School of Public Health

Nutritional and Pharmacological Regulation of Cerebral Capillary Function

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Declaration

To the best of my knowledge and belief this thesis titled "Nutritional and Pharmacological Regulation of Cerebral Capillary Function" contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Menuka Pallebage-Gamarallage

29 November 2012

Abstract

Alzheimer's disease (AD) is the most common cause of dementia pathologically characterised by neurovascular inflammation, extracellular proteinaceous deposits enriched in amyloid-β (Aβ) and formation of neurofibrillar tangles. The cerebrovasculature in subjects with AD is also significantly altered and is first indicated by the progressive dysfunction of the cerebral capillary vessels. Despite evidence of an underlying vascular contribution to onset and progression of AD, most research focuses on factors regulating the more advanced pathological processes.

Clinical and experimental evidence suggest vascular risk factors influence the onset and progression of AD. Several studies have demonstrated that atherosclerosis, cardiovascular disease, hypertension, dyslipidemia and insulin resistance are positively associated with AD risk. Furthermore, population studies have found that chronic ingestion of pro-inflammatory diets enriched in saturated fatty acids (SFA), trans-fatty acids and cholesterol are positive risk factors for AD and markedly exacerbate cerebral pathology in animal models. A putative mechanism for the dietary-fat/amyloidogenic pathway was demonstrated in recent animal studies where chronic consumption of diets enriched in SFA and cholesterol significantly compromised blood-brain barrier (BBB) function in wild-type (WT) mice, resulting in substantial blood-to-brain delivery of circulating Aβ and potential exacerbation of cerebral amyloid load. Significant plasma Aβ is derived from liver and small intestine, secreted into circulation associated with triglyceride-rich lipoproteins (TRLs). Dietary lipid regulation of TRL-Aβ was equivocally demonstrated in WT mice fed an SFA enriched diet, where intestinal Aβ biogenesis and secretion was enhanced. Given that humans are predominantly in an absorptive state, the cumulative cerebrovascular effects of transient exposure to dietary stimulated plasma Aβ may be AD-risk relevant. However, a dietary-lipid/Aβ axis for cerebrovascular function could also provide therapeutic opportunities to reduce AD risk.

Observational studies suggest that calorie restriction and regular consumption of diets which supress inflammation may delay development and progression of AD.

Recent findings have demonstrated that consumption of diets rich in monounsaturated fatty acids (MUFAs) and ω-3 polyunsaturated fatty acids (PUFAs), in particular docosahexaenoic acid (DHA), may reduce risk for AD via suppression of inflammatory pathways. Furthermore, beneficial effects of unsaturated fatty acids on prevention of cerebrovascular dysfunction have been demonstrated *in vivo*. The association between dietary fat intake, hypercholesterolemia and increased AD prevalence suggests the possibility that lipid-modulating agents might also delay onset and progression of AD. Some epidemiological studies have provided evidence of a lower prevalence of diagnosed AD in patients with hypercholesterolaemia treated with statins (3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors). In one clinical study, an older generation cholesterol lowering agent probucol was found to stabilise cognitive impairment in elderly AD subjects. The mechanisms for the pharmacological effects of lipid lowering agents in prevention of AD are unclear, but may include vascular benefits associated with lower plasma lipids, regulation of Aβ biogenesis and kinetics or indicative of anti-oxidant and anti-inflammatory properties of the agents.

At present, there is lack of effective strategies for the treatment of AD. Given that cerebral capillary disturbances is amongst the earliest indicators of disease onset, it seems a reasonable proposition to suggest that recognition of and timely intervention to restore capillary integrity would be therapeutically beneficial. This thesis explores the novel hypothesis that *"dietary fat induced cerebral capillary dysfunction can be corrected by timely provision of an anti-inflammatory diet enriched in ω-3 fatty acids, or lipid lowering agents that attenuate cerebrovascular exposure to peripheral Aβ and inflammation"*. The hypothesis presented is supported by a substantial literature review in Chapter 1 of this thesis.

As a 'proof of concept' approach to the indicated hypothesis, the effects of lipid lowering agents, atorvastatin, pravastatin and probucol, for 'prevention' of dietary fat induced BBB dysfunction was determined (Chapter 1: article 5). Wild-type mice were chronically fed an SFA diet supplemented with atorvastatin and pravastatin. In addition, the effects of probucol on BBB integrity were determined in mice fed a cholesterol supplemented diet. All mice were maintained on their respective diets for 12 weeks. Cerebral extravasation of plasma protein

immunoglobulin-G (IgG) was assessed utilising 3-dimensional (3-D) immunofluorescent microscopy. Immunoglobulin-G was used as a surrogate marker of non-specific cerebrovascular permeability. Significant BBB dysfunction and enhanced cerebral extravasation of IgG was evident in mice fed SFA and cholesterol diets. Atorvastatin, a lipid soluble HMG-CoA reductase inhibitor prevented SFA induced parenchymal extravasation of IgG at 12 weeks when incorporated into the diet. In contrast, hydrophilic pravastatin had no effect on BBB integrity. In cholesterol supplemented mice, probucol maintained BBB function and extravasation of IgG was not evident. These findings suggest that some lipid lowering agents may effectively prevent dietary fat induced BBB dysfunction and their efficacy may be dependent on solubility. The drug effects on BBB integrity were independent of significant modifications on plasma lipid homeostasis. These findings support the hypothesis that pleiotropic properties of lipid lowering agents maintain BBB integrity.

Evidence of protective effect of lipid modulating agents on BBB function was concomitant with an attenuation in TRL-Aβ biogenesis is reported in Chapter 2. The article presented in this chapter investigated *in vivo* the putative effects of probucol on in TRL-Aβ homeostasis in absorptive epithelial cells of the small intestine in WT mice. Intestinal Aβ abundance was stimulated in mice fed a high-fat (HF) diet enriched in SFA and cholesterol for 4 weeks. Drug effects were determined in mice given low-fat (LF) and HF diets supplemented with 1% probucol. Quantitative immunofluorescent microscopy determined intestinal Aβ and apolipoprotein B (apo B) abundance. Apo B is an obligatory structural component of TRL originating from liver and absorptive epithelial cells of the small intestine. Apo B staining was detected in both the perinuclear region of the enterocytes and the lacteals in all groups. However, HF feeding and probucol treatment increased secretion of apo B into the lacteals without any change in net villi abundance. On the other hand, HF induced enterocytic perinuclear Aβ was significantly attenuated by probucol. No significant changes in Aβ were observed within the lacteals. These findings support the notion that probucol normalised the HF induced intestinal Aβ biogenesis and availability of TRL-Aβ for secretion and may confer protection against AD by reducing exposure to plasma TRL-Aβ.

Chronic ingestion of SFAs compromise BBB integrity, leading to cerebral extravasation of apo B lipoproteins enriched in Aβ. In contrast, diets enriched in PUFA oils had no detrimental effect. Rather, ω-3 and ω-6 fatty acids generally confer protection via suppression of inflammation. Chapter 3 investigated in WT mice if a PUFA diet enriched in DHA restored BBB integrity and attenuated parenchymal apo B abundance induced by chronic ingestion of SFA. Cerebrovascular leakage of apo B was quantitated utilising immunofluorescent staining. The plasma concentration of brain-derived S100B was measured as a measure of brain-to blood leakage and complimentary marker of BBB function. In mice fed SFA for 12 weeks, provision thereafter of a DHA enriched diet exacerbated parenchymal apo B retention, concomitant with a significant increase in plasma cholesterol and S100B. In contrast, provision of a LF diet following chronic SFA feeding had no effect on SFA induced parenchymal apo B abundance. In the established mouse model of BBB dysfunction with a heightened state of cerebrovascular inflammation, the provision of unsaturated fatty acids may be detrimental, possibly as a consequence of a greater susceptibility for lipid peroxidation.

The 'proof of concept' study presented in Chapter 1 (article 5) reported that the SFA induced parenchymal accumulation of plasma proteins could be prevented by co-administration of some lipid lowering agents. Restoration of BBB function is clinically relevant in a therapeutic context. Chapter 4 explored whether lipid-lowering agents could reverse BBB disturbances induced by chronic SFA feeding. Wild-type mice were fed an SFA diet for 12 weeks to induce BBB dysfunction and then randomised to receive atorvastatin, pravastatin or ibuprofen in combination with the SFA-rich diet for an additional 2 or 8 weeks. Abundance of plasma-derived IgG and apo B lipoproteins within brain parenchyme were quantified utilising immunofluorescence microscopy. Atorvastatin treatment for 2 and 8 weeks restored BBB integrity, indicated by a substantial reduction of IgG and apo B, particularly within the hippocampus. Pravastatin, a water-soluble statin was less effective than atorvastatin (lipid-soluble). Statin effects were independent of changes in plasma lipid homeostasis. Ibuprofen, a lipid-soluble cyclooxygenase (COX) inhibitor attenuated cerebral accumulation of IgG and apo B as effectively as atorvastatin.

These findings are consistent with the drug effects being independent of plasma lipid homeostasis.

The outcomes presented in this thesis provide novel insight into the positive effects of lipid lowering agents and non-steroidal anti-inflammatory drugs (NSAIDs) on regression of SFA induced BBB dysfunction. The effects of lipid lowering statins on BBB dysfunction were similar to that of ibuprofen. In addition, statin effects were independent of significant modifications in plasma lipid homeostasis, suggesting pleiotropic anti-inflammatory anti-oxidative effects. Moreover, statins enhance endothelial nitric oxide (NO) bioavailability that is essential for regulation of cerebral perfusion and improved endothelial function. In the established mouse model of BBB dysfunction induced by SFA feeding, provision of diet enriched in ω-3 fatty acid DHA exaggerated BBB dysfunction. At high concentrations, ω-3 fatty acids are subject to lipid peroxidation and could notionally promote oxidative stress under certain conditions. At a heightened state of inflammation, provision of ω-3 fatty acid may exacerbate BBB dysfunction. Given the importance of cerebral capillary vessels in AD pathophysiology, it is our contention that animal model studies investigating the putative role of anti-inflammatory agents on restoration of BBB integrity will be exceedingly informative for clinical research studies.

Acknowledgements

First and foremost, I would like to give my utmost gratitude to my supervisor Prof. John Mamo for his guidance and support throughout my candidacy. I have always appreciated his 'holistic' approach in research life and for being 'cool'. I must also thank Associate Prof. Satvinder Dhaliwal for his contribution to my project and have always enjoyed his company.

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Last but not least, I would like to express my deepest appreciation to my dearest parents, Mr. P.G. Somapala and Mrs. Jayantha Somapala, for their unconditional love and patience. Thank you for all the encouragement you have given me throughout my life. I will not forget my lovely sisters, Ishani and Sachini, for just being 'sisters'.

List of primary publications

This PhD-by publication thesis comprises of 4 first author peer-reviewed publications supported by 4 co-author publications. These articles have been published in the following journals. Please refer to Appendix A for signed declarations for author contributions and copyright authorisation.

1. **Pallebage-Gamarallage MMS**, Takechi R, Lam V, Galloway S, Dhaliwal S & Mamo JCL. (2010) Post-prandial lipid metabolism, lipid-modulating agents and cerebrovascular integrity: Implications for dementia risk. *Atheroclerosis Supp.* 11**,** 49-54.

   [Impact factor: 3.794]

2. **Pallebage-Gamarallage M**, Galloway S, Takechi R, Dhaliwal S & Mamo J. (2012a) Probucol suppresses enterocytic accumulation of amyloid-β induced by saturated fat and cholesterol feeding. *Lipids*. 47, 27-34.

   [Impact factor: 2.129]

3. **Pallebage-Gamarallage M**, Lam V, Takechi R, Galloway S & Mamo JCL. (2012b) A diet enriched in docosahexanoic acid exacerbates brain parenchymal extravasation of apo B lipoproteins induced by chronic ingestion of saturated fats. *Int J Vasc Med.* 2012, 647689.

4. **Pallebage-Gamarallage M**, Lam V, Takechi R, Galloway S, Clark K & Mamo J. (2012c) Restoration of dietary-fat induced blood-brain barrier dysfunction by anti-inflammatory lipid-modulating agents. *Lipids Health Dis.* 11, 117.

   [Impact factor: 2.17]

List of secondary publications

The following publications were not specific outcomes for my candidacy outline. Rather, these articles demonstrate broader but complimentary intellectual input of research studies relevant to the hypothesis presented in the thesis.

1. Takechi R, Galloway S, **Pallebage-Gamarallage MMS**, Wellington CL, Johnsen RD, Dhaliwal SS & Mamo JCL. (2010a) Differential effects of dietary fatty acids on the cerebral distribution of plasma-derived apo B lipoproteins with amyloid-β. *Br J Nutr.* 103**,** 652-662.

   [Impact factor: 3.013]

2. Galloway S, Takechi R, **Pallebage-Gamarallage M**, Dhaliwal S & Mamo J. (2009) Amyloid-beta colocalizes with apolipoprotein B in absorptive cells of the small intestine. *Lipids Health Dis.* 8**,** 46.

   [Impact factor: 2.17]

3. Takechi R, Galloway S, **Pallebage-Gamarallage MMS**, Lam V & Mamo JCL. (2010b) Dietary fats, cerebrovasculature integrity and Alzheimer's disease risk. *Prog Lipid Res.* 49**,** 159-170.

   [Impact factor: 10.667]

4. Lam V, Takechi R, **Pallebage-Gamarallage MMS**, Galloway S & Mamo JCL. (2011) Colocalisation of plasma derived apo B lipoproteins with cerebral proteoglycans in a transgenic-amyloid model of Alzheimer's disease. *Neurosci Lett.* 492**,** 160-164.

   [Impact factor: 2.105]

Introduction and thesis outline

Introduction

Alzheimer's disease is the most common cause of dementia and presently is estimated to affect 40 million individuals worldwide (Barnes and Yaffe 2011). Furthermore, prevalence is expected to triple by the year 2050 because of increasing life expectancy in both developed and developing nations (Abbott 2011, Barnes and Yaffe 2011, Brookmeyer et al. 2007). Health care costs associated with dementia treatment and care is expected to increase by 85% by 2030 (Abbott 2011). Indicative of the significant disease burden, in Australia $4.9 billion per annum is spent on dementia related health care. Presently, dementia is the third leading cause for deaths in Australia, however it has been predicted to be the most significant cause of morbidity within 20 years (Abbott 2011, Brookmeyer et al. 2007). Current strategies for treatment of AD have been largely unsuccessful in altering disease progression (Barnes and Yaffe 2011, O'Brien and Burns 2011, Green et al. 2009, Quinn et al. 2010). Clearly, developing strategies to reduce AD risk; delaying disease onset and progression; or restoring neurovascular integrity and function in subjects with AD is increasingly a global health priority.

Hallmark pathological features of AD include the extracellular deposition of protein deposits enriched in Aβ within brain parenchyme (Fukuoka et al. 2004, Pastorino and Lu 2006); sporadic neurofibrillary tangles consisting of hyperphoshporylated fibrillar microtubule-associated protein *tau* (Perl 2010, Mattson 2004); neuronal degeneration (Mattson 2004) and significant cerebrovascular abnormalities (Ellis et al. 1996, Dickstein et al. 2010, Miyakawa 2010). The aetiology for AD is uncertain. Vascular disturbances generally precede frank amyloidosis and tangle formation (Kalaria 1992, Pluta and Amek 2008) suggesting a vascular origin for the disease. However, amyloid plaque and neurofibrillary tangles are found predominantly within the cortex (CTX) and hippocampal regions of the brain that are essential for learning and memory (Mattson 2004). Moreover, end-stage neuronal death and loss of glial cell function are associated with amyloidosis (Mattson 2004).

Amyloid-β is generated from the proteolytic degradation of the amyloid precursor protein (APP) expressed on the plasma membrane of neuronal cells (Fukuoka et al. 2004). Amyloid-transgenic rodent models support the notion that exaggerated fibril formation of Aβ stimulated by over-production results in neuronal oxidative stress and apoptosis (Takeuchi et al. 2000, Shie et al. 2002). However, there is no evidence for increased Aβ production in sporadic, late-onset AD, the most common form of AD-dementia.

Significant Aβ is in circulation derived from vascular smooth muscle cells and endothelial cells (Goldgaber et al. 1989, Haass et al. 1992) and from blood platelets (Chen et al. 1995). In addition, lipogenic organs including liver and small intestine secrete significant Aβ associated with TRLs (very-low density lipoproteins and chylomicrons, respectively) (Koudinov et al. 1994, Galloway et al. 2007). Amyloid-β is lipophilic and in blood therefore chaperoned by either lipid-macromolecules, or in association with amphiphylic proteins such as apo E or albumin (Biere et al. 1996, LaDu et al. 1997). Several studies (including published studies that support my thesis submission) suggested that exaggerated cerebrovascular exposure to plasma-derived Aβ, may significantly contribute to brain capillary disturbances that feature in early-AD. Thereafter, blood-to-brain delivery of Aβ could conceivably exacerbate cerebral amyloid load (Pluta and Amek 2008, Pluta et al. 1996, Pluta et al. 2006) and exacerbate deposition on extracellular matrices. Consistent with the latter, we reported significant colocalisation of plasma lipoprotein-Aβ with specific proteoglycans and with cerebral amyloid deposits in amyloid transgenic mice (Lam et al. 2011).

The BBB describes hallmark characteristics of brain capillary vessels pivotal for maintaining cellular homeostasis. A normal adult brain constitutes ~400 miles of capillary vessels with a total surface area of approximately 30 m^2 essential for the efficient transport and exchange of molecules between blood and the brain (Zlokovic 2005). The cerebral capillary vessels are lined by endothelial cells that are tightly apposed via abundant expression of tight-junction and adherens-junction proteins that ordinarily prevent blood-to-brain extravasation of lipophilic large macromolecules such as lipoproteins (Abbott et al. 2010, Ballabh et al. 2004). The

BBB protects the brain from systemically derived harmful neurotoxic and pro-inflammatory agents (Hawkins and Davis 2005), whilst allowing diffusion of small gaseous molecules (O_2 and CO_2), and active transport of essential nutrients (glucose, amino acids and vitamins) via specific protein transporters (Hawkins and Davis 2005, Abbott 2002). The cerebral capillary endothelium is lined by the basement membrane separating astrocytes and pericytes of the outer lining of the BBB (Zlokovic 2005). The astrocytes modulate tight junction protein expression and angiogenesis, which are vital for maintaining endothelial structural integrity (Zlokovic 2005, Lee et al. 2003, Balabanov and Dore-Duffy 1998), synaptic signal transmission and neuronal plasticity (Zlokovic 2005, Newman 2003, Nedergaard et al. 2003). Glial cells which are resident cells with astrocytes providing critical immune functionality to the cerebral capillary unit, however with persistent activation and respiratory activity could notionally increase oxidative stress.

The cerebrovasculature in subjects with AD shows pathological alterations including vascular endothelial and smooth muscle cell proliferation (Ellis et al. 1996). Blood plasma proteins have been detected in the parenchyma of AD brains (Kalaria 1992, Wisniewski et al. 1997) and inflammatory sequelae are commonly reported (Cullen 1997), observations that are consistent with breakdown of the BBB. Deposition of extracellular proteoglycans and collagen result in loss of vascular distensibility and may significantly alter brain perfusion (Dickstein et al. 2010, Miyakawa 2010). In advanced AD, biochemical and morphological studies show arteriosclerosis, focal constrictions and necrosis of the cerebral endothelium contributing to loss of the perivascular nerve plexus and a variety of alterations occurring within intracranial vessels and perforating arteries including significant convolutional abnormalities and “collapsed” capillaries (Dickstein et al. 2010, Miyakawa 2010, Zhang et al. 1994). Clinical imaging studies have demonstrated significant cerebral hypoperfusion and hypometabolism that precede the onset of dementia (Grammas et al. 2011).

Recent studies suggest that dietary lipids and metabolic factors are related to disrupted BBB integrity. A population study found both atherogenic and metabolic dyslipidemia to be more prevalent in AD subjects with BBB dysfunction than in subjects without BBB impairment (Bowman et al. 2012). Hypercholesterolemia is

considered a pro-inflammatory condition associated with increased microglial activation and leukocyte infiltration in the brain (Streit and Sparks 1997). Others have studied a number of circulating pro-inflammatory mediators that increase BBB permeability *in vivo* and *in vitro*. These include inflammatory cells, proteases, chemokines, cytokines and free radicals (McColl et al. 2008, Abbott 2000, Abbott 2002, Denes et al. 2011, Zhou et al. 2011). Putative mechanisms of plasma lipid-induced inflammation mediated BBB dysfunction include activation of vascular endothelium and glial cells (Denes et al. 2011). Furthermore, proteolysis of the tight junction proteins and basal lamina (McColl et al. 2008), would notionally amplify capillary vascular disturbances.

The positive association between dietary fat intake, hypercholesterolemia and AD raises the possibility that lipid modulating agents may delay onset or attenuate the progression of AD. Some population studies have provided evidence of lipid-lowering agents in the prevention of AD (Crisby et al. 2002, Wolozin 2004). However, the mechanisms for this association are not clear. A BBB axis for benefit is supported by the findings of Kalayci et al. (2005) who reported in a hypertensive mouse model that BBB damage was attenuated when rats were treated with atorvastatin. In the same study, atorvastatin was found to increase plasma anti-oxidant concentration and the expression of BBB tight junction proteins. In cell culture studies, pitavastatin was reported to strengthen the barrier integrity in primary cultures of rat brain endothelial cells (Morofuji et al. 2010). These findings support the concept that lipid-lowering agents such as statins may positively influence cerebral capillary function.

Thesis outline

This PhD thesis “by publication” is generated according to the “Guidelines for Thesis by Publication” detailed by the Curtin University Office of Research and Development.

(http://research.curtin.edu.au/local/docs/graduate/TE-ThesisByPubGuidelines.pdf).

In the first phase of my candidacy I contributed significantly to several publications that were pivotal to the generation of the hypothesis presented in this thesis. In Chapter 1, a summary of each of the supporting co-authored articles, describing the prevailing dogma at time of publication; the primary objectives; findings and relevance to candidacy is provided for consideration. Whilst each article cites the broader literature, the synopsis provided for each publication refers to key points of interest and articles referenced. The synopsis also considers relevant articles published thereafter. My contribution to each manuscript is detailed and clearly acknowledged by the co-authors and provided as Appendix A.

Chapters 2-4 for this thesis provide first-author manuscripts that explored key elements of the hypothesis presented. The experimental design for each first authored paper, completion of experiments, interpretation of data and genesis of manuscript was principally completed by the candidate. The contributions of co-authors are detailed as Appendix A. The first authored articles representing the main body of work supporting my candidacy are provided with a synopsis relevant to current literature. The thesis is then concluded with a "Discussion, limitations and future studies" chapter which explores the key findings in the context of the original hypothesis.

Hypothesis

The central hypothesis presented in my candidacy is:

Dietary-fat induced cerebral capillary dysfunction can be corrected by the timely provision of either: an anti-inflammatory diet enriched in ω-3 fatty acids; lipid-lowering agents that reduce vascular exposure to plasma Aβ; and/or lipid-lowering agents that pleiotropically attenuate inflammation.

Objectives

The objectives leading to and testing the indicated hypothesis were:

Objective 1 – in a proof-of concept context: To determine whether selected lipid-lowering agents (atorvastatin, pravastatin and probucol) could prevent cerebral capillary dysfunction. The functional integrity of the BBB was investigated in WT mice randomized to either a LF diet (control); a diet enriched in SFA (positive control); or a diets enriched in SFA plus one of three lipid-lowering agents (treatment groups: atorvastatin, pravastatin or probucol). Groups of mice were maintained for 12 weeks on the indicated diets and BBB integrity assessed principally by immunofluorescence microscopy.

Objective 2 – Based on the findings from objective 1: To determine if probucol modulates post-prandial lipoprotein associated secretion of Aβ. Wild-type mice were randomized to receive either a LF (control) diet; a diet enriched in SFA predicted to increase enterocytic lipoprotein-Aβ secretion (positive control); or a SFA diet containing probucol. The latter 'treatment' group was predicted to have substantially attenuated secretion of postprandial lipoprotein Aβ secretion, ordinarily stimulated by SFA. Groups of mice were maintained on the indicated diets for 4 weeks. Intestinal epithelial and lacteal abundance of Aβ relative to chylomicron abundance (indicated by apo B), was determined principally by immunofluorescence microscopy.

Objective 3 – Based on the findings of objective 1: To investigate in WT mice whether brain parenchymal extravasation of plasma proteins induced by chronic ingestion of a diet enriched in SFA, is reversible when mice were subsequently provided with either LF diet free from SFA (treatment 1), or a diet enriched in DHA (treatment group 2). Groups of mice were maintained for 12 weeks on the positive SFA diet. The treatment 'restoration' diets were then provided for 8 weeks. BBB integrity was assessed principally by immunofluorescence microscopy.

Objective 4 – Based on the findings of objectives 1-3: To investigate in WT mice, maintained on an SFA diet for 12 weeks to induce cerebral capillary disturbances, whether provision of either lipid-lowering statins with anti-inflammatory properties (atorvastatin and pravastatin), or provision of a NSAID (ibuprofen) restored BBB function. The treatment arms following 12 weeks of SFA feeding were for 2 weeks or 8 weeks on either a LF diet, or in combination with continued provision of the SFA diet.

Chapter outline

Chapter 1 – Literature review

The content in this chapter essentially describes background information that led to generation of the novel hypotheses provided. Several years prior to the commencement of my candidacy, there was a paucity of studies that had investigated the role of diet and specifically dietary fats on BBB function. The co-authored papers indicated below, discussed in detail in Chapter 1, provide the main foundation for the proposal that 'regression of BBB dysfunction' may notionally be achievable with the provision of a 'healthy' (anti-inflammatory) diet or with the provision of pharmacological agents which positively regulate plasma lipoprotein Aβ homeostasis and or cerebral capillary inflammation.

Takechi R, Galloway S, **Pallebage-Gamarallage MMS**, Wellington CL, Johnsen RD, Dhaliwal SS & Mamo JCL. (2010a) Differential effects of dietary fatty acids on the cerebral distribution of plasma-derived apo B lipoproteins with amyloid-β. *Br J Nutr.* 103**,** 652-662.

[Cited by 17]

Galloway S, Takechi R, **Pallebage-Gamarallage M**, Dhaliwal S & Mamo J. (2009) Amyloid-beta colocalizes with apolipoprotein B in absorptive cells of the small intestine. *Lipids Health Dis.* 8**,** 46.

[Cited by 5]

Takechi R, Galloway S, **Pallebage-Gamarallage MMS**, Lam V & Mamo JCL. (2010b) Dietary fats, cerebrovasculature integrity and Alzheimer's disease risk. *Prog Lipid Res.* 49**,** 159-170. (Review)

[Cited by 27]

Lam V, Takechi R, **Pallebage-Gamarallage MMS**, Galloway S & Mamo JCL. (2011) Colocalisation of plasma derived apo B lipoproteins with cerebral proteoglycans in a transgenic-amyloid model of Alzheimer's disease. *Neurosci Lett.* 492**,** 160-164.

[Cited by 3]

At the commencement of my PhD, there was substantial interest in the use of lipid-lowering agents for prevention of AD. In the context that AD may have a vascular origin first indicated by cerebral capillary aberrations and importantly as a preface to considering 'restoration' focussed experiments, I felt it was appropriate to take a 'proof of concept' approach. The literature review (Chapter 1) includes the first of my primary authored papers where I explored the effects of the lipid-lowering agents, atorvastatin and pravastatin and probucol, on 'prevention' of SFA induced BBB dysfunction in WT mice. The indicated manuscript addresses objective 1 of my candidacy.

Pallebage-Gamarallage MMS, Takechi R, Lam V, Galloway S, Dhaliwal S & Mamo JCL. (2010) Post-prandial lipid metabolism, lipid-modulating agents and cerebrovascular integrity: Implications for dementia risk. *Atheroclerosis Supp.* 11**,** 49-54.

[Cited by 6]

Chapter 2 – The effect of probucol on enterocytic amyloid-β abundance in mice maintained on diets enriched in saturated-fat and cholesterol

The contents of this chapter are covered by the article:

> **Pallebage-Gamarallage M**, Galloway S, Takechi R, Dhaliwal S & Mamo J. (2012a) Probucol suppresses enterocytic accumulation of amyloid-β induced by saturated fat and cholesterol feeding. *Lipids*. 47, 27-34.
>
> [Cited by 1]
>
> **The following objectives were explored in this article:**
>
> **Objective 2 -** To determine if probucol modulates post-prandial lipoprotein associated secretion of Aβ.

Chapter 3 – The effect of docosahexaenoic acid on saturated fatty acid induced brain parenchymal extravastation of apolipoprotein-B

The contents of this chapter is covered by the manuscript:

> **Pallebage-Gamarallage M**, Lam V, Takechi R, Galloway S & Mamo JCL. (2012b) A diet enriched in docosahexanoic acid exacerbates brain parenchymal extravasation of apo B lipoproteins induced by chronic ingestion of saturated fats. *Int J Vasc Med.* 2012, 647689.
>
> [Cited by 2]
>
> **The following objectives were explored in this article:**
>
> **Objective 3 -** To investigate in WT mice whether brain parenchymal extravasation of plasma proteins induced by chronic ingestion of a diet enriched in SFA, is reversible when mice are subsequently provided with either LF diet free from SFA (treatment 1), or a diet enriched in DHA (treatment group 2).

Chapter 4 – Restoration of dietary fat induced cerebrovascular dysfunction by anti-inflammatory lipid modulating agents

The contents of this chapter is covered by the manuscript:

> **Pallebage-Gamarallage M**, Lam V, Takechi R, Galloway S, Clark K & Mamo J. (2012c) Restoration of dietary-fat induced blood-brain barrier dysfunction by anti-inflammatory lipid-modulating agents. *Lipids Health Dis.* 11, 117.
>
> [Cited by 1]
>
> **The following objectives were explored in this article:**
>
> **Objective 4 -** To investigate in WT mice maintained on an SFA diet for 12 weeks to induce cerebral capillary disturbances, whether provision of either lipid-lowering statins with anti-inflammatory properties (atorvastatin and pravastatin), or provision of a NSAID (ibuprofen) restored BBB function.

Chapter 5 – Discussion, limitations and future studies

This chapter provides a general discussion of the findings presented in Chapters 2-4. The putative mechanisms lipid-lowering agents may provide cerebrovascular protection through their pleiotropic anti-inflammatory anti-oxidative properties and systemic TRL-Aβ metabolism are discussed. The outcomes presented in Chapter 3 demonstrated detrimental effects of PUFA enriched diets exaggerate BBB dysfunction and mechanisms of this action were attributed to their susceptibility for oxidation. In addition, limitations that were encountered during experimental procedures and may have influenced the outcomes are indicated. Taking into consideration the limitations, prospective studies to strengthen the findings presented in this thesis and opportunities for extending to clinical context for AD treatment are suggested.

Abbreviations

3-dimensional	3-D
3-hydroxy-3-methylglutaryl coenzyme A	HMG-CoA
Alzheimer's disease	AD
Amyloid-β	Aβ
Amyloid precursor protein	APP
Apolipoprotein	Apo
Blood-brain barrier	BBB
Bovine aortic endothelial cells	BAEC
Central nervous system	CNS
Cerebrospinal fluid	CSF
Cortex	CTX
Cyclooxygenase	COX
Deoxyribonucleic acid	DNA
Docosahexaenoic acid	DHA
Dynamic contrast-enhanced magnetic resonance imaging	DCE-MRI
Eicosapentaenoic acid	EPA
Endoplasmic reticulum	ER
Heparan sulphate proteoglycan	HSPG
High-fat	HF
Hippocampal formation	HPF
Human vascular endothelial cells	HUVEC
Immunoglobulin-G	IgG
Interferon-γ	IFN-γ
Interleukin	IL
Low-density-lipoprotein receptor-related protein	LRP
Low-fat	LF
Mammalian target of rapamycin	mTOR
Messenger ribonucleic acid	mRNA
Monounsaturated fatty acid	MUFA

Nicotinamide adenine dinucleotide phosphate	NADPH
Nitric oxide	NO
Nitric oxide synthase	NOS
Non-steriodal anti-inflammatory drug	NSAID
Polyunsaturated fatty acid	PUFA
Porcine pulmonary artery endothelial cells	PPAEC
Presenilin-1	PS1
Reactive oxygen species	ROS
Receptor for advanced glycation end product	RAGE
Saturated fatty acid	SFA
Transforming growth factor-β	TGF-β
Triglyceride-rich lipoprotein	TRL
Tumour necrosis factor-α	TNF-α
Vascular dementia	VaD
Very-low density lipoprotein	VLDL
Wild-type	WT

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CHAPTER 1

Chapter 1: Literature review

This chapter is covered by the following co-author and first author articles:

Takechi R, Galloway S, **Pallebage-Gamarallage MMS**, Wellington CL, Johnsen RD, Dhaliwal SS & Mamo JCL. (2010a) Differential effects of dietary fatty acids on the cerebral distribution of plasma-derived apo B lipoproteins with amyloid-β. *Br J Nutr.* 103**,** 652-662.

Galloway S, Takechi R, **Pallebage-Gamarallage M**, Dhaliwal S & Mamo J. (2009) Amyloid-beta colocalizes with apolipoprotein B in absorptive cells of the small intestine. *Lipids Health Dis.* 8**,** 46.

Takechi R, Galloway S, **Pallebage-Gamarallage MMS**, Lam V & Mamo JCL. (2010b) Dietary fats, cerebrovasculature integrity and Alzheimer's disease risk. *Prog Lipid Res.* 49**,** 159-170. (Review)

Lam V, Takechi R, **Pallebage-Gamarallage MMS**, Galloway S & Mamo JCL. (2011) Colocalisation of plasma derived apo B lipoproteins with cerebral proteoglycans in a transgenic-amyloid model of Alzheimer's disease. *Neurosci Lett.* 492**,** 160-164.

Pallebage-Gamarallage MMS, Takechi R, Lam V, Galloway S, Dhaliwal S & Mamo JCL. (2010) Post-prandial lipid metabolism, lipid-modulating agents and cerebrovascular integrity: Implications for dementia risk. *Atherosclerosis Supp.* 11**,** 49-54.

Article 1:

Takechi R, Galloway S, **Pallebage-Gamarallage MMS**, Wellington CL, Johnsen RD, Dhaliwal SS & Mamo JCL. (2010a) Differential effects of dietary fatty acids on the cerebral distribution of plasma-derived apo B lipoproteins with amyloid-β. *Br J Nutr.* 103**,** 652-662.

Synopsis

Background:

Accumulating evidence supports the hypothesis that vascular risk factors may contribute to AD onset and progression. Epidemiological studies have identified significant association with increased AD prevalence and several cardiovascular risk factors such as diabetes, obesity, hypertension and hypercholesterolemia (Kivipelto et al. 2001, Skoog et al. 1999). In addition, lifestyle factors such as poor nutrition, physical inactivity, smoking and alcohol consumption are considered positive risk factors for AD (Flicker 2010). Dietary lipids have also been implicated as a risk factor for AD onset and progression (Grant 1997, Kalmijn et al. 1997, Petot and Friedland 2004, Refolo et al. 2000, Sparks et al. 1994). The earliest cross-sectional study by Grant (1997) demonstrated that fat and total caloric supply had the highest correlations with AD prevalence in Western countries compared to Africa and Asia. Several population studies in humans have found that HF diets are a positive risk factor (Kalmijn et al. 1997, Petot and Friedland 2004), and HF feeding markedly exacerbates Alzheimer's-like cerebral pathology in animal models of AD (Refolo et al. 2000, Sparks et al. 1994). Conversely, diets rich in ω-3 PUFA, ω-6 PUFA and MUFA consumption was found to be protective against neurodegenerative diseases (Grant 1997, Kalmijn et al. 1997, Barberger-Gateau et al. 2002, Morris et al. 2003). The mechanisms for the HF diet/AD link are presently unclear.

Pathological features of AD are characterised by extracellular Aβ deposition, neurofibrillary tangle formation and neurodegeneration (Pastorino & Lu 2006). Cerebrovascular dysfunction is commonly reported in subjects with AD and with vascular dementia (VaD) concomitant with inflammatory sequelae. Pathological alterations of the BBB include loss of vascular endothelial tight junction proteins,

endothelial cell damage and smooth muscle cell proliferation. Furthermore, plasma proteins have been detected in AD brains (Kalaria 1992, Wisniewski et al. 1997). The latter observations are consistent with BBB breakdown. It has also been suggested that BBB dysfunction precedes amyloidosis (Attems et al. 2004, Kalaria 1992).

Previous research demonstrated that neuronal overproduction of Aβ resulted in accumulation in the extracellular space of the brain, increased propensity for Aβ fibrillar formation and eventually, aggregation to form stable 'senile'-plaques. It has also been proposed that decreased clearance of Aβ from the cerebrospinal fluid (CSF) may contribute to Aβ accumulation (Crossgrove et al. 2005, Deane et al. 2009, Deane et al. 2005). Evidence that dietary lipids influence amyloidosis comes from studies in amyloid transgenic mice maintained on a pro-atherogenic HF, high-cholesterol diet and in New-Zealand rabbits provided with supplementary dietary cholesterol (Refolo et al. 2000, Sparks et al. 1994, Shie et al. 2002). Refolo et al. (2000) suggested that increased amyloidosis may have been a consequence of increased Aβ synthesis in response to chronically elevated plasma cholesterol.

There is no evidence supporting the notion of exaggerated cerebral Aβ synthesis in sporadic late onset AD which accounts for 95% of cases (Cummings et al. 1998). Some studies suggest there is reduced clearance of efflux as a consequence of diminished CSF turnover (Crossgrove et al. 2005, Deane et al. 2009, Deane et al. 2005, Grimmer et al. 2009). However, the epithelial cells of the choroid plexus are remarkably equipped with proteases that avidly degrade CSF Aβ and efflux to plasma is considered to be relatively insignificant.

Blood-to-brain delivery of Aβ has been demonstrated via receptor and non-receptor processes. The receptor for advanced glycation end product (RAGE) can translocate Aβ from chaperone plasma Aβ carriers to brain parenchyme (Donahue et al. 2006), but rates of transport are thought to be insignificant compared to neural production rates for Aβ. Conversely, particularly at the basal junction of adjacent endothelial cells of brain capillary vessels, the low-density-lipoprotein receptor-related protein (LRP) facilitates transport of Aβ from brain-to-blood. Donahue et al. (2006) reported that LRP expression may be compromised in subjects with AD.

Studies by Mackic et al. (2002) first suggested that brain parenchymal extravasation of plasma Aβ occurs if BBB is significantly compromised. Equivocal evidence of this phenomenon was provided by Takechi et al (2010a), moreover in a dietary-fat induced model of BBB dysfunction (Mackic et al. 2002, Takechi et al. 2010a).

Physiological levels of Aβ in circulation can be sourced from vascular smooth muscle, endothelial cells and from platelets. Significant Aβ also appears to be derived from liver and intestine, secreted into circulation associated with TRL (very-low density lipoproteins (VLDL) and chylomicrons, respectively) (Biere et al. 1996, Galloway et al. 2007, James et al. 2003). Studies by Galloway et al. (2007) showed that TRL-associated Aβ was regulated by dietary fats. Wild-type mice maintained on a diet enriched with SFA and cholesterol had substantially greater enterocytic Aβ. However, in a subsequent study we showed that this effect was specific for SFA (Pallebage-Gamarallage et al. 2009). Several studies are consistent with the hypothesis that increased vascular exposure to plasma TRL-Aβ may contribute to increased amyloidosis and AD risk. Mamo et al. (2008) reported that TRL-Aβ was increased in subjects with AD concomitant with evidence of postprandial dyslipidemia. Furthermore, Burgess et al. (2006) found that the onset and progression of AD-like pathology in three strains of amyloid transgenic mice was strongly associated with TRL-Aβ secretion into plasma. Furthermore, changes in plasma TRL-Aβ levels preceded cerebral amyloidosis (Burgess et al. 2006).

The mechanisms involved in a putative TRL-Aβ induced risk for AD risk are unclear. A number of studies provide evidence that cytotoxic properties of circulating Aβ may result in significant cerebrovascular dysfunction. Amyloid-β is vasoconstrictive and vessels treated with Aβ show significant endothelial cell damage with changes in the cell membrane, cytoplasm, nucleus and other organelles (Thomas et al. 1997). Moreover, any such Aβ induced cerebrovascular abnormalities could potentially result in cognitive impairment, for example by contributing toward decreased cerebral perfusion. Previous studies where Aβ was intravascularly administered involved acute single injections and investigated transportation across, or sequestration within, brain capillaries (Giri et al. 2002,

Thomas et al. 1997). Long-term administration of Aβ significantly disrupted the BBB and activated central nervous system (CNS) glial cells (George et al. 2004).

The BBB is a tightly regulated interface between the CNS and the peripheral circulatory system. The cerebrovasculature consists of endothelial cells that are tightly bound by tight junction proteins, providing a barrier effect for unsolicited transport of proteins or macromolecules that may be inflammatory or neurotoxic (Hawkins and Davis 2005). Notionally, in the absence of specific transporter mechanisms, cerebral extravasation of plasma TRL-Aβ would only occur if BBB integrity becomes compromised.

The manuscript titled "Differential effects of dietary fatty acids on the cerebral distribution of plasma-derived apo B lipoproteins with amyloid-β" was key to the evolution of the hypothesis presented in my candidacy. The article describes a study which explored whether chronic ingestion of diet enriched in SFA compromise BBB integrity and leads to cerebral extravasation of circulating TRL-Aβ, *in vivo*. The intellectual contribution I made to this complimentary manuscript, which supports my specific PhD candidacy objectives were: assisting in animal care, tissue collection and appraisal of the findings and the manuscript.

Methods in brief:

The effects of chronic dietary fatty acid consumption on cerebrovascular integrity were determined in WT mice fed SFA, PUFA, MUFA or a LF control diet. Cerebrovascular dysfunction was determined utilising 3-D immunofluorescence microscopy. Cerebrovascular permeability was determined with immunofluorescence quantitation of parenchymal extravasation of plasma derived IgG and apo B. Apolipoprotein B is an exclusive marker for hepatic and intestinally derived TRLs. The plasma concentration of S100B was used as a secondary surrogate marker for BBB dysfunction. S100B is found enriched in CSF and elevated plasma S100B suggests exaggerated brain-to-blood delivery of CSF proteins. Furthermore, BBB structural changes were detected by double immunofluorescence staining of tight junction protein occludin-1. The SFA induced aberrations in BBB and purported TRL-Aβ disturbances in kinetics were also considered in APP/presenilin-

1(APP/PS1) amyloid transgenic mice, an established murine model with AD-like cerebral amyloidosis.

Results in brief:

Significant BBB dysfunction and abnormalities in protein kinetics were identified in mice maintained on an SFA enriched diet. Cerebral extravasation of plasma proteins IgG and apo B was concomitant with a significant reduction in endothelial tight junction protein occludin-1. Furthermore, significant increase in brain derived S100B in plasma was consistent with brain-to-blood efflux. In contrast, there was no evidence of BBB disruption in mice fed either MUFA or PUFA diet.

To consider the SFA induced effects on BBB integrity and TRL-Aβ in the context of amyloidosis and AD risk, cerebral TRL-Aβ distribution was assessed in an amyloid transgenic AD mouse model (APP/PS1). In the transgenic mouse model of AD, the pattern of cerebral extravasation of plasma proteins was indistinguishable to that found in WT mice on the SFA diet. In addition, double immunofluorescence labelling demonstrated significant colocalisation of Aβ with apo B within the amyloid plaques consistent with the notion of TRL-Aβ delivery from blood-to-brain. The APP/PS1 mice have approximately a 5-fold higher concentration of Aβ, above endogenous levels in control mice. However, the murine isoforms of Aβ are not prone to fibrillar formation because of differences in the amino acid sequence compared to human isoforms (Duff & Suleman 2004). Amyloid transgenic mice overexpress human isoforms of Aβ (Duff & Suleman 2004).

Discussion and conclusion in brief:

Chronic ingestion of SFA may compromise BBB integrity and function, resulting in non-specific brain parenchymal extravasation of plasma proteins including Aβ enriched apo B lipoproteins. Diets enriched in PUFA and MUFA appeared to have no detrimental effect on BBB function in this murine model.

Analogous to the inflammatory sequelae that occur in response to the subendothelial retention of cholesterol rich lipoproteins in coronary arteries, extracellular entrapment of apo B lipoprotein-Aβ may trigger inflammatory events. Activated glial cells and a respiratory burst would promote oxidative stress pathways

leading to possible production of toxic oxidised proteins and lipid peroxidation (Studzinski et al. 2009, Proudfoot et al. 2009, Ronti et al. 2006). Other lines of evidence suggest that SFAs are more likely to trigger mitochondrial and endoplasmic reticulum (ER) 'stress' pathways (Diakogiannaki et al. 2008, Wang et al. 2006). Cell culture studies suggest that incubation with longer chain unsaturates has an antagonistic effect on inflammatory stress pathways induced by SFAs (Diakogiannaki et al. 2008).

The principal finding of an SFA induced disturbance in BBB function are consistent with epidemiological studies that have investigated an association of dietary fats and AD prevalence. Limited evidence of a purported protective effect of ω-3 fatty acids in population (Laitinen et al. 2006, Roberts et al. 2010, van Gelder et al. 2007) and clinical studies (Morris et al. 2003, Quinn et al. 2010), coupled with the substantive evidence that ω-3 fatty acids generally suppress inflammation, was the basis for putting forward objective 3 (Chapter 3) for my candidacy: "to investigate in WT mice whether brain parenchymal extravasation of plasma proteins induced by chronic ingestion of a diet enriched in SFA, is reversible when mice are subsequently provided with either LF diet free from SFA, or a diet enriched in DHA".

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Differential effects of dietary fatty acids on the cerebral distribution of plasma-derived apo B lipoproteins with amyloid-β

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Some dietary fats are a risk factor for Alzheimer's disease (AD) but the mechanisms for this association are presently unknown. In the present study we showed in wild-type mice that chronic ingestion of SFA results in blood–brain barrier (BBB) dysfunction and significant delivery into the brain of plasma proteins, including apo B lipoproteins that are endogenously enriched in amyloid-β (Aβ). Conversely, the plasma concentration of S100B was used as a marker of brain-to-blood leakage and was found to be increased two-fold because of SFA feeding. Consistent with a deterioration in BBB integrity in SFA-fed mice was a diminished cerebrovascular expression of occludin, an endothelial tight junction protein. In contrast to SFA-fed mice, chronic ingestion of MUFA or PUFA had no detrimental effect on BBB integrity. Utilising highly sensitive three-dimensional immunomicroscopy, we also showed that the cerebral distribution and co-localisation of Aβ with apo B lipoproteins in SFA-fed mice are similar to those found in amyloid precursor protein/presenilin-1 (APP/PS1) amyloid transgenic mice, an established murine model of AD. Moreover, there was a strong positive association of plasma-derived apo B lipoproteins with cerebral Aβ deposits. Collectively, the findings of the present study provide a plausible explanation of how dietary fats may influence AD risk. Ingestion of SFA could enhance peripheral delivery to the brain of circulating lipoprotein–Aβ and exacerbate the amyloidogenic cascade.

Alzheimer's disease: Amyloid-β: Blood–brain barrier: SFA: TAG-rich lipoproteins

An accumulating body of evidence is consistent with the concept that the onset and progression of Alzheimer's disease (AD) is influenced by lifestyle factors including nutrition[1]. Several population studies have found that SFA are a positive risk factor for AD[2–3] and in animal models of AD, SFA or cholesterol feeding markedly exacerbates cerebral pathology[4–5]. However, the mechanisms that link dietary fat to the pathogenesis of AD are unclear.

The cerebrovasculature in subjects with AD shows pathological alterations including vascular endothelial and smooth muscle cell proliferation[6]. Blood plasma proteins have been detected in the parenchyma of AD brains[7–8] and inflammatory sequalae are commonly reported[9–10], observations that are consistent with breakdown of the blood–brain barrier (BBB). Yet despite evidence supportive of AD having an underlying vascular component, most research focuses on damage of neurons[11].

A major neuropathological marker of AD is amyloid-β (Aβ) deposition in the cerebrovasculature and in the cerebral parenchyma. Derived from amyloid precursor protein (APP), Aβ is the predominant component of amyloid plaque[12]. The source of cerebral Aβ deposits in AD is uncertain, though there is little evidence for increased cerebral Aβ production in sporadic, late-onset AD which accounts for over 96 % of AD cases. Rather, decreased Aβ clearance across the BBB via receptor pathways and/or via the choroid plexus has been suggested as an initiating pathway for amyloidosis[13–15]. More recent has been evidence of blood-to-brain delivery of circulating Aβ, a process that would conceivably exacerbate parenchymal load in the absence of compensatory clearance pathways[16].

Plasma Aβ can be derived from vascular smooth muscle cells and endothelial cells, or from blood platelets. However, another significant source of plasma Aβ may be from lipogenic organs such as the small intestine and liver[17–19]. Hepatocytes and absorptive epithelial cells of the small intestine (enterocytes) secrete Aβ as a lipoprotein complex, and in the small intestine this pathway is under dietary regulation[17–20].

Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; APP, amyloid precursor protein; BBB, blood–brain barrier; BS, brain stem; CTX, cortex, excluding the hippocampus; 3-D, three-dimensional; HPF, hippocampal formation; PS1, presenilin-1; TRL, TAG-rich lipoprotein; vWF, von-Willebrand factor.

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Chronic ingestion of SFA was reported to significantly enhance enterocytic abundance of Aβ and conversely the protein could not be detected when animals were fasted[17].

Distributional analysis of plasma lipoprotein–Aβ in normal subjects and those with AD or mild cognitive impairment (MCI) is consistent with the notion that dietary-induced Aβ may contribute to the aetiology of AD[21]. In control and in AD/MCI subjects, greater than 60 % of plasma lipoprotein–Aβ was found to be associated with TAG-rich lipoproteins (TRL); however, this was significantly increased in AD/MCI subjects *v.* controls. Moreover, whilst the AD subjects were normolipidaemic and had similar plasma levels of hepatically derived lipoproteins, the concentration of apo B48 (an exclusive marker of chylomicrons) was elevated more than three-fold in post-absorptive AD subjects. Increased apo B48 is indicative of postprandial dyslipidaemia, an exaggerated rise in plasma chylomicrons that occurs following the ingestion of dietary fats[22]. Consistent with the notion of an Aβ postprandial response, ingestion of a lipid-enriched meal was found to cause a transient increase in the plasma concentration of APP and of Aβ in otherwise healthy subjects[23].

Evidence of a causal link between plasma lipoprotein–Aβ and AD also comes from studies in animal models of AD. In transgenic mice that over-express APP, plasma Aβ concentration correlated with secretion rates into the blood of TRL, which was increased 3- to 8-fold above wild-type controls[24]. Furthermore, there was a positive association between plasma TRL–Aβ secretion with onset of cerebrovascular and parenchymal amyloidosis[25].

The mechanisms by which circulating Aβ increases AD risk are presently unclear. However, several studies have provided evidence of a vasoactive role of Aβ, with pathological manifestations before Aβ deposition[26–28]. Aβ is vasoconstrictive and vessels treated with Aβ show significant endothelial cell damage, with changes in the cell membrane, cytoplasm, nucleus and other organelles.

We hypothesise that SFA may adversely influence BBB function, because of exaggerated exposure to plasma lipoprotein–Aβ that leads to greater rates of plasma to cerebral Aβ delivery. In previous studies, integrity of the BBB has been assessed in experimental colitis[29] and following acute intravenous injection of solubilised, exogenous Aβ[26–28]. Consistent with the notion that lipoproteins have an important role in central nervous system diseases, Kay *et al.* reported significant remodelling of cerebrospinal fluid lipoproteins after subarachnoid haemorrhage[30]. However, the potential effect of SFA on plasma Aβ homeostasis and BBB function have not been reported. If this hypothesis is correct, it may explain epidemiological data that link SFA intake with AD risk. In the present study we directly explore BBB integrity in wild-type mice fed fatty acid-enriched diets.

Materials and methods

Animals

Female C57BL/6J mice, aged 6 weeks, were purchased from the Animal Resource Centre (Perth, WA, Australia). Mice were randomly allocated to either the control or one of three fatty acid treatment groups (see Dietary intervention in wild-type mice section). Mice were maintained in an accredited animal holding facility with regulated temperature, air pressure and lighting (12 h light–12 h dark). Mice had *ad libitum* access to feed and water. At 3 and 6 months following commencement of the dietary intervention, six mice from each group were killed by cardiac exsanguination under complete anaesthesia. The C57BL6J mice were considered an appropriate wild-type strain because the transgenic APP/presenilin-1 (APP/PS1) mice are a C57BL6J × C3H strain.

Double transgenic APP/PS1 mice develop AD-like brain pathology after 20 weeks of age. In the present study APP/PS1 mice were fed a standard low-fat rodent chow and killed at 12 months of age.

All experimental procedures used in this project were approved by a National Health and Medical Research Council (Australia) accredited Animal Ethics Committee (Curtin University approval no. R34/08).

Dietary intervention in wild-type mice

The feed preparations were made by Glenn Forest Stock Feeders (Perth, WA, Australia). The low-fat control diet was a standard American Institute of Nutrition AIN-93M rodent chow containing < 4 % (w/w) fat as polyunsaturates, with < 1 % total digestible energy as lipids and was free of cholesterol (for details, see Table 1). All of the fat-enriched diets comprised 40 % total digestible energy as lipids (or 20·3 %, w/w). For the SFA intervention group, the principal fatty acid types were palmitic (16 : 0) and stearic (18 : 0) (13 %, w/w) but the SFA diet also contained some oleic acid (18 : 1*n*-9, 6 %, w/w). The MUFA diet contained approximately 16 % as oleic acids, approximately 1·5 % as SFA and only trace amounts of PUFA. The PUFA-enriched diet was principally made up of DHA (22 : 6*n*-3), EPA (20 : 5*n*-3) and oleic acid. SFA and MUFA accounted for less than 4 % (w/w) of the PUFA-enriched diet. The MUFA and PUFA diets reflected blends of Sunola™ oil and fish oils, respectively.

Tissue and plasma sample collection

Following dietary intervention for 3 or 6 months, mice were anaesthetised with pentobarbitone and blood samples obtained by cardiac puncture. Plasma was separated by low-speed centrifugation and stored immediately at −80°C. Brains were carefully removed and washed in chilled PBS. For immunofluorescent microscopy, the right hemisphere was segmented and fixed in 4 % paraformaldehyde for 24 h followed by cryoprotection in 20 % sucrose solution for 3 d at 4°C. Tissues were then frozen in isopentane/dry ice and stored at −80°C.

Plasma S100B and amyloid-β analysis

Plasma S100B was measured by ELISA (CosmoBio, Tokyo, Japan) according to the instructions provided by the manufacturer. Briefly, 20 μl of plasma samples, or of the S100B standards (0, 98, 197, 394, 1575, 3150 and 6300 pg/ml) were incubated overnight at 4°C in ninety-six-well microplates coated with the primary antibody. Thereafter, plates were incubated with the conjugated secondary

Table 1. Dietary composition data sheet*
(Percentages)

Diet…	Control	SFA	MUFA	PUFA
Total fat	4	20·3	20·3	20·3
Total digestible energy from lipids	n/a	40	40	40
SFA, 12 : 0 and less	n/a	n/a	Not detected	n/a
Myristic acid (14 : 0)	Trace	0·05	0·02	0·54
Pentadecanoic acid (15 : 0)	n/a	0·01	n/a	0·16
Palmitic acid (16 : 0)	0·20	5·16	0·85	3·26
Magaric acid (17 : 0)	n/a	0·05	n/a	0·18
Stearic acid (18 : 0)	0·10	7·31	0·87	0·92
Arachidic acid (20 : 0)	n/a	0·24	n/a	0·06
Behenic acid (22 : 0)	n/a	0·04	n/a	n/a
Tetracosanoic acid (24 : 0)	n/a	0·03	n/a	n/a
Palmitoleic acid (16 : 1)	Trace	0·05	0·02	0·66
Heptadecenoic acid (17 : 1)	n/a	0·01	n/a	0·10
Oleic acid (18 : 1*n*-9)	2·40	6·62	15·70	2·25
Gadoleic acid (20 : 1)	Trace	0·01	0·07	0·18
Linoleic acid (18 : 2*n*-6)	0·80	0·67	2·42	0·23
α-Linolenic acid (18 : 3*n*-3)	n/a	0·05	0·13	0·09
γ-Linolenic acid (18 : 3*n*-6)	0·40	Not detected	n/a	0·08
Stearidonic acid (18 : 4*n*-3)	n/a	n/a	0·08	n/a
Arachidonic acid (20 : 4*n*-6)	Trace	Not detected	0·20	0·46
EPA (20 : 5*n*-3)	Trace	Not detected	Not detected	2·00
DPA (22 : 5*n*-3)	n/a	Not detected	Not detected	0·30
DHA (22 : 6*n*-3)	Trace	Not detected	Not detected	8·22

n/a, Not applicable.
* Detailed dietary compositions of the diet of low-fat control, saturated fat (SFA), monounsaturated fat (MUFA) and polyunsaturated fat (PUFA) groups are given.

antibody for 2 h, followed by 2 h incubation with streptavidin–horseradish peroxidase. Finally samples were incubated with substrate solution for 20 min and the reaction was terminated with stopping solution. The optical absorbance was measured at 490 nm.

Plasma concentrations of mouse $A\beta_{1-40}$ and $A\beta_{1-42}$ were measured utilising Biosource ELISA kits (KMB3441; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Plasma (100 μl) or Aβ standards ($A\beta_{1-40}$: 0, 7·8, 15·6, 31·3, 62·5, 125, 250, 500 pg/ml; $A\beta_{1-42}$: 0, 3·12, 6·25, 12·5, 25, 50, 100, 200 pg/ml) were dispensed into wells and incubated for 2 h at room temperature, then thoroughly washed. The primary antibody for either $A\beta_{1-40}$ or $A\beta_{1-42}$ was then added and incubated for 1 h at room temperature. Anti-rabbit IgG–horseradish peroxidase was added for 30 min, then incubated with stabilised chromogen for an additional 30 min in darkness. The optical absorbance was measured at 450 nm.

Immunofluorescent detection of IgG, apo B, occludin and von Willebrand factor in cerebral tissue

Cryosection specimens of 18 μm were prepared from the right hemisphere of the brain of each mouse. For IgG detection, sections were incubated with polyclonal goat anti-mouse IgG antibody conjugated with Alexa 488 fluorochrome (1:100; Abcam, Inc., Cambridge, MA, USA) for 2 h at room temperature. Subsequently, the sections were imaged utilising an inverted fluorescent microscope (Zeiss AxioVert 200M; Carl Zeiss, Jena, Germany) and AxioVision software (version 4.6; Carl Zeiss).

Apo B lipoproteins were detected by overnight incubation with polyclonal rabbit anti-apo B antibody (1:200; Abcam, Inc.). The primary antibody was then visualised with goat-anti-rabbit IgG conjugated with Alexa 488 (Invitrogen).

Negative controls were included for all immunofluorescent experiments and included replacement of the primary antibody with buffer, or an irrelevant serum. Fluorescent staining was not observed for any negative control tissue preparations.

Fluorescent image capture and semi-quantitative measurement

All fluorescent images were captured with a fluorescent microscope (AxioVert 200M) coupled to an MRm digital camera and managed by AxioVision software (version 4.6; Carl Zeiss). Three-dimensional (3-D) images were taken with ApoTome optical sectioning methodology (Carl Zeiss). Quantification was determined within the cortex excluding the hippocampus (CTX), hippocampal formation (HPF) and brain stem (BS).

For each mouse killed, a minimum of three cryosection specimens was prepared from the right hemisphere of the brain. For each specimen, up to seven 3-D ApoTome images were randomly taken within each designated region of the brain. For IgG and apo B quantitative measurement, images were captured at × 200 magnification (430 × 322 μm). Each 3-D image consisted of six to thirteen Z-stack images and the distance between Z-stack slices was 1·225 μm optimised by Nyquist theory (2 × oversampling in axial direction). The optical densitometric sum for the protein of interest was determined in three dimensions (1388 × 1040 pixel two-dimensional planes) utilising the automated optical density measurement tool (AxioVision; Carl Zeiss).

Double immunofluorescent detection with two polyclonal antibodies

In order to detect two different proteins with two polyclonal antibodies raised in the same species, a double immunofluorolabelling method was used as we have previously described[31]. For the co-localisation of Aβ with perivascular leakage of plasma apo B, rabbit polyclonal anti-$A\beta_{1-40/42}$ (Chemicon; Millipore, Billerica, MA, USA) and rabbit polyclonal anti-apo B antibodies were used. For the BBB tight junction protein occludin and the vascular endothelial cell marker von Willebrand factor (vWF), these were detected with rabbit polyclonal anti-occludin-1 (Invitrogen) and rabbit polyclonal anti-vWF antibodies (Abcam, Inc.). Briefly, 10 μm sections were fixed in acetone for 3 min at −20°C and heat-mediated antigen retrieval was achieved by incubating in a water-bath at 60°C for 3 h. Endogenous biotin was blocked with avidin in egg white and biotin in skimmed milk. In order to avoid the cross-reaction of the two polyclonal antibodies, the concentration of the first antibody was diluted so that it was undetectable with conventional secondary antibody detection, but detectable after the signal amplification with the biotin–avidin reaction. Subsequently the second protein of interest was detected with standard secondary detection.

For the co-localisation analysis of Aβ and apo B in cerebral tissue of wild-type mice given different fatty acid-enriched diets, fifteen 3-D images were captured at × 400 magnification (222 × 166 μm). Each of the 3-D images contained thirty-two to seventy-one Z-stack images and the distance between Z-stack slices was 0·275 μm. From a total of 695 two-dimensional images, co-localisation of Aβ and apo B was then determined with AxioVision software and data were expressed as Manders' and Pearson's correlation coefficients. Manders' correlation coefficient estimates the co-localisation independent of fluorescent intensity, whereas Pearson's correlation coefficient explores if there is also an association in fluorescent intensities.

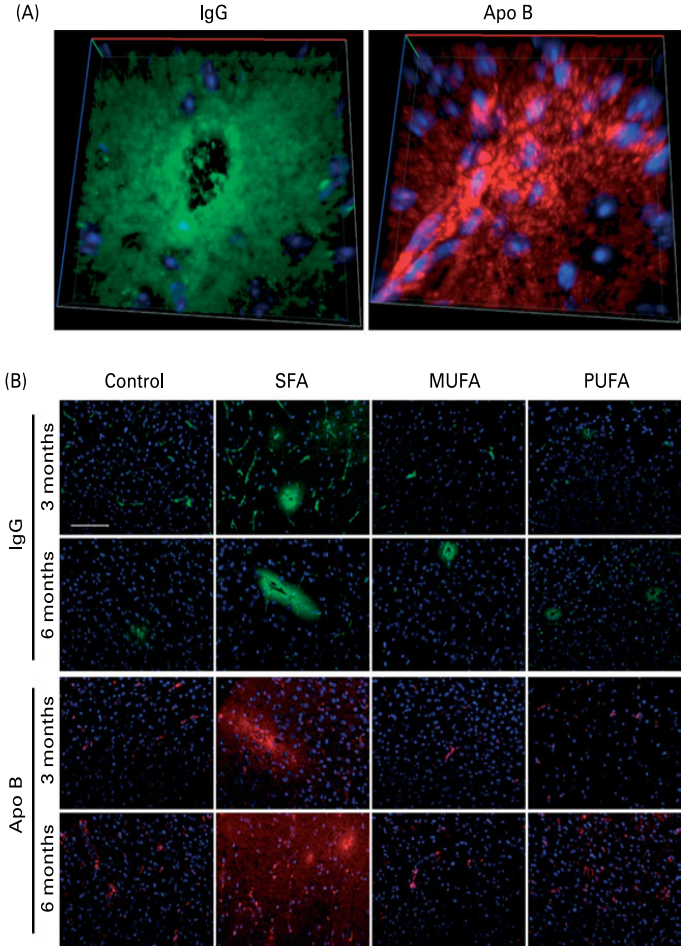

Fig. 1. Three-dimensional (3-D) immunodetection of cerebral IgG and apo B extravasation. Perivascular leakage of plasma IgG and apo B into the brain was captured in 3-D utilising highly sensitive immunofluorescent microscopy techniques. IgG is shown in green and apo B is in red. 6-Diamidino-2-phenylindole (DAPI)-counterstained nuclei are shown in blue. (A) Representative 3-D images of plasma IgG and apo B leakage observed in mice fed a SFA-rich diet for 6 months. Scales of *x* (red), *y* (red) and *z* (green) axes are 90 × 80 × 10 μm. (B) Low magnification images of cerebral IgG and apo B immunoreactivity. Significant cerebral extravasation of IgG and apo B was seen in both 3 and 6 month SFA groups. The scale bar indicates 100 μm.

For vWF and occludin measurement, 3-D ApoTome images were taken at × 200 magnification. Each 3-D image consisted of a minimum of three and up to seven Z-stack images and distance between Z-stack slices was 1·225 μm. To measure the relative abundance of vWF, the optical densitometric sum within the image was determined and expressed as per vWF per volume of tissue. In order to measure occludin abundance specific for the cerebrovasculature, only occludin staining which co-localised with vWF was measured.

Co-localisation of apo B with cerebral amyloid plaques

To examine the co-localisation of apo B with amyloid plaques in 12-month-old APP/PS1 transgenic mice, the immunofluorescent double labelling method was used as described for the wild-type mice given fat-enriched diets. In the APP/PS1 mice, 50 μm thick cryosections were fixed with 4 % paraformaldehyde for 1 h and heat-mediated antigen retrieval was done by incubating the sections in 60°C water for 3 h. A mixture of rabbit-derived anti-apo B and mouse monoclonal anti-Aβ antibodies was applied and incubated for 3 d at 4°C. Following washing with PBS, the primary antibodies were detected with anti-rabbit IgG Alexa 488 and anti-mouse IgG1 Alexa 680, respectively. Immunofluorescent 3-D images were captured at × 400 magnification with ApoTome.

Statistical analysis

There were twelve mice in each dietary group studied. For the immunodetection of IgG, apo B, occludin and vWF, up to seven 3-D images were obtained for each of the three regions of the brain studied (CTX, HPF and BS). Each 3-D image was generated from a stack of two-dimensional images, consisting of between twenty-two and 181 sequential images. The 3-D stacks represented 4·9–18·5 μm tissue thickness. For co-localisation analysis of apo B with Aβ in SFA-fed mice and in APP/PS1 mice, three to six 3-D images were generated per animal, with ten mice in the SFA group and six mice in the APP/PS1 group. A total of 694 images were analysed in SFA-fed mice and 834 images in transgenic APP/PS1 mice. Data were normally distributed and compared by one-way ANOVA followed by Tukey's *post hoc* test or Student's *t* test using SPSS (SPSS, Inc., Chicago, IL, USA). Data were expressed as mean values with their standard errors.

Results

The fatty acid-supplemented diets given to mice were well tolerated. There was no difference in plasma lipids or body weight following 3 or 6 months of dietary intervention (data not shown).

IgG (150 kDa) immunoreactivity was used as a generic marker of blood-to-brain influx of plasma proteins. In mice fed the SFA diet for 3 months, we identified significant perivascular leakage of IgG into the CTX, BS and to a lesser extent, within the HPF (Figs. 1 and 2). At 6 months of feeding, IgG leakage was more evident than at 3 months (Figs. 1 and 2). In contrast, in the mice given the low-fat control diet, or either of the unsaturated fatty acid diets, there was no evidence of cerebral IgG immunoreactivity (Fig. 1).

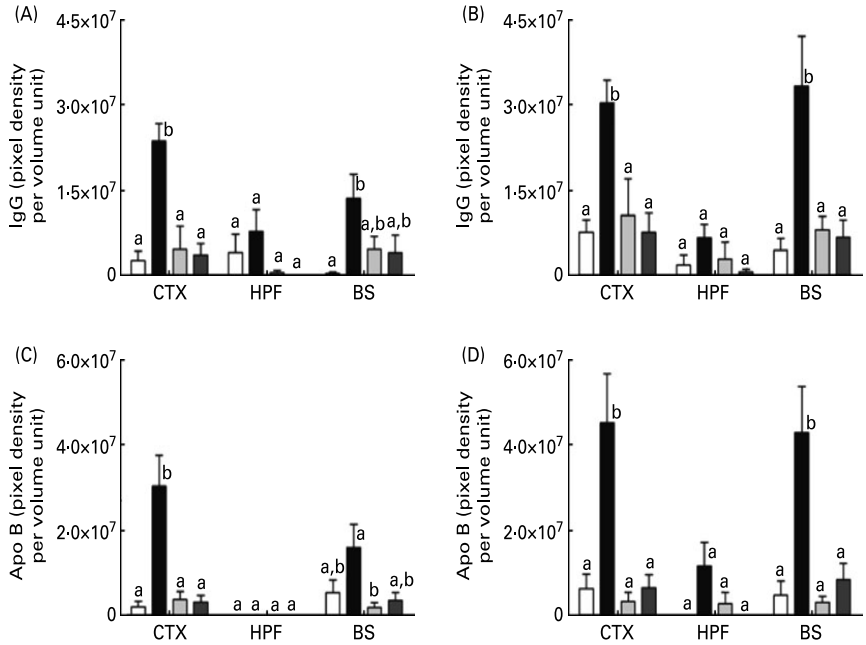

Fig. 2. Three-dimensional (3-D) semi-quantitative analysis of cerebral IgG (A and B) and apo B (C and D) extravasation in mice fed a control diet (□), a SFA-rich diet (■), a MUFA-rich diet (▨) or a PUFA-rich diet (▩) for 3 months (A and C) or 6 months (B and D). The extent of cerebral IgG and apo B abundance was determined in 3-D based on the optical pixel density. Optical pixel densities were measured in three major brain regions of the cortex (CTX), hippocampal formation (HPF) and brain stem (BS) and expressed as per volume unit. Values are means, with standard errors represented by vertical bars. [a,b] Mean values, within a region, with unlike letters were significantly different ($P < 0·05$; one-way ANOVA).

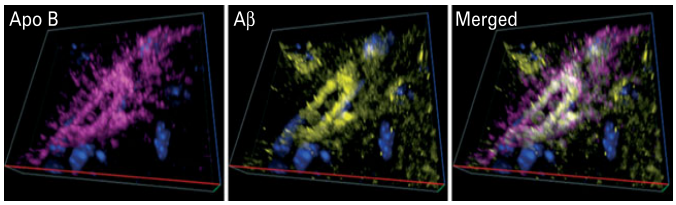

Fig. 3. Co-localisation of amyloid-β (Aβ) with perivascualr apo B influx in SFA-fed mice. Significant immunoreactivity of Aβ was detected concomitant with perivascular leakage of apo B lipoproteins in SFA-fed mice, consistent with blood-to-brain delivery of lipoprotein–Aβ. The immunofluorescent images were captured in three dimensions (x, y, $z = 70 \times 60 \times 11\,\mu$m), and separated single images of apo B (magenta) and Aβ (yellow), and the merged image are shown. The number of co-localising pixels of different fluorescent dyes was measured by AxioVision software™ (Carl Zeiss, Jena, Germany) utilising Manders' correlation analysis (coefficient = 0·843 (SEM 0·01); n 695).

Apo B was used as a marker of intestinal and hepatically derived lipoprotein influx into the brain. Native apo B lipoproteins are large macromolecules of 3–50 million kDa molecular weight and a diameter of > 23 nm. In SFA-fed mice, apo B immunoreactivity was visible within the CTX and BS at 3 months following commencement of the diet (Fig. 1). However, ingestion of SFA for 6 months exacerbated apo B extravasation and apo B became more evident within the HPF. Analogous to the findings for IgG, mice maintained on the MUFA-enriched, PUFA-enriched or control diet showed no apo B cerebral immunoreactivity at either 3 or 6 months of feeding (Figs. 1 and 2).

Evidence consistent with the possibility that SFA feeding leads to peripheral delivery to the brain of apo B lipoprotein-associated Aβ is shown in Fig. 3. Perivascular distribution of Aβ is clearly seen coinciding with the distribution of apo B lipoproteins (Manders' correlation coefficient 0·843 (SEM 0·01); $P < 0·0001$).

To explore if the pattern of cerebral apo B/Aβ distribution in SFA mice could be relevant to AD aetiology, we also investigated IgG and apo B distribution in 12-month-old APP/PS1 transgenic animals. As found in SFA wild-type mice, the APP/PS1 mice have significant cerebral IgG and apo B extravasation primarily within the CTX, but with significant amounts also within the HPF (Figs. 4 and 5). In addition, utilising highly sensitive 3-D immunodetection, apo B lipoproteins were clearly visible co-localised with Aβ plaque (Manders' correlation coefficient 0·85 (SEM 0·004); $P < 0·0001$; Fig. 6) and plaque abundance positively correlated with apo B immunoreactivity (Pearson's correlation coefficient 0·49 (SEM 0·037)).

BBB integrity was also assessed by determining the abundance of occludin (a tight junction protein) relative to vWF. Both in SFA-fed mice and in APP/PS1 transgenic mice, occludin expression was substantially attenuated compared with controls (Figs. 7 and 8). Further evidence that BBB integrity was compromised was suggested by changes in the plasma concentration of S100B (Fig. 9). In the cerebrospinal fluid, S100B level is orders of magnitude greater than in plasma and therefore serves as a useful surrogate marker of

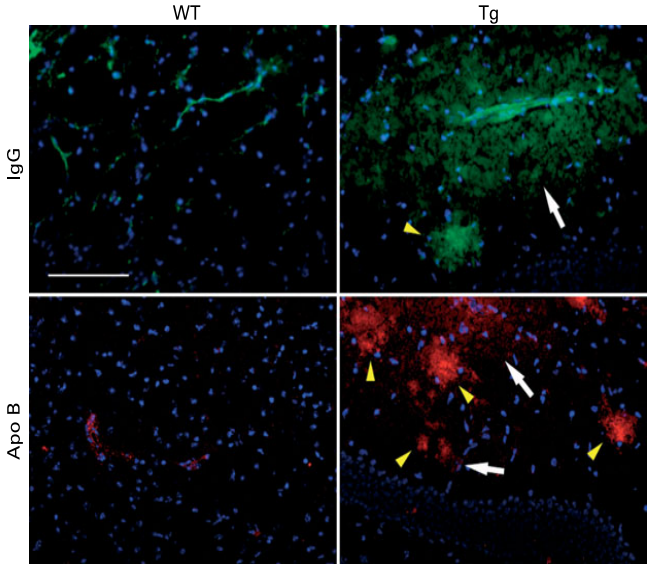

Fig. 4. Three-dimensional (3-D) quantitative immunomicroscopy of cerebral IgG and apo B extravasation in amyloid precursor protein/presenilin-1 (APP/PS1) amyloid transgenic (Tg) mice. The cerebral leakage of plasma IgG and apo B were quantitatively measured by 3-D immunofluorescent detection in the Tg mice. Substantial influx of plasma IgG and apo B into the brain was observed in the Tg mice (white arrows) while no leakages were seen in age-matched wild-type (WT) control mice. Notably, apo B immunoreactivity in the Tg mice strongly co-localised with amyloid plaques (see Fig. 6) while only occasional co-localisation of IgG with plaques was seen (yellow arrow heads). IgG, apo B and nuclei are shown in green, red and blue, respectively. The scale bar indicates 100 μm.

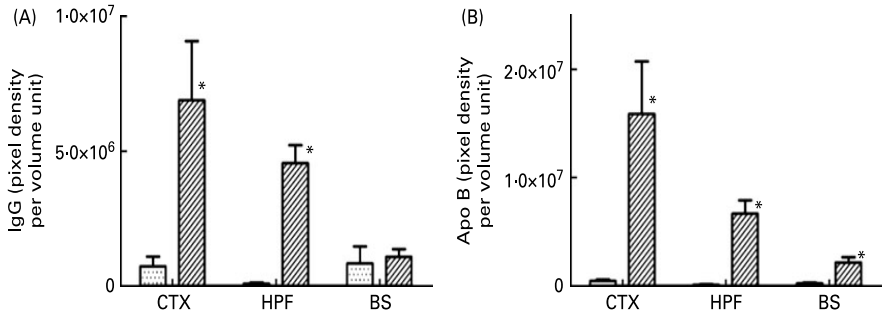

Fig. 5. Three-dimensional semi-quantitative analysis of cerebral IgG (A) and apo B (B) extravasation in amyloid precursor protein/presenilin-1 (APP/PS1) amyloid transgenic (Tg; ▨) mice. Optical pixel densities were measured in three major brain regions of the cortex (CTX), hippocampal formation (HPF) and brain stem (BS) and expressed as per volume unit. Values are means, with standard errors represented by vertical bars. * Quantitative analysis based on the pixel density suggested significantly increased immunoreactivity of IgG and apo B in the brain of Tg mice compared with wild-type control mice (⊠) ($P<0{\cdot}05$; *t* test).

brain-to-blood efflux[32–33]. Mice fed SFA had an 80 % increase in plasma S100B compared with low fat-fed mice, but there was no difference in mice fed either of the unsaturated fatty acid diets (Fig. 9). Similarly, plasma S100B was more than doubled in APP/PS1 mice.

The breakdown of the BBB and increased influx of TRL–Aβ in SFA-fed mice did not appear to be a consequence of exaggerated exposure to circulating Aβ, on the basis that the plasma concentration of $A\beta_{1-40}$ and $A\beta_{1-42}$ were similar for all groups of wild-type mice (Fig. 10).

Discussion

The present study suggests that chronic ingestion of SFA compromises BBB integrity, resulting in blood-to-brain delivery of plasma proteins, including apo B lipoproteins that may be endogenously enriched in Aβ. Consistent with the notion of BBB dysfunction in SFA-fed mice, there was evidence of cerebrospinal fluid-to-plasma efflux, with a doubling in the plasma concentration of S100B. The detrimental effect of SFA on BBB function seemed to be specific, as no differences were observed between control animals and mice supplemented with either MUFA or PUFA. The cerebral distribution and co-localisation of Aβ with apo B lipoproteins in SFA-fed mice was also found to be remarkably similar to that in APP/PS1 amyloid transgenic mice and in the latter there was a positive association of plasma-derived apo B lipoproteins with cerebral Aβ deposits. The cerebral distribution of apo B and IgG was greater at both 3 and 6 months of SFA feeding within the CTX than the HPF or BS regions, suggesting a site-specific effect. The observation is consistent with the hypothesis of enhanced blood-to-brain delivery of peripheral lipoprotein–Aβ, because the CTX has a more substantial capillary network. Kawai *et al.* reported that 60–77 % of amyloid plaques were associated with capillaries and relevant to the hypothesis presented in the present study, there was significantly greater vessel density within a 10 μm border surrounding plaques compared with unaffected grey matter[34]. Collectively, the findings of the present study provide one explanation of how dietary fats may influence AD risk. Ingestion of SFA could enhance peripheral delivery to the brain of circulating lipoprotein–Aβ and exacerbate the amyloidogenic cascade.

The fatty acid-enriched diets provided to mice were well tolerated and there were no significant differences in weight gain, plasma cholesterol or plasma TAG between groups. Therefore changes to BBB integrity in SFA-fed mice were not a consequence of dietary induced dyslipidaemia. The diets were physiologically relevant. Palmitic (16 : 0) and stearic (18 : 0) acid content of the mouse diets of 5·2 and 7·1 % of energy intake, respectively, is comparable with Western patterns of consumption of between 5 and 7 % for each. Total saturates of the mouse chow were also similar to Western dietary patterns (13 % mouse SFA chow *v.* 14 % in human diets) as well as total digestible energy as lipids (40 % for fatty acid-supplemented chow *v.* 37 % in Western diets).

We put forward the hypothesis that an SFA-induced elevation in plasma Aβ might compromise BBB function on

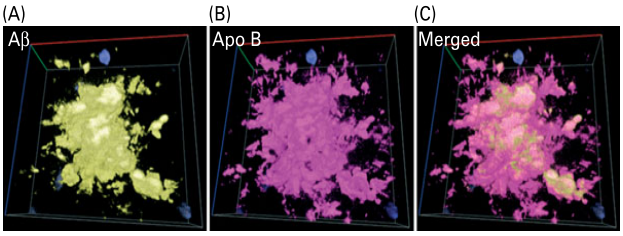

Fig. 6. Co-localisation of apo B with cerebral amyloid plaques. An immunofluorescent double-labelling method was utilised to explore the co-localisation of apo B with amyloid plaques in amyloid precursor protein/presenilin-1 (APP/PS1) transgenic Alzheimer's disease model mice. The distribution of apo B and amyloid-β (Aβ) for the same tissue specimen are shown separately (A and B). The co-location of apo B with Aβ is indicated in the merged image (C). For the latter, Aβ is shown in yellow and apo B is shown in magenta. Scales of *x* (red), *y* (blue) and *z* (green) axes are 70 × 70 × 22 μm.

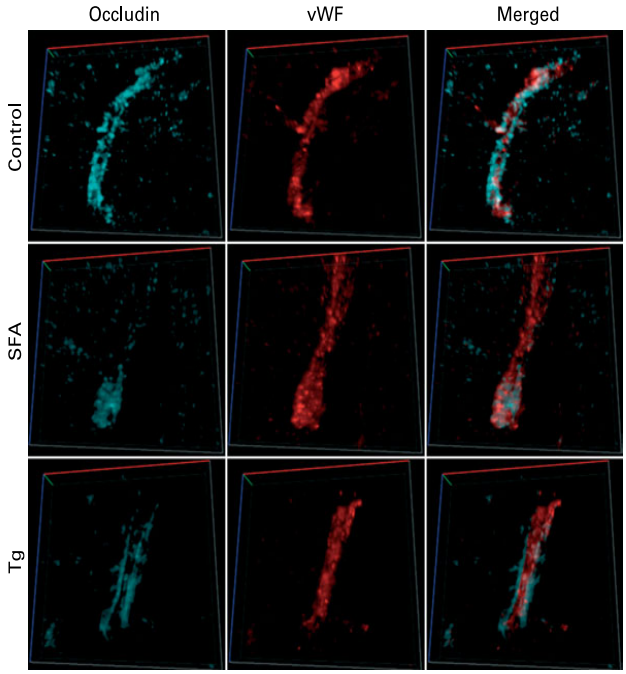

Fig. 7. Blood–brain barrier (BBB) three-dimensional (3-D) detection of the tight junction protein occludin. The expression of occludin-1 relative to epithelial cell abundance (expressed as von-Willebrand factor (vWF)) was quantitatively determined using double-labelling immunofluorescent microscopy. Representative 3-D images of occludin and vWF images are shown (x, y, $z = 80 \times 80 \times 7\,\mu$m). Significantly decreased expression of BBB occludin was found in SFA-fed mice compared with control mice, consistent with amyloid precursor protein/presenilin-1 (APP/PS1) amyloid trangenic (Tg) mice.

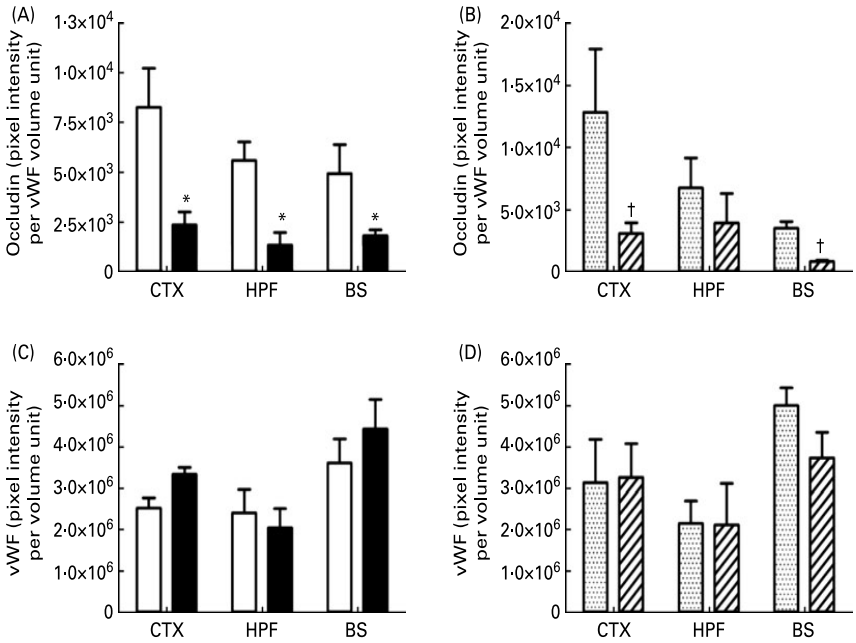

Fig. 8. Three-dimensional semi-quantitative analysis of cerebrovascular occludin (A and B) and von-Willebrand factor (vWF) (C and D) in control (□), SFA-fed (■), wild-type (▨) and amyloid precursor protein/presenilin-1 (APP/PS1) amyloid trangenic (▨) mice. Optical pixel intensities were measured in three major brain regions of the cortex (CTX), hippocampal formation (HPF) and brain stem (BS). Immunoreactivities of blood–brain barrier occludin-1 are expressed as per vWF volume unit. The net abundance of vWF is indicated per unit tissue. Values are means, with standard errors represented by vertical bars. * Mean value was significantly different from that of the control mice ($P < 0{\cdot}05$; t test). † Mean value was significantly different from that of the wild-type mice ($P < 0{\cdot}05$; t test).

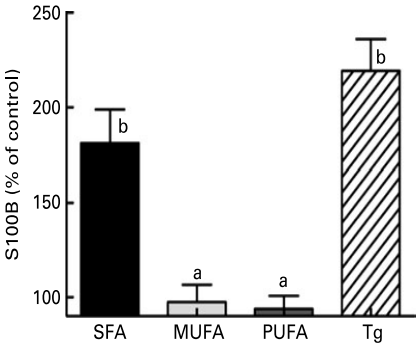

Fig. 9. Plasma level of S100B. Plasma S100B was determined by ELISA as a surrogate marker of brain-to-blood leakage. Mice fed SFA for 3 months had a significantly higher S100B level compared with the control, MUFA- and PUFA-fed mice. Similarly, amyloid precursor protein/presenilin-1 (APP/PS1) amyloid trangenic (Tg) mice had a doubling in plasma S100B compared with wild-type controls. Values are means, with standard errors represented by vertical bars. [a,b] Mean values with unlike letters were significantly different ($P<0{\cdot}01$; one-way ANOVA).

the basis that exogenous administration of this protein showed disturbing vasoreactive properties *in vivo*. However, in the present study the plasma concentrations of $A\beta_{1-40}$ and $A\beta_{1-42}$ were similar for all groups of wild-type mice. We cannot equivocally rule out an elevation in the lipoprotein pool of Aβ because some studies suggest that lipids block detection of lipoprotein-bound Aβ[35,36].

The SFA diet may have also compromised BBB function mechanisms independent of lipoprotein–Aβ concentration, including enhanced production of reactive oxygen species, increases in intracellular Ca or activation of endoplasmic reticulum stressors[37–40]. Clinical evidence suggests that inflammatory pathways can become activated because of impaired postprandial lipid metabolism[41]. Important differences in the cytotoxic effects of fatty acids have been reported, with longer-chain SFA being the most potent and the MUFA and PUFA being cytoprotective[37]. Morgan[37] suggests that the underlying toxicity of SFA is a consequence of disturbances in protein processing and endoplasmic reticulum dysfunction, for example, apoptotic induction. Consistent with this hypothesis, Patil *et al.* reported a palmitic acid-induced region-specific damage because of a higher fatty acid-metabolising capacity of cortical astroglia[42]. Conversely, cell-culture

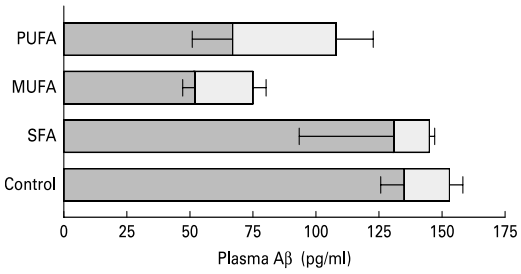

Fig. 10. Plasma amyloid-β (Aβ) concentration in control and high fatty acid-fed mice. Plasma levels of $A\beta_{1-40}$ (▨) and $A\beta_{1-42}$ (□) were measured with ELISA. Values are means, with standard errors represented by horizontal bars. There was no significant difference between the control group and any of the high-fat treatment groups.

studies suggest that incubation, particularly with longer-chain unsaturates, has an antagonistic effect on endoplasmic reticulum-centred stress pathways[43].

Dietary 'lipotoxicity' refers to the processes leading to end-organ damage and/or dysfunction following excess exposure to fatty acids and was first coined in the context of fat-induced insulin resistance[44]. Since then, however, the process has also been implicated in endothelial dysfunction and atherosclerosis, heart failure, kidney failure, steatohepatitis and liver failure, autoimmune inflammatory disorders, susceptibility to infections, cancer and ageing. The BBB disturbances identified in the present study may reflect a broader dietary toxic phenomenon.

SFA may also have compromised BBB function by secondary pathways other than elevations in plasma Aβ, or as a consequence of interactive effects with genes involved in Aβ metabolism and BBB function. For example, Deane *et al.* showed that apoE isoforms differentially regulate Aβ clearance from the brain by routing free Aβ through alternate receptors at the BBB. Apo E4-facilitated efflux was slower than apo E3- or apo E2-mediated clearance[45,46]. The effect of SFA on apo E isoforms was not explored in the present study.

Apo B immunoreactivity in senile plaque of subjects with AD was reported some years ago[47] but there was no evidence that this association was causal. However, several lines of study are consistent with the concept that peripheral delivery of lipoprotein–Aβ may contribute to AD risk[5,18,48–50]. Firstly, Aβ is tightly bound to TRL, the secretion of which is positively associated with the onset and progression of cerebrovascular and parenchymal amyloidosis[20–21]. Indirect evidence for the possibility of enhanced lipoprotein-mediated blood-to-brain delivery of Aβ is suggested by the study of Kreuter *et al.* who demonstrated enhanced drug transport into the brain when nanoparticles were covalently attached to apolipoproteins (including apo B)[51]. In clinical studies significantly greater levels of apo B are found in AD patients[52] and indirect evidence also comes from studies investigating the pro-atherogenic properties of apo B lipoproteins, a disease that shares some pathological similarities to AD. In arterial tissue, apo B lipoproteins and particularly the post-hydrolysed remnants of apo B lipoproteins have significant affinity for extracellular matrices and, if trapped, induce an inflammatory response[53]. The uptake by resident macrophages of apo B lipoproteins triggers a respiratory burst compromising cell viability leading to atherosclerotic plaque instability[54]. Obesity and diabetes also significantly increase risk for AD[55]. Profenno *et al.*[55] suggested that physiological changes common to obesity and diabetes plausibly promote AD. Resistance to the action of insulin in obese/diabetic individuals results in hepatic and intestinal apo B lipoprotein overproduction, diminished clearance of apo B lipoproteins from blood and postprandial dyslipidaemia[56]. It is possible that the cerebral parenchymal entrapment of lipoprotein–Aβ and formation of fibrillar deposits share pathways in common with the initiation of progression of atherosclerosis.

Nelson & Alkon[57] suggested that fibrillar formation of Aβ is prevented by proteins such as apo B that have affinity for the Aβ hydrophobic domain. Using a phage display system to explore protein–protein interaction, they found that Aβ binds to proteins primarily involved in LDL and cholesterol

transport. However, given that apo B is a protein not normally found in cerebrospinal fluid it is unlikely to serve as a suppressor of amyloidosis. Rather, cerebrovascular remodelling of apo B lipoprotein–Aβ delivered to the brain could exacerbate amyloidogenesis as a result of Aβ release, or enhanced epitope exposure[30].

There is an accumulating body of literature consistent with the concept that the onset and progression of AD is influenced by lifestyle factors including nutrition. Population studies support a role of dietary fats in AD. Laitinen *et al.* reported that intake of unsaturated fats is protective, whereas intake of saturates increases risk of AD[58]. In the Framingham study, the top quartile of plasma DHA was associated with a 47 % reduction in risk of all-cause dementia[59]. Furthermore, evidence continues to come from animal studies including by Oksman *et al.* who confirmed that saturates increase, while DHA decreases, cerebral Aβ levels compared with a soya oil diet[60].

The present study provides novel insight into how dietary fats might influence AD risk and reports for the first time differential effects of dietary fatty acids on BBB integrity. Mice chronically fed SFA show significant blood-to-brain delivery, retention and accumulation of apo B lipoproteins, primarily within the CTX of SFA-fed mice, observations consistent with the distribution of apo B in amyloid in brain specimens from subjects with AD. In the present study, chronic ingestion of SFA in wild-type mice also replicated the pattern of BBB dysfunction and of TRL–Aβ distribution observed in an established model of AD (APP/PS1 transgenics) maintained on normal chow. How dietary behaviour influences BBB function and the propensity for amyloidosis may prove helpful in the context of AD prevention.

Acknowledgements

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R. T. conducted the experimental procedures and prepared the manuscript. S. G. contributed to the animal care and experimental data collection. M. M. S. P.-G. contributed to experimental data collection. C. L. W. contributed to animal sample preparation. R. D. J. contributed to the development of methods. S. S. D. contributed to the experimental design and statistical assessment. J. C. L. M. contributed to the project concept, research funding, experimental design, data collection and writing of the manuscript.

The authors declare no conflicts of interest.

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Article 2:

Galloway S, Takechi R, **Pallebage-Gamarallage M**, Dhaliwal S & Mamo J. (2009) Amyloid-beta colocalizes with apolipoprotein B in absorptive cells of the small intestine. *Lipids Health Dis.* 8**,** 46.

Synopsis

Background:

Derived from the proteolytic processing of the APP, Aβ is the predominant component of amyloid plaques (Fukuoka et al. 2004). Cerebral amyloid plaque formation is a key pathological feature of AD commonly associated with end-stage neuronal death (Mattson 2004). Although, the source of cerebral Aβ deposits in AD is uncertain, recent evidence suggest blood-to-brain delivery of circulating Aβ, a process which may exacerbate cerebral amyloid load (Donahue et al. 2006). Circulatory Aβ could be derived from vascular smooth muscle cells and endothelial cells (Goldgaber et al. 1989, Haass et al. 1992) or from blood platelets. In addition, lipogenic organs such as liver and small intestine secrete Aβ as a lipoprotein complex (Koudinov et al. 1994).

Studies from our laboratory demonstrated Aβ in absorptive epithelial cells of the small intestine (enterocytes) of WT mice (Galloway et al. 2007). Enterocytic Aβ was substantially increased with the ingestion of a HF diet, but in contrast, was completely abolished by fasting (Galloway et al. 2007), clearly showing dietary regulation. Significant Aβ staining was found within the perinuclear region of the enterocytes, the site of chylomicron production. It was hypothesised that enterocytic Aβ is delivered into circulation associated with postprandial lipoproteins (chylomicrons) (Galloway et al. 2007). Chylomicrons are TRLs that are synthesised exclusively by the enterocytes of the small intestine in response to ingested fats. Several lines of evidence from *in vitro* and *in vivo* studies support the concept of chylomicron-Aβ metabolism (James and Mamo 2005, James et al. 2003, Mamo et al. 2008). Significant plasma Aβ was found enriched in the TRL fraction of plasma

(Mamo et al. 2008), including chylomicrons. Moreover, TRL-Aβ was increased in subjects with AD concomitant with evidence of postprandial dyslipidemia.

The mechanisms Aβ may be associated with chylomicrons are unclear. It is possible that chylomicrons interact with APP during generation of Aβ, or at the time of lipoprotein assembly. Enterocytic Aβ may be associated with chylomicrons during the lipidation of apo B within the ER and Golgi-apparatus of the enterocytes. Apolipoprotein B is the obligatory structural component necessary for synthesis and secretion of chylomicrons.

This manuscript explored the notion that enterocytic Aβ becomes associated with chylomicrons within the Golgi-apparatus in the absorptive epithelial cells of the small intestine and secreted into circulation.

Methods in brief:

Double immunofluorescence labelling of enterocytic Aβ and apo B within the Golgi-apparatus was determined in WT mice fed a LF and SFA diets. Correlation and colocalisation of these proteins were determined with Pearson's correlation coefficient and Mander's coefficient, respectively.

Results in brief:

Double immunofluorescence staining demonstrated that Aβ and chylomicron apo B colocalise within the perinuclear region of the enterocytes and within the lacteals. Chronic SFA feeding significantly enhanced enterocytic Aβ concomitant with increased apo B abundance. However, there was no evidence that relative abundance of the two proteins were associated.

Discussion and conclusion in brief:

These findings support the concept that enterocytic Aβ is secreted as a lipoprotein complex. Furthermore, Aβ biogenesis and secretion was found to be stimulated by dietary SFA. The findings raise the possibility that exaggerated cerebral capillary exposure to TRL-enriched Aβ contributes to BBB disturbances in mice chronically fed diets enriched in SFA (Takechi et al. 2010a) (article 1).

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Amyloid-β colocalizes with apolipoprotein B in absorptive cells of the small intestine

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Abstract

Background: Amyloid-β is recognized as the major constituent of senile plaque found in subjects with Alzheimer's disease. However, there is increasing evidence that in a physiological context amyloid-β may serve as regulating apolipoprotein, primarily of the triglyceride enriched lipoproteins. To consider this hypothesis further, this study utilized an in vivo immunological approach to explore in lipogenic tissue whether amyloid-β colocalizes with nascent triglyceride-rich lipoproteins.

Results: In murine absorptive epithelial cells of the small intestine, amyloid-β had remarkable colocalization with chylomicrons (Manders overlap coefficient = 0.73 ± 0.03 (SEM)), the latter identified as immunoreactive apolipoprotein B. A diet enriched in saturated fats doubled the abundance of both amyloid-β and apo B and increased the overlap coefficient of the two proteins (0.87 ± 0.02). However, there was no evidence that abundance of the two proteins was interdependent within the enterocytes (Pearson's Coefficient < 0.02 ± 0.03), or in plasma (Pearson's Coefficient < 0.01).

Conclusion: The findings of this study are consistent with the possibility that amyloid-β is secreted by enterocytes as an apolipoprotein component of chylomicrons. However, secretion of amyloid-β appears to be independent of chylomicron biogenesis.

Background

Amyloid-β is recognized as the principal protein in senile plaques in subjects with Alzheimer's disease (AD) [1]. Generated from the slicing of amyloid precursor protein (βAPP) by secretases, the synthesis of amyloid-β can be differentially modulated by cellular lipid homeostasis. Studies in cell culture and in vivo suggest that cholesterol inhibits amyloid-β biogenesis [2-4], although this effect may be dependent on the distribution of free and esterified cholesterol within the plasma membrane and within lipid rafts [5]. In contrast, in vivo studies found that chronic ingestion of diets enriched in saturated-fats (SFA) had a potent stimulatory effect on enterocytic amyloid-β abundance [6].

Several lines of evidence suggest that one physiological role for amyloid-β is as a regulating apolipoprotein, particularly of the triglyceride-rich lipoproteins (TRL's). Koudinov et al reported that amyloid-β is secreted by hepatocytes as a lipoprotein complex [7]. Significant plasma abundance of amyloid-β was also found in the TRL fraction of control subjects and amyloid-β enrichment in TRL's was evident in subjects with AD, or with mild cognitive impairment [8]. Ingestion of a lipid rich meal also causes a transient increase in plasma of soluble APP, concomitant with postprandial lipaemia [9] and when injected intravenously associated with TRL-emulsions, amyloid-β increased uptake in fat-rich tissues relative to liver [10].

The βAPP is expressed on the plasma membrane of a number of tissues including lipogenic organs such as liver [6]. Proteolytic cleavage of βAPP generally results in the extracellular release of amyloid-β which is then chaperoned by transporter proteins [11-14]. However, hydrophobic domains within amyloid-β [15] results in rapid folding of amyloid-β that make it unlikely to readily associate with lipoproteins already secreted into circulation. Rather, immunhistochemistical studies show amyloid-β abundance within the perinuclear region of hepatocytes and absorptive epithelial cells of the small intestine [4,6,7,16], suggesting that amyloid-β may form part of the primordial lipoprotein during the lipidation process. Consistent with the latter, using a phage display Nelson and Alkon showed that amyloid-β bound tightly with several apolipoproteins found commonly with TRL [17]. To further consider the possibility that amyloid-β becomes associated with nascent lipoproteins, in this study we utilized sensitive three-dimensional (3D) immunofluorescent (IF) microscopy to explore if enterocytic abundance of amyloid-β is associated with chylomicrons. Apolipoprotein (apo) B, an obligatory component of TRL secreted by intestine and liver, was used as a marker of enterocytic chylomicron distribution and plasma abundance of TRL.

Materials and methods

Diet and animals

The protocols described in this study were approved by an accredited National Health and Medical Research Council of Australia Animal Ethics Committee (Curtin University Animal Experimentation and Ethics Committee Reference number R02-07). Six-week-old female C57BL/6J mice (Animal resources centre, Murdoch, Western Australia) were divided randomly into a low-fat (LF) or saturated fatty acid (SFA) diet group. Low fat mice were given chow that contained 3.6% (w/w) as unsaturated fat and 0.4% SFA (AIN93M, Specialty Feeds, Western Australia). The SFA enriched chow contained 12.9% (w/w) as saturated fats and 7.4% as unsaturated oils (SF07-50, Specialty feeds, Western Australia). Both diets were free of cholesterol. Digestible energy for LF and SFA feed were 15.1 MJ/kg and 18.8 MJ/kg respectively and feed was available *ad libitum*. After three-months of dietary intervention, mice were sacrificed by pentobarbital injection. The small intestine was isolated and flushed with chilled phosphate buffered saline (PBS, pH 7.4). A 2 cm segment of the small intestine distal to the duodenum was fixed in 4% paraformaldehyde for a minimum of 24 h, processed and longitudinal segments embedded in paraffin wax. Serial sections of 5 μm thick were cut on microtome and mounted on silanised slides for histology and immunofluorescence microscopy.

Antibodies

Anti-apo B, anti Golgi-apparatus (anti-Golgi 58 K), anti-rabbit IgG with Alexa488, and streptavidin-Alexa546 were obtained from Invitrogen (Melbourne, Victoria, Australia). Anti-rabbit IgG biotin conjugate was obtained from DAKO (Glostrup, Denmark). Rabbit anti-human amyloid-β was obtained from Chemicon International (Temecula, California, United States).

Double-immunofluorescent labelling

An established double IF labelling method was utilized as previously described [18]. Cross reactivity was prevented using a biotin-avidin amplification technique microscopy. The concentration of the primary antibody used with biotin-avidin amplification is substantially below the threshold required for detection by standard IF and does not interfere with detection of the second protein.

Anti-amyloid-β (1:1000) was added to sections overnight at 4°C, followed by addition of goat anti-rabbit IgG with biotin (1:200) for 1 h at room temperature. Thereafter, anti-Golgi-apparatus (1:10) was added overnight at 4°C. Immunofluorescence was detected by streptavidin-Alexa546 (1:100) and anti-rabbit IgG with Alexa488 (1:100) for amyloid-β and Golgi-apparatus respectively. Cell nuclei were detected using DAPI and slides were mounted using anti-fade mounting medium. The same method was used to achieve double apo B and Golgi-apparatus staining by substituting the anti-amyloid-β with anti-apo B (1:400).

Image capture

Digital images were captured using AxioCam mRM and ApoTome on a Zeiss Axiovert 200 M inverted microscope and visualized with Plan-NeoFluar lenses (Carl Zeiss, Oberkochen, Germany). Excitation and emission were achieved by using filters 43 (Ex BP545/25, beam splitter FT570 and Em BP605/70) and 38 (Ex BP470/40, beam splitter FT495 and Em BP525/50) to determine fluorescence of Alexa546 and Alexa488 respectively. Filter 49 (Ex G365, beam splitter FT 395 and Em BP445/50) was used to detect nuclei stain DAPI. Individual channels are

devoid of fluorescence from other emission sources and are therefore clear of bleed-through.

Three-dimensional images were captured using the ApoTome optical sectioning mode which allows the creation of a 3D image based on the 'stacking' of consecutive 2D images. Each 3D image consisted from 8-10 2D images, and the axial distance of Z-stack was 0.5 μm for 200×. There were 6 animals per group with a minimum of 40 images per mouse used for analysis. Fluorescent intensity and area were determined using the measurement and colocalization module available on AxioVision v4.7.1 software (Carl Zeiss, Oberkochen, Germany).

Quantification of fluorescent intensity and colocalization

There are several algorithms capable of achieving measures of colocalization or association via measurement of fluorescent pixel spatial orientation and pixel intensity. The Pearson's correlation coefficient (r) is a commonly used quantitative estimate of association (abundance) for proteins [19]. However, as Pearson's correlation is a measure of variance from the mean pixel intensity, it does not provide information of the area of overlap. A modification to Pearson's correlation coefficient developed by Manders et al (1993) eliminates the average grey values from the Pearson's formula to allow the quantification of overlapping pixels from each channel [19]. The degree of colocalization for the proteins is positively related to the Manders coefficient, known commonly as the 'overlap coefficient' (OC). The AxioVision software utilizes an automated procedure based on spatial statistics to determine Pearson's correlation coefficient and Manders OC, thereby avoiding selection bias by manual selection methods.

Western blotting for plasma apolipoprotein B

Plasma samples were separated on NuPAGE 3-8% Tris-acetate gels (EA03752BOX, Invitrogen, Victoria, Australia) at 150 V (Biorad Model 20012.0) for 1 hr. Gels were then electrotransferred to PVDF membranes (PV4HY00010, Osmonics Inc, Minnesota U.S.A) at 40 V for 1 hr and blocked in 10% skim milk (in TBST) overnight at 4°C. The membranes were incubated with polyclonal rabbit anti-human apo B 1:100 (Q0497, Dakocytomation, Glostrup, Denmark), and then with donkey anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP) (Na934V, Amersham Bioscience, Buckinghamshire, UK). Proteins were detected using enhanced chemiluminescence reagent (ECL™) western blotting analysis system (RPN2108, Amersham Bioscience, Buckinghamshire, UK). Membranes were exposed to high performance chemiluminescence film (Amersham Hyperfilm™, Amersham Bioscience, Buckinghamshire, UK) and developed in an AGFA-Gevaert Rapidoprint X-Ray Developer (Septestraat, Belgium). Apo B48 bands were identified and quantified by densitometry against purified apo B48 protein of known mass (550 kDa for apo B-100 and 260 kDa for apo B48).

Amyloid-beta ELISA

Plasma amyloid-β 40/42 levels were measured using commercially available ELISA kits (Biosource, Camarillo CA).

Statistics

Enterocytic colocation of amyloid-β with apo B was determined by an automated procedure based on spatial statistics to determine Pearson's correlation coefficient and Manders OC (AxioVision 4.0). The association between total apo B, apo B48, and apo B100 with total amyloid-β, amyloid-β40 and amyloid-β42 were examined using Pearson's and Spearman's correlation. Spearman's correlation was used when the assumptions of the analysis were violated due to the presence of outliers. P-values less than 5% were considered as statistically significant and the data was analysed using SPSS version 17.0.

Results

Enterocytic chylomicrons were detected by determining the distribution of apo B, an obligatory structural component of chylomicrons. Significant amounts of amyloid-β and apo B were found to be enriched within the perinuclear region of cells. Amyloid-β and apo B colocalized with the Golgi-apparatus, towards the basolateral surface of the cell and within the lacteals (Figure 1). The patterns of distribution for amyloid-β and apo B remained essentially the same in LF and SFA fed mice (Figure 2), however abundance of each protein more than doubled in SFA fed mice compared to LF fed animals (Table 1, columns 1 and 2).

The colocalization of enterocytic amyloid-β and apo B was expressed as the OC (Manders overlap coefficient). The relative abundance of amyloid-β and apo B in LF and in SFA fed mice, given as mean densitometric sum. In LF mice, approximately 73% of immunodetectable amyloid-β colocated with apo B, but in SFA mice this was significantly increased ($p < 0.05$) to nearly 87% (Table 1, columns 3 and 4). Figure 2 shows the extent of colocalisation in three dimensions of amyloid-β relative to apo B under high magnification.

To explore if abundance of the amyloid-β was interdependent with TRL biogenesis and secretion, correlation analysis with apo B was determined within enterocytes and in plasma respectively. Pearson's correlation analysis found that just 2% of amyloid-β and apo B fluorescent intensities were positively associated in enterocytes of LF or any of the SFA fed mice (table 1). Similarly, in plasma there was no evidence that the principal isoforms of amy-

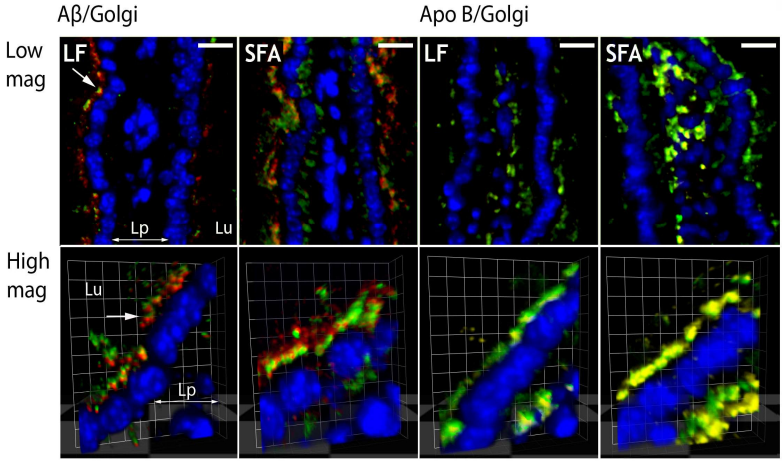

Figure 1

Enterocytic amyloid-β and apolipoprotein B colocalizes with Golgi-apparatus under LF and SFA feeding. The images depict the colocalization of Golgi-apparatus with amyloid-β (columns 1 and 2) and apo B (columns 3 and 4) in low-fat (LF) and saturated fat (SFA) fed mice. The upper row shows small intestinal villi at low magnification (mag) in two dimension, whilst the lower frames depicts enterocytes at high magnification in three dimensions. Amyloid-β as indicated in red, apo B as yellow, Golgi-apparatus as green, and nuclei as blue pixels. Where overlap of pixels occurs between amyloid-β (red) and Golgi-apparatus (green), an orange colour prevails. Similarly, the colocalization of apo B (yellow) with Golgi-apparatus (green) generates lime colour. Perinuclear (white arrow) and lamina propria (Lp) presence of amyloid-β and respective proteins are shown. Lu labels the lumen that represents the apical surface of the cell and Lp (lamina propria) is the direction of lacteals where lipoproteins are expelled via exocytosis. Scale: bar (2D images) = 10 μm; grid (3D images) = 3.63 μm.

loid-β (amyloid-β40 and 42) were associated with intestinal or hepatic apo B lipoproteins (figure 3).

Discussion

In this study the distribution and abundance of amyloid-β and apo B were detected in small intestinal enterocytes using an established double-labeled avidin-biotin IF microscopy technique [18]. Amyloid-β and chylomicron-apo B were remarkably colocalized in enterocytes, consistent with release of amyloid-β as a lipoprotein complex [7]. We also confirm that chronic consumption of SFA increases enterocytic amyloid-β and now show that this occurrence is concomitant with a substantially greater abundance of enterocytic apo B [6]. However, there was no evidence from this study that the biogenesis of amyloid-β and apo B are inter-dependent based on Pearson's correlation analysis within enterocytes and in plasma.

The biosynthesis of chylomicrons occurs in a multi-step process that requires the progressive lipidation of apo B an obligatory structural component of primordial lipoproteins secreted by the small intestine [20,21]. A number of proteins are reported to associate with nascent chylomicrons prior to secretion, including apo A-I, A-IV, apo J, apo D, apo E and small molecular weight proteins such as apo C-II. Nascent chylomicrons are then transported via the Golgi-apparatus to the basolateral membrane and secreted into lymphatics. The results from this study suggest that amyloid-β is secreted from small intestinal enterocytes as an apolipoprotein of chylomicrons.

Immunoreactivity for amyloid-β and apo B was found selectively within the ER/Golgi-apparatus and not on the plasma membrane. The findings are consistent with biogenesis of amyloid-β at the ER and translocation to primordial lipoproteins, rather than as a consequence of β APP processing. Similar results in hepatocyte cultures with secretion of amyloid-β also occurring exclusively as a lipoprotein complex [7].

Dietary SFA promote chylomicron biogenesis by stimulating apo B lipidation [22,23], an essential step to avoid post-translational degradation by intracellular proteases [24]. Greater lipid substrate availability (as a result of SFA ingestion) reduces the proportion of apo B that would otherwise be degraded. The SFA dietary intervention used in this study essentially doubled enterocytic apo B and a similar increase in amyloid-β abundance was observed.

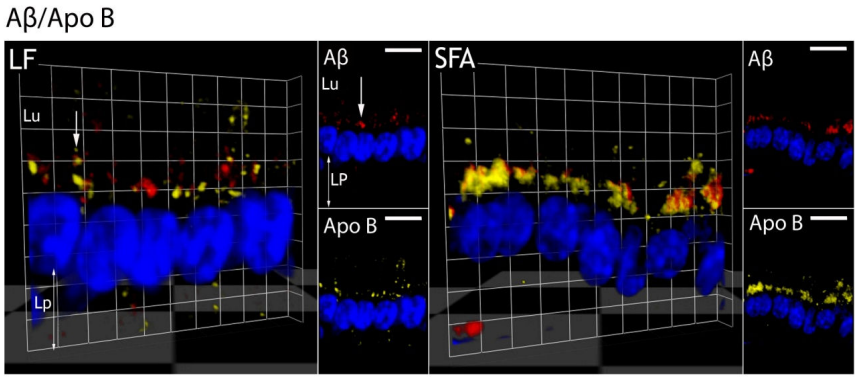

Figure 2
Enterocytic colocalization of amyloid-β with apo B under LF and SFA feeding. The enterocytic colocalization of amyloid-β (Aβ) with apolipoprotein B (apo B) in low-fat (LF) and saturated fat (SFA) fed mice is shown in three dimensions. The inset images depict the separate channel view for Aβ and apo B respectively. Amyloid-β is seen in red pixels, apo B as yellow and nuclei as blue. The perinuclear region (white arrow) and lacteal (Lp) orientation of enterocytes is indicated. Lu labels the lumen that represents the apical surface of the cell and Lp (lamina propria) is the direction of lacteals where lipoproteins are expelled via exocytosis. Scale: bar (2D inset images) = 10 μm; grid (3D images) = 3.63 μm.

However, the mechanisms by which SFA stimulate amyloid-β abundance and association with nascent chylomicrons are less clear. Saturated-FA may have a broader non-specific effect on enterocytic protein synthesis and consistent with the possibility of substrate driven biogenesis, Patil (2006) [25] found in neurons treated with palmitic acid resulted in increased upregulation BACE, a key enzyme complex involved in the processing of β *APP*. Alternatively, amyloid-β is an amphiphatic protein with a C-terminal domain that avidly binds with negatively charged hydrophobic lipids [15]. Increased substrate availability and synergistic lipidation of amyloid-β and apo B may promote the incorporation of amyloid-β into nascent chylomicrons and subsequently stimulate further synthesis of the proteins.

The SFA induction and secretion of enterocytic amyloid-β may be important in the context of AD risk. Recent studies suggest that blood-to-brain delivery of amyloid-β may contribute to amyloidosis, particularly when the concentration of circulating amyloid-β is chronically elevated [26-28]. This study suggest that SFA's increase synthesis and secretion of TRL associated amyloid-β concomitant with deterioration in blood-brain barrier integrity [29]. Indeed, the hypothesis is supported by studies in transgenic mice that over-express amyloid-β. In β APP/presenilin 1 transgenic mice, the plasma concentration correlated with secretion rates into blood of TRL's, which was increased 3-8 fold above wild-type mice [27]. Moreover, there was a positive association between plasma TRL-amyloid-β secretion with onset of cerebrovascular and parenchymal amyloidosis [29].

Conclusion

In this study, evidence in vivo that amyloid-β is secreted as a chylomicron complex and is stimulated by dietary SFA's is presented. Exploring this phenomenon in the context of plasma amyloid-β homeostasis and lipoprotein kinetics may provide insight into the putative association of high-fat diet with AD risk.

Table 1: Effect of SFA feeding on concentration and colocalization of enterocytic amyloid-β with apo B.

	Apo B*		Amyloid-β*		Overlap Coefficient		Pearson's Coefficient	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
LF	7013	790	5403	404	0.730	0.033	0.020	0.027
SFA	15840^	1812	13224^	1002	0.872^	0.022	0.015	0.023

*Mean enterocytic pixels value is expressed as mean densitometric sum and standard error of mean (SEM).
^Statistical significance was observed between LF and SFA groups with a p-value of at least less than 5%.

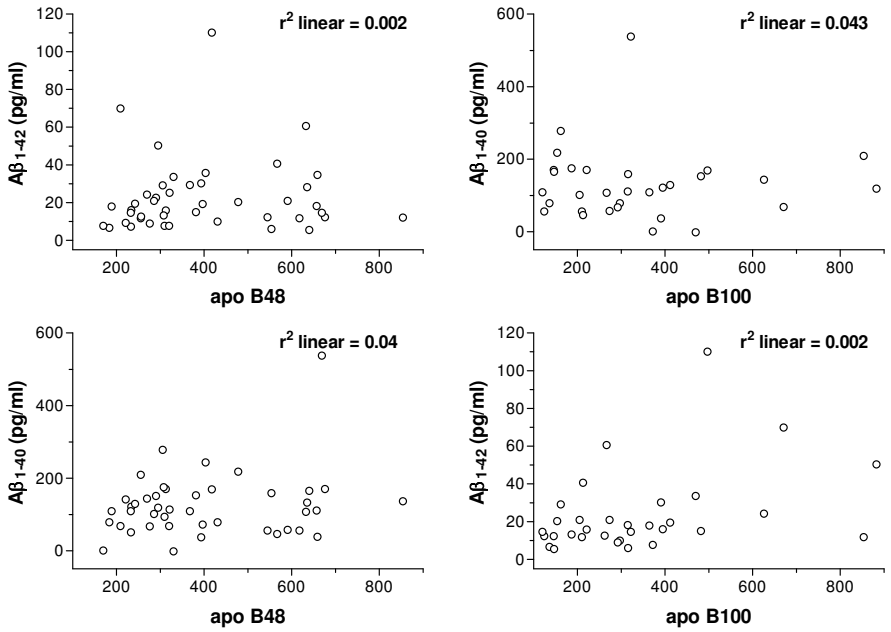

Figure 3
Correlation analysis of plasma amyloid-β40/42 with plasma apo B48/100. Correlation coefficients were determined with Pearson's correlation analysis where no outliers were identified.

List of Abbreviations

AD: Alzheimer's disease; apo: apolipoprotein; βAPP: β-amyloid precursor protein; IF: immunofluorescence; LF: low-fat; OC: overlap coefficient; PBS: phosphate buffered saline; SFA: saturated-fatty-acid; TRL: triglyceride-rich-lipoprotein

Competing interests

The authors acknowledge that there is no conflict of interest of any prior publication of any materials presented herein. All authors have seen and support the publication of this manuscript.

Authors' contributions

SG carried out the design of project, collection of data, immunofluorescence, statistical analysis and drafting of the manuscript. RT and MP-G assisted in the collection of tissues, interpretation of data and critically analyzing the manuscript content. SD helped in the statistical analysis of data and critically analyzing the manuscript content. JM conceived the study, helped in the interpretation of data, drafting of the manuscript, acquiring funding and role in general supervision of the research group. All authors have approved submission of the manuscript.

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Article 3:

Takechi R, Galloway S, **Pallebage-Gamarallage MMS**, Lam V & Mamo JCL. (2010b) Dietary fats, cerebrovasculature integrity and Alzheimer's disease risk. *Prog Lipid Res.* 49**,** 159-170. (Review)

Synopsis

Increasing evidence is consistent with the concept that chronic consumption of pro-atherogenic dietary lipids influence AD risk, however the underlying mechanisms are unclear. Studies have suggested that cerebrovascular disturbances precede plaque formation and may play a vital role in AD pathology (Attems et al. 2004, Kalaria 1992). This review article addresses the possible role of the BBB in contributing to AD development and progression. Furthermore, the putative mechanisms how dietary lipids influence AD risk via modulation of cerebrovascular integrity were also discussed.

Section 2.2 of the manuscript discussed that dietary lipids regulate TRL-Aβ synthesis and secretion from the absorptive epithelial cells of the small intestine. These findings support the notion that consumption of SFA enriched diets increase plasma TRL-Aβ and as a consequence AD risk. The latter is supported by *in vivo* experimental models that demonstrated a correlation between plasma TRL-Aβ and AD.

Section 3 and 4 of the manuscript explores the putative pathways by which postrprandial TRL-Aβ modulates cerebrovascular integrity. It was suggested that dietary SFA increase circulating TRL-Aβ and exaggerated exposure significantly compromise BBB integrity. Circulating Aβ may induce vascular endothelial damage due to their vasoactive properties (Thomas et al. 1997, Su et al. 1999). *In vivo* and *in vitro* studies demonstrated that exposure to exogenous Aβ resulted in significant endothelial cell damage leading to significant loss of vascular endothelial function, enhanced vasoconstriction and reduced vasodialation (Thomas et al. 1997, Su et al. 1999). In addition to TRL-Aβ metabolism, SFA induced lipotoxicity and oxidant pathways may also trigger vascular endothelial dysfunction. Long chain fatty acids

may induce significant endothelial cell apoptosis via disturbances in protein processing and ER dysfunction (Diakoginnaki et al. 2008, Morgan et al. 2009). Other studies have also demonstrated significant cellular dysfunction as a result of heightened protein oxidation and lipid peroxidation in response to diets enriched in SFA and cholesterol (Studzinski et al. 2009, Peng et al. 1979). These findings provide evidence that dietary lipids significantly influence BBB integrity and as a consequence exaggerate blood-to-brain delivery of TRL-Aβ contributing to cerebral amyloid load.

The article also discusses the possibility that apo E isoforms regulate TRL-Aβ metabolism and influence AD risk. Evidence shows a significant increase in AD risk in individuals inheriting one or two alleles of apo E4 isoforms compared to individuals with hetero- or homo-zygous for E2 and E3 isoforms (Strittmatter and Roses 1996).

Growing evidence suggest that proteoglycans, in particular the heparan sulphate proteoglycans (HSPGs), assist in the formation and stability of cerebral amyloid plaques (van Horssen et al. 2003). However, their contribution to the entrapment of peripherally derived TRL-Aβ has not been considered. Proteoglycans are a major component of brain extracellular matrix (Nelson and Cox 2005) and have high-affinity for apo E and apo B lipoproteins. The section 6 of this manuscript demonstrated the involvement of several HSPGs in TRL-Aβ entrapment within the amyloid plaques.

Collectively, this review addressed the importance of dietary lipids in modulating cerebrovascular integrity resulting in blood-to-brain delivery of circulating TRL-Aβ. Concluding remarks also suggest the importance of pharmacological strategies for attenuation of BBB dysfunction in AD treatment.

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Review

Dietary fats, cerebrovasculature integrity and Alzheimer's disease risk

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ABSTRACT

An emerging body of evidence is consistent with the hypothesis that dietary fats influence Alzheimer's disease (AD) risk, but less clear is the mechanisms by which this occurs. Alzheimer's is an inflammatory disorder, many consider in response to fibrillar formation and extracellular deposition of amyloid-beta (Aβ). Alternatively, amyloidosis could notionally be a secondary phenomenon to inflammation, because some studies suggest that cerebrovascular disturbances precede amyloid plaque formation. Hence, dietary fats may influence AD risk by either modulating Aβ metabolism, or via Aβ independent pathways. This review explores these two possibilities taking into consideration; (i) the substantial affinity of Aβ for lipids and its ordinary metabolism as an apolipoprotein; (ii) evidence that Aβ has potent vasoactive properties and (iii) studies which show that dietary fats modulate Aβ biogenesis and secretion. We discuss accumulating evidence that dietary fats significantly influence cerebrovascular integrity and as a consequence altered Aβ kinetics across the blood–brain barrier (BBB). Specifically, chronic ingestion of saturated fats or cholesterol appears to results in BBB dysfunction and exaggerated delivery from blood-to-brain of peripheral Aβ associated with lipoproteins of intestinal and hepatic origin. Interestingly, the pattern of saturated fat/cholesterol induced cerebrovascular disturbances in otherwise normal wild-type animal strains is analogous to established models of AD genetically modified to overproduce Aβ, consistent with a causal association. Saturated fats and cholesterol may exacerbate Aβ induced cerebrovascular disturbances by enhancing exposure of vessels of circulating Aβ. However, presently there is no evidence to support this contention. Rather, SFA and cholesterol appear to more broadly compromise BBB integrity with the consequence of plasma protein leakage into brain, including lipoprotein associated Aβ. The latter findings are consistent with the concept that AD is a dietary-fat induced phenotype of vascular dementia, reflecting the extraordinary entrapment of peripherally derived lipoproteins endogenously enriched in Aβ. Rather than being the initiating trigger for inflammation in AD, accumulation of extracellular lipoprotein-Aβ may be a secondary amplifier of dietary induced inflammation, or possibly, simply be consequential. Clearly, delineating the mechanisms by which dietary fats increase AD risk may be informative in developing new strategies for prevention and treatment of AD.

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Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; apo, apolipoprotein; BBB, blood–brain barrier; CSF, cerebrospinal fluid; CVD, cardiovascular disease; DHA, docosahexanoic acid; EPA, eicosapentaenoic acid; GAG, glycosaminoglycans; HSPG, heparin sulphate proteoglycan; IgG, immunoglobulin G; LDL-r, low density lipoprotein receptor; LRP1, lipoprotein receptor related protein-1; MCI, mild cognitive impairmend; MUFA, monounsaturated fatty acid; PUFA, poly-unsaturated fatty acid; RAGE, receptor for advanced glycosylation end products; SFA, saturated fatty acid; TAG, triacylglycerol; TRL, TAG rich lipoprotein; VLDL, very low density lipoprotein.

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1. Introduction

Hallmark pathological characteristics of advanced Alzheimer's disease (AD) include hyperphosphorylation of the microtubular protein tau in neurons and extracellular deposits of protein that are enriched in the protein amyloid-beta (Aβ) [1,2]. The formation of tau-tangles results in neuronal synapse dysfunction and eventually loss of cell-cell communication, whereas disturbed Aβ kinetics may be pivotal to pro-inflammatory pathways that compromise cellular integrity [1,3,4]. Despite a substantive body of research, it is presently difficult to equivocally delineate if these pathological features of AD are causal or consequential [5,6], emphasising the therapeutic challenge of identifying the inflammatory triggers that compromise cellular integrity.

Earlier research primarily focussed on the neuronal biogenesis of Aβ in the context that overproduction may initiate formation of fibrillar Aβ deposits and thereafter inflammation [7–10]. All mutations known to cause AD increases the production of Aβ peptide. However, in sporadic and late onset AD, the most common form of AD, Aβ biosynthesis is comparable to otherwise healthy individuals [11]. Alternatively, insufficient removal of Aβ from cerebrospinal fluid (CSF) has also been proposed as a mechanism for Aβ oligomerization [12–14]. However, the brain seems potently equipped with substantive efflux processes that would otherwise prevent this. It is estimated that CSF is replenished some three times daily via the choroid plexus and indeed the epithelial cells of the choroid plexus host an array of enzymes which effectively hydrolyse potentially toxic proteins including Aβ [15]. In addition, the endothelial cells of the cerebrovasculature host receptor-proteins that permit reciprocal transfer of Aβ across the blood–brain barrier (BBB) [16–20]. Collectively, there seems to be exquisite cerebral Aβ homeostatic mechanisms and therefore the concept that cerebral Aβ-overload triggers inflammatory pathways seems physiologically unlikely.

Alzheimer's disease is a chronic disorder and shares risk factors with other diseases such as non-insulin dependent diabetes and cardiovascular disease (CVD) [21–25]. However, chronic diseases are often 'spectrum disorders' with multiple aetiology. For example, obesity is a major risk factor for diabetes and CVD [26,27], but not a requisite feature per se and 40% of subjects who experience a coronary event are normolipaemic [26,27]. Indeed, cholesterol infiltration is not always found in atherosclerotic plaque and there is substantial heterogeneity in the extent of smooth muscle cell proliferation and tissue calcification [28,29]. Such paradoxes raise the possibility that amyloidosis is simply one of many 'triggers' for dementia per se.

Common to chronic disorders, there is ample evidence that lifestyle influences AD risk and progression. Good nutrition, physical activity and environmental enrichment confer synergistic reduction in AD risk [30–37]. However, in a therapeutic context, less is known of the efficacy of lifestyle interventions on disease progression, perhaps confounded by the diversity of dementia phenotypes. Given that within 20 years the expected global health burden for dementia, of which AD accounts for 80%, will exceed treatment of any other chronic disease [38–40], exploring lifestyle therapies has become as much an economic imperative as a therapeutic priority.

Most AD research has focused on damage of neurons, however there is an increasing effort to understand the possibility of cerebrovascular dysfunction as a primary risk factor for AD. This paradigm shift is arguably warranted because vascular alterations including endothelial and smooth muscle cell proliferation precede frank amyloidosis [41]. Blood plasma proteins have been detected in the parenchyma of AD brains [42,43] and inflammatory sequelae are commonly reported [44,45], observations that are consistent with breakdown of the BBB. Targeting vascular disturbances rather than Aβ deposition may therefore be an appropriate first-focus strategy for prevention and treatment of AD.

It is reasonable to suggest that diet is important in maintaining cerebrovascular integrity [46] particularly given the overwhelming evidence that it contributes substantially to coronary artery health and CVD risk [47–53]. Population studies also generally support this contention. Saturated fats and cholesterol are both positively associated with AD risk [54–60] and in animal models, including amyloid transgenic mice, saturated fat (SFA) and cholesterol induce or exacerbate cerebral amyloidoisis [61–64]. The studies in transgenic amyloid mice are certainly consistent with a vascular contribution to disease over and above exaggerated Aβ biogenesis.

The purpose of this review is to provide contemporary consideration of the mechanisms by which dietary fats influence AD risk. Specifically, this article will focus on the putative interrelationship between plasma lipoproteins, peripheral Aβ kinetics and cerebrovasculature integrity.

2. Dietary fats and Alzheimer's disease risk

2.1. Population, clinical and animal model studies

Population studies support a role of dietary fats in AD, although this remains controversial. Laitinen reported that intake of unsaturated fats is protective, whereas intake of saturates increases risk of AD [60]. In the Framingham study, the top quartile of plasma docosahexanoic acid (DHA) (profoundly influenced by diet) was

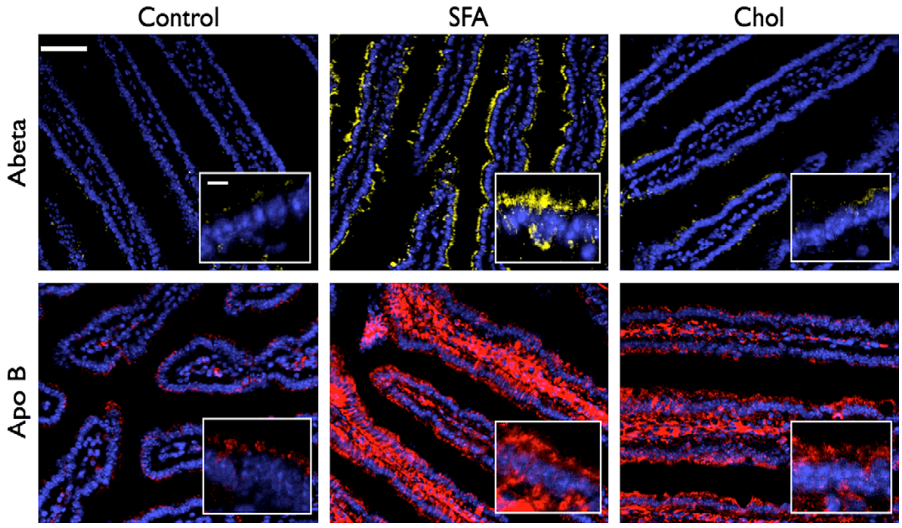

Fig. 1. Small intestine enterocytic abundance of amyloid-β and apolipoprotein B in saturated fat or cholesterol supplemented mice. The enterocytic perinuclear production and secretion into the lacteal of amyoid-β (Aβ) (top row) and apo B (bottom row) are shown in mice fed either a low-fat control, saturated fat enriched (SFA) or cholesterol supplemented (Chol) diet for 6 months. There was a significant increase of Aβ abundance concomitant with apo B in SFA-fed mice compared to control group. The cholesterol supplemented diet increased apo B abundance but had no significant effect on Aβ. Scale bar indicates 50 μm. Magnified images are also shown in each insert with 5 μm scale bar.

associated with a 47% reduction in risk of all-cause dementia [65]. Strong evidence continues to come from animal studies. Many studies show that cerebral amyloid burden and Alzheimer-like pathology is attenuated by a diet enriched in DHA in amyloid double transgenic [62,66–69]. Not surprisingly such findings have stimulated research to explore the benefits of diets low in SFA and cholesterol, such as the Mediterranean diet [47,51,70,71]. The omega 3 and 6 poly-unsaturated fatty acids (*n*3/*n*6 PUFA) have attracted particular interest. DHA and eicosapentaenoic acid (EPA) make up some 40–50% of cerebral fatty acids and are critical to neuronal plasticity and in addition may be therapeutically beneficial because of anti-inflammatory properties [69,72]. An increasing number of clinical studies demonstrate better cognitive performance in subjects with AD receiving *n*3 fatty acid supplementation [73] and meta-analysis is consistent with such a purported benefit [56,70]. However, the evidence that *n*3/6 intake reduces prevalence of AD is presently less convincing [74]. The mechanisms by which *n*3/6 confer protection have been elegantly summarized and will not be detailed here, suffice to say that these include the prevention of neuronal cell death, regulation of gene expression and anti-oxidative and anti-inflammatory functions [75–80].

2.2. Saturated fatty acids, amyloid-beta and the small intestine

The mechanisms by which dietary fats such as SFA increase AD risk may seem less of a scientific priority to delineate compared to dietary compounds that confer protection. Yet in some chronic disorders this approach has proven pivotal to developing effective therapeutic strategies for prevention and treatment of disease. For example, elucidating the role of cholesterol in atherosclerosis and cardiovascular disease led to the evolution of relatively safe and effective cholesterol-lowering drugs. Hence, significant attention will be provided in this article of possible pathways by which dietary fats increase AD risk and as a hypothesis-generating exercise.

Amyloid-beta is an amphiphylic protein normally chaperoned by transporter proteins [81]. However, strong hydrophobic domains have made distributional analysis of Aβ in blood and tissues difficult, because lipids often mask the immunodetection methodologies used to measure concentration [82,83]. Exogenous addition to blood of pre-solubilised Aβ suggests that less than 5% of Aβ binds to lipoproteins [84]. However, plasma lipoprotein fractionation and delipidation reveal that significant quantities of endogenous Aβ are associated with lipoproteins, particularly those enriched in triacylglycerol (TAG) [85]. The latter is also supported by studies in cell culture, which demonstrated that hepatocytes secrete Aβ as a lipoprotein complex [86].

Absorptive epithelial cells of the small intestine were more recently identified as another potential significant source of plasma Aβ [87–90] (Fig. 1). Enterocytic Aβ is enriched in the perinuclear region and within the lacteals, the site of chylomicron assembly and secretion respectively [90,91] and Aβ colocalizes with apolipoprotein (apo) B, an obligatory component of nascent chylomicrons (Fig. 1). Oral fat challenges in healthy subjects demonstrate a post-prandial Aβ response [92] and plasma kinetics of chylomicron-Aβ exactly parallels the metabolic pathway of the lipoprotein particle [93]. Indeed, given that Aβ significantly inhibits hepatic uptake of chylomicrons, one of its physiological roles is probably as a regulating apolipoprotein of TAG-rich lipoproteins (TRLs).

Dietary-fat regulation of enterocytic Aβ production and secretion was demonstrated in wild-type mice fed a SFA-enriched diet. Compared to low-fat fed controls, SFA substantially increased enterocytic Aβ, whereas fasting completely abolished Aβ immunoreactivity [87]. On the basis that SFA also suppress expression of receptors that are responsible for clearance of apo B lipoproteins [94–96], the findings raise the intriguing notion that dietary SFA may induce a state of post-prandial-hyperamyloidemia. Aberrations in chylomicron kinetics have been commonly reported in subjects with or at risk of CVD including otherwise normolipaemic subjects [97,98]. Chylomicron remnants are found in atherosclerotic plaque and may contribute significaintly to cholesterol deposition and inflammatory pathways [28,99,100]. Several lines of evidence are consistent with the notion that chylomicron-hyperamyloidemia may also contribute directly to amyloidosis and AD risk. Firstly, in clinical studies,

subjects with AD or mild cognitive impairment (MCI) have significantly greater plasma Aβ in a plasma TRL fraction that includes chylomicrons [85]. Moreover, plasma apo B_{48} (an exclusive marker of chylomicrons) was increased fourfold in post-absorptive AD subjects compared to age matched controls [85]. In amyloid transgenic mice that are established models of AD, onset and progression of disease was found to strongly correlate with secretion into blood of TRL-Aβ and plasma TRL-Aβ concentration [101]. In addition, studies showed apo B immunoreactivity associated with amyloid plaque of human brain specimens [102]. However the latter was not considered in the context that it may have reflected blood-to-brain delivery of plasma lipoprotein derived Aβ.

3. Blood-to-brain delivery of triacylglycerol-rich-lipoprotein amyloid-beta

3.1. Receptor mediated cerbrovascular amyloid-beta kinetics

The receptor for advanced glycosylation end products (RAGE) is one endothelial cell protein found to facilitate Aβ transfer from blood-to-brain [16,17]. However, there is no evidence that TRL or lipoproteins per se bind to RAGE, requiring therefore transfer of Aβ from the lipoprotein particle to the aqueous mileu prior to transport via this pathway. However, in vivo and in vitro studies suggest that Aβ binds tightly to TRL and is not shed or transferred to other chaperone transporters [93], so RAGE-mediated transfer of lipoprotein derived Aβ seems unlikely.

The low-density-lipoprotein receptor related protein-1 (LRP1) is expressed within the cerebrovascular endothelial cell junctions and binds with substantial affinity to both 'free' Aβ as well as to TRL-remnant lipoproteins that have become depleted of TAG. However, LRP1 is considered to be principally involved in cerebral efflux of Aβ to blood rather than influx of Aβ from plasma [12,13,17]. In AD subjects and animal models there appears to be a shift in RAGE relative to LRP expression [13,17,103,104], consistent with the possibility of a gradient shift of Aβ kinetics from blood-to-brain, but it is presently unclear if this pathway is relevant to TRL-Aβ metabolism.

3.2. Vasoactive active properties of amyloid-beta

Cerebral extravasation of TRL-Aβ may also occur non-specifically because of broader disruption of the cerebrovasculature. Plasma proteins, including apo B, have been reported in CSF and parenchyma of subjects with AD [42,44,102,105]. Indeed, raised TRL-Aβ may be responsible of the vascular disturbances which lead to increased rates of peripheral delivery [101,105,106]. Indirect evidence that TRL-Aβ may have vasoactive properties is provided by cell culture and animal model studies which investigated the effect of exogenous Aβ administration. Intravascular administration of solubilised Aβ induces significant endothelial cell damage with changes in the cell membrane, cytoplasm, nucleus and other organelles [107]. Sequestration of Aβ within brain capillaries was reported in several studies [18–20] and longer term administration of Aβ (2 weeks), resulted in a significantly compromised BBB and activated central-nervous-system glial cells [108].

The exposure of blood vessels to exogenous Aβ induces enhanced vasoconstriction and diminished vasodilation accompanied with lack of elasticity that are commonly seen in aged animals. In a study by Thomas et al. loss of endothelial function was demonstrated with acute Aβ exposure of bovine cerebral middle artery [107]. This vascular damage was prevented by the anti-oxidant enzyme superoxide-dismutase and the free radical scavenger PBN12, suggesting that reactive oxygen species may be involved in the vasoconstrictive action of Aβ. Morphological disturbances including necrotic cell damage accompanied the inflammatory response induced by Aβ exposure.

Animal model studies confirm blood-to-brain delivery of Aβ when the plasma concentration is chronically elevated. In study by LaRue et al., transport of Aβ across the BBB was increased eight fold in amyloid transgenic mice compared to wild-type controls [109], a process that could be inhibited by immunization [12].

3.3. Dietary saturated fats and blood–brain delivery of lipoprotein associated amyloid-beta

The vasoactive properties of exogenous Aβ and in hyperamyloidic transgenic mice led us to explore the hypothesis that dietary SFA increases plasma TRL-Aβ and that with chronic ingestion this consequently leads to blood-to-brain delivery of TRL-Aβ. In a recent study, wild-type mice were fed diets modified diets enriched in either SFA, monounsaturated (MUFA) or poly-unsaturated (PUFA) fatty acids and compared with low-fat fed controls [106] (Fig. 2). Following 3 months of dietary intervention there was remarkable parenchymal colocalization of Aβ with apo B immunoreactivity in SFA-supplemented mice. Six months of SFA feeding increased immunoreactive Aβ/apo B compared to the 3 month fed group and the pattern of distribution was remarkably similar to Aβ/apo B colocalization in APP/PS1 amyloid transgenic mice with cortex > brain stem > hippocampal formation. However, there was no evidence that TRL-Aβ delivery to brain occurred in either MUFA, PUFA or low-fat fed mice.

A shift in receptor-mediated transport across the BBB may have contributed to extravasation of apo B-Aβ in SFA mice. However, several other markers suggest that was more likely to be a non-specific phenomenon. Immunoglobulin G (IgG), a large molecular weight plasma protein, was evident in parenchyma of SFA-fed mice and occludin expression, the primary endothelial tight junction protein was substantially reduced compared to controls. In addition, the plasma concentration of S100B, a CSF abundant protein, was increased in plasma suggesting bidirectional disturbances in protein transport across the BBB.

4. Saturated fatty acid induced disturbances in blood–brain barrier integrity

4.1. Triacylglycerol-rich-lipoprotein amyloid-beta-induced cerbrovascular disturbances

It is proposed that post-prandial hyperamyloidemia is one possible mechanism for SFA-induced BBB dysfunction and delivery of TRL-Aβ from blood-to-brain, but presently this remains to be substantiated. Rather, we found that the plasma concentration of $A\beta_{1-40}$ and $A\beta_{1-42}$ in SFA-fed mice was similar to mice maintained on either MUFA, PUFA or low-fat (control) diets [106] (Fig. 2). However, caution must be exercised with this interpretation. Post-prandial hyperamyloidemia may not have been apparent in those studies because the mice had been deprived of food for approximately 6 h before blood was sampled. Alternatively, repetitive but transient (post-meal) exposure to post-prandial-Aβ may be sufficiently damaging to endothelial integrity, without inducing a state of basal hyperamyloidemia. Consistent with this concept, in non-demented participants significant variation in CSF-Aβ levels of 1.5- to 4-fold were detected over 36 h of serial sampling. Amyloid-β_{1-40} and $A\beta_{1-42}$ were highly correlated over time indicating that similar processes regulate the concentration of these isoforms. On average, the fluctuations of Aβ levels appeared to be time of day or activity dependent [110]. Methodological limitations may also be a confounder in interpretation. It is possible that the immunoassays used to measure plasma Aβ are not sensitive to the

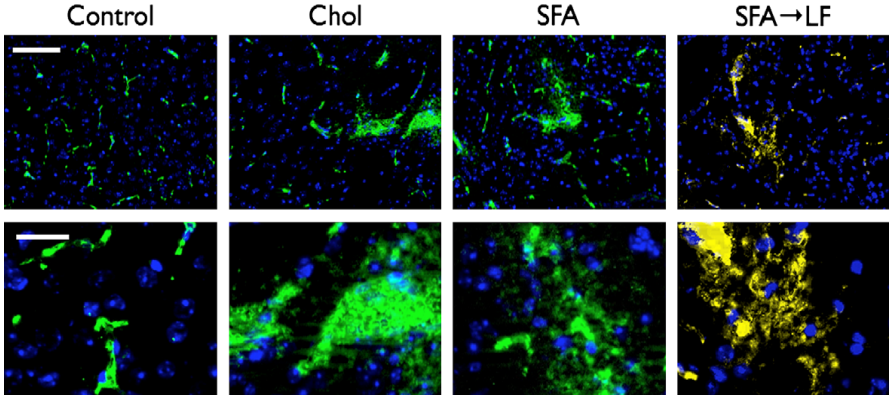

Fig. 2. Integrity of blood–brain barrier shown by the cerebral distribution of plasma immunoglobulin G. The cerebral distribution of immunoglobulin G (IgG) is shown in mice fed either a low-fat control, saturated fat enriched (SFA) or cholesterol (Chol) supplemented diet for 60 days. Significant peri-vascular leakage of IgG was seen in both SFA and cholesterol fed mice consistent with BBB dysfunction. Fourteen days after replacing the SFA diet with the low-fat diet (SFA → LF) IgG extravasation persisted. Scale bar in top and bottom row represents 100 μm and 30 μm, respectively.

lipidated form of Aβ and are unable to detect the putative SFA-induced increase in this pool of Aβ.

4.2. Saturated fatty acid induced amyloid-beta independent cerebrovacular disturbances

Several non-Aβ mediated pathways could also contribute to SFA-induced cerebrovascular disturbances. Dietary 'lipotoxicity' refers to the processes leading to end-organ damage and/or dysfunction following excess exposure to fatty acids identified in the context of fat-induced insulin resistance [111]. However, the process has also been implicated in endothelial dysfunction and atherosclerosis, heart failure, kidney failure, steatohepatitis and liver failure, autoimmune inflammatory disorders, susceptibility to infections, cancer and ageing. Significant differences in the cytotoxic effects of fatty acids have been reported, with longer chain SFA's being the most potent and the mono- and poly-unsaturated fatty acids being cytoprotective [112]. Morgan [112] suggests that the underlying toxicity of SFA is a consequence of disturbances in protein processing and endoplasmic reticulum dysfunction, for example apoptotic induction. One relevant example was a study by Patil et al. who found that palmitic acid induced region-specific cerebral damage because of higher fatty acid-metabolizing capacity of cortical astroglia [113]. Conversely, cell culture studies suggest that incubation, particularly with longer chain unsaturates has an antagonistic effect on endoplasmic reticulum-centred stress pathways [114]. If this were the case, then amyloidosis may be a phenomenon secondary to cerebrovascular inflammation.

Animal feeding studies have shown that typical Western diets substantially increase protein oxidation and lipid peroxidation [115,116]. In APP/PS1 mice, this occurred in the absence of increased Aβ levels [115]. In addition, differences in membrane lipid status as a consequence of diet may influence the propensity for Aβ oligomerization to occur [117]. Exogenous fatty acid supplementation results in significant shifts in neuronal phospholipids and in lipid raft composition [118–120], key regulators of cell protein transport and inflammation. Dietary fats also influence expression of critical genes involved in Aβ kinetics, for example the scavenger protein transthyretrin [77]. An alternate perspective is provided by Hooijmans and colleagues, who suggested that dietary fats influence AD risk because of chronic changes in cerebral hemodynamics [68]. In APP/PS1 mice fed DHA, plaque burden was attenuated probably because of greater blood circulation in the brain due to vasodilation. In contrast a Western diet rich in saturated fats and cholesterol increased amyloidoisis but without any changes to net blood volume or flow.

4.3. Cholesterol-induced disturbances in blood–brain barrier integrity

Studies by Ghbiri et al. found that like SFA, dietary cholesterol results in BBB dysfunction in New Zealand white rabbits [121]. Chronic dietary cholesterol supplementation also results in cerebral amyloidosis in wild-type rabbits, but this was not explored in the context of raised plasma TRL-Aβ [61]. However, indirect evidence that aberrant lipoprotein metabolism is involved in NZ-White rabbits fed cholesterol is suggested by the observation that the animals become grossly hypercholesterolemic as a consequence of apo B lipoprotein accumulation.

We have confirmed that modest dietary supplementation with cholesterol disturbs BBB function and, like SFA, extravasation of apo B/Aβ is observed within the brain parenchyma [106]. However, unlike the rabbit studies, mice were normolipaemic. Dietary cholesterol supplementation also had no measurable effect on plasma $A\beta_{1-40}$ or $A\beta_{1-42}$ in wild-type mice (albeit with the caveats in measurement discussed), consistent with the concept that the effects on BBB function were plasma Aβ independent. Cell culture studies suggest several mechanisms by which dietary cholesterol may be toxic. Frears et al. observed that, in the presence of cholesterol, human AβPP transfected HEK cells secrete greater quantities of Aβ [122]. However, the effects of cholesterol on Aβ biosynthesis are uncertain because cholesterol lowered Aβ synthesis in primary cell cultures of rat embryo hippocampal neurones [123] and dietary cholesterol reduces enterocytic abundance of Aβ [89]. Clearly, the effects of dietary cholesterol on net TRL-Aβ secretion in vivo need to be established. Alternatively, Subasinghe et al. showed that cholesterol can enhance Aβ induced toxicity because of increased protein binding to the plasma membrane and accelerated oligomerization of Aβ [124]. Yao and colleagues suggest that like SFA, excess cholesterol causes ER and mitochondrial stress that can lead to apoptosis [125,126]. Mitochondrial activity or lysosomal processing can result in the production of oxidized lipids including cholesterol. A number of studies support the contention that oxidized lipids compromise tissue integrity and exacerbate inflammatory pathways [127,128]. Interestingly, Stanyer and colleagues reported that plasma lipoproteins, particularly when oxidized, promote Aβ polymerization [129].

5. Apolipoprotein E phenotype, apo B-amyloid beta metabolism and Alzheimer's disease risk

5.1. Apolipoprotein E isoforms and Alzheimer's disease risk

Inheriting one or two alleles for apo E4 increase the risk of AD by 17% and 43%, respectively, compared to individuals hetero- or homo-zygous for apo E2 and E3 isoforms [130]. A number of hypotheses have been put forward for the positive association of AD with apo E4 and reviewed extensively in the literature [92,131–139]. Briefly, key concepts include; poorer sequestration of soluble Aβ and hence a propensity for oligomers to form; increased Aβ biosynthesis by regulating the activities of APP cleavage enzymes of beta- and gamma-secretase; disturbances in cholesterol homeostasis, which in turn will regulate Aβ biogenesis; pro-inflammatory and oxidative stress triggers; improper maintenance of BBB integrity; and defective neuronal growth. In this review, we wish to also consider the possibility that apo E isoforms influence AD risk via differential modulation of TRL metabolism.

5.2. Apolipoprotein E isoforms and triacylglycerol-rich-lipoprotein metabolism

More than 98% of plasma apo E exists in a lipidated form, principally bound to post-hydrolyzed TRL-remnants [140,141]. Apo E is the principal protein of chylomicrons, making up approximately 65% of total protein mass [142]. The acquisition by TRL of apo E inhibits interaction with endothelial lipases, serving instead as the binding ligand to high affinity receptors involved in TRL-remnant uptake [143–145]. The primary receptor responsible for TRL-remnant uptake is the low-density-lipoprotein receptor (LDL-r), however if this pathway becomes rate-limiting other high affinity processes such as lipoprotein–receptor-related protein one LRP1 may partially compensate.

In man, the three principal apo E isoforms are differentially distributed, probably because of differences in lipophilicity. Curiously, apo E4 is distributed with remnant lipoproteins that contain relatively more TAG (principally chylomicrons), whereas apo E2 and apo E3 tend to be primarily associate with hepatically derived TRL remnants, (i.e. intermediate density lipoproteins) [146].

There are several pathways by which apo E or specific variants may synergistically influence TRL-Aβ mediated AD risk. Firstly, apo E4 does not support proper BBB functionality compared to apo E2 and E3 [103] which may result in amplified blood-to-brain delivery of plasma proteins including TRL-Aβ. Apo E per se has significant affinity for extracellular matrices in particular the heparin sulphate proteoglycans (HSPG). Physiologically this is an important function as it facilitates interaction with proteins involved in receptor-mediated uptake. Binding of apo E to HSPG is an initial step in the localization of TRL-remnant to the surface of several different types of cells. Thereafter, the TRL-remnants are transported into the cell by receptor-mediated pathways, or by direct uptake of apoE-containing lipoprotein-HSPG complex [135]. Studies by Libeu et al. found that apo E has an HSPG-binding site highly complementary to heparin sulphate rich in *N*- and *O*-sulfo groups in the brain and liver [147].

The physiological effect of apo E variant on HSPG binding is difficult to predict although mutations in apo E have demonstrated potentially substantial differences in affinity [148,149], Arg-142 [150,151], Arg-145 [151], and Lys-146 [152]. However, the dissociation constant of equilibrium K_D of the principal apoE isoforms and glycosaminoglycans (GAGs) was found to be similar [153]. Collectively, apo E may mediate extracellular retention of TRL-Aβ if delivered from blood-to-brain but presently there is no clear evidence to suggest this would be exacerbated in individuals who express the apo E4 variant.

In atherosclerotic plaque, retention of apo B/E lipoproteins within the subendothelial space is considered the triggering event for monocyte infiltration. Activated macrophages are potently equipped with an array of receptors capable of internalizing TRL-Aβ [154]. Apo E serves as the principal lipoprotein binding ligand for many of these uptake pathways including the LDL-r and LRP1. Activated macrophages will secrete substantial quantities of apo E to enhance the efficiency of lipoprotein internalization [155]. Oxidative modification of lipoproteins may occur particularly if retention is prolonged. Modification, enables macrophage internalization by additional apo E mediated pathways, such as via the scavenger receptor [156] and the oxidized LDL receptor LOX-1 [157]. Evidence that apo E variants may influence the inflammatory pathway comes from primary cultures of macrophages. Macrophages expressing apo E4 enhanced atherosclerotic pathways compared to apo E3 macrophages, by promoting LDL-r mediated lipoprotein uptake [158]. Moreover, apo E4 was also found to be less efficient at conferring oxidative protection than apo E3. In another study the murine monocyte–macrophage cell line (RAW 264.7) was stably transfected to produce equal amounts of human apoE3 or apoE4. Following lipopolysaccharide stimulation, apoE4-macrophages showed higher and lower concentrations of tumour necrosis factor alpha (pro-inflammatory) and interleukin 10 (anti-inflammatory). In addition, increased expression of heme oxygenase-1 (a stress-induced anti-inflammatory protein) was observed in the apoE4-cells. The apoE4-macrophages also had an enhanced transactivation of the key redox sensitive transcription factor NF-κB.

A number of studies have shown that TRL-remnants are efficiently degraded by macrophages. If uptake occurs, a mitochondrial respiratory burst and lysosomal exocytosis results in the release of potent cytotoxic compounds such as superoxide, which compromise cellular integrity [159]. Proteinaceous deposits may be formed if cell death occurs and it is likely this exacerbates inflammatory pathways. The latter would suggest that amyloidosis is a secondary inflammatory trigger but pivotal to a subsequent cyclic phenomenon. Glial cell activation is the hallmark of inflammation in the brain [160]. Activated microglia produce inflammatory molecules such as cytokines, growth factors and complement proteins [161–163]. These mediators of inflammation in turn activate other cells to produce additional signalling molecules that further activate microglia in a positive feedback loop to perpetuate and amplify the inflammatory signalling cascade [164].

Apo E is an important ligand for binding of TRL-remnants to LRP1, a key endothelial junction protein thought to primarily facilitate cerebral efflux of Aβ. However, in cultured 293 cells, LRP1 had approximately equal affinity for apo E2/E3 and E4 [165], suggesting that cerebrovascular-mediated efflux of Aβ via LRP would not be unduly different in subjects with apo E4 alleles.

6. Apolipoprotein B/amyloid beta association with proteoglycans in a murine model of Alzheimer's disease

6.1. Apolipoprotein B association with agrin, perlecan, biglycan and decorin

Proteoglycans are major components of the extracellular matrices, comprised of one or more glycosaminoglycans chains covalently attached to a core protein [166]. Proteoglycans may serve as binding sites for receptors, or as mediators of cell adhesion, migration and proliferation [166]. Studies over the past decade suggest that proteoglycans, in particularly heparin sulfate proteo-

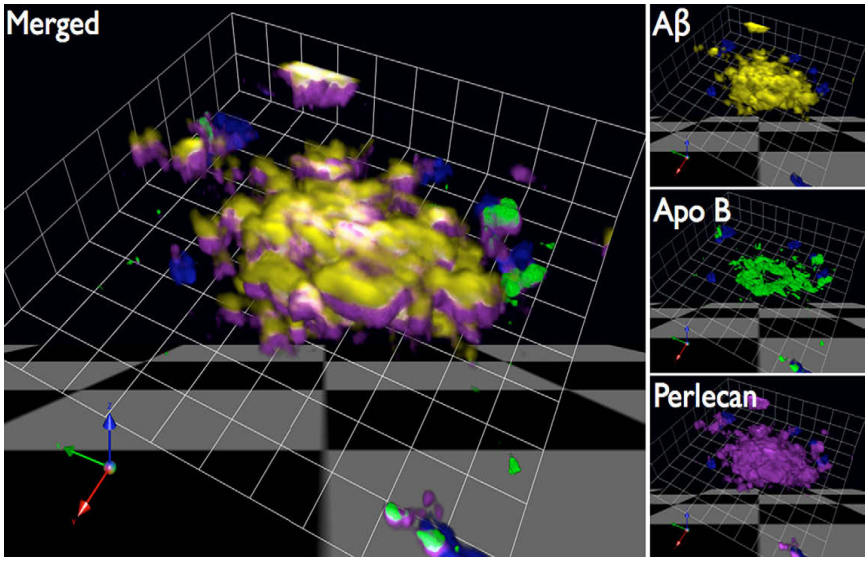

Fig. 3. The colocalization of apolipoprotein B, perlecan with cerebral amyloid plaques in amyloid transgenic mice. A 3-D triple immunofluorolabelling technique was utilized to investigate the colocalization of apolipoprotein B (apo B) and the proteoglycans perlecan, biglycan, agrin and decorin in the amyloid plaques of APP/PS1 transgenic amyloid mice. A representative image of apo B and perlecan colocalization is shown. There was significant colocalization of all four proteoglycans with amyloid plaque, but only perlecan, biglycan and decorin were positively associated with apo B lipoprotein retention (Lam, Takechi and Mamo unpublished observations). One unit of scale is indicative of 5 μm.

glycans, contribute towards the formation and thereafter stability of amyloid plaques [167]. However, their putative role in lipoprotein-Aβ entrapment has not been considered.

Proteoglycans bind apo E and apo B via ionic interactions with the core protein of proteoglycans [168,169] and proteoglycan mediated retention of apo B/E lipoproteins within the subendothelial space of arterial vessels is considered as the initiating event for atherosclerosis [170,171].

We suggest four proteoglycans that may be of particular importance to parenchymal binding of TRL-Aβ. Agrin is an extracellular matrix-associated HSPG pivotal for the development and the maintenance of the BBB and the formation of the neuromuscular junction [172]. Agrin exhibits structural similarity to perlecan, a proteoglycan reported to bind apo B lipoproteins in the hepatic sinusoidal space [173].

Perlecan, the largest extracellular matrix HSPG, has the capacity to facilitate the interaction of apo B and E lipoproteins with receptor-mediated pathways [173,174]. Perlecan exhibits structural homology to the ligand binding region of LDL-r, the primary pathway for apo B and apo E rich particle internalization [173,175]. Perlecan over-expression within the subendothelial space of coronary vessels has been implicated in the pathogenesis of atherosclerosis as a consequence of increased lipoprotein retention [176].

Cerebral biglycan expression in AD has not been reported. However, biglycan has significant affinity for apo B and E containing lipoproteins [177,178] and may contribute to the cerebral retention of TRL-Aβ. In atherosclerotic tissue, biglycan abundance is substantially greater in comparison to healthy tissue [179,180]. Moreover, there is a positive association between biglycan and arterial accumulation of apo B and E containing lipoproteins [177,178]. Decorin exhibits structural homology to biglycan (57%), including the apo B/E binding domain [173].

An immunhistological approach was used to investigate the putative colocalization of apo B/Aβ with agrin, perlecan, biglycan and decorin in an established murine model of AD. Double transgenic amyloid mice (APP/PS1) have an eightfold higher concentration of Aβ compared to wild-type mice and develop cerebral amyloid plaque by 6 months of age [105]. In APP/PS1 mice, focal accumulation of apo B lipoproteins was found with Aβ-plaque (Fig. 3). We found enrichment in cerebral amyloid deposits of the proteoglycans, agrin, perlecan, biglycan and decorin within the core of dense Aβ-plaque and an example of the perlecan/apo B/Aβ collocation is shown in Fig. 3. The Pearson's correlation coefficient was used as a measure of interdependent proteoglycan/apo B/Aβ association [181]. Of the four proteoglycans investigated, perlecan, biglycan and decorin were all positively associated with apo B lipoprotein abundance and with Aβ (Lam, Takechi and Mamo, unpublished data). These findings suggest that some proteoglycans contribute to Aβ retention and by extension amyloidosis, whilst other proteoglycans may have different functions, for example plaque stabilization [174,182,183].

7. Do hepatic and intestinally-derived apo B lipoproteins both contribute to Alzheimer's disease risk via increased blood-to-brain delivery and extracellular entrapment?

7.1. Apolipoprotein B isoforms and triacylglycerol-rich-lipoprotein kinetics

In man, hepatically derived TRL can be distinguished from chylomicrons based on the apo B_{100} and apo B_{48} isoforms respectively [184,185]. Apo B_{48} is synthesized in enterocytes as a consequence of mRNA processing and essentially represents half of the apo B_{100} amino acid sequence. It's not clear why this editing process occurs specifically in absorptive epithelial cells of the small intestine of man, suffice to say that this may be responsible for constitutive rates of chylomicron biogenesis in the absence of ingested fats.

Nascent TRL secreted from liver and intestine share similar metabolic pathways but there are some significant differences in metabolism which may be important in understanding AD risk. Chylomicrons and very low density lipoprotein (VLDL) interact

with endothelial lipases and become progressively depleted in TAG. The apo E rich post-hydrolyzed remnants then bind to receptors responsible for internalization. Chylomicrons are generally larger than VLDL and contain more TAG, yet hydrolysis to the remnant form is quicker. Once in circulation, chylomicron lipolysis and clearance is generally complete within about 15 min. Hydrolysis of VLDL TAG may take up several hours and approximately half of the VLDL-remnants will persist in circulation to become cholesterol rich (and apo E poor) LDL.

Several, but not all clinical studies suggest that fasting plasma apo B, which is primarily indicative of hepatically-derived lipoproteins, may be increased in subjects with AD [186]. Thus far only one study reported apo B_{48} in AD/MCI subjects and this was found to be substantially increased in the post-absorptive state [85]. Whilst the latter is consistent with post-prandial chylomicronemia, classical oral fat challenge tests have not yet been reported in AD/MCI subjects.

Unlike man, the liver of mice primarily secretes apo B_{48} and so there is no clearly distinguishing difference per se between lipoproteins of intestinal or hepatic origin in this species. Preliminary studies suggest that SFA supplementation in wild-type mice does not have the same stimulatory effect on Aβ and apo B abundance in hepatocytes as that observed for enterocytes (Galloway and Mamo, unpublished observations). This finding suggests that whilst the BBB disturbances reported in wild-type mice because of SFA feeding was specifically a post-prandial phenomenon, it does not rule out a role for hepatically-derived lipoproteins per se. Presently, there is no rationale to suggest that elevated apoB_{100} lipoprotein-Aβ would be any less challenging to cerebrovascular integrity than apo B_{48} lipoprotein-Aβ. A fundamental question then is whether extracellular entrapment by proteoglycans of apo B_{100} lipoproteins substantially differs from apo B_{48} lipoproteins. Both isoforms of apo B bind to heparin proteoglycans with significant affinity however the amino acid residues responsible differ substantially for the two isoforms [168,171]. Subtle differences in lipid composition can profoundly affect lipoprotein interaction with receptors and extracellular matrices so it is impossible to unequivocally say if there is a generic difference between apo B lipoproteins of hepatic and intestinal origin. In human atherosclerotic plaque both apo B_{100} and apo B_{48} are found [28,175], however there seems to be substantially more apo B_{48} than apo B_{100} relative to the plasma concentration of the two lipoprotein subtypes [187]. In LDL-r deficient rabbits that have massively elevated levels of apo B_{100} and apo B_{48} lipoprotein, only the latter was significantly increased in atherosclerotic plaque compared to healthy arterial tissue [188]. On the other hand, over-expression of human apo B (excluding brain) induces severe neurodegeneration in transgenic mice concomitant with elevated plasma TAG and Aβ deposition [189]. Collectively, there is some evidence to suggest that intestinally-derived apo B lipoproteins may be more prone to extracellular retention. However, their concentration in blood is typically much less than that of hepatically derived apo B lipoproteins. Therefore, information about the relative distribution of apo B isoforms in brain parenchyma and amyloid plaque would be informative.

8. Conclusion

The critical observations considered in this review are that dietary saturated fats and cholesterol cause BBB dysfunction, resulting in the blood-to-brain delivery of apo B lipoprotein-Aβ. In some individuals, dietary-induced disturbances in BBB integrity may be the initiating event for AD. If cerebrovascular disturbances are central to AD aetiology and progression, then considering strategies to positively influence integrity is a therapeutic priority. Presently, drug strategies used to treat AD are focussed on maintaining cell-cell communication rather than cerebrovascular function.

Some, but not all, clinical studies suggest that statins may reduce AD risk and progression [190–192] although the mechanisms for this putative effect are unclear. Relevant to the focus of this review, possibilities include reduced TRL-Aβ secretion; enhanced clearance from blood of TRL-remnants containing Aβ; maintenance of BBB function and anti-inflammatory properties. Fibrates can profoundly reduce TRL-secretion, but their efficacy in the context of BBB function and AD risk has not been considered.

Understanding the mechanisms by which dietary fats influence AD risk reinforces and substantiates the good nutrition public health strategies for prevention of disease. In a treatment context there may also be substantial value in knowing these mechanisms. However, developing nutritional/lifestyle or drugs which potentially may confer cerebrovascular benefit is not likely to be useful unless environmental and endogenous cerebrovascular 'insults' are synergistically considered.

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Article 4:

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Synopsis

Background:

Proteoglycans are major components of the extracellular matrix of the brain and serve as binding sites for receptors. They facilitate cell adhesion, migration, proliferation and the development of the BBB (Nelson and Cox 2005). In addition, HSPGs play a vital role in the pathogenesis of AD by enhancing the formation and stability of amyloid plaques (van Horssen et al. 2003). The significant affinity for apo B lipoproteins suggests a putative role in TRL-Aβ entrapment within brain parenchyme.

Our previous findings support the concept that extravasation and retention of plasma derived Aβ may contribute to cerebral amyloid load and the propensity to form insoluble fibrils (Takechi et al. 2010a). Heparan sulphate proteoglycans may contribute to TRL-Aβ accumulation because of affinity for apolipoproteins such as apo B, apo E or perhaps Aβ per se. This study was designed to explore the putative association between apo B lipoproteins and primary cerebral proteoglycans (agrin, perlecan, biglycan and decorin) within amyloid plaques of transgenic amyloid mice.

Methods in brief:

3-D immunofluorescence microscopy was utilised to demonstrate the association between apo B lipoproteins and proteoglycan expression within amyloid plaques in APP/PS1 transgenic mice, and were compared to their WT controls of similar age. The degree of colocalisation and quantitative protein abundance was determined by the Mander's overlap coefficient and Pearson's correlation coefficient, respectively.

Results in brief:

Accumulation of apo B was found within the core of the amyloid plaques in APP/PS1 mice. Furthermore, proteoglycans perlecan, biglycan and decorin were positively correlated with apo B lipoprotein abundance within the Aβ deposits. However, there was no correlation between agrin with apo B or Aβ within the plaques.

Discussion and conclusion in brief:

The findings of this study show that proteoglycans perlecan, biglycan and decorin are an integral component of amyloid deposits in APP/PS1 mice. These proteoglycans may contribute to parenchymal retention of plasma derived TRL-Aβ and aggravate amyloid plaque formation. The findings are consistent with a causal effect in the aetiology of AD.

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Colocalisation of plasma derived apo B lipoproteins with cerebral proteoglycans in a transgenic-amyloid model of Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is characterized by cerebral proteinaceous deposits comprised of amyloid beta (Aβ). Evidence suggests that enhanced blood-to-brain delivery of Aβ occurs when plasma concentration is increased, exacerbating amyloidosis. In blood, significant Aβ is associated with apolipoprotein (apo) B lipoproteins. In this study, immunofluorescent microscopy was utilised to explore if there is an association between apo B lipoproteins and proteoglycan expression within Aβ-rich plaques in transgenic-amyloid mice. Focal accumulation of apo B was found with Aβ-plaque in APP/PS1 mice. There was enrichment in the proteoglycans, agrin, perlecan, biglycan and decorin within the core of dense Aβ-plaque. Perlecan, biglycan and decorin were positively associated with apo B lipoprotein abundance within amyloid plaque consistent with a cause-for-retention effect. These findings show that proteoglycans are an integral component of Aβ deposits in APP/PS1 mice. This study suggests that some proteoglycans contribute to Aβ retention, whilst other proteoglycans have different functions in the aetiology of AD.

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Amyloid beta (Aβ), is a primary component of cerebral proteinaceous deposits that are found within brain parenchyma in subjects with Alzheimer disease (AD) [39]. Neuronal cells synthesize Aβ, however there is no evidence that accelerated biogenesis occurs in sporadic late onset AD, the most common form of this disorder [6]. Cerebral Aβ homeostasis is also dependent on degradation and clearance pathways that occur within the epithelial cells of the choroid plexus and via receptor mediated efflux of Aβ on vascular endothelial cells, [28,38]. Several studies suggest that degradation and efflux of Aβ may be impaired in subjects with AD, enhancing the propensity for Aβ-oligomer formation to occur [5,7]. Amyloid-β burden may also be exacerbated if cerebrovascular integrity is compromised, a common pathological co-morbidity in subjects with AD. Recent studies showed exaggerated blood-to-brain delivery of Aβ in amyloid transgenic mice and in wild-type mice and rabbits with dietary induced amplification of amyloidosis [30,31,11].

In blood, significant Aβ is associated with apolipoprotein (apo) B lipoproteins of intestinal and hepatic origin [14,16], a pathway of amyloid biogenesis that is regulated by the ingestion of fats [10]. In wild-type mice, saturated fat-feeding and cholesterol feeding independently and synergistically compromise cerebrovascular integrity, resulting thereafter in exaggerated parenchymal infiltration and retention of apo B lipoprotein-associated-Aβ [20,32]. Burgess et al. found in amyloid transgenic mice that the onset and progression of amyloidosis was positively associated with the plasma concentration of Aβ [2]. The latter correlated with the hepatic and intestinal secretion of apo B-lipoproteins. Apo B immunoreactivity has been reported in the brain parenchyma of post-mortem AD subjects and models of AD [30,40,17].

Proteoglycans are major components of the extracellular matrix, characterized by a core protein to which glycosaminoglycan side chains are covalently attached [18]. Proteoglycans serve as binding sites for receptors and as mediators of cell adhesion, migration and proliferation [18]. Proteoglycans, in particularly heparan sulfate proteoglycans, have an important role in the pathogenesis of AD by enhancing the formation and stability of amyloid plaques [36]. However, their putative role in lipoprotein-Aβ entrapment has not been considered.

Agrin is an extracellular matrix HSPG pivotal for the development of the BBB [1]. Agrin exhibits structural similarity to perlecan [13], a proteoglycan reported to bind apo B lipoproteins in the hepatic sinusoidal space and murine models of atherosclerosis [33]. Perlecan, exhibits structural homology to the ligand binding region of the apo B/E lipoprotein receptor, the primary pathway for lipoprotein internalization [25,22]. Perlecan over-expression within the sub-endothelial space of coronary arteries has been implicated in the pathogenesis of atherosclerosis [15]. A number of studies have reported an increased abundance of agrin and per-

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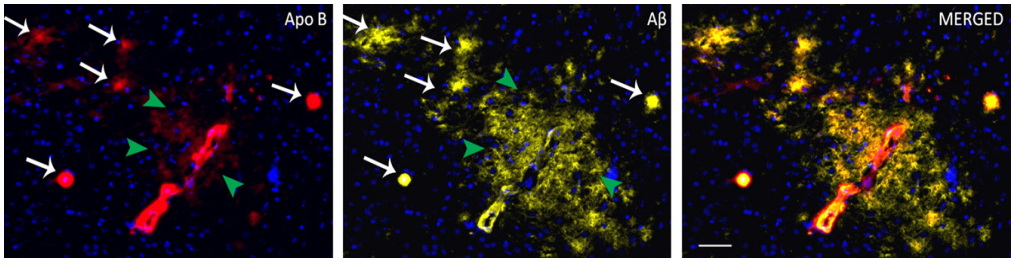

Fig. 1. Colocalisation of Aβ-plaques and apolipoprotein B in APP/PS1 mice. The immunofluroescent images captured demonstrate the perivascular leakage of plasma apolipoprotein B (green arrowheads) concomitant with Aβ-plaque staining (white arrows). Scale bar indicates 50 μm.

lecan in amyloid plaque [27,8,35], however the putative association with lipoprotein retention was not explored.

Cerebral biglycan expression in AD tissue has not been reported. Biglycan has affinity for apo B lipoproteins and its expression is overtly increased in atherosclerotic lesions [19,21]. Decorin exhibits 57% structural homology with biglycan [13], including the apo B/E binding domain. Biglycan and decorin may mediate retention of parenchymal apo B-Aβ.

The hypothesis underpinning this study is that the proteoglycans agrin, perlecan, biglycan or decorin can bind amyloid enriched apo B lipoproteins that have penetrated brain parenchyma and contribute to amyloid plaque formation.

Twelve-month male amyloid transgenic and C57BL/6J (control) mice were maintained on a standard rodent chow diet for 12 months. Cerebral tissue samples were collected and the distribution and abundance of Aβ, apo B and proteoglycans determined by 3-D-immunofluorescent microscopy [30]. Double immunolabelling was achieved utilizing a biotin–avidin amplification method [29]. Core protein antibodies against agrin, biglycan, decorin and perlecan were from Santa Cruz, USA. Mouse monoclonal anti-Aβ 6E10 and rabbit polyclonal anti-apolipoprotein B antibodies (Abcam, UK) were used as markers of Aβ and apo B-lipoproteins.

Cryosections (10–20 μm) were fixed in 4% paraformaldehyde followed by antigen retrieval at 60 °C. Non specific binding was blocked with 20% goat serum in PBS. For apo B, sections were incubated with anti-apo B (1:50,000) for 20 h. The sections were incubated with anti-rabbit IgG conjugated with biotin for 1 h (1:200). Following washing, antibodies directed against Aβ (6E10; 1:200) and the selected proteoglycan were applied for 20 h at 4 °C. Premixed fluorescently conjugated secondary antibodies were streptavidin Alexa 546 (1:100) for the detection of apo B, anti-rabbit IgG-Alexa 488 (1:100) for each of the proteoglycans and anti-mouse IgG_1-Alexa 680 (1:100) for Aβ (6E10) (Invitrogen). Nuclear counterstain was with DAPI.

Images were acquired with a fluorescent microscope (AxioVert 200M), coupled to a digital camera (AxioCam mRM) and managed by AxioVision Software, (Carl Zeiss, Germany). Up to six tissue slides were prepared for each mouse ($n = 4$). Three-D images of plaque were then constructed using the ApoTome optical sectioning module and each 3D image consisted of between 10 and 75 2D images optimised by the Nyquist theory. The colocalisation between amyloid beta, proteoglycans and apo B was determined for each 2D image via the AxioVision colocalisation module. The degree of colocalisation is indicated by the Mander's overlap coefficient whereas Pearson's correlation coefficient provides a quantitative estimate of protein abundance [30,29].

Apo B is an exclusive marker of nascent triglyceride-rich lipoproteins. The apo B moiety remains with the particle throughout the catabolic cascade and serves as a useful surrogate marker of distribution and abundance. We confirm that apo B immunoreactivity was found in all amyloid enriched parenchymal plaque of 12 month APP/PS1 mice (Fig. 1).

Cerebrovascular abundance of agrin was found in the basement membrane. However, transgenic mice also showed focal sites of enrichment that colocalised with amyloid plaque (Fig. 2). The Mander's coefficient given in Table 1 demonstrates significant colocalisation of agrin with apo B lipoproteins and with amyloid plaques. However, based on the Pearson's coefficient, there was no evidence that accumulation of apo B or Aβ was dependent on agrin abundance per se (Table 1).

An abundance of perlecan was observed within the blood vessels of control and APP/PS1 mice. Perlecan immunoreactivity in transgenic tissue was significant and clearly evident within the core and at the periphery of amyloid plaques (Fig. 3). Pearson's correlation indicated a significant association in perlecan expression and apo B lipoprotein immunoreactivity (Table 1) and with Aβ abundance, consistent with the notion of cause for retention.

Biglycan was found expressed in neurons and blood vessels of wild type and APP/PS1 mice. In addition, significant biglycan staining was associated with amyloid plaques. Biglycan was expressed within the core and periphery of dense amyloid plaques and within regions of diffuse amyloid deposits (Fig. 4). Apo B lipoprotein and Aβ abundance was positively associated with biglycan expression (Table 1).

The endothelium of blood vessels were positively stained with decorin, but in addition decorin was observed within the core and the periphery of the cerebral amyloid plaques (Fig. 5). As for biglycan, decorin abundance positively correlated with apo B lipoprotein retention (Pearson's coefficient, Table 1) and with Aβ abundance.

Collectively, Pearsons' correlation supports the hypothesis that perlecan, biglycan or decorin can bind amyloid enriched apo B lipoproteins that have penetrated brain parenchyma and contribute to plaque formation in APP/PS1 mice. However, the association of apo B lipoproteins with agrin does not appear to be causal.

Proteoglycans may be important in the fibrillisation of soluble Aβ and/or in plaque stabilization [36,8,3,4]. In vitro and in vivo

Table 1
Pearson's correlation coefficient analysis and Mander's overlap coefficient analysis in APP/PS1 mice. (mean ± standard error of mean).

	Agrin	Perlecan	Decorin	Biglycan
Pearson's				
Aβ vs PG	0.1 ± 0.08	0.41 ± 0.10	0.34 ± 0.09	0.34 ± 0.10
Aβ vs Apo B	0.57 ± 0.64	0.54 ± 0.10	0.55 ± 0.09	0.51 ± 0.10
PG vs Apo B	0.27 ± 0.08	0.69 ± 0.08	0.53 ± 0.10	0.45 ± 0.13
Mander's				
Aβ vs PG	0.96 ± 0.01	0.96 ± 0.01	0.87 ± 0.06	0.94 ± 0.02
Aβ vs Apo B	0.98 ± 0.01	0.96 ± 0.01	0.95 ± 0.02	0.96 ± 0.01
PG vs Apo B	0.97 ± 0.01	0.97 ± 0.01	0.92 ± 0.04	0.95 ± 0.02

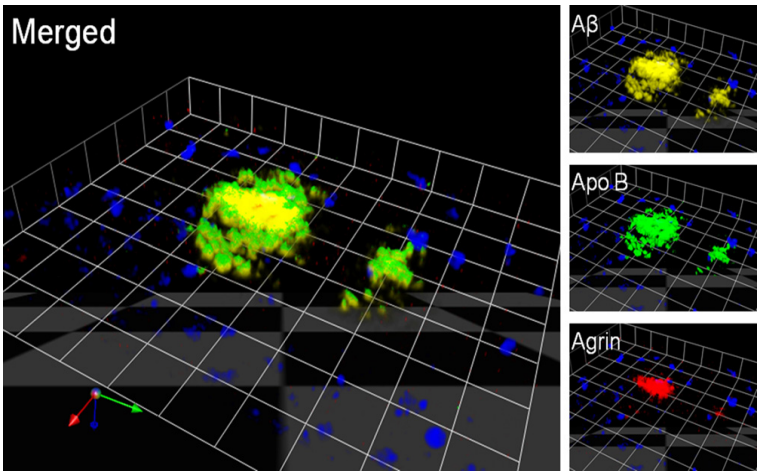

Fig. 2. The immunodetection of Aβ, agrin and apo B in APP/PS1 mice in three dimensions. The colocalisation of apo B and agrin in amyloid plaques is represented in the figure. Immunoreactivity of agrin was the most prominent within the inner core of amyloid plaque whereas siginifcant colocalisation was evident between apo B and Aβ. One unit of scale represents 22.24 μm.

studies have demonstrated a strong affinity between some proteoglycans and Aβ [3,4]. In the present study, the abundance of apo B coincided with the expression of proteoglycans which reportedly bind Aβ, consistent with the notion of entrapment of plasma derived lipoprotein-Aβ [30,31,2]. The findings should however be considered in the context of possible species differences in functional and structural significance of proteoglycans [33].

In APP/PS1 mice, there is a five-fold increase in CSF-Aβ concomitant with an eight-fold increase in the plasma Aβ concentration [2]. The exaggerated concentration of CSF-Aβ in APP/PS1 mice reflects disturbances in production [24], decreased degradation by choroid-plexus epithelial cells [38,7], decreased CSF turnover [5] and exaggerated blood-to-brain delivery. The association of proteoglycans with parenchymal retention of apo B lipoproteins and amyloid plaque may therefore be an interactive consequence of several aberrant processes.

Intense staining of agrin was seen within the core of amyloid plaque, however there was no association of agrin expression with apo B lipoproteins or Aβ abundance, suggesting that the colocalisation was consequential rather than casual. The results are consistent with the findings of Timmer, who reported minimal co-deposition of agrin with plaques [34]. Alternatively, agrin may play a role in protecting amyloid plaques from proteolytic degradation [4,37].

Substantial colocalisation was evident between perlecan, apo B lipoproteins and Aβ-plaque. Perlecan may mediate the binding of apo B lipoproteins via its affinity for apo B [26]. The significant association between perlecan and apo B lipoprotein is consistent with a role in the entrapment of Aβ. Expression of perlecan and Aβ appears to be reciprocally regulated in a cyclical pathway which may exacerbate amyloidosis. The extracellular residency of parenchymal Aβ enhances the expression of perlecan which this study suggests leads to exaggerated retention of apo B lipoprotein-Aβ.

The significant correlation between biglycan and decorin with apo B lipoproteins is consistent with a causal role in cerebral amyloidosis. Biglycan exhibits significant affinity with the carboxyl terminal domain of apo B, a mechanism thought to be responsible for lipoprotein 'anchoring' within the subendothelial space of

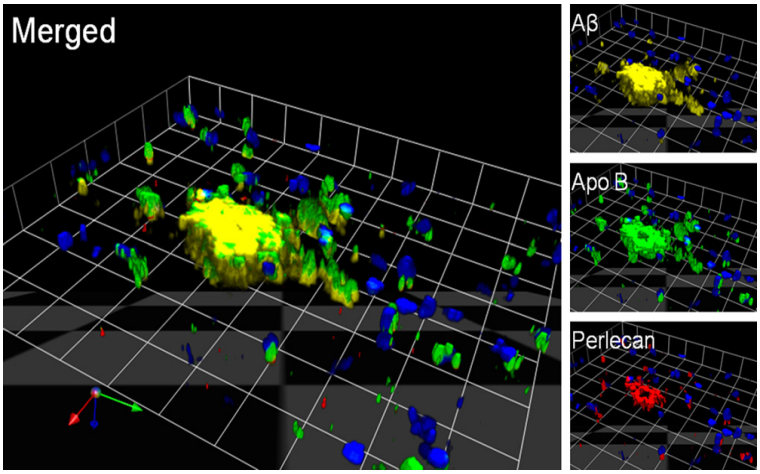

Fig. 3. 3-D triple immunodetection of Aβ, perlecan and apo B in APP/PS1 mice. A representative image of apo B and perlecan colocalisation among amyloid plaques is shown. Perlecan expression appeared to be substantially colocalised with deposits of Aβ and apo B. One unit of scale indicates 22.24 μm.

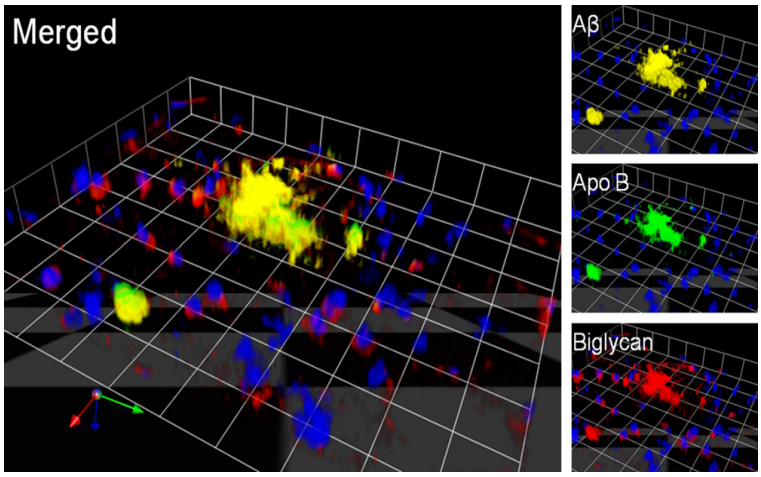

Fig. 4. 3-D triple immunodetection of Aβ, biglycan and apo B in APP/PS1 mice. This image denotes the colocalisation between apo B and biglycan. Immunoreactivity of biglycan was mostly colocalized with the core of the plaque. Siginifcant colocalisation was evident between apo B and Aβ; apo B staining seemed almost identical to the staining of Aβ deposits. One unit of scale is representative of 22.24 μm.

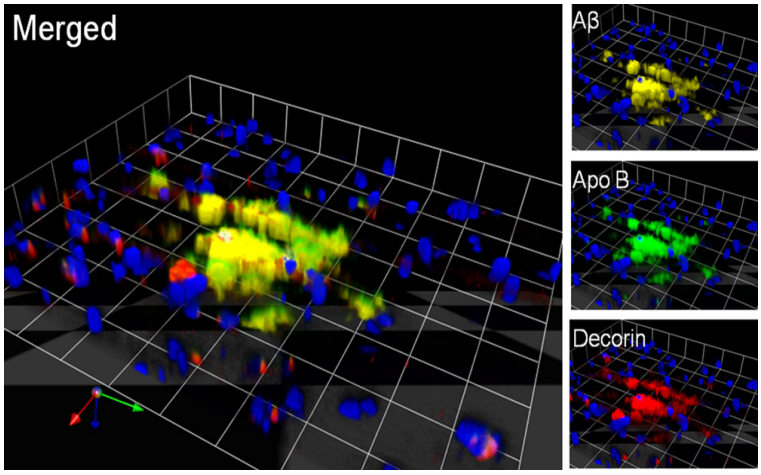

Fig. 5. 3-D triple immunodetection of Aβ, decorin and apo B lipoprotein in APP/PS1 mice. A representative image of apo B and decorin colocation. Decorin staining was observed primarily within the core and periphery of amyloid plaques. Substantial colocalisation was evident between apo B and Aβ. One unit of scale represents 22.24 μm.

coronary vessels [22,19]. Decorin is also commonly expressed in atherosclerotic tissue [12]. The cerebral distribution of biglycan and decorin colocalised with apo B lipoproteins, in small and diffuse as well as in the core of dense deposits consistent with a role in the initiation and maturation of amyloid plaques.

Sources of proteoglycans include smooth muscle cells, inflammatory and endothelial cells [1,19]. A cyclical phenomenon between Aβ entrapment and inflammation is probable, but what triggers this cascade is unclear. In atherosclerosis, enhanced arterial residency of cholesterol rich lipoproteins induces proinflammatory cytokines and activation of inflammatory pathways with downstream effects on proteoglycan expression and enhanced lipoprotein entrapment [22,23]. Cytokines secreted by inflammatory cells also stimulate elongation of the glycosaminoglycan chains which promotes lipoprotein binding [9].

Collectively, this study supports the concept that proteoglycans are an integral component of cerebral Aβ-plaque. These findings suggest that of the four proteoglycans studied, perlecan, biglycan and decorin, may be important in parenchymal entrapment of peripherally derived plasma apo B lipoproteins commonly enriched in Aβ.

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Article 5:

Pallebage-Gamarallage MMS, Takechi R, Lam V, Galloway S, Dhaliwal S & Mamo JCL. (2010) Post-prandial lipid metabolism, lipid-modulating agents and cerebrovascular integrity: Implications for dementia risk. *Atherosclerosis Supp.* 11**,** 49-54.

Synopsis

Background:

The findings by Takechi et al. (2010a, 2010b), Galloway et al. (2009) and Lam et al. (2011) support the hypothesis that dietary SFA cause BBB dysfunction, thereafter resulting in blood-to-brain delivery and brain accumulation of systemic TRL-Aβ. A lipoprotein-Aβ axis for cerebral capillary dysfunction suggests therapeutic opportunities to positively influence BBB function and reduce AD onset or progression.

There is substantial interest in using lipid lowering agents for the prevention and treatment of AD, however inconsistent results as to their purported efficacy have derived from population, clinical and animal studies. Some population and clinical studies suggest reduced AD risk and VaD in patients with hypercholesterolemia treated with statins (Crisby et al. 2002, Wolozin 2004, Haag et al. 2009, Rosenberg et al. 2008). Statins are HMG-CoA reductase inhibitors commonly used in hypercholesterolemic patients to lower the plasma cholesterol levels (Shobab et al. 2005). Several statins including atorvastatin, lovastatin and simvastatin are lipophilic and can cross the BBB, whereas cerivastatin, fluvastatin and pravastatin are hydrophilic with little or no BBB penetrance. In addition to the lipid-lowering and by extension possible lipoprotein-Aβ modulating effects, statins may enhance BBB function via pleiotropic anti-inflammatory properties. Consistent with the latter, Kalayci et al. (2005) reported that atorvastatin prevented BBB disturbances in normolipidemic hypertensive mice. Moreover, atorvastatin was found to increase plasma anti-oxidant concentration and the expression of BBB tight junction proteins. Pitavastatin was reported to strengthen the barrier integrity in rat brain endothelial cells *in vitro* (Morofuji et al. 2010).

Probucol is an older generation cholesterol-lowering agent once commonly prescribed to reduce atherosclerosis and coronary artery disease risk. However, probucol has other properties that could positively influence cerebrovascular integrity. Probucol is a potent lipid soluble anti-oxidant that provides cytoprotection against oxidative stress *in vivo* and *in vitro* (Li et al. 2007). Interestingly, in elderly AD subjects there was a stabilisation of cognitive symptoms given probucol (Poirier 2005). Whilst the mechanisms for this effect were not determined, animal models show that probucol suppresses glial cell activation and severity of amyloidosis (Poirier 2003).

At the commencement of my PhD, the potential for lipid pharmacotherapies to influence dietary lipid/lipoprotein induced disturbances in cerebral capillary function had not been investigated. As a 'proof of concept' approach, this first-authored manuscript explored the effects of the lipid lowering agents, atorvastatin, pravastatin and probucol, for the 'prevention' of SFA and cholesterol induced BBB dysfunction in WT mice.

Methods in brief:

To determine the preventative effects of statins on SFA induced BBB dysfunction, WT mice were given SFA containing diets with atorvastatin and pravastatin for 12 weeks. The effects of each agent were compared against control groups of mice fed LF and SFA diets. Utilising an additional dietary lipid induced model of BBB dysfunction, the effect of probucol (1%, w/w) was determined in cholesterol fed mice. Cerebrovascular permeability of plasma protein IgG was determined with 3-D immunofluorescence microscopy.

Results in brief:

The SFA induced cerebral extravasation of plasma proteins was prevented in mice given lipid-soluble atorvastatin. In contrast, water soluble pravastatin had no beneficial effect on BBB dysfunction. In mice given supplementary dietary cholesterol, hydrophobic probucol supplementation maintained cerebrovascular integrity. The diet and drug effects on the BBB were independent of dyslipidemia.

Discussion and conclusion in brief:

Our findings suggest that solubility of the pharmacological agents may be of importance as they are more likely to penetrate the BBB. Suppression of inflammation may be the primary modality for efficacy as neither agent significantly influcenced plasma lipid homeostasis. The drug effects on TRL-Aβ metabolism are discussed in Chapter 2.

The key findings reported in this study suggest beneficial effects of lipophilic lipid lowering drugs in preventing dietary lipid induced cerebrovascular dysfunction. The effects of these drugs on restoration of cerebrovascular dysfunction are important to consider in the context of disease regression. The principle objective of my candidacy was to determine the effects of anti-inflammatory lipid lowering agents in reversing dietary lipid induced BBB dysfunction. The primary objective findings are provided in Chapter 4.

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Post-prandial lipid metabolism, lipid-modulating agents and cerebrovascular integrity: Implications for dementia risk

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Abstract

Amyloid-β (Aβ) is secreted as an apolipoprotein of nascent triglyceride-rich lipoproteins (TRL) derived from both liver and intestine, but is better recognized as the principal protein component of senile plaque in subjects with Alzheimer's disease. Recent studies suggest that exaggerated exposure to plasma Aβ can compromise cerebrovascular integrity, resulting thereafter in blood to brain delivery of plasma proteins including TRL-Aβ. Parenchymal deposits of Aβ show significant immunoreactivity to apolipoprotein B (apo B), consistent with the notion of lipoprotein-Aβ entrapment. In wild type mice chronically fed physiologically relevant diets, saturated fats (SFA) enhance chylomicron-Aβ concomitant with disturbances in blood–brain barrier integrity. Similarly, dietary cholesterol promotes cerebrovascular extravasation of apo B lipoprotein-Aβ. In this study, we investigated the effects of Atorvastatin, Pravastatin and Probucol on dietary-fat induced disturbances in BBB function. Atorvastatin, a lipid soluble HMG-CoA reductase inhibitor prevented SFA induced parenchymal extravasation of apo B-Aβ at 28 days when incorporated into the diet at 20 mg/kg. In contrast, Pravastatin a water soluble agent had no effect on BBB integrity at an equivalent dose. In cholesterol supplemented mice, Probucol maintained BBB function and extravasation of apo B-Aβ was not evident. The findings suggest that some lipid-modulating agents may be effective in ameliorating the negative effects of saturated fats and cholesterol on cerebrovascular integrity.
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Keywords: Chylomicrons; Amyloid-beta; Alzheimer's disease; Blood–brain barrier

1. Alzheimer's disease, cerebrovasculature and dietary fat link

Alzheimer's disease (AD) is the most common cause of dementia and prevalence is expected to quadruple by the year 2050 [1]. Growing evidence supports the hypothesis that vascular disease risk factors may also contribute to AD onset and progression. Clinical, epidemiological and cross sectional studies have demonstrated a positive association between AD and atherosclerosis [2] and common risk factors include hypercholesterolaemia hypertension, sedentary lifestyle and poor nutrition [3]. Population studies have shown that dietary fats influence risk and progression of age-related diseases including AD, diabetes and cardiovascular disease. Grant [4] reported that the prevalence of AD in the >65 age population for 11 countries correlated with fat intake and was higher in Europe and North America, compared to Africa or Asia. Consumption of saturated fat, trans-fatty acids and cholesterol are positively associated with increased risk [4,5] through mechanisms which may include dyslipidemia, endothelial dysfunction, inflammation and oxidative stress. In contrast, populations with greater consumption of fats as poly- or mono-unsaturated oils (PUFA and MUFA, respectively) have lower rates of chronic diseases [4–7]. Other data show that dyslipidemia, a modifiable risk factor, is associated with a higher risk of dementia. Some studies report elevated serum levels of total cholesterol, low-density lipoprotein cholesterol and apolipoprotein B (apo B) and lower plasma high-density lipoprotein cholesterol in AD

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subjects [8,9]. These findings support the hypothesis that dietary saturated-fats (SFA) and cholesterol, or dietary induced dyslipidemia are causally associated with AD risk.

Alzheimer's disease is pathologically characterized by substantial neuronal loss and chronic inflammation that is associated with cerebrovascular and parenchymal accumulation of proteinaceous deposits enriched in amyloid-beta (Aβ) [10]. Presently, the source of cerebrovascular Aβ deposits in AD is uncertain, though there is little evidence for increased cerebral Aβ production in sporadic, late-onset AD. Rather, decreased Aβ clearance across the BBB via receptor pathways and/or via the choroid plexus has been suggested as an initiating pathway for amyloidosis [11,12]. More recent, has been evidence of blood-to-brain delivery of circulating Aβ, a process which would conceivably exacerbate parenchymal load in the absence of compensatory clearance pathways [13].

2. Plasma amyloid-beta, dietary lipids and blood–brain barrier integrity

Several studies have provided evidence of a vasoactive role of Aβ, with pathological manifestations prior to Aβ deposition. Furthermore, Aβ is vasoconstrictive and vessels treated with Aβ show significant endothelial cell damage [14]. However, studies where Aβ was intravascularly administered involved acute single injections and investigated transportation across, or sequestration within brain capillaries [15,16]. Longer term administration of Aβ resulted in a significantly compromised BBB and activated central-nervous-system glial cells [17]. Whilst these studies demonstrate regulatory responses following exogenous administration of Aβ, their physiological significance is not established.

Significant peripheral Aβ metabolism also occurs in association with post-prandial lipoproteins. In wild-type mice maintained on a low-fat diet containing 4% (w/w) as polyunsaturated fats, Aβ is seen within the perinuclear region of enterocytes, the site of chylomicron assembly [18]. When mice are fed a diet enriched in SFA, Aβ abundance is substantially increased commensurate with an increase in apo B_{48}, an exclusive structural component of nascent chylomicrons. In human studies, distributional analysis of plasma lipoprotein-Aβ found that >60% was associated with a triglyceride-rich-lipoprotein (TRL) which included chylomicrons and that this was significantly greater in subjects with AD [19]. Moreover, the concentration of apo B_{48} was substantially elevated in AD subjects (17.4 ± 5.0 versus 5.4 ± 1.1 respectively), concomitant with the raised plasma Aβ. Increased apo B_{48} is indicative of postprandial dyslipidemia, an exaggerated but transient rise in plasma chylomicrons that occurs following the absorption of dietary fats [20]. Consistent with this notion, post-prandial amyloidaemia was demonstrated in normal subjects following an oral fat challenge [21]. Collectively, these findings raise the intriguing notion that dietary fat induced elevations in plasma Aβ contribute to BBB dysfunction and thereafter exaggerated cerebral delivery. This hypothesis is supported by studies in transgenic animal models that over-express Aβ in neurons [22]. In these animals, an SFA/cholesterol enriched diet accelerates and increases amyloid burden, demonstrating that circulatory effects influence cerebrovascular deposition.

3. Apolipoprotein E, triglyceride-rich lipoproteins and blood–brain barrier integrity

Inheriting one or two alleles for apo E4 substantially increases onset and progression of AD, compared to individuals with hetero- or homo-zygous for apo E2 and E3 isoforms. In blood, apo E4 is distributed with remnant lipoproteins that contain relatively more triglycerides (principally chylomicrons), whereas apo E2 and apo E3 tend to be primarily associated with hepatically derived TRL remnants. Studies in apo E knockout mice demonstrate the importance of apo E in maintaining BBB integrity, however apo E4 does not support functionality as effectively as apo E2 or E3 isoforms [23,24]. It is our contention that subjects with apo E4 have exaggerated blood-to-brain transport of TRL-Aβ and extracellular entrapment [25,26].

4. Dietary fatty acids and blood–brain barrier integrity

The vasoactive properties of exogenous Aβ led us to explore the hypothesis that dietary SFA increases plasma TRL-Aβ and that with chronic ingestion this consequently leads to parenchymal Aβ accumulation. In a recent study, wild-type mice were fed modified diets enriched in either SFA, MUFA or PUFA fatty acids and compared with low-fat fed controls [27]. Three months after commencement of the lipid enriched diets, there was remarkable cerebral leakage and parenchymal colocalization of Aβ with apo B lipoproteins in SFA-supplemented mice. Duration of diet effect was reported. Mice fed for six months had significantly greater abundance of plasma derived proteins compared to the 3 month fed group. Greatest abundance was seen in cortex > brain stem > hippocampal formation. However, there was no evidence of apo B lipoprotein or Aβ immunoreactivity in brains from mice fed either MUFA, PUFA or low-fat diets. Several markers suggest that delivery of peripheral apo B-Aβ was a non-specific phenomenon because IgG, a large molecular weight plasma protein, was evident in parenchyma of SFA-fed mice and occludin expression, the primary endothelial tight junction protein was substantially reduced compared to controls [27]. The plasma concentration of S100B, a CSF abundant protein, was also increased in plasma suggesting bidirectional disturbances in protein transport via the BBB [27].

Further evidence supporting the hypothesis that circulating apo B lipoprotein-Aβ contributes to BBB dysfunction and cerebral amyloidosis comes from studies in amyloid

transgenic mice. In three murine models of AD, plasma Aβ concentration correlated with secretion rates into blood of TRLs and was increased 3–8 fold above wild-type controls. Moreover, plasma TRL-Aβ was positively associated with the onset of cerebrovascular and parenchymal amyloidosis [22]. In an extension of that study, we investigated BBB integrity and showed that there was substantial apo B co-localization with cerebral amyloid plaque [27]. We have proposed that postprandial hyperamyloidemia is one possible mechanism for SFA-induced BBB dysfunction and delivery of apo B lipoprotein-Aβ from blood to brain. Consistent with this concept, in non-demented participants significant variation in CSF-Aβ levels of up to 4-fold was detected over 36 h of serial sampling. $A\beta_{1-40}$ and $A\beta_{1-42}$ were highly correlated over time indicating that similar processes regulate the concentration of these isoforms. The fluctuations of Aβ levels appeared to be time of day dependent [28].

Several non-Aβ mediated pathways could also contribute to SFA-induced cerebrovascular disturbances. Significant differences in the cytotoxic effects of fatty acids have been reported, with longer chain SFAs being the most potent and the MUFA and PUFA being cytoprotective [29]. Morgan [29] suggests that the underlying toxicity of SFA is a consequence of disturbances in protein processing and endoplasmic reticulum (ER) dysfunction. Conversely, cell culture studies suggest that incubation with longer chain unsaturates has an antagonistic effect on stress pathways [30]. Western diets substantially increase protein oxidation and lipid peroxidation and in amyloid transgenic mice, this occurred in the absence of increased Aβ levels [31]. Exogenous fatty acid supplementation results in significant shifts in neuronal phospholipids and in lipid raft composition [32,33], key regulators of cell protein transport and inflammation.

5. Lipid lowering therapy for the prevention and treatment of Alzheimer's disease

The critical observations presented are that dietary saturated fats and cholesterol cause BBB dysfunction, resulting in the blood-to-brain delivery and parenchymal accumulation of apo B lipoprotein-Aβ. If cerebrovascular disturbances are indeed central to AD aetiology and progression, then considering strategies to positively influence integrity is a therapeutic priority. Presently, drug strategies used to treat AD are focussed on maintaining cell–cell communication rather than cerebrovascular function.

Population studies support a role for lipid lowering in the prevention of AD. The 3-City Study represents a cohort of approximately 9000 subjects examining the association of plasma cholesterol, lipid-lowering agent (LLA) intake and apo E genotype with dementia prevalence [34]. In that cohort, 2% of participants were demented at baseline. Overall 32.4% of participants had hyperlipidemia; 15.6% were taking statins and 13.7% fibrates. After adjusting for age, gender, education the odds ratio (OR) for dementia was lower among LLA users (OR = 0.61) compared with subjects taking no LLA. There was no differential between statin and fibrate users. The odds for dementia were increased in subjects with hyperlipidemia (OR = 1.43) and the authors reported that further adjustment for potential confounders did not modify these associations. In addition, the association between LLA intake and dementia was not modified by apo E genotype. This particular observational study provides evidence that LLAs are associated with decreased risk of dementia.

There is substantial interest in using lipid pharmacotherapies for prevention and treatment of AD, however paradoxical results as to their purported efficacy have come from population, clinical and animal studies. Lipids have diverse, differential effects on Aβ metabolism and cerebrovascular integrity. Hence, the effectiveness of lipid lowering drugs for reducing AD risk, or slowing disease progression would notionally be dependent on their suitability for correcting lipid-induced aberrations in metabolism.

6. Statins, Alzheimer's and blood–brain barrier integrity

Some, but not all population and clinical studies suggest that statins may reduce AD risk and progression of AD [35,36]. Possible mechanisms include reduced Aβ secretion; enhanced clearance from blood of apo B lipoproteins; maintenance of BBB function and/or anti-inflammatory properties. Consistent with the latter, Atorvastatin was shown to prevent BBB dysfunction in normolipidaemic spontaneously hypertensive rats [37] and was found to increase plasma anti-oxidant concentration and the expression of BBB tight junction proteins. We now present evidence that Atorvastatin (20 mg/kg) prevents cerebrovascular dysfunction in wild-type mice maintained on an SFA enriched diet (20%, w/w) for 90 days (Fig. 1). However, the solubility of the statin may be important because no benefit was observed with Pravastatin at an equipotent dose. Lipid soluble agents such as Atorvastatin are more likely to penetrate the BBB.

7. Probucol, Alzheimer's and blood–brain barrier integrity

A recent clinical study using Probucol in elderly AD subjects revealed a stabilisation of cognitive symptoms [38]. Studies in animal models suggest that Probucol could stimulate cerebral efflux of Aβ and suppress of glial activation [39]. In addition, Probucol is a hydrophobic agent delivered into blood in association with chylomicrons. Probucol significantly increases hepatic uptake of TRL's and reduces sub-endothelial entrapment of apo B lipoproteins within arterial intima [40]. The putative effect of Probucol on BBB function was explored in wild type mice maintained on chow supplemented with 1% (w/w) cholesterol for 90 days. In this

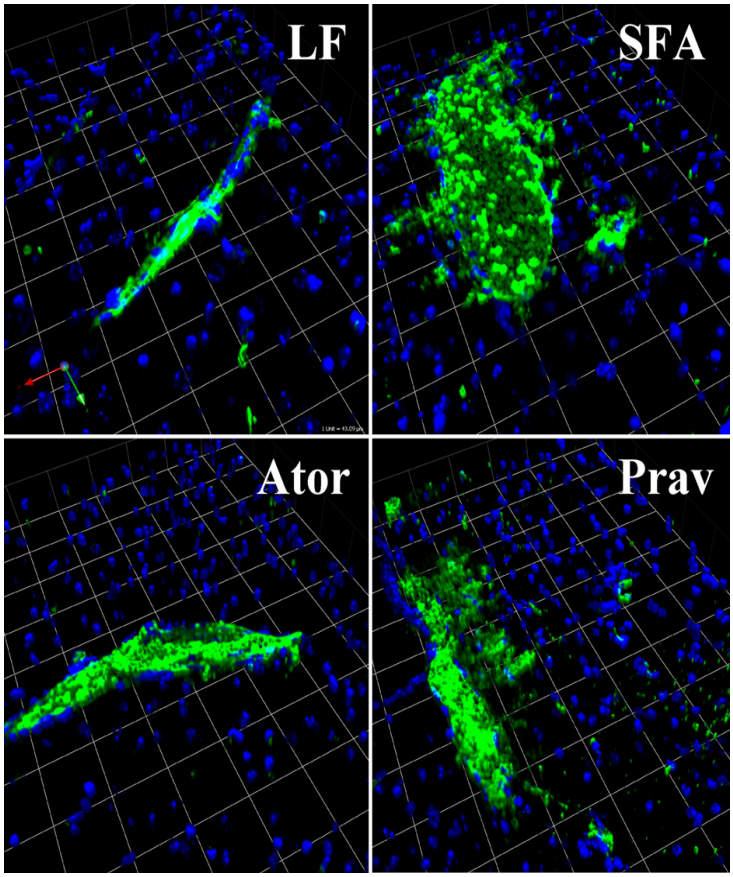

Fig. 1. Blood–brain barrier (BBB) integrity is demonstrated by extravasation of plasma protein immunoglobulin G (IgG) within the cerebral tissue. Wild-type mice fed saturated-fat (SFA) diet for 90 days had significant perivascular IgG leakage compared to the mice on the low-fat (LF) diet. Atorvastatin (Ator) prevented the SFA induced extravasatino of IgG, whereas Pravastatin (Prav) had no significant effect. Scale: 1 unit = 43.09 μm

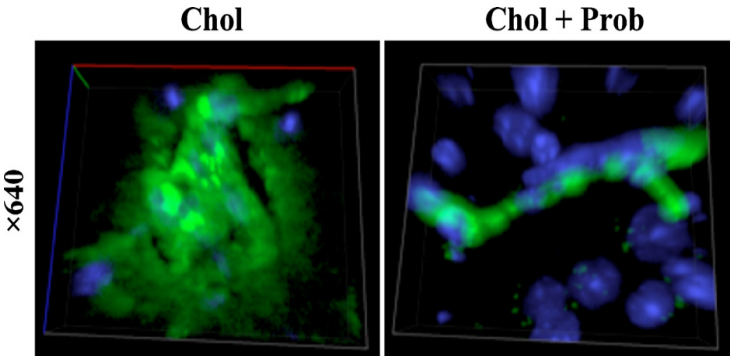

Fig. 2. Wild-type mice supplemented with cholesterol (1%, w/w) (Chol) for 90 days had showed significant cerebbrovascular leakage of IgG into brain parenchyma. Mice give Probucol (Prob) concomitant with dietary cholesterol had no evidence of IgG perivascular leakage.

model, Probucol appeared to prevent the cholesterol-induced disturbances in BBB function (Fig. 2). The protective effect of Probucol may be related to a marked reduction of TRL in serum and/or inhibition of inflammation. Probucol is a potent antioxidant.

8. Summary and conclusions

Dementia will become the world's most significant cause of morbidity and mortality within 30 years. Common to AD (the most common form of dementia) and other demen-

tia's is significant cerebrovascular aberrations, characterized by chronic inflammatory processes that compromise tissue integrity and ultimately cognitive function. Presently, there is an arsenal of drugs that notionally could interfere with cerebrovascular inflammation, however few have been methodically considered in this context. Rather, drugs designed to treat AD have primarily focussed on maintaining neuronal cell communication and these have not been particularly successful in maintaining cognition.

Accumulating evidence is consistent with the hypothesis that dietary fats and postprandial lipoprotein metabolism influence AD risk and progression, but not clear is the mechanisms by which this occurs. AD is an inflammatory disorder, possibly in response to fibrillar formation and extracellular deposition of amyloid-beta (Aβ). Alternatively, given that cerebrovascular disturbances may precede amyloid plaque formation, amyloidosis could be a secondary phenomenon that exacerbates pre-existing inflammatory processes. Fat-feeding studies are providing valuable insight with respect to the mechanisms underlying the lipids-AD risk paradigm and preliminary studies suggest that lipid-induced cerebrovascular disturbances are potentially reversible if mice have a timely return to a low-fat diet (i.e. lacking SFA or cholesterol). By extension, agents which address lipid-induced aberrations in cerebrovascular function would notionally accelerate recovery or prevent disease onset.

Conflicts of interest

The authors have no conflicts of interest to declare in relation to this article.

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CHAPTER 2

Chapter 2: The effect of probucol on enterocytic amyloid-β abundance in mice maintained on diets enriched in saturated-fat and cholesterol

Content of this chapter is covered by the article:

Pallebage-Gamarallage M, Galloway S, Takechi R, Dhaliwal S & Mamo J. (2012a) Probucol suppresses enterocytic accumulation of amyloid-β induced by saturated fat and cholesterol feeding. *Lipids.* 47, 27-34.

Synopsis

Background:

Alzheimer's disease is pathologically characterised by significant Aβ deposition within the extracellular matrices of the brain parenchyma and the cerebrovasculature. However, the source of cerebrovascular Aβ deposits in AD is unclear. Several studies have demonstrated a positive association between plaque formation and increased neuronal Aβ production (Citron et al. 1997, Tomita et al. 1998). However, overproduction does not seem to be causally related to amyloidosis in sporadic and late onset AD, the most common form of this disorder. Accumulating evidence suggests that enhanced blood-to-brain delivery of circulating Aβ may contribute to cerebrovascular amyloid pathology. Circulating Aβ may be derived from lipogenic organs such as the liver and the small intestine in association with TRLs (TRL-Aβ) (Koudinov and Koudinova 1997, Galloway et al. 2009) exacerbated by the provision of diets enriched in SFA (Galloway et al. 2007).

A putative role of systemic derived TRL-Aβ in the aetiology of AD was supported by clinical studies which reported that individuals with AD have increased TRL-Aβ and post-absorptive chylomicronemia compared to age-matched controls (Mamo et al. 2008). Moreover, in amyloid transgenic mice, the onset and progression of cerebral amyloidosis strongly correlated with secretion of circulating TRL-Aβ (Burgess et al. 2006). The demonstration of the significant colocalisation of apo B and Aβ in amyloid plaques of human and transgenic mouse brain specimens (Namba et al. 1992, Takechi et al. 2010a) are consistent with the notion of a vascular contribution to amyloidosis and AD aetiology.

The putative association between dietary lipids, TRL-Aβ and AD suggests selective therapeutic opportunities for lipid-lowering agents in reducing AD risk. Some clinical and population studies support reduced AD risk among the users of lipid-lowering agents (Dufouil et al. 2005, Ancelin et al. 2012, Jick et al. 2000), although the underlying mechanisms for this association are presently unclear.

Probucol is a potent cholesterol-lowering agent which enhances receptor mediated lipoprotein clearance (Mamo et al. 1993, Mellies et al. 1980). Probucol is

hydrophobic and delivered into circulation as part of the chylomicron moiety (Mamo et al. 1993). Probucol may influence biogenesis of Aβ, or secretion as nascent chylomicrons. Beneficial effects of probucol in AD were demonstrated in a clinical study where cognitive decline was improved in subjects with mild-cognitive impairment treated with probucol (Poirier 2003, Poirier 2005). Furthermore, probucol has shown to reduce amyloidosis via modulation of Aβ metabolism (Poirier 2003).

The primary objective of this study was to investigate the effects of probucol on the production and secretion of apo B containing TRL-Aβ in enterocytes of WT mice maintained on a high-fat diet previously shown to substantially increase enterocytic abundance of Aβ.

Methods in brief:

Mice were randomised to four groups and fed either a LF, LF+Probucol, HF or HF+Probucol diet. Enterocytic abundance of Aβ and apo B were detected via immunofluorescence microscopy. Abundance of Aβ and apo B were quantitated within the perinuclear region (site of Aβ biogenesis) of the enterocytes and the lacteals (site where TRL-Aβ are secreted for circulation). Plasma lipids were also analysed to observe the effects of the HF diet and probucol on plasma lipid homeostasis.

Results in brief:

Significant apo B was identified within the perinuclear regions of the enterocytes and the lacteals in all groups. Secretion of apo B into the lacteal was increased in response to HF feeding and probucol treatment (LF+Probucol and HF+Probucol). Perinuclear Aβ was significantly enhanced by HF feeding and attenuated by probucol without significant changes in lacteal Aβ.

Discussion and conclusion in brief:

Findings in this study confirm that HF feeding enhances enterocytic Aβ abundance. Furthermore, HF feeding enhanced apo B secretion in the lacteals, but without a concomitant increase in lacteal Aβ. Collectively, these findings suggest that the HF effects on enterocytic Aβ is likely to be a as a consequence of increased

production, rather than reduced rates of secretion. The latter observations are consistent with previous studies that reported HF induced enterocytic Aβ accumulation is reduced once the food is withdrawn (Galloway et al. 2007).

Probucol co-administration with the HF diet resulted in significant reduction in HF induced enterocytic Aβ abundance independent of significant changes in lacteal Aβ. These findings support the notion that probucol attenuated HF induced enterocytic Aβ biogenesis and possibly reduced the available pool of Aβ for secretion.

Clinical and animal studies demonstrated reduced AD risk and reduced amyloidosis by probucol treatment (Champagne et al. 2003, Poirier 2003, Poirier 2005). Our findings suggest a putative mechanism whereby probucol could confer AD protection via reduced vascular exposure to TRL-Aβ, or other lipid mediated inflammatory agents.

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ORIGINAL ARTICLE

Probucol Suppresses Enterocytic Accumulation of Amyloid-β Induced by Saturated Fat and Cholesterol Feeding

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Abstract Amyloid-β (Aβ) is secreted from lipogenic organs such as intestine and liver as an apolipoprotein of nascent triacylglycerol rich lipoproteins. Chronically elevated plasma Aβ may compromise cerebrovascular integrity and exacerbate amyloidosis—a hallmark feature of Alzheimer's disease (AD). Probucol is a hypocholesterolemic agent that reduces amyloid burden in transgenic amyloid mice, but the mechanisms for this effect are presently unclear. In this study, the effect of Probucol on intestinal lipoprotein-Aβ homeostasis was explored. Wild-type mice were fed a control low-fat diet and enterocytic Aβ was stimulated by high-fat (HF) diet enriched in 10% (w/w) saturated fat and 1% (w/w) cholesterol for the duration of 1 month. Mice treated with Probucol had the drug incorporated into the chow at 1% (w/w). Quantitative immunofluorescence was utilised to determine intestinal apolipoprotein B (apo B) and Aβ abundance. We found apo B in both the perinuclear region of the enterocytes and the lacteals in all groups. However, HF feeding and Probucol treatment increased secretion of apo B into the lacteals without any change in net villi abundance. On the other hand, HF-induced enterocytic perinuclear Aβ was significantly attenuated by Probucol. No significant changes in Aβ were observed within the lacteals. The findings of this study support the notion that Probucol suppresses dietary fat induced stimulation of Aβ biosynthesis and attenuate availability of apo B lipoprotein-Aβ for secretion.

Keywords Apolipoprotein B · Amyloid-β · Probucol · Small intestine · Saturated fat · Cholesterol

Abbreviations

AD	Alzheimer's disease
Aβ	Amyloid-β
Apo B	Apolipoprotein B
HF	High-fat
LF	Low-fat
TAG	Triacylglycerol(s)

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Introduction

Alzheimer's disease (AD) is the most common cause of dementia, characterized by neuronal cell loss and amyloid-beta (Aβ) deposition on extracellular matrices and within the cerebrovasculature [1]. Amyloid-β is present at sub-nanomolar levels in most biological fluids, such as cerebrospinal fluid and plasma [2] and, at physiological levels, regulates cell growth [3–5]. A hydrophobic protein, Aβ may undergo oligomerisation when it becomes disassociated from chaperone proteins that ordinarily facilitate kinetics and metabolism. It is the fibrillar form of Aβ that is thought to trigger pro-inflammatory pathways that compromise neuronal integrity [6, 7].

The origin of cerebrovascular Aβ deposits in AD is presently unclear. Amyloid-β may be generated as a

consequence of proteolytic processing of the amyloid-precursor-protein, which is expressed in significant quantities on the plasma membrane of neuronal cells [8]. However, biogenesis of Aβ is not increased in sporadic and late onset AD—the most common phenotype of AD [9, 10]. Rather, accumulating evidence suggests that enhanced blood-to-brain delivery relative to efflux, or via Aβ degradative pathways within the choroid plexus results in extracellular retention of Aβ and thereafter, inflammatory sequelae [11, 12].

Significant Aβ in blood is associated with apolipoprotein B (apo B) lipoproteins, particularly those enriched in triacylglycerol (TAG). Subjects with AD have greater apo B lipoprotein-Aβ relative to age-matched controls [13], and in transgenic amyloid mice, onset and progression of cerebral amyloidosis is associated strongly with the secretion into and concentration of plasma apo B lipoprotein-Aβ [14]. Apolipoprotein B immunoreactivity is evident in parenchymal amyloid plaque from human cadaver specimens [15]; and in Aβ-transgenic mice, cerebral apo B distribution and abundance strongly colocalise with extracellular deposits of Aβ [16]—observations consistent with a vascular contribution to disease aetiology.

A range of lipoprotein (lipid)-lowering agents are commonly used in clinical practice for the prevention and treatment of cardiovascular disease and may reduce AD risk by reducing cerebrovascular exposure to apo B lipoprotein-Aβ. The hydroxy-methyl-glutaryl coenzyme A reductase inhibitors lower plasma cholesterol by enhancing apo B lipoprotein clearance via high affinity receptor pathways and inhibiting apo B lipoprotein biogenesis [17–19]. Similarly, fibrates reduce plasma TAG by suppressing lipogenesis, a driver for the secretion of apo B lipoproteins [20]. Population and clinical studies generally support a risk reduction for all forms of dementia in subjects taking lipid-lowering agents [21, 22], although the mechanisms for this association are not clear.

Probucol is an older generation cholesterol-lowering agent that reduces plasma cholesterol by enhancing uptake via receptor pathways [23, 24]. However, other properties of Probucol make this a particularly interesting lipid-lowering agent relative to the plasma kinetics and metabolism of apo B-lipoprotein-Aβ. Probucol is hydrophobic and secreted into blood incorporated within the nascent TAG-rich apo B lipoproteins, a phenomenon that may influence Aβ association with, and secretion of, these macromolecules [23, 25]. In addition, lipoproteins that contain Probucol are cleared from circulation almost exclusively by the liver and consequently vascular retention is substantially reduced [23]. A small clinical study suggested that Probucol reduced cognitive decline in subjects with mild cognitive impairment [26, 27] and, consistent with the human findings, studies in transgenic amyloid mice showed that Probucol reduced the severity of amyloidosis [26]. In the latter, enhanced Aβ-efflux was put forward as one possible mechanism for the Probucol-induced effects.

The putative effects of Probucol on Aβ biogenesis and lipoprotein synthesis in lipogenic organs have not been reported. This study utilised an in vivo high-fat (HF) feeding model previously shown to stimulate enterocytic abundance of Aβ, to determine if Probucol modulates the secretion of apo B lipoprotein-Aβ from absorptive epithelial cells of the small intestine—a major site of Aβ biosynthesis [28–30].

Methods and Materials

Animals and Diet Conditions

The Curtin University Animal Experimentation and Ethics Committee approved the animal housing, handling and experimental procedures described. Seven-week-old female wild-type mice (C57BL/6J) were housed in groups and randomised into the diet or drug treatment groups ($n = 8$ mice per group). All mice were maintained on a 12-h light and dark cycle room, at 22°C and with free access to water and food. The low-fat control diet was standard AIN93M rodent chow containing <4% (w/w) fat as polyunsaturates, with <1% total digestible energy as lipids and was free of cholesterol (Glen Forrest Stockfeeders, Perth, Western Australia). To stimulate enterocytic Aβ production, the control feed was replaced with a HF diet enriched in saturated fats 10% (w/w) and 1% (w/w) cholesterol (Glen Forrest Stockfeeders). The principal fatty acid types in the HF treatment group were palmitic (16:0) and stearic (18:0) (total of 13% w/w) and oleic acid (18:1n-9, 6% w/w). Mice treated with Probucol (Sanofi-Aventis, Paris, France) had the drug incorporated into the chow at 1% (w/w) at the time of feed manufacture in order to achieve an estimated dose rate of 30 mg/day [23].

Tissue Collection and Sample Preparation

Mice were maintained for 32 days on the diets indicated and weighed weekly. Thereafter, mice were anaesthetised with pentobarbitone (45 mg/kg i.p.) and exsanguinated by cardiac puncture. Blood was collected into heparin tubes and stored in ice. Plasma was separated by short-speed centrifugation at 4°C and stored at −80°C.

A 2 cm segment of the small intestine duodenum at the proximal end was isolated, flushed with chilled phosphate buffer saline (PBS, pH 7.4) and fixed in 10% buffered formal saline for a minimum of 24 h. The tissues were then processed and longitudinal segments embedded in paraffin

wax. Serial sections of 5 μm thick were cut and mounted on silanised slides for histology and immunofluorescence.

Amyloid-β and Apolipoprotein B Immunofluorescence

Intestinal Aβ and apo B were detected by an immunofluorescent amplification method as previously described [31]. Intestinal tissue sections (5 μm) were deparaffinised, rehydrated and antigen-retrieval was carried out in boiling deionised water for 15 min. Briefly, all sections were permeabilised in PBS and incubated in blocking serum (20% goat serum).

For Aβ staining, polyclonal rabbit anti-human A$\beta_{1\text{-}40/42}$ antiserum (AB5076, Chemicon Temecula, CA), diluted to 1:2,000 in PBS was incubated overnight at 4°C. The specificity of the antibodies was previously established [28]. Sections were washed in PBS and incubated with biotinylated goat anti-rabbit secondary antibody (1:2,000 dilution) (E0432, DAKO, Carpentaria, CA) at room temperature for 1 h, followed by incubation with Streptavidin-Alexa Fluor® 546 (1:300 dilution) (S11225, Invitrogen, Victoria, Australia) for another hour in the dark for amplification. The nuclei were counterstained with DAPI (1:1,000 dilution) (Invitrogen, Victoria, Australia) for 5 min. The sections were then mounted with antifade mounting medium.

Enterocytic apo B was determined essentially as described for Aβ detection. Polyclonal rabbit anti-mouse apo B (ab20737, Abcam, Cambridge, UK) as primary and the biotinylated goat anti-rabbit secondary antibody was used at 1:2,250 dilutions.

Imaging

Digital images for photo microscopy were acquired through AxioCam HRm camera (Zeiss, Jena, Germany) with an AxioVert 200 M inverted microscope by Zeiss at 200× magnification (Plan Neofluar ×20 objective, 1.3 numerical aperture). Excitation and emission were achieved by using filters 43 (Ex BP545/25, beam splitter FT570 and Em BP605/70) and filter 49 (Ex G365, beam splitter FT395 and Em BP445/50) to determine fluorescence of Alexa Fluor® 546 and DAPI, respectively. Individual channels are free from fluorescence from other emission sources and are therefore clear of overlap. Each image was captured under identical exposure times utilising AxioVision software (version 4.7.1) to avoid artificial modification in pixel intensity.

Quantitative Immunofluorescent Imaging and Analysis

Images were collected at 200× magnification and approximately 30–50 images were captured per group showing at least four villi in each image (1,388 × 1,040 pixels per image). Pixel intensity for each fluorescent dye was obtained by calculating the densitometric sum by Automatic Measurement Program in AxioVision (Software version 4.7.1). Densitometric sum was calculated for each image staining intensity of Aβ, apo B and DAPI (nuclei).

For each image, either apo B or Aβ pixel intensities were standardised with total DAPI pixel intensity to normalise for cell number in the image, and expressed as per DAPI. Staining intensity in the perinuclear region within the enterocytes was calculated and expressed as perinuclear intensity per total DAPI for the image (perinuclear apo B/total DAPI, perinuclear Aβ/total DAPI). Lacteal staining intensity was obtained by subtracting perinuclear staining intensity from the whole villi intensity (lacteal apo B/total DAPI, lacteal Aβ/total DAPI). The data were then collated and final results are expressed as mean intensity ± standard error of mean per area unit.

Plasma Cholesterol and Triacylglycerol Analysis

Plasma Cholesterol and TAG were determined in duplicate by enzymatic assays (Randox Laboratories, Crumlin, UK) according to the manufacturers' instructions.

Statistical Analysis

All data was analysed by either parametric or non-parametric one-way analysis of variance (ANOVA) to assess the main effects of dietary fat and Probucol treatment and their two-way interactions. Post-hoc comparison of means was done if the associated main effect or interaction was statistically significant within the ANOVA procedure. P values < 0.05 were considered to be statistically significant.

Results

The distribution and abundance of immunoreactive apo B, an exclusive marker for nascent chylomicrons, was determined by quantitative immunofluorescent microscopy as previously described. Perinuclear enterocytic and lacteal abundance were utilized as surrogate markers of production and secretion, respectively. In all groups, the majority of immunoreactive apo B (≈80%) was located within the lacteals (Fig. 1, 2a), indicative of the efficient packaging and secretory pathway of dietary lipids with chylomicrons. Provision of an HF diet for 32 days resulted in a 60% increase of secreted apo B commensurate with decreased perinuclear apo B (Fig. 1, 2a), but there was no significant change in net villi apo B abundance (perinuclear + lacteal). Incorporation of Probucol in the LF diet, like the HF

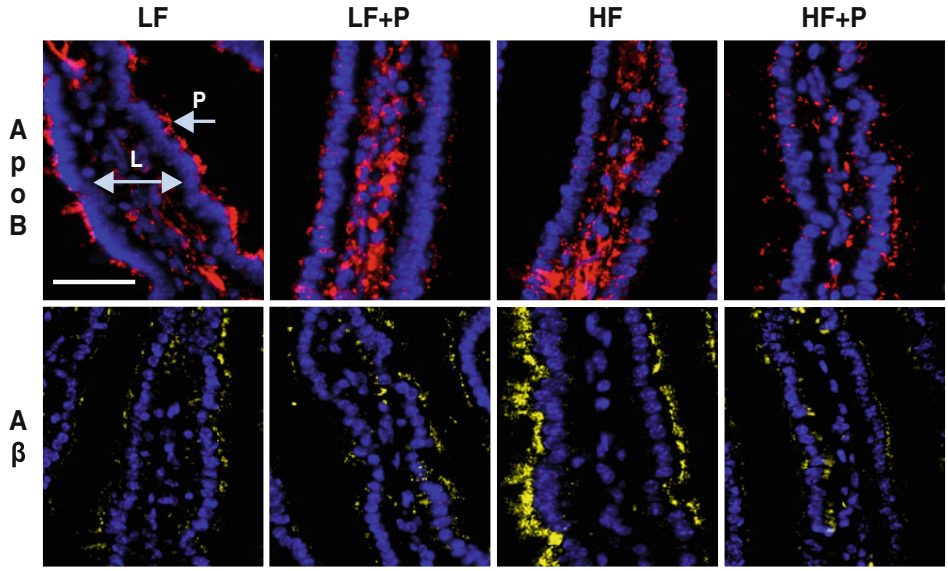

Fig. 1 Images showing apo B (*red*) and Aβ (*yellow*) in the intestinal villi. The nuclei are stained *blue*. The villi are lined with a single layer of absorptive epithelial cells. Amyloid-β staining is concentrated at the perinuclear region of the enterocytes (*P arrow*) with very little staining in the lacteals (*L arrow*). In contrast, a high concentration of the apo B is found within the lacteals of all the groups. *Bar* 30 μm, *LF* low-fat, *HF* high-fat, *P* probucol

diet, stimulated secretion of apo B. However, there did not appear to be a synergistic effect of HF + *P*. The findings of similar net villi abundance of apo B between treatment groups and a strong negative association between the perinuclear- versus lacteal-apo B distribution (Fig. 2c), is consistent with studies suggesting that enterocytic apo B is synthesized constitutively, whereas the secretion of the nascent lipoproteins is modifiable in response to the availability of dietary lipids [32].

The perinuclear and lacteal distribution of Aβ was qualitatively identical to that of apo B. Indeed, co-localisation analysis confirms that Aβ secreted from enterocytes was associated with chylomicrons. However, there were substantial differences in the relative villi abundance of Aβ and apo B and in the pattern of secretion between the two proteins. In contrast to apo B, approximately 70–80% of total intestinal villi Aβ was observed within the baso-lacteal nuclear region of the absorptive epithelial cells, suggesting that only small quantities of the total enterocytic Aβ pool were being secreted (Fig. 1, 2b). The HF-enriched diet doubled intestinal villi Aβ abundance but, in contrast to apo B, this was reflected predominantly in increased enterocytic abundance with virtually no change in the secreted component (Fig. 2b). Incorporation of Probucol in the HF diet normalized enterocytic Aβ to levels that were comparable to the LF control, in the absence of a reduction in lacteal Aβ. The latter suggests that Probucol reduced enterocytic Aβ primarily as a consequence of lower rates of biosynthesis. However, correlation analysis of perinuclear versus lacteal Aβ identified a relatively weak but nonetheless positive association (Fig. 2d), suggesting that increased rates of Aβ production also lead to modest increases in apo B lipoprotein-Aβ secretion.

The effects of HF- or Probucol-supplemented diets on plasma cholesterol, plasma TAG and body weight gain for each group of mice is given in Table 1. The mice fed the HF enriched diet had an increase in plasma cholesterol of more than two-fold compared to the LF control; however, the incorporation of Probucol completely abolished this effect. Indeed, the HF + *P* group had comparable plasma cholesterol to the LF + *P* treated mice. Probucol also significantly reduced plasma cholesterol in LF mice. In contrast, there was no appreciable effect of HF feeding, or Probucol, on plasma TAG in any treatment group.

Mice maintained on the HF diet were found to have a greater rate of body weight gain compared to mice maintained on the LF diet. Probucol had a synergistic stimulatory effect on body weight gain. Mice on LF + *P* were similar in weight to mice maintained on the HF diet, and mice on the HF + *P* were significantly heavier in weight than mice on HF alone.

Discussion

In absorptive epithelial cells of the small intestine, dietary fats regulate enterocytic abundance of Aβ profoundly, reflecting either changes in Aβ biogenesis, or in the

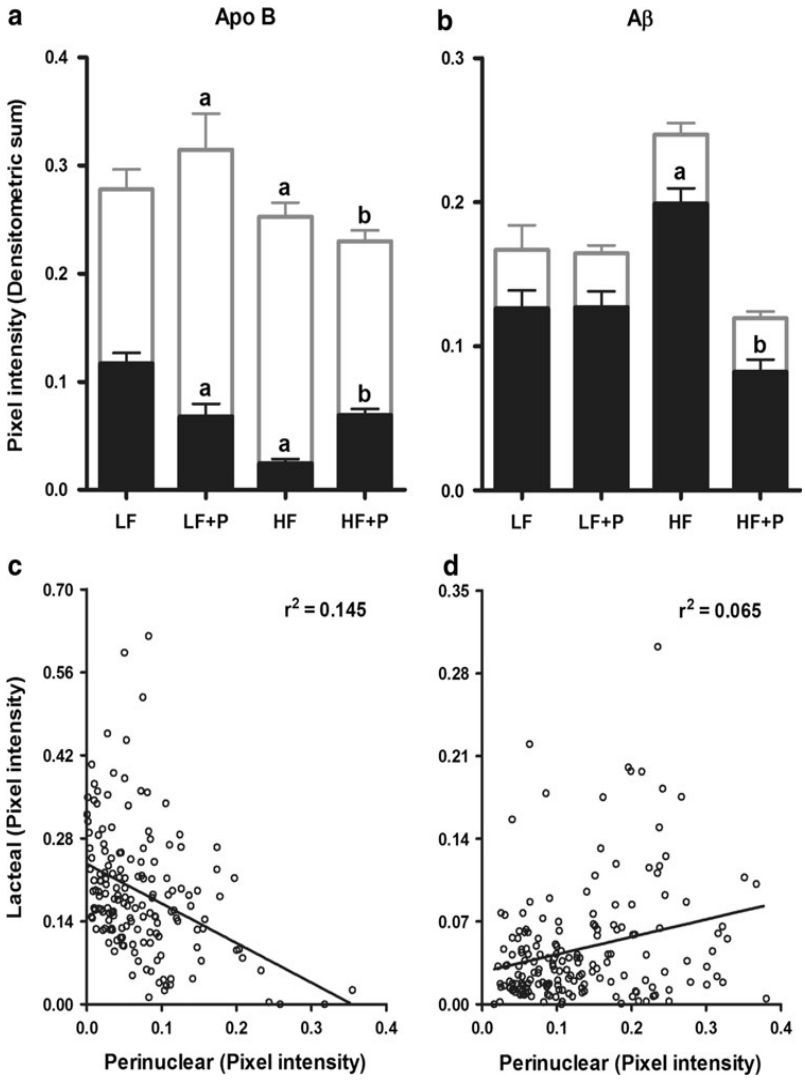

Fig. 2 **a**, **b** *Bar graphs* showing quantitative analysis of perinuclear (*filled squares*) and lacteal (*open squares*) apo B and Aβ staining intensities. Pixel intensity is expressed as densitometric sum as a ratio of nuclei intensity to normalise for nuclei number. **c**, **d** Scatter plots showing the distribution of apo B and Aβ between the perinuclear and lacteal regions of all groups. Pearson's correlation analysis (r^2 values) for apo B and Aβ was 0.145 ($P < 0.0001$) and 0.065 ($P = 0.0006$), respectively. **a** Statistically significant in comparison to the LF group ($P < 0.05$). **b** Statistically significant in comparison to the HF group ($P < 0.05$). *LF* low-fat, *HF* high-fat, *P* probucol

secretion of lipoproteins containing Aβ [28, 29]. Several lipid-lowering agents, including statins and fibrates, have been shown to suppress apo B secretion [17, 18, 20]; however, Probucol may have pleiotropic benefits post-secretion, including enhanced hepatic clearance apo B lipoprotein-Aβ and anti-oxidant activity.

In this study, an established in vivo murine model was used to investigate if Probucol modulates the effects of a HF diet on enterocytic Aβ and its secretion thereafter, with apo B lipoproteins. The study confirms that a HF diet substantially increases enterocytic perinuclear abundance of Aβ. Apolipoprotein B lipoprotein secretion is enhanced by HF feeding, but without evidence of a concomitant increase in lacteal Aβ staining. Therefore, the HF-mediated effect on enterocytic Aβ abundance is likely to be a consequence of greater rates of Aβ synthesis, rather than diminished rates of secretion. Previous studies reported that a HF-induced accumulation of enterocytic Aβ is progressively depleted in the post-absorptive state, or once food is withdrawn [28]. Hence, a dietary fat induced stimulation in Aβ biogenesis with constitutive rates of secretion as suggested in this study, would result in extended post-prandial amyloidemia. Clinical studies in normal healthy subjects consuming a mixed lipid meal are consistent with a transient single meal effect [34]. A phenomenon of extended exposure may be important in modulation vascular function. Co-administration of Probucol with the HF diet completely abolished the HF-induced effect on enterocytic Aβ abundance in the absence of a significant stimulatory effect on apo B lipoprotein-Aβ secretion. The findings are consistent with Probucol normalising enterocytic Aβ biogenesis, rather than promoting enterocytic secretion of Aβ.

Table 1 Effect of various feedings regimes on the average weight and plasma lipids in wild-type mice (C57BL/6J)

Feeding regime[c]	Body weight (g)[d]		Plasma lipids (mM)[e]	
	Final	Weight gain	TC	TAG
LF ($n = 8$)	20.56 ± 0.27	2.00 ± 0.16	1.86 ± 0.06	0.55 ± 0.05
LF + *P* ($n = 8$)	22.23 ± 0.36[a]	4.55 ± 0.16[a]	0.83 ± 0.04[a]	0.65 ± 0.06
HF ($n = 8$)	22.48 ± 0.33[a]	4.36 ± 0.19[a]	2.88 ± 0.17[a]	0.63 ± 0.10
HF + *P* ($n = 8$)	23.58 ± 0.32	5.82 ± 0.19[b]	0.67 ± 0.05[b]	0.39 ± 0.04

Data represented as mean ± standard error of the mean (SEM), numbers (n) indicate total number of samples used

LF low-fat, *HF* high-fat, *P* probucol, *TC* total serum cholesterol, *TAG* serum triglyceride

Values with lower case [a] and [b] indicate statistical significance in comparison with LF and HF groups, respectively, where $P < 0.05$

[c] Wild-type mice (C57BL/6J) were randomised to four different feeding regimens ($n = 8$ mice per group) and were fed their respective diets for 32 days. Weights, total serum cholesterol and triglyceride levels at the end of the experiment were compared between the groups with Post-hoc comparison of means within the ANOVA procedure and $P < 0.05$ was considered significant

[d] The average final body weights (per mouse) for LF + *P* and HF groups were significantly greater in comparison to the LF group; however, HF + *P* group final body weight was similar to that of the HF group. There was significant weight gain by all groups (vs LF) and there was also weight gain by the HF + *P* treated mice in comparison to the HF group

[e] Total plasma cholesterol level was significantly reduced by probucol treatment alone (vs LF), also by the HF + *P* treated group in comparison to the HF group. Fat feeding significantly have increased the circulating cholesterol level. On the other hand, neither the probucol treatment nor fat feeding did not affect the triglyceride concentration

Indeed, whilst Probucol was found to stimulate apo B lipoprotein secretion in LF-fed mice, there was no evidence that this translated into significantly increased enterocytic release of Aβ.

The HF diet utilized in this study contained both SFA and cholesterol, provided together in a context that it is physiologically relevant in comparison to commonly consumed atherogenic diets. The effects of Probucol on Aβ synthesis and secretion reported in this study must therefore be considered in the context of a mixed dietary lipid setting. Regulation by Probucol may change depending on the interactive effects of dose and duration of dietary lipids.

Several studies have shown synergistic stimulatory effects of fatty acids and cholesterol on apo B lipoprotein secretion [35, 36]. Therefore, the finding of increased apo B lipoprotein secretion shown in this study in HF-fed mice is to be expected. However, previous studies in the same strain of mice fed SFA, or cholesterol, found stimulatory and suppressive effects, respectively, on enterocytic abundance of Aβ [29, 33]. Saturated fatty acids were shown to have a profound stimulatory effect on enterocytic Aβ abundance, whereas dietary cholesterol was inhibitory. The reasons for the paradoxical differences between SFA and cholesterol have not been determined but may include differential regulation of Aβ biogenesis, transfer and association of Aβ with apo B lipoproteins and/or changes in intracellular degradation of Aβ or apo B lipoproteins.

In this study, saturated fats presented at 20% of digestible energy combined with 1% (w/w) cholesterol increased enterocytic abundance by approximately 75% above control mice given the LF diet alone. Hence, it would appear that the effects of SFA on enterocytic Aβ homeostasis were substantially greater than that of dietary cholesterol. How SFA influence Aβ biogenesis and association with apo B lipoproteins is not known. One possibility is increased lipidation of Aβ, a process found to protect other lipophilic apoproteins from proteolytic degradation.

Several studies suggest that the intracellular distribution between free cholesterol may be important in modulating Aβ homeostasis and intracellular kinetics. Inhibition of cholesterol trafficking in neuronal cells decreased β-secretase but enhanced γ-secretase processing of Aβ precursor protein [37]. The substantial increase in γ-secretase resulted in an increased intracellular concentration of Aβ [37]. Whilst in enterocytes Aβ biogenesis does not appear to occur at the plasma membrane, the subcellular distribution of cholesterol might nonetheless induce critical changes in the cell membranes of intracellular compartments such as within the endoplasmic reticulum and Golgi or re-localise enzymes responsible for Aβ synthesis, or its association with primordial lipoproteins. The notion that Probucol regulates enterocytic biogenesis of Aβ or association with apo B lipoproteins via modulation of intracellular pools of cholesterol is supported by the findings of Tawara et al. [38], who reported that Probucol stimulates cholesterol biosynthesis in absorptive epithelial cells of the small intestine, a process that would suppress Aβ biogenesis.

The HF diet resulted in greater body weight gain compared to LF-fed mice, presumably as a consequence of increased caloric intake and, somewhat surprisingly, Probucol also enhanced body weight gain in LF-fed mice. However, there was no evidence that body weight was associated with perinuclear or lacteal abundance of Aβ, or

of apo B lipoprotein-Aβ, so it is unlikely there is a causal association.

Clinical and animal studies suggest that Probucol may reduce AD risk and attenuate amyloidosis [26, 27, 39]. Suggested mechanisms include enhanced cerebrovascular efflux of soluble Aβ and neuro-protection as a consequence of suppression of oxidative pathways. Other indirect lines of evidence suggest that Probucol could confer AD protection by reducing vascular exposure to cytotoxic compounds including exaggerated plasma cholesterol, fatty acids or a reduction in inflammatory proteins including Aβ. The findings of this study support the latter notion and show that Probucol appears to suppress dietary fat induced stimulation of Aβ biosynthesis.

The 1 month dietary intervention study described in this study did not identify any significant increase in secretion of apo B lipoprotein-Aβ per se, and hence may reflect a localised phenomenon that is not particularly relevant to AD risk. Clearly, longer term feeding studies with an emphasis on the effects of Probucol on blood–brain barrier integrity and plasma Aβ homeostasis are warranted.

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Conflict of interest The authors have no conflicts of interest to declare in relation to this article.

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CHAPTER 3

Chapter 3: The effect of docosahexaenoic acid on saturated fatty acid induced brain parenchymal extravasation of apolipoprotein-B

Content on this chapter is covered by the article:

Pallebage-Gamarallage M, Lam V, Takechi R, Galloway S & Mamo JCL. (2012b) A diet enriched in docosahexanoic acid exacerbates brain parenchymal extravasation of apo B lipoproteins induced by chronic ingestion of saturated fats. *Int J Vasc Med.* 2012, 647689.

Synopsis

Background:

Population and clinical studies have suggested that regular intake of SFA, trans-fatty acids and cholesterol are positive risk factors for AD (Grant 1997, Kalmjin et al. 1997). In contrast, individuals with increased consumption of diets rich in PUFAs and MUFAs have lower prevalence of AD and vascular-based dementia (Barberger-Gateau et al. 2002, Morris et al. 2003, Ferrucci et al. 2006). Alzheimer's disease is characterised by brain parenchymal accumulation of Aβ associated with chronic inflammation leading to neuronal loss (Pastorino & Lu 2006). Presently, the exact sources of cerebrovascular Aβ deposits are unclear. However, growing evidence suggests parenchymal Aβ accumulation is exaggerated if cerebrovascular integrity is compromised and blood-to-brain delivery of circulating TRL-Aβ is increased (Takechi et al. 2010a, Thomas et al. 1997, Zlokovic et al. 1993).

We previously reported the role of dietary fatty acids on cerebrovascular integrity (Takechi et al. 2010a). We observed exacerbated cerebrovascular permeability in response to chronic SFA consumption in WT mice associated with significant loss of endothelial tight junction protein occludin-1. Furthermore, BBB dysfunction was concomitant with significant cerebral extravasation of plasma derived TRL-Aβ. The latter was supported by significant apo B colocalisation with amyloid plaques in transgenic mouse brain specimens. In contrast, mice fed MUFA and PUFA diets had no significant cerebrovascular dysfunction and were comparable to mice maintained on LF control diet.

Dietary fatty acid induced BBB dysfunction may be as a result of their cytotoxic properties. Saturated fatty acid mediated mechanisms include a mitochondrial respiratory burst resulting in oxidative stress and ER dysfunction (Morgan 2009, Proudfoot et al. 2009). In contrast, PUFAs generally antagonise the cytotoxic effects of SFA and are contended to provide cytoprotection because of potent anti-inflammatory effects. In particular the ω-3 PUFA, DHA, consumption was found to be protective against neurodegenerative disorders (Barberger-Gateau et al. 2002, Grant 1997, Kalmjin et al. 1997, Morris et al. 2003). Prasad et al. (1998) showed that in AD subjects, in cerebral regions including hippocampus, para-

hippocampal gyrus and the inferior parietal lobule, had significantly lower concentrations of DHA, arachidonic acid and stearic acid. These results indicate that the essential fatty acid components, in particular DHA, are important regulators for neuroinflammation and oxidative stress.

This chapter explores the hypothesis that a PUFA diet enriched in DHA can reverse SFA induced BBB dysfunction and attenuate cerebral extravasation of Aβ containing apo B lipoproteins. WT mice were initially maintained on a SFA enriched diet for 12 weeks to induce BBB dysfunction, followed by randomisation to either a LF or a PUFA diet for 8 weeks.

Methods in brief:

3-D immunofluorescence quantitation was utilised to determine brain parenchymal extravasation of apo B in WT mice. In addition, plasma concentration measurement of brain derived S100B was used as a secondary surrogate marker for disturbed BBB kinetics. The protein S100B is an inflammatory mediator produced exclusively by the astrocytes of the CNS and commonly used as a surrogate marker of brain-to-blood leakage.

Results in brief:

Our findings confirm previous studies where chronic SFA feeding enhanced cerebral extravasation of apo B. Mice randomised to LF diet following SFA feeding had no effect on the SFA induced parenchymal extravasation of apo B. Paradoxically, the SFA fed mice switched to a DHA enriched diet had increased cerebral accumulation of apo B compared to mice maintained on SFA alone. The detrimental effect of the DHA diet following chronic ingestion of SFA was supported by the findings of S100B concentration in plasma. The SFA-to-DHA randomised mice showed a 2-fold/significant increase in plasma S100B concentration compared to SFA control mice. In addition, DHA supplementation significantly increased plasma cholesterol concentration.

Discussion and conclusion in brief:

This study explored if provision of DHA enriched diet would attenuate BBB dysfunction induced by chronic SFA consumption. The principle outcome was to

quantitate cerebral extravasation of apo B lipoproteins that transport significant systemic derived Aβ. We conclude that the substantial provision of PUFA diet in a significantly heightened state of inflammation may initially be detrimental to BBB integrity because of heighted oxidative stress and PUFA susceptibility to lipid peroxidation (Barden et al. 2009). Other potential mechanisms include the hypercholesterolemic effect of the DHA diet. Others have demonstrated that cholesterol may be pro-inflammatory and can cause ER and mitochondrial stress resulting in cellular apoptosis (Almeida et al. 2007, Hennig et al. 2001, Yao and Tabas 2001). The latter may contribute to increased cerebral extravasation of circulating TRL-Aβ.

The findings presented in this chapter are consistent with paradoxical observations as to the purported benefits of ω-3 fatty acids for prevention and treatment of vascular-based disorders (Egert and Stehle 2011, Kris-Etherton et al. 2002, Albert et al. 1998, Ascherio et al. 1995, Kromhout et al. 1996, Mackay et al. 2012). Clearly, further studies are required to substantiate the potential detrimental effects of ω-3 PUFAs on BBB and whether this occurs via a a lipid peroxidation and oxidative stress axis. Collectively, the findings of this study suggest that introduction of PUFA in a pre-existing heightened state of inflammation needs to be carefully considered.

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Research Article

A Diet Enriched in Docosahexanoic Acid Exacerbates Brain Parenchymal Extravasation of Apo B Lipoproteins Induced by Chronic Ingestion of Saturated Fats

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Chronic ingestion of saturated fatty acids (SFAs) was previously shown to compromise blood-brain barrier integrity, leading to brain parenchymal extravasation of apolipoprotein B (apo B) lipoproteins enriched in amyloid beta. In contrast, diets enriched in mono- or polyunsaturated (PUFA) oils had no detrimental effect. Rather, n3 and n6 oils generally confer protection via suppression of inflammation. This study investigated in wild-type mice if a PUFA diet enriched in docosahexanoic acid (DHA) restored blood-brain barrier integrity and attenuated parenchymal apo B abundance induced by chronic ingestion of SFA. Cerebrovascular leakage of apo B was quantitated utilising immunofluorescent staining. The plasma concentration of brain-derived S100β was measured as a marker of cerebrovascular inflammation. In mice fed SFA for 3 months, provision thereafter of a DHA-enriched diet exacerbated parenchymal apo B retention, concomitant with a significant increase in plasma cholesterol. In contrast, provision of a low-fat diet following chronic SFA feeding had no effect on SFA-induced parenchymal apo B. The findings suggest that in a heightened state of cerebrovascular inflammation, the provision of unsaturated fatty acids may be detrimental, possibly as a consequence of a greater susceptibility for oxidation.

1. Introduction

Accumulating evidence supports the hypothesis that dietary behaviour and in particular ingestion of fats contribute to Alzheimer's disease (AD) onset and progression. The work in [1] reported that the prevalence of sporadic and late-onset AD in >65 years of age subjects correlated with fat intake and was higher in Western countries, compared to Africa or Asia. Population and clinical studies also suggest that regular consumption of saturated fatty acids (SFAs) and trans-fatty acids as well as cholesterol is synergistically and positively associated with increased risk of AD [1, 2] through mechanisms that include dyslipidemia, endothelial dysfunction, inflammation, and oxidative stress. In contrast, populations with greater consumption of fats as poly- or mono-unsaturated oils (PUFA and MUFA, resp.) have lower prevalence of AD and vascular based dementias [1–4], probably as a consequence of lower levels of systemic inflammation [5–7].

In AD, chronic inflammation leading to neuronal loss appears to be primarily associated with cerebrovascular and brain parenchymal deposits of amyloid beta (Aβ) [8]. Derived from the amyloid precursor protein, Aβ is the predominant component of "amyloid" (or senile) plaques [9, 10]. Key triggers of cerebrovascular amyloidosis are thought to include enhanced proteolytic processing of the precursor protein on the plasma membrane of neuronal cells [11–13], a phenomenon more common in early-onset AD. In addition, fibrillar formation of Aβ and deposition upon extracellular matrices may also reflect decreased degradation and efflux by epithelial cells of the choroid plexus [14, 15]. Alternatively, cerebral parenchymal Aβ load may be exacerbated if cerebrovascular integrity is compromised and

blood-to-brain delivery of peripheral Aβ is increased [16, 17]. Moreover, the latter typically results in the activation of astro-glial cells and oxidation of proteins and lipids [18, 19].

Significant plasma Aβ is found associated with triglyceride-rich lipoproteins (TRLs) and cell culture and immunohistochemical studies confirm secretion of Aβ as a TRL from hepatocytes and absorptive epithelial cells of the small intestine [20–22]. In humans there is a transient increase in plasma Aβ concentration, following the consumption of a mixed lipid meal and kinetic studies in vivo showing that Aβ serves as a regulating apolipoprotein of TRLs [23]. However, several lines of evidence suggest that persistent disturbances in the TRL-Aβ pathway may contribute to AD risk. In three strains of amyloid transgenic mice, secretion into plasma of TRL-Aβ was strongly associated with onset and progression of amyloidosis [24]. Moreover, significant cerebrovascular disturbances were reported preceding plaque formation in amyloid transgenic mice [25]. Consistent with the concept of disease induction in response to exaggerated exposure, subjects with AD were reported to have significantly elevated plasma TRL-Aβ concomitant with evidence postprandial dyslipidaemia [26]. Moreover, in human cadaver and in transgenic-amyloid mice brain specimens, significant apolipoprotein B (apo B) immunoreactivity colocalized with early diffused amyloid plaque [27, 28].

To explore directly the hypothesis of a dietary fat modulation of TRL-Aβ and cerebrovascular integrity axis, wild-type (WT) mice were fed diets enriched in either SFA, MUFA, or PUFAs [28]. Within 12 weeks of dietary intervention, mice maintained on the SFA diet showed substantial parenchymal extravasation of plasma proteins including apo B lipoproteins enriched with Aβ. The endothelial tight junction protein occludin was substantially attenuated in SFA-fed mice concomitant with substantially increased enterocytic abundance of Aβ [28, 29]. In contrast, mice maintained on either the MUFA or PUFA diets had no cerebrovascular aberrations and penetration of plasma proteins in these two groups was comparable to low-fat- (LF-) fed controls.

The potential cytotoxic properties of SFA are established and the principal mechanisms include mitochondrial respiratory burst resulting in oxidative stress and endoplasmic reticulum dysfunction [30]. Polyunsaturates on the other hand and in particular docosahexanoic acid (DHA) generally antagonise the effects of SFA and are purported to confer cytoprotection because of potent anti-inflammatory effects [5, 31–33]. However, unsaturated fatty acids such as DHA are highly susceptible to lipid peroxidation and if inflammation is already established, then oxidative damage may be paradoxically amplified with the provision of unsaturated fatty acids such as DHA. To explore the hypothesis that polyunsaturated fatty acids confer benefit and not risk in a cerebrovascular proinflammatory state, we explored parenchymal extravasation of apo B lipoproteins in WT mice initially maintained on an SFA-enriched diet for 3 months, followed by randomization to either an LF- or a PUFA-enriched diet for 2 months.

2. Methods

2.1. Animals and Diet Conditions. The animal housing, handling, and experimental procedures described for this study were approved by the Curtin University Animal Experimentation and Ethics Committee. Six-week-old female WT mice (C57BL/6J) were housed in groups and randomized into their respective diets ($n = 6$ mice per group). All mice were maintained on a 12 h light and dark cycle room, at 22°C and with free access to water and food.

Mice were fed an SFA diet (SF07-050, Glen Forrest Stockfeeders, Glen Forrest, Western Australia) for 3 months and then randomised to receive either an LF (AIN-93M, Glen Forrest Stockfeeders), PUFA-enriched (SF07-049, Glen Forrest Stockfeeders), or maintained on SFA for a further 2 months. Fatty acid composition for each diet is shown in Table 1. Digestible energy for the SFA diet was 18.8 MJ/kg and contained palmitic (16 : 0) and stearic (18 : 0) as the principle saturated fats (13%, w/w). The LF diet contained 4% (w/w) as total fat and 15.1 MJ/kg of digestible energy and only <1% of total digestible energy from lipids. The PUFA diet contained 8.22% (w/w) of DHA and 2.00% (w/w) eicosapentaenoic acid (EPA) sourced from NUMEGA fish oil. The diet generated 18.8 MJ/kg of digestible energy where 40% of energy derived from lipids. All diets had sufficient essential fatty acids.

2.2. Tissue Collection and Sample Preparation. Blood and brain samples were collected as previously described by Takechi et al. [28]. Mice were anaesthetised with pentobarbitone (45 mg/kg i.p.), and exsanguinated by cardiac puncture. Blood was collected into K-2 EDTA tubes and stored in ice. Plasma was separated by short speed centrifugation at 4°C and stored at −80°C.

Brain tissues were carefully isolated, washed with chilled phosphate buffer saline (PBS, pH 7.4), right hemispheres were separated, and fixed in 4% paraformaldehyde for 24 h. The tissues were then cryoprotected with 20% sucrose solution at 4°C for 72 h, frozen in isopentane with dry ice and stored at −80°C. For histology and fluorescence microscopy, serial cryosections of 18 μm were cut from the right cerebral hemispheres for each mouse and mounted on Polysine slides (LabServ, Australia).

2.3. Cerebral Apo B Immunofluorescence. Cerebrovascular leakage of apo B was detected as previously described [28]. Brain cryosections (18 μm) were air-dried for 30 min, rehydrated with PBS and incubated in blocking serum (10% goat serum) for 30 min prior to the application of the antibodies. Cerebral apo B was detected by overnight incubation with the primary antibody polyclonal rabbit anti-apo B (ab20737, Abcam, Cambridge, UK) at 1 : 500 dilution, at 4°C. Postovernight incubation and washing with PBS, primary antibody was labelled at room temperature with the secondary goat anti-rabbit IgG-Alexa 488 fluorochrome conjugate (A-11034, Invitrogen) for 2 h. The sections were then washed with PBS and the nuclei were counterstained with DAPI (1 : 1000) for 5 min at room temperature. Thereafter, the stained sections were mounted with antifade mounting

Table 1: Dietary composition.

	SFA diet	LF diet	DHA diet
Calculated nutritional parameters (%)			
Protein	13.6	13.6	13.6
Total fat	20.3	4	20.3
Crude fibre	4.7	4.7	4.7
Acid detergent Fibre	4.7	4.7	4.7
Digestible energy	18.8 MJ/kg	15.1 MJ/kg	18.8 MJ/kg
% Digestible energy from lipids	40	n/a	40
% Digestible energy from protein	15	n/a	15
Calculated fat composition (%)			
Myristic acid 14:0	0.05	Trace	0.54
Pentadecanoic acid 15:0	0.01	n/a	0.16
Palmitic acid 16:0	5.16	0.2	3.26
Megaric acid 17:0	0.05	n/a	0.18
Stearic acid 18:0	7.31	0.1	0.92
Arachidic acid 20:0	0.24	n/a	0.06
Behenic acid 22:0	0.04	n/a	0.05
Tetracosanoic acid 24:0	0.03	n/a	0.05
Palmitoleic acid 16:1	0.05	Trace	0.66
Heptadecenoic acid 17:1	0.01	n/a	0.10
Oleic acid 18:1 n9	6.62	2.4	2.25
Gadoleic acid 20:1	0.01	n/a	0.18
Lenoleic acid 18:2 n6	0.67	0.8	0.23
a Linolenic acid 18:3 n3	0.05	0.4	0.09
g Linolenic acid 18:3 n6	Not detected	n/a	0.08
Arachadonic acid 20:4 n6	Not detected	Trace	0.46
EPA 20:5 n3	Not detected	Trace	2.00
DHA 22:6 n3	Not detected	Trace	8.22

The total fatty acid composition of SFA, LF, and DHA diets. Vitamin and mineral contents were balanced in all diets.

medium. Primary antibody was replaced with buffer or an irrelevant serum for negative control tissues.

2.4. Quantitative Immunofluorescent Imaging and Analysis. Digital images for photomicroscopy were acquired through AxioCam HRm camera (Carl Zeiss, Germany) with an AxioVert 200 M inverted microscope by Zeiss (Germany) at ×200 magnification (Plan Neofluar ×20 objective, 1.3 numerical aperture). Three-dimensional (3D) images were captured through ApoTome optical sectioning methodology (Carl Zeiss). Each 3D image consisted of 6–10 two-dimensional (2D) images and the distance between Z-stack slices was 1.225 μm optimised by Nyquist. A minimum of nine 3D images were randomly captured per mouse, which include 5 images within the cortex (CTX) and 2 images each from brainstem (BS) and hippocampal formation (HPF).

Cerebrovascular leakage of plasma protein apo B was quantified within the CTX excluding the hippocampus, BS, and HPF. The pixel intensity of the protein of interest for each 3D image was calculated utilising the automated optical intensity measurement tool in Volocity (Software version 5.5, Perkin Elmer, Melbourne, Australia) and expressed as per unit volume. The investigator was blinded during imaging and quantitation.

2.5. Plasma Cholesterol, Triglyceride, and NEFA. Plasma cholesterol and triglycerides were determined in duplicate by enzymatic assays (Randox Laboratories LTD, UK). Non-esterified fatty acids (NEFAs) were determined with NEFA-C (ASC-ACOD method, Wako Pure Chemical Industries, Osaka, Japan).

2.6. Plasma S100β Analysis. Plasma S100β is used as a marker of cerebrovascular inflammation and was measured using ELISA kits according to manufacturers' instructions (CosmoBio, Tokyo, Japan). Plasma S100β was measured with 30 μL of plasma sample or standard and incubated in precoated microtitre well plates at 4°C overnight. Plates were then incubated with biotinylated secondary antibody and Streptavidin-HRP for 2 h, each. Colour generated with substrate and optical density determined at 492 nm. After adjusting for sample dilution, final concentrations of plasma S100β were extrapolated from standard curve.

2.7. Statistical Analysis. This study utilised $n = 6$ mice per group and minimum of nine 3D images were captured per mouse for detection of apo B leakage. Each 3D image was constructed by stacking of sequential 2D images, therefore generating 324–540 two-dimensional images per group. All

Table 2: Effects of various feeding regimens on plasma lipids in wild-type (C57BL/6J) mice.

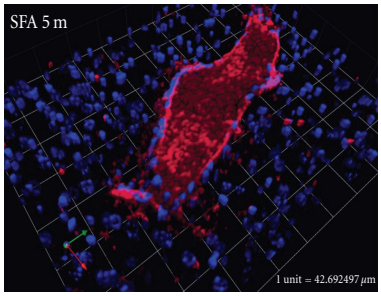
	SFA 5 m	+LF 2 m	+DHA 2 m
TG (mM)	0.39 ± 0.04	0.54 ± 0.03*	0.43 ± 0.04
TC (mM)	1.45 ± 0.23	1.69 ± 0.17	3.11 ± 0.12**
NEFA (mEq/L)	0.42 ± 0.03	0.45 ± 0.03	0.53 ± 0.05
Body weight			
Final	25.65 ± 0.64	23.72 ± 0.96	20.21 ± 0.50**
Weight gain	8.41 ± 0.51	5.7 ± 1.12	3.3 ± 0.51**

Plasma total cholesterol (TC), triglycerides (TGs), and non-esterified fatty acids (NEFAs) were measured at the end of the feeding regimen in mice fed saturated fats (SFA 5 m) and SFA-fed mice switched to an LF (+LF 2 m) and DHA diet (+DHA 2 m). Final body weight and weight gain were also calculated. Data represented as mean ± standard error of mean. Means were compared with one-way ANOVA, where $P < 0.05$ considered statistically significant (*).

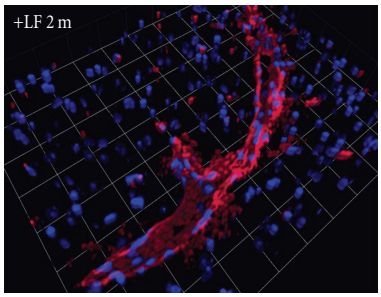
data was analysed by either parametric or nonparametric one-way Analysis of Variance to assess the main effects of LF and DHA on dietary SFA-induced blood-brain barrier (BBB) dysfunction and their two-way interactions. Post-hoc comparison of means was done if the associated main effect or interaction was statistically significant within the Analysis of Variance procedure. P values < 0.05 were considered statistically significant.

3. Results

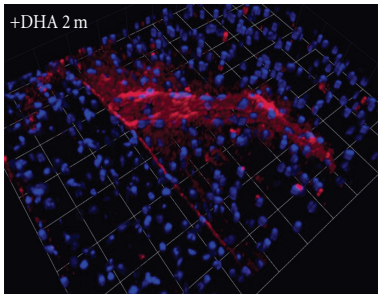
We confirm previous studies showing significant CTX > BS > HPF extravasation of apo B lipoproteins in WT mice maintained on an SFA diet for a total of 5 months (3 months plus randomization to SFA for an additional 2 months: Figures 1 and 2). Mice randomized to an LF diet following 3 months of SFA feeding had comparable levels of parenchymal apo B lipoprotein abundance to mice maintained on SFA feeding alone (Figures 1 and 2). However, in mice randomized to the DHA-enriched diet following 3 months of SFA feeding, parenchymal apo B abundance was markedly increased (Figures 1 and 2). The cerebrovascular effects of the DHA diet occurred commensurate with a 2-fold increase in plasma cholesterol compared to mice maintained on the SFA diet (Table 2). In contrast, the LF diet had no significant impact on plasma lipid homeostasis. Disturbances in BBB integrity and function were supported by the findings of substantially increased plasma S100β in the SFA → DHA mice compared to SFA alone (Figure 3). The protein S100β is commonly used as a surrogate marker of brain-to-blood leakage. The S100β is a cytokine produced exclusively by the astrocytes of the central nervous system. Following randomization, differences in food consumption were identified. Mice maintained on SFA or randomized to the LF consumed on average 3 g/day, whereas consumption of the DHA-enriched diet was reduced to 2 g/day. The lower caloric intake of mice on the DHA enriched diet resulted in a slower rate of growth following randomization (Table 2).



(a)



(b)



(c)

Figure 1: Three-D immunofluorescent staining of cerebral extravasation of apo B. Parenchymal leakage of apo B lipoproteins (red) is observed surrounding the cerebral microvessels. Nuclei are shown in blue. The 3D images are from mice maintained on saturated-fat diet for 5 months (SFA 5 m) and SFA fed mice randomised to LF (+LF 2 m) and DHA (+DHA 2 m) diet for further 2 months. Scale: 1 unit = 42.7 μm.

4. Discussion

This study was designed to explore if provision of a diet enriched in DHA attenuated cerebrovascular dysfunction induced by chronic ingestion of an SFA diet. The primary outcome measure was to determine the abundance of brain parenchymal apo B lipoproteins that transport significant endogenous Aβ. Cerebral capillary vessels normally have tightly apposed endothelial cells that ordinarily prevent transport of plasma proteins and macromolecules [34].

The primary finding of this study showed that provision of a PUFA diet principally enriched in DHA exacerbated brain parenchymal extravasation of apo B lipoproteins that had been initially induced by chronic ingestion of SFA. Previous studies exploring the effect on cerebrovascular integrity

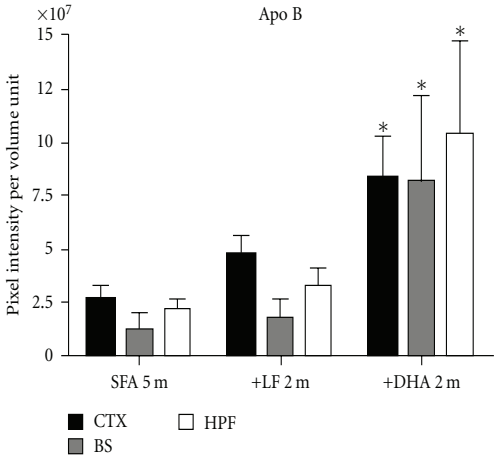


Figure 2: Three-dimensional (3D) quantitative analysis of apo B leakage in C57BL/6J mouse brain. Pixel intensity of apo B lipoproteins surrounding the cerebrovasculature was quantitated in 3D images from mice maintained on saturated-fat diet for 5 months (SFA 5 m) and SFA-fed mice randomised to LF (+LF 2 m) and DHA (+DHA 2 m) diet for further 2 months. The extent of apo B pixel intensity was measured in the cortex (CTX), brain stem (BS), and hippocampal formation (HPF) and expressed as per unit volume. The bars represent mean intensity and standard error of mean, where $P < 0.05$ considered statistically significant (* Kruskal Wallis t-test). The C57BL/6J mice randomised to DHA diet (+DHA) had elevated apo B intensities in all regions of the brain.

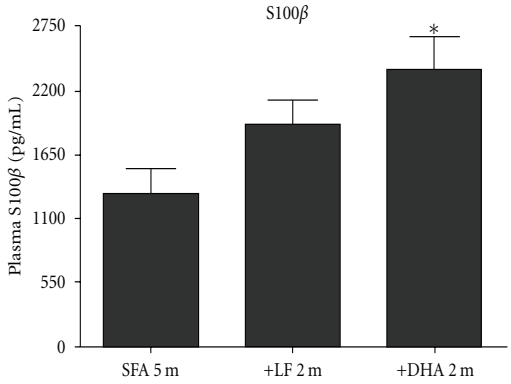


Figure 3: Effect of dietary fatty acids on plasma S100β concentration in C57BL/6J mice. The brain abundant protein S100β was measured in plasma as a surrogate marker of BBB leakage. High plasma S100β concentration in SFA-fed mice correlate with significant BBB dysfunction. In comparison to SFA 5 m group, plasma S100β was significantly increased in mice switched to the DHA diet (+DHA 2 m). The bars represent mean plasma concentration (pg/mL) and standard error of mean, where $P < 0.05$ considered statistically significant (* one-way ANOVA).

and function by the SFA and PUFA diets described here, as well as an MUFA-enriched diet, showed in C57BL/6J mice that only the SFA diet induced parenchymal accumulation of apo B lipoproteins [28]. Therefore, the paradoxical effects of the PUFA diet are likely to be a consequence of amplification of proinflammatory pathways induced as a consequence of chronic SFA ingestion. Consistent with this concept, SFA-fed mice randomized to an LF diet showed similar parenchymal apo B abundance and plasma S100β as mice that were maintained on SFA alone.

Several studies have provided evidence of a vasoactive role of Aβ, with pathological manifestations prior to Aβ deposition. Exogenous administration of Aβ is vasoconstrictive and vessels treated with Aβ show significant endothelial cell damage with changes in the cell membrane, cytoplasm, nucleus, and other organelles [16]. Takechi et al. [28] suggested that that chronic ingestion of SFA may increase TRL-Aβ secretion and that repeated postprandial excursions may eventually disrupt BBB function. Consistent with this possibility, SFAs were shown to stimulate enterocytic Aβ abundance and released into the circulation associated with postprandial TRL and a similar phenomenon may also occur in liver [20, 35]. Moreover, diets enriched in SFA reduce high affinity clearance pathways of TRL-remnant lipoproteins and this may contribute to increased postprandial lipaemia and plasma Aβ [36, 37].

In this study, parenchymal apo B abundance did not correlate with plasma triglyceride concentration. Mice fed the DHA-enriched diet had comparable triglycerides to the SFA group and plasma triglycerides were greatest in SFA mice randomized to LF. Saturated fats often increase plasma NEFA concentration compared to low-fat diets, whereas DHA generally accelerates TRL clearance by facilitating LPL-mediated lipolysis [38, 39]. However, in this study, there was no significant increase in net concentration of plasma NEFA in mice randomized to the DHA diet versus those maintained on SFA alone (0.53 ± 0.05 versus 0.42 ± 0.03 mEq/L, resp.). Nonetheless, a role of fatty acids in modulating cerebrovascular integrity cannot be ruled out because fatty acid phenotype may be critically important. Many studies suggest significant vascular effects of fatty acids. Human aortic endothelial cells treated with TRL and lipoprotein lipase (LPL) were highly permeable, whilst cells treated with TRL or LPL alone were not [40]. Furthermore, LPL-mediated TRL lipolysis initiated degradation of the tight junction protein ZO-1 and induced an endothelial apoptotic cascade.

The most significant lipid effect of randomization to DHA following chronic ingestion of SFA in this study was a twofold increase in plasma cholesterol compared to the mice maintained on the SFA diet, or mice randomied to the LF diet. A number of animal and clinical studies have shown that DHA-enriched diets can increase plasma cholesterol associated with low- and high-density lipoproteins [41–43]. Hypercholesterolemia has been associated with many vasculature abnormalities including endothelial dysfunction, decreased vascular reactivity, and enhanced expression of adhesion molecules [44, 45]. Cell culture studies suggest several mechanisms by which cholesterol may be pro-inflammatory and some of these appear to be analogous to the effects of dietary SFA. Yao and colleagues reported that excess cholesterol causes endoplasmic reticulum and mitochondrial stress that can lead to apoptosis [46]. Mitochondrial activity or microsomal processing also results in the production

of oxidized lipids that trigger and exacerbate inflammatory pathways [47].

The anti-inflammatory properties of particularly the n3 and n6 fatty acids have been unequivocally demonstrated in many studies. However, the propensity for PUFA to oxidize may under some circumstances amplify oxidative stress sequelae. Diets enriched in SFA enhance oxidation as a consequence of stimulated mitochondrial function in activated macrophages [46]. Dietary SFA diminish the proper function of the cerebrovascular endothelial cells and are thereafter likely to activate astro-glial cells which encompass cerebral capillary vessels. It's plausible that enhanced interaction of plasma PUFA's in DHA-fed mice with activated inflammatory cells may be a primary mechanism by which the effects of SFA are amplified. Consistent with this possibility, Kuo et al. [48] showed a dose effect of dietary DHA on BBB permeability in mice supplemented with 12% fish oil for 6 months, versus mice fed 3% fish oil. Similarly, rats consuming fish oil exhibited increased lipid peroxidation [49, 50] and oxidative stress-induced damage of DNA in the absence of dietary antioxidants [51]. In vitro, DHA and EPA enhanced lipid peroxidation and triggered cellular apoptosis [52, 53].

5. Summary

Chronic ingestion of diets enriched in SFA commonly causes vascular dysfunction, including in capillary vessels of the brain. The effects of SFA could be described as a response-to-injury phenomenon induced by exaggerated exposure to plasma triglyceride, cholesterol, NEFA, or harmful inflammatory products of lipid metabolism, such as lipid peroxides. Many studies support a role of n3 and n6 fatty acids in the prevention of vascular, based disorders primarily via suppression of inflammatory cascades. Less clear however are the benefits of polyunsaturated oils in the presence of profound inflammation, because of the propensity to generate lipid peroxidation products.

In an established model of cerebrovascular dysfunction induced by chronic ingestion of an SFA-enriched diet, provision of DHA amplified the harmful effects. Probable mechanisms include hypercholesterolemia and perhaps fatty acid-induced cytotoxicity. The data suggests that introduction of n3/n6 fatty acids in metabolic conditions that are characterized by heightened systemic inflammation needs to be carefully considered in the context of paradoxical detrimental effects that could occur.

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CHAPTER 4

Chapter 4: Restoration of dietary-fat induced cerebrovascular dysfunction by anti-inflammatory lipid modulating agents

Content of this chapter is covered by the article:

Pallebage-Gamarallage M, Lam V, Takechi R, Galloway S, Clark K & Mamo J. (2012c) Restoration of dietary-fat induced blood-brain barrier dysfunction by anti-inflammatory lipid-modulating agents. *Lipids Health Dis.* 11, 117.

Synopsis

Background:

Accumulating evidence support the concept that onset and progression of AD is influenced by vascular-risk factors. Studies have demonstrated detrimental effects on cerebrovascular integrity and increased risk of AD in populations consuming diets rich in SFAs, trans-fatty acids and cholesterol (Kalmijn et al. 1997, Laitinen et al. 2006, Petot et al. 2004, Takechi et al. 2010a). Furthermore, others have demonstrated significant association in cerebrovascular dysfunction with ageing in the absence of other comorbidities (Beckmann et al. 2003, Nagayama et al. 1999). Given the increase in ageing population worldwide, identifying strategies for prevention and regression of cerebrovascular dysfunction is a therapeutic priority.

Dietary lipid induced cerebrovascular pathological alterations are commonly associated with an heightened inflammatory response (Candore et al. 2010, Devore et al. 2009, Morris et al. 2006, Rossi et al. 2008). Several lines of evidence suggest that supressing cerebral capillary inflammation may confer protection in AD prevention and progression. Reducing the systemic concentration of cytokines and other pro-inflammatory proteins is concomitant with a delay in AD development (Candore et al. 2010, Devore et al. 2009, Moriss et al. 2006, Rossi et al. 2008). Although controversial, some human epidemiological studies suggest that long-term use of NSAIDs may prevent AD (Breitner et al. 2011, Imbimbo et al. 2010, In 'T Veld et al. 2001, Jaturapatporn et al. 2012, Vlad et al. 2008). Some epidemiological studies have shown a lower prevalence of AD among users of NSAIDs independent of modulating amyloidosis (In T' Veld et al. 2001, Vlad et al. 2008). Furthermore, among the individual NSAIDs, ibuprofen was found to be most protective (Vlad et al. 2008). In a primary prevention study (ADAPT trial) demonstrated chronic use of non-selective COX naproxen protected individuals from AD onset (Imbimbo et al. 2010). However, adverse events were detected at later stages of AD (Breitner et al. 2011). These findings suggest that chronic use of NSAIDs may benefit at early stages of AD. Given that accumulating evidence suggests that cerebrovascular dysfunction precedes amyloidosis, we contend that anti-inflammatory properties of NSAIDs may protect BBB against damage.

The positive association between dietary fat intake, hypercholesterolemia and AD raises the possibility that lipid modulating agents might delay onset or attenuate progression of AD. Some epidemiological studies have provided evidence of a lower prevalence of diagnosed AD and VaD in patients with hypercholesterolemia treated with statins (Crisby et al. 2002, Wolozin 2004). Statins are HMG-CoA reductase inhibitors commonly used in hypercholesterolemic patients to lower the plasma cholesterol levels (Shobab et al. 2005). However, the mechanisms for this association are not clear. In addition to beneficial effects on plasma lipids and TRL-Aβ, the purported efficacy of statins may reflect pleiotropic anti-inflammatory properties.

Restoration of cerebral capillary integrity would be beneficial in delaying AD onset and/or progression. However, currently, there is insufficient evidence to recommend statins for treatment of dementia once the disease is developed. Studies detailed in Chapter 1 (article 5) and Chapter 2 demonstrated beneficial effects of atorvastatin and probucol in the prevention of SFA/cholesterol induced BBB dysfunction. However, the beneficial effects of anti-inflammatory agents on restoration of cerebrovascular dysfunction were not explored. This chapter investigated the effects of atorvastatin, pravastatin and ibuprofen in restoration of SFA induced disturbances of the cerebrovascular integrity in WT mice.

Methods in brief:

Mice initially maintained on a SFA enriched diet for 12 weeks to induce BBB damage, were randomised to diets containing either atorvastatin, pravastatin or ibuprofen for 2 or for 8 weeks. Immunofluorescence microscopy was utilised for quantitative analysis of cerebral extravasation of plasma proteins IgG and Aβ enriched apo B lipoproteins. Plasma lipid levels were also measured to determine whether the diet and drug effects were dependent on lipid homeostasis.

Results in brief:

Our findings indicate that atorvastatin was most effective in attenuating cerebral extravasation of IgG and apo B independent of duration of treatment. Water soluble pravastatin was less effective in reducing cerebral IgG and apo B at 2 weeks of treatment. However, the pravastatin effects were more significant following 8 weeks of intervention. The lipid soluble COX inhibitor ibuprofen effects were

similar to that of atorvastatin. The statin effects were independent of significant changes in plasma lipid profile.

Discussion and conclusion in brief:

Consistent with previous observations, chronic SFA feeding significantly compromised BBB integrity resulting in cerebral extravasation of plasma proteins IgG and apo B (Takechi et al. 2010a). A significant finding of this study was that atorvastatin completely abolished cerebral extravasation of IgG and apo B within 8 weeks of treatment and restoration commenced within 2 weeks of intervention. Similarly, ibuprofen effectively attenuated brain parenchymal accumulation of IgG and apo B, however the effects were not as pronounced. In contrast to atorvastatin, hydrophilic pravastatin effectively attenuated cerebrovascular permeability only following the longer-term 8-week intervention. The difference in statin effects may be due to their difference in solubility. Lipophilic atorvastatin can passively diffuse through BBB allowing rapid and widespread tissue distribution (Shitara and Sugiyama 2006), whereas, hydrophilic pravastatin is delivered to the cerebral tissue via an active transport system (Shitara & Sugiyama 2006).

The statin effects were independent of lipid metabolism suggesting that the beneficial effects of statins on restoration of BBB integrity were mediated via regulation of inflammation. The latter is supported by the findings with ibuprofen. Furthermore, the drug effects were substantial within the hippocampal formation (HPF), the vital brain region involved in learning and memory (Tejada-Simon et al. 2005, Wang et al. 2006). Previous studies demonstrated potent SFA induced cerebrovascular dysfunction particularly within the HPF (Takechi et al. 2010a).

Collectively, findings in this chapter suggest that the anti-inflammatory properties of atorvastatin, pravastatin and ibuprofen may be responsible for the reversal of SFA induced BBB dysfunction.

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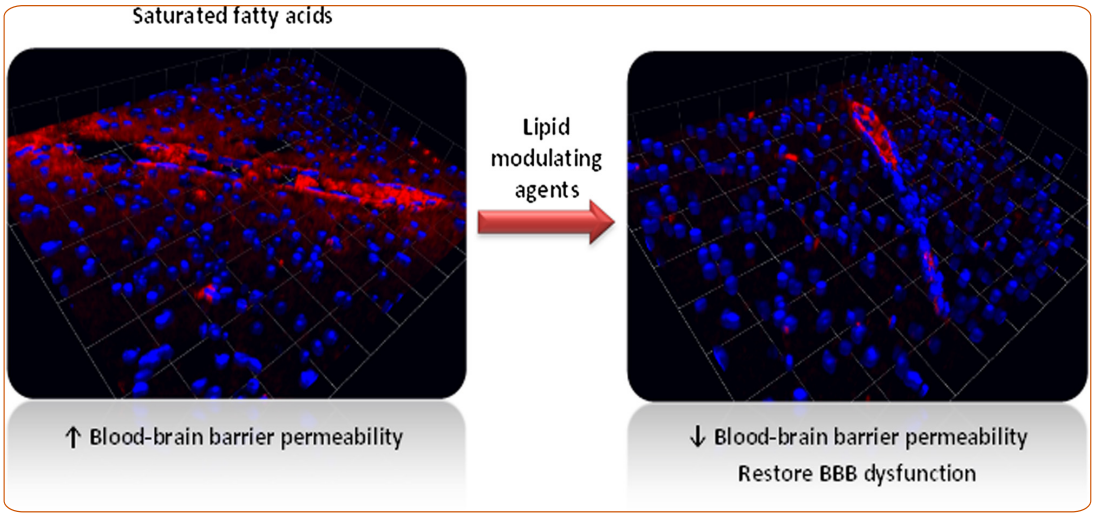

Restoration of dietary-fat induced blood–brain barrier dysfunction by anti-inflammatory lipid-modulating agents

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LIPIDS IN HEALTH AND DISEASE

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Restoration of dietary-fat induced blood–brain barrier dysfunction by anti-inflammatory lipid-modulating agents

Menuka Pallebage-Gamarallage[1,2], Virginie Lam[1,2], Ryusuke Takechi[1,2], Susan Galloway[1,2], Karin Clark[1,2] and John Mamo[1,2*]

Abstract

Background: Several studies have identified use of non-steroidal-anti-inflammatory drugs and statins for prevention of dementia, but their efficacy in slowing progression is not well understood. Cerebrovascular disturbances are common pathological feature of Alzheimer's disease. We previously reported chronic ingestion of saturated fatty acids (SFA) compromises blood–brain barrier (BBB) integrity resulting in cerebral extravasation of plasma proteins and inflammation. However, the SFA-induced parenchymal accumulation of plasma proteins could be prevented by co-administration of some cholesterol lowering agents. Restoration of BBB dysfunction is clinically relevant, so the purpose of this study was to explore lipid-lowering agents could reverse BBB disturbances induced by chronic ingestion of SFA's.

Methods: Wild-type mice were fed an SFA diet for 12 weeks to induce BBB dysfunction, and then randomised to receive atorvastatin, pravastatin or ibuprofen in combination with the SFA-rich diet for 2 or 8 weeks. Abundance of plasma-derived immunoglobulin-G (IgG) and amyloid-β enriched apolipoprotein (apo)-B lipoproteins within brain parenchyme were quantified utilising immunofluorescence microscopy.

Results: Atorvastatin treatment for 2 and 8 weeks restored BBB integrity, indicated by a substantial reduction of IgG and apo B, particularly within the hippocampus. Pravastatin, a water-soluble statin was less effective than atorvastatin (lipid-soluble). Statin effects were independent of changes in plasma lipid homeostasis. Ibuprofen, a lipid-soluble cyclooxygenase inhibitor attenuated cerebral accumulation of IgG and apo B as effectively as atorvastatin. Our findings are consistent with the drug effects being independent of plasma lipid homeostasis.

Conclusion: Our findings suggest that BBB dysfunction induced by chronic ingestion of SFA is reversible with timely introduction and sustained treatment with agents that suppress inflammation.

Keywords: Alzheimer's disease, Blood–brain barrier, Atorvastatin, Pravastatin, Ibuprofen, Saturated-fatty acids

Background

Accumulating evidence is consistent with the concept that the onset and progression of Alzheimer's disease (AD) is influenced by vascular-risk factors. A number of studies have demonstrated a positive association between AD and atherosclerosis, cardiovascular disease, dyslipidaemia, hypertension and insulin resistance [1,2]. Population studies have also demonstrated that consumption of diets which compromise vascular integrity, such as those enriched in saturated-fatty acids, trans-fatty acids, or cholesterol are also associated with increased risk of AD [3-5]. Moreover, recent animal model and clinical studies suggest that cerebral capillary dysfunction may develop with ageing in the absence of other significant comorbidities [6-8]. Clearly, identifying strategies to prevent or regress this age-induced effect on cerebrovascular function is a therapeutic priority given the aging population of developed and developing countries.

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Accumulating evidence suggests that cerebral capillary dysfunction precedes amyloidosis, a hallmark pathological protein marker for Alzheimer's disease [9]. Common vascular pathological alterations prior to amyloid deposition include a reduction of cerebral capillary endothelial tight junction proteins and increased endothelial pinocytic activity, which in combination result in parenchymal extravasation of plasma proteins within brain parenchyma [10-13]. Activation of glial cells and mitochondrial respiration are markedly increased, altering the phenotypic properties of astrocytes. In response to cytokine production by the latter, parenchymal penetrance of circulating monocytes may subsequently occur [10]. Thereafter, deposition of extracellular proteoglycans and collagen reduce arterial distensibility and may cause gross convolutional abnormalities including total capillary collapse with significant alterations in brain blood perfusion [14,15].

Several lines of evidence are consistent with the hypothesis that suppressing cerebral capillary inflammation may confer benefit to AD onset, or disease progression. Reducing the plasma concentration of cytokines and pro-inflammatory proteins by the regular consumption of foods or vitamin supplements that suppress inflammation is associated with a delay for development of dementia [16-19]. Furthermore, attenuation of cerebral capillary inflammatory processes by inhibition of cyclooxygenase (COX) via the use of non-steroidal anti-inflammatory drugs (NSAIDs) may aid in prevention and treatment of AD. Although beneficial properties of NSAIDs in prevention of AD remain controversial [20], some human epidemiological studies suggest that long-term uses of NSAIDs are protective against AD [21]. The adjusted odds ratios (OR) for AD among NSAID users decreased from 0.98 for less than or equal to one year of use, to 0.76 for greater than five years of use [21]. For users of ibuprofen, the OR decreased substantially from 1.03 to 0.56. In a primary prevention study (ADAPT trial) of naproxen (a non-selective COX inhibitor) and celecoxib (a COX-2 selective inhibitor), a 4-year follow-up assessment revealed that subjects previously exposed to naproxen were protected from the onset of AD by 67% compared to placebo [22]. Further analysis of the study identified reduced AD incidence in asymptomatic individuals. However, there were adverse effects at later stages of AD [23]. Therefore, it could be hypothesised that the chronic use of selected and non-specific NSAIDs may be beneficial in the early stages of AD. Direct evidence of an early preventive effect comes from animal studies, which have shown that a range of both COX-1 and COX-2 inhibitors can reduce plaque burden in AD mice and improve cognition in others [24,25].

Population studies also support a role for anti-inflammatory lipid lowering agents in the prevention of dementia. The 3-City Study represents a cohort of more than 9,000 subjects examining the association of plasma cholesterol and lipid-lowering agent intake with dementia prevalence [26]. In that cohort, 2% of participants were demented at baseline. Of the remainder, 30% of the subjects had been prescribed either HMGCoA reductase inhibitors (statins) or peroxisome proliferator activated receptor agonists (fibrates). The HMGCoA reductase inhibitors are widely used lipid-lowering agents that effectively reduce cardiovascular disease risk by not only a reduction in plasma cholesterol, but additionally pleiotropic anti-inflammatory properties. The study observed the OR for dementia was significantly lower among users of lipid lowering agents (OR = 0.61) compared with subjects taking no lipid lowering agents; the effect was similar between statin and fibrate users. The odds for dementia were increased in subjects with hyperlipidemia that were not treated with statins (OR = 1.43). This particular observational study suggests that anti-inflammatory lipid lowering agents could be associated with decreased risk of dementia. However, following adjusting for multiple co-founders, no association was found between lipid lowering agent intake in late life and reduced risk of dementia [27].

Presently, there is insufficient evidence to recommend statins for the treatment of dementia once disease is established or advanced [28]. However, in a cohort of approximately 3,100 subjects, the adjusted hazards ratio for developing AD was substantially reduced in subjects who commenced statin therapy at an earlier age [29]. This finding may explain paradoxical clinical studies, with only some statin interventions demonstrating benefit in subjects with AD [30,31]. Interestingly, a study in normolipemic spontaneously hypertensive rats supports the notion that statins have beneficial cerebral capillary effects; where atorvastatin was shown to prevent blood–brain barrier (BBB) dysfunction [32]. In culture studies of rat brain endothelial cells, pitavastatin was reported to strengthen the BBB integrity [33].

In a clinical context restoration of cerebral capillary integrity would be therapeutically beneficial for slowing or delaying AD progression, although presently this is a difficult phenomenon to assess *in vivo*. Despite this difficulty, proof-of-concept data using surrogate markers of cerebrovascular inflammation in relevant animal models would provide information on the putative efficacy of selected and targeted interventions. Our preliminary investigations demonstrated beneficial effects of atorvastatin in preventing saturated-fatty-acid (SFA) induced cerebrovascular dysfunction [34]. However, restoration by anti-inflammatory agents of BBB function has not been previously investigated. In this study, following, dietary SFA-induced disturbances of BBB integrity, mice were randomised to receive atorvastatin, pravastatin, or

ibuprofen. The brain parenchymal extravasation of large molecular weight plasma proteins, including apolipoprotein (apo)-B lipoproteins that are endogenously enriched in amyloid-β (Aβ) was assessed.

Results

Our results confirm the significant abundance of immunoglobulin-G (IgG) and distribution within the cortex (CTX), brainstem (BS) and hippocampal formation (HPF) in SFA fed wild-type (WT) (Figure 1 and Figure 2). The SFA fed mice had a five-fold greater abundance of IgG compared to the low fat (LF)-control mice, with the majority of this accumulation being indicated within the CTX > HPF > BS. However, HPF had the largest increase in IgG as a consequence of SFA feeding compared to the LF-control fed mice. Following SFA feeding for 12 weeks, provision of atorvastatin, pravastatin or ibuprofen for 2 weeks generally reduced the total parenchymal IgG abundance, however there were differential effects of the agents with respect to efficacy and tissue distribution. The abundance of IgG was essentially completely reversed with atorvastatin, a lipid soluble drug and notably, completely ameliorated the HPF accumulation. In contrast, the IgG distribution in mice given water-soluble pravastatin was not significantly different compared to the SFA treated group. Ibuprofen attenuated IgG as effectively as mice provided with atorvastatin. There was no statistical evidence of an interactive effect of drug with diet regimen (i.e., LF vs SFA) with drug.

The longer duration of SFA feeding significantly increased the apo B distribution in the parenchyma (compare y-axis, Figure 1 frame B vs. Figure 3), and this occurred primarily within the HPF. The efficacy of a longer period of intervention with atorvastatin, pravastatin or ibuprofen on apo B parenchymal abundance is depicted in Figure 3 and Figure 4. Provision of atorvastatin, pravastatin and also ibuprofen for 8 weeks completely suppressed the SFA-induced effect (Figure 3). This marked improvement with all three agents included normalisation of the HPF accumulation of apo B lipoproteins. There was no evidence of an interactive effect of drugs with diet.

The SFA diet was generally well tolerated consistent with no significant changes in plasma total cholesterol or triglyceride compared to LF controls (Tables 1 and 2). Indeed, the LF control had modestly higher plasma triglyceride compared to other intervention groups. Weight gain was also similar between all treatment groups relative to duration of experimental design (data not shown). Some differential agent effects were observed. Mice randomised to LF with atorvastatin (LF + At) diet for 8 weeks had significantly lower plasma cholesterol, triglyceride and non-esterified fatty acids (NEFA) concentrations (vs. LF control, Table 2), but this was not observed with the provision of atorvastatin with the SFA diet. Ibuprofen and pravastatin had no substantial effects on plasma lipid or NEFA concentrations. Pearson's correlation analysis found no association between plasma lipid homeostasis and the parenchymal abundance or distribution of IgG or apo B for any of the interventions described (data not shown).

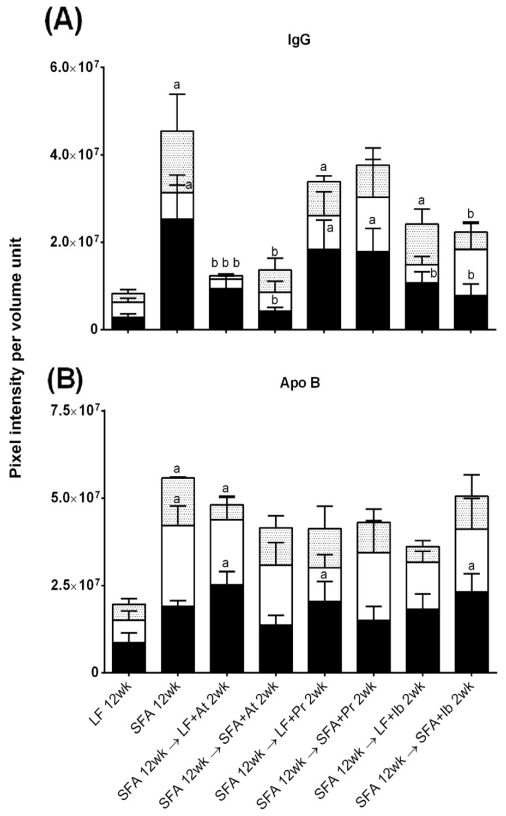

Figure 1 Quantition of cerebral IgG and apo B in mice randomised to drug diets for 2 weeks. The bar graphs show 3-dimensional (3D) quantitative analysis of IgG (frame A) and apo B (frame B) leakage of wild-type mouse brains at 2 weeks of drug consumption. Pixel intensity of IgG and apo B was quantitated surrounding the cerebrovasculature in mice fed low-fat (LF), saturated-fatty acids (SFA) and SFA mice randomised to diets containing atorvastatin (LF + At, SFA + At), pravastatin (LF + Pr, SFA + Pr) and ibuprofen (LF + Ib, SFA + Ib). Immunoglobulin-G and apo B pixel intensity was measured in the cortex (CTX, black column), brain-stem (BS, white column) and hippocampal formation (HPF, dotted column) and expressed as per unit volume. Symbol → indicate that mice were fed SFA diet for 12 weeks and then the diet was replaced (→) with a drug containing diet. The bars represent mean intensity and standard error of mean, where $P < 0.05$ considered statistically significant. a: statistically significant in comparison to LF. b: statistically significant in comparison to SFA.

Discussion

We confirm that SFA feeding significantly disrupts BBB integrity and function [35], resulting in exaggerated

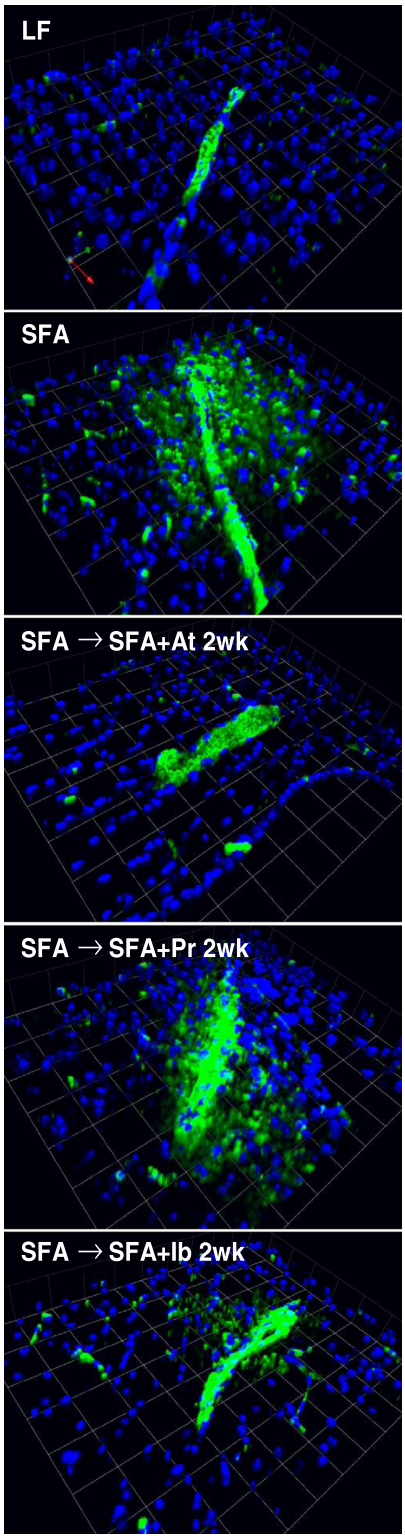

Figure 2 3-D immunofluorescent staining of cerebral IgG in mice randomised to diet + drug for 2 weeks. Parenchymal leakage of IgG (green) is observed surrounding the cerebral microvessels. Nuclei are shown in blue. The 3D images are from mice fed low-fat (LF), saturated-fatty acids (SFA) and SFA mice randomised to drug diets containing atorvastatin (LF + At, SFA + At), pravastatin (LF + Pr, SFA + Pr) and ibuprofen (LF + Ib, SFA + Ib) for 2 weeks. Scale: 1 unit = 42.7 μm.

cerebral extravasation of IgG and apo B, but now extend those findings and show an association of severity of dysfunction with duration of SFA feeding. Immunoglobulin-G and apo B lipoproteins are derived from peripheral circulation with molecular weights of approximately 166 kDa and 2.2-20 × 10^6 kDa, respectively, and are commonly used as a surrogate markers for BBB permeability. The measurement of cerebral abundance of different molecular weight proteins approximates the extent of vascular permeability in cerebral tissue [36]. Wild-type mice fed a SFA diet for 12 weeks had a 5-fold increase in parenchymal abundance of IgG compared to the LF control and about a 2-fold increase in apo B abundance. However, 20 weeks of SFA consumption resulted in >3-fold increase in cerebral apo B abundance when compared to the LF control.

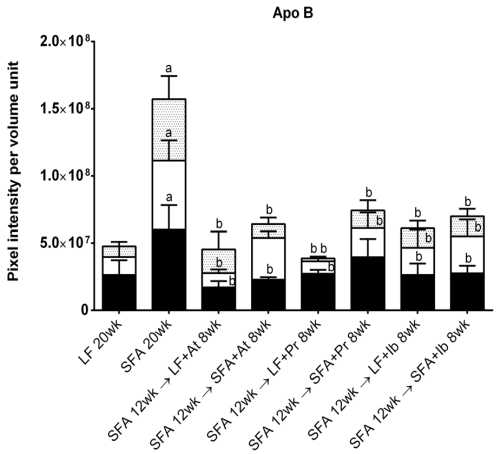

Figure 3 Quantitation of cerebral apo B in mice randomised to diet + drug for 8 weeks. The bar graphs show 3-dimensional (3D) quantitative analysis of apo B leakage of wild-type mouse brains. Pixel intensity of apo B was quantitated surrounding the cerebrovasculature in mice fed low-fat (LF), saturated-fatty acids (SFA) and SFA mice randomised to diets containing atorvastatin (LF + At, SFA + At), pravastatin (LF + Pr, SFA + Pr) and ibuprofen (LF + Ib, SFA + Ib). Apo B pixel intensity was measured in the cortex (CTX, black column), brain-stem (BS, white column) and hippocampal formation (HPF, dotted column) and expressed as per unit volume. Symbol → indicate that mice were fed SFA diet for 12 weeks and then the diet was replaced (→) with a drug containing diet.The bars represent mean intensity and standard error of mean, where $P < 0.05$ considered statistically significant. a: statistically significant in comparison to LF. b: statistically significant in comparison to SFA.

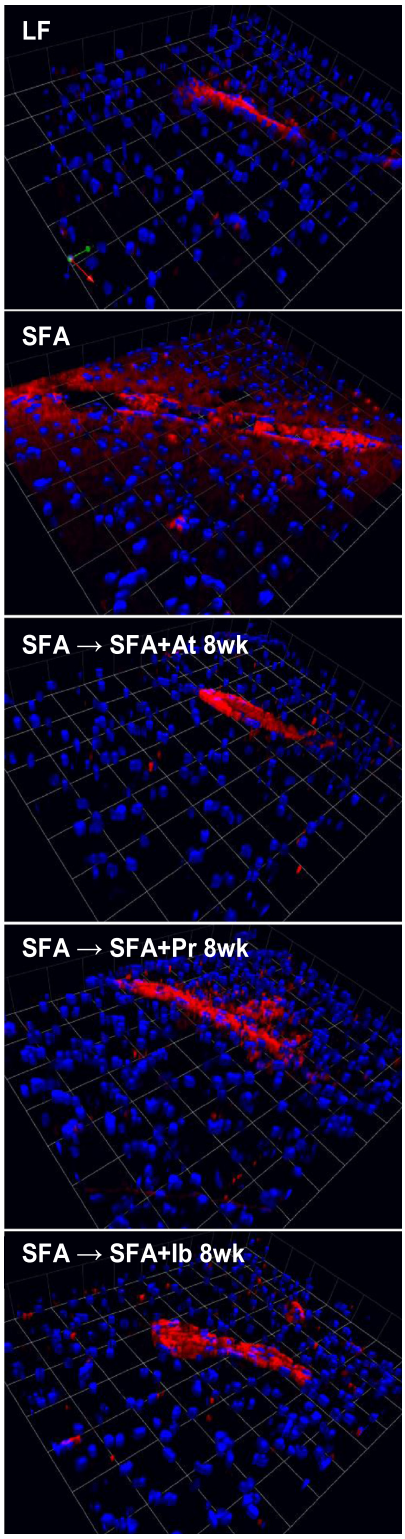

Figure 4 3-D immunofluorescent staining of cerebral apo B in mice randomised to diet + drug for 8 weeks. Parenchymal leakage of apo B (red) is observed surrounding the cerebral microvessels. Nuclei are shown in blue. The 3D images are from mice fed low-fat (LF), saturated-fatty acids (SFA) and SFA mice randomised to drug diets containing atorvastatin (LF + At, SFA + At), pravastatin (LF + Pr, SFA + Pr) and ibuprofen (LF + Ib, SFA + Ib) for 8 weeks. Scale: 1 unit = 42.7 μm.

Therefore, our findings suggest that the longer the duration of SFA feeding, the greater degree of vascular permeability.

The protective role of statins and NSAIDs on dietary-induced vascular disorders suggests properties that may attenuate cerebral capillary dysfunction [37,38]. The objective of this study was to assess whether dietary SFA-induced cerebral extravasation of IgG and apo B could be reversed by atorvastatin, pravastatin, or ibuprofen in WT mice. A key finding was that atorvastatin completely abolished the accumulation of brain parenchymal IgG and apo B within 8 weeks of treatment and that restoration of function had commenced within just 2 weeks of treatment. This atorvastatin-induced reversal phenomenon occurred independent of whether the mice were maintained on the LF or SFA diet, suggesting potent effects in the continued presence of a potent dietary vascular insult. Similarly, ibuprofen had regressed cerebral abundance of IgG at 2 weeks of intervention, however the magnitude of its effects were not as profound as atorvastatin. A part systemic-mediated phenomenon is supported by the finding that pravastatin, a water soluble statin with poor diffusion properties through the BBB showed efficacy, albeit at 8 weeks of treatment. Atorvastatin, being lipophilic, can passively diffuse through the BBB allowing more rapid and widespread tissue distribution [39]. Pravastatin requires a rate limiting active transport system for cerebral delivery to occur [39].

Evidence that the beneficial effects of atorvastatin and pravastatin on BBB integrity and function were mediated via regulation of inflammation and not lipid metabolism are suggested by the findings with ibuprofen, a non-selective COX inhibitor. Parenchymal abundance of IgG was substantially reduced following 2 weeks of treatment in mice that had been fed an SFA-enriched diet for 12 weeks. Apo B abundance was also significantly reduced with ibuprofen therapy after 8 weeks, analogous to the findings with atorvastatin.

Regional differences in statin or ibuprofen-induced restoration were observed. Atorvastatin was most effective in normalising hippocampal IgG load at 2 weeks and apo B at 8 weeks of intervention, with only partial restoration in CTX and BS with longer-term (8 week) treatment. Treatment with pravastatin or ibuprofen, although less potent than atorvastatin, nonetheless resulted in a

Table 1 Plasma lipid profile of SFA mice randomised to SFA diet + drugs for 2 weeks

					SFA 12wk ↓			
	LF 12wk	SFA 12wk	LF + At 2wk	SFA + At 2wk	LF + Pr 2wk	SFA + Pr 2wk	LF + Ib 2wk	SFA + Ib 2wk
Cholesterol (mM)	1.83 ± 0.20	1.54 ± 0.20	1.68 ± 0.11	1.69 ± 0.05	1.64 ± 0.13	1.91 ± 0.13	1.93 ± 0.12	1.59 ± 0.13
Triglycerides (mM)	0.74 ± 0.08	0.34 ± 0.04[a]	0.39 ± 0.03[a]	0.34 ± 0.02[a]	0.36 ± 0.01[a]	0.40 ± 0.04[a]	0.42 ± 0.03[a]	0.31 ± 0.02[a]
NEFA (mEq/L)	0.85 ± 0.08	0.68 ± 0.04	0.93 ± 0.05	0.82 ± 0.04	1.02 ± 0.05[b]	0.90 ± 0.08	1.05 ± 0.10[b]	0.79 ± 0.05

The table shows the effects of various feeding regimens on plasma lipids. Plasma total cholesterol, triglycerides and non-esterified fatty acids (NEFA) were measured at the end of the feeding regimen. Data represented as mean ± standard error of mean. Means were compared with non-parametric independent t test, where $P < 0.05$ considered statistically significant.
a: statistically significant in comparison to LF;
b: statistically significant in comparison to SFA.
Abbreviations: LF, low-fat; SFA, saturated fatty acids; At, atorvastatin; Pr, pravastatin; Ib, ibuprofen; wk, weeks.

uniform reduction of IgG and apo B abundance within HPF, CTX and BS. Ibuprofen showed evidence of efficacy by 2 weeks in HPF and CTX, but pravastatin had no significant effect within these regions at 2 weeks. Collectively, these findings indicate that atorvastatin was most effective in regression of hippocampal plasma protein abundance independent of duration of intervention. Longer-term intake of pravastatin and ibuprofen was required to ameliorate accumulation of plasma proteins within the HPF.

The HPF is the vital brain region involved in learning, memory and some suggest, particularly vulnerable to oxidative damage induced by inflammation [40,41]. Pathological changes reported in human brain specimens indicate that capillary and larger vessel disturbances occur within the HPF and precede the structural abnormalities that then develop within the temporal cortex and other regions of the brain [42-44]. The findings of previous studies demonstrating the potent effects of an SFA diet on BBB function principally within the HPF and of this study, showing the restorative effects of atorvastatin are therefore potentially of clinical relevance.

The mechanisms by which atorvastatin, pravastatin and ibuprofen influenced BBB integrity were not specifically explored in this study. However, the data show that the improvements in BBB function were not associated with plasma lipid homeostasis. These findings suggest that the diets were well tolerated in our mouse model. Consistent with our previous observations where SFA induced BBB dysfunction was independent of hypercholesterolemia [35]. Many studies have demonstrated pleiotropic effects of statins mediated principally via anti-inflammatory, or suppression of oxidative stress pathways, including endothelial specific protection [32,45,46]. In the context of these findings, the statins were comparable, or in some instances more effective than the commonly utilised COX inhibitor, ibuprofen. Similar findings on BBB protection in other models were reported for atorvastatin in a hypertensive rat model [32]. They demonstrated that disturbance in BBB permeability improvement with atorvastatin were associated with abundance of cerebrovascular tight junction proteins; zonula occludens and occludin; plasma nitric oxide concentration and anti-oxidant homeostasis. Similarly, pravastatin was shown to reduce micro-vascular permeability and restore vascular endothelial function via modulation of endothelial nitric oxide synthase level in micro-vessels of rat mesentery [47].

We previously demonstrated that SFA induced BBB leakage was associated with significant reduction in the tight junction protein occludin [35]. Furthermore, studies have demonstrated increased oxidative stress [48-51] and inflammatory cytokine [52,53] associated vascular endothelial dysfunction in rodents maintained on SFA enriched diets. In the model used in this study, statins and ibuprofen probably enhanced the expression of tight

Table 2 Plasma lipid profile of SFA mice switched to diets + drug for 8 weeks

					SFA 12wk ↓			
	LF 20wk	SFA 20wk	LF + At 8wk	SFA + At 8wk	LF + Pr 8wk	SFA + Pr 8wk	LF + Ib 8wk	SFA + Ib 8wk
Cholesterol (mM)	1.64 ± 0.14	1.43 ± 0.08	1.17 ± 0.11[a]	1.55 ± 0.14	1.52 ± 0.17	1.37 ± 0.09	1.81 ± 0.26	1.19 ± 0.11[a]
Triglycerides (mM)	0.80 ± 0.12	0.55 ± 0.14	0.40 ± 0.04[a]	0.63 ± 0.09	0.89 ± 0.09[b]	1.00 ± 0.17[b]	0.57 ± 0.07	0.80 ± 0.14
NEFA (mEq/L)	0.70 ± 0.04	0.61 ± 0.07	0.50 ± 0.07[a]	0.62 ± 0.05	0.62 ± 0.06	0.51 ± 0.04[a]	0.52 ± 0.04[a]	0.52 ± 0.06[a]

The table shows the effects of various feeding regimens on plasma lipids. Plasma total cholesterol, triglycerides and non-esterified fatty acids (NEFA) were measured at the end of the feeding regimen. Data represented as mean ± standard error of mean. Means were compared with non-parametric independent t test, where $P < 0.05$ considered statistically significant.
a: statistically significant in comparison to LF.
b: statistically significant in comparison to SFA.
Abbreviations: LF, low-fat; SFA, saturated fatty acids; At, atorvastatin; Pr, pravastatin; Ib, ibuprofen; wk, weeks.

junction proteins, suggesting structural stabilisation and repair of this pivotal capillary network.

A paradoxical finding was that abundance of apo B lipoproteins within brain parenchyme required a longer duration of treatment with statins or ibuprofen to be reduced compared IgG, yet apo B lipoproteins have a molecular weight of up to 100 times greater than IgG. Notionally, penetrance into brain parenchyme of macromolecules such as apo B lipoproteins would become evident more quickly than large proteins such as IgG. However, previous findings have shown significant retention of apo B lipoproteins associated with extracellular matrices including heparin-sulphate proteoglycans, biglycan and decorin [54]. The exaggerated presence of apo B lipoproteins compared to IgG in SFA mice treated with statins or ibuprofen, may be indicative of a relatively slow turnover through brain parenchyma, compared to proteins that more readily diffuse into cerebrospinal fluid, such as IgG. Interestingly, apo B lipoproteins that are enriched with amyloid-beta as a consequence of chronic SFA ingestion show remarkable colocalisation with amyloid-plaque in rodent models of AD [35].

Conclusion

Dysfunction of the BBB is increasingly recognised in neurodegenerative diseases with cerebral capillary disturbances including AD [55]. Although statin and NSAID use have demonstrated benefits for the prevention of AD, appropriate treatment following disease development is understudied and controversial. The majority of studies focus on pathological accumulation of Aβ within the brain parenchyma and neuronal death.

Evidence showing cerebral capillary dysfunction preceding amyloid deposition is growing. This study provides novel insight into the effects of atorvastatin, pravastatin and ibuprofen on regression and prevention of SFA-induced BBB permeability and preceding amyloidosis. Further studies are required to ascertain the underlying mechanisms of how statins and ibuprofen modulate BBB integrity.

Methods

Animals and diet conditions

The Curtin University Animal Experimentation and Ethics Committee approved housing, handling and experimental procedures described for this study. Six-week-old female WT mice (C57BL/6 J) were housed in groups and randomised into the diet or drug treatment groups (6 mice per group). All mice were maintained in a 12 h light and dark cycle room, at 22°C and with free access to water and food. Mice were weighed weekly and average daily diet consumption was recorded.

The LF control group of mice were fed a semi-purified diet (AIN93M, Glen Forrest Stockfeeders, Glen Forrest, Western Australia) containing 4% (w/w) total fat (derived from canola oil) and <1% of total digestible energy from lipids. As previously demonstrated, mice were fed SFA diet containing 20% (w/w) cocoa butter (SF07-050, Glen Forrest Stockfeeders) to induce BBB damage [35]. The SFA diet contained palmitic (16:0) and stearic (18:0) acids as the primary saturated fats (13% w/w). Digestible energy for LF and SFA diets were 15.1 MJ/kg and 18.8 MJ/kg, respectively (Table 3).

To determine the putative restorative effects of atorvastatin (At), pravastatin (Pr) and ibuprofen (Ib) on BBB damage, WT mice were initially fed with SFA diet for a period of 12 weeks to induce damage to the BBB. The SFA fed mice were then switched to SFA diets containing atorvastatin (SFA → SFA + At), pravastatin (SFA →

Table 3 Dietary composition

	SFA diet	LF diet
Calculated nutritional parameters (%)		
Protein	13.6	13.6
Total Fat	20.3	4
Crude Fibre	4.7	4.7
Acid Detergent Fibre	4.7	4.7
Total Carbohydrate	50	64.9
Digestible Energy	18.8 MJ/kg	15.1 MJ/kg
% Digestible Energy from Lipids	40	n/a
% Digestible Energy from Protein	15	n/a
Calculated fat composition (%)		
Myristic Acid 14:0	0.05	Trace
Pentadecanoic Acid 15:0	0.01	n/a
Palmitic Acid 16:0	5.16	0.2
Megaric Acid 17:0	0.05	n/a
Stearic Acid 18:0	7.31	0.1
Arachidic Acid 20:0	0.24	n/a
Behenic Acid 22:0	0.04	n/a
Tetracosanoic Acid 24:0	0.03	n/a
Palmitoleic Acid 16:1	0.05	Trace
Heptadecenoic Acid 17:1	0.01	n/a
Oleic Acid 18:1 n9	6.62	2.4
Gadoleic Acid 20:1	0.01	n/a
Lenoleic Acid 18:2 n6	0.67	0.8
a Linolenic Acid 18:3 n3	0.05	0.4
g Linolenic Acid 18:3 n6	Not detected	n/a
Arachadonic Acid 20:4 n6	Not detected	Trace
EPA 20:5 n3	Not detected	Trace
DHA 22:6 n3	Not detected	Trace

The table shows the total fatty acid composition of SFA and LF. Vitamin and mineral content were balanced in all diets. The agents were incorporated into either LF or SFA chow at a concentration of 20 mg/kg (w/w) atorvastatin, 23.4 mg/kg (w/w) pravastatin and 333.3 mg/kg (w/w) ibuprofen.

SFA + Pr) or ibuprofen (SFA → SFA + Ib) to determine the effect of drugs with insult (SFA diet). Mice switched to LF diets containing identical doses of atorvastatin (SFA → LF + At), pravastatin (SFA → LF + Pr) or ibuprofen (SFA → LF + Ib) to determine the effects of drugs in the absence of dietary insult. Animals that were initially fed SFA diet for 12 weeks were switched to the drug containing diets (LF + drug or SFA + drug). They were then sacrificed at two time points, at 2 weeks and 8 weeks after drug intervention, to observe any progressive effects of the drugs on BBB restoration. Mice given LF or SFA alone were run parallel with all experiments and sacrificed at each end point.

The agents were incorporated into either LF or SFA chow at a concentration of 20 mg/kg (w/w) atorvastatin (Lipitor, Pfizer, Australia), 23.4 mg/kg (w/w) pravastatin sodium (Lipostat®, Australia) and 333.3 mg/kg (w/w) ibuprofen (I110, Sigma-Aldrich, New South Wales, Australia). Based on measured consumption rates, the daily ingested dose for each agent approximated three times the highest recommended dose for human studies per unit body. However, the bioavailability of atorvastatin and pravastatin has been reported to be reduced when consumed with food [56,57].

Tissue collection and sample preparation

Mice were maintained on the indicated diets and weighed weekly. Tissue samples were collected as previously described by Takechi et al. [35]. Mice were anesthetised with pentobarbitone (45 mg/kg i.p.) and were exsanguinated by cardiac puncture. Blood was collected into K-2 EDTA tubes and stored on ice. Plasma was separated by short time, high speed centrifugation at 4°C and stored at −80°C.

Brain tissues were carefully isolated, washed with chilled phosphate buffered saline (PBS, pH 7.4), and the right hemispheres were separated and fixed in 4% paraformaldehyde for 24 h. The tissues were then cryoprotected with 20% sucrose solution at 4°C for 72 h, frozen in isopentane with dry ice and stored at −80°C. For histology and fluorescence microscopy, serial cryo-sections of 18 μm were cut from the right cerebral hemispheres for each mouse and mounted on Polysine slides [35].

Immunoglobulin-G and apolipoprotein B immunofluorescence

Cerebrovascular leakage of IgG and apo B were detected as previously described [35]. Brain cryosections (18 μm) were air-dried for 30 min, rehydrated with PBS and incubated in blocking serum (10% goat serum) prior to application of the antibodies.

For IgG staining, tissues were incubated with goat anti-mouse IgG-Alexa 488 flurochrome conjugated antibody (Invitrogen) at 1:100 dilution, overnight at 4°C. The sections were then washed with PBS and nuclei were counterstained with DAPI (1:1000) for 5 min at room temperature. Thereafter, the stained sections were mounted with anti-fade mounting medium.

Cerebral apo B was detected by overnight incubation with polyclonal rabbit anti-apo B as the primary antibody (ab20737, Abcam, Cambridge, UK) at 1:500 dilution, at 4°C. Post-overnight incubation, primary antibody was labelled at room temperature with the secondary goat anti-rabbit IgG-Alexa 488 conjugate (Invitrogen) for 2 h. The tissues were then counterstained with DAPI and mounted as per IgG staining method [35].

Immunofluorescent imaging of and quantitative analysis of cerebral IgG and apo B

Digital images for photomicroscopy were acquired through AxioCam HRm camera (Zeiss Germany) with an AxioVert 200 M inverted microscope by Zeiss (Germany) at × 200 magnification (Plan Neofluar x20 objective, 1.3 numerical aperture). Three-dimensional (3-D) images were captured through ApoTome optical sectioning methodology (Carl Zeiss) [35,58]. Each 3-D image consisted of 6–10 two-dimensional images and the distance between Z-stack slices was 1·225 μm optimised by Nyquist. A minimum of nine 3-D images were randomly captured per mouse, which include 5 images within the CTX and 2 images each from BS and HPF.

Cerebrovascular leakage of plasma proteins IgG and apo B were quantified within the CTX, BS and HPF. The pixel intensity of protein of interest surrounding the blood vessels for each 3-D image was quantitated utilising the automated optical intensity measurement tool in Volocity (Software version 5.5, Perkin Elmer, Melbourne, Australia) and expressed as per unit volume. The investigator was blinded during image capturing and quantitative analysis.

Plasma cholesterol, triglyceride and NEFA

Plasma Cholesterol and triglycerides were determined in duplicate by enzymatic assays (Randox Laboratories LTD, UK). Non-esterified fatty acids were determined with NEFA-C (ASC-ACOD method, Wako Pure Chemical Industries, Osaka, Japan).

Statistical analysis

This study utilised 6 mice per group and minimum of nine 3-D images were captured per mouse for detection of IgG and apo B leakage within the CTX, BS and HPF. In each group, 324–540 two-dimensional images were generated for adequate statistical comparison.

Normally distributed data were analysed by parametric one-way analysis of variance to assess the main effects of the dietary SFA, atorvastatin, pravastatin and ibuprofen treatment. The Kruskall-Wallis test was utilised if data was not-normally distributed. Post-hoc comparison of

means was done if the associated main effect or interaction was statistically significant within the analysis of variance procedure. *P*-values < 0.05 were considered to be statistically significant.

Abbreviations

(AD): Alzheimer's disease; (Aβ): Amyloid-β; (apo B): Apolipoprotein B; (At): Atorvastatin; (BBB): Blood–brain barrier; (BS): Brainstem; (CTX): Cortex; (COX): Cyclooxygenase; (HPF): Hippocampal formation; (Ib): Ibuprofen; (IgG): Immunoglobulin; (LF): Low fat; (SFA): Saturated fatty acid; (NEFA): Non-esterified fatty acids; (NSAIDs): Non-steroidal anti-inflammatory drugs; (OR): Odds ratio; (PBS): Phosphate buffered saline; (Pr): Pravastatin; (WT): Wild-type.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MPG carried out the design of project, data collection, immunofluorescence, statistical analysis and drafting of the manuscript. VL and RT assisted in tissue collection and interpretation of data. SG helped in the collection of tissues. KC assisted in critically analysing and drafting of the manuscript. JM conceived the study, helped in data interpretation, drafting of the manuscript, obtaining funding and general supervision of the research group. All authors have approved manuscript for submission.

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CHAPTER 5

Chapter 5: Discussion, limitations and future studies

This chapter presents a general discussion of the key findings presented in this thesis by publication. Chapter 1 (article 5) and Chapter 4 demonstrated beneficial effects of lipid-lowering agents in prevention and regression of dietary SFA induced cerebrovascular dysfunction. In contrast, findings in Chapter 3 demonstrated detrimental effects PUFA enriched diets in exaggerated BBB dysfunction. This chapter discusses in detail the putative mechanisms lipid-lowering agents may provide cerebrovascular protection, namely through their pleiotropic anti-inflammatory anti-oxidative properties and systemic TRL-Aβ metabolism (Chapter 2). Furthermore, the putative mechanisms by which PUFA exaggerate BBB dysfunction are also considered. Finally, study limitations are discussed in the context of prospective studies that could be considered.

5.1 Cerebrovascular dysfunction in Alzheimer's disease and vascular dementia

The cerebrovasculature is an integral part of the CNS and plays a vital role in regulation of the bi-directional transport of nutrients, electrolytes and metabolic waste products between systemic circulation and the cerebral tissue (Salmina et al. 2010). Cerebral capillaries are lined by a mono-layer of endothelial cells tightly opposed by tight- and adherence-junction proteins. The outer lining of endothelial cells is reinforced by a basement membrane and astroglial end-feet. Compact organisation of tight- and adherens-junctions between adjacent endothelial cells normally restricts the passage of large hydrophilic substances from the blood to the brain. However, carrier-mediated systems of the cerebrovascular endothelium actively control the passage of essential nutrients, including sugars, amino acids and vitamins into the CNS (Grammas et al. 2011). Structural, chemical, or functional disturbances of the cerebrovascular endothelium may trigger onset or progression of cerebrovascular and several neurodegenerative disorders including AD, Parkinson's disease and Multiple Sclerosis (Zlokovic 2008).

Morphological abnormalities of cerebral capillaries and deficient cerebral circulation have been reported in several neurodegenerative and inflammation-related diseases, in particular AD and VaD (Persidsky et al. 2006). Vascular based disturbances in cognition takes into consideration the consequence of a variety of cerebrovascular lesions and/or impaired brain perfusion (Dikstein et al. 2010, Miyakawa et al. 2010, Zlokovic 2008). Characterized by longer survival, subcortical ischemic VaD is the most significant subtype, involving substantial small vessel disease (Menon and Kelley 2009, Roman et al. 2002). In comparison, in AD vascular lesions coexist with proteinaceous deposits (Bowman et al. 2012, Dickstein et al. 2010). Nonetheless, even in prospectively assessed AD subjects, entirely pure neurodegenerative pathology is infrequent. Rather, autopsied brains of AD subjects typically show cerebrovascular degenerative microangiopathy and cerebral infarcts concomitant with cerebral amyloid angiopathy (Ellis et al. 1996, Vasilevko et al. 2010).

Accumulating evidence suggests that cerebrovascular dysfunction precedes amyloidosis and neurodegeneration (Grammas et al. 2011, Kanoski and Davidson 2011, Ujiie et al. 2003). Compromised BBB would allow brain to become vulnerable to exposure to circulating potentially pro-inflammatory macromolecules. The latter supported by findings where BBB leakage of several plasma proteins such as prothrombin, IgG, albumin and lipoproteins were detected in AD brains (Takechi et al. 2009, Takechi et al. 2010a, Zipser et al. 2007). More importantly, disrupted BBB can also facilitate blood-to-brain delivery of circulating TRL-Aβ and contribute to amyloid plaque formation (Takechi et al. 2010a) perhaps contributing to end-stage neurodegeneration.

Several post-mortem human and animal experiments have demonstrated significant structural changes of the cerebrovascular microanatomy commonly associated with neuroinflammation in AD (Grammas 2011). The BBB breakdown coincided with substantial endothelial cell necrosis (Claudio 1996). In addition, loss of tight junction proteins such as occludin, claudin and ZO-1, and adherence junction proteins have also been reported (Takechi et al. 2010a, Zlokovic 2011). Subsequently, increased basement membrane thickening concomitant with fibrosis (Farkas et al. 2000), significant collagen accumulation (Claudio 1996) and altered

brain perfusion may occur (Grammas 2011). The latter supported by clinical imaging studies that demonstrated significant cerebral hypoperfusion and hypometabolism preceding onset of dementia (Grammas 2011). Similarly, dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) demonstrated increased hippocampal BBB permeability in subjects with mild-cognitive impairment compared to age-matched controls (Wang et al. 2006a).

Alterations in the cerebrovascular function co-exist with heightened state of cerebral inflammation with the activation of microglia and astrocytes (Zlokovic 2011), resulting in enhanced secretion of neurotoxic and inflammatory mediators (Zlokovic 2011). Early expression of inflammatory triggers in AD by non-neuronal cells, including endothelial cells, is likely to lead to the development of disease (Grammas 2011). Several i*n vitro* studies have demonstrated that microvessels derived from AD brains release significantly higher levels of various inflammatory factors including NO, thrombin, tumour necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interleukin (IL)-1β, 6, 8 and matrix metalloproteinases compared to their age-matched controls (Dorheim et al. 1994, Grammas et al. 2011, Grammas and Ovase 2001, Grammas and Ovase 2002, Thirumangalakudi et al. 2006). These inflammatory factors were shown to negatively influence cerebrovascular structural and functional integrity by directly acting on endothelial cells (Deli et al. 1995) and enhancing tight junction protein breakdown (Bolton et al. 1998) leading to hypoperfusion (Abbott 2000).

5.2 Dietary lipid induced cerebrovascular dysfunction

Accumulating evidence is consistent with the notion that the onset and progression AD is influenced by lifestyle factors including nutrition. The cross-sectional study by Grant et al. (1997) in USA, Europe and China demonstrated that fat and total caloric supply had the greatest association with AD prevalence. Subsequently, several population studies have found that pro-inflammatory diets enriched in SFA and cholesterol are positive risk factors for VaD/AD (Kalmijn et al. 1997, Petot and Friedland 2004, Perez et al. 2012). Although the mechanisms of this association are unclear, dietary lipids may contribute to onset and progression of dementia via modulation of cerebrovascular integrity.

Recent findings suggest that dietary lipids and metabolic dyslipidemia are related to disrupted BBB integrity. A population study found both atherogenic and metabolic dyslipidemia more prevalent in AD subjects with BBB dysfunction than in subjects without BBB impairment (Bowman et al. 2012). Significant association was observed between plasma triglyceride and CSF albumin levels (Bowman et al. 2012). In addition, increased cerebrovascular dysfunction associated with significant loss of tight junction proteins and increase in reactive astrocytes and microglia in response to chronic SFA and cholesterol consumption were demonstrated in several animal models (Chen et al. 2008, Freeman and Granholm 2012, Takechi et al. 2010a, Kanoski et al. 2009). Freeman and Granholm (2012) evaluated long-term effects of a diet rich in SFA and cholesterol in a rat model. They observed that chronic feeding reduced BBB integrity with increased microgliosis in the hippocampus and loss of vascular tight junction protein occludin. Similarly, findings made in our laboratory demonstrated physiologically relevant Western diet enriched in SFA enhanced cerebrovascular permeability in WT mice, resulting in significant extravasation plasma proteins IgG and apo B lipoproteins concomitant with loss of occludin (Takechi et al. 2010a). In addition, Kanoski et al. (2010) showed that Wistar rats fed high-energy diet enriched in SFA reduced the expression of several tight junction proteins occludin, claudin 5 and claudin 12. Furthermore, increased BBB permeability was evident with significant extravasation of sodium fluorescein within the hippocampus (Kanoski et al. 2010). Observations coincided with significant impairment in hippocampal-dependent learning and memory in rats maintained on high-energy diets compared to rats given standard chow (Kanoski et al. 2009). These findings suggest that consumption of SFA enriched diets may compromise BBB integrity in a manner that may eventuate to decline in neurological function.

5.2.1 Dietary lipids, inflammation, oxidative stress and blood-brain barrier

The mechanisms underlying SFA mediated BBB disturbances are unclear. However, dietary SFA induced cerebrovascular dysfunction may be dependent on the fatty acid phenotype. Current evidence suggests that endothelial toxicity was dependent on the length and the dose of the SFAs (Zhang et al. 1992, Harvey et al. 2010). The most potent palmitic (16:0) and stearic (18:0) acids significantly impacted cell growth and viability on several endothelial cell lines *in vitro* by

stimulating pro-inflammatory pathways (Harvey et al. 2010, Zhang et al. 1992). The role of dietary lipids on inflammation and oxidative stress mediated vascular endothelial dysfunction was further supported by observations made by Van Oostrum et al. (2003). They demonstrated that postprandial lipemia increased plasma IL-8 and hyperperoxides, the latter was associated with significant endothelial dysfunction in subjects given fat enriched high-energy diets (Van Oostrum et al. 2003). Animal feeding studies have shown that SFA enriched diets increase protein oxidation and lipid peroxidation (Studinzki et al. 2009, Ronti et al. 2006). Morgan et al. (2009) suggests that one mechanism underlying toxicity of SFA is a consequence of disturbances in protein processing and ER dysfunction. Other mechanisms for SFA induced alterations in BBB function include stimulation of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase derived reactive oxygen species (ROS) generated by activated microglial cells (Kahles et al. 2007), or modulation of mTOR (mammalian target of rapamycin), a key signal transduction protein which regulates vascular endothelial fenestration (Laplante and Sabatini 2009). Patil et al. (2006) concluded that dietary palmitic acid induced region-specific cerebral damage because of higher fatty acid-metabolizing capacity of cortical astroglia. These findings suggest that SFA induced pro-inflammatory and oxidative changes may be relevant for the pathogenesis of cerebrovascular dysfunction.

5.2.2 Dietary lipids, nitric oxide and blood-brain-barrier

Studies have demonstrated the role of NO in regulation of cerebrovascular integrity (Hurst and Clark 1997, Kalayci et al. 2005, Mayhan 2000, Mayhan and Didion 1996, Tan et al. 2004, Thiel and Audus 2001). Nitric oxide is synthesised from NO synthase (NOS) is constitutively produced by cerebrovascular endothelial cells and glial cells for regulation of cerebrovascular tone, vasodilation (Hurst and Clark 1997, Thiel and Audus 2001, Aliev et al. 2010) and mediates vascular responses to various stimuli (Aliev et al. 2010). Altered production has been implicated in BBB permeability (Kalayci et al. 2005, Thiel and Audus 2001, Janigro et al. 1994). Nitric oxide overproduction in response to cytokines and other inflammatory mediators (Thiel and Audus 2001, Hurst and Clark 1997) have been implicated in AD (Smith et al. 1997, Aliev et al. 2010). In contrast, chronic inhibition of NO production enhanced endothelial cell permeability and has been implicated in

several vascular disorders (Aliev 2002, Aliev et al. 2010, Moncada and Higgs 1995, Moncada et al. 1991, Sessa 1994, Weyerbrock et al. 2010, Zhu et al. 2004). Several experimental models have explored the effect of SFA on NO dependent endothelial dysfunction (Javeshghani et al. 2009, Zhang et al. 2012). Javeshghani et al. (2009) observed that HF diet aggravated vasodilation and vascular remodelling by altered NO in oestrogen deficient mice. Furthermore, endothelial NO production and endothelium-dependent relaxation was reduced in apo E-knockout mice (d'Uscio et al. 2001, Wu and Meininger 2002) and subjects with type II diabetes fed a Western type HF diet (Fard et al. 2000, Wu and Meininger 2002). Roberts et al. (2005) demonstrated inhibition of NOS protein expression in brain and other vascular tissues in rats fed HF/sucrose diet. Similarly, endothelial NOS expression in aortic rings was abolished in rats fed HF diet (Zang et al. 2012). The effect of dietary lipids on NO induced BBB permeability is unclear. However, these findings suggest that dietary SFA may influence cerebrovascular endothelial NO by inhibiting NOS expression and compromising its integrity thereafter.

5.3 Effect of anti-inflammatory-lipid modulating agents on blood-brain barrier dysfunction

An accumulating body of evidence support a positive role of lipid lowering agents in prevention of cerebrovascular disorders (Laws et al. 2004). These include statin interventions demonstrating benefit in subjects with AD/VaD (Jick et al. 2000, Li et al. 2007, Wolozin et al. 2000). The latter observations were independent of lipid lowering effects (Jick et al. 2000), rather through actions on inflammation, oxidative pathways and on endothelial dysfunction (Cordle and Landreth 2005) and (Cimino et al. 2007, Jick et al. 2000). Role of oxidative stress and inflammation in enhanced BBB permeability suggest that pleiotropic properties of statins may confer cerebrovascular protection.

In Chapter 4, we explored the effects of atorvastatin and pravastatin on SFA induced BBB dysfunction reversibility. Our findings demonstrated that statin effectively attenuated BBB permeability of plasma proteins independent of plasma lipid changes. The latter effects were similar to that of ibuprofen suggesting that pleiotropic properties of statins modulating cerebrovascular dysfunction. Here we

discuss putative mechanisms of statins involved in ameliorating BBB dysfunction.

5.3.1 Statins, inflammation, oxidation and blood-brain barrier

The therapeutic potential of statins for treatment of AD/VaD via protection of cerebrovascular function has been demonstrated *in vitro* and *in vivo* (Kalayci et al. 2005, Kurata et al. 2012, Morofuji et al. 2010, Tong et al. 2009). In a cell culture study, pitavastatin enhanced tight junction protein claudin-5 expression to strengthen the barrier integrity in primary cultures of rat brain endothelial cells (Morofuji et al. 2010). A study in normolipemic spontaneously hypertensive rats, atorvastatin prevented BBB permeability and dysfunction (Kalayci et al. 2005). The authors reported that statin treatment significantly increased tight junction proteins ZO-1 and occludin immunoreactivity (Kalayci et al. 2005). Furthermore, in APP transgenic mouse models of AD, significant cerebrovascular dysfunction was attenuated in response to atorvastatin, pitavastatin and simvastatin treatment (Tong et al. 2009, Kurata et al. 2012). Statin treatment resulted in improved cerebrovascular reactivity and cerebral blood flow (Kurata et al. 2012).

Statins may provide cerebrovascular protection via anti-inflammatory mechanisms including glial cell inactivation. Consistent with the latter, Kalayci et al. (2005), Tong et al. (2009) and Kurata et al. (2012) reported that improved structural and functional cerebrovascular integrity by statin treatment was concomitant with attenuated inflammation and oxidative stress. Furthermore, statins inhibit the production of cytotoxic ROS, NO, COX-2 and cytokines such as IL-1β and TNF-α by the activated glial cells (Cimino et al. 2007, Hopkins and Rothwell 1995, Raghavendra et al. 2003, Tong et al. 2009). The role of these inflammatory mediators in disrupting cerebrovascular integrity has been previously explored (Abbott 2000, Huber et al. 2001). Several studies have demonstrated exposure of vasoactive cytokines TNF-α, IL-1β and interferon-γ (IFN-γ) markedly increased BBB permeability (Abbott 2000). Cytokines such as TNF-α and IL-1β have shown to modulate cerebrovascular permeability by directly acting on the endothelium (Deli et al. 1995) and enhancing the breakdown of the tight junction proteins (Bolton et al. 1998), respectively. These findings suggest that statin treatment may attenuate inflammation and restore normal glial cell function essential for preservation of BBB

structural integrity.

The role of oxidative stress induced by increased production and release of ROS in cerebrovascular endothelial dysfunction has been demonstrated (Kahles et al. 2007, Wassmann et al. 2002). The latter includes generation of ROS and superoxides via COX and NADPH-oxidase pathways (Wassmann et al. 2002, De Sotomayor et al. 2005). Several lines of evidence suggest that statins provide neuroprotection and endothelial protection by their anti-oxidative properties (Erdös et al. 2006, Hong et al. 2006, Wassmann et al. 2002). Otto et al. (2006) showed that chronic rosuvastatin treatment attenuated vascular superoxide formation via down-regulation of NADPH-oxidative stress pathway in endothelial NOS-knockout mice. Similarly, rosuvastatin inhibited NADPH-oxidative stress dependent superoxide production in cerebral arteries improved cerebrovascular function in Zucker obese rats (Erdös et al. 2006). Atorvastatin pretreatment attenuated cerebral infarcts and NADPH-oxidative stress dependent ROS formation (Hong et al. 2006). *In vivo* and *in vitro* observations by Wassmann et al. (2007) and Hong et al. (2006) suggests that statins effectively down-regulate the messenger ribonucleic acid (mRNA) expression of essential NADPH-oxidase subunits and contribute to vasoprotective role of statins.

5.3.2 Statins, nitric oxide and blood-brain barrier

In addition to the anti-inflammatory and anti-oxidant properties, statins enhance NO bioavailability that is essential for regulation of cerebral perfusion and improved endothelial function (Cimino et al. 2007). Altered NO levels have been implicated in vascular endothelial dysfunction. A cohort clinical study in patients with dementia demonstrated a correlation between lower serum NO level and cognitive decline in individuals with VaD (Corzo et al. 2007). Nitric oxide mediated improvement in cerebrovascular function by statin treatment has been implicated in cerebrovascular disorders including VaD (Jick et al. 2000, Sterzer et al. 2001). In addition, *in vivo* and *in vitro* experiments demonstrated atorvastatin (Kalayci et al. 2005) and fluvastatin (Kuhlmann et al. 2006) treatment enhanced NO bioavailability associated with improved cerebrovascular integrity.

Several lines of evidence have shown that statins increase NO production by upregulating endothelial NOS (Jick et al. 2000, Di Napoli et al. 2002), Desotomayor et al. 2005). The significance of endothelial NOS activity in the cerebrovascular structural integrity has been demonstrated in knockout mice, lacking the endothelial NOS gene, had significantly larger cerebral infarcts following middle cerebral artery occlusion, compared to their WT controls (Huang et al. 1996). In addition, other cerebrovascular aberrations including reduced endothelial dysfunction, smooth muscle proliferation and impaired cerebral perfusion have also been demonstrated in the same mouse model (Atochin and Huang 2010).

Jick et al. (2000) demonstrated that statin treatment had favourable effects on endothelial NOS and modulation of cerebrovascular function. Furthermore, others have indicated that statins may provide neuroprotection during cerebral schemia via modulation of cerebral endothelial NOS (Di Napoli et al. 2002). Beneficial effects of simvastatin, rosuvastatin and lovastatin on improving endothelial dysfunction via upregulation of endothelial NOS expression have been demonstrate *in vitro* and in animal models (De Sotomayor et al. 2005, Laufs et al. 2002, Laufs et al. 1998). Collectively, statin mediated increase in NO levels by upregulation of endothelial NOS expression may improve cerebrovascular endothelial function.

5.3.3 Statins, amyloid-β and blood-brain barrier

Growing evidence suggest that compromised BBB may facilitate blood-to-brain delivery of circulating TRL-Aβ contributing to cerebral amyloid load. Statins may be beneficial in treatment and regression of cerebrovascular dysfunction, attenuate plasma TRL-Aβ extravasation and reduce AD/VaD progression. In addition to the anti-inflammatory and anti-oxidative effects on the BBB, statins may lower circulating Aβ and contribute to reduction in cerebral Aβ load. Others have demonstrated cytotoxic properties of Aβ (Atwood et al. 2003, Behl et al. 1994, Gschwind and Huber 1995, Morishima et al. 2001), thus interaction of circulating Aβ may be detrimental to the cerebrovascular endothelium.

Although statin effects on circulating Aβ is controversial, one clinical study by Buxbaum et al. (2002) demonstrated that lovastatin treatment significantly

lowered serum Aβ in subjects with AD. Decrease in Aβ levels may be as a consequence of statin mediated inhibition of Aβ synthesis and secretion. Ostrowski et al. (2007) observed *in vitro* that simvastatin and lovastatin treatment in murine neuroblastoma cell culture inhibited Aβ production and secretion. It was suggested that the statin effects were attributed to the inhibition of protein isoprenylation of APP (Ostrowski et al. 2007, Wolozin et al. 2006). In addition, others suggested the potential of statins in Aβ degradation. Recent investigations by Tamboli et al. (2010) observed that lovastatin treatment enhanced extracellular Aβ degradation by insulin degradation enzyme release by microglia *in vitro*. Moreover *in vivo*, serum insulin degradation enzyme was enhanced in WT mice injected with lovastatin (Tamboli et al. 2010) and may facilitate Aβ degradation in circulation. *In vivo* experimental model in WT mice demonstrated fluvastatin treatment resulted in decreased cerebral Aβ as a result of increased intracellular lysomal degradation of APP-C terminal fragment (Shinohara et al. 2010).

Although, the effect of statins on circulating TRL-Aβ was not explored in Chapter 4, we observed that the non-statin lipid-lowering agent probucol attenuated enterocytic Aβ production and secretion into circulation thereafter (Chapter 2) (Pallebage-Gamarallage et al. 2012a). These findings indicate that statins may attenuate circulating TRL-Aβ by decreased production, secretion and increased degradation. The latter may reduce Aβ induced cerebrovascular damage, and attenuate availability and cerebral extravasation of TRL-Aβ.

5.4 Effect of polyunsaturated fatty acids on blood-brain barrier dysfunction

Clinical and epidemiological studies suggest that consumption of diets enriched in PUFAs, in particular the ω-3 fatty acids, not only prevents but may also be useful in treatment of AD (Hooijmans et al. 2012, Jicha and Markesbery 2010). In addition to the lipid lowering, PUFA consumption has reduced the incidence of cardiovascular events by improved vascular endothelial integrity and function (Egert and Stehle 2011, Goodfellow et al. 2000, Wang et al. 2006c). The ability of ω-3 PUFAs in lowering cellular oxidative stress, inflammatory pathways and preventing atherosclerotic events provide vascular endothelial protection (Cuevas and Germain 2004). The latter suggests that consumption of ω-3 fatty acids may benefit in

treatment of AD by attenuating cerebrovascular endothelial dysfunction. Therefore in Chapter 3, we explored the putative effects of PUFA diet enriched in DHA on reversing SFA induced BBB dysfunction.

As described in Chapter 3, chronic consumption of PUFA diet enriched in DHA surprisingly worsened SFA induced BBB permeability. The paradoxical findings are in contrast to majority of evidence that support the positive role of ω-3 fatty acids in prevention and treatment of cardiovascular disorders and vascular endothelial protection (Angerer and von Schacky 2000, Egert and Stehle 2011, Kinsella et al. 1990, Kris-Etherton et al. 2002). However, in some clinical studies ω-3 fatty acid consumption failed to provide cardiovascular protection (Albert et al. 1998, Ascherio et al. 1995, Kromhout et al. 1996, Mackay et al. 2012). The putative detrimental effects of ω-3 PUFAs in this model can be attributed perhaps to their susceptibility to lipid peroxidation. Polyunsaturated fatty acids interact with ROS resulting in lipid peroxidation and increased risk of oxidative stress (Calder et al. 2010, Walters et al. 2010). Crosby et al. (1996) observed that ω-3 fatty acids, eicosapentaenoic acid (EPA) and DHA, treatment of human vascular endothelial cells (HUVEC) resulted in a 3-4 fold increase in lipid peroxidation. Similar observations were made in cultured porcine pulmonary artery endothelial cells (PPAEC) and bovine aortic endothelial cells (BAEC).

Peroxidation of PUFAs is known to alter membrane fluidity (Dobretsov et al. 1977, García et al. 1997) and enhance BBB permeability (Chan et al. 1984, Tayarani et al. 1987). In addition, PUFA mediated increase in lipid peroxidation and its' oxidative products have shown to cause deoxyribonucleic acid (DNA) damage resulting in cellular dysfunction *in vivo* (Fang et al. 1996, Jenkinson et al. 1999, Nair et al. 1997). The damaging effects were only observed after chronic consumption of 15% PUFA diet (Jenkinson et al. 1999). In contrast, DNA damage was attenuated when 5% PUFA was consumed (Jenkinson et al. 1999). The latter findings suggest that detrimental effects of PUFA are observed at high concentrations.

Several other factors characteristic to the cerebral endothelial cells increase susceptibility for oxidative stress. The brain is exposed to large quantity of oxygen and the abundance of mitochondria in the cerebrovascular endothelial cells increase

opportunity for oxidative stress (Freeman and Keller 2012, Grammas et al. 2011, Shulman et al. 2004). The BBB endothelial mitochondria are essential for maintaining various energy-dependent transport mechanisms (Grammas et al. 2011). Increased cerebrovascular endothelial oxidative stress associated BBB dysfunction have been demonstrated (Haorah et al. 2005, Plateel et al. 1995, Skowronska et al. 2012). Furthermore, the abundance of ω-3 fatty acids in neuronal membrane may increase PUFA mediated lipid peroxidation in the brain. An increase in cytotoxic products from lipid peroxidation has been implicated in AD aetiology (Butterfield et al. 2010, Markesbery and Lovell 1998, Montine et al. 2002, Skoumalova et al. 2011). Collectively, these findings suggest that the physical properties of the BBB increase its' vulnerability to PUFA induced lipid peroxidation and dysfunction thereafter.

5.5 Conclusion

Blood-brain barrier dysfunction is frequently observed in neurodegenerative disorders including AD. Cerebrovascular aberrations are commonly associated with inflammatory changes that compromise tissue integrity and result in cognitive impairment. Presently, the majority of research focuses on preventative strategies for AD. Studies have suggested protective effects of statins, NSAIDs, ω-3 and ω-6 fatty acid consumption for reducing AD risk. However, a number of treatments designed to treat AD have focussed on maintaining neuronal function have not been particularly successful in improving memory and cognition. Accumulating evidence suggests that BBB disturbances precede amyloid deposition, so vascular focussed strategies are increasingly being considered for treatment.

Our early observations hypothesised that chronic feeding of diets enriched in SFA resulted in significant cerebrovascular dysfunction in WT mice (Takechi et al. 2010a). The role of lipids in cerebrovascular dysfunction suggested that lipid-modulating agents may be beneficial in treatment (Takechi et al. 2010b). The latter supported by our findings where atorvastatin and pravastatin reversed BBB permeability given the timely intervention and their effects were similar to that of ibuprofen (Pallebage-Gamarallage et al. 2012c). In addition, the efficacy of the drugs may be dependent on their solubility. Furthermore, their effects were independent of lipid lowering, rather through the pleiotropic anti-inflammatory and anti-oxidative

properties of statins. Further studies are required to ascertain their mechanisms of action in restoration of cerebrovascular integrity.

Many studies support the beneficial effects of ω-3 fatty acids providing vascular protection in cardiovascular disorders. In an established mouse model of BBB dysfunction induced by SFA feeding, provision of diet enriched in ω-3 fatty acid DHA exaggerated the detrimental effects on the BBB (Pallebage-Gamarallage et al. 2012b). Given at high concentrations, ω-3 fatty acids are highly susceptible to lipid peroxidation and promoting oxidative stress. Therefore, at a heightened state of inflammation provision of ω-3 fatty acid may induce cytotoxicity and exacerbate BBB dysfunction.

The potential benefits of anti-inflammatory lipid modulating statins and NSAIDs in restoring cerebrovascular integrity has been indicated by findings from this study. However, the use of ω-3 fatty acids in cerebrovascular dysfunction treatment should be carefully considered in the context of paradoxical damaging effects that can occur due to their propensity to enhance inflammation. The data presented in this thesis suggest that the putative role of anti-inflammatory pharmacotherapy on BBB integrity will be exceedingly informative for translation of appropriate clinical intervention studies. Non-invasive i*n vivo* human studies using DCE-MRI may assist in elucidating the association between cerebrovascular permeability and anti-inflammatory treatment. Further studies, *in vivo* and *in vitro*, are warranted to elucidate the association between the specific mechanisms discussed in this chapter and cerebral capillary dysfunction.

5.6 Limitations of the study

The WT mouse models used in this study are the most widely used inbred strain and have previously demonstrated to enhance cerebrovascular permeability in response to chronic SFA feeding. However, in this established model of cerebrovascular dysfunction the mice remain normolipemic. Similarly, plasma lipid parameters were not attenuated in response to statin treatment. The latter suggests that the SFA diet and statins are well tolerated and their effects may differ from that of humans and other experimental rodent models. Studies in other animal models

may be warranted to elucidate whether the diet and drug effects on the dependency of plasma lipid homeostasis in contributing to cerebrovascular dysfunction. Furthermore, it is to be noted that the dietary effects on plasma lipids may be dependent on the types of fatty acids consumed and statin effects may be dose dependent.

The WT rodent models have not effectively demonstrated the generation of cerebral Aβ deposits. Therefore, the use of transgenic mice that exhibit amyloid pathology may provide further insight in the context of AD. Furthermore, translation of findings from animal studies to human diseases needs to be carefully considered. Mammalian animal models have been commonly used to model human diseases due to their remarkable homology in the genome and similarities in cell biology and physiology (Lieschke and Currie 2007). However, differences in metabolism and protein expression between mammalian species may account for significant variations in pathophysiology and aetiology of the disease. Hence, further studies in a different mammalian model may be beneficial in validating current findings.

Cerebral abundance of plasma proteins IgG and apo B were used as markers for BBB permeability. The two proteins are vastly different in size and possess other potentially important biophysical properties such as affinity for extracellular matrices. Measurement of brain parenchymal abundance of plasma-derived proteins or of other soluble tracers (eg. sodium fluorescein) are indicative only of BBB dysfunction. It would be useful to explore the structural features of the BBB to further confirm our findings.

5.7 Future studies

This thesis for the first time presents evidence for statins and NSAIDs in reversing SFA induced cerebrovascular permeability. The data may be informative in AD research and may contribute to the development of treatment strategies. The principal findings also suggest that lipid solubility of cerebrovascular anti-inflammatories may be an important consideration. The potency of the agent in correcting for BBB disturbances also needs to be considered in dose and duration studies.

In contrast to the statin effects, we observed that a diet enriched in ω-3 fatty acid DHA did not restore SFA induced cerebrovascular dysfunction. The dose of the PUFA diet used was significant compared to clinical recommendations (relative to weight). Others have demonstrated that effects of PUFA were dependent on the dose used, with only lower doses shown to provide anti-oxidative protection (Jenkinson et al. 1999). Additional studies are necessary to consider the paradoxical observations reported (Pallebage-Gamarallage et al. 2012b).

Studies are warranted to determine plasma oxidative and inflammatory changes and within the cerebral tissue to confirm our findings. Measurement of specific vasoactive cytokines such as TNF-α, IL-1β and IFN-γ, which are known stimulators of cerebrovascular permeability are of interest (Abbott 2000, Deli et al. 1995). In addition to the permeability of plasma proteins, their actions on tight junction protein breakdown must also be elucidated (Bolton et al. 1998). Moreover, specific markers of lipid peroxidation can be generated from individual fatty acids (Barden et al. 2009, Guichardant et al. 2004). Measurements of lipid peroxidation products and oxidative stress markers may broaden our understanding into the detrimental properties of SFA and PUFA on cerebrovascular dysfunction.

Additional studies are essential for identification of specific pathways by which anti-inflammatory/anti-oxidative treatment may attenuate dietary lipid induced cerebrovascular dysfunction. The outcomes may then be extended to human experiments for AD treatment.

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APPENDICES

Appendix A: Author statement of contribution and copyright authorisation

This thesis presents 4 first-author and 4 additional peer-reviewed articles that were published in quality international scientific journals. In this appendix, author and co-author contributions for each paper are clearly stated and signed. The final version of the manuscripts were read and approved by all authors prior to their submission and publication.

Copyright authorisation was obtained, where necessary, for all the articles included in this thesis and their letters of authorisation are provided.

First-author manuscripts

Article 1:

Pallebage-Gamarallage MMS, Takechi R, Lam V, Galloway S, Dhaliwal S & Mamo JCL. (2010) Post-prandial lipid metabolism, lipid-modulating agents and cerebrovascular integrity: Implications for dementia risk. *Atherosclerosis Supp.* 11**,** 49-54.

Pallebage-Gamarallage MMS was responsible for writing of the manuscript, preparation image, collating and analysis of data required for generating the manuscript. Takechi R provided figures and assited in appraisal of the manuscript. Lam V and Galloway S assisted in data collection. Dhaliwal S was responsible for statistical analysis of data. Mamo JCL contributed to consideration of data, presentation format and critical appraisal of the manuscript.

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Pallebage-Gamarallage M was responsible for the study design, the experimental procedures, tissue collection, data collection, data interpretation and manuscript preparation. Galloway S and Takechi R assisted in tissue sample collection. Dhaliwal S contributed to statistical analysis of the data. Mamo J discussed the study design, data interpretation and review of the manuscript.

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Pallebage-Gamarallage M, Lam V, Takechi R, Galloway S & Mamo JCL. (2012b) A diet enriched in docosahexanoic acid exacerbates brain parenchymal extravasation of apo B lipoproteins induced by chronic ingestion of saturated fats. *Int J Vasc Med.* 2012, 647689.

Pallebage-Gamarallage M generated the study design and was responsible for tissue collection, data collection, statistical analysis, interpretation of data and development of the manuscript. Lam V assisted in animal care, tissue collection, experimental procedures and appraisal of the manuscript. Takechi R helped in tissue collection and appraisal of the manuscript. Galloway S supported in tissue sample collection. Mamo JCL assisted in study design and was responsible for data interpretation and critical review of the manuscript.

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Pallebage-Gamarallage M, Lam V, Takechi R, Galloway S, Clark K & Mamo J. (2012c) Restoration of dietary-fat induced blood-brain barrier dysfunction by anti-inflammatory lipid-modulating agents. *Lipids Health and Dis.* 11, 117.

Pallebage-Gamarallage M carried out the project design, data collection, statistical analysis and drafting of the manuscript. Lam V assisted in animal care, tissue collection and experimental procedures. Takechi R helped in tissue collection and interpretation of data. Galloway S helped in collection of tissues. Clark K assisted in critically analysis and drafting of the manuscript. Mamo J was assisted in study design, data interepretation and drafting of the manuscript.

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Takechi R, Galloway S, **Pallebage-Gamarallage MMS**, Wellington CL, Johnsen RD, Dhaliwal SS & Mamo JCL. (2010a) Differential effects of dietary fatty acids on the cerebral distribution of plasma-derived apo B lipoproteins with amyloid-β. *Br J Nutr.* 103**,** 652-662.

Takechi R developed the methods and was responsible for generation and interpretation of the data, and preparation of the manuscript. Galloway S contributed to study design, the animal care, tissue collection and review of the manuscript. Pallebage-Gamarallage MMS assisted in animal care, sample collection and appraisal of manuscript. Wellington CL supplied transgenic mouse tissue samples and was involved in manuscript appraisal. Johnsen RD provided advice on method development and statistical advice was given by Dhaliwal SS. Mamo JCL assisted in study design, consideration of the data and manuscript analysis.

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Galloway S, Takechi R, **Pallebage-Gamarallage M**, Dhaliwal S & Mamo J. (2009) Amyloid-beta colocalizes with apolipoprotein B in absorptive cells of the small intestine. *Lipids Health Dis.* 8**,** 46.

Galloway S carried out project design, data collection, immunofluorescence microscopy, statistical analysis and generating manuscript draft. Takechi R and Pallebage-Gamarallage M assisted in the tissues collection, data interpretation and critical analysis of manuscript. Dhaliwal S was responsible for statistical analysis of data and manuscript appraisal. Mamo J assisted in study design, helped in the interpretation of data, drafting and critical appraisal of the manuscript.

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Takechi R, Galloway S, **Pallebage-Gamarallage MMS**, Lam V & Mamo JCL. (2010b) Dietary fats, cerebrovasculature integrity and Alzheimer's disease risk. *Prog Lipid Res.* 49**,** 159-170. (Review)

Takechi R was responsible for generating the review and contributed substantially to the interpretation of the literature. Galloway S, Pallebage-Gamarallage MMS and Virginie Lam supplied images and critical analysis of the manuscript. Mamo JCL contributed to the discussions of the literature and appraisal of the manuscript.

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Article 4:

Lam V, Takechi R, **Pallebage-Gamarallage MMS**, Galloway S & Mamo JCL. (2011) Colocalisation of plasma derived apo B lipoproteins with cerebral proteoglycans in a transgenic-amyloid model of Alzheimer's disease. *Neurosci Lett.* 492**,** 160-164.

Lam V was responsible for method development, sample analysis, data collection, interpretation and generation of the manuscript. Takechi R assisted in method development, interpretation of data and appraisal of the manuscript. Pallebage-Gamarallage MMS assisted in data interpretation and appraisal of the manuscript. Galloway S involved in review of the manuscript. Mamo JCL contributed to the study design, interpretation of the data and critical appraisal of the manuscript.

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Appendix B: Conference abstracts

Appendix C contains detailed list of abstracts presented at international conferences during candidacy, followed by a copy of the abstracts.

2009 **International Congress on Vascular Dementia 2009**, Barcelona, Spain.
Pallebage-Gamarallage M, Takechi R, Galloway S, Mamo J. "Effect of statins on blood-brain barrier integrity and inflammation."

2010 **International Symposium on Chylomicron in Disease II,** Rotterdam, Netherlands.
Pallebage-Gamarallage M, Takechi R, Galloway S, Lam V, Dhaliwal S, Mamo J. "The effect of atorvastatin, pravastatin and ibuprofen on dietary fat induced disturbances in blood-brain barrier function."
*Presentation award and travel award.

2010 **Asian-Pacific Society of Atherosclerosis and Vascular Disease,** Cairns, Australia.
Pallebage-Gamarallage MM, Takechi R, Galloway S, Lam V, Dhaliwal S, Mamo JCL. "The effect of atorvastatin, pravastatin and ibuprofen on dietary saturated-fat induced disruptions in blood-brain barrier function."
*Best student poster presentation award.

Title: Effect of statins on blood-brain barrier integrity and inflammation.

Authors: Menuka Pallebage-Gamarallage[1], Ryusuke Takechi[1], Susan Galloway[1] and John Mamo[1]

[1]The Australian Technology Network Centre for Metabolic Fitness, School of Public Health, Curtin Health Innovation Research Institute, Perth, Western Australia.

Background:

Dietary saturated fatty acids (SFA) and cholesterol are risk factors for Alzheimer's disease (AD). Recent studies from our laboratory suggest that one possibility for this association is because of compromised blood-brain-barrier (BBB) integrity and enhanced delivery of amyloid-beta (Aβ) from blood to brain.

The 'statin' group of drugs reduce AD and stroke via several pathways. However, in spontaneously hypertensive rats, atorvastatin was found to prevent BBB dysfunction.

Aims:

In this study, we explored if atorvastatin (AT) and pravastatin (PR) prevents BBB disturbances induced by SFA.

Methods:

Wild-type mice were fed an SFA enriched diet (20% w/w) for 3 months ± AT or PR. In a second experiment, AT or PR were provided after the 3 months of SFA feeding. Restoration of BBB function was compared to mice withdrawn from the SFA diet. BBB integrity was assessed by immunofluorescence microscopy and included cerebral extravasation of plasma proteins (IgG), intestinal and hepatically derived apo B lipoproteins naturally enriched in Aβ and endothelial tight junction proteins (occludin).

Results:

The SFA mice showed cerebral extravasation of IgG and apo B lipoproteins and the expression of endothelial tight junction proteins were suppressed. Both AT and PR given synergistically with SFA, attenuated the effect. Statins introduced after 3 months of SFA feeding showed accelerated recovery compared to mice subsequently maintained on LF.

Conclusion:

AT and PR confer protection to SFA induced BBB damage in wild-type mice chronically fed an SFA enriched diet. Lipid solubility of the statins may be important in mediating the protective effect.

Main topic: The Blood-Brain Barrier in Dementia

The effect of Atorvastatin, Pravastatin and Ibuprofen on dietary-fat induced disturbances in blood-brain barrier function.

Pallebage-Gamarallage M[1,2,3], Takechi R[1,2,3], Galloway S[1,2,3], Lam V[1,2,3], Dhaliwal S[1,2,3] & Mamo J[1,2,3]

[1] School of Public Health and [2]Curtin Health Innovation Research Institute, Curtin University of Technology, Perth, Western Australia.
[3]Australian Technology Network, Centre for Metabolic Fitness, Australia.

Introduction:
Dietary saturated fatty acids (SFA) have been associated with increased risk of developing Alzheimer's disease (AD). Cerebrovascular degeneration is a hallmark pathological feature of AD and blood plasma proteins have been detected within brain parenchyma. In clinical studies we reported that plasma amyloid-β (Aβ) was significantly enriched in the triglyceride-rich-lipoprotein (TRL) fraction. Moreover, in subjects with AD TRL-Aβ was increased, concomitant with evidence of postprandial dyslipidemia. In animal models, we found SFA increases Aβ secretion in association with chylomicrons. In addition, wild-type mice chronically fed an SFA enriched diet had significant cerebral extravasation of plasma proteins and an influx of apo B lipoprotein-Aβ. The positive association between dietary fat intake, hypercholesterolemia, oxidative stress and AD raises the possibility that lipid modulating and anti-inflammatory agents might delay onset or attenuate progression of AD. In addition, epidemiological studies have shown a lower prevalence of AD among users of non-steroidal anti-inflammatory drugs (NSAIDs).

Aims:
To investigate the effects of Atorvastatin (AT), Pravastatin (PR) and Ibuprofen (IB) on blood-brain barrier (BBB) function in wild-type mice maintained on an SFA enriched diet.

Methods:
We previously reported that chronic SFA feeding resulted in significant BBB permeability with increased cerebral influx of plasma proteins. To determine if AT, PR or IB attenuates or restores SFA induced disturbances in BBB function, six-week old wild-type mice were first fed an SFA enriched diet (20% w/w) for 3 months before being randomised to low-fat diet containing one of the indicated agents. Restoration of BBB function was compared to mice randomized to a low-fat (LF) diet alone. Mice maintained on a LF or SFA diet served as negative and positive controls, respectively. BBB integrity was assessed by immunofluorescence microscopy and included cerebral extravasation of plasma proteins (IgG), intestinal and hepatically derived apo B lipoproteins naturally enriched in Aβ and endothelial tight junction proteins (occludin).

Results:
Mice fed SFA for 90 days showed significant cerebral extravasation of IgG and apo B lipoproteins and the abundance of endothelial occludin was profoundly suppressed compared to negative controls. Mice fed an SFA enriched diet followed by randomisation to an LF-diet containing either AT or IB showed complete restoration of BBB function after 2 weeks. SFA mice randomized to LF alone, showed modest restoration of BBB integrity. Pravastatin appeared to have no significant effect on BBB function for the duration of study in this model.

Conclusion:
AT and IB substantially accelerated restoration of BBB function induced by chronic feeding of SFA. Mice given SFA were normolipidemic and IB has no significant effect on plasma lipids, so the findings appear to be independent of triglyceride and cholesterol homeostasis. Atorvastatin and IB cross the BBB readily, whereas Pravastatin does not. Hence delivery of anti-inflammatory agents may explain differences in efficacy.

THE EFFECT OF ATORVASTATIN, PRAVASTATIN AND IBUPROFEN ON DIETARY SATURATED-FAT INDUCED DISRUPTIONS IN BLOOD-BRAIN BARRIER FUNCTION

Pallebage-Gamarallage MM[1,2,3], Takechi R[1,2,3], Galloway S[1,2,3], Lam V[1,2,3], Dhaliwal S[1,2,3] & Mamo JCL[1,2,3]
[1]School of Public Health and [2]Curtin Health Innovation Research Institute, Curtin University of Technology, Perth, Western Australia; [3]Australian Technology Network, Centre for Metabolic Fitness, Australia.

Background: Dietary saturated fatty acids (SFA) are positively associated with Alzheimer's disease (AD) risk. We have shown that dietary SFA compromise cerebrovascular integrity possibly as a consequence of exaggerated exposure to plasma amyloid-β (Aβ) that is complexed to apo B lipoproteins. Disturbances in blood-brain-barrier (BBB) function result in cerebral extravasation of plasma proteins and extracellular retention of apo B lipoprotein-Aβ.
Objective: In this study, we investigated if Atorvastatin (AT), Pravastatin (PR) or Ibuprofen (IB) restored blood-brain barrier (BBB) function in wild-type (WT) mice that had been maintained on an SFA enriched diet.
Methods: Six-week old WT mice were fed an SFA diet for 3 months to induce BBB dysfunction. Mice were then randomised to either low-fat (LF-control) diet, or LF containing either AT, PR or IB. Cerebral extravasation of plasma proteins (IgG) and of apo B was determined by 3D-immunofluorescent microscopy
Results: Mice fed SFA for 3 months (positive control) showed significant cerebral extravasation of plasma proteins compared to LF mice. Cerebral IgG and apo B abundance was not reduced in SFA mice that were randomized to the LF diet for 2 weeks. In contrast, Atorvastatin treated mice showed no persistent retention of IgG or apo B. Ibuprofen reduced abundance of IgG and apo B by about half, whereas Pravastin had no significant effect.
Conclusion: Only AT and IB accelerated restoration of BBB function. Mice given SFA were normolipidemic and IB has no significant effect on plasma lipids, so the findings appear to be independent of triglyceride and cholesterol homeostasis. Hence, anti-inflammatory properties of these agents may explain the differences in efficacy.