**School of Public Health**

# Disruption of Blood-Brain Barrier Function by Chronic Intake of Saturated Fat and Cholesterol: Implications for Alzheimer's Disease Risk

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# Declaration

The research presented contains original papers considering the putative effects of chronic intake of saturated fat and cholesterol on blood-brain barrier function and integrity. To the best of my knowledge and belief this thesis contains no materials previously published by any other person except where due acknowledgement has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Ryusuke Takechi

10th November 2009

# Acknowledgements

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# Abstract

It has been reported that lifestyle including diet is associated with Alzheimer's disease (AD) risk and progression. Population studies indicate that the chronic consumption of diets enriched in saturated fats (SFA) and cholesterol significantly increase the risk of AD onset and progression. However, the mechanisms underlying the association of AD risk with dietary fat intake are presently unclear

Proteinaceous deposits enriched in amyloid-β (Aβ) within the cerebral parenchyma (amyloid plaque) and in the cerebrovasculature (cerebral amyloid angiopathy) are the hallmark pathological features of AD. Several animal and cell culture studies suggest that high-fat diets exacerbate amyloidosis by promoting Aβ secretion by neurons and increasing the propensity for oligomerization to occur. However, there is little evidence consistent with cerebral Aβ overproduction in AD. Rather, recent studies in animal models of AD suggest that efflux of Aβ relative to its delivery from the blood is pivotal to cerebral Aβ homeostasis.

Two lines of evidence led me to develop the hypothesis that dietary fats may influence AD risk by modulating cerebrovascular exposure to circulating Aβ (Paper1 presented as Literature review of thesis). Firstly, Aβ has potent vasoactive properties and blood vessels treated with exogenous Aβ show substantial structural damage. Moreover, exaggerated plasma Aβ could occur because of chronic ingestion of diets enriched in fats. Dietary SFA were found to significantly increase Aβ abundance within the absorptive cells of small intestine (enterocytes) and thereafter, substantial plasma Aβ remains associated with triglyceride rich lipoproteins (TRLs). It is my contention that cerebrovascular integrity is compromised by the ingestion of fats which increases the plasma concentration of Aβ.

An immunohistochemical approach was developed to explore the effects of dietary SFA and cholesterol on cerebrovascular integrity and Aβ kinetics at the blood-brain barrier (BBB) (Paper 2 presented as Chapter 2 of thesis). Wild-type (WT) mice were used for the dietary intervention studies and appropriate comparisons were made

with amyloid precursor protein/presenilin-1 (APP/PS1) amyloid transgenic mice, an established model of AD (Paper 3-5 presented as Chapters 3-5). Critical to the primary scientific objectives, three-dimensional colocalization analysis using double immunofluorescent microscopy was developed (Paper 2). This double immunofluorescent labelling technique enabled the simultaneous detection of two proteins utilizing polyclonal antibodies derived from the same species. Briefly, in order to avoid the cross-reactivity of two polyclonal antibodies that originate from the same species, the concentration of one of the primary antibodies was reduced, so that it was undetectable with conventional secondary antibody methodologies. Rather, avidin-biotin amplification that was specific for the diluted primary antibody was utilized to identify its specific immunoreactivity. The double labelling of proteins with certainty that cross-reactivity did not occur, enabled consideration of protein distribution and association within tissues and cells.

In some cells Aβ is generated following processing of a precursor protein (APP) embedded within the plasma membrane. However, Chapter 5 of this thesis (Paper 5) shows that within enterocytes of the upper small intestine, Aβ genesis occurs within the Golgi apparatus and is likely secreted associated with nascent postprandial lipoproteins (chylomicrons). Apolipoprotein B immunoreactivity was used as a surrogate marker of enterocytic chylomicron distribution as it is an obligatory component of these macromolecules.

Evidence that plasma derived apo B lipoproteins containing Aβ may contribute to the aetiology of AD is presented in Chapter 3 (Paper 3). Consistent with the hypothesis presented, APP/PS1 mice were previously reported to have significantly increased secretion of TRL-Aβ as a consequence of the genetically induced over-expression of Aβ. However, this study expanded on that finding and explored if there was evidence of blood-to-brain delivery of apo B and if this was positively associated with amyloid plaque distribution and abundance. In transgenic APP/PS1 mice, immunoreactive apo B was detected in the core and periphery of cerebral amyloid plaques with significant colocalization coefficience (Manders' overlap coefficient =

0.85±0.004). The findings are consistent with the notion that cerebrovascular exposure to plasma TRL-Aβ is causally associated with cerebral amyloidosis.

Chapter 4 (Paper 4) investigates the differential effects of dietary fatty acids on BBB integrity. WT mice were fed either low-fat (LF) control chow, or physiologically relevant diets enriched in SFA, monounsaturated fatty acid (MUFA) or polyunsaturated fatty acid (PUFA) for either 3 or 6 months. Blood-to-brain delivery of apo B was found in SFA fed mice and exaggerated with a longer duration of feeding. The distribution of cerebral apo B in SFA fed mice, closely paralleled with the distribution of Aβ, consistent with blood-to-brain delivery as a lipoprotein complex. The cerebral extravasation of apo B was more evident in the cortex region (CTX) than in hippocampal formation (HPF). Mice fed the LF control, MUFA or PUFA diets for 3 or 6 months showed no evidence of apo B/Aβ parenchymal extravasation. The cerebral distribution of immunoglobulin G (IgG) was used as a surrogate marker of non-specific plasma protein leakage into the brain. In SFA fed mice alone, significant peri-vascular leakage of IgG was observed, suggesting that the endothelial dysfunction induced by SFA feeding was a non-specific or leakage phenomenon. Consistent with the latter, plasma S100B, a marker of brain-to-blood protein kinetics, was significantly increased in SFA fed mice but not in LF control, MUFA or PUFA supplemented mice. SFA group mice also had significantly attenuated occludin-1 expression, the primary BBB endothelial tight junction protein. Apo B and IgG extravasation greater in CTX than in HPF, increased plasma S100B, and decreased occludin-1 abundance were also observed in APP/PS1 amyloid transgenic mice. Hence the findings in SFA fed mice are consistent with a causal role in cerebral amyloidosis.

The data presented in this thesis and consideration of other studies reported particularly since commencement of my candidacy are then presented in Chapter 5, which was published as a review in Progress in Lipid Research (Paper 5). Briefly, several studies suggest that significant plasma Aβ is associated with TRLs secreted by the small intestine as chylomicrons and from the liver as very low density lipoproteins. Evidence presented in this thesis of apo B colocalization within amyloid plaques is consistent with the concept that plasma derived lipoprotein-Aβ may be causally

associated with cerebral amyloidosis. However, for delivery of lipoprotein-Aβ from blood to brain to occur, the breakdown of BBB function would be required as the cerebrovasculature architecture normally prevents transport of large macromolecules such as lipoproteins. In this study, chronic consumption by WT mice of food enriched in SFA resulted in non-specific leakage of plasma proteins within the brain parenchyma, analogous to that observed in an established transgenic murine model of AD (APP/PS1 mice). Within the review, dietary cholesterol is shown to elicit the same response. The mechanisms by which SFA and cholesterol cause the BBB dysfunction are presently unclear. Increased BBB exposure to circulating TRL-Aβ is one possibility, however, there was no significant difference in fasting plasma Aβ in mice maintained on SFA or cholesterol supplemented diets compared to LF control. Postprandial transient increases in plasma TRL-Aβ, not necessarily detected in fasting blood, may have been sufficient to cause the BBB dysfunction, but this was not specifically investigated. Alternatively SFA and cholesterol may have compromised cerebrovascular integrity via Aβ independent mechanisms, many of which are considered in depth in the review article. The review manuscript also discusses the potential mechanisms that could contribute to the extracellular retention of apo B lipoproteins enriched in Aβ and the inflammatory sequelae that may ensue thereafter. There is a positive association of apo B/Aβ retention with the abundance of the heparin-sulphate proteoglycans; perlecan, biglycan and decorin. A number of studies show that apo B lipoproteins are avidly metabolized by inflammatory cells and indeed under certain conditions may trigger the inflammatory cascade. In the review article, this paradigm is discussed in the context of apo B lipoproteins containing Aβ and putative interaction with activated glial cells.

Collectively, the results presented in this thesis suggest that dietary SFA and cholesterol may increase the risk of AD and/or accelerate the progression of disease by compromising cerebrovascular integrity and promoting the cerebral delivery of Aβ from the blood. The findings support the contention that diet is an important consideration in the context of disease prevention, but also raise the intriguing notion that nutritional intervention approaches could be developed to treat AD.

# List of Publications Included

This thesis contains the following 5 peer-reviewed scientific publications. These articles have been published or accepted for publication in quality scientific journals. Signed declarations of the contributions of coauthors are provided as Appendix A (pages 87-100).

1. **Takechi R**, Galloway S, Pallebage-Gamarallage MM, Wellington C, Mamo JC. (2008) Chylomicron β-amyloid in the aetiology of Alzheimer's disease. *Atheroscler Suppl*. 9, 19-25 [Impact factor: 6.6]
2. **Takechi R**, Galloway S, Pallebage-Gamarallage MM, Johnsen RD, Mamo JC. (2008) Three-dimensional immunofluorescent double-labelling using polyclonal antibodies derived from the same species: Enterocytic colocalization of chylomicrons with Golgi apparatus. *Histochem Cell Biol*. 129, 779-84 [Impact factor: 2.3]
3. **Takechi R**, Galloway S, Pallebage-Gamarallage MM, Wellington C, Johnsen R, Mamo JC. (2009) Three-dimensional colocalization analysis of plasma-derived apolipoprotein B with amyloid plaques in APP/PS1 transgenic mice. *Histochem Cell Biol*. 131, 661-6 [Impact factor: 2.3]
4. **Takechi R**, Galloway S, Pallebage-Gamarallage MM, Wellington CL, Johnsen RD, Dhaliwal SS, Mamo JC. (2009) Differential effects of dietary fatty acids on the cerebral distribution of plasma derived apo B lipoproteins with amyloid-β. *Br J Nutr*. (in press) [Impact factor: 2.8]
5. **Takechi R**, Galloway S, Pallebage-Gamarallage MMS, Lam V, Mamo JCL. (2009) Dietary fats, cerebrovascular integrity and Alzheimer's disease risk. *Prog Lipid Res*. (in press) [Impact factor: 11.2]

# List of Additional Publications

2 additional papers that compliment but are not considered central to the hypothesis presented in this thesis are listed and provided as Appendix B (pages 101-118).

1. Galloway S, Pallebage-Gamarallage MM, **Takechi R**, Jian L, Johnsen RD, Dhaliwal SS, Mamo JC. (2008) Synergistic effects of high fat feeding and apolipoprotein E deletion on enterocytic amyloid β abundance. *Lipids Health Dis*. 7, 15 [Impact factor: 2.1]
2. Galloway S, **Takechi R**, Pallebage-Gamarallage MM, Dhaliwal SS, Mamo JC. (2009) Amyloid-β colocalizes with apolipoprotein B in absorptive cells of the small intestine. *Lipids Health Dis*. 8, 46 [Impact factor: 2.1]

# Introduction and Structure of Thesis

## Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder resulting in the progressive decline of cognitive and physical functions. It has been almost a century since the first case of AD was diagnosed by the German psychiatrist and neuropathologist, Alois Alzheimer. However the aetiology of AD is still unclear and the disease remains incurable. Currently 25 million individuals have been diagnosed with AD and the number is estimated to double within the next 20 years (2008; Bickel 2001; Brookmeyer *et al.* 2007). In Australia, approximately 1% of the population have dementia primarily as AD and this is predicted to increase to 3% by 2050. Within the next decade, dementia will be the major cause of disability in Australia, greater than either cancer or cardiovascular disease. By 2030 the economic health burden for dementia will be approximately $9.5 billion p.a., exceeding the treatment of cancers by 30% and equivalent to the combined costs for the prevention and treatment of cardiovascular disease. Clearly, the development of prevention and treatment strategies for AD are urgently required.

A hallmark pathological feature of AD is the abnormal accumulation of amyloid-β (Aβ) peptide in the cerebral parenchyma (amyloid plaque) and in the cerebrovasculature (cerebral amyloid angiopathy) (Joachim *et al.* 1988a; Joachim *et al.* 1988b). The aggregation and fibrillar formation of Aβ forms an insoluble neurotoxic aggregate (plaque) that may trigger inflammatory sequelae including neuronal cell death. However, the origin of Aβ found in cerebral amyloidosis is controversial. Aβ is derived from the enzymatic cleavage of a precursor protein (amyloid-precursor-protein or APP) (Joachim *et al.* 1988a). Neurons produce Aβ and several genes related to increased neuronal Aβ biogenesis have been reported in familial AD (Citron *et al.* 1997; Hardy and Higgins 1992; Kirkitadze and Kowalska 2005; Tomita *et al.* 1998). However, presently there is little evidence that Aβ synthesis is increased in sporadic and late onset AD, the most common form of this disorder (Cummings *et al.* 1998). Rather, an insufficient Aβ clearance across the blood-brain barrier (BBB) via receptor pathways

and/or via the choroid plexus has been suggested as an alternative pathway for amyloidosis (Crossgrove *et al.* 2005; Deane *et al.* 2009; Deane *et al.* 2005). Moreover, recent evidence that Aβ may also be delivered from blood-to-brain could conceivably exacerbate the parenchymal load in the absence of compensatory clearance pathways (Donahue *et al.* 2006).

The BBB is characterized by endothelial cells of the cerebrovasculature and basement membranes of the lamina rara externa, lamina densa, and lamina rara interna that are tightly apposed (Farkas and Luiten 2001). The properties of the BBB enable the brain vascular endothelium to exclude neurotoxic and inflammatory agents such as proteins, ions, non-electrolytes and water, and to allow for the highly selective exchange of substances between the systemic circulation and the interstitial fluid of the brain (Deane *et al.* 2003; Donahue *et al.* 2006; Maness *et al.* 1994; Martel *et al.* 1996; Zlokovic *et al.* 1993). In AD subjects, pathological alterations of the cerebrovasculature including vascular endothelial and smooth muscle cell proliferation are often reported (Ellis *et al.* 1996). Plasma derived proteins have been detected in the parenchyma of AD brains (Kalaria 1992; Wisniewski *et al.* 1997), and inflammatory sequelae are commonly reported (Cullen 1997; Itagaki *et al.* 1989), observations consistent with breakdown of the BBB. Yet despite evidence supportive of AD having an underlying vascular component, most research focuses on damage of neurons.

Recent studies have suggested that lifestyle including nutrition is associated with AD risk, age of onset and disease progression (Abbott *et al.* 2004; Kivipelto and Solomon 2008; Larson *et al.* 2006; Pasinetti and Eberstein 2008; Podewils *et al.* 2005; Santana-Sosa *et al.* 2008; van Gelder *et al.* 2004; Weuve *et al.* 2004). Based on population and animal studies, high-fat diets that are characterized by significant quantities of saturated fats (SFA) and cholesterol are a risk factor of AD (Engelhart *et al.* 2002; Kalmijn 2000; Laitinen *et al.* 2006; Luchsinger and Mayeux 2004; Morris *et al.* 2003; Oksman *et al.* 2006; Refolo *et al.* 2000; Shie *et al.* 2002; Solfrizzi *et al.* 2005; Solfrizzi *et al.* 2009; Sparks *et al.* 1994). In contrast, diets enriched in polyunsaturated fats, particularly the omega 3 oils, may confer protection against AD risk, age of onset

and progression (Cole and Frautschy 2006; Hooijmans *et al.* 2007; Hooijmans *et al.* 2009; Lim *et al.* 2005; Oksman *et al.* 2006; Schaefer *et al.* 2006).

The mechanisms by which dietary fats influence AD risk were the stimulus for my PhD candidacy. One commonly held view is that diets enriched in cholesterol stimulate Aβ biogenesis in the brain resulting in the abnormal accumulation of Aβ peptides. However, paradoxical studies in cell culture models suggest that cholesterol may be both stimulatory and inhibitory (Abad-Rodriguez *et al.* 2004; Frears *et al.* 1999), and there is no evidence of cerebral Aβ overproduction per se in AD (Cummings *et al.* 1998).

In blood, 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 (Biere *et al.* 1996; Koudinov and Koudinova 1997; Mamo *et al.* 2008). 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 fat regulation (Boyt *et al.* 1999; Galloway *et al.* 2007; Galloway *et al.* 2008; James *et al.* 2003; Pallebage-Gamarallage *et al.* 2009; Takechi *et al.* 2008a; Takechi *et al.* 2008b). 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 (Galloway *et al.* 2007). Distributional analysis of plasma lipoprotein-Aβ in normal and AD subjects found that greater than 60% of plasma-lipoprotein-Aβ was associated with triglyceride-rich liporpteins (TRLs) and this was significantly increased in those with AD or subjects with mild-cognitive-impairment (Mamo *et al.* 2008). Whilst AD subjects are typically normolipidemic, the concentration of apolipoprotein (apo) B48 (an exclusive marker of chylomicrons) was elevated more than 3-fold in post-absorptive AD subjects (Mamo *et al.* 2008). Increased apo B48 is indicative of post-prandial dyslipidemia, an exaggerated rise in plasma chylomicrons that occurs following the ingestion of dietary fats. Consistent with the notion of an Aβ post-prandial response, ingestion of a lipid enriched meal was found to cause a transient increase in the plasma concentration of the APP and of Aβ in otherwise healthy subjects (James *et al.* 2003).

The mechanisms by which circulating Aβ could increase the risk of AD are presently unclear. However, several studies have provided evidence of a vasoactive role of Aβ with pathological manifestations prior to Aβ deposition (George *et al.* 2004; Thomas *et al.* 1997). Amyloid-β is vasoconstrictive and vessels treated with exogenous Aβ show significant endothelial cell damage with changes in the cell membrane, cytoplasm, nucleus and other organelles. In previous studies, significant breakdown of the BBB was reported following acute or chronic intravenous injection of Aβ.

## Hypothesis

The broad hypothesis on which my candidacy is based is that dietary SFA and cholesterol compromise BBB function because of exaggerated exposure to plasma lipoprotein-Aβ. As a consequence of these specific dietary induced cerebrovascular aberrations, rates of lipoprotein-Aβ delivery from blood to brain are exaggerated. Lipoproteins endogenously enriched in Aβ then become retained on extracellular matrices initiating an inflammatory response.

## Objectives

This thesis puts forward an innovative perspective of the putative mechanisms underlying the link between dietary fats and AD risk. The primary objective of this study was to investigate the effects of chronic ingestion of different fatty acids on the integrity of the BBB in a wild-type (WT) mouse model fed either low-fat (LF) control, SFA, monounsaturated fats (MUFA) or polyunsaturated fats (PUFA). A response to duration model was utilized exploring the putative effects at 3 and 6 months of feeding and comparisons were made with amyloid transgenic mice that are commonly used as a model of AD pathology. The integrity of the BBB was investigated by determining whether plasma proteins were present within the brain parenchyma. The approach

utilized for this objective was state-of-art 3-dimensional (3-D) immunofluorescent microscopy developed as part of my candidacy and selected biochemical markers.

The specific objectives of my candidacy were:

**Objective 1:** To develop 3-dimensional double immunofluoroscent microscopy using polyclonal antibodies raised in the same species

**Objective 2:** To establish 3-dimensional semi-quantitative colocalization analysis methodologies with double immunofluorescent labelling

**Objective 3:** To investigate the 3-dimensional localization of plasma derived TRL-Aβ relative to cerebral amyloid plaque abundance

**Objective 4:** To evaluate the differential effects of dietary fatty acids on BBB integrity in wild-type mice and to compare this with an established murine model of AD (amyloid transgenic mice)

**Objective 5:** To provide informative consideration of whether disturbances in cerebrovascular integrity explain in part the positive association between dietary SFA/cholesterol ingestion and AD risk

## Structure of Thesis

This thesis consists of 5 peer-reviewed papers that have been published or accepted for publication. The first paper is a review article essentially summarising the literature at the commencement of my PhD which led to the genesis of the hypothesis presented. The second published manuscript is a methods paper describing the key approach used to investigate Aβ metabolism and cerebrovascular integrity by immunomicroscopy. Paper 3 reports the colocalization of apo B with cerebral amyloid

plaque in an established murine model of AD and provides direct evidence that circulating TRL-Aβ may contribute to the formation of amyloid plaques. The possibility that dietary fats modulate cerebrovascular integrity and blood-to-brain kinetics of plasma derived TRL-Aβ is reported in Paper 4. This particular study showed that chronic ingestion of SFA induced significant breakdown of the BBB and delivery from blood to brain of plasma derived apo B lipoprotein-Aβ, whereas MUFA and PUFA had no detrimental effects. The fifth manuscript which serves as the general discussion of my thesis presented additional studies which show that dietary cholesterol induces analogous disturbances as SFA on BBB function and apo B/Aβ kinetics across the BBB. The review also shows for the first time, retention of apo B lipoprotein containing Aβ with specific heparin sulphate proteoglycans. Possible mechanisms by which SFA and dietary cholesterol cause endothelial dysfunction are discussed in detail and take into consideration a significant number of relevant studies.

## Chapter 1 – Introduction and literature review

**The content of this chapter is covered by Paper 1:**

**Takechi R**, Galloway S, Pallebage-Gamarallage MM, Wellington C, Mamo JC. (2008) Chylomicron β-amyloid in the aetiology of Alzheimer's disease. *Atheroscler Suppl*. 9, 19-25

Chapter 1 is an introduction and literature review of the association between dietary fats and AD aetiology. Key papers from clinical, population, cell culture and animal studies are reviewed synthesizing the key elements which underpin the hypothesis and the objectives of the study.

It is suggested that dietary SFA increases the risk of progression of AD. However the mechanisms for this association are presently unclear. This review takes into consideration the findings that SFA enhance enterocytic abundance of Aβ and by extension may increase the concentration of plasma Aβ. The latter may challenge cerebrovascular integrity resulting in a shift from blood to brain of circulating Aβ. The

putative SFA induced elevations in plasma Aβ may exacerbate or indeed trigger the amyloidogenic cascade. Other key published findings which led to the hypothesis were clinical studies which have revealed that significant plasma Aβ is associated with TRL particles, and the plasma TRL-Aβ level is elevated after a fat meal intake.

## Chapter 2 – Development of 3-dimensional double immunofluorescent labelling and semi-quantitative colocalization analysis methodology

**The content of this chapter is covered by Paper 2:**

**Takechi R**, Galloway S, Pallebage-Gamarallage MM, Johnsen RD, Mamo JC. (2008) Three-dimensional immunofluorescent double-labelling using polyclonal antibodies derived from the same species: Enterocytic colocalization of chylomicrons with Golgi apparatus. *Histochem Cell Biol*. 129, 779-84

**Thesis objectives addressed in this chapter:**

**Objective 1:** To develop 3-dimensional double immunofluoroscent microscopy using polyclonal antibodies raised in the same species

**Objective 2:** To establish 3-dimensional semi-quantitative colocalization analysis methodologies with double immunofluorescent labelling

In this chapter, key methodologies pivotal to the scientific approach chosen to interrogate the Hypothesis that dietary SFA compromises BBB integrity resulting in blood-to-brain delivery of Aβ were established. A highly specific immunofluorescent double labelling method using polyclonal antibodies derived from the same species was demonstrated, and 3-D semi-quantitative colocalization analysis was developed.

Multiple labelling immunofluorescent microscopy has been used to consider the abundance and distribution of two or more proteins within cells and tissues. Briefly, the method ordinarily involves incubation of cells with two or more antibodies that will

only recognize highly conserved amino acid sequences of specific proteins (antigen). Following a washing procedure to remove excess primary antibodies not bound to antigen, the distribution of the respective primary antibody-antigen complexes are identified by incubating with secondary antibodies that bind exclusively to one of the primary antibodies. Visualisation of the two proteins is possible because the secondary antibodies are differentially conjugated to fluorochromes. In order to avoid cross reactivity between secondary antibodies and primary antibodies, the primary antibodies are normally derived from two alternate species. However, commercially available antibodies for the proteins of interest in this study were found to be in most instances limited to just one species (rabbit). In order to meet the objective of exploring associations in protein distribution and abundance, methods had to be developed to avoid cross-reactivity of the secondary antibodies with the primary antibodies. The approach developed required a dilution of one of the primary antibodies so that it could not be detected by conventional incubation with conjugated secondary antibodies. Rather, for the diluted primary antibody, the fluorescent emission following interaction with its specific secondary antibody had to be amplified utilizing an amplifying protocol (biotin-avidin). Critically, the biotin-avidin amplification process is specific for the diluted primary antibody interaction. The successful development of this technique was published in a highly ranked immunohistochemistry journal. To demonstrate the methodology, the manuscript reported on the colocalization of apo B and Golgi apparatus within enterocytes of the small intestine. The primary antibodies for apo B and Golgi were both derived from rabbits.

A second important methodological approach for this study was to develop measures of protein abundance (i.e. proportional to fluorescent intensity) and distribution in three dimensions. The published manuscript demonstrates the application of newly released microscopy hardware and software configuration (Zeiss's ApoTome optical sectioning technologies and AxioVision image analysis software) and shows in 3-D the colocalization of apo B within the enterocytic Golgi apparatus.

## Chapter 3 – Colocalization of apolipoprotein B with cerebral amyloid plaques of Alzheimer's disease

**The content of this chapter is covered by Paper 3:**

**Takechi R**, Galloway S, Pallebage-Gamarallage MM, Wellington C, Johnsen R, Mamo JC. (2009) Three-dimensional colocalization analysis of plasma-derived apolipoprotein B with amyloid plaques in APP/PS1 transgenic mice. *Histochem Cell Biol*. 131, 661-6

**Thesis objective addressed in this chapter:**

**Objective 3:** To investigate the 3-dimensional localization of plasma derived TRL-Aβ relative to cerebral amyloid plaque abundance

In this chapter, the 3-D colocalization of plasma derived TRL-Aβ and the amyloid plaques of the transgenic AD mouse model was demonstrated.

The formation of amyloid plaques in the parenchyma of the brain is a hallmark feature of AD, however, the origin of Aβ is still unclear. Several lines of evidence suggest that the plasma concentration of Aβ is positively associated with AD risk and progression. One mechanism by which this may occur is that exaggerated cerebrovascular exposure to Aβ compromises BBB integrity resulting in a shift in Aβ transport from blood to brain. My research group previously reported that significant Aβ in plasma is associated with TRLs and stimulated in response to the ingestion of particular fats. TRL-Aβ may contribute to BBB dysfunction and cerebral Aβ accumulation if plasma concentration is increased. To explore this possibility further, cerebral immunoreactivity to apo B was investigated in amyloid transgenic mice. The APP/PS1 transgenic mice have an 8-fold greater concentration of TRL-Aβ in plasma than wild type mice.

The 3-D double immunofluorescent colocalization analysis technique described in Chapter 2 showed immunoreactivity of apo B within the core and the periphery of

amyloid plaques of APP/PS1 mice, providing direct evidence that peripheral Aβ enriched in TRL may contribute to the cerebral amyloidosis.

## Chapter 4 – Differential effects of fatty acids on blood-brain barrier integrity

**The content of this chapter is covered by Paper 4:**

**Takechi R**, Galloway S, Pallebage-Gamarallage MM, Wellington CL, Johnsen RD, Dhaliwal SS, Mamo JC. (2009) Differential effects of dietary fatty acids on the cerebral distribution of plasma derived apo B lipoproteins with amyloid-β. *Br J Nutr*. (in press)

**Thesis objective addressed in this chapter:**

**Objective 4:** To evaluate the differential effects of dietary fatty acids on BBB integrity in wild-type mice and to compare this with an established murine model of AD (amyloid transgenic mice)

The capillary network of the brain effectively separates blood from cerebral spinal fluid (CSF). Tightly apposed endothelial cells bound via tight junction proteins characterize what is commonly referred to as the BBB and effectively exclude neurotoxic and inflammatory agents. In addition. plasma proteins and large macromolecules such as lipoproteins are prevented from entering brain parenchyma. However, in Chapter 3 strong immunohistochemical staining for apo B lipoproteins within amyloid plaque was reported in APP/PS1 mice, suggesting that plasma TRL-Aβ may be a source of Aβ found in the amyloid plaques of the AD brain. In the absence of specific transporter mechanisms, blood-to-brain delivery of plasma apo B lipoprotein-Aβ could only occur if BBB integrity becomes compromised.

The primary contention of the hypothesis presented for my candidacy is that chronic ingestion of SFA compromise cerebrovascular integrity resulting in delivery from blood to brain of TRL-Aβ. To investigate this, normal WT mice were randomized

to receive either a LF control diet, or chow enriched in SFA, MUFA or PUFA. The integrity of the BBB was assessed by detecting the distribution in brain parenchyma of the plasma proteins immunoglobulin G (IgG) and of apo B containing lipoproteins. Colocalization of the apo B with Aβ is reported as is the abundance of the endothelial tight junction protein, occludin-1. In the context of amyloidosis and AD risk, comparisons were made with amyloid transgenic mice (APP/PS1), an established model of AD.

In WT mice fed an SFA enriched diet, significant immunoreactivity of IgG was detected in the cerebral perivascular regions concomitant with a reduction of occludin-1 expression and elevated plasma S100B level. The results suggest that SFA induced cerebrovascular dysfunction and aberrations in protein kinetics across the BBB. In contrast, there was no evidence of BBB disruption in mice fed a control diet, or chow enriched in either MUFA or PUFA. Perivascular leakage of apo B was clearly seen in the SFA fed mice, particularly within the cortex and the hippocampal formation and this was exacerbated with a longer duration of feeding. Moreover, the 3-D double immunofluorescent labelling technique established in Chapter 2 demonstrated the significant colocalization of Aβ with apo B consistent with the notion of apo B lipoprotein/Aβ delivery from blood to brain.

The pattern of cerebrovascular disturbances and relative distribution of apo B/Aβ within particular regions of the brain paralleled that found in APP/PS1 amyloid transgenic mice, that had substantial elevations in plasma TRL-Aβ because of amyloid over-expression.

Collectively, the data presented in this manuscript is consistent with the hypothesis that chronic ingestion of SFA may cause disruption of BBB function which may result in significant blood-to-brain delivery of TRL-Aβ. The findings provide one possible explanation for epidemiological studies that show a positive association in AD risk with dietary SFA. The differential effects of fatty acids on BBB function and apo B/Aβ cerebral distribution were published in the British Journal of Nutrition. Parallel

studies investigating the effects of dietary cholesterol supplementation on BBB function are presented in Chapter 5.

## Chapter 5 – Discussion and conclusion

**The content of this chapter is covered by Paper 5:**

**Takechi R**, Galloway S, Pallebage-Gamarallage MM, Lam V, Mamo JC. (2009) Dietary fats, cerebrovascular integrity and Alzheimer's disease risk. *Prog Lipid Res*. (in press)

**Thesis objectives addressed in this chapter:**

**Objective 5:** To provide informative consideration of whether disturbances in cerebrovascular integrity in part explain the positive association between dietary SFA/cholesterol ingestion and AD risk

The principal findings of immunoreactive apo B within cerebral plaque (Chapter 3) and the differential effects of fatty acids on BBB integrity (Chapter 4) attracted significant attention with invitations to present the findings at several conferences (see Appendix C). The Chief Editor of the prestigious journal Progress in Lipid Research invited me to submit a review (impact factor: 11.2) to present my hypothesis in greater detail. The review was written in an hypothesis generating context and takes into consideration a substantial body of literature (192 references) and additional studies done by me.

Key concepts discussed in the review is the suggestion that dietary SFA and cholesterol induce cerebrovascular disturbances that can be independent of an increased concentration in plasma Aβ. Synergistic effects of apo B lipoproteins with apo E is considered because of the significant differences in AD risk with apo E isoforms and because of the importance of apo E in TRL-lipoprotein metabolism. Evidence for the association of apo B/Aβ containing lipoproteins with specific heparin-sulphate

proteoglycans is presented and consideration of how this occurs is discussed. The review supports the contention that AD is characterized by significant cerebrovascular disturbances preceding frank amyloidosis and that the latter is more likely to exacerbate inflammatory pathways.

# Abbreviations

| | |
|---|---|
| Aβ | Amyloid-β |
| AD | Alzheimer's Disease |
| ANOVA | Analysis of Variance |
| Apo | Apolipoprotein |
| APP | Amyloid-Precursor-Protein |
| BBB | Blood-Brain Barrier |
| BS | Brain Stem |
| CH | Cholesterol |
| CSF | Cerebral Spinal Fluid |
| CTX | Cortex |
| CVD | Cardiovascular Disease |
| DHA | Docosahexanoic Acid |
| EPA | Eicosapentaenoic Acid |
| GAG | Glycosaminoglycans |
| HF | High-Fat |
| HPF | Hippocampal Formation |
| HRP | Horseradish Peroxidase |
| HSPG | Heparin Sulphate Proteoglycan |
| IF | Immunofluorescence |
| IgG | Immunoglobulin G |
| IHC | Immunohistochemistry |
| KO | Knockout |
| LDL | Low Density Lipoprotein |
| LF | Low-Fat |
| LRP1 | Lipoprotein Receptor Related Protein-1 |
| MCI | Mild Cognitive Impairment |
| MUFA | Monounsaturated Fatty Acid |

| | |
|---|---|
| OC | Overlap Coefficient |
| PBS | Phosphate Buffered Saline |
| PS1 | Presenilin-1 |
| PUFA | Polyunsaturated Fatty Acid |
| RAGE | Receptor for Advanced Glycosylation End Products |
| SFA | Saturated Fatty Acid |
| TAG | Triacylglycerol |
| TC | Total Cholesterol |
| Tg | Transgenic |
| TG | Triglycerides |
| TRL | Triglyceride Rich Lipoprotein |
| TSA | Tyramide Signal Amplification |
| VLDL | Very Low Density Lipoprotein |
| vWF | von-Willebrand Factor |
| WT | Wild-Type |
| 3-D | 3-Dimensional |

# Table of Content

# CHAPTER 1

# Chapter 1

## Introduction and Literature Review

**The content of this chapter is provided as the manuscript titled:**

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# Chylomicron amyloid-beta in the aetiology of Alzheimer's disease

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**Abstract**

Alzheimer's disease is characterized by inflammatory proteinaceous deposits comprised principally of the protein amyloid-beta (Aβ). Presently, the origins of cerebral amyloid deposits are controversial, though pivotal for the prevention of Alzheimer's disease.

Recent evidence suggests that in blood, Aβ may serve as a regulating apoprotein of the triglyceride-rich-lipoproteins and we have found that the synthesis of Aβ in enterocytes and thereafter secretion as part of the chylomicron cascade is regulated by dietary fats.

It is our contention that chronically elevated plasma levels of Aβ in response to diets rich in saturated fats may lead to disturbances within the cerebrovasculature and exaggerated blood-to-brain delivery of circulating Aβ, thereby exacerbating amyloidosis. Consistent with this hypothesis we show that enterocytic Aβ is increased concomitant with apolipoprotein B48. Furthermore, cerebral extravasation of immunoglobulin G, a surrogate marker of plasma proteins is observed in a murine model of Alzheimer's disease maintained on a saturated-fat diet and there is diminished expression of occludin within the cerebrovasculature, an endothelial tight junction protein.
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*Keywords:* Chylomicrons; Amyloid-beta; Alzheimer's disease; Blood–brain barrier

## 1. Cerebrovascular integrity in Alzheimer's disease

Based on epidemiologic studies, there are statistically significant correlations between the prevalence of Alzheimer's disease (AD) and diabetes, hypercholesterolemia, hypertension, hyperhomocysteinemia, dietary saturated fats, cholesterol, antioxidants, alcohol consumption, smoking, physical activity, atherosclerotic disease, and the plasma concentration of some hemostatic factors. Most of the risk factors found to be associated with AD are age-dependent, and the prevalence of AD increases with age. Therefore, the association could simply be attributed to aging. On the other hand, common pathogenetic mechanisms for diseases such as AD and atherosclerosis, such as inflammation and the generation of free radicals, suggest a causal link. If this is the case, the identification of modifiable risk factors for dementia becomes a research priority and early intervention aimed at reducing those risk factors by therapeutic imperative.

An accumulating body of evidence is consistent with the concept that the onset and progression of sporadic and late-onset AD is significantly influenced by lifestyle factors including nutrition. Several population studies in humans have found that high-fat diets are a positive risk factor [1,2], and high-fat feeding markedly exacerbates Alzheimer's-like cerebral pathology in animal models of AD [3,4]. The mechanisms for the high-fat diet/AD link are presently unclear, but we will present in this article a novel hypothesis that may explain this effect. We contend that further studies are urgently needed to delineate the relationship between diet and AD. Indeed, by 2030 the expected global health burden for dementia will exceed treatment of cancers by 30% and will be equivalent to the combined costs for the prevention and treatment of cardiovascular disease.

The cerebrovasculature in subjects with AD shows pathological alterations including vascular endothelial and smooth muscle cell proliferation [5]. Blood plasma proteins have been detected in the parenchyma of AD brains [6,7] and inflammatory sequelae are commonly reported [8,9], observations that are consistent with breakdown of the blood–brain barrier (BBB). Despite the increasing evidence supportive of AD having an underlying vascular component, most research focuses on damage of neurons [10].

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A major neuropathological marker of AD is amyloid-beta (Aβ) deposition in the cerebrovasculature. However, clarification of its putative role in the aetiology of AD has been more elusive. The pivotal question is whether cerebral and cerebrovascular Aβ is a final neurotoxic, common to all forms of AD; a toxic by-product of an independent primary metabolic lesion that is also neurotoxic; or an inert by-product of an independent primary neurotoxic reaction [11].

Derived from the amyloid precursor protein (APP), Aβ is the predominant component of amyloid plaque [12]. The putative source of cerebrovascular Aβ deposits in AD is controversial. There is little evidence for increased Aβ production in sporadic and late-onset AD. Rather, decreased Aβ clearance from the brain has been suggested [13]. In addition, there is accumulating evidence of blood-to-brain delivery of circulating Aβ, contributing to total parenchymal load [14]. Consistent with the concept of a vascular origin for cerebral Aβ was the finding that intravenous injection of anti-Aβ-IgG completely blocked the influx of peripheral Aβ across the BBB [13].

Plasma Aβ could be derived from vascular smooth muscle cells and endothelial cells, or from blood platelets which are an established and significant source of Aβ. However, in recent studies, we reported that the absorptive epithelial cells of the small intestine (enterocytes) also have substantial abundance of Aβ, secreted into blood associated with chylomicrons [15]. Our observations are the first evidence of significant tissue Aβ abundance in the absence of pathological amyloidosis. Interestingly, enterocytic Aβ was substantially increased with the ingestion of a diet enriched in saturated fat and cholesterol, but in contrast, was completely abolished by fasting. These findings may help to explain the mechanisms underlying epidemiological studies and animal feeding studies that have demonstrated a strong positive relationship between fat intake and accelerated amyloid pathology in AD [1–4,16–18]. We will describe in this article how dietary induced elevations in plasma Aβ could compromise BBB integrity, resulting in altered cerebral Aβ homeostasis and inflammatory sequelae. Our hypothesis is supported by studies in transgenic animal models developed to over-express Aβ particularly, but not exclusively in neurons. In these animals, a high-fat diet substantially exacerbates amyloid burden demonstrating that cerebrovascular deposition is influenced by circulatory effects. LaRue et al. [19] showed in one strain of mice (Tg2576), a greater than eightfold increase in peripheral delivery of Aβ across the BBB to the brain and in other studies by Giri et al. [20] it was found that exposure of brain endothelial cells to Aβ promotes migration of inflammatory cells.

## 2. Circulating amyloid-beta and blood–brain barrier integrity

Several studies have provided evidence of a vasoactive role of Aβ, with pathological manifestations prior to Aβ deposition. Aβ is vasoconstrictive and vessels treated with Aβ show significant endothelial cell damage with changes in the cell membrane, cytoplasm, nucleus and other organelles [21]. Soluble Aβ in contact with the cerebrovasculature may play a pathophysiological role, which accompanies or precedes Aβ deposition. 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 only acute single injections and investigated transportation across, or sequestration within, brain capillaries [22–24]. Longer term administration of Aβ (2 weeks), resulted in a significantly compromised BBB and activated CNS glial cells [25]. Whilst these studies demonstrate regulatory responses following exogenous administration of Aβ, the physiological significance of these studies is presently unclear.

## 3. Chylomicron assimilation of amyloid-beta, blood and BBB kinetics

There is a growing body of evidence supporting the hypothesis that the BBB permeability is influenced by a variety of factors including humoral, endocrine and inflammatory mediators. However, the potential effect of diet on the BBB has to our knowledge not been studied. A number of centrally mediated changes in physiology and behaviour occur in response to diet. How these changes are mediated is not currently established. One way for diet to alter function in the central nervous system is through a disruption of the BBB, through the influence of circulating inflammatory cytokines, hormones or other mediators. Disruption of the BBB could allow inflammatory mediators or other circulating products to enter the brain parenchyma. However, to date BBB permeability has only been assessed in experimental colitis [26], or following acute intravenous injection of solubilised, exogenous Aβ [22–24].

The secretion, interaction with chaperone molecules, clearance and metabolism of Aβ are unclear. Koudinov et al. [27] found that Aβ in cerebrospinal fluid (CSF) was associated with a lipoprotein whose density was similar to plasma high-density-lipoproteins. Biere et al. [28] monitored the association of Aβ with plasma proteins in vitro and found that 5% bound to lipoproteins. Schwarzman and Goldgaber [29] incubated Aβ with CSF proteins and identified a peak of Aβ within very-dense-lipoproteins rich in precipitable transthyretin and Ghiso et al. [30] suggested that apolipoprotein (apo) J might be a primary vehicle of Aβ transport. Note however, that these studies using exogenous Aβ may be confounded because of the solubilising conditions used and because of the non-physiological introduction of Aβ into the brain or peripheral fluids.

We now present several lines of evidence suggesting that significant peripheral Aβ metabolism occurs in association

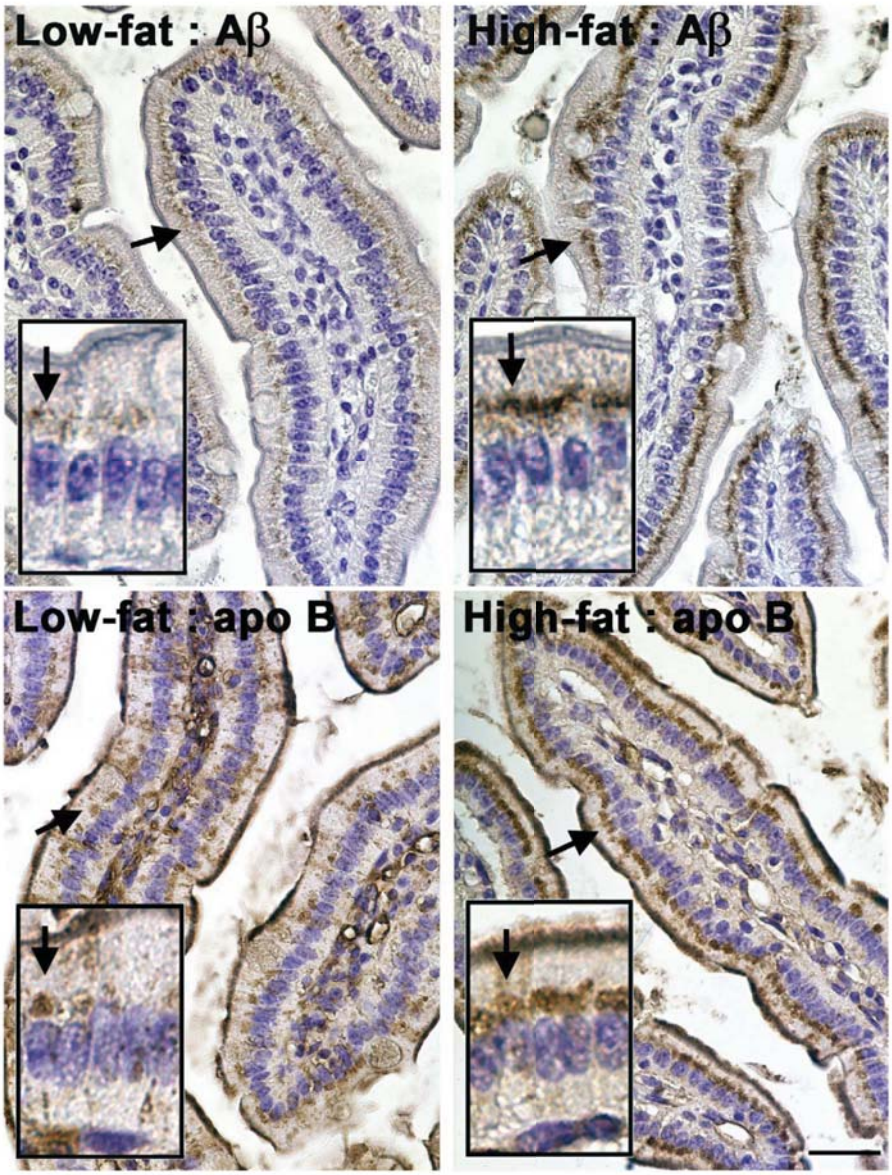


Fig. 1. Amyloid-beta (Aβ) is observed (by immunohistochemistry staining) within the perinuclear region of absorptive cells of the small intestine of wild-type mice maintained on a low-fat (4% (w/w) as poly-unsaturates) diet. Mice given saturated fats (16% (w/w)) and 0.5% cholesterol (w/w) had substantially greater abundance of amyloid-beta concomitant with increased apolipoprotein B48. Inset shows enterocytes at higher magnification. Bar indicates 20 μm.

with chylomicrons. In wild-type mice, there is abundance of Aβ in the epithelial (absorptive) cells of villi and in the crypts of the upper small intestine. Amyloid-beta expression is enhanced with saturated-fat feeding commensurate with an increase and apo B48, an obligatory component of nascent chylomicrons (Fig. 1). We found that a combined high saturated fat and cholesterol diet substantially enhanced enterocytic abundance of Aβ within the perinuclear region of the cell, consistent with exaggerated chylomicron biogenesis. A second line of evidence comes from clinical studies, where we found that following ingestion of a fatty meal, there is a transient increase in the plasma concentration of both the precursor protein and of Aβ, the latter in a triglyceride-rich plasma chylomicron fraction [31]. If Aβ, as our data suggest, is associated with chylomicrons, then a physiological function of Aβ may be to modulate the metabolism of dietary fats. Previous studies are consistent with this concept. Amyloid-beta attenuated hepatic uptake of chylomicron-like emulsions by 75%, promoting uptake by fat rich tissues including adipose and bone marrow [32].

Kinetic analysis in blood of doubly labelled chylomicron-Aβ showed that the majority of Aβ was cleared from plasma with uptake of the chylomicron particles, however, there was some delay in the clearance of Aβ relative to chylomicrons at later time points [32]. A divergence in kinetics of Aβ relative to the chylomicron particle is consistent with some apolipoprotein transfer and in the context of AD risk, this may be pathologically significant. In man the 'post-prandial' chylomicron excursion in response to a single standard meal, lasts for approximately 6–8 h. However, the transfer or shedding of Aβ from chylomicrons would suggest the possible genesis, or supplementation of a secondary pool of plasma Aβ with even longer plasma residency time. Given that humans are almost always in an absorptive state, the cumulative effect of high saturated fat diets on plasma Aβ homeostasis and thereafter BBB integrity may be significant.

## 4. Plasma lipoprotein amyloid-beta distribution

Clinically determining chylomicron-Aβ and indeed the plasma lipoprotein distribution of Aβ has proven to be difficult because hydrophobic lipids bind tightly to Aβ and mask antigenic epitopes [33]. Indeed, we found that delipidation leads to substantial loss of Aβ because of the tight binding of the protein with neutral lipids. Recently, we reported the distributional analysis of plasma lipoprotein-Aβ in normal subjects and those with probably AD [34]. For all subjects, we found the majority of plasma-Aβ to be associated with triglyceride-rich-lipoproteins (TRLs) inclusive of chylomicrons and their post-hydrolyzed remnants (lipoprotein fraction with $\rho \leq 1.019$ g/ml). For all lipoprotein groups (including LDL ($1.020 < \rho < 1.064$ g/ml) and HDL ($1.064 < \rho < 1.21$ g/ml)), $A\beta_{1-40}$ was the predominant isoform accounting for approximately 50% of the total and thereafter, equivalent amounts of the isoforms 1–42, 2–40, 1–38, 1–37 and 1–39 were found. Notably, the $A\beta_{1-37}$, $A\beta_{1-38}$ and $A\beta_{2-40}$ isoforms were significantly enriched within the TRL fraction of AD subjects and similar trends were observed for isoforms $A\beta_{1-39}$, $A\beta_{1-40}$ and $A\beta_{1-42}$. Interestingly, whilst AD subjects were normolipidemic, the concentration of apo B48 (an exclusive marker of chylomicrons) was significantly elevated in the post-absorptive state ($17.4 \pm 5.0$ plasma vs $5.4 \pm 1.1$ μg/ml). Increased apo B48 is usually indicative of post-prandial dyslipidemia, an exaggerated but transient rise in plasma chylomicrons that occurs following the ingestion of dietary fats [35].

It is unclear how Aβ might become associated with chylomicrons or other chylomicron-apoprotein moieties. Amyloid-beta forms part of the amyloid multi-domain precursor protein and its predicted structure indicates that APP resembles a trans-membrane cell surface receptor. Amyloid-precursor-protein possesses a cysteine-rich motif not unlike several lipoprotein receptors that bind with very high affinity to apo E containing lipoproteins. Therefore, it is possible that chylomicrons assimilate endogenously produced Aβ during the proteolytic processing of APP in enterocytes. Once secreted into circulation, chylomicrons interact with lipases located on the surface of endothelial cells. As triglycerides are hydrolyzed, the chylomicrons accumulate apo E, which is known to bind Aβ. The association of chylomicrons with the plasma membrane and acquisition of apo E, may also contribute to Aβ acquisition. Furthermore, Aβ may exchange between chaperone macromolecules, not unlike the normal transfer of many apolipoproteins. However, none of these possibilities fully explain the significant intracellular abundance of Aβ found within the peri-nuclear vicinity of enterocytes. Rather, this and other studies which demonstrate Aβ within the rough endoplasmic reticulum (rER) and Golgi compartments, raise the possibility that Aβ associates directly with primordial lipoproteins during their biosynthesis. Indeed in cell culture exudates, Aβ is secreted exclusively as a lipoprotein complex [33].

Apolipoprotein B48, an obligatory structural component of chylomicrons requires successful 'lipidation,' specifically the addition of cholesteryl-ester for lipoprotein secretion to occur and at this point other apoproteins bind to the nascent lipoprotein. It is possible that intestinal secretion of Aβ might occur with the ingestion of cholesterol and other dietary fats.

## 5. Transgenic animal models of Alzheimer's disease

Indirect evidence for a role of TRLs in AD also comes from studies conducted in various strains of transgenic mice over-expressing the amyloid precursor protein (predominantly in neurons). In a recent study by Burgess et al. [36] plasma Aβ was substantially increased and this was positively related to plasma triglyceride concentration and with the onset of cerebrovascular and parenchymal amyloidosis. Moreover, plasma Aβ correlated with lipoprotein triglyceride secretion rates, possibly because of an overproduction phenomenon of lipoprotein-Aβ.

Consistent with the concept that circulating lipoprotein-Aβ may compromise BBB integrity and exacerbate amyloidosis, we now present preliminary evidence of plasma protein extravasation in APP/PS1 transgenic mice (Fig. 2). Significant immunoglobulin G (IgG) was observed in transgenic mice with enrichment associated with amyloid plaque. In contrast, wild-type mice only showed modest IgG staining in some blood vessels. Moreover, occludin expression (an endothelial tight junction protein) was substantially less in transgenic mice compared to wild-type controls (Fig. 2), consistent with diminished endothelial integrity.

## 6. Chylomicron amyloid-beta metabolism and apo E genotype in man

Considerable interest has focused on the putative relationship between Aβ kinetics and apo E, because of strong genetic evidence that links increased incidence of sporadic

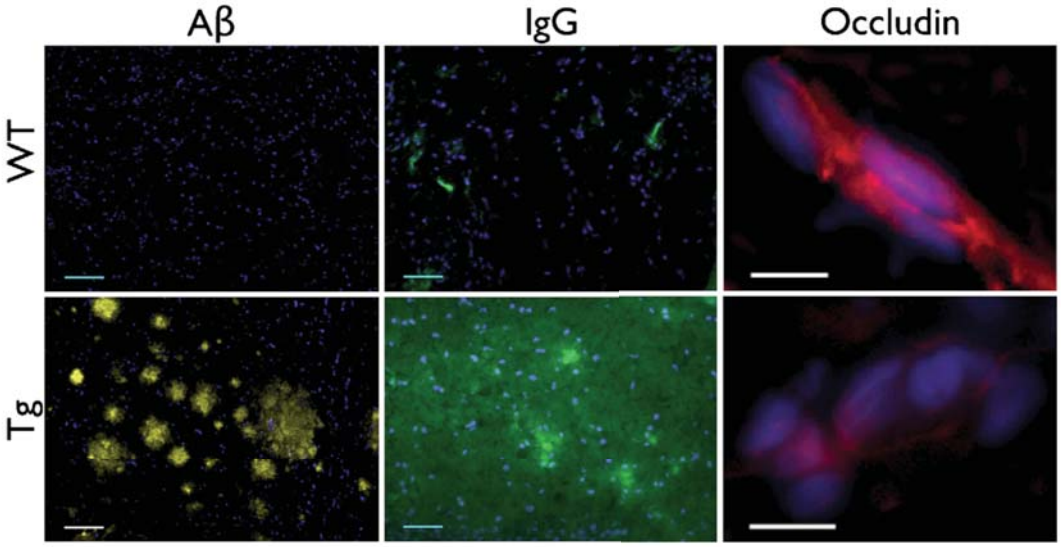


Fig. 2. Cerebral amyloid plaque is seen in a murine model of Alzheimer's disease (transgenic amyloid-precursor-protein/presenelin mice maintained on a high-fat diet), but not in wild-type controls. Concomitant with amyloidosis is widespread extravasation of immunoglobulin G, a surrogate marker of plasma proteins, with accentuated staining seen associated with amyloid deposits. Expression of occludin, an endothelial tight junction protein is attenuated in the transgenic mice compared to controls. Bars indicate 50 μm.

and familial late-onset AD in subjects with E4 isoforms [37] (of which there are three in man (apo-E4, -E3 and -E2)). Furthermore, apo E is found in dietary fat induced extracellular amyloid deposits, including vascular deposits. Greater than 90% of plasma apo E is associated with remnants of chylomicrons and hepatic very-low-density-lipoproteins (VLDL), that is, particles that have undergone triglyceride hydrolysis by endothelial lipases. However, interestingly there is a preferential distribution of apo E4 compared to apo E3 or apo E2 amongst remnant fractions containing greater amounts of residual triglyceride. Chylomicron remnants contain approximately twofold more triglyceride than hepatic VLDL-remnants and therefore presumably accumulate more apo E4 in subjects expressing this apolipoprotein isoform. Apo E4 may enhance Alzheimer's risk via several mechanisms, but we now introduce the concept that this may also be linked to aberrant chylomicron-Aβ metabolism. It has been proposed that lipidated and free apo E functions as a pathological chaperone of Aβ. LaDu [38] reported that apo E3 lipoproteins bound approximately 20 times more Aβ than apo E4 lipoproteins. If Aβ binding to chylomicrons is modulated by apo E, then based on the findings of LaDu, we would predict that in man, chylomicron remnants enriched in apo E4 will retain less Aβ, a phenomenon which may be detrimental to BBB integrity if the non-chylomicron bound form of the protein is more 'cytotoxic' than the lipoprotein bound form (Fig. 3).

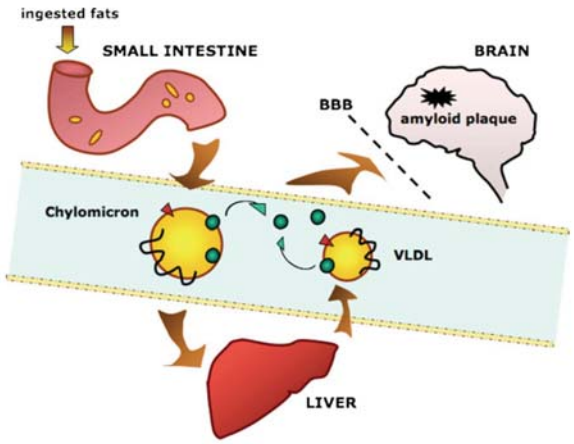


Fig. 3. Model for dietary induced modulation of blood–brain barrier integrity and amyloidosis. Saturated fats stimulate synthesis and secretion of chylomicron-associated amyloid-beta (Aβ). Dietary saturated fats may also increase hepatic secretion of very-low-density-lipoprotein (VLDL)-Aβ. Overproduction, coupled with diminished clearance by receptor pathways of remnant lipoproteins results in chronically elevated levels of plasma Aβ. Cerebrovascular integrity is compromised and there is increased blood-to-brain delivery of circulating Aβ.

## 7. Summary and conclusion

Population studies continue to support a role of dietary fats in Alzheimer's (AD). Laitinen et al. [39] reported that intake of unsaturated fats is protective, whereas intake of saturates increases risk of AD. In the Framingham study, the top quartile of plasma docosahexanoic acid (DHA) (profoundly influenced by diet) was associated with a 47% reduction in risk of all-cause dementia [40]. Irrefutable evidence continues to come from animal studies including by Oksman et

al. [41] who confirmed that saturates increased, whilst DHA decreased Aβ levels compared to soy oil diet. In cell culture studies, fatty acids increased presenilin 1, gamma secretase and Aβ independent of any increase in cellular cholesterol [42] and other evidence consistent with our hypothesis comes from Cullen et al. [43], who showed in human studies that Aβ deposits co-localized with blood proteins and microhemorrhages.

Elevated total cholesterol, LDL and triglyceride with normal HDL and TC/HDL ratio characterize the lipid profile in AD. However, MMSE (mini mental-state examination) does not correlate with lipid parameters suggesting no interaction between cholesterol and cognition in AD [44]. Inhibitors of HMG-CoA reductase (statins) reduce cardiovascular diseases and exhibit pleiotropic effects independent of lipid modification. Some of these effects may improve outcome or ameliorate symptoms of neurological disorders including AD, but the appropriateness of initiating statin therapy is not established at this time [45].

Recent evidence indicates that insulin resistance is associated with an increased relative risk for AD [46] and this notion was directly explored in Tg2576 mice which model AD-like neuropathology [47]. Ho et al. found that diet-induced insulin resistance promoted amyloidogenic Aβ. However, consistent with the hypothesis proposed in this article, the murine model would also have exaggerated plasma TRL-Aβ.

How dietary fats influence BBB function and the propensity for amyloidosis we contend would be useful in the context of AD prevention and quite possibly, in the development of nutrition-based intervention strategies. Collectively, accumulating evidence suggests that chronic consumption of foods rich in saturated fats may increase AD risk but the mechanisms for this are presently unknown. It is our contention that increased secretion and diminished clearance of lipoprotein associated Aβ in response to saturated fats leads compromised BBB integrity and a shift in Aβ kinetics from blood-to-brain. Evidence consistent with this hypothesis may provide novel nutrition and/or pharmacological strategies to reduce the prevalence or progression of Alzheimer's disease.

## Conflicts of interest

None.

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# CHAPTER 2

# Chapter 2

# Development of 3-Dimensional Double Immunofluorolabelling and Semi-quantitative Colocalization Analysis Methodology

**The content of this chapter is provided as the manuscript titled:**

**Takechi R**, Galloway S, Pallebage-Gamarallage MM, Johnsen RD, Mamo JC. (2008) Three-dimensional immunofluorescent double-labelling using polyclonal antibodies derived from the same species: Enterocytic colocalization of chylomicrons with Golgi apparatus. *Histochem Cell Biol*. 129, 779-84 [Impact factor: 2.3]

**Thesis objectives addressed in this chapter:**

**Objective 1:** To develop 3-dimensional double immunofluoroscent microscopy using polyclonal antibodies raised in the same species

**Objective 2:** To establish 3-dimensional semi-quantitative colocalization analysis methodologies with double immunofluorescent labelling

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ORIGINAL PAPER

# Three-dimensional immunofluorescent double labelling using polyclonal antibodies derived from the same species: enterocytic colocalization of chylomicrons with Golgi apparatus

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**Abstract** Double immunolabelling is a useful technique to determine cellular colocalization of proteins, but is prone to false-positive staining because of cross-reactivity between antibodies. In this study, we established a simple and quick method to demonstrate the immunofluorescent double labelling with two rabbit-derived polyclonal antibodies. The principle used was to establish a dilution of primary antibody for the first protein of interest, which would only be detectable following biotin–avidin amplification. Thereafter, the second protein of interest was assessed via standard secondary antibody detection, ensuring no cross-reactivity with the first protein antibody–antigen complex. We successfully demonstrated the three-dimensional colocalization of enterocytic apolipoprotein B, an equivocal marker of intestinal lipoproteins with Golgi apparatus. Colocalization of apo B and Golgi apparatus (75.2 ± 8.5%) is consistent with the purported mode of secretion of these macromolecules.

**Keywords** Double immunofluorolabelling · Three-dimensional colocalization · Polyclonal antibodies · Intestinal Golgi apparatus · Chylomicron

## Introduction

Immunofluorescent double labelling is an increasingly used histological technique to examine colocalization of alternate antigens (Nishimura et al. 2006; Soldani et al. 2007). To avoid cross-reactivity of primary antibodies, the ideal approach for multiple immunolabelling is to either utilize polyclonal antibodies raised in different species or to utilize monoclonal antibodies comprising different immunoglobulin isoforms (Hermiston et al. 1992). However, these two options for double immunolabelling are often not possible, because commercially available polyclonal antibodies are commonly raised in the same species, or monoclonal antibodies are either the entire IgG or the cross-reactive isoforms of the immunoglobulin. One approach for avoiding cross-reactivity and false-positive signalling is to differentially conjugate primary antibodies with labels such as enzymes, biotin or fluorochromes, prior to their application on tissue (Uchihara et al. 1995; Tsurui et al. 2000). The latter method generally provides reliable detection of two or more antigens; however, the sensitivity of preconjugation is generally lower compared to when primary antibodies are applied and detected thereafter with labelled secondary antibodies. Furthermore, preconjugation typically requires larger quantities of antibodies; the procedure can be rather complex and not always successful (Uchihara et al. 1995).

**Electronic supplementary material** The online version of this article (doi:10.1007/s00418-008-0404-0) contains supplementary material, which is available to authorized users.

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Several laboratories have demonstrated labelling of two or more proteins by saturating the residual binding sites following application of the first antibody, prior to detection of the second protein of interest (Kroeber et al. 1998; Tornehave et al. 2000). Saturation is typically achieved with serum and/or by microwave heating. However, this approach may be limited by diminished sensitivity and may also be confounded by quenching (Bauer et al. 2001).

Shindler and Roth (1996) developed a novel, sensitive immunofluorescent double-labelling technique utilizing a tyramide-signal-amplification method (TSA). Briefly, the primary antibody to the first protein of interest is diluted to an extent that it can only be detected via TSA amplification. Hence, the signal strength as a result of cross-reactivity during the second cycle of protein staining is below the threshold level of detection. Utilizing a similar approach, Uchihara et al. (2003) demonstrated multilabelling utilizing a catalyzed-reporter-amplification technique.

In the current study, we developed a simple and quick method for polyclonal antibody-based double immunofluorescent detection using a biotin–avidin amplification method. Biotin–avidin binding is commonly used in immunohistological staining of singular proteins and in biochemical assays such as for enzyme-linked-immuno-absorption (Rambozzi et al. 2004). However, biotin–avidin has not been assessed in the context of utilizing amplification of one protein to avoid cross-reactivity with alternate antigen–antibody complexes. The specific binding affinity of biotin–avidin ($K_d = 10^{-15}$ M) is almost 1 million times greater than that of an antigen–antibody and hence substantially more sensitive than the standard approach of secondary antibody detection of a primary antibody. Binding of biotin–avidin is also rapid (complete within 20–30 min) enabling significant sample throughput. To demonstrate the potential usefulness of this approach, in this study, we show double-immunofluorescent labelling of chylomicrons, which are lipoproteins synthesized in enterocytes, within the Golgi apparatus. Moreover, we demonstrate applicability with rabbit-derived polyclonal antibodies for apolipoprotein B (apo B—an equivocal marker of chylomicrons) and Golgi apparatus.

The intestinal chylomicron synthetic pathway is reasonably well established, essentially by extension of studies of hepatic lipogenic pathways (Hussain 2000). Briefly, apo B is enzymatically lipidated generating a nascent spherical lipoprotein complex. Thereafter, the chylomicrons are thought to migrate via the Golgi apparatus towards the basolateral surface of the cell and are secreted into the lacteals of intestinal villi. The intracellular distribution of apo B in intestinal epithelial cells has to date not been histologicaly demonstrated. In the current study, we present the three-dimensional colocalization of apoB and Golgi apparatus in mouse enterocytes.

## Materials and methods

### Animals and tissue preparation

The experimental procedure in this study was approved by a National Health and Medical Research Committee (Australia)-accredited Animal Ethics Committee (Curtin University approval R02-07). Mouse intestinal samples were collected from 12-week-old female C57BL/6J mice. Mice were fed a high fat diet containing 10% (w/w) of saturated fat and 2% (w/w) of cholesterol for 4 weeks to enhance chylomicron biogenesis. Tissue samples were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin as previously described (Galloway et al. 2007).

### Antibodies

Anti-Golgi 58K protein antibody, anti-apolipoprotein B antibody and anti-rabbit IgG with FITC were purchased from Abcam (Cambridge, UK). DAPI and streptavidin-Alexa 546 were obtained from Invitrogen (VIC, Australia). Anti-rabbit IgG biotin conjugate was obtained from DAKO (Denmark).

### Optimization of antibody concentrations

For apo B detection, a series of dilutions of the primary antibody were compared to identify a concentration, which would only be detectable by biotin–avidin secondary antibody amplification. Briefly, 5-μm-thick tissue sections were prepared from the duodenum and collected on silane-coated microscope slides. After the permeabilization of sections with PBST (PBS with 0.2% Triton X-100), non-specific binding sites were blocked with 10% goat serum in PBST for 30 min. Alternate concentrations of rabbit polyclonal anti-apoB antibody (1:100–1:4,000 diluted in PBST supplemented with 10% goat serum) was then applied onto tissue sections and incubated overnight at 4°C. Apo B detection was compared between a standard secondary detection approach [goat anti-rabbit IgG conjugated with FITC (1:100 for 1 h), versus biotin–avidin amplification (1:200 for 1 h), followed by streptavidin with Alexa 546 (1:100 for 1 h)]. Negative controls included tissue specimens treated with rabbit sera or phosphate-buffered saline in lieu of anti-apo B. No immunostaining was observed in negative controls.

The same approach to compare anti-apo B dilutions was also used to assess dilutions of anti-Golgi protein with and without the amplification (1:10–1:300). By collecting the data from these preliminary studies, the final procedure for double labelling method was determined and summarized in Fig. 1.

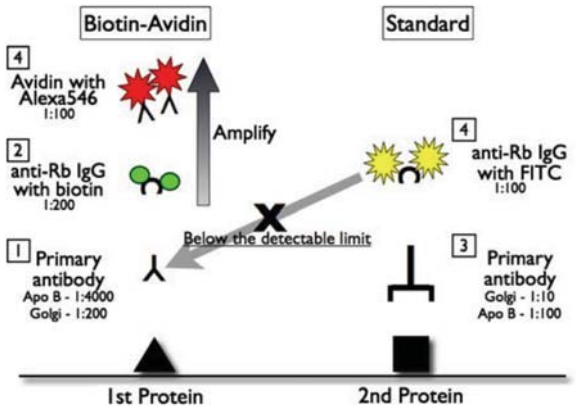


**Fig. 1** Summary of double labelling method. The diagram summarizes the principle of double immunofluorescent labelling established for this study. Potential cross-reactivity of rabbit-derived antibodies of the first protein of interest with standard secondary antibody detection (anti-rabbit IgG) was avoided by using a dilution below the level of detection (apo B 1:4,000; Golgi protein 1:200). Rather, apo B or Golgi apparatus is detected by biotin–avidin amplification prior to second protein immunodetection, which is done without amplification

### Double-immunofluorescent labelling of Golgi apparatus and apo B

Following optimization of the biotin–avidin approach, which ruled out cross-reactivity of apo B with Golgi apparatus detection, double labelling of these two proteins was done. Tissue sections were incubated with anti-apo B (1:4,000) overnight at 4°C. Goat anti-rabbit IgG with biotin was then added for 1 h. After the thorough wash of sections with PBST, the sections were incubated with anti-Golgi protein antibody (1:10) overnight at 4°C. Finally, the signals were visualized with premixed fluorochromes of streptavidin-Alexa 546 (1:100) and anti-rabbit IgG with FITC (1:100). The cell nuclei were counterstained with DAPI. Sections were mounted with anti-fade mounting medium. Fluorescent images were digitally captured at 200 or 400 times magnification (Plan-NeoFluar 40× objective lens with 1.3 numerical aperture), using Axiovert 200M, AxioCam MRm and AxioVision imaging software ver. 4.6.3.0, Zeiss. The filters used for FITC and Alexa546 detection were the recommended Zeiss filter sets 38 and 43, respectively, for double capturing of these fluorochromes (excitation and emission data available at http://www.micro-shop.zeiss.com). Importantly, for the exposure time used in this study (<300 ms), there was no detectable fluorescence overlap. The filter set for DAPI was Ziess set 49.

### Three-dimensional double immunofluorescent imaging

For 3D distributional immuno-analysis of apo B and Golgi apparatus, the mouse intestine was sectioned to 10–12 μm in thickness. The sections were subjected to double immunofluorescent labelling as described. Three-dimensional fluorescent images were captured through the optical sectioning mode using ApoTome (Zeiss) at 400 times magnification under oil immersion. Forty to sixty consecutive optical sectioning images were captured and reconstructed to three-dimensional image by AxioVIsion.

### Estimation of colocalization rates of intestinal Golgi apparatus and apo B

The colocalization of chylomicron apo B with Golgi apparatus was determined in enterocytes utilizing the AxioVision colocalization software module (Zeiss), which determines Pearson's correlation using Nyquist oversampling. The degree of colocalization for proteins is positively related to the coefficient. The AxioVision software automatically divides the entire 2D image (1,024 × 767 pixels) to each single pixel and analyzes the number of pixels by which two different dyes are colocalized. Enterocytic colocalization of apo B and Golgi apparatus was determined. Tissue specimens from four mice with three images per animal were determined.

## Results

### Intestinal morphology and localization of apo B and Golgi apparatus

An example image to demonstrate the intestinal morphology and the localization of apo B is presented in Fig. 2a, b. In this image, apo B was visualized with rabbit polyclonal anti-apo B (1:100) and anti-rabbit IgG with FITC (1:100, 1 h). The morphology image of the intestine was taken through bright-field mode with DIC phase 3 filter (Zeiss) and overlapped onto the fluorescent image. Specific perinuclear staining of apo B was clearly detected in the enterocytes throughout the villi and enriched within the lacteals (Fig. 2a, b).

Immunofluorescent detection of Golgi-58K in the enterocytes was also done utilizing standard nonamplified secondary antibody techniques (anti-rabbit IgG with FITC, 1:100 for 1 h). As for apo B, Golgi-58K protein was observed primarily within the perinuclear region of enterocytes (Fig. 2c).

### Optimization of biotin–avidin amplification of apo B immunofluorescent detection

Biotin–avidin-amplified apo B staining was determined at alternate concentrations of the primary antibody (1:100–1:4,000) and compared with nonamplified staining

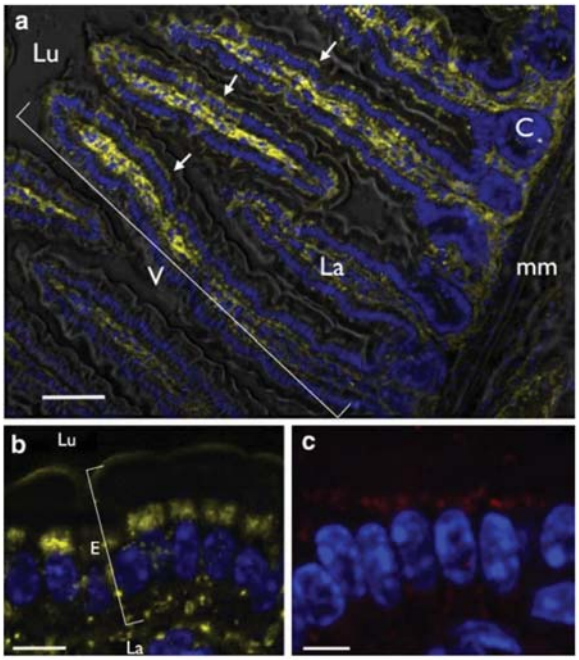


**Fig. 2** Apolipoprotein B and Golgi apparatus immunofluorescent detection and morphology in small intestine. Anti-apo B antibody was visualized with anti-rabbit IgG FITC conjugate (*yellow*). Golgi apparatus were detected by polyclonal rabbit anti-Golgi 58K protein antibody and anti-rabbit IgG with FITC (*red*). Cell nuclei were counterstained with DAPI (*blue*). Histological morphology was captured on bright-field mode through phase 3 DIC filter. **a** Apo B staining was seen within the perinuclear region of enterocytes (*arrows*) and within the lacteals, where chylomicron secretion occurs (*scale bar* 50 μm). Significant perinuclear staining of apo B (**b**) and Golgi apparatus (**c**) was seen in high-magnified images of enterocytes (*scale bar* 10 μm). *C* crypts; *E* enterocyte; *La* lacteal; *Lu* lumen; *mm* mascularis mucosa; *V* villus

(Fig. 3a). Perinuclear staining of apo B within the enterocytes in the absence of biotin–avidin amplification was only slightly detectable at 1:1,000–1:2,000. At the primary antibody concentration of 1:4,000, fluorescence could not be seen utilizing standard secondary antibody detection. However, biotin–avidin significantly amplified apo B signal at all concentrations of the primary antibody and was readily apparent at a concentration of 1:4,000 of anti-apo B. It was concluded that the latter could be used to determine apo B with other enterocytic proteins detected without amplification.

The Golgi apparatus was also compared with and without amplification at a series of primary antibody concentrations ranging 1:10 to 1:300. At 1:200 dilution of anti-Golgi protein antibody, perinuclear staining was undetectable; however, it was clearly detected with amplification (Fig. 3b).

### Colocalization of intestinal Golgi apparatus and apo B

Utilizing biotin–avidin-based amplification of apo B, coupled with standard immunolabelling of Golgi-58K, as explained in Fig. 1, colocalization of these two proteins in enterocytes was explored in two- and three-dimensions (Figs. 4 and 5, respectively; also see Fig. S1 in electronic supplementary material). We found that $75.2 \pm 8.5\%$ of Golgi apparatus in the enterocytes was colocalized with apo B.

## Discussion

We have demonstrated the three-dimensional double immunofluorescent labelling of mouse enterocytic apo B and Golgi apparatus using two polyclonal antibodies raised in the same species. Immunofluorescent staining of apo B with standard secondary anti-rabbit IgG with FITC found that the optimal dilution of rabbit polyclonal anti-apo B antibody was 1:100. At a dilution of 1:4,000, apo B was not seen utilizing standard secondary antibody detection. However, apo B was clearly visualized at an anti-apo B dilution of 1:4,000 when amplified with biotin–avidin. Collectively, these results show that anti-apo B at a concentration of 1:4,000 would not cross-react with the detection of Golgi apparatus utilizing antisera generated from the same species without amplification. The biotin–avidin amplification was also demonstrated for anti-Golgi apparatus, and we contend generally that it is applicable for the detection of two proteins using polyclonal anti-sera derived from the same species. This study showed that the biotin–avidin amplification is sufficiently sensitive to detect proteins following substantial dilution of the primary antibody, in this study up to 40 times less than when detected without amplification.

To achieve double immunolabelling using antibodies raised in the same species, the key principle demonstrated in this study was to set the concentration of the first antigens primary antibody to be lower than the detectable range by standard secondary antibody staining. Biotin–avidin-based amplification offers a comparatively cheap and less laborious alternative to other amplification methods such as TSA, though the latter may more sensitive (Uchihara et al. 2003; Hunyady et al. 1996). With this relatively simple and quick double-immunofluorescent labelling approach, the colocalization of intestinal apo B and Golgi apparatus was investigated. Lipidation of apo B within the Golgi apparatus is a requisite for proper chylomicron assembly and secretion, but has not been previously visualized. In this study, three-dimensional double immunofluorescent labelling of apo B and Golgi apparatus was captured using

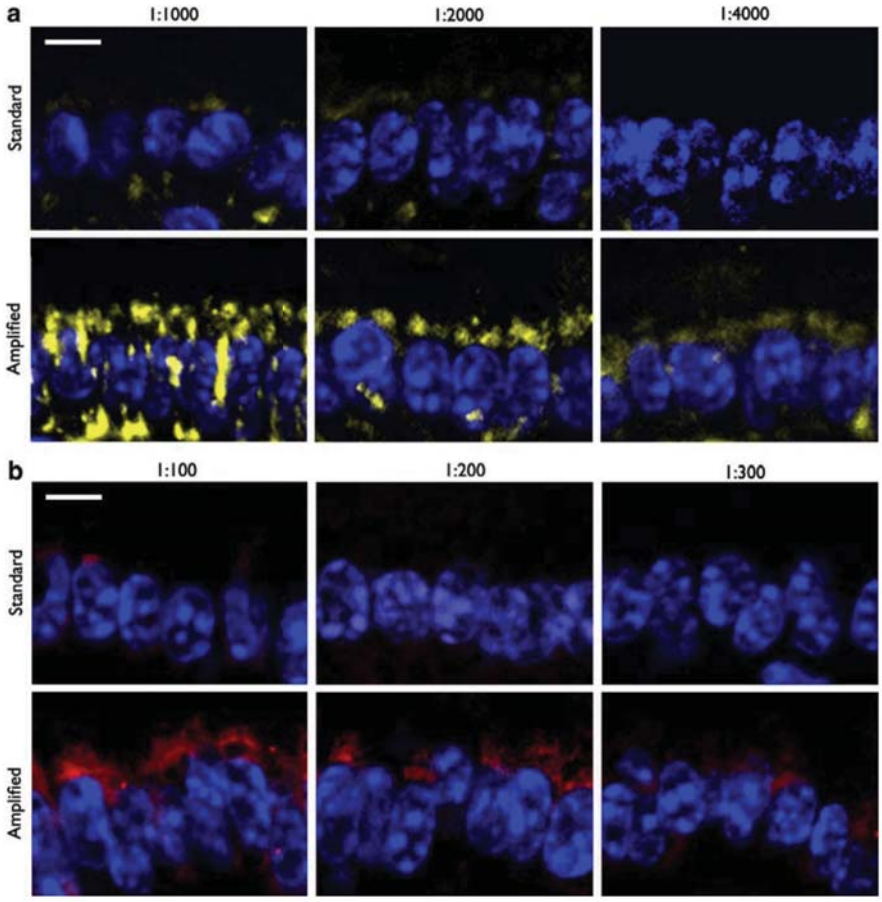


**Fig. 3** Biotin–avidin amplification of apolipoprotein B and Golgi apparatus immunofluorescent detection. Enterocytic apo B was visualized with and without the biotin–avidin amplification. Amplification enhanced signal intensity at all concentrations of the primary antibody. However, in the absence of amplification, apo B could not be detected at a primary antibody concentration of 1:4,000. Similarly, Golgi apparatus was not detected at a primary antibody concentration of 1:200–1:300 without amplification, whereas the signal was clearly detected after the amplification. Apo B is shown in *yellow*, Golgi apparatus in *red* and cell nuclei in *blue* (DAPI). *Bar* in the image indicates 10 μm

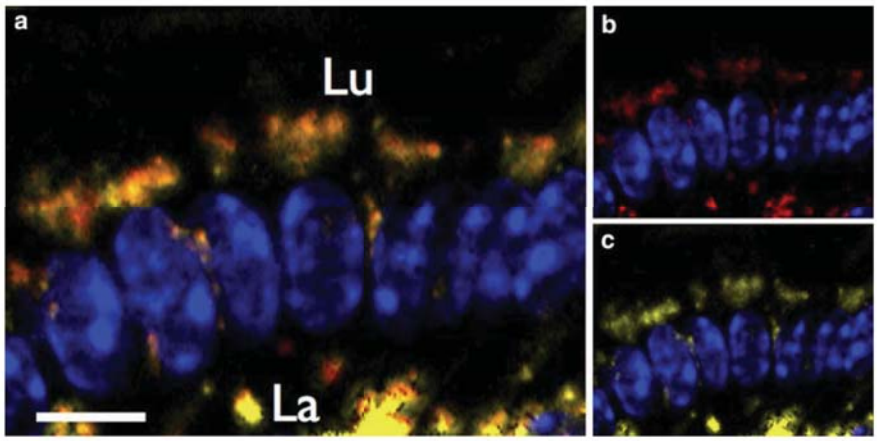


**Fig. 4** Enterocytic distribution of apolipoprotein B and Golgi apparatus. Double immunofluorescent labelling of apo B (*yellow*) and Golgi apparatus (*red*) is depicted using the differential biotin–avidin amplification procedure described in Fig. 1. The *orange* color shows significant colocalization of apo B and Golgi apparatus (**a**). Separated images for Golgi apparatus (**b**) and apoB (**c**) are shown. *Scale bar* indicates 10 μm. *Lu* lumen; *La* lacteal

differential-filter-processing (ApoTome), and the colocalization of the two proteins was compared. The data show a strong correlation of apo B with Golgi apparatus, consistent with the purported assembly and secretion of intestinal lipoproteins.

In summary, three-dimensional double immunofluorescent labelling with polyclonal antibodies raised in the same species was successful by using biotin–avidin amplification to avoid cross-reactivity. The method demonstrated in this study was simple, and the same approach could be applied

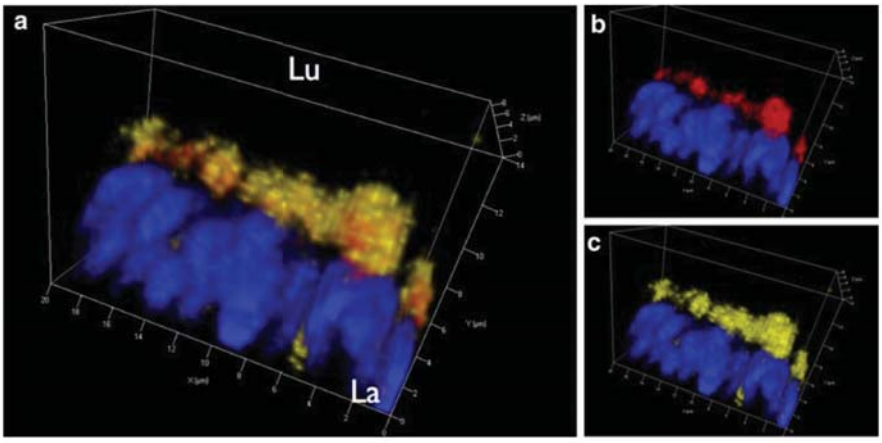


**Fig. 5** Three-dimensional colocalization of intestinal Golgi apparatus and apo B. Double immunofluorescent labelling of apo B (*yellow*) and Golgi apparatus (*red*) is depicted in three-dimension using the differential biotin–avidin amplification procedure described in Fig. 1. Separated images of Golgi apparatus (**b**) and apo B (**c**) are also shown. Lengths of *x*, *y* and *z* axes are 20, 14 and 9 μm, respectively. *Lu* lumen; *La* lacteal

to other proteins of interest. Our results showing significant colocalization of enterocytic apo B with Golgi apparatus is consistent with the purported assembly of chylomicron.

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# CHAPTER 3

# Chapter 3

# Colocalization of Apolipoprotein B with Cerebral Amyloid Plaques of Alzheimer's Disease

**The content of this chapter is provided as the manuscript titled:**

**Takechi R**, Galloway S, Pallebage-Gamarallage MM, Wellington C, Johnsen R, Mamo JC. (2009) Three-dimensional colocalization analysis of plasma-derived apolipoprotein B with amyloid plaques in APP/PS1 transgenic mice. *Histochem Cell Biol*. 131, 661-6

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**Thesis objective addressed in this chapter:**

**Objective 3:** To investigate the 3-dimensional localization of plasma derived TRL-Aβ relative to cerebral amyloid plaque abundance

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# Three-dimensional colocalization analysis of plasma-derived apolipoprotein B with amyloid plaques in APP/PS1 transgenic mice

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**Abstract** Parenchymal accumulation of amyloid-beta (A$\beta$) is a hallmark pathological feature of Alzheimer's disease. An emerging hypothesis is that blood-to-brain delivery of A$\beta$ may increase with compromised blood–brain barrier integrity. In plasma, substantial A$\beta$ is associated with triglyceride-rich lipoproteins (TRLs) secreted by the liver and intestine. Utilizing apolipoprotein B as an exclusive marker of hepatic and intestinal TRLs, here we show utilizing an highly sensitive 3-dimensional immunomicroscopy imaging technique, that in APP/PS1 amyloid transgenic mice, concomitant with substantially increased plasma A$\beta$, there is a significant colocalization of apolipoprotein B with cerebral amyloid plaque. The findings are consistent with the possibility that circulating lipoprotein-A$\beta$ contributes to cerebral amyloidosis.

**Keywords** 3-Dimensional colocalization microscopy · Alzheimer's disease · Amyloid-beta · Apolipoprotein B · Blood–brain barrier

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## Introduction

A hallmark neuropathological marker of Alzheimer's disease (AD) is amyloid-beta (A$\beta$) deposition in the cerebrovasculature and brain parenchyma. Why A$\beta$ accumulates in AD is uncertain, although there is little evidence for increased cerebral A$\beta$ production in sporadic, late-onset AD. Rather, diminished clearance of A$\beta$ via the blood–brain barrier (BBB) or choroid plexus may occur with aging (Deane et al. 2005; Crossgrove et al. 2005). There is also emerging evidence of blood-to-brain delivery of A$\beta$ (Mackic et al. 2002), a process that may be exaggerated as a consequence of BBB dysfunction.

The cerebrovasculature in subjects with AD shows pathological alterations including vascular endothelial and smooth muscle cell proliferation (Ellis et al. 1996). Blood derived proteins have been detected in brain parenchyma of AD subjects (Wisniewski et al. 1997) and inflammatory sequealae are reported (Cullen 1997), observations that are consistent with the breakdown of the BBB.

In blood, significant A$\beta$ is associated the triglyceride-rich lipoproteins (TRLs) (Mamo et al. 2008) consistent with the findings by Koudinov and Koudinova (1997), who found that hepatocytes secrete A$\beta$ exclusively as a lipoprotein complex. In recent studies, we reported that the absorptive epithelial cells of the small intestine (enterocytes) have substantial abundance of A$\beta$, secreted into blood associated with dietary induced lipoproteins (chylomicrons) (Galloway et al. 2007). Enterocoytic A$\beta$ levels were substantially increased by the ingestion of a high-fat diet but completely abolished by fasting, suggesting that the secretion of lipoprotein-A$\beta$ from the small intestine and liver is regulated by dietary lipids. This finding may help explain the mechanisms underlying epidemiological studies and animal feeding studies that demonstrate an association between the

amount and type of fats ingested and AD risk (Kalmijn 2000; Sparks et al. 1994). Fat-induced elevations of plasma Aβ may contribute to compromised BBB integrity and thereafter, result in increased Aβ transport. Our hypothesis is supported by studies in transgenic animal models that over-express the amyloid precursor protein (Levin-Allerhand et al. 2002). In these animals, a high-fat diet exacerbates Aβ burden demonstrating that cerebrovascular deposition is influenced by circulatory effects. Indeed, LaRue et al. (2004) showed that the Tg2576 model of AD has a >8-fold increase in peripheral delivery of Aβ from blood-to-brain.

Several studies have provided evidence of a vasoactive role of Aβ, with pathological manifestations prior to Aβ deposition. Amyloid-beta 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). Soluble Aβ in contact with the cerebrovasculature may have a pathophysiological role, which accompanies or precedes Aβ deposition. Previous studies where Aβ was intravascularly administered involved acute single injections and investigated transportation across, or sequestration within brain capillaries. Two weeks of peripherally administered Aβ resulted in a significantly compromised BBB and reactive gliosis (Su et al. 1999). These studies demonstrate regulatory responses following exogenous administration of Aβ.

Evidence of plasma-derived TRL-Aβ in brain comes is suggested in a study by Namba et al. (1992) who found apo B immunoreactivity in brains of patients with AD. Extending on this initial finding by Namba et al., in this study, we utilized highly sensitive 3-dimensional immuno-fluorescence microscopy technique to investigate the putative colocalization of plasma-derived apo B lipoproteins with cerebral amyloid plaque.

## Methods

### Materials

Primary antibodies of mouse monoclonal anti-Aβ (6E10) and rabbit polyclonal anti-apolipoprotein B were purchased from Abcam (UK). Secondary Alexa fluorochrome antibody conjugates were purchased from Invitrogen (US). Other major laboratory reagents were purchased from Sigma (US). Plasma Aβ was determined by ELISA (Biosource, US).

### Tissue collection

The protocols used in this study were approved by a National Health and Medical Research Council of Australia accredited Animal Ethics Committee (approval no. R34/08). Twelve-month-old transgenic amyloid precursor protein/presenilin-1 (APP/PS1) mice over-expressing human APP and PS1 and C57BL/6 (wild-type control mice) were maintained on a standard rodent chow diet. The APP/PS1 transgenic mice are widely used and develop AD like amyloid plaques at the age of 36-40 weeks. Relevant to the hypothesis presented, these mice have an eight- to tenfold increase in plasma Aβ (Burgess et al. 2006). Three transgenic and four wild-type mice were anesthetized (45 mg/kg of pentobarbital), and brains were isolated, washed with PBS and segmented into right and left hemisphere. Specimens were embedded with OCT compound and frozen in liquid nitrogen.

### Immunofluorescent labeling of Aβ and apolipoprotein B

Apolipoprotein B and Aβ were determined using immunofluorescent microscopy. For the broad distribution of apo B and Aβ detection, 10 μm frozen cryosections were prepared from the right hemisphere of the brain. Following blocking with 10% goat serum for 30 min, apo B and Aβ were detected with rabbit polyclonal anti-apo B (1/200, 20 h) and mouse monoclonal anti-Aβ (6E10) (1/200, 20 h) respectively. Cerebral apo B was then identified with anti-rabbit IgG conjugated to Alexa488 (1/200, 1 h) and Aβ using anti-mouse $IgG_1$-Alexa680 (1/100, 1 h). The nuclei were counterstained with DAPI.

The colocalization of apo B with Aβ was similarly demonstrated using double immunofluorolabeling method. A 50 μm frozen brain cryosections were fixed in 4% paraformaldehyde for 30 min and kept for 12 h in deionized water at 60°C in order to help the penetration of antibodies into the plaques. After blocking with 10% goat serum for 1 h, a mixture of anti-Aβ and anti-apo B antibodies were applied to the sections and incubated for 3 days at 4°C. The antibodies were subsequently visualized with anti-rabbit IgG-Alexa488 and anti-mouse $IgG_1$-Alexa680. Negative control specimens for each experiment were tissue treated as described, but with the omission of the primary antibodies.

### Imaging and analysis

The immunofluorescent images were captured utilizing the optical sectioning mode with ApoTome (Zeiss, Germany) at ×100 and magnification for entire apo B distribution analysis (Zeiss Axiovert 200 M and AxioVision 4.7 imaging software). Colocalization analysis was based on our previous study (Takechi et al. 2008b). Briefly, the 3-dimensional images were taken with ApoTome at ×400 magnification (Zeiss Plan-Neofluar, numerical aperture 1.3 with oil immersion). Each 3-D image consisted of 24–126 two-dimensional images collected in the axial plane at 0.275 μm that was optimized by Nyquist theory (2× oversampling).

Three-dimensional (3D) images were generated with Volocity 4.2 software (Improvision).

A minimum of five 3-D images were sampled from each mouse and data are from 3 APP/PS1 transgenic mice (more than 800 2-D images in total). The AxioVision software measures stochastic fluctuations of fluorescence in a well-defined volume element and determines the number of different dye pixels that colocalize. The Pearson's correlation coefficient ($r$) is a common quantitative estimate of colocalization that depends on the amount of colocalized signals within two channels (Manders et al. 1992), but may be less reliable if there is significant divergence in fluorescence between channels. The most widespread approach for quantitative colocalization measurements via fluorescence microscopy is to determine the colocalized fraction of each fluorochrome species. Developed by Manders et al. (1993), colocalization coefficients M1 and M2 require a threshold value for each channel, which is then used as a cut off between specific staining versus non-specific. The overlapping regions between both channels that are above cut off are then considered as colocalized regions, and the proportions of signal for each channel inside those areas are defined as colocalization coefficients. However, the setting thresholds based on visual assessment may be misleading because of inconsistencies in selection (Costes et al. 2004). The AxioVision software utilizes an automated procedure based on spatial statistics, thus eliminating potential user error. Other methods of colocalization have recently been developed for images with a high-density of particles (Comeau et al. 2006). However, colocalization algorithms

**Table 1** The plasma concentration of murine and human Aβ in control and transgenic APP/PS1 mice is shown

| | Plasma murine Aβ (pg/ml) | | Plasma human Aβ (pg/ml) | |
|---|---|---|---|---|
| | $A\beta_{1-40}$ | $A\beta_{1-42}$ | $A\beta_{1-40}$ | $A\beta_{1-42}$ |
| Wild type | 97 ± 36.2 | 26 ± 18.9 | N/A | N/A |
| APP/PS1 | 118 ± 29.0 | 32 ± 23.0 | 725 ± 291.2 | 372 ± 70.2 |

Transgenic mice had substantially elevated plasma concentration of the beta-amyloid isoforms 1–40 and 1–42 compared to wild-type control mice

Data represents mean ± standard deviation of $n = 12$ animals per group

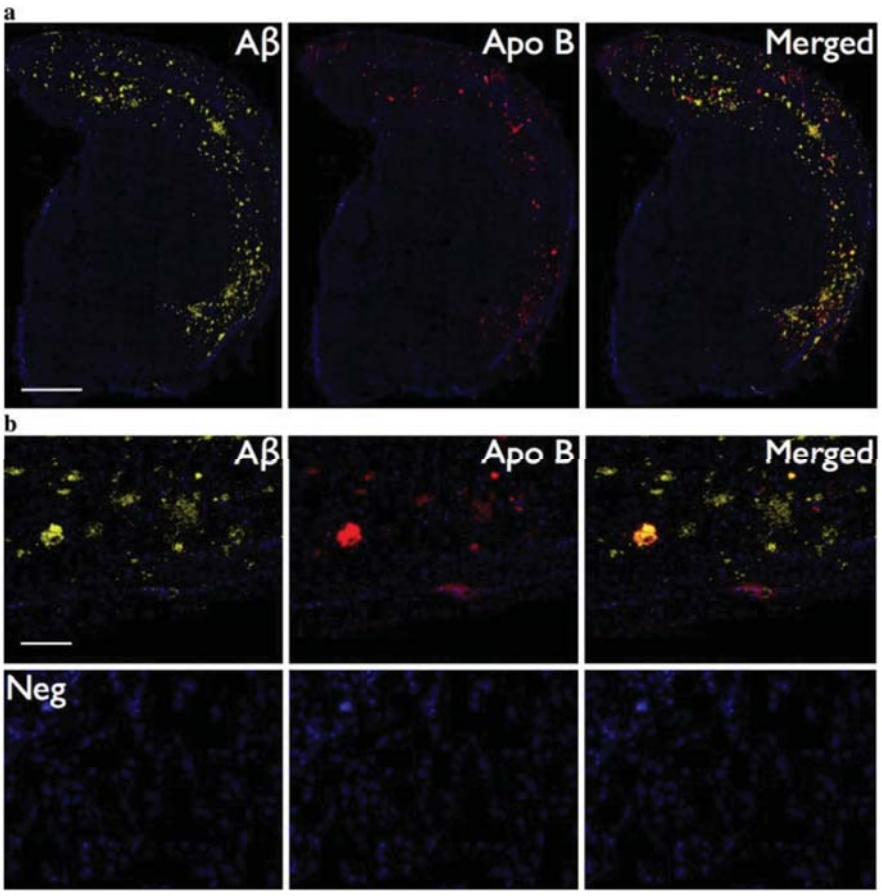


**Fig. 1** Broad distribution of apolipoprotein B and Aβ in APP/PS1 amyloid transgenic mouse brains were shown with immunofluorescent technique. **a** Aβ was detected with monoclonal anti-Aβ antibody (6E10) and Alexa 680 conjugated with anti-mouse $IgG_1$ (*yellow*). Nuclei were counterstained with DAPI (*blue*). Apolipoprotein B was detected with polyclonal anti-apolipoprotein B antibody and Alexa 488 conjugated with anti-rabbit IgG (*red*). *Scale bar* indicates 2 mm. **b** Co-localizations are shown at higher magnifications (×100) as well as their negative controls at the *bottom frames*. *Scale bar* indicates 100 μm

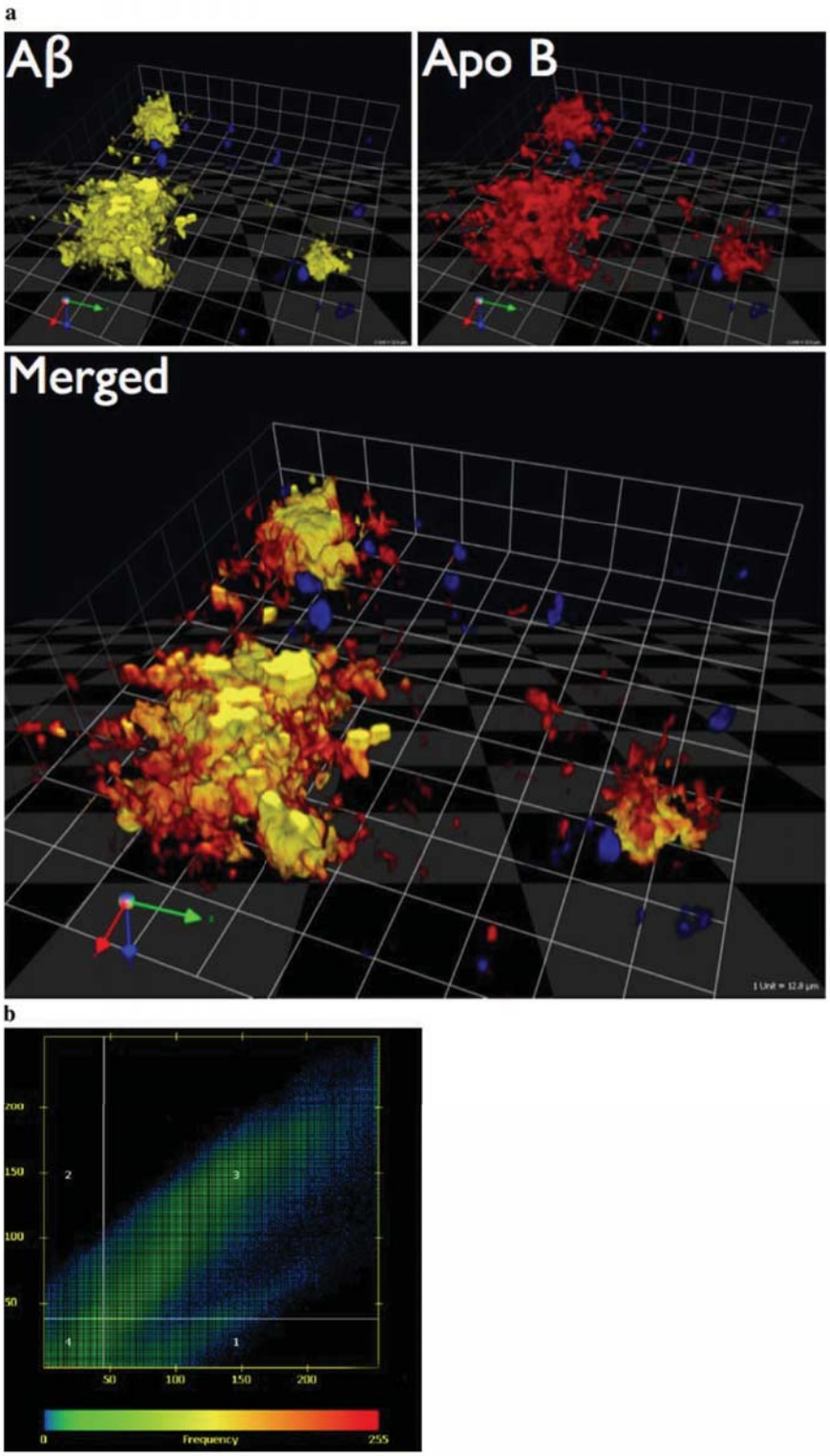


**Fig. 2** Colocalization of A$\beta$ and apolipoprotein B were detected with double immunofluorescent labeling in 3-D. **a** The two *top frames* show the separate images of A$\beta$ and apolipoprotein B for the same tissue specimen in three-dimensions. Co-localization correlation coefficient was $0.49 \pm 0.037$ on Pearson-based analysis and was $0.85 \pm 0.004$ on Manders-based analysis. Nuclei were counterstained with DAPI (*blue*). Scales of *X* (*green*), *Y* (*red*) and *Z* (*blue*) axis are 140, 120 and 20 µm respectively. **b** An example of scatter plot of colocalization analysis is shown. *Box* 1 and 2 are the each channel of A$\beta$ or apolipoprotein B, *box 3* is the colocalization area, and *box 4* is the background. The appropriate threshold was determined using automatic function of the software with minor manual modification

have proven to be effective and adequate in a qualitative context (Bolte and Cordelieres 2006). In this study, Pearson's and the colocalization coefficients are presented.

## Results

Twelve-month-old APP/PS1 amyloid transgenic mice had substantial plaque formation primarily distributed in the cortex area (Fig. 1), which occurred concomitant with a significant increase in the plasma concentration of human A$\beta$ (Table 1). In contrast, wild-type mice showed no formation of amyloid plaques. To explore if A$\beta$ in parenchymal amyloid deposits could be derived from circulating TRL, in this study we investigated the cerebral distribution of apo B, a marker of triglyceride and cholesterol-rich lipoproteins synthesised exclusively by liver and intestine, relative to amyloid plaques. Analogous to the distribution of amyloid plaques, we show that apo B was also primarily distributed in the cortex (Fig. 1), however there was no immunoreactivity in wild-type mice. Figure 2 shows the colocalization of apo B with dense neuritic amyloid plaque and more broadly surrounding focal sites of A$\beta$ accumulation in three-dimensions. To estimate the colocalization of apo B and A$\beta$ independent of abundance of the two proteins, Manders analysis was determined for 824 images and found to be highly significant (overlap coefficient = $0.85 \pm 0.004$). Moreover, we report a positive association between A$\beta$ and apo B abundance (Pearson's Correlation coefficient = $0.49 \pm 0.037$). The colocalization coefficients for A$\beta$ and apo B were $0.79 \pm 0.067$ and $0.64 \pm 0.099$, respectively.

## Discussion

Chronically exaggerated plasma A$\beta$ has been suggested to compromise BBB integrity, resulting in enhanced blood-to-brain delivery of A$\beta$ and thereafter, accelerated amyloidosis. In this study, we used a 3D immuno-microscopy approach to unequivocally demonstrate the colocation of plasma lipoproteins enriched in A$\beta$ with cerebral amyloid plaque.

Significant peripheral A$\beta$ metabolism occurs in association with the dietary-derived lipoproteins produced by the small intestine (chylomicrons) and the liver [very-low-density-lipoproteins (VLDL)]. Recently, we reported the distributional analysis of endogenous plasma lipoprotein-A$\beta$ in normal subjects and those with AD or mild-cognitive impairment (MCI) (Mamo et al. 2008). We found in both control and in AD/MCI subjects, that approximately 60% of lipoprotein-A$\beta$ was associated a TRL fraction that included chylomicrons and hepatically derived VLDL, with lesser amounts for low-density- and high-density-lipoproteins (25 and 15% respectively). The TRL-A$\beta$ concentration was greater in AD/MCI subjects and there was evidence of post-prandial dyslipidemia. The concentration of plasma chylomicrons was $17.4 \pm 5.0$ µg/ml in the post-absorptive state (expressed as apolipoprotein B48) and $5.4 \pm 1.1$ µg/ml for AD versus control subjects respectively.

Evidence that TRL-A$\beta$ contributes to cerebrovascular abnormalities and accelerated amyloidosis comes from a recent study in transgenic mice that over-express the amyloid precursor protein. Burgess et al. (2006) reported that in three alternate strains of transgenic amyloid mice, plasma A$\beta$ correlated with secretion rates into blood of TRLs and was increased three- to eightfold above wild-type controls. Plasma A$\beta$ was positively associated with the onset of cerebrovascular and parenchymal amyloidosis (Burgess et al. 2006) and direct evidence of BBB breakdown was the finding of significant cerebral immunoglobulin G extravasation and a substantial reduction of occludin expression (an endothelial tight junction protein) (Takechi et al. 2008a). Immunoglobulin G, is an abundant plasma protein not normally present in cerebrospinal fluid.

In this study, we now show that the significant colocalization of apo B with amyloid plaque in APP/PS1 amyloid mice consistent with the hypothesis of enhanced blood-to-brain delivery of apo B lipoprotein-A$\beta$ and its accumulation in the amyloid plaques. This study provides insight of putative mechanisms by which the circulating lipoprotein-A$\beta$ might influence AD risk. Further evidence that cerebral amyloidosis is modulated by plasma lipoprotein-A$\beta$ kinetics may offer novel intervention strategies to slow AD disease progression.

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# CHAPTER 4

# Chapter 4

# Differential Effects of Fatty Acids on Blood-Brain Barrier Integrity

**The content of this chapter is provided as the manuscript titled:**

**Takechi R**, Galloway S, Pallebage-Gamarallage MM, Wellington CL, Johnsen RD, Dhaliwal SS, Mamo JC. (2009) Differential effects of dietary fatty acids on the cerebral distribution of plasma derived apo B lipoproteins with amyloid-β. *Br J Nutr*. (in press)

[Impact factor: 2.8]

**Thesis objectives addressed in this chapter:**

**Objective 4:** To evaluate the differential effects of dietary fatty acids on BBB integrity in wild-type mice and to compare this with an established murine model AD (amyloid transgenic mice)

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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**

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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).



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*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.

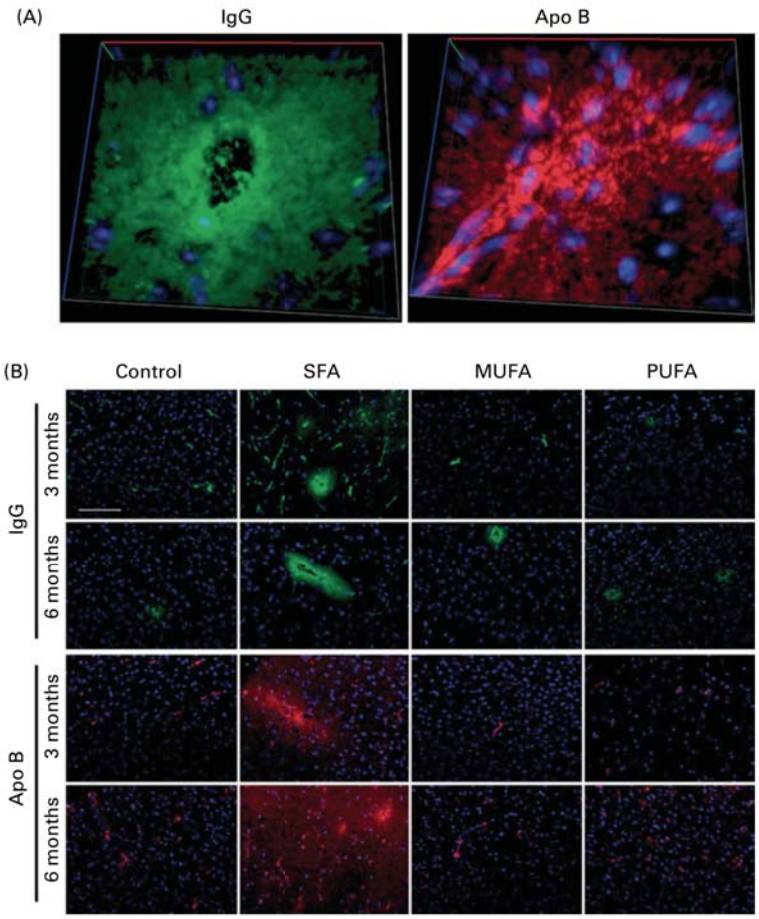


**Fig. 1.** Three-dimensional (3-D) immunodetection of cerebral IgG and apo B extravasation. Perivascular leakage of plasma IgG and apo B into the brain was captured in 3-D utilising highly sensitive immunofluorescent microscopy techniques. IgG is shown in green and apo B is in red. 6-Diamidino-2-phenylindole (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.



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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).

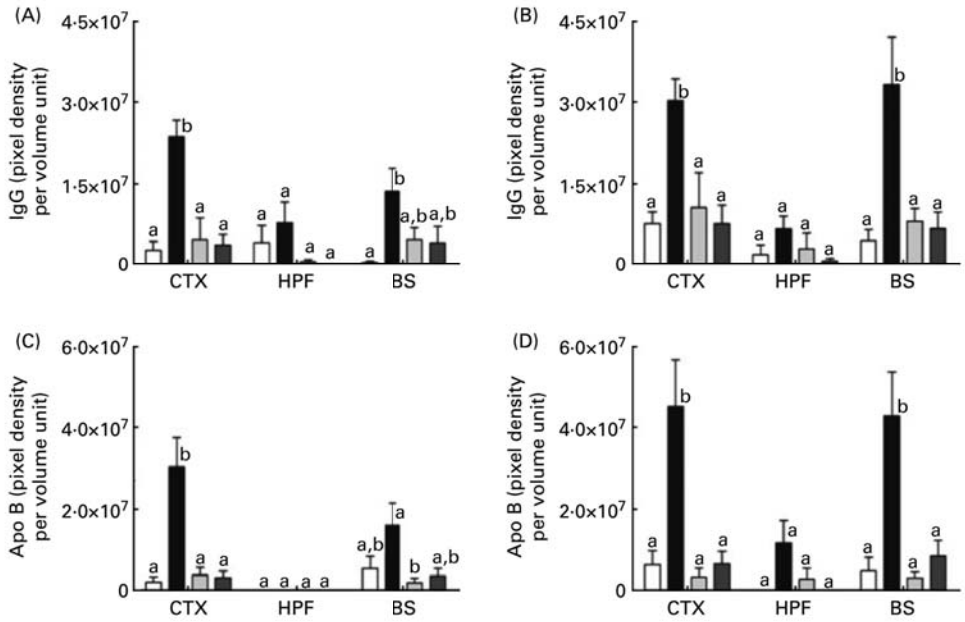


**Fig. 2.** Three-dimensional (3-D) semi-quantitative analysis of cerebral IgG (A and B) and apo B (C and D) extravasation in mice fed a control diet (□), a SFA-rich diet (■), a MUFA-rich diet (▨) or a PUFA-rich diet (■) for 3 months (A and C) or 6 months (B and D). The extent of cerebral IgG and apo 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).

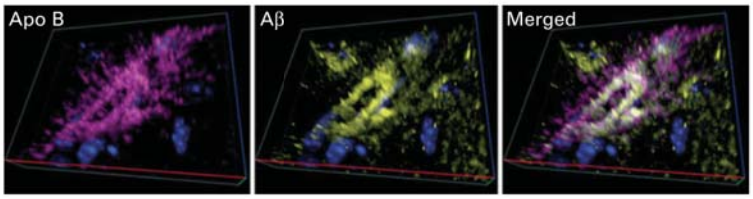


**Fig. 3.** Co-localisation of amyloid-β (Aβ) with perivascualr apo B influx in SFA-fed mice. Significant immunoreactivity of Aβ was detected concomitant with perivascular leakage of apo B lipoproteins in SFA-fed mice, consistent with blood-to-brain delivery of lipoprotein–Aβ. The immunofluorescent images were captured in three dimensions ($x, y, z = 70 \times 60 \times 11$ μm), 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

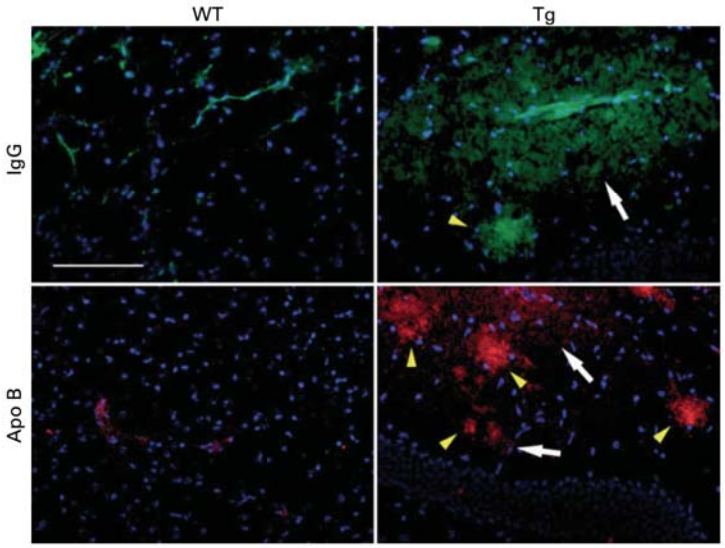


**Fig. 4.** Three-dimensional (3-D) quantitative immunomicroscopy of cerebral IgG and apo B extravasation in amyloid precursor protein/presenilin-1 (APP/PS1) amyloid transgenic (Tg) mice. The cerebral leakage of plasma IgG and apo B were quantitatively measured by 3-D immunofluorescent detection in the 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.



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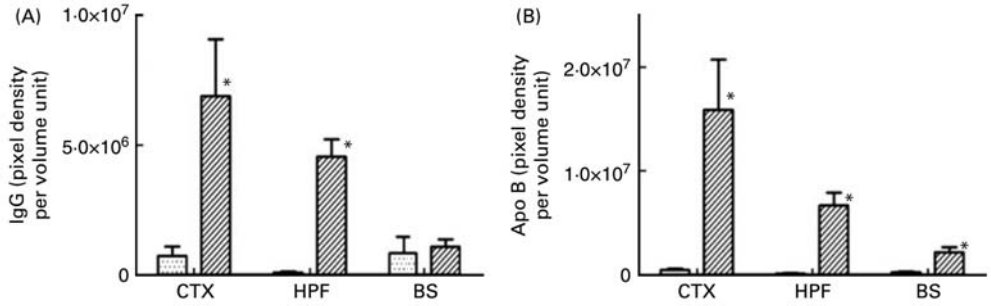


**Fig. 5.** Three-dimensional semi-quantitative analysis of cerebral IgG (A) and apo B (B) extravasation in amyloid precursor protein/presenilin-1 (APP/PS1) amyloid transgenic (Tg; ▨) mice. Optical pixel densities were measured in three major brain regions of the cortex (CTX), hippocampal formation (HPF) 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

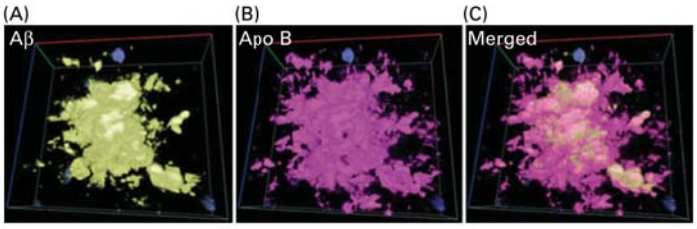


**Fig. 6.** Co-localisation of apo B with cerebral amyloid plaques. An immunofluorescent double-labelling method was utilised to explore the co-localisation of apo B with amyloid plaques in amyloid precursor protein/presenilin-1 (APP/PS1) transgenic Alzheimer's disease model mice. The distribution of 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.



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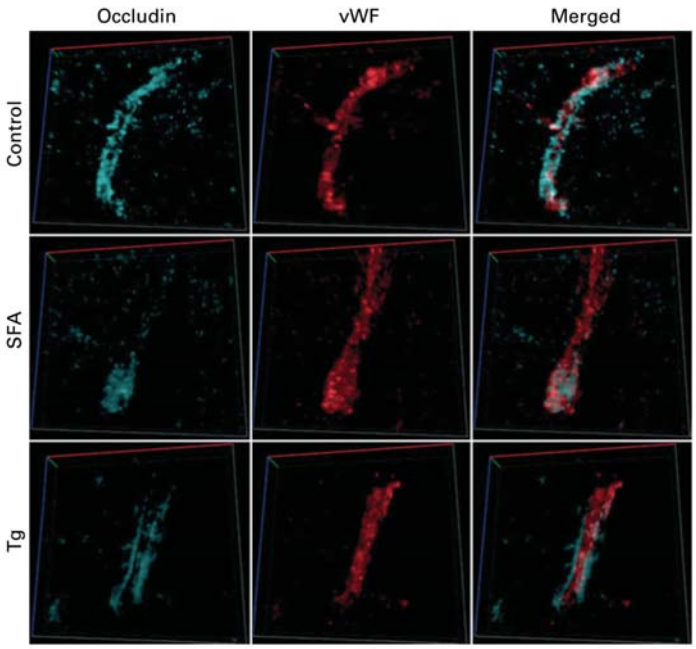


**Fig. 7.** Blood–brain barrier (BBB) three-dimensional (3-D) detection of the tight junction protein occludin. The expression of occludin-1 relative to epithelial cell abundance (expressed as von-Willebrand factor (vWF)) was quantitatively determined using double-labelling immunofluorescent microscopy. Representative 3-D images of occludin and vWF images are shown ($x$, $y$, $z = 80 \times 80 \times 7$ μm). 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.

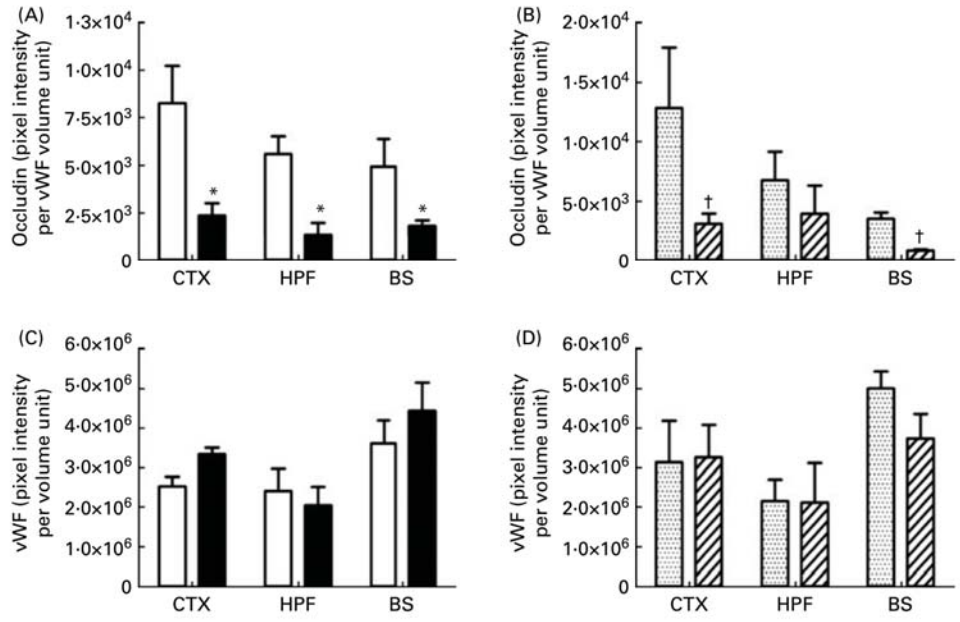


**Fig. 8.** Three-dimensional semi-quantitative analysis of cerebrovascular occludin (A and B) and von-Willebrand factor (vWF) (C and D) in control (□), SFA-fed (■), wild-type (▨) and amyloid precursor protein/presenilin-1 (APP/PS1) amyloid trangenic (▩) mice. Optical pixel intensities were measured 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 ($P<0·05$; $t$ test). † Mean value was significantly different from that of the wild-type mice ($P<0·05$; $t$ test).

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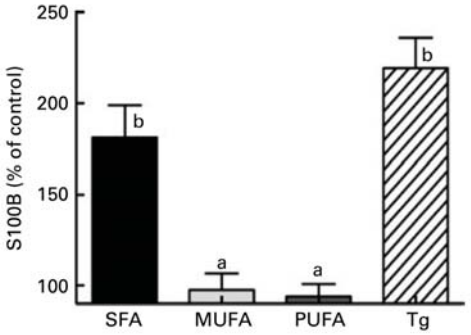


**Fig. 9.** Plasma level of S100B. Plasma S100B was determined by ELISA as a surrogate marker of brain-to-blood leakage. Mice fed SFA for 3 months had a significantly higher S100B level compared with the control, MUFA- and PUFA-fed mice. Similarly, amyloid precursor protein/presenilin-1 (APP/PS1) amyloid 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

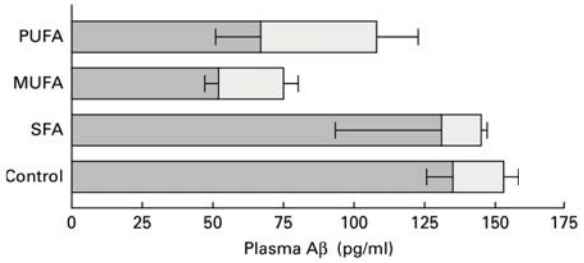


**Fig. 10.** Plasma amyloid-β (Aβ) concentration in control and high fatty acid-fed mice. Plasma levels of $A\beta_{1-40}$ (▨) and $A\beta_{1-42}$ (□) were measured with ELISA. Values are means, with standard errors represented by horizontal bars. There was no significant difference between the control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

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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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# CHAPTER 5

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# Discussion and Conclusion

**The content of this chapter is provided as the manuscript titled:**

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**Thesis objectives addressed in this chapter:**

**Objective 5:** To provide informative consideration of whether disturbances in cerebrovascular integrity in part explain the positive association between dietary SFA/cholesterol ingestion and AD risk

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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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**Contents**



*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 amyloidosis [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

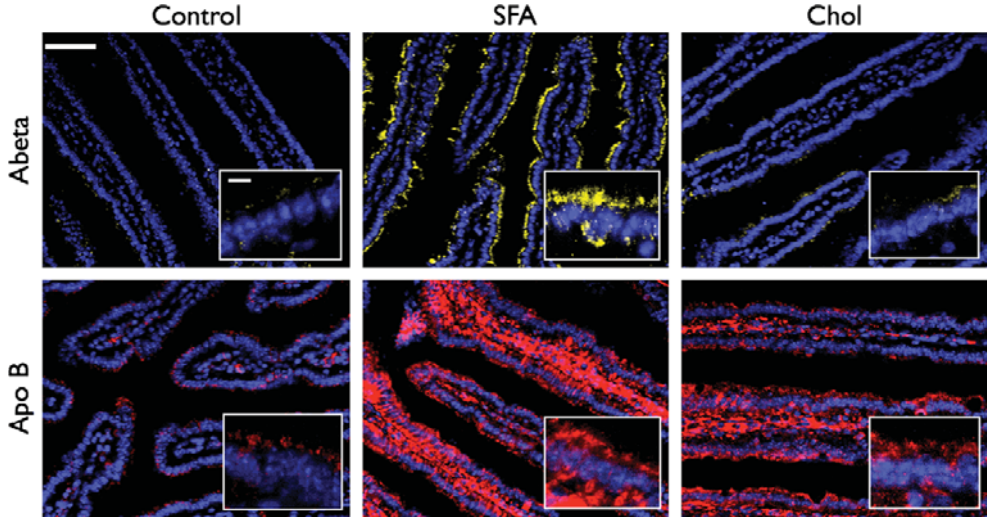


**Fig. 1.** Small intestine enterocytic abundance of amyloid-β and apolipoprotein B in saturated fat or cholesterol supplemented mice. The enterocytic perinuclear production and secretion into the lacteal of amyoid-β (Aβ) (top row) and apo B (bottom row) are shown in mice fed either a low-fat control, 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 $Aβ_{1-40}$ and $Aβ_{1-42}$ 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 $Aβ_{1-42}$ 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 lipi-

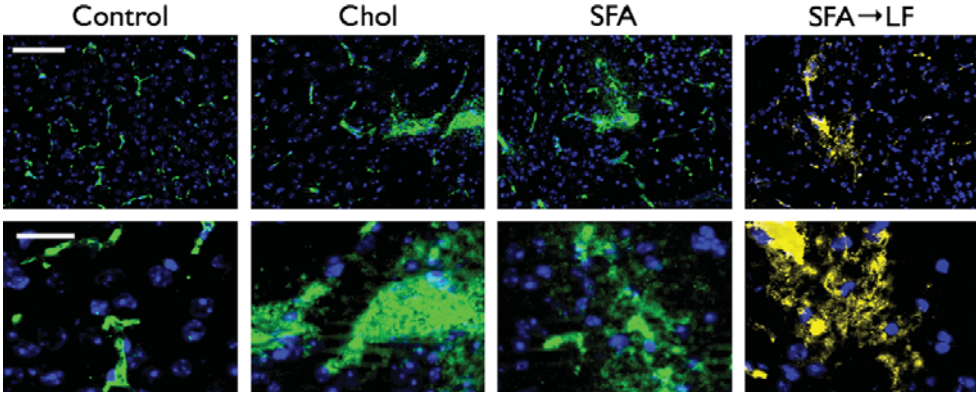


**Fig. 2.** Integrity of blood–brain barrier shown by the cerebral distribution of plasma immunoglobulin G. The cerebral distribution of immunoglobulin G (IgG) is shown in mice fed either a low-fat control, saturated fat enriched (SFA) or cholesterol (Chol) supplemented diet for 60 days. Significant 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.

dated 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-

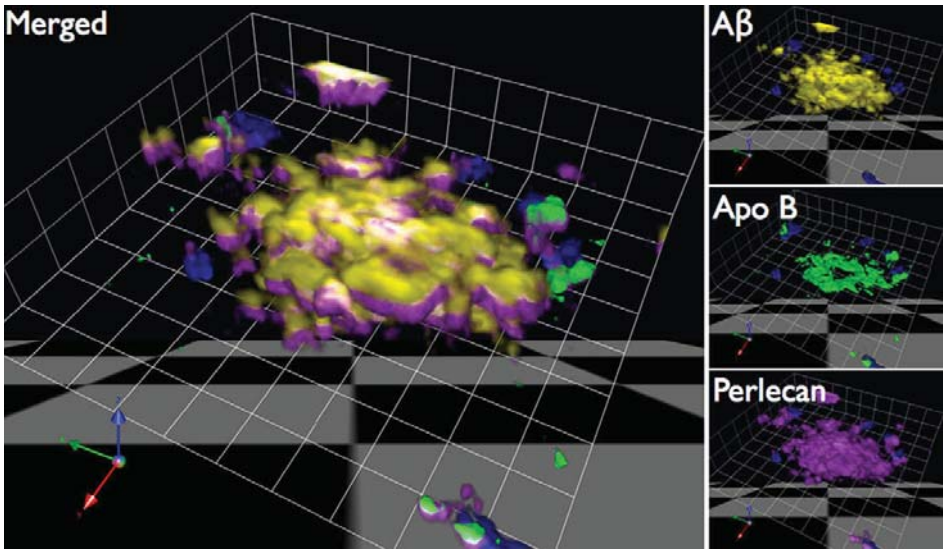


**Fig. 3.** The colocalization of apolipoprotein B, perlecan with cerebral amyloid plaques in amyloid transgenic mice. A 3-D triple immunofluorolabelling technique was utilized to investigate the colocalization of apolipoprotein B (apo B) and the proteoglycans perlecan, biglycan, agrin and decorin in 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 apo$B_{100}$ 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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# GENERAL DISCUSSION
# AND
# FUTURE STUDIES

# General Discussion and Future Studies

## General Discussion and Conclusion

An emerging body of evidence is consistent with the hypothesis that dietary fats influence AD risk (Engelhart *et al.* 2002; Morris *et al.* 2003; Solfrizzi *et al.* 2009), 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 Aβ (Duyckaerts *et al.* 2009; Joachim *et al.* 1988a; Selkoe 2002). Alternatively, amyloidosis could notionally be a secondary phenomenon to inflammation, because some studies suggest that cerebrovascular disturbances precede amyloid plaque formation (Cullen 1997; Ellis *et al.* 1996; Itagaki *et al.* 1989). Hence, dietary fats may influence AD risk by either modulating Aβ metabolism, or via Aβ independent pathways. Studies included in this thesis explore 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.

In Chapter 3, the study demonstrated the significant colocalization of plasma-derived apo B lipoprotein and cerebral amyloid plaques in an established AD model animal. The data provides the evidence of the severe breakdown of BBB which lead to the blood-to-brain delivery of peripheral lipoprotein-Aβ and its contribution toward cerebral amyloidosis. Recent studies provide accumulating evidence that dietary fats significantly influence cerebrovascular integrity and as a consequence altered Aβ kinetics across the BBB (Ghribi *et al.* 2006; Takechi *et al.* 2009). In Chapter 4 and 5, it has been shown that chronic ingestion of saturated fats or cholesterol appears to result 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 to 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.

## Limitations of study

Even though the wild-type and transgenic animal models utilized are established and widely used, clinical studies confirming the key findings are warranted. Mice are herbivorous, hence tolerance and susceptibility to diets enriched with saturated fats or cholesterol may differ from humans.

There are metabolic differences between mammalian species and indeed even between strains of mice. Subtle differences in protein expression may be responsible for substantial differences aetiology, disease progression or pathology. Hence the findings in mice presented in this thesis must be interpreted with caution when considering their relevance to human. Studies in other mammalian species would also be helpful in considering generalisability of the findings.

Immunological based methods were used to quantitate key proteins such as Aβ and to determine their distribution in tissues and indeed within cells. One methodological limitation relevant to this study was the potential confounding effects of lipids that may bind tightly to proteins of interest. Lipids may interfere with binding of antibodies with protein epitopes consequently underestimating protein abundance or

distribution (James and Mamo 2005; Yanagisawa *et al.* 1997). In published article 4, there was no difference in the plasma concentration of Aβ between mice fed low-fat or saturated-fatty acids. The findings suggest that dietary saturated-fat induced disturbances in BBB integrity occur independently of change in plasma Aβ concentration However, as discussed in chapter 5, significant changes in plasma lipoprotein Aβ as a consequence of SFA feeding may not have been detected because of the confounding effects of lipids. In some instances, lipids can be separated from the protein of interest, however this often requires complex procedures (Mamo *et al.* 2008).

## Future studies

This thesis presented a quality series of evidence to investigate the partial mechanisms of the link between dietary fat and AD risk. Moreover, the studies for the first time demonstrated the effects of diet on BBB integrity. These data may be informative for future AD research and may contribute to the development of strategy for AD prevention and treatment. The data presented in this thesis have suggested that the dietary saturated fatty acids and cholesterol may increase the AD risk or exacerbate its pathology by causing the dysfunction of BBB which may lead to the blood-to-brain leakage of plasma neurotoxic and neuroinflammatory agents including TRL-Aβ. The findings are hypothesis generating and substantially more research is required to delineate the role of dietary fats and nutrition per se in AD risk. Some key areas of interest would be to explore the possible reversibility of SFA/cholesterol induced disturbances in BBB function. Moreover, the interactive effects of diet with genetic dyslipidemia or with lipid lowering drugs deserve attention. Several abnormal mental and physical conditions including head injury, stroke and mental stress are reported as a risk factor for BBB dysfunction (Ghabriel *et al.* ; Sandoval and Witt 2008). In some instances these conditions may be transient which could be informative in comparison to chronic dietary induced disturbances.

Perhaps of most interest is an accumulating body of evidence which shows that biological agents with anti-oxidative and anti-inflammatory properties maintain and in

some instances restore vascular function (Allen and Bayraktutan 2009; Kuhlmann *et al.* 2008). Investigating the role of such agents on cerebrovascular integrity may be pivotal for prevention and treatment of AD and other vascular forms of dementia.

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# APPENDICES

# Appendix A

## Author Contribution and Copyright Authorization

This thesis presents, in fulfillment of my PhD candidacy requirements, 5 first author peer-reviewed papers published in quality international scientific journals. In this appendix, the contributions of co-authors for each paper are clearly stated and signed. All authors have read and approved the final version of manuscripts before their submission and publication.

Copyright authorization to include the manuscripts within this thesis was sought where necessary and the letters of authority are provided.

## Paper 1:

**Takechi R**, Galloway S, Pallebage-Gamarallage MM, Wellington C, Mamo JC. (2008) Chylomicron β-amyloid in the aetiology of Alzheimer's disease. *Atheroscler Suppl*. 9, 19-25

Ryusuke Takechi undertook the literature review, the primary writing of the manuscript, data collation and data analysis, presentation format and preparation of the figures. Susan Galloway and Menuka M. Pallebage-Gamarallage contributed to the preparation of figures, data presentation and appraisal of the manuscript. Cheryl Wellington provided the sample tissue for the data collection and appraisal of the manuscript. John C. Mamo contributed to consideration of the data and appraisal of the manuscript.

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## Paper 2:

**Takechi R**, Galloway S, Pallebage-Gamarallage MM, Johnsen RD, Mamo JC. (2008) Three-dimensional immunofluorescent double-labelling using polyclonal antibodies derived from the same species: Enterocytic colocalization of chylomicrons with Golgi apparatus. *Histochem Cell Biol*. 129, 779-84

Ryusuke Takechi was responsible for study design, method development, sample collection, the experimental procedures, data collection, data interpretation and preparation of manuscript. Susan Galloway and Menuka M. Pallebage-Gamarallage were involved in the tissue sample collection procedures and appraisal of the drafted manuscript. Russell D. Johnsen contributed to the consideration of method development. John C. Mamo discussed study design of and interpretation of data.

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**Takechi R**, Galloway S, Pallebage-Gamarallage MM, Wellington C, Johnsen R, Mamo JC. (2009) Three-dimensional colocalization analysis of plasma-derived apolipoprotein B with amyloid plaques in APP/PS1 transgenic mice. *Histochem Cell Biol*. 131, 661-6

Ryusuke Takechi was responsible for the study design of study, method development, experimental procedures and preparation of manuscript. Susan Galloway and Menuka M. Pallebage-Gamarallage contributed to discussion of the experimental approach and appraisal of the manuscript. Cheryl Wellington provided the tissue samples of transgenic mice and contributed to discussion of the manuscript discussion. Russell Johnsen contributed to the method development. John C. Mamo assisted with the study design and the appraisal of the manuscript.

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**Paper 4:**

**Takechi R**, Galloway S, Pallebage-Gamarallage MM, Wellington CL, Johnsen RD, Dhaliwal SS, Mamo JC. (2009) Differential effects of dietary fatty acids on the cerebral distribution of plasma derived apo B lipoproteins with amyloid-β. *Br J Nutr*. (in press)

Ryusuke Takechi was responsible for method development, generation and interpretation of the data, and the preparation of the manuscript. Susan Galloway contributed to study design, the animal care, sample collection and appraisal of the manuscript. Menuka M. Pallebage-Gamarallage contributed to the animal care, sample collection and appraisal of manuscript. Cheryl L. Wellington provided the tissue samples of transgenic mice and contributed to the manuscript appraisal. Russell D. Johnsen advised on method development and Satvinder S Dhaliwal provided statistical advice. John C. Mamo contributed to study design and consideration of the data.

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## Paper 5:

**Takechi R**, Galloway S, Pallebage-Gamarallage MMS, Lam V, Mamo JCL. (2009) Dietary fats, cerebrovascular integrity and dementia risk. *Prog Lipid Res*. (in press)

Ryusuke Takechi established the review structure and contributed substantially to the interpretation of the literature. Susan Galloway, Menuka M. Pallebage-Gamarallage and Virginie Lam provided images and appraisal of the manuscript. John C. Mamo contributed to the discussions on the published literature.

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# Appendix B

## Additional Articles

This thesis contains 2 additional articles that do not form the main body of the thesis but provide significant information to support and enhance the interpretation and discussion of the results presented. These include published peer-reviewed papers as well as articles submitted and close to its publication.

## Article 1:

Galloway S, Pallebage-Gamarallage MM, **Takechi R**, Jian L, Johnsen RD, Dhaliwal SS, Mamo JC. (2008) Synergistic effects of high fat feeding and apolipoprotein E deletion on enterocytic amyloid β abundance. *Lipids Health Dis*. 7, 15 [Impact factor: 2.1]

This study investigated the difference in enterocytic amyloid-β abundance in apo E knockout (KO) mice and WT control when the mice are fed either LF control or HF diet containing 16% SFA and 1% cholesterol. Ryusuke Takechi contributed to the data collection and analysis of the results.

Isoforms of apo E are associated with AD risk. Many mechanisms have been put forward to explain this association including modulation of Aβ synthesis and secretion, Aβ kinetics and cerebrovascular integrity. However, apo E is also critically involved in the metabolism and homeostasis of TRL including their biogenesis, clearance and binding to extracellular matrices. In this study, the putative role of apo E on enterocytic Aβ abundance was investigated. The intestinal expression of Aβ was determined in wild type mice and apo E KO mice under LF or HF feeding regimens.

The study reports that enterocytic Aβ was substantially increased by SFA feeding in WT and in apo E KO mice. However, the SFA induced effect was greater in the apo E KO mice, possibly because of a greater enterocytic absorptive capacity. The villi length of the upper small intestine was greater in apo E KO mice. These data suggest that the greater availability of dietary fat may stimulate the enterocytic Aβ biogenesis, and apo E may be synergistically involved in this phenomenon.

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# Synergistic effects of high fat feeding and apolipoprotein E deletion on enterocytic amyloid-beta abundance

Susan Galloway[1], Menuka MS Pallebage-Gamarallage[1], Ryusuke Takechi[1], Le Jian[1], Russell D Johnsen[2,3], Satvinder S Dhaliwal[1] and John CL Mamo*[1]

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## Abstract

**Background:** Amyloid-β (Aβ), a key protein found in amyloid plaques of subjects with Alzheimer's disease is expressed in the absorptive epithelial cells of the small intestine. Ingestion of saturated fat significantly enhances enterocytic Aβ abundance whereas fasting abolishes expression. Apolipoprotein (apo) E has been shown to directly modulate Aβ biogenesis in liver and neuronal cells but it's effect in enterocytes is not known. In addition, apo E modulates villi length, which may indirectly modulate Aβ as a consequence of differences in lipid absorption. This study compared Aβ abundance and villi length in wild-type (WT) and apo E knockout (KO) mice maintained on either a low-fat or high-fat diet. Wild-type C57BL/6J and apo E KO mice were randomised for six-months to a diet containing either 4% (w/w) unsaturated fats, or chow comprising 16% saturated fats and 1% cholesterol. Quantitative immunohistochemistry was used to assess Aβ abundance in small intestinal enterocytes. Apo E KO mice given the low-fat diet had similar enterocytic Aβ abundance compared to WT controls.

**Results:** The saturated fat diet substantially increased enterocytic Aβ in WT and in apo E KO mice, however the effect was greater in the latter. Villi height was significantly greater in apo E KO mice than for WT controls when given the low-fat diet. However, WT mice had comparable villi length to apo E KO when fed the saturated fat and cholesterol enriched diet. There was no effect of the high-fat diet on villi length in apo E KO mice.

**Conclusion:** The findings of this study are consistent with the notion that lipid substrate availability modulates enterocytic Aβ. Apo E may influence enterocytic lipid availability by modulating absorptive capacity.

## Background

Net concentration of cerebral Aβ is determined by the presence of apolipoprotein (apo) E with a dose dependent gene effect of apo E -/- < apo E -/+ < apo E +/+ on hippocampal senile plaques [1,2]. Animals and cell culture studies show that apo E regulates the production, transport,

clearance and solubility of Aβ [1-8]. Apolipoprotein E may modulate cerebral Aβ homeostasis by regulating cerebral Aβ efflux via the low-density-lipoprotein-receptor-related protein (LRP), relative to the influx of Aβ via transporters such as the receptor for advanced-glycation-end-products (RAGE) [9]. In addition, apo E can also directly influence Aβ biogenesis via regulation of α- and β-secretases activity [10], or indirectly, by influencing the intracellular pool of regulating lipids [11].

Apolipoprotein E critically regulates cholesterol metabolism and lipid homeostasis. The apo E protein is the primary receptor ligand for dietary-derived lipoproteins synthesized by the small intestine (chylomicrons) and triglyceride-rich lipoproteins (very-low-density lipoproteins (VLDL)), synthesized from liver [12]. Several lines of evidence support a link between aberrations in lipid metabolism and AD risk [7,11,13]. Epidemiological and clinical studies suggest that a high intake of saturated fat and/or cholesterol accelerate onset and progression of AD, whereas some polyunsaturated fatty acids may be protective [13-17]. Moreover, strong evidence of a causal relationship between dietary fats and AD comes from feeding studies in mice or rabbits. Animals given saturated-fat diets show significant immuno-detectable cerebral Aβ burden [18-20], although the mechanisms by which this occurs are presently unclear.

Our laboratory recently reported that absorptive epithelial cells of the small intestine secrete Aβ associated with dietary-derived lipoproteins (chylomicrons) [21]. A diet enriched in saturated fats and cholesterol was found to markedly increase enterocytic Aβ, whereas fasting completely abolished Aβ production. Chronic ingestion of saturated-fat may lead to sustained elevations in blood of lipoprotein-bound Aβ, because of overproduction and thereafter, reduced clearance from blood. Moreover, recent studies suggest that exaggerated exposure to circulating Aβ may compromise blood-brain-barrier integrity and exacerbate cerebral amyloidosis [22]. In normal subjects, approximately 60% of lipoprotein-bound plasma Aβ is associated with the triglyceride-rich-lipoproteins (TRL's) and in subjects with AD, post-absorptive accumulation of chylomicrons has been identified [23].

Apolipoprotein E is pivotal for the interaction of TRL with high affinity clearance pathways [12] including the low-density-lipoprotein-receptor (LDL-r) and LRP and will therefore significantly influence plasma lipoprotein-Aβ concentration and kinetics. However, apo E may also influence plasma Aβ homeostasis by modulating synthesis and secretion of the lipoprotein-Aβ complex from either the intestine and/or liver. To explore this concept further, in this study we compared enterocytic Aβ homeostasis in wild-type mice versus animals devoid of apo E (apo E knockouts). Mice were given either a low-fat, or high saturated-fat diet to explore synergistic effects. We find that apo E modulates intestinal morphology in a manner which may influence lipid absorptive capacity and has a synergistic effect with dietary fats on enterocytic Aβ homeostasis.

## Results

### *High-fat feeding induced hypercholesterolemia in apo E KO mice*

Apo E KO mice given low-fat chow had significantly elevated plasma cholesterol compared to WT mice on the low-fat diet (table 1), however plasma triglycerides were not significantly affected because of the gene deletion. In WT mice the high saturated fat diet had no significant affect on plasma cholesterol or triglycerides (table 1). However, in apo E KO mice hypercholesterolemia was substantially exacerbated and some two-fold greater than the apo E KO mice given low-fat chow. All groups of mice gained weight during the intervention and there was no significant difference between treatment groups (data not shown).

### *Immunolocalisation of Aβ in the small intestine of apolipoprotein E KO mice: synergistic effects of high fat feeding*

For all groups of mice, Aβ immunostaining was demonstrated within the perinuclear region of absorptive columnar epithelial cells of the small intestine mucosa (insert, figure 1). With low-fat feeding, WT and apo E KO mice

**Table 1: Plasma lipids in wild-type and apolipoprotein E knockout mice fed low and high fat diets**

| Diet | Gene | Cholesterol (mM) mean ± S.E.M | Triglyceride (mM) mean ± S.E.M |
|---|---|---|---|
| LF | WT | 2.1 ± 0.05 | 0.69 ± 0.19 |
| LF | Apo E KO | *6.95 ± 1.97 | 0.68 ± 0.09 |
| HF | WT | 2.2 ± 0.46 | 0.42 ± 0.12 |
| HF | Apo E KO | *14.3 ± 0.01 | 0.38 ± 0.12 |

*$P < 0.05$
S.E.M = standard error of the mean
Table shows plasma cholesterol and triglyceride concentrations (mean ± SEM, n = 6 mice per group) in C57BL/6J WT mice and apo E KO mice maintained on either LF or HF diet for six-months. Apo E KO mice had significantly elevated levels of plasma cholesterol compared to WT controls under both feeding regimens ($P < 0.05$). High-fat feeding further exacerbated the elevation of cholesterol in apo E KO mice compared to HF-WT ($P < 0.001$) and LF-APOE KO mice ($P < 0.05$). Plasma triglyceride was not significantly different between groups.

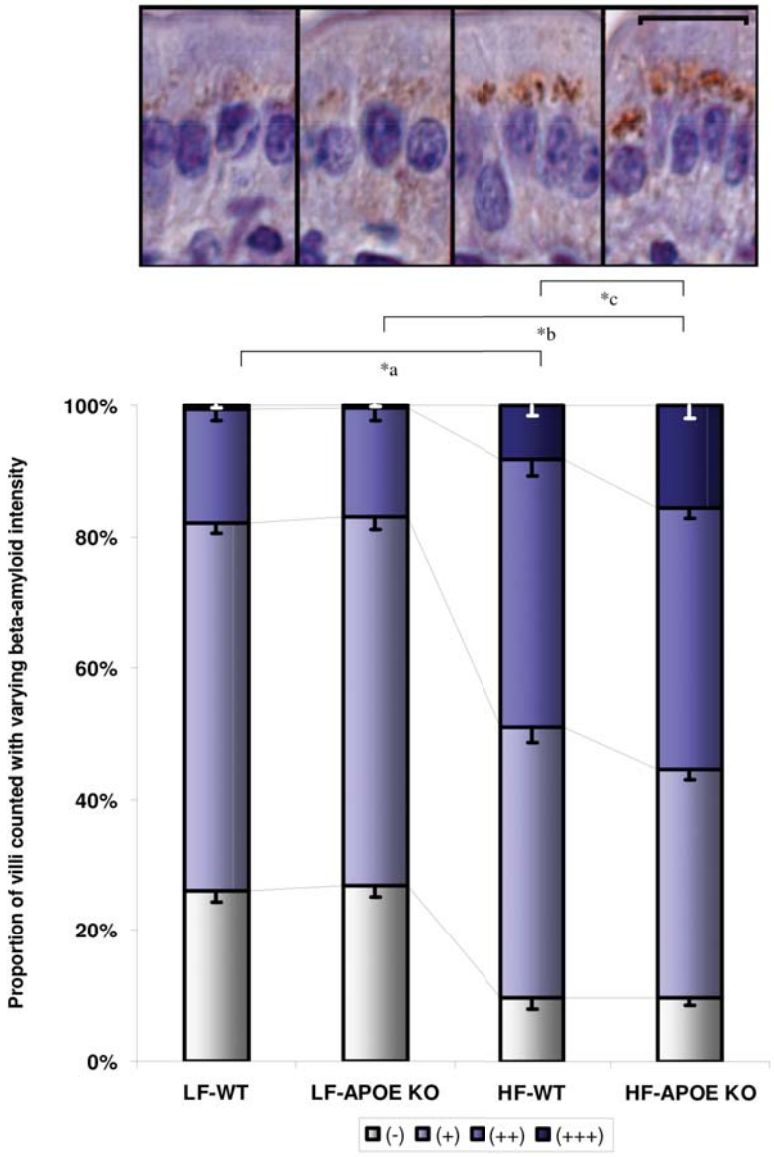


**Figure 1**

**Enterocytic Aβ in wild-type and apolipoprotein E knockout mice given a high fat diet**. Figure shows proportion of small intestinal epithelial cells with different staining intensity for Aβ. Score as follows: (-) no granular coloration, (+) modest with 1–2 granules, (2+) moderate with 3–4 granules or (3+) high, containing larger intense granules. Data was collected for six mice per group, with a minimum of four tissue sections per mouse studied. A minimum of 200 cells per section were scored and statistical significance was determined by one-way ANOVA with post-hoc Bonferroni test. LF-WT and LF-APOE KO mice have significantly ($p < 0.05$) fewer cells which stained positive for Aβ compared to mice fed high fats (HF-WT and HF-APOE KO *a and *b respectively). Under high-fat feeding, apo E KO mice had significantly greater proportion of cells which expressed Aβ at higher intensity compared to high-fat fed WT mice (*c, $p < 0.05$). The inset micrograph shows high-magnification of enterocytes from groups corresponding to graphs below. Beta-amyloid colocalized within the perinuclear regions of the cell containing Golgi and ER within enterocytes from all groups. (Scale bar = 20 μm).

exhibited positive staining of Aβ relatively evenly distributed throughout the mucosa epithelium. Apo E KO mice on low-fat diets showed a similar distribution of Aβ compared to WT controls (figure 1). The effect of high-fat feeding on enterocytic Aβ in WT and apo E KO mice is also given in figure 1. Both WT and apo E KO mice had significantly greater enterocytic Aβ abundance, however the effect was more pronounced in the apo E knockout group, notably with more enterocytes showing intense (3+) staining (double asterisks, figure 1).

***Villi height in apo E KO mice and effects of high fat feeding***

Small intestinal villi length was determined as a surrogate marker of intestinal absorptive capacity. Apo E KO mice on the low-fat diet had significantly greater mean villi length compared to WT controls (figure 2). High-fat feeding was found to substantially increase villi length in control animals and was comparable to apo E KO mice. High-fat feeding had no synergistic influence on villi length in the absence of apo E expression (figure 2).

## Discussion

This study shows that in the absence of apo E, intestinal villi length is significantly greater than WT mice. The absence of apo E coupled with chronic ingestion of a saturated fat and cholesterol diet, increased enterocytic Aβ abundance compared to WT mice on a low-fat diet. This may have simply been a dietary-fat induced effect independent of apo E, because apo E KO mice on a low-fat diet showed similar levels of enterocytic Aβ compared to WT controls. On the other hand, the absence of apo E with a high-fat diet was found to enhance Aβ abundance above that observed in WT mice given saturates and cholesterol. The latter is consistent with modulation of Aβ by apo E that is lipid-threshold dependent.

Apolipoprotein E serves as a TRL ligand for both the LDL receptor and LRP [24,25]. The liver is a major source of apo E, however other tissues including the small intestine express apo E [24,26,27]. Apo E KO mice [28-30] accumulate TRL's because they are unable to bind and be cleared by receptor processes [31]. Under low-fat feeding, apo E KO mice had a greater than three-fold increase in plasma. High-fat feeding exacerbated plasma cholesterol accumulation in apo E KO mice, presumably because of exaggerated lipoprotein production and indeed hypercholesterolemia was increased two-fold above low-fat fed apo E KO mice. Clearance of TRL's from blood is a two-step process requiring triglyceride lipolysis by lipases to produce a depleted apo E rich 'remnant' lipoprotein [24]. Thereafter, remnants are cleared by receptor pathways utilizing apo E as the ligand. There is no hydrolytic defect in apo E KO mice, which explains why these mice were not hypertriglyceridemic.

The mechanisms by which the absence of apo E increased enterocytic Aβ in high-fat fed mice are unclear, although studies in cell culture provide clues. Irizarry *et al* (2004) found that incubation of neuronal cells with apo E resulted in a reduced synthesis of Aβ by lowering the gamma secretase activity [4]. Rough endoplasmic reticulum (rER) and the Golgi compartments are where early endoplasmic cleavage of the Aβ precursor protein occurs, the latter consistent with increased enterocytic perinuclear Aβ immunostaining in apo E KO mice.

This study and others [32,33] found longer villi length in apo E KO mice, suggestive of greater absorptive capacity. Greater substrate availability might stimulate Aβ biogenesis and this hypothesis is supported by the increase in Aβ abundance in high-fat WT mice which also had a marked increase in villus length. Greater Aβ abundance would have been expected in apo E KO mice given the low-fat diet compared to WT controls, because villus length was comparatively greater in the absence of the apo E gene. However, if lipid absorption is already efficient with the low-fat feeding regimen; the deletion of apo E (and increased villus length) would not necessarily have had the expected stimulatory effect on enterocytic Aβ.

Chylomicron synthesis occurs within the ER and Golgi requiring the progressive lipidation of apolipoprotein $B_{48}$ (apo $B_{48}$) [34,35]. Dietary fats transiently stimulate chylomicron synthesis and secretion [36,37] and in clinical studies post-prandial elevations in the Aβ-precursor protein have been reported synergistic with the lipaemic response [3]. How Aβ binds and is secreted with chylomicron is unclear, although the protein is known to bind avidly with negatively charged hydrophobic lipids [5,38]. Cell culture studies also support a lipoprotein mediated secretory pathway because in hepatocyte media, Aβ is found associated with lipoprotein complexes [11].

In animal models and in cell cultures, apo E has confounding effects on hepatic secretion of VLDL. Apo E will normally suppress apo B production, but this is contradicted in the presence of lipids which strongly stimulate lipoprotein biogenesis [39]. In this study, enterocytic Aβ abundance was not significantly different in low-fat apo E KO mice compared to controls, suggesting that chylomicron synthetic rates were not different between these two groups of mice. The increased availability of dietary lipids when animals were fed the high-fat diet would promote chylomicron production and by extension, perhaps Aβ genesis. However, whilst enhanced enterocytic abundance of Aβ was seen in both WT and apo E KO mice given the high-fat diet, the effect was greater in the latter. One explanation is the finding that apo E normally suppresses triglyceride secretion from liver. Therefore, the enhanced effect on enterocytic Aβ seen in apo E KO given high-fat

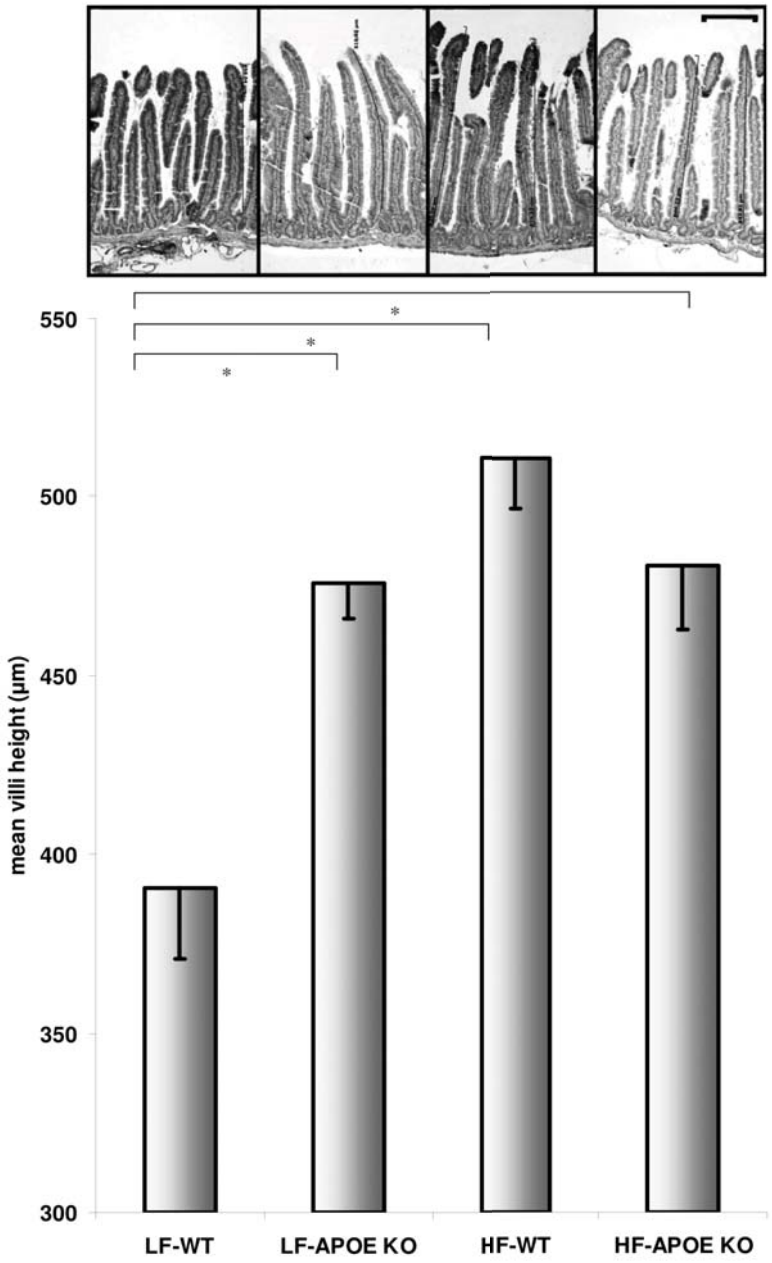


**Figure 2**
**Villi height in wild-type and apolipoprotein E knockout mice given a high fat diet**. Mean villi height (mm) in WT and apo E KO mice fed low- and high-fat chow. LF-WT group had significantly (*$p < 0.05$) shorter villi height compared to other groups. The inset micrograph shows low-magnification of intestinal villi height for each group. (Scale bar = 200 µm).

may have been indicative of amplification in the presence of greater cytosolic lipids [39,40].

## Conclusion

Many studies have demonstrated the central role of apo E in maintaining cerebral Aβ homeostasis including modulation of production, as a chaperone protein, and in maintaining efflux and influx pathways across the blood brain barrier. Furthermore, apo E profoundly influences the kinetics in blood of Aβ containing lipoproteins as well as their secretion from liver. This study now demonstrates that apo E may also regulate intestinal Aβ metabolism.

## Materials and methods

### *Animals*

The protocols described were approved by an ethics committee accredited by the National Health and Medical Research Council of Australia (Curtin University ethics approval N 55-04). Six-week-old female C57BL/6J apolipoprotein E gene knockout (apo E KO) and wild-type (WT) mice weighing approximately 16 g were obtained from the Animal ARC, Perth, Western Australia. Mice were divided and randomly allocated into a low-fat or high-fat diet group. Mice were housed separately in a well-ventilated room that was maintained at 22°C on a 12:12-h light/dark cycles. Body weight was measured weekly.

### *Dietary regimen*

Chow was purchased from Rodent Diet Specialty Feeds (Glen Forrest, Western Australia). The low-fat (control) group of mice was given chow that contained 4.0% (w/w) as unsaturated fat (AIN93M standard rodent diet) and the diet was free of cholesterol. Mice on the high-fat diet were given chow containing 1.0% (w/w) as unsaturated fat and 16.0% (w/w) as saturated fat (SF00–245 high-fat mouse diet). In addition, the high-fat feed was supplemented with 1% (w/w) cholesterol and 0.5% (w/w) cholate, the latter to aid in absorption. The digestible energy for low-fat and high-fat feed were 15.2 MJ/kg and 18.7 MJ/kg respectively. Food and water were available *ad libitum*.

### *Sample collection*

After six-months of dietary interventions, mice were anaesthetized with an intraperitoneal injection of Phenobarbital (45 mg/kg). Mice were exsanguinated by cardiac punctureand blood was collected into ethylene-diaminetetracetic acid (EDTA)-tubes. Plasma was separated by low speed centrifugation and stored at -80°C (under an atmosphere of argon).

### *Tissue processing*

A small intestine segment measuring 2 cm was cut and isolated from the rest of the digestive tract at the proximal duodenal sphincter. The contents were flushed *in-situ* with phosphate buffered saline (PBS, pH = 7.4), and placed into 10% buffered formalin (ph = 7.4) for fixation. Tissues were fixed for 24 h and processed for immunohistochemistry (IHC).

### *Immunohistochemistry*

Tissue sections (5 μm) were deparaffinised, rehydrated and IHC analysis was done as previously described [21]. Briefly, the sections were exposed to 3% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase activity, washed and incubated in blocking serum (20% goat serum) prior to overnight incubation at 4°C with polyclonal rabbit anti-human $A\beta_{1-40/42}$ antiserum (AB5076, Chemicon Temecula, CA), diluted to 1:1000 with 10% goat serum. We previously established specificity by replacing the primary antibody with an irrelevant serum or with PBS and by competition IHC analysis [21]. For the latter, the primary antisera were pre-mixed with solubilised Aβ. Cerebral tissues from transgenic mice (Tg2576sw) expressing familial human APP695 with established plaques were used as positive controls. Slides were washed in PBS and incubated with biotinylated goat anti-rabbit secondary antibody (1:1000 dilution) (E 0432, DAKO, Carpinteria, CA), followed by avidin-biotin-peroxidase complex (ABC/HRP) (K 0377, DAKO, Carpinteria, CA) for 45 min at room temperature. Positive immunostaining was established with liquid diaminobenzidine plus (DAB+) substrate chromogen kit (K 3467, DAKO, Carpinteria, CA). Sections were then counterstained with Harris's haematoxylin.

### *Imaging*

Digital images for photomicroscopy were acquired by AxioCam HRc camera (Zeiss Germany). Images were captured under identical settings utilising AxioVision software, version 4.5.

### *Quantitation of intestinal beta-amyloid abundance*

Six animals per group were investigated with a minimum of four tissue blocks prepared for each. From each slide, four images were captured randomly at low magnification (Zeiss AxioVert 200 M, Germany). The intensity of immunolabeling was quantified as previously described [41,42]. Labelling was considered adequate if it was mild (+), moderate (2+), or intense (3+), with adequately labeled positive controls and no labeling in negative controls. The total number of cells with different intensity of Aβ staining was counted by a blinded-to-group investigator in each villus and the data expressed as a percentage.

### *Measurements of intestinal villi height*

Total of 8 images was taken at low-magnification per group. Representative villi were selected by two independent investigators for height measurement (measurement tool, AxioVision program 4.5).

### *Plasma lipid measurements*

Plasma lipids were measured immediately following plasma isolation via commercial absorbance-based assays. Triglyceride was determined by measurement of glycerol liberated following enzymatic hydrolysis of triglyceride (TR 1697, Randox laboratories, U.K). Total plasma cholesterol concentration was determined via the cholesterol esterase/cholesterol oxidase technique (CH 201, Randox laboratories, U.K).

### *Statistical analysis*

The effect of high-fat feeding and apo E gene on Aβ abundance, intestinal villi height, plasma triglyceride and total cholesterol was assessed by univariate analysis. Post-hoc comparisons of means were performed using Bonferroni tests and if equal variance was not found, then Games-Howell test was used to compare difference between individual groups. P-value $< 0.05$ was considered a statistically significant.

## List of abbreviations

Aβ: beta-amyloid; AD: Alzheimer's disease; Apo: apolipoprotein; APP: amyloid precursor protein; CH: cholesterol; IHC: immunohistochemistry; KO: knockout; TG: triglycerides; TRL: triglyceride-rich-lipoprotein; WT: wild-type.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

SG participated in the design of the study, carried out the study, performed the IHC and lipid analysis and helped to draft the manuscript. MMSPG helped to collect data for results analysis. RT helped to collect data for results analysis. LJ participated in the design of study and performed the statistical analysis. RDJ participated in design of study and helped to draft the manuscript. SSD helped with performing statistical analysis. JCLM participated in the design of the study, performed statistical analysis, and coordinated and helped to draft the manuscript. All authors read and approved the final manuscript.

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**Article 2:**

Galloway S, **Takechi R**, Pallebage-Gamarallage MM, Dhaliwal SS, Mamo JC. (2009) Amyloid-β colocalizes with apolipoprotein B in absorptive cells of the small intestine. *Lipids Health Dis*. 8, 46 [Impact factor: 2.1]

In this study, the colocalization of Aβ and apo B within the Golgi-apparatus of small intestinal absorptive cells was investigated. Ryusuke Takechi has contributed to the tissue sample collection, data analysis and manuscript preparation.

Previous studies suggested that enterocytes produce and secrete Aβ in association with nascent chylomicrons. One of the main steps of chylomicron assembly and secretion is the lipidation of apo B within the endoplasmic reticulum and Golgi apparatus. In this study, the 3-D immunofluorescent colocalization analysis technique described in Chapter 2 was used to determine the colocalization of Aβ and apo B within the Golgi compartment of enterocytes.

Double immunofluorescent staining demonstrated that both Aβ and apo B in the enterocytes were located within the Golgi apparatus that is located in the perinuclear region of the cells. Immunoreactivity of apo B and Aβ was also observed in the basolateral region of enterocytes and within the lacteals. 3-D colocalization analysis showed substantial colocalization of Aβ and apo B including in lacteals, consistent with the concept that Aβ is secreted as a nascent chylomicron. However, there was no evidence that relative abundance of the two proteins were correlated

The finding of this study is consistent with the notion that Aβ is synthesized and secreted into the circulation as apoprotein of chylomicrons via similar pathway to the chylomicron metabolism, that supports the hypothesis of this thesis.

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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-

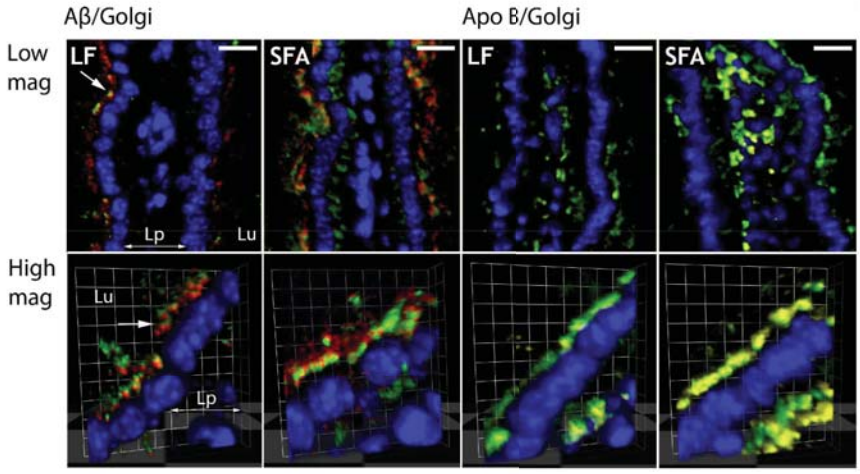


**Figure 1**

**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.

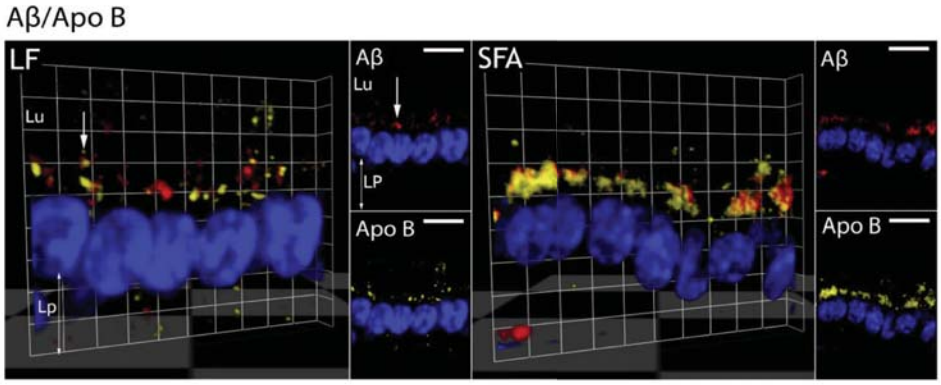


**Figure 2**
**Enterocytic colocalization of amyloid-β with apo B under LF and SFA feeding**. The enterocytic colocalization of amyloid-β (Aβ) with apolipoprotein B (apo B) in low-fat (LF) and saturated fat (SFA) fed mice is shown in three dimensions. The inset images depict the separate channel view 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%.

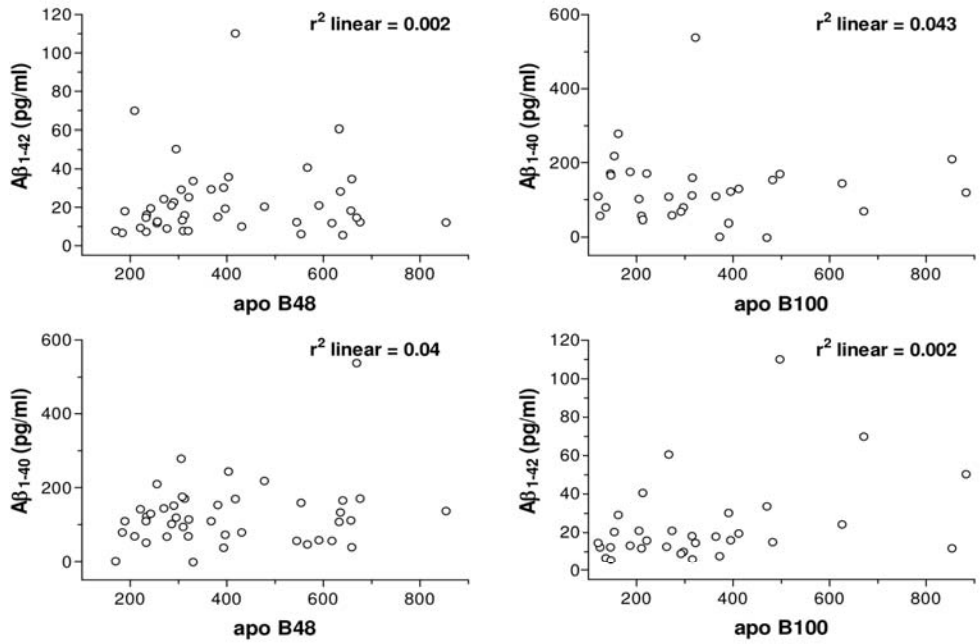


**Figure 3**
**Correlation analysis of plasma amyloid-β40/42 with plasma apo B48/100**. Correlation coefficients were determined with Pearson's correlation analysis where no outliers were identified.

## 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.

## Acknowledgements

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# Appendix C

## Conference Abstracts

Abstracts presented at international conferences during my candidacy are listed, and the abstracts are attached.

**2008** **International Symposium on Chylomicron in Disease,** Alberta, Canada

**Takechi R,** Galloway S, Pallebage-Gamarallage M, Johnsen R, Mamo J. "Immunohistological analysis of intestinal apolipoprotein B and its 3-dimensional colocalization with Golgi apparatus."

* awarded Young Scholar Travel Award

**2009** **The 41st Annual Scientific Meeting of Japan Atherosclerosis Society**, Yamaguchi, Japan

**Takechi R**, Galloway S, Pallebage-Gamarallage M, Wellington C, Johnsen R, Mamo J. "New insight of Alzheimer's disease-high fat diet link."

*awarded Young Tainee Travel Grant

**2009** **Annual Scientific Meeting of Australian Atherosclerosis Society 2009**, Victoria, Australia

**Takechi R**, Galloway S, Pallebage-Gamarallage M, Mamo J. "Differential effects of dietary fatty acids on the cerebral distribution of plasma derived apo B lipoproteins with amyloid-β."

*awarded Best Poster Presentation Award

**2009** **International Congress on Vascular Dementia 2009**, Barcelona, Spain

**Takechi R**, Galloway S, Pallebage-Gamarallage M, Wellington C, Johnsen R, Mamo J. "Chronic ingestion of saturated fats induce the disruption of BBB and increase blood-to-brain delivery of lipoprotein Aβ."

**IMMUNOHISTOLOGICAL ANALYSIS OF INTESTINAL APOLIPOPROTEIN B AND ITS 3-DIMENSIONAL COLOCALIZATION WITH GOLGI APPARATUS**

Takechi R, Galloway S Johnsen R and Mamo J.
*Australian Technology Network, Centre for Metabolic Fitness and the School of Public Health, Curtin University of Technology, Western Australia.*

Background: The assembly and secretion of chylomicrons has been investigated in cell culture and animal model systems and extrapolations have been made from studies detailing hepatic lipoprotein biosynthesis. Chylomicrons are synthesized continuously even with prolonged absence of dietary lipids. On the other hand, some dietary fats appear to regulate chylomicron secretion. Apolipoprotein B48 (apo B48) requires lipidation to generate a nascent primordial lipoprotein a process which is probably completed within the endoplasmic reticulum (ER) and Golgi apparatus. In this study, we have investigated chylomicron assembly and secretion utilizing novel three-dimensional immunohistological methodologies.

Results 1: Distribution of apo B in mouse small intestine
The distribution of apo B in the small intestine was determined by immunofluorescent microscopy. Substantial immunoreactivity was seen within the perinuclear region of enterocytes. There was also significant apo B staining within lacteals, consistent with secretion into lymphatics.
Results 2: Colocalization of apo B and Golgi
Three dimensional immunofluorescent colocalization studies were done utilizing ApoTome based microscopy techniques (Zeiss). Within enterocytes, apo B strongly co-localized with Golgi 75.2±8.5% consistent with the purported assembly of chylomicrons.

Conclusion: Immunofluorescence microscopy can provide novel insight into small intestinal chylomicron homeostasis.

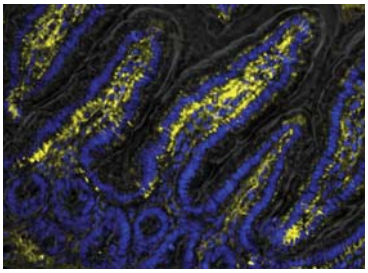
Figure 1

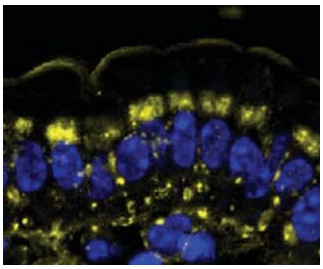
Figure 2

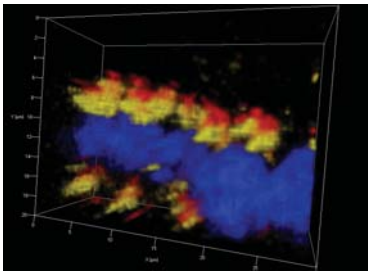
Figure 3

Figure 1. Distribution of small intestinal apo B. Significant staining of apo B was observed within the enterocytes. Apo B staining was also observed in the lacteals.
Figure 2. Enterocytic apo B. Strong staining of apo B was observed in the perinuclear areas of enterocytes.
Figure 3. 3-dimensional colocalization of apo B and Golgi apparatus. The figure represents the 3-dimensional colocalization of Golgi and apo B within enterocytes. Of total enterocytic apo B, 75% was associated within the ER/Golgi apparatus.

**Saturated fats disrupt blood-brain barrier integrity resulting in blood-to-brain delivery of lipoprotein-amyloid-β; Implications for Alzheimer's disease risk.**

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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. A major neuropathological marker of AD is amyloid-β (Aβ) deposition in the parenchyma of brain. However the origin of this protein still remains controversial. In this study we show in wild-type mice that chronic ingestion of saturated fats (SFA) results in blood-brain barrier (BBB) dysfunction and significant leakage into brain of dietary-derived lipoproteins enriched in Aβ. In contrast, chronic ingestion of monounsaturated or polyunsaturated fatty acids had no detrimental effect on BBB integrity. Utilizing highly sensitive 3-dimenisional immuno-microscopy, we also show in a murine model of AD, a positive association of intestinal and hepatic derived apolipoprotein B lipoproteins with cerebral amyloid plaque. Collectively, the findings of this study provide a plausible explanation of how dietary fats influence AD risk. Ingestion of saturated fats may enhance peripheral delivery to brain of circulating lipoprotein-Aβ and exacerbate the amyloidogenic cascade.**

**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 unknown. In a murine model we show that chronic ingestion of saturated fats (SFA) results in blood-brain barrier (BBB) dysfunction and delivery into brain of apo B lipoproteins that are endogenously enriched in amyloid-β (Aβ). Apo B delivery was greatest in regions of brain with greater capillary density. Consistent with BBB dysfunction, pPlasma S100B (a marker of brain-to-blood leakage) was doubled in SFA mice and expression of the endothelial tight junction protein occludin was reduced. Utilizing sensitive 3-dimenisional immuno-microscopy, we also show that the cerebral distribution and colocalization of Aβ with apo B lipoproteins in SFA fed mice, is similar to that found in 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. Ongoing studies suggest that the deterioration in BBB function in SFA fed mice may be reversible and that some lipid modulating agents may confer a level of protection. Collectively, the findings of this study provide a plausible explanation of how dietary fats may influence AD risk. Ingestion of SFA could enhance peripheral delivery to brain of circulating lipoprotein-Aβ and exacerbate the amyloidogenic cascade.

**Chronic ingestion of saturated fats induce the disruption of BBB and increase blood-to-brain delivery of lipoprotein-Aß**

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Background: Saturated fats (SFA) and cholesterol are risk factors for Alzheimer's disease (AD), but the mechanisms for this effect are not clear. We reported that dietary SFA stimulate synthesis and secretion of lipoprotein bound amyloid-ß (Aß) from the small intestine and in clinical studies found that AD subjects have exaggerated plasma Aß bound to apolipoprotein (apo) B containing lipoproteins. It is our hypothesis that dietary induced elevations in plasma Aß compromise BBB integrity and exacerbate amyloidosis.

Aim: to investigate in wild-type mice the differential effects of dietary fatty acids on BBB function.

Methods: C57BL/6J mice were fed chow enriched in either low fat (LF control) SFA, monounsaturates (MUFA) or polyunsaturates (PUFA) for 3 months. BBB integrity was assessed by immunofluorescence microscopy and included cerebral extravasation apo B lipoproteins naturally enriched in Aß as well as IgG. Expression of the endothelial tight junction protein occluding was measured and plasma S100B was used as a marker of brain-to-blood leakage.

Results: SFA fed mice had significant cerebral extravasation of IgG and apo B containing lipoproteins. Occludin expression was substantially attenuated in SFA mice and plasma levels of S100B increased 2-fold. Cerebral apo B colocalized with Aß immunoreactivity. Mice fed MUFA, PUFA or LF diets showed no cerebrovascular abnormalities.

Conclusion: Our findings are consistent with the hypothesis that dietary fats influence AD risk by modulating BBB function.