occurs in AD [581].

Interestingly I have found that SIRT2 is upregulated in the AD brain and that many of the SIRT2 binding partners identified in Chapter 3 are involved in metabolism (including glycolysis). Also in Chapter 4, I show that glycolysis is upregulated when glia are exposed to AD plasma. Therefore this data, taken together, suggests the possibility that some of the metabolic changes observed upon exposure to AD plasma may be mediated by SIRT2 and perhaps also other sirtuins as well.

6.5 Brain pathology in AD: offense vs defence

In the pathogenesis of AD, there is a strong interplay between how much of the disease is due to pathological processes and stressors and how much might be due to insufficient defensive strategies. Therefore, reinforcing defensive strategies such as preventing amyloid aggregation via the use of compounds such as polyphenols, might prove a potent way to avoid/postpone disease onset.

Aβ, the product of the proteolytic cleavage of the APP into 38, 40, or 42 amino acid peptides, is generally viewed as a toxic mediator of AD [669]. The generation of Aβ is orchestrated by the β- and γ-secretases in a sequential manner such that inhibition of either secretase prevents Aβ generation. The oligomerisation of Aβ is toxic to neurons *in vitro* and *in vivo* and produces synaptic dysfunction, calcium dysregulation, oxidative stress, and neuroinflammation. Additionally, Aβ (specifically $A\beta_{42}$) is the primary component of the senile plaques that become deposited in the brains of AD patients and are a hallmark pathological feature of the disease [669]. Alternative processing of APP, through α-secretase cleavage (followed by that of γ-secretase), produces a soluble segment of APP that may be neuroprotective. This "non-amyloidogenic" pathway deters the processing of APP through the β- and γ-secretases and thus inhibits the formation of toxic Aβ species. Interestingly, recent evidence indicates that SIRT1, along with its varied role in the regulation of ageing, is also a key regulator of α-secretase, and thus of amyloidogenic processing [340,669]. SIRT1 hyperactivity is capable of reducing AD pathologies both *in vitro* and *in vivo* through upregulation of the ADAM10 gene [340]. Laboratory upregulation of the sirtuin pathway, via sirtuin-activating compounds like resveratrol, have also effectively reversed the ageing process and its comorbidities in animal models [200,202,479]. SIRT1 was upregulated in AD temporal lobe (Chapter 3, Figure 3.4), which may be a homeostatic response to the pathology. Consequently SIRT1 upregulation may provide a possible therapeutic (or preventative) avenue for age-related diseases such as AD, and polyphenolic compounds are potential modulators of sirtuin activity.

Polyphenols, which include resveratrol, epigallocatechin gallate and curcumin, have gained considerable interest in AD research for their ability to reduce many of the main pathological hallmarks of AD and their potential to slow down cognitive decline [252,389,634]. Polyphenols exhibit remarkable antioxidant and anti-inflammatory activities that may exert a leading role in reducing age-related oxidative stress and inflammation, and thus hampering the onset and progression of degenerative diseases [379,635]. Oxidative stress is a key hallmark in AD. More

recently polyphenols have been shown to produce other important effects including anti-amyloidogenic activity, metal chelation and modulation of the sirtuin proteins [365,619,634]. Numerous polyphenols from natural products such as turmeric, grapes, red wine and green tea, have been reported to inhibit Aβ aggregation and toxicity and show promise *in vivo*, including EGCG, curcumin and resveratrol [183,190,620-626]. The major tea polyphenol, EGCG, shows neuroprotective activity against neurological disorders, and inhibits toxicity induced by Aβ and α-synuclein oligomers [183]. Moreover, using isothermal titration calorimetry at different EGCG and salt concentrations, it has been shown that the interactions between Aβ1-42 and EGCG are mainly H-bonds in the region 1–16 and hydrophobic in the region 17–42 [621,627]. Experimentally, EGCG has been reported to generate unstructured, off-pathway Aβ1-42 oligomers [620].

Brain accessible polyphenols with multiple effects on pathways involved in neurodegeneration and ageing may therefore prove efficacious in the treatment of age-related diseases such as AD, although the evidence for this so far is limited. The toxic effects of amyloid aggregates to exposed cells, includes nonspecific membrane permeabilization, oxidative stress, mitochondria impairment and eventually apoptosis [26,610,621]. Polyphenols are characterized by the presence of several phenolic hydroxyl groups with acidic properties and their planar structure forms hydrogen bonds with peptides [646]. Thus the use of polyphenols is promising, as agents that are capable to protect cells against oxidative stress, inhibit amyloid formation at its earliest stages and disrupt the fibrillar structures.

The various types of damage seen in AD including glycation, protein oxidation, lipid peroxidation and nucleic acid oxidation, almost all result directly or indirectly from metal catalysed hydroxyl radical production [43,128]. Redox active metals therefore play a primary role in the oxidative damage associated with AD [43,128]. Considering that metals such as iron and copper induce aggregation of Aβ and facilitate free radical-mediated oxidative stress reactions, metal chelation has gained increasing popularity as a means of reducing cell damage in AD. Metal chelation involves introducing compounds to bind metal ions, making them nonbioavailable and facilitating their elimination [650]. Consequently the redox active metals are no longer available to bind other larger ligands such as Aβ, promoting its deposition, nor to promote oxidative stress reactions, through participation in Fenton type reactions [650].

Therefore, in Chapter 5, I explored the ability of three common polyphenols to prevent the neurotoxicity caused by amyloid and Fenton chemistry on astrocytes using a brain cell model for oxidative stress and amyloid toxicity. I also investigated the effects of polyphenol treatment on amyloid aggregation using electron microscopy and the metal chelating properties of these compounds using mass spectrometry. The polyphenols, EGCG, resveratrol and curcumin displayed neuroprotective properties against amyloid toxicity in cultured astrocytes by preventing the loss of cell viability caused by amyloid in the presence of transition metals iron and copper and preventing the effects of oxidative stress via Fenton chemistry. Electron microscopy images of the amyloid:polyphenol complexes showed that they have the ability to bind directly to amyloid forming non-structured, non-toxic aggregates. Other studies have also shown this effect and found EGCG to bind to amyloid and form highly stable structures which are not in the β-sheet formation [651]. TEM analysis also showed that curcumin also had a similar effect as EGCG. Resveratrol however displayed slightly different structure to the other two TEM images, but was also neuroprotective as shown via the cell viability effects in my amyloid and oxidatives stress cellular model. EGCG and resveratrol were also able to chelate iron as seen from the mass spectrometry results. Thus these compounds have the dual ability to provide neuroprotection by binding to amyloid directly and by chelation of metal ions minimizing oxidative stress. I found treatment with EGCG, resveratrol and curcumin also minimised mitochondrial dysfunction caused by incubation of cells with amyloid and metals.

As for many other drugs developed to counteract AD, the role of these compounds in ameliorating disease progression is still to be clinically verified. First of all, bioavailability of polyphenols is in general low, and some concerns still remain regarding their capacity to bypass the blood brain barrier. Clinical trials conducted with these compounds have also failed to demonstrate efficacy in slowing the progression of the disease in patients affected by AD [659,660]. Furthermore, although many attempts have been made to elucidate the molecular mechanism of natural polyphenols against amyloidogenesis, the structure-activity relationship is still obscure and remains to be further explored.

6.6 Future Studies

An important aspect of sirtuin biology is that their deacetylase activity is nicotinamide and NAD^+ dependent, establishing a direct link between their function and energy metabolism. MRM mass spectrometry can not only be utilised for the quantification of sirtuin proteins as demonstrated in Chapter 2, but MRM can also be applied to measure sirtuin and energy related metabolites such as metabolites of the NAD salvage pathway and ATP. Thus targeted mass spectrometry methodologies have the potential to not only validate and complement currently established methods for investigation of sirtuin protein expression changes but can also help build a larger view of sirtuin biology in the normal brain and in disease conditions by linking sirtuin protein expression with NAD^+ metabolism. It has been shown that NAD^+ declines with age, reducing sirtuin activity [670]. This could potentially contribute to the pathogenesis of age-related diseases. Further exploration of sirtuin related metabolites such as the metabolites of the NAD salvage pathway will enhance our understanding of the interplay between energy metabolism and sirtuin function in the process of ageing and neurodegeneration. It can also serve to investigate the efficacy of potential treatments such as polyphenols and NAD^+ intermediates such as nicotinamide mononucleotide and nicotinamide riboside, which are currently under study for their longevity promoting effects.

My preliminary data showing changes to SIRT1 and SIRT2 in AD *post mortem* brain tissues will need to be expanded to include a larger cohort of control and AD samples to validate these findings. This is to overcome any inevitable inter-individual variations in protein expression, as well as potentially variable tissue quality. Future work investigating sirtuin changes in the hippocampus of control and AD subjects would also be interesting as it will highlight how sirtuins change in this especially vulnerable part of the brain. Looking at brain tissue samples from younger, middle aged, older, and exceptionally old indivdiuals would also be helpful in discovering changes that happen during normal ageing versus those that occur more specifically during neurodegenerative diseases such as AD. Combining my proteomics data with metabolomics data would be another avenue to explore in order to gain further insights into the biological effects and importance of sirtuins. Sirtuin proteins are linked to NAD and thus energy metabolism. By looking at changes to NAD and other related metabolites in the same samples quantifying sirtuin protein will validate corresponding metabolite changes that are occurring as a result of differential sirtuin protein expression. Finally it would also be important to look at sirtuin activation by polyphenols, both

individually and also to investigate combination therapy using polyphenols and possibly NAD related metabolites to protect CNS cells against oxidative stress.

My SIRT2 binding partner work revealed some interesting proteins for further investigation. iTC methodology will help confirm the binding properties of some of these novel proteins such as ATP synthase with SIRT2. It would also be ideal to repeat the pulldowns and analysis for the other 6 sirtuins as this may uncover further new or overlapping roles for these sirtuins and novel sites of action.

The use of SIRT2 knock out mouse models would be another possible avenue to explore biological functions of this abundant brain sirtuin. These animals would allow us to further investigate metabolic and proteomic changes that occur as a result of the loss of SIRT2 in the brain. I predict that such a knockout would leave the brain more vulnerable to pathology, particularly to oligodendrocytes, where SIRT2 is most abundant.

Results from the work on the effects of AD plasma on cell viability, mitochondrial function and glycolysis can also be further explored, using neuronal cells to investigate toxicity differences between different cell types. It would also be interesting to measure the metabolites of some of the glycolytic enzymes we found upregulated to validate the proteomics data using measures that reflect functional changes. Further fractionation of the AD plasma would also allow deeper investigation into the toxic proteins/factors involved in the loss of cell viability seen in the cultured cells.

The next step in connecting to my polyphenolics work, would be to look at sirtuin activation following treatment of CNS cell lines with various polyphenols, alone and in combination. The combination of polyphenols along with NAD precursors would also be an interesting avenue to explore. With the use of transgenic AD mouse models, the neuroprotective role of polyphenol and/or sirtuin activation could be further explored. Animal models would also allow us to investigate how treatment with polyphenols and sirtuins may affect amyloid deposition and aggregation.

6.7 Conclusions

In conclusion, I have successfully applied MRM mass spectrometry for the detection and quantification of sirtuin proteins in the central nervous system, paving the way for more quantitative and functional studies. Collectively, my results add further insights to the limited

data which currently exist regarding the expression of sirtuins in the human CNS. I found differential expression of SIRT1 in plasma and brain tissue, SIRT2 being the most abundant sirtuin and elevated in the occipital lobe of AD brain. This technique provides a powerful tool and helps improve upon the limitations of current protocols. While mass spectrometry based assays for protein quantification may still have some barriers to overcome before they can be used in a clinical setting due to low throughput and expense compared to methods such as ELISA, MS has great value in investigating lower abundance proteins such as sirtuins in complex samples such as the brain with great sensitivity and specificity.

This work has also demonstrated the utility of examining effects of disease plasma on cell culture systems, to facilitate the study of both disease markers and disease mechanisms. I found AD plasma caused loss of cell viability and mitochondrial function (Figure 6.1). I showed that plasma expression levels of acute phase proteins are altered in AD and MCI, supporting a role for increased inflammatory activity in this disease which is detectable in the plasma (Figure 6.1). Cells exposed to AD plasma show an upregulation of glycolysis possibly as a compensatory response to compromised mitochondrial function. I also found SIRT2 to be elevated in the AD brain and having the ability to interact with a number of proteins involved in metabolism (particularly glycolysis). Together these observations support an emerging body of evidence that inflammation and metabolism are closely linked processes, which are regulated by transcriptional and protein translation events [601,602]. This study showed that the use of biological assays in combination with proteomic analysis may help uncover possible mechanisms of disease and may be complementary techniques to validate cellular changes and effects in a range of biological samples.

Finally I have investigated the anti-oxidant, anti-aggregation and chelation properties of a number of common polyphenols (Figure 6.1) and with future work investigating these compounds efficacy in activating sirtuins could provide promising results for the development of combination therapies targeting various pathways involved in AD such as oxidative stress, sirtuin activation, mitochondrial dysfunction and energy metabolism for use in transgenic animal models and/or human pilot clinical trials.

Ultimately, an efficient harnessing of the beneficial effects of the sirtuin pathway will certainly be of great therapeutic importance in the general population, where AD and ageing both suffer a lack of preventative measures. This work helps add further insight to the changes and distribution of mammalian sirtuins in the CNS during ageing and AD and some

of their potential targets within the brain, revealing evidence for novel roles for these proteins in the brain and revealing new pathways for future research.

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Appendix

Publications:

<u>Jayasena T</u>, Poljak A, Smythe G, Braidy N, Münch G, Sachdev P (2013). The role of polyphenols in the modulation of sirtuins and other pathways involved in Alzheimer's disease. Ageing Research Reviews 12(4):867-883

<u>Jayasena T</u>, Poljak A, Braidy N, Zhong L, Rowlands B, Muenchhoff J, Grant R, Smythe G, Teo C, Raftery M, Sachdev P (2016). Application of Targeted Mass Spectrometry for the Quantification of Sirtuins in the Central Nervous System. Scientific Reports 2016 Oct 20;6:35391.

<u>Jayasena T</u>, Poljak A, Braidy N, Smythe G, Raftery M, Hill M, Brodaty H, Trollor J, Kochan N, Sachdev P (2015). Upregulation of glycolytic enzymes, mitochondrial dysfunction and increased cytotoxicity in glial cells treated with Alzheimer's disease plasma. PLoS One 10(3):e0116092.

Review

The role of polyphenols in the modulation of sirtuins and other pathways involved in Alzheimer's disease

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a b s t r a c t

Alzheimer's disease (AD) is characterised by extracellular amyloid deposits, neurofibrillary tangles, synaptic loss, inflammation and extensive oxidative stress. Polyphenols, which include resveratrol, epi-gallocatechin gallate and curcumin, have gained considerable interest for their ability to reduce these hallmarks of disease and their potential to slow down cognitive decline. Although their antioxidant and free radical scavenging properties are well established, more recently polyphenols have been shown to produce other important effects including anti-amyloidogenic activity, cell signalling modulation, effects on telomere length and modulation of the sirtuin proteins. Brain accessible polyphenols with multiple effects on pathways involved in neurodegeneration and ageing may therefore prove efficacious in the treatment of age-related diseases such as AD, although the evidence for this so far is limited. This review aims to explore the known effects of polyphenols from various natural and synthetic sources on brain ageing and neurodegeneration, and to examine their multiple mechanisms of action, with an emphasis on the role that the sirtuin pathway may play and the implications this may have for the treatment of AD.

Contents

1. Introduction

The accumulation of oxidative damage from free radical pro-duction during normal oxidative metabolism increases with age

and is thought to be linked to age-related diseases (Harman, 1956). Oxidative stress results when pro-oxidant and anti-oxidant activ-ities in the body are not occurring at a balanced rate, leading to the production of excess reactive oxygen species, such as peroxi-des and free radicals (Barnham et al., 2004). The generation of free radicals is catalysed by redox-active metals such as copper and iron (Smith et al., 1997a). For example, hydrogen peroxide in the pres-ence of copper or iron produces hydroxyl radicals via the Fenton reaction (Buettner and Jurkiewicz, 1996). Brain tissue is vulnerable

to oxidative damage due to its high oxygen consumption, relatively low antioxidant levels, high content of redox-active metals (copper and iron), and limited regenerative capacity, and thus free radicals have been hypothesised to play an important role in the brain age-ing process (Honda et al., 2004; Reiter, 1995; Romano et al., 2010; Smith et al., 1996).

Ageing is also the main risk factor in the development of Alzheimer's disease (AD), with the vast majority of cases developing after the age of sixty-five. Thus, oxidative damage is also thought to be an important factor in the pathogenesis of this disease and indeed seems to be one of the earliest events (Jomova et al., 2010; Nunomura et al., 2001; Smith et al., 1996). Markers of free-radical damage such as increased protein and DNA oxidation, enhanced lipid peroxidation, advanced glycation end products, carbonyls, dityrosine, malondialdehyde, and peroxynitrite damage have all been reported to occur in AD (Aksenov et al., 2001; Greilberger et al., 2008; Hensley et al., 1998; Lovell and Markesbery, 2007; Montine et al., 2002; Munch et al., 1997; Sasaki et al., 1998; Smith et al., 1997b). Furthermore, decreased levels of plasma antioxidants have been seen in AD patients (Kim et al., 2006).

A hallmark of AD is the presence in the brain of extracellular amyloid plaques, mainly containing aggregated amyloid (A) peptides. These A plaques are another source of oxidative stress in AD as they sequester divalent metals such as copper and iron, which have the potential to catalyse free radical formation (Atwood et al., 2003). Copper and iron in abnormally high concentrations and markers representing oxidative stress have been found in amyloid plaques, and are elevated in the neocortex of the AD brain (Jomova et al., 2010; Lovell et al., 1998; Maynard et al., 2005; Roberts et al., 2012; Smith et al., 2000). Iron has been shown to facilitate the aggregation and deposition of A and also induce aggregation of the major constituent of neurofibrillary tangles, hyperphosphory-lated tau (Mantyh et al., 1993; Yamamoto et al., 2002). Amyloid plaques are sites of chronic inflammation in the AD brain, and this represents another source of oxidative stress due to the release of superoxide and nitric oxide (Butterfield, 2002; Reynolds et al., 2007; Wang et al., 2004).

Polyphenols may hold potential therapeutic benefits in age-related disorders due to their potent free-radical scavenging and antioxidant effects (Salah et al., 1995). Polyphenols are secondary plant metabolites that are involved in plant defence against pathogens and ultraviolet damage (Manach et al., 2004). They are found in many fruits, herbs and vegetables and thus serve as important dietary micronutrients. Chemically, polyphenols include a wide variety of biomolecules which contain several hydroxyl groups on one or more aromatic rings. They can be divided into var-ious groups according to chemical structure – including flavonoids, stilbenes and lignans (D'Archivio et al., 2007; Tsao, 2010). Natural phenolics are compounds which contain hydroxyl groups on only a single aromatic ring and therefore are smaller in size than polyphe-nols. They include phenolic acids and phenolic alcohols (see Table 1 for further details).

Recently, the potential role of polyphenols in ageing and neurodegeneration has widened with discoveries that they can modulate various important pathways in the pathogenesis of AD by reducing amyloid aggregation and inflammation and modulating a class of proteins called sirtuins which are involved in longevity and cell survival. This review aims to highlight some of these new effects of polyphenols with a focus on how polyphenols may influence amyloid aggregation and the sirtuin family of proteins.

2. Modulation of oxidative stress by polyphenols

Polyphenols are well known as antioxidants and direct scaven-gers of free radicals (Lodovici et al., 2001; Robak and Gryglewski,

1988; Salah et al., 1995). In addition, polyphenols can act as metal chelators (Brown et al., 1998; Hider et al., 2001; Moridani et al., 2003), which adds to the antioxidant effects of these compounds through inhibition of transition metal-catalysed free radical for-mation (Jomova et al., 2010). Chelation of transition metals such as Fe^{2+} can directly reduce the rate of the Fenton reaction thus preventing oxidation caused by highly reactive hydroxyl radicals (Cheng and Breen, 2000; Lopes et al., 1999). Epigallocatechin gal-late (EGCG), a green tea polyphenol, for example has also been shown to be a potent chelator of transition metals such as copper and iron (Mandel et al., 2008a), and inhibits over 90% of iron-mediated DNA damage caused by Fe^{2+} and H_2O_2 (Mandel et al., 2011). It has also been found that polyphenols are involved in the regeneration of essential vitamins (Mandel et al., 2008c; Pedrielli and Skibsted, 2002). Polyphenols can induce antioxidant enzymes such as gluthathione peroxidase, catalase, superoxide dismutase, as well as hydrogen peroxide and superoxide anions, and inhibit the expression of enzymes such as xanthine oxidase, which is involved in the generation of free radicals (Alvarez-Suarez et al., 2011; Frei and Higdon, 2003; Lee et al., 2006; Luceri et al., 2002; Luczaj et al., 2004; Mandel et al., 2008b, 2011; Moskaug et al., 2005; Sanbongi et al., 1997). These multiple antioxidant effects prevent oxidative damage to important cellular components and have lead to inves-tigation of polyphenols as therapeutic agents for the treatment of AD, to combat the increase in oxidative stress seen in this disease.

3. Effects of polyphenols on amyloid and tau aggregation

The A deposited in AD is derived from the larger amyloid pre-cursor protein (APP). Surface APP can be endocytosed, and then undergo various forms of processing, one of which results in the formation of A peptides (Selkoe, 1998). These peptides have the ability to aggregate and generate the amyloid that is found in the AD brain. The toxic potential of the A peptide depends on its con-formational state and peptide length, with A (1–42) being more toxic than A (1–40) and A (1–42) oligomers are thought to be the most toxic form (Walsh and Selkoe, 2007). Metal ions such as copper, iron and zinc also influence the aggregation state of A peptides (Mantyh et al., 1993).

Several studies have suggested that polyphenols may have anti-amyloidogenic effects. A number of polyphenols including tannic acid, quercetin, kaempferol, curcumin, catechin and epicatechin were shown to dose-dependently inhibit the formation of A fib-rils as well as their elongation (Ono et al., 2003, 2004). In addition, polyphenols can bind directly to A or mature aggregates and impair their stability, as all the compounds tested destabilised pre-formed A fibrils (Ono et al., 2003). EGCG significantly inhibits A aggregation and has the ability to remodel large A fibrils into smaller aggregates which display no toxic effects (Bieschke et al., 2010) (see Fig. 1). Fish oil has been shown to have a synergistic effect in combination with EGCG with co-treatment leading to a reduc-tion in A plaque formation and levels of A (1–40) and A (1–42) in AD transgenic Tg2576 mice (Giunta et al., 2010).

Tannic acid was shown to reduce A deposits as well as A species including oligomers in the transgenic AD mouse brain (Mori et al., 2012; Ono et al., 2003). Tannic acid decreased cleavage of the -carboxyl-terminal APP fragment, lowered APP- production, and attenuated neuroinflammation (Mori et al., 2012). It is thought to work by inhibiting amyloidogenic APP metabolism as a result of reducing -site APP cleaving enzyme 1 expression and lowering -secretase activity (Mori et al., 2012). EGCG was also shown to down-regulate APP levels and cerebral amyloidosis in Alzheimer transgenic mice (Rezai-Zadeh et al., 2005).

Curcumin, found in the spice turmeric, has been shown to pre-vent brain lipid peroxidation in rats and increase gluthathione

Table 1
Classification and dietary sources of polyphenols and phenolics.
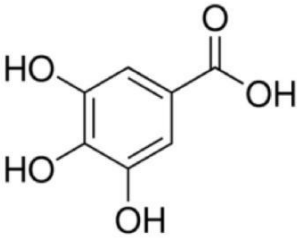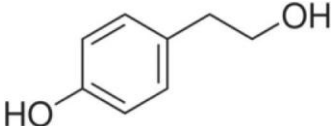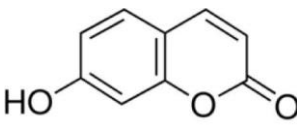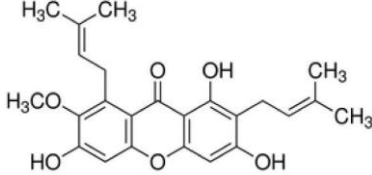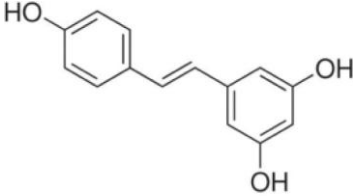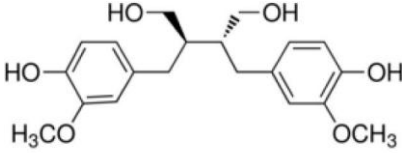
Polyphenol class	Examples	Structure	Plant source
Phenolic acids	Gallic acid Caffeic acid Ferulic acid Curcumin (derivative of ferulic acid)	(Gallic Acid)	Clove 7.8 g/kg (Shan et al., 2005), Chestnut 4.8 g/kg (Do Carmo Barbosa Mendes De Vasconcelos et al., 2007), Red wine 7.8–70.8 mg/L (Burns et al., 2000), Blackberries 80–270 mg/kg (Manach et al., 2004)
Phenolic alcohols	Tyrosol Oleuropein	(Tyrosol)	Black olives 144 mg/kg, olive oil 5.3 mg/kg (Perez-Jimenez et al., 2010)
Coumarins	Umbelliferone Aesculetin Scopoletin	(Umbelliferone)	Wine 0.05 mg/L (Alonso et al., 2004)
Xanthones	Mangostin Mangiferin	(α-mangostin)	Mangosteen 0.91–55.1 g/kg (Walker, 2007); Mango 31.7 mg/kg (Sulaiman and Ooi, 2012)
Stilbenes	Resveratrol Piceatannol	(Resveratrol)	Red wine 1.9–27.8 mg/L (Vitrac et al., 2005), Cranberry 19.2 mg/kg (Ehala et al., 2005), Peanut 11.2 mg/kg (Chukwumah et al., 2007)
Lignans	Secoisolariciresinol Matairesinol Lariciresinol	(Secoisolariciresinol)	Linseed 3.7 g/kg, Flax seed 3.35 g/kg (Penalvo et al., 2005)

Table 1 (Continued)
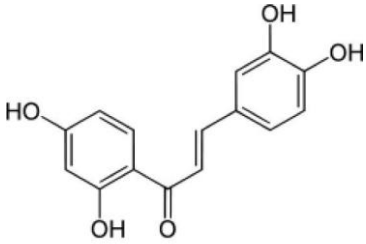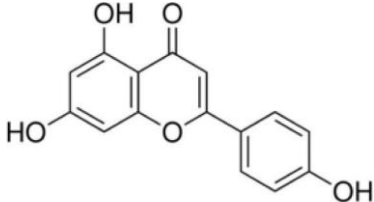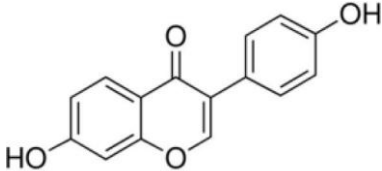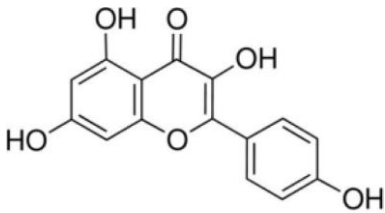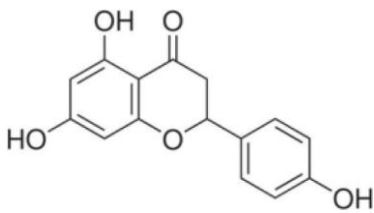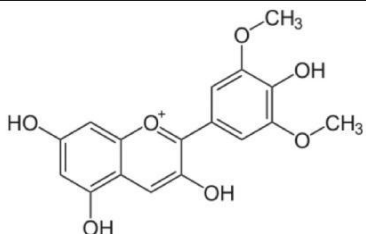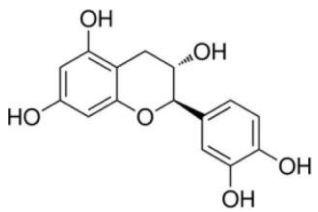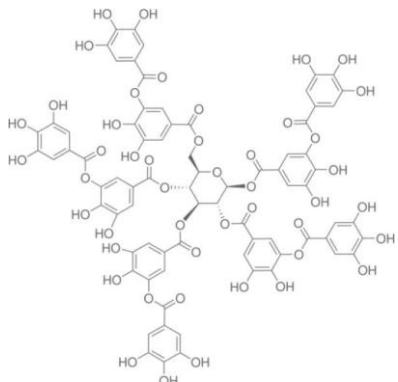
Polyphenol class	Examples	Structure	Plant source
Chalcones	Butein Cardamonin Phloridzin	(Butein)	Apple 6.4–91.1 mg/kg (Vrhovsek et al., 2004)
Flavones	Apigenin Luteolin Diosmetin	(Apigenin)	Parsley 0.24–1.85 g/kg, Celery 20–140 mg/kg, Capsicum 5–10 mg/kg (Justesen et al., 1998; Manach et al., 2004)
Isoflavones	Daidzein Genistein Glycitein	(Daidzein)	Soybeans 0.2–0.9 g/kg, Miso 0.25–0.9 g/kg, Tofu 0.08–0.7 g/kg, Tempeh 0.43–0.53 g/kg (Manach et al., 2004)
Flavonols	Kaempferol Quercetin Myricetin Fisetin	(Kaempferol)	Kale 0.3–0.6 g/kg, Onion 0.35–1.2 g/kg, Ginseng 4.3–6.6 g/kg (Justesen et al., 1998; Manach et al., 2004)
Flavanones	Naringenin Hesperidin	(Naringenin)	Grapefruit juice 100–650 mg/L, lemon juice 50–300 mg/kg (Justesen et al., 1998; Manach et al., 2004)

Table 1 (Continued)

Polyphenol class	Examples	Structure	Plant source
Anthocyanidins	Malvidin Cyanidin Pelargonidin	(Malvidin)	Cherry 0.35–4.5 g/kg, Rhubarb 2.0 g/kg, Strawberry 0.15–0.75 g/kg (Manach et al., 2004)
Flavanols (monomer)	Catechin Epicatechin Gallocatechin Epigallocatechin gallate	(Catechin)	Beans 350–550 mg/kg, Apricot 100–250 mg/kg, Green tea 100–800 mg/L, Chocolate 460–610 mg/kg, Red wine 203–805 mg/L (Arts et al., 2000a,b; Manach et al., 2004; Pellegrini et al., 2000)
Flavanols (polymer)	Tannic acid Proanthocyanidins	(Tannic acid)	Cinnamon 81 mg/kg, chocolate 16 mg/kg, grape seed 35 mg/kg (Gu et al., 2004)
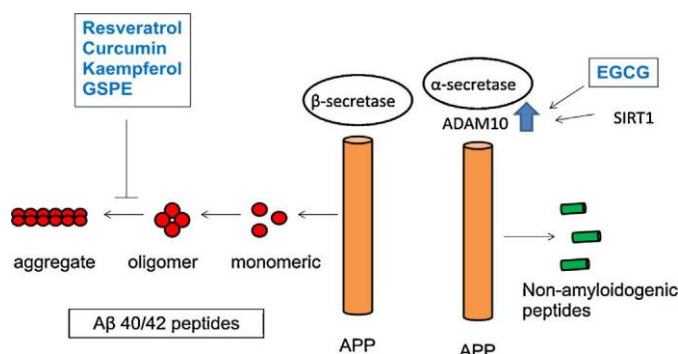
Fig. 1. Influence of SIRT1 and polyphenols on amyloid precursor protein (APP) processing and amyloid formation. SIRT1 and the green tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) activate ADAM10 and thus enhance -secretase APP processing leading to the generation of non-amyloidogenic peptides (Donmez et al., 2010; Obregon et al., 2006). Resveratrol is able to selectively remodel toxic amyloid aggregates into non-toxic species (Ladiwala et al., 2010). Polyphenols such as curcumin, kaempferol and grape seed extract polyphenols (GSEP) also have the ability to reduce A levels in the brain (Ono et al., 2003).

levels and the activity of other detoxifying enzymes (Jena et al., 2012; Khurana et al., 2012). Curcumin has also been shown to pro-tect cells against A -induced oxidative insult (Huang et al., 2012). When administered to AD transgenic mice, curcumin was found to prevent protein oxidation and inflammation and reduce the forma-tion of soluble and insoluble A by up to 50% (Lim et al., 2001).

Luteolin, a citrus polyphenol was found to reduce A peptide production in cultured neurons and decreased amyloidogenic - secretase APP processing (Rezai-Zadeh et al., 2009). In the AD mouse model Tg2576 mice, luteolin along with a structurally simi-lar compound, diosim, were both shown to reduce soluble A levels (Rezai-Zadeh et al., 2009).

In a recent study anthocyanin-enriched fractions of bilberry and blackberry extracts were found to modulate APP processing and significantly alter A 40 and A 42 levels. Both compounds also alleviated spatial working memory deficits in a mouse model of AD (Vepsalainen et al., 2013). Cinnamon extract was also found to reduce A aggregation and plaque formation as well as improve cognitive behaviour in AD transgenic mice (Frydman-Marom et al., 2011).

Abnormal folding of the microtubule-associated protein tau leads to aggregation of tau into neurofibrillary tangles, another major pathological hallmark of AD. It has been shown that

polyphenols derived from grape seed extract reduce tau pathology in mouse models of AD (Ksiezak-Reding et al., 2012; Santa-Maria et al., 2012; Wang et al., 2010a). These polyphenols also enhanced disaggregation of neurofibrillary filaments, and improved struc-tural stability (Ksiezak-Reding et al., 2012; Wang et al., 2010a). EGCG has also been shown to modulate tau pathology in a mouse model of Alzheimer's disease (Rezai-Zadeh et al., 2008). Tannic acid has recently been shown to dose-dependently inhibit tau aggrega-tion and polymerisation through formation of a hairpin binding motif (Yao et al., 2013).

4. Role of sirtuins in AD and longevity

Sirtuins are a class of proteins that possess deacetylase or mono-ribosyltransferase activity and play critical roles in cell survival in response to oxidative stress and caloric restriction (CR) regimes (Horio et al., 2011; Yamamoto et al., 2007). In mammals, seven sirtuins (SIRT1-7) have been identified. All mammalian sirtuins contain a conserved NAD-binding and catalytic domain, but dif-fer in their N and C-terminal domains (Horio et al., 2011). They have different specific substrates including histones, transcriptio-nal regulators and enzymes (Yamamoto et al., 2007). They also have various biological functions, are localised in specific cell compart-ments, and play various roles in health and disease including ageing and neurodegeneration (Yamamoto et al., 2007) (see Table 2).

Sirtuins can affect ROS production and increase resistance to its damaging effects. Oxidative stress has been shown to decrease SIRT1 expression in the rat hippocampus and cortex (Wu et al., 2006). SIRT1 overexpression prevents oxidative stress-induced apoptosis and increases resistance to oxidative stress through reg-ulation of the FOXO family of forkhead transcription factors (Brunet et al., 2004). The only cytoplasmic sirtuin protein SIRT2, has been shown to increase in response to oxidative stress but promotes cell death through FOXO proteins (Wang et al., 2007). SIRT3, a mito-chondrial protein, reduces oxidative stress through activation of superoxidase dismutase activity (Qiu et al., 2010). Recent work has shown that SIRT3 mRNA expression is elevated in response to increased mitochondrial ROS production in primary mouse hip-pocampal cells and the SIRT3 over-expression increases neuronal lifespan (Weir et al., 2012). In AD transgenic mice SIRT3 expres-sion was shown to mirror the pattern of A deposition (Weir et al., 2012). This study also showed a significant increase in SIRT3 expression in the AD temporal cortex of human post-mortem brain tissue (Weir et al., 2012). SIRT6, like the founding member of the sirtuin family SIRT1, is a nuclear protein which is involved in oxidative-stress induced DNA repair through its activation of the PARP-1 DNA repair enzyme (Mao et al., 2011) (for a summary see Table 2 and Fig. 2).

Interest in sirtuins increased greatly after the discovery that the sirtuin gene, Sir2 (SIRT1 being the equivalent in mammals) extends yeast lifespan up to 30% (Kaeberlein et al., 1999). A sim-ilar effect was found in nematodes and flies and it was thought the effect may occur through a pathway related to caloric restric-tion (Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). Recent work however suggests that the effects on longevity may not be as strong as were observed in the early studies as controlling for genetic background removed any effects on longevity (Burnett et al., 2011). The sirtuin system is strongly influenced by caloric restriction (CR), which refers to a reduction in calorie intake with-out malnutrition (Crujeiras et al., 2008). CR has been shown to have a wide range of benefits in humans (Fontana et al., 2004; Holloszy and Fontana, 2007; Omodei and Fontana, 2011) and life span exten-sion in many species from yeast, fruit flies, nematodes, rodents to primates (Fontana et al., 2010; Lin et al., 2004; Partridge et al., 2005; Qin et al., 2006a). Furthermore, CR for 3 months was found

to improve memory functions in the elderly (Witte et al., 2009). CR is also known to modulate neuroinflammation and oxidative stress in various animal models (Merry, 2004; Morgan et al., 2007; Patel et al., 2005).

The mechanism by which CR increases life span is still unclear. Some theories include reduced cellular divisions, reduced metabolic rate, reduced production of ROS and hormesis (Heilbronn et al., 2006; Lin et al., 2002; Mattson, 2008; Schulz et al., 2007). Hormesis is used to describe the beneficial actions resulting from the response of an organism to a low-intensity biological stressor (Mattson, 2008). The hormesis theory of CR proposes that diet imposes a low-intensity biological stress on the organism, which causes a defence response that helps protect it against the causes of ageing (Mattson, 2008). Therefore, it is highly likely that CR places the organism in a defensive state, and enables it to survive adver-sity, leading to improved health and life span.

Evidence suggests that some of the beneficial effects of CR occur through its influence on sirtuins. The functions of mitochondrial sirtuins suggest that they control activity of several metabolic enzymes and play a crucial role in metabolic adaptation to dietary conditions, such as CR and fasting (Li and Kazgan, 2011) (see Table 2). It has also been reported that CR does not extend lifespan in SIRT1-deficient mice, suggesting that CR works via activation of SIRT1 (Bordone et al., 2007; Qin et al., 2006a). Conversely, trans-genic mice overexpressing SIRT1 display a number of phenotypic features, such as increased metabolic activity, glucose tolerance, and reduced body weight and cholesterol levels (Bordone et al., 2007). The ability of SIRT1 to mimic the anti-ageing effects of CR is most likely linked to its regulation of various proteins and genes involved in oxidative stress, inflammation, amyloidogenesis and synaptic plasticity (see Table 2 and Fig. 2).

CR prevents A neuropathology in animal models of AD and modulation of SIRT1 expression or activity may be a mechanism by which CR influences AD-type neuropathology (Qin et al., 2006a,b, 2008). In one study 30% CR resulted in significantly lower levels of A (1–40) and A (1–42) in the temporal cortex of Squirrel monkeys relative to control fed monkeys. The cortical A levels were inversely correlated with SIRT1 protein concentrations in the same brain region, and there was also a selective elevation of - but not - or -secretase activity (Qin et al., 2006a). As mentioned previously, the cleavage of the membrane-bound APP leads to a range of protein fragments that have a role in AD pathogenesis (Kojro and Fahrenholz, 2005). Cleavage of APP by -secretase leads to the formation of APPs- , and together with -secretase leads to the generation of the A peptides (1–40) and (1–42). The lat-ter is thought to be the main toxic component in AD (Kojro and Fahrenholz, 2005). If APP is processed by -secretase, then the for-mation of these pathogenic peptides is prevented, and instead a fragment APPs- that is associated with a neuroprotective effect is generated (Donmez et al., 2010; Thornton et al., 2006). SIRT1 is able to modulate APP processing by activating the expression of the - secretase gene ADAM10, thereby reducing brain levels of A (1–42) as well as A (1–40) (Donmez et al., 2010) (see Fig. 1). Moreover, deacetylation of the retinoic acid receptor (RAR) by SIRT1 can induce transcription of the ADAM10 gene and suppress the pro-duction of the neurotoxic A (1–42) peptide (Donmez et al., 2010). There is however, no published literature to date on the effects of CR or sirtuin activation in AD using human clinical trials.

5. Polyphenols, sirtuins and ageing

The most studied polyphenol which shows effects on the sirtuin family of proteins is resveratrol which is found in its highest levels in grape seed and skin as well as in red wine. It has been discov-ered that apart from its free radical scavenging and antioxidant

Table 2
Role of the sirtuin family in calorie restriction, ageing and neurodegeneration.

Name	Location	Involvement in calorie restriction, ageing and neurodegeneration
SIRT1	Nucleus	Suppresses amyloid production by promoting -secretase cleavage of APP and Induces the Notch pathway, involved in repair of neuronal damage in the brain (Donmez et al., 2010).
		Ameliorates learning impairment and neurodegeneration in mouse models (Kim et al., 2007).
		Regulates proteins and genes involved in antioxidant response (FOXO3), apoptosis (p53) and anti-inflammatory response (NF B) (Brunet et al., 2004; Chen et al., 2005; Langley et al., 2002; Yeung et al., 2004).
		Modulates memory formation and synaptic plasticity. Activation of SIRT1 in brain enhances, and its deletion impairs, memory function and synaptic plasticity in mice (Michan et al., 2010).
		Protects neurons against metabolic imbalance (Ramadori et al., 2011).
		Reduction in SIRT1 is associated with accumulation of A and tau (Julien et al., 2009; Qin et al., 2006b).
		Promotes feeding behaviour during dietary limited conditions including calorie restriction or fasting (Cohen et al., 2009).
		Mouse knock-outs show impaired cognitive abilities, decreased dendritic branching, length and complexity, lower levels of oxidised proteins and lipids in the brain, lower growth hormone levels, and shortened lifespan (Akieda-Asai et al., 2010; Li et al., 2008a; Michan et al., 2010).
SIRT2	Cytoplasm	Enhances apoptosis and atrophy, SIRT2 inhibition is neuroprotective (Hasegawa et al., 2010; Liu et al., 2012; Outeiro et al., 2007; Spires-Jones et al., 2012).
		A tubulin deacetylase which regulates cell division and differentiation and accumulates with age (Maxwell et al., 2011).
		Impairs neurite outgrowth and oligodendrocyte differentiation and promotes axonal degeneration (Harting and Knoll, 2010; Li et al., 2007; Suzuki and Koike, 2007a).
		Small molecule inhibitor of SIRT2 showed neuroprotection via cholesterol-reducing properties in a Huntington's disease model (Luthi-Carter et al., 2010; Taylor et al., 2011).
		Upregulates in response to calorie restriction and oxidative stress, and promotes cell death under severe stress conditions via interaction with FOXO3a (Wang et al., 2007).
		Involved in myelin formation (Beirowski et al., 2011).
SIRT3	Mitochondria	Over-expressed in response to oxidative stress and significantly elevated in human AD post mortem brain tissue (Weir et al., 2012).
		Protects against excitotoxic injury in neurons (Kim et al., 2011).
		Plays a role in CR-mediated reduction in oxidative stress and decreases reactive oxygen species by increasing activity of superoxide dismutase (Bell and Guarente, 2011; Qiu et al., 2010; Someya et al., 2010).
		Variability of the SIRT3 gene may have a role in human longevity (Halaschek-Wiener et al., 2009; Rose et al., 2003).
		SIRT3 upregulates fatty acid oxidation and lowers ATP levels during fasting (Hirschey et al., 2010).
		Involved in the maintenance of mitochondrial function by preventing premature opening of the mitochondrial permeability transition pore (Hafner et al., 2010).
SIRT4	Mitochondria	Downregulates glutamate dehydrogenase activity, and controls insulin secretion in response to calorie restriction (Haigis et al., 2006).
		Negatively regulates fatty acid oxidation in combination with SIRT1 (Nasrin et al., 2010).
SIRT5	Mitochondria	SIRT5 promoter polymorphism is associated with a higher brain molecular ageing (Glorioso et al., 2011).
		Involved in regulation of the urea cycle during fasting and calorie restriction (Nakagawa et al., 2009; Nakamura et al., 2012; Ogura et al., 2010)
SIRT6	Nucleus	Involved in DNA double strand break repair following oxidative stress by inducing PARP1 activity (Lombard et al., 2008; Mao et al., 2011, 2012).
		SIRT6 associates with telomeric chromatin and depletion of SIRT6 results in premature cellular senescence and telomere dysfunction (Kawahara et al., 2011; Michishita et al., 2009).
		Prevention of transforming growth factor- -induced cell senescence in human epithelial cells (Minagawa et al., 2011).
		Neuronal SIRT6 is involved in regulation of somatic growth and obesity prevention in mice (Schwer et al., 2010).
		SIRT6 genes suppress NF-kappaB signalling and thus can delay the ageing process and extend lifespan (Kawahara et al., 2009).
		SIRT6-knockout mice exhibit genomic instability, ageing-like phenotypes and die from hypoglycaemia by 4–5 weeks. Stem cells that are deficient in SIRT6 show genomic instability (Mostoslavsky et al., 2006).
SIRT7	Nucleus	SIRT7 overexpression in fibroblasts inhibits cell growth and proliferation and promotes cell cycle arrest (Vakhrusheva et al., 2008a).
		SIRT7 deficient mice exhibit heart hypertrophy, inflammatory cardiomyopathy and a reduced lifespan (Vakhrusheva et al., 2008b).
		Positive regulator of RNA polymerase I transcription (Ford et al., 2006).

properties, resveratrol has many of the same benefits as calorie restriction. Resveratrol is associated with neuroprotective effects (Liu et al., 2011; Moriya et al., 2011; Narita et al., 2011), and other beneficial effects including maintenance of mitochondrial integrity, reduction of insulin-like growth factor-1 expression, activation of SIRT1, and increased longevity in some animals models (Baur and Sinclair, 2006; Borra et al., 2005; Brown et al., 2010; Chandrashekara and Shakarad, 2011; Dave et al., 2008; Howitz et al., 2003; Lagouge et al., 2006; Valenzano et al., 2006; Wood et al., 2004), though life span effects are controversial (Kaeberlein et al., 2005; Miller et al., 2011; Pearson et al., 2008). Epidemiological evidence suggests red wine, which is rich in resveratrol, reduces the risk of dementia (Truelsen et al., 2002).

Resveratrol has also been shown to improve outcomes after ischaemic episodes in the heart and brain of mice by a SIRT1-mediated process (Mukhopadhyay et al., 2010; Wang et al., 2012). Resveratrol strongly stimulates SIRT1 deacetylase activity in a dose-dependent manner by increasing its binding affinity to both

the acetylated substrate and NAD^+ (Chung et al., 2010). Whether resveratrol is a direct or indirect activator of SIRT1 remains con-troversial (Gertz et al., 2012; Pacholec et al., 2010). Resveratrol and CR have also been shown to modulate mitochondrial sirtuins (Gertz et al., 2012; Guarente, 2008; Qiu et al., 2010; Someya et al., 2010). Resveratrol-fed rats showed increased expression of SIRT3 and SIRT4 in the heart compared to control-fed mice (Mukherjee et al., 2009), however the opposite effect was seen in the liver of a zebrafish model (Schirmer et al., 2012). Resveratrol also increased expression of SIRT3 in adipocytes (Rayalam et al., 2008). Resver-atrol mediated changes to sirtuin expression in the brain have yet to be intensively studied. Investigation of polyphenolic sirtuin-activating compounds are thought to hold promise for enhancing longevity (Howitz et al., 2003; Quideau, 2004).

A variety of polyphenols (or foods rich in polyphenolic com-pounds) have been investigated for their effects on AD and longevity (see Table 3). Interestingly, many of these compounds influence sirtuins through direct or indirect pathways. For example,

Table 3
Influence of polyphenols on sirtuins and pathways involved in ageing and AD.

Polyphenol and source	Effect on sirtuins	Influence in AD	Influence in ageing
Resveratrol (grapes, red wine, peanuts)	Increased SIRT1, SIRT2, SIRT3, SIRT4 and SIRT7 (Lagouge et al., 2006; Mukherjee et al., 2009; Schirmer et al., 2012; Suzuki and Koike, 2007b; Yu et al., 2009); Normalised SIRT1 and SIRT3 ethanol-induced elevation in rat liver tissue (Oliva et al., 2008); Inhibits human SIRT3 and stimulates SIRT5 (Gertz et al., 2012);	Selectively remodels toxic amyloid aggregates (Ladiwala et al., 2010); Reduces Aβ plaques and toxicity in animal models (Karuppagounder et al., 2009; Porquet et al., 2012)	Increases longevity in a variety of organisms from yeast to mammals (Agarwal and Baur, 2011; Barger et al., 2008b; Chandrashekara and Shakarad, 2011; Fernandez and Fraga, 2011; Mouchiroud et al., 2010; Porquet et al., 2012; Wood et al., 2004)
Quercetin (tea, onions, apples)	Increased SIRT1 (Davis et al., 2009)	Reduces Aβ-mediated cyctotoxicity and oxidative stress (Ansari et al., 2009; Belinha et al., 2007; Heo and Lee, 2004); decreases neuronal β-secretase activity (Shimmyo et al., 2008)	Extends life span in worms and yeast (Howitz et al., 2003; Pietsch et al., 2009)
Butein (Chinese lacquer tree)	Increased SIRT1 (Chung et al., 2010; Howitz et al., 2003)	Neuroprotective and anti-inflammatory effects (Cho et al., 2012); protects cells against oxidative stress through influence on antioxidant enzyme expression (Yang et al., 2011)	Lengthens life span in yeast (Howitz et al., 2003)
Fisetin (strawberries, blueberries, cucumber)	Increased SIRT1 (Howitz et al., 2003)	Regulates levels of glutathione and mitochondrial function during oxidative stress (Maher, 2009); inhibits Aβ fibril formation (Akaishi et al., 2008)	Extends life span in yeast, worms and flies (Howitz et al., 2003; Kampkotter et al., 2007; Wood et al., 2004)
Kaempferol (tea, leek, tomato)	Increased SIRT3 (Marfe et al., 2009)	Inhibit Aβ formation and toxicity as well as reversed Aβ-induced impaired performance in mice (Kim et al., 2010; Ono et al., 2003; Roth et al., 1999)	Extends life span in worms (Kampkotter et al., 2007)
Catechins (chocolate, bitter melon, green tea)	Increased SIRT1 and SIRT3 in mice (Nerurkar et al., 2011) and increased Sir2 activity in yeast	Attenuates oxidative stress and neuroinflammation (Nerurkar et al., 2011); Neuroprotective and metal chelating properties (Mandel et al., 2008a); Inhibits formation of fibrillar Aβ (Mandel et al., 2008b,c); modulates tau and enhances cognition (Rezai-Zadeh et al., 2008); modulates APP cleavage (Rezai-Zadeh et al., 2005)	Increases lifespan in worms, flies and mice (Kumari et al., 1997; Li et al., 2008b; Zhang et al., 2009) and delays memory regression in animal models of ageing (Li et al., 2009a; Unno et al., 2007)
Curcumin (turmeric)	Not known	Reduces Aβ levels through influence on APP in AD mice (Zhang et al., 2010) and enhances memory in AD rat model (Ahmed et al., 2010)	Extends life span in flies and mice (Kitani et al., 2007; Lee et al., 2010)
Phloridzin (apple)	Increased Sirt1 activity in yeast (Xiang et al., 2011)	Upregulates superoxide dismutase and catalase genes (Xiang et al., 2011)	Extend lifespan of flies (Peng et al., 2011)
Procyanidins (apple, cocoa)	No extension of lifespan in worms lacking Sir2.1 (Sunagawa et al., 2011)	Increased resistance to oxidative stress in yeast and worms; improved cognition in elderly human subjects and aged rats (Bisson et al., 2008; Desideri et al., 2012; Xu et al., 2011)	Increase lifespan of worms, flies and rats (Peng et al., 2011; Sunagawa et al., 2011)
Proanthocyanidins (persimmon, cinnamon, grape, blueberry, blackcurrant)	Increased SIRT1 expression (Yokozawa et al., 2011)	Suppress brain oxidative stress (Asha Devi et al., 2011); inhibit formation of advanced glycation end products (Peng et al., 2010); inhibits tau aggregation (Peterson et al., 2009); improves learning and memory in mice (Yokozawa et al., 2011); modulates APP processing as well as spatial working memory deficits in a mouse model of AD (Vepsalainen et al., 2013)	Increase lifespan in worms and mice (Wilson et al., 2006; Yokozawa et al., 2009)
Astragaloside (astragalus root)	Not known	Prevents amyloid induced memory and neuronal loss in AD mouse model (Li et al., 2009b; Tohda et al., 2006)	Reduced DNA damage after oxidative stress and increased telomere length in mice fibroblasts (Lei et al., 2003; Wang et al., 2010b)
Naringin (centella asiatica, citrus fruits)	Normalised ethanol-induced increase in SIRT1 in rat liver tissue (Oliva et al., 2008)	Cognitive enhancing effects (Kumar et al., 2010; Veerendra Kumar and Gupta, 2003); reduces brain Aβ levels and is protective against Aβ-induced neurotoxicity (Dhanasekaran et al., 2009).	Not known
Xanthones (mango, mangosteen)	Not known	Improved learning ability and memory retention in mice and prevented cell damage (Biradar et al., 2012); Inhibits Aβ aggregation and Aβ-induced cytotoxicity (Moongkarndi et al., 2010; Wang et al., 2012); Prevents free radical induced oxidative damage in rat brain tissue (Marquez-Valadez et al., 2009)	Inhibits telomerase activity in cancer cells (Wu et al., 2004)
Apigenin (celery)	Not known	Reversed cognitive deficits in aged mice (Patil et al., 2003); Reduce levels of Aβ by inhibiting β-secretase activity (Shimmyo et al., 2008)	Not known
Isoflavones (soy)	Increased expression of SIRT1 (Rasbach and Schnellmann, 2008)	Protects against Aβ induced toxicity and inhibits aggregation (Gutierrez-Zepeda et al., 2005; Ma et al., 2009; Yang et al., 2010)	Enhanced cognitive performance in senescence-accelerated mice (Yang et al., 2010)
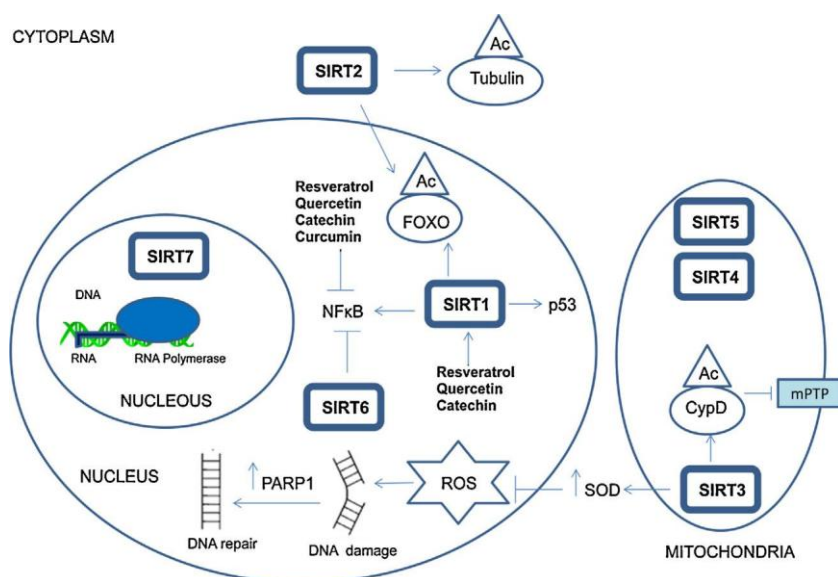
Fig. 2. Potential roles and mechanisms of action of SIRT1-7 and the influence of polyphenols. SIRT1 induces a neuroprotective effect through its interaction with FOXO proteins, p53 and NF B (Brunet et al., 2004; Chen et al., 2005; Langley et al., 2002). SIRT1 is also directly activated by resveratrol. SIRT2 is involved in regulation of the cell cycle as well as neurite outgrowth and axonal degeneration, possibly through its ability to deacteylate tubulin (Maxwell et al., 2011; Wang et al., 2007). SIRT2 is upregulated in response to calorie restriction and oxidative stress, and promotes cell death under severe stress conditions via interaction with FOXO3a (Wang et al., 2007). SIRT3 reduces oxidative stress by increasing superoxide dismutase activity (Qiu et al., 2010). It also deacetylates cyclophilin D (which regulates opening of the mPTP) and thus prevents age-related increase in mitochondrial swelling due to increased opening of the mPTP (Hafner et al., 2010). Very little is known about the other two mitochondrial sirtuins, SIRT4 and SIRT5. SIRT6 is involved in DNA repair following oxidative stress through activation of the DNA repair enzyme PARP-1 (Mao et al., 2011). SIRT7 is thought to be involved in RNA transcription as it activates RNA polymerase 1 transcription (Ford et al., 2006). Resveratrol has been found to activate SIRT1, SIRT2, SIRT3, SIRT4 and SIRT7 protein and/or gene expression in cells and animal models (Lagouge et al., 2006; Mukherjee et al., 2009; Schirmer et al., 2012; Suzuki and Koike, 2007b; Vakhrusheva et al., 2008b; Yu et al., 2009). SIRT1 has also been found to be activated by other polyphenols including quercetin, catechins and kaempferol (Chung et al., 2010; Davis et al., 2009; Marfe et al., 2009; Nerurkar et al., 2011). The anti-inflammatory activity of phenolic compounds has been demonstrated in a number of in vitro and in vivo studies. Polyphenols including catechins, resveratrol and curcumin can reduce inflammation through down-regulation of NF B function (Capiralla et al., 2012; Choi et al., 2009).

in a recent study, the functional food, bitter melon was found to attenuate oxidative stress and neuroinflammation in mice on a high fat diet (Nerurkar et al., 2011). Functional foods are foods or food components which beyond providing basic nutritional needs, also have physiological/pharmacological benefits or reduce the risk of chronic diseases (Arai, 1996). Bitter melon was also found to regulate sirtuin protein and mRNA expression. A high fat diet significantly reduced SIRT1 mRNA expression by 20%, and brain SIRT1 protein levels were reduced by 25% in C57BL/6 female mice (Nerurkar et al., 2011). Feeding of bitter melon normalised both. Interestingly, bitter melon not only normalised SIRT3 levels but also up-regulated SIRT3 mRNA by 20% (Nerurkar et al., 2011). Bitter melon also attenuated oxidative stress and neuroinflamma-tory marker levels caused by the high fat diet. The bitter melon extract contains a variety of polyphenols including gallic acid, gentistic acid, chlorogenic acid and epicatechin, but the most abundant polyphenol isolated was catechin (Nerurkar et al., 2011). Catechins are also most abundant in tea, cocoa and berries and daily consump-tion of catechin increases life span and delays memory regression in animal models of ageing (Li et al., 2009a; Saul et al., 2009).

Another traditional food Centella asiatica has been reported to have a variety of beneficial effects on A -mediated toxicity, and to promote longevity (Dhanasekaran et al., 2009). It contains numer-ous polyphenols including quercetin, kaempferol, catechin, rutin and naringin, some of which are potent antioxidants and have demonstrated effects on sirtuins (Table 3). Therefore C. asiatica may have the ability to modulate the effects of oxidative stress in the brain directly, and through regulation of sirtuin activity since several of its polyphenols influence the activity and expression of sirtuins. Most of the work to date has focused on SIRT1 as it is the mammalian homolog of yeast Sir2, which was the first organism in which sirtuin functions were studied. SIRT1 was also the first sirtuin which was shown to be activated by a polyphenol, namely

resveratrol. Future investigation into the interaction between sir-tuins (especially SIRT2-7) and polyphenols may help determine which polyphenols are specific to which sirtuins and which if any have a general effect on the whole group of proteins.

6. Protective effects of polyphenols against inflammation

Excessive inflammation is considered a critical factor in the pathogensis of AD. The AD brain has an increased expression of pro-inflammatory cytokines such as interleukin-1 and tumour necrosis factor and evidence of microglial activation (Mrak and Griffin, 2005). Several inflammatory genes are associated with an increased risk of AD, and epidemiological studies on AD patients using non-steroidal anti-inflammatory drugs suggest some benefits (Johnston et al., 2011). Inflammation is also another source of oxidative stress as superoxide and nitric oxide are released.

The anti-inflammatory activity of phenolic compounds has been demonstrated in a number of in vitro and in vivo studies (Santangelo et al., 2007). Polyphenols may affect inflammation not only as antioxidants, but also as modulators of inflammatory redox signalling pathways. Polyphenols can work as modifiers of signal transduction pathways to elicit their beneficial effects (Santangelo et al., 2007). These compounds express anti-inflammatory activity by modulating the expression of pro-inflammatory genes such as cyclooxygenase, lipoxygenase, nitric oxide synthases and several important cytokines, mainly acting through nuclear factor-kappaB and mitogen-activated protein kinase signalling (Ahmed et al., 2002; Bhardwaj et al., 2007; Capiralla et al., 2012; Khan et al., 2006; Santangelo et al., 2007; Xie et al., 1993).

In a transgenic mouse model of AD, treatment with pomegranate extract was found to reduce microgliosis and A deposition. Significant behavioural improvements were also seen and correlated with decreased tumour necrosis factor

concentration and lower nuclear factor of activated T-cell activ-ity in the pomegranate-fed mice. Pomegranate polyphenols punicalagin and ellagic acid were also shown to decrease A - induced tumour necrosis factor in cultured mouse microglia (Rojanathammanee et al., 2013).

Polyphenols may also modulate programmed cell death path-ways. Resveratrol, EGCG and luteolin have been shown to significantly inhibit the activation of caspase-3 and are able to modulate mitogen-activated protein kinases known to play an important role in neuronal apoptosis (Bastianetto et al., 2011).

7. Protective effects of polyphenols on mitochondrial dysfunction

Changes to the mitochondria seem to be another patholog-ical characteristic of AD. The AD brain shows a reduction in a number of mitochondrial enzyme activities including dehydroge-nase enzymes such pyruvate dehydrogenase, and cyclooxygenase enzymes (Parihar and Brewer, 2007). The cause is unknown, although oxidative stress is thought to play a role. Furthermore the number of intact, normal appearing mitochondria has been shown to decline in the AD brain, particularly in hippocampal neurons (Parihar and Brewer, 2007). Elevation of intracellular A levels may also potentially alter mitochondrial function and lead to increased reactive oxygen species generation (Casley et al., 2002).

Polyphenols have also been proposed to protect mitochondrial function, as has been shown for quercetin, rutin and resveratrol (Carrasco-Pozo et al., 2012). Apple peel polyphenols have similar protective effects by preventing mitochondrial complex 1 inhibi-tion (Carrasco-Pozo et al., 2011). Grape seed extract has been shown to protect the enzyme activities of the mitochondrial respiratory electron transport chain (complexes I and II) and pyruvate dehy-drogenase against oxidative stress (Long et al., 2009). EGCG and luteolin have been found to restore amyloid-induced mitochon-drial dysfunction by reducing reactive oxygen species production and normalising mitochondrial membrane potential and cellular ATP levels (Dragicevic et al., 2011). Moreover, polyphenols have also been reported to exert their anti-apoptotic action by inhibiting apoptosis-inducing factor release from mitochondria, thus pro-viding a new mechanism of action for polyphenols (Panickar and Anderson, 2011).

8. Involvement of polyphenols in telomere maintenance

The role of a number of polyphenols in the maintenance of telomere length has been investigated (see Table 3). Telomeres are specialised nucleoprotein structures that protect the ends of eukaryotic chromosomes from unscheduled DNA repair reactions and degradation. Due to the intrinsic inability of the DNA repli-cation machinery to copy the end of linear molecules, and the endogenous DNA end-degrading activity, telomeres become pro-gressively shorter after every cell division cycle. When a critical number of replications are reached, the cells enter senescence (Herbig et al., 2004) or the end of their replicative ability (Hayflick limit).

In humans, several studies have described an inverse correlation between telomere length and age or age-related disease (Cawthon et al., 2003; Panossian et al., 2003; Takubo et al., 2010). Telomere length shortens with normal ageing, life stress, inflammation and chronic diseases (Herbig et al., 2004) and telomere dysfunction is linked to the pathogenesis of age-related diseases including AD (Guan et al., 2008; Panossian et al., 2003). Various nutrients such as nicotinamide and vitamins A, D, C and E are positively associated with telomere length (Kang et al., 2006; Richards et al., 2007, 2008; Xu et al., 2009) via mechanisms that reflect their

role in cellular functions, including DNA repair and chromosome maintenance, DNA methylation, inflammation, oxidative stress modulation and activity of the enzyme telomerase which repairs chromosome ends by adding telomeric repeat subunits. Healthy lifestyles and diets are positively correlated with telomere length. Changes in diet and lifestyle can modulate telomerase activity in peripheral blood mononuclear cells, although it is not yet clear if this translates to changes in telomere length (Ornish et al., 2008).

Resveratrol has been shown to induce telomere maintenance factor WRN helicase (Uchiumi et al., 2011). Another study showed that habitual tea drinkers have longer telomeres in peripheral blood cells than those who drink tea less often (Chan et al., 2010). This may possibly be due to the anti-inflammatory effect of tea polyphenols (De Bacquer et al., 2006). Similarly, the dietary admin-istration of grape seed polyphenols to mice resulted in a trend for longer telomeres compared with controls (Thomas et al., 2009). It is clear that diet and lifestyle can together play a pivotal role in influencing inflammation, oxidative stress and psychological stress, all of which cause telomere attrition. A healthy lifestyle with a diet rich in fruits and vegetables combined with exercise and lower body mass index is indeed associated with longer telomeres (Mirabello et al., 2009). EGCG, quercetin and carvediol were shown to prevent telomere shortening and oxidative stress markers in rat cardiomyocytes after cardiac hypertrophy (Sheng et al., 2011). Two isomers, 4-Hydroxy-5-hydroxymethyl-[1,3]dioxolan-2,6 -spirane-5 ,6 ,7 ,8 -tetrahydro-indolizine-3 -carbaldehyde (HDTIC-1 and HDTIC-2) extracted from Astragalus membranaceus root have been shown to extend lifespan of human foetal lung fibroblasts (Wang et al., 2010b). Cells pre-treated with these compounds show a significant reduction in DNA damage after exposure to oxidative stress. The authors hypothesised that the Astragalus root extract related delay in replicative senescence was due to the combined effects of: (1) reduction in telomere shortening rate,(2) reduced DNA damage and (3) improvement in DNA repair (Wang et al., 2010b). A small-molecule activator of telomerase known as TA-65 has been purified from the root of the Astragalus, and is capable of increasing average telomere length and decreasing the percentage of critically short telomeres, and attenuating DNA damage in mouse fibroblasts (Bernardes de Jesus et al., 2011).

More recently, SIRT1 was reported to be a positive regulator of telomere length in vivo and to attenuate age related telomere shortening, an affect dependent on telomerase activity (Palacios et al., 2010). By contrast, another study found that SIRT1 inhibition was associated with increased telomerase activity in human cells (Narala et al., 2008). These findings link SIRT1 to telomere biol-ogy and global DNA repair. Furthermore this effect does not appear to be specific to SIRT1 alone. SIRT6 is associated with telomeric chromatin, and depletion of SIRT6 has been shown to result in pre-mature cellular senescence and telomere dysfunction (Michishita et al., 2008).

9. Therapeutic potential

As the human population continues to age, interventions that improve the quality of life and delay the onset of age-related diseases are highly desirable. Polyphenols are candidates for such interventions through their diverse biochemical roles including free radical scavenging, protein homeostasis, metal chelation, telomere maintenance and stimulation of sirtuins. Their potent effects in combination with little or no known toxicity may give them a therapeutic advantage. In the past, clinical trials using vitamins and antioxidants have frequently had equivocal out-comes in the treatment of AD. One explanation for the failure of single drugs or natural compounds in AD is that this disorder has multiple aetiologies, involving various pathological and molecular

events occurring in parallel or sequentially. This has lead to the assumption that a single drug targeting two or more active neu-roprotective moieties or a combination of compounds, targeting multiple pathological aspects of the disease, may offer a superior therapeutic benefit over a drug targeting one pathway alone. For example, a treatment regime using a mixture of polyphenols which together exhibit antioxidant, metal chelating, anti-amyloidogenic and sirtuin activation properties may prove to be superior. Initial studies using polyphenol combination therapy or "cocktails" have shown some promising preliminary results (Barger et al., 2008a; Parachikova et al., 2010).

Another important issue is the bioavailability of such com-pounds. Most polyphenols have poor bioavailability following oral ingestion (Scheepens et al., 2010). Those found in food take the form of glycosides, esters or polymers which cannot be absorbed directly and therefore must be hydrolysed by microflora or enzymes of the small intestine (Manach et al., 2004). A further confounder for the central nervous system is that polyphenols cannot pass easily through the blood brain barrier. There are however, vari-ous strategies currently under investigation to improve bioactivity. It has been found that some polyphenols can specifically mod-ify metabolic and transport processes that govern bioavailability (Scheepens et al., 2010). Therefore, specific synergistic combina-tions and interactions could be designed to improve entry into the brain. Another promising method is the use of nanoparticle carriers coupled to polyphenols (Souto et al., 2013). Brain per-meable nanoparticles may be used to enhance entry of poorly absorbed phenolic compounds into the brain (Bonda et al., 2012; Doggui et al., 2012; Sahni et al., 2011). Creation and administra-tion of EGCG nanolipidic complexes were found to double the oral bioavailability of EGCG in male Sprague-Dawley rats and also improve the EGCG's ability to enhance -secretase activity in vitro (Smith et al., 2010). Cocrystallisation is another tech-nique which could increase solubility and thus bioavailability of polyphenols. This procedure used with quercetin has shown promising results with cocrystals of quercetin showing signifi-cant increases in solubility and oral bioavailability (Smith et al., 2011).

10. Conclusion

The emerging view that polyphenols may exert a variety of mod-ulatory actions which influence the pathogenesis of AD has brought attention to functions beyond their usual antioxidant activities. A clearer understanding of the mechanisms of action of polyphe-nols, as modulators of cell signalling, their role in amyloid and tau disaggregation, telomere and mitochondria maintenance and modulation of sirtuins, and the influence of their metabolism on these properties, are pivotal for the evaluation of these potent biomolecules as inhibitors of neurodegeneration, and in developing effective treatment regimes for AD. This appears to be a promising area for future work.

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SCIENTIFIC REP⚙RTS

Application of Targeted Mass Spectrometry for the Quantification of Sirtuins in the Central Nervous System

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Sirtuin proteins have a variety of intracellular targets, thereby regulating multiple biological pathways including neurodegeneration. However, relatively little is currently known about the role or expression of the 7 mammalian sirtuins in the central nervous system. Western blotting, PCR and ELISA are the main techniques currently used to measure sirtuin levels. To achieve sufficient sensitivity and selectivity in a multiplex-format, a targeted mass spectrometric assay was developed and validated for the quantification of all seven mammalian sirtuins (SIRT1-7). Quantification of all peptides was by multiple reaction monitoring (MRM) using three mass transitions per protein-specific peptide, two specific peptides for each sirtuin and a stable isotope labelled internal standard. The assay was applied to a variety of samples including cultured brain cells, mammalian brain tissue, CSF and plasma. All sirtuin peptides were detected in the human brain, with SIRT2 being the most abundant. Sirtuins were also detected in human CSF and plasma, and guinea pig and mouse tissues. In conclusion, we have successfully applied MRM mass spectrometry for the detection and quantification of sirtuin proteins in the central nervous system, paving the way for more quantitative and functional studies.

Sirtuins are a class of proteins that possess histone deacetylase or mono-ribosyltransferase activity and play crit-ical roles in cell survival in response to oxidative stress and caloric restriction (CR) regimes[1]. In mammals, seven sirtuins (SIRT1-7) have been identified. All mammalian sirtuins contain a conserved NAD-binding and catalytic domain, but differ in their N and C-terminal domains. They have different specific substrates including histones, transcriptional regulators and enzymes. They localise to cell compartments which regulate cellular structure, metabolism and gene expression, including the cytoskeleton (SIRT2), mitochondria (SIRT3, SIRT4 and SIRT5) and nucleus/nucleolus (SIRT1, SIRT6 and SIRT7), and play important roles in health and disease[1]. SIRT1 is the best characterized and has the broadest substrate specificity. Sirtuins have emerged as critical modulators of met-abolic adaptive responses, and their activities have been linked to ageing and multiple diseases, from metabolic abnormalities to neurodegeneration.

Sirtuins can affect reactive oxygen species (ROS) production and promote resistance to their damaging effects. Oxidative stress has been shown to decrease SIRT1 expression in the hippocampus and cortex, possibly by direct degradation by ROS[2]. SIRT1 overexpression prevents oxidative stress-induced apoptosis and increases resistance to oxidative stress through regulation of the FOXO family of forkhead transcription factors[3]. The cytoplasmic sirtuin protein SIRT2, has been shown to increase in response to oxidative stress but promotes cell death through FOXO proteins[4]. SIRT3, a mitochondrial protein, reduces oxidative stress through activation of superoxidase dis-mutase[5]. SIRT6 and SIRT7, like the founding member of the sirtuin family SIRT1, are nuclear proteins involved in oxidative-stress induced DNA repair through activation of the PARP-1 DNA repair enzyme.

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Name	Areas detected in CNS	Function in CNS	Techniques used
SIRT1	Human hippocampus and cortex[6]. Has also been detected in human serum at approx 8.16 ng/µl [8]. Mouse neural stem cells and adult mouse brain[7]. Porcine brain[21].	Modulates memory formation and synaptic plasticity. Reduces with age in mice. Metabolic sensor.	TR-qPCR [6], immunohistochemistry [41], western blotting, surface plasmon resonance and ELISA [8].
SIRT2	Mouse neural stem cells and adult mouse brain[7]. Porcine brain[21]. Human serum[9].	Inhibitor of microglia-mediated inflammation and neurotoxicity [18]. Impairs neurite outgrowth and oligodendrocyte differentiation. Involved in myelin formation.	Mouse knockouts [18], western blotting.
SIRT3	Cortex, Hippocampus and Cerebellum[10]. Mouse neural stem cells and adult mouse brain[7]. Porcine brain[21]. Rat brain. Human serum[9].	Responses to oxidative stress and involved in maintenance of mitochondrial function.	Western blotting and qPCR[10].
SIRT4	Mouse neural stem cells and adult mouse brain[7]. Porcine brain[21]. Rat cortical cells and brain tissue[42].	Regulation of glial development[42]; involved in glutamate transport and protective role against excitotoxicity[43].	qPCR, western blotting and immunofluorescence [21,42]
SIRT5	Mouse neural stem cells and adult mouse brain[7]. Porcine brain [21]	SIRT5 gene polymorphism may promote molecular brain aging and be a risk factor for mitochondrial dysfunction-related diseases[44].	qPCR [21]
SIRT6	Mainly localised in the nucleus in the cortical layers[45]. Mouse neural stem cells and adult mouse brain[7,40,47]. Rat brain. Porcine brain[21].	Regulator of somatic growth by modulating neural chromatin and gene activity[40]. Modulated DNA repair in the brain[46]. Suppresses proinflammatory gene expression.	Brain specific mouse knockout models and primary brain cell models[40,46,47]; immunohistochemistry[45]; western blotting, immunofluorescence[47].
SIRT7	Mouse neural stem cells and adult mouse brain[7]. Porcine brain[21].	Positive regulator of RNA polymerase I transcription.	Mouse knockout [46]

Table 1. Expression of sirtuins in the CNS and current methods used for analysis.

SIRT1 is expressed in the adult brain, in the cortex, hippocampus, cerebellum, and hypothalamus, and in lower levels in the white matter[6]. Among the brain cell types, SIRT1 is predominantly expressed in neurons and viewed as a nuclear protein[6]. The mRNAs for all seven sirtuins have been identified in mouse brain tissue and also neural stem cells [7]. SIRT1, SIRT2 and SIRT3 have also been detected in human serum, and levels were shown to decline with age and were linked to frailty[8,9]. SIRT3 is elevated at both the mRNA and protein levels in Alzheimer's disease (AD) *post mortem* brain tissue compared to controls[10].

The most common techniques currently utilised for detecting a change in sirtuin levels at the mRNA or pro-tein level are PCR and western blotting, respectively (see Table 1). Other studies have used methods such as immunohistochemistry, surface plasmon resonance and ELISA assays (see Table 1). The majority of these meth-ods are only semi-quantitative with moderate sensitivity, use antibodies which may not have sufficient specificity or detect expression at the mRNA level which may not reflect protein expression. Furthermore, there is no current assay which detects multiple sirtuins simultaneously.

Quantitative expression analysis of mammalian sirtuin proteins (especially SIRT2-7), across cell and tissue types is limited in the current literature. Previous studies have shown an increase in SIRT3 in AD post- mortem brain tissue using western blotting for protein expression and multiplex qPCR to assay SIRT3 mRNA levels[10]. Both SIRT3 protein and mRNA were shown to be significantly elevated in the AD group[10]. Another recent paper detected SIRT1 in human serum samples using western blotting, surface plasmon resonance and ELISA to meas-ure SIRT1 protein levels[8]. SIRT1 declines with age and is more dramatically reduced in MCI and AD patients compared to age matched controls, suggesting that SIRT1 may warrant further investigation as a potential plasma biomarker for AD[8]. SIRT1 and SIRT3 levels in serum were found to be significantly lower in frail subjects as com-pared to the non - frail[9]. Another study reported a decrease in SIRT1 and SIRT2 mRNA levels using quantitative real time PCR in the primary motor cortex of human post-mortem amyotrophic lateral sclerosis brain tissue[11].

Mass spectrometry has been used to successfully assay metabolites of SIRT1 activator drugs in plasma and urine[12], but to date has not been used to quantify protein levels directly. Mass spectrometry has a great advantage over the conventionally used antibody based methods, such as western blotting, in that it offers greater specificity, linearity, reproducibility and typical limits of quantification down to the low fmol range. It also removes some of the antibody specificity issues that are associated with methods such as western blotting and ELISA, as peptides unique to each protein are measured. Targeted MRM based mass spectrometry may provide a more specific and sensitive method to detect and quantify sirtuin expression at the protein level, providing a tool for comprehensive sirtuin protein expression analysis. The aim of this study was to develop a targeted mass spectrometry method using multiple reaction monitoring (MRM) to quantify the seven human sirtuins at the protein level and to apply the assay to CNS biological samples.

Results

Peptide standards. Standard curves for sirtuin peptides are shown in Fig. 1. The LOD and LOQ were deter-mined when the signal to noise ratio of the transition with the highest intensity was approximately 3:1 and 10:1 respectively. All peptides had good linearity in the 1–200 fmol/µl range and intra- and inter- assay variance was <10% and less than < 14% respectively. Representative chromatograms for each of the 14 peptides are shown in Supplementary Figure S1.

Sirtuin peptide standard curves spiked with buffer only (0.1% formic acid and no gel bit), blank gel bits and gel bits containing Hu6 depleted plasma had very similar regression equations and slopes, indicating modest matrix effects (Supplementary Figure S2). Sirtuin recoveries (from the gel spike experiment, Supplementary Figure S8), relative to sirtuin run in buffer only achieved 61–96% recovery and 58–131% recovery for the 5 µg and 2 µg sirtuin levels respectively (Supplementary Table S1).

Quantification of sirtuin expression in cells and tissues. In all the human primary brain cell types and human brain cell lines SIRT1, 2, 3, 6 and 7 were detected and quantified (Fig. 2). All seven sirtuins were detected

in human control brain tissue, with SIRT2 highly expressed (Fig. 3, Panel A). Detergent fractionation into subcel-lular groups improved identification of mitochondrial sirtuins (SIRT3–5) (Fig. 3, Panel B).

We were also able to quantify some sirtuins in guinea pig and mouse tissue due to their common sequences with human sirtuin peptides. SIRT1-3 levels in the guinea pig (Fig. 4, Panel A) and SIRT 1 and 3 levels in mouse organs (Fig. 4, Panel B) were quantified. Furthermore the method could be adapted for all sirtuins from other species with purchase of the appropriate synthetic peptides and their heavy internal standards. Supplementary Table S2 shows the level of homology (or identity) of human SIRT1-7 peptide sequences with mouse and guinea pig. All samples were fractionation on a 1D-SDS-PAGE to reduce sample complexity and improve detection sen-sitivity. A workflow of the sample preparation procedure can be found in Supplementary Figure S3.

Another important issue is the existence of splice variants or isoforms of the sirtuin proteins. Supplementary Table S3 lists the sequences of all sirtuin isoforms and the location of the two MRM peptides within each sequence. The majority of sirtuin isoforms contain both peptides, and all contain at least one of the two peptide sequences used for quantification in this study.

Validation of MRM method with established protocols.
Human brain cells and tissue samples were compared with established protocols for sirtuin detection such as immunohistochemical staining, PCR and west-ern blotting. Immunohistochemical staining showed that all seven sirtuins are expressed in astrocytes (Fig. 5, panel A, rows 1–4) and control human frontal lobe brain tissue (Fig. 5, panel A, row 5). PCR identified SIRT1 and SIRT2 mRNA in control human frontal, occipital and hippocampus brain tissue (Fig. 5, panel B). Western blotting confirmed the identification of SIRT1, 2 and 3 in control human frontal lobe brain tissue (Fig. 5, panel C and Supplementary Figure S4).

Quantification of sirtuins in CSF and immunodepleted plasma.
SIRT1 was quantified in the CSF of five control patients and in control plasma immunodepleted of the six most abundant proteins. SIRT1 was the only sirtuin detected and was found to range from 4.30 ± 0.19 to 5.09 ± 0.53 fmol/µg total protein in CSF and 8.68 ± 0.35 fmol/µg total protein in plasma. Individual data can be found in Supplementary Figure S5. SIRT2-7 in CSF and immunodepleted plasma were found to be below the detection limits of the assay. All samples were fractionated by 1D-SDS-PAGE to reduce sample complexity and a workflow of the sample preparation procedure can be found in Supplementary Figure S3.

Discussion

The rapid expansion of instruments and software in the field of targeted protein quantification by MRM is expected to have vast applications in quantitative protein biochemistry[13,14]. Most of the currently used protocols to measure sirtuin changes in the CNS have ultilised methods such as ELISA, surface Plasmon resonance, western blotting and fluorescent staining. Mass spectrometry provides a platform that overcomes some of the limitations of antibody based approaches for protein quantification, in particular providing a level of specificity not avail-able with the other approaches. Isotopically labelled peptide standards allow for quantification of protein levels and also provide the ability to monitor stability of analytes throughout the sample processing steps. This greatly simplifies the development of assays compared with standard immunological formats such as ELISA where well-characterised antibodies are needed. Furthermore MRM facilitates multiplexed analysis, identifying several proteins in a single run, thereby maximising information obtained per sample, minimising assay time while at the same time conserving precious samples. It measures several transitions per quantified protein, thus gener-ating several independent measurements, and with the use of heavy peptide standards allows for generation of standard curves and accurate quantification. This approach has advantages over methods such as western blotting and staining techniques which are semi-quantitative at best and lack the level of structural specificity achieved by mass spectrometry. Our MRM approach is also complementary to PCR which targets mRNA levels only.

Perhaps the major limitation to mass spectrometry based protein quantification is throughput and cost. Immunological assays can be performed in 96 well plate formats and plate readers allow measurement of an entire plate in a single run. Mass spectrometry requires each sample to be run individually with longer run times. However one of the main advantages of mass spectrometry is its ability to specifically and accurately distinguish different isoforms or modified forms of proteins, even in complex samples. The sensitivity of mass spectrome-try is very high but limitations to sensitivity of quantitative assays are often caused by the dynamic range of the proteins in the sample. Future developments in mass spectrometer analysers and detectors may help address this limitation. Even so, it is probable that improved sample purification/fractionation procedures will continue to be a vital element of the most challenging and complex proteomics problems, and to achieve the required sensitivity for robust quantification. For example in this study all samples were fractionation on a 1D-SDS-PAGE gel and gel bands excised and tryptic digested prior to MRM analysis to reduce sample complexity. A workflow of the sample preparation procedure can be found in Supplementary Figure S3.

MRM mass spectrometry was successfully utilised in this study to quantify seven mammalian sirtuins in human brain cells, tissues and fluid. Furthermore the method can be used to measure sirtuin expression in animal tissues, such as mouse and guinea pig, in cases where peptide sequences are identical to the human standards. In line with previous data, our results affirm that there is significant divergence in abundance amongst members of the sirtuin family of proteins in the brain. We report SIRT1 and SIRT2 to be the most abundant sirtuins in cultured brain cells with SIRT1 highest in neurons and SIRT2 highest in oligodendrocytes, validating previous studies showing SIRT2 to be highly expressed in oligodendrocytes[15]. Our study and others have also found SIRT2 expressed in neurons and glial cells[16,17]. Further validation with higher sample numbers and across a wider range of cell lines may help elucidate differences between cell types for the lower abundant sirtuins.

We found SIRT2 to be the most abundant sirtuin in the adult human frontal lobe and cortex and cerebel-lum homogenates from the guinea pig. SIRT2 is known to co-localise with microtubules and functions as a
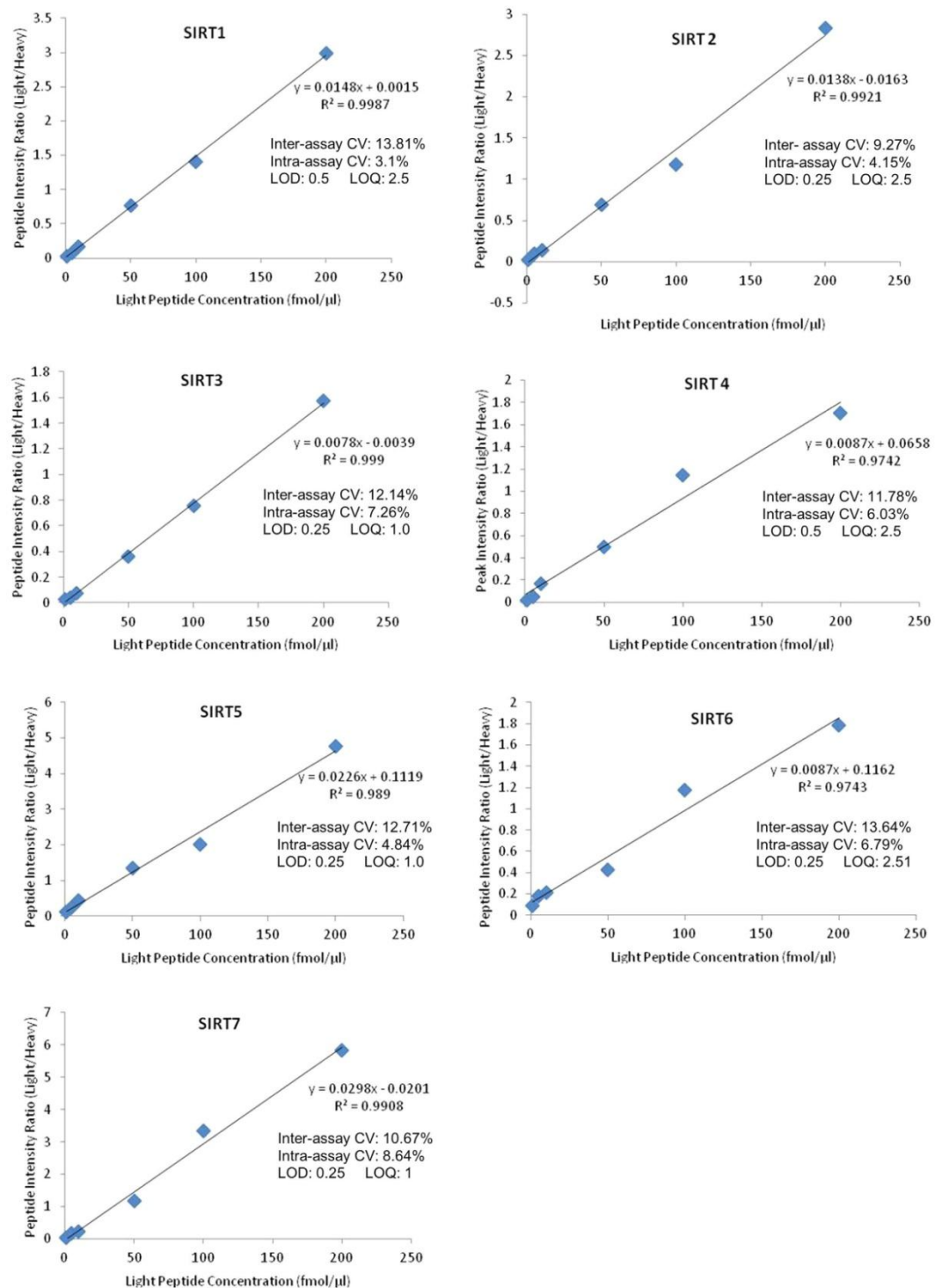
Figure 1. Sirtuin peptide standard curves, variance and limits of detection and quantification. Sirtuin peptide standard curves (average of the peak area ratios for the two peptides, in triplicate for each sirtuin, with heavy peptide spike of 100 fmol/µl). Both inter- and intra-assay variance were calculated for three replicates and the LOD and LOQ are shown in fmol/ul. CVs calculated using peptide peak area ratios (light/heavy) at the 100 fmol/µl peptide concentration level. Individual chromatograms for each of the 14 peptides can be found in Supplementary Figure S1.

tubulin-deacetylase. It is a suppressor of microglial activation and brain inflammation, with reduced levels of SIRT2 leading to increased production of free radicals and neurotoxicity, while its overexpression inhibits brain inflammation[18]. In other studies, however, it has been shown to increase in cells with oxidative stress and promote death cell[4]. In addition, SIRT2 inhibition has been shown to protect against Parkinson's disease and Huntington's
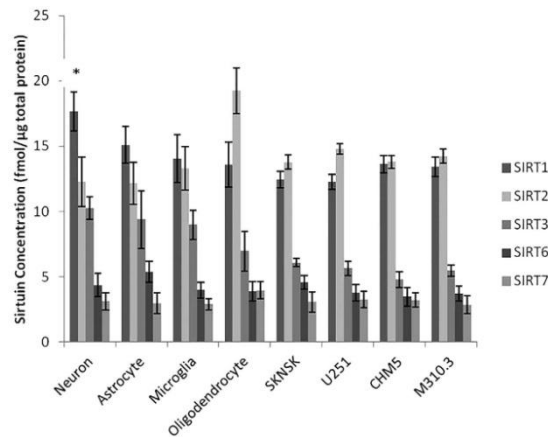
Figure 2. Sirtuin expression in primary cultured brain cells and cell lines. In primary neurons SIRT1 was found to be the most abundant sirtuin (*p < 0.05) compared to other neuronal sirtuins (n =3). SIRT2 was found to be abundant in primary oligodendrocytes (*p <0.05) compared to other cell types (n = 3). SIRT1 and SIRT2 were the most abundant in all the cell cultures. SIRT4 and SIRT5 were below the detection limits and only small amounts of SIRT6 and SIRT7 were detected.
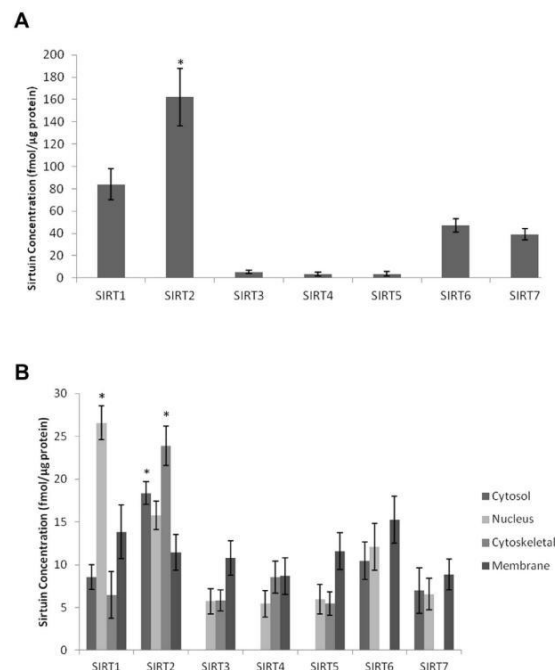
Figure 3. Sirtuin expression in human frontal lobe brain tissue. Sirtuin expression in unfractionated (Panel A) and fractionated (Panel B) human frontal lobe brain tissue. All seven sirtuins were detected in unfractionated human frontal lobe brain tissue with SIRT2 the most abundant (*p <0.05) compared to all other sirtuins (Panel A). The mitochondrial sirtuins (SIRT3-5) were found to be close to the LOQ, but their signals improved following fractionation of the tissue into cytosolic, nuclear, cytoskeletal and membrane fractions (Panel B). Fractionation showed SIRT1 and SIRT2 expressed in all fractions, whereas SIRT3-5 were below the LOD in the cytosol and SIRT6 and SIRT7 were below the LOD in the cytoskeletal fraction. SIRT1 was most abundant in the nucleus and SIRT2 in the cytoskeletal fraction (*p < 0.05, Panel B). In the cytosol, SIRT2 was the most abundant sirtuin (*p <0.05), and all sirtuins were captured in the membrane fraction.

disease[19,20]. Previously published results have shown SIRT2 to be abundant in the brain and serum[9,11,21,22] and our study provides further evidence for this and shows that it is also abundant in the guinea pig brain.

SIRT1 was the second-most abundant sirtuin in the brain and the only sirtuin detected in the CSF and plasma using our method. SIRT1 is a neuroprotective factor in a variety of models of neurodegenerative diseases, includ-ing Huntington's disease, Multiple Sclerosis and AD[23,24]. It increases following exposure to cellular stressors, including energy/nutrient depletion and has been linked to increased lifespan in animal models[25]. We were only
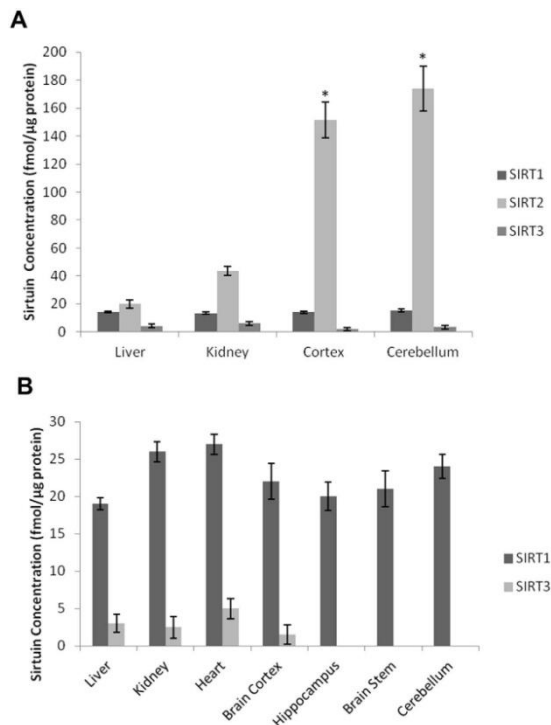
Figure 4. SIRT1-3 protein expression in animal organs. SIRT1-3 protein expression in guinea pig (Panel A) and mouse organs (Panel B). SIRT2 was found to be the most abundant sirtuin in guinea pig, with higher levels expressed in the brain (*p <0.01, n =2) compared to liver and kidney. SIRT2 in mouse was not quantified due to peptide sequence difference with the human peptide standards.

able to detect SIRT1 in both CSF and plasma while Kumar *et al.* have recently identified SIRT1, SIRT2 and SIRT3 in human serum using surface plasmon resonance[9]. The use of more sensitive mass spectrometry instrumen-tation, and/or fractionation or enrichment of plasma proteins, may improve sensitivity for the other sirtuins in plasma.

The mitochondrial sirtuins SIRT3 -5 were found at lower levels in human brain but detection was improved after detergent fractionation. Further fractionation of samples or purification/enrichment of mitochondria may facilitate improved mitochondrial sirtuin quantification. Our data also show that SIRT6 and SIRT7 are the third and fourth most abundant members of the sirtuins family in the adult human frontal lobe. Both SIRT6 and SIRT7 have been associated with the maintenance of DNA stability and promotion of DNA repair following exposure to cellular stressors[26,27].

We also confirm that mammalian sirtuins are localised to several subcellular compartments. While SIRT1, SIRT6 and SIRT7 are predominantly found in nuclear fractions, they are also detected in cytosol, cytoskeleton and membrane fractions, albeit at much lower levels. Similarly, although SIRT2 is the only human sirtuin primar-ily localised in the cytoplasm, it may also be found at lower levels in the nucleus and cellular membrane. Our data confirm that SIRT1 and SIRT2 may interact with both the nuclear and cytoplasmic subcellular compartments. Similarly, while the mitochondrial sirtuins (SIRT3-5) were previously reported to be exclusively localised to the mitochondria, other studies have reported the localisation of several variants in the nucleus as well as the cyto-plasm, in line with our study[28]. SIRT5 has recently been found to have both desuccinylase and de-malonylase activities in both the mitochondria and cytosol[29,30]. The expression patterns of sirtuins in brain cells remains controversial and it is unclear whether certain sirtuins are specific to cell types. Our MRM study indicates that the majority of sirtuins (SIRT1, 2, 6 and 7) are present across all the main brain cell types.

For validation, analyses of the samples by immunohistochemical staining confirmed that all seven sirtuins are found in human primary astrocytes (Fig. 5, panel A row 1–5). PCR detected SIRT1 and SIRT2 mRNA in frontal, occipital and hippocampal brain tissue (Fig. 5, panel B). Western blotting identified protein levels in frontal lobe brain tissue with SIRT2 showing the strongest bands (Fig. 5, panel C and supplementary Figure S4), in good agreement with the results from our MRM assay (Fig. 3).

Various studies have demonstrated that abnormal changes to sirtuin proteins occur during disease states as well as ageing. SIRT2 accumulates with age in the mouse brain and spinal cord[22]. Other recent studies have shown that SIRT1 decreases with age in the rat brain[31] and SIRT2 is upregulated in brain tumours[32]. Hence, there is great value in developing robust quantitative assays such as MRM mass spectrometry to accurately quantify these proteins in control vs disease states. Another important aspect of sirtuin biology is that their deacetylase activity is nicotinamide and NAD^+ dependent, establishing a direct link between their function and energy metabolism. To improve sensitivity, we found our fractionation method of running the samples on SDS PAGE and excising gel bands corresponding to the molecular weights of the intact sirtuin proteins to improve detection sensitivity.
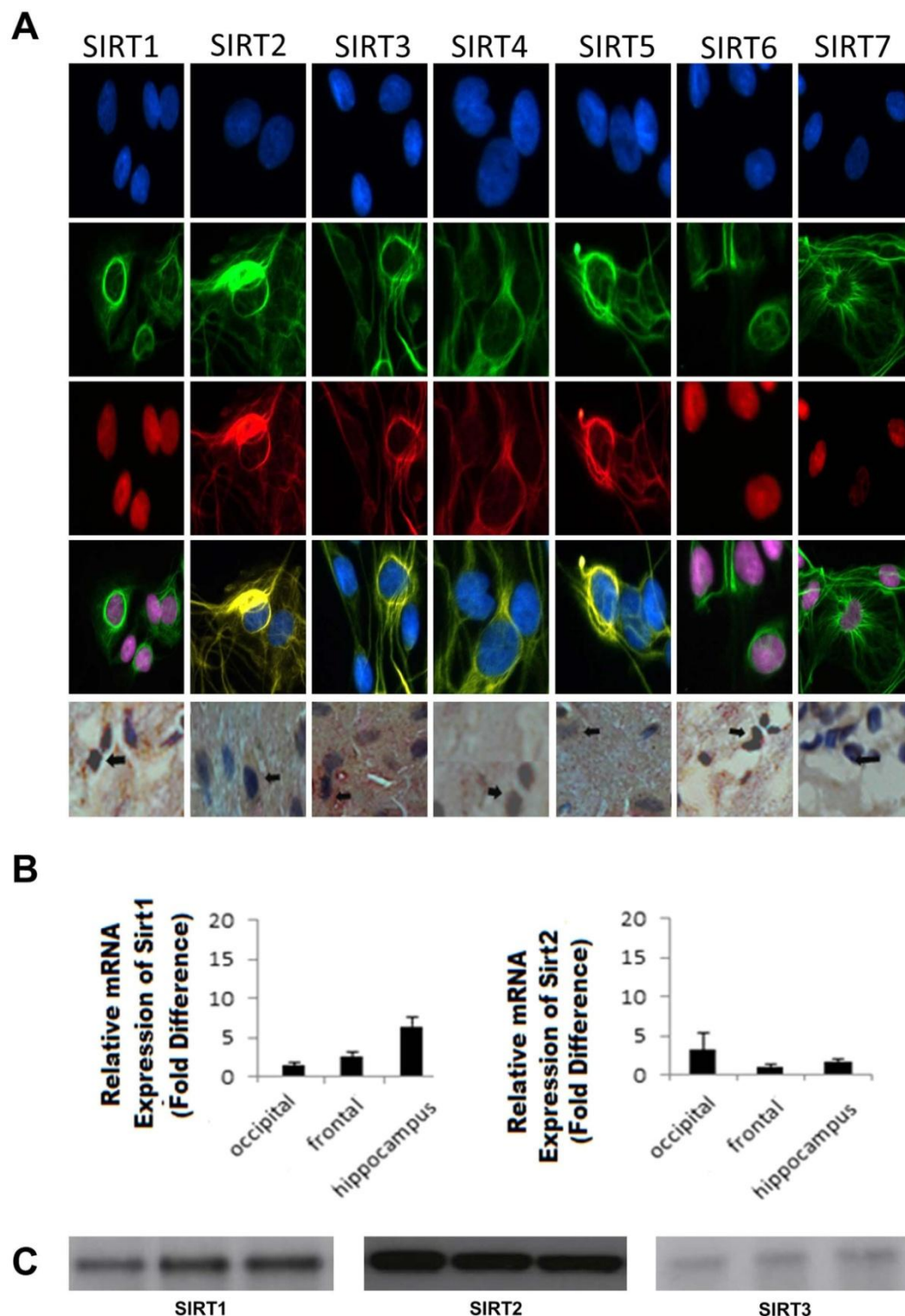
Figure 5. Sirtuin expression in human brain cells and tissue using immunohistochemical staining of astrocytes (Panel A row 1–4) and human frontal lobe brain tissue (Panel A, row 5), PCR of SIRT1 and SIRT2 mRNA in occipital, frontal and hippocampal human control brain (Panel B) and cropped images of western blotting of SIRT1-3 protein expression in human control frontal lobe brain tissue (n = 3) at molecular weights of approx 40 kDa, 50 kDa and 30 kDa respectively (Panel C). Full length blots are presented in Supplementary Figure S4.

The use of more sensitive mass spectrometers with high resolution MRM capability may improve detection of low abundant sirtuins and/or allow removal of the gel fractionation step. This would allow for sample lysates to be digested and analysed directly and decrease sample processing time. Using hybrid mass spectrometers capable

of high resolution and accurate mass measurements such as the quadrupole-orbitrap may overcome these issues and yield better sensitivity for high-complexity samples. This will help future validation of levels in large cohorts which may be useful for the identification of sirtuins as potential biomarkers.

Collectively, our results add further insights to the limited data which currently exist regarding the expression of sirtuins in the human CNS. This technique provides a powerful tool and helps improve upon the limitations of current protocols. While mass spectrometry based assays for protein quantification may still have some bar-riers to overcome before they can be used in a clinical setting due to low throughput and expense compared to methods such as ELISA, they have great value in investigating lower abundance proteins such as sirtuins in com-plex samples such as the brain with great sensitivity and specificity. Furthermore the current approach could be extended to develop isotope specific sirtuin assays, or extended to other species by use of appropriate synthetic peptide standards. MRM mass spectrometry can also be utilised for the quantification of sirtuin related metabo-lites and thus targeted mass spectrometry methodologies have the potential to not only validate and complement currently established methods for investigation of sirtuin protein expression changes but can also help build a larger view of sirtuin biology in the normal brain and in disease conditions by linking sirtuin protein expression with metabolism.

Methods

Selection of target sirtuin peptides.   Recombinant protein standards were purchased for seven human sirtuins (Cayman Chemical, USA), and 5 µg of each was run by 1D SDS-PAGE gel and colloidal coomassie stained. Sirtuin bands were excised, trypsin digested overnight followed by LC-MS/MS analysis on a QToF Ultima API hybrid tandem mass spectrometer (Micromass, UK) as previously described[33–35] and a detailed method is provided in Supplementary Protocols. The two peptides with the highest signal intensity for each sirtuin were cross-referenced with Skyline software (MacCoss Lab Software, USA). Peptide sequences were checked to ensure no overlap with other sirtuins. Sirtuin standards were run using MRM LC-MS/MS on a 4000 Q TRAQ (SCIEX, USA) mass spectrometer to ensure good signals were detected for all 14 peptides selected for the final list (two unique peptides for each sirtuin). See Supplementary Tables S2 and S4 and supplementary protocols for peptide sequences and light and heavy product ions for all 14 peptides. The full list of transitions and corresponding col-lision energies and MRM method details are provided in Supplementary Table S5.

Targeted mass spectrometry.   MRM analyses were performed on a 4000 Q TRAP hybrid triple quadru-pole linear ion trap mass spectrometer (SCIEX, USA) interfaced with a nanospray ion source, operating in posi-tive ion mode and controlled by Analyst 1.5 software. Peptides were concentrated and desalted onto a micro C18 precolumn (500 µm × 2 mm, Michrom Bioresources, USA) with $H_2O:CH_3CN$ (98:2, 0.05% TFA) at 15 µl/min. After a 4 min wash, the pre-column was automatically switched (10 port valve, Valco, USA) into line with a nano column (as described in the previous section). Peptides were eluted using a linear gradient of $H_2O:CH_3CN$ (98:2, 0.1% formic acid) to $H_2O:CH_3CN$ (36:64, 0.1% formic acid) at ~300 nl/min over 30 minutes. Samples were ana-lyzed with an ion spray voltage of 2.4 kV, curtain gas flow of 12 and nebulizing gas flow of 5 L/min. Quadrupoles were operated in the low resolution mode, and the dwell time was 50 ms. For validation runs, the MRM experi-ment triggered MS/MS spectrum acquisition. MS/MS spectra were acquired in the trap mode (enhanced product ion) with dynamic fill time, Q1 was operated using low resolution. Each sirtuin protein (SIRT1-7, 2 peptides per protein) were run with 12 transition ions per run, per protein and with a 50 ms dwell time. See Supplementary Table S5 for a full list of transitions for each peptide and corresponding collision energies (estimated using Skyline software).

Sirtuin Peptide Standards.   Multiple point calibration was used where a series of standard 'light' peptides with known concentrations together with fixed amounts of stable isotope-labelled 'heavy' peptides (100 fmol/µl) were used to generate calibration curves (Fig. 1). The curves were expressed as ratios of light/heavy peak area (y-axis) versus concentration of light peptide (x-axis) for each of the 14 peptides selected. A commercially avail-able stable isotope-labelled peptide standard (AQUA peptide, Sigma, USA) was used for absolute quantification of proteins. This heavy surrogate of each of the peptide standards is added at a constant level to all samples and standards and is used for correction of sample losses during workup and normalisation across runs, allowing accurate quantification of the target protein in samples. Peptides labelled with a stable isotope (^{13}C and ^{15}N) are chemically identical to their native counterparts and have identical chromatographic behaviour but can be distin-guished from the calibration standards based on a small specific mass difference.

Preparation of Cell Cultures, Tissues, CSF and Plasma.   *Cells.*   Adult human primary micro-glia, astrocytes, neurons and oligodendrocytes were cultured from resected normal adult brain tissue follow-ing removal of brain tumour with informed consent at the Minimally Invasive Cancer Centre, Prince of Wales Hospital, Sydney, Australia. Astrocytes were prepared from the mixed brain cell cultures using a protocol previ-ously described by Guillemin *et al.*[36]. Cell culture procedures are described in detail in Supplementary Protocols. All cells and cell lines were lysed using RIPA buffer followed by probe sonication and cell debris removed by centrifugation at 10,000RPM for 5 min. Total protein concentration was assayed in the supernatant using the Pierce BCA protein assay kit (Life Technologies, Australia), and three 10 µg replicates were run by 1D SDS-PAGE followed by colloidal coomassie staining.

Human Tissues.   20 µg of protein from five individual control post mortem brain tissue samples were extracted as described for cells in the previous section, and run on a 1D SDS-PAGE gel and coomassie stained. Detailed patient information including age, sex and post-mortem tissue collection times can be found in Supplementary

Table S6. For the fractionated samples, 200 mg of frontal lobe brain tissue from five control subjects were pooled and differential detergent fractionation performed to obtain cytosol, nucleus, cytoskeletal and membrane protein fractions[37]. Each fraction (20 μg) was run on a 1D SDS-PAGE gel and coomassie stained.

Animals.   Female guinea pigs (Dunkin–Hartley) and C57BL6 mice were housed in temperature-controlled rooms (21–22 °C; 49–55% humidity) with 12 h light-dark cycle (lights on 7:00–19:00). Food and water was avail-able *ad libitum*. Wild type mice and guinea pig organs were lysed (RIPA buffer) by probe sonication. Proteins (20 μg/lane) were run by 1D SDS-PAGE and coomassie stained. Protein concentrations were determined using the Pierce BCA protein assay kit (Life Technologies, Australia).

CSF and plasma.   CSF samples were collected by standard lumbar puncture from five patients assessed as clin-ically well after investigation for suspected meningitis (Supplementary Figure S5), returning normal results for routine CSF pathology markers (white cell count, protein, glucose and bacterial sterility). Aliquots (50 μl) from each patient were obtained for our study. Control human plasma from a healthy individual was immunodepleted of the six most abundant plasma proteins using an Hu6 column (Agilent, USA) according to manufacturer's instructions[38]. For immunodepletion using the Multiple Affinity Removal System Hu6 column and buffer kit (Agilent, Santa Clara, CA, USA). 24 μl EDTA plasma was diluted into 120 μl Buffer A. 100 μl of this diluted EDTA plasma was injected onto the Hu6 column connected to a HP 1090 HPLC system (Agilent, Santa Clara, CA, USA) and the low abundance protein fraction was collected following manufacturer's instructions. The low abundance protein fractions from six injections were pooled, buffer exchanged and concentrated into 45 mM $NaHCO_3$ using Amicon 3 kDa centrifugal devices (Millipore, Billerica, MA, USA). CSF protein (5 μg) and depleted plasma pro-tein (20 μg) as determined by a BCA protein assay were run on a 1D SDS-PAGE gel and colloidal coomassie stained.

For all samples, the band corresponding to the molecular weight for each sirtuin was excised (see Supplementary Figure S6), de-stained and trypsin digested overnight with heavy sirtuin peptides added as internal standards to all samples prior to digestion (the internal standard was added at a constant level to all samples and standards, and was at about the midrange of the standard curve). The tryptic peptides were dried under vacuum (Savant Speedvac, Thermo Scientific, USA), reconstituted in 0.1% formic acid (5 μl), injected into the mass spectrometer (1 μL) and analysed using MRM. Peak area ratios (light/heavy) were calculated for each endogenous peptide (light) relative to the spiked isotope- labelled (heavy) peptide using Skyline MRM analysis software. Protein concentrations were determined using calibration curves. All samples were fractionated by 1D-SDS-PAGE to reduce sample complexity. A workflow of the sample preparation procedure can be found in Supplementary Figure S3.

To test for matrix effects relevant to the in-gel processed sirtuins, Hu6 depleted plasma was run by SDS-PAGE (see Supplementary Figure S7) and sirtuin peptide standard curves prepared by spiking with excised gel bits taken from the expected migration position of the intact sirtuin. Additional matrices tested included buffer only (0.1% formic acid, no gel bit), a blank gel bit, and the spiked standard curves are shown in Supplementary Figure S2.

To determine recoveries of sirtuin proteins, commercial intact sirtuin standards were run by SDS PAGE (5 μg per lane). Hu6 high abundance depleted plasma spiked with 2 ug and 5 μg of the sirtuin standard for SIRT1, 2, 3, 5 and 6 were also run on the same gel (Supplementary Figure S8). Sirtuin 5 μg spike recoveries were calculated in the LAP spike relative to the sirtuin 5 μg standard protein in buffer only samples. The sirtuin 2 μg spike recoveries were calculated in the LAP spike relative to values extrapolated from the results of the sirtuin 5 μg standard pro-tein in buffer only samples (Supplementary Table S1).

Sirtuin mRNA Expression in Human Brain Tissue using PCR.
For the gene expression studies RNA was extracted from human brain cells using RNeasy mini kits (Qiagen, Germany). The cDNA was prepared using SuperScript III First-Strand Synthesis System and random hexamers (Invitrogen Corporation, USA) as previously described[39]. Detailed protocol and primer sequences are described in Supplementary Protocols and Supplementary Table S7 respectively.

Sirtuin Expression in Human Brain Cells and Tissue using immunohistochemical staining.
Post mortem brain tissue from a male patient aged 63 years was obtained from the Sydney Brain Bank. Immunohistochemical staining was performed as previously published[40] and was performed using anti-human sirtuin (1:250) primary antibodies (raised in rabbit). The full protocol description can be found in Supplementary Protocols.

Sirtuin Protein Expression in human control brain tissue using western blotting.
Protein from three individual control post mortem frontal brain tissue samples were extracted as described in the previous section, and run on a 1D SDS-PAGE gel (20 μg protein per lane), and western blotted with antibodies for SIRT1, SIRT2 and SIRT3 (details provided in Supplementary Protocols and list of antibodies used in Supplementary Table S8).

Statistics.
Sirtuin concentrations are presented as means ± SEM using peak area ratios of light and heavy peptides obtained from Skyline MRM Proteomics software v3.1 (MacCoss Lab, USA). Sirtuins were quantified based on an average of data from both peptides used for each sirtuin where possible. Statistical comparisons were performed using two-tailed student t-tests assuming equal variance. Differences between groups were considered statistically significant at the $p < 0.05$ level.

Ethics.   All human and animal brain tissue samples were obtained and experiments conducted in accordance with the guidelines of the National Health and Medical Research Council of Australia and were approved by the University of New South Wales Human Research Ethics Committee (human brain tissue reference number HC12563) and the University of New South Wales Animal Care Ethics Committee (guinea pig tissue reference number 14/40B and mice 13/39B). Adult human primary microglia, astrocytes, neurons and oligodendrocytes were cultured from resected normal adult brain tissue following removal of brain tumour with informed consent at the Minimally Invasive Cancer Centre, Prince of Wales Hospital, Sydney, Australia (reference number X12-0314 and HREC/12/RPAH/481). All control CSF samples were obtained with ethics approval from the Sydney Adventist Hospital, Sydney Australia, with informed consent obtained from all subjects (reference number SAHHREC #13-02).

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Author Contributions

P.S., A.P., T.J. and G.S. conceived and planned the project. T.J. performed experimental work (MRM assay development and validation, cell culture, protein extraction, 1D SDS/PAGE) and wrote the main manuscript text. A.P., L.Z. and M.R. assisted with mass spectrometry instrumentation setup, maintenance and optimisation. N.B. performed all experimental work for and prepared Figure 5, Panel A and B. B.R. was involved in animal care of the guinea pig and mice used. J.M. performed Hu6 depletion of high abundance plasma proteins. R.G. was involved in collection of CSF samples. C.T. surgically removed brain tumors and provided samples. All authors reviewed the manuscript.

Additional Information

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SUPPLEMENTARY INFORMATION

Application of Targeted Mass Spectrometry for the Quantification of Sirtuins in the Central Nervous System

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SUPPLEMENTARY PROTOCOLS

a) Selection of Peptides

Digested peptides were separated by nano-LC using a Cap-LC autosampler system (Waters, USA). Samples (5 µl) were concentrated and desalted onto a micro C18 precolumn (500 µm x 2 mm, Michrom Bioresources, Auburn, CA) with $H_2O:CH_3CN$ (98:2, 0.05% HFBA) at 15 ul/min. After a 4 min wash, the pre-column was automatically switched (Valco 10 port valve, Houston, TX) into line with a fritless nano column (75 µm x ~12 cm) containing Magic C18 (~10cm, 200Å, Michrom). Peptides were eluted using a linear gradient of $H_2O:CH_3CN$ (98:2, 0.1% formic acid) to $H_2O:CH_3CN$ (55:45, 0.1% formic acid) at ~300 nl/min over 30 min. The precolumn was connected via a fused silica capillary (10 cm, 25 µ) to a low volume tee (Upchurch Scientific) where high voltage (2400 V) was applied and the column tip positioned ~1 cm from the Z-spray inlet of an QTof Ultima API hybrid tandem mass spectrometer (Micromass, UK). Positive ions were generated by electrospray and the QTof operated in data dependent acquisition mode (DDA). A Tof MS survey scan was acquired (m/z 350-1700, 1 s) and the two largest multiply charged ions (counts > 20) were sequentially selected by Q1 for MS-MS analysis. Argon was used as collision gas and an optimum collision energy chosen (based on charge state and mass). Tandem mass spectra were accumulated for up to 2 s (m/z 50-2000). Peak lists were submitted to the database search program Mascot (Matrix Science, UK) to confirm identification of sirtuin peptides.

b) Sirtuin standards and calibration curves

Isotopically labelled internal standards are excellent tools to normalise the variability in sample handling, injection volume and the performance of the mass spectrometer. Synthetic light and heavy peptides were purchased from GL Biochem (China) and Sigma-Aldrich (USA) respectively. Calibration curves in the range of 1-200 fmol/µl (three replicates using three transitions) were prepared. Heavy peptide (100 fmol/µl) was added to all standards and peptide ratios (light/heavy) were obtained using Skyline MRM analysis software. Average inter- and intra- assay %CV values (n=3) were calculated for each of the seven sirtuins. The linear range, limit of detection (LOD) and limit of quantification (LOQ) were determined. A signal-to-noise ratio of >3:1 and >10:1 was used to define LOD and LOQ respectively.

Cell Culture

Cells were cultured in medium RPMI 1640 supplemented with 10% foetal bovine serum, 1% 1-glutamax, 1% antibacterial/antifungal, and 0.5% glucose. Cells were maintained at 37°C in a

humidified atmosphere containing 95% air/5% CO_2. Cells were seeded into 24-well tissue culture plates to a density of 1×10^5 cells 24 hours prior to experimentation. Neurons were prepared from the same mixed brain cell cultures as previously described (Guillemin et al., 2007). Briefly, cells were plated in 24-well culture plates coated with Matrigel (1/20 in Neurobasal) and maintained in Neurobasal medium supplemented with 1% B-27 supplement, 1% Glutamax, 1% antibiotic/antifungal, 0.5% HEPES buffer, and 0.5% glucose. Microglia were prepared from the mixed brain cell cultures using a published protocol (Guillemin et al., 2001). Briefly, the original mixed brain cell culture was shaken at 220 rpm for 2 h, floating cells were centrifuged, transferred to Permanox chamber slides (NUNC) at a density of 5×10^5 cells/ml and grown in DMEM supplemented with 10% fetal calf serum (FCS), 1% L-glutamax, 0.5% glucose, IL-3 (300 IU/ml), macrophage colony-stimulating factor (M-CSF) (50 IU/ml), and 1% antibiotic/antifungal. Oligodendrocytes were prepared from the mixed brain cell cultures using a published protocol (Guillemin et al., 2001). Briefly, cells were seeded and grown in flasks coated with poly-L-lysine to confluence at 34°C, the permissive temperature, in DMEM/F12 supplemented with 10% FBS, 1% antibiotic/antifungal, 1% of G418 (Invitrogen, Melbourne, Australia) and then shifted to 39°C, the non-permissive temperature that leads to the 'differentiated' state for 72 hours. Cells were cultured in medium RPMI supplemented with 1% myelin binding protein, 1% 1-glutamax, and 1% antibacterial/antifungal. CNS cell lines of neurons (SKNSH), astrocytes (U251), oligodendrocytes (M310) and microglia (CHM5) were cultured in medium RPMI supplemented with 10% foetal bovine serum, 1% 1-glutamax, 1% antibacterial/antifungal, and 0.5% glucose. All cells were maintained at 37°C in a humidified atmosphere containing 95% air/5% CO_2. Cells were seeded into 24-well tissue culture plates (3 wells per cell type) to a density of 1×10^5 cells 24 hours prior to experimentation. Wells were pooled prior to cell lysis and probe sonication and all measurements were performed in triplicate.

PCR

Briefly, for each reaction 2 μl of diluted cDNA, 10 μL of SYBR green master mix, 0.15 μL of 10 μM forward and reverse primers and 7.7 μl of nuclease-free water was used making a total volume of 20 μl. Q-PCR was carried out using the Mx3500P Real-Time PCR system (Stratagene, Australia). The primer sequences are shown in Supplementary Table S7. The relative expression levels of sirtuin transcripts were calculated using a mathematical model based on the individual Q-PCR primer efficiencies and the quantified values were normalized against the housekeeping gene 18S. From

these values, fold-differences in the levels of transcripts between individual cell cultures were calculated according to the formula $2^{-\Delta\Delta Ct}$

Immunohistochemical Staining

Formalin fixed blocks from the hippocampal region were embedded in paraffin and cut into 5 μm sections on superfrost plus slides. For sirtuin staining, sections were deparaffinised in xylene for 20 min and rehydrated through graded alcohol treatment. Antigen retrieval was performed through the use of Target Retrieval solution pH 6 (DAKO, Denmark) and autoclaving at 121°C for 20 min. Endogenous peroxidase was blocked using 3% w/v hydrogen peroxide for 5 min and endogenous biotin was blocked as described previously. After incubation with 10% horse serum for 30 min to block non-specific binding, sections were incubated with anti-human sirtuin (1:250) primary antibodies (raised in rabbit), for 2 hours at ambient temperature. After three rinses, sections were incubated with secondary antibodies–biotinylated goat-anti rabbit or mouse (Vector Laboratories, USA, 1:200) for 30 min at ambient temperature followed by 30 min treatment with avidin-biotin-complex (ABC) elite (Vector). Labelling was visualized with liquid DAB and sections were counterstained and mounted. Negative controls for non-specific staining included replacement of the primary antibody by normal rabbit or mouse IgG.

Western Blotting

The gel was electroblotted (20V, 110mA, 90mins) onto a nitrocellulose membrane in transfer buffer of 50mM tris, 40mM glycine, 1.3mM SDS, 20% methanol, pH 9.2. After transfer of proteins, the membrane was incubated in 10% skim milk powder solution containing primary sirtuin antibody. SIRT1, SIRT2 and SIRT3 rabbit polyclonal antibodies were used (1:1000 dilution, Abcam, UK), followed by a secondary antibody (1:100,000 dilution of anti-mouse IgG, Pierce, USA). See Supplementary Table S8 for full antibody details and dilutions. Chemiluminescence blots were developed using 1:1 solution of Super Signal West Femto luminol and peroxide buffer (Pierce, USA) according to manufacturer's instructions.

References

Guillemin, G.J. *et al* Characterization of the kynurenine pathway in human neurons. *J Neurosci* **27**, 12884-92 (2007).

Guillemin, G. J. *et al.* Kynurenine pathway metabolism in human astrocytes: a paradox for neuronal protection. *J Neurochem* **78**, 1-13 (2001).

Supplementary Table S1. Sirtuin recovery using the full protocol (sirtuins run by SDS/PAGE) with spiking into matrices consisting of either buffer only (5μg sirtuin) or low abundance plasma (LAP) proteins with either 5μg or 2μg sirtuin spiked. Sirtuin 5μg spike recoveries were calculated in the LAP spike relative to sirtuin 5μg standard protein in buffer only samples. Sirtuin 2μg spike recoveries were calculated in the LAP spike relative to values extrapolated from the results of the sirtuin 5μg standard protein in buffer only samples. The peak ratios and percentage recovery values is from three transitions used for each of the two sirtuin peptides per sirtuin protein and were obtained from one gel lane per sirtuin protein. The gel on which these samples were run is shown in Figure S8.

	Avg Peak Intensity Ratio (Light/Heavy)	Avg Peak Intensity Ratio (Light/Heavy)	% Recovery	%Recovery
	DINTIEDAVK	TSVAGTVR	DINTIEDAVK	TSVAGTVR
SIRT1 Std 5μg	0.0106	0.0196		
SIRT1 Std 2μg (extrapolated)	0.0042	0.0078		
SIRT1 Std 5μg + 20μg LAP	0.0080	0.0178	76.10	90.79
SIRT1 Std 2μg + 20μg LAP	0.0046	0.0101	108.50	128.83
	LLDELTLEGVAR	IFSEVTPK	LLDELTLEGVAR	IFSEVTPK
SIRT2 Std 5μg	0.0656	0.0514		
SIRT2 Std 2μg (extrapolated)	0.0262	0.0206		
SIRT2 Std 5μg + 20μg LAP	0.0523	0.0496	79.70	96.34
SIRT2 Std 2μg + 20μg LAP	0.0245	0.0219	93.51	106.31
	LYTQNIDGLER	VSGIPASK	LYTQNIDGLER	VSGIPASK
SIRT3 Std 5μg	0.1015	0.0085		
SIRT3 Std 2μg (extrapolated)	0.0406	0.0034		
SIRT3 Std 5μg + 20μg LAP	0.0620	0.0067	61.08	78.80
SIRT3 Std 2μg + 20μg LAP	0.0236	0.0035	58.13	102.9
	VVVITQNIDELHR	NLLEIHGSLFK	VVVITQNIDELHR	NLLEIHGSLFK
SIRT5 Std 5μg	0.0224	0.0271		
SIRT5 Std 2μg (extrapolated)	0.00896	0.01084		
SIRT5 Std 5μg + 20μg LAP	0.01812	0.0198	80.89	73.33
SIRT5 Std 2μg + 20μg LAP	0.0093	0.0104	103.80	95.94

	FLVSQNVDGLHVR	LVIVNLQPTK	FLVSQNVDGLHVR	LVIVNLQPTK
SIRT6 Std 5µg	0.0789	0.0837		
SIRT6 Std 2µg (extrapolated)	0.0316	0.0335		
SIRT6 Std 5µg + 20µg LAP	0.0571	0.0743	72.37	88.76
SIRT6 Std 2µg + 20µg LAP	0.0326	0.0459	103.16	131.14

	FLVSQNVDGLHVR	LVIVNLQPTK	FLVSQNVDGLHVR	LVIVNLQPTK

Supplementary Table S2. Human sirtuin peptide homology with guinea pig and mouse. Sequence variations from the human are highlighted in red.

Sirtuin	Human Peptide	Homology in Guinea Pig and Mice
SIRT1	DINTIEDAVK	Identical sequence found in both Guinea Pig and Mouse
	TSVAGTVR	In Mice: TSVAETVR In GP: TSVAETVR
SIRT2	LLDELTLEGVAR	Identical sequence found in Guinea PigIn Mice: LLDELTLEGVTR
	IFSEVTPK	In mice: IFSEATPR In GP: LFSDVTPK
SIRT3	LYTQNIDGLER	Identical sequence found in both Guinea Pig and Mouse
	VSGIPASK	In mice: ASGIPASK In GP: ASGIPASK
SIRT4	RPIQHGDFVR	In mice: RPIQHIDFVR In GP: RPIQHSDFVR
	FILTAWEK	In mice: FILTAREQ In GP: FTLTAQDK
SIRT5	VVVITQNIDELHR	Identical sequence found in Mouse In GP: VAVITQNIDELHLR
	NLLEIHGSLFK	In mice: NLLEIHGTLFK In GP: NLVEIHGTIFK
SIRT6	FLVSQNVDGLHVR	Identical sequence found in both Guinea Pig and Mouse
	LVIVNLQPTK	Identical sequence found in both Guinea Pig and Mouse
SIRT7	LLAESADLVTELQGR	In mice: LLAESEDLVTELQGR In GP: LLAESEDLVTELQGR
	DTIVHFGER	Identical sequence found in both Guinea Pig and Mouse

Supplementary Table S3. Sequences of all 7 human sirtuins and their isoforms (obtained from UniProt). The peptides used for MRM quantification experiments are highlighted in red and in larger font size. All sirtuin isoforms contain at least one of the peptides used for quantification, and both peptides are represented in the majority of isoforms.

SIRT1 Isoform 1	>sp|Q96EB6|SIR1_HUMAN NAD-dependent protein deacetylase sirtuin-1 OS=Homo sapiens GN=SIRT1 PE=1 SV=2 MADEAALALQPGGSPSAAGADREAASSPAGEPLRKRPRRDGPGLERSPGEPGGAAPER EVPAAARGCPGAAAAALWREAEAEAAAAGGEQEAQATAAAGEGDNGPGLQGPSREP PLADNLYDEDDDDEGEEEEAAAAAIGYRDNLLFGDEIITNGFHSCESDEEDRASHASSS DWTPRPRIGPYTFVQQHLMIGTDPRTILKDLLPETIPPPELDDMTLWQIVINILSEPPKRK KRK**DINTIEDAVK**LLQECKKIIVLTGAGVSVSCGIPDFRSRDGIYARLAVDFPDLPDPQA MFDIEYFRKDPRPFFKFAKEIYPGQFQPSLCHKFIALSDKEGKLLRNYTQNIDTLEQVAGIQ RIIQCHGSFATASCLICKYKVDCEAVRGDIFNQVVPRCPRCPADEPLAIMKPEIVFFGENL PEQFHRAMKYDKDEVDLLIVIGSSLKVRPVALIPSSIPHEVPQILINREPLPHLHFDVELLGD CDVIINELCHRLGGEYAKLCCNPVKLSEITEKPPRTQKELAYLSELPPTPLHVSEDSSSPERT SPPDSSVIVTLLDQAAKSNDDLDVSESKGCMEEKPQEVQTSRNVESIAEQMENPDLKNV GSSTGEKNER**TSVAGTVR**KCWPNRVAKEQISRRLDGNQYLFLPPNRYIFHGAEVYSDS EDDVLSSSSCGSNSDSGTCQSPSLEEPMEDESEIEEFYNGLEDEPDVPERAGGAGFGTDG DDQEAINEAISVKQEVTDMNYPSNKS
SIRT1 Isoform 2	>sp|Q96EB6-2|SIR1_HUMAN Isoform 2 of NAD-dependent protein deacetylase sirtuin-1 OS=Homo sapiens GN=SIRT1 MADEAALALQPGGSPSAAGADREAASSPAGEPLRKRPRRDGPGLERSPGEPGGAAPER EVPAAARGCPGAAAAALWREAEAEAAAAGGEQEAQATAAAGEGDNGPGLQGPSREP PLADNLYDEDDDDEGEEEEAAAAAIGYRDNLLFGDEIITNGFHSCESDEEDRASHASSS DWTPRPRIGPYTFVQQHLMIGTDPRTILKDLLPETIPPPELDDMTLWQIVINILSEPPKRK KRK**DINTIEDAVK**LLQECKKIIVLTGAGVSVSCGIPDFRSRDGIYARLAVDFPDLPDPQA MFDIEYFRKDPRPFFKFAKEIYPGQFQPSLCHKFIALSDKEGKLLRNYTQNIDTLEQVAGIQ RIIQCHGSFATASCLICKYKVDCEAVRGDIFNQVVPRCPRCPADEPLAIMKPEIVFFGENL PEQFHRAMKYDKDEVDLLIVIGSSLKVRPVALIPSNQYLFLPPNRYIFHGAEVYSDSEDDV LSSSSCGSNSDSGTCQSPSLEEPMEDESEIEEFYNGLEDEPDVPERAGGAGFGTDGDDQ EAINEAISVKQEVTDMNYPSNKS
SIRT2 Isoform 1	>sp|Q8IXJ6|SIR2_HUMAN NAD-dependent protein deacetylase sirtuin-2 OS=Homo sapiens GN=SIRT2 PE=1 SV=2 MAEPDPSHPLETQAGKVQEAQDSDSDSEGGAAGGEADMDFLRNLFSQTLSLGSQKER **LLDELTLEGVAR**YMQSERCRRVICLVGAGISTSAGIPDFRSPSTGLYDNLEKYHLPYPE AIFEISYFKKHPEPFFALAKELYPGQFKPTICHYFMRLLKDKGLLLRCYTQNIDTLERIAGLE QEDLVEAHGTFYTSHCVSASCRHEYPLSWMKEK**IFSEVTPK**CEDCQSLVKPDIVFFGES LPARFFSCMQSDFLKVDLLLVMGTSLQVQPFASLISKAPLSTPRLLINKEKAGQSDPFLG MIMGLGGGMDFDSKKAYRDVAWLGECDQGCLALAELLGWKKELEDLVRREHASIDAQ SGAGVPNPSTSASPKKSPPPAKDEARTTEREKPQ

SIRT2 Isoform 2 >sp|Q8IXJ6-2|SIR2_HUMAN Isoform 2 of NAD-dependent protein deacetylase sirtuin-2 OS=Homo sapiens GN=SIRT2

MDFLRNLFSQTLSLGSQKER**LLDELTLEGVAR**YMQSERCRRVICLVGAGISTSAGIPDF
RSPSTGLYDNLEKYHLPYPEAIFEISYFKKHPEPFFALAKELYPGQFKPTICHYFMRLLKDKG
LLLRCYTQNIDTLERIAGLEQEDLVEAHGTFYTSHCVSASCRHEYPLSWMKEK**IFSEVTP
K**CEDCQSLVKPDIVFFGESLPARFFSCMQSDFLKVDLLLVMGTSLQVQPFASLISKAPLST
PRLLINKEKAGQSDPFLGMIMGLGGGMDFDSKKAYRDVAWLGECDQGCLALAELLGW
KKELEDLVRREHASIDAQSGAGVPNPSTSASPKKSPPPAKDEARTTEREKPQ

SIRT2 Isoform 3 >sp|Q8IXJ6-3|SIR2_HUMAN Isoform 3 of NAD-dependent protein deacetylase sirtuin-2 OS=Homo sapiens GN=SIRT2

MPLAECPSCRCLSSFRSVDFLRNLFSQTLSLGSQKER**LLDELTLEGVAR**YMQSERCRR
VICLVGAGISTSAGIPDFRSPSTGLYDNLEKYHLPYPEAIFEISYFKKHPEPFFALAKELYPGQ
FKPTICHYFMRLLKDKGLLLRCYTQNIDTLERIAGLEQEDLVEAHGTFYTSHCVSASCRHE
YPLSWMKEK**IFSEVTPK**CEDCQSLVKPDIVFFGESLPARFFSCMQSDFLKVDLLLVMGT
SLQVQPFASLISKAPLSTPRLLINKEKAGQSDPFLGMIMGLGGGMDFDSKKAYRDVAWL
GECDQGCLALAELLGWKKELEDLVRREHASIDAQSGAGVPNPSTSASPKKSPPPAKDEA
RTTEREKPQ

SIRT2 Isoform 4 >sp|Q8IXJ6-4|SIR2_HUMAN Isoform 4 of NAD-dependent protein deacetylase sirtuin-2 OS=Homo sapiens GN=SIRT2

MAEPDPSHPLETQAGKVQEAQDSDSDSEGGAAGGEADMDFLRNLFSQTLSLGSQKER
LLDELTLEGVARYMQSERCRRVICLVGAGISTSAGIPDFRSPSTGLYDNLEKYHLPYPE
AIFEISYFKKHPEPFFALAKELYPGQFKPTICHYFMRLLKDKGLLLRCYTQNIDTLERIAGLE
QEDLVEAHGTFYTSHCVSASCRHEYPLSWMKEK**IFSEVTPK**CEDCQSLVKPDIVFFGES
LPARFFSCMQSDFLKVDLLLVMGTSLQGRGLAG

SIRT2 Isoform 5 >sp|Q8IXJ6-5|SIR2_HUMAN Isoform 5 of NAD-dependent protein deacetylase sirtuin-2 OS=Homo sapiens GN=SIRT2

MAEPDRRRVICLVGAGISTSAGIPDFRSPSTGLYDNLEKYHLPYPEAIFEISYFKKHPEPFFA
LAKELYPGQFKPTICHYFMRLLKDKGLLLRCYTQNIDTLERIAGLEQEDLVEAHGTFYTSHC
VSASCRHEYPLSWMKEK**IFSEVTPK**CEDCQSLVKPDIVFFGESLPARFFSCMQSDFLKV
DLLLVMGTSLQVQPFASLISKAPLSTPRLLINKEKAGQSDPFLGMIMGLGGGMDFDSKK
AYRDVAWLGECDQGCLALAELLGWKKELEDLVRREHASIDAQSGAGVPNPSTSASPKK
SPPPAKDEARTTEREKPQ

SIRT3 Isoform 1 >sp|Q9NTG7|SIR3_HUMAN NAD-dependent protein deacetylase sirtuin-3, mitochondrial OS=Homo sapiens GN=SIRT3 PE=1 SV=2

MAFWGWRAAAALRLWGRVVERVEAGGGVGPFQACGCRLVLGGRDDVSAGLRGSHG
ARGEP
LDPARPLQRPPRPEVPRAFRRQPRAAAPSFFFSSIKGGRRSISFSVGASSVVGSGGSSDKG
KLSLQDVAELIRARACQRVVVMVGAGISTPSGIPDFRSPGSGLYSNLQQYDLPYPEAIFEL
PFFFHNPKPFFTLAKELYPGNYKPNVTHYFLRLLHDKGLLLR**LYTQNIDGLERVSGIPA
SK**LVEAHGTFASATCTVCQRPFPGEDIRADVMADRVPRCPVCTGVVKPDIVFFGEPLPQ
RFLLHVVDFPMADLLLILGTSLEVEPFASLTEAVRSSVPRLLINRDLVGPLAWHPRSRDVA

	QLGDVVHGVESLVELLGWTEEMRDLVQRETGKLDGPDK
SIRT3 Isoform 2	>sp|Q9NTG7-2|SIR3_HUMAN Isoform 2 of NAD-dependent protein deacetylase sirtuin-3, mitochondrial OS=Homo sapiens GN=SIRT3 MVGAGISTPSGIPDFRSPGSGLYSNLQQYDLPYPEAIFELPFFFHNPKPFFTLAKELYPGNY KPNVTHYFLRLLHDKGLLLR**LYTQNIDGLERVSGIPASK**LVEAHGTFASATCTVCQR PFPGEDIRADVMADRVPRCPVCTGVVKPDIVFFGEPLPQRFLLHVVDFPMADLLLILGTS LEVEPFASLTEAVRSSVPRLLINRDLVGPLAWHPRSRDVAQLGDVVHGVESLVELLGWT EEMRDLVQRETGKLDGPDK
SIRT4	>sp|Q9Y6E7|SIR4_HUMAN NAD-dependent protein lipoamidase sirtuin-4, mitochondrial OS=Homo sapiens GN=SIRT4 PE=1 SV=1 MKMSFALTFRSAKGRWIANPSQPCSKASIGLFVPASPPLDPEKVKELQRFITLSKRLLVM TGAGISTESGIPDYRSEKVGLYARTDR**RPIQHGDFVR**SAPIRQRYWARNFVGWPQFS SHQPNPAHWALSTWEKLGKLYWLVTQNVDALHTKAGSRRLTELHGCMDRVLCLDCGE QTPRGVLQERFQVLNPTWSAEAHGLAPDGDVFLSEEQVRSFQVPTCVQCGGHLKPDV VFFGDTVNPDKVDFVHKRVKEADSLLVVGSSLQVYSGYR**FILTAWEK**KLPIAILNIGPTR SDDLACLKLNSRCGELLPLIDPC
SIRT5 Isoform 1	>sp|Q9NXA8|SIR5_HUMAN NAD-dependent protein deacylase sirtuin-5, mitochondrial OS=Homo sapiens GN=SIRT5 PE=1 SV=2 MRPLQIVPSRLISQLYCGLKPPASTRNQICLKMARPSSSMADFRKFFAKAKHIVIISGAGV SAESGVPTFRGAGGYWRKWQAQDLATPLAFAHNPSRVWEFYHYRREVMGSKEPNAG HRAIAECETRLGKQGRR**VVVITQNIDELHR**KAGTK**NLLEIHGSLFK**TRCTSCGVVA ENYKSPICPALSGKGAPEPGTQDASIPVEKLPRCEEAGCGGLLRPHVVWFGENLDPAILE EVDRELAHCDLCLVVGTSSVVYPAAMFAPQVAARGVPVAEFNTETTPATNRFRFHFQG PCGTTLPEALACHENETVS
SIRT5 Isoform 2	>sp|Q9NXA8-2|SIR5_HUMAN Isoform 2 of NAD-dependent protein deacylase sirtuin-5, mitochondrial OS=Homo sapiens GN=SIRT5 MRPLQIVPSRLISQLYCGLKPPASTRNQICLKMARPSSSMADFRKFFAKAKHIVIISGAGV SAESGVPTFRGAGGYWRKWQAQDLATPLAFAHNPSRVWEFYHYRREVMGSKEPNAG HRAIAECETRLGKQGRR**VVVITQNIDELHR**KAGTK**NLLEIHGSLFK**TRCTSCGVVA ENYKSPICPALSGKGAPEPGTQDASIPVEKLPRCEEAGCGGLLRPHVVWFGENLDPAILE EVDRELAHCDLCLVVGTSSVVYPAAMFAPQVAARGVPVAEFNTETTPATNRFSHLISISS LIIIKN
SIRT5 Isoform 3	>sp|Q9NXA8-3|SIR5_HUMAN Isoform 3 of NAD-dependent protein deacylase sirtuin-5, mitochondrial OS=Homo sapiens GN=SIRT5 MRPLQIVPSRLISQLYCGLKPPASTRNQICLKMARPSSSMADFRKFFAKAKHIVIISGAGV SAESGVPTFRGAGGYWRKWQAQDLATPLAFAHNPSRVWEFYHYRREVMGSKEPNAG HRAIAECETRLGKQGRR**VVVITQNIDELHR**KAGTK**NLLEIHGSLFK**TRCTSCGVVA ENYKSPICPALSGKGCEEAGCGGLLRPHVVWFGENLDPAILEEVDRELAHCDLCLVVGTS SVVYPAAMFAPQVAARGVPVAEFNTETTPATNRFRFHFQGPCGTTLPEALACHENETV S
SIRT5 Isoform 4	>sp|Q9NXA8-4|SIR5_HUMAN Isoform 4 of NAD-dependent protein deacylase

	sirtuin-5, mitochondrial OS=Homo sapiens GN=SIRT5 MGSKEPNAGHRAIAECETRLGKQGRR**VVVITQNIDELHR**KAGTK**NLLEIHGSLFK**T RCTSCGVVAENYKSPICPALSGKGAPEPGTQDASIPVEKLPRCEEAGCGGLLRPHVVWF GENLDPAILEEVDRELAHCDLCLVVGTSSVVYPAAMFAPQVAARGVPVAEFNTETTPAT NRFRFHFQGPCGTTLPEALACHENETVS
SIRT6 Isoform 1	>sp|Q8N6T7|SIR6_HUMAN NAD-dependent protein deacetylase sirtuin-6 OS=Homo sapiens GN=SIRT6 PE=1 SV=2 MSVNYAAGLSPYADKGKCGLPEIFDPPEELERKVWELARLVWQSSSVVFHTGAGISTAS GIPDFRGPHGVWTMEERGLAPKFDTTFESARPTQTHMALVQLERVGLLR**FLVSQNV DGLHVR**SGFPRDKLAELHGNMFVEECAKCKTQYVRDTVVGTMGLKATGRLCTVAKA RGLRACRGELRDTILDWEDSLPDRDLALADEASRNADLSITLGTSLQIRPSGNLPLATKRR GGR**LVIVNLQPTK**HDRHADLRIHGYVDEVMTRLMKHLGLEIPAWDGPRVLERALPPL PRPPTPKLEPKEESPTRINGSIPAGPKQEPCAQHNGSEPASPKRERPTSPAPHRPPKRVKA KAVPS
SIRT6 Isoform 2	>sp|Q8N6T7-2|SIR6_HUMAN Isoform 2 of NAD-dependent protein deacetylase sirtuin-6 OS=Homo sapiens GN=SIRT6 MSVNYAAGLSPYADKGKCGLPEIFDPPEELERKVWELARLVWQSSSVVFHTGAGISTAS GIPDFRGPHGVWTMEERGLAPKFDTTFESARPTQTHMALVQLERVGLLR**FLVSQNV DGLHVR**SGFPRDKLAELHGNMFVEECAKCKTQYVRDTVVGTMGLKATGRLCTVAKA RGLRACRNADLSITLGTSLQIRPSGNLPLATKRRGGR**LVIVNLQPTK**HDRHADLRIHGY VDEVMTRLMKHLGLEIPAWDGPRVLERALPPLPRPPTPKLEPKEESPTRINGSIPAGPKQ EPCAQHNGSEPASPKRERPTSPAPHRPPKRVKAKAVPS
SIRT7 Isoform 1	>sp|Q9NRC8|SIR7_HUMAN NAD-dependent protein deacetylase sirtuin-7 OS=Homo sapiens GN=SIRT7 PE=1 SV=1 MAAGGLSRSERKAAERVRRLREEQQRERLRQVSRILRKAAAERSAEEGR**LLAESADLV TELQGR**SRRREGLKRRQEEVCDDPEELRGKVRELASAVRNAKYLVVYTGAGISTAASIP DYRGPNGVWTLLQKGRSVSAADLSEAEPTLTHMSITRLHEQKLVQHVVSQNCDGLHLR SGLPRTAISELHGNMYIEVCTSCVPNREYVRVFDVTERTALHRHQTGRTCHKCGTQLR**D TIVHFGER**GTLGQPLNWEAATEAASRADTILCLGSSLKVLKKYPRLWCMTKPPSRRPKL YIVNLQWTPKDDWAALKLHGKCDDVMRLLMAELGLEIPAYSRWQDPIFSLATPLRAGE EGSHSRKSLCRSREEAPPGDRGAPLSSAPILGGWFGRGCTKRTKRKKVT
SIRT7 Isoform 2	>sp|Q9NRC8-2|SIR7_HUMAN Isoform 2 of NAD-dependent protein deacetylase sirtuin-7 OS=Homo sapiens GN=SIRT7 MAAGGLSRSERKAAERVRRLREEQQRERLRQVSRILRKAAAERSAEEGR**LLAESADLV TELQGR**SRRREGLKRRQEEVCDDPEELRGKVRELASAVRNAKYLVVYTGAGISTAASIP DYRGPNGVWTLLQKGRSVSAADLSEAEPTLTHMSITRLHEQKLVRALGGWYTCQGPGR APWCPVGN
SIRT7 Isoform 3	>sp|Q9NRC8-3|SIR7_HUMAN Isoform 3 of NAD-dependent protein deacetylase sirtuin-7 OS=Homo sapiens GN=SIRT7 MPGPRRRSPSACPQVSRILRKAAAERSAEEGR**LLAESADLVTELQGR**SRRREGLKRR

QEEVCDDPEELRGKVRELASAVRNAKYLVVYTGAGISTAASIPDYRGPNGVWTLLQKGR
SVSAADLSEAEPTLTHMSITRLHEQKLVQHVVSQNCDGLHLRSGLPRTAISELHGNMYIE
VCTSCVPNREYVRVFDVTERTALHRHQTGRTCHKCGTQLRDTIVHFGERGTLGQPLNW
EAATEAASRADTILCLGSSLKVLKKYPRLWCMTKPPSRRPKLYIVNLQWTPKDDWAALKL
HGKCDDVMRLLMAELGLEIPAYSRVL

Supplementary Table S4. List of 14 unique sirtuin peptides selected for sirtuin protein quantification. Arg and Lys amino acids highlighted in bold used in 'heavy' peptides, labelled with a stable isotope (^{13}C and ^{15}N). Precursor charge state for all peptides was 2^{+}.

Sirtuin	Peptide	Light Precursor Ion *m/z*	Heavy Precursor Ion *m/z*
SIRT1	DINTIEDAVK	559.2904	563.2975
	TSVAGTVR	395.7245	400.7286
SIRT2	LLDEELTLEGVAR	664.8746	669.8788
	IFSEVTPK	460.758	464.7651
SIRT3	LYTQNIDGLER	661.341	666.3451
	VSGIPASK	379.724	383.7311
SIRT4	RPIQHGDFVR	612.8334	617.8376
	FILTAWEK	504.2817	508.2888
SIRT5	VVVITQNIDELHR	512.6229	515.9589
	NLLEIHGSLFK	635.8613	639.8613
SIRT6	FLVSQNVDGLHVR	742.4044	747.4086
	LVIVNLQPTK	562.8555	566.8626
SIRT7	LLAESADLVTELQGR	807.9385	812.9426
	DTIVHFGER	537.2724	542.2765

Supplementary Table S5. List of product and transition ions with collision energies used for all 14 selected sirtuin peptides. Each sirtuin protein (SIRT1-7, with 2 peptides per protein) was run with 12 transition ions per run, per protein and with a 50ms dwell time. Precursor charge state for all peptides was 2^+.

Precursor Ion *m/z*	Transition Ion	Peptide	Declustering Potential	Collision Energy
559.2904	889.4625	SIRT1.DINTIEDAVK.y8.light	71.9	27.6
559.2904	775.4196	SIRT1.DINTIEDAVK.y7.light	71.9	27.6
559.2904	674.3719	SIRT1.DINTIEDAVK.y6.light	71.9	27.6
563.2975	897.4767	SIRT1.DINTIEDAVK.y8.heavy	71.9	27.6
563.2975	783.4338	SIRT1.DINTIEDAVK.y7.heavy	71.9	27.6
563.2975	682.3861	SIRT1.DINTIEDAVK.y6.heavy	71.9	27.6
395.7245	602.362	SIRT1.TSVAGTVR.y6.light	60	18.3
395.7245	503.2936	SIRT1.TSVAGTVR.y5.light	60	18.3
395.7245	432.2565	SIRT1.TSVAGTVR.y4.light	60	18.3
400.7286	612.3703	SIRT1.TSVAGTVR.y6.heavy	60	18.3
400.7286	513.3019	SIRT1.TSVAGTVR.y5.heavy	60	18.3
400.7286	442.2648	SIRT1.TSVAGTVR.y4.heavy	60	18.3
664.8746	858.5043	SIRT2.LLDELTLEGVAR.y8.light	79.6	33.6
664.8746	745.4203	SIRT2.LLDELTLEGVAR.y7.light	79.6	33.6
664.8746	644.3726	SIRT2.LLDELTLEGVAR.y6.light	79.6	33.6
669.8788	868.5126	SIRT2.LLDELTLEGVAR.y8.heavy	79.6	33.6
669.8788	755.4285	SIRT2.LLDELTLEGVAR.y7.heavy	79.6	33.6
669.8788	654.3809	SIRT2.LLDELTLEGVAR.y6.heavy	79.6	33.6
460.758	807.4247	SIRT2.IFSEVTPK.y7.light	64.7	22
460.758	660.3563	SIRT2.IFSEVTPK.y6.light	64.7	22
460.758	573.3243	SIRT2.IFSEVTPK.y5.light	64.7	22
464.7651	815.4389	SIRT2.IFSEVTPK.y7.heavy	64.7	22
464.7651	668.3705	SIRT2.IFSEVTPK.y6.heavy	64.7	22
464.7651	581.3385	SIRT2.IFSEVTPK.y5.heavy	64.7	22
661.341	944.4796	SIRT3.LYTQNIDGLER.y8.light	79.3	33.4
661.341	816.421	SIRT3.LYTQNIDGLER.y7.light	79.3	33.4
661.341	702.3781	SIRT3.LYTQNIDGLER.y6.light	79.3	33.4
666.3451	954.4879	SIRT3.LYTQNIDGLER.y8.heavy	79.3	33.4
666.3451	826.4293	SIRT3.LYTQNIDGLER.y7.heavy	79.3	33.4
666.3451	712.3863	SIRT3.LYTQNIDGLER.y6.heavy	79.3	33.4
379.724	659.3723	SIRT3.VSGIPASK.y7.light	58.8	17.4
379.724	572.3402	SIRT3.VSGIPASK.y6.light	58.8	17.4
379.724	515.3188	SIRT3.VSGIPASK.y5.light	58.8	17.4
383.7311	667.3865	SIRT3.VSGIPASK.y7.heavy	58.8	17.4

383.7311	580.3544	SIRT3.VSGIPASK.y6.heavy	58.8	17.4
383.7311	523.333	SIRT3.VSGIPASK.y5.heavy	58.8	17.4
612.8334	1068.559	SIRT4.RPIQHGDFVR.y9.light	75.8	30.7
612.8334	971.5057	SIRT4.RPIQHGDFVR.y8.light	75.8	30.7
612.8334	858.4217	SIRT4.RPIQHGDFVR.y7.light	75.8	30.7
617.8376	1078.567	SIRT4.RPIQHGDFVR.y9.heavy	75.8	30.7
617.8376	981.514	SIRT4.RPIQHGDFVR.y8.heavy	75.8	30.7
617.8376	868.4299	SIRT4.RPIQHGDFVR.y7.heavy	75.8	30.7
504.2817	747.4036	SIRT4.FILTAWEK.y6.light	67.9	24.5
504.2817	634.3195	SIRT4.FILTAWEK.y5.light	67.9	24.5
504.2817	533.2718	SIRT4.FILTAWEK.y4.light	67.9	24.5
508.2888	755.4178	SIRT4.FILTAWEK.y6.heavy	67.9	24.5
508.2888	642.3337	SIRT4.FILTAWEK.y5.heavy	67.9	24.5
508.2888	541.286	SIRT4.FILTAWEK.y4.heavy	67.9	24.5
512.6229	896.4585	SIRT5.VVVITQNIDELHR.y7.light	68.5	23
512.6229	782.4155	SIRT5.VVVITQNIDELHR.y6.light	68.5	23
512.6229	669.3315	SIRT5.VVVITQNIDELHR.y5.light	68.5	23
515.9589	906.4667	SIRT5.VVVITQNIDELHR.y7.heavy	68.5	23
515.9589	792.4238	SIRT5.VVVITQNIDELHR.y6.heavy	68.5	23
515.9589	679.3397	SIRT5.VVVITQNIDELHR.y5.heavy	68.5	23
635.8613	930.5043	SIRT5.NLLEIHGSLFK.y8.light	77.5	32
635.8613	801.4618	SIRT5.NLLEIHGSLFK.y7.light	77.5	32
635.8613	688.3777	SIRT5.NLLEIHGSLFK.y6.light	77.5	32
639.8684	938.5185	SIRT5.NLLEIHGSLFK.y8.heavy	77.5	32
639.8684	809.4759	SIRT5.NLLEIHGSLFK.y7.heavy	77.5	32
639.8684	696.3919	SIRT5.NLLEIHGSLFK.y6.heavy	77.5	32
742.4044	1124.581	SIRT6.FLVSQNVDGLHVR.y10.light	85.2	38.1
742.4044	909.4901	SIRT6.FLVSQNVDGLHVR.y8.light	85.2	38.1
742.4044	696.3787	SIRT6.FLVSQNVDGLHVR.y6.light	85.2	38.1
747.4086	1134.589	SIRT6.FLVSQNVDGLHVR.y10.heavy	85.2	38.1
747.4086	919.4984	SIRT6.FLVSQNVDGLHVR.y8.heavy	85.2	38.1
747.4086	706.387	SIRT6.FLVSQNVDGLHVR.y6.heavy	85.2	38.1
562.8555	912.5513	SIRT6.LVIVNLQPTK.y8.light	72.1	27.8
562.8555	799.4672	SIRT6.LVIVNLQPTK.y7.light	72.1	27.8
562.8555	700.3988	SIRT6.LVIVNLQPTK.y6.light	72.1	27.8
566.8626	920.5655	SIRT6.LVIVNLQPTK.y8.heavy	72.1	27.8
566.8626	807.4814	SIRT6.LVIVNLQPTK.y7.heavy	72.1	27.8
566.8626	708.413	SIRT6.LVIVNLQPTK.y6.heavy	72.1	27.8
807.9385	915.5258	SIRT7.LLAESADLVTELQGR.y8.light	90	41.8
807.9385	802.4417	SIRT7.LLAESADLVTELQGR.y7.light	90	41.8
807.9385	703.3733	SIRT7.LLAESADLVTELQGR.y6.light	90	41.8

812.9426	925.5341	SIRT7.LLAESADLVTELQGR.y8.heavy	90	41.8
812.9426	812.45	SIRT7.LLAESADLVTELQGR.y7.heavy	90	41.8
812.9426	713.3816	SIRT7.LLAESADLVTELQGR.y6.heavy	90	41.8
537.2724	958.5105	SIRT7.DTIVHFGER.y8.light	70.3	26.4
537.2724	857.4628	SIRT7.DTIVHFGER.y7.light	70.3	26.4
537.2724	744.3787	SIRT7.DTIVHFGER.y6.light	70.3	26.4
542.2765	968.5188	SIRT7.DTIVHFGER.y8.heavy	70.3	26.4
542.2765	867.4711	SIRT7.DTIVHFGER.y7.heavy	70.3	26.4
542.2765	754.387	SIRT7.DTIVHFGER.y6.heavy	70.3	26.4

Supplementary Table S6. Human frontal lobe brain tissue sample and subject details.

Patient	Sex	Age at death	Post mortem tissue collection time
1	Male	72	24h
2	Female	71	24h
3	Male	72	24h
4	Male	71	29h
5	Male	54	24h

Supplementary Table S7. Real-time primer sequences used for PCR analysis.

Primer name	Oligo name	Sequence (5'→3')
SIRT1-Forw	NM_012238-Forw	CAC-CAG-AAA-GAA-CTT-CAC-CAC-CAG
SIRT1-Rev	NM_012238-Rev	ACC-ATC-AAG-CCG-CCT-ACT-AAT-CTG
SIRT2-Forw	AJ505014-Forw	AGG-GAC-AAG-GAG-CAG-GGT-TC
SIRT2-Rev	AJ505014-Rev	GAA-GAG-AGA-CAG-CGG-CAG-GAC
SIRT3-Forw	NM_12239-Forw	GAG-GTT-CTT-GCT-GCA-TGT-GGT-TG
SIRT3-Rev	NM_12239-Rev	AGT-TTC-CCG-CTG-CAC-AAG-GTC
SIRT4-Forw	NM_012240-Forw	TTG-TGC-CAG-CAA-GTC-CTC-CTC
SIRT4-Rev	NM_012240-Rev	GTC-TCT-TGG-AAA-GGG-TGA-TGA-AGC
SIRT5-Forw	NM_12241-Forw	TCC-AGC-GTC-CAC-ACG-AAA-CC
SIRT5-Rev	NM_12241-Rev	AAC-ACC-AGC-TCC-TGA-GAT-GAT-GAC
SIRT6-Forw	NM_016539-Forw	GCT-GGA-GCC-CAA-GGA-GGA-ATC
SIRT6-Rev	NM_016539-Forw	AGT-AAC-AAA-GTG-AGA-CCA-CGA-GAG
SIRT7-Forw	NM_016538-Forw	GAG-CCA-ACC-CTC-ACC-CAC-ATG
SIRT7-Rev	NM_016538-Rev	ACG-CAG-GAG-GTA-CAG-ACT-TCA-ATG
GAPDH-Forw	NM_017008-Forw	TGG-AGT-CTA-CTG-GCG-TCT-T
GAPDH –Rev	NM_017008-Rev	TGT-CAT-ATT-TCT-CGT-GGT-TCA

Supplementary Table S8. Antibodies and dilutions used for western blotting.

Antibody	Dilution	Manufacturer
Rabbit Polyclonal SIRT1	1:1000	Abcam (Cambridge, UK)
Rabbit Polyclonal SIRT2	1:1000	Abcam (Cambridge, UK)
Rabbit Polyclonal SIRT3	1:1000	Abcam (Cambridge, UK)

Supplementary Figure S1. Representative chromatograms for all 14 sirtuin peptides used in the MRM method, showing transition ions, retention times and signal intensities. The vertical dotted lines either side of the peak clusters indicate the boundaries for peak area integration.
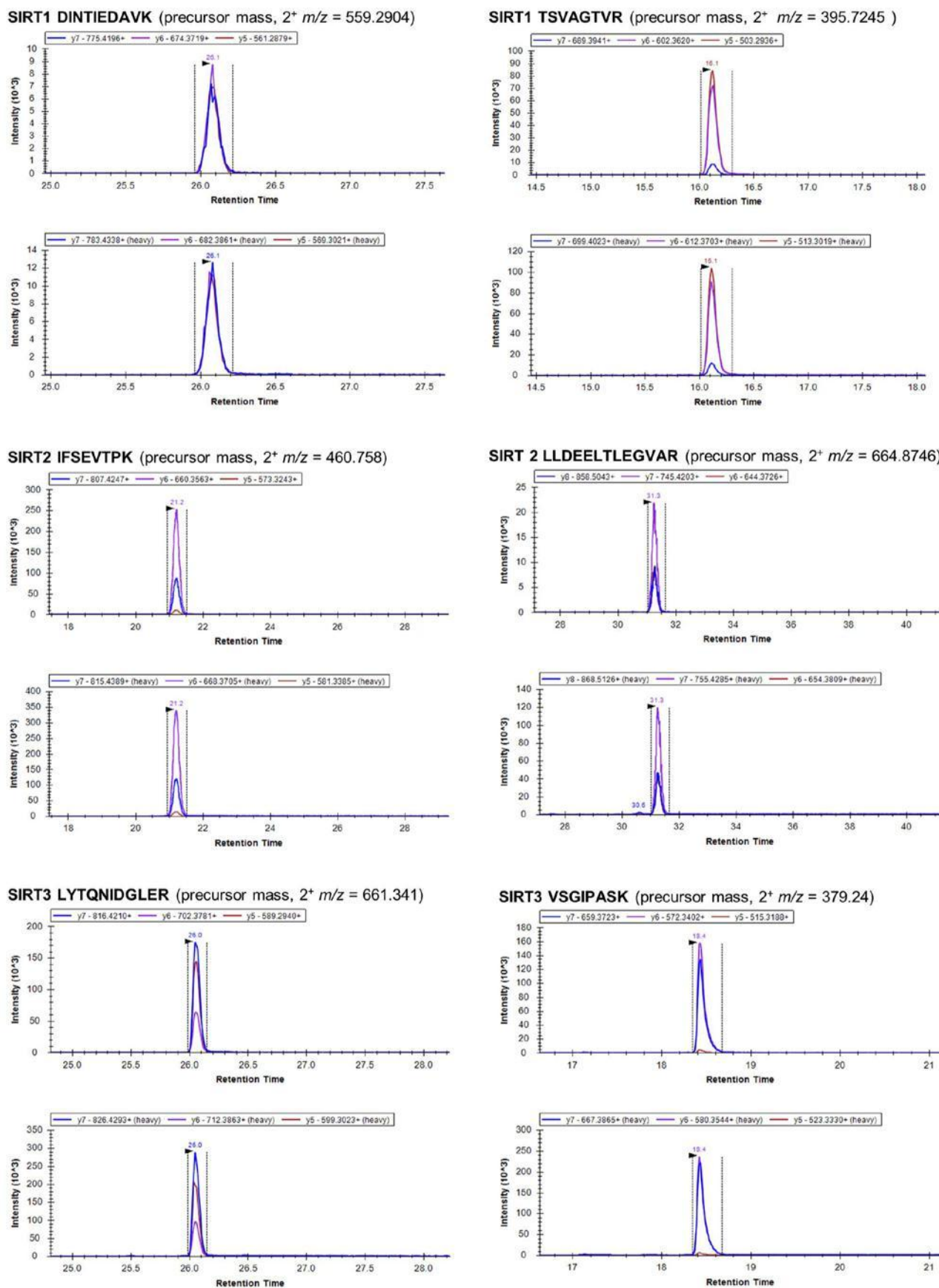
Supplementary Figure S1 continued.

SIRT4 FILTAWEK (precursor mass, 2+ m/z = 612.8334)
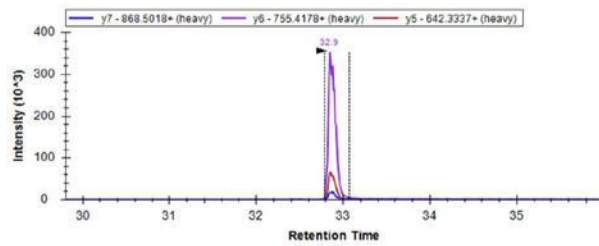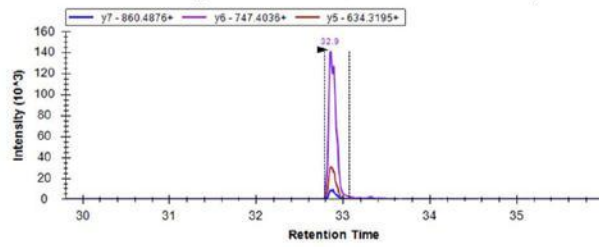
SIRT5 VVVITQNIDELHR (precursor mass, 2+ m/z = 512.6229)
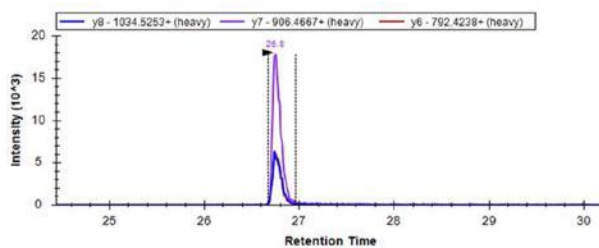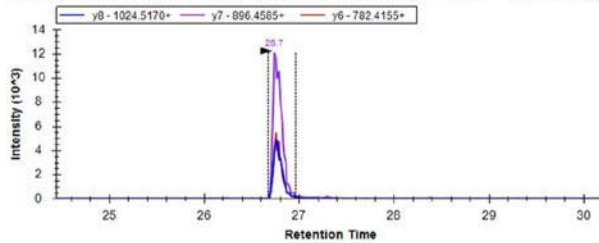
SIRT5 NLLEIHGSLFK (precursor mass, 2+ m/z = 635.8613)
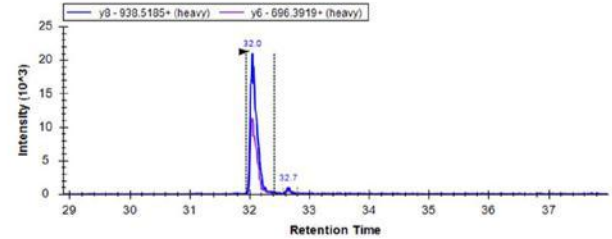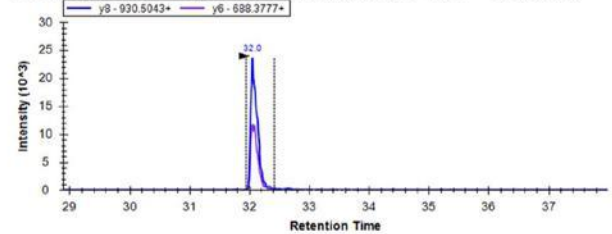
SIRT6 FLVSQNVDGLHVR (precursor mass, 2+ m/z = 742.4044)
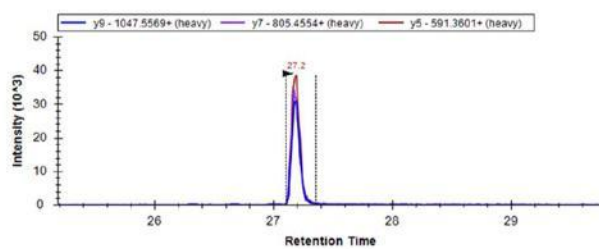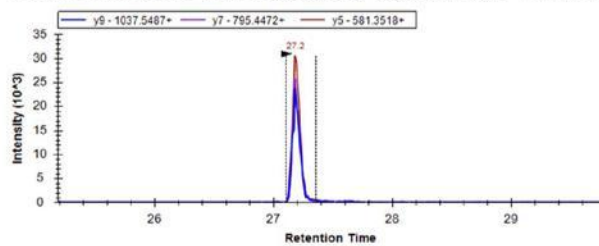
SIRT6 LVIVNLQPTK (precursor mass, 2+ m/z = 562.8555)
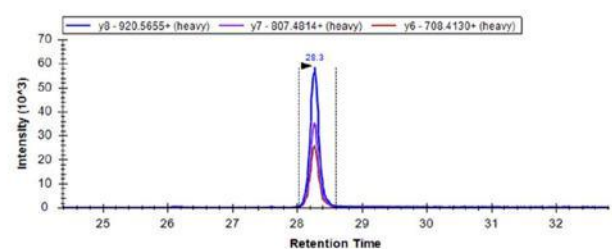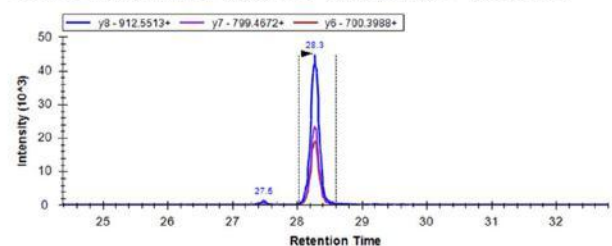
Supplementary Figure S1 continued.

SIRT7 DTIVHFGER (precursor mass, 2⁺ *m/z* = 537.2724)
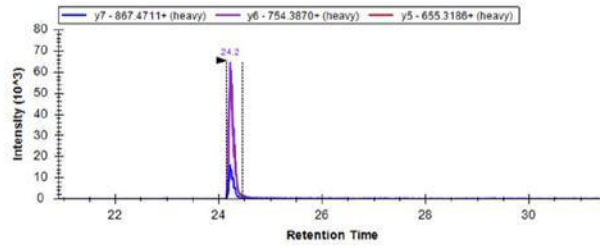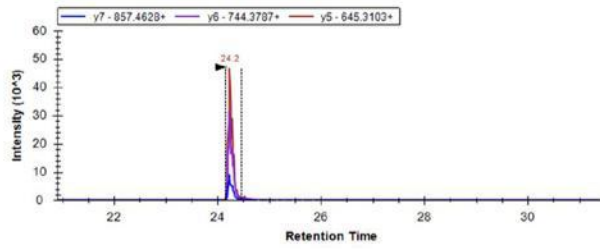
SIRT7 LLAESADLVTELQGR (precursor mass, 2⁺ *m/z* = 807.9385)
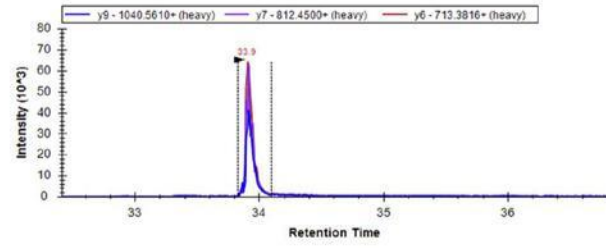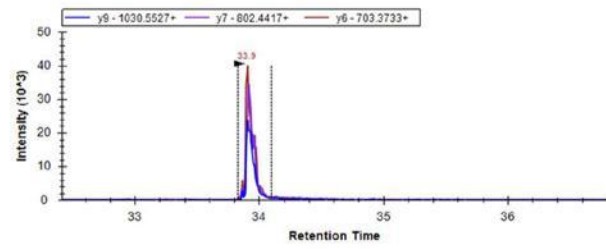
Supplementary Figure S2. Sirtuin peptide standard curves prepared in several matrices, including buffer only, a blank gel control, a gel containing low abundance plasma proteins (see Supplementary Figure S7 for the gel used for the gel matrices).
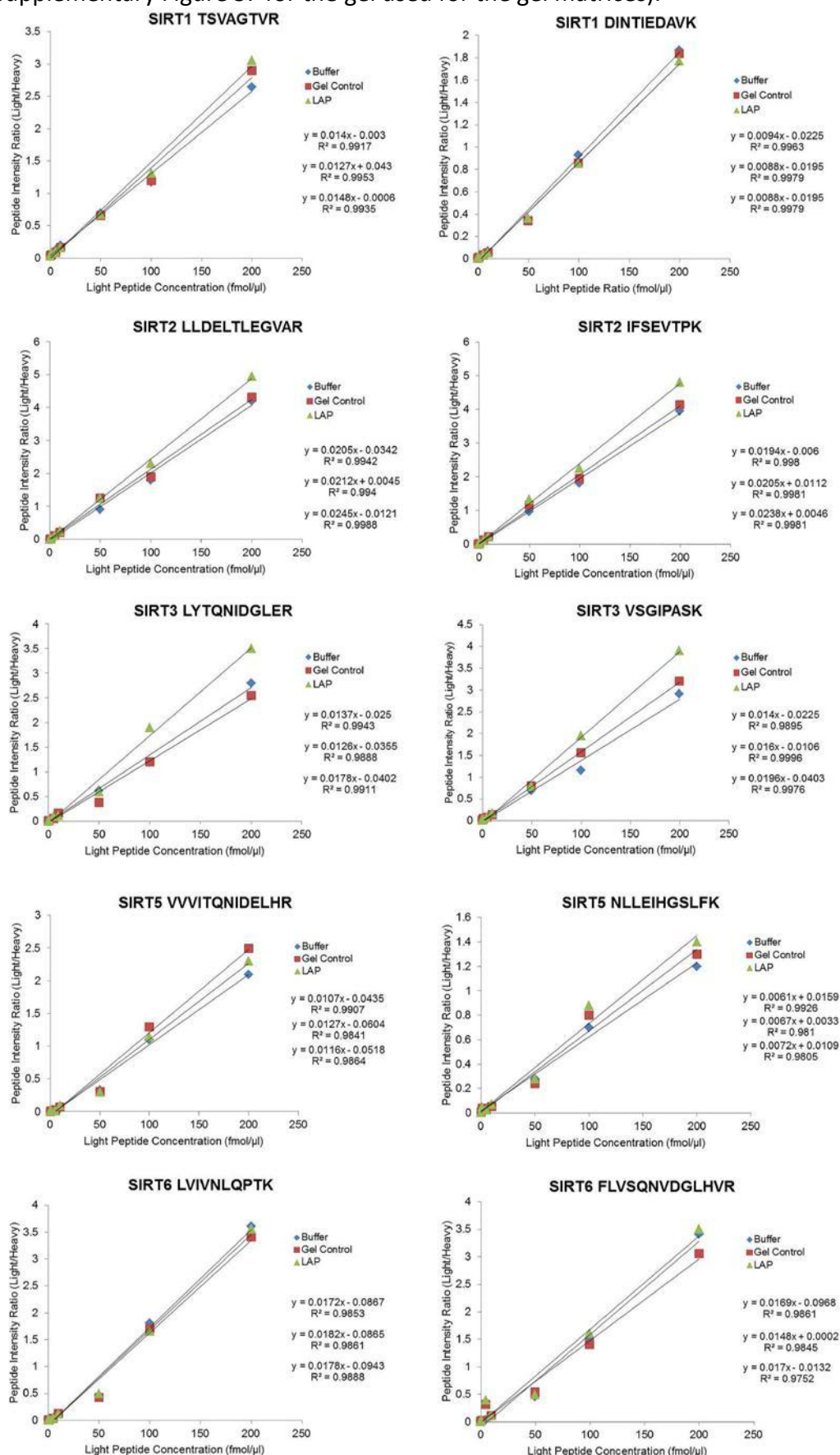
Supplementary Figure S3. Sample fractionation and MRM workflow.

1. Run known amount of protein sample on 1D electrophoresis gel.

2. Cut out bands corresponding to molecular weight of sirtuins.

3. Add trypsin to digest the proteins within each gel bit. Heavy isotope labelled internal standards were also added at this time. Incubate overnight at 37°C.

4. Vacuum dry peptides and reconstitute in 0.1% formic acid followed by MRM-MS analysis on 4000QTRAP.
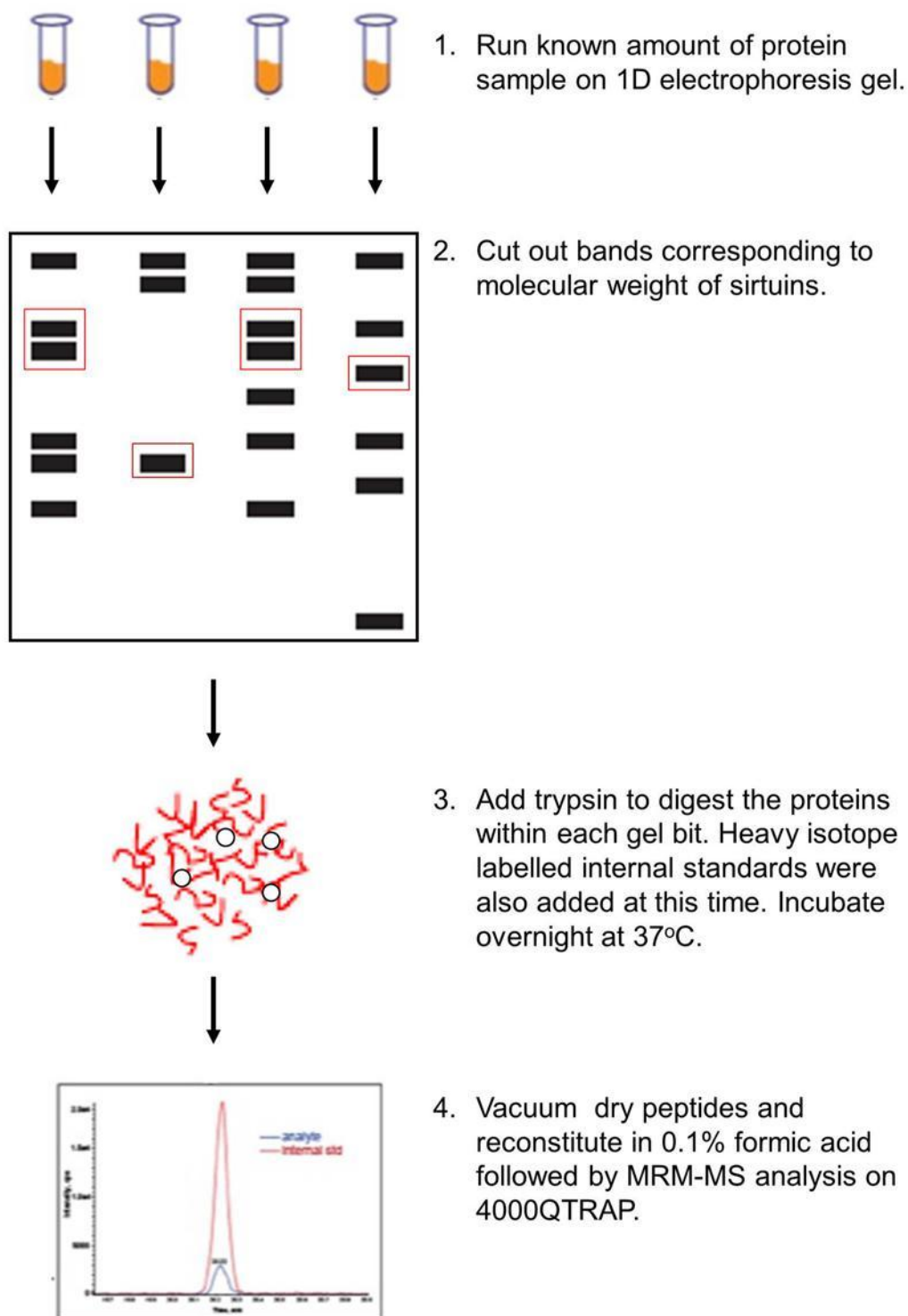
Supplementary Figure S4. Full length western blots of SIRT1-3 protein expression in human control frontal lobe brain tissue (n=3 individuals) at molecular weights of approx 40kDa, 50kDa and 30kDa respectively. All blots were run under the same experimental protocols with 1:1000 dilution of primary antibodies. The exposure times shown are 3hrs, 30sec and overnight for SIRT1, SIRT2 and SIRT3 respectively.
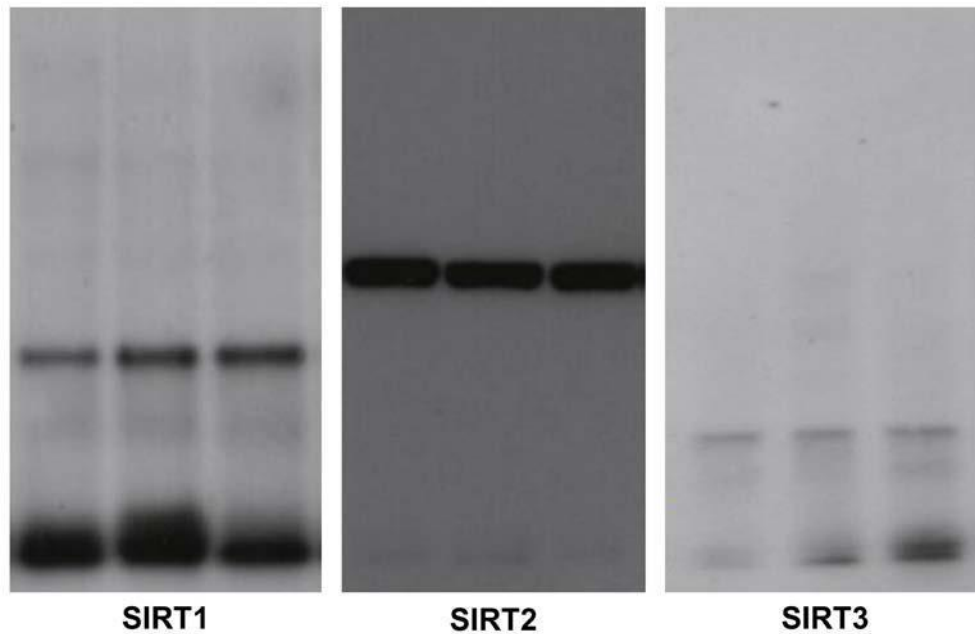
Supplementary Figure S5. SIRT1 levels in CSF and depleted plasma. SIRT1 levels in CSF (n=5) samples and a plasma sample depleted of the six most abundant plasma proteins (high abundance protein removal was achieved using an Agilent Hu6 affinity column).
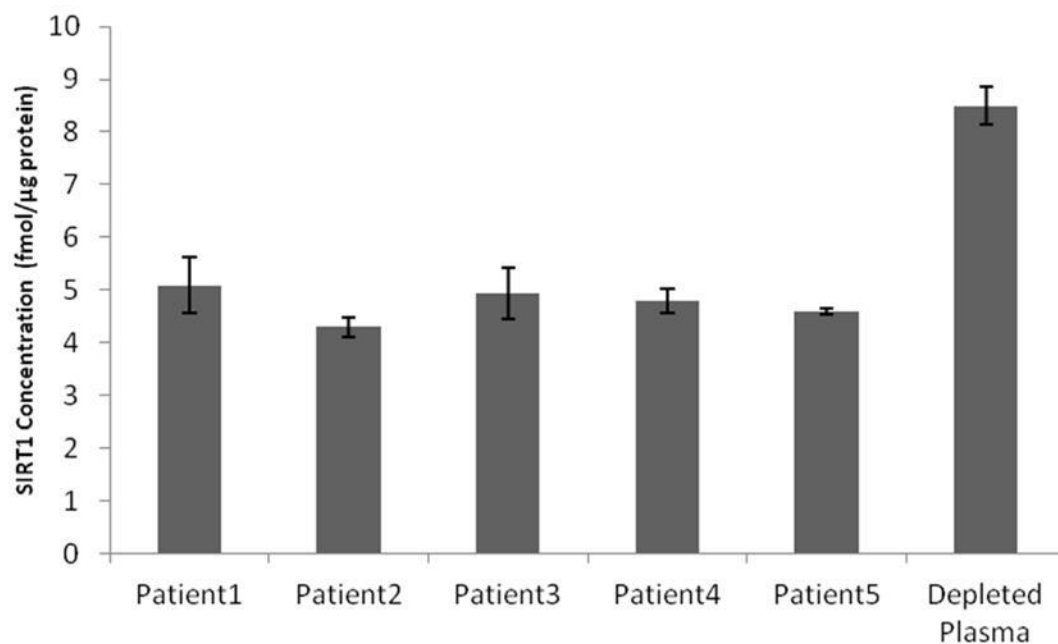
Supplementary Figure S6. Representative colloidal coomassie stained SDS PAGE gel of recombinant sirtuin protein standards highlighted in red boxes with the bands cut corresponding to the molecular weight of each full length sirtuin. Note the presence of host cell contaminants in most of the preparations, especially SIRT7, and the particularly low yield of SIRT4. These preparations were adequate to validate good signal intensity for each of the two peptides per sirtuin used for MRM quantification. SIRT1, 2, 3, 5 and 6 were also used to estimate recoveries using the full protocol (see Supplementary Figure S7 and Supplementary Table S1), in buffer only *vs* LAP spiked samples.
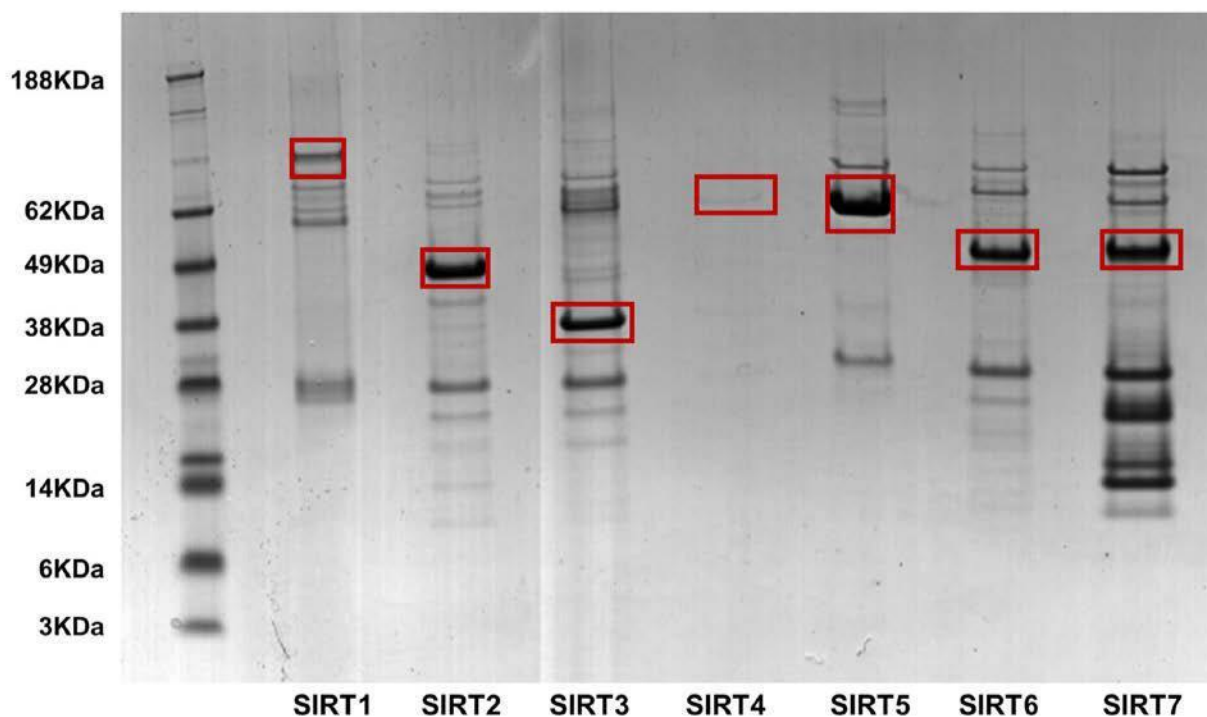
Supplementary Figure S7. A typical colloidal coomassie stained SDS/PAGE gel used to assess matrix effects on standard curves. Bands were cut from positions at which intact sirtuin proteins would typically be expected, and used to test matrix effects on the synthetic peptide standards. In addition to this, intact commercial recombinant sirtuin preparations were spiked into depleted plasma and run by SDS/PAGE (see Supplementary Figure S8).

Depleted plasma
(20μg per lane)

Blank Gel Lanes
(Gel Control)
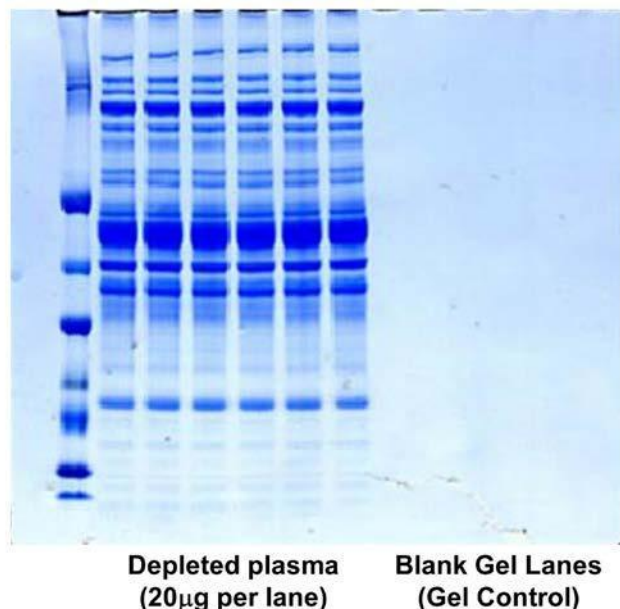
Supplementay Figure S8. Colloidal oomassie stained SDS/PAGE gel used for sirtuin recovery experiments. Lane 1: prestained molecular weight markers; Lanes 2-6: commercial SIRT1, 2, 3, 5 and 6 recombinant sirtuin protein standards (5ug per lane. This amount was based on the manufacturer's indicated quantification of vial contents.); Lanes 8-12: 20ug Depleted plasma spiked with 5ug of sirtuin standards 1, 2, 3, 5 and 6; Lanes 14-18: Depleted plasma spiked with 2ug of sirtuin standards 1, 2, 3, 5 and 6. Sirtuins 4 and 7 were not used for this experiment as the SIRT4 yield was very low, and SIRT7 was particularly impure (see Supplementary Figure S6).
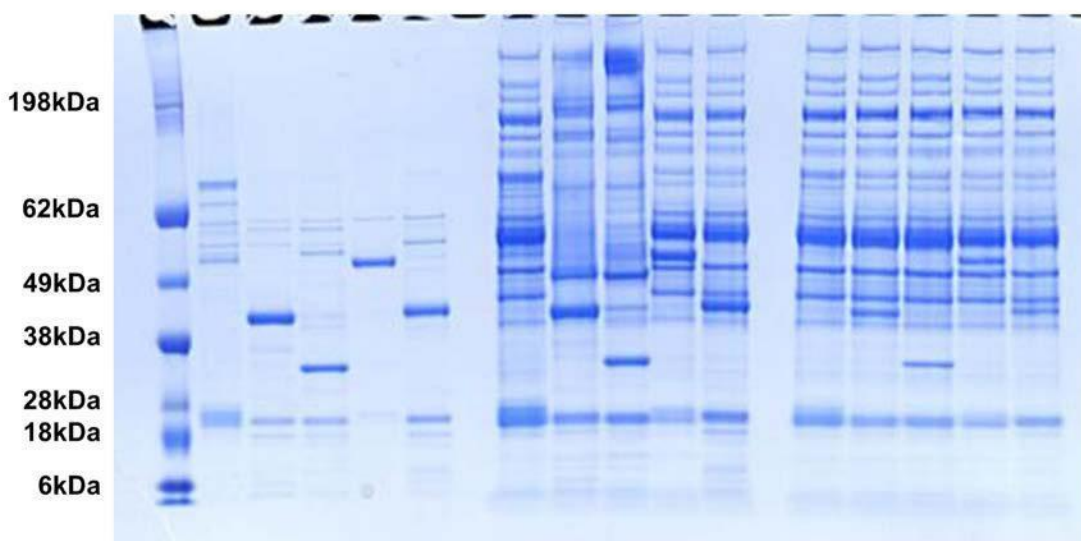
Upregulation of Glycolytic Enzymes, Mitochondrial Dysfunction and Increased Cytotoxicity in Glial Cells Treated with Alzheimer's Disease Plasma

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder associated with increased oxidative stress and neuroinflammation. Markers of increased protein, lipid and nucleic acid oxidation and reduced activities of antioxidant enzymes have been reported in AD plasma. Amyloid plaques in the AD brain elicit a range of reactive inflammatory responses including complement activation and acute phase reactions, which may also be reflected in plasma. Previous studies have shown that human AD plasma may be cytotoxic to cultured cells. We investigated the effect of pooled plasma (n = 20 each) from healthy controls, individuals with amnestic mild cognitive impairment (aMCI) and Alzheimer's disease (AD) on cultured microglial cells. AD plasma and was found to significantly decrease cell viability and increase glycolytic flux in microglia compared to plasma from healthy controls. This effect was prevented by the heat inactivation of complement. Proteomic methods and isobaric tags (iTRAQ) found the expression level of complement and other acute phase proteins to be al-tered in MCI and AD plasma and an upregulation of key enzymes involved in the glycolysis pathway in cells exposed to AD plasma. Altered expression levels of acute phase reactants in AD plasma may alter the energy metabolism of glia.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that results in the progressive and irreversible loss of cholinergic neurons in specific areas of the brain [1]. Amnestic Mild Cognitive Impairment (aMCI) is considered to be a pre-dementia stage of AD [2], with a proportion of aMCI cases progressing to AD with time. AD is characterised by an abnormal accumulation of amyloid β (Aβ) and tau proteins, increased oxidative stress and redox metals in the brain all of

which are associated with an immunological response [3]. Aβ primarily accumulates extracellularly and eventually leads to the formation of amyloid plaques, the main pathological hall-mark of AD. The accumulation of Aβappears also appears to occur in synaptic mitochondria leading to impaired respiration and increased oxidative stress [4].

Damage to the blood-brain barrier is thought to occur in AD, and this may increase move-ment of proteins between the brain and the vasculature [5]. It is therefore possible that AD and its precursor, MCI, may be associated with the presence of specific biomarkers detectable in plasma and recent work has successfully used a panel of plasma biomarkers to predict disease severity and progression from MCI to dementia [6]. There are a number of proposed plasma biomarkers for AD, some of which reflect increased protein, lipid and nucleic acid oxidation and reduced activities of antioxidant enzymes in the AD brain [7–13]. AD has been reported to be associated with reduced plasma levels of vitamin A, C and E [9]. Isoprostanes, which arise from free-radical-mediated peroxidation of polyunsaturated fatty acids, are elevated in the AD brain, CSF and plasma [11]. 4-hydroxynonenal, another product of lipid peroxidation, is also increased in AD plasma [8].

A variety of inflammatory markers are increased with the onset of AD pathology, including cytokines and chemokines, coagulation factors and acute-phase reactive proteins as well as reactive astrocytes and activated microglial cells, the main cells involved in the neuroinflammation process [3,14]. Previous studies have shown that upregulation of the acute phase protein clusterin in plasma, is associated with prevalence, rate of progression, brain atrophy and disease severity in AD patients [15,16]. Other studies however have found no difference and suggest against the idea that acute phase protein changes in the CNS can be detected in plasma [17,18]. Alternative-ly, AD may be associated with a more widespread immune dysregulation, detectable in plasma.

Previous studies investigating the effects of human AD plasma on cells in culture have found differential effects on protein expression and cell biology. One study aimed to determine if exposure to serum from AD patients would affect markers for AD brain lesions [19], and found that 24 hour exposure to AD serum increased four molecular markers characteristic of AD senile plaques and neurofibrillary tangles (NFTs) in rat hippocampal neurons [19]. These markers were Alz-50, beta-amyloid, MAP2 and ubiquitin [19]. This stimulation of AD markers by human serum suggests that the genesis of both neuronal plaques and tangles may arise from exposure of susceptible neurons to toxic serum factors and/or failure to detoxify these factors. Another study found that antibodies in serum of patients with AD caused immunolysis of cho-linergic nerve terminals from the rat cerebral cortex, supporting the hypothesis that autoim-mune mechanisms may operate in the pathogenesis of AD [20].

Other studies have shown that serum of multiple sclerosis patients causes demyelination in rat CNS explant cultures and also induces cytotoxicity in rat oligodendrocytes in culture [21,22]. Demyelination was present not only in multiple sclerosis sera but was also found in sera from patients with other neurological diseases and complement was shown to be a factor involved in the effect [23,24]. In yet another study, human serum from patients with septic shock was shown to induce apoptosis in human cardiac myocytes [25]. This work demonstrat-ed the utility of examining effects of disease plasma on cell culture systems, to facilitate the study of both disease markers and disease mechanisms.

Since previous studies indicate that AD plasma may contain oxidative stress markers as well as cytotoxic factors, we investigated the effect of the addition of pooled control, MCI and AD plasma from 20 individuals each on a microglial cell line. Cell viability, proliferation and mito-chondrial function were investigated following 48 hour treatment with non heat-inactivated plasma and plasma in which complement proteins had been deactivated. We also tested the ef-fect of commercially purchased complement factors alone and in combination on cultured glia. We then undertook proteomic analysis of the plasma from each group and iTRAQ quantitative

proteomic analysis of cell extracts exposed to plasma from each group to investigate possible plasma protein alterations unique to MCI or AD, to detect any protein aberrations within the cells treated with the plasma and to correlate these finding to cell viability and mitochondrial function assays measured in vitro.

Materials and Methods

Subjects

Age matched healthy control (n = 20), amnestic mild cognitive impairment (aMCI, n = 20), and probable AD (n = 20) plasmas were pooled and used in both the cell culture and plasma proteomics experiments. AD patients were recruited from the Memory Clinic of the Depart-ment of Old Age Psychiatry of the Prince of Wales Hospital and participants in a clinical drug trial of donepezil (Aricept). All met the NINCDS-ADRDA criteria [26] for probable AD. The aMCI subjects were recruited from the Memory and Ageing Study, a longitudinal study of community dwelling individuals aged 70–90 [27]. The diagnosis of aMCI was determined using the Petersen Criteria as follows [28]: (i) subjective complaint of memory impairment,
(ii) objective impairment in memory (performance >1.5 SD below normal for age on a stan-dardised memory test) (iii) essentially preserved general cognitive function (MMSE 24)
(iv) intact functional activities as indicated by instrumental activities of daily living; and (v) not meeting DSM-IV criteria for dementia. Healthy control subjects had a normal performance for age on a range of neuropsychological tests and intact functional activities. Ethics committee ap-proval was obtained from the University of New South Wales (UNSW) and the South Eastern Sydney Illawarra Area Health Service (SESIAHS) ethics committees and written informed con-sent was obtained from all participants.

Cell Culture

CHME-5 cells are a human microglial cell line obtained from embryonic fetal human microglia through transformation with SV-40 virus [29,30] and were a generous gift from Prof Gilles Guillemin (Macquarie University, Sydney, Australia). These cells express antigens present on adult human microglia, secrete pro-inflammatory cytokines upon activation, exhibit properties of primary human microglia and have been successfully used as a model of microglial activa-tion by others [29,31]. Cells were maintained in RPMI1640 cell culture medium, supplemented with 10% heat inactivated foetal bovine serum, 2 mM l-glutamine, and 1% penicillin/strepto-mycin, at 37°C in a humidified atmosphere containing 95% air/5% CO_2. Before experimenta-tion, cells were seeded into 24 or 96 well culture plates to a density of approximately $1x10^4$ or $2x10^3$ cells respectively. Cells were left overnight and then supplemented with up to 20% (by volume) heat-inactivated and non heat-inactivated control, MCI or AD plasma for 48 hours. For the cell viability and iTRAQ proteomic analyses cells were washed to remove all plasma, and lysed in RIPA buffer (Thermo Fisher Scientific, IL, USA) followed by sonication. For the complement factor experiments cells were seeded into plates and treated with 1, 5 or 10 µg of each complement factor or the complement standard (in a total volume of 200µL cell cul-ture media) and then incubated for 48 hours. These concentrations are within the physiological range of these proteins in plasma [32].

MTT Cell Proliferation Assay

In actively proliferating cells, an increase in 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) conversion in cells relative to controls represents an increase in cellular

proliferative activity. Conversely, in cells that are undergoing apoptosis, MTT conversion, and thus biological activity, decreases. Cell proliferation was analysed using established protocols [33,34].

NAD(H) Assay

Damaged cells show mitochondrial dysfunction, which results in decreased cellular nicotin-amide adenine dinucleotide (NAD) levels. Intracellular NAD(H) concentration was quantified using the thiazolyl blue microcycling assay established by Bernofsky and Swan and adapted here for the 24 well plate format [35].

Lactate Dehydrogenase (LDH) Leakage

Cytoplasmic enzyme leakage has been shown to be a useful tool for measuring early cellular damage or impairment [12], and has also been used as a sign of cytotoxicity [36,37]. LDH is re-leased from cells due to loss of membrane integrity. Therefore LDH was measured in the cell culture medium as well as in cell homogenates as another measure of cell viability.

XF24 Microplate-Based Respirometry as a measure of mitochondrial function

To determine the effect of human control, MCI and AD plasma on oxygen consumption rates (OCRs; an indicator of mitochondrial respiration) and extracellular acidification rates (ECARs; a measure of glycolytic flux) in the microglial cell line, the Seahorse XF24, extracellular flux an-alyzer (Seahorse Bioscience, North Billerica, MA, USA) was employed and assays performed as previously described [38–40]. The basal control ratio (BCR) and the uncoupling ratio (UCR) were determined as previously described [41]. Essentially, the BCR is a measure of how close the basal level of respiration is to the maximum level of respiration. The closer this ratio is to 1, the greater the mitochondrial malfunction. The UCR is a measurement of mitochondrial functional integrity and measures the ratio of uncoupled to physiologically normal respiration levels. The greater the maximum level of respiration, the greater the mitochondrial functional integrity.

Fractionation of Plasma

Control, MCI and AD plasma from 20 subjects was pooled and fractionated by two methods. To fractionate it into its protein and metabolite fractions, a PD10 column separation method was used. The PD10 column was washed with MilliQ water before the addition of 500 μL of plasma. The flow-through was collected as 750 μL fractions topping the column with MilliQ water. In total 20 fractions were collected and the absorbances read at 280nm. For the proteo-mics analysis, fractionation into low and high abundant protein fractions was undertaken using an MARS-Hu6 column (Agilent Technologies, CA, USA) according to the manufactur-er's instructions. The MARS-Hu6 column depletes the top 6 contaminating proteins (albumin, IgG, IgA, transferrin, haptoglobin and antitrypsin) from plasma. This eliminates the masking effect of highly abundant proteins so lower-abundant proteins can be more easily detected. The low abundance fraction produced was used for the proteomic analysis experiments.

Proteomics of MCI and AD plasma

A one dimensional SDS 4–12% NuPAGE (Thermo Fisher Scientific Inc, MA, USA) gel was run using the low abundance fraction from each of the three groups. The gel was colloidal coomas-sie stained [42], and the lanes uniformly cut into 7–8 bits using a gridcutter and mount from

The Gel Company, CA, USA. The gel bits were trypsin digested and then analysed using mass spectrometry as outlined in detail in S1 Methods.

Peak lists were generated by MassLynx (version 4.0 SP1, Micromass) using the Mass Mea-sure program and submitted to the database search program Mascot (version 2.2, Matrix Sci-ence, London, England). Search parameters were: Precursor and product ion tolerances ± 0.25 and 0.2 Da respectively; Met(O) and Cys-carboxyamidomethyl were specified as variable modi-fication, enzyme specificity was trypsin, one missed cleavage was possible on the NCBInr database.

Scaffold Q+ (version 4.3.4), Proteome Software, Portland, OR, USA) was used to identify any altered proteins between the groups. The Scaffold programme uses mass spectrometric data to identify protein changes in biological samples by collating Mascot data and using the ProteinProphet algorithm [43]. We compared the normalised total spectral count values from Scaffold [44] with the emPAI values from Mascot [45], which uses a different algorithm for spectral counting.

iTRAQ Proteomics of Cell Lysates Treated with Human Control, MCI and AD Plasma

Two biological replicates of cells treated with 10% (by volume) non-heat inactivated fetal bo-vine serum, human control, MCI or AD plasma were washed to remove all plasma/serum and then lysed using RIPA buffer and probe sonicated. Total protein concentrations were deter-mined using the Bicinchoninic acid (BCA) protein assay kit (Pierce, IL, USA). The total protein (120 µg) from each sample was reduced with 2 µl of 5mM tris-(2-carboxyethyl) phosphine (TCEP) for 60 min at 60°C followed by alkylation with 200 mM iodoacetamide (1 µL) for 10 min at room temperature. To remove any buffer components incompatible with mass spec-trometry a buffer exchange was performed with 50 mM $NaHCO_3$ using Microcon centrifugal filter devices with a 3,000 Da nominal molecular weight limit membrane (Millipore, MA, USA) to give a final protein concentration of 1 µg/µl.

For tryptic digestion 100 µg total protein from each sample was incubated overnight at 37°C with 4 ug of trypsin. Samples were labelled using the 8-plex iTRAQ kit (Applied Biosystems, CA, USA). Each iTRAQ reporter label was mixed with a biological replicate of cell lysate sample, pH adjusted to basic (ca pH = 9) with 2 µl of 50 mM of Na_2CO_3 and incubated for 2 hours. The re-porter masses for the samples were labelled as follows: fetal bovine serum; 113 and 117, human control plasma; 114 and 118, human MCI plasma; 115 and 119, human AD plasma; 116 and 121. Sample clean-up was performed using a strong cation exchange cartridge (Applied Biosys-tems, CA, USA) and a syringe pump at a flow rate of 9.5 ml/hr, and using the manufacturer's protocol. Sample was then passed through a C18 Peptide Macrotrap (Michrom Bioresources, CA, USA). The flow-through from the C18 step was passed through an Oasis cartridge (Waters, MA, USA) to maximise peptide recovery and the two eluants pooled and dried under vacuum, resuspended in 0.2% heptafluorobutyric acid and then analysed using mass spectrom-etry as outlined in detail in S1 Methods.

Protein identification and quantification was performed using the MS/MS data (WIFF files) and the Paragon algorithm as implemented in Protein Pilot v4.0 software (Applied Biosystems, CA, USA) using the NCBInr database. Only proteins identified with a ProteinPilot unused score of 1.3 (greater than 95% confidence in sequence identity) were accepted as previously described [46,47]. The unused score is a ProteinPilot generated value for the level of confidence in protein sequence identification. As an approximate guide, ProteinPilot unused scores give the following percentage levels of confidence; score 1.3(95% confidence), score 2(99% confidence), score 3 (99.9% confidence) [46]. The only fixed modification used was

Biological Effects of Exposure to Alzheimer's Disease Plasma in Glia

Table 1. Cell viability as measured by MTT absorbance (abs) at 570nm, LDH release and intracellular NAD levels of microglial cells after 48 hour incubation in pooled, non heat inactivated and heat inactivated MCI and AD plasma.

		Non Heat Inactivated Plasma Control MTT abs	Control NAD (ng)	Control LDH Activity (U/L)	MCI MTT abs	MCI NAD (ng)	MCI LDH Activity (U/L)	AD MTT abs	AD NAD (ng)	AD LDH Activity (U/L)	Heat Inactivated Plasma Control MTT abs	Control NAD (ng)	MCI MTT abs	MCI NAD (ng)	AD MTT abs	AD NAD (ng)
5% plasma	Mean	0.21	10.71		0.23	10.84		0.14	10.3							
	SEM	0.05	3.84		0.05	1.26		0.03*	1.82							
10% plasma	Mean	0.13	3.61	Media 3.35 Lysate 11.4	0.09	2.35	Media 2.7 Lysate 13.06	0.04	0.89	Media 6.09 Lysate 7.76	0.13		0.11		0.11	
	SEM	0.03	1.25	Media 0.36 Lysate 1.19	0.02	0.85	Media 0.3 Lysate 1.22	0.01**	1.3*	Media 1.05* Lysate 1.0	0.03		0.04		0.02	
20% plasma	Mean	0.06	0.79		0.06	0.56		0.03	0			14.02		12.3		12.1
	SEM	0.01	0.14		0.004	0.07		0.004**	0**			1.15		0.60		0.65

p 0.05 vs Control,

p 0.01 vs Control

Cell viability was determined by measurement of cell proliferation, intracellular NAD levels and LDH activity in cell culture media and cell lysate homogenates. n = 9 (nine replicates) for cell proliferation measurements, n = 6 (six replicates) for NAD concentration and LDH activity. Plasma used for the measurements were obtained from the pooled plasma of 20 patients from each of the three groups (Control, MCI and AD) investigated. Three concentration levels of plasma were tested: 5%, 10% and 20% plasma as a percentage of total media volume.

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iodoacetamide alkylation of cysteine residues. Mass tolerances were set to 50ppm for the pre-cursor and 0.2 Da for the fragment ions. Autobias correction was applied to correct for any sys-tematic bias in total protein concentration during sample pooling. Both biological replicates for the three human plasma types (control, MCI and AD) were compared to the fetal bovine serum control (iTRAQ reporter 117) and data exported to Microsoft Excel software (Micro-scoft, WA, USA).

Protein interactions between dysregulated proteins were determined using the web-based bioinformatics tool STRING v9.1 (http://string-db.org). STRING has a database that collates information on protein-protein interactions and associations. It scores and weights connec-tions and provides predicted interaction network maps from literature mining searches. The 27 proteins which were significantly deregulated in glia treated with AD plasma, but not deregu-lated in either control or MCI plasma treated glia were analysed in STRING v9.1. MCL cluster-ing was used with the 2 clusters option picked and with the confidence view selected to display the strength of evidence for protein associations and analysis of enrichment was also performed.

Statistics

All cell viability values are presented as means ± SEM. Statistical comparisons were performed using two-tailed student t-tests assuming equal variance. Differences between treatment groups were considered statistically significant at the $p < 0.05$ level. Scaffold values are represented as normalised total spectral counts and p-values for significantly altered proteins were obtained using the ProteinProphet algorithm of the Scaffold Q+ software (Proteome Software, OR, USA). All iTRAQ values are presented as ratios of cells treated with human plasma to cells treated with fetal bovine serum control. Ratios and p-values for significantly altered proteins were obtained through the Paragon algorithm of the Protein Pilot v4.0 software (Applied Bio-systems, CA, USA).

Results

Cell Proliferation

Cells treated with non heat-inactivated pooled control and AD plasma for 48 hours showed a significant decrease in cell proliferation in cells treated with AD plasma compared to controls (Table 1). The addition of MCI plasma to the cells also caused a similar drop in cell prolifera-tion, though not reaching statistical significance (Table 1). Mild heat treatment at 56°C for 30 minutes is an established approach for inactivating complement proteins [48,49]. Such heat treatment prevented the effects of MCI and AD plasma on cell proliferation (Table 1).

To determine whether the effect on cell proliferation was exclusively due to the protein component or to both the protein and low molecular weight components of plasma we separat-ed proteins and metabolites. Plasma was fractionated using a PD10 column into its protein and metabolite portions to determine which portion of the plasma was causing the cytotoxic effect (Fig. 1). Addition of these two fractions to the cells showed that it was exclusively the protein portion which was initiating the reduction in cell proliferation (Fig. 1). Protein fractions of both MCI and AD plasma were found to significantly reduce cell proliferation (Fig. 1). The me-tabolite fraction of the plasma had no significant effect on microglial proliferation (Fig. 1).

Treatment of the cells with complement factors C1q, C1 inhibitor, C4, C5 and C9 effected a downward trend in cell proliferation with increasing concentration, but did not reach signifi-cance (Table 2). In combination, the factors were found to reduce cell proliferation at the high-est concentration tested. The human complement standard which contains the factors C1q,
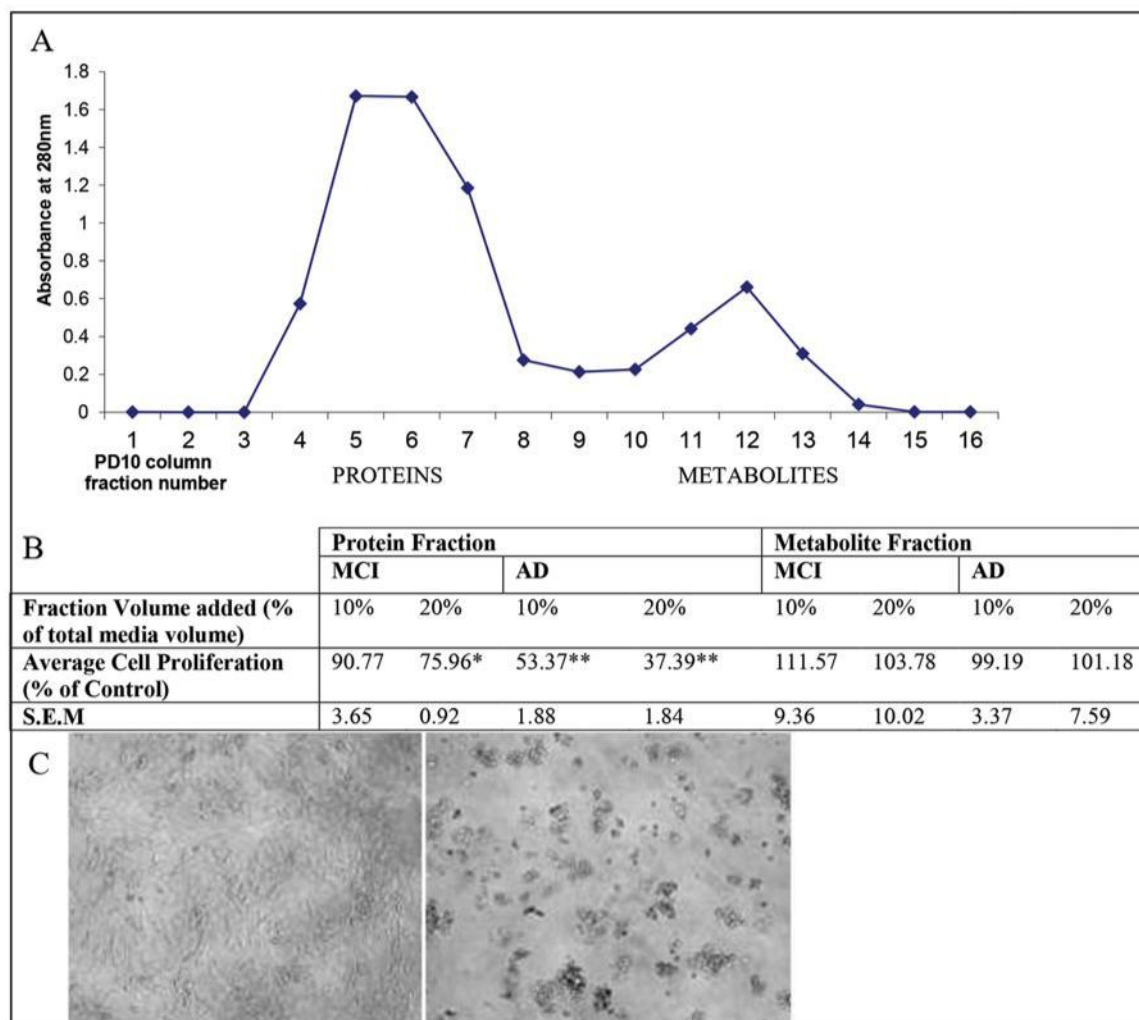
Fig 1. Fractionation of non heat inactivated control plasma into protein and metabolite fractions and the effects of plasma treatment on cell proliferation. Panel A: Fractionation of non heat inactivated control plasma into protein and metabolite fractions using PD10 column Panel B: Effect of these fractions on cell proliferation. Three replicates were performed. Plasma used for the measurements were obtained from the pooled plasma of 20 patients from each of the three groups (Control, MCI and AD). * p 0.01 vs Control, ** p 0.001 vs Control. Panel C: Images of microglia after 48 hour incubation with non heat inactivated 20% control plasma (left) and 20% AD plasma (right), showing increased toxicity and reduced cell proliferation in the AD plasma treated cells.

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C2, C3, C4, C5, C6, C7, C8, C9 and factor B was found to be the most potent at preventing cell proliferation (Table 2).

NAD levels and LDH Leakage

Incubation with non heat inactivated plasma caused a significant drop in cell viability as re-flected in lower NAD levels, for cells treated with both MCI and AD plasma, compared to con-trols (Table 1). This result was again prevented by plasma heat inactivation (Table 1). The addition of human complement standard containing C1q, C2, C3, C4, C5, C6, C7, C8, C9 and factor B was found to significantly reduce NAD levels in the microglia (Table 2).

A significant increase in LDH leakage into the cell culture medium was seen in cells incubat-ed with non heat inactivated AD plasma (Table 1). A concurrent decrease was seen in the amount of intracellular LDH in these same cells (Table 1).

Table 2. Cell viability of microglial cells after 48 hour incubation with human complement components (C1q, C1 inhibitor, C4, C5 and C9), both individually and in combination with each other; and a human complement standard containing complement components C1q, C2, C3, C4, C5, C6, C7, C8, C9 and factor B.

Cell Proliferation (abs at 570nm)		Complement C1q	Complement C1 inhibitor	Complement C4	Complement C5	Complement C9	C1q + C4	C5 + C9	C1q, C1inhib, C4, C5 + C9	Complement Standard	Complement Standard NAD concentration (ng)
Control (0µg/µl)	Average	0.317	0.609	0.454	0.375	0.324	0.384	0.356	0.378	0.511	0.114
	SEM	0.019	0.07	0.025	0.031	0.014	0.025	0.018	0.013	0.004	0.003
0.005µg/µl	Average	0.3	0.593	0.458	0.382	0.333	0.377	0.371	0.377	0.426	0.104
	SEM	0.042	0.02	0.019	0.028	0.0098	0.019	0.025	0.019	0.031	0.010
0.025µg/µl	Average	0.31	0.571	0.461	0.364	0.336	0.366	0.359	0.331*	0.210**	0.094
	SEM	0.018	0.03	0.02	0.025	0.0096	0.025	0.021	0.015	0.036	0.063
0.05µg/µl	Average	0.267	0.49	0.435	0.355	0.344	0.289*	0.265*	0.292*	0.129**	0.063*
	SEM	0.0075	0.05	0.019	0.03	0.021	0.027	0.013	0.024	0.018	0.0024

Cell viability was determined by MTT assay of cell proliferation and intracellular NAD levels (for complement standard samples). Replicates included n = 6 for cell proliferation measurements and, n = 3 for NAD concentrations.

* p $\leq$ 0.05 vs Control,

** p $\leq$ 0.01 vs Control

doi:10.1371/journal.pone.0116092.t002

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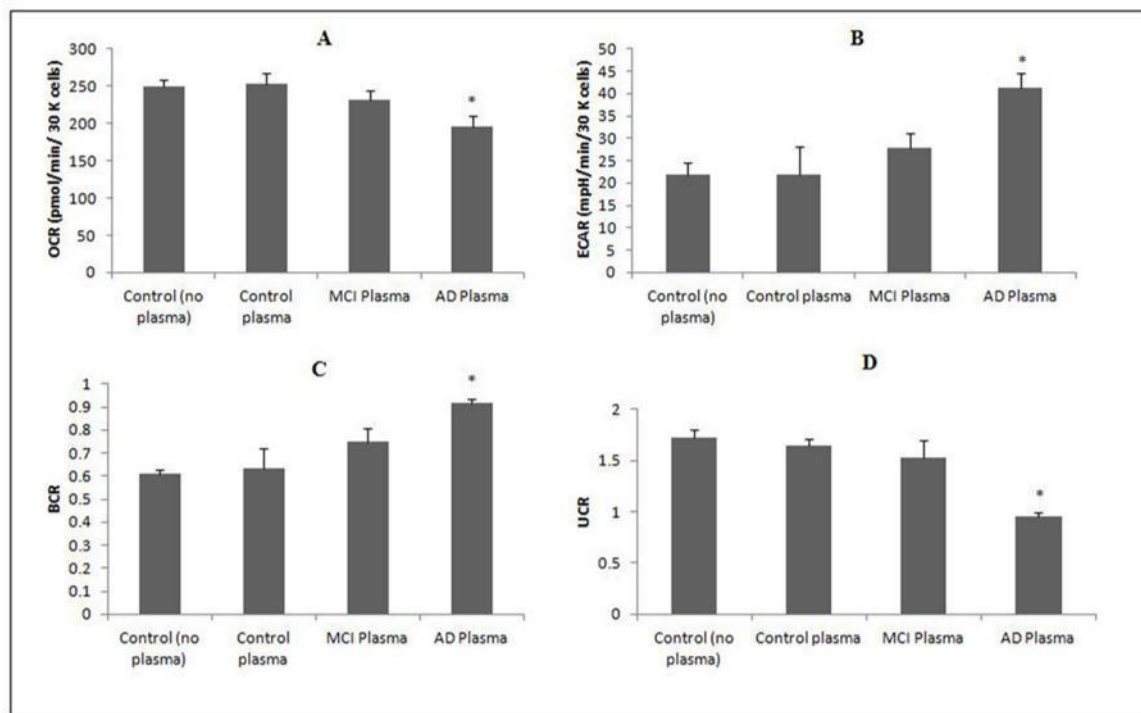
Fig 2. Effects of human plasma on cellular bioenergetics in a microglial cell line. (A) Effect of human plasma on oxygen consumption rates (OCR) in a microglial cell line for 48 hours. *$p<0.05$ compared to non-treated cells (control); ($n = 4$ for each treatment group). (B) Effect of human plasma on extracellular acidification rates (ECAR) in a microglial cell line for 48 hours. *$p<0.05$ compared to non-treated cells (control); ($n = 4$ for each treatment group). (C) Effect of human plasma on the basal control ratio (BCR) in a microglial cell line for 48 hours. *$p<0.05$ compared to non-treated cells (control); ($n = 4$ for each treatment group). (D) Effect of human plasma on the uncoupling ratio (UCR) in a microglial cell line for 48 hours. *$p<0.05$ compared to non-treated cells (control); ($n = 4$ for each treatment group).

doi:10.1371/journal.pone.0116092.g002

Mitochondrial Function and Cellular Bioenergetics

To determine whether mitochondrial bioenergetic mechanisms are associated with AD patho-genesis, we assessed mitochondrial function in glial cells treated with human plasma using the Seahorse XF24 (Seahorse Bioscience, MA, USA). We observed a significant decrease in OCRs and an increase in ECAR for cells treated with AD plasma and MCI plasma effected similar trends though did not achieve statistical significance (Fig. 2). We also observed a significant in-crease in the BCR and a decline in the UCR in microglial cells after 48 hour incubation with AD plasma (Fig. 2), consistent with impaired mitochondrial function and increased shift towards glycolysis.

Plasma fractionation and 1D gel electrophoresis

Fractionation using the MARS-Hu6 column provided a baseline separation of low and high abundant proteins (Fig. 3). These fractions were run on a 1D SDS NuPage gel and proteins were shown to be effectively separated with a substantial depletion of high abundant proteins, revealing many lower abundant protein bands in the low abundant fraction (Fig. 3).

Proteomics of MCI and AD plasma

Following proteomic analysis of pooled control, MCI and AD plasma, normalised total spectral counts using Scaffold software and emPAI values from Mascot showed complement compo-nent 2, fibronectin and fibrinogen to be significantly increased in the AD groups compared to
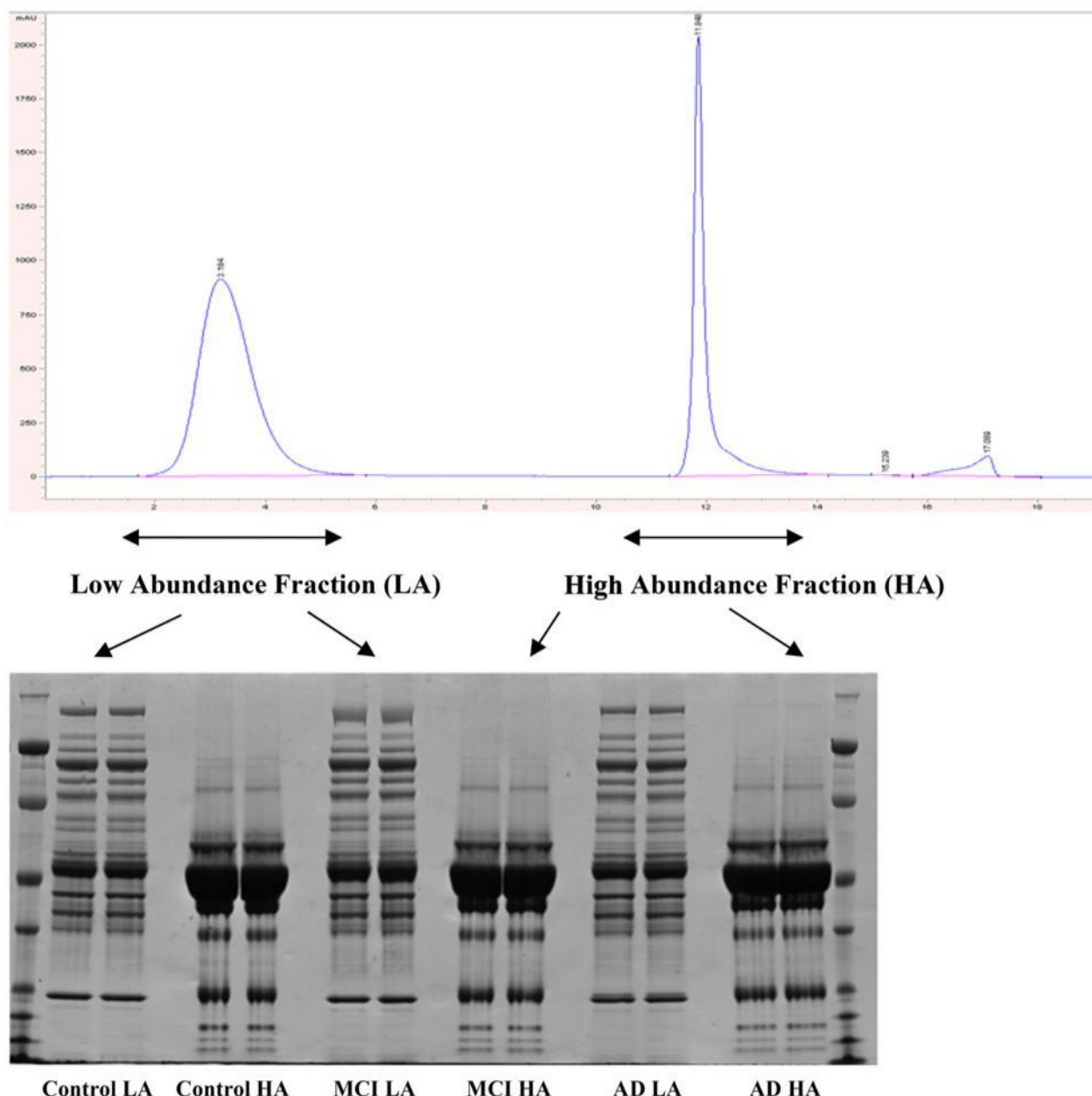
Fig 3. Chromatogram of fractionation using Hu6 column and 1D SDS/PAGE of these fractions. Low abundant proteins are eluted first (first peak on chromatogram) and high abundant proteins are eluted after (second peak). Gel shows significant depletion of high abundant proteins in the low abundant fractions. Loading was 50 µg/lane. First and last lanes contained molecular weight markers. Each fraction was run in duplicate.

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the control group (Table 3). Thrombin was decreased in the MCI and AD patients compared to controls (Table 3). A full list of proteins identified and normalised spectral counts in human control, MCI and AD plasma can be found in S1 Table and the peptide false discovery rate analysis can be found in S1 Fig.

Table 3. Average normalised spectral counts (obtained from Scaffold) and emPAI values (obtained from Mascot) of significantly deregulated proteins identified in pooled plasma samples between the Control, MCI and AD groups.

		Complement component 2 (gi|14550407)			Complement component 1 inhibitor (gi|114642584)			Complement component 4 binding protein (gi|4502503)			Fibronectin (gi|109658664)			Thrombin (gi|119588383)			Fibrinogen (gi|70906435)			Alpha-1B-glycoprotein (gi|46577680)		
		C	MCI	AD	C	MCI	AD	C	MCI	AD	C	MCI	AD	C	MCI	AD	C	MCI	AD	C	MCI	AD
Normalised Spectral Count	Average	0	0.82	2.77	9.98	7.45	11.30	4.18	3.38	4.51	18.9	15.1	32.2	10.34	5.57	6.29	39.78	35.93	43.19	6.08	8.32	7.64
	S.E.M	0	0.50	0.29	1.0	0.46	2.10	0.51	0.46	0.83	0.18	2.55	2.29	1.18	0.50	0.88	5.58	2.42	7.25	0.49	0.70	1.01
	p-value		0.015#	0.000074**		p = 0.06 vs AD						0.0025##	0.0012**		0.098*	0.033*					0.040*	
emPAI value	Average	0.08	0.06	0.145	0.74	0.62	0.95	0.35	0.18	0.26	0.39	0.30	0.64	0.83	0.46	0.48	5.40	4.74	7.87	0.78	0.86	0.75
	S.E.M	0.010	0.020	0.014	0.090	0.06	0.13	0.027	0.037	0.029	0.068	0.065	0.033	0.068	0.055	0.033	0.48	0.62	0.71	0.10	0.032	0.029
	p-value		0.014#	0.025*		p = 0.06 vs AD				0.013*		0.0071##	0.0018**		0.0057*	0.0034*		0.016#	0.036*		0.050#	

Values are averages from 4 replicates. Plasma used was obtained from the pooled plasma of 20 patients from each of the three groups (Control, MCI and AD) with depletion of high abundance proteins.

* p 0.05 vs Control
** p 0.01 vs Control
# p 0.05 vs AD
p 0.01 vs AD

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Biological Effects of Exposure to Alzheimer's Disease Plasma in Glia

iTRAQ proteomic analysis of cell lysates treated with Control, MCI and AD plasma

Differential protein expression in glial cells treated with fetal bovine serum (control) or human plasma from control, MCI and AD subjects were analysed with two biological replicates performed using an 8-plex iTRAQ experimental design. In total, 791 proteins were identified with 95% or greater confidence in correct protein sequence identification and 750 proteins with a false discovery rate of 5% (see S2 Table for full summary of identified proteins and S3 Table for full false discovery rate analysis). Fourty-one proteins were found altered between the MCI and AD groups of cellular lysates (Table 4). The highest numbers of dysregulated proteins were found in the cells treated with AD plasma (Table 4, 27 proteins highlighted in bold). Interest-ingly a significant number of proteins involved in the glycolysis cycle were shown to be upregu-lated in this group, namely glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, enolase, aldolase and pyruvate kinase. These enzymes catalyse five of the ten enzyme re-actions of the pathway and their functions are shown in Fig. 4. Transketolase was also shown to be significantly elevated in both the AD plasma treated cell sample replicates. This enzyme is part of the pentose phosphate pathway and connects this pathway to glycolysis. Analysis of protein interactions of the 27 dysregulated glial proteins treated with AD plasma using the on-line STRING v9.1 tool confirmed a significant enrichment of proteins involved in glucose me-tabolism (Fig. 5 and Table 5).

Discussion

A variety of studies have looked at the effects of plasma on cell cultures in different diseases. For example, Brewer et al found 24 hr exposure of human serum from AD patients to rat hippocam-pal neurons increased four molecular markers characteristic of Alzheimer senile plaques and neurofibrillary tangles [19]. Another study has shown that Parkinsonian serum has comple-ment-dependent toxicity to rat dopaminergic neurons [50]. A study using a differentiated neuro-nal cell line investigated the susceptibility of neuronal cells to human complement. It was found that human serum caused lysis of the neurons by complement, as tested by cell viability. The ef-fect was lost when cells were treated with complement-depleted serum by heat inactivation [51].

Our cell culture results also showed that the loss of cell viability and reduction in cell prolif-eration caused by AD plasma can be prevented by inhibiting the activity of plasma complement proteins. Alterations in peripheral proteins may reflect changes in the brain, especially since damage to the blood-brain-barrier (BBB) resulting in increased permeability has been reported in MCI and AD [52,53]. This suggests that complement may have the capacity to play a role in the cell loss seen in AD. Complement factors may work synergistically to cause loss in cell via-bility. We observed reduced cell viability when cells were exposed to the complement standard mixture, as compared to addition of single complement factors (Table 2). However in all cases we observed a downward trend in cell viability as complement concentration levels increased regardless of the number of complement proteins present. The data achieved statistical signifi-cance with exposure to as few as two complement factors (Table 2), indicating that the full spectrum of complement proteins are not necessary for cytotoxicity. Indeed it has been found that treatment of a transgenic mouse model with an agonist to a single complement receptor, C5aR, decreased pathology and improved behavioural performance [54].

There is significant evidence for the involvement of inflammation in the pathogenesis of Alzheimer's disease. In the AD brain, damaged neurons and highly insoluble Aβ peptide de-posits and NFTs provide stimuli for inflammation [3,14]. Various neuroinflammatory media-tors including complement activators and inhibitors, chemokines, cytokines, radical oxygen species and inflammatory enzymes have been shown to be altered in AD [3,14]. Another

Table 4. Dysregulated proteins in glial cells treated with human control, MCI and AD plasma compared to FBS (non human serum control) following iTRAQ analysis.

Protein Function	Accession #	Name	Replicate 1 Ctrl:FBS	PVal	Replicate 2 Ctrl:FBS	PVal	Replicate 1 MCI:FBS	PVal	Replicate 2 MCI:FBS	PVal	Replicate 1 AD:FBS	PVal	Replicate 2 AD:FBS	PVal
Glycolysis	gi|7669492	glyceraldehyde-3-phosphate dehydrogenase	0.97	0.524	1.02	0.603	0.98	0.676	0.98	0.655	1.27	3.4E-5*	1.32	1.0E-4*
	gi|4505763	phosphoglycerate kinase 1	1.07	0.175	1.05	0.322	1.05	0.279	1.06	0.207	1.2	7.0E-5*	1.31	1.1E-4*
	gi|4503571	alpha-enolase isoform 1	0.93	0.12	0.98	0.753	0.99	0.719	0.98	0.553	1.19	2.7E-4*	1.22	1.0E-4*
	gi|342187211	fructose-bisphosphate aldolase A	0.99	0.829	1	0.989	1.02	0.836	1	0.991	1.2	0.004*	1.22	0.001*
	gi|33286418	pyruvate kinase isozymes M1/M2	0.98	0.746	1.02	0.726	1.01	0.869	1.03	0.6	1.22	0.008*	1.22	0.002*
	gi|4507521	transketolase isoform 1	0.98	0.686	1.01	0.881	1.04	0.323	0.99	0.749	1.13	0.022*	1.16	0.002*
Chaperone	gi|20070125	protein disulfide-isomerase precursor	0.98	0.566	0.96	0.352	0.93	0.057	1.01	0.821	0.93	0.026*	0.98	0.478
	gi|153792590	heat shock protein HSP 90-alpha	1.03	0.679	1.05	0.601	1.06	0.512	1.06	0.473	1.3	0.036*	1.2	0.059
	gi|66933005	calnexin precursor	1	0.942	0.98	0.7	0.94	0.315	0.93	0.149	0.92	0.266	0.88	0.010*
	gi|5031973	protein disulfide-isomerase A6 precursor	0.97	0.429	0.98	0.662	0.94	0.134	1	0.952	0.96	0.546	0.91	0.019*
	gi|21361657	protein disulfide-isomerase A3 precursor	0.96	0.427	0.98	0.653	0.9	0.013*	1	0.871	0.92	0.117	0.89	0.06
	gi|16507237	78 kDa glucose-regulated protein precursor	0.99	0.806	0.97	0.729	0.92	0.038*	0.98	0.643	0.91	0.101	0.91	0.108
Cytoskeletal	gi|4505257	moesin	1.08	0.265	1.14	0.243	1.12	0.106	1.03	0.721	1.22	0.021*	1.2	0.028*
	gi|21614499	ezrin	1.06	0.498	0.95	0.718	1.05	0.555	1.13	0.446	1.31	0.033*	0.9	0.712
	gi|38176300	nestin	0.99	0.773	1	0.984	0.97	0.518	1.04	0.354	0.94	0.173	0.85	0.004*
	gi|44680105	caldesmon isoform 1	0.91	0.154	0.97	0.773	0.9	0.174	0.94	0.478	0.87	0.085	0.86	0.027*
	gi|19920317	cytoskeleton-associated protein 4	0.85	0.049*	0.77	0.012*	0.92	0.229	0.79	0.032*	0.74	1.0E-4*	0.77	0.019*
	gi|62414289	vimentin	0.97	0.23	1	0.965	0.9	2.4E-4*	0.99	0.778	0.97	0.261	0.96	0.277

(Continued)

Table 4. (Continued)

Protein Function	Accession #	Name	Replicate 1 Ctrl:FBS	PVal	Replicate 2 Ctrl:FBS	PVal	Replicate 1 MCI:FBS	PVal	Replicate 2 MCI:FBS	PVal	Replicate 1 AD:FBS	PVal	Replicate 2 AD:FBS	PVal
Proteolysis	gi|4506713	ubiquitin-40S ribosomal protein S27a	0.98	0.738	0.82	0.159	1.06	0.286	1.04	0.594	0.84	0.066	0.77	0.022*
	gi|66346681	plasminogen activator inhibitor 1	1.04	0.587	0.99	0.922	1	0.999	0.93	0.476	0.97	0.612	0.79	0.023*
	gi|54792069	small ubiquitin-related modifier 2	1.15	0.291	1.02	0.948	1.46	0.083	1.41	0.052	1.23	0.26	1.29	0.043*
	gi|109637759	calpastatin isoform f	0.86	0.228	0.75	0.138	0.81	0.24	0.88	0.46	0.82	0.206	0.74	0.045*
Translation	gi|4503471	elongation factor 1-alpha 1	0.97	0.493	1.02	0.76	0.93	0.225	0.97	0.563	1.13	0.024*	1.24	0.014*
	gi|17158044	40S ribosomal protein S6	0.95	0.62	0.54	0.063	0.83	0.091	0.7	0.172	0.74	0.041*	0.72	0.178
	gi|15431288	60S ribosomal protein L10a	1.14	0.206	1.31	0.037*	1.19	0.098	1.27	0.048*	1.41	0.079	1.06	0.732
	gi|214010226	40S ribosomal protein S24 isoform d	0.68	0.062	0.66	0.015*	0.75	0.011*	0.66	0.085	0.6	0.063	0.64	0.022*
	gi|124494254	proliferation-associated protein 2G4	0.97	0.897	0.99	0.974	1.36	0.040*	1.22	0.194	1.24	0.276	1.15	0.146
Transcription	gi|4885379	histone H1.4	0.86	0.158	0.78	0.053	0.79	0.063	0.96	0.61	0.58	0.006*	0.79	0.349
	gi|4885377	histone H1.3	1.04	0.538	1.21	0.386	0.94	0.438	1.18	0.369	0.75	0.012*	0.96	0.613
	gi|4885381	histone H1.5	0.83	0.011*	0.77	0.057	0.79	0.006*	0.9	0.164	0.65	3.4E-4*	0.85	0.205
	gi|4885375	histone H1.2	0.87	0.059	0.73	0.002*	0.74	0.002*	0.95	0.468	0.54	0.001*	0.76	0.102
Immune response	gi|4502101	annexin A1	1	0.999	0.99	0.848	0.93	0.161	1.02	0.794	0.87	0.023*	0.83	0.042*
	gi|50845388	annexin A2 isoform 1	1.04	0.339	1.1	0.021*	0.89	8.0E-4*	0.99	0.647	0.95	0.192	0.93	0.1
	gi|48255891	glucosidase 2 subunit beta	1.04	0.679	1.13	0.292	1.14	0.081	1.16	0.012*	1.01	0.914	1.05	0.584
Antioxidant	gi|32189392	peroxiredoxin-2 isoform a	1.34	0.096	1.2	0.268	1.28	0.15	1.16	0.454	1.19	0.026*	1.28	0.146
	gi|4505591	peroxiredoxin-1	1.01	0.871	0.95	0.379	1.01	0.871	0.96	0.479	1.07	0.223	1.17	0.021*
Cell Growth Regulation	gi|4503057	alpha-crystallin B chain	1.04	0.429	1.02	0.718	1.04	0.45	0.99	0.931	1.24	0.002*	1.26	0.043*
	gi|19743823	integrin beta-1 isoform 1A precursor	1.32	0.102	1.05	0.81	1.14	0.259	1.52	0.038*	1.35	0.069	1.08	0.829
Fatty Acid Metabolism	gi|4557585	fatty acid-binding protein, brain	0.85	0.22	0.84	0.176	0.8	0.228	0.87	0.353	0.9	0.247	0.83	0.049*
	gi|4758504	3-hydroxyacyl-CoA dehydrogenase	1.17	0.445	1.09	0.795	1.33	0.097	1.57	0.015*	1.18	0.49	1.14	0.381

(Continued)

Table 4. (Continued)

Protein Function	Accession #	Name	Replicate 1 Ctrl:FBS	PVal	Replicate 2 Ctrl:FBS	PVal	Replicate 1 MCI:FBS	PVal	Replicate 2 MCI:FBS	PVal	Replicate 1 AD:FBS	PVal	Replicate 2 AD:FBS	PVal
Energy Metabolism	gi|19923437	GTP:AMP phosphotransferase	1.4	0.083	1.27	0.371	1.46	0.047*	1.14	0.44	1.43	0.052	1.43	0.149

Cells were incubated with plasma in two 24 well plates, 3 wells for each of the plasma types were pooled from each plate to obtain two biological replicates for the 8-plex iTRAQ experiment. iTRAQ reporter ratios and p-values for altered proteins are shown for both replicates. Proteins found to be dysregulated in MCI and AD treated cells are shown in table. Proteins dysregulated only in cells treated with AD plasma are highlighted in bold. Full list of identified proteins can be found in S2 Table.
* p   0.05 vs Fetal Bovine Serum Control

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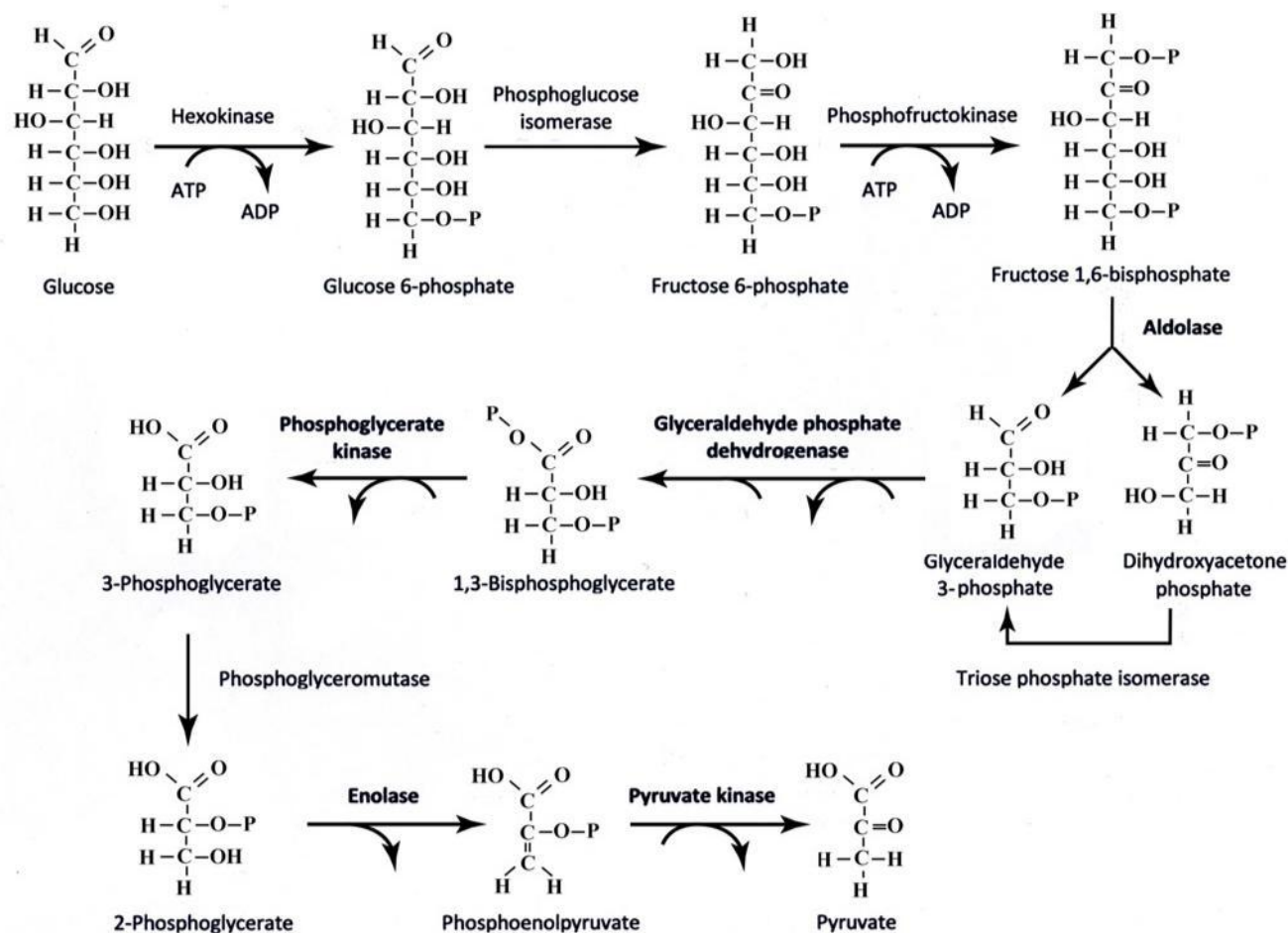
Biological Effects of Exposure to Alzheimer's Disease Plasma in Glia

Fig 4. Glycolysis Pathway highlighting enzymes which were shown to be upregulated in cells treated with AD plasma.

prominent feature of AD neuropathology is the association of activated proteins of the classical complement pathway with the lesions. The full-range of classical pathway complement pro-teins from C1q to C5b-9, known as the membrane attack complex, has been found highly local-ised with Aβ deposits in neuritic plaques [55,56]. It is also present in dystrophic neurites in AD. The fact that complement activation has progressed until the final membrane attack com-plex stage and the observation that complement regulators have also been found in association with the AD lesions indicates a disturbance in the regulatory mechanisms controlling comple-ment activation in this disease [57–60].

Aβ itself can induce complement-mediated toxicity against neurons in culture, suggesting that Aβ-induced complement activation may contribute to the neuropathogenesis of AD [56,61]. Hyperphosphorylated tau protein, the main component of NFTs, is also a potent stimulator of the complement cascade. Purified NFTs have been shown to activate the complement system in plasma, resulting in a significant increase in levels of membrane attack complexes [62]. Tau and Aβ are both able to increase inflammatory responses and cytokine production. Since the comple-ment system is strongly activated in AD, it could possibly participate either in the exacerbation or amelioration of the pathology. Because Aβ deposits and extracellular NFTs are present during early preclinical until terminal stages of AD, their ability to activate complement provides a mechanism for initiating and sustaining chronic, low-level inflammatory responses that may
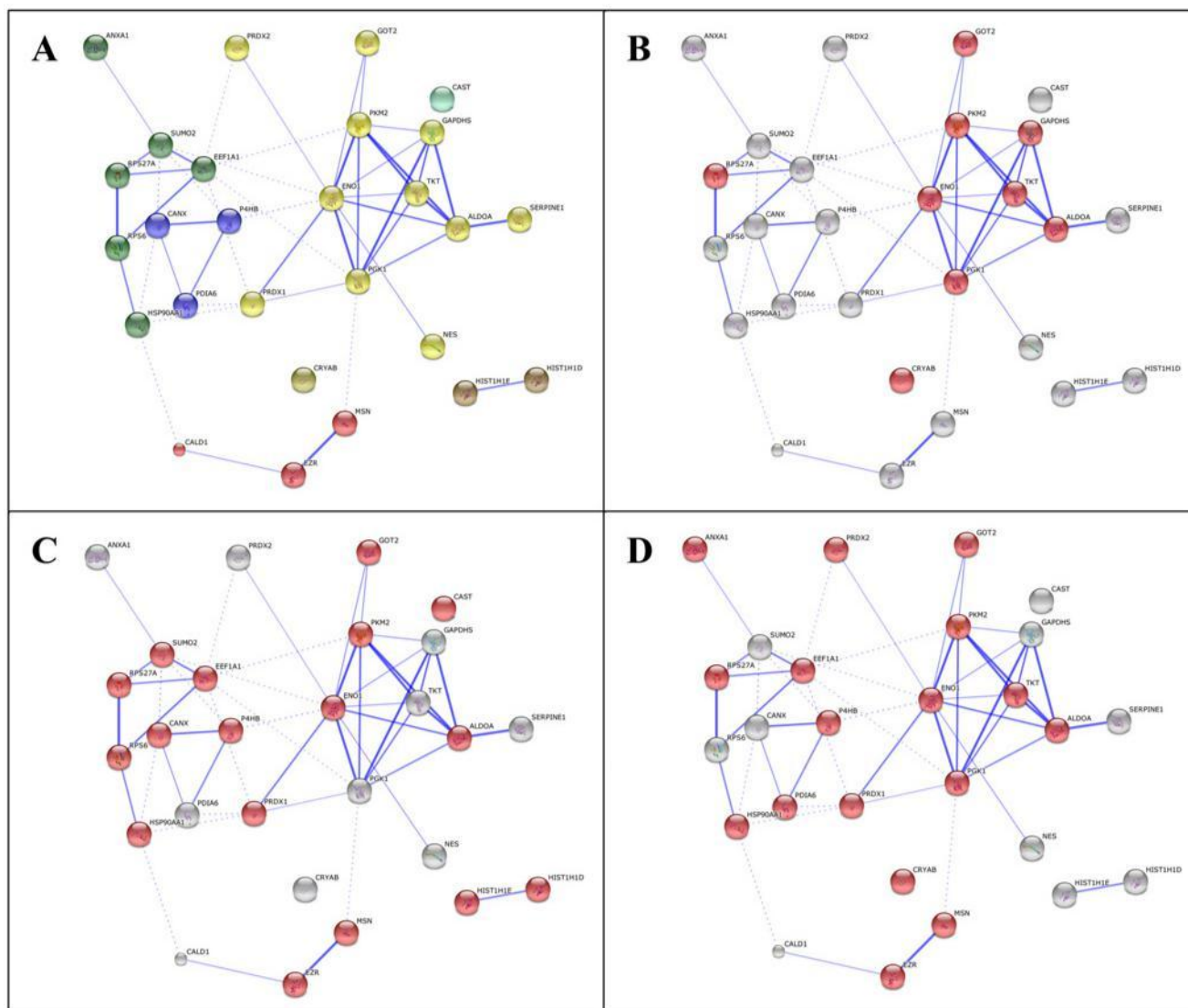
Fig 5. The 27 proteins which were significantly deregulated in glia treated with AD plasma, but not deregulated in either control or MCI plasma treated glia (shown in Table 4) were analysed in STRING v9.1. MCL clustering was used with the 2 clusters option picked and with the confidence view selected to display the strength of evidence for protein associations (panel A). Analysis of enrichment was also performed and the most significantly enriched biological process was glucose metabolic process (FDR p-value = 1.759x10E-8, with the 9 proteins involved in this process shown in panel B). Other distinct biological processes which were also significantly enriched included response to hydrogen peroxide (FDR p-value = 3.559x10E-2 with 4 proteins involved; ANXA1, PRDX1, PRDX2, CRYAB) and membrane to membrane docking (FDR p-value = 4.299x10E-2 with 2 proteins involved; MSN, EZR). Several molecular functions were also enriched, the most significant being RNA binding (FDR p-value = 5.340x10E-9 with 17 proteins involved shown in panel C). Another distinct and significantly enriched molecular function is thioredoxin peroxidase activity (FDR p-value = 9.220x10E-3 with 4 proteins involved; PRDX1, PRDX2). Several cellular components were also enriched, the most significant of these being extracellular vesicle exosome (FDR p-value = 5.019x10E-9 with 17 proteins involved as shown in panel D). Multiple other significantly enriched cellular components were also observed, and all enriched protein groups are shown in Table 5.

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accumulate over the disease course. This supports the idea that the complement system cascade intervention might be a useful pharmacological approach to treat early stages of AD.

Proteomic analysis of MCI and AD plasma in this study revealed a number of proteins which were significantly altered between the three groups. The majority of these proteins were acute phase reactants, including proteins which were related to the complement system (Table 3). This supports the results from other published studies using mainly proteomic techniques which have

Table 5. STRING v9.1 analysis of the 27 proteins deregulated only in glia exposed to AD plasma (shown in Table 4). for enrichment in gene ontology biological processes. Glucose metabolism was found to be the most significant biological process, and is also highlighted in the STRING network map (Fig. 5).

Molecular Function Enrichment GO_ID	Term	Number of Proteins	p-value	p-value FDR	p-value Bonferroni
GO:0003723	RNA Binding	17	1.94E-12	5.34E-09	7.51E-09
GO:0044822	Poly(A) RNA Binding	16	2.75E-12	5.34E-09	1.07E-08
GO:0008379	Thioredoxin peroxidase activity	2	8.88E-06	9.22E-03	3.45E-02
GO:0003676	Nucleic acid binding	16	9.50E-06	9.22E-03	3.69E-02
GO:0051920	Peroxiredoxin activity	2	2.95E-05	2.29E-02	1.15E-01
Biological Process Enrichment GO_ID	Term	Number of Proteins	p-value	p-value FDR	p-value Bonferroni
GO:0006006	Glucose metabolic process	9	1.42E-12	1.76E-08	1.76E-08
GO:0019318	Hexose metabolic process	9	7.28E-12	4.51E-08	9.02E-08
GO:0005996	Monosaccharide metabolic process	9	3.63E-11	1.50E-07	4.49E-07
GO:0046364	Monosaccharide biosynthetic process	6	2.23E-10	6.89E-07	2.75E-06
GO:0016051	Carbohydrate biosynthetic process	7	7.56E-10	1.87E-06	9.35E-06
GO:0006094	Gluconeogenesis	5	5.43E-09	1.12E-05	6.73E-05
GO:0019319	Hexose biosynthetic process	5	9.29E-09	1.64E-05	1.15E-04
GO:0042542	Response to hydrogen peroxide	4	2.30E-05	3.56E-02	2.85E-01
GO:0022614	Membrane to membrane docking	2	3.13E-05	4.30E-02	3.87E-01
GO:0016584	Nucleosome positioning	2	6.56E-05	8.12E-02	8.12E-01
Cellular Component Enrichment GO_ID	Term	Number of Proteins	p-value	p-value FDR	p-value Bonferroni
GO:0070062	Extracellular vesicular exosome	17	1.04E-11	5.02E-09	1.51E-08
GO:0044421	Extracellular region part	18	1.23E-09	4.44E-07	1.77E-06
GO:0005576	Extracellular region	18	1.08E-07	3.06E-05	1.57E-04
GO:0031988	Membrane-bounded vesicle	15	1.27E-07	3.06E-05	1.84E-04
GO:0031982	Vesicle	15	1.83E-07	3.77E-05	2.64E-04
GO:0043233	Organelle lumen	16	2.50E-07	4.32E-05	3.62E-04
GO:0042470	Melanosome	5	2.98E-07	4.32E-05	4.32E-04
GO:0005829	Cytosol	15	1.22E-06	1.47E-04	1.77E-03
GO:0070013	Intracellular organelle lumen	13	5.33E-05	5.93E-03	7.71E-02

GO:0031254	Cell trailing edge	2	7.00E-05	6.76E-03	1.01E-01
GO:0001931	Uropod	2	7.00E-05	6.76E-03	1.01E-01
GO:0030016	Myofibril	4	1.41E-04	1.28E-02	2.05E-01
GO:0016323	Basolateral plasma membrane	4	1.51E-04	1.29E-02	2.19E-01
GO:0043292	Contractile fiber	4	1.66E-04	1.33E-02	2.40E-01
GO:0060205	Cytoplasmic membrane-bound vesicle lumen	3	2.64E-04	1.91E-02	3.82E-01
GO:0031983	Vesicle lumen	3	2.64E-04	1.91E-02	3.82E-01
GO:0031528	Microvillus membrane	2	2.98E-04	2.05E-02	4.31E-01
GO:0016023	Cytoplasmic membrane-bounded vesicle	7	3.68E-04	2.42E-02	5.32E-01
GO:0005719	Nuclear euchromatin	2	5.70E-04	3.43E-02	8.24E-01
GO:0000791	Euchromatin	2	7.98E-04	4.62E-02	1.00E+00

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shown changes in complement protein levels and other acute phase proteins in AD plasma [5,47,63,64]. A summary of proteins found altered in such studies is provided in Table 6 and some of the proteins found in our study overlap with those found by other groups using larger cohorts of patients. One study has also shown a correlation between brain hippocampal volume changes and plasma levels of acute phase proteins, including complement [63].

Interestingly one of the complement proteins that was reduced in the AD plasma, comple-ment 4 binding protein (C4BP) is a complement inhibitor which is detected in Aβ plaques and on apoptotic cells in the AD brain [65]. In vitro, C4BP binds apoptotic and necrotic but not via-ble brain cells. It also binds to Aβ(1–42) peptide directly and limits the extent of complement activation by Aβ [65]. C4BP levels in CSF of dementia patients and controls were low com-pared to levels in plasma and correlated with CSF levels of other inflammation-related factors [65]. Therefore it possibly protects against excessive complement activation in AD brains.

Fibronectin is present in plaques of AD brains and may modify biosynthesis of APP in microglia [66]. Addition of Aβ to cultured astrocytes has been shown to induce a marked in-crease in the production of fibronectin [67]. This suggests that in vivo fibronectin accumulation in senile plaques may be the result, at least in part, of the response of reactive astrocytes to the presence of Aβ. Fibrinogen is associated with an increased risk of AD and vascular dementia [68]. Our study found fibronectin and fibrinogen to be significantly increased in the AD group compared to controls (Table 3).

Furthermore, we have also shown that treatment with AD plasma can affect cellular bioener-getics in a microglial cell line, by increasing glycolysis to compensate for declining oxygen con-sumption and mitochondrial respiration (Fig. 2). The reduction in cerebral glucose metabolism, as measured by FDG-PET, is a common diagnostic tool for AD. Positron emission tomography (PET) imaging has identified a strong correlation between the spatial distribution of increased glycolysis, and Aβplaques in the AD brain [69]. It is estimated that aerobic glycolysis accounts for up to 90% of glucose consumed [70]. By contrast, a recent neuroimaging study which

Table 6. Summary of previous studies showing changes in acute phase proteins in Alzheimer's disease.

Name	Site of effect	Function	Modification	Reference
Alpha-2-macroglobulin	plasma	Inhibitor of coagulation; inhibitor of fibrinolysis	Increased in MCI and AD	[5,47]
Complement C3	plasma	Most abundant protein of the complement system, enhances response	Increased in AD	[101–104]
Complement C4	plasma	Protein involved in the complement system and undergoes cleavage	Increased in AD	[5,105]
C4b-binding protein	plasma	Inhibits C4 and binds necrotic cells	Decreased in MCI and AD	[47]
Complement C5	plasma	Fifth component of the complement pathway	Increased in MCI	[47]
Complement C9	mRNA and protein levels, vascular amyloid deposits	Involved in MAC formation	Increased in AD brain areas, increased deposition in vascular plaques	[101,102,106]
Complement factor H	plasma	Regulation of alternative pathway of the complement system, ensuring no damage to host tissue	Increased in AD	[5,107]
Fibrinogen	plasma	Involved in blood clotting	Decreased in AD	[47,104,108,109]
Haptogloblin	Plasma, CSF	Binds free haemoglobin thereby reducing its oxidative activity	Increased in AD plasma, decreased in CSF of MCI and AD patients. Other studies show increase in CSF of AD	[110,111]
Hemopexin	plasma and CSF	Binds heme, preserves iron levels in the body	Increased in AD	[105,112,113]
Thrombin	Brain tissue, amyloid plaques, neurofibrillary tangles	Coagulation protein that converts fibrinogen into fibrin, also catalyses other coagulation related reactions	Increased in AD	[114–116]
Transthyretin	Plasma, CSF	Carrier of the thyroid hormone thyroxine	Decreased in AD	[47,112,117,118]

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correlated multimodal neuronal parameters including glucose metabolism and hippocampal volume with Aβ deposition in cognitively normal older individuals, did not find any association between the multimodal neurodegenerative biomarkers [71]. However, diminished neuronal integrity and cognitive function correlated with an increased Aβ burden in brain regions that are most affected by AD pathology [71]. Increased glycolysis was associated with better verbal epi-sodic memory in individuals with elevated amyloid levels in another study [72]. The increased shift towards glycolysis may occur in regions of the brain most vulnerable to insult, or may occur in response to Aβ accumulation during ageing. Loss of this protective mechanism may increase the vulnerability of certain brain regions to Aβ-induced neurotoxicity.

Quantitative iTRAQ analysis of glial cells treated with human AD plasma showed the high-est number of dysregulated proteins (Table 4). The most significantly enriched biological pro-cess was glucose metabolism (Fig. 5 and Table 5), and a significant number of upregulated glycolytic proteins were found (highlighted in bold in Fig. 4), which is in agreement with the ECAR effect we found in cellular biogenetics using mitochondrial function assays (Fig. 2). In-creased expression of glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, enolase, aldolase and pyruvate kinase may increase glycolytic flux leading to the accumulation of pyruvate and thus stimulating anaerobic metabolism to lactic acid. We found an increased level of LDH activity in the cell culture media and an increase in extracellular acidification, as indicated by the increase in ECAR in the microglial cells exposed to human AD plasma (Fig. 2). These findings suggest a role for mitochondrial bioenergetic deficits in AD pathogene-sis. Our study is consistent with previous PET metabolic analyses in individuals with AD, MCI, or incipient to late AD [73]. Our findings are also consistent with microarray analyses and ac-tivity assays of ageing, incipient AD, and AD human samples and rodent models which indi-cate that genes and the catalytic activity of several glycolytic enzymes are altered in AD or MCI patients [74,75]. Similarly, increased amyloid production and nerve cell atrophy have been shown to induce mitochondrial dysfunction [76]. Overexpression of pyruvate dehydrogenase kinase and lactate dehydrogenase in neurons has been shown to provide resistance to Aβ toxic-ity and reduces mitochondrial respiration and oxidative stress [77]. Previous proteomic studies have also revealed that enzymes involved in energy metabolism show altered oxidative modifi-cation in the AD brain [78]. A recent study has also shown a number of proteins significantly oxidised in the Down syndrome brain with and without AD pathology [79]. A significant num-ber of proteins involved in energy metabolism were identified including some of the glycolysis enzymes which we found altered in our study.

We also report an increase in the protein expression of the enzyme transketolase in microglial cells in response to human AD plasma. Transketolase is a thiamine-dependent enzyme which ca-talyses the first reaction in the pentose phosphate pathway. Transketolase alterations have been previously identified in (i) several probable AD patients regardless of age-of-onset and severity of disease; (ii) all early-onset AD patients and APOE ε4/4 carriers; and (iii) nearly half of asymp-tomatic AD relatives [80]. Increased transketolase activity has also been correlated with increased levels of BACE1, the key rate-limiting enzyme for the production of the Aβ peptide [80].

Increased oxidative stress, mitochondrial dysfunction and alterations to energy metabolism have all been implicated as early events in the pathogenesis of AD. Cellular models for AD have been shown to display functional impairment of the mitochondrial respiratory chain, and a de-crease in oxygen respiration and ATP production [81]. All functional measures highlight biogenetic impairment of the AD cells with a correlation to the accumulation of amyloid peptides [81].

Factors which induce the upregulation of glycolysis in glial cells treated with AD plasma re-main unclear. Apart from the acute phase proteins found altered in our study, small molecules in plasma may also play a role in the upregulation of glycolytic enzymes. A recent study of metabolomic profiling of plasma from an AD mouse model found significant changes to sever-al metabolites involved in energy metabolism, linking neuroinflammation with metabolic dis-turbance in AD [82]. Since oxidative stress is thought be an important factor in the pathogenesis of AD, the effects of this may also be detected in the circulation in levels of mark-ers such as isoprostanes. Isoprostanes have been shown to be elevated in the AD brain and CSF [83], however recent evidence suggests this may not be reflected in plasma [84], consequently additional work is warranted. Aerobic glycolysis has also been correlated spatially with amyloid deposition in AD brains [69]. It has also been shown that elevated levels of the enzymes pyru-vate dehydrogenase kinase and lactate dehydrogenase provide resistance to amyloid and other neurotoxins [77]. The ability of the brain to maintain expression of these enzymes involved in

mitochondrial energy metabolism may explain why some individuals could show high levels of amyloid deposition without neurodegeneration [77,85–87]. In our study, the indicators of in-creased glycolysis in microglia may be a compensatory action caused by the loss of cell viability and mitochondrial function following exposure to AD plasma. Altered activities of key glyco-lytic enzymes have also been found in hippocampal, frontal and temporal cortex of AD brains and thought to possibly be related to the astrocytosis that occurs in AD [74].

Chaperone proteins are thought to be involved in the pathogenesis of several neurodegenera-tive and amyloidogenic diseases [88,89]. A number of chaperone proteins such as protein disulfide isomerases were found to be downregulated in cells treated with MCI and AD plasma. Protein di-sulfide isomerases can inhibit the aggregation of misfolded proteins and are also involved in mod-ulating apoptosis and endoplasmic reticulum redox balance [90]. It has been shown that the proinflammatory activation of microglia suppresses mitochondrial function and increases glycoly-sis and overexpression of mitochondrial chaperone mortalin can attenuate this effect [91]. Heat shock proteins are chaperone proteins which have a important impact on the proteotoxic effects of tau and Aβ accumulation. Immunohistochemical studies and expression analyses in AD brain tissue have shown that expression levels of a number of heat shock proteins are upregulated and it has been hypothesised that this effect may be due to a hybridisation of activated glia and dysregu-lated/stressed neurons [92,93]. Dysregulated chaperone proteins in the cells in our study may re-flect a homeostatic attempt to clear toxic plasma proteins and protect mitochondrial function.

Cytoskeletal proteins are another group of altered proteins identified in our iTRAQ proteo-mics data (Table 4). Nestin expression is seen during pathological situations and is a marker of cell proliferation and is reduced in the cells treated with AD plasma, supporting our MTT data (Table 1 and Fig. 1). Ezrin and moesin are involved in crosslinking actin filaments with plasma membranes and stabilising microtubules respectively [94,95] and both were found to be upre-gulated in cells treated with AD plasma. The antioxidant proteins peroxiredoxins were also found to be elevated in cells exposed to AD plasma which may also be another indication of a compensatory mechanism to attempt to attenuate the toxic effects of AD plasma.

In conclusion, this study shows that plasma expression levels of acute phase proteins are al-tered in AD and MCI, supporting a role for increased inflammatory activity in this disease which is detectable in the plasma. Cells exposed to AD plasma show an upregulation of glycoly-sis possibly as a compensatory mechanism in response to compromised mitochondrial function. Together our observations lend support to an emerging body of evidence that inflammation and metabolism are closely linked processes, which are regulated by transcriptional and protein translation events [96,97]. In the CNS, complement proteins are synthesised by a variety of cells including neurons, microglia, astrocytes, oliogendrocytes and endothelial cells [98]. Since dis-ruptions in the blood-brain-barrier have been reported in AD there is a possible source of in-creased complement levels in the AD brain from plasma. It is however likely that there may be other thermolabile factors in disease plasma which facilitate the cytotoxic and glycolytic effects in microglia, one example may be micro RNAs as they are emerging as important factors in neu-rogenesis, synaptic plasticity and AD [99,100]. Other yet to be characterised substances may also make a significant contribution. This study shows that the use of biological assays in combina-tion with proteomic analysis may help uncover possible mechanisms of disease and may be com-plementary techniques to validate cellular changes and effects in a range of biological samples.

Supporting Information

S1 Fig. Scaffold peptide false discovery analysis. Peptide FDR analysis using Scaffold v4 with peptide ROC curve.
(PDF)

S1 Methods. Detailed mass spectrometry protocols. Detailed mass spectrometry protocols for proteomics of depleted control, MCI and AD plasma and iTRAQ proteomic analysis of microglial cell lysates treated with control, MCI and AD plasma.
(PDF)

S1 Table. Protein summary of human control, MCI and AD plasma. Name and accession of proteins identified in human control, MCI and AD plasma and normalised spectral counts for replicates from Scaffold v4 software.
(PDF)

S2 Table. Proteomic of glial cells treated with human plasma. Protein summary of identified proteins with unused score 1.3 in glial cells treated with control, MCI and AD plasma, showing unused score, percentage coverage, accession number, name, number of peptides, iTRAQ ratios and p-values for both biological replicates. Significantly upregulated proteins are highlighted in red and downregulated proteins in blue.
(PDF)

S3 Table. False discovery rate analysis summary for iTRAQ. FDR analysis summary at the protein, peptide and spectral levels for iTRAQ experiment from Protein Pilot v4.
(PDF)

Acknowledgments

Author Contributions

Conceived and designed the experiments: TJ AP NB GS MH PS. Performed the experiments: TJ NB. Analyzed the data: TJ AP NB. Contributed reagents/materials/analysis tools: AP MR HB JT NK PS. Wrote the paper: TJ AP NB GS PS. Design of study and diagnoses: HB JT NK PS. Editing/Feedback on Manuscript: AP NB GS MR MH HB JT NK PS.

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Scaffold False Discovery Rate Analysis

Scaffold Version: Scaffold_4.3.4

Protein Grouping Strategy: Experiment-wide grouping with binary peptide-protein weights
Peptide Thresholds: 95.0% minimum

Protein Thresholds: 99.0% minimum and 2 peptides minimum
107 Proteins identified at 99.0% minimum

Peptide FDR: 5.2% (Prophet)

Figure S1 - Scaffold Peptide ROC Plot
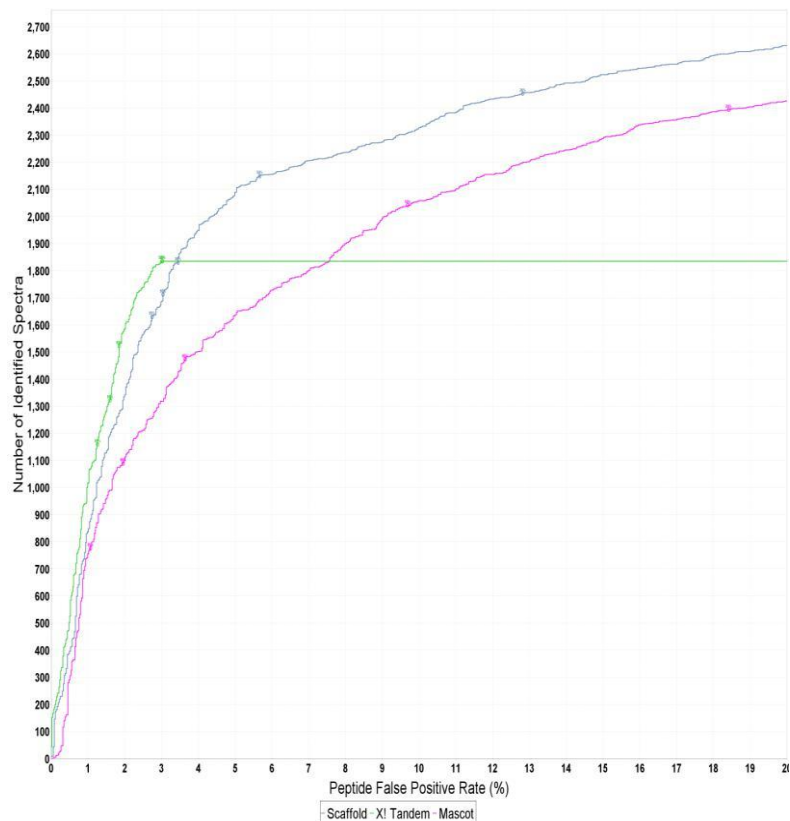
Supplementary Methods S1

Detailed Mass Spectrometry Protocol for Proteomic Analysis of Human Control, MCI and AD Plasma

Digested peptides were separated by nano-LC using a Cap-LC autosampler system (Waters, Milford MA). Samples (5 µl) were concentrated and desalted onto a micro C18 precolumn (500 µm x 2 mm, Michrom Bioresources, Auburn, CA) with $H_2O:CH_3CN$ (98:2, 0.05 % HFBA) at 15 ul/min. After a 4 min wash the pre-column was automatically switched (Valco 10 port valve, Houston, TX) into line with a fritless nano column (75 µm x ~12 cm) containing Magic C18 (~10cm, 200Å, Michrom) manufactured according to Gatlin [35]. Peptides were eluted using a linear gradient of $H_2O:CH_3CN$ (98:2, 0.1 % formic acid) to $H_2O:CH_3CN$ (55:45, 0.1 % formic acid) at ~300 nl/min over 30 min. The precolumn was connected via a fused silica capillary (10 cm, 25 µ) to a low volume tee (Upchurch Scientific) where HV (2400 V) was applied and the column tip positioned ~ 1 cm from the Z-spray inlet of a QTof Ultima API hybrid tandem mass spectrometer (Micromass, Manchester, UK). Positive ions were generated by electrospray and the QTof operated in data dependent acquisition mode (DDA). A Tof MS survey scan was acquired (m/z 350-1700, 1 s) and the 2 largest multiply charged ions (counts > 20) were sequentially selected by Q1 for MS-MS analysis. Argon was used as collision gas and an optimum collision energy chosen (based on charge state and mass). Tandem mass spectra were accumulated for up to 2 s (m/z 50-2000).

Detailed Mass Spectrometry Protocol for iTRAQ Proteomic Analysis of Cell Lysates Treated with Human Control, MCI and AD Plasma

Chromatography was performed using an LC Packings capillary HPLC system (Dionex, Amsterdam, Netherlands), comprised of an Ultimate pump system, Switchos valve unit and Famos autosampler. Samples (iTRAQ labelled peptide mixtures) were captured onto a C18 pre-column cartridge (500 µm x 2 mm, Michrom Bioresources, Auburn, CA). After a 10 min wash to remove any residual buffers or salts, the pre-column was automatically switched into line with a capillary column (75 µm x ~12 cm) containing C18 reverse phase packing material (ReproSil-Pur 120 C18-AQ, 1.9 µM, Dr Maisch, Ammerbuch-Entringen, Germany). Peptides were eluted using a 240 min linear gradient of $H_2O:CH_3CN$ (98:2, 0.1 % formic acid) to $H_2O:CH_3CN$ (20:80, 0.1 %

formic acid) at ~300 nl/min. The precolumn was connected via a fused silica capillary (10 cm, 25 µ) to a low

volume tee (Upchurch Scientific, WA, USA) where high voltage (2300 V) was applied and the column tip

positioned ~ 1 cm from the spray inlet of a TripleTOF 5600 hybrid tandem mass spectrometer (ABSciex, CA,

USA). Positive ions were generated by electrospray and the instrument operated in information dependent

acquisition (IDA) mode. A TOF MS survey scan was acquired (m/z 350-1700, 0.75 s) and the three largest

multiply charged ions (counts > 20, charge state ≥ 2 and ≤ 4) were sequentially selected by Q1 for MS-MS

analysis. Nitrogen was used as collision gas and an optimum collision energy automatically chosen (based on

charge state and mass). Tandem mass spectra were accumulated for up to 2.5 s (m/z 65-2000).

Table S1 - Normalised Total Spectra Counts for Control, MCI and AD plasma using Scaffold v4.3.4 (significantly altered proteins in bold)

#	Identified Proteins (107)	Accession Number	AD plasma replicates				MCI Plasma replicates				Control plasma replicates			
			AD1	AD2	AD3	AD4	MCI1	MCI2	MCI3	MCI4	C1	C2	C3	C4
1	Alpha-2-macroglobulin precurso	gi|112911 (+2)	100.27	99.586	119.25	107.24	131.22	99.621	116.67	104.21	149.75	162.1	122.29	98.57
2	apolipoprotein B precursor [Ho	gi|105990532 (+1)	93.535	112.74	89.439	89.941	131.95	124.53	90.063	73.558	74.876	93.006	57.683	95.39
3	Chain A, Human Serum Albumin	gi|122920512 (+4)	86.052	115.24	83.051	65.726	137.05	132.62	75.735	67.428	81.562	90.349	73.834	71.543
4	complement component 3 prec	gi|115298678	94.283	84.554	112.86	103.78	110.08	104.6	112.58	112.38	116.33	102.31	124.6	111.29
5	keratin 1 [Homo sapiens], gi|39	gi|11935049	53.876	57.622	83.051	53.619	35.721	39.226	96.204	149.16	52.815	47.832	101.52	71.543
6	fibrinogen, alpha polypeptide is	gi|11761629 (+2)	39.659	30.69	40.461	32.863	32.076	29.886	47.078	34.736	31.421	30.559	41.532	41.336
7	proapolipoprotein	gi|178775	41.155	35.701	27.684	34.593	46.657	39.848	28.656	28.606	63.511	53.146	23.073	31.797
8	hCG2001591 [Homo sapiens]	gi|119623974 (+2)	41.155	35.074	29.813	39.782	39.367	43.584	30.703	32.692	36.77	39.195	29.995	42.926
9	Chain A, Trypsin In Complex Wit	gi|110590762 (+3)	23.945	17.537	10.648	17.296	29.889	29.264	12.281	6.1298	40.112	35.209	16.151	22.258
10	**fibrinogen, beta chain prepropr**	**gi|70906435**	**36.666**	**30.69**	**63.885**	**41.511**	**34.263**	**31.754**	**34.797**	**42.909**	**32.758**	**27.902**	**50.761**	**47.695**
11	keratin 10 (epidermolytic hyper	gi|119581085 (+2)	26.938	40.711	53.238	13.837	18.225	15.566	47.078	63.342	28.079	20.594	64.605	28.617
12	similar to Keratin, type I cytoske	gi|114667176	19.455	21.295	27.684	20.756	13.851	17.434	32.75	67.428	20.725	21.923	34.61	12.719
13	fibrinogen gamma chain, isofor	gi|119625320 (+4)	29.183	23.174	12.777	13.837	28.431	25.528	20.469	12.26	28.079	30.559	18.459	25.437
14	keratin 2 [Homo sapiens], gi|64	gi|47132620	29.183	27.558	63.885	19.026	19.683	21.169	55.266	55.169	31.421	24.58	46.146	27.027
15	ceruloplasmin (ferroxidase), iso	gi|119599289 (+3)	23.945	21.921	17.036	13.837	23.328	20.547	18.422	12.26	16.713	17.937	13.844	17.488
16	**Fibronectin 1 [Homo sapiens], g**	**gi|109658664 (+11)**	**32.924**	**38.206**	**29.813**	**27.674**	**19.683**	**18.056**	**14.328**	**8.1731**	**18.719**	**19.266**	**18.459**	**19.078**
17	Complement factor H precursor	gi|158517847 (+3)	20.204	13.779	23.425	22.485	19.683	7.4716	22.516	20.433	14.039	18.601	23.073	19.078
18	inter-alpha (globulin) inhibitor H	gi|119606782 (+3)	19.455	22.548	8.518	15.567	9.4771	10.585	12.281	8.1731	11.365	15.944	11.537	12.719
19	hemopexin [Homo sapiens], gi|	gi|11321561 (+6)	11.224	12.527	10.648	5.1889	15.309	14.943	14.328	8.1731	16.045	16.608	11.537	9.539
20	inter-alpha (globulin) inhibitor H	gi|119585668 (+3)	20.952	10.648	17.036	13.837	14.58	11.83	14.328	10.216	13.371	12.622	9.2293	20.668
21	Chain A, Crystal Structure Of Th	gi|28373620 (+1)	14.217	11.9	8.518	10.378	11.664	13.075	8.1875	12.26	15.376	13.951	9.2293	15.898
22	inter-alpha (globulin) inhibitor H	gi|119585664 (+1)	13.469	20.042	12.777	10.378	10.206	12.453	16.375	16.346	7.3539	11.294	16.151	11.129
23	Chain A, Tertiary Structures Of T	gi|2098257 (+3)	15.714	12.527	17.036	15.567	17.496	13.698	10.234	10.216	16.713	15.28	18.459	17.488
24	Complement factor B [Homo sa	gi|13278732 (+2)	11.224	8.7686	4.259	5.1889	6.5611	9.9621	2.0469	4.0866	10.028	11.294	4.6146	6.3594
25	Chain I, 2.5a Crystal Structure O	gi|52695711 (+1)	12.721	11.274	8.518	12.107	13.122	11.207	6.1407	6.1298	10.028	7.972	6.922	17.488
26	apolipoprotein A-IV precursor [	gi|178757 (+2)	11.224	9.3949	2.1295	1.7296	10.206	8.7168	4.0938	4.0866	9.3595	8.6363	2.3073	4.7695
27	gelsolin isoform 32	gi|114626427 (+2)	11.972	7.5159	4.259	10.378	10.206	9.9621	8.1875	10.216	8.691	9.9649	4.6146	6.3594

#	Protein	1	2	3	4	5	6	7	8	9	10	11	12
28	Chain A, Crystal Structure Of Cle gi|443345	6.7345	5.6369	12.777	19.026	5.1031	6.8489	14.328	10.216	4.0112	6.6433	16.151	12.719
29	**complement component 1 inhi gi|114642584 (+8)**	**9.7276**	**7.5159**	**10.648**	**17.296**	**8.0191**	**7.4716**	**6.1407**	**8.1731**	**8.0225**	**9.9649**	**9.2293**	**12.719**
30	Complement component 5 [Ho gi|109731812 (+2)	5.9862	6.2633	4.259	8.6482	5.1031	5.6037	4.0938	6.1298	8.0225	8.6363	2.3073	11.129
31	**Alpha-1B-glycoprotein precurso gi|46577680 (+1)**	**6.7345**	**6.2633**	**10.648**	**6.9185**	**8.0191**	**6.8489**	**10.234**	**8.1731**	**6.6854**	**6.6433**	**4.6146**	**6.3594**
32	plasminogen gi|387026 (+1)	6.7345	8.7686	2.1295	0	4.3741	8.7168	4.0938	0	5.3483	8.6363	6.922	1.5898
33	Kininogen 1 [Homo sapiens] gi|37748641 (+2)	8.9794	6.2633	6.3885	12.107	9.4771	4.981	2.0469	6.1298	4.6798	5.979	11.537	6.3594
34	apolipoprotein E mutant E3K gi|364011 (+1)	6.7345	5.6369	10.648	5.1889	4.3741	5.6037	8.1875	6.1298	4.0112	5.3146	9.2293	9.539
35	alpha-1-microglobulin/bikunin p gi|4502067 (+1)	6.7345	6.8896	4.259	5.1889	5.8321	6.2263	4.0938	2.0433	5.3483	4.6503	6.922	3.1797
36	**coagulation factor II (thrombin) gi|119588383 (+4)**	**4.4897**	**5.6369**	**6.3885**	**8.6482**	**5.8321**	**6.2263**	**4.0938**	**6.1298**	**8.691**	**9.3006**	**13.844**	**9.539**
37	Angiotensinogen (serpin peptid gi|15079348 (+10)	9.7276	8.1422	10.648	8.6482	8.0191	7.4716	10.234	8.1731	8.0225	8.6363	4.6146	7.9492
38	alpha-1-acid glycoprotein 1 prec gi|1197209 (+2)	6.7345	6.2633	4.259	3.4593	7.2901	5.6037	4.0938	4.0866	2.0056	5.979	2.3073	6.3594
39	Alpha-2-HS-glycoprotein precur gi|112910 (+2)	6.7345	3.758	6.3885	6.9185	8.0191	4.3584	8.1875	2.0433	6.6854	5.3146	11.537	11.129
40	haptoglobin, isoform CRA_a [Ho gi|119579598	9.7276	2.5053	2.1295	3.4593	6.5611	7.4716	4.0938	2.0433	3.3427	1.993	2.3073	1.5898
41	apolipoprotein H precursor [Ho gi|153266841 (+2)	5.238	4.3843	0	1.7296	2.916	4.3584	2.0469	0	2.6742	5.3146	0	4.7695
42	apolipoprotein J precursor gi|178855 (+6)	4.4897	1.879	2.1295	1.7296	3.645	2.4905	4.0938	4.0866	2.6742	1.993	0	7.9492
43	Chain A, The Structure Of Penta gi|576259	4.4897	5.0106	4.259	3.4593	3.645	3.1132	6.1407	4.0866	4.0112	5.979	2.3073	3.1797
44	apolipoprotein D, apoD [human gi|619383	2.9931	2.5053	4.259	3.4593	2.916	1.8679	4.0938	2.0433	2.6742	2.6573	4.6146	0
45	retinol binding protein 4, plasm gi|119570453 (+6)	2.9931	2.5053	2.1295	1.7296	1.458	3.7358	0	0	2.6742	2.6573	2.3073	1.5898
46	paraoxonase 1 [Homo sapiens], gi|19923106	4.4897	2.5053	4.259	1.7296	5.1031	2.4905	4.0938	4.0866	4.0112	3.3216	4.6146	1.5898
47	Vitronectin [Homo sapiens], gi| gi|13477169 (+2)	2.9931	3.1316	6.3885	3.4593	3.645	4.3584	4.0938	6.1298	3.3427	4.6503	6.922	3.1797
48	**complement component 4 bind gi|4502503**	**3.7414**	**3.1316**	**4.259**	**6.9185**	**3.645**	**3.7358**	**4.0938**	**2.0433**	**4.6798**	**2.6573**	**4.6146**	**4.7695**
49	histidine-rich glycoprotein precu gi|4504489	4.4897	6.8896	0	1.7296	1.458	1.8679	4.0938	4.0866	2.0056	1.993	6.922	3.1797
50	Keratin, type I microfibrillar 48 k gi|125090	0	0	0	17.296	0	6.2263	0	0	0.6685	3.986	0	0
51	similar to afamin gi|114594313 (+1)	2.9931	3.758	2.1295	0	1.458	3.1132	2.0469	4.0866	1.3371	1.993	4.6146	3.1797
52	complement component 6, isof gi|119576417 (+4)	4.4897	3.1316	0	6.9185	2.916	1.8679	4.0938	0	2.6742	2.6573	4.6146	1.5898
53	unnamed protein product [Hom gi|158261613 (+1)	4.4897	3.1316	2.1295	1.7296	3.645	3.7358	2.0469	2.0433	3.3427	2.6573	0	7.9492
54	type II intermediate-filament pr gi|246276	0	0	0	19.026	0	4.981	0	0	0	0	0	0
55	complement component 7, isof gi|119576411 (+4)	3.7414	4.3843	2.1295	3.4593	1.458	3.7358	4.0938	2.0433	2.6742	3.3216	0	1.5898
56	heparin cofactor II isoform 1 [Pa gi|114685230 (+5)	1.4966	1.2527	2.1295	0	1.458	0.6226	0	2.0433	0.6685	1.3287	2.3073	4.7695
57	complement component 9, isof gi|119576390 (+3)	5.238	3.758	2.1295	3.4593	4.3741	3.7358	4.0938	2.0433	2.6742	2.6573	4.6146	6.3594
58	complement component 1, q su gi|150036344 (+5)	2.9931	1.879	2.1295	1.7296	2.916	1.8679	2.0469	2.0433	2.0056	2.6573	0	3.1797
59	serine (or cysteine) proteinase i gi|114665618 (+6)	2.2448	1.879	2.1295	3.4593	1.458	1.8679	4.0938	2.0433	2.0056	0.6643	6.922	7.9492
60	unnamed protein product [Hom gi|16553735 (+2)	2.2448	1.2527	4.259	3.4593	1.458	1.8679	4.0938	2.0433	1.3371	2.6573	4.6146	4.7695

#	Protein	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
61	inter-alpha (globulin) inhibitor H gi|119585666 (+5)	1.4966	1.2527	2.1295	1.7296	2.187	1.8679	2.0469	0	1.3371	1.3287	2.3073	3.1797
62	Complement component 8, gam gi|109731764 (+4)	2.2448	1.2527	2.1295	1.7296	1.458	1.2453	4.0938	2.0433	2.6742	1.3287	0	3.1797
63	unnamed protein product [Hom gi|1335054	2.2448	0.6263	0	0	1.458	1.2453	8.1875	6.1298	2.0056	1.993	2.3073	3.1797
64	serum amyloid A4, constitutive gi|10835095 (+3)	2.9931	1.2527	2.1295	0	0	1.8679	0	2.0433	0.6685	0.6643	0	1.5898
65	keratin 5 [Homo sapiens], gi|14 gi|119395754 (+1)	0	10.021	4.259	0	0	0	6.1407	6.1298	5.3483	0	2.3073	6.3594
66	hCG2040284 [Homo sapiens] gi|119598461 (+2)	2.2448	2.5053	0	0	1.458	2.4905	0	0	2.0056	1.3287	0	0
67	alpha-1-acid glycoprotein 2 prec gi|29170378 (+2)	1.4966	1.879	4.259	1.7296	3.645	3.7358	2.0469	0	2.0056	3.986	0	1.5898
68	Complement C1r subcomponen gi|115204 (+6)	2.2448	2.5053	0	1.7296	0.729	1.8679	0	2.0433	1.3371	2.6573	0	1.5898
69	Chain A, Human Apolipoprotein gi|157831482	0	0	0	5.1889	0	0	2.0469	0	0	0	2.3073	0
70	alpha S1 casein gi|159793187 (+6)	0	0	4.259	0	0	0	6.1407	8.1731	0	0	6.922	0
71	similar to Ficolin (collagen/fibrin gi|114555009 (+4)	1.4966	1.879	2.1295	1.7296	1.458	1.2453	2.0469	0	1.3371	1.3287	2.3073	1.5898
72	C-type lectin domain family 3, m gi|156627579 (+2)	0.7483	1.879	0	1.7296	0.729	1.2453	2.0469	0	2.0056	1.3287	2.3073	3.1797
73	protein S (alpha) gi|114588068 (+5)	0	0.6263	0	0	0	1.2453	4.0938	0	0.6685	1.3287	0	1.5898
74	Kallikrein B, plasma (Fletcher fa gi|109659056 (+4)	0	3.1316	0	0	0	1.2453	0	0	2.0056	3.3216	0	0
75	hair type II keratin intermediate gi|1308	0	0	0	20.756	0	8.0942	0	0	0	2.6573	0	0
76	alpha-2-glycoprotein 1, zinc gi|114614917 (+6)	2.2448	1.2527	0	0	1.458	0.6226	0	2.0433	0.6685	0.6643	0	1.5898
77	hypothetical protein LOC50742 gi|134085840	0	0	0	17.296	0	7.4716	0	0	0	0.6643	0	0
78	alpha-2-plasmin inhibitor [Hom gi|115583663 (+5)	0	1.2527	0	0	1.458	0.6226	0	0	1.3371	0.6643	2.3073	0
79	apolipoprotein M [Homo sapien gi|22091452 (+1)	0.7483	0.6263	0	0	0	1.8679	0	0	0.6685	0	0	0
80	Rubber elongation factor protei gi|132270	0.7483	0	0	1.7296	0	0	4.0938	2.0433	0	1.3287	2.3073	1.5898
81	complement factor B isoform 1 gi|109070536	7.4828	6.8896	2.1295	3.4593	0	6.8489	0	0	6.6854	6.6433	0	0
82	coagulation factor XII (Hageman gi|119605410 (+6)	1.4966	0.6263	0	1.7296	0	0	0	0	0	0	0	1.5898
83	hypothetical protein LOC53959 gi|149642659	0	0	0	17.296	0	7.4716	0	0	0	0	0	0
84	Chain A, Thyroxine-Binding Glob gi|114793604 (+5)	0.7483	1.2527	0	0	0.729	1.2453	0	0	0.6685	0.6643	0	0
85	**complement component 2 prec gi|14550407 (+4)**	**2.9931**	**2.5053**	**2.1295**	**3.4593**	**0**	**1.2453**	**0**	**2.0433**	**0**	**0**	**0**	**0**
86	keratin 6A [Homo sapiens] gi|114644568 (+5)	6.7345	11.274	0	0	0	0	0	6.1298	8.0225	0	0	4.7695
87	pregnancy-zone protein isoform gi|114643429 (+4)	0	0	0	10.378	14.58	12.453	14.328	12.26	18.051	17.273	0	9.539
88	hypothetical protein isoform 2 gi|114654573 (+4)	1.4966	1.2527	0	0	2.187	1.8679	0	0	0.6685	0	0	1.5898
89	Chain A, Structural Changes Of T gi|1065033 (+143)	0.7483	0.6263	2.1295	0	0.729	1.2453	0	0	0.6685	0.6643	2.3073	0
90	hemoglobin alpha-1 globin chai gi|13650074 (+22)	0.7483	0.6263	0	1.7296	0	1.2453	2.0469	0	0	0	0	3.1797
91	lumican isoform 1 gi|114646184 (+4)	0.7483	1.879	0	0	0	0	0	0	0	0	2.3073	0
92	unnamed protein product [Hom gi|158257796 (+3)	0	2.5053	0	0	0	0	0	0	1.3371	2.6573	0	0
93	complement component 8, beta gi|119627047 (+4)	1.4966	1.2527	0	1.7296	0.729	0	2.0469	0	0	0	0	1.5898

#	Protein	Accession												
94	similar to Fibrinogen beta chain	gi|119908847	2.9931	3.1316	0	0	0	3.1132	0	0	3.3427	1.993	6.922	0
95	similar to apolipoprotein F	gi|114644115 (+3)	0.7483	0	0	0	0	1.2453	2.0469	0	0	0.6643	0	1.5898
96	lipoprotein CIII	gi|224917	0	0.6263	4.259	3.4593	0	0	0	0	0	0	4.6146	0
97	Human Vitamin D Binding Prote	gi|18655424 (+1)	13.469	11.9	8.518	10.378	0	13.075	0	12.26	14.039	0	0	15.898
98	complement component 8, alph	gi|114556800 (+5)	0.7483	1.2527	0	1.7296	0	0.6226	0	0	0.6685	0	0	0
99	attractin, isoform CRA_b [Homo	gi|119630936 (+6)	0	0	0	0	0	0	2.0469	2.0433	0	1.3287	2.3073	0
100	keratin associated protein 13.1	gi|41350563	0	0	0	6.9185	0	0.6226	0	0	0	0	0	0
101	hair keratin A1	gi|115495167	0	0	0	13.837	0	6.8489	0	0	0	0	0	0
102	fibulin 1 isoform 3	gi|109094533 (+5)	0.7483	0.6263	0	0	0	0.6226	0	0	1.3371	0	0	0
103	similar to fibrinogen, gamma ch	gi|73977992 (+2)	0	8.1422	0	6.9185	0	0	0	0	0	0	0	12.719
104	hypothetical protein	gi|119922176 (+2)	0	0	0	6.9185	0	0.6226	0	0	0	0	0	0
105	Chain A, Apo-Human Serum Tra	gi|110590597 (+6)	0	1.879	0	0	0	0	0	0	0	0	0	0
106	Chain A, Yeast Alcohol Dehydro	gi|112491285 (+4)	0	1.2527	0	0	0	0	0	0	0	0	0	0
107	Keratin-associated protein 11-1	gi|52783049	0	0	0	5.1889	0	0	0	0	0	0	0	0

Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analysed using Mascot (Matrix Science, London, UK). Mascot was set up to search the NCBInr database (33055681 entries). Mascot was searched with a fragment ion mass tolerance of 0.20Da and a parent tolerance of 0.25Da. Oxidation of methionine and iodoacetaminde derivative of cysteine were specified as variable modifications.

Scaffold was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.

Table S2 - Protein Summary for iTRAQ Experiment with mircoglia treated with human control, MCI and AD plasma from Protein Pilot v4.0

114: Miroglia treated with Control Plasma replicate 1; **115**: Microglia treated with MCI plasma replicate 1; **116**: Microglia treated with AD plasma replicate 1 **117**: Microglia treated with Fetal Bovine Serum
118: Microglia treated with Control plasma replicate 2; **119**: Microglia treated with MCI plasma replicate 2; **121**: Microglia treated with AD plasma replicate 2

Upregulated proteins are shown in red and downregulated proteins are shown in blue

Unused	%Cov (95)	Accession	Name	Peptides (95%)	114:117	PVal 114:117	115:117	PVal 115:117	116:117	PVal 116:117	118:117	PVal 118:117	119:117	PVal 119:117	121:117	PVal 121:117
	72.54	gi|7669492	glyceraldehyde-3-phosphate dehydrogenase isoform 1 [Homo sapiens]	62	0.97414	0.52389	0.9826	0.676093	1.273859	3.43E-05	1.02487	0.60275	0.976086	0.65477	1.31801	0.0001
64.83	76.27	gi|4503571	alpha-enolase isoform 1 [Homo sapiens]	62	0.9323	0.11964	0.98826	0.719005	1.192321	0.00027	0.98271	0.75327	0.97853	0.55307	1.22469	0.000105
	55.88	gi|4505763	phosphoglycerate kinase 1 [Homo sapiens]	27	1.07208	0.17501	1.0474	0.279231	1.203913	7.01E-05	1.04557	0.32215	1.062029	0.20679	1.30616	0.000111
	61.48	gi|342187211	fructose-bisphosphate aldolase A isoform 2 [Homo sapiens]	27	0.98961	0.82864	1.01971	0.836449	1.204599	0.00446	1.00107	0.9894	1.000761	0.99085	1.2183	0.001405
77.64	69.11	gi|33286418	pyruvate kinase isozymes M1/M2 isoform a [Homo sapiens]	49	0.97698	0.74623	1.00909	0.869057	1.22076	0.00752	1.02407	0.72615	1.027908	0.59976	1.2245	0.001615
49.96	46.39	gi|4507521	transketolase isoform 1 [Homo sapiens]	34	0.9812	0.68558	1.04368	0.323134	1.130957	0.02218	1.01055	0.88087	0.987174	0.74884	1.15666	0.001681
74.49	23.07	gi|38176300	nestin [Homo sapiens]	42	0.98677	0.77329	0.96911	0.518102	0.943442	0.17346	0.99888	0.98412	1.036204	0.35449	0.85376	0.004498

		gi	Protein	Count												
37.03	38.85	gi|66933005	calnexin precursor [Homo sapiens]	22	0.99713	0.94162	0.94451	0.31514	0.924302	0.26575	0.9798	0.70005	0.932549	0.14875	0.87883	0.010136
	72.51	gi|4503471	elongation factor 1-alpha 1 [Homo sapiens]	54	0.96687	0.49315	0.93464	0.225032	1.131237	0.0241	1.02401	0.75967	0.967436	0.56314	1.24135	0.014366
	29.73	gi|19920317	cytoskeleton-associated protein 4 [Homo sapiens]	17	0.84689	0.04873	0.92014	0.229067	0.736276	0.0001	0.76848	0.01158	0.78562	0.03204	0.76711	0.018819
30.5	47.73	gi|5031973	protein disulfide-isomerase A6 precursor [Homo sapiens]	22	0.9654	0.42869	0.944	0.133813	0.957242	0.54622	0.97927	0.66221	1.002759	0.95171	0.90975	0.019165
20.99	59.3	gi|4505591	peroxiredoxin-1 [Homo sapiens]	15	1.01122	0.8714	1.00843	0.871252	1.071734	0.22303	0.95462	0.37925	0.963405	0.47862	1.1723	0.021238
	13.15	gi|214010226	40S ribosomal protein S24 isoform d [Homo sapiens]	4	0.67916	0.06245	0.75061	0.010725	0.60023	0.06339	0.66105	0.01456	0.664317	0.08515	0.63548	0.021809
18.45	46.15	gi|4506713	ubiquitin-40S ribosomal protein S27a precursor [Homo sapiens]	11	0.98274	0.73773	1.05902	0.285887	0.836245	0.0664	0.82362	0.15854	1.037765	0.59399	0.77292	0.022464
	19.9	gi|66346681	plasminogen activator inhibitor 1 RNA-binding protein isoform 2 [Homo sapiens]	7	1.04273	0.58745	1.00011	0.999016	0.966927	0.6123	0.98868	0.92202	0.927851	0.47639	0.78652	0.023336
21.89	15.51	gi|44680105	caldesmon isoform 1 [Homo sapiens]	11	0.90827	0.1536	0.89843	0.174139	0.874096	0.0854	0.97455	0.77315	0.936821	0.47815	0.85601	0.026671
41.45	38.13	gi|4505257	moesin [Homo sapiens]	23	1.07974	0.26539	1.11677	0.105786	1.224702	0.02088	1.13547	0.24289	1.029872	0.72063	1.20124	0.028037
38.53	62.14	gi|4502101	annexin A1 [Homo sapiens]	23	1.00008	0.99855	0.92705	0.161224	0.872665	0.02261	0.98702	0.84843	1.015028	0.79418	0.8338	0.042088
17.57	59.43	gi|4503057	alpha-crystallin B chain [Homo sapiens]	12	1.03866	0.42864	1.04427	0.449927	1.237261	0.00212	1.02433	0.71835	0.994397	0.93124	1.26081	0.042824
2.32	12.63	gi|54792069	small ubiquitin-related modifier 2 isoform a precursor [Homo sapiens]	2	1.14799	0.29051	1.45937	0.083382	1.231096	0.26043	1.01658	0.94786	1.407796	0.05217	1.28827	0.043238

		accession	protein	count												
9.54	13.73	gi|109637759	calpastatin isoform f [Homo sapiens]	7	0.86253	0.228	0.80906	0.239705	0.817004	0.20571	0.74525	0.1378	0.884395	0.46022	0.74361	0.045267
	22.73	gi|4557585	fatty acid-binding protein, brain [Homo sapiens]	4	0.85065	0.21974	0.79912	0.227698	0.90105	0.24714	0.84292	0.17597	0.873763	0.35343	0.83176	0.049333
	24.27	gi|4503143	cathepsin D preproprotein [Homo sapiens]	10	0.95791	0.58116	1.0536	0.628932	1.106723	0.5541	1.06446	0.69411	0.9615	0.74084	1.16945	0.058726
50.95	32.2	gi|153792590	heat shock protein HSP 90-alpha isoform 1 [Homo sapiens]	33	1.03008	0.67865	1.06218	0.512408	1.299528	0.03574	1.04757	0.60122	1.055205	0.4726	1.20463	0.058983
45.71	56.44	gi|21361657	protein disulfide-isomerase A3 precursor [Homo sapiens]	37	0.9619	0.42744	0.89756	0.012721	0.924691	0.11729	0.98181	0.65258	0.995083	0.87086	0.89188	0.059792
14.84	80	gi|5032057	protein S100-A11 [Homo sapiens]	16	1.04077	0.52822	0.94971	0.423999	0.863005	0.05467	1.04763	0.46558	0.98643	0.82636	0.86681	0.060101
15.61	63.49	gi|31543380	protein DJ-1 [Homo sapiens]	8	0.87038	0.05065	1.0421	0.587302	1.041927	0.75125	0.95154	0.45415	0.994383	0.95366	1.16939	0.069841
	57.3	gi|66392203	NME1-NME2 protein [Homo sapiens]	11	1.05003	0.63669	1.05967	0.740323	1.119333	0.36696	1.10984	0.37772	1.046986	0.7303	1.22055	0.086404
	38.67	gi|20149594	heat shock protein HSP 90-beta [Homo sapiens]	33	0.94769	0.31531	1.04297	0.30697	1.114919	0.12151	1.03159	0.49331	1.024515	0.70189	1.12563	0.088957
11.8	13.04	gi|56699409	RNA-binding motif protein, X chromosome isoform 1 [Homo sapiens]	6	0.92096	0.49133	0.87494	0.158879	0.877598	0.12292	1.0088	0.9293	0.966318	0.74655	0.87719	0.094431
	48.24	gi|5803225	14-3-3 protein epsilon [Homo sapiens]	16	1.02601	0.76029	0.91136	0.328953	1.11497	0.15435	0.96656	0.78874	0.960346	0.44181	1.14985	0.097089
52.59	69.47	gi|50845388	annexin A2 isoform 1 [Homo sapiens]	52	1.03601	0.33861	0.89261	0.000799	0.952177	0.19231	1.09683	0.02102	0.986273	0.64679	0.93349	0.100108
7.85	53.99	gi|4885375	histone H1.2 [Homo sapiens]	16	0.87375	0.05937	0.7433	0.00219	0.542623	0.00103	0.73003	0.00159	0.947048	0.46755	0.75976	0.101682

24.44	gi|4506661	60S ribosomal protein L7a [Homo sapiens]	6	1.12561	0.37809	1.10248	0.529482	1.115835	0.16495	1.03566	0.61818	1.010542	0.93949	1.25406	0.104774
65.26 54.13	gi|16507237	78 kDa glucose-regulated protein precursor [Homo sapiens]	42	0.99068	0.80644	0.91924	0.037995	0.914667	0.10089	0.97414	0.72851	0.975383	0.64284	0.90792	0.107537
65.68	gi|21735621	malate dehydrogenase, mitochondrial precursor [Homo sapiens]	21	0.98306	0.72628	0.96187	0.550987	1.082983	0.19161	0.99879	0.98249	1.003288	0.95569	1.09901	0.108722
6.298	gi|4504763	integrin alpha-V isoform 1 precursor [Homo sapiens]	6	1.10731	0.34169	1.50407	0.12905	0.922235	0.67208	1.35084	0.4102	1.65825	0.06426	1.25584	0.110681
3.27 3.004	gi|38327625	citrate synthase, mitochondrial precursor [Homo sapiens]	2	0.90179	0.42891	1.04244	0.800114	0.948916	0.85177	1.10885	0.48796	0.896501	0.31286	1.30983	0.112072
1.04	gi|4505541	general vesicular transport factor p115 [Homo sapiens]	1	1.03	0.9444	1.16908	0.359291	1.468988	0.16016	1.34375	0.59047	1.403052	0.25749	1.86084	0.114754
11.64 4.447	gi|223029410	talin-1 [Homo sapiens]	7	0.7748	0.08765	1.01838	0.916424	0.810045	0.62668	0.53988	0.04862	0.830302	0.16782	0.80491	0.115327
15.01 30.56	gi|24431933	reticulon-4 isoform B [Homo sapiens]	9	1.03261	0.79202	0.92584	0.443218	0.86613	0.3369	0.98962	0.93193	0.999494	0.99403	0.75832	0.118577
40.45	gi|4502389	barrier-to-autointegration factor [Homo sapiens]	3	1.01481	0.90604	0.79065	0.497285	0.785352	0.47056	0.77148	0.48763	1.065285	0.63782	0.60144	0.122476
21.8	gi|15431297	60S ribosomal protein L13 isoform 1 [Homo sapiens]	6	1.00852	0.94051	1.04466	0.512794	0.999406	0.99678	0.99211	0.96695	1.008986	0.93835	0.85176	0.122519
6 11.89	gi|169404009	translocon-associated protein subunit alpha precursor [Homo sapiens]	3	0.95232	0.61818	0.87112	0.397806	0.856702	0.23079	1.06776	0.59316	0.959057	0.73145	0.80544	0.122538

		Description													
4.09 37.5	gi|51036603	guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12 precursor [Homo sapiens]	3	0.82618	0.40915	0.66391	0.07069	0.823514	0.43842	0.91099	0.71981	0.765134	0.40552	0.64208	0.124749
60.61	gi|10863927	peptidyl-prolyl cis-trans isomerase A [Homo sapiens]	21	0.98345	0.72502	1.04141	0.522254	1.157293	0.14108	1.04379	0.67672	1.040531	0.7547	1.13552	0.125378
14.61 62.86	gi|4826898	profilin-1 [Homo sapiens]	11	0.93252	0.25277	0.9354	0.272724	1.1113	0.10339	0.95098	0.4023	0.930043	0.40442	1.13055	0.127681
18.74	gi|20127408	trifunctional enzyme subunit alpha, mitochondrial precursor [Homo sapiens]	9	0.93017	0.43003	0.88367	0.537465	0.804996	0.40295	1.0888	0.67464	0.886635	0.51654	0.85087	0.130116
20	gi|24797067	HLA class I histocompatibility antigen, A-1 alpha chain precursor [Homo sapiens]	8	0.88901	0.29354	0.94676	0.494894	0.929581	0.37731	0.97793	0.82815	0.935571	0.41568	0.85741	0.130658
6.37 14.08	gi|7019415	protein transport protein Sec61 subunit alpha isoform 1 [Homo sapiens]	5	0.79339	0.19075	0.97595	0.796917	0.744264	0.37933	0.62065	0.1723	0.702107	0.27466	0.70581	0.136075
6.289	gi|68509926	putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15 [Homo sapiens]	5	0.62423	0.18212	0.47583	0.334467	1.130629	0.43257	0.77012	0.46447	0.811996	0.64092	0.49002	0.13646
4.664	gi|38201710	probable ATP-dependent RNA helicase DDX17 isoform 1 [Homo sapiens]	3	0.79915	0.61738	0.91094	0.620215	0.750311	0.21345	0.80939	0.28139	0.780581	0.24321	0.63829	0.138851

5.383	gi|4757732	apoptosis-inducing factor 1, mitochondrial isoform 1 precursor [Homo sapiens]	2	1.38271	0.07172	1.19782	0.407886	1.088847	0.41412	1.28989	0.41554	1.162864	0.38478	1.34436	0.140307
24.62	gi|238776833	THO complex subunit 4 [Homo sapiens]	6	0.83702	0.04946	0.94848	0.403375	0.86749	0.31273	0.87675	0.42273	0.897684	0.40794	0.88435	0.14495
43.94	gi|32189392	peroxiredoxin-2 isoform a [Homo sapiens]	10	1.34013	0.09637	1.27568	0.14954	1.190778	0.02567	1.19692	0.26779	1.15698	0.45387	1.28334	0.145991
2.03	gi|124494254	proliferation-associated protein 2G4 [Homo sapiens]	1	0.96538	0.89731	1.35889	0.039772	1.239406	0.2757	0.98931	0.97425	1.219182	0.19368	1.14722	0.146018
14.1	gi|19923437	GTP:AMP phosphotransferase, mitochondrial isoform a [Homo sapiens]	3	1.39625	0.08317	1.46051	0.046626	1.432712	0.05166	1.27401	0.37104	1.138681	0.44	1.42747	0.148779
20.35 55.61	gi|21361091	ubiquitin carboxyl-terminal hydrolase isozyme L1 [Homo sapiens]	12	0.94536	0.55362	1.07884	0.312914	1.099702	0.14413	0.92686	0.5639	0.969563	0.73363	1.14064	0.149277
11.48	gi|14141159	heterogeneous nuclear ribonucleoprotein H3 isoform b [Homo sapiens]	5	0.8342	0.1545	0.96127	0.711873	0.817383	0.18652	0.86804	0.22181	0.778232	0.09066	0.71379	0.153676
51.55	gi|5729877	heat shock cognate 71 kDa protein isoform 1 [Homo sapiens]	39	1.02418	0.63104	0.9707	0.403987	1.061664	0.19288	0.9861	0.86555	0.965753	0.41121	1.06446	0.153957
23.65	gi|4557032	L-lactate dehydrogenase B chain [Homo sapiens]	6	0.93851	0.60795	1.08169	0.304175	1.185435	0.06437	0.88422	0.54126	1.059711	0.73119	1.12483	0.154941

5.92	32	gi|48255933	non-histone chromosomal protein HMG-14 [Homo sapiens]	3	0.91603	0.6829	0.98843	0.959112	0.811181	0.18646	0.82704	0.40778	0.919272	0.33885	0.87101	0.160101
13	45.56	gi|4507651	tropomyosin alpha-4 chain isoform 2 [Homo sapiens]	13	0.9197	0.35893	0.94139	0.238868	0.978656	0.86419	0.77351	0.09697	0.898614	0.18298	0.88635	0.160554
2.03	11.8	gi|15451856	caveolin-1 isoform alpha [Homo sapiens]	1	0.99581	0.97294	0.88967	0.446766	0.866877	0.38527	0.89103	0.45106	0.978481	0.86208	0.68415	0.162168
7.77	28.87	gi|4506761	protein S100-A10 [Homo sapiens]	6	1.05949	0.39457	0.98038	0.763376	0.915157	0.21142	0.9942	0.92867	1.068271	0.33669	0.83821	0.162865
	34.94	gi|94721252	vesicle-associated membrane protein-associated protein A isoform 2 [Homo sapiens]	8	1.227	0.01851	0.97704	0.71228	1.185619	0.34812	1.26298	0.03566	1.170574	0.09287	1.22005	0.164682
8.4	33.14	gi|4507669	translationally-controlled tumor protein [Homo sapiens]	6	1.08576	0.52472	1.09398	0.508183	1.116665	0.46569	1.13494	0.37263	1.084082	0.68352	1.23937	0.170834
	10.25	gi|25188179	voltage-dependent anion-selective channel protein 3 isoform 1 [Homo sapiens]	3	1.02508	0.91757	1.16013	0.376609	1.484604	0.36729	1.26373	0.25659	0.81173	0.69302	1.54434	0.177514
	10.84	gi|17158044	40S ribosomal protein S6 [Homo sapiens]	2	0.95076	0.6196	0.82852	0.091434	0.738315	0.04109	0.53979	0.06305	0.69574	0.1722	0.71755	0.1779
	4.09	gi|33350932	cytoplasmic dynein 1 heavy chain 1 [Homo sapiens]	13	1.57248	0.29762	1.57923	0.217808	1.282865	0.35371	1.80211	0.13401	1.017224	0.96817	1.5064	0.181445
	7.064	gi|50592988	cytochrome b-c1 complex subunit 2, mitochondrial precursor [Homo sapiens]	2	0.81533	0.4452	0.69962	0.31094	0.740066	0.414	0.68865	0.20942	0.423321	0.20012	0.70978	0.181631

		gi	Protein													
	12.75	gi|24307939	T-complex protein 1 subunit epsilon [Homo sapiens]	6	0.88391	0.83878	1.03728	0.930669	1.416377	0.6002	1.44063	0.48371	1.130974	0.78207	0.69751	0.182052
25.85	24.2	gi|19923142	importin subunit beta-1 [Homo sapiens]	17	1.05472	0.72744	1.03584	0.67655	1.133016	0.19697	1.16673	0.15872	0.998602	0.98495	1.13234	0.185815
6.49	17.1	gi|38372925	basigin isoform 2 [Homo sapiens]	4	1.01305	0.85925	1.00488	0.960318	0.988465	0.91265	0.947	0.49923	1.10776	0.58434	0.89814	0.189596
	14.29	gi|78214522	60S ribosomal protein L38 [Homo sapiens]	1	0.96672	0.84305	0.91545	0.719896	0.828098	0.3865	0.66688	0.42456	1.096426	0.88091	0.40804	0.191292
3.66	11.28	gi|5729875	membrane-associated progesterone receptor component 1 [Homo sapiens]	3	1.29932	0.22776	1.19393	0.255245	0.922448	0.82759	1.2013	0.35792	1.273844	0.11659	1.30413	0.191907
1.96	2.809	gi|5803023	vesicular integral-membrane protein VIP36 precursor [Homo sapiens]	1	1.25437	0.44629	0.9238	0.572269	1.003053	0.9813	1.24652	0.57488	0.725378	0.19242	0.72577	0.192623
19.62	28.1	gi|5032093	neutral amino acid transporter B(0) isoform 1 [Homo sapiens]	14	0.99773	0.98539	0.97139	0.597192	0.993195	0.94792	1.06033	0.30024	1.012247	0.93042	0.90298	0.192785
	12.15	gi|7657581	calcium-binding mitochondrial carrier protein Aralar2 isoform 2 [Homo sapiens]	5	1.24309	0.38294	1.04897	0.552228	1.137596	0.35039	1.21757	0.22643	1.018496	0.82502	0.77457	0.197527
	34.01	gi|194248072	heat shock 70 kDa protein 1A/1B [Homo sapiens]	20	1.00579	0.94158	1.09057	0.121139	1.049526	0.65628	1.07026	0.41274	0.957757	0.53605	1.1032	0.200776
	27.06	gi|11321585	guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 [Homo sapiens]	7	0.73007	0.19568	0.64127	0.141431	0.681516	0.19847	0.71464	0.34485	1.037866	0.77339	0.74014	0.204888

11.47	43.36	gi	4885381	histone H1.5 [Homo sapiens]	15	0.83443	0.01052	0.79034	0.005847	0.651536	0.00034	0.77306	0.05702	0.897578	0.16378	0.84588	0.205013
	14.2	gi	156523968	poly [ADP-ribose] polymerase 1 [Homo sapiens]	10	1.21558	0.14288	1.17066	0.120212	1.051162	0.47741	1.0265	0.87789	1.061355	0.76352	1.172	0.207123
3.04	3.187	gi	77404397	staphylococcal nuclease domain-containing protein 1 [Homo sapiens]	3	1.17405	0.49812	1.22773	0.468987	1.488215	0.12081	1.08531	0.69621	1.110144	0.54598	1.36789	0.210295
	5.495	gi	4503583	epoxide hydrolase 1 precursor [Homo sapiens]	2	0.84869	0.29741	0.72711	0.197375	1.081442	0.45904	0.87687	0.24494	0.884422	0.60119	0.86167	0.213117
	24.41	gi	9845502	40S ribosomal protein SA [Homo sapiens]	6	1.03724	0.74771	1.08735	0.352101	1.152088	0.1949	1.2666	0.02453	0.950464	0.75669	1.16312	0.215801
	24.35	gi	4506631	60S ribosomal protein L30 [Homo sapiens]	2	1.14158	0.69687	1.10043	0.803557	1.054163	0.80851	1.05796	0.56652	1.038772	0.81646	1.12587	0.218553
8.3	22.18	gi	11559923	eukaryotic translation initiation factor 4H isoform 1 [Homo sapiens]	5	1.12264	0.68798	1.25954	0.340565	1.042103	0.8514	1.01228	0.88499	1.066275	0.614	1.09901	0.225424
	13.87	gi	40068518	6-phosphogluconate dehydrogenase, decarboxylating [Homo sapiens]	5	0.81545	0.14339	0.94935	0.623335	1.02256	0.78535	0.79003	0.11995	0.8653	0.35679	0.77777	0.227224
2.11	3.371	gi	24797106	FAS-associated factor 2 [Homo sapiens]	1	1.06334	0.65017	1.45771	0.417112	1.365147	0.33565	1.01105	0.94592	1.360478	0.20061	1.30254	0.231693
	9.594	gi	5453549	peroxiredoxin-4 precursor [Homo sapiens]	3	1.09696	0.59119	1.16346	0.599874	1.087432	0.78277	1.19647	0.61772	1.284313	0.55549	1.31451	0.235158
3.24	7.754	gi	23397429	eukaryotic translation initiation factor 3 subunit M [Homo sapiens]	2	0.89871	0.47538	1.31111	0.222737	1.013742	0.93117	0.81456	0.2861	1.138485	0.56671	1.28961	0.236077

	4.537	gi|162329583	cleavage and polyadenylation specificity factor subunit 6 [Homo sapiens]	1	0.90214	0.50643	1.11391	0.867037	1.458042	0.54873	1.06447	0.65983	1.296901	0.68157	1.30871	0.236755
	13.39	gi|237681109	sodium/potassium-transporting ATPase subunit alpha-1 isoform c [Homo sapiens]	18	1.02552	0.80461	1.00024	0.998588	0.844812	0.12679	0.91605	0.50142	0.914821	0.38021	0.91843	0.239598
	21.58	gi|14251209	chloride intracellular channel protein 1 [Homo sapiens]	6	0.90088	0.30114	0.95147	0.772095	1.09574	0.46797	0.96541	0.77434	0.984818	0.91828	0.88861	0.24316
4.53	6.239	gi|109589363	glucose-6-phosphate 1-dehydrogenase isoform a [Homo sapiens]	2	1.20426	0.33865	1.43066	0.189014	1.624618	0.2636	1.55257	0.22793	1.448891	0.23758	1.30435	0.249744
6.35	24.44	gi|7657532	protein S100-A6 [Homo sapiens]	3	0.98566	0.85809	1.09844	0.296211	1.059034	0.49517	1.03444	0.67966	1.031681	0.7023	1.1115	0.250352
21.3	42.45	gi|5803227	14-3-3 protein theta [Homo sapiens]	17	0.97905	0.87629	1.0435	0.605723	0.966232	0.87085	0.92408	0.61253	1.178148	0.16	1.1743	0.251827
2	8.744	gi|88953571	PREDICTED: POTE ankyrin domain family member I isoform 2 [Homo sapiens]	34	0.86459	0.70652	0.90932	0.524006	0.832067	0.32213	0.85508	0.3682	0.725671	0.19692	0.78485	0.253517
32.32	35.84	gi|5031877	lamin-B1 isoform 1 [Homo sapiens]	18	0.94349	0.56293	0.90136	0.275431	0.918949	0.44907	0.93501	0.4858	0.893712	0.32872	0.89476	0.254337
5.39	11.56	gi|4501853	3-ketoacyl-CoA thiolase, peroxisomal isoform a [Homo sapiens]	3	2.16834	0.1858	1.18441	0.641874	1.62163	0.13731	1.42343	0.17432	1.2076	0.45654	1.25897	0.259562
	9.246	gi|7661920	eukaryotic initiation factor 4A-III [Homo sapiens]	2	1.29956	0.34769	1.01607	0.902547	1.212463	0.31844	1.27806	0.42026	0.783348	0.76584	0.79002	0.262826
50.42	31.55	gi|55956788	nucleolin [Homo sapiens]	29	1.05759	0.23708	1.02976	0.572142	0.98183	0.74371	1.09084	0.15784	1.007521	0.87671	1.05735	0.263211

2.95	3.655	gi|308818200	dihydropyrimidinase-related protein 3 isoform 1 [Homo sapiens]	2	1.34309	0.06857	1.2031	0.161372	0.967412	0.85001	1.52203	0.03584	1.210626	0.5645	1.23319	0.263459
	32.08	gi|23308577	D-3-phosphoglycerate dehydrogenase [Homo sapiens]	17	1.06596	0.47514	0.97881	0.832898	1.203403	0.08204	1.02448	0.76084	1.029579	0.60888	1.15234	0.267369
25.86	13.07	gi|4758012	clathrin heavy chain 1 [Homo sapiens]	16	0.99052	0.92065	0.94667	0.522496	1.13722	0.24734	0.88892	0.13223	0.916398	0.48145	1.09911	0.268588
	30.23	gi|4502985	cytochrome c oxidase subunit 6B1 [Homo sapiens]	2	0.90839	0.35899	0.88557	0.684512	0.735976	0.10006	0.76604	0.22395	0.772901	0.17148	0.81212	0.274825
	8.225	gi|153945728	microtubule-associated protein 1B [Homo sapiens]	17	0.94326	0.58955	0.9836	0.825151	0.952414	0.62219	0.87584	0.26177	0.900398	0.23709	0.88394	0.275407
	84.33	gi|62414289	vimentin [Homo sapiens]	111	0.96853	0.22969	0.8979	0.00024	0.966613	0.26142	0.99839	0.96544	0.992586	0.77803	0.96001	0.276668
	84.33	gi|62414289	vimentin [Homo sapiens]	111	0.96853	0.22969	0.8979	0.00024	0.966613	0.26142	0.99839	0.96544	0.992586	0.77803	0.96001	0.276668
	27.31	gi|5453555	GTP-binding nuclear protein Ran [Homo sapiens]	5	0.98859	0.87907	1.14585	0.42636	1.194501	0.25584	1.03131	0.69113	0.94485	0.71941	1.14543	0.276881
6.52	14.19	gi|4507171	SPARC precursor [Homo sapiens]	3	0.9158	0.47886	1.02166	0.864754	0.936253	0.51281	0.94402	0.83367	0.974326	0.78471	0.73391	0.27843
32.05	53.69	gi|32189394	ATP synthase subunit beta, mitochondrial precursor [Homo sapiens]	26	1.06947	0.33782	1.02526	0.810252	1.085629	0.19352	1.06683	0.36414	1.062145	0.36963	1.05613	0.282311
	32.7	gi|4502551	calumenin isoform a precursor [Homo sapiens]	11	1.0541	0.35248	0.92044	0.374923	1.046819	0.65407	1.02137	0.84663	1.013005	0.8751	0.90294	0.28484
30.99	46.28	gi|4757900	calreticulin precursor [Homo sapiens]	22	0.97558	0.48559	0.9151	0.110982	0.924536	0.15201	1.02055	0.6353	1.000256	0.99424	0.94295	0.285087

		ID	Protein	N	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12
	13.38	gi|4506619	60S ribosomal protein L24 [Homo sapiens]	2	0.88742	0.57195	0.87413	0.254582	0.97148	0.78669	0.90032	0.34225	0.858709	0.57737	0.81159	0.285288
4.33	12.64	gi|19557691	surfeit locus protein 4 [Homo sapiens]	3	1.07383	0.75128	0.79406	0.111418	0.815363	0.65159	1.00065	0.99896	0.826807	0.64556	0.68096	0.286924
14.4	25.05	gi|34147630	elongation factor Tu, mitochondrial precursor [Homo sapiens]	8	1.24054	0.2593	1.00401	0.978324	1.262414	0.32398	1.23422	0.17278	1.179966	0.2319	1.20203	0.287698
	20	gi|262118227	cytochrome c oxidase subunit 7A2, mitochondrial precursor [Homo sapiens]	2	1.1199	0.72902	0.90201	0.486512	0.756671	0.2722	1.38307	0.18831	0.946541	0.67701	1.2207	0.292914
	32.21	gi|5901912	calmodulin [Homo sapiens]	4	0.97733	0.85572	1.0233	0.851035	1.04108	0.74264	0.91374	0.49865	1.008276	0.91537	0.83326	0.292997
	5.497	gi|157694492	myb-binding protein 1A isoform 2 [Homo sapiens]	5	1.00363	0.98643	0.9421	0.906806	0.905539	0.52855	1.10364	0.52725	0.925595	0.78667	0.80372	0.296452
4.38	38.46	gi|4504253	histone H2A.x [Homo sapiens]	55	1.02226	0.91213	0.96013	0.770314	0.821872	0.25679	1.0363	0.92006	0.957424	0.76937	0.90772	0.304709
	17.58	gi|4506003	serine/threonine-protein phosphatase PP1-alpha catalytic subunit isoform 1 [Homo sapiens]	6	1.48714	0.13004	1.10011	0.713846	1.277277	0.52397	1.33377	0.25545	1.172138	0.4535	1.28387	0.30728
11.93	21.53	gi|42794771	thioredoxin domain-containing protein 5 isoform 1 precursor [Homo sapiens]	7	1.14981	0.0688	1.00059	0.996582	1.070191	0.40696	0.96876	0.79451	0.947137	0.80295	1.09618	0.309544
2.19	4.61	gi|4504619	insulin-like growth factor-binding protein 7 isoform 1 precursor [Homo sapiens]	1	0.84172	0.29423	0.98065	0.834212	1.054621	0.66737	1.60677	0.49731	1.126455	0.67446	0.83734	0.310771

	7.745	gi|4557809	ornithine aminotransferase, mitochondrial isoform 1 [Homo sapiens]	6	1.24849	0.12014	1.16548	0.620637	1.100635	0.88714	0.97603	0.91645	1.240592	0.14432	1.12319	0.31102
	16.48	gi|5803013	endoplasmic reticulum resident protein 29 isoform 1 precursor [Homo sapiens]	4	0.84553	0.25641	1.10238	0.275949	1.055268	0.87182	0.88294	0.27119	0.94896	0.7498	1.17577	0.31394
6.07	16.79	gi|4505415	NAD(P)H dehydrogenase [quinone] 1 isoform a [Homo sapiens]	5	1.36635	0.37502	1.01123	0.930016	1.33663	0.34932	1.45685	0.29703	1.027479	0.83314	1.57378	0.314236
2.22	7.487	gi|4505621	phosphatidylethanolamine-binding protein 1 preproprotein [Homo sapiens]	1	0.96277	0.8957	1.09319	0.533952	0.903624	0.4929	1.02145	0.91865	1.219476	0.35161	1.19588	0.321973
	4.412	gi|358356404	60S ribosomal protein L15 isoform 1 [Homo sapiens]	1	0.87189	0.36029	0.93705	0.674173	1.013222	0.93016	1.11807	0.32778	1.005237	0.93799	0.93831	0.322112
	31.86	gi|13904870	40S ribosomal protein S5 [Homo sapiens]	7	0.9506	0.72658	1.0316	0.603441	0.924649	0.46719	0.99064	0.95691	1.001791	0.98677	1.091	0.322428
	48.28	gi|4501881	actin, alpha skeletal muscle [Homo sapiens]	84	0.70402	0.19171	0.88213	0.452415	0.895226	0.49113	0.9076	0.68294	0.910421	0.74174	0.83025	0.335221
	61.46	gi|4504517	heat shock protein beta-1 [Homo sapiens]	14	1.03033	0.75301	1.09164	0.398213	1.129264	0.45398	1.07204	0.36605	1.008009	0.93325	1.14863	0.335319
3	15.63	gi|23308579	prostaglandin E synthase 3 [Homo sapiens]	2	1.09274	0.60681	1.33151	0.112159	1.200575	0.47968	1.17833	0.45949	1.215746	0.48534	1.12312	0.341192
	23.31	gi|100913206	ATP-dependent RNA helicase A [Homo sapiens]	25	1.01038	0.85557	0.98092	0.810188	1.026626	0.71249	0.99051	0.9034	0.908446	0.21786	0.93231	0.343017

6	15.03	gi|5453597	F-actin-capping protein subunit alpha-1 [Homo sapiens]	3	1.08743	0.8451	1.19702	0.574836	1.148574	0.63926	0.8406	0.67537	1.190998	0.50429	1.17969	0.344035
	8.013	gi|7705855	estradiol 17-beta-dehydrogenase 12 [Homo sapiens]	2	1.35665	0.14963	1.58221	0.299346	1.327157	0.60819	0.82147	0.54246	2.201652	0.16227	1.17915	0.348877
32.68	55.25	gi|4885379	histone H1.4 [Homo sapiens]	18	0.86188	0.15805	0.79458	0.062715	0.581451	0.00631	0.783	0.05277	0.955935	0.61036	0.78812	0.349009
	32.02	gi|4503529	eukaryotic initiation factor 4A-I isoform 1 [Homo sapiens]	13	1.04269	0.74889	1.03994	0.678853	1.271338	0.05202	1.0321	0.8665	0.954919	0.82177	1.16915	0.351373
	14.38	gi|4506691	40S ribosomal protein S16 [Homo sapiens]	2	0.97562	0.80498	1.08435	0.534558	0.91199	0.46498	0.90795	0.25085	1.061548	0.63913	1.08996	0.352086
5.35	21.47	gi|89903012	cell division control protein 42 homolog isoform 1 [Homo sapiens]	4	0.84946	0.37036	0.96503	0.661392	0.873435	0.40147	0.85893	0.56613	0.93952	0.65745	0.8919	0.363234
4.28	8.456	gi|4826659	F-actin-capping protein subunit beta isoform 1 [Homo sapiens]	2	0.67802	0.35754	0.69959	0.172607	0.851274	0.65389	0.86143	0.37487	0.66671	0.15342	0.85952	0.369847
16.25	8.025	gi|7669550	vinculin isoform meta-VCL [Homo sapiens]	7	0.88768	0.17609	0.86082	0.075983	0.892825	0.45546	0.90485	0.43053	0.975215	0.86887	0.89204	0.37485
	23.14	gi|35493916	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2 isoform 1 precursor [Homo sapiens]	16	0.97184	0.85688	0.82452	0.080194	0.845658	0.09154	0.94712	0.71073	0.862415	0.34484	0.82828	0.378348
14	22.43	gi|4758112	spliceosome RNA helicase DDX39B [Homo sapiens]	11	0.76734	0.20451	1.11319	0.323403	0.756887	0.24072	1.25882	0.36395	0.926787	0.45626	0.75424	0.37859
11.59	15.71	gi|7549809	plastin-3 isoform 1 [Homo sapiens]	9	1.12989	0.64507	0.90527	0.733379	1.317888	0.17397	0.86056	0.69041	0.96376	0.83803	1.20141	0.379589

11.33	36.02	gi|4758504	3-hydroxyacyl-CoA dehydrogenase type-2 isoform 1 [Homo sapiens]
3.4	19.47	gi|4503253	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1 [Homo sapiens]
3.13	8.871	gi|5902076	serine/arginine-rich splicing factor 1
14.51	18.85	gi|21361399	serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform [Homo sapiens]

			isoform 1 [Homo sapiens]
4.595		gi|4826686	ATP-dependent RNA helicase DDX1 [Homo sapiens]
11.88		gi|55956899	keratin, type I cytoskeletal 9 [Homo sapiens]
24		gi|4506633	60S ribosomal protein L31 isoform 1 [Homo sapiens]
4.981		gi|5453710	LIM and SH3 domain protein 1 [Homo sapiens]
21.59		gi|4506723	40S ribosomal protein S3a isoform 1 [Homo sapiens]

9	0.85201	0.64513	0.93269	0.807974	1.291011	0.53085	1.19777	0.7306	1.030423	0.92072	1.31892	0.380861
5	1.16862	0.44494	1.33119	0.096689	1.177838	0.49	1.0947	0.79475	1.573738	0.01477	1.13974	0.381154
2	0.97294	0.92271	1.26859	0.287512	1.130249	0.50102	1.09105	0.81061	0.985191	0.90638	1.15704	0.386139
2	0.80818	0.51448	0.95702	0.88235	0.88861	0.73781	0.93516	0.86581	0.90713	0.71945	0.74119	0.387206
2	0.96152	0.7616	0.50291	0.169483	0.979637	0.92474	1.28961	0.57091	0.61504	0.12877	0.86852	0.391631
6	0.859	0.1927	0.99136	0.910421	1.174134	0.18032	1.15622	0.23457	0.965199	0.70957	1.10427	0.395095
3	1.23708	0.47402	0.95535	0.724579	1.41888	0.35286	0.98525	0.96622	1.213987	0.63144	1.1551	0.397981
1	0.7038	0.17472	0.74995	0.303498	0.81862	0.29299	1.06878	0.74451	0.721986	0.50264	0.74921	0.401414
6	1.12182	0.58683	1.07692	0.62046	1.139729	0.42901	1.17579	0.43977	1.065154	0.63398	1.10216	0.401429

25.7	38.78	gi|4506243	polypyrimidine tract-binding protein 1 isoform a [Homo sapiens]	18	1.06292	0.50756	1.06923	0.515736	1.03194	0.7921	1.05954	0.60728	1.071681	0.34712	1.09726	0.405096
9.37	13.6	gi|4503249	protein DEK isoform 1 [Homo sapiens]	6	0.90486	0.7789	0.82548	0.362847	0.887533	0.5517	0.78952	0.49732	0.937237	0.82238	0.72629	0.405633
14.64	25.24	gi|213511508	B-cell receptor-associated protein 31 isoform a [Homo sapiens]	10	0.9788	0.86518	0.94279	0.594634	0.912101	0.48864	1.007	0.95315	1.037329	0.68442	0.92084	0.409043
7.42	23.37	gi|219555707	eukaryotic translation initiation factor 5A-1 isoform A [Homo sapiens]	12	0.93983	0.54649	0.9442	0.764423	0.988686	0.90673	0.86285	0.22899	0.906192	0.37119	0.80789	0.411159
1.55	4.286	gi|151101292	thioredoxin-related transmembrane protein 1 precursor [Homo sapiens]	1	1.06382	0.75255	0.97657	0.857522	0.819195	0.30702	0.88399	0.46597	0.94309	0.88688	0.87165	0.412176
2.73	1.905	gi|65506891	4F2 cell-surface antigen heavy chain isoform c [Homo sapiens]	1	0.98317	0.93864	0.84624	0.244296	1.115642	0.53319	0.93078	0.55737	1.007492	0.96461	1.15408	0.413802
	12.29	gi|4504897	importin subunit alpha-2 [Homo sapiens]	4	1.62496	0.16839	1.58207	0.135682	1.24339	0.36447	1.51981	0.50696	1.039731	0.89515	1.5101	0.415434
	68.18	gi|226529917	triosephosphate isomerase isoform 2 [Homo sapiens]	18	0.89284	0.09459	0.9824	0.725009	1.039563	0.50875	0.86402	0.04131	0.996977	0.94683	1.0504	0.4184
	45.92	gi|10835063	nucleophosmin isoform 1 [Homo sapiens]	27	1.05093	0.20853	0.98486	0.687201	0.932746	0.16808	1.0139	0.77562	0.993499	0.88674	0.96765	0.418435
	52.68	gi|4506667	60S acidic ribosomal protein P0 [Homo sapiens]	10	1.07767	0.28966	0.92404	0.551593	0.973779	0.83745	1.06728	0.4878	1.123923	0.35376	1.08972	0.4209
9.86	54.29	gi|11128019	cytochrome c [Homo sapiens]	7	1.02581	0.90995	1.1736	0.485643	1.168768	0.23072	1.206	0.51323	1.136284	0.41989	1.14466	0.422321

1.67	3.499	gi	5803181	stress-induced-phosphoprotein 1 [Homo sapiens]	1	0.71762	0.41633	0.77017	0.230487	0.873029	0.40131	0.75715	0.48774	0.672277	0.46885	1.13446	0.423418
6.23	5.735	gi	149999606	mannosyl-oligosaccharide glucosidase isoform 1 [Homo sapiens]	4	1.08059	0.53739	1.14425	0.238759	1.1575	0.39612	1.20626	0.22942	1.027046	0.82377	1.22927	0.424115
2.06	1.744	gi	13375618	delta(24)-sterol reductase precursor [Homo sapiens]	1	0.96975	0.95351	1.02975	0.912942	0.726643	0.58232	1.01279	0.96941	0.809533	0.46642	0.69083	0.428542
4.59	33.59	gi	6912616	histone H2A.V isoform 1 [Homo sapiens]	9	0.95702	0.75379	0.85738	0.31234	0.799618	0.39941	0.95914	0.85394	0.973601	0.77491	0.91318	0.430038
12.27	17.53	gi	47132620	keratin, type II cytoskeletal 2 epidermal [Homo sapiens]	10	0.94036	0.69134	1.17098	0.215737	0.949491	0.52853	1.22133	0.14278	1.251278	0.05142	1.10158	0.431077
29.14	48.39	gi	24119203	tropomyosin alpha-3 chain isoform 2 [Homo sapiens]	17	1.09868	0.52294	1.13547	0.264856	1.02411	0.77098	1.07533	0.58977	1.025008	0.85158	1.10599	0.4331
4.54	1.504	gi	7305053	myoferlin isoform a [Homo sapiens]	3	1.03642	0.91549	1.07562	0.753873	0.677725	0.15841	1.17281	0.35344	0.804299	0.27121	0.80158	0.434082
5.17	11.54	gi	71772415	40S ribosomal protein S15a [Homo sapiens]	2	1.08261	0.30057	0.86993	0.526937	1.005628	0.96992	1.06918	0.64765	0.895366	0.53786	0.9438	0.435165
19.01	71.69	gi	5031635	cofilin-1 [Homo sapiens]	13	0.98335	0.79485	1.06505	0.609521	1.073792	0.50449	0.94654	0.66373	1.006219	0.93333	1.08085	0.437716
4.87	17.72	gi	5031741	dnaJ homolog subfamily A member 2 [Homo sapiens]	4	1.02126	0.95399	0.95642	0.65181	0.777159	0.21533	1.08462	0.83024	1.354666	0.12297	1.18166	0.440861
3.65	23.53	gi	20357529	guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 [Homo sapiens]	5	1.12035	0.48505	1.20046	0.396539	1.116905	0.28669	0.87631	0.53161	1.27117	0.30243	1.06684	0.441087
3.09	9.509	gi	38201714	ELAV-like protein 1 [Homo sapiens]	3	1.41868	0.19346	1.17549	0.435049	1.144978	0.407	1.38872	0.18715	1.15082	0.57414	1.18829	0.441784

		Accession	Description	Count												
	30.82	gi|148833484	poly(rC)-binding protein 2 isoform c [Homo sapiens]	10	0.8692	0.5888	0.79919	0.127357	1.023241	0.9611	0.89854	0.74636	0.88669	0.61133	0.80637	0.442107
60.22	77.33	gi|4501887	actin, cytoplasmic 2 [Homo sapiens]	122	1.04669	0.76057	0.9772	0.835104	1.156228	0.46372	1.12886	0.57932	0.952823	0.68722	1.13584	0.443001
64.06	45.78	gi|27436946	lamin isoform A [Homo sapiens]	33	1.00508	0.89186	0.97829	0.718695	0.922786	0.10936	0.99433	0.90623	0.986285	0.69962	0.95111	0.446249
	20.35	gi|4502847	cold-inducible RNA-binding protein [Homo sapiens]	4	0.77481	0.23807	0.96341	0.8403	0.974154	0.9343	0.84858	0.47177	0.883339	0.43224	0.88827	0.446678
2.34	40	gi|15617199	histone H2A type 3 [Homo sapiens]	42	1.09822	0.74304	0.87151	0.415005	0.765686	0.38284	1.16096	0.38969	1.043543	0.75415	0.88391	0.448851
1.81	6.704	gi|7706495	dnaJ homolog subfamily B member 11 precursor [Homo sapiens]	2	0.93474	0.87702	0.53691	0.101191	0.806413	0.2753	0.5963	0.38777	0.827227	0.3501	0.79794	0.450096
	25.6	gi|4506707	40S ribosomal protein S25 [Homo sapiens]	4	1.05992	0.71046	0.99801	0.984935	0.891834	0.22233	1.00881	0.92385	1.001775	0.9825	0.93387	0.453378
	31.16	gi|195972866	keratin, type I cytoskeletal 10 [Homo sapiens]	18	0.99279	0.96579	1.17686	0.25241	1.082097	0.65185	1.1831	0.40404	1.176889	0.26041	1.15457	0.453813
1.82	8.824	gi|72534660	serine/arginine-rich splicing factor 7 isoform 1 [Homo sapiens]	2	1.01438	0.96006	1.32191	0.462731	1.03348	0.93519	0.9051	0.57763	1.129918	0.43756	1.25841	0.454052
	3.239	gi|21361282	serine/arginine-rich splicing factor 4 [Homo sapiens]	2	0.86496	0.58839	0.9941	0.985146	0.989135	0.93152	0.69451	0.50631	0.947242	0.68143	0.83973	0.454583
	24.34	gi|11968182	40S ribosomal protein S18 [Homo sapiens]	4	0.9108	0.17598	0.9615	0.565209	0.836623	0.29264	0.96303	0.84958	1.048824	0.83849	0.92731	0.46041
9.36	13.58	gi|62750354	matrin-3 isoform a [Homo sapiens]	11	1.04516	0.83428	1.19344	0.652837	1.008595	0.97584	0.94902	0.77475	1.150566	0.455	1.23023	0.463137
	9.359	gi|71773106	AP-2 complex subunit beta isoform a [Homo sapiens]	6	1.03159	0.80628	0.90953	0.514151	0.860359	0.37066	0.88525	0.43526	0.974676	0.93431	0.80142	0.464299

5.49	20.15	gi|51599151	calpain small subunit 1 [Homo sapiens]	3	1.0993	0.90564	1.2801	0.713734	1.693094	0.48935	1.52327	0.58126	0.975995	0.97003	1.2446	0.464515
6.79	1.756	gi|91208426	pre-mRNA-processing-splicing factor 8 [Homo sapiens]	4	0.74541	0.52013	0.93731	0.762332	0.802356	0.39367	0.788	0.63705	0.875564	0.80852	0.82589	0.46519
	8.029	gi|13904866	60S ribosomal protein L28 isoform 2 [Homo sapiens]	1	1.1852	0.61711	1.19004	0.350877	1.095949	0.54876	1.1129	0.49895	1.056091	0.72529	1.26634	0.469429
	22.18	gi|4506663	60S ribosomal protein L8 [Homo sapiens]	3	1.34108	0.1674	1.18917	0.596438	0.934418	0.82729	1.33352	0.31409	1.327422	0.05463	1.3069	0.473156
59.02	46.15	gi|4503483	elongation factor 2 [Homo sapiens]	39	0.94928	0.36084	1.04053	0.380718	1.053971	0.35544	0.9786	0.75631	0.929768	0.18854	1.04154	0.473738
4.45	4.67	gi|197276600	microtubule-associated protein 4 isoform 4 [Homo sapiens]	4	1.27162	0.35766	1.30783	0.138875	0.971935	0.77141	1.24298	0.34491	1.142233	0.44348	1.17	0.47386
	62.4	gi|20070125	protein disulfide-isomerase precursor [Homo sapiens]	36	0.9796	0.56599	0.92769	0.057465	0.928316	0.02639	0.96119	0.35188	1.007923	0.82127	0.97761	0.477783
8.82	44.44	gi|5031749	non-histone chromosomal protein HMG-17 [Homo sapiens]	7	1.02829	0.76222	0.94558	0.568522	0.847099	0.15126	0.96638	0.60275	0.990646	0.88526	0.95379	0.477923
8.82	44.44	gi|5031749	non-histone chromosomal protein HMG-17 [Homo sapiens]	7	1.02829	0.76222	0.94558	0.568522	0.847099	0.15126	0.96638	0.60275	0.990646	0.88526	0.95379	0.477923
	9.854	gi|48762932	T-complex protein 1 subunit theta [Homo sapiens]	4	0.92715	0.48706	0.96602	0.82075	0.929325	0.69985	0.87594	0.5857	1.082928	0.61024	0.86331	0.478863
	2.383	gi|54112121	splicing factor 3B subunit 3 [Homo sapiens]	3	0.81032	0.48085	0.98602	0.920135	1.05857	0.67167	0.5949	0.49536	1.079662	0.84245	0.89031	0.48195

	7.161	gi|4758756	nucleosome assembly protein 1-like 1 [Homo sapiens]	2	1.062	0.82004	1.2756	0.571647	1.408068	0.46674	1.48367	0.24859	1.196134	0.33178	1.30001	0.484108
	62.57	gi|5453740	myosin regulatory light chain 12A [Homo sapiens]	12	1.03618	0.53408	1.07574	0.396769	1.10096	0.25569	1.06196	0.4021	1.059293	0.3758	0.9605	0.484747
	11.87	gi|373251164	glutaminase kidney isoform, mitochondrial isoform 2 [Homo sapiens]	6	0.74804	0.21256	0.77018	0.315563	0.807316	0.31161	0.7773	0.4977	0.717889	0.29766	0.84106	0.487298
2.85	1.876	gi|41393608	reticulon-3 isoform b [Homo sapiens]	2	0.80302	0.36014	0.86762	0.389095	0.664497	0.27998	0.77081	0.7731	0.900955	0.48418	0.7174	0.493207
2.9	8.914	gi|156564403	pyruvate dehydrogenase E1 component subunit beta, mitochondrial isoform 1 precursor [Homo sapiens]	2	1.45512	0.16682	1.33691	0.487042	1.523417	0.43403	1.45412	0.24868	1.41206	0.69537	1.298	0.49854
51.12	71.17	gi|29788785	tubulin beta chain [Homo sapiens]	51	0.90405	0.36942	0.91409	0.416252	1.033772	0.7416	0.9256	0.47261	0.917076	0.43044	1.07465	0.499031
	8.385	gi|17402900	far upstream element-binding protein 1 [Homo sapiens]	6	1.03031	0.66506	1.10446	0.493208	0.912083	0.35392	1.08702	0.37794	1.001769	0.9928	1.14004	0.499632
	14.91	gi|5453832	hypoxia up-regulated protein 1 precursor [Homo sapiens]	10	1.03014	0.81577	0.97909	0.8833	0.952214	0.64282	0.98593	0.92964	0.952431	0.60746	0.87514	0.500821
5.32	5.253	gi|66933016	inosine-5'-monophosphate dehydrogenase 2 [Homo sapiens]	4	1.03226	0.89524	1.09494	0.50848	1.268973	0.42403	1.3762	0.15979	1.245079	0.48519	1.19792	0.502222

2.63	5.122	gi|4504957	lysosome-associated membrane glycoprotein 2 isoform A precursor [Homo sapiens]	2	0.54855	0.55156	1.08974	0.849683	0.484158	0.57545	0.37086	0.39568	0.6991	0.54638	0.51917	0.506245
4.23	31.03	gi|4826848	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5 [Homo sapiens]	2	1.46828	0.41391	1.14203	0.815197	0.917116	0.54645	1.22387	0.73155	1.096227	0.88499	1.28746	0.506837
6.17	6.658	gi|4503131	catenin beta-1 [Homo sapiens]	3	0.41141	0.15644	0.66533	0.51162	0.503723	0.46341	0.41106	0.25461	0.599967	0.48447	0.41318	0.507001
63.94	40.4	gi|12025678	alpha-actinin-4 [Homo sapiens]	36	0.97761	0.81249	1.05219	0.64193	1.086704	0.45213	1.05716	0.61573	0.980235	0.82704	1.07302	0.511242
	15.43	gi|332801090	heterogeneous nuclear ribonucleoprotein D-like isoform b [Homo sapiens]	6	1.00871	0.91679	1.05032	0.446338	0.913776	0.26441	0.98511	0.90239	1.054689	0.41219	0.89372	0.512474
	44.33	gi|4507677	endoplasmin precursor [Homo sapiens]	42	0.9961	0.93353	0.94848	0.248495	0.968327	0.51543	0.9863	0.81623	0.976286	0.57443	0.96779	0.512923
	4.478	gi|5803149	transmembrane emp24 domain-containing protein 2 precursor [Homo sapiens]	1	1.41839	0.40694	1.2308	0.287656	1.26119	0.2614	1.52491	0.1496	1.161351	0.65161	1.39668	0.513323
2.036	2.25	gi|5174735	tubulin beta-4B chain [Homo sapiens]	42	0.85065	0.43007	0.9246	0.575418	0.790037	0.41365	0.90389	0.49652	1.074749	0.66239	1.09878	0.518424
11.66	16.6	gi|19923653	PC4 and SFRS1-interacting protein isoform 2 [Homo sapiens]	8	1.17679	0.73143	1.56402	0.358592	0.968929	0.9434	1.46777	0.32185	1.363601	0.25718	1.28947	0.522585

5.85	10.4	gi|60279268	splicing factor U2AF 65 kDa subunit isoform b [Homo sapiens]	4	1.2201	0.66253	1.02211	0.952784	1.130686	0.83639	0.93141	0.83825	1.173532	0.5247	1.13973	0.52352
	7.914	gi|112380628	lysosome-associated membrane glycoprotein 1 precursor [Homo sapiens]	3	0.92406	0.43426	1.07253	0.481096	0.862072	0.21099	0.78483	0.09717	0.937752	0.51208	0.78737	0.525828
17.81	20.36	gi|4758138	probable ATP-dependent RNA helicase DDX5 [Homo sapiens]	9	1.02008	0.93999	1.03103	0.885579	1.087795	0.60812	0.90254	0.59855	0.900662	0.697	0.85903	0.527072
2.84	15.89	gi|22208971	high mobility group protein HMG-I/HMG-Y isoform a [Homo sapiens]	2	1.16637	0.22879	1.11889	0.314787	0.942611	0.76233	0.96675	0.72794	1.098282	0.38348	1.06576	0.530562
2.52	2.347	gi|30581111	minor histocompatibility antigen H13 isoform 3 [Homo sapiens]	1	0.96889	0.88579	1.0853	0.672652	0.932619	0.85235	0.80742	0.27991	0.966258	0.79095	0.57797	0.530633
	16.9	gi|5031755	heterogeneous nuclear ribonucleoprotein R isoform 2 [Homo sapiens]	12	1.1786	0.36141	1.03955	0.91766	1.411819	0.05493	1.20407	0.33353	1.512006	0.06272	1.34337	0.531745
	3.639	gi|21361361	acetolactate synthase-like protein [Homo sapiens]	2	0.96311	0.95496	1.11329	0.773986	1.2958	0.74466	1.02802	0.94228	0.89748	0.78731	1.2972	0.533523
	4.592	gi|4506609	60S ribosomal protein L19 [Homo sapiens]	1	1.28745	0.58224	1.12827	0.259487	1.136556	0.75982	1.21129	0.29878	1.386144	0.3114	1.23701	0.533725
24.42	42.35	gi|5453539	multifunctional protein ADE2 isoform 2 [Homo sapiens]	14	1.00356	0.9713	1.00013	0.998729	1.148299	0.30568	1.09789	0.39572	0.967332	0.82108	1.06801	0.541305

33.86	6.371	gi|126032350	DNA-dependent protein kinase catalytic subunit isoform 2 [Homo sapiens]	22	1.20563	0.32156	1.04779	0.533682	1.107957	0.44994	1.05744	0.66483	1.165061	0.4342	1.10473	0.543078
6.03	17.34	gi|4502969	catechol O-methyltransferase isoform MB-COMT [Homo sapiens]	4	0.93424	0.61858	0.80864	0.279603	0.927369	0.90939	1.13765	0.41947	0.713404	0.1827	0.77919	0.546947
7.11	29.53	gi|5031851	stathmin isoform a [Homo sapiens]	5	1.00604	0.96349	1.00856	0.924299	1.159543	0.4121	0.99103	0.96627	0.904638	0.20568	1.16846	0.547923
26.6	25.31	gi|119395750	keratin, type II cytoskeletal 1 [Homo sapiens]	19	0.9346	0.43611	0.95497	0.621952	1.108099	0.40979	1.19886	0.10667	1.200578	0.05109	1.04931	0.549805
1.66	10.73	gi|4505641	proliferating cell nuclear antigen [Homo sapiens]	3	0.95874	0.74344	0.76238	0.351143	0.672644	0.18698	0.43342	0.07491	0.500235	0.27736	0.56731	0.556316
17.5	14.43	gi|24234750	interleukin enhancer-binding factor 3 isoform a [Homo sapiens]	10	1.2214	0.42885	1.08192	0.713069	1.193625	0.35476	1.21833	0.49341	0.91235	0.70867	1.04385	0.557202
17.62	37.67	gi|4501891	alpha-actinin-1 isoform b [Homo sapiens]	33	1.14984	0.38813	1.13194	0.32002	1.205048	0.21244	1.01236	0.92856	1.162788	0.20884	1.08371	0.560889
2.12	7.034	gi|4826734	RNA-binding protein FUS isoform 1 [Homo sapiens]	4	0.94968	0.70164	0.86002	0.376919	0.888291	0.45267	0.98973	0.93593	0.969715	0.81381	0.92116	0.568145
4.09	5.954	gi|295842307	splicing factor 1 isoform 4 [Homo sapiens]	3	0.94439	0.91318	1.24603	0.673259	0.931656	0.89132	1.241	0.722	1.023873	0.94625	1.28342	0.570229
5.48	6.397	gi|311078508	histone-binding protein RBBP7 isoform 1 [Homo sapiens]	3	1.10172	0.52866	0.99141	0.969002	0.914266	0.62674	0.9097	0.7392	1.184806	0.19987	0.89257	0.571518
9.97	6.485	gi|33620775	kinectin isoform a [Homo sapiens]	8	1.05966	0.69111	1.19364	0.149146	1.159778	0.26147	0.79517	0.22887	0.90011	0.62253	0.90417	0.572624

	1.025	gi|32967311	ephrin type-A receptor 2 precursor [Homo sapiens]	1	0.9746	0.94795	1.05692	0.85878	1.210665	0.32138	1.1077	0.56043	1.387491	0.37876	1.08739	0.574518
40.89	35.24	gi|6005942	transitional endoplasmic reticulum ATPase [Homo sapiens]	25	0.97357	0.71935	0.95857	0.555716	1.12462	0.17086	1.01768	0.8572	0.946802	0.38291	0.95645	0.575353
	21.4	gi|78000183	60S ribosomal protein L14 [Homo sapiens]	5	1.04335	0.72706	0.99861	0.994141	0.968287	0.89286	1.18263	0.33665	0.920667	0.68297	0.89227	0.575757
	1.903	gi|4504747	integrin alpha-3 isoform a precursor [Homo sapiens]	2	1.226	0.2114	0.8335	0.417073	0.827589	0.60291	1.01131	0.98103	1.119362	0.30086	1.06507	0.578152
	10.41	gi|91199540	dihydrolipoyl dehydrogenase, mitochondrial precursor [Homo sapiens]	4	1.1821	0.51111	1.02828	0.834679	1.282727	0.58737	1.05334	0.70556	1.129976	0.66848	1.19902	0.58019
2.25	3.558	gi|25777612	26S proteasome non-ATPase regulatory subunit 3 [Homo sapiens]	2	0.8381	0.48744	1.10067	0.583873	1.099142	0.73254	0.63133	0.3464	0.970719	0.81572	0.63441	0.581959
11.11	11.62	gi|48255891	glucosidase 2 subunit beta isoform 2 precursor [Homo sapiens]	6	1.03946	0.67934	1.13566	0.081367	1.012411	0.9142	1.13026	0.29235	1.162264	0.01179	1.05193	0.584009
18.97	17.57	gi|4503841	X-ray repair cross-complementing protein 6 [Homo sapiens]	11	0.97292	0.81085	1.00878	0.880034	0.998476	0.98044	0.92188	0.2548	1.058863	0.64942	0.9691	0.58695
	20.54	gi|4506455	reticulocalbin-1 precursor [Homo sapiens]	4	0.9615	0.61475	1.05458	0.619085	0.942759	0.64057	1.04202	0.67339	0.937237	0.68695	0.92712	0.58783
11.48	49.46	gi|7661678	ras-related protein Rap-1b isoform 1 precursor [Homo sapiens]	9	1.04781	0.8059	0.91308	0.460369	1.104292	0.36436	1.11936	0.62485	0.863622	0.22512	1.13417	0.589483

		Accession	Protein	Count												
	18.53	gi|57863257	T-complex protein 1 subunit alpha isoform a [Homo sapiens]	6	0.99365	0.97173	1.10198	0.768095	0.820472	0.46263	0.92611	0.65421	1.094258	0.72633	1.15002	0.592124
4	19.33	gi|4506697	40S ribosomal protein S20 isoform 2 [Homo sapiens]	2	1.16724	0.3705	1.38228	0.267105	1.193196	0.66439	1.42943	0.38635	1.142585	0.66025	1.2463	0.592284
	5.183	gi|70995211	delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial precursor [Homo sapiens]	1	1.02368	0.95347	1.20449	0.732047	0.8886	0.71644	0.98045	0.87509	1.373089	0.27118	1.07641	0.594433
	15.23	gi|68160922	40S ribosomal protein S14 [Homo sapiens]	2	0.89167	0.72053	1.24591	0.353475	1.058112	0.75346	1.02913	0.93338	1.107532	0.68517	1.16347	0.601089
23.39	50	gi|4758950	peptidyl-prolyl cis-trans isomerase B precursor [Homo sapiens]	15	0.96317	0.36944	0.91885	0.055881	0.933665	0.26808	0.99647	0.94972	0.960674	0.37431	0.96843	0.603144
	9.423	gi|154355000	far upstream element-binding protein 2 [Homo sapiens]	8	1.08689	0.50836	1.05982	0.68563	0.932229	0.61558	1.1761	0.473	1.185571	0.19675	1.09494	0.609018
3.71	14.94	gi|62244044	OCIA domain-containing protein 2 isoform 1 [Homo sapiens]	2	1.06593	0.75131	1.07831	0.465847	0.860725	0.46251	0.71107	0.43904	0.881257	0.32917	0.84963	0.609097
6.07	43.44	gi|4885377	histone H1.3 [Homo sapiens]	14	1.04454	0.53785	0.93529	0.437735	0.754462	0.01216	1.21122	0.38623	1.178831	0.3691	0.96051	0.612951
	19.31	gi|4506695	40S ribosomal protein S19 [Homo sapiens]	3	1.18345	0.60921	1.09019	0.861537	0.929737	0.8499	1.1507	0.69475	1.276557	0.41324	1.20276	0.61489
	22.49	gi|354721184	ras-related protein Rab-5C isoform b [Homo sapiens]	5	0.9894	0.89493	1.06862	0.461529	1.002682	0.98651	0.96801	0.8683	0.969061	0.76529	1.08543	0.617855
	19.23	gi|4506743	40S ribosomal protein S8 [Homo sapiens]	7	1.17932	0.49985	1.18919	0.299259	1.224889	0.46026	1.20005	0.31305	1.077038	0.79546	1.1569	0.618747

25.41		gi|40254924	leucine-rich repeat-containing protein 59 [Homo sapiens]	7	1.17331	0.02623	1.05979	0.327971	0.949168	0.65078	1.1888	0.01927	1.124964	0.16462	1.04358	0.620272
46.54	43.1	gi|4758304	protein disulfide-isomerase A4 precursor [Homo sapiens]	29	0.94767	0.13639	0.94301	0.132998	0.926221	0.06328	0.91517	0.09507	0.961057	0.40127	1.02263	0.620681
183.84	45.56	gi|12667788	myosin-9 [Homo sapiens]	131	0.98833	0.75974	0.97703	0.492793	1.006742	0.82829	0.98619	0.77237	0.960691	0.2	1.01558	0.622695
10.97		gi|61743954	neuroblast differentiation-associated protein AHNAK isoform 1 [Homo sapiens]	12	0.96465	0.7331	1.01859	0.841517	0.948976	0.63379	0.99368	0.95963	1.066503	0.5159	0.95444	0.625648
4.3 8.462		gi|42734430	polymerase I and transcript release factor [Homo sapiens]	3	0.95085	0.86297	0.89311	0.699758	0.803026	0.67284	0.87765	0.59893	0.805946	0.69619	0.89028	0.628441
17.87		gi|34147513	ras-related protein Rab-7a [Homo sapiens]	5	0.95351	0.73379	0.76061	0.213252	0.854884	0.35362	0.81528	0.41645	1.001302	0.98901	0.94089	0.631549
17.26		gi|228008400	heterogeneous nuclear ribonucleoprotein Q isoform 6 [Homo sapiens]	14	1.21817	0.42309	1.24361	0.309094	1.08515	0.76039	1.27522	0.33853	1.195927	0.2873	1.09644	0.632009
35.05		gi|4506741	40S ribosomal protein S7 [Homo sapiens]	6	1.00409	0.9778	0.96526	0.812921	0.909794	0.66104	0.9513	0.54511	0.966876	0.7708	0.92787	0.634911
60.5		gi|4757826	beta-2-microglobulin precursor [Homo sapiens]	6	1.26003	0.50144	0.8665	0.306054	1.14102	0.77709	1.42354	0.55364	0.945291	0.79075	0.8252	0.638802
9.813		gi|223890243	60S ribosomal protein L10 isoform a [Homo sapiens]	2	1.14824	0.20993	1.03798	0.754032	1.268798	0.08304	1.15213	0.28899	1.087423	0.68281	1.04897	0.641001

16.25	45.18	gi|153070260	myristoylated alanine-rich C-kinase substrate [Homo sapiens]	9	0.983	0.80578	0.9737	0.694966	0.988063	0.84891	0.99415	0.92598	0.997562	0.96911	1.03013	0.641041
34.42	55.7	gi|156071459	ADP/ATP translocase 2 [Homo sapiens]	29	1.03376	0.68358	0.9262	0.376592	0.988751	0.95299	0.97673	0.78837	0.96945	0.77703	0.9515	0.641079
2.17	0.4867	gi|54607053	translational activator GCN1 [Homo sapiens]	1	1.28676	0.24123	1.07193	0.906543	0.741843	0.68752	0.71728	0.66122	1.331493	0.53099	0.83414	0.644476
2.39	5.917	gi|4502981	cytochrome c oxidase subunit 4 isoform 1, mitochondrial precursor [Homo sapiens]	1	0.86484	0.25424	0.99029	0.964486	1.020714	0.83282	0.8429	0.23318	1.026772	0.81804	1.0846	0.645195
1.43	0.9151	gi|29826341	calmodulin-binding transcription activator 2 isoform 1 [Homo sapiens]	2	1.02264	0.97348	1.1667	0.370435	1.013866	0.97412	1.00199	0.99841	0.997553	0.99617	0.82669	0.646072
18	89.63	gi|4504981	galectin-1 [Homo sapiens]	13	0.94609	0.56748	1.06686	0.337019	0.998775	0.99189	0.98275	0.86625	0.982519	0.85361	0.96945	0.646657
30.18	31.63	gi|34932414	non-POU domain-containing octamer-binding protein isoform 1 [Homo sapiens]	18	1.02179	0.71872	0.95014	0.56712	0.987357	0.85793	1.00228	0.97442	0.979062	0.76057	0.95323	0.661185
20.86	21.51	gi|18201905	glucose-6-phosphate isomerase isoform 2 [Homo sapiens]	14	0.8891	0.11886	0.99188	0.914596	1.007987	0.93038	0.92749	0.67746	1.005971	0.94939	1.03989	0.662333
1.69	10.67	gi|4758040	cytochrome c oxidase subunit 6C proprotein [Homo sapiens]	2	0.85609	0.37626	1.19072	0.565045	0.999303	0.99786	1.33632	0.67643	1.130728	0.64572	1.06285	0.662841
6.13	6.647	gi|87196351	ATP-dependent RNA helicase DDX3X isoform 1 [Homo sapiens]	3	1.18019	0.51813	1.21877	0.602725	0.919683	0.75389	0.73428	0.20041	1.18035	0.41588	1.05972	0.665489

	gi	Protein	Count												
58.07	gi|14043072	heterogeneous nuclear ribonucleoproteins A2/B1 isoform B1 [Homo sapiens]	24	0.99729	0.99214	0.89746	0.632954	0.951845	0.82147	1.04794	0.90827	0.952007	0.78644	0.91491	0.666499
15.36	gi|15055539	40S ribosomal protein S2 [Homo sapiens]	4	0.98191	0.94926	1.00864	0.979743	0.878588	0.57863	1.02254	0.91929	0.956993	0.88491	1.1123	0.670417
11.41	gi|4506649	60S ribosomal protein L3 isoform a [Homo sapiens]	3	0.86881	0.71238	0.92019	0.286429	0.938384	0.8218	0.99791	0.9954	0.829736	0.47181	0.90558	0.673162
3.95 2.855	gi|122939155	NAD(P) transhydrogenase, mitochondrial [Homo sapiens]	3	1.00797	0.94948	1.13779	0.597416	1.143784	0.49699	0.8888	0.4475	0.881076	0.48103	1.13072	0.673925
29.93	gi|63252900	tropomyosin alpha-1 chain isoform 4 [Homo sapiens]	10	1.08825	0.37639	1.04674	0.732007	1.130557	0.28152	1.16461	0.14713	0.981288	0.89392	1.04555	0.674296
22.02 20.65	gi|4826998	splicing factor, proline- and glutamine-rich [Homo sapiens]	19	0.97461	0.91414	1.03224	0.88877	0.887348	0.62919	0.97859	0.93981	1.022428	0.92594	0.90595	0.674537
17.62	gi|251831110	cytochrome c oxidase subunit II [Homo sapiens]	3	0.80403	0.69103	0.86297	0.299261	0.722846	0.28015	0.6475	0.10402	0.866816	0.66375	0.91801	0.675132
19.68	gi|4506607	60S ribosomal protein L18 isoform 1 [Homo sapiens]	3	0.96035	0.7603	0.87805	0.465616	0.962498	0.839	0.88382	0.53872	1.032148	0.81521	0.89035	0.675519
74.51	gi|4504523	10 kDa heat shock protein, mitochondrial [Homo sapiens]	8	0.99268	0.88544	0.97341	0.600927	0.974775	0.61892	0.97012	0.56798	0.974523	0.6162	1.02344	0.675919
19.44	gi|67189747	60S ribosomal protein L6 [Homo sapiens]	5	1.02062	0.76923	0.84308	0.303084	1.054461	0.51107	1.04638	0.62651	0.978707	0.85244	0.95504	0.677122
29.17	gi|67944630	60S ribosomal protein L9 [Homo sapiens]	5	1.15467	0.38103	1.15642	0.137453	1.229212	0.2788	0.96642	0.85104	1.288677	0.12373	0.93631	0.677782

2.18	2.71	gi|19923193	hsc70-interacting protein [Homo sapiens]	1	0.95037	0.83989	0.96798	0.821141	0.800799	0.76442	1.21002	0.30454	0.833078	0.31592	0.84992	0.678324
13.2	29.47	gi|55956921	heterogeneous nuclear ribonucleoprotein A/B isoform b [Homo sapiens]	9	1.0958	0.21875	0.95419	0.514181	0.998728	0.98187	1.06164	0.42607	1.077823	0.36657	1.03855	0.682577
4.49	15.75	gi|217330646	activated RNA polymerase II transcriptional coactivator p15 [Homo sapiens]	2	1.00545	0.95064	0.93514	0.513028	0.866581	0.5256	0.9196	0.27018	0.941666	0.70261	0.93123	0.689454
7.02	12.25	gi|10835067	lupus La protein [Homo sapiens]	4	0.89702	0.81509	1.02335	0.970048	0.912524	0.7839	0.7299	0.73114	0.740666	0.69847	0.84575	0.690483
4.6	48.99	gi|156071462	ADP/ATP translocase 3 [Homo sapiens]	27	1.12804	0.72644	1.12233	0.745559	1.261938	0.52519	1.17129	0.68662	1.088808	0.69381	1.11963	0.691645
4.14	16.17	gi|39725636	transmembrane emp24 domain-containing protein 9 precursor [Homo sapiens]	3	1.09319	0.53296	1.04374	0.761259	1.333761	0.26105	0.73663	0.19907	0.970606	0.87339	1.08416	0.691946
4.38	12.82	gi|16117789	60S ribosomal protein L34 [Homo sapiens]	2	0.87852	0.5603	0.88838	0.470693	0.772053	0.45763	0.59708	0.13049	0.960721	0.77356	0.75407	0.692607
28.84	30.2	gi|50345984	ATP synthase subunit alpha, mitochondrial isoform a precursor [Homo sapiens]	21	0.95209	0.43118	0.97584	0.817633	0.944751	0.56027	0.99797	0.98171	0.942627	0.43909	1.03342	0.693136
20.15	55.88	gi|4504297	histone H3.1 [Homo sapiens]	20	1.11344	0.49506	0.89675	0.505913	0.998635	0.99393	1.19646	0.59173	0.925642	0.59705	0.94698	0.696575
19.47	39.46	gi|221307584	prohibitin-2 isoform 1 [Homo sapiens]	14	1.03343	0.73666	0.93609	0.6425	0.962379	0.72427	0.95351	0.83651	0.943864	0.59801	1.0389	0.697615
18.66	33.42	gi|93141020	core histone macro-H2A.1 isoform 2 [Homo sapiens]	12	1.03657	0.80111	0.98151	0.885185	1.032144	0.80074	0.93467	0.77516	1.039574	0.78897	0.94747	0.698714

		Accession	Protein	Count												
	17.45	gi|47132595	phosphate carrier protein, mitochondrial isoform b precursor [Homo sapiens]	5	1.06729	0.32103	0.95402	0.461468	1.101687	0.16278	0.99838	0.98638	1.049733	0.44725	1.04113	0.699173
	27.91	gi|17017988	cytochrome c oxidase subunit 5B, mitochondrial precursor [Homo sapiens]	4	1.01292	0.91838	1.27302	0.344714	0.87403	0.55306	0.53226	0.34154	1.014991	0.92652	0.92066	0.701532
	11.59	gi|48255935	CD44 antigen isoform 1 precursor [Homo sapiens]	9	0.99745	0.96815	1.05606	0.470139	0.974859	0.77069	1.02163	0.80288	0.952887	0.7142	0.95638	0.702046
6.26	41.78	gi|83376130	elongation factor 1-beta [Homo sapiens]	5	1.06939	0.52176	0.99888	0.998173	1.26491	0.11474	1.26122	0.61166	1.299958	0.42094	1.17718	0.702982
24.64	16.54	gi|23510340	ubiquitin-like modifier-activating enzyme 1 [Homo sapiens]	17	0.86945	0.13185	0.89243	0.154395	1.047217	0.59847	0.8588	0.06201	0.98632	0.84641	1.03481	0.70466
2.08	13.04	gi|5453559	ATP synthase subunit d, mitochondrial isoform a [Homo sapiens]	1	1.54649	0.1424	1.23522	0.663545	1.086338	0.55674	1.28814	0.2495	1.425763	0.40471	1.21003	0.705296
12.34	2.626	gi|41322916	plectin isoform 1 [Homo sapiens]	10	0.95217	0.8024	1.34488	0.401625	1.034696	0.93365	1.19649	0.63772	1.423922	0.15327	1.12662	0.707928
	32.53	gi|14043070	heterogeneous nuclear ribonucleoprotein A1 isoform b [Homo sapiens]	23	1.05591	0.75886	0.96459	0.690319	1.149365	0.41417	1.20924	0.58318	1.019104	0.89852	1.07093	0.708404
4.16	12.32	gi|6912634	60S ribosomal protein L13a isoform 1 [Homo sapiens]	3	0.95479	0.81055	0.87337	0.259546	1.140071	0.34559	0.89491	0.55672	1.120049	0.45983	0.95566	0.711813
10.8	26.28	gi|21614499	ezrin [Homo sapiens]	15	1.06182	0.49832	1.05333	0.555483	1.307711	0.03294	0.94707	0.71822	1.133167	0.4463	0.89818	0.712349

	gi	Protein	Count												
21.58	gi|4506675	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 precursor [Homo sapiens]	12	1.05826	0.57928	0.982	0.858546	0.991824	0.94215	1.03226	0.81042	0.962578	0.72136	1.03882	0.713397
14.9	gi|19743875	fumarate hydratase, mitochondrial [Homo sapiens]	5	1.23102	0.54474	0.98192	0.924664	1.139205	0.30063	0.85337	0.73457	1.058899	0.90011	1.1213	0.713871
15.3	gi|52630342	HLA class I histocompatibility antigen, Cw-1 alpha chain precursor [Homo sapiens]	6	0.97171	0.88809	1.02558	0.936087	1.027002	0.79844	0.88162	0.32984	0.853302	0.58819	0.90592	0.715453
65.58	gi|380837121	vesicle-trafficking protein SEC22b precursor [Homo sapiens]	20	1.10024	0.05719	1.03229	0.495044	1.038818	0.5283	1.06556	0.32572	0.996953	0.97062	1.03531	0.723297
5.752	gi|394025723	very long-chain specific acyl-CoA dehydrogenase, mitochondrial isoform 3 [Homo sapiens]	3	0.74699	0.59182	0.76043	0.55318	0.988837	0.95255	0.65897	0.26758	0.911972	0.79251	0.72594	0.725427
22.11	gi|42476281	voltage-dependent anion-selective channel protein 2 isoform 2 [Homo sapiens]	7	0.98794	0.90701	0.94137	0.558142	0.967298	0.81086	1.19774	0.20741	0.999744	0.998	0.97538	0.726116
7.612	gi|21536286	creatine kinase B-type [Homo sapiens]	2	0.93056	0.85493	0.75114	0.745502	0.851942	0.75556	0.70757	0.23455	0.726465	0.72965	0.86017	0.728049
22.58	gi|15431288	60S ribosomal protein L10a [Homo sapiens]	5	1.13923	0.20613	1.19379	0.097845	1.405531	0.07869	1.30502	0.03712	1.272751	0.04776	1.06428	0.731592

6.093	gi|4885281	glutamate dehydrogenase 1, mitochondrial precursor [Homo sapiens]	3	1.03466	0.82658	1.31687	0.384031	0.988878	0.95217	1.03104	0.84229	0.9713	0.90753	1.04757	0.734971
39.92	gi|378548190	40S ribosomal protein S3 isoform 1 [Homo sapiens]	10	1.05379	0.67765	0.90108	0.325676	0.998495	0.98928	0.95251	0.73441	1.080897	0.54587	1.05154	0.736446
28.89	gi|4502205	ADP-ribosylation factor 4 [Homo sapiens]	5	1.12509	0.85061	0.88053	0.643544	1.213056	0.76438	0.7777	0.57084	0.860941	0.67797	0.83245	0.740092
11.04	gi|52632383	heterogeneous nuclear ribonucleoprotein L isoform a [Homo sapiens]	6	1.1947	0.19772	1.03175	0.790467	1.158331	0.37641	1.06595	0.63342	0.987748	0.89712	1.04132	0.742688
10.65	gi|321117084	RPS10-NUDT3 protein [Homo sapiens]	3	1.01139	0.94355	1.0143	0.970992	0.922407	0.5861	1.00405	0.96666	1.059227	0.64926	0.96851	0.743459
50.71	gi|4506605	60S ribosomal protein L23 [Homo sapiens]	5	0.87865	0.74322	0.95354	0.771566	0.815871	0.53752	1.04347	0.881	0.760368	0.11783	0.92817	0.750798
18.67	gi|190885499	cytochrome c oxidase subunit 5A, mitochondrial precursor [Homo sapiens]	3	1.04812	0.65287	1.12766	0.567805	0.993509	0.97116	0.77747	0.09934	1.026415	0.78893	1.04016	0.752238
27.27	gi|194688135	high mobility group protein B2 [Homo sapiens]	7	1.06464	0.85993	1.01726	0.972515	1.015592	0.97689	1.02219	0.96616	0.999257	0.99421	0.91965	0.760339
21.92	gi|98986464	transmembrane emp24 domain-containing protein 10 precursor [Homo sapiens]	6	0.88786	0.72101	0.78503	0.248243	0.818962	0.61315	0.91388	0.79432	0.896156	0.53912	0.92621	0.763469
4.637	gi|19913406	DNA topoisomerase 2-alpha [Homo sapiens]	7	1.19997	0.81665	1.39591	0.184234	1.329365	0.30032	1.49119	0.45954	1.127594	0.8554	1.14999	0.764389

			Protein	Count												
19.02		gi|5803036	heterogeneous nuclear ribonucleoprotein A0 [Homo sapiens]	5	1.05968	0.83796	0.97227	0.854566	1.114078	0.49743	1.07709	0.79112	1.012741	0.96513	1.05259	0.765253
19.03	48.66	gi|4758638	peroxiredoxin-6 [Homo sapiens]	9	0.96744	0.76701	1.1843	0.204936	1.193197	0.07085	0.98306	0.94703	1.00903	0.97102	1.07972	0.767286
10.43		gi|4502285	sarcoplasmic/endoplasmic reticulum calcium ATPase 2 isoform a [Homo sapiens]	7	1.10151	0.66208	1.04782	0.815902	1.335057	0.31594	1.0606	0.84338	1.01103	0.93739	1.06943	0.767545
10.29	26.43	gi|73760405	thymopoietin isoform beta [Homo sapiens]	7	1.05181	0.61144	1.12399	0.350742	0.91077	0.54197	0.84554	0.35827	0.975897	0.80234	0.96391	0.768285
73.91		gi|4506671	60S acidic ribosomal protein P2 [Homo sapiens]	7	1.08556	0.34581	1.0435	0.605096	1.058493	0.49566	1.11581	0.23312	1.056699	0.50924	1.0239	0.768286
5.06	12.77	gi|163644321	cytochrome b-c1 complex subunit Rieske, mitochondrial [Homo sapiens]	3	0.75455	0.71922	0.57239	0.407115	0.771567	0.45298	0.86607	0.39779	0.948623	0.69406	0.84441	0.772512
4.75	14.44	gi|311771647	actin-related protein 2/3 complex subunit 4 isoform c [Homo sapiens]	2	1.08265	0.87616	0.96313	0.925078	1.106502	0.78565	0.72989	0.67988	0.828008	0.34231	1.1508	0.773513
65.79		gi|41399285	60 kDa heat shock protein, mitochondrial [Homo sapiens]	57	1.00869	0.83404	1.0126	0.787426	1.000185	0.99618	0.99905	0.9821	0.998881	0.96892	1.00977	0.773751
36.93		gi|14110414	heterogeneous nuclear ribonucleoprotein D0 isoform c [Homo sapiens]	11	0.99292	0.95083	0.93943	0.765849	0.999996	1	0.90384	0.55038	0.911699	0.637	0.96899	0.774905
31.82		gi|14277700	40S ribosomal protein S12 [Homo sapiens]	5	1.01814	0.89192	0.83278	0.636779	1.036141	0.79088	1.09946	0.53195	0.947092	0.70597	1.03925	0.774943

43.03		gi|4506597	60S ribosomal protein L12 [Homo sapiens]	5	1.12104	0.41768	0.99867	0.991624	1.142792	0.27762	1.15111	0.36571	1.168818	0.22156	0.97404 0.775656
2.14	1.183	gi|69354671	ATP-binding cassette sub-family F member 1 isoform a [Homo sapiens]	1	0.97085	0.91323	1.37039	0.194106	1.471155	0.16004	0.99946	0.99909	1.19076	0.84283	0.88838 0.777605
15.77		gi|38455427	T-complex protein 1 subunit delta isoform a [Homo sapiens]	9	1.00106	0.99697	0.9324	0.78378	0.94696	0.89777	0.73934	0.19109	0.953969	0.86496	0.88244 0.781297
3.55	3.568	gi|25777600	26S proteasome non-ATPase regulatory subunit 1 isoform 1 [Homo sapiens]	2	0.5996	0.2571	0.72519	0.44323	0.696634	0.17297	0.78612	0.64533	0.951232	0.78597	0.90594 0.783538
37.56		gi|4758988	ras-related protein Rab-1A isoform 1 [Homo sapiens]	6	0.99181	0.96521	0.92253	0.748232	0.935789	0.76268	0.90523	0.4775	0.908909	0.59598	0.95635 0.786256
16.67		gi|7706244	protein CutA isoform 2 precursor [Homo sapiens]	2	0.67103	0.589	1.19653	0.60623	1.002143	0.99217	0.80534	0.65969	0.637288	0.47229	0.87021 0.787415
2.13	1.915	gi|338827685	AP-2 complex subunit alpha-2 isoform 1 [Homo sapiens]	2	0.71448	0.18213	1.31353	0.221365	1.383797	0.18813	0.99862	0.9911	0.962428	0.76468	0.92112 0.789026
10.09		gi|20070197	dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit precursor [Homo sapiens]	4	1.04981	0.70743	0.9536	0.607867	1.072354	0.48364	1.0482	0.65371	1.145003	0.10928	0.95791 0.795514
5.68	1.27	gi|114155142	nucleoprotein TPR [Homo sapiens]	3	0.95437	0.71923	1.55827	0.139745	1.144505	0.69242	1.20935	0.80201	1.199909	0.83418	1.21081 0.797233
5.361		gi|42544159	heat shock protein 105 kDa [Homo sapiens]	5	1.03791	0.83751	0.71001	0.495345	0.921183	0.61123	0.87039	0.52533	0.997984	0.98476	0.95339 0.800479

31.56	gi|4502491	complement component 1 Q subcomponent-binding protein, mitochondrial precursor [Homo sapiens]	sapiens]
5.75	7.296	gi|21361368	delta-1-pyrroline-5-carboxylate synthase isoform 1 [Homo sapiens]
18.54		gi|4506685	40S ribosomal protein S13 [Homo sapiens]
30.7		gi|4502923	calponin-3 [Homo sapiens]
8.48	9.269	gi|29029559	exportin-2 isoform 1 [Homo sapiens]
8.48	9.269	gi|29029559	exportin-2 isoform 1 [Homo sapiens]
14.77	50.49	gi|77539758	histone H4 [Homo sapiens]
4.48	43.75	gi|4506613	60S ribosomal protein L22 proprotein [Homo sapiens]
51.1		gi|4505773	prohibitin [Homo sapiens]
18.99		gi|4503481	elongation factor 1-gamma [Homo sapiens]
18.97		gi|7330335	chloride intracellular channel protein 4 [Homo sapiens]
30.6		gi|20544151	chromobox protein homolog 3 [Homo

9	1.00508	0.9556	1.08064	0.456452	3	1.08761	0.76023	1.14249	0.606873	0.916121	0.50274	1.29959	0.53768	0.975734	0.92692	1.07487	0.821474
	1.010557	0.92983	1.20337	0.33467													
	1.019807	0.90324	0.91517	0.802382													

3	0.9781	0.9669	0.85497	0.50659	0.897332	0.73533	0.78013	0.6573	0.641257	0.47964	1.06857	0.80268
3	0.99031	0.93996	0.84468	0.333348	1.051426	0.71728	0.7756	0.29608	0.912275	0.4263	0.97195	0.802778
15	1.02544	0.70414	1.05062	0.341917	0.941943	0.35536	0.95816	0.58435	1.044902	0.49742	1.01242	0.806483
5	1.09982	0.8148	0.8955	0.765058	1.387637	0.56142	0.88907	0.77044	0.874732	0.85614	1.12938	0.809937
5	1.09982	0.8148	0.8955	0.765058	1.387637	0.56142	0.88907	0.77044	0.874732	0.85614	1.12938	0.809937
28	1.11512	0.252	0.94207	0.44027	1.094707	0.25106	1.24563	0.0212	0.995098	0.94742	1.09363	0.814591
5	0.92582	0.39406	0.88941	0.431866	0.985671	0.87993	1.04055	0.82157	0.807588	0.15141	1.03663	0.815932
12	0.96556	0.73689	1.04412	0.592809	1.132968	0.46259	1.04172	0.8169	1.082056	0.61104	0.97867	0.817195
6	1.03165	0.89414	0.94509	0.835196	1.099946	0.45211	0.90037	0.57359	1.077196	0.62388	1.04039	0.819465
4	1.02164	0.91147	1.13859	0.422729	1.466823	0.16472	1.11304	0.48267	1.016055	0.92994	0.97107	0.819961

8.04	9.255	gi|5730027	KH domain-containing, RNA-binding, signal transduction-associated protein 1 [Homo sapiens]	5	1.06558	0.65957	0.93595	0.427594	0.998715	0.99478	0.95233	0.74678	0.92066	0.80624	0.96775	0.822087
3.383		gi|19743823	integrin beta-1 isoform 1A precursor [Homo sapiens]	3	1.32055	0.10167	1.13929	0.259019	1.350968	0.06939	1.04784	0.8101	1.521222	0.03788	1.08382	0.82873
172.41	48.02	gi|160420317	filamin-A isoform 2 [Homo sapiens]	119	0.97842	0.41197	0.99518	0.85802	0.967653	0.27161	0.98363	0.61147	0.984986	0.55125	0.99244	0.828854
20.61		gi|16579885	60S ribosomal protein L4 [Homo sapiens]	7	1.07615	0.33021	1.01311	0.924931	1.084191	0.48575	1.04919	0.72858	0.997751	0.98856	0.9789	0.833723
32.11		gi|4504041	guanine nucleotide-binding protein G(i) subunit alpha-2 isoform 1 [Homo sapiens]	9	1.13704	0.56315	0.96894	0.813532	1.020694	0.96158	0.93776	0.64576	1.055722	0.81975	1.02806	0.8344
16.02		gi|5802974	thioredoxin-dependent peroxide reductase, mitochondrial isoform a precursor [Homo sapiens]	4	0.96798	0.81028	0.95634	0.515702	1.033963	0.62264	1.22076	0.55074	0.991637	0.90516	1.02995	0.835193
9.494		gi|4506681	40S ribosomal protein S11 [Homo sapiens]	2	0.99409	0.97465	1.13149	0.545044	1.047803	0.88522	1.08859	0.77372	0.982615	0.92298	1.06109	0.835841
8.42	9.772	gi|154354966	mitochondrial inner membrane protein isoform 3 [Homo sapiens]	5	1.0206	0.93464	1.03532	0.871746	0.988825	0.96777	0.9628	0.86904	1.0296	0.89968	0.96498	0.837598
4.45	61.11	gi|28173554	histone H2B type 3-B [Homo sapiens]	43	1.02072	0.90655	0.96056	0.861582	1.037087	0.84719	1.18373	0.44769	0.948548	0.65963	1.0357	0.838179
18.29		gi|4506901	serine/arginine-rich splicing factor 3 [Homo sapiens]	3	0.9852	0.96279	1.1985	0.438019	1.078372	0.69505	1.06264	0.90255	0.984337	0.93061	0.92951	0.838933

18.42		gi|4507793	ubiquitin-conjugating enzyme E2 N [Homo sapiens]	1	1.16792	0.65472	1.21634	0.809184	1.932162	0.44384	1.52095	0.40788	0.920573	0.83344	1.21791	0.840396
1.927.042		gi|5174449	histone H1x [Homo sapiens]	1	0.85343	0.28064	0.85981	0.345885	0.741735	0.07861	0.83554	0.26722	0.963425	0.82536	1.04754	0.841578
38.34		gi|10835049	transforming protein RhoA precursor [Homo sapiens]	8	0.99703	0.98135	0.79242	0.729798	0.851819	0.80345	1.07826	0.72817	0.687668	0.33072	0.822	0.841902
3.96	3.659	gi|166795299	solute carrier family 2, facilitated glucose transporter member 1 [Homo sapiens]	2	1.04935	0.91109	0.94918	0.915556	0.754969	0.25409	1.11376	0.68062	1.154167	0.39811	1.02541	0.847455
20.38		gi|5803137	putative RNA-binding protein 3 [Homo sapiens]	1	1.08481	0.88279	0.85746	0.624625	0.849487	0.80461	1.20187	0.86022	0.779833	0.50567	0.93073	0.84798
2.204		gi|7661936	scaffold attachment factor B2 [Homo sapiens] aspartate aminotransferase,	2	1.01899	0.91967	1.04755	0.836359	1.058761	0.80586	0.94253	0.81642	1.236486	0.51996	0.95485	0.848025
16.74		gi|73486658	mitochondrial precursor [Homo sapiens]	5	0.96036	0.87103	0.97537	0.860831	1.070459	0.6887	1.07102	0.52078	1.011362	0.91313	1.03523	0.849832
6.79	29.52	gi|50592994	thioredoxin isoform 1 [Homo sapiens] voltage-dependent	4	0.87285	0.42193	1.06975	0.476514	1.075307	0.44631	0.97736	0.91538	0.937442	0.49301	0.9463	0.854211
23.82	61.48	gi|4507879	anion-selective channel protein 1 [Homo sapiens]	14	0.97731	0.69263	0.92668	0.34112	0.913339	0.34029	0.97341	0.74774	0.978336	0.79238	1.0166	0.858966
3.32	12.62	gi|4758018	calponin-2 isoform a [Homo sapiens] mesencephalic astrocyte-derived	3	1.19158	0.71105	1.19474	0.741549	0.728561	0.79122	1.01345	0.96606	0.868569	0.78228	1.14008	0.860278
26.37		gi|299523086	neurotrophic factor precursor [Homo sapiens]	5	1.11545	0.57209	1.11679	0.585957	1.029277	0.91557	1.01628	0.94928	1.076269	0.66921	1.04584	0.860548

	20.63	gi|34740329	heterogeneous nuclear ribonucleoprotein A3	10	1.10445	0.17571	0.9032	0.316306	1.145317	0.40173	1.07986	0.8933	1.122994	0.34016	1.02019	0.860986
	10.77	gi|117168248	[Homo sapiens] 3-hydroxyacyl-CoA dehydratase 3 [Homo sapiens]	4	0.84957	0.44745	0.8334	0.161112	1.038632	0.8902	0.95966	0.90221	1.045877	0.88382	0.9249	0.862774
	24.93	gi|14141152	heterogeneous nuclear ribonucleoprotein M isoform a [Homo sapiens]	17	1.08628	0.35293	1.03725	0.695026	1.026806	0.73823	0.99633	0.96725	1.016342	0.83824	0.98941	0.873553
3.23	12.07	gi|170763500	protein SET isoform 1 [Homo sapiens]	4	1.0848	0.69043	1.03571	0.725061	1.088743	0.67097	0.98089	0.84488	1.116083	0.53368	0.97681	0.873979
12.24	64.24	gi|88999583	myosin light polypeptide 6 isoform 2 [Homo sapiens]	15	0.94267	0.343	0.9248	0.245371	0.932394	0.26936	0.99883	0.9843	0.985798	0.8209	0.9908	0.876608
2.04	11.88	gi|63025214	vacuolar ATPase assembly integral membrane protein VMA21 [Homo sapiens]	1	0.90619	0.51417	0.99561	0.972821	1.057661	0.75898	1.18775	0.61068	1.014332	0.97255	0.96105	0.878343
7.9	9.387	gi|29789090	protein RCC2 [Homo sapiens]	4	1.11316	0.66394	1.06546	0.807756	1.060682	0.8303	0.96863	0.86843	1.225477	0.32522	1.03343	0.879579
27.24	41.98	gi|117190254	heterogeneous nuclear ribonucleoproteins C1/C2 isoform b [Homo sapiens]	17	1.02698	0.67678	0.92177	0.182329	0.974944	0.74372	1.04255	0.69517	0.976558	0.74628	0.99	0.88068
18.26	15.39	gi|189458819	transferrin receptor protein 1 [Homo sapiens]	11	1.11446	0.42576	0.99792	0.991522	1.124781	0.5508	1.14668	0.4871	0.988161	0.94215	0.94789	0.881912
8.03	10.46	gi|63162572	T-complex protein 1 subunit gamma isoform a [Homo sapiens]	4	0.82695	0.56059	0.81169	0.577272	1.153473	0.80554	0.82045	0.75663	1.050539	0.85656	0.93205	0.890922

5.536	gi|11056044	inorganic pyrophosphatase [Homo sapiens]	1	1.04929	0.9395	0.99655	0.995222	1.184591	0.80537	1.15813	0.82207	1.036608	0.79363	1.15318	0.891584
0.6877	gi|56676335	telomere-associated protein RIF1 isoform 1 [Homo sapiens]	1	1.08279	0.66024	0.87066	0.777724	0.618766	0.34994	0.55636	0.52788	0.984399	0.9835	0.92906	0.892763
16.95	gi|315221152	60S ribosomal protein L11 isoform 2 [Homo sapiens]	4	1.06126	0.56421	0.99761	0.975118	1.065146	0.43782	0.95199	0.536	1.085682	0.32948	0.98499	0.89321
27.44	gi|4504425	high mobility group protein B1 [Homo sapiens]	8	1.025	0.85145	0.92685	0.563656	0.927637	0.65527	1.01212	0.93795	0.957028	0.6268	0.97689	0.893937
13.77 28.61	gi|24308201	adipocyte plasma membrane-associated protein [Homo sapiens]	10	0.83601	0.21067	1.06307	0.479644	0.886176	0.51837	0.86221	0.20778	0.968326	0.63722	1.03196	0.894531
26.57	gi|4506701	40S ribosomal protein S23 [Homo sapiens]	3	0.87112	0.40648	0.72728	0.199117	0.817343	0.42284	0.7103	0.30555	1.074612	0.66576	1.01694	0.896721
45.19	gi|4506693	40S ribosomal protein S17 [Homo sapiens]	6	0.99431	0.97242	0.97255	0.722537	1.038886	0.75248	1.03874	0.67553	1.034698	0.66409	0.98707	0.899802
20.93	gi|5453603	T-complex protein 1 subunit beta isoform 1 [Homo sapiens]	6	1.09951	0.79704	1.08657	0.641929	1.428159	0.05029	1.03563	0.83282	0.912354	0.81703	1.02791	0.899862
46.15	gi|21464101	14-3-3 protein gamma [Homo sapiens]	15	1.02568	0.83239	1.03997	0.769753	0.969569	0.85613	0.90003	0.52358	0.991797	0.94948	0.96655	0.900023
16.61 9.229	gi|256222411	filamin-B isoform 1 [Homo sapiens]	20	0.98336	0.8992	1.02555	0.853117	1.118949	0.54084	0.99812	0.9923	0.991695	0.90764	0.97554	0.900285
4.3 11.45	gi|388240801	lamin-B2 [Homo sapiens]	7	0.87942	0.71958	1.10445	0.77971	0.78914	0.61079	0.91952	0.83412	0.95001	0.85965	0.94256	0.901109
2.058	gi|39753957	torsin-1A-interacting protein 1 isoform 2 [Homo sapiens]	1	0.83348	0.33185	0.96138	0.867541	1.103016	0.62859	0.87927	0.53707	1.083103	0.74121	1.05113	0.903757

22.16	gi|14141193	40S ribosomal protein S9 [Homo sapiens]	5	0.99992	0.99954	0.92862	0.763386	0.945508	0.76024	0.95503	0.83905	1.075762	0.60233	1.01905	0.904019
8.11 27.47	gi|34098946	nuclease-sensitive element-binding protein 1 [Homo sapiens]	5	1.07413	0.56558	1.14988	0.476996	1.06747	0.59239	1.0434	0.82634	1.220969	0.45264	1.0157	0.90823
2.15 8.894	gi|42822874	ribonuclease inhibitor [Homo sapiens]	2	0.80302	0.27929	0.90655	0.700213	1.344467	0.6081	1.12269	0.45072	1.136887	0.41847	0.98588	0.909263
2.452	gi|225543215	antigen KI-67 isoform 2 [Homo sapiens]	4	1.03006	0.8092	0.87668	0.728422	1.203502	0.26766	1.24771	0.44123	1.27859	0.18565	1.03634	0.910268
3.52 50.74	gi|4885385	histone H3.3 [Homo sapiens]	11	0.96893	0.81563	0.94274	0.67602	1.054957	0.7012	0.9351	0.87652	1.046815	0.7414	1.02837	0.910912
22.4	gi|4506457	reticulocalbin-2 precursor [Homo sapiens]	5	1.38471	0.39167	1.2916	0.445955	1.296941	0.47927	1.43781	0.41555	1.536593	0.26422	1.06997	0.911035
2.682	gi|50659095	nucleolar RNA helicase 2 isoform 1 [Homo sapiens]	2	1.01952	0.95996	1.38491	0.187652	1.017043	0.89225	1.16703	0.36259	0.984875	0.91557	0.97993	0.915927
3.63 9.016	gi|5031777	isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial precursor [Homo sapiens]	2	1.16688	0.3754	1.19345	0.716846	1.497555	0.3531	1.59903	0.20131	1.23718	0.57148	1.03029	0.917579
25	gi|15431301	60S ribosomal protein L7 [Homo sapiens]	7	0.92425	0.48551	1.03374	0.876283	0.95133	0.5483	0.99818	0.99234	1.082148	0.57258	1.0118	0.917983
24	gi|256222019	ras-related protein Rab-10 [Homo sapiens]	5	0.94102	0.6384	0.90518	0.403307	0.902896	0.68559	0.80889	0.38432	0.987171	0.95238	0.95457	0.918074

3.12	2.979	gi|22749415	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A [Homo sapiens]	1	1.06993	0.62139	1.02313	0.947688	0.891197	0.48906	1.44778	0.43354	1.22369	0.29182	0.94878	0.91885
	5.682	gi|4758788	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial precursor [Homo sapiens]	1	1.09954	0.75239	1.00194	0.98818	1.04381	0.83939	1.10044	0.84765	1.189316	0.20548	1.01421	0.919937
	21.92	gi|4503607	electron transfer flavoprotein subunit alpha, mitochondrial isoform a [Homo sapiens]	7	0.745	0.58946	1.25831	0.663793	0.924426	0.79947	0.83736	0.64983	0.555631	0.57613	0.93131	0.921689
	7.143	gi|4759160	small nuclear ribonucleoprotein Sm D3 [Homo sapiens]	1	0.90276	0.57568	0.9279	0.468609	0.88426	0.48308	0.81171	0.52085	0.831009	0.17558	0.98806	0.923847
	23.08	gi|4758302	enhancer of rudimentary homolog [Homo sapiens]	3	1.03392	0.80889	1.12985	0.460855	1.232816	0.30435	1.13608	0.57846	0.945932	0.6978	1.02672	0.926136
2.46	3.648	gi|5031703	ras GTPase-activating protein-binding protein 1 [Homo sapiens]	1	0.92013	0.57087	0.85965	0.452881	0.880045	0.48158	0.95338	0.81138	0.917742	0.69749	0.95912	0.926288
4.52	7.756	gi|56549640	septin-2 [Homo sapiens]	3	0.99332	0.99483	1.23575	0.823806	1.353351	0.76378	1.72484	0.58133	1.284647	0.70863	0.93565	0.926332
1.68	5.263	gi|4758356	flap endonuclease 1 [Homo sapiens]	1	1.15011	0.39223	1.10884	0.675855	1.067793	0.6274	1.00338	0.98366	0.94599	0.67436	1.01114	0.929123
11.37	23.19	gi|4506725	40S ribosomal protein S4, X isoform X isoform [Homo sapiens]	5	0.97449	0.7355	0.92138	0.507416	0.964998	0.73662	1.12599	0.23149	0.953784	0.56743	1.00613	0.930998

		Accession	Description	#	1	2	3	4	5	6	7	8	9	10	11	12
6.34	23.31	gi|7305503	stomatin-like protein 2 [Homo sapiens]	5	1.17661	0.76853	1.01792	0.956275	0.970179	0.89195	1.06593	0.66163	0.877609	0.82736	1.03079	0.931181
10.33	10.26	gi|55770844	catenin alpha-1 [Homo sapiens]	6	1.03773	0.77724	1.42203	0.622809	1.56616	0.3328	1.23645	0.37736	0.997016	0.98129	1.04962	0.932653
	46.12	gi|4507953	14-3-3 protein zeta/delta [Homo sapiens]	15	0.89929	0.60586	0.98527	0.856421	1.099533	0.64591	0.782	0.35808	0.893897	0.20654	1.01769	0.935496
	42.89	gi|50592996	tubulin beta-3 chain isoform 1 [Homo sapiens]	29	0.78939	0.25372	1.16165	0.795319	0.842534	0.33496	0.84947	0.34915	0.973308	0.83089	1.03767	0.937168
	5.112	gi|332688210	catenin delta-1 isoform 1AC [Homo sapiens]	4	1.02514	0.97096	0.55873	0.469676	1.062146	0.89209	1.20211	0.85366	0.898966	0.87101	0.97397	0.939865
6.98	58.88	gi|4507729	tubulin beta-2A chain [Homo sapiens]	41	0.948	0.76834	0.9999	0.99978	0.903984	0.61965	0.88799	0.79613	0.752115	0.29768	0.96998	0.940554
16.43	40.26	gi|24234747	interleukin enhancer-binding factor 2 isoform 1 [Homo sapiens]	13	1.0511	0.7347	0.91691	0.632643	0.969474	0.89625	0.95433	0.83549	0.978358	0.87143	0.98794	0.942942
	25.2	gi|4505753	phosphoglycerate mutase 1 [Homo sapiens]	8	0.96526	0.90588	1.29984	0.237936	0.99391	0.9809	1.07441	0.81875	0.812698	0.54835	1.02478	0.944817
	5.972	gi|15991831	hexokinase-1 isoform HKI-ta/tb [Homo sapiens]	4	1.10822	0.50056	1.13319	0.676442	1.011951	0.97474	1.03129	0.9428	0.989503	0.97605	0.98934	0.947689
	14.19	gi|4506625	60S ribosomal protein L27a [Homo sapiens]	2	0.99323	0.96296	1.09852	0.728854	0.889413	0.73997	1.01465	0.96828	0.90663	0.53302	1.02292	0.950836
5.31	19.58	gi|4757908	calcyphosin isoform a [Homo sapiens]	4	0.7789	0.18217	1.03856	0.851888	0.898004	0.30411	0.85694	0.39045	0.957459	0.62455	0.98766	0.952912
3.08	4.58	gi|11386147	proactivator polypeptide isoform a preproprotein [Homo sapiens]	2	0.7416	0.68952	1.1469	0.767531	0.803347	0.76691	0.88676	0.90596	1.279128	0.79946	1.05125	0.953217

36.71	60.31	gi	4502107	annexin A5 [Homo sapiens]	28	1.02188	0.71923	1.04762	0.283796	1.041221	0.53751	1.02478	0.79402	1.003106	0.9381	1.00371	0.960815
	11.11	gi	4758984	ras-related protein Rab-11A isoform 1 [Homo sapiens]	2	1.05913	0.823	0.92678	0.757553	1.219468	0.2448	1.02015	0.94044	0.980833	0.91924	1.01765	0.961985
	9.559	gi	127139033	NADPH--cytochrome P450 reductase [Homo sapiens]	5	1.46665	0.26812	1.35078	0.78343	1.750845	0.28485	1.50179	0.58197	1.396446	0.19557	1.01903	0.963064
	9.428	gi	304555583	elongation factor 1-delta isoform 1 [Homo sapiens]	4	1.05626	0.86542	1.13667	0.76818	1.069415	0.889	1.01608	0.96552	1.236347	0.55616	1.01694	0.964416
19.2	40	gi	4504245	histone H2A type 1-C [Homo sapiens]	42	1.03017	0.86464	0.91872	0.699991	0.933817	0.74092	1.19479	0.32328	0.974854	0.83978	0.99492	0.967249
25.54	15.89	gi	38202257	neutral alpha-glucosidase AB isoform 2 precursor [Homo sapiens]	14	1.04371	0.617	1.12201	0.3039	1.067465	0.42923	1.09288	0.44729	1.079871	0.33047	1.00402	0.967442
	32.07	gi	5031753	heterogeneous nuclear ribonucleoprotein H [Homo sapiens]	18	0.97527	0.83475	0.82622	0.394135	1.084716	0.50783	0.92009	0.68814	0.934815	0.65578	0.99293	0.972627
	17	gi	31621305	leucine-rich PPR motif-containing protein, mitochondrial precursor [Homo sapiens]	16	1.03366	0.87957	0.98367	0.912034	1.06177	0.80072	1.01847	0.89318	1.135167	0.43715	0.99557	0.974808
	17	gi	31621305	leucine-rich PPR motif-containing protein, mitochondrial precursor [Homo sapiens]	16	1.03366	0.87957	0.98367	0.912034	1.06177	0.80072	1.01847	0.89318	1.135167	0.43715	0.99557	0.974808
	80.9	gi	4507357	transgelin-2 [Homo sapiens]	44	0.94206	0.15285	0.98498	0.707045	0.982281	0.65704	0.95133	0.34752	0.973675	0.50977	0.99881	0.976356
	33.33	gi	4506715	40S ribosomal protein S28 [Homo sapiens]	2	1.06887	0.59919	1.27592	0.261705	1.038603	0.6719	1.2457	0.32914	1.089153	0.60128	1.00461	0.986721

			Protein	N												
37.3	37.85	gi|24234688	stress-70 protein, mitochondrial precursor [Homo sapiens]	26	0.98254	0.76659	1.0399	0.634538	0.983587	0.83881	1.00545	0.95484	0.989735	0.89661	1.00057	0.990667
30.94	67.46	gi|18105048	histone H2B type 1-K [Homo sapiens]	47	1.08079	0.28917	0.9607	0.567832	0.967995	0.66044	1.16835	0.15734	1.040797	0.56995	0.99872	0.990732
16.64	14.34	gi|10863945	X-ray repair cross-complementing protein 5 [Homo sapiens]	8	1.01635	0.9172	0.98945	0.940811	1.036516	0.81601	0.87213	0.45505	1.077628	0.58733	0.99791	0.992423
2.67	8.974	gi|4826852	acyl carrier protein, mitochondrial precursor [Homo sapiens]	1	0.9768	0.97217	0.99151	0.99244	1.00298	0.99757	1.00566	0.99457	1.070932	0.8989	1.00317	0.993962
	39.52	gi|14165435	heterogeneous nuclear ribonucleoprotein K isoform b [Homo sapiens]	15	0.95513	0.29475	0.95093	0.441387	0.970964	0.63696	0.97352	0.67837	1.055012	0.42691	1.00023	0.997159
	20.75	gi|5803048	ER lumen protein retaining receptor 1 [Homo sapiens]	3	1.05895	0.89643	0.89579	0.811545	0.803206	0.31489	1.08902	0.89204	0.932645	0.86832	1.00017	0.999305
	34.67	gi|74136883	heterogeneous nuclear ribonucleoprotein U isoform a [Homo sapiens]	34	0.71668		0.61704		0.608761		0.57835		0.824188		0.60273	
49.17	64.97	gi|57013276	tubulin alpha-1B chain [Homo sapiens]	63	0.93023		1.05083		0.983988		1.04234		0.83438		1.05425	
	7.121	gi|4506787	ras GTPase-activating like protein IQGAP1 [Homo sapiens]	9	0.81002		0.8467		1.040909		0.76514		1.094655		0.71155	
11.38	8.671	gi|5453998	importin-7 [Homo sapiens]	8												

11.28	gi|28178832	isocitrate dehydrogenase [NADP], mitochondrial precursor [Homo sapiens]	5	1.30054	0.97425	1.043614	0.95524	1.064548	1.35882
12.65	gi|285002233	glycerol-3-phosphate dehydrogenase, mitochondrial precursor [Homo sapiens]	8	0.63612	0.43177	0.605118	0.60499	0.806922	0.5378
9.02 44.56	gi|28395033	rho-related GTP-binding protein RhoC precursor [Homo sapiens]	6	1.92031	0.99363	1.700983	1.06466	1.078723	1.42801
8.89 4.503	gi|38201623	eukaryotic translation initiation factor 4 gamma 1 isoform 5 [Homo sapiens]	9	0.66325	1.16796	0.670461	0.73769	1.420639	0.48499
6.069	gi|4507943	exportin-1 [Homo sapiens]	4						
8 79.82	gi|4506669	60S acidic ribosomal protein P1 isoform 1 [Homo sapiens]	7	1.06983	0.97872	1.0065	1.03349	0.986462	0.95046
8.063	gi|45387945	extended synaptotagmin-2 [Homo sapiens]	4						
7.63 13.58	gi|4507353	TATA-binding protein-associated factor 2N isoform 2 [Homo sapiens]	5						
7.35 56.82	gi|11056061	thymosin beta-4 [Homo sapiens]	5	0.92823	1.03146	1.020059	0.8738	0.987755	1.09835

		gi	Protein	Count						
7.22	4.92	gi|4503509	eukaryotic translation initiation factor 3 subunit A [Homo sapiens]	5	2.39279	4.4105	2.463701	3.54179	2.01806	2.10314
7.069		gi|385298680	116 kDa U5 small nuclear ribonucleoprotein component isoform c [Homo sapiens]	6						
6.54	3.137	gi|40217847	U5 small nuclear ribonucleoprotein 200 kDa helicase [Homo sapiens]	6	1.14194	1.62901	0.86916	0.90805	1.30212	0.33775
4.864		gi|21361370	glycogen phosphorylase, brain form [Homo sapiens]	4	0.74647	0.88615	0.815465	0.71197	0.782035	1.25844
3.298		gi|54112117	splicing factor 3B subunit 1 isoform 1 [Homo sapiens]	3						
6.23	5.349	gi|71773329	annexin A6 isoform 1 [Homo sapiens]	3	4.50272	3.54499	4.594796	3.09253	3.811192	5.69826
5.579		gi|109148542	alanine--tRNA ligase, cytoplasmic [Homo sapiens]	3	0.82244	0.9845	0.950517	0.84237	0.686344	1.03448
20.49		gi|7706322	UPF0568 protein C14orf166 [Homo sapiens]	5						
25.17		gi|194097323	enoyl-CoA hydratase, mitochondrial [Homo sapiens]	7	2.03732	1.74468	2.134784	1.98122	1.267649	3.15589
28.65		gi|222352151	poly(rC)-binding protein 1 [Homo sapiens]	8	0.92485	0.81808	1.163378	1.00833	0.545206	1.00614
30.2		gi|50593002	U2 small nuclear ribonucleoprotein A' [Homo sapiens]	6	1.12271	0.91509	1.310521	1.0248	1.543845	1.43563

9.605	gi|4502643	T-complex protein 1 subunit zeta isoform a [Homo sapiens]	4	0.66609	0.49984	1.35727	1.1912	0.776804	0.82225
4.98	6.839	gi|45827806	atlastin-3 [Homo sapiens]	3						
	6.071	gi|38327039	heat shock 70 kDa protein 4 [Homo sapiens]	3						
4.75	27.57	gi|6912238	peroxiredoxin-5, mitochondrial isoform a precursor [Homo sapiens]	5	0.7021	1.18907	1.478837	0.4313	1.39936	1.21253
	7.559	gi|4758786	NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial isoform 1 precursor [Homo sapiens]	2						
4.63	6.966	gi|18677735	protein PRRC1 [Homo sapiens]	3						
	14.94	gi|23110942	proteasome subunit alpha type-5 isoform 1 [Homo sapiens]	3						
4.46	12.4	gi|269914128	OCIA domain-containing protein 1 isoform 4 [Homo sapiens]	2	1.21801	1.37961	1.346004	1.48184	1.117946	1.18723
4.44	10.44	gi|4502049	aldose reductase [Homo sapiens]	4	1.47454	1.36634	1.409654	0.99615	0.923845	1.00292
	9.053	gi|5453595	adenylyl cyclase-associated protein 1 [Homo sapiens]	4	1.57866	1.2196	2.380994	1.61705	1.994657	2.52747
4.4	5.615	gi|4506341	ATP-binding cassette sub-family D member 3 isoform a [Homo sapiens]	3	0.32324	0.75219	1.198383	1.30105	1.203778	1.54799

		Protein	Count						
3.725	gi|6005757	FACT complex subunit SPT16 [Homo sapiens]	2						
6.442	gi|56788366	phosphatidylinositol-binding clathrin assembly protein isoform 1 [Homo sapiens]	3	0.84523	0.80269	1.0253	0.9725	0.986006	0.97061
4.32 6.795	gi|308818195	dihydropyrimidinase-related protein 2 isoform 1 [Homo sapiens]	2						
6.369	gi|4505891	procollagen-lysine,2-oxoglutarate 5-dioxygenase 3 precursor [Homo sapiens]	5						
4.17 1.681	gi|153946395	tenascin precursor [Homo sapiens]	2						
5.479	gi|319655561	alpha-aminoadipic semialdehyde dehydrogenase isoform 2 [Homo sapiens]	3						
4.14 3.894	gi|17999541	vacuolar protein sorting-associated protein 35 [Homo sapiens]	3						
6.225	gi|116805340	glycine--tRNA ligase precursor [Homo sapiens]	2	0.80401	0.7306	0.752112	0.67938	0.6988	0.817
4.12 5.368	gi|302344762	non-specific lipid-transfer protein isoform 6 proprotein [Homo sapiens]	2						

2.758	gi|148536855	coatomer subunit alpha isoform 1 [Homo sapiens]	3	0.84495	1.14378	0.879331	1.20518	0.78377	0.92041
12.28	gi|53692187	actin-related protein 2 isoform a [Homo sapiens]	3						
6.528	gi|5803187	transaldolase [Homo sapiens]	3						
6.742	gi|6598323	rab GDP dissociation inhibitor beta isoform 1 [Homo sapiens]	2	1.31164	1.35366	1.719467	1.22879	1.907645	1.82516
4.11 17.34	gi|5454090	translocon-associated protein subunit delta isoform 2 precursor [Homo sapiens]	2	1.09614	1.01594	1.240244	1.2115	1.02137	1.06552
4.1 2.276	gi|21361794	cullin-associated NEDD8-dissociated protein 1 [Homo sapiens]	3						
2.816	gi|38569421	ATP-citrate synthase isoform 1 [Homo sapiens]	2	1.47681	1.78721	2.177375	1.43963	1.056422	1.85165
12.5	gi|19923315	serine hydroxymethyltransferase, mitochondrial isoform 1 precursor [Homo sapiens]	5	2.17269	1.45205	1.733907	1.84592	1.6707	1.17943
3.904	gi|109134349	coatomer subunit gamma-2 [Homo sapiens]	2						
7.196	gi|195539395	26S protease regulatory subunit 10B [Homo sapiens]	2	0.78017	0.46331	1.391888	1.14351	0.599761	1.27963

	14.64	gi|30410792	proteasome activator complex subunit 2 [Homo sapiens]	3	1.35076	1.30523	0.949045	1.36713	1.264915	0.92517
	8.119	gi|166795301	prenylcysteine oxidase 1 precursor [Homo sapiens]	3	1.05175	0.98525	1.0761	0.91641	0.986455	0.77613
4.02	4.662	gi|8922712	septin-11 [Homo sapiens]	2						
	5.568	gi|37537716	eukaryotic translation initiation factor 5 [Homo sapiens]	3						
4.01	29.13	gi|17933772	protein S100-A16 [Homo sapiens]	2	0.91551	0.57837	1.177884	1.32834	1.245144	1.04009
4	64.97	gi|393715091	tubulin alpha-1A chain isoform 1 [Homo sapiens]	61	0.86763	1.09195	1.091364	0.95509	0.999499	1.17204
4	17.76	gi|151108473	mitochondrial fission 1 protein [Homo sapiens]	2						
4	41.07	gi|7705501	transmembrane protein 14C [Homo sapiens]	2						
	27.97	gi|21956645	myotrophin [Homo sapiens]	3						
3.85	4.626	gi|25777602	26S proteasome non-ATPase regulatory subunit 2 [Homo sapiens]	3						
	1.638	gi|110611220	ribosome-binding protein 1 [Homo sapiens]	2						
3.68	4.777	gi|4758648	kinesin-1 heavy chain [Homo sapiens]	4						
	19.44	gi|4759212	tubulin-specific chaperone A [Homo sapiens]	4	0.72985	0.79919	0.8261	0.89374	0.765348	0.87837

	gi ID	Description	Count						
6.383	gi|4501993	alkyldihydroxyacetonephosphate synthase, peroxisomal precursor [Homo sapiens]	3	1.3973	1.20706	1.229661	1.30968	0.870439	0.9839
6.713	gi|9951915	adenosylhomocysteinase isoform 1 [Homo sapiens]	3	0.91699	1.54572	0.960423	0.51959	1.187545	1.25412
3.47 11.58	gi|397138571	PREDICTED: putative trypsin-6 isoform 2 [Homo sapiens]	6	0.6452	0.71299	0.688758	0.84167	0.871762	0.4976
5.369	gi|4503971	rab GDP dissociation inhibitor alpha [Homo sapiens]	2						
3.41 3.436	gi|56676330	heterochromatin protein 1-binding protein 3 [Homo sapiens]	3	0.93177	1.38266	0.985569	0.63015	1.159698	0.87353
16.67	gi|28933465	syntaxin-12 [Homo sapiens]	3	0.65228	1.28782	1.08027	0.56742	0.870332	1.17819
8.754	gi|14591909	60S ribosomal protein L5 [Homo sapiens]	2	0.96889	1.29402	0.971669	1.24532	1.17296	0.77783
3.212	gi|45827771	enhancer of mRNA-decapping protein 4 [Homo sapiens]	2						
6.25	gi|38150007	small nuclear ribonucleoprotein-associated proteins B and B' isoform B' [Homo sapiens]	2	0.94294	0.69364	0.869764	0.63095	0.803134	0.49355
1.404	gi|148529014	DNA damage-binding protein 1 [Homo sapiens]	2						

		Accession	Description	N						
	10.32	gi|4503139	cathepsin B preproprotein [Homo sapiens]	2	0.70471	0.80279	0.654314	0.69681	0.634219	0.7471
	11.01	gi|346644849	DNA-(apurinic or apyrimidinic site) lyase [Homo sapiens]	3	1.2326	1.04638	1.573288	1.95319	1.46752	1.26419
3.15	13.95	gi|7705696	thioredoxin domain-containing protein 12 precursor [Homo sapiens]	2						
3.06	9.44	gi|6005721	erlin-2 isoform 1 [Homo sapiens]	3	1.05746	1.13755	0.971272	1.06971	0.825092	1.21269
	3.714	gi|157389005	calpain-2 catalytic subunit isoform 1 [Homo sapiens]	2						
2.93	20.61	gi|5802966	destrin isoform a [Homo sapiens]	3	0.86667	1.08644	1.071251	0.54751	0.656449	0.63406
2.93	6.395	gi|217272851	prolyl 4-hydroxylase subunit alpha-1 isoform 3 precursor [Homo sapiens]	2	1.05682	0.51686	1.175281	1.00522	0.697477	1.48189
	0.8478	gi|194595509	spectrin alpha chain, brain isoform 1 [Homo sapiens]	2						
	9.562	gi|5454088	acidic leucine-rich nuclear phosphoprotein 32 family member B [Homo sapiens]	2						
2.81	18.62	gi|7657257	mitochondrial import receptor subunit TOM20 homolog [Homo sapiens]	2	1.12647	0.96503	0.830369	1.03807	0.875227	0.89786
2.71	9.603	gi|386781571	radixin isoform 1 [Homo sapiens]	6	0.81532	1.25499	1.601472	0.54108	0.565041	1.1877

			Count						
9.375	gi|42761474	CD59 glycoprotein preproprotein [Homo sapiens]	1	0.86805	0.97073	0.997505	1.10319	0.866608	0.84686
2.65 1.65	gi|9257257	WD repeat-containing protein 1 isoform 1 [Homo sapiens]	1						
2.64 7.125	gi|365192532	myosin-10 isoform 1 [Homo sapiens]	15	0.66064	0.65413	1.10042	0.93428	0.603853	0.98694
5.534	gi|219283152	apoptosis inhibitor 5 isoform a [Homo sapiens]	4	0.85311	1.16414	1.634897	1.38697	1.133337	1.00298
2.61 7.116	gi|30410796	proteasome activator complex subunit 3 isoform 2 [Homo sapiens]	3	0.63085	0.92394	0.72022	0.36527	0.9777	0.76639
12.16	gi|4507231	single-stranded DNA-binding protein, mitochondrial precursor [Homo sapiens]	3	1.58337	1.341	1.461128	1.51503	1.563343	1.89598
7.182	gi|7657441	28 kDa heat- and acid-stable phosphoprotein [Homo sapiens]	1	0.85503	0.87906	0.343909	0.40988	0.740731	1.13019
2.58 10.82	gi|13569879	acidic leucine-rich nuclear phosphoprotein 32 family member E isoform 1 [Homo sapiens]	3						
2.51 1.262	gi|283436220	ATPase family AAA domain-containing protein 3A isoform 1 [Homo sapiens]	1	0.57696	0.62027	0.424078	0.20236	0.411318	0.31566
2.492.839	gi|4885409	vigilin isoform a [Homo sapiens]	2						

2.45	5.919	gi|12056465	rRNA 2'-O-methyltransferase fibrillarin [Homo sapiens]	2	0.84699	1.04848	0.77802	0.72476	0.814099	1.04849
2.43	7.67	gi|312283701	malate dehydrogenase, cytoplasmic isoform 1 [Homo sapiens]	2	0.88475	0.75548	0.903711	0.51043	0.991972	0.72992
2.42	16.39	gi|21040371	ATP-dependent RNA helicase DDX39A [Homo sapiens]	5						
2.42	9.394	gi|19923181	PDZ and LIM domain protein 4 isoform 1 [Homo sapiens]	3	0.74782	1.03132	1.006054	0.97864	1.449987	1.22062
2.4	32.52	gi|4507949	14-3-3 protein beta/alpha [Homo sapiens]	14	1.25318	1.06152	1.807213	1.40418	0.817041	1.31435
2.38	1.307	gi|11225260	DNA topoisomerase 1 [Homo sapiens]	1	0.66957	0.99773	1.318882	0.92511	1.109218	1.38873
2.36	5.966	gi|7657649	tropomodulin-3 [Homo sapiens]	1	0.54916	0.58171	0.468889	0.03686	0.571124	0.66021
2.35	14.81	gi|4505357	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4 [Homo sapiens]	1	1.14039	0.96507	1.457507	0.9558	1.061194	1.20029
2.34	1.223	gi|124494238	unconventional myosin-Ic isoform a [Homo sapiens]	1	0.62802	0.55643	0.50495	0.18695	0.410643	0.63169
2.33	1.135	gi|30581135	structural maintenance of chromosomes protein 1A [Homo sapiens]	2						

	Description	Count						
2.57	gi|21536326 heterogeneous nuclear ribonucleoprotein U-like protein 1 isoform a [Homo sapiens]	1	0.27416	0.26981	0.297418	0.21473	0.517851	0.40407
4.837	gi|38202214 protein transport protein Sec23A [Homo sapiens]	2						
1.844	gi|10190742 ubiquitin carboxyl-terminal hydrolase 29 [Homo sapiens]	1						
2.3 12.24	gi|66737374 protein S100-A13 [Homo sapiens]	1	1.52857	0.65298	0.87031	1.73263	1.020324	1.2015
2.29 10.38	gi|373432684 ubiquitin-conjugating enzyme E2 L3 isoform 4 [Homo sapiens]	3						
2.29 6.407	gi|4759034 eukaryotic peptide chain release factor subunit 1 [Homo sapiens]	2	0.79436	0.80778	0.666296	0.44907	0.246555	0.56426
2.28 4.723	gi|316983160 NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial isoform 5 [Homo sapiens]	2						
23.2	gi|4502203 ADP-ribosylation factor 3 [Homo sapiens]	4	1.20672	1.21644	1.051839	1.28095	0.886377	1.39553
1.534	gi|33356174 pinin [Homo sapiens]	1	0.85067	0.83291	1.13135	0.86094	1.075418	0.5121
2.26 4.172	gi|33469968 DNA replication licensing factor MCM7 isoform 1 [Homo sapiens]	2	0.66264	0.98625	1.075919	0.92704	0.936778	0.82464

		Accession	Protein	Count						
2.25	1.436	gi|296317244	extended synaptotagmin-1 isoform 1 [Homo sapiens]	2						
	13.1	gi|4506687	40S ribosomal protein S15 [Homo sapiens]	2	0.72722	0.74515	0.691769	0.38782	0.804124	0.74194
2.25	7.981	gi|4502303	ATP synthase subunit O, mitochondrial precursor [Homo sapiens]	2	1.18023	1.17369	0.871478	0.88042	0.799421	0.82527
2.25	10.62	gi|31563374	eukaryotic translation initiation factor 6 isoform c [Homo sapiens]	1						
	1.957	gi|11863154	coatomer subunit delta isoform 1 [Homo sapiens]	1						
	1.579	gi|51944971	nodal modulator 2 isoform 1 precursor [Homo sapiens]	1						
2.24	1.062	gi|21237808	SWI/SNF complex subunit SMARCC2 isoform b [Homo sapiens]	1						
	4.889	gi|7661922	ras-related protein Rab-21 [Homo sapiens]	1	1.2084	0.76928	1.115088	0.71112	0.929048	0.73102
	0.9009	gi|27477134	nuclear pore membrane glycoprotein 210 precursor [Homo sapiens]	1						
2.23	1.794	gi|24797086	importin-5 [Homo sapiens]	1	1.02307	1.20567	1.213528	1.21964	0.937203	0.83983

2.22	1.463	gi|61742777	leucyl-cystinyl aminopeptidase isoform 1 [Homo sapiens]	1						
	2.968	gi|4826960	glutamine--tRNA ligase [Homo sapiens]	2						
2.21	1.286	gi|31377697	procollagen galactosyltransferase 1 precursor [Homo sapiens]	1	0.89469	0.93139	1.864245	1.28507	2.11273	1.20325
	1.372	gi|19924129	DNA repair protein RAD50 [Homo sapiens]	1						
	2.111	gi|14043022	methionine--tRNA ligase, cytoplasmic [Homo sapiens]	1						
	5.882	gi|4506623	60S ribosomal protein L27 [Homo sapiens]	1	1.10035	0.91496	0.965733	1.13191	0.864173	1.03142
2.17	1.894	gi|6912482	LETM1 and EF-hand domain-containing protein 1, mitochondrial precursor [Homo sapiens]	1	0.71105	0.92143	1.40973	1.03453	0.92632	0.9231
2.17	1.799	gi|4504373	beta-hexosaminidase subunit beta preproprotein [Homo sapiens]	1						
2.16	0.8333	gi|21264365	nuclear pore complex protein Nup98-Nup96 isoform 1 [Homo sapiens]	1						

0.65		gi|52630326	chromodomain-helicase-DNA-binding protein 3 isoform 1 [Homo sapiens]	1						
2.16	7.489	gi|38505222	protein disulfide-isomerase TMX3 precursor [Homo sapiens]	3	0.79121	0.71153	0.996528	0.97663	0.65292	0.96825
2.16	6.297	gi|4504511	dnaJ homolog subfamily A member 1 [Homo sapiens]	1	0.7664	1.08557	0.631588	0.45396	0.520625	0.50215
2.15	0.742	gi|21040326	protein SON isoform F [Homo sapiens]	2	0.50701	0.5684	0.514403	1.07732	0.679063	1.01611
2.15	1.086	gi|112382237	synemin isoform A [Homo sapiens]	1						
	0.8696	gi|22202611	carboxypeptidase D isoform 1 precursor [Homo sapiens]	1	0.98697	0.81086	1.696009	1.31237	1.248185	1.63565
	1.732	gi|17149828	N-alpha-acetyltransferase 15, NatA auxiliary subunit [Homo sapiens]	1						
	0.7216	gi|254939537	unconventional myosin-XIX isoform 2 [Homo sapiens]	1						
2.15	1.514	gi|333033787	nascent polypeptide-associated complex subunit alpha isoform a [Homo sapiens]	1	1.09531	0.98972	1.223688	0.95587	1.039843	0.96309

2.15	17.17	gi|27436901	39S ribosomal protein L12, mitochondrial [Homo sapiens]	2	1.43183	1.09845	1.298743	1.5196	0.923348	0.5195
2.15	3.024	gi|5730023	ruvB-like 2 [Homo sapiens]	1						
2.15	3.024	gi|5730023	ruvB-like 2 [Homo sapiens]	1						
	5.155	gi|4505119	methyl-CpG-binding domain protein 3 [Homo sapiens]	1						
2.14	16.38	gi|7524346	adenylate kinase 2, mitochondrial isoform b [Homo sapiens]	4	1.3076	1.68282	1.177596	1.40287	1.297324	1.02061
2.12	4.242	gi|30089972	peroxisomal acyl-coenzyme A oxidase 1 isoform a [Homo sapiens]	3	0.68112	0.64966	1.304939	0.76658	0.415005	1.11262
2.12	0.7937	gi|62241042	bifunctional glutamate/proline--tRNA ligase [Homo sapiens]	1						
	3.109	gi|37594471	3-hydroxyisobutyryl-CoA hydrolase, mitochondrial isoform 1 precursor [Homo sapiens]	1	1.03883	1.04724	0.897887	0.7685	0.744229	0.95015
	0.8649	gi|7662180	centrosomal protein of 104 kDa [Homo sapiens]	1	0.93559	0.78752	0.838642	0.90171	0.888156	0.80893
2.12	8.264	gi|4826964	UV excision repair protein RAD23 homolog A isoform 1 [Homo sapiens]	1						

2.12	3.587	gi|237820620	glutamate-rich WD repeat-containing protein 1 [Homo sapiens]	1						
2.11	5.419	gi|5453629	dynactin subunit 2 isoform 1 [Homo sapiens]	2	1.03997	0.76595	1.000128	0.98143	0.874996	1.13523
2.11	6.579	gi|7706497	UMP-CMP kinase isoform a [Homo sapiens]	2	0.84369	0.62042	1.25376	1.12914	1.027527	0.9796
2.11	12.78	gi|5901926	cleavage and polyadenylation specificity factor subunit 5 [Homo sapiens]	1	1.40339	1.13078	2.007566	1.89092	1.45933	1.30963
2.1	2.178	gi|4506103	interferon-induced, double-stranded RNA-activated protein kinase isoform a [Homo sapiens]	1						
2.1	16.81	gi|5902102	small nuclear ribonucleoprotein Sm D1 [Homo sapiens]	1	1.06963	1.11104	1.238307	1.14907	0.987501	1.01882
2.09	10.23	gi|153791352	POTE ankyrin domain family member F [Homo sapiens]	45						
2.09	1.441	gi|27477136	zinc finger CCCH-type antiviral protein 1 isoform 1 [Homo sapiens]	1						
2.09	5.329	gi|45827712	protein quaking isoform HQK-7B [Homo sapiens]	2						

2.08	7.322	gi|5031631	lysosome membrane protein 2 isoform 1 precursor [Homo sapiens]	2						
	7.717	gi|21389315	tricarboxylate transport protein, mitochondrial isoform a precursor [Homo sapiens]	1						
	2.143	gi|4826870	nucleobindin-2 precursor [Homo sapiens]	1						
2.08	6.186	gi|223555917	protein LYRIC [Homo sapiens]	1						
2.08	4.658	gi|14719402	ribosome biogenesis regulatory protein homolog [Homo sapiens]	1	1.01198	0.95753	0.794942	1.12435	1.176603	0.75437
2.08	3.464	gi|255958289	perilipin-3 isoform 2 [Homo sapiens]	1						
2.08	10.11	gi|4506203	proteasome subunit beta type-7 proprotein [Homo sapiens]	2						
	15.66	gi|4826760	heterogeneous nuclear ribonucleoprotein F [Homo sapiens]	10						
2.07	63.64	gi|10863895	thymosin beta-10 [Homo sapiens]	4	0.95092	1.06945	1.129401	0.97323	1.04227	1.02178
2.07	1.448	gi|133925811	transportin-1 isoform 1 [Homo sapiens]	1						
	2.82	gi|20070228	nucleobindin-1 precursor [Homo sapiens]	1						

2.07	3.761	gi|42542379	serine/arginine repetitive matrix protein 1 [Homo sapiens]	2						
	10.68	gi|83641885	transcription factor BTF3 isoform A [Homo sapiens]	2	0.84445	0.94586	0.965018	1.10656	0.886324	1.11327
	5.515	gi|17402904	exosome complex component MTR3 [Homo sapiens]	1	0.73338	0.7553	0.937293	1.04302	0.94509	1.08138
2.06	3.664	gi|117938759	protein ALEX XLas [Homo sapiens]	2	0.79719	1.3224	0.93709	1.06557	1.270463	1.04841
	0.7087	gi|57164975	treacle protein isoform b [Homo sapiens]	1	0.78893	0.91721	0.515189	0.61893	0.519783	0.68702
2.06	10.12	gi|291621647	membrane-associated progesterone receptor component 2 [Homo sapiens]	2	1.41716	1.55355	1.276417	1.66223	1.337127	1.38874
2.06	3.593	gi|284448551	NADH-cytochrome b5 reductase 3 isoform 3 [Homo sapiens]	1	0.67991	0.59962	1.002967	0.87319	0.970829	0.83486
2.06	2.756	gi|4758340	phenylalanine--tRNA ligase alpha subunit [Homo sapiens]	1						
2.06	1.923	gi|48762920	6-phosphofructokinase, liver type [Homo sapiens]	1						
2.06	2.917	gi|19913424	V-type proton ATPase catalytic subunit A [Homo sapiens]	1						

6.557	gi|13376840	WD repeat-containing protein 61 [Homo sapiens]	1	1.08326	0.77932	0.993702	1.19976	0.904646	0.81726
4.157	gi|109452587	protein FAM98B isoform 1 [Homo sapiens]	1	0.92196	1.00054	1.440914	1.02121	1.427297	0.86005
2.06 20.33	gi|212276121	malignant T-cell-amplified sequence 1 isoform 2 [Homo sapiens]	2						
1.351	gi|217416374	coiled-coil domain-containing protein 164 [Homo sapiens]	1						
2.05 2.848	gi|83700233	eukaryotic translation initiation factor 3 subunit C [Homo sapiens]	1						
2.116	gi|123173757	ribonucleoprotein PTB-binding 1 [Homo sapiens]	1						
1.729	gi|21361659	importin-9 [Homo sapiens]	1						
7.317	gi|371872778	transmembrane and coiled-coil domain-containing protein 1 isoform b [Homo sapiens]	1	1.24359	1.33452	1.106128	0.63591	1.539528	0.99547
2.05 5.414	gi|13027602	DDRGK domain-containing protein 1 precursor [Homo sapiens]	1						
2.146	gi|148596949	nucleolar and coiled-body phosphoprotein 1 [Homo sapiens]	1	0.95672	0.96839	0.740136	1.56721	0.678062	0.57317
8.072	gi|209969700	coiled-coil domain-containing protein 124 [Homo sapiens]	2						

2.05	3.07	gi|4506753	ruvB-like 1 [Homo sapiens]	1						
	8.974	gi|4506181	proteasome subunit alpha type-2 [Homo sapiens]	2	1.01666	1.14002	0.840779	0.73922	0.93359	0.91044
2.05	6	gi|83921614	cytochrome b5 type B [Homo sapiens]	1	1.75772	4.65792	2.081941	1.80761	1.731172	1.11116
	1.815	gi|150378533	ubiquitin carboxyl-terminal hydrolase 7 [Homo sapiens]	1						
2.04	3.695	gi|4506209	26S protease regulatory subunit 7 isoform 1 [Homo sapiens]	1	0.55722	1.89039	2.196407	2.30166	1.325583	1.15757
	6	gi|42491362	inhibitor of nuclear factor kappa-B kinase-interacting protein isoform 2 [Homo sapiens]	1	1.33451	0.97453	0.847668	1.18622	1.052938	0.72269
	2.044	gi|21070997	stromal interaction molecule 1 precursor [Homo sapiens]	1						
	3.795	gi|81295407	acyl-coenzyme A thioesterase 9, mitochondrial isoform a precursor [Homo sapiens]	1						
2.04	9.302	gi|215490089	histone deacetylase complex subunit SAP18 [Homo sapiens]	1						
2.04	4.984	gi|16445421	secretory carrier-associated membrane protein 3 isoform 2 [Homo sapiens]	1						

2.04	16.13	gi|13775198	SH3 domain-binding glutamic acid-rich-like protein 3 [Homo sapiens]	1	1.17325	1.93406	1.533712	0.74937	2.796483	1.02523
2.04	5.556	gi|4885413	histidine triad nucleotide-binding protein 1 [Homo sapiens]	1	1.08696	1.08068	1.320304	1.09545	0.955904	1.13904
2.03	0.8224	gi|12083581	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-1 isoform a [Homo sapiens]	1						
2.03	6.912	gi|24308295	grpE protein homolog 1, mitochondrial precursor [Homo sapiens]	2						
2.493		gi|47458820	signal transducer and activator of transcription 3 isoform 3 [Homo sapiens]	1						
2.03	2.513	gi|33457336	interferon regulatory factor 2-binding protein-like [Homo sapiens]	1	0.85583	0.76036	1.050461	0.80003	0.814673	0.70424
1.183		gi|112789528	cohesin subunit SA-2 isoform a [Homo sapiens]	1	0.43056	0.45479	0.738483	0.83165	0.690343	0.78841
2.03	2.108	gi|156416003	succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial [Homo sapiens]	1	1.91777	2.26216	2.547209	1.10328	2.323811	1.762

2.03 1.116	gi|375298678	tripartite motif-containing protein 46 isoform 3 [Homo sapiens]	1						
7.303	gi|5031597	actin-related protein 2/3 complex subunit 3 [Homo sapiens]	1	1.13189	1.17068	1.715736	1.03642	0.949944	1.34422
2.03 4.286	gi|156631005	26S proteasome non-ATPase regulatory subunit 8 [Homo sapiens]	1						
1.783	gi|20127450	protein kinase C beta type isoform 2 [Homo sapiens]	1						
2.03 4.306	gi|5729991	26S protease regulatory subunit 6B isoform 1 [Homo sapiens]	1						
4.375	gi|46593007	cytochrome b-c1 complex subunit 1, mitochondrial precursor [Homo sapiens]	2						
2.03 5.389	gi|156416000	ubiquitin-fold modifier-conjugating enzyme 1 [Homo sapiens]	1	1.02711	0.9376	1.189223	1.25373	0.952967	1.2758
2.243	gi|164519136	endothelin-converting enzyme 1 isoform 3 [Homo sapiens]	1	0.46947	1.08796	0.734908	0.60167	0.984969	0.70464
2.919	gi|27262628	nuclear autoantigenic sperm protein isoform 2 [Homo sapiens]	1	1.17216	1.29347	1.231107	1.42517	0.964626	1.11173

6.757	gi|8922331	protein mago nashi homolog 2 [Homo sapiens]	1	0.96856	0.79949	0.987411	0.42568	0.737719	0.60271
1.11	gi|82799484	cAMP-specific 3',5'-cyclic phosphodiesterase 4B isoform 3 [Homo sapiens]	1						
6.667	gi|23065552	glutathione S-transferase Mu 3 [Homo sapiens]	1						
2.02 3.957	gi|42476108	neuronal membrane glycoprotein M6-a isoform 1 [Homo sapiens]	1	0.96611	0.86918	0.528097	0.77639	0.964402	0.7135
7.282	gi|4502419	flavin reductase (NADPH) [Homo sapiens]	1	0.82492	0.76531	0.45059	0.59814	0.256164	0.86739
2.02 7.627	gi|4759158	small nuclear ribonucleoprotein Sm D2 isoform 1 [Homo sapiens]	1	0.81977	0.97189	0.852765	1.01776	0.888338	1.01979
2.02 10.87	gi|118402586	lactoylglutathione lyase [Homo sapiens]	1						
12.95	gi|31543397	phosphoglycerate kinase 2 [Homo sapiens]	6						
4.326	gi|40538799	ubiquilin-4 [Homo sapiens]	2	1.18846	1.60339	0.711944	1.67087	1.463316	1.57243
2.414	gi|262359914	excitatory amino acid transporter 1 isoform 2 [Homo sapiens]	2	1.73275	2.19773	1.971571	2.32741	1.602276	1.20143
3.667	gi|5031599	actin-related protein 2/3 complex subunit 2 [Homo sapiens]	1	2.27054	1.63589	2.338493	1.29921	0.955917	0.99613

2.01	4.645	gi|4506387	UV excision repair protein RAD23 homolog B isoform 1 [Homo sapiens]	2						
	2.193	gi|48526509	mitochondrial import inner membrane translocase subunit TIM50 [Homo sapiens]	1						
2.01	7.339	gi|10801345	eukaryotic translation initiation factor 3 subunit K [Homo sapiens]	2						
2.01	5.859	gi|21361565	ATP synthase subunit b, mitochondrial precursor [Homo sapiens]	1						
	9.434	gi|4506629	60S ribosomal protein L29 [Homo sapiens]	1	1.17387	1.89628	1.432563	1.65658	1.126862	1.46405
	11.5	gi|5032133	eukaryotic translation initiation factor 1 [Homo sapiens]	1						
2	21.54	gi|4507951	14-3-3 protein eta [Homo sapiens]	10						
2	5.545	gi|109240550	paraspeckle component 1 [Homo sapiens]	3	0.87887	1.04839	0.999175	0.77477	0.444983	0.87935
2	9.79	gi|5453599	F-actin-capping protein subunit alpha-2 [Homo sapiens]	2	1.68511	1.74422	1.030252	0.81911	1.008209	0.92859
2	11.84	gi|8923557	protein C20orf11 [Homo sapiens]	2						

2	6.806	gi|4504001	gap junction alpha-1 protein [Homo sapiens]	2						
2	1.751	gi|16306548	serine--tRNA ligase, cytoplasmic [Homo sapiens]	1						
2	4.595	gi|40806190	TMEM189-UBE2V1 fusion protein [Homo sapiens]	2	1.07323	0.77767	0.860442	0.77759	0.910226	1.2448
2	2.063	gi|32307144	procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 precursor [Homo sapiens]	2						
2	4.665	gi|31377663	armadillo repeat-containing protein 10 isoform a [Homo sapiens]	1						
2	8.78	gi|22538465	proteasome subunit beta type-3 [Homo sapiens]	2						
2	3.169	gi|116256489	septin-9 isoform c [Homo sapiens]	1						
2	3.409	gi|24430151	26S protease regulatory subunit 4 [Homo sapiens]	2						
2	2.318	gi|14589889	cadherin-2 preproprotein [Homo sapiens]	1						
2	8.553	gi|4505893	proteolipid protein 2 [Homo sapiens]	1	1.02496	0.98235	1.004735	1.11342	0.972551	1.01897
2	16.76	gi|13376717	optic atrophy 3 protein isoform b [Homo sapiens]	1						
2	2.195	gi|112789562	gamma-interferon-inducible protein 16 isoform 2 [Homo sapiens]	1	1.037	0.5475	0.795671	0.88866	0.763422	0.71412

2	4.583	gi|149193321	G-rich sequence factor 1 isoform 1 [Homo sapiens]	1						
2	3.788	gi|58761500	obg-like ATPase 1 isoform 1 [Homo sapiens]	2						
2	10.14	gi|7705636	vesicle transport protein GOT1B [Homo sapiens]	1	6.49616	2.27653	4.414598	4.37636	2.95415	3.01594
2	5.234	gi|4506491	replication factor C subunit 4 [Homo sapiens]	1						
2	9.418	gi|5174723	mitochondrial import receptor subunit TOM40 homolog [Homo sapiens]	2						
2	3.65	gi|21264355	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1 [Homo sapiens]	1						
2	11.96	gi|4507129	small nuclear ribonucleoprotein E [Homo sapiens]	3	0.9772	0.90978	1.049372	1.20251	1.077493	0.90789
2	7.246	gi|70608174	tumor protein D52 isoform 2 [Homo sapiens]	1						
2	12.82	gi|13491174	MARCKS-related protein [Homo sapiens]	2	1.18191	1.19468	1.390571	1.32367	1.278538	1.18775
2	9.028	gi|260763955	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 13 [Homo sapiens]	1						

2	4.016	gi|222080062	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial precursor [Homo sapiens]	1					
2	6.771	gi|93277094	protein FAM210B [Homo sapiens]	1	1.49494	1.14174	2.094259	3.25341	2.226591	2.03697
2	4.864	gi|217035102	cleavage and polyadenylation specificity factor subunit 7 isoform 1 [Homo sapiens]	1					
2	10.56	gi|206725530	peptidyl-prolyl cis-trans isomerase FKBP2 precursor [Homo sapiens]	1					
2	15.79	gi|4507133	small nuclear ribonucleoprotein G [Homo sapiens]	1					
2	6.18	gi|6005794	PRA1 family protein 2 [Homo sapiens]	1	1.1698	1.31058	1.179651	1.26741	1.467394	1.16345
2	7.568	gi|6005884	translocon-associated protein subunit gamma [Homo sapiens]	1	1.20281	1.13479	1.173929	1.2955	0.868648	0.98349
2	14.74	gi|11024700	mitochondrial import inner membrane translocase subunit Tim13 [Homo sapiens]	4					
2	10.11	gi|5453704	PRA1 family protein 3 [Homo sapiens]	1	2.88799	0.96158	2.671536	0.63546	1.864812	1.05061
2	7.254	gi|4507605	CD70 antigen [Homo sapiens]	1					

2	3.175	gi|5870893	sodium-coupled neutral amino acid transporter 3 [Homo sapiens]	1	0.79604	1.03163	0.501218	0.83569	0.968156	0.28512
2	3.686	gi|25470886	DAZ-associated protein 1 isoform b [Homo sapiens]	1						
2	5.24	gi|282165814	transmembrane emp24 domain-containing protein 5 isoform 1 precursor [Homo sapiens]	1						
2	8.553	gi|4758714	microsomal glutathione S-transferase 3 [Homo sapiens]	2	1.07214	0.77882	1.157946	0.94076	1.049032	1.0345
2	15.23	gi|5453678	epididymal secretory protein E1 precursor [Homo sapiens]	1						
2	4.58	gi|18379349	synaptic vesicle membrane protein VAT-1 homolog [Homo sapiens]	2						
2	10.78	gi|46276863	parathymosin [Homo sapiens]	1	0.75017	1.47755	1.608629	1.14413	1.249661	1.06365
2	20.2	gi|14211889	protein dpy-30 homolog [Homo sapiens]	1	1.06193	0.74713	0.785391	1.0061	0.706888	0.70462
2	8.791	gi|7657176	protein canopy homolog 2 isoform 1 precursor [Homo sapiens]	1						
2	14.53	gi|7657609	signal peptidase complex catalytic subunit SEC11A [Homo sapiens]	1						

2	4.911	gi|4503727	peptidyl-prolyl cis-trans isomerase FKBP3 [Homo sapiens]	1	0.6618	0.88653	0.711606	0.64077	0.791742	0.88282
2	5.425	gi|5032069	splicing factor 3B subunit 4 [Homo sapiens]	1						
2	4.032	gi|221316575	lymphocyte function-associated antigen 3 isoform 2 [Homo sapiens]	1	1.10848	0.95693	1.071785	0.83056	0.940833	0.95787
2	8.475	gi|341915584	PREDICTED: prothymosin alpha-like [Homo sapiens]	1	1.05324	1.3211	1.186233	1.03384	1.033843	1.26186
	2.64	gi|259155315	mitochondrial 2-oxoglutarate/malate carrier protein isoform 2 [Homo sapiens]	1	1.82798	2.05946	1.756294	1.94721	1.415	1.33975
1.93	11.86	gi|5729850	guanine nucleotide-binding protein G(k) subunit alpha [Homo sapiens]	3	0.56981	0.66885	0.601442	0.40964	0.354968	0.60163
	6.077	gi|4502227	ADP-ribosylation factor-like protein 1 [Homo sapiens]	1						
1.85	7.519	gi|4503659	ubiquitin-like protein fubi and ribosomal protein S30 precursor [Homo sapiens]	1	1.93251	0.52726	0.873157	1.16378	0.939255	1.03131
1.83	1.74	gi|52485606	formin-like protein 2 [Homo sapiens]	2						
	3.93	gi|4505585	platelet-activating factor acetylhydrolase IB subunit beta isoform a [Homo sapiens]	1						

1.8	2.545	gi|68508955	CD97 antigen isoform 3 preproprotein [Homo sapiens]	1						
1.79	3.438	RRRgi|45021	REVERSED annexin A5 [Homo sapiens]	1						
1.77	10.26	gi|73912720	mitochondrial inner membrane organizing system protein 1 isoform a [Homo sapiens]	1	1.21962	0.9844	0.784586	0.64946	1.253601	1.76032
1.76	1.531	gi|157388967	coiled-coil domain-containing protein 87 [Homo sapiens]	2	0.85902	0.99372	1.045406	0.93941	1.111446	0.61127
1.7	35.73	gi|14141161	heterogeneous nuclear ribonucleoprotein U isoform b [Homo sapiens]	34	1.76342	1.10382	1.33401	1.0886	1.327369	1.53358
1.7	11.64	gi|334688844	glial fibrillary acidic protein isoform 3 [Homo sapiens]	7	0.91713	0.81444	1.222358	1.12989	1.145062	1.32143
1.7	5.932	gi|6005890	transcription elongation factor B polypeptide 2 isoform a [Homo sapiens]	1						
1.7	11.64	gi|334688844	glial fibrillary acidic protein isoform 3 [Homo sapiens]	7	0.91713	0.81444	1.222358	1.12989	1.145062	1.32143
1.69	19.01	gi|4506587	replication protein A 14 kDa subunit [Homo sapiens]	1						
1.68	4.93	gi|50659074	ATP synthase lipid-binding protein, mitochondrial isoform A precursor [Homo sapiens]	1	0.92016	0.90905	0.935273	1.02621	1.145511	0.92555

1.66	11.2	gi|7705477	tRNA methyltransferase 112 homolog [Homo sapiens]	1					
1.229		gi|122939157	gem-associated protein 4 [Homo sapiens]	2					
1.62	4.93	gi|4504347	hemoglobin subunit alpha [Homo sapiens]	1	1.59508	0.66166	1.879694	0.99311	1.061659	0.51581
5.797		gi|6678271	TAR DNA-binding protein 43 [Homo sapiens]	2					
1.143		gi|116256464	uncharacterized protein C2orf55 [Homo sapiens]	2					
2.105		gi|28178838	isocitrate dehydrogenase [NAD] subunit gamma, mitochondrial isoform b precursor [Homo sapiens]	1					
2.256	RRR	gi|166235	REVERSED DNA damage-regulated autophagy modulator protein 2 [Homo sapiens]	1					
1.57	0.5336	gi|297207099	AT-rich interactive domain-containing protein 1B isoform 2 [Homo sapiens]	1					
1.55	3.918	gi|311893345	copine-1 isoform c [Homo sapiens]	1					
0.503		gi|61676188	E3 ubiquitin-protein ligase HUWE1 [Homo sapiens]	1	1.09776	1.27215	0.877703	1.14276	0.851826	0.89419

		Protein							
7.469		gi|4506193	proteasome subunit beta type-1 [Homo sapiens]	1	1.43887	2.04849	1.395369	1.22527	1.514252	1.1148
3.981		gi|4557237	acetyl-CoA acetyltransferase, mitochondrial precursor [Homo sapiens]	1	1.24819	1.1585	1.084654	0.95572	0.973047	1.16623
3.233		gi|68799814	AP-2 complex subunit mu isoform b [Homo sapiens]	1
1.5	0.8475	gi|30578410	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3B [Homo sapiens]	1
1.5	0.8706	gi|39780588	pre-rRNA-processing protein TSR1 homolog [Homo sapiens]	1	0.90888	1.05537	0.932494	0.94253	1.05303	1.22672
1.5	1.675	gi|333360851	serpin H1 precursor [Homo sapiens]	1	0.72935	0.7743	0.915738	0.72061	0.754026	0.75084
4.762		gi|70906441	deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial isoform 1 precursor [Homo sapiens]	1	2.0039	0.45709	1.823647	0.98667	1.09756	1.21117
1.48	4.02	gi|312596881	26S protease regulatory subunit 8 isoform 2 [Homo sapiens]	1
1.47	1.849	gi|18426915	drebrin isoform a [Homo sapiens]	1

		gi	Description	Count						
1.47	1.98	gi|4503915	trifunctional purine biosynthetic protein adenosine-3 isoform 1 [Homo sapiens]	1	1.39568	1.34603	0.916473	0.99879	1.676535	1.02895
1.44		gi|296841091	protein kinase C and casein kinase substrate in neurons protein 2 isoform A [Homo sapiens]	1						
1.626		gi|139394599	hypermethylated in cancer 2 protein [Homo sapiens]	1	1.13664	1.05848	0.96557	1.0482	1.011859	0.96627
1.559		gi|8051636	exportin-T [Homo sapiens]	1						
1.241		gi|359279861	dihydropyrimidinase-related protein 5 [Homo sapiens]	1						
1.44	3.081	gi|4503519	eukaryotic translation initiation factor 3 subunit F [Homo sapiens]	1						
7.634		gi|4757926	RNA-binding protein 39 isoform b [Homo sapiens]	2						
4.233		gi|115430112	receptor expression-enhancing protein 5 [Homo sapiens]	1	0.81179	1.07698	1.398986	0.48126	1.126422	1.05482
1.31	59.91	gi|14389309	tubulin alpha-1C chain [Homo sapiens]	53						
1.31	3.704	gi|52426743	luc7-like protein 3 [Homo sapiens]	1						

| 1.3 | 3.965 | gi\|8923390 | coiled-coil-helix-coiled-coil-helix domain-containing protein 3, mitochondrial precursor [Homo sapiens] | 1 | 0.85987 | 0.85484 | 0.750112 | 1.52743 | 0.919463 | 0.55202 |