Investigating the association between hepcidin and brain amyloid-β burden in cognitively normal elderly individuals

Maryam Mohammadi

A thesis submitted in fulfilment of the requirements for the degree of Master of research

Supervisor:

Professor Ralph Martins

Macquarie University

Faculty of Medicine and Health sciences

Department of Biomedical Sciences

MACQUARIE University

Submitted: October 2018

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Abstract

Introduction: Metal dyshomeostasis is one of the predominant pathways in the pathogenesis of Alzheimer's disease (AD). Hepcidin is a protein synthesised in the liver, and it is known to play a key role in iron regulation. Hepcidin level alteration has been previously observed in the brain and blood samples of patients with AD, however, it has not been investigated yet in preclinical AD, i.e. prior to cognitive impairment.

Objective: Investigate the association of serum hepcidin with neocortical amyloid load (NAL). Also, investigate its association with AD risk factors, cognitive performance and other iron related proteins.

Methods: Serum hepcidin concentration was measured by using the enzyme-linked immunosorbent assay in participants from the Kerr Anglican Retirement Village Initiative in Ageing Health cohort. Participants were aged 65–90 years and they were cognitively normal based on the neuropsychological tests. Subjects were categorised into the high NAL (n=35) and low NAL (n=65) groups via positron emission tomography (PET) scans using a standard uptake value ratio cutoff = 1.35.

Results: Serum hepcidin levels were significantly higher in participants with high NAL compared to those with low NAL. Moreover, a significant positive association was observed between the serum hepcidin and NAL. To evaluate the potential of hepcidin in distinguishing the low NAL and high NAL groups, receiver operating characteristic (ROC) curves were generated using the logistic regression. The area under the curve slightly increased from 0.78 in the 'base model' to 0.81 in the 'base+hepcidin' model.

Conclusion: Current findings show that increased serum hepcidin is an early event in AD pathogenesis. Therefore, hepcidin can be considered as a potential biomarker within a diagnostic panel of markers for AD, and could be helpful in the process of identifying therapeutic targets for AD.

Declaration of originality

I certify that the work in this thesis entitled “Investigating the association between hepcidin and brain amyloid-β burden in cognitively normal elderly individuals” has not been previously submitted for a degree nor as part of requirements for a degree to any other university or institution other than Macquarie University. I certify that the thesis is an original piece of research and it has been accomplished by myself. Any help and assistance that I have received during my research work and toward the preparation of the thesis have been appropriately acknowledged. I also certify that all information sources and literature used in the thesis are properly cited.

All the ethics required for this study have been received from the Macquarie University Human Research Ethics Committees (Ref. No. 5201701078) and the Bellberry Human Research Ethics Committee of Australia.

Maryam Mohammadi

Department of Biomedical Sciences

Faculty of Medicine and Health Sciences

Macquarie University

Date of Submission

21/10/2018

This thesis comprises a research work conducted within 10-month from January to October 2018, in partial fulfilment of the requirements for the Master of Research Degree at Macquarie University.

Acknowledgements

Firstly, I would like to express my sincere gratitude to my principal supervisor Prof Ralph Martins for the continuous support of my MRES study, his patience, motivation, and immense knowledge. I am also deeply grateful to Dr Pratishtha Chatterjee for providing directions to undertake this thesis, that without her support and encouragement this study could not be accomplished.

My sincere thanks also go to Associate Prof Hamid Sohrabi and Dr Tejal shah who provided me with the invaluable advices in all steps of the research and writing of this thesis. I would also like to extend my thanks to all of the members of Martins group in Macquarie University who made me feel welcome and who helped me learn the skills I needed.

Great supports of Macquarie Medical Imaging for providing MRI and PET scans, Laverty Pathology for blood examinations, KaRaMind Institute and Anglicare Retirement Village for managing the KARVIAH cohort are all highly acknowledged.

I must express my very profound gratitude to my parents for providing me with unfailing support and continuous encouragement throughout my years of study and in my whole life.

Last but not the least, I would like to thank my lovely husband for his support through the process of researching and writing this thesis. This accomplishment would not have been possible without him.

Abbreviations

AD	Alzheimer's disease
AChEI	Acetylcholinesterase inhibitors
ADAD	Autosomal dominant AD
APOE	Apolipoprotein E
APP	Amyloid-β precursor protein
AUC	Area under the curve
Aβ	Amyloid-β
BBB	Blood brain barrier
BMI	Body Mass Index
BMPR	Bone morphogenetic protein 6 receptor
BNT	Boston Naming Test
CDR	Clinical dementia rating
CHAMPS	Community Healthy Activities Model Program for Seniors
COWAT	Controlled Oral Word Association
DASS	Depression Anxiety and Stress Scales
DAT	Dementia of the Alzheimer type
Dcytb	Duodenal cytochrome b
DMT1	Divalent metal ion transporter-1
DSST	Digit Symbol Substitution Test
ELISA	Enzyme-linked immunosorbent assay
FBB	F^{18}-Florbetaben
FDG	Fluorodeoxyglucose
FFQ	Food Frequency Questionnaire
Fpn	Ferroportin
GDS	Geriatric depression scale
GSK3	Glycogen synthase kinase 3
HAMP	Hepcidin antimicrobial peptide gene
HAMP	Hepcidin antimicrobial peptide
HO	Heme oxygenase
hs-CRP	High sensitivity C reactive protein
Hsp 90	Heat shock protein 90
IDE	Insulin degrading enzyme
IFN	Interferon
IP–MS–IL	Immunoprecipitation–mass spectrometry interleukin
IQCODE	Informant Questionnaire on Cognitive Decline in the Elderly
IREs	Internal ribosome entry site
Jak	Janus kinase
KARVIAH	Kerr Anglican Retirement Village Initiative in Aging Health
MAC-Q	Memory Assessment Clinical-Q
MCD	Multicentric Castleman's disease
MCI	Mild cognitive impairment
MCP	Monocyte chemoattractant Protein
MMSE	Mini-Mental State Examination
MoCA	Montreal Cognitive Assessment Score

MRI	Magnetic resonance imaging functional MRI
NAL	Neocortical amyloid load
NFT	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
PET	Positron emission tomography
PIB	Pittsburgh compound B
PQSI	Pittsburgh Quality Sleep Index
PSEN	Presenilin
p-tau	Phosphorylated tau
RAVLT	Rey Verbal Learning Test
RCFT	Rey Complex Figure Test
ROC	Receiver operating characteristic
ROI	Regions-of-interest
SAD	Sporadic alzheimer's disease
SEC-ICP-MS	Size exclusion chromatography–inductively coupled Plasma–mass spectrometry
SMC	Subjective memory complainers
SPGR	Spoiled gradient recall
Stat	Signal transducer and activation of transcription
SUVR	Standardized uptake value ratio
T2D	Type 2 diabetes
Tf	Transferrin
TFR	Transferrin-transferrin receptor
TfR1	Transferrin receptor 1
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
t-tau	Total tau
wr-CRP	Wide-range C reactive protein
WTAR	Wechsler Test of Adult Reading

Chapter 1
Literature review

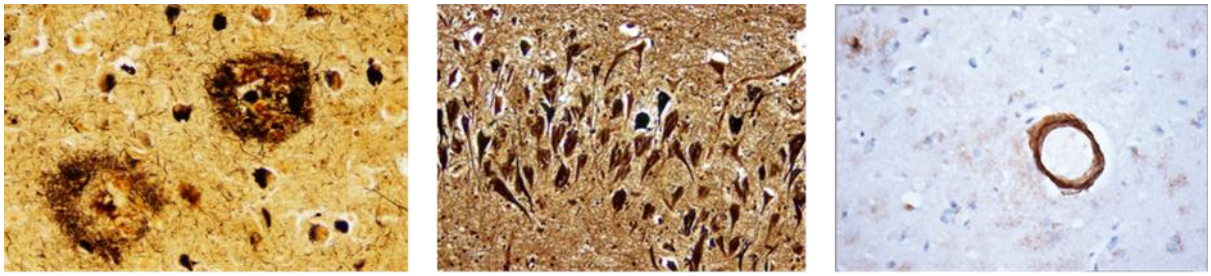
1.1. Alzheimer's disease

Alzheimer's disease (AD) is the most common cause of dementia which has been estimated to affect 150 million people globally by 2050 [1]. According to the Alzheimer's Association, currently one person develops AD every 65 seconds, in the United States. By the end of 2050, it is predicted that every 33 seconds a new AD case will occur resulting in one million new AD cases every year which will adversely influence the healthcare system and costs. Based on official death certificates in 2014, AD was the sixth leading cause of death in United States and the fifth leading cause of death in Americans over 65 years old [2]. It is estimated that currently over 400,000 individuals are living with dementia in Australia. Dementia is the second leading cause of death in men in Australia and it is also the first leading cause of death in Australian women [3].

AD is a progressive neurodegenerative disease where an overall loss of synapses and neurons in different regions of the brain, particularly the cerebral cortex and hippocampus, occur [4]. AD is characterised by memory impairment and neuropsychiatric symptoms including depression, agitation, anxiety and aggression [5].

1.2. Neuropathological hallmarks of AD

AD is mainly characterised by three neuropathological features: 1. extracellular plaques of aggregated amyloid-β (Aβ) [6], 2. intracellular neurofibrillary tangles (NFT) primarily constituting hyperphosphorylated tau [7], and 3. cerebral amyloid angiopathy (CAA) caused by the deposition of Aβ within cerebral vessels [8]. Other prominent features of the disease include oxidative modification of biomolecules in the brain, neuronal glucose metabolism impairment and the imbalance in metal ion homeostasis such as zinc, copper, iron and calcium [9]. Aβ plaques (which include senile plaques), NFT which accumulates within neurons and CAA that accumulates within the blood vessels of the brain of AD patients are shown in Figure 1.2.



(1) (2) (3)

Figure 1.1. Neuropathological hallmarks of Alzheimer's disease. (1) Amyloid beta (Aβ) plaques (2), Neurofibrillary Tangles (NFT) [10], (3) Cerebral amyloid angiopathy (CAA) [11].

1.2.1. Amyloid plaques

Aβ, is derived from amyloid-β precursor protein (APP) which is sequentially cleaved by a series of enzymes named secretases. APP is a transmembrane protein cleaved by two different pathways, amyloidogenic and non-amyloidogenic pathways. In the non-amyloidogenic pathway, APP is cleaved by α-secretase within the Aβ sequence preventing the formation of Aβ. However, in amyloidogenic pathway APP is cut firstly by the β-secretase and secondly by γ-secretase to produce Aβ peptides (Figure 1.2). Depending on the precise cleavage point, 37 to 49 amino acid residues long Aβ isoforms are generated, with Aβ40 and Aβ42 as the most dominant forms. Although Aβ40 is the most common form, two additional hydrophobic amino acids to the existing C-terminal in Aβ40 makes Aβ42 more aggressive toward AD pathogenesis than Aβ40 [6].

According to the amyloid hypothesis, Aβ overload and deposition are the main causes of AD pathogenesis [12, 13]. The excessive amount of Aβ in the brain occurs as a result of either overproduction of the peptide or impairment in its clearance from the brain. Aβ peptides are hydrophobic and notorious for their tendency to aggregate into the smaller soluble oligomers. These oligomers are believed to be the most toxic form of Aβ which could then form insoluble large fibrils. Accumulation and deposition of the insoluble fibrils and soluble oligomers in form of plaques in the cerebral cortex followed by subsequent events including oxidative stress and inflammation are believed to play crucial roles in neuron loss and dysfunction.

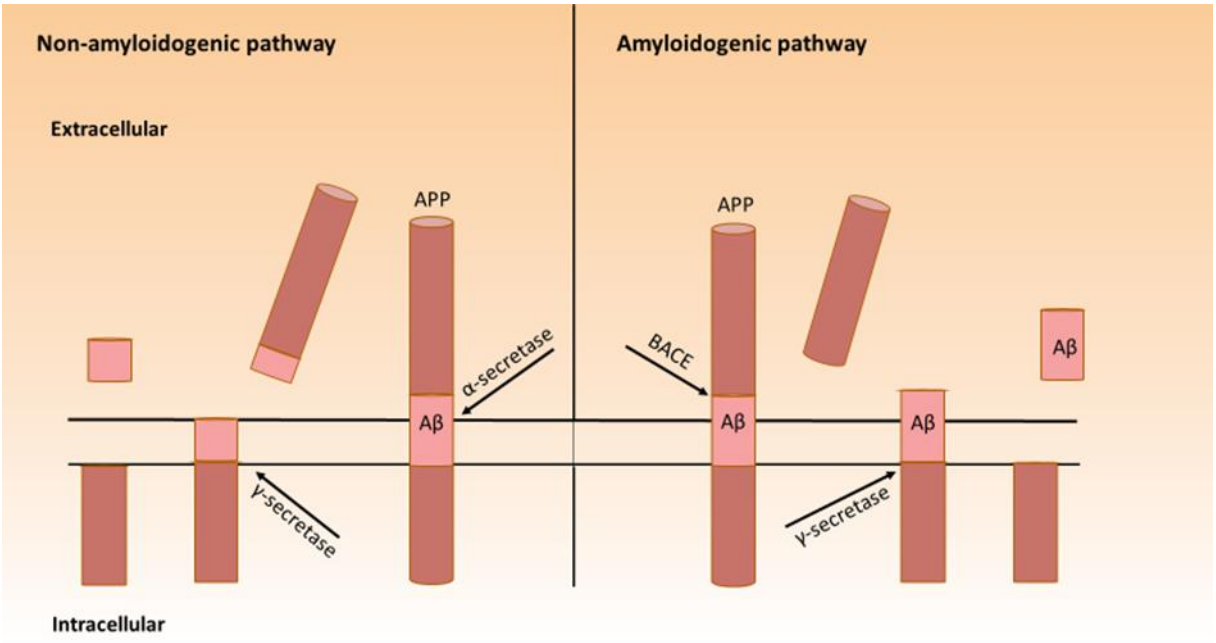


Figure 1.2. Non-amyloidogenic and Amyloidogenic pathways in amyloid-β precursor protein (APP) process. In non-amyloidogenic pathway α-secretase cleaves APP in the middle of Aβ sequence preventing the formation of Aβ. However, in amyloidogenic pathway, APP is first cleaved by β-cut APP-cleaving enzyme (BACE) and then by γ-secretase leading to Aβ peptides generation. Arrows in the figure show the cut-off points of the enzymes.

1.2.2. Neurofibrillary tangles

Tau is a protein, predominantly found in the axon of neurons, which is associated with the stability structure of microtubule and axonal signalling [14]. Tau is normally found in a phosphorylated form, however in AD abnormal hyperphosphorylation of tau affects its biological functions [7]. In fact, hyper phosphorylated tau destabilizes the microtubules and aggregates into helical filaments forming intraneuronal tangles namely NFT which are associated with synaptic and neuronal loss [15]. Recent studies suggest that Aβ and NFT synergistically interact to develop AD wherein NFT increases the aggregation of Aβ oligomers and Aβ increases the production of NFT [16].

1.2.3. Cerebral amyloid angiopathy

CAA is characterized by the deposition of Aβ in the walls of cerebral blood vessels [17]. It is an aged-related process that occurs in 10% to 40% of the normal aged brains while its prevalence in AD cases is up to 98% [18]. Cerebral Aβ deposits are mostly found in the cortical arteries while it is less observed in the veins and capillaries [19]. CAA lesions are usually deposited in the outer basement membrane of the vessels or between the smooth muscle cells of lamina media. CAA is accompanied with the destruction of vessel walls, loss of smooth muscle cells and fibrinoid necrosis. Severe degeneration in vessel walls can be accompanied by hemorrhages and cerebrovascular infarction [8].

1.3. Types of AD

There are two types of AD, namely autosomal dominant AD (ADAD) and sporadic AD (SAD). ADAD is caused by genetic mutations in *APP* [20], presenilin-1 (*PSEN1*) [21] and presenilin-2 (*PSEN2*) [22] genes and constitutes less than 1% of all Alzheimer's cases. *PSEN1* and *PSEN2* genes encode the presenilin proteins that form the essential component of the γ-secretase enzyme. These mutations affect APP processing and result in an increase in Aβ production or an increase in Aβ42: Aβ40 ratio [23]. Mutations in *PSEN1* are the most common cause of ADAD and patients with *PSEN1* mutation develop the most aggressive form of AD in early ages (as early as 25 years old) [24]. However, *PSEN2* mutation result in a wider range of age of disease onset (39-83 years) compared to *PSEN1* mutation carriers (25-65 years). On the other hand, SAD is the most common type of AD in people over 65 years and occurs in patients without a direct pattern of inheritance [3].

1.4. Genetic and non-genetic risk factors

Numerous genetic and lifestyle modifiers have been suggested to be associated with AD, such as carriage of the apolipoprotein (*APOE*) ε4 allele [25], age [26], diabetes [27, 28], hypertension [29], smoking [30], body weight [31-33], alcohol consumption [34, 35], plasma lipid levels [36], cerebrovascular disease, metabolic syndrome and traumatic the brain injury.

1.4.1. Apolipoprotein E allele

APOE plays an important role in lipid transport and metabolism in the brain, providing essential lipids which are required for synaptic plasticity as well as neuronal growth, maintenance and repair [37]. *APOE* has three alleles namely ε2, ε3 and ε4. Structural differences in APOE proteins, based on allelic differences, result in altered functionality between different isoforms. For instance, a study by Rapp et al. showed that *APOE* ε4 is less efficient at transporting brain cholesterol compared to *APOE* ε3 which could negatively influence synaptic integrity and plasticity in ε4 carriers versus ε3 carriers [38].

APOE ε4 has been well established as the strongest genetic risk factor for sporadic AD by several studies [39]. Carrying the ε4 allele is also associated with an increased risk of AD onset at early ages [40]. For instance, a meta-analysis by Farrer et al. reported that individuals with one or two copies of the ε4 allele have a greater risk of developing AD compared to ε2 or ε3 allele carriers, while the ε2 allele provides protection against AD [41]. Although the mechanism by which *APOE* ε4 increases the risk of AD is not completely understood, it has been reported that ε4 affects Aβ metabolism and clearance. By binding to Aβ, *APOE* ε4 accelerates Aβ aggregation and deposition in the form of senile plaques. In addition, it has been proposed that *APOE* ε4 decreases the clearance of Aβ [42].

1.4.2. Age

Aging is the biggest risk factor of AD. The level of Aβ in the cerebral cortex of cognitively normal individuals seems to have a positive correlation with age [43]. A study by Miner et al. showed that the level of insoluble Aβ42 increases progressively with age in human brain tissue [44]. In addition, age-

related production of free radicals contributes to increased oxidative stress and inflammation responses causing impaired neurotransmission and synaptic damage [45]. Moreover, age-related iron accumulation in the brain has been shown to be associated with AD progression [46, 47]. A recent study has investigated the effect of aging on pathological AD hallmarks and the expression of iron transporters and regulators in rat brains [48]. The results showed that the level of hyperphosphorylated tau, Aβ oligomers and APP-derived soluble fragments significantly increase in the hippocampus and cerebral cortex with ageing. Also, they have seen the association between age and alterations of iron transporters and regulators in the rat brain with a region-specific pattern [48].

1.4.3. Type 2 diabetes

Type 2 diabetes (T2D) is a well-established risk factor for AD. Several epidemiological studies on elderly populations have reported an increased risk of dementia among T2D patients [49-51]. Moreover, a link between T2D and cognitive impairment has been reported in AD and vascular dementia [52]. T2D has been found to increase the risk of AD via several mechanisms including dysregulation in glucose metabolism and insulin resistance [53]. Dysregulation in glucose metabolism can cause hyperglycaemia which is a toxic condition in the brain [54]. Hyperglycaemia can cause an imbalance in the generation and scavenging of reactive oxygen species, and lead to microvascular changes in the brain [54]. On the other hand, insulin regulates the metabolism of β-amyloid and tau [53]. High insulin levels during insulin resistance have been seen to accelerate the deposition of Aβ and phosphorylated tau in the brain [55]. Insulin degrading enzyme can be also another link between T2D and AD. IDE influences the clearance of Aβ in the brain wherein Aβ and insulin are present as two competing substrates [56]. An *in vivo* study on a mouse model with homozygous deletions of the IDE gene showed a decrease in Aβ degradation in the brain [56]. The mouse model also showed increased cerebral Aβ accumulation as well as hyperinsulinemia and glucose tolerance. Insulin deficiency may also cause the loss of protection against toxicity of Aβ oligomers [57]. Interestingly, some of the anti-diabetic medicines such as thiazolidinedione and incretin hormone analogues have been shown to decrease Aβ pathology and cognitive decline in AD transgenic mouse models [57].

1.4.4. Hypertension

Several studies on the relationship between hypertension and risk of AD found that elevated blood pressure in mid-life increases the risk of developing AD [58, 59]. Hypertension is suggested to reduce the integrity of the BBB causing the extravasation of proteins into the brain tissue. Protein extravasation may lead to neuronal damage, dysfunction and apoptosis [60].

1.5. Modifiable lifestyle factors

Lifestyle parameters such as diet, physical and mental activities, education and sleep have been reported to influence the risk of developing AD [61]. For instance, an observational study in an elderly cognitively normal cohort reported that participants with higher levels of physical activity obtained

higher scores in cognitive tests compared to participants with low levels of physical activity [62]. Moreover, this study showed that high intensity physical activity was more beneficial than the quantity of activity [63]. In addition, recent rodent studies suggest that physical activity reduced aggregation and deposition of Aβ in the brain [64, 65].

Studies also suggest that a Mediterranean diet comprising high intake of fruits and vegetables and low intake of red meat, reduces the risk of AD [66, 67]. A study by Solfrizzi and colleagues concluded that high consumption of fish, fruits, vegetables and supplements consisting of antioxidants, vitamin B, vitamin C, polyphenols and polyunsaturated fatty acids are associated with reduced risk of AD development [66].

1.6. Alzheimer's disease biomarkers

Biomarkers are invaluable for diagnosing the disease and determining the risk of AD. The current established biomarkers for AD are described below.

1.6.1. CSF biomarkers

Since there is free transport of proteins between the CSF and the brain, the level of total tau (t-tau), phosphorylated tau (p-tau) and Aβ42 in the CSF, reflect the levels of the aforementioned proteins (Aβ and tau) in the brain, enabling the early diagnosis of the disease [68]. Numerous studies showed that in mild cognitive impairment (MCI) and AD, CSF level of Aβ decreases whereas the levels of t-tau and p-tau increase compared to cognitively normal individuals [69]. Although the relationship between CSF biomarkers and the brain amyloid load and NFT remains unclear, it is suggested that the decrease in CSF Aβ level occurs as a result of Aβ sequestration in brain amyloid plaques [70]. Although a study found a positive correlation between CSF p-tau and NFT in individuals with AD [71], others have not [72, 73]. The sensitivity and specificity of these CSF biomarkers have been tested in few studies, for example Parnetti et al. reported a sensitivity of 75% and specificity of 96% [74]. Despite the high accuracy of CSF biomarkers [75], it is not commonly used in routine clinical practice because of the invasive nature of CSF collection.

1.6.2. Imaging biomarkers

1.6.2.1. Structural magnetic resonance imaging (MRI)

By using structural MRI in AD, atrophy in the medial temporal lobe especially in the hippocampus and amygdala is detectable. In AD, atrophy is also found in posterior regions including the posterior cortex, occipital lobes, posterior cingulate and precuneus [76]. Atrophy in the hippocampus and entorhinal cortex leads to progressive memory impairment and an increased risk of AD [77]. However, these structural changes are not specific to AD and may occur in other neurodegenerative diseases as well as normal aging. Several studies proposed structural MRI biomarkers as a valuable diagnostic tool. A study by Chetelat et al. showed that among MCI patients, those converting to AD develop higher atrophy in the hippocampus and the inferior and middle temporal gyri compared to those who do not

convert to AD [78]. It is also suggested that atrophy in the corpus callosum may help to discriminate AD from frontotemporal dementia where the posterior area shows greater atrophy [79].

1.6.2.2. Functional MRI

In functional MRI (fMRI), neuronal activity during rest or a cognitive task in a specific brain region is investigated. Blood oxygenation level dependent-fMRI (BOLD-fMRI) is the most common method in which alterations in blood flow based on changes in deoxyhemoglobin concentration are measured [80]. BOLD signals in the medial temporal lobe, parietal lobe and hippocampal areas have been seen to be lower in AD patients compared to controls during a cognitive task [80, 81]. Another study observed different task-associated neuronal activity patterns in patients with MCI compared to the healthy controls [82]. Since the default mode network of the brain is disrupted in AD, resting state fMRI can reflect the functional connectivity deficits in AD [83]. In addition, some studies showed that fMRI has potential in discriminating between AD and MCI as well as MCI and controls [84].

1.6.2.3. Positron emission tomography (PET)

Positron emission tomography (PET) is regarded as a reliable diagnostic tool for dementia with high diagnostic and prognostic capabilities. Numerous studies have investigated several PET ligands targeting Aβ, tau and glucose uptake activities [85]. Pittsburgh compound B (PIB) was the first specific amyloid imaging probe which selectively bound to Aβ plaques [86]. Retention of PIB positively correlated with fibrillary amyloid plaques at the brain autopsy of people diagnosed with AD [87] while correlated inversely with the CSF Aβ42 levels in AD patients [88]. Although, some studies found significant PIB retention in people with no cognitive impairment [89], further studies are required to explain whether this retention could characterise the preclinical AD. Recently two amyloid imaging agents, Florbetaben (18F-BAY94-9172) and Florbetapir (18F AV-45), have been developed with similar binding pattern to PIB but with longer half-life, which also have the potential to distinguish AD from controls with high sensitivity [88]. The 18F-fluorodeoxyglucose (FDG) is also another PET ligand that reflects cerebral glucose metabolism. Several studies found that cerebral glucose metabolism decreases in MCI and AD compared to control groups [90]. FDG-PET measurements predict cognitive decline in dementia with a high sensitivity (94%) but a low specificity (73-78%) [91]. Lastly, molecular PET-imaging ligands indicating microglial activation are being investigated for AD diagnosis [92]. The 11C-(R)-PK11195 is the most common PET probe which has shown an increase retention in AD and MCI in comparison with age-matched healthy individuals [92].

1.6.3. Blood biomarkers

Currently, the only definite clinical way for AD diagnosis is the post-mortem criteria or autopsy. Moreover, the current gold standard markers are amyloid-PET neuroimaging and CSF biomarkers; however they are uneconomical and invasive to implement for population wide screening. Hence, the search for less invasive and more economical biomarkers (e.g. blood biomarkers) with diagnostic and prognostic values for AD has significantly grown over the last decade. It has been suggested that blood-

based biomarkers (such as Aβ) can be considered as the first step in the diagnostic process of AD, similar to diagnosis protocols used in other pathologies such as cancer and cardiovascular diseases [93]. In physiological circumstances, the steady-state level of brain Aβ is adjusted by the production and deposition of Aβ in the brain as well as through platelets in the periphery. Consequently, in cognitively normal people the brain Aβ loads reflect the plasma Aβ concentrations [61].

Interestingly, the results of studies investigating the alteration of plasma Aβ as an AD biomarker have been inconsistent [94]. However, a recent promising study by Nakamura et al. has reported that plasma Aβ concentrations (and APP669–711/Aβ 1–42, Aβ 1–40/Aβ 1–42) measured by immunoprecipitation–mass spectrometry (IP–MS) were over 90% accurate in predicting the brain Aβ load [95]. Further research is required to investigate the diagnostic reproducibility and prognostic value of this biomarker. A number of studies have evaluated the diagnostic value of other potential blood biomarkers such as cholesterol, homocysteine, iron related protein including ferritin and inflammation related protein such as C-reactive protein, interleukin (IL)-1b, tumour necrosis factor (TNF) and IL-6, although further validation studies are required [61].

1.7. Treatment strategies

Despite significant efforts of pharmaceutical industries and research studies to identify the pathophysiology of AD, there is still a lack of effective treatment to cure AD or to inhibit the progression of the symptoms. Currently, there are only four approved medicines which are being clinically administered for AD related dementia, while their efficacy is still controversial [96]. Three drugs, namely Donepezil, Galantamine and Rivastigmine are acetylcholinesterase inhibitors (AChEI), and the fourth named Memantine is an antagonist of the N-methyl-D-aspartate (NMDA) receptors. AChEI drugs could increase the cognitive capacity of AD patients by inhibiting the acetylcholinesterase in synapses and increasing the cholinergic transmission [97]. However, Memantine decreases the excitotoxicity degeneration of the glutaminergic system by blocking the NMDA receptors [98]. Although all four drugs have been approved for use in moderate to severe AD, there is not enough evidence for their curative effects [96]. Nevertheless, clinical trials show that these medicines can generally be used as a palliative therapy relieving symptoms and slowing down the progression of AD. Currently, more efforts are being focused on developing drugs which target Aβ and NFT and enable prevention of the disease in the early stages.

1.7.1. Anti-amyloid strategies

Anti-amyloid therapeutics aim to target different aspects of Aβ metabolism including Aβ production, aggregation and clearance.

1.7.1.1. Targeting Aβ production

Gamma (γ) secretase plays a critical role in the formation of Aβ40 and Aβ42 proteins, and therefore the development of their associated inhibitors has been considered to be a breakthrough [99].

However, γ-secretase is involved in the processing of other proteins such as Notch protein which is essential for cell proliferation and development [100]. Therefore, non-specific inhibition of this enzyme can lead to serious side effects in AD patients, which has resulted in the discontinuation of drugs such as Semagacestat and Avagacestat in different phases of clinical trials [101, 102].

Beta (β) secretase plays a key role in initiating the amyloidogenic pathway of APP processing (as already shown in Figure 1.2). Developing β-secretase (BACE1) inhibitors appeared to be challenging given the number of substrates they had, including neuregulin-1 which is essential for the myelination of nerves [103]. Therefore, non-specific inhibiting of this enzyme could result in severe side effects as well. E2609 and MK-8931 are two β-secretase inhibitors showing significant decrease in Aβ40 and Aβ42 production [104], however they still need to undergo clinical trials [104].

1.7.1.2. Aβ aggregation inhibitors

The neurotoxic and synaptotoxic features of Aβ oligomers make them good targets for therapeutic purposes. Tramiprosate is an Aβ aggregation inhibitor which inhibits the interaction between Aβ and endogenous glucosamino-glycans [105]. Although Tramiprosate showed promising results in inhibiting the formation of aggregated Aβ fibrils, the negative results in phase 3 clinical trial led to suspension of the study.

1.7.1.3. Targeting Aβ clearance

In AD, the mechanisms that are involved in Aβ clearance, such as enzyme-mediated Aβ degradation, appear to become impaired [106]. Results from an animal study suggested that inhibitors of an enzyme named plasminogen activator inhibitor 1, which is involved in Aβ degradation, can reduce the level of Aβ oligomers in the plasma and brain [107]. In addition, few studies showed that somatostatin regulates the clearance of Aβ by activating neprilysin [108].

1.7.2. Targeting Neurofibrillary tangles

Given that tau protein is normally produced in neurons to stabilize the microtubule structure of axons, it could be a good target for therapeutic interventions. Numerous drugs were developed focusing on tau protein metabolism including tau phosphorylation inhibitors, tau oligomerisation inhibitors and tau degradation inhibitors [109].

1.7.2.1. Tau phosphorylation inhibitors

Glycogen synthase kinase 3 (GSK3) is one of the enzymes involved in the phosphorylation of tau protein [23]. It has been suggested that lithium and valproate can inhibit GSK3 action and consequently inhibit tau hyperphosphorylation [110]. Tideglusib is a GSK3 inhibitor which has recently completed a phase 2 clinical trial [111].

1.7.2.2. Enhancing tau degradation

Heat shock protein 90 (Hsp 90) has been suggested to play a role in tau protein degradation [111]. A study on transgenic mice showed that curcumin supresses the formation of tau tangles and increases the solubility of formed tangles by inhibiting the Hsp 90 [112].

1.8. Clinical and preclinical stages of AD

Several studies on cognitively normal elderly individuals as well as those who are genetically predisposed to AD suggest that the onset of disease pathogenesis begins at least two decades before the clinical symptoms begin to appear [113, 114]. Individuals with previously defined brain biomarkers of AD who have not presented any cognitive symptoms of dementia are considered as preclinical AD. As the disease progresses, individuals with preclinical AD present subtle cognitive decline which is detectable only via sensitive neuropsychological tests. MCI is defined when cognitive decline in individuals becomes noticeable by the individuals, themselves, or their family members while it has not affected their daily functioning yet. When the cognitive decline progresses in a way that affects the daily function in individuals, they meet AD dementia criteria [113].

Evidence from clinical studies show that Aβ accumulation and the associated functional and structural brain alterations in MCI are consistent with the pattern of abnormalities in AD patients [113]. However, some elderly individuals with the pathophysiological biomarkers of AD may not represent any symptom during their lifetime. Therefore, it is vital to identify biomarkers which can predict the progression from preclinical stages to MCI and from MCI to AD, given that the preclinical stage of AD provides an opportunity for the intervention with disease modifying strategies when neuronal damage is not yet irreversible and treatment-resistant.

According to the diagnostic guidelines from National Institute on Aging-Alzheimer's Association workgroups, preclinical AD is categorised into three stages [115], as explained below. It is notable that some individuals may not progress beyond stage 1 or stage 2 while those in stage 3 are more likely to progress to MCI and AD.

Stage 1: Asymptomatic cerebral amyloidosis

In this stage, the elevated Aβ accumulation observed via brain PET scans and decreased $A\beta_{42}$ level in CSF is detectable while there is no evidence of neurodegeneration or cognitive decline in individuals.

Stage 2: Amyloid deposition plus evidence of synaptic dysfunction or early neurodegeneration

In this stage, in addition to the increased amyloid accumulation, evidence of neuronal damage also appears. Neuronal damage can be detected through a number of markers such as increased CSF phosphorylated tau, hypometabolism seen on FDG-PET images with similar pattern to AD, as well as grey matter loss seen on MRI, particularly in the lateral and medial parietal, posterior cingulate, lateral temporal and hippocampus regions.

Stage 3: Amyloid deposition plus evidence of neurodegeneration and subtle cognitive decline

Individuals with elevated amyloid deposition in the brain, together with evidence of neurodegeneration and subtle cognitive decline are in the last stage of preclinical AD and are nearly approaching to meet the criteria for MCI.

1.9. Oxidative stress and metal dyshomeostasis in AD

Over the last few decades, several studies have investigated the role of Aβ in AD pathogenesis. Based on the "amyloid hypothesis" many drugs have been developed to inhibit the formation of Aβ. However, previous trials have shown that amyloid drugs as a standalone therapy failed to treat AD [116-118] indicating that there are other pathogenic pathways involved in AD such as oxidative stress and metal dyshomeostasis. Understanding other biological pathways involved in AD will not only add value to the diagnosis of AD but will also help identify therapeutic targets. Metal dyshomeostasis is a pathway observed to play an important role in AD pathogenesis [119]. The human brain contains high amounts of metal ions, particularly copper (Cu), zinc (Zn) and iron (Fe) as critical cofactors required for metabolic processes of the nervous tissue such as myelin synthesis and neurotransmission [120]. Although these metal ions play a critical role in neuron signalling, apoptosis and proliferation, small variations in their concentrations may cause shifts from beneficial to toxic effects [120, 121]. Despite a few studies indicating non-significant changes in the level of Fe, Zn and Cu in individuals with AD compared to age-matched controls [122], the majority of the studies have shown elevated concentrations of these metals [123, 124] in the brain of AD patients.

It is now proven that oxidative stress is one of the earliest steps in the AD pathogenesis trajectory occurring few decades prior to the appearance of symptoms. Findings of significantly elevated concentrations of metals such as Fe, Zn and Cu, and in the brain regions with Aβ plaques and neurofibrillary tangles suggest that these metals contribute to AD pathogenesis given their ability to generate free radicals resulting in toxicity and aggregation of Aβ plaques and tau tangles [123, 124].

1.10. The role of iron in healthy individuals

Iron plays a crucial role in maintaining some of the biological functions in the human body including mitochondrial respiration, oxygen transformation, cell growth and differentiation [125]. In healthy adults, the total iron levels are between 3.5 to 5 g, stored mainly in red blood cells, liver, macrophages, muscles and bone marrow, including small amounts in the brain and kidney [126]. In the brain, iron participates in vital cellular functions such as mitochondrial respiration, myelin synthesis as well as neurotransmitter synthesis and metabolism [127]. Although distribution of iron in the brain is heterogenous, the highest concentrations are normally found in the substantia nigra and basal ganglia [127, 128]. The aging process in healthy people is accompanied by iron accumulation in specific brain

regions [129]. However, whether iron accumulation is a primary or secondary incident in aging related neuronal damage, as well as the mechanism responsible for these changes are still unclear.

1.10.1. Peripheral iron uptake

In the peripheral iron regulatory system, four different cells are involved including intestinal enterocytes, erythroblasts, splenic macrophages and hepatocytes [130]. Iron uptake starts from intestinal enterocytes where absorbed (dietary) ferric iron (Fe^{3+}) gets reduced to ferrous iron (Fe^{2+}) which is then imported from the duodenum lumen into enterocytes via the divalent metal ion transporter-1 (DMT1) (Figure 1.3). Fe^{2+} within the enterocytes is either stored in ferritin after oxidisation as a soluble, non-toxic and bioavailable form or exported into extracellular fluids via ferroportin at the basal membrane site. Before being exported into extracellular fluid, iron is required to be oxidised from the Fe^{2+} form to the Fe^{3+} form by the enzyme hephaestin. As an iron transporter protein, transferrin only binds to ferric iron ions and transfers them to the periphery. Iron homeostasis is generally regulated by circulating hepcidin to interact with ferroportin when iron is abundant, resulting in ferroportin degradation thus blocking iron exportation from cells. When the level of circulating iron decreases, the synthesis of hepcidin decreases thereby increasing iron exportation by ferroportin.

1.10.2. Brain iron uptake

In the brain, iron passes through the BBB endothelial cells via the transferrin-transferrin receptor (TFR) system [131]. The way iron is exported to the abluminal membrane is not clear, but it is suggested to involve ferroportin or other transporters [130]. The released iron is then taken up by other cells such as neurons and astrocytes through two pathways: (a) as a low molecular weight component or (b) by binding to transferrin and using the transferrin-TFR 1 pathway in neurons [132]. Iron frequently transfers between neurons, astrocytes and microglia, while the mechanism of uptake and release of iron is not fully understood. It has been suggested that neurons mostly acquire iron via ferroportin as both ferroportin and TFR are expressed by neurons. Iron is then required to be transferred down the axons to the synapses through unknown mechanism(s). However, TFR is not expressed in astrocytes and these cells might take the required iron from the BBB via DMT1 [133]. Oligodendrocytes need significant amount of iron for axon myelination which is either extracted from adjacent blood vessels or up taken from ferritin via the ferritin receptors [130]. Although iron metabolism is not clearly known in microglia, it is assumed that during microglial activation, iron uptake increases while iron exportation decreases [130].

In vitro studies indicate that inflammatory stimulation lead to iron accumulation only in neurons and microglia while no changes are detected in iron levels of astrocytes [134]. This accumulation seems to be related to changes in two iron transporter proteins, DMT1 and ferroportin. Additionally, following inflammatory stimuli, hepcidin concentrations in neurons, astrocytes and microglia increase significantly, leading to a decrease in ferroportin expression in these cells [134].

1.11. Iron dysregulation and its role in AD pathogenesis

Dysregulation of iron homeostasis in AD was first reported by Goodman and his colleagues in 1953, who observed the deposition of iron in NFT and other regions of the brain in AD patients [135]. Further studies also demonstrated the relation of elevated brain iron with senile plaques, NFT and ferritin in the surrounding glia cells [46, 136]. Advance brain MRI images of AD patients indicate that iron deposition is not only present in senile plaques and NFT but also occurs in parietal cortex, motor cortex and hippocampus [137, 138].

A recent study showed a decrease in plasma iron in AD patients based on the desaturation of transferrin [139]. Another study by Guerreiro et al. reported a significant decrease in the expression of aconitase 1, ceruloplasmin and *APP* in peripheral blood cells in AD in comparison with the control group, indicating down-regulation of cellular iron export in AD patients [140].

It has been reported that iron induces the oligomerization, aggregation and amyloidosis of Aβ peptides [141]. Also, the interaction of iron with Aβ peptides was toxic in cultured cells [141, 142]. Moreover, some studies proposed that Aβ might not be toxic in the absence of redox metals [142], as oxidative damage in AD may occur due to the high binding affinity of Aβ to iron, and its capacity to reduce the metal ions and produce the catalytic hydrogen peroxide resulting in oxidative stress [143]. Furthermore, the association of iron with tau phosphorylation and aggregation has also been shown [144, 145]. The accumulation of tau protein in the form of NFT induces the production of heme oxygenase (HO) which contributes to ferrous iron release and free radical formation [131]. The role of iron in AD pathogenesis is not only because of iron accumulation but more importantly due to the imbalance of iron levels in different brain regions. Such an imbalance causes an iron overload in the brain regions with Aβ plaques and NFTs, which contributes to oxidative stress and neuronal damage, while the reduced iron levels in other brain areas lead to neuronal dysfunction [130].

Interestingly, some studies show that cellular iron levels regulate the translation of *APP* by identifying the Iron-responsive elements (IREs) in the untranslated region of its mRNA, indicating a relationship between iron homeostasis and AD [146]. Consequently, increased brain iron levels modulate *APP* expression and induce Aβ production. A study by Bodovitz et al. reported a modulatory effect of iron on APP cleavage by α-secretase [147].

The association between brain iron metabolism and AD has also been corroborated by a longitudinal study by Ayton et al. who reported a negative correlation between CSF ferritin levels and cognitive performance [148].

1.12. Ferritin and its alteration in AD

Ferritin is a cytosolic protein known as a crucial intracellular iron storage protein which is also found in the CSF and serum. Low plasma ferritin levels reflect a reduction in body iron stores, while increased

plasma ferritin is caused not only by elevated iron storage but also by inflammation in individuals with normal iron levels [130].

The relationship between ferritin and AD progression has been investigated in few studies. For instance, a longitudinal study by Ayton et al. reported a negative correlation between CSF ferritin levels and cognitive performance over 7 years, such that increased ferritin in CSF was associated with poorer cognitive performance and higher risk of converting from MCI to AD. This study also showed a strong association between ferritin concentrations and *APOE* Ɛ4, suggesting that elevated ferritin is a risk factor for AD [148].

Elevated ferritin in brain tissue of AD patients has been observed previously [149, 150]. Furthermore, elevated blood ferritin has been reported in AD patients. For instance, Faux et al. reported a significantly higher frequency of individuals having ferritin levels above the normal range in AD compared to healthy controls [151]. A study by Goozee et al. on individuals in the preclinical stage of AD, characterised by high neocortical amyloid-β load (NAL), reported that ferritin levels in serum and plasma of individuals with high NAL were significantly higher than that in healthy controls [152]. This study also indicated a strong positive correlation between NAL and either serum or plasma ferritin. Results of this study suggested that increased plasma (and serum) ferritin levels could serve as an important marker of preclinical AD occurring two or three decades before the symptoms of the disease appear.

1.13. Hepcidin and its biological function

Hepcidin is a twenty-five amino acid protein synthetised by the liver. It targets several cells including enterocytes, macrophages, hepatocytes and has recently also been found in brain cells [153]. Hepcidin is an iron regulatory hormone which regulates iron levels by binding to ferroportin and reducing its activity. Ferroportin is a transmembrane protein found on the basolateral surface of enterocytes in the duodenum, hepatocytes and macrophages in the reticuloendothelial system. By suppressing this iron transporter, hepcidin reduces the release of stored iron from hepatocytes, and decreases the release of recycled iron from macrophages as well as absorbed dietary iron from duodenum into the plasma [154, 155]. Figure 1.3 illustrates the regulation of systemic iron homeostasis and the critical role of hepcidin in regulating plasma iron levels.

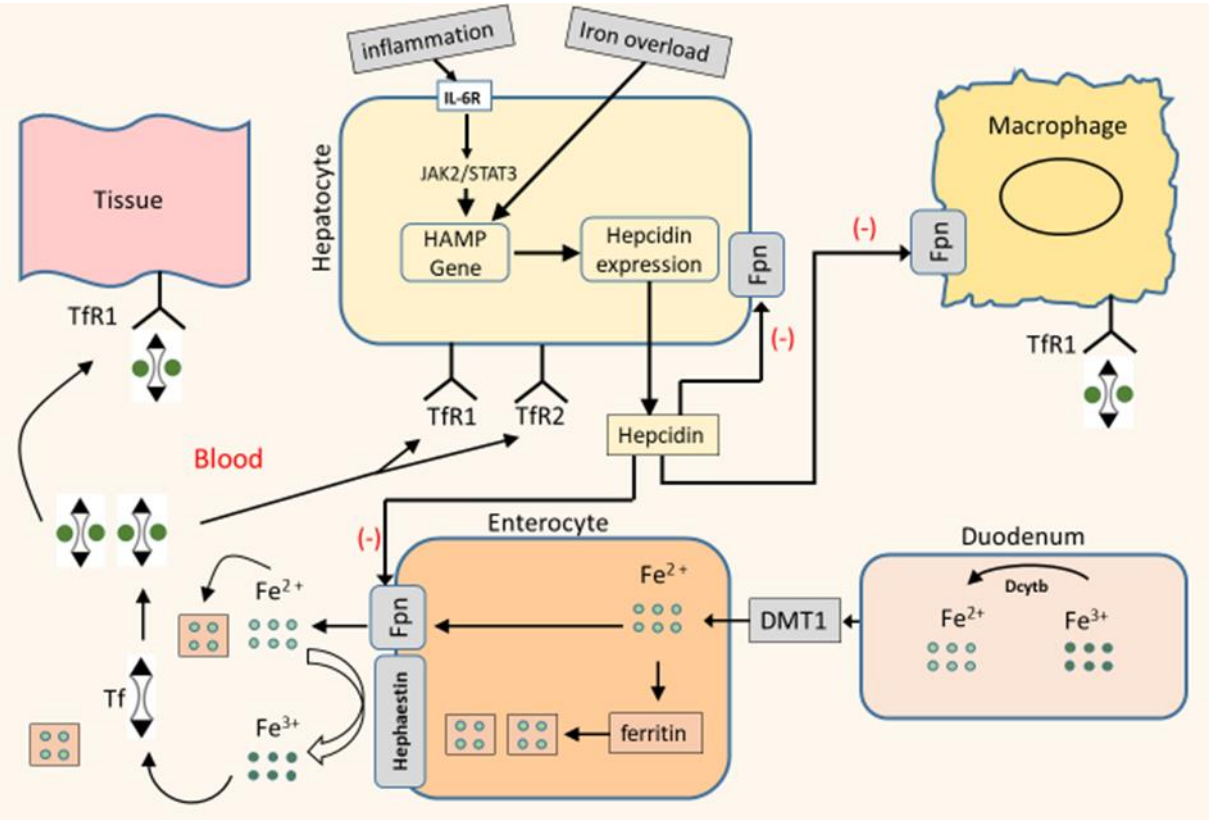


Figure 1.3. Regulation of systemic iron. Dietary iron absorbs as ferrous, ferric or heme iron in the duodenum. Ferric iron (Fe^{3+}), is first reduced to ferrous iron (Fe^{2+}) by duodenal cytochrome b (Dcytb) and transported through the divalent metal transporter-1 (DMT1) in the apical membrane of the enterocyte. Absorbed ferrous iron in enterocytes is either stored in the form of ferritin or exported to the blood via ferroportin (Fpn) transporter in the basolateral membrane. In blood, exported ferrous iron is oxidised to ferric form by the ferroxidase hephaestin. Ferric iron can then bind to plasma transferrin (Tf) and be transferred to different tissues by binding of Tf to the transferrin receptor 1 (TfR1). Hepatocytes monitor body iron levels by detecting iron bound to transferrin in plasma via TfR1 and transferrin receptor 2 (TfR2). Hepatocytes regulate iron levels by controlling the expression of hepcidin antimicrobial peptide gene (*HAMP*) and producing hepcidin protein. In the case of iron overload, the expression and release of hepcidin from hepatocytes is elevated which can then bind to Fpn (in macrophages, enterocytes and hepatocytes) and decrease its activity. Hepcidin expression is also induced by inflammation through the Janus kinase 2 /signal transducer and activation of transcription 3 (Jak 2/Stat 3) pathway leading to inflammation anaemia. However, iron deficiency reduces hepcidin expression and allows Fpn to release iron into plasma**.**

As it has been shown in Figure 1.3, hepcidin expression is induced in response to either the body's iron load or inflammatory stimulation. In the case of iron overload, bone morphogenetic protein 6 receptor (BMPR) in the liver's endothelial cells is activated by a series of molecules, which then stimulates the hepcidin antimicrobial peptide gene (*HAMP*) and increases hepcidin expression [156]. On the other hand, inflammation controls hepcidin expression through Janus kinase 2 (Jak 2)/signal transducer and activation of transcription 3 (Stat 3) pathway [157, 158]. Consequently, due to the elevation of hepcidin levels during inflammation, iron is trapped in the macrophages and hepatocytes, resulting in lower serum iron concentrations and anaemia [159].

On the other hand, decreased hepcidin expression is usually observed as a result of erythroferrone produced by erythrocyte precursors to conserve more iron for erythropoiesis [160]. Further, other factors such as vitamin D, hypoxia, heparin and estrogen that have also been reported to inhibit hepcidin expression [161-164].

Reports from studies on the expression of hepcidin in the brain have been inconsistent. A study by Krause et al. showed that brain hepcidin expression is low compared to the expression of hepcidin in the liver and heart [165]. The expression of hepcidin in different brain regions and cell types has been also investigated. A study on rat brains showed that the expression of hepcidin is not uniform in different areas of the brain, such that higher expression is observed in the cortex, striatum and cerebellum [166]. Also, hepcidin has been reported to be present in mature astrocytes localised in the lateral ventricle walls as well as in immature glial cells. Under physiological conditions, circulating hepcidin is responsible for regulating iron homeostasis whereas the role of brain hepcidin seems to be limited to controlling iron transportation from the blood to the brain [167].

1.14. The role of hepcidin alteration in AD

Given that AD is accompanied with systemic brain iron dysregulation, hepcidin as a main iron regulator can have a pivotal role in AD pathogenesis. This hypothesis is further supported by observations of hepcidin localised in brain regions with amyloid plaques in AD patients [167]. Moreover, a recent genetic study suggested that specific genetic variations accompanying decreased expression of ferroportin could serve as risk factors for AD [168]. Interestingly, given that AD is associated with advanced aging, a study by Lu et al. found that hepcidin expression in rat brains also increased with aging [48].

Only a few studies have investigated the role of hepcidin in AD pathogenesis. In a study by Raha et al., the alteration of hepcidin and ferroportin in AD brains were investigated to explore the role of these proteins on iron dysregulation in association with AD [167]. In this study, human brain tissue of six patients diagnosed with AD were analysed in comparison with brain tissue of six age-matched healthy adults. The levels of hepcidin and ferroportin analysed by western blotting indicated significant decrease in brain hepcidin levels of the AD patients compared to the control group. However, high

hepcidin distribution around amyloid plaques and surviving neurons was observed. This study also explored the variation of these two proteins in an *APP* transgenic mouse model of AD (Tg2576) at different stages of the disease. Results from immunohistochemistry showed that the expressions of both hepcidin and ferroportin decrease further as the disease progresses such that, in the late stages of AD, the expression of ferroportin becomes restricted to axons while hepcidin expression is limited to glial cells. Moreover, as the disease progresses and Aβ plaques form, ferritin light chain increases in plaques cores, further indicating iron dysregulation in these regions.

The systematic disturbance of hepcidin in AD has recently been investigated in a study by Sternberg et al. [169]. They compared the levels of hepcidin in 37 AD patients, MCI and 24 healthy individuals using enzyme-linked immunosorbent assay (ELISA). Results of this study showed that hepcidin serum levels in AD patients were significantly higher than the control group. Although the mean serum hepcidin levels of the MCI group were higher than the control grop, these differences were not statistically significant. Also, the study found significantly higher levels of ferritin in AD patients compared to the healthy individuals.

However, the above-mentioned studies on brain and blood hepcidin alteration have not yet been reproduced. Additionally, both studies were conducted on small sample sizes, which is a considerable limitation. More importantly, alterations in hepcidin levels within the preclinical stages of AD, prior to cognitive impairments have not been investigated previously.

Given that the neuronal damage within the MCI or AD stages of the disease trajectory may become irreversible and treatment-resistant [170], understanding the pathobiological changes occurring in the preclinical stages of the disease will aid disease diagnosis in the early stages when treatment might be more effective. Moreover, understanding the biological pathways involved in the disease pathogenesis process might help identify new drug targets that may enable management of early symptoms for delaying the onset of AD.

1.15. Hypothesis and aims

1.15.1. Aims

The main aims of this study are described below:

1. Investigating the serum hepcidin concentrations in preclinical AD by comparing serum hepcidin levels between cognitively normal participants with low and high NAL
2. Investigating the associations between serum hepcidin levels and NAL, and between serum hepcidin levels and cognitive performance
3. Exploring the association of serum hepcidin with other iron related proteins such as ferritin, transferrin and ceruloplasmin
4. Investigating the association between serum hepcidin and AD risk factors such as age, gender, *APOE* ε4 status and BMI
5. Evaluation of serum hepcidin as a potential biomarker that can differentiate between low NAL and high NAL participants

1.15.2. Hypothesis

Based on the literature, it is hypothesized that serum hepcidin level will be elevated in individuals with preclinical AD, characterised by high NAL compared to those at no apparent risk to AD or low NAL.

Chapter 2
Materials and methods

2.1. Participants

Participants of the current study belong to the Kerr Anglican Retirement Village Initiative in Aging Health (KARVIAH) cohort who were residents of Anglicare (Sydney, Australia). Written informed consent was obtained from each participant prior to study enrolment. To be recruited in the KARVIAH cohort, all participants (N=206) were required to meet a set of inclusion and exclusion criteria.

The inclusion criteria to be eligible for the KARVIAH cohort were as follows:

1. Age range of 65-90 years old, good general health and no serious cerebrovascular disorders
2. Living in independent living units, or similar accommodations
3. Fluent in English
4. Satisfactory vision and hearing to undertake the test
5. No objective memory impairment based on cognitive tests
6. Normal general cognitive function as determined by the Montreal Cognitive Assessment Score (MoCA) ≥26. MoCA scores lying between 18 and 25 were assessed on a case-by-case basis by a team of neuropsychologists. Eligibility was determined on a case-by-case basis following stratification of the MoCA scores considering the age and education [171].
7. No significant functional impairment or behavioural problem as specified by the Informant Questionnaire on Cognitive Decline in the Elderly (IQCODE) and the 36-Item Short Form Health Survey (SF-36)
8. Minimum or no impairment in daily life activities as determined by a clinical interview

The exclusion criteria related to the current study were as follows:

1. Diagnosis of dementia according to the revised criteria from the National Institute on Aging—Alzheimer's Association [172]
2. A MoCA score ≤17. Individuals with a MoCA score of 18-25 were discussed by a team of neuropsychologists; eligibility was determined on a case-by-case basis following stratification of the MoCA score according to the age and education [171]
3. Significant functional impairments or behavioural problems as indicated by IQCODE and the 36-Item Short Form Health Survey
4. Existence of acute functional psychiatric disorder (such as a history of schizophrenia or bipolar disorders)

5. History of stroke prior to study
6. History of alcohol or drug abuse within 2 years of screening
7. History of regular alcohol consumption exceeding 7 drinks per week for women and 14 drinks per week for men (1 drink = 5 ounces of wine or 12 ounces of beer or 1.5 ounces of hard liquor) within 6 months of screening
8. Presenting severe depression (according to the Depression, Anxiety, Stress Scales; DASS)
9. Existence of bleeding risk factors such as using warfarin within 4 weeks of screening
10. Non-fluency in English
11. Hearing or visual impairments sufficient to inhibit cognitive testing
12. Obstruction of biliary tract
13. Contraindication to MRI, including those with a pacemaker, presence of metallic fragments near the eyes or spinal cord, or cochlear implant (Dental fillings do not present a risk for MRI)
14. History of closed angle glaucoma, or related conditions
15. Uncontrolled hypertension with either systolic blood pressure >170 mmHg or diastolic blood pressure >100 mmHg
16. Any significant systemic illness or unstable medical condition that could lead to difficulty complying with the protocol including: history of myocardial infarction in the past or unstable or severe cardiovascular disease including angina or congestive heart disease, chronic renal failure, chronic hepatic disease, severe pulmonary disease
17. Clinically significant and unstable gastrointestinal disorder such as ulcer disease or a history of active or occult gastrointestinal bleeding within two years

2.1.2. Demographic data collection

Demographic data of residents who wished to participate in KARVIAH study were collected by a staff member of the research facility. These data included: date of birth, address, education level, marital status, gender, occupation and retirement history.

2.1.3. Medical history

Comprehensive health history was collected from the participants, including questions detailing prior illness or surgery, and medication use. Family history of diseases and lifestyle choices associated with the high risk of AD were extensively documented, including; diabetes, stroke, hypertension, hyperlipidaemia, obesity, alcohol consumption and smoking status.

From one hundred and thirty-four participants who met the aforementioned inclusion and exclusion criteria, 29 either declined neuroimaging or withdrew from the study. The remaining one hundred and five volunteers underwent physical assessment, neuroimaging, comprehensive neuropsychological examination, and blood collection. Participants also completed three questionnaires; Community Healthy Activities Model Program for Seniors (CHAMPS); Pittsburgh Quality Sleep Index (PQSI); and the Cancer Council of Victoria Food Frequency Questionnaire (CCVFFQ).

2.1.4. Physical assessment

General physical information was conducted including documentation of weight, height, girth, body mass index (BMI), blood pressure pulse, abdominal and chest examination.

2.2. Neuropsychological tests

All participants underwent a comprehensive battery of validated psychometric and neuropsychological tests which are known to be sensitive to the presence of MCI and cognitive decline in participants with pre-dementia. These tests were administered by an experienced research assistant, under the supervision of a neuropsychologist, taking approximately 1.5 hours to perform the whole battery.

2.2.1. Psychometrics battery

The psychometrics battery comprised the following tests: Depression Anxiety and Stress Scales (DASS), Clinical Dementia Rating (CDR), Geriatric Depression Scale (GDS) – 15 item version, Wechsler Test of Adult Reading (WTAR) and Mini-Mental State Examination (MMSE), and Memory Assessment Clinical-Q (MAC-Q). Nevertheless, MMSE was primarily used to examine cognitive abilities of the KARVIAH cohort. MMSE is a commonly used 30 items screening of orientation, attention, memory, language and praxis and MMSE≥24 usually indicates the normal cognition. The MAC-Q test consists of 6 questions asking the individual to rate their current abilities compared to their past abilities. The MAC-Q has a cut off score of 25, so participants with MAC-Q≥25 are considered as subjective memory complainers (SMC).

2.2.2. Neuropsychological tasks

The battery included the following well-known and well validated neuropsychological tasks. Memory and learning tasks such as Rey Verbal Learning Test (RAVLT) and Wechsler Logical Memory –II; Language tasks including Category Fluency, Controlled Oral Word Association (COWAT) and Boston Naming Test (BNT); working memory task comprising Digit Span forward and backwards; Digit Symbol attention task (WAIS III): Stroop Test (Victoria version), Rey Complex Figure Test (RCFT), Digit Symbol Substitution Test (DSST) WAIS–III; and D-KEFS Category Fluency (Boys Names) and Switching (Fruits and Furniture) Tasks.

Composite scores for KARVIAH participants were created for verbal and visual episodic memory functions, and for working memory and executive function. To calculate the verbal and visual episodic memory composite score, the mean of the z-scores of RAVLT List A, RAVLT short delay, RAVLT long delay, Logical Memory (LM I), LM II, Rey Complex Figure Test (RCFT) 3 min and RCFT 30 min were considered. The working memory and executive function composite scores were created from the mean of the z-scores of Digit Span backward, DSST, D-KEFS, Boys names and Fruits and Furniture Switching tasks.

2.3. Neuroimaging

All participants, upon passing all inclusion and exclusion criteria, underwent MRI imaging and PET scans at Macquarie Medical Imaging, Sydney within 3 months of blood collection.

2.3.1. PET imaging

Participant preparation involved simple intravenous catheterization. Prior to PET imaging, 5±0.5 mCi of [F-18] fluorodeoxyglucose (FDG) or 10 mCi of 18F-Florbetaben ([18F] BAY94-9172) as an amyloid ligand was administrated slowly over 30 seconds via an intravenous line inserted into a vein in the arm of the participant. Following injection of the ligand, participants were moved to a quiet room and rested for thirty minutes. Forty to fifty minutes after injection of the tracer, participants were positioned in the PET scanner following which a 20 minute scan was acquired.

2.3.2. MRI imaging

In addition to PET scans, all participants underwent an anatomical MRI scanning for anatomical localization of regions-of-interest (ROI). The participants were positioned in a standard head coil and a brief scout T1-weighted image obtained. A volumetric spoiled gradient recall (SPGR) sequence and a T2 sequence were performed which maximised the contrast among grey matter, white matter, and CSF. This provided a high-resolution delineation of the cortical and subcortical structures. The MRI were administered over 20 minutes and the data were used to create individualised anatomic maps for the selection of ROIs used in the analysis of the PET data.

2.3.3. Image analysis

Data obtained from MRI and PET scans were analysed to calculate some characteristics such as hippocampal volume and standardized uptake value ratio (SUVR) in participants. Hippocampal volume was determined from the images obtained and was normalised with the total intracranial volume containing the CSF, grey matter and white matter volumes.

To calculate NAL, the image processing software, CapAIBL, was used. Neocortical amyloid load was calculated as the mean SUVR of the ligand FBB in the frontal, superior parietal, lateral temporal, lateral occipital, and anterior and posterior cingulate regions normalised with that in the cerebellum [173, 174].

2.4. Blood collection

A sample of 80 mL blood was collected from participants who fasted for at least 10 hours overnight via venipuncture. Vital signs such as weight, height, blood pressure and pulse were also collected. Tubes containing the blood were placed on rocker for 20 minutes. Blood sample processing and fractionation took place within 3.5 hours after the collection.

Tubes were spun at 1800 rpm for 15 minutes at 20˚C with brake in a centrifuge. Serum was then removed from the tube, aliquoted into cryovials and frozen immediately at -80˚C.

A 53 mL sample of the total blood taken was then stored at -80˚C for research purposes after being fractionated [3]. However, the remaining 27 mL was sent to a clinical pathology laboratory (Laverty Pathology, Kotara, NSW, Australia) for whole blood examinations such as measuring the level of serum ferritin, transferrin, iron and haemoglobin.

2.4.1. *APOE* genotype determination

To determine the *APOE* genotype, purified genomic DNA extracted from 0.5 mL blood was used. Each sample was examined for the three *APOE* variants (ε2, ε3, ε4) based on TaqMan SNP genotyping assays for rs7412 (C 904973) and rs429358 (C 3084793) according to the manufacturer's instructions.

2.4.2. Measurement of iron, transferrin, ferritin, C-reactive protein

The level of transferrin and iron in serum were measured using immunoturbidometric and colorimetric methods at Laverty Pathology. Serum and plasma ferritin concentrations were also measured at Laverty Pathology, by using a sandwich immunoassay with chemiluminometric technology. C-reactive serum level was determined using COBAS Tina-quant immunoturbidimetric high-sensitivity CRP (hs-CRP) and ADVIA wide-range CRP (wr-CRP) method in the same laboratory [152].

2.5. Categorising study participants

One hundred participants (out of a total of 206) were considered as cognitively normal for this study based on their MMSE scores (MMSE≥26). Based on the SUVR cut-off score of 1.35, participants were categorised into two groups: low NAL with SUVR<1.35, and high NAL with SUVR≥1.35. Therefore, the study cohort comprised 65 participants recognised as low NAL group (no apparent risk to AD) and 35 participants as high NAL group (preclinical AD). Serum hepcidin concentrations were measured in all 100 cognitively normal participants by using ELISA method.

2.6. ELISA as a quantitative analysis method for hepcidin

ELISA is a biochemical technique employed to detect and quantify substances such as proteins, peptides, antibodies and hormones. In this method the targeted antigen is attached to the assay plate either directly or indirectly via a capture antibody which has been allocated to the plate surface. In the next step, the antigen is detected directly by a primary antibody or indirectly by using a secondary antibody. Then, an enzyme is added such that it attaches to the primary or secondary antibody, and after interaction with the substrate it generates a detectable signal. Several enzymes can be employed in an ELISA including galactosidase, glucose oxidase, peroxidase, and alkaline phosphatase. The substrate is chosen based on the selected enzyme which could produce the yellow or brown colour after interaction with enzyme. In the last step, by using sodium hydroxide, hydrochloric acid or sulfuric acid the interaction is stopped and the results are read via spectrophotometer. ELISA can use 4 different principles for antigen-antibody binding that include direct, indirect, sandwich and competitive ELISA which have been demonstrated in Figure 2.1.

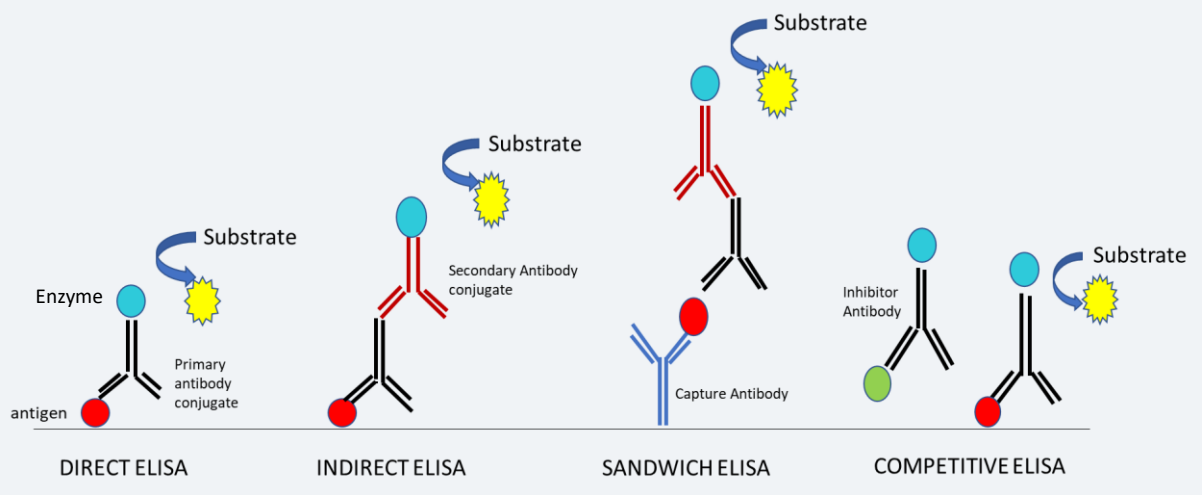


Figure 2.1. Common formats of ELISA, enzyme-linked immunosorbent assay.

Direct ELISA was developed by Engvall et al. in 1971 and is suitable for determining the concentration of high molecular weight peptides [175]. In this method, the sample containing antigen is directly placed in the well and the appropriate antibody is subsequently added after the incubation. In indirect ELISA introduced in 1978 by Lindstrom et al. [176], after preparing an antigen-antibody complex in the wells, a secondary antibody is added which could then bind to the complex as well as enzyme.

The most common ELISA format is the sandwich ELISA (s-ELISA) in which the target antigen is bound between the capture antibody and the detection antibody [177]. This has been reported as the most sensitive type of ELISA method in which the developed colouration directly relates to the concentration of analyte. However, in c-ELISA, the sample and the enzyme-tagged antibody are simultaneously placed into the well of ELISA kit such that they compete to bind to the coated antibody in the well. In this case, there is an inverse relation between analyte concentration and the intensity of coloration. The DRG hepcidin ELISA kit, employed within the current study, is designed based on the c-ELISA principle, and is a highly sensitive method to measure hepcidin 25, which is the bioactive form of hepcidin, in serum or plasma.

2.6.1. Hepcidin DRG ELISA kit

Hepcidin-25 (bioactive) DRG ELISA kits (EIA-5782, detection range 0.153 ng/mL-81 ng/mL, sensitivity 0.153ng/mL, LoD 0.304 ng/mL and LoQ 1.149 ng/mL) were purchased from Bio-Strategy (Figure 2.2). The reagents provided by the company have been listed in Table 2.1.

Table 2.1. Reagents in the Hepcidin-25 DRG ELISA kit

Reagents	Contents	Preparation
96 Microtiter wells plate	wells coated with anti-hepcidin-25 monoclonal antibody	Ready to use
Standards	Standard 0-5	Preparation required
Controls	Control low and high	Preparation required
Sample diluent		Ready to use
Enzyme conjugate	Hepcidin-25 conjugated to biotin	Ready to use
Enzyme complex	Streptavidin conjugated to horseradish peroxidase	Ready to use
Substrate solution	Tetramethylbenzidine (TMB)	Ready to use
Stop solution	H_2SO_4	Ready to use
Wash solution	-	Preparation required

2.6.2. Reagents preparation

To prepare standards and controls, 0.5 mL=500 µL of deionised water was added to the lyophilised contents of each vial and mixed by vortex several times before use. Wash solution was also prepared by diluting 30 mL of the concentrated wash solution with 1170 mL deionised water to a final volume of 1200 mL.

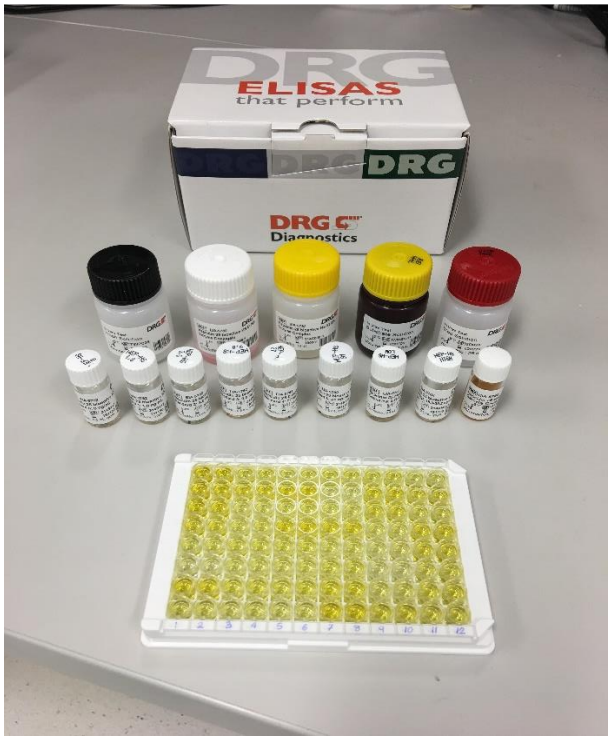


Figure 2.2. Hepcidin-25 DRG ELISA kit plate and reagents.

2.6.3. Hepcidin ELISA test procedure

DRG hepcidin ELISA kits were stored at 4˚C until the test day when it was taken out of the cold room and brought to room temperature in approximately 30 min. Next, 40 serum samples were taken out of the -80˚C freezer and placed on ice for 30 minutes in order to maintain sample integrity while thawing. Standards, controls and wash solution were then prepared based on the manufacturer protocol. According to the protocol provided by the manufacturer, each ELISA run went through following steps:

1. 20 µL of each standard, control and samples were dispensed into appropriate wells of a pre-coated antibody plate in duplicate format.
2. 50 µL of the enzyme conjugate was dispensed into each well and was mixed thoroughly for 10 seconds. In this step, endogenous hepcidin-25 of a patient sample competes with a hepcidin-25-biotin conjugate (enzyme conjugate) for binding to the coated antibody.
3. The plate was incubated at room temperature for 60 minutes.
4. Completing the incubation, the contents of the wells were briskly shaken out and the wells were washed with 300 µL of diluted wash solution for 4 times. After each wash, the residual droplets in the wells were removed by striking the wells sharply on absorbent paper. By washing the microtiter plate in this step, the competition reaction was stopped.
5. Next, 100 µL of enzyme complex was dispensed into appropriate wells.
6. In this step, the plate was incubated for 30 minutes at room temperature. Therefore, the bound biotin molecules were detected with streptavidin peroxidase (enzyme complex).
7. Following the incubation, the contents of the wells were briskly shaken out and the plate was washed manually for 4 times with 300 µL of diluted wash solution (similar to step 4).
8. Then, 100 µL of substrate solution was added to each well.
9. The plate was then incubated for another 20 minutes at room temperature. The intensity of the colour developed after incubation was inversely proportional to the concentration of hepcidin-25 in the patient sample.
10. Finally, by adding 100 µL of stop solution to each well the enzymatic reaction was stopped.
11. According to the manufacturer's protocol within 10 minutes of adding the stop solution, the absorbance of each well is to be determined with a microtiter plate reader at 450±10 nm. The current study employed the PHERAstar FS plate reader.

2.6.4. Analysing the results

The average absorbance of each set of standards, controls and patient samples were calculated. To create a standard curve, a 4-parameter curve fit (symmetrical sigmoidal) was used and the concentration of each sample was calculated from the equation obtained from the standard curve. To

normalise the three ELISA assays, the average concentrations of the low and high control samples provided within the kit were used, and the calculated factor was taken into account.

2.7. Statistical Analyses

Descriptive statistics comprising means and standard deviations were calculated for high and low NAL groups. To compare gender and *APOE* ε4 carrier status between high and low NAL groups, chi-square tests were applied. For analysing the results of this study, general linear models were used to compare continuous variables between high NAL and low NAL, with and without adjusting for covariates age, gender, *APOE* ε4 carrier status. Logistic regression with high/low NAL as response was used to evaluate predictive models. To analyse the approximate normality, variance homogeneity and an appropriate transformed scale, continuous response variables were checked as required. To investigate the correlation between hepcidin concentrations and all other continues variables, Pearson's correlation coefficient was used. P-value<0.05 was considered as significant. All analyses were carried out by using IBM SPSS version 25 (IBM, Armonk, NY, USA). All graphs were created using GraphPad version 7.02. Receiver operating characteristic (ROC) curves were generated by using the package Deducer on R version 3.2.5.

Chapter 3
Results

3.1. Participant demographics

KARVIAH participant demographics including age, gender, years of education, BMI and *APOE* ε4 carrier status have been presented in Table 3.1. In addition, other characteristics of the cohort such as MMSE scores, neocortical SUVR, hippocampal volumes and inflammation factors including hs-CRP and wr-CRP have been presented in the same table. As described before, based on the SUVR of ligand F^{18}-Florbetaben (FBB) in the neocortical regions in PET scans, study participants were categorised as low NAL (SUVR<1.35) and high NAL (SUVR≥1.35).

As expected, the frequency of *APOE* ε4 carriers were significantly higher in participants with high NAL compared to the participants with low NAL (P=0.000008). However, no significant differences were detected in age, gender, education and BMI between low NAL and high NAL participants. Similarly, MMSE scores, hippocampal volumes, and hs-CRP and wr-CRP levels were not significantly different between the two groups.

Table 3.1. Characteristics of KARVIAH cohort participants.

	All subjects	**Low NAL**	**High NAL**	**P value**
Gender (M/F)	32/68	19/46	13/22	0.419
Age (years, mean± SD)	78.18±5.52	77.62±5.56	79.23±5.38	0.165
Education (years, mean± SD)	14.42±3.26	14.85±3.37	13.64±2.92	0.078
BMI (mean± SD)	27.62±4.56	27.38±4.47	28.05±4.73	0.486
***APOE* ε4 carriers (%)**	17.4	7.69	45.71	<0.001
MMSE (mean± SD)	28.61±1.14	28.51±1.16	28.80 ±1.11	0.225
n SMC (%)	62.8	51(78.5%)	25(71.4%)	0.432
Neocortical SUVR (mean ± SD)	1.35±0.31	1.16±0.08	1.71±0.26	–
Hippocampal volume (%)				
Left lobe (mean± SD)	0.194±0.02	0.195±0.02	0.194±0.02	0.805
Right lobe (mean± SD)	0.199±0.02	0.199±0.021	0.199±0.018	0.891
Hs-CRP (mg l^{-1} ,mean± SD)	2.49±4.41	2.16±2.23	3.09±6.84	0.317
Wr-CRP (mg l^{-1} ,mean± SD)	3.10±6.08	2.68±3.18	3.88±9.37	0.349

Abbreviations: F, female; M, male; BMI, body mass index; *APOE*, apolipoprotein E; MMSE, Mini Mental State Examination; n SMC, number of subjective memory complainers based on the Memory Assessment Clinic-Questionnaire (MAC-Q); NAL, neocortical amyloid-β load; SUVR, standard uptake value ratio; Hs-CRP, high-sensitivity C-reactive protein; Wr-CRP, wide range C-reactive protein.

Characteristics have been compared between high NAL (n=35) and low NAL (n=65) participants. Chi-square tests or linear models were used as appropriate. P<0.05 was considered as significant.

3.2. Association of hepcidin with AD risk factors

Associations between hepcidin and AD risk factors including age, gender, *APOE* ε4 status, BMI and years of education in the study cohort were investigated (Table 3.2). However, no significant correlation was observed between hepcidin and age.

Table 3.2. Correlation between hepcidin and AD risk factors in KARVIAH participants.

	r	P-value
Age	0.022	0.827
BMI	0.054	0.594
Education	-0.139	0.169

Abbreviations. BMI, body mass index; r, correlation co-efficient. Pearson correlation was used to calculate P-value and correlation coefficient. P<0.05 was considered as significant.

Although the mean±SD serum hepcidin levels in females (26.95±17.31) was higher than that in males (22.54±17.36), differences in hepcidin between males and females were not statistically significant (P=0.238) (Figure 3.1).

No significant differences were observed in serum hepcidin levels between *APOE* ε4 carriers and non-carriers (P=0.836) (Figure 3.1). Interestingly, on stratifying study participants into *APOE* ε4 carriers and non-carriers, serum hepcidin levels continued to remain significantly higher in the high NAL non-carriers compared to the low NAL non-carriers (P=0.035).

In addition, there was no significant correlation between hepcidin and BMI, and hepcidin and education (Table 3.2 and Figure 3.2).

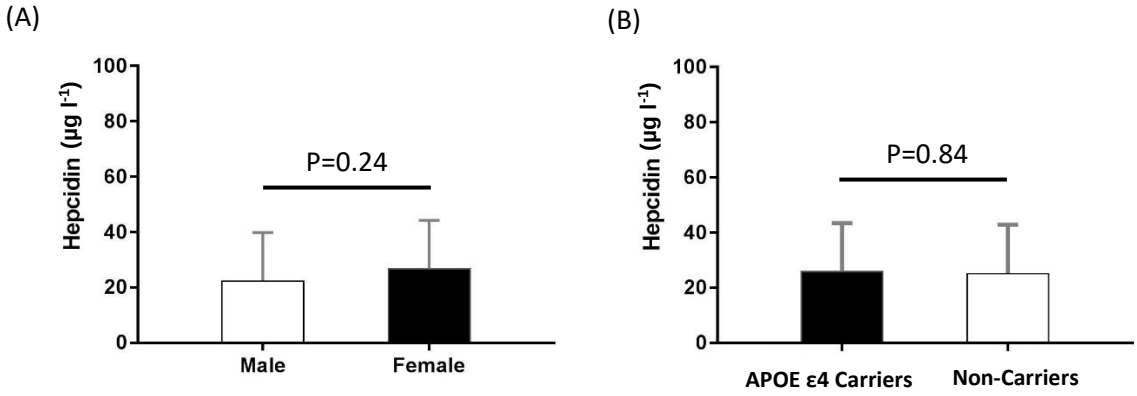


Figure 3.1. Association between (A) serum hepcidin level and gender, (B) serum hepcidin level and *APOE* ε4 status. There are no significant differences observed in serum hepcidin levels between males and females. Similarly, no significant differences are detected in serum hepcidin levels between *APOE* ε4 carries and non-carriers. Error bars in the graphs represent standard deviations.

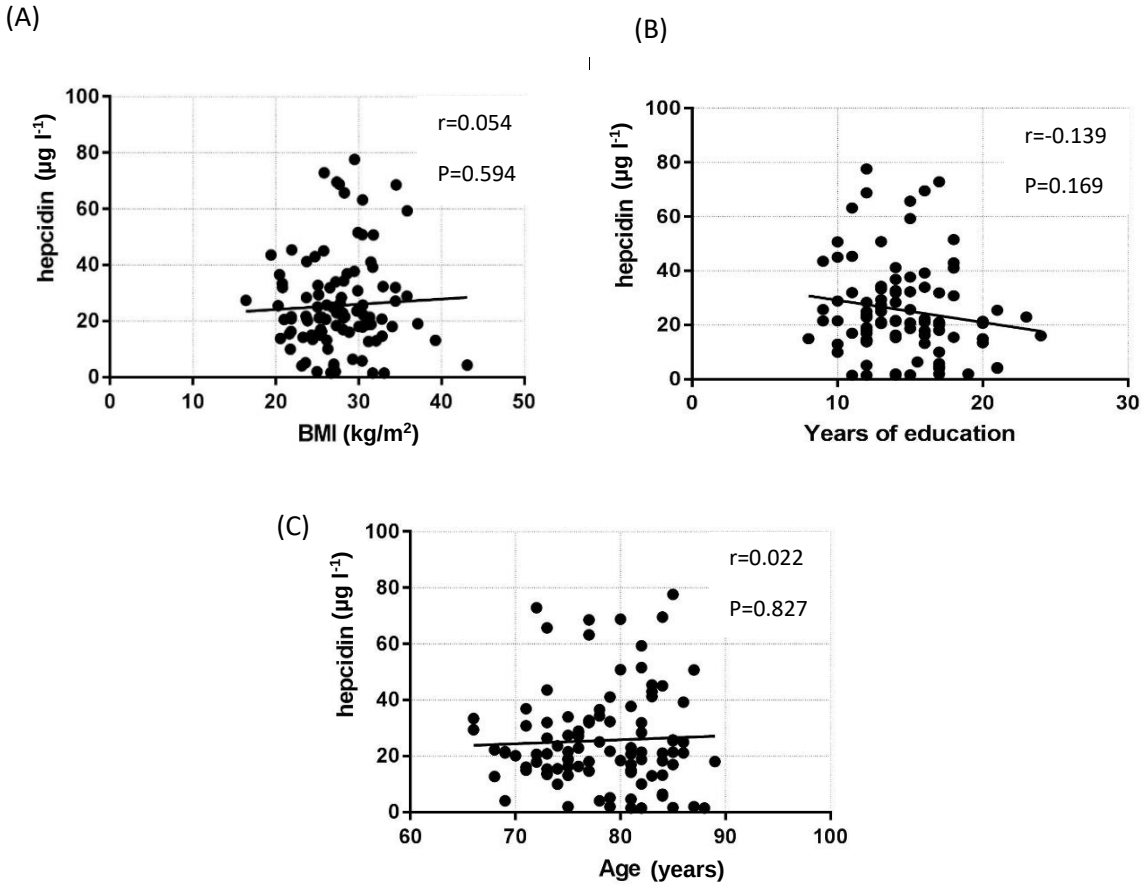


Figure 3.2. Association between (A) serum hepcidin level and BMI, (B) serum hepcidin level and years of education, (C) serum hepcidin level and age. No significant associations were observed between serum hepcidin and BMI, serum hepcidin and years of education, serum hepcidin and age.

3.3. Association of hepcidin and other iron related proteins with NAL

3.3.1. Comparison of serum hepcidin in high NAL versus low NAL participants

The differences between hepcidin levels in high NAL group versus low NAL group with and without adjusting for covariates including age, gender, *APOE* ε4 and years of educations are presented in Table 3.3. Serum hepcidin levels were significantly higher in participants with high NAL compared to those with low NAL with (Figure 3.3) and without correcting for age, gender and *APOE* ε4 status. Even when adjusted for age, gender, *APOE* ε4 status and education, serum hepcidin remained significantly higher in the high NAL participants compared to the low NAL participants indicating that elevated serum hepcidin level could be an early indicator of preclinical AD, characterised by NAL.

Interestingly, on stratifying study participants into males and females, serum hepcidin levels continued to remain significantly higher in males with high NAL compared to males with low NAL (P=0.022). However, in female, the differences in serum hepcidin level between high NAL and low NAL were not significant (P=0.278).

Also, by splitting study participants into *APOE* ε4 carriers and non-carriers, serum hepcidin levels continued to remain significantly higher in the high NAL non-carriers compared to the low NAL non-carriers (P=0.035). The differences between low NAL versus high NAL ε4 carriers were not significant (P=0.455).

Additionally, the correlation between NAL and serum hepcidin levels in the study cohort was further investigated, and it was found that serum hepcidin levels positively correlate with NAL (r=0.244, p=0.014) (Figure 3.4).

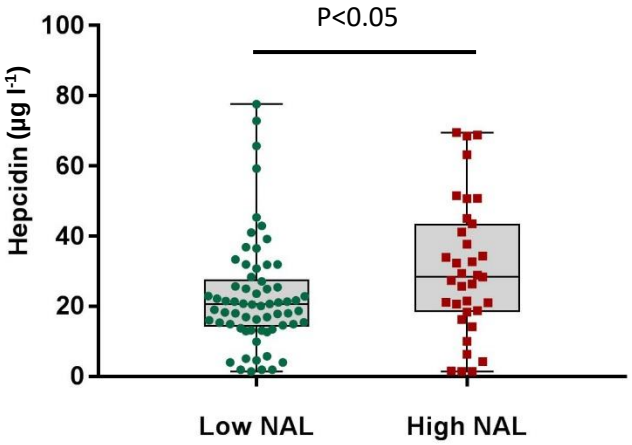


Figure 3.3. Comparison of serum hepcidin concentrations between participants with low and high neocortical amyloid-β load (NAL). Serum hepcidin concentrations were compared between participants with high and low NAL using linear models. Serum hepcidin levels were significantly higher in the high NAL (n=35) compared to the low NAL group (n=65). Error bars in the graphs represent the range of data in each group and the line segment within each box plot represents the median within each box plot.

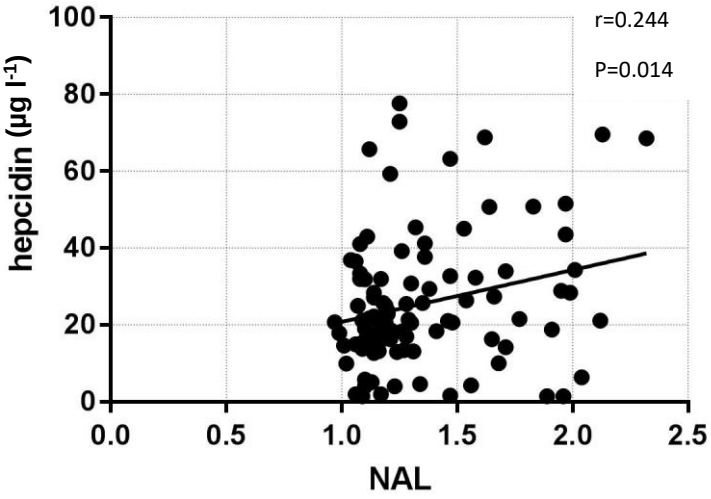


Figure 3.4. Association between serum hepcidin concentration and neocortical amyloid-β load (NAL). Significant correlation was observed between hepcidin concentration and NAL in the study cohort (r=0.244, p=0.014). The correlation coefficient was calculated using Pearson's correlation coefficient.

3.3.2. Levels of iron and iron related proteins in high NAL versus low NAL participants

Levels of iron and iron related proteins including hepcidin, plasma ferritin, serum ferritin, transferrin and saturated transferrin, haemoglobin, and ceruloplasmin in low NAL participants in comparison to the high NAL participants were further investigated, either with or without adjusting for age, gender, *APOE* ε4 status and years of education (Table 3.3).

Serum and plasma ferritin were significantly higher in participants with high NAL compared to those with low NAL whether or not corrected for covariates (Figure 3.5).

However, no further significant differences exhibited in serum iron, transferrin, saturated transferrin, hemoglobin and ceruloplasmin between the two groups (Figure 3.6 and Figure 3.7).

Table 3.3. Hepcidin and Iron related proteins in participants with low NAL versus high NAL

	Low NAL	High NAL	P-value	P-value[a]	P-value[b]
Serum hepcidin (µg l^{-1})	22.88±15.72	30.49±19.34	0.036	0.018	0.027
Serum iron (µmol l^{-1})	17.04±5.48	16.75±4.84	0.803	0.768	0.767
Plasma ferritin (µg l^{-1})	113.53±86.57	198.59±131.99	0.0003	0.003	0.005
Serum ferritin (µg l^{-1})	113.75±87.23	176.00±123.55	0.005	0.012	0.023
Serum transferrin (µmol l^{-1})	34.46±6.05	32.82±6.13	0.211	0.129	0.161
Transferrin saturation (%)	25.76±7.86	27.09±9.25	0.461	0.149	0.130
Hemoglobin (g l^{-1})	138.49±11.25	133.51±16.33	0.080	0.162	0.235
Ceruloplasmin (g l^{-1})	0.25±0.48	0.24±0.45	0.640	0.947	0.829

Abbreviations: NAL, neocortical amyloid load; Aβ, amyloid β. All data represented as mean±SD. General linear model was used to analyse data between low NAL (n=65) and high NAL (n=35) participants. P<0.05 was considered as significant. P-value[a] has been adjusted for age, gender and *APOE* ε4 status. P-value[b] has been adjusted for age, gender, *APOE* ε4 status and years of education.

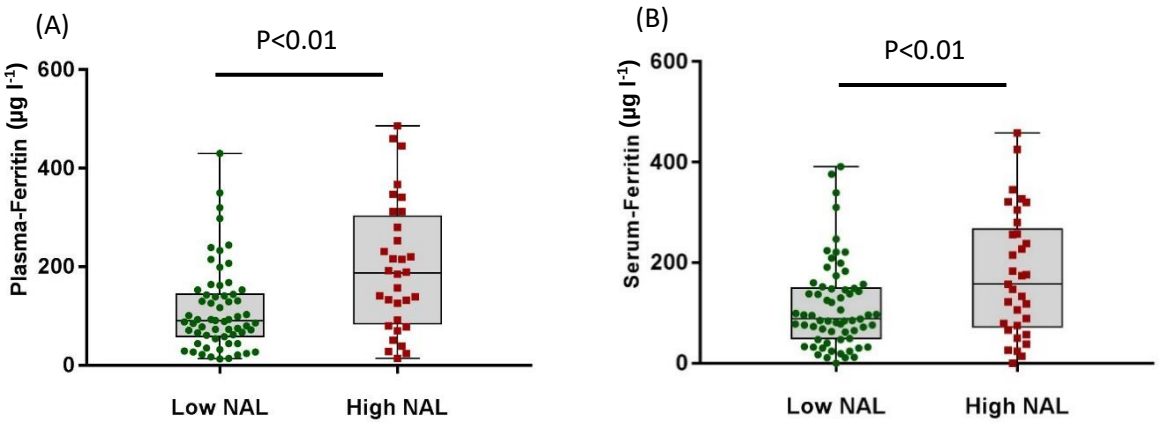


Figure 3.5. Comparison of (A) plasma ferritin concentrations, (B) serum ferritin concentrations between participants with low and high neocortical amyloid-β load (NAL). Plasma and serum ferritin concentrations were compared between participants with high and low NAL using linear models. Ferritin levels (serum and plasma) were significantly higher in the high NAL (N=35) compared to the low NAL group (N=65). Error bars in the graphs represent the range of data in each group and the line segment within each box plot represents the median within each box plot.

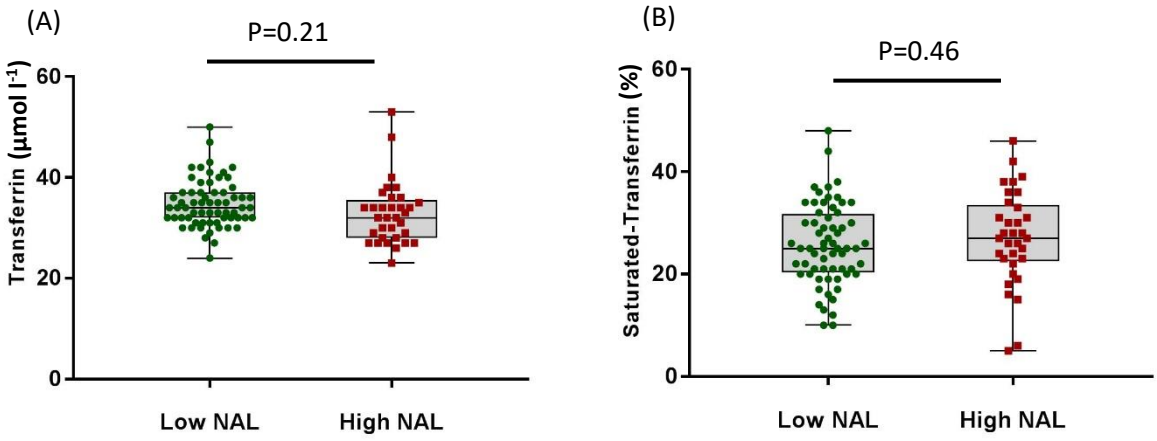


Figure 3.6. Comparison of (A) transferrin concentrations, (B) saturated transferrin concentrations between participants with low and high neocortical amyloid-β load (NAL). Transferrin and transferrin saturation concentrations were compared between participants with high and low NAL using linear models. No significant differences were detected in transferrin and saturated transferrin in the high NAL group (N=35) compared to the low NAL group (N=65). Error bars in the graphs represent the range of data in each group and the line segment within each box plot represents the median within each box plot.

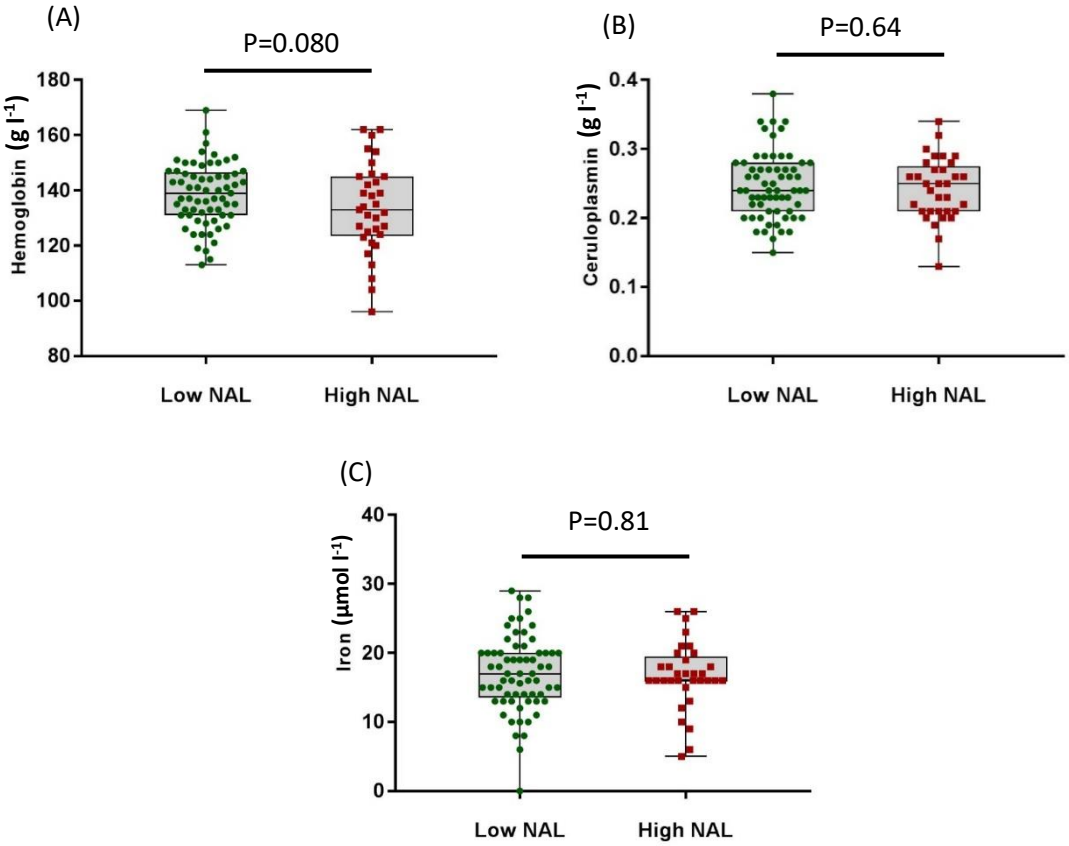


Figure 3.7. Comparison of (A) hemoglobin levels, (B) ceruloplasmin concentrations, (C) iron levels between participants with low and high neocortical amyloid-β load (NAL). Hemoglobin, iron and ceruloplasmin concentrations were compared between participants with high and low NAL using linear models. No significant differences were detected in hemoglobin, iron and ceruloplasmin

concentrations between the two groups. Error bars in the graphs represent the range of data in each group and the line segment within each box plot represents the median within each box plot.

3.4. Hepcidin association with iron and iron related proteins

The association of hepcidin with iron and other iron-related proteins including ferritin, transferrin, saturated transferrin, ceruloplasmin and hemoglobin were further investigated in the current study (Table 3.4).

Interestingly, the correlation between hepcidin and ferritin (both serum and plasma) was strongly significant (Figure 3.8).

Further, serum hepcidin levels were observed to inversely correlate with serum transferrin, and positively correlate with saturated transferrin concentrations (Figure 3.9).

As illustrated in Figure 3.10, a significant positive correlation was observed between serum hepcidin and ceruloplasmin concentrations in the current study.

However, no significant correlation was observed between hepcidin and hemoglobin in the study cohort. Also, no significant correlation was observed between hepcidin concentrations and iron levels (Figure 3.10).

Table 3.4. Correlation between hepcidin and iron related proteins.

	r	P-value
Hepcidin-serum iron	0.188	0.064
Hepcidin-plasma ferritin	0.695	<0.001
Hepcidin-serum ferritin	0.717	<0.001
Hepcidin-serum transferrin	-0.409	<0.001
Hepcidin-saturated transferrin	0.344	0.001
Hepcidin-ceruloplasmin	0.250	0.014
Hepcidin-hemoglobin	-0.002	0.988

Pearson's correlation coefficient (r) was used to calculate the above correlation coefficients and P-values. P<0.05 was considered as significant.

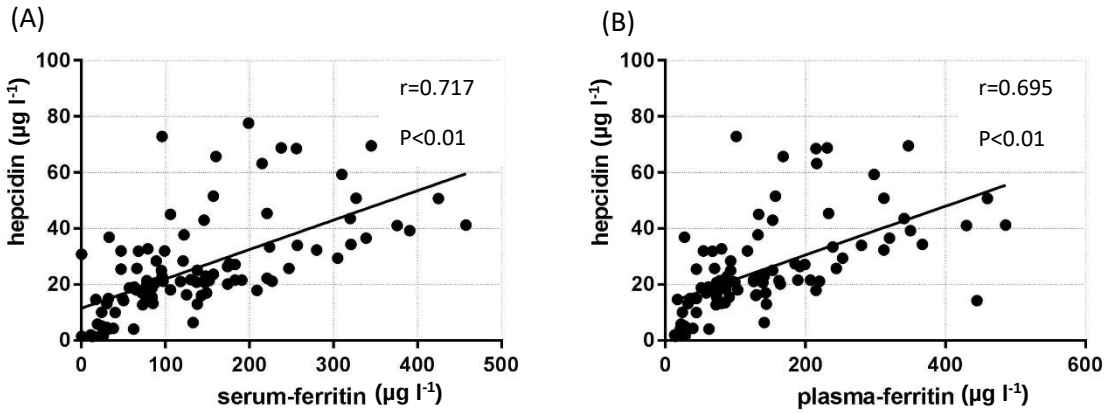


Figure 3.8. Correlations between (A) hepcidin and serum ferritin (B) hepcidin and plasma ferritin. Significant positive correlations were observed between hepcidin and serum ferritin (r=0.717, P<0.001), and hepcidin and plasma ferritin (r=0.695, P<0.001). Correlation coefficients and P-values were calculated using Pearson's correlation coefficient (r).

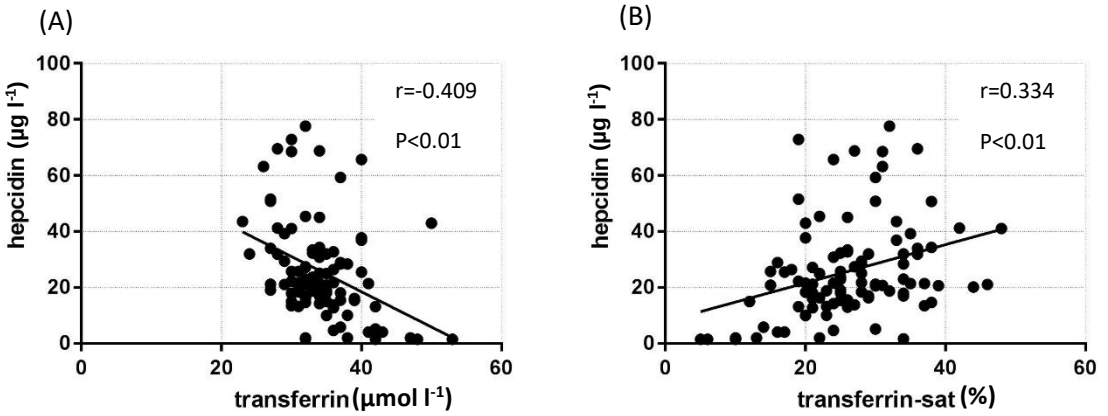


Figure 3.9. Correlations between (A) hepcidin and transferrin (B) hepcidin and saturated transferrin. A significant negative correlation was observed between hepcidin and transferrin (r=-0.409, P<0.001), while the correlation between hepcidin and saturated transferrin was positively significant (r=0.344, P=0.001). Correlation coefficients and P-values were calculated using Pearson's correlation coefficient (r).

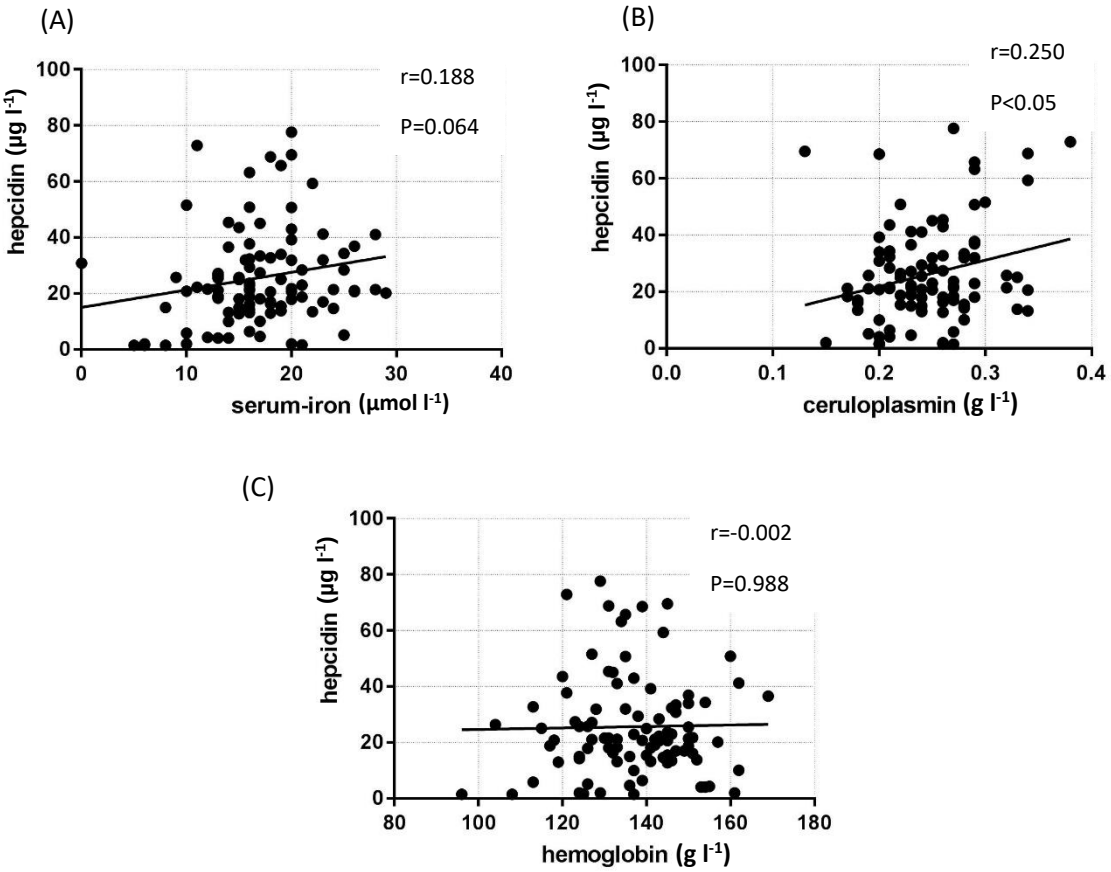


Figure 3.10. Correlations between (A) hepcidin and iron (B) hepcidin and ceruloplasmin (C) hepcidin and hemoglobin (HB). No significant correlation was observed between hepcidin and serum iron (r=0.188, P=0.064). However, there was positive correlation between hepcidin and ceruloplasmin (r=0.250, P=0.014). There was no significant association between hepcidin and hemoglobin (r=-0.002, p=0.988). Correlation coefficients and P-values were calculated using Pearson's correlation coefficient (r).

3.5. Association of hepcidin and inflammatory proteins

The association between hepcidin and inflammation indicator proteins including hs-CRP and wr-CRP were further analysed in the current study. Wr-CRP is a protein which shows increase in the case of inflammation and it has been detected around amyloid plaques in the brain of patients with dementia. On the other hand, hr-CRP is a sensitive marker identifying low levels of inflammation. Hs-CRP has been established as a sensitive marker for cardiovascular disease, however, some studies showed the relationship between hs-CRP and dementia [178]. So in this study the relationship between hepcidin and both types of CRP were investigated.

Notably, results demonstrated significant associations between hepcidin and wr-CRP, and hepcidin and hs-CRP (Table 3.5 and Figure 3.11).

Table 3.5. Correlation between hepcidin and inflammatory proteins.

	r	P-value
Hepcidin-wr-CRP	0.240	0.016
Hepcidin-hs-CRP	0.233	0.020

Abbreviations: hs-CRP, high-sensitivity C-reactive protein; wr-CRP, wide range C-reactive protein. R, correlation co-efficient. Pearson correlation was used to calculate the P-value and correlation coefficient. P<0.05 was considered as significant.

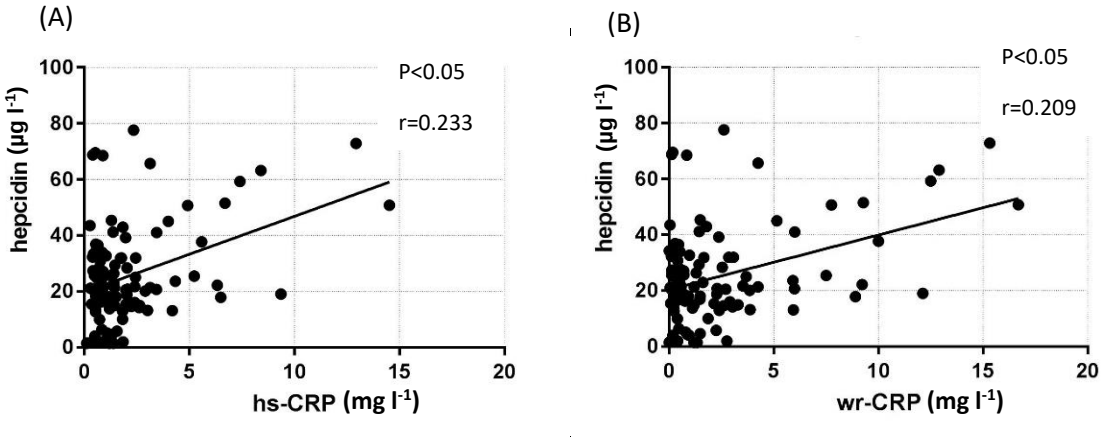


Figure 3.11. Correlations between (A) hepcidin and hs-CRP, high-sensitivity C-reactive protein (B) hepcidin and wr-CRP, wide range C-reactive protein. Significant correlations were detected between hepcidin and hs-CRP (r=0.233, P=0.020), and hepcidin and wr-CRP (r=0.240, P=0.016). Correlation coefficients and P-values were calculated using Pearson's correlation coefficient (r).

3.6. Hepcidin association with hippocampal volume

No significant association was detected between hepcidin and left hippocampal volume, and hepcidin and right hippocampal volume (Table 3.6 and Figure 3.12).

Table 3.6. Correlation between hepcidin and hippocampal volume.

	r	P-value
Hepcidin-left hippocampal volume	0.145	0.160
Hepcidin-right hippocampal volume	0.149	0.147

Correlation coefficients and P-values were calculated using Pearson's correlation coefficient (r).

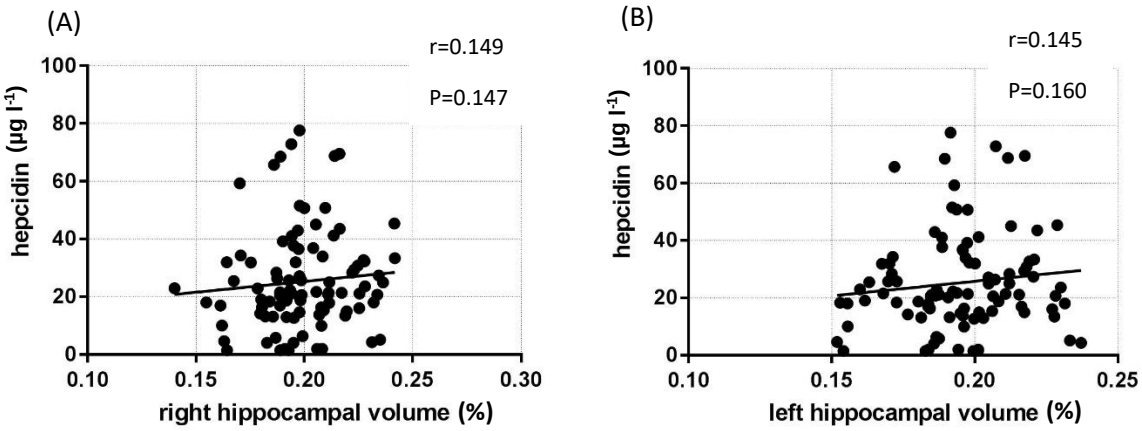


Figure 3.12. Correlations between (A) hepcidin and right hippocampal volume, (B) hepcidin and left hippocampal volume. No significant correlations were detected between hepcidin and right hippocampal volume (r=0.149, P=0.147), and hepcidin and left hippocampal volume (r=0.145, P=0.160). Correlation coefficients and P-values were calculated using Pearson's correlation coefficient (r).

3.7. Correlation between hepcidin and neuropsychological tests

In the current study, the association between hepcidin and neuropsychological test scores including MMSE, MAC-Q, verbal and visual episodic memory and, working memory and executive function scores were also analysed (Table 3.7).

The association between hepcidin and MMSE scores, and hepcidin and MAC-Q scores were not statistically significant. Similarly, no significant association was detected between hepcidin and composite verbal and visual episodic memory scores, as well as between hepcidin and working memory and executive function composite scores in KARVIAH cohort (Figure 3.13).

Additionally, no significant differences were obtained between serum hepcidin levels in subjective memory complainers and non-complainers (P=0.489) (Figure 3.14).

Table 3.7. Hepcidin association with neuropsychological tests.

	r	P-value
MAC-Q scores	0.068	0.503
MMSE scores	0.154	0.127
composite verbal and visual memory	0.104	0.301
composite working memory	-0.015	0.885

Abbreviations: MAC-Q, Memory Assessment Clinic-Questionnaire; MMSE, Mini Mental State Examination. r, correlation co-efficient. Pearson correlation was used to calculate the P-value and correlation coefficient. P<0.05 was considered as significant.

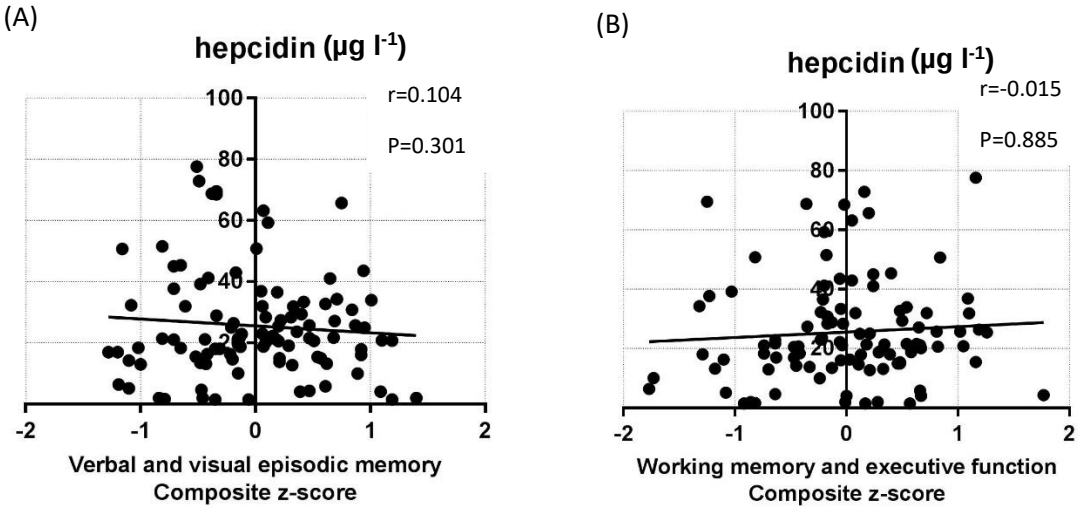


Figure 3.13. Correlations between serum hepcidin levels and cognition. No significant associations were observed between (A) hepcidin and verbal and visual episodic memory (r=0.104, p=0.301), (B) hepcidin and working memory and executive function (r=-0.015, p=0.885). Correlation coefficients and P-values were calculated using Pearson's correlation coefficient (r).

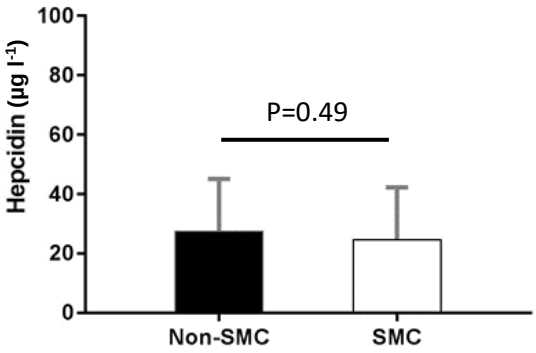


Figure 3.14. Differences between serum hepcidin level in subjective memory complainers (SMC) and non-complainers. Differences in serum hepcidin level between SMC and non-SMC groups are not statistically significant (P=0.489). SMC is defined based on the MAC-Q scores, and participants with MAC-Q≥25 were considered as subjective memory complainers.

3.8. Evaluation of serum hepcidin as a potential AD biomarker

Finally, serum hepcidin was evaluated as a potential biomarker to differentiate between low and high NAL groups. To this purpose, receiver operating characteristic (ROC) curves were generated by using logistic regression as described previously in Chapter 2. The 'Base' model comprising the major risk factors of AD, namely, age, gender and *APOE* ε4 status was generated. When serum hepcidin was added to the 'base' model (base model+serum hepcidin), a modest increase in the area under the curve (AUC) was observed, wherein the AUC of the 'base+serum hepcidin' model increased to 81.3% compared to the 'base' model with an AUC of 78.7%. Additionally, at a sensitivity of 80%, the specificity slightly increased from 61% in the 'base' model to 66% in the 'base+serum hepcidin' model in better distinguishing low NAL participants from high NAL participants. The ROC curves for both 'base' and 'base+serum hepcidin' models are presented in Figure 3.15.

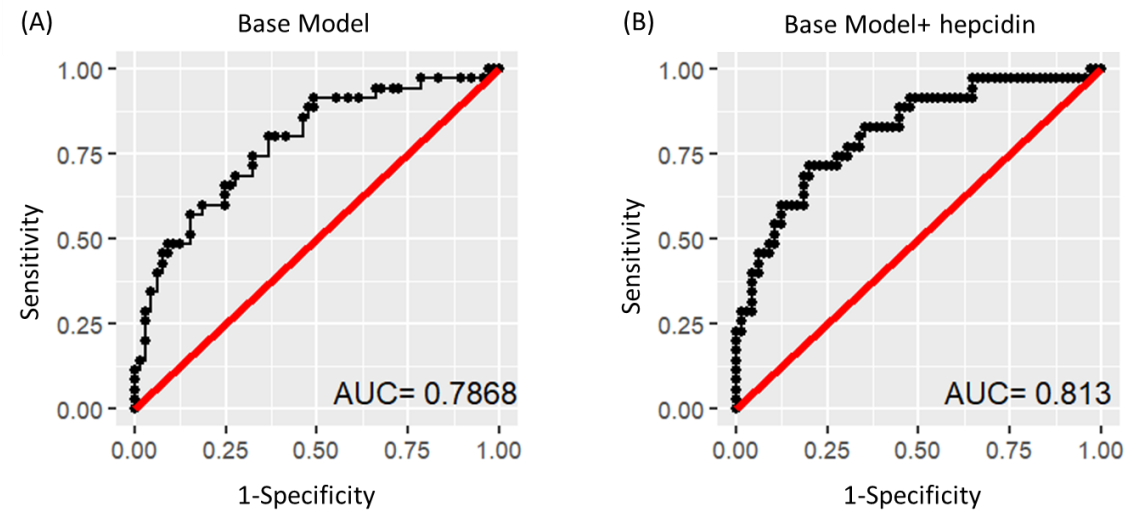


Figure 3.15. Receiver operating characteristic (ROC) curves for the prediction of high neocortical amyloid-β load in cognitively normal participants. ROC curves of logistic regression modelling show that the AUC of the 'base' model (A) including major risk factors such as age, gender and *APOE* ε4 allele status was slightly increased by adding the serum hepcidin into the 'base + hepcidin' model (B). Logistic regression models were employed to perform the analyses. *APOE*, apolipoprotein E; AUC, area under the curve.

Chapter 4
Discussion

4.1. Main findings and implications

Iron dysregulation is one of the predominant pathological features of neurodegenerative conditions such as AD and Parkinson's disease. Iron dyshomeostasis in AD does not only occur in brain regions where disease pathology has mostly been observed, but is also apparent in the periphery wherein alterations in blood proteins that play a critical role in iron metabolism have been seen [179].

In the present study, the alteration of the protein hepcidin, that plays a central role in maintaining systemic iron homeostasis, was investigated in preclinical AD to further characterise the abnormalities in iron metabolism. Based on the literature, this is the first study reporting an association between concentrations of serum hepcidin and NAL, a gold standard biomarker of AD, in cognitively normal elderly individuals. Additionally, the association of hepcidin with other iron related proteins such as ferritin, transferrin and saturated transferrin were explored. Finally, serum hepcidin was evaluated as a potential biomarker which along with the known risk factors for AD, namely, age, gender and *APOE* ε4 status distinguished between low and high NAL participants with a specificity of 66% at 80% sensitivity.

Hepcidin, a disulfide-rich peptide produced by hepatocytes, is a major regulator of systemic iron homeostasis, and the main mediator of anaemia of inflammation. By inhibiting intestinal iron absorption, placental iron transport and release of recycled iron from macrophages, hepcidin effectively decreases the delivery of iron into maturing erythrocytes in the bone marrow. Association between hepcidin localisation and the localisation of amyloid plaques in the brain of AD patients, suggests the possible role of hepcidin in AD pathogenesis [167]. Moreover, a recent study has confirmed that the disruption in hepcidin haemostasis in AD is reflected in the periphery as well [169], providing evidence that such changes may also be detectable in the preclinical phases of AD.

The main finding of this study is that in preclinical AD, participants with high NAL were observed to have significantly higher serum hepcidin levels compared to participants with low NAL. Furthermore, a significant positive correlation is observed between serum hepcidin concentrations and NAL. Consequently, it can be argued that elevated serum hepcidin levels contribute to preclinical AD. The current findings are consistent with a previous study in which increased serum hepcidin levels were observed in AD patients [169]. However, these findings show that serum hepcidin elevations occur in the early stages of the disease, perhaps a few decades before any significant hippocampal atrophy and cognitive impairment. The observation of increased hepcidin levels in aged rats also corroborate the findings of the current study, given that aging is a major risk factor for AD [48].

Raha et al., examined the levels of hepcidin in the brain of AD patients compared to age matched controls [167]. In contrast to my findings of elevated hepcidin levels in the serum, Raha and colleagues observed decreased hepcidin levels in the brain. While, it is important to consider that their study employed a considerably small sample size (6 AD patients and 6 aged-matched controls) and they investigated hepcidin expression in the brain tissue of AD patients while our study has investigated hepcidin in the blood in preclinical AD, the findings of Raha and colleagues are consistent with the accumulation of iron in the brain in AD pathogenesis [135].

Interestingly, higher serum levels in high NAL compared to low NAL remained significant in the *APOE* ε4 non-carrier subset of the study cohort. It is noteworthy to mention that the *APOE* ε4 allele is a major risk factor for AD, and findings from the current study indicate that elevated hepcidin concentrations observed in the high NAL group are not dependent to carrying the *APOE* ε4 allele.

Moreover, based on the ROC results, adding serum hepcidin to a base model consisting of age, gender and *APOE* ε4 status increases the specificity from 61% to 66% at a sensitivity of 80%. However hepcidin contribution alone is relatively minor compared with the base model. Although hepcidin does not seem to be a strong biomarker compared to other reported biomarkers in the literature [152] in distinguishing the low NAL from the high NAL participants, more studies on larger, longitudinal studies with and without clinically diagnosed patients are required to further investigate whether it could serve as a potential AD biomarker within a diagnostic panel of markers for AD.

According to the hepcidin regulatory pathway, which was presented in Figure 1.3, current findings of elevated hepcidin levels in preclinical AD may either be attributed to inflammation or impaired iron metabolism. Inflammation and iron dyshomeostasis are two common incidents in AD pathogenesis which will be further discussed below.

4.2. Hepcidin association with inflammation

To investigate whether the elevated hepcidin levels observed in the current study were related to inflammation, the correlations between serum hepcidin levels and CRP concentrations in the study cohort were analysed. Interestingly, a significant association was observed between hepcidin levels and CRP concentrations (both wr-CRP and hs-CRP) which indicates the possible role of inflammation in hepcidin alterations. However, even after adjusting for CRP measures, elevated hepcidin levels continued to remain significant (P=0.055). Given that CRP is a general marker of inflammation, further studies are required to investigate the association between hepcidin and inflammation. Future studies on specific inflammation markers such as IL-6 and TNF-α may illuminate the role of inflammation in elevated hepcidin level in preclinical AD.

CRP is elevated during inflammation, and it has been detected around amyloid plaques in the brain of patients with dementia [180]. Hr-CRP is a sensitive marker for identifying low levels of inflammation.

Altered CRP concentrations in AD patients have been reported previously [181], however in the current study, CRP concentrations were not significantly different between the high NAL and low NAL participants.

4.2.1. Critical role of inflammation in AD pathogenesis

The critical role of neuroinflammation within the early stages of AD pathogenesis has been demonstrated in many studies [182]. Generation of amyloid plaques in the early stages of AD is associated with the secretion of acute phase proteins (monocyte chemoattractant Protein (MCP)-1) and proinflammatory cytokines (IL-6) in brain regions with Aβ deposition, demyelination and neuronal injury [183]. For instance, it has been suggested that the over-expression of IL-1β in microglia occurs after Aβ deposition in the brain and leads to chronic neuroinflammation and neuronal dysfunction [184].

Administration of anti-inflammatory drugs such as tumour necrosis factor alpha (TNF-α) monoclonal antibody in a rodent study has shown positive effects in decreasing Aβ plaques and tau phosphorylation [185]. In addition, the significant functional improvement in AD patients after administration of interferon (IFN)-1β1α antagonists suggests that decreasing proinflammatory cytokine concentrations may have positive effects on AD symptoms [186].

Several studies reported the elevation of proinflammatory markers such as IL-1β, IL-2, IL-18, homocysteine, hs-CRP and IFN-γ in the blood of AD patients compared to the controls [187, 188]. For instance, a systematic review and meta-analysis by Lai et.al reviewed 175 studies on the alteration of peripheral inflammatory factors in AD patients and healthy controls [189]. Increased concentrations of peripheral interleukins (IL-1β, IL-2, IL-6, IL-18), interferon-γ, homocysteine, hs-CRP, TNF-α converting enzyme, soluble TNF receptors, leptin, 1 and 2C-X-C motif chemokine-10, epidermal growth factor and vascular cell adhesion molecule-1 were observed in 13,334 patients with AD compared to 12,912 healthy controls [189]. Elevated blood concentrations of the aforementioned cytokines and acute phase reactants in AD patients may indicate the presence of a systemic chronic inflammation associated with the disease.

4.2.2. Interleukin-6 induces hepcidin expression

Evidence from several studies suggest that hepcidin expression is regulated through three major pathways: regulation by inflammation depending on IL-6 secretion, regulation by iron concentration depending on other factors (probably saturated transferrin [190]) and suppression of hepcidin production caused by hypoxia and erythropoiesis [191]. In fact, elevated blood iron increases the expression of hepcidin without requirement for IL-6, while during inflammation IL-6 is essential for hepcidin mRNA expression. The critical role of IL-6 in hepcidin secretion was further supported by observing *in vitro* stimulation of fresh human hepatocytes with a panel of cytokines, where hepcidin mRNA was induced by IL-6 (but not IL-1α nor TNF-α) [192]. In addition, Nemeth et al. showed that IL-

6 is required for hepcidin induction in microbial stimuli *in vitro* as well as hepcidin induction and hypoferremia during inflammation in mice [193]. The same study reported that within two hours after IL-6 infusion in humans, hepcidin excretion increased while serum iron and transferrin saturation decreased accordingly indicating the role of IL-6 in hepcidin production.

Numerous rodent and clinical studies have investigated the role of IL-6 in the neurodegenerative process of AD [187, 194, 195]. IL-6 is a proinflammatory cytokine produced by lymphoid cells and macrophages in the periphery and can induce an acute response during the early phase of inflammation [182]. Although few studies reported decreased [196] or non-significant [197] changes in IL-6 peripheral levels in AD patients, most of the studies have shown a significant increase of this inflammation marker in AD [187, 198, 199]. For instance, a recent review paper reported significantly elevated levels of IL-6 in AD patients compared to healthy controls. Within the same study, IL-6 levels were also seen to inversely correlate with the mean MMSE scores in AD patients [189]. These findings suggest that IL-6 plays a critical role in the inflammatory cascade of AD and may correlate with other pathological pathways of the disease, predominantly, iron dyshomeostasis.

As it has been previously demonstrated in Figure 1.3, IL-6 regulates hepcidin expression by activation of STAT3 protein which binds directly to the hepcidin promoter and induces its expression [200]. As a result of increased hepcidin levels, the release of iron from macrophages (which recycle iron from senescent erythrocytes) is blocked and hence plasma iron concentration reduces rapidly. Given that developing erythrocytes is an iron-consuming process, decrease in the iron supply affects hemoglobin synthesis and causes anaemia due to inflammation. This is in agreement with the reported result of overexpression of IL-6 in mice [201], and repeated administration of IL-6 in rats where anaemia was observed. Conclusively, long-term treatment with an anti-IL-6 receptor antibody in patients with multicentric Castleman's disease (MCD) results in hepcidin down regulation [202].

Considering all the evidences, it is suggested that the current findings of elevated hepcidin levels may be attributed to the inflammation cascade, as a consequence of over expression of IL-6 which occurs in early stages of AD pathogenesis [203]. However, future investigations are required to further understand the association between hepcidin alteration and inflammation in the preclinical stages of the disease.

4.3. Association between hepcidin and iron dysregulation

Increased iron accumulation in the affected areas of AD brains, such as hippocampus and cerebral cortex with Aβ plaques and NFT tangles, is a pathological feature of AD, which has been consistently reported since 1953 [135]. Iron accumulation could potentially promote neurodegeneration through oxidative damage to sensitive subcellular compartments. Iron plays a central role in oxidative stress in AD, as it is a redox-active transition metal switching between Fe^{2+} and Fe^{3+} states in biological systems

[179]. Iron deposition in the brain seems to occur decades before brain atrophy, as has been observed in the brain tissues of individuals within the MCI and preclinical stages of AD [204].

However, the results of studies regarding blood iron alteration in AD patients are inconsistent. Although several studies reported decreased iron levels in AD patients [205, 206], a few studies have not observed significant alterations in plasma iron levels in AD patients [207]. For instance, a study by Basun et.al reported significantly lower plasma iron levels in 24 individuals with dementia of the Alzheimer type (DAT) compared to 28 healthy volunteers [205]. Similarly, in a study by Vural et.al, variation of some metal elements such as zinc, copper, magnesium, iron and selenium were investigated in AD patients, where they reported significant reduction in plasma iron level in AD patients compared to the healthy group [206]. However, a recent meta-analysis by Wang et al showed no significant alteration in serum iron levels in AD patients, and a strong association between serum iron level and mean age of subjects. Therefore, they suggested that the variation of the iron found in some studies can be caused by the diverse mean age of study cohorts [207].

In my study, no significant differences were observed in serum iron levels between low NAL and high NAL participants. Hepcidin elevation in blood is expected to be accompanied by a decrease in blood iron levels due to the suppression of ferroportin and prevention of iron secretion from the liver and macrophages. Given that the observed alterations in serum hepcidin level were not accompanied with corresponding alterations in serum iron levels, I propose that the decrease in blood iron levels may occur later in the AD pathogenesis pathway. This notion is supported by the observed significant decline in plasma iron and hemoglobin concentrations in AD patients, and the reported strong association between anaemia and AD [36]. However, further studies are required to distinguish the sequence of events in the brain and periphery, considering Aβ deposition in the brain as well as the circulating hepcidin and tissue iron deposition in preclinical AD.

4.3.1. Hepcidin association with ferritin

Ferritin is an intracellular protein which is mainly involved in iron storage and delivery. Serum ferritin is also a marker of inflammation and cellular damage as it has been reported as a leakage product of the damaged cells [208]. Although hepatocytes are the main source of cellular ferritin, it is also found in macrophages where recycled iron from red blood cells is stored in ferritin before reutilisation for developing erythrocytes.

Elevated ferritin levels have been already observed in the hippocampal tissue of AD patients [46]. Moreover, Majkusiak et al. reported a significant positive association between the concentration of ferritin light chain and senile plaques in subiculum [209]. Disruption in ferritin homeostasis in AD has been confirmed to reflect in the periphery levels as well [210]. For instance, Sternberg et al. reported a significant higher concentration of serum ferritin in AD patients compared to the control group which is consistent to our current results [169].

Since recent reports highlight differences in ferritin measurements between plasma and serum samples [211, 212], therefore, ferritin level were measure in both serum and plasma to investigate whether both plasma and serum ferritin concentrations were altered in cognitively normal elderly individuals with high NAL. In the current study cohort, both serum and plasma ferritin levels were significantly higher in the high NAL group compared to the low NAL group [152]. These results of higher ferritin in plasma preclinical AD verify the results of previous studies where elevated serum ferritin was observed in AD patients [169], suggesting that this elevation occurs at early stages of the disease. Increased blood ferritin concentrations could also reflect an elevated brain ferritin level and leakage of ferritin from the brain due to compromised BBB integrity [213]. Current results may also be attributed to impaired iron mobilisation which was previously corroborated by the higher serum hepcidin concentrations observed in the high NAL compared to the low NAL group. However, the increase of ferritin levels in the high NAL versus low NAL groups were not accompanied by the corresponding alteration in serum iron levels.

Importantly, a strong positive correlation was observed between the serum hepcidin and serum (and plasma) ferritin levels in the study cohort ($p<0.001$). This strong association implies that the increased serum ferritin levels may reflect tissue iron loading [214]. This is attributed to the fact that elevated serum hepcidin, observed in my study, inhibits iron release from specific tissues so that the trapped iron is stored within the tissues in the form of ferritin. This has been further supported by a previous study reporting iron deposition in preclinical AD brain tissue [204].

4.3.2. Hepcidin association with transferrin and saturated transferrin

Transferrin is an iron binding protein, and under physiological circumstances most of the iron in circulation is bound to transferrin. Normally, around 30% of transferrin is saturated by iron, and in diseases with iron overload in tissues, transferrin saturation is elevated [215]. Based on an early study, transferrin is distributed homogenously around the senile plaques and is found in the astrocytes (rather than oligodendrocytes) in the cerebral cortex of AD brain tissues [46]. Interestingly, a relatively recent study found that transferrin concentrations are not significantly altered in AD patients compared to heathy controls [139], while a decrease has been observed in the total transferrin-bound iron. The aforementioned study reported transferrin desaturation as a cause of decreased plasma iron in AD patients.

In our study, although there was no significant difference in transferrin and saturated transferrin between low and high NAL participants, a strong inverse association was found between hepcidin and transferrin ($p<0.001$). In addition, significant positive correlation was observed between serum hepcidin and transferrin saturation which is consistent with the observation of a tight relationship between liver hepcidin expression and transferrin saturation in mouse models with anaemia [216, 217].

In summary, in my study a strong positive correlation has been observed between serum hepcidin and serum ferritin while an inverse correlation has been seen between serum hepcidin and serum transferrin. Therefore, it can be posited that in AD, the body tends to store iron in the tissues and iron is not adequately mobilised via transferrin in the circulation. This is consistent with the observation of iron accumulation in the neocortex in individuals in preclinical and AD stages [204].

4.3.3. Hepcidin association with ceruloplasmin

Ceruloplasmin is the major copper transporter protein in the blood which also plays a critical role in iron metabolism by oxidation of Fe^{2+} (ferrous iron) into Fe^{3+} (ferric iron) [218]. Ceruloplasmin is also essential for cellular iron efflux by oxidizing ferrous iron exported from ferroportin. Elevated ceruloplasmin levels in critical brain areas in AD have been reported previously [219], and this disruption has been observed in the periphery as well [220]. For instance, a study reported significant increase in ceruloplasmin levels in AD patients while it is not associated with increased copper levels [218]. In my study, there was no significant difference in serum ceruloplasmin levels between the high and low NAL groups which suggests that this alteration happens later in pathological pathway of AD. Interestingly, a significant association was obtained between the concentration of hepcidin and ceruloplasmin in current study which may be attributed to the metal dyshomeostasis in AD. However, future studies are required to further investigate the relation between hepcidin and ceruloplasmin in preclinical AD.

4.4. Limitations

Limitations acknowledged in the current study include the followings:

- Small sample size particularly in the high NAL group, and cross-sectional nature of the study. Therefore, future longitudinal studies in an independent larger cohort are required to validate the current findings.
- Uneven gender and *APOE* ε4 carrier status distribution between the low NAL and high NAL groups. Nevertheless, in this study no differences were observed in serum hepcidin levels between males and females or between *APOE* ε4 carriers and non-carriers (Figure 3.1).
- Other considerable limitation of this study is associated with the measurement method used to quantify serum hepcidin concentrations. In the present study ELISA was used as an analytical method because it was cost effective and easy to run in our laboratory. However, some studies reported that ELISA might not be as sensitive as other quantitative methods such as LC-MS, as there is a possibility of cross reaction between various hepcidin isoforms (hepcidin-20, -22 and -25) in ELISA [193]. However, hepcidin DRG ELISA kit (EIA-5782) is based on the c-ELISA principal which is designed to specifically detect the active isoform of hepcidin (hepcidin-25) in human samples [221].

4.5. Future direction

Possible future direction includes:

- Conducting a longitudinal study on a larger cohort including healthy and cognitively normal individuals with low and high NAL, MCI and AD patients. The results of such study will help evaluate the current findings of increased hepcidin serum level as an early AD biomarker.
- Current findings confirm iron dysregulation in preclinical AD. However, the sequence of events in the brain tissue and periphery could be investigated in a mouse model of AD with respect to amyloid deposition. This model can also explore the relation between hepcidin elevation and alteration of other iron related proteins such as ferritin and transferrin.
- The relation between hepcidin and inflammatory factors specifically IL-6 can be further investigated in preclinical and clinical AD patients.
- Given that the activity of ferroportin is regulated by hepcidin, the alteration of ferroportin expression in response to hepcidin elevation in preclinical and clinical AD can also be investigated in future studies.
- Based on the ROC results, hepcidin does not seem to be a strong biomarker compared to other reported biomarkers in the literature. Therefore, more studies on larger cohorts with and without clinically diagnosed AD are needed to further investigate whether it could serve as a potential biomarker within a diagnostic panel of markers for AD.

4.6. Conclusion

The present study provides evidence which underlies the progressive and dynamic changes in iron homeostasis in preclinical AD. AD is a complex disease which is developed over few decades and the only definite clinical way for its diagnosis is an autopsy. Currently, there are two major biomarkers for AD including cerebral Aβ load measured via PET scans and CSF tau concentrations requiring a lumbar puncture for CSF collection. However, the use of these biomarkers is uneconomical and invasive for population-wide screening. Therefore, current studies are focussing on finding blood biomarkers which can reflect brain Aβ load and be easily translated for use in pathology laboratories. The current study for the first time demonstrated that elevated serum hepcidin is a feature of preclinical AD which occurs few decades prior to cognitive impairment. The results may also add a value to a panel of biomarkers which reflects the brain Aβ load of individuals in preclinical stages. The current findings will help understand the biological pathways involved in the early stages of the disease by providing more insight into the systemic disruption of metal homeostasis. Therefore, these findings warrant further investigation, can improve diagnostic accuracy, and could be helpful in the process of identifying therapeutic targets for AD as well.

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