

School of Public Health

Curtin Health Innovation Research Institute

Regulation of the brain lipidome in health and disease

Corey Steven Giles

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Declaration

To the best of my knowledge and belief, this thesis titled ‘Regulation of the brain lipidome in health and disease’, contains no material previously published by any other person except where due acknowledgement has been made. This thesis contains no material that has been accepted for the award of any other degree or diploma in any university.

The research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes 8th edition (2013). The proposed research study received animal ethics approval from the Curtin University Animal Ethics Committee, Approval Number #AEC_2014_27.

Signature:

A handwritten signature in black ink, appearing to be 'C. A. G.', written in a cursive style.

Date: 26th August 2017

Abstract

The brain is the most lipid abundant organ in the body, with the exception of adipose tissue. Composed of several thousand distinct lipid species, the brain lipidome is unique from other organs. Brain lipids support the structural, biochemical and signalling functions required of the central nervous system (CNS). Strict maintenance of the brain lipidome is necessary for synaptic plasticity, membrane fluidity and signal transduction. Small perturbations in lipid composition can result in large phenotypic consequences, such as impaired learning, memory and cognitive ability.

Neurodegenerative diseases, cognitive and behavioural disorders have been associated with dysregulated cerebral lipid metabolism. A growing body of evidence links alterations in sphingolipids, plasmalogens and numerous phospholipid classes with neurodegenerative disorders such as Alzheimer's disease (AD). However, it is not known whether these changes are causally related with the onset and progression of disease, or a consequence of progressive disease pathology. Indeed, the factors that regulate the cerebral lipidome in physiological systems are still largely unknown.

The brain is continuously exposed to plasma lipids through an extensive network of blood micro vessels. The brain has an obligatory requirement for selected plasma lipids that cannot be produced *de novo*. Moreover, *in situ* synthesis of fatty acids is considered to be inadequate for the brains requirements.

Circulating lipids are found as triacylglycerols, phospholipids and cholesterol esters packaged within plasma lipoproteins, in addition to non-esterified fatty acids associated with chaperone proteins, principally albumin. The composition and abundance of circulating lipids are significantly influenced by dietary lipids. Epidemiological, clinical and animal model studies show that plasma lipids are associated with vascular-based neurodegenerative disorders, consistent with causality. Nonetheless, it is not established that changes in the brain lipidome precede frank evolution of neurodegenerative diseases such as AD.

This thesis explores the plasma-brain axis in ‘Regulation of the brain lipidome in health and disease’. The hypothesis is supported by an in-depth review of the literature presented in Chapter 1. Methodological considerations for the complex analytical techniques and statistical modelling are presented in Chapter 2. The main experimental objectives of this thesis were to investigate the plasma lipidome as a regulator of the cerebral lipidome, presented in Publication 1. The second primary objective of this thesis was to explore the potential regulatory capacity of circulating sphingolipids in modulating cerebral sphingolipid homeostasis, as presented in Publication 2.

To explore the physiological regulatory capacity of circulating lipids on the cerebral lipidome, wild-type mice were subjected to a diet low in fat, or a diet modestly enriched in saturated fatty acids (Publication 1). Following six months on their respective diets, the hippocampus, cerebral cortex and plasma lipidome were characterised using a comprehensive lipidomics platform. Long-term consumption of saturated fatty acids (SFA) caused substantial alterations in the abundance of numerous circulating lipid classes, while the cerebral lipidome remained remarkably stable. An investigation of individual lipid species revealed a remodelling of acyl-chain composition of cerebral phospholipids. Long-term consumption of the SFA diet was associated with a reduction in phospholipids containing highly unsaturated fatty acids (6 or 7 total double bonds). Concomitantly, phospholipids containing 4 or 5 double bonds were generally increased. The hippocampus (HPF) and cerebral cortex (CTX) were similarly affected suggesting both regions were regulated by a common mechanism. The plasma lipidome was investigated as a regulator through multivariate-multivariable predictive models. Strong associations were observed between plasma lipid species containing highly unsaturated fatty acids with cerebral phospholipids containing similar highly unsaturated species. Similar associations were observed with putative arachidonic acid containing species, whereas those containing five double bonds showed complex associations. The overall findings from this study demonstrate a physiological mechanism through which circulating lipids regulate the cerebral lipidome. Moreover, the data indicates that long-term consumption of well-tolerated diets can alter the cerebral lipidome, highlighting a potential therapeutic pathway for some neurological disorders.

Elevated levels of circulating ceramides have been proposed to pathologically accumulate in the brain, leading to neurodegenerative disease initiation and progression. However, several sphingolipid metabolic pathways intersect at ceramides, potentially leading to accumulation. To investigate the regulation and interaction of circulating and cerebral ceramides, wild-type mice were randomised to receive dietary manipulations and sphingolipid modulating agents (Publication 2). Mice consuming an SFA enriched diet showed elevated levels of circulating ceramides and the precursor, dihydroceramides. Inhibiting sphingolipid *de novo* synthesis prevented this increase. By contrast, cerebral ceramides were unaffected by the SFA diet. Sphingolipid *de novo* synthesis inhibition reduced ceramides in the cerebral cortex, but not in the hippocampus. Agonism of sphingosine-1-phosphate receptors reduced hippocampal ceramides, but not CTX or plasma ceramides. Unexpectedly, negative associations were observed between HPF and plasma ceramides, whereas small positive correlations were observed between CTX and plasma. The data suggest circulating ceramides were highly regulated through *de novo* synthesis, whereas cerebral ceramide levels are tightly regulated. The principal findings of this study indicate that increased circulating ceramides do not lead to accrual of ceramides in the brain.

The primary findings presented in Publication 1 of this thesis support the broad hypothesis that the plasma lipidome regulates a specific subset of lipids in the brain. However, the results of Publication 2 highlight a regulatory axis through which only specific lipid classes modulate cerebral lipid composition and metabolism. Taken together, the experimental data from the animal studies provide novel insight into regulatory mechanisms, which is informative in the context of neurodegenerative disease processes. The findings emphasize the importance of identifying plasma lipids that regulate CNS function through modulation of the cerebral lipidome.

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List of primary publications

The PhD thesis includes 2 first author peer-reviewed publications to address my candidacy objectives. The following articles have been published in each of the journals listed below. Author contributions and copyright authorisation for each publication is detailed in Appendix I.

1. **Giles, C.**, Takechi, R., Mellett, N. A., Meikle, P. J., Dhaliwal, S., & Mamo, J. C. (2016). The Effects of Long-Term Saturated Fat Enriched Diets on the Brain Lipidome. *PLoS One*, 11(12). doi:10.1371/journal.pone.0166964

[Impact Factor: 4.411]

2. **Giles, C.**, Takechi, R., Mellett, N. A., Meikle, P. J., Dhaliwal, S., & Mamo, J. C. (2017). Differential regulation of sphingolipid metabolism in plasma, hippocampus, and cerebral cortex of mice administered sphingolipid modulating agents. *Journal of Neurochemistry*. doi:10.1111/jnc.13964

[Impact Factor: 3.842]

List of secondary publications

The following publications are not directly linked to my candidacy objectives, however are complimentary and demonstrate my research productivity and engagement with other relevant research activities during the course of my project.

1. Gogoi-Tiwari¹, J., Köhn-Gaone, J., **Giles, C.**, Schmidt-Arras, D., Gratte, F. D., Elsegood, C. L., McCaughan G. W., Ramm, G. A., Olynyk, J. K., & Tirnitz-Parker, J. E. (2017). The Murine Choline-Deficient, Ethionine-Supplemented (CDE) Diet Model of Chronic Liver Injury. *Journal of Video Experiments. In press.*

[Impact Factor: 1.1]

2. Snelson, M., Mamo, J. C., Lam, V., **Giles, C.**, & Takechi, R. (2017). Differential effects of high protein diets derived from soy and casein on blood-brain barrier integrity in wild-type mice. *Frontiers in Nutrition*. doi:10.3389/fnut.2017.00035

3. Mamo, J. C., Lam, V., **Giles, C.**, Coulson, S. H., Fimognari, N., Mooranian, A., Al-Salami, H., & Takechi, R. (2017). Antihypertensive agents do not prevent blood-brain barrier dysfunction and cognitive deficits in dietary-induced obese mice. *International Journal of Obesity*. doi:10.1038/ijo.2017.57

[Impact Factor: 5.337]

4. **Giles, C.**, Albrecht, M. A., Lam, V., Takechi, R., & Mamo, J. C. (2016). Biostatistical analysis of quantitative immunofluorescence microscopy images. *Journal of Microscopy*, 264(3), 321-333. doi:10.1111/jmi.12446

[Impact Factor: 2.136]

5. Lam, V., Takechi, R., Pallabage-Gamarallage, M., **Giles, C.**, & Mamo, J. C. (2015). The vitamin D, ionised calcium and parathyroid hormone axis of cerebral capillary function: therapeutic considerations for vascular-based neurodegenerative disorders. *PLoS One*, 10(4), e0125504. doi:10.1371/journal.pone.0125504

[Impact Factor: 4.411]

6. Heidari Nejad, S., Takechi, R., Mullins, B. J., **Giles, C.**, Larcombe, A. N., Bertolatti, D., Runchev, K., Dhaliwal, S., & Mamo, J. (2015). The effect of diesel exhaust exposure on blood-brain barrier integrity and function in a murine model. *Journal of Applied Toxicology*, 35(1), 41-47. doi:10.1002/jat.2985

[Impact Factor: 2.722]

7. Elahy, M., Lam, V., Pallegage-Gamarallage, M. M., **Giles, C.**, Mamo, J. C., & Takechi, R. (2015). Nicotine Attenuates Disruption of Blood-Brain Barrier Induced by Saturated-Fat Feeding in Wild-Type Mice. *Nicotine & Tobacco Research*, 17(12), 1436-1441. doi:10.1093/ntr/ntv044

[Impact Factor: 3.811]

8. Elahy, M., Jackaman, C., Mamo, J. C., Lam, V., Dhaliwal, S. S., **Giles, C.**, Nelson, D., & Takechi, R. (2015). Blood-brain barrier dysfunction developed during normal aging is associated with inflammation and loss of tight junctions but not with leukocyte recruitment. *Immunity & Ageing*, *12*, 2. doi:10.1186/s12979-015-0029-9

[Impact Factor: 2.708]

9. Takechi, R., Pallegage-Gamarallage, M. M., Lam, V., **Giles, C.**, & Mamo, J. C. (2014). Long-term probucol therapy continues to suppress markers of neurovascular inflammation in a dietary induced model of cerebral capillary dysfunction. *Lipids in Health and Disease*, *13*, 91. doi:10.1186/1476-511X-13-91

[Impact Factor: 2.137]

10. Takechi, R., Pallegage-Gamarallage, M. M., Lam, V., **Giles, C.**, & Mamo, J. C. (2013a). Aging-related changes in blood-brain barrier integrity and the effect of dietary fat. *Neurodegenerative Diseases*, *12*(3), 125-135. doi:10.1159/000343211

[Impact Factor: 2.937]

11. Takechi, R., Pallegage-Gamarallage, M. M., Lam, V., **Giles, C.**, & Mamo, J. C. (2013b). Nutraceutical agents with anti-inflammatory properties prevent dietary saturated-fat induced disturbances in blood-brain barrier function in wild-type mice. *Journal of Neuroinflammation*, *10*, 73. doi:10.1186/1742-2094-10-73

[Impact Factor: 4.667]

12. Lam, V., Albrecht, M. A., Takechi, R., **Giles, C.**, James, A. P., Foster, J. K., & Mamo, J. C. (2013). The Serum Concentration of the Calcium Binding Protein S100B is Positively Associated with Cognitive Performance in Older Adults. *Frontiers in Aging Neuroscience*, *5*, 61. doi:10.3389/fnagi.2013.00061

[Impact Factor: 4.348]

Abbreviations

AD	Alzheimer's Disease
ALA	alpha-Linolenic acid (18:3n3)
AMI	Amitriptyline
ANOVA	Analysis of variance
ApoE	Apolipoprotein E
ARA	Arachidonic acid (20:4n6)
aSMase	Acidic-sphingomyelinase
BBB	Blood-brain barrier
BMI	Body mass index
BMP	Bis(monoacylglycerol)phosphate
CE	Cholesterol ester
Cer	Ceramide
Cer(d18:0)	Dihydroceramide
Cer(d18:1)	Ceramide
CNS	Central nervous system
COH	Cholesterol
CTRL	Control diet

CTX	Cerebral Cortex
DG	Diacylglycerol
DHA	Docosahexaenoic acid (22:6n3)
dhCer	Dihydroceramide
DHC	Dihexosylceramide
DPA	Docosapentaenoic acid (22:5n6)
EPA	Eicosapentaenoic acid (20:5n3)
ESI	Electrospray ionisation
FDR	False discovery rate
FTY	FTY 720; Fingolimod
FWER	Family-wise error rate
GalCer	Galactosylceramide
GluCer	Glucosylceramide
GM3	G _{M3} Ganglioside (monosialodihexosylgangliosides)
HexCer	Monohexosylceramide
Hex2Cer	Dihexosylceramide
Hex3Cer	Trihexosylceramide
HPF	Hippocampal formation; Hippocampus

HPLC	High-performance liquid chromatography
LA	Linoleic acid (18:2n6)
LC	Liquid chromatography
LPC	Lysophosphatidylcholine
LPC(O)	Lysoalkylphosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPI	Lysophosphatidylinositol
LV	Latent variable
MCI	Mild cognitive impairment
Mfsd2a	Major facilitator superfamily domain-containing protein 2A
MG	Monoacylglycerol
MHC	Monohexosylceramide
MMSE	Mini mental state examination
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MUFA	Mono-unsaturated fatty acids
MYR	Myriocin
NEFA	Non-esterified fatty acids

nSMase	Neutral-sphingomyelinase
PC	Phosphatidylcholine
PC(O)	Alkylphosphatidylcholine
PC(P)	Alkenylphosphatidylcholine
PD	Parkinson's Disease
PE	Phosphatidylethanolamine
PE(O)	Alkylphosphatidylethanolamine
PE(P)	Alkenylphosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PLS	Partial least squares; Projection to latent structures
PS	Phosphatidylserine
PUFA	Poly-unsaturated fatty acids
ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate
SD	Standard deviation
SFA	Saturated fatty acids
SM	Sphingomyelin

SM4	Sulfatide
SPT	Serine-palmitoyltransferase
TG	Triacylglycerol
THC	Trihexosylceramide

Table of Contents

Declaration	ii
Abstract	iii
Acknowledgements	vi
List of primary publications	vii
List of secondary publications	viii
Abbreviations	xii
Table of Contents	xvii
Chapter 1: Review of the Literature	1
1.1 Introduction	1
1.1.1 Lipids in the Brain	1
1.2 Mechanisms of circulating lipid uptake by the brain	5
1.2.1 Lipoprotein mediated uptake	6
1.2.2 Non-esterified fatty acid uptake	8
1.2.3 Lyso-phospholipids	10
1.2.4 Cerebral selectivity of blood-derived non-esterified fatty acids.	10
1.3 Regulation of cerebral lipid composition and dietary fat composition	11
1.3.1 Deficiency and supplementation of dietary n-3/n-6 fatty acids	11
1.3.2 Saturated fat enriched diets	14
1.4 Involvement of circulating lipids in neurodegenerative diseases	17
1.4.1 Sphingolipids	18
	xvii

1.4.2	Plasmalogens	22
1.4.3	n-3 fatty acids	25
1.4.4	Blood-based Lipid Biomarker Panels	26
1.5	Conclusion	27
Thesis hypothesis and specific objectives		28
Chapter 2: Methodological approaches to lipidomics and analysis		31
2.1	Introduction	31
2.2	Sample preparation	32
2.3	Mass spectrometry	34
2.3.1	Liquid chromatography	34
2.3.2	Ionization technique	36
2.3.3	Mass spectrometer	37
2.3.4	Fragmentation pattern of lipids	39
2.3.5	Isotope distribution of lipids	40
2.4	Quantitative lipidomics	42
2.4.1	Internal standards	42
2.4.2	Quality controls	44
2.5	Challenges with lipidomics datasets	44
2.6	Statistical procedures in lipidomics research	46
2.6.1	Univariate statistical tests	46
2.6.2	Multiple testing correction	47

2.6.3	Regression methods	49
2.6.4	Regularization methods	50
2.6.5	Latent Variable methods	51
2.7	Procedures in predictive modelling	53
2.7.1	Cross validation	53
2.7.2	Permutation testing	56
2.8	Latent variable perspective of the interaction between plasma and brain lipidomes	58
2.9	Conclusions	60

Chapter 3: Investigation into the effects of long term consumption of saturated fat enriched diets on the brain lipidome **62**

3.1	Article synopsis	62
3.1.1	Background	62
3.1.2	Experimental design in brief	63
3.1.3	Results in brief	63
3.1.4	Discussion and conclusion in brief	63
3.2	Manuscript	65
3.3	Supplementary material	86

Chapter 4: Circulating ceramides and cerebral ceramide homeostasis **94**

4.1	Article synopsis	94
4.1.1	Background	94

4.1.2	Experimental design in brief	95
4.1.3	Results in brief	95
4.1.4	Discussion and conclusion in brief	96
4.2	Manuscript	98
Chapter 5: General discussion, limitations and future directions		109
5.1	General Discussion	109
5.1.1	Uptake of circulating lipids by the brain	110
5.1.2	Associated changes of the brain lipidome relative to plasma	112
5.1.3	Regulation of circulating and cerebral ceramides	119
5.2	Limitations of the present studies	124
5.3	Future directions for research	125
Bibliography		128
Appendix I: State of contribution by others and copyright authorisation		200
Appendix II: R Code for isotope distribution, partial least squares, double cross validation and permutation tests		212

Chapter 1

Chapter 1: Review of the Literature

1.1 Introduction

The brain is highly enriched in lipids, supporting structural, biochemical and cell signalling functions (Cermenati et al., 2015). Correct lipid composition is pivotal for central nervous system (CNS) homeostasis by modulating neurotransmission (Allen, Halverson-Tamboli, & Rasenick, 2007; Heron, Shinitzky, Hershkowitz, & Samuel, 1980), synaptic plasticity (Bazan, 2003a; Koudinov & Koudinova, 2001), ion channel activities (Chen & Gross, 1994; Tillman & Cascio, 2003), gene expression (de Urquiza et al., 2000) and inflammation (Farooqui, Horrocks, & Farooqui, 2007). A strong body of evidence from both animal and human studies suggest abnormal cerebral lipid metabolism is associated with neurodegenerative diseases (Cheng, Wang, Li, Cairns, & Han, 2013; Han, 2010), behavioural disorders (Muller et al., 2015; Schwarz et al., 2008) and cognitive dysfunction (Coetzee et al., 1996). Therefore, a thorough understanding of the role of circulating lipids in regulating the cerebral lipidome may provide insight into therapies, biomarkers and diagnostic tests for said conditions.

1.1.1 Lipids in the Brain

Lipid composition of the brain is unique from other organs and biofluids. The brain contains numerous highly specialised cell types, with strict demands on the functional properties of membrane bilayers (Piomelli, Astarita, & Rapaka, 2007; Rohrbough & Broadie, 2005). To achieve the specialized roles, cells require a complex mixture of lipids in membranes. For instance, neuronal and synaptic junction membranes require different lipid membrane composition than oligodendrocytes and myelin. Myelin is a lipid-rich and multilaminar structure that encloses segments of axons in the brain (**Figure 1**), greatly enhancing propagation of action potentials. Constituting a large part of white matter, myelin is comprised of approximately 80% lipid by dry weight (O'Brien & Sampson, 1965b). Due to the structural role that myelin plays, the lipid composition reflects a stiffer, less fluid

membrane – enriched in cholesterol (COH), galactosylceramides (GalCer), sulfatides (SM4), plasmalogens and overall, a higher percentage of esterified saturated fatty acids (SFA) (Macala, Yu, & Ando, 1983; O'Brien & Sampson, 1965a). In contrast, neuronal and synaptic junction membranes require very high fluidity, due to lateral diffusion of receptors and repeated endocytotic and exocytotic events (Allen et al., 2007; Choquet & Triller, 2003). Therefore, neuronal membranes are enriched in unsaturated fatty acids such as docosahexaenoic acid (DHA; 22:6n-3) (Breckenridge, Gombos, & Morgan, 1972; Cotman, Blank, Moehl, & Snyder, 1969).

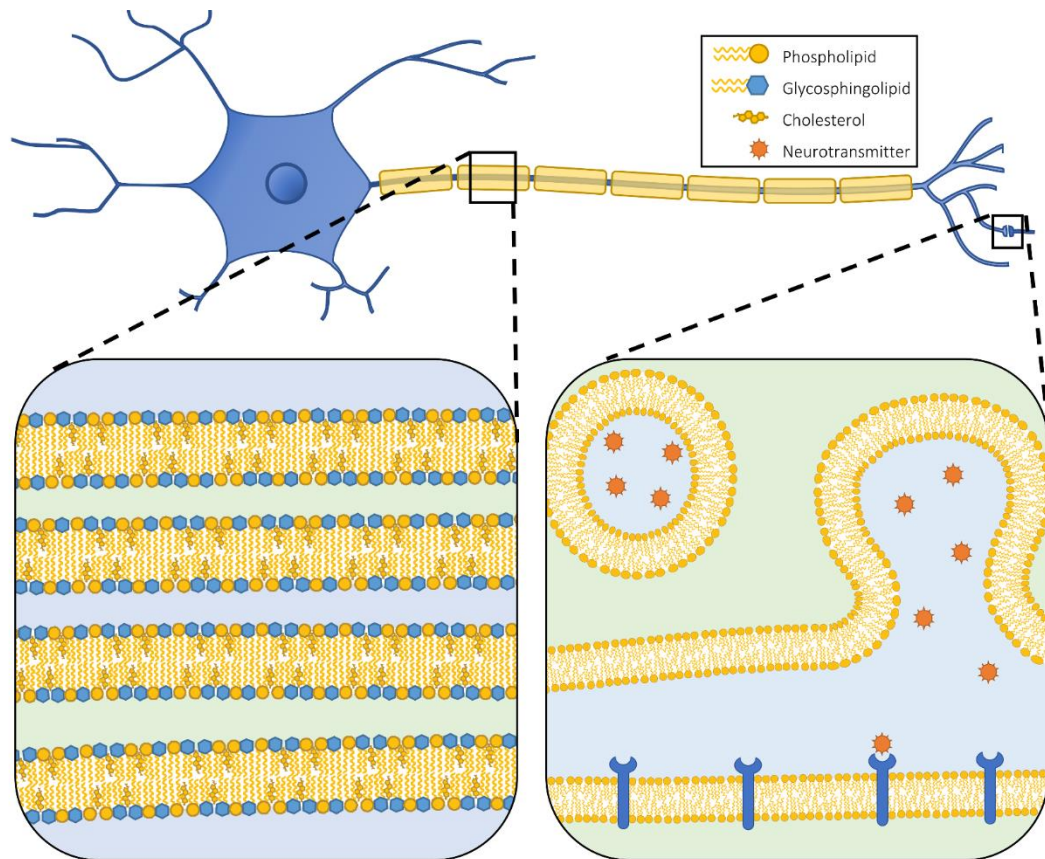


Figure 1. Structural and functional organisation of lipids in the brain. *Top*, diagram of neuronal cell body, axon and synapse. *Left*, multilaminar structure of myelin. Lipid membranes are highly enriched in cholesterol, glycosphingolipids and saturated acyl chains. *Right*, lipid membranes and vesicles at synapses. Lipid membranes are enriched with unsaturated acyl chains and exhibit a fluid behaviour.

A major component of brain lipids are membrane phospholipids (O'Brien & Sampson, 1965b). Due to their amphipathic nature, phospholipids form integral components of membrane bilayers. A majority of phospholipids in the brain are of the class glycerophospholipids, containing a glycerol backbone and up to two acyl chains (**Figure 2**). It is common for the sn-1 position to contain saturated or monounsaturated fatty acids, while the sn-2 position contains polyunsaturated fatty acids (Beermann, Mobius, Winterling, Schmitt, & Boehm, 2005). The sn-3 position is occupied by one of several head groups, including phosphocholine (phosphatidylcholine, PC), phosphorylethanolamine (phosphatidylethanolamine, PE), phosphoserine (phosphatidylserine, PS) and phosphoinositol (phosphatidylinositol, PI). Compared to peripheral tissues, a substantial proportion of glycerophospholipids within the brain contain an ether or vinyl-ether linkage at the sn-1 position (**Figure 2**), constituting a lipid class collectively known as plasmalogens. Most frequently, these ether-lipids contain a phosphocholine or phosphorylethanolamine headgroup. Within the brain, plasmalogens represent up to 85% of the ethanolamine phospholipid pool in brain tissue (Han, Holtzman, & McKeel, 2001).

The brain also contains a substantial quantity of sphingolipids, containing a sphingoid base rather than the glycerol backbone (**Figure 2**). The sphingoid bases contain a long-chain aliphatic tail, replacing the equivalent sn-1 position of glycerolipids. A restricted subset of fatty acids can be amide-linked to the sphingoid base, forming the central sphingolipid metabolite ceramides (Cer). Complex sphingolipids are produced through O-linking of headgroups to the 1-hydroxyl group, such as phosphocholine (sphingomyelin, SM) or through a glycosidic linkage to glucose (glucosylceramide, GluCer) or galactose (galactosylceramides, GalCer). Addition monosaccharides and sialic acid can be added to GluCer to produce higher-order oligosaccharide headgroups, forming many glycosphingolipid classes referred to as gangliosides (Schnaar, Suzuki, & Stanley, 2009). However, over 95% of gangliosides in the human brain are comprised of the classes GM1, GD1a, GD1b and GT1b (Svennerholm & Fredman, 1980). While GalCer is a stereoisomer of GluCer, they are not interchangeable in the brain. Sulfatides are produced through sulfation of GalCer and both have been shown to be irreplaceable for myelin function (Suzuki, Vanier, Coetzee, & Popko, 1999).

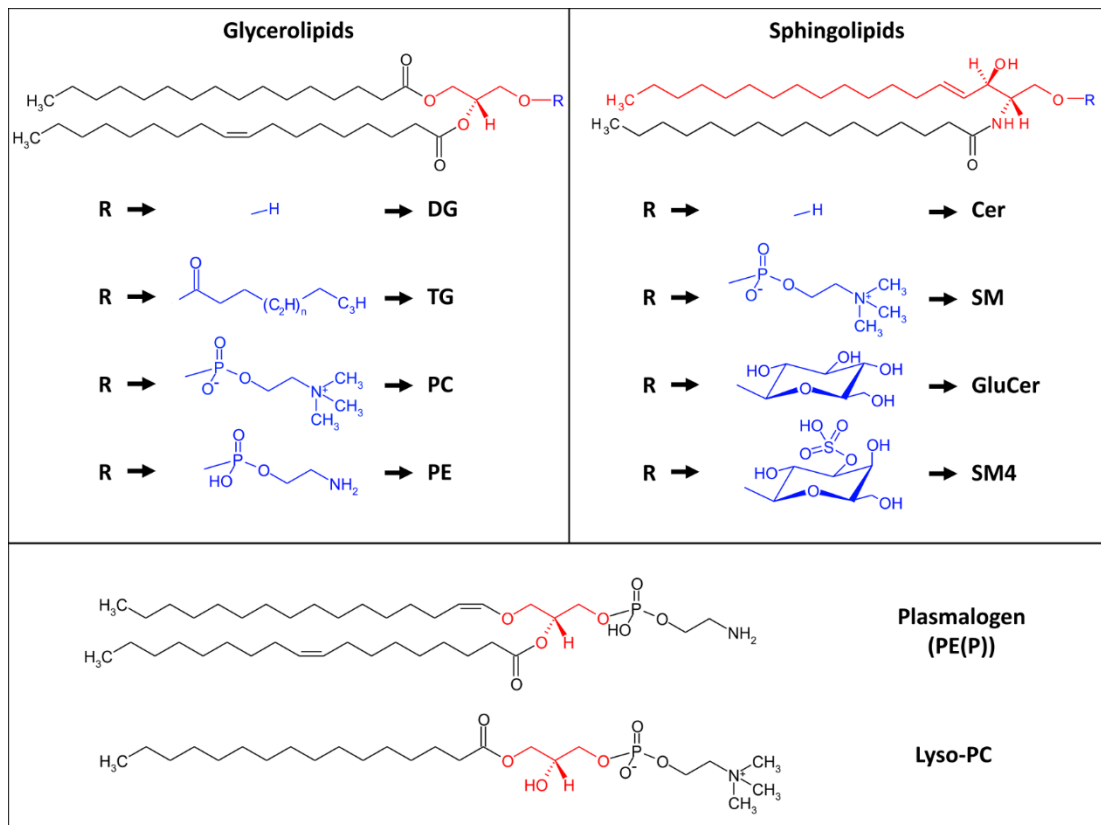


Figure 2. Structure and diversity of lipid classes. *Left*, glycerolipids are characterized by presence of a glycerol backbone (red). Numerous head groups afford a multitude of biological roles. *Right*, sphingolipids are characterized by presence of a sphingoid backbone (red). *Bottom*, plasmalogens contain a vinyl ether linkage in the sn-1 position of the glycerol backbone, while lyso-phospholipids lack an acyl chain in either the sn-1 or sn-2 position.

The unique fatty acid composition of the brain has been known for a long time. The brain is enriched in specific saturated, monounsaturated and polyunsaturated fatty acids, while being devoid of others. Furthermore, of the fatty acids in the cerebral cortex, n-6 and n-3 fatty acids comprise ~8.5% and ~15%, respectively (Fraser, Tayler, & Love, 2010). Of these species, arachidonic acid (ARA; 20:4n-6) comprises approximately 60% of the n-6 fatty acids, while DHA comprises approximately 95 % of the n-3 fatty acids. However, humans are unable to de novo synthesis n-6 and n-3 fatty acids, therefore they must be consumed in the

diet and subsequently taken up by the brain. The structure of non-esterified fatty acids is illustrated in **Figure 3**.

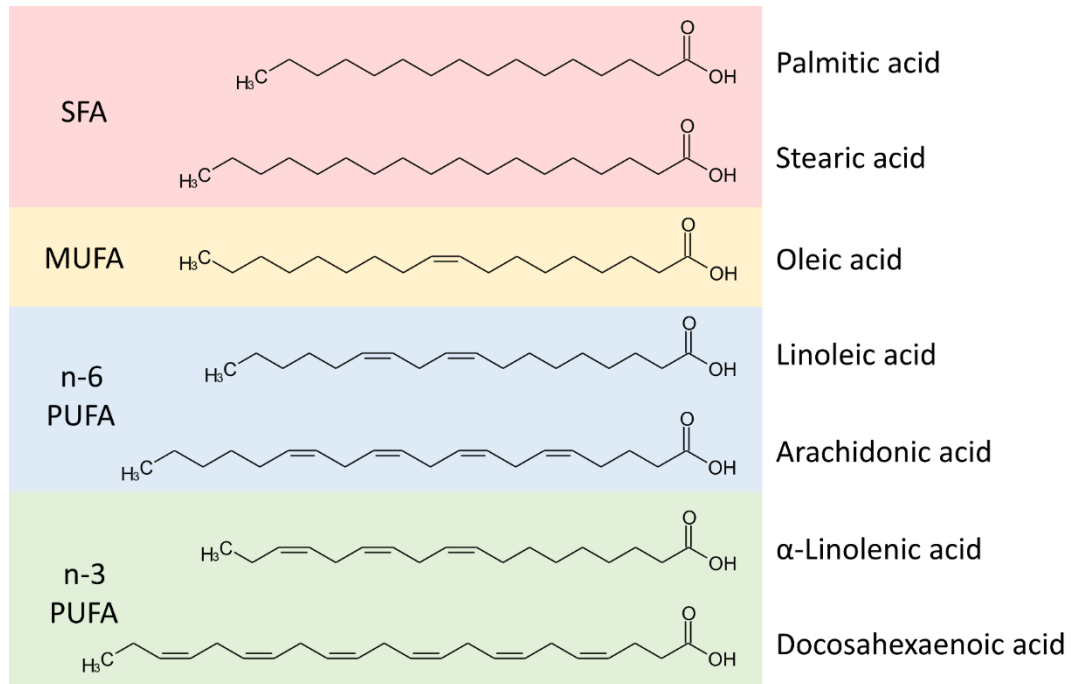


Figure 3. Structure of non-esterified fatty acids. Fatty acids can be classified into saturated (SFA), mono-unsaturated (MUFA) and poly-unsaturated (PUFA) fatty acids. Polyunsaturated fatty acids can be further separated based on the position of the first double bond from the methyl end (n-6 and n-3).

1.2 Mechanisms of circulating lipid uptake by the brain

The brain is the most lipid abundant organ, next to adipose tissue. Therefore, the uptake and synthesis of lipids in the brain is paramount to maintaining the structural and functional requirements of the brain. In contrast to peripheral organs, the brain has a vast vascular network that serves as a physical protective barrier between capillaries and the CNS (Ballabh, Braun, & Nedergaard, 2004). The blood-brain barrier (BBB) is the first major obstacle for lipid uptake from the circulation into the brain. Endothelial cells serve as the interfacing cell, tightly apposed through

tight junction complexes. This tight apposition restricts paracellular transport across the BBB and forces substances to move transcellularly through endothelial cells using passive diffusion or active transport systems (Abbott, Ronnback, & Hansson, 2006). However, the principal mechanism and rates of lipid transport across the BBB is still not agreed upon and highly debated. It has been suggested that circulating lipoproteins (Edmond, 2001; Spector, 2001), lysophospholipids (Nguyen et al., 2014) and non-esterified fatty acids (NEFA) (Chen et al., 2015) contribute to the pool of lipids available for the brain (**Figure 4**).

1.2.1 Lipoprotein mediated uptake

Within the aqueous environment of blood, lipids are found principally associated with lipoproteins, as circulating lysophospholipids and as NEFA. It has been suggested that lipoproteins serve as a substrate pool for cerebral lipid requirements but the mechanisms for this putative kinetic process is presently unclear. Although lipoprotein receptors are found on capillary endothelial cells of the brain (Martin-Nizard, Meresse, Cecchelli, Fruchart, & Delbart, 1989; Meresse, Delbart, Fruchart, & Cecchelli, 1989; Wyne, Pathak, Seabra, & Hobbs, 1996), few experimental studies suggest uptake of peripherally derived lipoproteins occurs in mammals (Chen, Ma, Kim, Mount, & Bazinet, 2008; Purdon, Arai, & Rapoport, 1997; Rahman et al., 2010). When circulating lipoproteins are internalised through receptor mediated processes, they are often targeted for endosomal-lysosomal degradation (Brown & Goldstein, 1979). Edmond (2001) proposed this internalization and processing as an explanation for the apparent selectivity of the brain for specific fatty acids. However, some evidence suggests transcytosis of intact lipoproteins may be an alternative mechanism for lipid uptake into the brain. Indeed, lipoproteins have been shown to cross the BBB in drosophila (Brankatschk & Eaton, 2010) and cell culture models are supportive of lipoprotein transcytosis (Balazs et al., 2004; Candela et al., 2008; Goti et al., 2001).

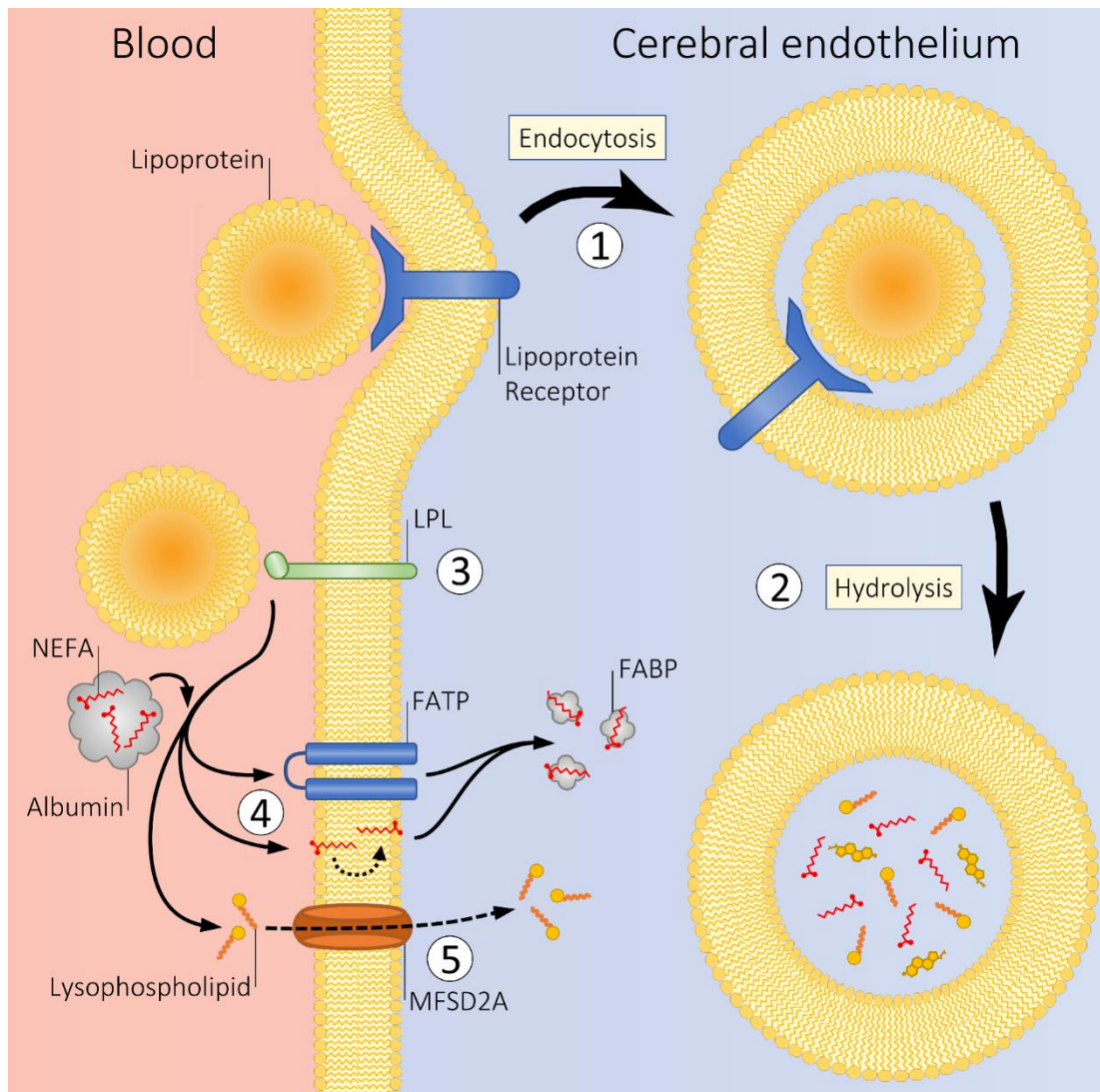


Figure 4. Mechanisms of lipid uptake from plasma into the brain through the blood-brain barrier. (1) Lipoproteins are endocytosed through interaction with lipoprotein receptors or non-specific pinocytotic processes. (2) Lipoprotein components are hydrolysed. Non-esterified fatty acids (NEFA) and lysophospholipids may diffuse into the brain, while cholesterol is secreted into plasma. (3) Circulating lipoproteins are hydrolysed by lipoprotein lipase (LPL), releasing NEFA and lysophospholipids. (4) NEFA may cross membrane bilayers through a passive 'flip-flop' mechanism or fatty acid transport proteins (FATP). Dissociation of NEFA from membranes is enhanced by fatty acid binding proteins (FABP). (5) Lysophospholipid uptake is enhanced by the MFSD2A transporter.

The Edmond model of lipoprotein uptake into cerebral endothelial cells is supported based on the apparent lack of evidence that circulating cholesterol enters the brain. When incorporated into lipoproteins, cholesterol has been shown to be unable to cross the blood-brain barrier (Pardridge & Mietus, 1980). A study by Edmond, Korsak, Morrow, Torok-Both, and Catlin (1991) fed deuterium labelled cholesterol to rat pups at different concentrations. The approach indicated ensures that labelled cholesterol is naturally incorporated into native lipoproteins and that the bio-distribution is physiologically relevant. The abundance of deuterium labelled cholesterol in peripheral tissue was increased proportionally with the concentration of deuterium labelled cholesterol provided in the diet. However, the concentration of labelled cholesterol within brain was minimal and residual amounts were suggested to be within cerebral capillaries rather than brain parenchyme.

Using LDL receptor knockout mice, Chen et al. (2008) explored if LDL uptake is a major contributor to brain fatty acid composition. Compared to wild-type mice, LDLr^{-/-} mice showed no differences in the fatty acid profile of two regions of the brain. Comparable results were later shown for VLDL receptor knockout mice (Rahman et al., 2010). These studies suggest that attenuated lipoprotein uptake via capillary endothelial cells does not significantly influence the brain lipidome in ordinary physiological conditions. An alternate interpretation is that cerebral capillary lipoprotein uptake does contribute to cerebral lipid homeostasis, however redundant pathways are capable of compensating for its absence (**Figure 4**).

1.2.2 Non-esterified fatty acid uptake

Several reports have suggested NEFA are readily taken up by the brain. Using radiolabelled fatty acids, the *in vivo* incorporation rate of circulating NEFA into the brain has been estimated. Rapoport et al. (1997) found that a rapid equilibrium is established between plasma fatty acid and cerebral fatty acyl-CoA concentrations. Following intravenous infusion of radiolabelled palmitate, the abundance of brain phospholipids containing radiolabelled palmitate increased with duration of infusion (Grange et al., 1995). The uptake is rapid, with radiolabel

appearing in the brain within 45 seconds. Of the radiolabelled palmitate measured in the brain, 80% is found esterified to phospholipids within 2 minutes (Rapoport et al., 1997). The indicated experimental data suggests that NEFA are rapidly taken up by the brain and incorporated into phospholipids. However, the mechanism through which this occurs is presently not established.

Two prevailing theories have evolved to explain the rapid uptake of NEFA by the brain: Fatty acids may passively diffuse through membranes using a ‘flip-flop’ mechanism (Hamilton, 2003; Higgins, 1994); or alternatively specific protein-mediated transport mechanisms exist to facilitate fatty acid transport, such as fatty acid translocases and binding proteins (Glatz, Luiken, & Bonen, 2010; Schwenk, Holloway, Luiken, Bonen, & Glatz, 2010) (**Figure 4**). Indeed, several candidate fatty acid transporters have been identified in the brain and other organs. Most of the transporters identified also show long-chain-fatty-acid-CoA synthase activity and therefore may function in ‘metabolically trapping’ NEFA (Coe, Smith, Frohnert, Watkins, & Bernlohr, 1999; Falcon et al., 2010). The passive diffusion model of NEFA uptake from blood into the brain involve several general steps. (1) The NEFA enter the basolateral leaflet from blood, which may be preceded by liberation from albumin or lipoprotein lipase mediated hydrolysis of circulating lipoproteins (Libby et al., 2015). (2) Translocation from the basolateral leaflet to the apical layer. (3) Dissociation from the membrane to the cytosol or fatty acid binding proteins. These processes have been reviewed in detail, previously (Bazinet & Laye, 2014; Chen, Green, Orr, & Bazinet, 2008; Mitchell & Hatch, 2011; Rapoport, Chang, & Spector, 2001; Spector, 2001). It has been speculated that transporters are required to achieve the rates of incorporation observed in experimental studies (Mitchell, On, Del Bigio, Miller, & Hatch, 2011). However, experiments using artificial membranes, which do not contain membrane proteins, indicate that NEFA passive diffusion through bilayers is not rate limiting (Kamp, Zakim, Zhang, Noy, & Hamilton, 1995). Furthermore, Ouellet et al. (2009) has shown that DHA and eicosapentaenoic acid (EPA; 20:5n3) diffusion into the brain is not saturable up to supra-physiological concentrations. Indeed, most studies using intravenous radiolabelled NEFA support the idea that the passive diffusion model of NEFA uptake is sufficient to maintain the brain’s lipid requirement (Chen et al., 2015).

1.2.3 Lyso-phospholipids

It has been known for a long time that lyso-phosphatidylcholine (LPC) species in blood are taken up by brain (Thiès, Delachambre, Bentejac, Lagarde, & Lecerf, 1992). Further, it was suggested that DHA in the form of LPC was a preferential carrier of DHA for the brain (Lagarde et al., 2001). Recently, the orphan receptor Mfsd2a (major facilitator superfamily domain-containing protein 2A) was identified as a transporter for DHA in the form of LPC in the brain (Nguyen et al., 2014) (**Figure 4**). Mfsd2a is found to be exclusively expressed in cerebral capillary endothelial cells. Furthermore, Mfsd2a knockout mice show significantly reduced DHA concentrations in the brain. The findings are consistent with the concept that Mfsd2a regulates, in part, blood-to-brain kinetics of DHA.

1.2.4 Cerebral selectivity of blood-derived non-esterified fatty acids.

The selectivity of the brain for different NEFA was proposed in the early 1990s (Anderson, Tso, & Connor, 1994). This arose due to the apparent retention of a specific subset fatty acids, while other fatty acids were excluded from the brain. In these experiments, multiple radiolabelled fatty acids were fed through a gastric tube and the enrichment measured in the brain and peripheral organs (Edmond, Higa, Korsak, Bergner, & Lee, 1998). Peripheral organs were enriched between 4-40 % for the various fatty acids, while the brain showed no detectable fatty acid enrichment except for the essential poly-unsaturated fatty acid (PUFA) linoleic acid (LA; 18:2n6). It was speculated that the brain contained a mechanism to selectively take up only essential fatty acids. Selective fatty acid transport proteins were proposed as the principle mechanism, however, subsequent studies provided evidence to the contrary (Anderson, Hohimer, & Willeke, 1993). The cerebral fatty acid composition appears to be principally regulated through metabolic processing (Chen et al., 2013). Briefly, once a fatty acid enters a cell, it can become 'metabolically trapped' by esterification to coenzyme A (CoA), through action of an acyl-CoA synthetase. The newly formed acyl-CoA can now be directed down several metabolic pathways: esterified to a vacant position of a lysophospholipid; n-acylated to sphingosine; fatty acid elongation and desaturation; or oxidised for energy production.

The brain is capable of synthesizing only a few non-essential fatty acids. Therefore, the primary source for many non-essential fatty acids and all essential fatty acids is circulating lipids. Upon entry into the brain, PUFA such as ARA and DHA are activated and preferentially esterified into phospholipids (Anderson & Connor, 1988). Whereas, non-essential fatty acids and other PUFA, such as LA, alpha-linolenic acid (ALA; 18:3n3) and EPA are preferentially beta-oxidised (Chen et al., 2013). Therefore, it is apparent that the regulation of cerebral lipids is controlled by a complex interplay between circulating lipids and the cerebral metabolic activities.

1.3 Regulation of cerebral lipid composition and dietary fat composition

1.3.1 Deficiency and supplementation of dietary n-3/n-6 fatty acids

The brain is highly enriched in n-3 fatty acids, which are critical for cell structure and function. Therefore, it is not surprising that much of the literature on dietary modulation of cerebral lipids has focused on n-3 fatty acids. Epidemiological studies suggest consumption of n-3 fatty acids or foods containing them are associated with a lower risk of neuroinflammatory disorders such as Alzheimer's disease (AD), Parkinson's disease (PD) and major depression (Messamore & McNamara, 2016; Wu et al., 2015; Zhang et al., 2016). However, current technology platforms cannot measure the dynamic changes in brain n-3 fatty acids in humans. Therefore, many of the assumptions regarding the role of dietary n-3 fatty acids in the regulation of cerebral lipids come from post-mortem studies, or relevant animal models.

Studies have indicated that DHA is involved in learning and memory (Yurko-Mauro, Alexander, & Van Elswyk, 2015), however, the cellular and molecular mechanisms involved are not yet understood. Some of the first described effects of DHA include the maintenance of membrane bilayer fluidity (Neuringer, Anderson, & Connor, 1988); stimulating neurogenesis and neurite outgrowth (Kawakita,

Hashimoto, & Shido, 2006); and promoting neuronal survival (Akbar, Calderon, Wen, & Kim, 2005). In addition, oxidized DHA metabolites in the brain, such as neuroprotectin D1, also support neuronal cell homeostasis possibly by reducing oxidative stress (Lukiw et al., 2005).

Founding studies revealed cognitive and behavioural abnormalities in animals on n-3 fatty acid deficient diets (Carrie, Clement, de Javel, Frances, & Bourre, 2000a; Ikemoto et al., 2001; Lim & Suzuki, 2000; Moriguchi & Salem, 2003). In rodent models 15 weeks of treatment on an n-3 deficient diet reduced brain DHA concentration by approximately 40 % (Kim, Rao, Rapoport, & Igarashi, 2011). Dietary deficiency of n-3 fatty acids, using an artificial rearing system from post-natal day 2, reduces cerebral DHA as much as 60 % in rats (Lim, Hoshiba, & Salem, 2005). Results of these studies suggest strong homeostatic adaptations in the brain exist to conserve DHA. To prevent loss of DHA, the rate of phospholipid deacylation-reacylation (membrane recycling) of DHA containing membrane phospholipids is reduced, while the incorporate rate of DHA from plasma is increased (Contreras et al., 2000). These homeostatic adaptations have been estimated to increase the half-life of DHA in the brain from 33 to 90 days (DeMar, Ma, Bell, & Rapoport, 2004a).

To explore cerebral homeostatic mechanisms for maintaining brain DHA, Kim et al. (2011) fed rats graded n-3 fatty acid deficient diets. Six diets were prepared, ranging from 4.6 % ALA (n-3 sufficient) down to 0.2 % ALA (n-3 deficient). Following 15 weeks of feeding, the fatty acid composition of blood, brain and livers of the rats were assessed. Despite a significant reduction in plasma DHA, the brain remained stable in lipid composition until dietary ALA was reduced to 0.8 %. At that level of dietary intake, total plasma DHA was reduced by 54 %, while the brain was reduced by 11 %. This study suggests that the brain lipidome remains relatively stable over a wide range of dietary n-3 fatty acid availability. It is important to note that the n-3 deficient diets used in many animal studies may be physiologically extreme and may not be clinically relevant given that n-3 deficiency in humans is uncommon (Anderson & Connor, 1989; Bjerve, Mostad, & Thoresen, 1987). A lack of DHA in the diet is compensated for by elongation and desaturation of ALA in the liver (Rapoport & Igarashi, 2009). However, this pathway is

considered to be inefficient in humans and it has been questioned whether it would be sufficient to supply the human brain. Several recent reviews have covered this in detail (Domenichiello, Kitson, & Bazinet, 2015).

A frequent observation during n-3 deprivation, is the replacement of DHA by fatty acids that are usually uncommon in the brain (Lim et al., 2005). The n-6 fatty acid, docosapentaenoic acid (DPA; 22:5n-6), is produced by elongation and desaturation of ARA and usually found in very low concentrations in the brain. During n-3 fatty acid deficiency, cerebral DPA concentrations increase generally in proportion to the DHA reduction, notionally enabling similar amounts of total PUFA within the brain (Kim et al., 2011). However, the latter does not necessarily protect the animal from CNS deficits that may occur with n-3 deficiency (Lim et al., 2005). Studies of artificial membranes containing DHA or DPA indicate slight differences in membrane dynamics, which could influence the function of membrane proteins or synaptic signal transduction (Eldho, Feller, Tristram-Nagle, Polozov, & Gawrisch, 2003).

In studies where compensatory increases in brain DPA relative to DHA were indicated, the plasma concentration of DPA and DHA showed parallel changes in concentration. It was not known whether the increased DPA content of the brain was the result of increased availability due to hepatic production or the lack of competition of DHA. A study by Lim et al. (2005) provided artificially reared rat pups with an n-3 deficient diet containing preformed DPA, DHA or a mixture of the two. Pups receiving DHA or the DPA/DHA mixture had unchanged brain DHA levels, similar to dam reared pups. However, pups fed the DPA containing diet had a 36 % loss of cerebral DHA, while DPA was 10.5-fold higher. Further studies by Stark, Lim, and Salem (2007) confirmed that DPA does not compete with DHA for incorporation into brain phospholipids. These studies confirm the requirement and specificity of the brain for DHA and indicate DPA only replaces DHA in the absence of sufficient DHA.

The studies of dietary n-3 fatty acids and brain concentrations provide insight into the organ requirements of DHA, utilization of alternative fatty acids and the putative effectiveness of the brain to regulate DHA concentrations over a wide range

of blood lipid concentrations. However, the brain is composed of many different fatty acids, of which, ARA is significant. Deficiency of ARA is almost non-existent due to the high rate of consumption of the precursor fatty acid, LA. However, chronic deprivation of n-6 fatty acids has been explored in rats (Lin et al., 2015). Reducing dietary intake of LA from 28 % to 2 % reduces brain ARA concentrations by 15-28 %, while increasing DHA by 11-18 %. Concomitant with the decrease in ARA in the brain, EPA was found to be increased. Similarly, Igarashi et al. (2009) could not detect EPA in brain of rats receiving regular chow, but n-6 deprivation for 15 weeks increased EPA to detectable levels. These studies highlight the complex metabolic processes involved in regulating cerebral n-3 and n-6 PUFA composition (Alashmali, Hopperton, & Bazinet, 2016).

In the adult brain, ARA and DHA do not need to be further accumulated. Therefore, uptake into the brain serves the purpose of replacing fatty acids lost to beta-oxidation or those converted to active metabolites (Rapoport et al., 2001). However, numerous animal studies have reported that brain DHA levels can be increased by chronic consumption of DHA (Bascoul-Colombo et al., 2016; Labrousse et al., 2012; Skorve et al., 2015). A recent study by Bascoul-Colombo et al. (2016) fed mice a diet with 10-fold greater abundance of n-3 fatty acids, through DHA supplementation. The lipid composition of the hippocampus, cerebral cortex and cerebellum revealed that ARA was partially replaced by DHA in phospholipids. Furthermore, young and old mice fed a diet enriched in EPA and DHA for 2 months showed greater abundance of n-3 fatty acids in the brain (Labrousse et al., 2012). These studies suggest cerebral fatty acid composition may be partially malleable to chronic exposure to n-3 fatty acids.

1.3.2 Saturated fat enriched diets

Most studies exploring dietary regulation of the cerebral lipidome have focused principally on n-3 fatty acids, generally with severe dietary restriction models. Fewer studies have investigated the putative regulatory effects of pro-inflammatory diets, for example diets enriched in SFA. The latter is an important

physiological consideration based on epidemiological studies that show an association between SFA intake and increased risk for neurodegenerative and indeed behavioural disorders (Kalmijn et al., 1997; Laitinen et al., 2006; Morris et al., 2006).

Saturated fat enriched diets have been associated with numerous peripheral organ disturbances, including insulin resistance, cardiomyopathy, non-alcoholic fatty liver disease and systemic inflammation (Kennedy, Martinez, Chuang, LaPoint, & McIntosh, 2009; Leamy, Egnatchik, & Young, 2013; Shi et al., 2006). Indeed, chronic consumption of high fat diets, particularly those enriched in SFA, can induce obesity and metabolic syndrome (Davis, Gabler, Walker-Daniels, & Spurlock, 2008; Rosqvist et al., 2014). The mechanism through which high fat diets induce metabolic disturbances is thought to include the exposure organs to excess fatty acids. Lipotoxicity is a term that describes the excessive lipid accumulation that occurs in non-adipose tissue in response to fatty acid oversupply. This ectopic lipid accumulation results in metabolic stress, cellular dysfunction and eventually cell death (Holland et al., 2011; Mayer & Belsham, 2010). Considering the widely-accepted association between excessive lipid accumulation and cellular dysfunction, it appears to be a plausible mechanism that explains dietary fats and risk of neurodegenerative diseases.

A substantive body of evidence suggests long-chain SFA are pro-inflammatory and induce cellular stress in the brain (Marwarha, Claycombe, Schommer, Collins, & Ghribi, 2016; Milanski et al., 2009; Park et al., 2010; Takechi, Pallegage-Gamarallage, Lam, Giles, & Mamo, 2013b). Long term consumption of high fat diets has been shown to impair rats in a range of learning and memory tasks (Greenwood & Winocur, 1990; Winocur & Greenwood, 2005). In fact, short term exposure of the brain to lipids through intravenous infusion is sufficient to impair hippocampal energy metabolism in humans (Emmanuel et al., 2013). It is speculated that when the brain is exposed to high concentrations of fatty acids in the blood, inflammatory pathways are activated and excess fatty acids can form lipotoxic substrates. Saturated fatty acids, such as palmitate, are well documented to cause intracellular accumulation of lipids, such as long-chain acyl-CoA, Cer and diacylglycerols (DG) – all documented to cause insulin resistance and cellular stress

(Cooney, Thompson, Furler, Ye, & Kraegen, 2002; Erion & Shulman, 2010; Summers, 2006). Furthermore, it has been suggested that the rate of ceramide synthesis is primarily controlled by availability of palmitoyl-CoA, a substrate for the enzyme serine-palmitoyl transferase (Hanada, 2003).

Several recent papers have explored the response of the cerebral lipidome in response to chronic ingestion of high fat diets. Borg, Omran, Weir, Meikle, and Watt (2012) fed mice a high fat diet (60 % energy from fat) for 8 weeks and measured lipids in the hypothalamus. Mice consuming the high fat diet showed increased abundance of Cer, dihydroceramides (dhCer), dihexosylceramides (DHC), DG, triacylglycerols (TG), alkylphosphatidylcholine (PC(O)) and bis-monoacylglycerophosphate (BMP). As expected, this was accompanied by activation of inflammatory pathways. In another study, Posey et al. (2009) showed that a high fat diet (60 % energy from fat) caused the accumulation of saturated long-chain acyl-CoAs in the hypothalamus. Both studies examined the hypothalamus, a region of the brain proposed to have roles in 'fatty acid sensing' for modulating energy intake (Pocai et al., 2006). The latter may predispose the region to have altered susceptibility for lipid accumulation (Martinez de Morentin et al., 2010).

The hippocampal formation (HPF) is a region of the brain important for memory and shown to be adversely affected by high fat diets. Stranahan, Cutler, Button, Telljohann, and Mattson (2011) assessed the HPF lipidome in rats fed a high fat diet with high-fructose corn syrup in their water. After three months on the diet, the HPF of rats on the high fat diet has higher abundance of unesterified cholesterol, GalCer, Cer, SM4 and several isoforms of SM. It should be noted that only hippocampus tissue of animals whose serum cholesterol levels fell into the highest third were selected; limiting generalizability of results.

Changes in the phospholipid fatty acyl composition has potential to highly influence synaptic function, independently of changes in the abundance of lipid classes (Spector & Yorek, 1985). Yu et al. (2010) performed a multi-generation feeding study with two high fat diets in mice. The cerebral fatty acid composition of mice fed a SFA enriched diet showed reduced PUFA and significantly greater abundance of SFA. Whereas the cerebral fatty acid composition of mice consuming a

PUFA enriched diet remained unchanged. Comparable results were demonstrated by Cintra et al. (2012), however mice fed the SFA enriched diet also showed greater abundance of MUFA in the hypothalamus. Partial substitution of dietary SFA with MUFA or PUFA corrected the disturbances in the cerebral lipidome, suggesting dietary SFA, rather than total fat load, is responsible for these observations.

1.4 Involvement of circulating lipids in neurodegenerative diseases

Early pathogenic aberrations in many neurological and neurodegenerative diseases show significant alterations in cerebral membrane lipids (Han, 2010; Schwarz et al., 2008). Earlier studies principally relied on post-mortem analysis of brain tissue, however more recent studies are now also describing peripheral lipid changes prior to onset and during progressive stages of disease (Han et al., 2011; Huynh, Martins, & Meikle, 2017; Mielke, Haughey, et al., 2010; Mielke et al., 2011). Lipidomic technologies readily enable quantitative lipidomic profiling of blood, however in the context of cerebral physio/pathological considerations, the relevance of said measures requires careful consideration of how lipids in circulation might regulate the brain lipidome. Potential mechanisms include via alteration of capillary architecture and function; a consequence of altered blood-to-brain kinetics; via changes in pool size; via altered cerebral metabolism of lipids; or indirectly, for example via changes in redox homeostasis, synthesis or response to bioactive proteins.

This section provides an overview of circulating lipids that have been implicated as biomarkers or suggested to play an active role in some neurodegenerative diseases. **Figure 5** illustrates associations observed between circulating lipids and the time course of neurodegenerative disease processes. Where appropriate, discussion of the evidence for these lipids as regulators of cerebral lipids is provided.

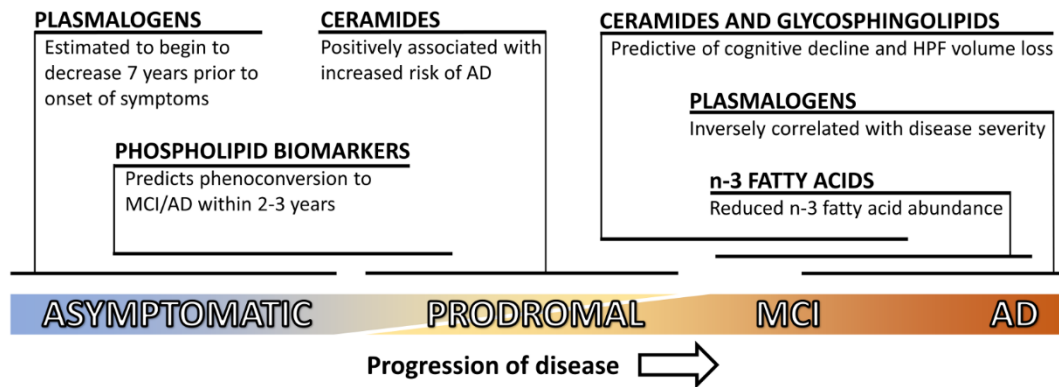


Figure 4. Associations found between circulating lipids and time course of neurodegenerative disease processes. Circulating lipids have been found to estimate risk of-, stratify-, and predict phenoconversion to- Alzheimer’s disease.

1.4.1 Sphingolipids

Accumulating evidence suggests that sphingolipid metabolism is perturbed early in the development of many neurodegenerative diseases, including AD, PD and Multiple Sclerosis (Bradley-Whitman & Lovell, 2015; Farmer, Smith, Hayley, & Smith, 2015; Ginsberg, Rafique, Xuereb, Rapoport, & Gershfeld, 1995; Moyano et al., 2013). Post-mortem lipidomics analysis of AD patients brain tissue has suggested a range of sphingolipid abnormalities, including elevations in Cer (Cutler et al., 2004; Han, M. Holtzman, W. McKeel, Kelley, & Morris, 2002; Katsel, Li, & Haroutunian, 2007); increases in sphingosine (He, Huang, Li, Gong, & Schuchman, 2010); decreases in SM (He et al., 2010); decreased sphingosine-1-phosphate (S1P) (Couttas et al., 2014; He et al., 2010); and changes glycosphingolipids such as GalCer and SM4 (Han, M. Holtzman, et al., 2002; Wallin, Gottfries, Karlsson, & Svennerholm, 1989). It is well established that maintenance of sphingolipid metabolism is critical for neuronal function and survival (Buccoliero & Futerman, 2003; Hirabayashi & Furuya, 2008; Mencarelli & Martinez–Martinez, 2013). Further, Cer have been implicated to play a key role in neurodegeneration. These studies highlight the importance of sphingolipid metabolism for CNS function.

1.4.1.1 Ceramides

While the regulation of sphingolipids within the brain is implicated in progression of neurodegenerative processes, circulating sphingolipids have been proposed to influence the risk of developing neurodegenerative diseases (Han et al., 2011). Several prospective studies have suggested that higher baseline levels of Cer are predictive of future memory impairment and increased risk of developing AD (Mielke et al., 2012; Mielke et al., 2016). Furthermore, increases in circulating Cer are associated with HPF volume loss and worsening of white matter microstructure (Gonzalez et al., 2016; Mielke, Haughey, et al., 2010). These studies suggest that circulating Cer may have a biological role in the pathogenesis of neurodegenerative disorders, cognitive decline and brain atrophy.

Presently, the physiological function of circulating Cer is unclear. The hepatic ceramide-brain axis has been proposed to link several metabolic stresses to neurodegeneration (Lyn-Cook et al., 2009a). In principle, extra-cerebral sources of Cer, such as liver, intestine or adipose tissue can contribute to the initiation or pathogenesis of neurodegenerative diseases. This hypothesis suggests that circulating Cer can directly influence the brain, although the pathway through which circulating Cer pass the BBB is not known. Ceramides found in blood as a constituent of lipoproteins. Several lines of evidence suggest that Cer are secreted nascently with lipoproteins and generated in the circulation by the action of serum-sphingomyelinases (Lightle et al., 2003; Merrill et al., 1995; Schissel et al., 1998; Wiesner, Leidl, Boettcher, Schmitz, & Liebisch, 2009). Both mechanisms have been shown to be upregulated during inflammation and insulin resistance; systemic pathologies that are risk factors for neurodegenerative diseases. It is not surprising that circulating Cer have been suggested to be a major factor for development of such conditions.

The regulatory effect of circulating Cer to influence cellular Cer homeostasis and cell function has been investigated using several models. Boyanovsky, Karakashian, King, Giltiy, and Nikolova-Karakashian (2003b) was the first to show that Cer-enriched LDL was capable of accumulating within human microvascular endothelial cells, which correlated with an increased incidence of apoptosis. To

investigate the biological role of circulating Cer on the brain, de la Monte et al. (2010) delivered alternative day intraperitoneal injections of short chain Cer for two weeks to rat pups. This repeat dosing protocol resulted in mild diabetic symptoms, hyperlipidaemia, cognitive deficits and accumulation of Cer in the brain. While the results are striking, a limitation of the study was the use of short-chain Cer, which are water soluble and highly permeable of cell membranes. An investigation using more physiological lipid preparations was considered by Boon et al. (2013). Low-density lipoproteins enriched with Cer were generated and infused into mice, leading to development of insulin resistance and inflammation - effects causally attributed to increased serum Cer. The findings by Boon et al. was the first evidence indicating that lipoprotein-associated Cer can independently exert biological effects *in vivo*. Interestingly, the induction of insulin resistance was not accompanied by an overt increase in skeletal muscle Cer, however there was a trend to increased plasma membrane Cer. In a follow-up experiment, LDL-ceramide was shown to increase Cer content of cultured myocytes independently of lipoprotein uptake. This suggests that lipoprotein-associated Cer may exert biological effects through lipid-soluble exchange between lipoproteins and cellular membranes. The latter suggests significant sequelae for the cerebrovasculature, where sphingolipid homeostasis is highly indicative of protective versus dysfunctional states (Sathishkumar et al., 2005; Spampinato et al., 2015; Testai et al., 2014; van Doorn, Nijland, Dekker, Witte, Lopes-Pinheiro, et al., 2012).

While epidemiological, cell and animal model studies support the notion that circulating Cer are directly involved in the initiation and progression of neurodegenerative diseases, few studies have explored whether endogenous circulating Cer can modulate cerebral Cer homeostasis per se. There have been several suggestions in the literature that plasma Cer are associated with cerebrospinal fluid Cer, although the relationship and strength have not been reported (Mielke, Haughey, et al., 2010; Mielke et al., 2011).

1.4.1.2 Glycosphingolipids

Neurodegenerative diseases often present with substantial changes in cerebral glycosphingolipid concentrations. Shotgun lipidomic analysis of post-mortem samples of pre-clinical AD subjects brain tissue have suggested that some of the earliest lipid abnormalities involve glycosphingolipids (Cheng et al., 2013). Sulfatides, an important component of myelin, is specifically depleted at the earliest clinically definable stage of AD and progresses with disease severity (Couttas et al., 2016). Interestingly, it has been found that apolipoprotein E (ApoE) plays a key role in SM4 transport and homeostasis (Han, Cheng, Fryer, Fagan, & Holtzman, 2003). Since the ApoE4 allele is a major risk factor for AD, it is thought that SM4 depletion may be mediated by aberrant lipoprotein kinetics within the brain (Cheng, Zhou, Holtzman, & Han, 2010; Han, 2007). In addition, several studies have identified differences in multiple gangliosides in various brain regions throughout the course of the disease (Chan et al., 2012a).

While there is substantial evidence for an involvement of cerebral glycosphingolipid disturbances with neurodegenerative diseases, evidence for circulating glycosphingolipids is only beginning to be elucidated. A recent study by Savica et al. (2016) observed higher levels of both Cer and MHC in the plasma of subjects with diagnosed AD and dementia with Lewy Bodies. Similarly, Mielke et al. (2013) found both lipid subclasses to be elevated in the plasma of PD patients. Extending these observations, Chan et al. (2017) performed a comprehensive plasma lipidomics analysis of PD subjects compared to control subjects. Elevations in MHC were confirmed, however they additionally observed increases in plasma monosialo-dihexosyl-gangliosides (GM3).

The indicated studies highlight the evidence supporting glycosphingolipid dysregulation in blood as well as brain in several neurodegenerative disorders. However, a mechanistic link to explain concomitant changes in glycosphingolipids in blood and brain is missing. It is possible that changes in these complex lipids are the result of increased Cer levels. Alternatively, it has been suggested that in PD, mutations in the enzyme glucocerebrosidase result in reduced hydrolysis of GluCer to Cer (Sidransky et al., 2009). The incidence of glucocerebrosidase mutations is

estimated to be between 7 and 15 % of PD patients (Sidransky et al., 2009), suggesting it might not entirely account for the association of GluCer with PD risk. Rather, it has been suggested that GluCer is an independent risk factor for PD (Mielke et al., 2013).

Currently, it is not known how circulating glycosphingolipids modulate CNS function. It is apparent that not all circulating glycosphingolipids have similar biological effects. In fact, GM1 ganglioside has been in use for treatment of many neurological conditions, including PD (Schneider et al., 2015; Schneider et al., 2013). The neuroprotective effects of GM1 has been demonstrated in many animal experiments (Fong, Neff, & Hadjiconstantinou, 1997). In addition, GM1 gangliosides have shown proficiency in ameliorating cerebrovascular disturbances (Battistin et al., 1985). Moreover, in adults it is not clear to what extent circulating glycosphingolipids can pass the BBB intact. Animal model studies using radiolabelled GM1 have shown small increases of GM1 in the brain following repeated dosing (Polo, Kirschner, Guidotti, & Costa, 1994).

1.4.2 Plasmalogens

The brain is highly enriched in ethanolamine plasmalogens (PE(P)), a unique glycerophospholipid characterised by the presence of a vinyl ether at the sn-1 position of PE. Ethanolamine plasmalogens are a major component of myelin, comprising greater than 80 % of the ethanolamine phospholipid pool (Norton & Poduslo, 1973). The acyl composition of PE(P) differ between myelin and neuronal membranes, with myelin containing less unsaturated fatty acids, providing the compact membrane conformation required. Whereas, grey matter requires a membrane with higher fluidity, which is represented by the greater abundance of highly unsaturated fatty acids in the sn-2 position. It is expected that ethanolamine plasmalogens are essential for signal transduction, membrane fusion and cell-cell communication in the brain.

Among the many membrane abnormalities documented in AD, plasmalogen deficiency is frequently indicated (Ginsberg et al., 1995; Guan et al., 1999; Han et

al., 2001; Igarashi et al., 2011b; Wood, Barnette, Kaye, Quinn, & Woltjer, 2015). Post mortem analysis of AD patients at various stages of the disease have suggested PE(P) deficiency occurs early in the disease (Wood et al., 2015). Han et al. (2001) reported that PE(P) were decreased up to 40 % in the white matter at a very early stage of AD and a progressive loss of grey matter plasmalogens with increasing disease severity. It is clear that cerebral plasmalogen deficiency is a consistent observation in AD subjects.

While there has been long standing evidence of cerebral plasmalogen deficiency in AD, it is only recently that decreasing plasma plasmalogens are being reported in AD and mild cognitive impairment (MCI) subjects. Goodenowe et al. (2007) examined the serum concentration of PE(P) in a cohort of 324 subjects (68 cognitive normal and 256 subjects with probably AD). Ethanolamine plasmalogens were decreased in the probable AD group, compared to controls and indeed, serum concentration was inversely correlated with AD disease severity. Linear regression suggested that circulating plasmalogens appear to decrease early in the initiation of AD. Estimates suggested reductions in plasmalogens begin to occur 7 years prior to the appearance of clinical symptoms. Further evidence was provided by Yamashita et al. (2016) who showed AD subjects had lower plasma concentrations of PE(P) compared to controls, particularly in PE(P) species containing DHA. Interestingly, Wood et al. (2016) three distinct groups could be stratified using plasma lipidomics in a cohort of AD and MCI patients. One of those groups was characterised by low plasma PE(P) levels.

Despite the apparent relationship between cerebral and circulating plasmalogens, little work has been done to specifically determine what putative functional role peripheral plasmalogens have in AD. Fundamentally, deficiency in plasmalogens can occur through two pathways, decreased synthesis or increased degradation. Plasmalogens are produced *de novo* through a non-redundant peroxisome-dependent pathway (Meikle & Summers, 2017) and it has been speculated that peroxisomal function is compromised in AD (Kou et al., 2011). Some preliminary evidence suggests the primary cause of peripheral plasmalogen deficiency occurs through decreased peroxisomal production in AD patients (Goodenowe et al., 2007). Alternatively, increased degradation mediated by

oxidative stress has been suggested (Guan et al., 1999). Indeed, plasmalogens act as anti-oxidants in cellular membranes (Kuczynski & Reo, 2006; Stadelmann-Ingrand, Favreliere, Fauconneau, Mauco, & Tallineau, 2001). Membrane plasmalogens dramatically delay oxidative degradation of polyunsaturated fatty acid. The oxidative products of the vinyl ether bond in plasmalogens does not propagate the peroxidation reaction of unsaturated fatty acids. Therefore, plasmalogens can serve as sacrificial lipids in membranes to prevent excessive membrane oxidative damage. It is thus conceivable that oxidative stress in the brain can lead to plasmalogen deficiency. While it is known that the brain contains all the necessary machinery to synthesize plasmalogens *in situ* ("*Biosynthesis of Plasmalogens in Brain*," 2008), it is not clear if circulating plasmalogens and their precursors are a significant source for the brain (Brites, Ferreira, Ferreira da Silva, et al., 2011; Das & Hajra, 1988; Das, Holmes, Wilson, & Hajra, 1992). It has been suggested that correcting peripheral plasmalogen deficiency might be sufficient to correct a CNS deficiency i.e. restoration of CNS plasmalogens (Goodenowe et al., 2007).

It is possible to augment plasmalogen levels through dietary supplementation of alkylglycerols. Providing this precursor avoids the requirement for *de novo* synthesis in the peroxisome. Dietary supplementation has been used in animal experiments as a therapeutic treatment in a number of conditions, including insulin resistance, inflammation, and atherosclerosis (Brites, Ferreira, da Silva, et al., 2011; Ifuku et al., 2012; Rasmiena et al., 2015; Zhang, Sun, Tang, Cai, & Qian, 2013). A recent double-blind, randomised control trial was conducted to assess whether supplementation with scallop-derived plasmalogens improved cognitive function in subjects with MCI or mild AD (Fujino et al., 2017). After 24 weeks of treatment, no difference in mini mental state examination-Japanese score between subjects taking plasmalogens versus those taking placebo was observed. Rather, subgroup analysis revealed mild effects only in younger female subjects with mild AD. It should be noted that the daily dose for this study was small, 1 mg/day. Interestingly, erythrocyte plasmalogens increased at only one-time point in the treatment group, while plasma plasmalogens tended to decrease over the course of the study.

1.4.3 n-3 fatty acids

Epidemiological studies and animal experiments have provided evidence that higher consumption of n3-fatty acids or n-3 fatty acid containing foods decrease the risk of developing AD (Wu et al., 2015; Zhang et al., 2016). With some inconsistency in findings, post mortem brain tissue of AD patients has generally supported a decrease of DHA containing phospholipids within selected brain regions (Grimm et al., 2011; Igarashi et al., 2011a). It is often suggested that loss of cerebral DHA is associated with cognitive dysfunction during AD (Astarita et al., 2010). Further, due notionally to anti-inflammatory and anti-apoptotic effects, some suggest that dietary supplementation with n-3 fatty acids could replace lost brain DHA and promote neuronal survivability (Belkouch et al., 2016). Indeed, a large number of prospective clinical studies have been conducted, involving supplementation of n-3 fatty acids in patients with MCI or AD (Freund-Levi et al., 2014; Phillips, Childs, Calder, & Rogers, 2015; Quinn et al., 2010). Unfortunately, clinical trials have found little evidence for the beneficial effects of n-3 fatty acid supplementation (Burckhardt et al., 2016).

Recent evidence is suggesting that the ApoE4 allele, the major genetic risk factor for late-onset AD, may play a role in blood-to-brain kinetics of DHA (Vandal et al., 2014). It was proposed that the reduced uptake of DHA into the brain in individuals with the ApoE4 allele explains the lack of effect of DHA in treating AD (Yassine et al., 2017). However, Yassine et al. measured the regional incorporation rates of DHA from blood into brain using positron emission tomography, to compare ApoE4 carriers and non-carriers. Contrary to the expected findings, the study revealed that carriers of the ApoE4 allele showed a greater incorporation rate of DHA in grey matter. It should be noted that higher rates of DHA uptake are not synonymous with DHA accrual. In fact, it is believed that DHA incorporation into the brain is related to the turnover of DHA in the brain (Rapoport, Ramadan, & Basselin, 2011). It is not known whether the loss of DHA from the brain is the result of higher oxidative stress, beta-oxidation or conversion of DHA to active metabolites. Indeed, carriers of ApoE4 tend to have slightly altered whole body DHA homeostasis compared to non-carriers, which supports the relationship between ApoE4 and decreased response to DHA (Hennebelle et al., 2014).

1.4.4 Blood-based Lipid Biomarker Panels

Recently, many studies have published blood-based lipid biomarker panels that distinguish study participants as healthy controls, MCI or AD (Fiandaca et al., 2015; Mapstone et al., 2014; Olazaran et al., 2015; Proitsi et al., 2017; Wang et al., 2014). Using metabolomic and lipidomic profiling of plasma/serum samples, predictive models can be generated to (1) provide an understanding of the biology underpinning a disease and (2) provide a clinically translatable model for diagnosis. An unfortunate side-effect of measuring large numbers of metabolites is the confounder of dimensionality, that is that the number of metabolites measured generally exceeds the number of subjects measured. Therefore, the imperative is to validate panels on independent cohorts.

Beyond stratifying subjects, Mapstone et al. (2014) described a panel of 10 plasma lipids that predicted the phenotypic conversion from healthy to MCI/AD within 2-3 years. Using the prospective nature of their study, they generated a predictive model on a discovery cohort and validated the model on subjects enrolled at a later timepoint. The proposed panel was found to be over 90 % accurate, with a validation C statistic of 0.92 in classifying phenotypic converters. However, when the biomarker panel underwent independent validation by other laboratories, the results were unable to be replicated. Li et al. (2017) assessed the predictive panel in plasma samples of a prospective cohort of African Americans. While the mean follow-up (7.3 years) was greater than Mapstone et al., the model achieved a C statistic of only 0.609. In a separate validation experiment, Casanova et al. (2016) assessed the model in two large independent cohorts. In the Baltimore Longitudinal Study of Aging cohort, the panel achieved a C statistic of 0.642. Whereas, in the Age, Gene/Environment Susceptibility-Reykjavik cohort, the panel achieved a C statistic of 0.395. Furthermore, using a suite of machine learning algorithms, Casanova et al. were unable to create a 'classifier' for both cohorts.

The panel proposed by Mapstone et al. consisted of the lipid species: PC 36:6, PC 38:0, PC 38:6, PC 40:1, PC 40:2, PC 40:6, alkylphosphatidylcholine (PC(O)) 40:6, lysophosphatidylcholine (LPC) 18:2, propionyl acylcarnitine and C16:1-OH. It is interesting to note that (1) several of the lipids likely contain DHA,

which has been associated with AD previously (Lin, Chiu, Huang, & Su, 2012); and (2) an ether lipid is decreased in those who phenoconvert, highlighting previous associations between peroxisome dysfunction and AD (Goodenowe et al., 2007). Future research could focus on circulating lipids and their relation to brain function.

1.5 Conclusion

There is a significant body of evidence which shows that the plasma lipidome has regulatory effects on the abundance of specific cerebral lipid species. Associated changes of the brain lipidome relative to plasma abundance may reflect changes in kinetics across the BBB; cerebral metabolic responses and/or changes in degradation and efflux through cerebrospinal fluid transport. Alterations in the cerebral lipidome are commonly indicated in neurodegenerative disorders and increasingly implicated in a diagnostic or causal context. However, there remains a paucity of studies which have interrogated the capillary axis central to lipid kinetics. With the evolution of contemporary lipidomic, neuroscience, neuroimaging and psychobiology technology platforms significant opportunities exist to explore the interrelationship between the blood and brain lipidome with risk for onset and progression of neurological disorders.

Thesis hypothesis and specific objectives

The animal model studies presented in this thesis were aimed to explore the associated changes in the brain lipidome relative to the plasma lipidome. A secondary objective was to consider whether targeted modulation of sphingolipid metabolism was associated with parallel modulation of peripheral and cerebral sphingolipids.

HYPOTHESIS: The plasma lipidome has a regulatory effect of the abundance and composition of cerebral lipids. Long term consumption of saturated fatty acids is associated with aberrant sphingolipid metabolism in plasma and the brain. Targeted modulation of sphingolipid metabolic pathways can restore aberrant metabolism.

The above hypothesis was investigated by the following objectives:

Objective 1

To explore the effects of long term consumption of SFA enriched diets on associated changes in plasma and brain lipidomes. Wild-type mice were randomised to diets containing differing composition of fats – a low-fat diet (4 % w/w) containing principally PUFA or a high-fat diet (20 % w/w) modestly enriched in SFA. After 6 months of ad libitum consumption, the lipid composition of plasma, HPF and cerebral cortex (CTX) was assessed by a comprehensive lipidomics platform (HPLC-ESI-MS/MS). Rigorous statistical analysis of lipidomes were utilised to identify plasma lipids that are jointly and independently associated with changes in cerebral lipids.

Objective 2

To investigate whether targeted modulation of key lipid metabolic pathways could restore aberrant lipid metabolism in plasma and brain. Wild-type mice were randomised to dietary and pharmacological interventions targeting enzymes and receptors regulating sphingolipid synthesis and degradative pathways. Mice were assigned to one of the following treatments: chow diet, SFA enriched diet with or without one of the following pharmacological agents – myriocin, an inhibitor of sphingolipid *de novo* synthesis; amitriptyline, an inhibitor of acidic sphingomyelinase; FTY 720, a S1P receptor agonist. Alterations to sphingolipids were assessed in plasma, HPF and CTX using HPLC-ESI-MS/MS. Putative associations between circulating sphingolipids and cerebral sphingolipids were assessed.

Chapter 2

Chapter 2: Methodological approaches to lipidomics and analysis

2.1 Introduction

Chapter 1 established a putative causal relationship between circulating lipids and regulation of the cerebral lipidome. To accomplish the objectives of this thesis, methods to accurately characterise changes in complex biological lipidomes must be utilized. Lipidomics is an emerging field involving the systematic study of lipids in biological systems (Bou Khalil et al., 2010; Wenk, 2005; Yang & Han, 2016). Cellular lipids are highly complex, with many thousands of unique lipid structures and abundance ranging over many orders of magnitude (Schmelzer, Fahy, Subramaniam, & Dennis, 2007; Shevchenko & Simons, 2010). The diverse nature of structure and physiochemical properties enable these compounds to fulfil many biological functions, including for energy storage; membrane structure or as critical signalling intermediates. However, this complexity poses many challenges for characterisation and quantitation.

While the goal of lipidomics is the analysis of ‘whole systems’, several technical and practical issues constrain this approach. Recent advances in analytical techniques have greatly enhanced depth of coverage, however lipidomic studies are still restricted to analysing only a subset of known lipids. Numerous complementary techniques are used for investigation of cellular lipidomes, including mass spectrometry (MS), nuclear magnetic resonance and vibrational spectroscopy (Carrasco-Pancorbo, Navas-Iglesias, & Cuadros-Rodríguez, 2009; Rolim, Henrique-Araujo, Ferraz, de Araujo Alves Dultra, & Fernandez, 2015). The development in electrospray ionisation (ESI) MS has accelerated lipidomics research in the past two decades and represents one of the most widely used technique. The versatility of ESI-MS systems allows development of methods to detect and quantify the large diversity of lipid species and classes (Blanksby & Mitchell, 2010; Wenk, 2005). However, highly targeted and specific approaches can preclude global analysis of many lipid classes. Indeed, experimental procedures are generally optimised for the lipid species or lipid class of interest (Merrill, Sullards, Allegood, Kelly, & Wang,

2005; Taguchi et al., 2005). Therefore, careful consideration of experimental procedures is required for characterisation of biological lipidomes.

To address the thesis hypothesis, accurate and broad analysis of circulating and cerebral lipids is required. This chapter describes the methodological considerations for methods utilised in the proceeding chapters. Discussion of the main sequences in a lipidomics workflow is presented, including preparation of samples, accurate quantitation of lipid species and statistical modelling (**Figure 6**).

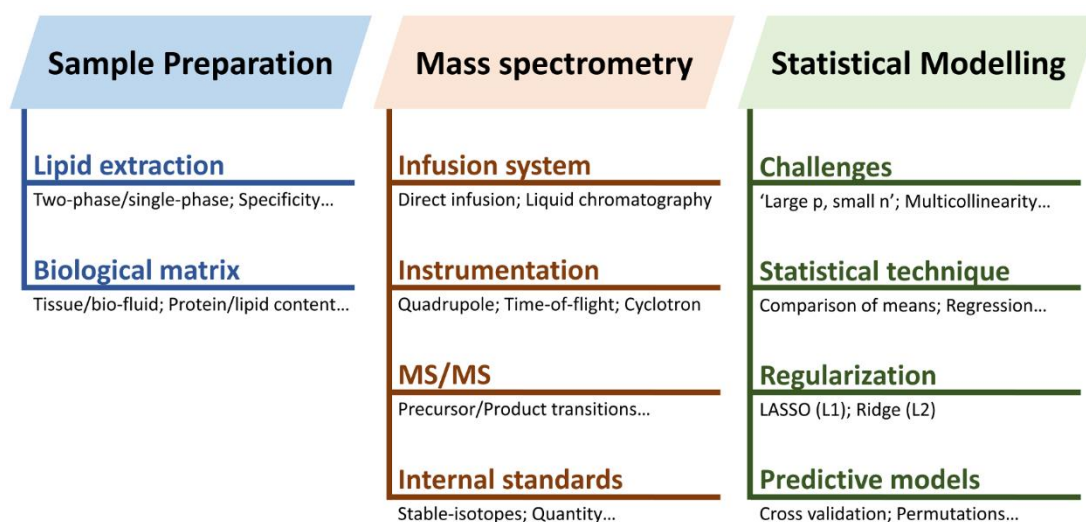


Figure 6. Methodological considerations for lipidomic experiments.

2.2 Sample preparation

To obtain an accurate reflection of the biological lipidome, correct sample preparation is essential. Ideally, sample preparation for lipidomics simultaneously accomplishes several tasks: extraction and isolation of lipids; removal of contaminating molecules; and preparation for MS. An appropriate sample preparation minimizes loss of lipids, maximises ionization efficiency, is reproducible and ensures longevity of instrumentation. Methods must be suitable for the extraction

of lipids from the biological matrix and be specific for lipids of interest (Furse, Egmond, & Killian, 2015).

Preparation of a biological sample for lipidomics generally involves the extraction and isolation of lipids. One of the most widely accepted methods of lipid extraction from biological specimens is the method of Folch, Lees, and Sloane Stanley (1957). The original method describes a two-phase liquid-liquid extraction process using chloroform, methanol and water. The biphasic separation forms two phases, a high density organic phase and an aqueous phase. High abundance proteins are denatured and aggregate, forming a protein 'disk' at the interface of the solvent phases. Lipids and hydrophobic metabolites are largely concentrated into the lower organic phase while hydrophilic metabolites partition into the upper aqueous phase. Isolation of the organic phase accomplishes the lipid extraction procedure. Modifications to Folch's procedure have been proposed (Bligh & Dyer, 1959), including enhanced recovery of lipid classes by acidification (Retra et al., 2008). However, caution should be taken in implementing strategies to enhance recovery of specific lipid classes, as the recovery of other lipid classes may be compromised. Acidification enhances the recovery of lysophospholipids and phosphatidic acid, however results in hydrolysis of plasmalogens (Murphy, Stephens, Jurkowitz-Alexander, & Horrocks, 1993; Shaikh, 1994).

Several challenges have been associated with Folch's procedure, including the reliance on halogenated solvents and partitioning of lipids to the lower organic phase. Chloroform has a higher density than water/methanol, is a known carcinogen and poses health risks for laboratory personnel (Torkelson, Oyen, & Rowe, 1976). Recently, several lipid extraction methods have been described which avoid the use of chloroform and other similar solvents. Two prominent examples include the method described by Matyash, Liebisch, Kurzchalia, Shevchenko, and Schwudke (2008) using methyl-tert-butyl ether and the method of Lofgren et al. (2012) using 1-butanol/methanol. Both systems are based on organic solvents with low density, therefore causing lipids to partition to the upper phase, allowing automation with pipetting robots. Several comparisons of lipid extraction procedures have been performed (Bjerve, Daae, & Bremer, 1974; Iverson, Lang, & Cooper, 2001; Lee, Kind, Yoon, Fiehn, & Liu, 2014; Patterson, Ducrocq, McDougall, Garrett, & Yost,

2015). Several publications suggest the newer methods perform as well as that of Folch et al., while others suggest the newer methods perform worse in their effectiveness to extract lower abundant lipid classes (Alshehry et al., 2015; Reis et al., 2013).

The lipid extraction procedures described above involve a phase separation between organic and aqueous liquids. Amphiphilic lipid classes are known to partition into the aqueous phase or the interface (Quarles & Folch-Pi, 1965; Schacht, 1981; Shaikh, 1994). This results in loss of those lipid classes if only the organic phase is collected. Additional extractions or chromatography is required to recover lipids loss during biphasic extractions. To overcome loss of lipids and additional complexity introduced with multiple extraction procedures, single-phase extractions can be considered. Single-phase extraction, which does not include a phase separation, is generally more efficient in terms of harvesting a more comprehensive capture of lipids with considerable differences in polarity (Alshehry et al., 2015; Liu, Rochfort, & Cocks, 2016). However, this method suffers from a higher content of aqueous impurities. In the procedure described by Folch et al., aqueous contaminants are removed by mixing the sample-chloroform-methanol mixture with one quarter volume of water. Following the procedure of Folch et al. but omitting this step avoids the loss of lipids into the aqueous phase, at the expense of aqueous impurities. However, water contained within tissue constitutes a tertiary component of the extraction mixture, which will cause phase separation if not considered. It is possible to avoid a phase separation if the ratio of solvent mixture to water is greater than 17:1 (Wilson et al., 2013). Therefore, a single-phase extraction yields an efficient procedure to extract lipids from biological specimens, if subsequent procedures are robust against the additional impurities.

2.3 Mass spectrometry

2.3.1 Liquid chromatography

Two principal methods are used for analysing lipidomic samples: direct infusion MS, also known as shotgun lipidomics; or chromatography based

lipidomics, such as liquid chromatography (LC) coupled MS. Shotgun lipidomics consists of infusing samples directly into a mass spectrometer (Kofeler, Fauland, Rechberger, & Trotsmuller, 2012; Wang, Wang, Han, & Han, 2016). As all lipids enter the mass spectrometer simultaneously, identification of lipids relies entirely on discrimination by mass analysers. Given that lipids are highly complex, with many isobaric species and non-unique precursor/product transitions, unambiguous identification and quantitation of complex lipid mixtures is a challenge. Recent advances have aimed to overcome some of these limitations, including the development of intra-source separation/selective ionization (Han et al., 2006) and use of high resolution mass spectrometers (Ekroos, Chernushevich, Simons, & Shevchenko, 2002). By contrast, chromatography based systems allow complex lipid mixtures to be separated based on chemical or physical properties prior to entering the mass spectrometer. As lipid species enter the mass spectrometer at different times, isobaric species can be separated and retention time used as an additional parameter for identification (Wenk, 2005). The latter overcomes the disadvantages of direct infusion systems, at the expense of longer analysis time. In addition, choices in column chemistry and mobile phase composition allow substantial flexibility in system design.

Appropriate sample preparation is required for both shotgun and chromatography based lipidomics. However, shotgun lipidomics has stricter requirements for removal of impurities (Yang & Han, 2016). Removal of salts and polar metabolites is critical to ensure the ionization of lipids is not suppressed (Han & Gross, 2005). By contrast, LC separation prior to MS is more tolerant of aqueous impurities. Following injection onto the column, lipids are retained, while polar metabolites and salts are eluted with the solvent front. These impurities can be eluted to waste prior to entering the mass spectrometer. Therefore, the single-phase extraction procedure described above is suitable for LC-MS, but not shotgun lipidomics.

Liquid chromatography uses a liquid mobile phase that passes through a column packed with a stationary phase. Many column chemistries are available, exploiting different physiochemical properties. Due to the hydrophobic nature of lipids, the most common separation system for LC-MS based lipidomics is reverse-

phase chromatography with a C18 column. Once injected onto the column, lipids adsorb to the hydrophobic stationary phase and are eluted based on the relative affinity with the stationary and mobile phases. Mobile phase composition can be formulated to achieve the required separation of lipid species (Cajka & Fiehn, 2016; Gao, Zhang, & Karnes, 2005; Ogiso, Suzuki, & Taguchi, 2008). The composition can be changed throughout the course of a run, increasing the hydrophobicity of mobile phase and therefore increasing the elution strength. This gradient based approach allows for controlled elution of lipids over a wide range of polarities. By contrast, greater separation of lipid species can be achieved if the mobile phase composition is held constant (isocratic flow) (Merlin, Gresti, Bellenger, & Narce, 2006; Weir et al., 2013b). However, this improvement in separation is only evident for a narrow range of polarities and can result in long run times. Indeed, the selection of LC parameters, including column chemistry, mobile phase composition and run time, are highly dependent on goal of the experiment. Recent approaches have adopted multiple chromatographic runs, leveraging the advantages of both gradient and isocratic based approaches (Weir et al., 2013b).

2.3.2 Ionization technique

For analysis and detection by a mass spectrometer, analytes must be ionized. Numerous techniques exist to produce ions for mass spectrometers, however ESI is the most common method of coupling LC systems to mass spectrometers. By applying a strong electric field to a liquid passing through a capillary tube, a fine aerosol is produced which evaporates to produce ions (Smith, Loo, Edmonds, Barinaga, & Udseth, 1990). The latter technique allows the constantly flowing eluent of LC systems to be directly coupled to a mass spectrometer. Electrospray ionisation is considered a 'soft ionisation' technique as it results in little fragmentation of molecules, thus preserving information of their intact mass (Fenn, Mann, Meng, Wong, & Whitehouse, 1990). Ions produced by ESI are often created by addition or loss of hydrogen. In addition to hydrogen, adducts can form with salts or buffer components i.e. Na⁺, K⁺ NH₄⁺. While large molecules can attain multiple charge

states, lipids tend to ionize with a single charge state. Therefore, ESI offers the capability to effectively ionize lipids with well characterized ionization products.

2.3.3 Mass spectrometer

Once lipids are ionized and enter a mass spectrometer, the ion signal as a function of m/z can be produced. Through carefully tuned electric fields, the path of ions can be controlled. Depending on the type of mass spectrometer, ions can be filtered by m/z using a quadrupole; accelerated along a flight path to measure their m/z ; or orbited around an electrode to measure m/z ratios. These different approaches offer advantages and disadvantages in resolution and ion throughput (Figeys & Aebersold, 1997; Kofeler et al., 2012; Winger, Hofstadler, Bruce, Udseth, & Smith, 1993).

Quadrupole mass analysers act as mass filters by only allowing ions within a small m/z window to pass through (Hager & Le Blanc, 2003). By applying radio frequency AC voltage with a DC offset, the transmissible m/z window can be tuned over a wide range. To acquire a mass spectrum, the quadrupole must scan over a range of m/z . By contrast, time-of-flight and cyclotron based mass analysers acquire a mass spectrum in a single scan (Guilhaus, 1995; Michalski et al., 2011). Time-of-flight mass analysers accelerate ions through a flight tube. Ions traverse the flight tube at different rates, based on mass, charge and electric field strength. Therefore, by measuring the arrival time of an ion at the detector, the m/z can be calculated. Cyclotron mass analysers capture ions in an orbit around an electrode. The movement of ions induces a charge which is detected and processing with a fourier transform to produce a mass spectrum. Consistent with the functioning of time-of-flight and cyclotron mass analysers, mass spectrums can only be acquired on batches of ions. Introduction of new ions into a batch disrupts the relationship between signal and m/z . By contrast, quadrupole mass analysers are capable of continuously filtering ions. Further differences in mass analysers are found in mass resolution, with a rough ordering: quadrupole < time-of-flight < cyclotron. Due to the filtering action of quadrupoles, smaller isolation windows (higher resolution) result in decreased

transmission efficiency (Kofeler et al., 2012). It is possible for ion transmission to decrease enough to result in poor signal-to-noise ratio. Time-of-flight instruments, on the other hand, can have orders of magnitude better mass resolution than quadrupole mass analysers. However, due to the low duty cycle, time-of-flight instruments have considerably lower levels of sensitivity. Compared to both quadrupole and time-of-flight mass analysers, cyclotron based mass analysers have the highest resolution. Interestingly, mass resolution can be increased though increasing scan time. However, due to the long scan times for high resolution mass spectra, these instruments are commonly used with direct infusion (Fhaner, Liu, Ji, Simpson, & Reid, 2012; Ghaste, Mistrik, & Shulaev, 2016; Kofeler et al., 2012; Schuhmann et al., 2012). Therefore, the higher resolution is a trade-off against prior chromatography separation.

Choice of mass analyser is highly dependent on the biological question to be answered. For discovery based experiments, the ability to sample entire mass spectrums simultaneously is advantageous for identifying unknown compounds. Indeed, time-of-flight and cyclotron mass analysers have shown substantial use in this area (Dunn et al., 2013). However, quantitative measurements of known compounds do not require full mass spectrums to be acquired. Rather, it is desirable to have the properties of quadrupole mass analysers: high sensitivity, high ion transmission and a large, linear dynamic range.

Mass analysers can be arranged sequentially to perform tandem mass spectrometry (MS/MS). The most common variant of these instruments being a triple quadrupole mass spectrometer. In this configuration, the first and third quadrupole act as mass filters, whereas the second quadrupole consists of a collision cell (Han, Yang, & Gross, 2012). Ions transmitted by the first quadrupole undergo collision-induced dissociation with an inert gas, causing fragmentation of molecules. Ions passed through the first quadrupole are referred to as precursor ions, while ions passing through the third quadrupole are referred to as product ions. Fragmentation products can provide structural information about the precursor ions. Therefore, unique precursor/product transitions can be used to infer identity, or to achieve greater selectivity for quantitative analysis (Kitteringham, Jenkins, Lane, Elliott, & Park, 2009). If unique precursor/product transitions are known for lipid species, the

mass spectrometer can cycle through the list and thus, measure the abundance of these species. With a chromatography system, the transitions can be scheduled for the approximate retention time of the lipid, therefore reducing unnecessary scans and greatly improving signal-to-noise ratio. This technique has allowed the measurement of several hundred lipid species within a single run, provided chromatographic separation is adequate (Weir et al., 2013b).

2.3.4 Fragmentation pattern of lipids

To create a list of precursor/product transitions, precursor and product ion scans can be performed on purified samples of each lipid specie. However, this is impractical when the intention is to measure many lipid species. Indeed, experimentally derived MS/MS spectral libraries have been compiled and freely available for researchers (METLIN) (Kind et al., 2009; Smith et al., 2005). However, these databases are still far from complete. Therefore, it is common to exploit knowledge of the fragmentation patterns that are consistent for a specific lipid class (Hsu & Turk, 2009; Pulfer & Murphy, 2003). As lipids are comprised of ‘building blocks’, there is a substantial consistency in fragmentation pattern between lipids of the same lipid class (Han et al., 2012). The dominant fragmentation patterns used in lipidomic research involve the loss of head group. For example, a lipid specie of the PC class will produce a strong product ion signal at 184.1 m/z – the phosphocholine headgroup (**Figure 7**) (Hsu & Turk, 2003). Whereas a PE lipid specie will produce a strong product ion signal of the precursor ion minus 141 Da – the phosphorylethanolamine headgroup (**Figure 7**) (Hsu & Turk, 2008). However, not all lipid classes can be analysed in this manner. Sphingolipids, with the exception of sphingomyelin, have a tendency to produce a characteristic fragment ion of 264.2 m/z – the long chain sphingoid base (Shaner et al., 2009a). In addition, DG and TG species do not show class specific fragmentation patterns, Rather, each specie must be characterised by the loss of an acyl chain (Hsu & Turk, 2010). That is, the precursor ion minus an acyl chain, or by the liberated acyl chain fragment. Negative ionisation mode can often be used to monitor the liberation of acyl chains as product ions (**Figure 7**). Based on these fragmentation patterns, an in-silico database with

MS/MS spectra has been created for over 200,000 lipid species (Kangas et al., 2012; Kind et al., 2013; Witting, Ruttkies, Neumann, & Schmitt-Kopplin, 2017). However, care should be taken to avoid indiscriminate use of such a database as instrument specific nuances can result in poor matches.

2.3.5 Isotope distribution of lipids

When measuring a lipid specie with a mass spectrometer, multiple m/z peaks can often be observed in close succession (**Figure 7**). These m/z peaks indicate the isotopic distribution of the molecule. In free-living biological systems, lipids contain chemical elements with isotope abundances reflecting those naturally occurring on earth. As most lipids are principally composed of only carbon, hydrogen, oxygen, phosphorus and nitrogen, it is important to account for the naturally occurring isotope abundance of each element. For these elements, the lightest isotopes are also the most abundant (Meija et al., 2016). The most abundant heavier isotope commonly found in lipids is carbon-13, comprising roughly 1.1 % of all carbon atoms. That is, for each atom of carbon, there is a 1.1 % chance it is carbon-13 rather than the more abundant carbon-12 (~98.9 %). Mass spectrometers are capable of distinguishing a lipid containing a single carbon-13 molecule from the same lipid without any carbon-13 atoms. While these molecules appear as separate peaks in a mass spectrum, the difference in biological activity is fundamentally negligible (Dufner & Previs, 2003). Therefore, the total abundance of a lipid specie is the sum of all isotope peaks (Fernandez, Des Rosiers, Previs, David, & Brunengraber, 1996).

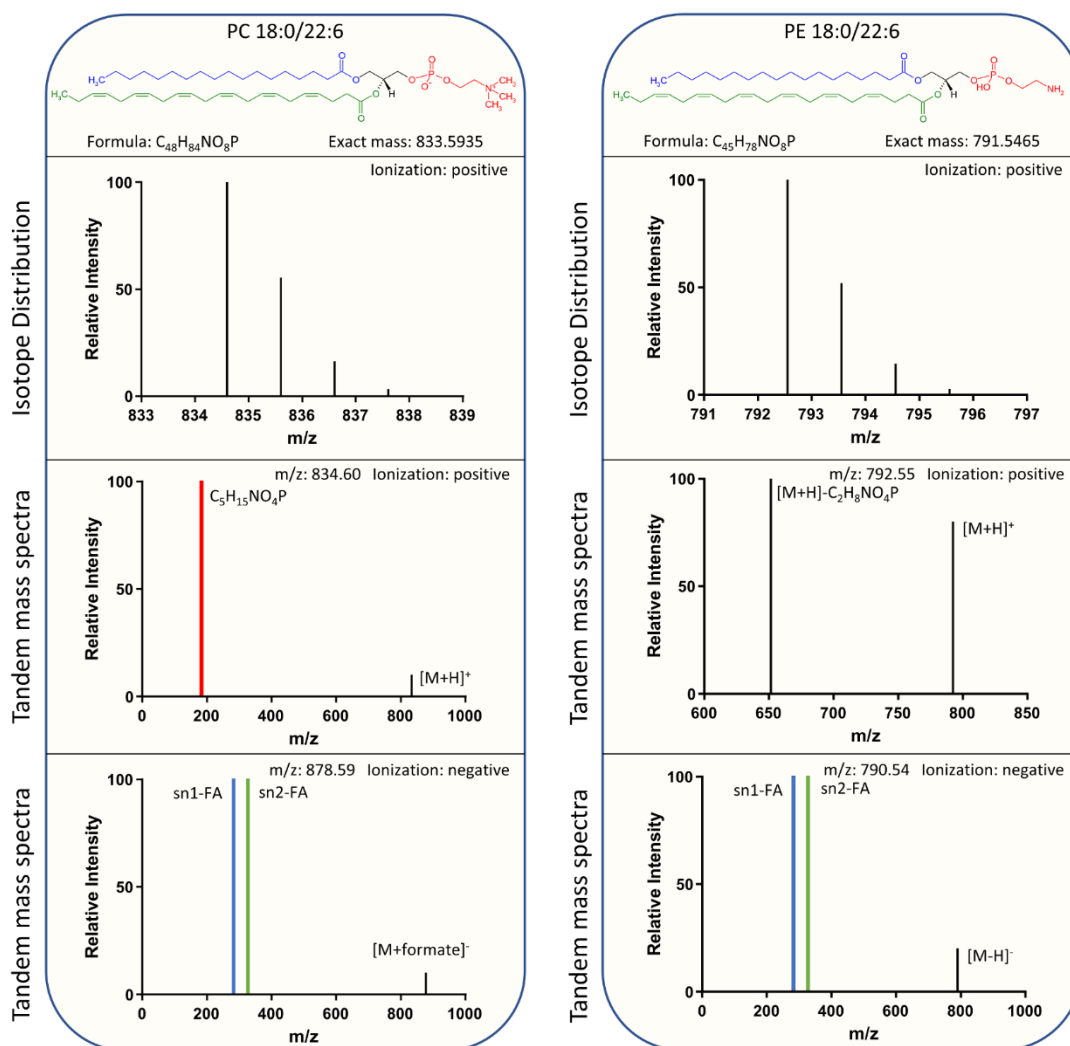


Figure 7. Mass spectra of two phospholipids. PC 18:0/22:6 and PE 18:0/22:6 differ in mass, as illustrated in their respective isotope distributions. PC lipids show a characteristic 184.1 m/z ion in positive mode product ion scans, representing the phosphocholine head group. PE lipids show a product ion of the precursor minus 141 m/z; the loss of phosphorylethanolamine headgroup. Negative ionisation product ion scans show the C18:0 and C22:6 fatty acids.

As lipids contain many atoms, each with a small percentage of being a heavier isotope, a single lipid can be observed as having a distribution of masses. For larger molecules, it is possible that the most abundant m/z peak does not relate to the mass of the molecule made up of the most abundant isotopes of each element. This

occurs due to the fact that as the number of atoms increases, the probability of the entire molecule containing at least a one heavy isotope increases (Senko, Beu, & McLafferty, 1995). As most lipids are small molecules, the monoisotopic peak (theoretical mass without heavy isotopes) generally appears as the predominant ion. However, the presence of isotopic species of lipids can complicate mass spectrometric acquisition and analysis. Due to small differences in mass between lipids differing in saturation (loss or gain of 2 hydrogens; 2 Da), the isotope envelope of an unsaturated specie can overlap a more saturated specie (Weir et al., 2013a). Significant differences in lipid specie abundance can cause the isotope envelope to mask the existence of another lipid specie or lead to inaccurate quantitation (Liebisch, Lieser, Rathenberg, Drobnik, & Schmitz, 2004). It is for this reason that chromatographic separation of lipids differing by a single double bond is essential (Weir et al., 2013a).

Code to calculate the exact mass and isotopic distribution for lipid species in the statistical programming language R is included in Appendix II. The probability of isotopic variants can be modelled by a binomial distribution (Valkenburg, Mertens, Lemièr, Witters, & Burzykowski, 2012). Extending this to the multinomial case allows determination of all possible isotope combinations (Yergey, 1983). Due to finite mass resolving power of mass spectrometers, isotope peaks can also be modelled by convolution of isotope probabilities with a Gaussian distribution of specified width.

2.4 Quantitative lipidomics

2.4.1 Internal standards

Accurate quantitation in lipidomics requires a method to control for variability in lipid extraction, ionization efficiency and systemic drift in the mass spectrometer. Addition of internal standards to samples allows compensation for these sources of variability (Wang, Wang, & Han, 2016). However, the choice of internal standards is critical for accurate quantification. Ideally, internal standards should have similar chemical and physical properties to the lipids they are being

compared against, but distinguishable in mass spectra. Therefore, a stable-isotope labelled internal standard for each lipid specie is the optimal option for accurate quantitation. However, lipidomic experiments are measuring several-hundred lipid species per run. Therefore, it is impractical to obtain stable-isotope labelled variants of each lipid specie. In practice, it is common to utilise one to two internal standards for each lipid class. Nonetheless, stable-isotope labelled internal standards are often not available for every lipid class (Schmelzer et al., 2007). Therefore, non-physiologically occurring lipid species (odd- or short-chain variants) can be used in their place (Lam, Tian, & Shui, 2017).

A concern with using one/two internal standards for a lipid class, is that they will not accurately reflect the physiochemical properties of all the lipid species in that class. Indeed, acyl-chain composition can range considerably across a lipid class. Lipid extractions are known to vary in their specificity of lipids based on hydrophobicity and acyl chain composition alters the fragmentation kinetics (Brouwers, 2011; Reis et al., 2013; Wang, Wang, & Han, 2016). Several studies have attempted to characterise the extent to which these issues contribute to quantitative inaccuracies (Khoury, El Banna, Tfaili, & Chaminade, 2016; Koivusalo, Haimi, Heikinheimo, Kostianen, & Somerharju, 2001). Indeed, the response factor of lipids have been shown to differ by acyl chain composition. However, careful optimization of ionization and fragmentation parameters for an instrument can reduce this effect to a negligible amount (Kim, Wang, & Ma, 1994; Koivusalo et al., 2001; Shaner et al., 2009b). A further concern has also been proposed for gradient based LC-MS/MS systems. Changes in mobile phase composition can have varying effects on ionization efficiency (Brouwers, 2011; Wang, Wang, & Han, 2016). If internal standards are eluted with a considerably different mobile phase composition than the analyte it is compared against, erroneous relative abundances can be obtained. An ideal chromatography solution achieves robust separation of lipid classes, while individual species of a class are eluted within a narrow window. This ensures analytes are eluted with a similar matrix as their internal standard.

2.4.2 Quality controls

In addition to internal standards, quality controls should be in place to ensure systematic variation does not occur over the course of an experiment (Huynh, Mundra, & Meikle, 2015). To avoid systematic variation from being misrepresented as group differences, samples should be randomised prior to sample preparation. While this helps to reduce the likelihood of false positive treatment effects, it does not identify or control systematic sources of variation. The simplest approach to identifying systematic variation is to utilise quality controls – the same sample analysed at different points throughout an experiment. Each quality control should be individually processed by the same experimental procedures as each sample. This ensures experimental variations arising from sample preparation and mass spectrometric acquisition are captured. In addition to quality controls, technical replicates should be assessed throughout an experiment (Huynh et al., 2015). Technical replicates attempt to isolate variation arising from a single process. By repeatedly analysing a pooled lipid extract interspersed with samples, variation from instrumentation can be isolated. Systematic issues such as HPLC column degradation or progressive loss of sensitivity in the mass spectrometer are identified by assessing variability between technical replicate samples (Kamleh, Ebbels, Spagou, Masson, & Want, 2012). An increase in systematic variation contributes to non-biological variance and reduces power to identify important features in the resulting dataset.

2.5 Challenges with lipidomics datasets

Data processing and interpretation can represent the most challenging and time-consuming aspects in lipidomic experiments. Lipidomics has advanced over the past decade and now it is routine to measure the abundance of several hundred lipids within a single run. In addition, the sensitivity of mass spectrometers has increased, allowed the detection of lipids over many orders of magnitude. While the depth of coverage is appealing to biomedical scientists, statistical and bioinformatic strategies need to be developed to cope with the specifics of the data generated. Several challenges need to be addressed when dealing with lipidomic data which, while not exhaustive, include:

- (1) Large 'p' small 'n'. In principle, this issue occurs whenever there are more predictors (p) than samples (n) i.e. $p > n$. The 'large p small n ' issue is one of the most frequently encountered problems with wide datasets. As is frequent in many lipidomic studies the number of predictors far exceeds the number of samples ($p \gg n$). While not immediately obvious why this may be an issue to those unfamiliar with the methodological approach, it poses large problems for statistical analysis (Rubingh et al., 2006). In fact, many of the commonly used statistical procedures fail with large datasets generated by lipidomics because no unique solution exists.

- (2) Dimensionality. The challenge of dimensionality is related to the 'large p small n ' problem, in that data with high-dimensions (many predictors and samples) can be computationally expensive to process. The time taken to process data with some algorithms can scale with the number of predictors squared or worse. Therefore, even with access to powerful computers, the processing time required can be prohibitive. Furthermore, high dimensional data can lead to non-convex solution spaces in many algorithms, causing premature stopping in local optima but not global ones (Floudas & Gounaris, 2009).

- (3) Multicollinearity. Multicollinearity is a phenomenon in which predictors are highly correlated with each other. In linear regression models, multicollinearity causes β -coefficients to become unstable and small amounts of noise can cause large changes in coefficient estimates (Kaduk et al., 2014). Unfortunately, this is commonly observed in lipidomic datasets, as the large variety of lipid species are all products of common biosynthetic pathways and precursors (Wong, Chan, Kingwell, Leckie, & Meikle, 2014). Therefore, robust methods capable of handling multicollinearity are important for lipidomic data analysis (Kaduk et al., 2014).

- (4) Concentrations differing by orders of magnitude. Due to the sensitivity of mass spectrometers, the concentrations of lipids can be measured over many orders of magnitude. From a scientist's point of view this is advantageous as lipids with a high concentration are not necessarily more or less important

than lipids present at low concentrations, and vice versa. Unfortunately, multivariate statistical techniques can overemphasize higher abundance lipids due to their, generally, higher variance (van den Berg, Hoefsloot, Westerhuis, Smilde, & van der Werf, 2006) – which leads the potential confounder of heteroscedasticity.

- (5) Heteroscedasticity. Many statistical techniques rely on the assumption of equal variances. Heteroscedasticity occurs when variance (or standard deviation) scales with the absolute value of the measurement i.e. increasing the abundance also increases the standard deviation. This is frequently observed in lipidomics datasets, particularly over the orders of magnitude differences in lipid concentrations measured. This can result from physiological biological variability or as a consequence of measuring lipids with a mass spectrometer – fundamentally a particle counter subject to poisson noise (Du et al., 2008).

The above highlight several of the challenges when working with lipidomic datasets. While there are indeed numerous ways of overcoming these challenges, they often are highly constrained to the type of biological questions a researcher would like to ask. Furthermore, the outcome and biological interpretation can be impacted by decisions made during the modelling process (van den Berg et al., 2006).

2.6 Statistical procedures in lipidomics research

2.6.1 Univariate statistical tests

One of the most common statistical methods using in the analysis of lipidomics data is the comparison of means between groups/treatments. This is typically accomplished through consideration of one variable at a time i.e. univariate analysis. Most frequently, the parametric independent samples t-test is used to compare means, due to it's easy of use and power (Sawilowsky, 2005). It should be

noted that there are several assumptions associated with this test, including the assumption of normally distributed data and the assumption of homogeneity of variances. Assumption of normality can be assessed using Shapiro-Wilk's test, Kolmogorov-Smirnov's test, Anderson-Darling's test or many others (Anderson & Darling, 1954; Lilliefors, 1967; Shapiro & Wilk, 1965). In the case of non-normally distributed data, transformation of data can be used – however, caution should be used when transforming data (Giles, Albrecht, Lam, Takechi, & Mamo, 2016). Assumption of homogeneity of variance is commonly tested using Levene's test or Browne-Forsythe test (Brown & Forsythe, 1974; Levene, 1960). Welch's t-test is an alternative to the traditional t-test that does not assume equality of variances (Welch, 1947). However, alternatives to parametric statistical tests exist and include the family of non-parametric tests. With less assumptions on the distribution of the data, non-parametric tests can show improved power with non-normally distributed data (Giles, Albrecht, et al., 2016). One of the most popular is the Mann-Whitney U test (Mann & Whitney, 1947).

For situations where there are more than two groups that researchers wish to compare, the analysis of variance (ANOVA) family of statistical tests are commonly utilised. In one of the simplest forms, the one-way ANOVA is formulated such that the null hypothesis is that all groups are random samples of the same population. Therefore, a true alternative hypothesis is that at least one sample is sufficiently different. In order to investigate which group this may represent, post-hoc tests are conducted following a rejection of the null hypothesis in an ANOVA test. A number of post-hoc tests are utilised, with some correcting for multiple comparisons error and others not (Dunnett, 1955; Tukey, 1949). Non-parametric alternatives to the ANOVA exist, such as the Kruskal-Wallis one-way ANOVA (Kruskal & Wallis, 1952).

2.6.2 Multiple testing correction

When performing many statistical tests, such as applying univariate tests to each lipid individually across a lipidomics dataset, the total probability of observing

false positives rises above the typically selected 5 % level ($\alpha = 0.05$). That is, the expectation of observing a false positive is the number of statistical tests performed multiplied by 0.05. For example, in a large lipidomics datasets consisting of 300 lipids measured across two groups, it is expected to observe statistically significant results (rejecting the null hypothesis) in 15 lipids which did not actually change (false positive). To address this issue, several approaches have been developed to account for multiple testing, the most common methods are family-wise error rate (FWER) correction and false discovery rate (FDR) correction (Krzywinski & Altman, 2014).

Family-wise error rate is the probability of making a type 1 error – false positive. Most biological scientists are familiar with at least some of the corrective procedures, which include the Bonferroni procedure, Tukey's procedure, Sidak's procedure and Dunnett's correction among others (Dunn, 1961; Dunnett, 1955; Tukey, 1949). These methods control the rate of false positives when conducting multiple hypothesis testing. However, controlling the false positive rate often comes at the expense of power, thereby greatly increasing the number of false negatives (type 2 error) (Krzywinski & Altman, 2014). In application to large datasets, the threshold for rejecting the null hypothesis becomes increasingly stringent. The simplest method, the Bonferroni correction, reduces the threshold for significance by dividing α by the number of tests performed (Dunn, 1961). In our hypothetical experiment above, the p-value threshold for significance is reduced to 0.000167 (0.05/300). Using methods to control FWER in lipidomics datasets can be overly conservative and lead to very inefficient study designs. In most cases, we are willing to accept a certain number of false positives. This is where methods that control the false discover rate offer advantages.

The false positive rate is defined as the number of positive results reported, that are actually false. Methods that control the false discovery rate attempt to keep the proportion of false positives low in comparison to the number of true positives (Storey, 2011). While this distinction may appear subtle, these methods have been shown to provide more power than FWER methods. The first FDR method described by Benjamini and Hochberg (1995b) has become a staple method in many fields of

biomedical science due to adoption of high-throughput technologies (Benjamini & Hochberg, 1995b).

2.6.3 Regression methods

Regression models offer the ability to use a single, or multiple, predictor variables to explain the variance in a dependent (outcome) variable. Interestingly, standard linear regression models are rarely used in lipidomic studies. This is likely due to the experimental designs commonly utilised. Rather, one of the most commonly used methods in the generation of regression models using lipidomics datasets is binary logistic regression (Meikle et al., 2011). Binary logistic regression is a statistical model where the dependent variable is categorical and can take on either one of two states i.e. healthy vs diseased. Lipid species are used as independent predictors, often alongside covariates such as age, sex, BMI etc. The resulting coefficients provide a measure through which the lipid species are associated with the outcome. In brief, logistic regression relates the log odds of the probability of the outcome to a linear combination of the predictor variables (Harrell, 2015). Quite often, researchers construct many logistic regression models, where each lipid is considered one at a time (Meikle et al., 2011; Proitsi et al., 2015; Weir et al., 2013b). The repeated application of logistic regression is still susceptible to multiple testing error and therefore correction is required. Furthermore, due to the challenges outlined above, when a greater number of predictors are included than subjects, a solution cannot be obtained without a form of regularization applied (Bickel et al., 2006).

Using lipid species as predictor variables is common. Whereas, it is rare to assign a lipid specie as a dependent variable (with the exception of testing for mean differences). Indeed, few studies have attempted to generate regression models explaining variation in lipidomic datasets.

2.6.4 Regularization methods

When there are a larger number of predictors than observations, multi-variable regression techniques do not work (Bickel et al., 2006). The matrix inversion in ordinary least squares solutions do not have a unique solution (ill-posed problem), therefore the algorithm fails. Regularization offers an attractive solution to this problem, as the addition of a regularizer term allows solutions to be attained, at the expense of a small amount of bias (Tibshirani, 1996). Regularization has been shown to reduce prediction errors, compared to unregularized models. In non-regularized models, beta coefficients are unconstrained and can easily multiply in value and hence, are susceptible to very high variance. If we impose a level of constraint of these coefficients i.e. limiting how large they can get, we are subject to a level of bias however we achieve a substantially reduced variance. The result is a model that can have smaller prediction error than a typical ordinary least squares regression and higher generalizability to other datasets.

Two regularization procedures are generally considered: least absolute shrinkage and selection operator (LASSO) (Tibshirani, 1996) and Tikhonov (L_2) (Le Cessie & Van Houwelingen, 1992) regularization. It is much more common for LASSO to be utilised in lipidomics studies due to perceived benefits as a ‘feature selector’. Incorporation of LASSO causes coefficients to ‘shrink’ and forces certain coefficients to equal zero. This effectively removes their influence, leading to a sparse/simpler model. However, model coefficients can vary significantly when the number of observations are small and correlated features are present. If we wish to derive information from model coefficients, the instability of LASSO estimates is an undesirable feature. By contrast, L_2 regularization does not perform any variable selection, but instead shrinks all coefficients towards zero without explicitly setting any to zero. This procedure allows regression to occur when the number of predictors exceed the number of samples and when a high degree of multicollinearity is present. For the purposes of predictive models, several papers suggest it outperforms LASSO (Steyerberg, Eijkemans, Harrell, & Habbema, 2000), but at the expense of estimating a large number of parameters (non-parsimonious model). Recently, a combination of the two paradigms was developed and called the elastic net (Zou & Hastie, 2005).

Lastly, an issue with regularization is the formation of the hyperparameter, lambda (λ). Lambda controls the amount of regularization and must be specified prior to analysis. Typically, this is accomplished through a cross validation framework which is discussed below.

2.6.5 Latent Variable methods

Multivariable linear models offer the ability to regress a continuous variable on a linear sum of independent variables i.e. lipid species. Regularization procedures allow evaluation of regression models despite multicollinearity or ill-posed models ($p > n$). Extending these models to multivariable-multivariate linear models allows the simultaneous evaluation of several independent variables. While this appears to achieve the goal of regressing whole lipidomes against each other, it comes with several costs. For every variable in the dependent dataset, coefficients for every independent variable are estimated. This leads to an exponential increase in the number of coefficients estimated for larger lipidomes. In fact, for lipidomes of size 100, the number of coefficients exceed 10,000. The considerable number of coefficients complicates interpretation and leads to overfitting.

One of the most commonly used multivariate procedures used in metabolomics and lipidomics are partial least squares (PLS) methods (Geladi & Kowalski, 1986; Wold, Sjöström, & Eriksson, 2001). Often referred to as a ‘chemometric’ technique, there are several variants of PLS, including PLS-regression, PLS-discriminant analysis (PLS-DA), orthogonal-PLS and multilevel-PLS-DA (Checa, Bedia, & Jaumot, 2015; Gromski et al., 2015; Westerhuis, van Velzen, Hoefsloot, & Smilde, 2010). Partial least squares attempts to find the relations between two data matrices; the independent variables (X) and the dependent variables (Y). To achieve this, latent variables are introduced to model the covariance structure between the two. One of the major advantages of PLS methods is that it effectively handles data that is highly collinear and when there are more variables than samples (Chong & Jun, 2005; Gromski et al., 2015). As discussed earlier, lipidomics datasets often contain many highly collinear features and

hundreds/thousands of variables. Traditional regression techniques fail in these cases, unless regularization techniques are introduced.

Construction of a PLS model involves the iterative formation of latent structures that capture data from many predictors in order to maximally explain variance in the Y matrix. As is often the case, a substantial proportion of variance can be explained by just a few latent components. This achieves a form of dimensionality reduction, not unlike principle component analysis. Using the constructed model, loading coefficients can be used to explore the importance of variables in the model (Afanador, Tran, & Buydens, 2013; Kvalheim & Karstang, 1989). Loading and score plots are often used to investigate the relationships between variables and groups. Additional methods of determining variable importance have also been suggested, such as variable importance on projection, S-S plots and bootstrapped loading coefficients (Afanador et al., 2013; Mehmood, Liland, Snipen, & Sæbø, 2012).

While the properties of PLS make it an attractive method for lipidomics analysis, there are important caveats. A PLS model can be constructed for any dataset, however, unlike other statistical procedures, there is no implicit measure of statistical significance. Indeed, it is common to see ‘goodness-of-fit’ measures (R^2 , Q^2) quoted as if to describe a ‘significance’ to a model (Szymanska, Saccenti, Smilde, & Westerhuis, 2012). Furthermore, it is common to read ‘clear separation between groups’ as the only performance measure when PLS-DA is used. This is one of the most concerning issues with use of PLS based methods. However, this is an issue with how the methods are used, rather than the technique itself. Indeed, validation can be accomplished using double cross validation (single cross validation is not sufficient) and statistical significance can be formalised through permutation tests. Neither of these techniques are commonly used in lipidomic research, or are used in an insufficient context.

2.7 Procedures in predictive modelling

Predictive modelling encompasses traditional statistical procedures with several principles that differ from explanatory modelling. A key difference is the models derived between these two paradigms are the predictive models are generally parsimonious, less optimistic and are not overfit (Shmueli, 2010). The advantage is that models generated may be more generalizable and applicable to datasets which the models were not created (Bleeker et al., 2003; Ivanescu et al., 2016).

2.7.1 Cross validation

Cross validation (CV) is often recommended as a method of assessing the predictive value of a statistical model. In brief, CV involves splitting a dataset into training and testing sets, using the training set to construct a model and assessing the performance on the testing set (**Figure 8**). In principle, CV provides an assessment of a model's performance on data that it has not been trained on – that is, how well does the model generalize to new data (Ivanescu et al., 2016). This powerful idea is one of the key principles underpinning predictive modelling and highly used in the field of machine learning (Shmueli, 2010).

Despite the simple idea of CV, complexity arises in alternative implementations of CV. The simplest approach to devising train/test splits is the 'leave-one-out' CV, where a single observation is predicted from a model generated from all other observations. If the number of observations is n , then n models are created from $n-1$ observations and the mean performance calculated over all models. Generalizing this approach, the dataset can be divided into k "folds" of equal size, where each fold is used as the testing set once. In the simplest approach, leave-one-out CV is k -folds CV where $k=n$. Simulation studies have suggested that higher numbers of k results in lower variance in the estimates of model performance at the expense of computational time (Steyerberg et al., 2001b).

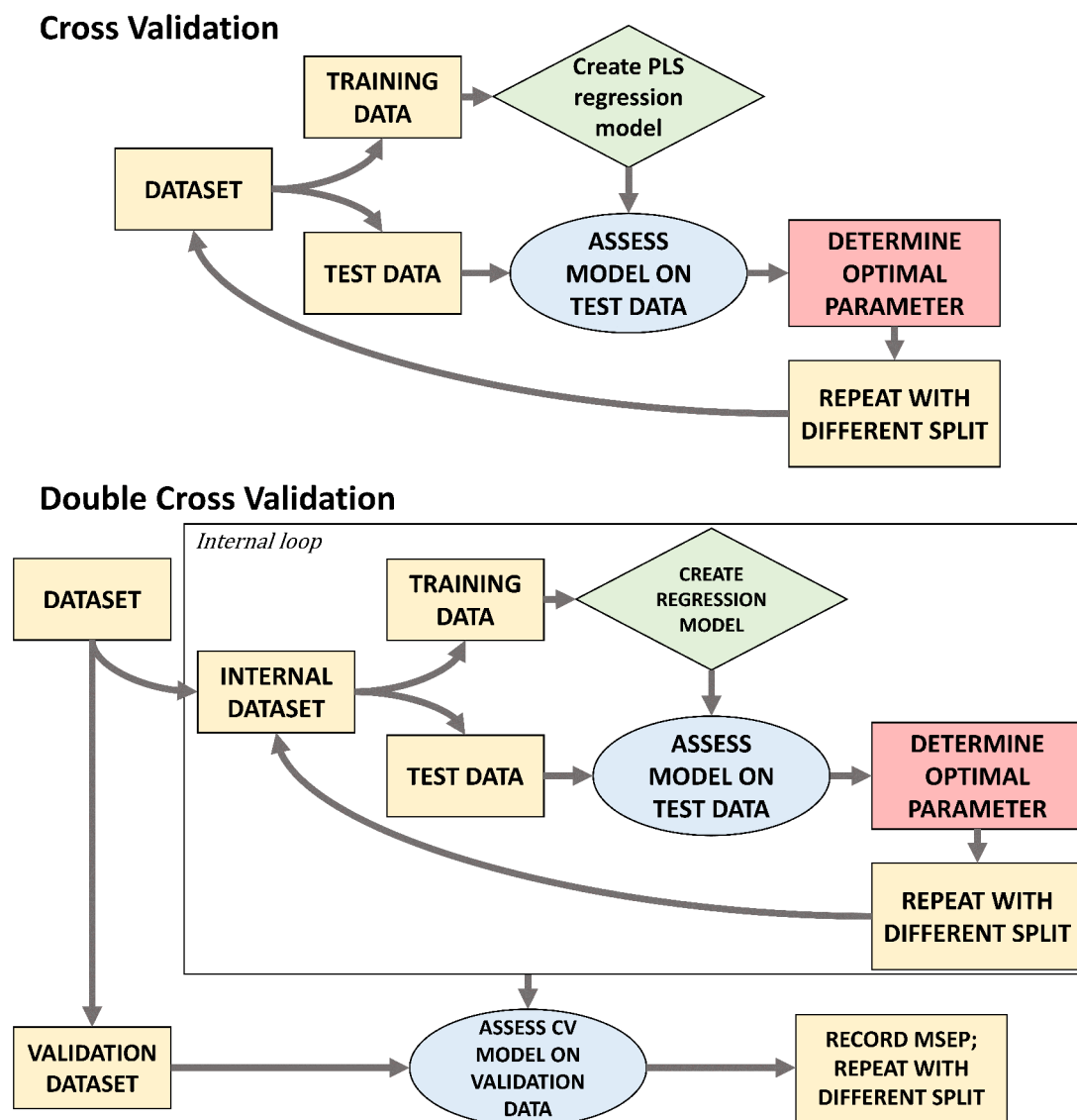


Figure 8. Schematic of cross validation and double cross validation procedures. *Top*, cross validation involves splitting data into test and train sets. A model is constructed on the train dataset and performance assessed on the test dataset. An error metric can be recorded or a hyperparameter can be determined (not both). *Bottom*, double cross validation allows optimization of hyperparameters and unbiased performance to be assessed.

Several measures of model performance can be assessed using CV, depending on the model used. In a regression context, we are principally interested in measuring the predictive accuracy of the model on a continuous outcome variable.

Therefore, mean squared error of prediction (MSEP) is a suitable performance metric to measure. The MSEP is the average value of the squared difference between the real value (test set) and the predicted value from the model. Small values of MSEP indicate a model with good predictive accuracy. Larger values suggest worse predictive accuracy and can indicate several issues, including overfitting or insufficient ability to capture underlying trends in the data.

2.7.1.1 Hyper parameters and double cross validation

Cross validation is not only used to measure the predictive accuracy of models. The CV framework also functions as a systematic way to determine the optimal value of hyperparameters. Hyperparameters are values that are specified for a statistical test, but are not specifically optimized during model creation. The classic example is the determination of λ in regularization procedures. In both LASSO and Ridge regression, the amount of regularization is continuous with λ , therefore there is a unique model for every value of λ . To determine the ‘optimal’ value of λ , CV is performed and λ tuned to provide the lowest MSEP. A similar approach can be used in latent variable models, such as PLS, to determine the optimal number of latent components. Using this approach with PLS prevents overfitting, compared with other *ad hoc* approaches.

It should be stated that using CV for hyper-parameter optimization is not the same as determining predictive accuracy using CV (Ambroise & McLachlan, 2002; Steyerberg, Bleeker, Moll, Grobbee, & Moons, 2003a). Indeed, stating the “predictive accuracy” of a model which was chosen as the best among many alternatives is not a reflection of the model’s true predictive ability. Unfortunately, this distinction is not often realised (Ambroise & McLachlan, 2002). The valid method is to use double CV, where an internal CV is performed within each iteration of an external CV (**Figure 8**) (Steyerberg et al., 2003a). Within each internal CV, the optimal number of latent components is determined and the predictive ability assessed at the external CV stage. Unfortunately, this procedure has not been readily taken up and many statistical packages do not offer such solutions.

Code to perform double CV in the statistical programming language R has been included in Appendix II.

2.7.2 Permutation testing

Permutation tests consist of a formalised approach to deriving statistical tests when traditional tests are not available. This approach constitutes a non-parametric statistical test by explicitly comparing a model against an appropriately generated distribution of null hypothesis test statistics. Through disrupting the association between independent and dependent variables, the possible values of the null hypothesis test statistic can be determined. By approximating the null distribution of the test statistic, the significance of the model can be determined. As PLS methods do not have an inherent statistical testing procedure, we can utilize permutation testing to determine the significance of a model (**Figure 9**).

Permutation tests are particularly relevant for PLS models. A common criticism of PLS-DA models is that two arbitrarily designated groups can be separated given a sufficient number of randomly generated variables. Indeed, larger numbers of random variables allows greater separation. This is often considered a negative trait of PLS methods. However, it shows the power of PLS models in finding patterns in data. To determine whether a model is obtained by chance (no association between X and Y matrices; null hypothesis), the true model must be able to perform better than data with no association between the X and Y matrices i.e. permuted data. This intrinsically incorporates a penalty for highly complex models by increasing the performance threshold when many variables are being used. Therefore, permutation tests for PLS models are guaranteed to have a consistent false positive rate, regardless of the number of variables or samples.

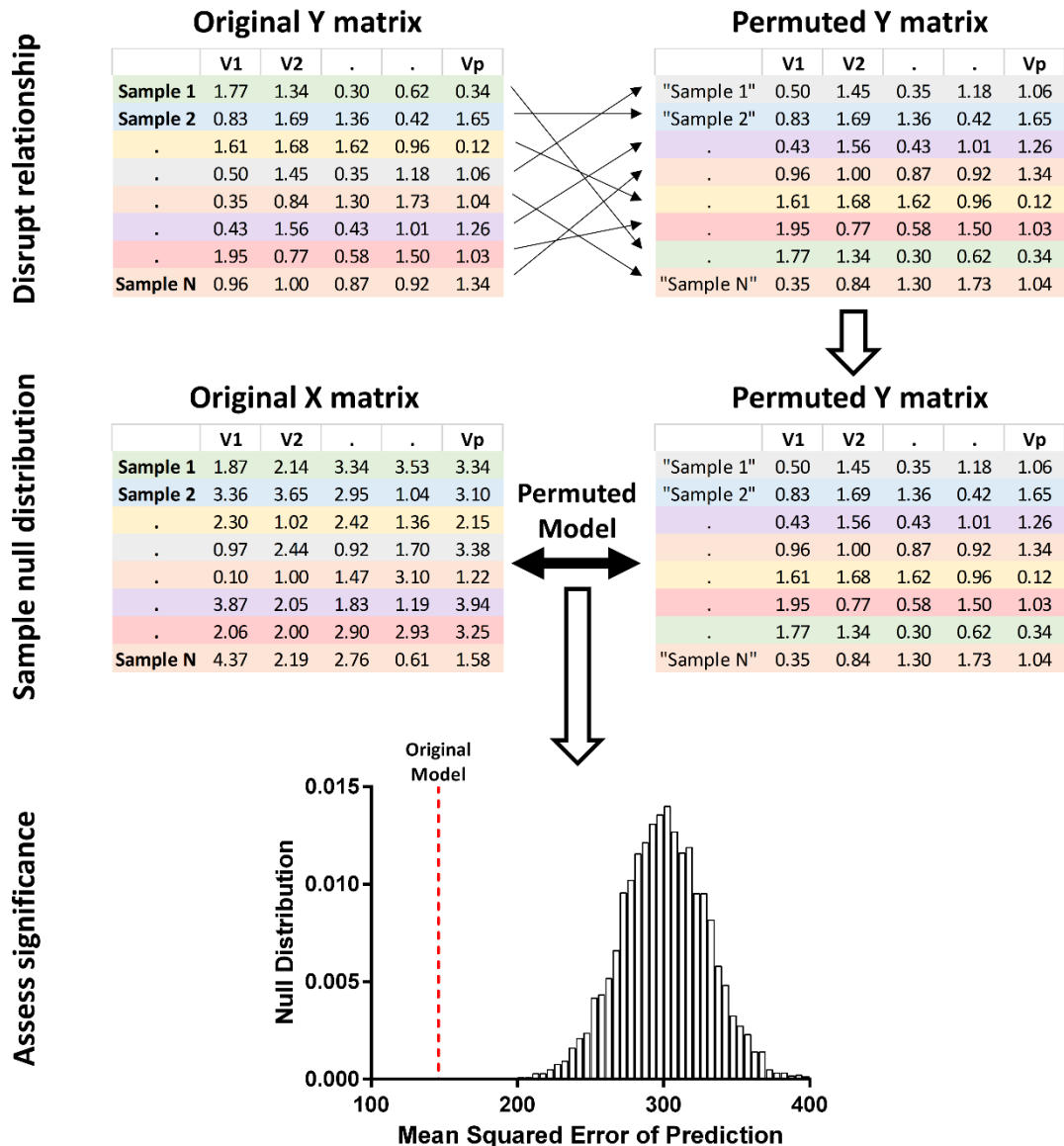


Figure 9. Construction of a permuted dataset and assessment of performance. *Top*, a permuted Y matrix is generated by randomising row order. *Middle*, a model is constructed using the original X matrix and the permuted Y matrix. Due to the randomised row order, any associations between X and Y matrices are due to chance. *Bottom*, the performance of the permuted models is determined across many permutations. The original model performance is assessed against the distribution of permuted model performances.

Two common problems are evident in the literature with the use of permutation tests. (1) An inappropriate null hypothesis is tested. For a permutation test to accurately reflect the null distribution of the test statistic, the entire statistical modelling procedure must be replicated, including variable selection and/or cross validation (Steyerberg, Bleeker, Moll, Grobbee, & Moons, 2003b; Steyerberg et al., 2001a). Omitting these steps leads to optimistic estimates of model significance (Harrell, 2015). (2) A single, or too few permutations are conducted. An adequate number of permutations is required to approximate the null distribution of the test statistic. Minimum number of permutations relates to the accuracy of p-value one wishes to attain. However, for complex models involving variable selection or double CV, the computational time required to perform many permutations can be limiting.

Code to perform permutation testing of PLS models in the statistical programming language R has been included in Appendix II.

2.8 Latent variable perspective of the interaction between plasma and brain lipidomes

As discussed previously, lipidomics datasets contain numerous correlated variables. Lipid species become correlated due to common biosynthesis pathways and precursors. Indeed, the correlation within lipidomes encode biological information. However, this correlation is often ignored. The latter is possibly due to difficulties in analysis and interpretation, but also due to lack of exposure of alternative procedures in the field of lipidomics. While traditional statistical procedures encourage removal of correlated features, PLS can leverage this covariance structure.

To consider potential changes in the cerebral lipidome as a result of circulating lipids, the data analysis approach presented in this thesis has been designed around putative physiological mechanisms. Therefore, the relationship between circulating and cerebral lipids is modelled as a latent variable model

(**Figure 10**). The independent variables consist of plasma lipids and the dependent variables are the cerebral lipids. Connecting the two sets are several latent components. The latent variables model the covariance structure between the two variable sets. In a biological perspective, the latent variables represent the transport of lipids in the brain; uptake and metabolism by the BBB; remodelling of lipids within the brain. This unique approach implies the latent variables have biological meaning and the loadings attached represent the importance of a lipid to that feature. Indeed, the use of PLS which incorporates covariance between lipids is particularly appropriate for the hypothesis central to this thesis.

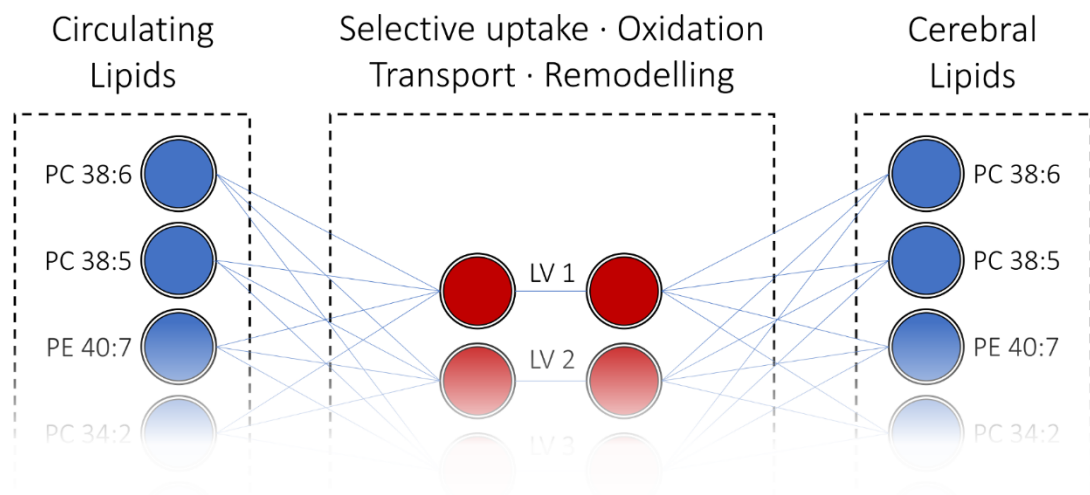


Figure 10. Latent variable model of the interaction between circulating and cerebral lipids. *Left*, circulating lipids are assigned loadings to each latent variable. *Middle*, latent variables represent physiological processes involved in coupling circulating and cerebral lipids. *Right*, cerebral lipids reflect the combined effect of circulating lipids and the metabolic processes involved in uptake, transport and remodelling.

2.9 Conclusions

There are few analytical approaches for comprehensive analysis of lipidomes. However, considerable methodological considerations are required given the large combinatorial options in instrument configuration, sample preparation and approach to statistical analysis. Indeed, each approach offers a compromise in sensitivity, specificity, accuracy, number of lipids analysed and time for sample throughput. In these studies, in order to obtain an unbiased extraction of lipids, single phase chloroform:methanol (2:1 v/v) extractions were utilized. For quantitative lipidomics, HPLC-ESI-MS/MS using triple quadrupole mass spectrometers was chosen as the preferred analytical approach. These instruments provide high sensitivity and large dynamic ranges which are critical in measuring lipids in biological systems. Furthermore, high specificity of quantitation could be accomplished through scheduled multiple reaction monitoring of established precursor/product transitions and retention time. To explore associations between the plasma cerebral lipidomes, PLS regression models were constructed. The number of latent components were determined through internal CV, while predictive accuracy was determined in the outer CV. Formalised regression significance was computed through permutation tests, incorporating the double CV procedure. The well-considered and justified approach indicated, encompass a coordinated approach to examine the thesis hypothesis and achieve the thesis objectives. Methodological details are provided in the relevant publications presented.

Chapter 3

Chapter 3: Investigation into the effects of long term consumption of saturated fat enriched diets on the brain lipidome

The content of this chapter is covered by Publication 1:

Giles, C., Takechi, R., Mellett, N. A., Meikle, P. J., Dhaliwal, S., & Mamo, J. C. (2016). The Effects of Long-Term Saturated Fat Enriched Diets on the Brain Lipidome. *PLoS One*, 11(12). doi:10.1371/journal.pone.0166964

3.1 Article synopsis

3.1.1 Background

Epidemiological studies have suggested diets high in saturated fats are associated with increased risk of neurodegenerative diseases, such as Alzheimer's Disease. Furthermore, dysregulation of the cerebral lipidome has been documented at every stage of the disease. However, it is not known whether these changes are causally related with the onset and progression of the disease or a consequence of the disease pathology. Indeed, the factors that regulate the cerebral lipidome in physiological systems are still largely unknown.

As discussed in Chapter 1, the brain relies on circulating lipids for the formation of new lipids and replacement of lost lipids. Pathological modulation of the cerebral lipidome has been demonstrated in severe dietary models, yet little is known about changes in the plasma and cerebral lipidomes for otherwise well tolerated diets. Despite this mechanistic insight, no previous studies had investigated associations between the blood and brain lipidomes, within the same subjects.

To investigate the ability of well tolerated diets to influence both the plasma and brain lipidomes and the associations between them, animal models were used.

3.1.2 Experimental design in brief

Wild-type C57Bl/6J mice were randomised to receive either a standard rodent chow (4 % w/w fat) or a saturated fat enriched diet (20 % w/w fat). Following six months on their respective diets, the hippocampus, cerebral cortex and plasma were collected. Lipids were extracted by single-phase chloroform/methanol (2:1) extraction and analysed by a state of the art lipidomics platform. Separation of lipids was performed by reverse-phase HPLC, followed by electrospray ionisation in positive mode and identification with tandem mass spectrometry. The abundance of lipid classes and individual species were compared between mice on either diet. Further, multivariate predictive models were developed to find plasma lipids jointly and independently associated with changes in the cerebral lipidomes.

3.1.3 Results in brief

Following six months on their respective diets, mice fed the saturated fat enriched diet maintained similar total abundance of lipid subclasses in both regions of the brain, despite substantial changes in plasma lipids. Comparison of individual lipid species in the brain revealed changes to the acyl composition within the lipid subclasses PC, PE, PC(O), PC(P), PE(O), PE(P), CE, DG, PI and PS. Multivariate predictive models revealed strong associations between plasma lipids and both HPF and CTX lipids.

3.1.4 Discussion and conclusion in brief

This study explored how long-term consumption of saturated fat enriched diets affects the cerebral lipidome. In the hippocampus and cerebral cortex, substantial alterations in the acyl chain composition of numerous lipid classes was observed. Long term consumption of saturated fat enriched diet was associated with a reduction in highly unsaturated phospholipids (6 or 7 double bonds) and an

increase in phospholipids containing a total of 4 or 5 double bonds. It would be expected that this alteration results in a considerably reduced membrane fluidity, affecting synaptic plasticity and neural activity.

Similar patterns of lipid change were observed in both regions of the brain, suggesting a common causal mechanism. It was shown that the multivariate predictive models, using the plasma lipidome, accounted for substantial changes in the brain lipidome. Collectively, these results strengthen the suggestion that plasma lipids are causally related to changes in the brain lipidome. This is the first time such an approach has been conducted, whereby in-depth lipidomics of both plasma and brain was collected in the same subject and associations between the two rigorously explored. The information gained from this study provides insight into the major plasma lipid species that influence cerebral lipid homeostasis.

RESEARCH ARTICLE

The Effects of Long-Term Saturated Fat Enriched Diets on the Brain Lipidome

Corey Giles^{1,2}, Ryusuke Takechi^{1,2}, Natalie A. Mellett³, Peter J. Meikle³, Satvinder Dhaliwal², John C. Mamo^{1,2*}

1 Curtin Health Innovation Research Institute, Curtin University, Perth, Western Australia, Australia, **2** School of Public Health, Faculty of Health Sciences, Curtin University, Perth, Western Australia, Australia, **3** Metabolomics Laboratory, Baker IDI Heart and Diabetes Institute, Melbourne, Victoria, Australia

* J.Mamo@curtin.edu.au



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Abstract

The brain is highly enriched in lipids, where they influence neurotransmission, synaptic plasticity and inflammation. Non-pathological modulation of the brain lipidome has not been previously reported and few studies have investigated the interplay between plasma lipid homeostasis relative to cerebral lipids. This study explored whether changes in plasma lipids induced by chronic consumption of a well-tolerated diet enriched in saturated fatty acids (SFA) was associated with parallel changes in cerebral lipid homeostasis. Male C57Bl/6 mice were fed regular chow or the SFA diet for six months. Plasma, hippocampus (HPF) and cerebral cortex (CTX) lipids were analysed by LC-ESI-MS/MS. A total of 348 lipid species were determined, comprising 25 lipid classes. The general abundance of HPF and CTX lipids was comparable in SFA fed mice versus controls, despite substantial differences in plasma lipid-class abundance. However, significant differences in 50 specific lipid species were identified as a consequence of SFA treatment, restricted to phosphatidylcholine (PC), phosphatidylethanolamine (PE), alkyl-PC, alkenyl-PC, alkyl-PE, alkenyl-PE, cholesterol ester (CE), diacylglycerol (DG), phosphatidylinositol (PI) and phosphatidylserine (PS) classes. Partial least squares regression of the HPF/CTX lipidome versus plasma lipidome revealed the plasma lipidome could account for a substantial proportion of variation. The findings demonstrate that cerebral abundance of specific lipid species is strongly associated with plasma lipid homeostasis.

Introduction

The brain is highly enriched in lipids, supporting structural, biochemical and cell signalling functions [1]. Bioactive lipids within the brain are shown to be pivotal for central nervous system homeostasis by modulating neurotransmission, synaptic plasticity, enzyme function, ion channel activities, gene expression and inflammation [2–4]. Changes in cerebral lipid homeostasis are widely reported to be associated with neurodegenerative disorders, and several studies also suggest significant changes in brain lipids with non-pathological ageing [5–10].

The regulation of the cerebral lipidome is poorly understood. Some studies suggest that the isolated organ status of brain limits substantial shifts in brain lipid homeostasis. However,

longer-term feeding studies (8–12 weeks duration) in animal models with fat formulated diets suggest a cerebral response within the brain lipidome [11–14]. With severe dietary n-3 fatty acid deficiency, several laboratories reported changes in the phospholipid composition of the brain [15–18]. In other studies, Rabiei et al. (2013) reported in a rodent model of stroke, that pre-treatment with dietary virgin olive oil influenced the brain lipidome in a dose dependent manner [19]. Such observations demonstrate that the brain lipidome is at least partially modulated as a consequence of changes in peripheral or dietary lipids [11, 20, 21].

The functional properties of brain capillaries ordinarily strictly regulate kinetics of plasma macromolecules such as lipoproteins between blood and brain [22]. Characterized by tightly apposed junctional proteins between adjacent capillary endothelial cells, non-specific intercellular kinetics across the capillary plasma membrane is normally restricted. However, the brain can rapidly take up free fatty acids from circulation and cerebral endothelial cells express lipases which can hydrolyse lipoprotein associated lipids for uptake. Other potential blood-to-brain lipid kinetic pathway includes transcytotic vesicles forming on the plasma membrane of capillary endothelia with extrusion occurring on the subluminal basolateral membrane [23]. Moreover, attenuated expression of endothelial junctional proteins may result in greater trans-endothelial rates of molecular transport. This phenomenon has been described in neurological disorders [24] and also in brain capillaries of aged rodent models [25, 26]. The latter was reported to be exacerbated by high fat feeding with diets enriched in saturated fats or cholesterol [27].

Chronic ingestion of Western styled diets enriched in saturated fats are causally associated with a range of neurodegenerative disorders including vascular dementia and Alzheimer's disease [28, 29]. The mechanisms underpinning this association are not completely understood but broadly include capillary dysfunction; neurovascular inflammation; altered redox state and heightened oxidative stress [30–33]. With chronic ingestion of pro-inflammatory fat enriched diets, it is a reasonable proposition to suggest that changes of the brain lipidome may be realized and causally associated with some of the indicated mechanistic pathways. Cerebral sequelae that may reflect changes in the brain lipidome following long-term consumption of high-fat diets may be broad and include compromised insulin signalling [21, 34], neuronal apoptosis [12], and poorer cognitive performance [35, 36].

The rapid development of lipidomics provides an unprecedented opportunity to consider the brain lipidome in the context of central nervous system function. Lipidomic studies show marked differences in brain lipid species in several neurodegenerative disorders [7, 8, 37–39], however it is unclear whether these changes are causally associated with the onset or progression of said disorders. The hippocampal formation (HPF) is central to episodic and associative memory and significantly compromised in cognitively deficient subjects [40]. The cerebral cortex contribute to executive functioning and semantic functioning [41]. To consider the putative changes within the brain lipidome in the physiological context of dietary behaviour, this study comprehensively explores cerebral lipid homeostasis in otherwise healthy wild-type mice maintained on ordinary chow, or a diet enriched with saturated fat (SFA) for six months. In addition, we consider the potential of the plasma lipidome to explain the variance observed in the HPF and CTX lipidomes.

Methods

Animals

All experimental procedures were approved by the Curtin University Animal Ethics Committee and conducted in accordance with National Health and Medical Research Council (NHMRC) guidelines. Twenty C57BL/6 mice were obtained from the Animal Resource

Centre (ARC; Murdoch). They were housed in an accredited animal holding facility with 12 hour light/dark cycles, at ambient temperature of 22 degrees Celsius. Mice had ad libitum access to water and their respective diets. At 8 weeks of age, mice were randomly assigned to one of two groups, receiving either AIN-93M control diet (9% energy from canola oil), or a modified AIN-93M chow containing 40% energy from cocoa butter (Glen Forrest Stock Feeds). Table 1 indicates the approximate fatty acid composition of the diets and quantity of fatty acids consumed per kilogram of diet.

Sample isolation and preparation

Mice were maintained on their respective diets for six months. One mouse was removed from the study prior to completion due to health issues unrelated to the study design. Mice were administered an intraperitoneal dose of pentobarbital and following complete anaesthesia, blood was collected through cardiac puncture into EDTA containing tubes. Plasma was separated through centrifugation and frozen at -80 degrees Celsius for further analysis. Following exsanguination, brains were rapidly excised, washed in ice-cold PBS and left hemispheres snap frozen in liquid nitrogen. Using a commercial brain block, the left frozen hemispheres were sectioned into 1 mm coronal slices. Under a stereotaxic microscope, sections of the S2 cerebral cortex and hippocampus were isolated and weights recorded. Isolated regions were diluted in 10 volumes of ice cold phosphate buffered saline (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl; pH 7.4), homogenized and frozen for further analysis.

Extraction of lipids

The extraction of lipids was performed by a single phase chloroform:methanol extraction as described previously [42]. In brief, 10 uL of plasma or 10 uL of brain homogenate (containing approximately 20 ug of protein) was transferred to an eppendorf tube with 10 uL of internal standard mixture [42]. The internal standard mix included lipid species from the lipid classes

Table 1. Lipid composition of regular chow and saturated fat enriched chow.

	Fatty acid composition (%)		Quantity per kg (g/kg)	
	Control diet	High fat diet	Control diet	High fat diet
C12 or less	0.2	4.7	0.1	9.6
C14:0	0.2	0.2	0.1	0.5
C16:0	4.4	24.4	1.7	49.7
C18:0	2.1	34.5	0.8	70.5
C20:0	0.5	1.1	0.2	2.3
C16:1	0.1	0.2	0.04	0.5
C18:1	60.6	31.3	24	64
C20:1	1.0	0.0	0.4	0
C18:2 n6	20	3.2	7.9	6.5
C18:3 n3	9.5	0.2	3.8	0.5
SFA	7.1	63.7	2.8	129.9
MUFA	62.3	32.8	24.9	66.9
PUFA	30.6	3.5	12.3	7.2

Approximate fatty acid composition of regular AIN-93M chow and saturated fat enriched chow used in this study. Percentage of fatty acid composition is relative to total fat content (4% w/w for regular chow and 20% w/w for SFA enriched chow). Accounting for difference in total fat content, the quantity of fatty acids in grams per kilogram of diet is shown.

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dihydroceramide (dhCer d18:0/8:0), ceramide (Cer d18:1/17:0), monohexosylceramide (MHC d18:1/16:0d3), dihexosylceramide (DHC d18:1/16:0d3), trihexosylceramide (THC d18:1/17:0), sphingomyelin (SM d18:1/12:0), phosphatidylcholine (PC 13:0/13:0), lysophosphatidylcholine (LPC 13:0), phosphatidylethanolamine (PE 17:0/17:0), lysophosphatidylethanolamine (LPE 14:0), phosphatidylserine (PS 17:0/17:0), phosphatidylglycerol (PG 17:0/17:0), cholesterol ester (CE 18:0d6), cholesterol (COHd7), diacylglycerol (DG 15:0/15:0), triacylglycerol (TG 17:0/17:0/17:0) and bis(monoacylglycerol)phosphate (BMP 14:0/14:0). Chloroform/methanol (2:1; 20 volumes) was added to each sample, followed by rotary mixing (10 minutes), sonication (30 minutes) and allowed to stand (20 minutes) at room temperature. Samples were centrifuged (16,000 x g, 10 minutes) and the supernatant collected and dried under nitrogen gas at 40°C. Samples were reconstituted with 50 μ L of water saturated butanol and sonicated (10 minutes), followed by 50 μ L methanol (with 10 mM ammonium formate). Extracts were centrifuged (3350 x g, 5 minutes) and the supernatant transferred to 0.2 mL glass vials with Teflon caps ready for analysis.

Mass spectrometric analysis of lipids

Analysis of lipids were conducted using liquid chromatography electrospray ionisation-tandem mass spectrometry on an Agilent 1200 UHPLC coupled to an AB Sciex Q/TRAP 4000 mass spectrometer with a turbo-ion spray source as comprehensively described previously [42, 43]. Liquid chromatographic separation was performed on a Zorbax C18 column (1.8 μ m, 50x2.1 mm; Agilent Technologies). Mobile phase solutions consisted of tetrahydrofuran:methanol:water (A, 30:20:50; B, 75:20:5) containing 10 mM ammonium formate. Solvent flow was set at 300 μ L/minute using the gradient: 0% B to 100% B in 8 minutes, a further 2.5 minutes at 100%, a return to 0% B over 0.5 minutes and a 3 minute hold at 0% B prior to the next injection. Diacylglycerols and triacylglycerols were separated by an isocratic flow at 85% B over 6 minutes. Briefly, detection of lipids was performed with scheduled multiple reaction monitoring (MRM) in positive ion mode using Analyst 1.5 (AB Sciex) (details in S1 Table). Precursor-product ion pairs (identified from precursor and neutral loss scans, as described previously [42]) were continuously scanned over their elution period with a 30 second window. The concentration of individual lipid species was determined by relating the integrated peak areas to the appropriate internal standards in MultiQuant 2.1 (AB Sciex). Quality control samples were included every 20 samples to assess repeatability and drift. In total, 348 lipids were detected and quantitated from each plasma, cerebral cortex and hippocampal sample.

Data analysis

Individual lipid specie concentrations were expressed as picomoles per mg wet weight for brain samples and picomoles per microliter for plasma samples. Total lipid concentration of each class was calculated by summing across individual species in that class. Pre-treatment of data included removal of lipids species below the limit of detection from the dataset. Univariate comparisons were conducted comparing lipids between the control diet fed mice against lipids observed in the saturated fat enriched diet fed group. For these comparisons, Welch's t-test was used, followed by Benjamini-Hochberg correction [44] to account for multiple comparisons.

For multivariate analysis, partial least squares regression (PLS) was used to generate a multivariate model which relates two data matrices. Using PLS, all plasma lipids are defined as predictor variables (X) and all HPF/CTX lipids as dependent variables (Y) simultaneously, in a multivariate model. The PLS model highlights the lipid species which are jointly and independently associated with the variation in the HPF/CTX. The final PLS model consists of plasma

lipid species that account for the maximum variation in all the HPF/CTX. Two PLS models were generated, a Hippocampus-Plasma regression model and a Cortex-Plasma regression model. For analysis by partial least squares regression, data was log-transformed, centred and unit variance scaled. Missing values in the datasets were imputed using the NIPALS algorithm [45]. Double cross-validated PLS models were generated and assessed for statistical significance through permutation tests [46, 47].

Formalising the regression significance was accomplished through double cross validation of permuted datasets. A single permutation was conducted by randomising the relationship between the dependent variables and independent predictors and performing double cross validation on the permuted dataset. A total of 1,000 permuted datasets were analysed for each regression model.

For the same lipid species in both the plasma and brain regions, in the PLS model, the loadings are represented as two-dimensional plots. Each axis of this two-dimensional plots represents the first latent variable (LV 1) for the plasma lipids plotted against the hippocampus/cortex lipids.

Data analysis was performed using R version 5.2 [48] and the package “mixOmics” [49].

Results

Following six months on their respective diets, the body mass of SFA fed mice was increased compared to CTRL fed mice ($p < 0.001$, 23.4 ± 0.3 vs 32.3 ± 1.5 g; CTRL vs SFA). Mice were normoglycemic (data not shown) and otherwise healthy.

Overview of lipid species analysed

A total of 348 molecular lipid species were scheduled into the MRM analysis. A total of 342 were above the limit of detection and identified in plasma, 333 in the HPF and 330 in the CTX. Fig 1A depicts in decreasing order, the molar abundance for 25 lipid classes in plasma of mice maintained on normal chow or an SFA enriched diet respectively. The most abundant lipid class in plasma of mice fed a regular chow diet was cholesterol ester, comprising 63.8% of the molar abundance of lipids. The next two most abundant lipid classes were phosphatidylcholine (PC) and lysophosphatidylcholine (LPC), comprising 19.2% and 6.8% of total plasma lipids in control fed mice respectively. Unesterified cholesterol (COH) comprised 5.9% of the molar abundance of plasma lipids in control fed mice, whilst sphingomyelin (SM) > triacylglycerol (TG) > phosphatidylinositol (PI) > phosphatidylethanolamine (PE) and lysophosphatidylethanolamine (LPE) cumulatively accounted for the remaining 4.3% of plasma lipids.

A summary of the lipid ‘species’, including the most abundant species within each lipid class reported for plasma HPF and CTX is indicated in Table 2. The lipid classes indicated contained between 1–51 individual species, differing principally in acyl-chain length or degree of unsaturation. Within some lipid classes, singular lipid species made up a significant proportion of the molar abundance. For example, the fatty acyl chain 20:4 (arachidonic acid) made up 35.7% of the molar abundance of all cholesteryl ester (CE) in the plasma of mice fed regular chow. The most diverse lipid class in plasma, HPF and CTX was PC, with 51 individual species of which the most abundant (36:2) comprised 15.3% of the molar abundance of all plasma PC species.

Provision of a diet enriched in SFA for six months was well tolerated. Absolute molar abundance of plasma lipids in mice fed SFA for each lipid class are indicated in Fig 1A. The dietary induced changes in plasma lipid classes as a proportion of that found in control mice is illustrated in Fig 2. In mice fed the SFA enriched diet, CE increased 41.8%; ($p = 0.005$) compared to control fed mice of same age. Of the neutral lipids, plasma TG was significantly decreased in

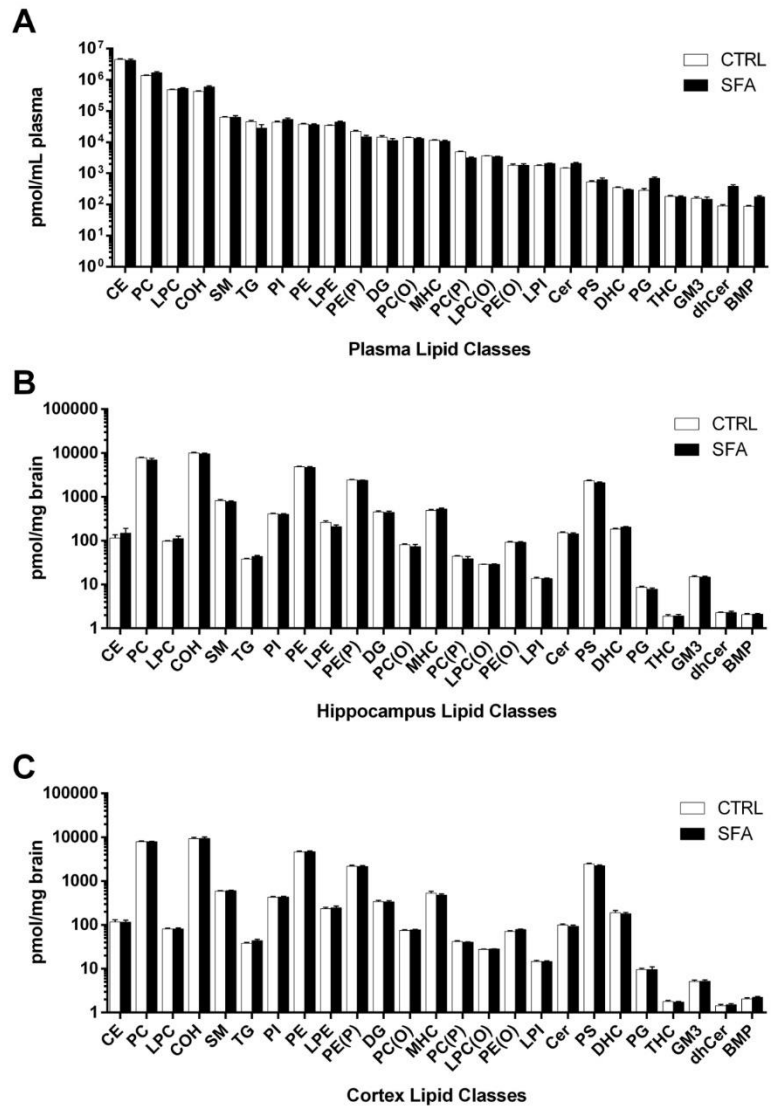


Fig 1. Molar abundance of lipid classes in plasma, hippocampus and cortex of mice fed regular chow or saturated fat enriched diet. Plasma, hippocampus and cortex was isolated from mice fed regular chow or an SFA enriched diet, following six months of feeding. Samples were analysed and lipids quantitated by LC-ESI-MS/MS. Molar sums were calculated by summing individual molar abundance for individual lipid species. A. Molar abundance for lipid classes in plasma in mice fed regular chow or a SFA enriched diet for six months. Order is decreasing abundance for regular chow fed animals. B. Molar abundance of lipid classes (pmol/mg wet weight) observed in the hippocampus of mice fed regular chow or an SFA enriched diet. C. Molar abundance of lipid classes (pmol/mg wet weight) observed in the cortex of mice fed regular chow or an SFA enriched diet. n = 9–10 per group. Mean ± SEM.

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SFA fed mice compared to controls (38%; p = 0.004). Plasma diacylglycerol (DG) and CE content did not change as a consequence of long-term SFA feeding. The abundance of several plasma phospholipid classes was significantly altered as a consequence of SFA feeding. Compared to controls, SFA fed mice had a 102% increase in plasma bis(monoacylglycerol)phosphate (BMP) (p < 0.001); a 144% increase in phosphatidylglycerol (PG) (p < 0.001) and heightened plasma PC 23.5% (p = 0.017). Of the lyso-phospholipids in plasma, in SFA fed mice there was a 15.4% increase in lysophosphatidylinositol (LPI) (p = 0.019) and a 28.9% increase in LPE (p = 0.0096). In contrast, lysoalkylphosphatidylcholine (LPC(O)) was 6.1% less in mice maintained on the SFA enriched chow diet compared to controls (p = 0.019). Significant changes in plasma alkenylphosphatidylethanolamine (PE(P)) and alkenylphosphatidylcholine (PC(P)) were identified in mice fed SFA. There was a 33.5% reduction in plasma PE

Table 2. Overview of lipid classes and most abundant species identified in mice fed regular chow.

Species Name	Abbreviation	No. of species	Plasma		HPF		CTX	
			Most abundant	% of lipid class	Most abundant	% of lipid class	Most abundant	% of lipid class
Free cholesterol	COH	1	-	-	-	-	-	-
Cholesterol ester	CE	25	20:4	35.7	20:4	41.1	20:4	38.1
Diacylglycerol	DG	26	36:2	51.0	38:4	25.3	38:4	27.1
Triacylglycerol	TG	44	54:3	36.4	48:0	15.7	54:3	14.9
Bis(monoacylglycerol)phosphate	BMP	1	36:2	100	36:2	100	36:2	100
Phosphatidylglycerol	PG	2	36:2	100	36:1	51.6	36:1	55.7
Phosphatidylserine	PS	5	40:6	65.8	40:6	60.7	40:6	64.4
Phosphatidylinositol	PI	16	38:4	54.9	38:4	56.2	38:4	48.3
Lysophosphatidylinositol	LPI	4	20:4	67.1	18:0	65.2	18:0	61.9
Phosphatidylethanolamine	PE	20	38:6	20.8	40:6	27.3	40:6	35.8
Alkylphosphatidylethanolamine	PE(O)	12	38:4	27.4	38:4	20.6	38:4	19.7
Alkenylphosphatidylethanolamine	PE(P)	11	40:5	41.1	38:5	23.3	40:6	21.1
Lysophosphatidylethanolamine	LPE	6	22:6	32.1	22:6	50.8	22:6	54.2
Phosphatidylcholine	PC	51	36:2	15.3	34:1	28.2	34:1	29.5
Alkylphosphatidylcholine	PC(O)	19	34:1	18.8	34:1	49.6	34:1	50.3
Alkenylphosphatidylcholine	PC(P)	13	38:5	32.1	32:0	24.1	34:3	22.5
Lysophosphatidylcholine	LPC	22	16:0	29.8	16:0	36.9	16:0	37.3
Lysoalkylphosphatidylcholine	LPC(O)	10	20:0	85.3	20:0	100	20:0	100
Sphingomyelin	SM	21	34:1	48.7	36:1	81.0	36:1	75.7
Ceramide	Cer	10	18:1	31.8	18:0	85.9	18:0	78.9
Dihydroceramide	dhCer	6	18:0	37.8	18:0	100	18:0	92.2
Monohexosylceramide	MHC	6	24:1	34.1	24:1	60.6	24:1	59.6
Dihexosylceramide	DHC	6	18:0	54.5	18:0	92.1	18:0	92.7
Trihexosylceramide	THC	6	18:0	100	18:0	100	18:0	100
GM3 ganglioside	GM3	6	24:1	52.7	18:0	84.6	18:0	82.9

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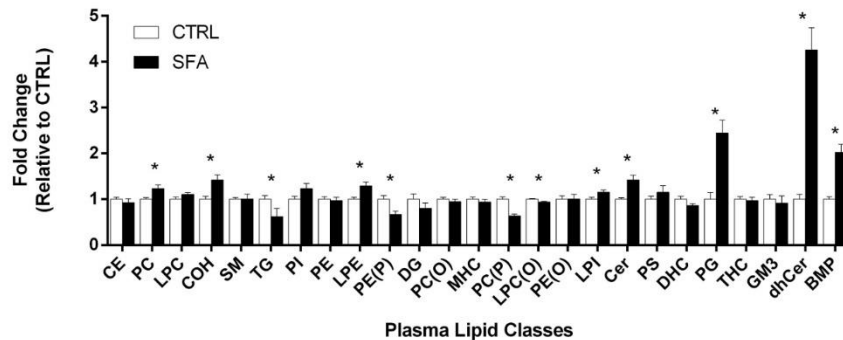


Fig 2. Fold changes in the molar abundance of lipid classes in plasma of mice fed regular chow or saturated fat enriched diet. Plasma was collected from mice fed a regular chow or saturated fat diet for six months. Individual lipid species were quantitated with LC-ESI-MS/MS and the molar sum across lipid classes calculated. Changes in the molar abundance of lipid classes in plasma of mice fed regular chow or saturated fat enriched diets. Fold changes in lipid classes are relative to mean lipid class concentration in mice fed regular chow diets. Lipid classes are in the order of decreasing abundance for regular chow fed animals. * indicates $p < 0.05$, data compared using Welch's T-test and corrected for multiple comparisons by Benjamini-Hochberg. $n = 9-10$ per group. Mean \pm SEM.

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(P) ($p = 0.005$) and PC(P) was 36.3% ($p < 0.001$) less than control fed mice. Of the sphingolipids analysed in plasma, the SFA diet resulted in an increase in ceramide (Cer) (42.3%, $p = 0.002$) and a substantial increase in the precursor class, dihydroceramide (dhCer) (326%, $p < 0.001$).

The brain lipidome in mice maintained on ordinary chow, or a diet enriched in SFA is depicted for HPF and CTX in Fig 1B and 1C, respectively. For both HPF and CTX, the brain lipidome was substantially different from that in plasma. Key qualitative differences between brain regions and plasma were proportionally greater abundance of PC, COH, PE, PE(P), monohexosylceramide (MHC), phosphatidylserine (PS) and Cer. Relative to total molar abundance in brain versus plasma; CE was 0.4% in HPF versus 63.8% plasma; PC 25.4% HPF versus 19.2% plasma, LPC 0.3% HPF versus 6.8% plasma, COH 32.5% HPF versus 5.9% plasma; SM 2.7% HPF versus 0.9% plasma, PE 15.8% HPF versus 0.5% plasma, PE(P) 7.9% HPF versus 0.3% plasma, PS 7.5% HPF versus 0.02% plasma, MHC 1.6% HPF versus 0.2% plasma and Cer 0.4% HPF versus 0.02% plasma. A similar relative abundance profile was observed for CTX samples, with relative abundance in decreasing order of COH (31.5%), PC (26.9%), PE (15.9%), PS (8.3%) and PE(P) (7.5%) of total lipids respectively.

Absolute changes in the brain lipidome as a consequence of long-term SFA feeding is indicated in Fig 1B and 1C. Unlike plasma, gross substantial changes in the abundance of lipid classes was not identified for HPF lipids and indeed limited to alkylphosphatidylethanolamine (PE(O)) for CTX (increased 10%; $p = 0.04$). However, significant changes in particular lipid species were identified within HPF and CTX as a consequence of SFA treatment (Fig 3). A total of 50 individual lipid species significantly changed and were restricted to the lipid classes PC, PE, alkylphosphatidylcholine PC(O), PC(P), PE(O), PE(P), CE, DG, PI and PS.

Total abundance of the PC class did not change following SFA treatment, however there were 11 PC species that significantly decreased in the HPF (Fig 3A); PC species 33:1, 34:2, 34:3, 36:5, 36:6, 38:6, 38:7, 39:6, 39:7, 40:6 and 40:7. Similarly in the CTX, nine PC species decreased (34:2, 34:3, 36:5, 36:6, 37:6, 36:6, 38:7 and 39:7) while PC 40:4 increased (Fig 3B).

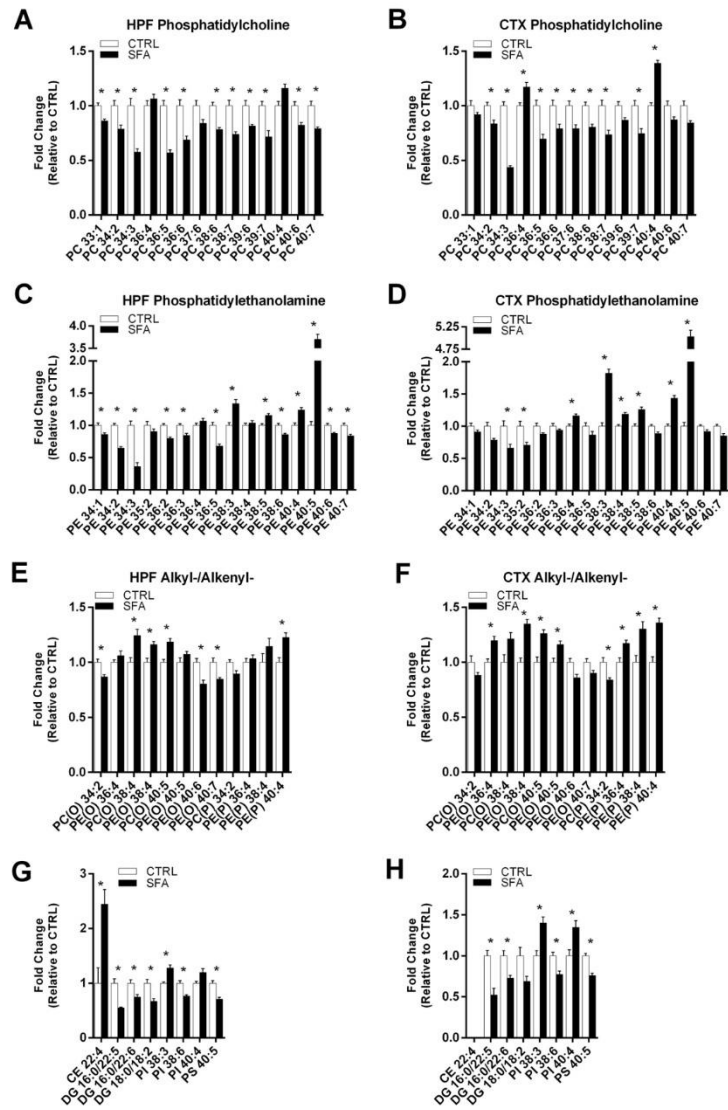


Fig 3. Fold change in individual lipid species in the hippocampus and cortex of mice fed a saturated fat enriched diet, relative to mice fed regular chow. Hippocampus and cortex was collected from mice fed a regular chow or saturated fat diet for six months. Individual lipid species were quantitated with LC-ESI-MS/MS. Fold changes in individual lipid species are relative to mean lipid species concentration in mice fed regular chow diets. Phosphatidylcholine species that significantly differed between mice in the (A) hippocampus and (B) cortex. Phosphatidylethanolamine species that significantly differed between mice in the (C) hippocampus and (D) cortex. Alkyl- and Alkenyl- phospholipid species that significantly differed between mice in the (E) hippocampus and (F) cortex. Other lipid species that significantly changed following consumption of a saturated fat enriched diet in the (G) hippocampus and (H) cortex. * indicates $p < 0.05$, data compared using Welch's T-test and corrected for multiple comparisons by Benjamini-Hochberg method. $n = 9-10$ per group. Mean \pm SEM.

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Of the second most abundant phospholipid class of the hippocampus, PE, species 34:1, 34:2, 34:3, 36:2, 36:3, 36:5, 38:6, 40:6 and 40:7 significantly decreased following high fat feeding, while 38:3, 38:5, 40:4 and 40:5 significantly increased (Fig 3C). Fewer species were significantly changed in the CTX; PE species 36:4, 38:3, 38:4, 38:5, 40:4 and 40:5 increased in SFA fed mice, while 34:3 and 35:2 significantly decreased (Fig 3D).

Within the HPF, a number of alkyl- and alkenyl- species changed following SFA treatment. Six alkyl- species changed in the HPF; PC(O) 34:2, PE(O) 40:6 and PE(O) 40:7 significantly decreased, while PC(O) 38:4, PC(O) 40:5 and PE(O) 38:4 significantly increased (Fig 3E). The only change in alkenyl- species was an increase in PE(P) 40:4. In contrast, there were seven species that significantly increased in the CTX of SFA fed mice; PC(O) 40:5, PE(O) 36:4, PE(O) 38:4, PE(O) 40:5, PE(P) 36:4, PE(P) 38:4 and PE(P) 40:4 (Fig 3F). The alkyl-/alkenyl- species to significantly decrease in the CTX was PC(P) 34:2 (Fig 3F).

Three additional HPF phospholipids were associated with SFA feeding; PI 38:6 and PS 40:5 significantly decreased, while PE 38:3 decreased (Fig 3G). Similar changes were observed in the CTX of SFA fed mice, except with the provision that PI 40:4 also significantly increased. Four neutral lipids changed in the HPF of SFA fed mice were identified; CE 22:4 increased while three DG species decreased (16:0/22:5, 16:0/22:6 and 18:0/18:2).

Partial least squares regression analysis was performed to explore the hypothesis that the regional specific changes in HPF and CTX lipidome were associated with changes in the plasma lipidome. Both PLS regression models were statistically significant as determined by permutation tests ($p < 0.001$). The first latent variable (LV 1) explained 46.4% of the variance in the hippocampus dataset and 46.0% of the variance in the cortex dataset. Score plots of PLS regression models (S1 Fig) and proportion of explained variance (R^2) of individual lipids are shown in S2 and S3 Tables.

Loading plots of the PLS models can be seen for PC species in Fig 4. Due to the large number of PC species identified, only the 22 most abundant plasma PC species are shown. Of these species, five (PC 36:5, PC 38:6, PC 40:7, PC 40:6 and PC 34:2) are positively correlated with the major source of variation in both hippocampus and cortex lipids. Within the plasma, PC 36:5, PC 38:6, PC 40:7 and PC 40:6 are strongly correlated with each other and also strongly correlation with the plasma species LPC 22:6. The only PC species with a strong negative correlation in the hippocampus is PC 38:5, although this was not observed in the cortex.

Fig 5 shows the loadings for PE species for both the HPF-Plasma and CTX-Plasma models. A group of four lipids, PE 36:5, PE 38:6, PE 40:7 and PE 40:6 correlate in both the HPF and plasma. Three lipids, PE 38:3, PE 38:5 and PE 40:5 are strongly negatively correlated to those lipids in both HPF and plasma. A number of PE species (PE 34:2, PE 36:2, PE 36:3 and PE 34:1) positively correlate with the PE 36:5, PE 38:6, PE 40:7 and PE 40:6 species in the HPF, however the plasma equivalents are positively correlated with the PE 38:3, PE 38:5 and PE 40:5 species. Similar observations are found in the CTX-Plasma model, except the strength of correlations for PE 40:6 is lower in plasma and PE 38:5 is lower in the CTX.

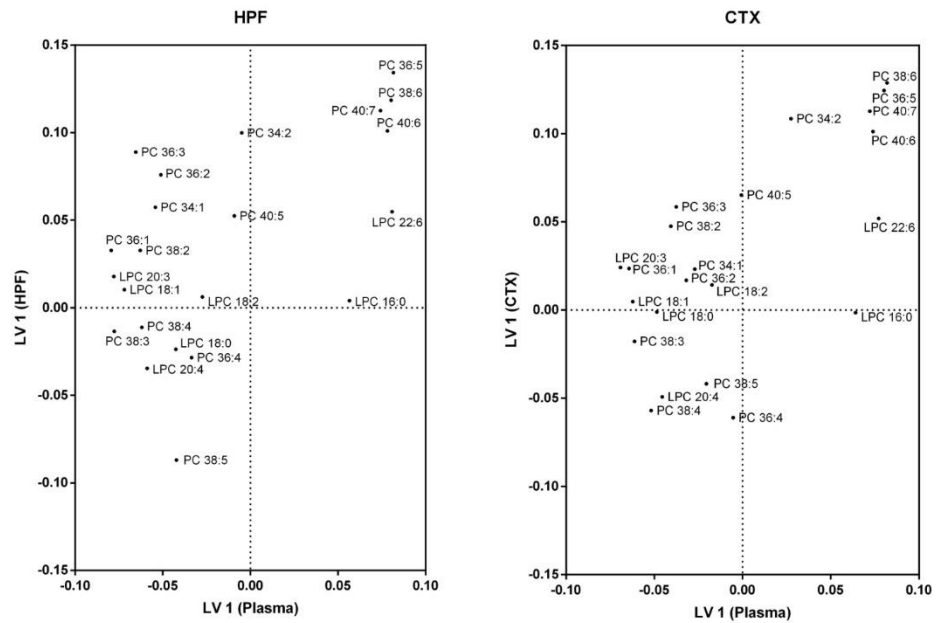


Fig 4. Partial least squares loading plot for phosphatidylcholine species. Wild-type mice were fed regular chow ($n = 10$) or a saturated fat enriched diet ($n = 9$) for six months. Plasma, hippocampus and cortex lipid species were quantitated with LC-ESI-MS/MS. Partial least squares regression was used to identify plasma lipid species that account for the major sources of variation within the (A) hippocampus and (B) cortex. Using the first latent variable for phosphatidylcholine species, loadings for plasma lipids are distributed on the X-axis, while loadings for hippocampus/cortex lipids are on the Y-axis. The X-axis is a measure of correlation to explaining the variation in the brain region. The Y-axis is a measure of correlation to the major source of variation in the brain region. Loadings are from the jack-knife resampled global model. Shown are the 95% most abundant PC species (22) in plasma.

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Loadings for the other major phospholipid classes, PS and PI, are shown in Fig 6. Two lipids (PI 38:6 and PI 38:3) contribute the most to the major source of variation in the HPF and correlate negatively with each other. Partial correlations are observed between PI 40:6 and PS 40:6 in the HPF and are partially negatively correlated with the species PI 38:2, PI 40:4 and PS 38:4. Within the CTX, PI 38:3 contributes less to the variation in the cerebral lipids, while PI 40:6 contributes more. In plasma, the PI species 38:6, 40:6, 40:5 and 38:5 strongly correlate, but PI 40:5 and PI 38:5 do not correlate with other lipids that make up the major source of variation in the CTX.

Discussion

In this study putative associations of changes in the brain lipidome with plasma lipids were determined in regular chow and saturated fat enriched diet fed mice utilizing a comprehensive lipidomics approach. Long term consumption of a SFA enriched diet realized substantial changes in the abundance of several lipid classes in plasma, including increases in PC, COH,

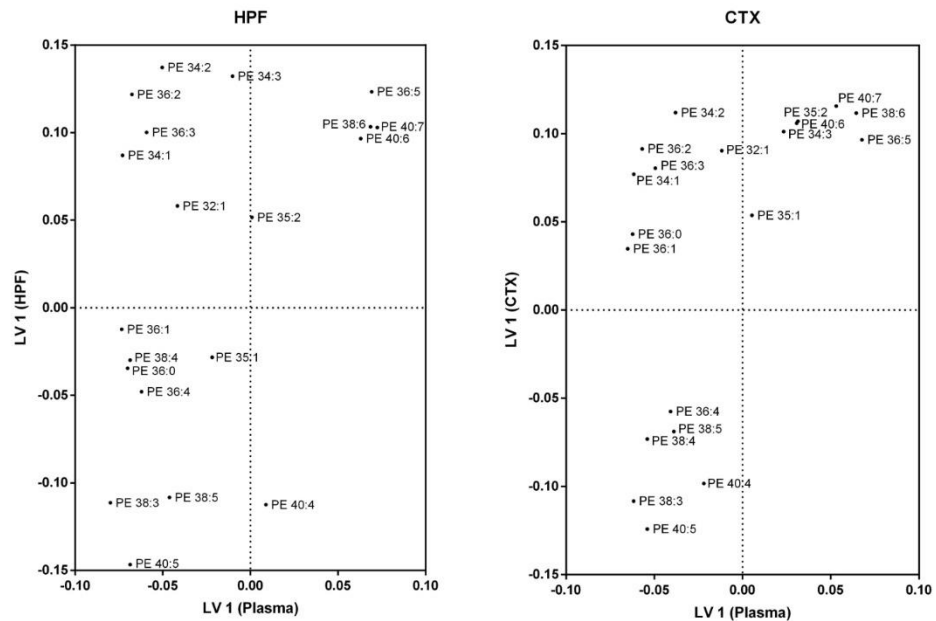


Fig 5. Partial least squares loading plot for phosphatidylethanolamine species. Wild-type mice were fed regular chow (n = 10) or a saturated fat enriched diet (n = 9) for six months. Plasma, hippocampus and cortex lipid species were quantitated with LC-ESI-MS/MS. Partial least squares regression was used to identify plasma lipid species that account for the major sources of variation within the (A) hippocampus and (B) cortex. Using the first latent variable for phosphatidylethanolamine species, loadings for plasma lipids are distributed on the X-axis, while loadings for hippocampus/cortex lipids are on the Y-axis. The X-axis is a measure of correlation to explaining the variation in the brain region. The Y-axis is a measure of correlation to the major source of variation in the brain region. Loadings are from the jack-knife resampled global model.

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LPE, LPI, Cer, PG, dhCer and BMP. Simultaneously, there was a decrease in the plasma abundance of TG, PE(P) and PC(P) lipid classes. In contrast, the cerebral lipidomes were relatively stable in the relative abundance of lipid classes. Hippocampus lipid classes were not different between the respective diets, while PE(O) increased slightly in CTX. Inspection of individual lipid species in the HPF/CTX revealed a total of 50 species that changed in concentration following long term consumption of the SFA enriched diet and were restricted to the lipid classes PC, PE, PC(O), PC(P), PE(O), PE(P), CE, DG, PI and PS. The regional specific changes in HPF and CTX lipidomes were assessed against associated changes in the plasma lipidome with partial least squares regression. Strong associations between a number of plasma lipids and changes in HPF and CTX lipidomes was observed. Our experiments demonstrate that well tolerated diets are capable of regulating the HPF and CTX lipidomes, while plasma lipids may be a causal mechanism for these changes. This paper suggests dietary lipids are capable of influencing cerebral lipid homeostasis, which may explain previously observed associations of dietary patterns with onset and progression of neurodegenerative disorders [50, 51].

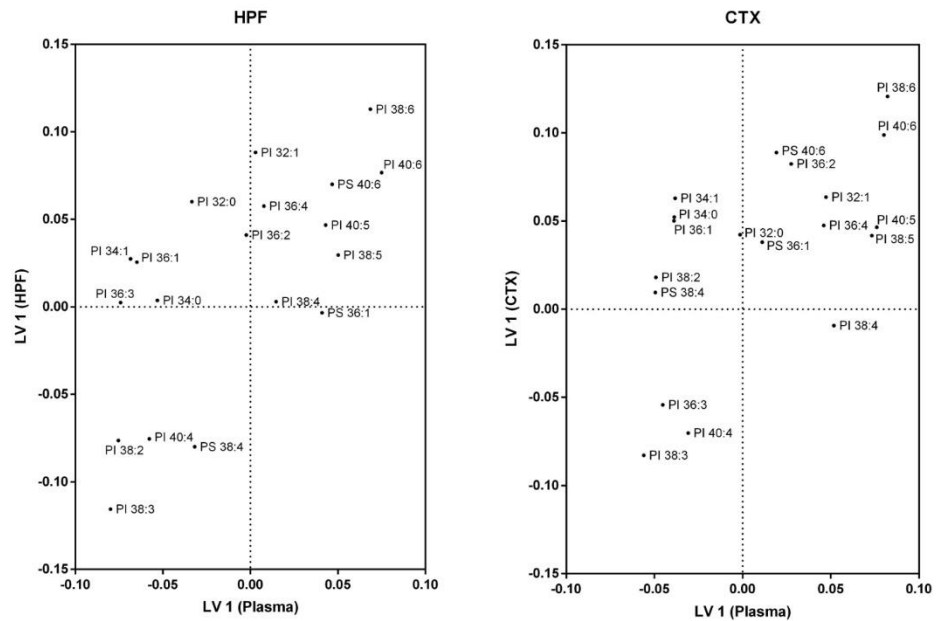


Fig 6. Partial least squares loading plot for phosphatidylinositol and phosphatidylserine species. Wild-type mice were fed regular chow (n = 10) or a saturated fat enriched diet (n = 9) for six months. Plasma, hippocampus and cortex lipid species were quantitated with LC-ESI-MS/MS. Partial least squares regression was used to identify plasma lipid species that account for the major sources of variation within the (A) hippocampus and (B) cortex. Using the first latent variable for phosphatidylinositol and phosphatidylserine species, loadings for plasma lipids are distributed on the X-axis, while loadings for hippocampus/cortex lipids are on the Y-axis. The X-axis is a measure of correlation to explaining the variation in the brain region. The Y-axis is a measure of correlation to the major source of variation in the brain region. Loadings are from the jack-knife resampled global model.

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The brain is highly enriched in lipids, where they influence neurotransmission, synaptic plasticity and inflammation. Changes in cerebral lipid homeostasis has been widely reported in a number of neurodegenerative and behavioural disorders, however, it is not known whether these changes are causally related with the onset or progression of such disorders or consequential to the disease pathology. Knowledge of factors that regulate the cerebral lipidome in a physiological context is still in its infancy. Further, animal models are routinely used to study the impact of western diets on neurological function/disorders, yet little is known about changes in the plasma and the cerebral lipidome for well tolerated diets. Herein, we explore in detail the influence a diet enriched in saturated fat has on the lipidome of the hippocampus and cerebral cortex, and the associated changes in plasma.

Following long term consumption of an SFA enriched diet, we observed an increase in the abundance of plasma PC lipids. It is not known whether bulk changes in plasma PC abundance has any effect on cerebral homeostasis. Individual PC species, on the other hand, have been noted to be associated with several neurodegenerative disorders [52, 53]. The bioactive PC isoform hypothesis may be because, next to cholesterol esters, PC lipids contain the largest

plasma proportion of circulating DHA, eicosapentaenoic acid (EPA) and arachidonic acid (AA) (data not shown)—fatty acids reported to influence risk of cognitive decline. Lysophosphatidylcholine, a product of enzyme hydrolysis of PC lipids, has been proposed to be a major route for peripheral uptake by the brain [54, 55], although the plasma abundance was not altered in these mice.

Plasma non-esterified cholesterol has long been known to be associated with many conditions, including coronary heart disease, Alzheimer's disease and vascular mortality [56]. Elevated plasma COH has previously been reported to be associated with alternations in cerebral lipid homeostasis [57]. Stranahan et al. speculated this was through an oxidative mechanism, however causal associations with plasma lipids was not assessed. A surprising result observed in this study was the decrease in plasma TG. Plasma TG concentrations are predominantly determined by the rate of production/secretion of triglyceride-rich lipoproteins and by the rate of hydrolysis/removal of circulating TG. Given our SFA enriched diet contains approximately 5-fold more dietary fat, we might expect to observe an increase in circulating TG. The contribution of circulating TG to brain lipid uptake is unclear, as a majority of research on brain uptake of peripheral lipids have focused on non-esterified fatty acids and LPC [55]. However, further research will be needed to clarify this difference, as triacylglycerol-rich lipoproteins and their lipolysis products in high physiological concentrations are known to cause endothelial injury and dysfunction in the periphery and in the cerebral circulation [33].

Sphingolipids have long been known to be responsive to SFA enriched diets and thought to be responsible for many of the deleterious effects, including insulin resistance, oxidative stress and inflammation [58, 59]. Herein, we observed increases in the plasma concentration of ceramides and its precursor dihydro-ceramides. Several cohort studies have identified circulating sphingolipids as potential biomarkers of neurodegenerative diseases [60–62]. Lyn-Cook et al. proposed that circulating ceramides might directly enter the brain, due to their hydrophobicity [63]. However, in this study, there was no observable change in cerebral sphingolipids.

An interesting finding in this study was a considerable decrease in the concentration of alkenylphosphatidylethanolamine and alkenylphosphatidylcholine classes in plasma following long term consumption of an SFA enriched diet. These lipid classes have been proposed to have a protective role against oxidative stress and lower concentrations associated with cardiovascular and neurodegenerative disorders [64, 65]. To the authors knowledge, this study is the first report of decreases in plasma PE(P) and PC(P) in mice fed a saturated fat enriched diet. This result is particularly interesting because serum PE(P) was noted to be able to predict severity of dementia in humans [66]. Further, serum PE(P) concentrations was shown to decrease prior to the detectable symptoms of dementia, suggesting changes in plasma ether lipids may have an early involvement in neurodegenerative disease progression.

Changes in the cerebral lipidome to dietary interventions have principally focused on n-3 fatty acid modulation and in many situations represent very severe restrictions [67]. The cerebral lipidome modulating ability of high-(saturated) fat feeding is considerably less well known. In our study, we report a significant increase in the abundance of PE(O) lipid species in the cerebral cortex of mice fed a modest saturated fat enriched diet. As PE(P) was not changed and PE(O) is a direct biosynthetic precursor to PE(P), this mismatch may be indicative of early perturbations in ether-lipid synthesis and/or peroxisomal stress [68]. The saturated ether linkage in PE(O) is not particularly reactive to oxidative stress, unlike the vinyl-ether linkage in PE(P), so increased PE(O) may be indicative of an increased flux through the pathway to replace PE(P) lost to reactive oxygen species (ROS) scavenging. To further test this hypothesis, studies examining the oxidative products of PE(P) species could be performed.

Recent findings also confirm that high-fat feeding can influence different regions of the brain. Borg et al. fed C57Bl/6 mice a 60% energy from fat diet for 8 weeks and observed

increases in DG, TG, PC(O), dhCer, DHC and BMP lipid classes in the hypothalamus [11]. Further, they note increases in a number of individual Cer species. The hypothalamic lipid accumulation in response to the high-fat diet was not ameliorated by exercise, which has been shown to reduce lipid accumulation in many peripheral tissues. The accumulation of TG, dhCer and Cer may indicate that the supply of lipids is surpassing the ability of neuronal cells to effectively handle the quantity of fatty acids. This observation is supported by Posey et al. whom observed an increase in saturated long chain acyl-CoA species in the hypothalamus of rats fed a similar diet i.e. accumulation of early lipid metabolism intermediates [13]. These studies examined the hypothalamus, which has roles in modulating energy intake through fatty acid sensing neurons within this region. This may predispose this region to being more susceptible to lipid accumulation. However, a study by Stranahan et al. using an aggressive high-fat diet in rats, showed an accumulation in the hippocampus of non-esterified cholesterol, galactosyl ceramide, ceramide sulfatide and several isoforms of sphingomyelin [57]. It should be noted that only hippocampus tissue of animals whose serum cholesterol levels fell into the highest third where selected; limiting generalizability of results. Nonetheless, there is a growing number of high-fat feeding studies which indicate sphingolipid species to be elevated in various brain regions.

In addition to the change in lipid class abundance, we observed a large number of changes in individual lipid species in both the hippocampus and cortex. Significant changes were observed in many phospholipid classes, as well as the DG class and one species changed in the cholesterol esters. A possible explanation for the large number of changes in phospholipid classes is due to their continuous turnover. Neuronal membrane phospholipids are repeatedly hydrolysed by phospholipase A1/2 enzymes and re-esterified—a cyclical process which is crucial for endo/exo-cytosis of vesicles during neurotransmitter release and membrane fusion events [69]. The deacylation/reacylation provides opportunity for phospholipid remodelling, where the local activity of enzymes and fatty acid concentrations interact and determine phospholipid composition [55]. We observed similar changes in both the hippocampus and cortex of mice fed the saturated fat enriched diet, suggesting a high degree of co-regulation between the regions. Considering the changes were broadly reflected in both regions, it is possible that peripheral supply of fatty acids is major factor in phospholipid composition.

Few studies have described in detail the changes in individual lipid species within the brain in high-fat feeding studies. However, some evidence suggests robust changes in phospholipid composition in response to high-fat feeding. Yu et al. performed a study in mice with multi-generation feeding with a high-lard diet and reported cerebral fatty acid composition using gas-chromatography [70]. Compared to control-diet fed mice, the high-lard diet group exhibited a fatty acid profile that contained significantly less polyunsaturated fatty acids and significantly more saturated fatty acids. This coincided with a halving in DHA content in the brain. Lepinay et al. fed pregnant wistar rats and their pups a high-fat diet through to adulthood and measured the fatty acid composition of PE lipids in the hippocampus [71]. The high-fat diet resulted in a significant reduction in n-3 fatty acids and an increase in n-6 fatty acids, while total polyunsaturated fatty acids did not change. This implicates a replacement of n-3 fatty acids with n-6 fatty acids is occurring within the PE class of the hippocampus.

Examination of the individual species in the brains of our mice which significantly differed, reveals that many of the lipids that putatively contain DHA decreased, and those containing AA increased. The replacement of DHA by AA has been reported previously in studies of severe n3-fatty acid deficiency, however these changes were principally only observed in PC and PE lipids [67]. By contrast, a recent study by Bascoul-Colombo et al. assessed cerebral lipids following long-term provision of a diet with 10-fold greater n3-fatty acids by way of DHA supplementation and reported a replacement of AA containing phospholipids by DHA in the

hippocampus, cortex and cerebellum [72]. While the relative composition of the different regions varied, the changes in individual lipids all trended in the same direction among the phospholipid classes assessed. This suggests that dietary lipids can influence the composition of many phospholipid classes simultaneously.

The most novel component of this study is the simultaneous multivariate analysis of whole lipidomes from both plasma and brain. Whereas there has been attempts to correlate cerebral lipids with circulating lipids, these have been limited to cerebral fatty acid profiles against measures of serum cholesterol [70]. By contrast, we explore relationships between all plasma lipids and all cerebral lipids observed in our detailed lipidomics study. This approach is particularly relevant for lipidomic studies as the complex relationships within and between different compartments (plasma vs brain) can be examined. Furthermore, changes in individual lipids in the brain are generally small because the influencing plasma lipids are metabolised and distributed among many lipids.

Partial least squares regression used in this study shows that DHA and AA containing phospholipids contribute, opposingly, to a major source of variation observed in our dataset. The plasma lipidome, as a whole, was able to account for a substantial proportion of variation in the brain, strengthening the suggestion that plasma lipids are causally related to lipidome changes in the brain.

A number of assumptions were made during this study: (1) the number of internal standards are limited compared to the number of species measured. It is assumed that one internal standard is representative of the entire class, with minimal differences in response factor with changes in fatty acid composition; (2) there may be some degradation of lipids during sample isolation and processing. However, the hippocampus and cortex were isolated from brain sections that were snap frozen immediately after collection; (3) the same mass spectrometry analysis of lipids was performed on both plasma and brain samples. While some studies have detailed novel and specific species in plasma [73] and brain [74], we have attempted to focus on the major species present in these samples. Further, the number of species identified, 348, provides an ample number to characterise a vast number of the major species present in the samples; (4) we did not perfuse the brain prior to sample collection. Plasma lipids will contribute to the cerebral lipids measured, however their relative abundance will be negligible given the volume of plasma within the brain compared to tissue mass. As evidenced by the differential composition of plasmalogens and cholesterol esters between plasma and brain regions. (5) Internal validation does not preclude the need for external validation of the PLS models generated. Further research is required to externally validate the findings [75]. Despite these potential limitations, the relative changes between groups are still accurate as each animal was processed the same. Furthermore, by utilizing multivariate analysis of plasma with the two brain regions from each animal allows correlations to be observed and biological phenomenon to be characterised independent of intra-sample systematic error [76].

Herein, we show that long-term feeding of SFA enriched diets in mice leads to changes in the hippocampus and cerebral cortex lipidomes. Similar patterns of change are observed between the two regions of the brain, suggesting a common causal mechanism. Whole lipidome multivariate analysis suggests the plasma lipidome can account for a substantial proportion of variation in both the hippocampus and cortex. Given that well tolerated diets can alter cerebral lipid composition, future clinical work should consider 'whole diets', rather than single dietary components i.e. DHA.

Supporting Information

S1 Fig. Scores scatter plot for partial least squares models. Wild-type mice were fed regular chow (n = 10) or a saturated fat enriched diet (n = 9) for six months. Plasma, hippocampus and cortex lipid species were quantitated with LC-ESI-MS/MS. Partial least squares regression was used to identify plasma lipid species that account for the major sources of variation within the (A) hippocampus and (B) cortex. Scores scatter plot of the first latent variable for mice fed regular chow (squares) or a saturated fat enriched diet (triangles). The first latent variable score for the independent lipids (plasma) on the X-axis and the first latent variable score for dependent lipids (hippocampus/cortex) on the Y-axis. Scores are from the jack-knife resampled global model.
(TIF)

S1 Table. Conditions for tandem mass spectrometry analysis of lipid species
(DOCX)

S2 Table. Proportion of variance explained (R^2) by the first latent variable for the first 25 individual lipids in the hippocampus and plasma lipids.
(DOCX)

S3 Table. Proportion of variance explained (R^2) by the first latent variable for the first 25 individual lipids in the cerebral cortex and plasma lipids.
(DOCX)

Author Contributions

Conceptualization: CG RT JM.

Formal analysis: CG SD.

Funding acquisition: RT JM.

Investigation: CG RT NM.

Methodology: CG RT NM PM JM.

Project administration: JM.

Resources: CG RT NM PM JM.

Software: CG SD.

Supervision: RT JM.

Visualization: CG RT SD JM.

Writing – original draft: CG RT JM.

Writing – review & editing: CG RT PM JM.

References

1. O'Brien JS, Sampson EL. Lipid composition of the normal human brain: gray matter, white matter, and myelin. *J Lipid Res.* 1965; 6(4):537–44. PMID: 5865382
2. Cermenati G, Mitro N, Audano M, Melcangi RC, Crestani M, De Fabiani E, et al. Lipids in the nervous system: from biochemistry and molecular biology to patho-physiology. *Biochim Biophys Acta.* 2015; 1851(1):51–60. doi: 10.1016/j.bbailip.2014.08.011 PMID: 25150974

3. Bazinet RP, Laye S. Polyunsaturated fatty acids and their metabolites in brain function and disease. *Nat Rev Neurosci*. 2014; 15(12):771–85. doi: 10.1038/nrn3820 PMID: 25387473
4. Horrocks LA, Faroqui AA. Docosahexaenoic acid in the diet: its importance in maintenance and restoration of neural membrane function. *Prostaglandins Leukot Essent Fatty Acids*. 2004; 70(4):361–72. doi: 10.1016/j.plefa.2003.12.011 PMID: 15041028
5. Cunnane SC, Schneider JA, Tangney C, Tremblay-Mercier J, Fortier M, Bennett DA, et al. Plasma and brain fatty acid profiles in mild cognitive impairment and Alzheimer's disease. *J Alzheimers Dis*. 2012; 29(3):691–7. PubMed Central PMCID: PMC3409580. doi: 10.3233/JAD-2012-110629 PMID: 22466064
6. Muller CP, Reichel M, Muhle C, Rhein C, Gulbins E, Kornhuber J. Brain membrane lipids in major depression and anxiety disorders. *Biochim Biophys Acta*. 2015; 1851(8):1052–65. doi: 10.1016/j.bbali.2014.12.014 PMID: 25542508
7. Chan RB, Oliveira TG, Cortes EP, Honig LS, Duff KE, Small SA, et al. Comparative lipidomic analysis of mouse and human brain with Alzheimer disease. *J Biol Chem*. 2012; 287(4):2678–88. PubMed Central PMCID: PMC3268426. doi: 10.1074/jbc.M111.274142 PMID: 22134919
8. Han X. Multi-dimensional mass spectrometry-based shotgun lipidomics and the altered lipids at the mild cognitive impairment stage of Alzheimer's disease. *Biochim Biophys Acta*. 2010; 1801(8):774–83. PubMed Central PMCID: PMC2886180. doi: 10.1016/j.bbali.2010.01.010 PMID: 20117236
9. Cunnane SC, Chouinard-Watkins R, Castellano CA, Barberger-Gateau P. Docosahexaenoic acid homeostasis, brain aging and Alzheimer's disease: Can we reconcile the evidence? *Prostaglandins Leukot Essent Fatty Acids*. 2013; 88(1):61–70. doi: 10.1016/j.plefa.2012.04.006 PMID: 22575581
10. Naudi A, Cabre R, Jove M, Ayala V, Gonzalo H, Portero-Otin M, et al. Lipidomics of human brain aging and Alzheimer's disease pathology. *Int Rev Neurobiol*. 2015; 122:133–89. doi: 10.1016/bs.irn.2015.05.008 PMID: 26358893
11. Borg ML, Omran SF, Weir J, Meikle PJ, Watt MJ. Consumption of a high-fat diet, but not regular endurance exercise training, regulates hypothalamic lipid accumulation in mice. *J Physiol*. 2012; 590(17):4377–89. PubMed Central PMCID: PMC3473292. doi: 10.1113/jphysiol.2012.233288 PMID: 22674717
12. Moraes JC, Coope A, Morari J, Cintra DE, Roman EA, Pauli JR, et al. High-fat diet induces apoptosis of hypothalamic neurons. *PLoS One*. 2009; 4(4):e5045. PubMed Central PMCID: PMC2661137. doi: 10.1371/journal.pone.0005045 PMID: 19340313
13. Posey KA, Clegg DJ, Printz RL, Byun J, Morton GJ, Vivekanandan-Giri A, et al. Hypothalamic proinflammatory lipid accumulation, inflammation, and insulin resistance in rats fed a high-fat diet. *Am J Physiol Endocrinol Metab*. 2009; 296(5):E1003–12. PubMed Central PMCID: PMC2681305. doi: 10.1152/ajpendo.90377.2008 PMID: 19116375
14. Herrero A, Portero-Otin M, Bellmunt MJ, Pamplona R, Barja G. Effect of the degree of fatty acid unsaturation of rat heart mitochondria on their rates of H₂O₂ production and lipid and protein oxidative damage. *Mech Ageing Dev*. 2001; 122(4):427–43. PMID: 11240164
15. Carrie I, Clement M, de Javel D, Frances H, Bourre JM. Specific phospholipid fatty acid composition of brain regions in mice. Effects of n-3 polyunsaturated fatty acid deficiency and phospholipid supplementation. *J Lipid Res*. 2000; 41(3):465–72. PMID: 10706594
16. Taha AY, Chang L, Chen M. Threshold changes in rat brain docosahexaenoic acid incorporation and concentration following graded reductions in dietary alpha-linolenic acid. *Prostaglandins Leukot Essent Fatty Acids*. 2016; 105:26–34. PubMed Central PMCID: PMC4752724. doi: 10.1016/j.plefa.2015.12.002 PMID: 26869088
17. Agrawal R, Gomez-Pinilla F. 'Metabolic syndrome' in the brain: deficiency in omega-3 fatty acid exacerbates dysfunctions in insulin receptor signalling and cognition. *J Physiol*. 2012; 590(10):2485–99. PubMed Central PMCID: PMC3424766. doi: 10.1113/jphysiol.2012.230078 PMID: 22473784
18. Tu WC, Muhlhauser BS, Yelland LN, Gibson RA. Correlations between blood and tissue omega-3 LCPUFA status following dietary ALA intervention in rats. *Prostaglandins Leukot Essent Fatty Acids*. 2013; 88(1):53–60. doi: 10.1016/j.plefa.2012.04.005 PMID: 22521090
19. Rabiei Z, Bigdeli MR, Rasouljan B. Neuroprotection of dietary virgin olive oil on brain lipidomics during stroke. *Curr Neurovasc Res*. 2013; 10(3):231–7. PMID: 23713733
20. Holland WL, Bikman BT, Wang LP, Yuguang G, Sargent KM, Bulchand S, et al. Lipid-induced insulin resistance mediated by the proinflammatory receptor TLR4 requires saturated fatty acid-induced ceramide biosynthesis in mice. *J Clin Invest*. 2011; 121(5):1858–70. PubMed Central PMCID: PMC3083776. doi: 10.1172/JCI43378 PMID: 21490391
21. Benoit SC, Kemp CJ, Elias CF, Abplanalp W, Herman JP, Migrenne S, et al. Palmitic acid mediates hypothalamic insulin resistance by altering PKC-theta subcellular localization in rodents. *J Clin Invest*.

- 2009; 119(9):2577–89. PubMed Central PMCID: PMC2735917. doi: 10.1172/JCI36714 PMID: 19726875
22. Edmond J. Essential polyunsaturated fatty acids and the barrier to the brain: the components of a model for transport. *J Mol Neurosci*. 2001; 16(2–3):181–93; discussion 215–21. doi: 10.1385/JMN:16-2-3:181 PMID: 11478373
 23. Dehouck B, Fenart L, Dehouck MP, Pierce A, Torpier G, Cecchelli R. A new function for the LDL receptor: transcytosis of LDL across the blood-brain barrier. *J Cell Biol*. 1997; 138(4):877–89. PubMed Central PMCID: PMC2138047. PMID: 9265653
 24. van de Haar HJ, Burgmans S, Jansen JF, van Osch MJ, van Buchem MA, Muller M, et al. Blood-Brain Barrier Leakage in Patients with Early Alzheimer Disease. *Radiology*. 2016:152244.
 25. Elahy M, Jackaman C, Mamo JC, Lam V, Dhaliwal SS, Giles C, et al. Blood-brain barrier dysfunction developed during normal aging is associated with inflammation and loss of tight junctions but not with leukocyte recruitment. *Immun Ageing*. 2015; 12:2. PubMed Central PMCID: PMC4362825. doi: 10.1186/s12979-015-0029-9 PMID: 25784952
 26. Takechi R, Pallegage-Gamarallage MM, Lam V, Giles C, Mamo JC. Aging-related changes in blood-brain barrier integrity and the effect of dietary fat. *Neurodegener Dis*. 2013; 12(3):125–35. doi: 10.1159/000343211 PMID: 23128303
 27. Takechi R, Galloway S, Pallegage-Gamarallage MM, Lam V, Dhaliwal SS, Mamo JC. Probucol prevents blood-brain barrier dysfunction in wild-type mice induced by saturated fat or cholesterol feeding. *Clin Exp Pharmacol Physiol*. 2013; 40(1):45–52. doi: 10.1111/1440-1681.12032 PMID: 23167559
 28. Kanoski SE, Davidson TL. Western diet consumption and cognitive impairment: links to hippocampal dysfunction and obesity. *Physiol Behav*. 2011; 103(1):59–68. PubMed Central PMCID: PMC3056912. doi: 10.1016/j.physbeh.2010.12.003 PMID: 21167850
 29. Kalmijn S, Launer LJ, Ott A, Witteman JC, Hofman A, Breteler MM. Dietary fat intake and the risk of incident dementia in the Rotterdam Study. *Ann Neurol*. 1997; 42(5):776–82. doi: 10.1002/ana.410420514 PMID: 9392577
 30. Breteler MM. Vascular risk factors for Alzheimer's disease: an epidemiologic perspective. *Neurobiol Aging*. 2000; 21(2):153–60. PMID: 10867200
 31. Sparks DL, Kuo YM, Roher A, Martin T, Lukas RJ. Alterations of Alzheimer's disease in the cholesterol-fed rabbit, including vascular inflammation. Preliminary observations. *Ann N Y Acad Sci*. 2000; 903:335–44. PMID: 10818523
 32. Hsu TM, Kanoski SE. Blood-brain barrier disruption: mechanistic links between Western diet consumption and dementia. *Front Aging Neurosci*. 2014; 6:88. PubMed Central PMCID: PMC4023063. doi: 10.3389/fnagi.2014.00088 PMID: 24847262
 33. Aung HH, Altman R, Nyunt T, Kim J, Nuthikattu S, Budamagunta M, et al. Lipotoxic brain microvascular injury is mediated by activating transcription factor 3-dependent inflammatory and oxidative stress pathways. *J Lipid Res*. 2016; 57(6):955–68. PubMed Central PMCID: PMC4878181. doi: 10.1194/jlr.M061853 PMID: 27087439
 34. Oh H, Boghossian S, York DA, Park-York M. The effect of high fat diet and saturated fatty acids on insulin signaling in the amygdala and hypothalamus of rats. *Brain Res*. 2013; 1537:191–200. doi: 10.1016/j.brainres.2013.09.025 PMID: 24076449
 35. Pipatpiboon N, Pintana H, Pratchayasakul W, Chattipakorn N, Chattipakorn SC. DPP4-inhibitor improves neuronal insulin receptor function, brain mitochondrial function and cognitive function in rats with insulin resistance induced by high-fat diet consumption. *Eur J Neurosci*. 2013; 37(5):839–49. doi: 10.1111/ejn.12088 PMID: 23240760
 36. Greenwood CE, Winocur G. Glucose treatment reduces memory deficits in young adult rats fed high-fat diets. *Neurobiol Learn Mem*. 2001; 75(2):179–89. doi: 10.1006/nlme.2000.3964 PMID: 11222059
 37. Lam SM, Wang Y, Duan X, Wenk MR, Kalaria RN, Chen CP, et al. Brain lipidomes of subcortical ischemic vascular dementia and mixed dementia. *Neurobiol Aging*. 2014; 35(10):2369–81. PubMed Central PMCID: PMC4099521. doi: 10.1016/j.neurobiolaging.2014.02.025 PMID: 24684787
 38. Wood PL, Barnette BL, Kaye JA, Quinn JF, Woltjer RL. Non-targeted lipidomics of CSF and frontal cortex grey and white matter in control, mild cognitive impairment, and Alzheimer's disease subjects. *Acta Neuropsychiatr*. 2015; 27(5):270–8. doi: 10.1017/neu.2015.18 PMID: 25858158
 39. Guan Z, Wang Y, Cairns NJ, Lantos PL, Dallner G, Sindelar PJ. Decrease and structural modifications of phosphatidylethanolamine plasmalogen in the brain with Alzheimer disease. *J Neuropathol Exp Neurol*. 1999; 58(7):740–7. PMID: 10411344
 40. Chetelat G, Desgranges B, de la Sayette V, Viader F, Berkouk K, Landeau B, et al. Dissociating atrophy and hypometabolism impact on episodic memory in mild cognitive impairment. *Brain*. 2003; 126(Pt 9):1955–67. doi: 10.1093/brain/awg196 PMID: 12821520

41. Kramer JH, Rosen HJ, Du AT, Schuff N, Hollnagel C, Weiner MW, et al. Dissociations in hippocampal and frontal contributions to episodic memory performance. *Neuropsychology*. 2005; 19(6):799–805. PubMed Central PMCID: PMCPMC1851935. doi: 10.1037/0894-4105.19.6.799 PMID: 16351355
42. Weir JM, Wong G, Barlow CK, Greeve MA, Kowalczyk A, Almasy L, et al. Plasma lipid profiling in a large population-based cohort. *J Lipid Res*. 2013; 54(10):2898–908. PubMed Central PMCID: PMCPMC3770102. doi: 10.1194/jlr.P035808 PMID: 23868910
43. Meikle PJ, Wong G, Barlow CK, Weir JM, Greeve MA, MacIntosh GL, et al. Plasma lipid profiling shows similar associations with prediabetes and type 2 diabetes. *PLoS One*. 2013; 8(9):e74341. PubMed Central PMCID: PMCPMC3785490. doi: 10.1371/journal.pone.0074341 PMID: 24086336
44. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate—a Practical and Powerful Approach to Multiple Testing. *J Roy Stat Soc B Met*. 1995; 57(1):289–300.
45. Folch-Fortuny A, Arteaga F, Ferrer A. PCA model building with missing data: New proposals and a comparative study. *Chemometr Intell Lab*. 2015; 146:77–88.
46. Szymanska E, Saccenti E, Smilde AK, Westerhuis JA. Double-check: validation of diagnostic statistics for PLS-DA models in metabolomics studies. *Metabolomics*. 2012; 8(Suppl 1):3–16. PubMed Central PMCID: PMCPMC3337399. doi: 10.1007/s11306-011-0330-3 PMID: 22593721
47. Rubingh CM, Bijlsma S, Derks EP, Bobeldijk I, Verheij ER, Kochhar S, et al. Assessing the performance of statistical validation tools for megavariable metabolomics data. *Metabolomics*. 2006; 2(2):53–61. PubMed Central PMCID: PMCPMC3906710. doi: 10.1007/s11306-006-0022-6 PMID: 24489531
48. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2015.
49. Le Cao KA, Gonzalez I, Dejean S. mixOmics: Omics Data Integration Project. R package version 5.2.0 ed2015.
50. Alles B, Samieri C, Fear C, Jutand MA, Laurin D, Barberger-Gateau P. Dietary patterns: a novel approach to examine the link between nutrition and cognitive function in older individuals. *Nutr Res Rev*. 2012; 25(2):207–22. doi: 10.1017/S0954422412000133 PMID: 22874455
51. Morris MC, Tangney CC. Dietary fat composition and dementia risk. *Neurobiol Aging*. 2014; 35 Suppl 2: S59–64. PubMed Central PMCID: PMCPMC4107296.
52. Schaefer EJ, Bongard V, Beiser AS, Lamon-Fava S, Robins SJ, Au R, et al. Plasma phosphatidylcholine docosahexaenoic acid content and risk of dementia and Alzheimer disease: the Framingham Heart Study. *Arch Neurol*. 2006; 63(11):1545–50. doi: 10.1001/archneur.63.11.1545 PMID: 17101822
53. Cheng ML, Chang KH, Wu YR, Chen CM. Metabolic disturbances in plasma as biomarkers for Huntington's disease. *J Nutr Biochem*. 2016; 31:38–44. doi: 10.1016/j.jnutbio.2015.12.001 PMID: 27133422
54. Lagarde M, Bernoud N, Brossard N, Lemaitre-Delaunay D, Thies F, Croset M, et al. Lysophosphatidylcholine as a preferred carrier form of docosahexaenoic acid to the brain. *J Mol Neurosci*. 2001; 16(2–3):201–4; discussion 15–21. doi: 10.1385/JMN.16.2-3:201 PMID: 11478375
55. Chen CT, Green JT, Orr SK, Bazinet RP. Regulation of brain polyunsaturated fatty acid uptake and turnover. *Prostaglandins Leukot Essent Fatty Acids*. 2008; 79(3–5):85–91. doi: 10.1016/j.plefa.2008.09.003 PMID: 18938067
56. Prospective Studies C, Lewington S, Whitlock G, Clarke R, Sherliker P, Emberson J, et al. Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths. *Lancet*. 2007; 370(9602):1829–39. doi: 10.1016/S0140-6736(07)61778-4 PMID: 18061058
57. Stranahan AM, Cutler RG, Button C, Telljohann R, Mattson MP. Diet-induced elevations in serum cholesterol are associated with alterations in hippocampal lipid metabolism and increased oxidative stress. *J Neurochem*. 2011; 118(4):611–5. PubMed Central PMCID: PMCPMC3137681. doi: 10.1111/j.1471-4159.2011.07351.x PMID: 21682722
58. Holland WL, Brozinick JT, Wang LP, Hawkins ED, Sargent KM, Liu Y, et al. Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. *Cell Metab*. 2007; 5(3):167–79. doi: 10.1016/j.cmet.2007.01.002 PMID: 17339025
59. Cutler RG, Kelly J, Storie K, Pedersen WA, Tammara A, Hatanpaa K, et al. Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. *Proc Natl Acad Sci U S A*. 2004; 101(7):2070–5. PubMed Central PMCID: PMCPMC357053. doi: 10.1073/pnas.0305799101 PMID: 14970312
60. Mielke MM, Bandaru VV, Haughey NJ, Xia J, Fried LP, Yasar S, et al. Serum ceramides increase the risk of Alzheimer disease: the Women's Health and Aging Study II. *Neurology*. 2012; 79(7):633–41. PubMed Central PMCID: PMCPMC3414665. doi: 10.1212/WNL.0b013e318264e380 PMID: 22815558

61. Mielke MM, Haughey NJ, Bandaru VV, Weinberg DD, Darby E, Zaidi N, et al. Plasma sphingomyelins are associated with cognitive progression in Alzheimer's disease. *J Alzheimers Dis.* 2011; 27(2):259–69. PubMed Central PMCID: PMC3218198. doi: 10.3233/JAD-2011-110405 PMID: 21841258
62. Mielke MM, Maetzler W, Haughey NJ, Bandaru VV, Savica R, Deuschle C, et al. Plasma ceramide and glucosylceramide metabolism is altered in sporadic Parkinson's disease and associated with cognitive impairment: a pilot study. *PLoS One.* 2013; 8(9):e73094. PubMed Central PMCID: PMC3776817. doi: 10.1371/journal.pone.0073094 PMID: 24058461
63. Lyn-Cook LE Jr., Lawton M, Tong M, Silbermann E, Longato L, Jiao P, et al. Hepatic ceramide may mediate brain insulin resistance and neurodegeneration in type 2 diabetes and non-alcoholic steatohepatitis. *J Alzheimers Dis.* 2009; 16(4):715–29. PubMed Central PMCID: PMC2893047. doi: 10.3233/JAD-2009-0984 PMID: 19387108
64. Wood PL, Mankidy R, Ritchie S, Heath D, Wood JA, Flax J, et al. Circulating plasmalogen levels and Alzheimer Disease Assessment Scale-Cognitive scores in Alzheimer patients. *J Psychiatry Neurosci.* 2010; 35(1):59–62. PubMed Central PMCID: PMC2799506. doi: 10.1503/jpn.090059 PMID: 20040248
65. Stenvinkel P, Diczfalusy U, Lindholm B, Heimbürger O. Phospholipid plasmalogen, a surrogate marker of oxidative stress, is associated with increased cardiovascular mortality in patients on renal replacement therapy. *Nephrol Dial Transplant.* 2004; 19(4):972–6. doi: 10.1093/ndt/gfh035 PMID: 15031358
66. Goodenowe DB, Cook LL, Liu J, Lu Y, Jayasinghe DA, Ahiahonu PW, et al. Peripheral ethanolanine plasmalogen deficiency: a logical causative factor in Alzheimer's disease and dementia. *J Lipid Res.* 2007; 48(11):2485–98. doi: 10.1194/jlr.P700023-JLR200 PMID: 17664527
67. Kim HW, Rao JS, Rapoport SI, Igarashi M. Regulation of rat brain polyunsaturated fatty acid (PUFA) metabolism during graded dietary n-3 PUFA deprivation. *Prostaglandins Leukot Essent Fatty Acids.* 2011; 85(6):361–8. PubMed Central PMCID: PMC3208751. doi: 10.1016/j.plefa.2011.08.002 PMID: 21880477
68. Brites P, Waterham HR, Wanders RJ. Functions and biosynthesis of plasmalogens in health and disease. *Biochim Biophys Acta.* 2004; 1636(2–3):219–31. doi: 10.1016/j.bbaliip.2003.12.010 PMID: 15164770
69. Sun GY, Xu J, Jensen MD, Simonyi A. Phospholipase A2 in the central nervous system: implications for neurodegenerative diseases. *J Lipid Res.* 2004; 45(2):205–13. doi: 10.1194/jlr.R300016-JLR200 PMID: 14657205
70. Yu H, Bi Y, Ma W, He L, Yuan L, Feng J, et al. Long-term effects of high lipid and high energy diet on serum lipid, brain fatty acid composition, and memory and learning ability in mice. *Int J Dev Neurosci.* 2010; 28(3):271–6. doi: 10.1016/j.ijdevneu.2009.12.001 PMID: 20015474
71. Lepinay AL, Larriéu T, Joffre C, Acar N, Garate I, Castanon N, et al. Perinatal high-fat diet increases hippocampal vulnerability to the adverse effects of subsequent high-fat feeding. *Psychoneuroendocrinology.* 2015; 53:82–93. doi: 10.1016/j.psyneuen.2014.12.008 PMID: 25614359
72. Bascoul-Colombo C, Guschina IA, Maskrey BH, Good M, O'Donnell VB, Harwood JL. Dietary DHA supplementation causes selective changes in phospholipids from different brain regions in both wild type mice and the Tg2576 mouse model of Alzheimer's disease. *Biochim Biophys Acta.* 2016; 1861(6):524–37. PubMed Central PMCID: PMC4847476. doi: 10.1016/j.bbaliip.2016.03.005 PMID: 26968097
73. Quehenberger O, Armando AM, Brown AH, Milne SB, Myers DS, Merrill AH, et al. Lipidomics reveals a remarkable diversity of lipids in human plasma. *J Lipid Res.* 2010; 51(11):3299–305. PubMed Central PMCID: PMC2952570. doi: 10.1194/jlr.M009449 PMID: 20671299
74. Guan Z. Discovering novel brain lipids by liquid chromatography/tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2009; 877(26):2814–21. PubMed Central PMCID: PMC2723173. doi: 10.1016/j.jchromb.2009.03.002 PMID: 19303823
75. Steyerberg EW, Bleeker SE, Moll HA, Grobbee DE, Moons KGM. Internal and external validation of predictive models: A simulation study of bias and precision in small samples. *J Clin Epidemiol.* 2003; 56(5):441–7. PMID: 12812818
76. Kaduk M, Hoefsloot HC, Vis DJ, Reijmers T, van der Greef J, Smilde AK, et al. Correlated measurement error hampers association network inference. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2014; 966:93–9. doi: 10.1016/j.jchromb.2014.04.048 PMID: 24851433

3.3 Supplementary material

S1 Table: Internal standards used in tandem mass spectrometry analysis of lipid classes

Lipid class or subclass	No. of species	Internal standard	Pmol ^a
Dihydroceramide (dhCer)	6	dhCer 8:0	100
Ceramide (Cer)	10	Cer 17:0	100
Monohexocylceramide (MHC)	6	MHC 16:0 <i>d</i> ₃	50
Dihexosylceramide (DHC)	6	DHC 16:0 <i>d</i> ₃	50
Trihexosylceramide (THC)	6	THC 17:0	50
G _{M3} ganglioside (GM3)	6	THC 17:0	50
Sphingomyelin (SM)	21	SM 12:0	200
Phosphatidylcholine (PC)	51	PC 13:0/13:0	100
Alkylphosphatidylcholine (PC(O))	19	PC 13:0/13:0	100
Alkenylphosphatidylcholine (PC(P))	13	PC 13:0/13:0	100
Lysophosphatidylcholine (LPC)	22	LPC 13:0	100
Lysoalkylphosphatidylcholine (LPC(O))	10	LPC 13:0	100
Phosphatidylethanolamine (PE)	20	PE 17:0/17:0	100
Alkylphosphatidylethanolamine (PE(O))	12	PE 17:0/17:0	100
Alkenylphosphatidylethanolamine (PE(P))	11	PE 17:0/17:0	100
Lysophosphatidylethanolamine (LPE)	6	PE 14:0/0:0	100
Phosphatidylinositol (PI)	16	PE 17:0/17:0	100
Lysophosphatidylinositol (LPI)	4	PE 14:0/0:0	100

Phosphatidylserine (PS)	5	PS 17:0/17:0	100
Phosphatidylglycerol (PG)	2	PG 17:0/17:0	100
Cholesterol ester (CE)	25	CE 18:0 <i>d</i> ₆	1000
Free cholesterol (COH)	1	COH <i>d</i> ₇	1000
Diacylglycerol (DG)	26	DG 15:0/15:0	200
Triacylglycerol (TG)	44	TG 17:0/17:0/17:0	100
Bis(monoacylglycero)phosphate (BMP)	1	BMP 14:0/14:0	100

^a Amount of internal standard per sample

S2 Table: Conditions for tandem mass spectrometry analysis of lipid species

Lipid class or subclass	Parent ion	Experiment ^b	Voltage settings ^c			
			DP	EP	CollE	CXP
dhCer	[M+H] ⁺	PIS, 284.3 <i>m/z</i>	90	30	28	10
Cer	[M+H] ⁺	PIS, 264.3 <i>m/z</i>	50	10	35	12
MHC	[M+H] ⁺	PIS, 264.3 <i>m/z</i>	77	10	50	12
DHC	[M+H] ⁺	PIS, 264.3 <i>m/z</i>	100	10	65	12
THC	[M+H] ⁺	PIS, 264.3 <i>m/z</i>	130	10	73	12
GM3	[M+H] ⁺	PIS, 264.3 <i>m/z</i>	155	10	105	16
SM	[M+H] ⁺	PIS, 184.1 <i>m/z</i>	65	10	35	12
PC	[M+H] ⁺	PIS, 184.1 <i>m/z</i>	100	10	45	11
PC(O)	[M+H] ⁺	PIS, 184.1 <i>m/z</i>	100	10	45	11
PC(P)	[M+H] ⁺	PIS, 184.1 <i>m/z</i>	100	10	45	11
LPC	[M+H] ⁺	PIS, 184.1 <i>m/z</i>	90	10	38	12
LPC(O)	[M+H] ⁺	PIS, 285.2 <i>m/z</i>	90	10	42	5
PE	[M+H] ⁺	NL, 141 Da	80	10	31	7
PE(O)	[M+H] ⁺	NL, 141 Da	80	10	31	7
PE(P)	[M+H] ⁺	NL, 141 Da	80	10	31	7
LPE	[M+H] ⁺	NL, 141 Da	80	10	31	7
PI	[M+NH ₄] ⁺	NL, 277 <i>m/z</i>	51	10	43	14
LPI	[M+NH ₄] ⁺	NL, 277 <i>m/z</i>	51	10	43	14

PS	[M+H] ⁺	NL, 185 Da	86	10	29	16
PG	[M+NH ₄] ⁺	NL, 189 Da	60	10	25	12
CE	[M+NH ₄] ⁺	PIS, 369.3 <i>m/z</i>	30	10	20	12
COH	[M+NH ₄] ⁺	PIS, 369.3 <i>m/z</i>	55	10	17	12
DG	[M+NH ₄] ⁺	NL, fatty acid	55	10	30	22
TG	[M+NH ₄] ⁺	NL, fatty acid	95	10	30	12
BMP	[M+NH ₄] ⁺	NL, fatty acid dependent	65	10	35	5

^b PIS = precursor ion scan, NL = neutral loss scan.

^c DP = declustering potential (volts); EP = entrance potential (volts); ColIE = collision energy (volts); CXP = collision cell exit potential (volts).

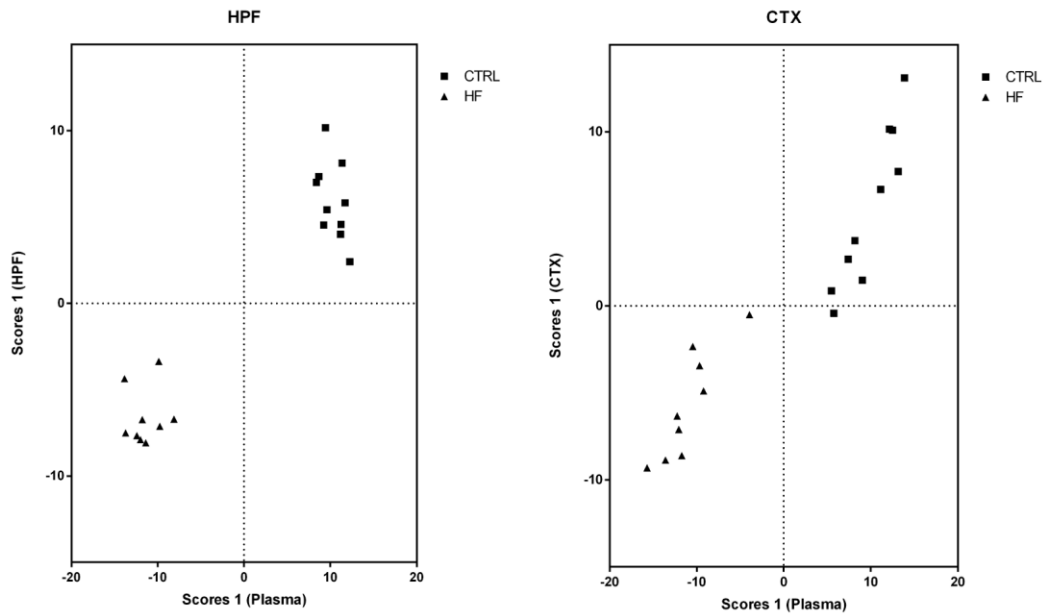
S3 Table. Proportion of variance explained (R^2) by the first latent variable for the first 25 individual lipids in the hippocampus and plasma lipids.

Hippocampus		Plasma	
Lipid	R^2	Lipid	R^2
PE 34:2	0.90	PC 39:6	0.96
PC 36:5	0.83	LPC 22:5	0.96
PE 40:5	0.80	LPC 20:5	0.95
PC 34:3	0.79	CE 20:5	0.94
PE 36:2	0.77	PC 36:5	0.94
PC 38:6	0.76	TG 18:1/18:1/22:6	0.94
PE 36:5	0.75	TG 16:1/18:1/18:2	0.94
PC 38:7	0.74	PC(P) 38:6	0.93
PI 38:6	0.74	PC 36:6	0.93
PC 36:6	0.74	PC 38:7	0.93
PC 40:7	0.72	TG 16:0/18:1/18:2	0.93
DG 16:0/22:5	0.70	PC(P) 38:5	0.93
PS 40:5	0.69	LPC 22:6	0.93
PC 33:1	0.68	TG 16:0/18:2/18:2	0.92
PE 34:3	0.68	PC(O) 40:7	0.92
PE(O) 40:7	0.61	TG 18:1/18:1/18:2	0.92
PE 40:7	0.61	PC(P) 36:5	0.91
PC 40:6	0.60	CE 20:2	0.90
PE 40:6	0.59	PC 38:6	0.90
PE 38:6	0.59	PC 34:5	0.89
PE(O) 40:6	0.58	CE 14:0	0.89
PC 39:6	0.56	PC 37:6	0.89
PE 36:3	0.55	TG 17:0/16:0/18:0	0.87
PC 34:2	0.51	PE(O) 40:5	0.87
DG 18:0/18:2	0.50	CE 17:0	0.87

S4 Table. Proportion of variance explained (R^2) by the first latent variable for the first 25 individual lipids in the cerebral cortex and plasma lipids.

Cortex		Plasma	
Lipid	R^2	Lipid	R^2
PC 38:6	0.87	PC 39:6	0.96
PE 34:2	0.85	PC 36:5	0.96
PC 40:7	0.85	PC 38:7	0.96
PC 38:7	0.80	CE 20:5	0.96
PC 28:0	0.80	PC 36:6	0.96
PE 40:7	0.79	PC 37:6	0.95
PI 38:6	0.79	PC(P) 38:6	0.94
PC 36:6	0.76	PC 31:0	0.94
PE(P) 38:6	0.73	TG 16:0/18:1/18:2	0.93
PC 40:6	0.73	PC(P) 38:5	0.93
PC 37:6	0.72	LPC 22:6	0.93
PS 40:5	0.71	PC(O) 40:7	0.92
PE(P) 40:6	0.70	CE 14:0	0.92
PC(P) 38:6	0.70	PC 38:6	0.92
PE(O) 40:6	0.70	PC 34:5	0.92
PE 40:6	0.69	TG 16:1/18:1/18:2	0.91
PE 38:6	0.69	PC(P) 36:5	0.91
PC 34:3	0.67	PI 40:6	0.91
PC 36:5	0.65	TG 16:0/18:2/18:2	0.91
PC(P) 34:2	0.65	TG 18:1/18:1/18:2	0.90
PI 40:6	0.65	TG 18:1/18:1/22:6	0.90
PC 39:6	0.64	PE(O) 40:5	0.89
PC 39:7	0.63	LPC 20:5	0.89
PC 34:2	0.62	LPC 22:5	0.88
PC 35:1	0.62	TG 16:0/16:1/18:1	0.88

S1 Figure. Scores scatter plot for partial least squares models.



Wild-type mice were fed regular chow (n=10) or a saturated fat enriched diet (n=9) for six months. Plasma, hippocampus and cortex lipid species were quantitated with LC-ESI-MS/MS. Partial least squares regression was used to identify plasma lipid species that account for the major sources of variation within the (A) hippocampus and (B) cortex. Scores scatter plot of the first latent variable for mice fed regular chow (squares) or a saturated fat enriched diet (triangles). The first latent variable score for the independent lipids (plasma) on the X-axis and the first latent variable score for dependent lipids (hippocampus/cortex) on the Y-axis. Scores are from the jack-knife resampled global model.

Chapter 4

Chapter 4: Circulating ceramides and cerebral ceramide homeostasis

The content of this chapter is covered by Publication 2:

Giles, C., Takechi, R., Mellett, N. A., Meikle, P. J., Dhaliwal, S., & Mamo, J. C. (2017). Differential regulation of sphingolipid metabolism in plasma, hippocampus, and cerebral cortex of mice administered sphingolipid modulating agents. *Journal of Neurochemistry*. doi:10.1111/jnc.13964

4.1 Article synopsis

4.1.1 Background

Aberrant sphingolipid metabolism has been implicated in the progression of neurodegenerative disorders. Accumulation of ceramide within the brain is suggested to participate in the substantial neuronal loss observed in Alzheimer's disease. Within the circulation, elevated ceramides are associated with cognitive decline and increased risk of developing AD. Combining the two lines of evidence, it has been suggested that circulating ceramides may play a key role in regulating cerebral ceramide abundance and therefore, influence the initiation of AD.

As ceramides are the central metabolite in the sphingolipid metabolic network, there are multiple pathways that regulate their abundance. Several key pathways have been identified that might be influential in regulating blood and brain ceramide concentrations. The two most well-known mechanism leading to ceramide accumulation are through de novo synthesis and activation of sphingomyelinases. In physiological systems, there is potential for trafficking of ceramides from other organs. A downstream metabolite of ceramides, sphingosine-1-phosphate has shown to protect cells from ceramide accumulation and lead to cell survival. It is now clear that most functions S1P are accomplished through activation of 5 extracellular facing receptors.

To consider the physiological roles of the different pathways that regulate ceramides, animal models were utilised. Using a suite of well-characterised sphingolipid modulating agents, the major pathways regulating ceramides can be assessed in a physiological setting.

4.1.2 Experimental design in brief

Male C57Bl/6J mice were randomised to one of five experiment groups: mice fed standard rodent chow; mice fed a saturated fat enriched diet (SFA); mice fed SFA with an inhibitor of sphingolipid de novo synthesis; mice fed SFA with an inhibitor of sphingomyelinase; mice fed SFA with a sphingosine-1-phosphate receptor agonist. Following treatment, the hippocampus, cerebral cortex and plasma were collected for detailed analysis of sphingolipids. The putative role of different sphingolipid metabolic pathways, including that of circulating ceramides, were assessed.

4.1.3 Results in brief

Ceramides and their precursors, dihydroceramides, were elevated in the plasma of mice consuming the SFA diet (~60 % increase), compared to chow fed mice. Inhibiting the sphingolipid de novo synthesis prevented this increase, bringing ceramide concentrations down to control animal levels. This decrease was accompanied by a substantially altered sphingolipidome, including a reduction in dihydroceramides, sphingomyelin and monohexosylceramides and elevation in dihexosylceramide and GM3 gangliosides. Inhibition of sphingomyelinase with amitriptyline did not affect the concentration of any sphingolipids in plasma, nor did the agonist of sphingosine-1-phosphate receptors. By contrast, the sphingolipids in the hippocampus and cerebral cortex did not change in response to the SFA diet. The concentration of ceramides in the cerebral cortex were reduced (~15 %) by inhibiting sphingolipid de novo synthesis, whereas hippocampal ceramides were reduced by S1P receptor agonism (~20 %).

Associations between circulating ceramides and cerebral associations were assessed individually and separated by chain length and saturation. There were multiple negative correlations between circulating ceramides and hippocampal ceramides. Whereas, several small positive correlations existed for circulating C24:1 ceramide (a high abundance plasma isoform) and several ceramide isoforms in the cerebral cortex.


4.1.4 Discussion and conclusion in brief

In this study, a suite of sphingolipid modulating agents and diets were used to investigate the regulation and interaction of circulating and cerebral ceramides. Using state-of-the-art lipidomics, alterations in abundance of sphingolipids could be measured in plasma, hippocampus and cerebral cortex of mice fed saturated fat enriched diets, an inhibitor of sphingolipid de novo synthesis, inhibitor of sphingomyelinase or an agonist of sphingosine-1-phosphate receptors. The results suggest that sphingolipids in plasma are highly regulated by de novo synthesis. Application of de novo synthesis inhibitor reduced all simple sphingolipids, whereas consumption of saturated fat (containing substrates for de novo synthesis) increased ceramides and dihydroceramides. By contrast, sphingolipids within the brain were generally tightly regulated. Small decreases in ceramides were observed in the cerebral cortex with inhibition of de novo synthesis and in the hippocampus with S1P receptor agonism.

A large body of work has implicated circulating ceramides in the development of cognitive decline and risk for neurodegenerative diseases. The hypothesis proposed is that circulating ceramides are capable of influencing ceramide concentration in the brain, possibly through direct deposition. However, to date, no studies have been conducted to explore these hypotheses, therefore this study was the first to explore relationships between circulating and cerebral sphingolipids. In contrast to expectations, there were a substantial number of negative correlations between circulating and hippocampal ceramides. While these results are not supportive of the hypothesis stated above, it does not preclude

circulating ceramides as influential mediators of neurodegenerative disease risk. Rather, the results are supportive of the notion that (1) increased circulating ceramides do not lead to accrual of ceramides in the brain; and (2) circulating ceramides are not indirect measures of cerebral ceramides.

The information gained from this study builds on the hypotheses generated from cross-sectional and longitudinal studies in humans suggesting a biological function of circulating ceramides in neurodegenerative disease risk. Rather than supporting these hypotheses, the results highlight a gap in our understanding of how these lipids influence the brain. Future research should endeavour to delineate the mechanisms through which this risk is pronounced, possibly with a focus on the BBB which has shown vulnerability for ceramide accrual.

ORIGINAL
ARTICLEDifferential regulation of sphingolipid metabolism
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mice administered sphingolipid modulating agentsCorey Giles,*†  Ryusuke Takechi,*† Natalie A. Mellett,‡ Peter J. Meikle,‡
Satvinder Dhaliwal† and John C. Mamo*†

*Curtin Health Innovation Research Institute, Curtin University, Perth, Western Australia, Australia

†School of Public Health, Faculty of Health Sciences, Curtin University, Perth, Western Australia,
Australia

‡Metabolomics Laboratory, Baker IDI Heart and Diabetes Institute, Melbourne, Victoria, Australia

Abstract

Accumulation of ceramide is implicated in mediating the cellular responses to stress and aberrant sphingolipid metabolism is frequently associated with metabolic and neurodegenerative diseases. It is often assumed that (i) peripheral disturbances in sphingolipid concentrations are reflective of processes occurring in the brain, or (ii) circulating sphingolipids directly influence cerebral sphingolipid abundance. In order to address these assumptions, this study explores, in a physiological system, the metabolic pathways regulating sphingolipid metabolism in the brain and plasma of mice. Male C57Bl/6 were maintained on a low fat (control diet) or saturated fat enriched (SFA) diet with, or without the provision of sphingolipid modulating agents. Following 6 months of feeding, the abundance of seven sphingolipid classes was assessed by LC-ESI-MS/MS in the hippocampus (HPF), cerebral cortex (CTX), and plasma. Long-term consumption

of the SFA diet increased ceramide and dihydroceramide in the plasma. Inhibiting de novo synthesis ameliorated this effect, while inhibition of acidic sphingomyelinase, or the sphingosine-1-phosphate receptor agonist did not. SFA feeding did not influence sphingolipid levels in either the HPF or CTX. De novo synthesis inhibition reduced ceramide in the CTX, while treatment with a sphingosine-1-phosphate receptor agonist reduced ceramides in the HPF. Analysis of the individual ceramide species revealed the effects were chain-length dependent. Both positive and negative correlations were observed between plasma and HPF/CTX ceramide species. The findings in this study show that HPF and CTX sphingolipid concentration are influenced by distinct pathways, independent of peripheral sphingolipid concentration.

Keywords: brain, ceramide, lipidomics, mass spectrometry, neurodegeneration.

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Sphingolipids are highly enriched in the central nervous system, contributing to the structural integrity and fluidity of cellular membranes and myelin sheaths. Accumulating evidence suggests that sphingolipid metabolism is perturbed early in the development of many neurodegenerative diseases, including Alzheimer's disease (AD) (Cutler *et al.* 2004), Multiple Sclerosis (Vidaurre *et al.* 2014), and Parkinson's disease (Mielke *et al.* 2013; Xing *et al.* 2016). Several postmortem studies have found that elevated levels of ceramides are found in the brain of AD patients (Han *et al.* 2002; Cutler *et al.* 2004; Filippov *et al.* 2012). The susceptibility of neurons to ceramide-induced pathology has been extensively reviewed (Jana *et al.* 2009; Mencarelli and Martinez-Martinez 2013). Interestingly, increases in plasma ceramides have been shown to predict cognitive decline and

hippocampal volume loss in patients with mild cognitive impairment (Mielke *et al.* 2010b). Furthermore, elevated circulating ceramides were associated with a worsening of

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Address correspondence and reprint requests to John C. Mamo, Curtin Health Innovation Research Institute, Curtin University, GPO Box U1987, Perth, WA 6845, Australia. E-mail: J.Mamo@curtin.edu.au

Abbreviations used: AD, Alzheimer's disease; AMI, amitriptyline; aSMase, acid sphingomyelinase; BBB, blood-brain barrier; Cer(d18:0), dihydroceramides; Cer(d18:1), ceramides; CTRL, control diet; CTX, cerebral cortex; FTY, FTY 720; GM3, GM3 ganglioside; Hex2Cer, dihexosylceramides; Hex3Cer, trihexosylceramides; HexCer, monohexosylceramides; HPF, hippocampus; MYR, myriocin; S1P, sphingosine-1-phosphate; SFA, saturated fat-enriched diet; SM, sphingomyelin; SPT, serine-palmitoyltransferase.

white matter microstructure in cognitively normal patients (Gonzalez *et al.* 2016). These studies highlight the importance of sphingolipid metabolism for central nervous system function and the potential for peripheral ceramides to influence the brain. Despite the accumulating evidence suggesting a pivotal role of sphingolipid deregulation in pathogenesis of many brain disorders, few studies have attempted pharmacological modulation of sphingolipid abundance in the brain, nor attempted to examine the potential of circulating ceramides to influence cerebral ceramide concentrations.

Ceramides have been implicated as a lipid mediator of cellular responses, including redox homeostasis, inflammation, and indeed apoptosis (Hannun 1996). Forming the central metabolite in sphingolipid metabolic network, ceramides exist as both ubiquitous modulators of membrane dynamics and as a second messenger (van Blitterswijk *et al.* 2003). Therefore, it is important to consider the multiple pathways that regulate ceramide metabolism. In cellular systems, two principle pathways have been extensively studied that are involved in ceramide accumulation – de novo synthesis, a non-reversible pathway catalyzed by the rate-limiting enzyme serine-palmitoyltransferase and sphingomyelin hydrolysis by sphingomyelinases. However, in physiological systems, there is potential for trafficking of circulating ceramides to organs through uptake of lipoprotein-associated ceramides and lipid soluble exchange during lipoprotein-cell interactions (Boyanovsky *et al.* 2003; Boon *et al.* 2013). There have been several suggestions that plasma ceramides are associated with cerebrospinal fluid (Mielke *et al.* 2010b, 2012; Mielke *et al.* 2011). Given that circulating ceramides appear early in the development of many neurodegenerative diseases, their contribution to deregulation of sphingolipid metabolism in the brain should be clarified.

A family of ceramide synthases (CerS), involved during de novo synthesis, are responsible for the differences in acyl-chain lengths across tissues (Levy and Futerman 2010). Six CerS catalyze the N-acylation of acyl-CoAs to the (dihydro) sphingosine backbone, each utilizing a restricted set of acyl-CoAs. In addition to their involvement in de novo synthesis, CerS also have central roles in the sphingolipid recycling or 'salvage pathway' (Kitatani *et al.* 2008). There have been reported differences in the acyl-chain composition of sphingolipids between blood, cerebrospinal fluid and brain tissue (Haughey *et al.* 2004; Mielke *et al.* 2015) and it's conceivable that circulating ceramides may exert their biological effects after delivery and deacylation/reacylation. The involvement and role of ceramide acyl chain length and ceramide synthases in neurodegeneration has recently been reviewed (Ben-David and Futerman 2010). An increase in the number of studies examining the role of sphingolipids in pathologies has occurred because of the discovery of pharmacological agents that modulate different pathways of

sphingolipid metabolism. These sphingolipid modulating agents are in use in animal and human research. Myricin (MYR), an inhibitor of serine-palmitoyltransferase, has been shown to decrease atherosclerosis in apo-E-deficient mice (Hojjati *et al.* 2005); ameliorate glucocorticoid-, saturated fat- and obesity-induced insulin resistance (Holland *et al.* 2007); and to reduce non-alcoholic fatty liver disease in high-fat fed rats (Kurek *et al.* 2014). Inhibitors of sphingomyelinases typically fall under two categories, acidic sphingomyelinases, or neutral sphingomyelinase inhibitors. Amitriptyline (AMI), an inhibitor of acid sphingomyelinase (aSMase), has been used in the study of cystic fibrosis (Teichgraber *et al.* 2008; Becker *et al.* 2010), renal injury during high-fat diets (Boini *et al.* 2010), and studied for the role of sphingomyelinase inhibition in anti-depressant drugs (Gulbins *et al.* 2013).

Although much research has focused on inhibiting ceramide accumulation, the phosphorylated sphingosine base, sphingosine-1-phosphate (S1P), opposes many of the cellular functions of ceramide. While no pharmacological agent is routinely in use to increase ceramide degradation, there are S1P mimetics in use in clinical trials. FTY720 (FTY) is the first oral drug approved for treatment of the relapsing-remitting form of multiple sclerosis and works through activation of sphingosine-1-phosphate receptors (Brinkmann *et al.* 2002). There is currently debate as to the physiological mechanism through which FTY abates multiple sclerosis and other neurodegenerative disorders. Whether the mechanism is principally through a peripheral immunomodulatory response, or through direct actions on neurons and cerebrovascular protective effects (van Doorn *et al.* 2012). Furthermore, the ceramide modulating effects of FTY have been noted in the periphery (Bruce *et al.* 2013) but require clarification, especially in the brain.

In this study, concentrations of multiple sphingolipid classes and species are measured in plasma, hippocampus (HPF), and cerebral cortex (CTX) of an animal model recognized for peripheral disturbances of sphingolipid metabolism and which shows early pathological symptoms of neurovascular inflammation (Takechi *et al.* 2014). To consider the putative role of the different pathways that regulate sphingolipids, a suite of sphingolipid modulating agents were utilized, shown to attenuate ceramide accumulation in peripheral organs through alternate pathways. In addition, the associations between plasma ceramides and HPF and CTX ceramides are reported.

Methods

Animals

All experimental procedures were approved by the Curtin University Animal Ethics Committee and completed in accordance with National Health and Medical Research Council (NHMRC) guidelines. Male C57BL/6 mice were obtained from the Animal

Resource Centre (ARC; Murdoch, Western Australia). They were housed in an accredited animal holding facility with 12 h light/dark cycles, at ambient temperature of 22 degrees Celsius. Mice had ad libitum access to water and their respective diets. At 8 weeks of age, mice were randomly assigned to one of five groups ($n = 10$ per group), receiving either a control diet (CTRL, AIN-93M), a modified AIN-93M chow containing 40% energy from cocoa butter (SFA), SFA diet with MYR, SFA diet with AMI or SFA diet with FTY. Myriocin was incorporated into chow at 1.5 mg/kg (w/w) to deliver a daily dose of approximately 0.3 mg/kg body weight. Amitriptyline and FTY were supplied through drinking water, at concentrations of 125 and 3.75 mg/L, respectively. Fresh drinking water solutions were prepared daily.

Sample isolation and preparation

Mice were maintained on their respective diets and treatments for 6 months. Mice were administered an intraperitoneal dose of pentobarbital and following complete anesthesia, blood was collected through cardiac puncture into EDTA containing tubes. Plasma was separated through centrifugation and frozen at -80°C for further analysis. Following exsanguination, brains were rapidly excised, washed in ice-cold phosphate-buffered saline and left hemispheres snap frozen in liquid nitrogen. Using a commercial brain block, the left frozen hemispheres were sectioned into 1 mm coronal slices. Under a stereotaxic microscope, sections of the S2 cerebral cortex and hippocampus were isolated and weights recorded. Isolated regions were diluted in 10 volumes of ice-cold phosphate-buffered saline (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl; pH 7.4), homogenized and frozen for further analysis.

Extraction of lipids

The extraction of lipids was completed by a single-phase chloroform:methanol extraction as described previously (Weir *et al.* 2013). In brief, 10 μL of plasma or 10 μL of brain homogenate (containing approximately 20 μg of protein) was transferred to an eppendorf tube with 10 μL of internal standard mixture (Weir *et al.* 2013). Chloroform/methanol (2 : 1; 20 volumes) was added to each sample, followed by rotary mixing (10 min), sonication (30 min), and standing (20 min) at 22°C . Samples were centrifuged (16 000 g, 10 min) and the supernatant collected and dried under nitrogen gas at 40°C . Samples were reconstituted with 50 μL of water saturated butanol and sonicated (10 min), followed by 50 μL methanol (with 10 mM ammonium formate). Extracts were centrifuged (3350 g, 5 min) and the supernatant transferred to 0.2 mL glass vials with Teflon caps ready for analysis.

Mass spectrometric analysis of lipids

Analysis of lipids was conducted using liquid chromatography electrospray ionization-tandem mass spectrometry on an Agilent 1200 UHPLC coupled to an AB Sciex Q/TRAP 4000 mass spectrometer with a turbo-ion spray source as extensively described previously (Weir *et al.* 2013). Briefly, detection of lipids was performed with scheduled multiple reaction monitoring in positive ion mode using Analyst 1.5 (AB Sciex; Framingham, MA, U.S.A.). The concentration of individual lipid species was determined by relating the integrated peaks to the appropriate internal standards in MultiQuant 2.1 (AB Sciex). Samples were randomized and quality control samples were analyzed each 20 samples to assess assay

performance. In total, 55 lipid species in seven lipid classes and subclasses were detected and quantitated from each plasma, cerebral cortex and hippocampal sample.

Data analysis

Individual lipid species concentrations were expressed as picomoles per mg wet weight for brain samples and picomoles per microliter for plasma samples. Total lipid concentration of each class was calculated by summing the individual species in that class. All lipid species detected within a bio-compartment (hippocampus, cerebral cortex or plasma) were used for calculating class sums.

Prior to statistical comparisons, all data were tested and confirmed for normality using Shapiro–Wilk test. Univariate comparisons were conducted comparing lipids between the high-fat diet fed mice against lipids observed in the control diet fed group and those receiving sphingolipid modulating agents. For these comparisons, independent samples *t*-tests were used. Correlations were assessed by Pearson's correlation.

Data analysis was performed using R version 3.3.1 (R Core Team 2015).

Results

Sphingolipid profiles were determined in the plasma, HPF, and CTX of mice following 6 months feeding with their respective diet/drug. Sphingolipids were classified as one of seven classes and subclasses: ceramides (Cer(d18:1)), dihydroceramide (Cer(d18:0)), sphingomyelin (SM), monohexosylceramide (HexCer), dihexosylceramide (Hex2Cer), trihexosylceramide (Hex3Cer), and $\text{G}_{\text{M}3}$ ganglioside (GM3). Table 1 details the estimated molar abundance of the sphingolipid classes in plasma.

Mice maintained on the SFA diet for 6 months developed an altered plasma sphingolipid composition in comparison to those on the CTRL diet. Mice on the SFA diet showed a 38.3% increase in total Cer(d18:1) in plasma compared to CTRL animals (Table 1; $p < 0.001$). Cer(d18:0), the de novo synthesis pathway precursor to Cer(d18:1), increased 326% in plasma of mice fed the SFA diet ($p < 0.001$). The plasma abundance of SM, HexCer, Hex2Cer, Hex3Cer, and GM3 did not change in response to the SFA diet.

Treatment of mice with MYR, an inhibitor of sphingolipid de novo synthesis, produced a substantially altered plasma sphingolipid profile. Myriocin reduced plasma Cer(d18:1) by 42.6% compared to mice on the SFA diet ($p < 0.001$). Similarly, Cer(d18:0) was reduced by 40.4% compared to SFA diet group ($p = 0.012$). Myriocin treatment reduced plasma SM compared to the CTRL group ($p = 0.024$), but did not reach statistical significance compared with SFA. Complex glycosphingolipids were also altered by treatment with MYR. Plasma HexCer was reduced compared to both the SFA and CTRL groups ($p < 0.001$), while plasma Hex2Cer ($p < 0.05$) and GM3 ($p < 0.001$) were increased. No change in plasma Hex3Cer was observed with treatment of MYR.

Table 1 Relative abundance of sphingolipid classes in plasma, hippocampus, and cerebral cortex

	CTRL	SFA	MYR	AMI	FTY
Plasma					
Cer(d18:1)	1.00 ± 0.14 [†]	1.62 ± 0.41*	0.93 ± 0.12 [†]	1.53 ± 0.27*	1.88 ± 0.60*
Cer(d18:0)	0.13 ± 0.04 [†]	0.57 ± 0.18*	0.34 ± 0.2 ^{††}	0.60 ± 0.29*	0.5 ± 0.19*
SM	68.3 ± 7.8	68.5 ± 20.7	60.7 ± 6*	60.5 ± 9.8	71.2 ± 24.5
HexCer	11.5 ± 1.6	10.7 ± 2	7.49 ± 0.83 ^{††}	10.5 ± 2.1	12.4 ± 4.6
Hex2Cer	0.33 ± 0.06	0.31 ± 0.04	0.42 ± 0.09 ^{††}	0.30 ± 0.03	0.31 ± 0.05
Hex3Cer	0.17 ± 0.03	0.16 ± 0.04	0.17 ± 0.03	0.16 ± 0.03	0.18 ± 0.03
GM3	0.12 ± 0.05	0.12 ± 0.06	0.25 ± 0.06 ^{††}	0.13 ± 0.05	0.17 ± 0.08
Hippocampus					
Cer(d18:1)	145 ± 21	136 ± 25	128 ± 28	129 ± 29	113 ± 21 ^{††}
Cer(d18:0)	4.71 ± 0.7	4.63 ± 0.8	4.53 ± 1.2	4.48 ± 0.9	4.28 ± 1.0
SM	839 ± 118	789 ± 83	786 ± 62	809 ± 70	812 ± 104
HexCer	493 ± 81	535 ± 58	493 ± 102	540 ± 102	525 ± 114
Hex2Cer	189 ± 25	210 ± 20	217 ± 39	224 ± 45*	216 ± 36
Hex3Cer	10.5 ± 2.9	10.6 ± 1.9	11.2 ± 2.5	11.2 ± 2.5	9.9 ± 2.8
GM3	13 ± 2.6	12.7 ± 2.0	12.0 ± 2.5	13 ± 2	13 ± 3.1
Cerebral Cortex					
Cer(d18:1)	94.1 ± 17.2	87.8 ± 15.6	76.3 ± 12.4*	81.5 ± 16.0	98.1 ± 21
Cer(d18:0)	3.37 ± 0.62	3.45 ± 0.54	3.21 ± 0.73	2.94 ± 0.64	3.5 ± 0.62
SM	601 ± 76	613 ± 50	584 ± 78	628 ± 101	615 ± 84
HexCer	532 ± 176	484 ± 84	564 ± 115	478 ± 166	447 ± 89
Hex2Cer	192 ± 70	186 ± 37	214 ± 35	176 ± 57	170 ± 43
Hex3Cer	9.21 ± 2.65	9.1 ± 2.05	9.99 ± 1.45	9.57 ± 2.73	8.23 ± 1.33
GM3	3.67 ± 1.04	3.77 ± 0.8	4.08 ± 1.69	3.98 ± 1.2	4.49 ± 0.97

Mean ± SD. Plasma, pmol/μL; Hippocampus, pmol/mg brain; Cerebral cortex, pmol/mg brain. CTRL, control diet fed group; SFA, saturated fat enriched diet fed group; MYR, myriocin-treated mice; AMI, amitriptyline-treated mice; FTY, FTY720-treated mice; Cer(d18:1), ceramide; Cer(d18:0), dihydroceramide; SM, sphingomyelin; HexCer, monohexosylceramide; Hex2Cer, dihexosylceramide; Hex3Cer, trihexosylceramide; GM3, GM₃ ganglioside. N = 9–10 per group. **p* < 0.05 versus CTRL. [†]*p* < 0.05 versus SFA.

Amitriptyline and FTY did not alter the plasma sphingolipid composition in comparison to SFA diet alone. Similar to the SFA only group, plasma Cer(d18:1) and Cer(d18:0) were elevated over CTRL animals (all comparisons, *p* < 0.001). Plasma SM, HexCer, Hex2Cer, Hex3Cer, and GM3 were not significantly altered in the AMI and FTY groups.

The HPF and CTX sphingolipid profile was characterized and differed substantially from that observed in plasma (Table 1). Comparison of sphingolipid class abundance between the HPF and CTX is shown in Fig. 1. Based on the abundance per milligram of tissue, the HPF is more enriched in sphingolipids than the CTX. Key differences between the two regions principally lie in the abundance of Cer(d18:1) (*p* < 0.001), Cer(d18:0) (*p* = 0.002), SM (*p* < 0.001) and GM3 (*p* < 0.001).

Compared to CTRL-fed mice, the SFA diet did not significantly alter the abundance of any sphingolipid class in either the HPF or CTX. Mice treated with MYR showed a decrease in the abundance of Cer(d18:1) within the CTX compared to CTRL animals (*p* = 0.016), although this did not reach statistical significance compared to the SFA only

group (*p* = 0.082). The abundance of the other sphingolipid classes was not altered by MYR in either the HPF or CTX.

Mice treated with AMI had similar levels of Cer(d18:1) in both the CTX and HPF. However, they showed increased concentrations of Hex2Cer in the HPF compared to CTRL animals (*p* = 0.05). The abundance of other sphingolipid classes was not altered by AMI in both regions of the brain.

Mice consuming the SFA diet and treated with FTY showed a decreased HPF abundance of Cer(d18:1) compared to both CTRL (*p* = 0.004) and SFA alone (*p* = 0.05). This effect was only observed in the HPF, as the CTX abundance of Cer(d18:1) was not different compared to CTRL or SFA. The abundance of other sphingolipid classes was not significantly altered by FTY in both regions of the brain.

The accumulation of different Cer(d18:1) species has been implicated in inducing different cellular functions, therefore the abundance of individual species is analyzed in the plasma, HPF, and CTX. The changes in plasma Cer(d18:1) were species specific (Fig. 2). Consumption of the saturated fat enriched diet caused substantial rearrangement of the plasma ceramide profile (Fig. 2). Ceramide species d18:1/16:0 (↑121%, *p* < 0.001), d18:1/20:0 (↑185%, *p* < 0.001),

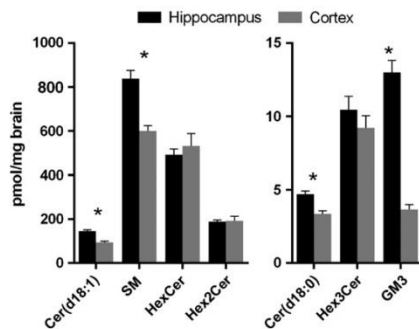


Fig. 1 Comparative abundance of sphingolipids between the hippocampus and cerebral cortex of control mice. The abundance of seven sphingolipid classes in the hippocampus (black bars) and cortex (grey bars) fed a low-fat diet for 6 months. Cer(d18:1), ceramides; SM, sphingomyelin; HexCer, monohexosylceramides; Hex2Cer, dihexosylceramides; Cer(d18:0), dihydroceramides; Hex3Cer, trihexosylceramide; GM3, GM3 ganglioside. $N = 10$, paired t -test. * $p < 0.05$. Mean \pm SEM.

d18:1/22:0 ($\uparrow 216\%$, $p < 0.001$), d18:1/22:1 ($\uparrow 86\%$, $p = 0.001$), d18:1/24:0 ($\uparrow 104\%$, $p < 0.001$), and d18:1/26:1 ($\uparrow 49\%$, $p = 0.002$) all increased following consumption of the SFA diet.

Treatment with MYR ameliorated much of the effects of the SFA diet on the plasma Cer(d18:1) profile, lowered elevated species to near or below CTRL levels. Treatment with AMI or FTY did not cause a significant change in the plasma ceramide profile in comparison to SFA diet alone.

The HPF (Fig. 3) and CTX (Fig. 4) showed a substantially different ceramide profile from that observed in plasma. In

CTRL mice, the ceramide isoform d18:1/18:0 accounted for 88% of HPF ceramides and 82% of ceramide abundance in CTX. Intermediate abundance species included d18:1/24:1 (5.3%, 9.9%; HPF, CTX), d18:1/16:0 (2.3%, 2.2%) and d18:1/20:0 (1.7%, 1.9%). The other isoforms constituted minor species and each make up less than 1% of total ceramides.

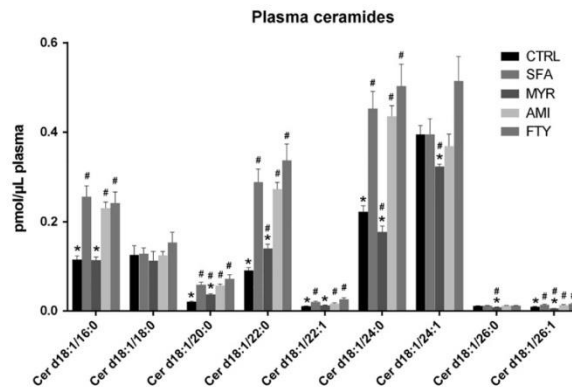
Mice fed the SFA diet did not exhibit an altered abundance of any ceramide species compared to CTRL animals, in both the HPF and CTX. Myriocin administration caused the abundance of several ceramide species to decrease in the CTX (Fig. 4). Amitriptyline treatment reduced abundance of several ceramide species in both the HPF and CTX. However, mice in the FTY group had a reduced total Cer (d18:1) abundance in the HPF and this corresponded to the reduction in several ceramide isoforms.

In order to investigate the putative associations between circulating plasma ceramides and cerebral ceramide species, a correlation matrix between plasma and the two regions of the brain was generated (Fig. 5). Between plasma and the hippocampus, there were numerous significant negative correlations, particularly involving plasma species d18:1/16:0, d18:1/20:0, d18:1/22:0, d18:1/22:1, and d18:1/24:0. The corresponding hippocampus species were d18:1/16:0, d18:1/18:0, d18:1/20:0, d18:1/22:0, d18:1/22:1, d18:1/24:0, and d18:1/24:1. In contrast, there was a trend to more positive correlations with the CTX. However, only one plasma specie, d18:1/24:1, was significantly correlated with any CTX species.

Discussion

In this study, a lipidomic approach was used to elucidate the metabolic pathways regulating sphingolipid concentrations in the brain and plasma of mice. Using sphingolipid modulating agents combined with an SFA-enriched diet,

Fig. 2 Altered abundance of ceramide species in plasma in response to saturated fat-enriched diet and sphingolipid modulating agents. Ceramide species separated by acyl chain length and degree of saturation. CTRL, control diet fed group; SFA, saturated fat enriched diet fed group; MYR, myriocin-treated mice; AMI, amitriptyline-treated mice; FTY, FTY720-treated mice. $N = 9-10$ per group. * $p < 0.05$ compared to SFA, # $p < 0.05$ compared to CTRL. Mean \pm SEM.



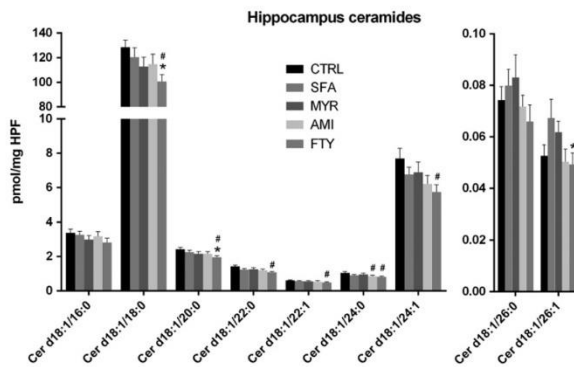


Fig. 3 Altered abundance of ceramide species in hippocampus of mice in response to sphingolipid modulating agents. Ceramide species separated by acyl chain length and degree of saturation. CTRL, control diet fed group; SFA, saturated fat enriched diet fed group; MYR, myriocin-treated mice; AMI, amitriptyline-treated mice; FTY, FTY720-treated mice. $N = 9-10$ per group. * $p < 0.05$ compared to SFA, # $p < 0.05$ compared to CTRL. Mean \pm SEM.

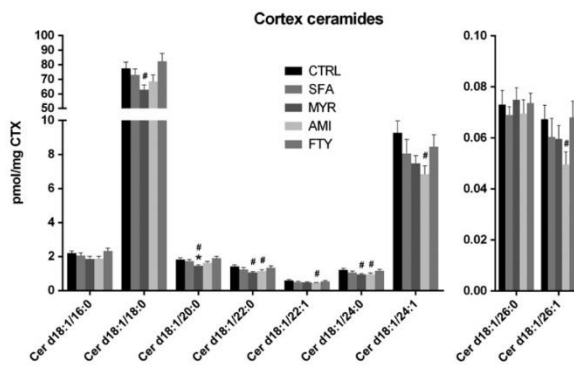


Fig. 4 Altered abundance of ceramide species in cerebral cortex of mice in response to sphingolipid modulating agents. Ceramide species separated by acyl chain length and degree of saturation. CTRL, control diet fed group; SFA, saturated fat enriched diet fed group; MYR, myriocin-treated mice; AMI, amitriptyline-treated mice; FTY, FTY720-treated mice. $N = 9-10$ per group. * $p < 0.05$ compared to SFA, # $p < 0.05$ compared to CTRL. Mean \pm SEM.

changes in the abundance of sphingolipid classes were assessed in plasma, the HPF, and CTX of each animal. Long-term consumption of the SFA diet increased Cer(d18:1) and Cer(d18:0) in plasma. Inhibiting de novo synthesis reversed these changes, while inhibiting aSMase or activating SIP receptors had no effect. In contrast to plasma, the SFA diet alone had no effect on the abundance of sphingolipids in the HPF and CTX. The data support the hypothesis that the HPF and CTX respond differently to the sphingolipid modulating agents. De novo synthesis inhibition reduced Cer(d18:1) in the cortex, while FTY reduced Cer(d18:1) in the HPF. Analysis of the individual Cer(d18:1) species revealed that changes are chain length dependant. In addition, aSMase inhibition reduced the abundance of several ceramide isoforms in both the HPF and CTX. Lastly, the correlation between circulating and cerebral Cer(d18:1) showed both positive and negative associations depending on the species and cerebral location. This study highlights the

associations, in a physiological model, between regional responses in the brain and plasma to sphingolipid modulating agents.

Circulating sphingolipids have been shown as predictive markers for risk and progression of neurodegenerative disorders. Mielke *et al.* (2010a) reported that elevated SM and Cer(d18:1) are early predictors of memory impairment in a longitudinal study. Another study revealed that higher plasma Cer(d18:1) were associated with poorer cerebral white matter microstructure (Gonzalez *et al.* 2016). These studies suggest that plasma sphingolipids may have a biological role in the pathogenesis in neurodegenerative disorders. It has been speculated that peripheral sphingolipids may (i) be reflective of changes in lipid concentrations that occurs in the brain; (ii) directly influence the functioning of the central nervous system, through uptake; or (iii) indirectly, by altering risks factors such as peripheral insulin resistance or vascular dysfunction. However, to date, no studies have

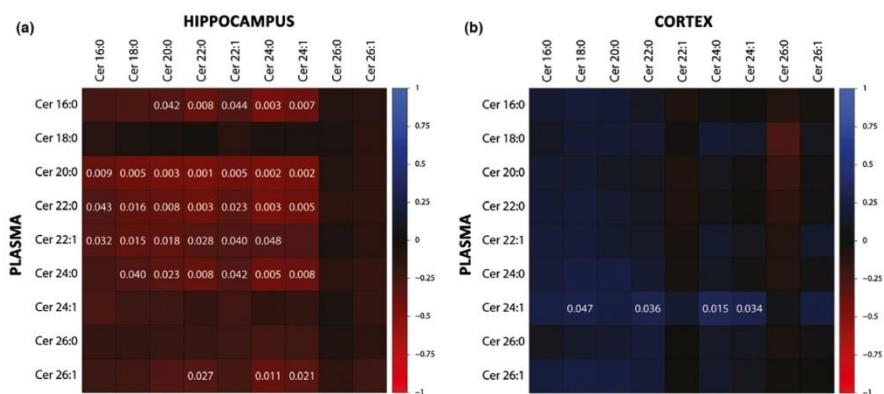


Fig. 5 Pearson correlation heatmap between plasma and cerebral ceramide species. Blue color depicts positive association, and red depicts negative association. Brightness indicates the strength of association (Pearson's r). Significant associations are indicated with the p -value in white. All animals were included in the analysis without stratifying by group. (a) Plasma ceramide species versus hippocampus ceramide species. (b) Plasma ceramide species versus cortex ceramide species.

reconciled these alternate hypotheses. To address this, three sphingolipid modulating agents are used in an SFA enriched diet mouse model and the plasma, HPF and CTX sphingolipidomes are compared.

Herein, the results provide confirmatory evidence that perturbations in plasma sphingolipid concentrations with SFA feeding are principally determined through de novo synthesis. Inhibiting de novo synthesis ameliorated the elevated Cer(d18:1) and Cer(d18:0), while inhibiting aSMase and activation of S1P receptors had no effect on plasma sphingolipids. This finding support the results of previous studies (Hojjati *et al.* 2005; Holland *et al.* 2007), however contrast with the suggestion of Boini *et al.* (2010) that aSMase is a major pathway leading to Cer(d18:1) accumulation in the plasma of high-fat fed mice. Changes in complex sphingolipids were not observed with the SFA diet, but were with long-term MYR treatment. Reduced de novo synthesis led to reduced SM and HexCer, both of which have been implicated in neurodegenerative disorders, atherosclerosis, and endothelial dysfunction (Glaros *et al.* 2008).

In contrast to plasma, there was no observed change in the total abundance of sphingolipid classes in either region of the brain, in response to SFA feeding. The finding is in contrast to Borg *et al.* (2012) who reported hypothalamic accumulation of Cer(d18:1), Cer(d18:0) and Hex2Cer in mice chronically fed a 60% fat diet. The hypothalamus is a region of the brain which has 'lipid sensing' functions and previously shown to accumulate long-chain acyl-CoA in response to short-term over-feeding (Pocai *et al.* 2006). Differences in the regional abundance and response of lipid

supply likely account for the differences in results reported. While there were no changes in response to the SFA-enriched diet, the response to the sphingolipid modulating agents revealed region-specific changes in the abundance of several sphingolipid classes. In mice chronically administered FTY, HPF ceramides were decreased, while CTX ceramides were unaffected. FTY has been shown to have bioactive functions in the brain, whereby it concentrates and exceeds blood concentrations several fold (Foster *et al.* 2007). FTY has been shown to inhibit ceramide synthases (Berdyshev *et al.* 2009), to inhibit acidic sphingomyelinase (Dawson and Qin 2011) and recently shown to reduce muscle ceramide concentration in response to high-fat feeding (Bruce *et al.* 2013). Within the CTX, inhibition of de novo synthesis with MYR reduced Cer(d18:1) concentration. Few studies have assessed the effects of MYR to influence cerebral sphingolipids. However, Saito *et al.* (2010) reported reductions in cerebral ceramides with intracerebroventricular injection of MYR in a mouse model of ethanol-induced apoptotic neurodegeneration, but did not explore the regions influenced. Inhibition of acidic sphingomyelinase, with AMI and fluoxetine, has been shown to dose dependently reduce ceramides in the hippocampus of ordinary mice. However, therapeutic treatment with fluoxetine in an induced stroke mouse model showed no effect on CTX ceramides. Similarly, in this study with SFA-fed mice, there was no observed change in total Cer(d18:1) in either brain region in response to AMI treatment.

It has been proposed that some of the biological effects of ceramides might be acyl-chain dependant (Grosch *et al.*

2012; Park *et al.* 2014). Ceramide acyl chain length is determined by a set of six ceramide synthases that are specific for varying acyl-CoA chain length. The loss of a single ceramide synthase, has been shown to lead to alterations in the abundance of sphingolipids in the brain, myelin deficiency, and neurodegeneration (Imgrund *et al.* 2009; Zhao *et al.* 2011). Given there is tight regulation on ceramide acyl chain length and recycling (Schiffmann *et al.* 2013), changes in individual ceramide species was assessed in plasma, hippocampus, and cortex. The data presented suggest that there is very tight regulation in maintaining the abundance of Cer(d18:1/18:0) in plasma. Interestingly, among the plasma ceramide species measured in a longitudinal study of elderly women, Cer(d18:1/18:0) was the poorest predictor of dementia and AD risk (Mielke *et al.* 2012). Acyl-chain-specific changes were also observed in both regions of the brain. Cer(d18:1/16:0) and Cer(d18:1/26:0) were unaffected by the SFA diet or the sphingolipid modulated agents, in both regions of the brain. As Cer(d18:1/18:0) was the predominant isoform, a reduction in this species likely accounts for the reduction in total ceramides. The ceramide species Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d18:1/22:0), Cer(d18:1/22:1), Cer(d18:1/24:0), Cer(d18:1/24:1), and Cer(d18:1/26:1) were reduced in at least two different groups receiving sphingolipid modulating agents, suggesting they reflect a more dynamic ceramide metabolic pool. Interestingly, while AMI treatment did not reduce total Cer(d18:1), it did reduce a number of the very long-chain ceramides in the CTX. Further, AMI reduced Cer(d18:1/24:0) in both the HPF and CTX. The latter may be significant because cerebral levels of Cer(d18:1/24:0) is frequently found to be elevated in AD and other neurodegenerative disorders (for review see Ben-David and Futerman 2010).

Presently, the physiological function of circulating ceramides is unclear, despite their purported central function for development and progression of neurodegenerative diseases. The hepatic ceramide-brain axis has been proposed to link several metabolic stresses to neurodegeneration (Lyn-Cook *et al.* 2009). de la Monte *et al.* (2010) suggested that short-chain ceramides may cross the blood-brain barrier and showed that intraperitoneal injection of a short-chain ceramide (Cer(d18:1/2:0)) increased the concentration of ceramides in the brains of Evans Long rat pups. Herein, we assessed associations between circulating and brain Cer(d18:1). Contrary to the hypothesis indicated, the strongest associations were indicated with multiple negative correlations between plasma and HPF ceramide species. Although circulating Cer(d18:1) do not directly correlate with brain Cer(d18:1), it does not mean they may not be good biomarkers for neurodegenerative diseases, as suggested by Mielke and Lyketsos (2010). Nonetheless, elevated circulating Cer(d18:1) may influence the brain or cerebrovasculature without overtly raising tissue levels of ceramides. A study by Boon *et al.* (2013) demonstrated that circulating Cer(d18:1)

was associated with biological effects in organs without elevating tissue levels per se. Through infusion of low-density lipoprotein-enriched with Cer(d18:1), Boon *et al.* were able to singularly isolate the effects of elevated circulating Cer(d18:1) on inducing skeletal muscle insulin resistance. This result is not surprising given that Cer(d18:1) constitute membrane lipids which coalesce to membrane rafts, which are capable of substantial modulation of cellular function (van Blitterswijk *et al.* 2003). Further, the contribution of the recycling pathway to remodeling of peripherally derived ceramide is not yet known and certainly, the uptake and metabolism of circulating Cer(d18:1) into the brain has yet to be quantified or studied.

Further research will be needed to understand the biological role of circulating sphingolipids in neurodegenerative diseases. If circulating ceramides influence the brain, it is important to distinguish the direct effects on CSF sphingolipids, amyloid-beta and tau (Mielke *et al.* 2014; Fonteh *et al.* 2015) and potential for inducing cerebrovasculature dysfunction, a major risk factor for neurodegenerative diseases (Takechi *et al.* 2010). The cerebral capillaries provide an active interface between blood and the brain and thus, regulates the passage of molecules into and out of the brain, including lipids. Sphingolipid signaling in cerebral endothelial cells appear to be highly selective of protective versus dysfunctional states (van Doorn *et al.* 2012; Testai *et al.* 2014; Prager *et al.* 2015). Furthermore, accumulation of ceramides has been shown to induce endothelial dysfunction in the periphery (Zhang *et al.* 2012).

A number of limitations applied to this study: (i) only one internal standard per sphingolipid class was used. It is assumed that minimal differences in response factor exist with changes in length and saturation of the fatty acyl chains. (ii) Small, but biologically significant, changes in some brain lipids may not be observable with the current sample sizes. A priori power analysis was performed to achieve a sensitivity sufficient for modest changes in Cer(d18:1), based on prior publications. (iii) Not all known sphingolipid classes were examined. Certainly, the number of sphingolipid classes is vast, each with considerable differences in chemical and biophysical properties. For this reason, development of LC-MS/MS protocols for simultaneous detection of all sphingolipid classes is impractical. Therefore, the focus of this paper was on the major classes present and those reported in previous studies. Only male mice were used in this study. Sex differences in circulating sphingolipid levels have been reported in both animals and humans (Hammad *et al.* 2010; Mielke *et al.* 2015). Currently, it is not known whether these differences correspond to altered susceptibility for neurodegenerative diseases.

In summary, using an array of sphingolipid modulating agents in mice fed a SFA-enriched diet, the results presented herein suggest: plasma sphingolipids are principally regulated through the de novo synthesis pathway; the HPF and

CTX regulate Cer(d18:1) abundance through different pathways; plasma Cer(d18:1) concentrations do not appear to reliably predict, nor influence the abundance of Cer(d18:1) in the HPF or CTX. Collectively, this study identifies the different metabolic pathways that regulate Cer(d18:1) concentrations in plasma and the brain.

Acknowledgements and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

References

- Becker K. A., Tummler B., Gulbins E. and Grassme H. (2010) Accumulation of ceramide in the trachea and intestine of cystic fibrosis mice causes inflammation and cell death. *Biochem. Biophys. Res. Commun.* **403**, 368–374.
- Ben-David O. and Futerman A. H. (2010) The role of the ceramide acyl chain length in neurodegeneration: involvement of ceramide synthases. *Neuromolecular Med.* **12**, 341–350.
- Berdyshev E. V., Gorshkova I., Skobeleva A., Bittman R., Lu X., Dudek S. M., Mirzapoozova T., Garcia J. G. and Natarajan V. (2009) FTY720 inhibits ceramide synthases and up-regulates dihydrosphingosine 1-phosphate formation in human lung endothelial cells. *J. Biol. Chem.* **284**, 5467–5477.
- van Blitterswijk W. J., van der Luit A. H., Veldman R. J., Verheij M. and Borst J. (2003) Ceramide: second messenger or modulator of membrane structure and dynamics? *Biochem. J.* **369**, 199–211.
- Boini K. M., Zhang C., Xia M., Poklis J. L. and Li P. L. (2010) Role of sphingolipid mediator ceramide in obesity and renal injury in mice fed a high-fat diet. *J. Pharmacol. Exp. Ther.* **334**, 839–846.
- Boon J., Hoy A. J., Stark R. et al. (2013) Ceramides contained in LDL are elevated in type 2 diabetes and promote inflammation and skeletal muscle insulin resistance. *Diabetes* **62**, 401–410.
- Borg M. L., Omran S. F., Weir J., Meikle P. J. and Watt M. J. (2012) Consumption of a high-fat diet, but not regular endurance exercise training, regulates hypothalamic lipid accumulation in mice. *J. Physiol.* **590**, 4377–4389.
- Boyanovsky B., Karakashian A., King K., Giltiy N. and Nikolova-Karakashian M. (2003) Uptake and metabolism of low density lipoproteins with elevated ceramide content by human microvascular endothelial cells: implications for the regulation of apoptosis. *J. Biol. Chem.* **278**, 26992–26999.
- Brinkmann V., Davis M. D., Heise C. E. et al. (2002) The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J. Biol. Chem.* **277**, 21453–21457.
- Bruce C. R., Risis S., Babb J. R., Yang C., Lee-Young R. S., Henstridge D. C. and Febbraio M. A. (2013) The sphingosine-1-phosphate analog FTY720 reduces muscle ceramide content and improves glucose tolerance in high fat-fed male mice. *Endocrinology* **154**, 65–76.
- Cutler R. G., Kelly J., Storie K., Pedersen W. A., Tammara A., Hatanpaa K., Troncoso J. C. and Mattson M. P. (2004) Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* **101**, 2070–2075.
- Dawson G. and Qin J. (2011) Gilenya (FTY720) inhibits acid sphingomyelinase by a mechanism similar to tricyclic antidepressants. *Biochem. Biophys. Res. Commun.* **404**, 321–323.
- van Doorn R., Nijland P. G., Dekker N. et al. (2012) Fingolimod attenuates ceramide-induced blood-brain barrier dysfunction in multiple sclerosis by targeting reactive astrocytes. *Acta Neuropathol.* **124**, 397–410.
- Filippov V., Song M. A., Zhang K., Vinters H. V., Tung S., Kirsch W. M., Yang J. and Duerksen-Hughes P. J. (2012) Increased ceramide in brains with Alzheimer's and other neurodegenerative diseases. *J. Alzheimers Dis.* **29**, 537–547.
- Fonteh A. N., Ormseth C., Chiang J., Cipolla M., Arakaki X. and Harrington M. G. (2015) Sphingolipid metabolism correlates with cerebrospinal fluid Beta amyloid levels in Alzheimer's disease. *PLoS ONE* **10**, e0125597.
- Foster C. A., Howard L. M., Schweitzer A. et al. (2007) Brain penetration of the oral immunomodulatory drug FTY720 and its phosphorylation in the central nervous system during experimental autoimmune encephalomyelitis: consequences for mode of action in multiple sclerosis. *J. Pharmacol. Exp. Ther.* **323**, 469–475.
- Glaros E. N., Kim W. S., Quinn C. M., Jessup W., Rye K. A. and Garner B. (2008) Myricin slows the progression of established atherosclerotic lesions in apolipoprotein E gene knockout mice. *J. Lipid Res.* **49**, 324–331.
- Gonzalez C. E., Venkatraman V. K., An Y. et al. (2016) Peripheral sphingolipids are associated with variation in white matter microstructure in older adults. *Neurobiol. Aging* **43**, 156–163.
- Grosch S., Schiffmann S. and Geisslinger G. (2012) Chain length-specific properties of ceramides. *Prog. Lipid Res.* **51**, 50–62.
- Gulbins E., Palmada M., Reichel M. et al. (2013) Acid sphingomyelinase-ceramide system mediates effects of antidepressant drugs. *Nat. Med.* **19**, 934–938.
- Hammad S. M., Pierce J. S., Soodavar F. et al. (2010) Blood sphingolipidomics in healthy humans: impact of sample collection methodology. *J. Lipid Res.* **51**, 3074–3087.
- Han X., M Holtzman D., McKeel D. W. Jr, Kelley J. and Morris J. C. (2002) Substantial sulfatide deficiency and ceramide elevation in very early Alzheimer's disease: potential role in disease pathogenesis. *J. Neurochem.* **82**, 809–818.
- Hannun Y. A. (1996) Functions of ceramide in coordinating cellular responses to stress. *Science* **274**, 1855–1859.
- Haughey N. J., Cutler R. G., Tamara A., McArthur J. C., Vargas D. L., Pardo C. A., Turchan J., Nath A. and Mattson M. P. (2004) Perturbation of sphingolipid metabolism and ceramide production in HIV-dementia. *Ann. Neurol.* **55**, 257–267.
- Hojjati M. R., Li Z., Zhou H., Tang S., Huan C., Ooi E., Lu S. and Jiang X. C. (2005) Effect of myricin on plasma sphingolipid metabolism and atherosclerosis in apoE-deficient mice. *J. Biol. Chem.* **280**, 10284–10289.
- Holland W. L., Brozinick J. T., Wang L. P. et al. (2007) Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. *Cell Metab.* **5**, 167–179.
- Imgrund S., Hartmann D., Farwanah H., Eckhardt M., Sandhoff R., Degen J., Gieselmann V., Sandhoff K. and Willecke K. (2009) Adult ceramide synthase 2 (CERS2)-deficient mice exhibit myelin sheath defects, cerebellar degeneration, and hepatocarcinomas. *J. Biol. Chem.* **284**, 33549–33560.
- Jana A., Hogan E. L. and Pahan K. (2009) Ceramide and neurodegeneration: susceptibility of neurons and oligodendrocytes to cell damage and death. *J. Neurol. Sci.* **278**, 5–15.

- Kitatani K., Idkowiak-Baldys J. and Hannun Y. A. (2008) The sphingolipid salvage pathway in ceramide metabolism and signaling. *Cell. Signal.* **20**, 1010–1018.
- Kurek K., Piotrowska D. M., Wiesiolek-Kurek P., Lukaszuk B., Chabowski A., Gorski J. and Zendzian-Piotrowska M. (2014) Inhibition of ceramide de novo synthesis reduces liver lipid accumulation in rats with nonalcoholic fatty liver disease. *Liver Int.* **34**, 1074–1083.
- Levy M. and Futerman A. H. (2010) Mammalian ceramide synthases. *IUBMB Life* **62**, 347–356.
- Lyn-Cook L. E., Lawton M., Tong M. *et al.* (2009) Hepatic ceramide may mediate brain insulin resistance and neurodegeneration in type 2 diabetes and non-alcoholic steatohepatitis. *J. Alzheimers Dis.* **16**, 715–729.
- Mencarelli C. and Martinez-Martinez P. (2013) Ceramide function in the brain: when a slight tilt is enough. *Cell. Mol. Life Sci.* **70**, 181–203.
- Mielke M. M. and Lyketsos C. G. (2010) Alterations of the sphingolipid pathway in Alzheimer's disease: new biomarkers and treatment targets? *Neuromolecular Med.* **12**, 331–340.
- Mielke M. M., Bandaru V. V., Haughey N. J., Rabins P. V., Lyketsos C. G. and Carlson M. C. (2010a) Serum sphingomyelins and ceramides are early predictors of memory impairment. *Neurobiol. Aging* **31**, 17–24.
- Mielke M. M., Haughey N. J., Bandaru V. V. *et al.* (2010b) Plasma ceramides are altered in mild cognitive impairment and predict cognitive decline and hippocampal volume loss. *Alzheimers Dement.* **6**, 378–385.
- Mielke M. M., Haughey N. J., Bandaru V. V., Weinberg D. D., Darby E., Zaidi N., Pavlik V., Doody R. S. and Lyketsos C. G. (2011) Plasma sphingomyelins are associated with cognitive progression in Alzheimer's disease. *J. Alzheimers Dis.* **27**, 259–269.
- Mielke M. M., Bandaru V. V., Haughey N. J. *et al.* (2012) Serum ceramides increase the risk of Alzheimer disease: the Women's Health and Aging Study II. *Neurology* **79**, 633–641.
- Mielke M. M., Maetzler W., Haughey N. J. *et al.* (2013) Plasma ceramide and glucosylceramide metabolism is altered in sporadic Parkinson's disease and associated with cognitive impairment: a pilot study. *PLoS ONE* **8**, e73094.
- Mielke M. M., Haughey N. J., Bandaru V. V. *et al.* (2014) Cerebrospinal fluid sphingolipids, beta-amyloid, and tau in adults at risk for Alzheimer's disease. *Neurobiol. Aging* **35**, 2486–2494.
- Mielke M. M., Bandaru V. V., Han D., An Y., Resnick S. M., Ferrucci L. and Haughey N. J. (2015) Demographic and clinical variables affecting mid- to late-life trajectories of plasma ceramide and dihydroceramide species. *Aging Cell* **14**, 1014–1023.
- de la Monte S. M., Tong M., Nguyen V., Setshedi M., Longato L. and Wands J. R. (2010) Ceramide-mediated insulin resistance and impairment of cognitive-motor functions. *J. Alzheimers Dis.* **21**, 967–984.
- Park J. W., Park W. J. and Futerman A. H. (2014) Ceramide synthases as potential targets for therapeutic intervention in human diseases. *Biochim. Biophys. Acta* **1841**, 671–681.
- Pocai A., Lam T. K., Obici S., Gutierrez-Juarez R., Muse E. D., Arduini A. and Rossetti L. (2006) Restoration of hypothalamic lipid sensing normalizes energy and glucose homeostasis in overfed rats. *J. Clin. Invest.* **116**, 1081–1091.
- Prager B., Spampinato S. F. and Ransohoff R. M. (2015) Sphingosine 1-phosphate signaling at the blood-brain barrier. *Trends Mol. Med.* **21**, 354–363.
- R Core Team (2015) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
- Saito M., Chakraborty G., Hegde M., Ohsie J., Paik S. M., Vadasz C. and Saito M. (2010) Involvement of ceramide in ethanol-induced apoptotic neurodegeneration in the neonatal mouse brain. *J. Neurochem.* **115**, 168–177.
- Schiffmann S., Birod K., Mannich J. *et al.* (2013) Ceramide metabolism in mouse tissue. *Int. J. Biochem. Cell Biol.* **45**, 1886–1894.
- Takechi R., Galloway S., Pallegage-Gamarallage M. M., Lam V. and Mamo J. C. (2010) Dietary fats, cerebrovasculature integrity and Alzheimer's disease risk. *Prog. Lipid Res.* **49**, 159–170.
- Takechi R., Pallegage-Gamarallage M. M., Lam V., Giles C. and Mamo J. C. (2014) Long-term probucol therapy continues to suppress markers of neurovascular inflammation in a dietary induced model of cerebral capillary dysfunction. *Lipids Health Dis.* **13**, 91.
- Teichgraber V., Ulrich M., Endlich N. *et al.* (2008) Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis. *Nat. Med.* **14**, 382–391.
- Testai F. D., Kilkus J. P., Berdyshev E., Gorshkova I., Natarajan V. and Dawson G. (2014) Multiple sphingolipid abnormalities following cerebral microendothelial hypoxia. *J. Neurochem.* **131**, 530–540.
- Vidaurre O. G., Haines J. D., Katz Sand I. *et al.* (2014) Cerebrospinal fluid ceramides from patients with multiple sclerosis impair neuronal bioenergetics. *Brain* **137**, 2271–2286.
- Weir J. M., Wong G., Barlow C. K. *et al.* (2013) Plasma lipid profiling in a large population-based cohort. *J. Lipid Res.* **54**, 2898–2908.
- Xing Y., Tang Y., Zhao L., Wang Q., Qin W., Ji X., Zhang J. and Jia J. (2016) Associations between plasma ceramides and cognitive and neuropsychiatric manifestations in Parkinson's disease dementia. *J. Neurol. Sci.* **370**, 82–87.
- Zhang Q. J., Holland W. L., Wilson L. *et al.* (2012) Ceramide mediates vascular dysfunction in diet-induced obesity by PP2A-mediated dephosphorylation of the eNOS-Akt complex. *Diabetes* **61**, 1848–1859.
- Zhao L., Spassieva S. D., Jucius T. J., Shultz L. D., Shick H. E., Macklin W. B., Hannun Y. A., Obeid L. M. and Ackerman S. L. (2011) A deficiency of ceramide biosynthesis causes cerebellar purkinje cell neurodegeneration and lipofuscin accumulation. *PLoS Genet.* **7**, e1002063.

Chapter 5

Chapter 5: General discussion, limitations and future directions

The final chapter of this thesis includes a general discussion to deliberate the major results reported in the animal model studies presented in Chapters 3 and 4. This chapter discusses potential mechanisms and significance of the findings in how circulating lipids have a homeostatic role in cerebral lipid metabolism. In addition, the limitations relevant to each of the studies presented as part of this thesis are addressed, followed by the consideration of potential future study directions.

5.1 General Discussion

The fundamental findings of Chapter 3 support the broad thesis hypothesis that the plasma lipidome have regulatory effects on the abundance of specific cerebral lipid species. Comprehensive lipidomics enabled the characterisation of associated changes in the cerebral lipidome relative to the plasma lipidome. The key results demonstrate a highly-regulated axis through which plasma lipids modulate a subset of cerebral lipids. For the first time, the plasma lipids that are jointly and independently responsible for this regulation capacity have been identified. The subset of cerebral lipids modulated are critical for many of the cellular functions of neurons and synaptic plasticity (Bazan, 2003b; Bazinet & Laye, 2014). Indeed, cerebral lipid homeostasis is associated with cognitive function and synaptic plasticity (Alashmali et al., 2016; Bazinet & Laye, 2014; Lim et al., 2005). Epidemiological studies have found associations between plasma lipids and cognitive and behavioural disorders as well as neurodegenerative disease risk (Huynh et al., 2017; Zhang et al., 2016). The results from this study provide a framework for interpreting said studies, by highlighting the interrelationships between blood and brain lipidomes.

The work presented in Chapter 4 investigated the regulatory effects of circulating sphingolipids in influencing cerebral sphingolipid homeostasis. Epidemiological, cellular and animal model studies suggested circulating

sphingolipids are involved in the initiation and progression of many neurological disorders (Mielke, Haughey, et al., 2010; Mielke et al., 2016). Using an animal model of perturbed peripheral sphingolipid metabolism in combination with sphingolipid modulating agents, we observed numerous inverse associations between circulating and HPF Cer. By contrast, a few positive associations were identified between circulating and CTX Cer. The results of this study highlight a gap in our understanding of how circulating sphingolipids are associated with brain lipid metabolic processes.

5.1.1 Uptake of circulating lipids by the brain

The brain is highly enriched in lipids, supporting structural, biochemical and cell signalling functions. Critical maintenance of cerebral lipid composition is pivotal for central nervous system function. It has been known for a long time that *in situ* synthesis of fatty acids is inadequate for the brain, therefore the transport, metabolism and uptake of fatty acids into the brain has been studied for more than a century (Chen et al., 2015; Grey, 1913; Rapoport, 2001). While much progress has been made, many fundamental questions are still unresolved.

The first major obstacle for lipid uptake by the brain is the BBB, a highly active interface between blood and the brain. The restrictive nature of the BBB allows a level of control over transport of metabolites. The mechanisms by which lipids are taken up by the brain are slowly being elucidated, however there is considerable debate. Three major routes of uptake have been suggested to occur in the brain: lipoprotein mediated uptake, receptor mediated uptake of lyso-phospholipids and uptake of non-esterified fatty acids. Most studies have attempted to quantify the rates of uptake through these alternative pathways using radiolabelled lipids (DeGeorge, Noronha, Bell, Robinson, & Rapoport, 1989; DeMar, Ma, Bell, & Rapoport, 2004b; Nariai et al., 1994; Rapoport, 2001). However, the interpretation of these studies is difficult due to complex interactions with endogenous lipids (Chen et al., 2015); metabolism and re-secretion of radiolabels in alternate forms (DeGeorge et al., 1989); and short time frame (seconds to hours) of these studies.

Understanding the major routes of uptake of circulating lipids into the brain, under different physiological conditions, is suggested to aid diagnosis and therapeutic strategies for neurological diseases (Hong, Belayev, Khoutorova, Obenaus, & Bazan, 2014; Jicha & Markesbery, 2010; Kim, 2014; Taha, Burnham, & Auvin, 2010). However, these studies do not address a critical issue: to what extent do circulating lipids regulate the metabolism of lipids in the brain. Many studies have focused on acute infusion of radiolabelled fatty acids to derive information on the metabolic fate of that lipid specie in the brain – be it esterification to specific phospholipid classes or oxidation as fuel. This approach relies on several methodological caveats and biological assumptions. (1) Intravenous infusion of radiolabelled lipids is generally limited to ‘simple’ lipids, such as NEFA or lyso-phospholipids, which do not require incorporation into lipoproteins. In fact, the appearance of radiolabelled fatty acids in multiple plasma pools is often considered an experimental confounder as the specific contribution of a lipid class is unclear. However, in physiological systems, circulating lipids are continuously metabolised by multiple organs; incorporated into and secreted associated with lipoproteins as phospholipids, TG and CE; temporarily stored as TG in adipose tissue, followed by hydrolysis and release as circulating NEFA. (2) The metabolism of fatty acids is influenced by the presence, abundance and composition of other fatty acids in the system. Indeed, the elongation and desaturation rate of n-3 fatty acids are highly influenced by the abundance of n-6 fatty acids (Harnack, Andersen, & Somoza, 2009). Furthermore, mixtures of fatty acids are known to result in altered cellular responses, contrasted with the individual fatty acids alone (Watt, Hoy, Muoio, & Coleman, 2012). Indeed, oleate regulates the channelling of palmitate down different metabolic pathways and can prevent palmitate induced lipotoxicity (Capel et al., 2016; Coll et al., 2008). Given the brain is continuously exposed to circulating lipids, understanding the physiological regulation of cerebral lipids by circulating lipids requires consideration of all circulating lipids and the interaction between them, rather than focusing on individual lipid species.

5.1.2 Associated changes of the brain lipidome relative to plasma

The plasma lipidome reflects the co-ordinated effort of a highly dynamic, multi-organ system responsible for synthesis, storage and delivery of lipids throughout the body. It is well established that the plasma lipidome is altered by the amount and composition of dietary fat consumed (Mattson & Grundy, 1985; Miao et al., 2015; Schwingshackl & Hoffmann, 2013). Immediately following a meal, dietary fat is digested and long-chain fatty acids esterified into TG within intestinal enterocytes. Intestinally derived lipoproteins, chylomicrons, are assembled and secreted for distribution of these dietary-derived lipids throughout the body (Mamo & Proctor, 1999). During this postprandial state, dietary fat composition directly influences the plasma lipidome. Indeed, the plasma lipidome is a product of transient influx of exogenous (dietary) lipids, *de novo* synthesis of fatty acids and constitutive recycling of lipids between the liver, adipose stores and tissues.

The role of dietary lipids in modulating cerebral lipid composition is considered using animal model studies. However, there is a paucity of studies that simultaneously consider both the plasma and cerebral lipidomes. Indeed, changes in cerebral lipid composition are often considered a response to diet and not mechanistically linked to circulating lipids. Further confusion can be had due to the vague descriptions of 'high-fat diets' that abound in the literature (Warden & Fislser, 2008). This missing mechanistic insight is exacerbated because plasma lipid composition is differentially altered depending on the source of lipids composing the diet (Buettner et al., 2006; Yaqoob et al., 1995).

While much of the research relating diet and cerebral lipid metabolism has focused on dietary n-3 fatty acids, a few studies have investigated pro-inflammatory diets, for example diets enriched in saturated fatty acids. Borg et al. (2012) investigated whether high fat diets induced lipotoxicity in the hypothalamus of mice. Based on observations of ectopic lipid accumulation in peripheral tissue of high fat fed mice, Borg et al. explored whether the same mechanism occurred in the brain. Mice fed the high fat diet (59 % energy from fat) showed accumulation of several lipid classes, including dhCer, DHC, BMP, PC(O), DG, TG and several isoforms of ceramide. Borg et al. findings of increased abundance of lipid classes contrasts with

results presented in Chapter 3. Several potential reasons may explain the differences in observations: (1) Borg used a diet with a much higher fat content, which may have led to greater exposure of the brain to circulating lipids. (2) The hypothalamus is known to regulate appetite through sensing of circulating lipids. Excess exposure of the hypothalamus to circulating lipids has been shown to blunt satiety signals. (3) The hypothalamus does not have an intact BBB, eliminating a large component of lipid processing for the brain. These features may predispose this region to be susceptible to accumulation of lipids. However, findings by Stranahan et al. (2011) suggest the higher abundance of fat in the diet could account for the accumulation of lipids. By feeding rats a high fat (60 % energy from fat) and high fructose diet, the accumulation of sphingolipids was observed in the hippocampus.

Accumulation of lipids in the brain suggests substantial dysregulation of lipid processing. Compared to peripheral tissue, the brain has a limited capacity to store lipids. In view of the lipid oversupply hypothesis, exposure to excess fatty acids results in accumulation of fatty acyl-CoAs and subsequently excess flux down glycerolipid and sphingolipid synthetic pathways. Indeed, accumulation of saturated long-chain acyl-CoAs have been observed in mice fed high fat diets (Posey et al., 2009). However, the results in Chapter 3 suggest lipid oversupply was not present with the diet utilised in this study. Although PE(O) was elevated in the cerebral cortex of mice fed the SFA diet, it is unlikely to be caused by lipid oversupply. Acyl chain abundance is not a rate limiting factor for ether phospholipid synthesis ("Biosynthesis of Plasmalogens in Brain," 2008). Rather, this increase in abundance might indicate an imbalance in processing in the endoplasmic reticulum – an early indicator of endoplasmic reticulum stress. Interestingly, Borg et al. observed an increase in PC(O) in their high fat feeding study. This may suggest a common mechanism through which high fat diets negatively influence the brain. Indeed, saturated fats have been well documented to induce endoplasmic reticulum stress in vitro (Watt, Hoy, et al., 2012).

The results of Chapter 3 suggest that remodelling of phospholipids principally occurs as a consequence of long-term consumption of a diet modestly enriched in saturated fat. This finding supports previous observations in the literature obtained using GC-MS to measure the cerebral fatty acid composition in high fat fed

mice. Yu et al. (2010) observed a decrease in the abundance of polyunsaturated fatty acids in the brain, while the proportion of saturated fatty acids increased. In the present study, individual lipid species were measured without prior liberation of fatty acids, allowing information of lipid classes to remain. Between the hippocampus and cerebral cortex, 50 individual lipid species were altered. Phospholipids of the classes PC, PC(O), PC(P), PE, PE(O), PE(P), PI and PS were increased or decreased depending on composition of their acyl chains. While the overall remodelling is complex, several patterns emerge upon consideration of the individual species. Phospholipids with a high number of double bonds (6 or 7) were generally reduced, while those containing four double bonds were increased. The number of double bonds and the reciprocal relationship suggests differences in the incorporation of DHA versus AA. The n-3 fatty acid DHA contains 22 carbons with 6 double bonds, while AA is an n-6 fatty acid containing 20 carbons and 4 double bonds. Individually, DHA and AA are the major n-3 and n-6 fatty acids in the brain and together account for most of PUFAs in the brain (O'Brien & Sampson, 1965a). Changes in phospholipids with five double bonds show a complex relationship between lipid class and total length of acyl chains. For instance, phospholipids with a total acyl chain length of 36 carbons decreased, while those with longer chain lengths increased. This is except for PS 40:5 which shows a considerable decrease in both regions of the brain. The complex relationship between acyl chain length and changes in abundance of phospholipids with 5 double bonds could represent a shift in the utilization of DPA (22:5n-6), rather than DHA (22:6n-3) and EPA (20:5n-3) (Alashmali et al., 2016). The brain normally contains very little EPA and DPA (Chen et al., 2013), however their concentrations have been shown to change with dietary manipulations (Igarashi et al., 2009; Igarashi, Kim, Chang, Ma, & Rapoport, 2012; Lin et al., 2015). The changes in phospholipids with 5 double bonds might reflect losses of EPA and increases in DPA.

Changes in the length and degree of saturation of phospholipids in the brain is known to cause substantial changes in the functioning of the brain (Lim et al., 2005). Deficiency of DHA in the brain leads to cognitive and learning deficits in animals (Carrie et al., 2000a; Hajjar et al., 2012; Ikemoto et al., 2001; Moriguchi, Harauma, & Salem, 2013) and humans (Kuratko, Barrett, Nelson, & Salem, 2013). The specificity for DHA has been previously demonstrated in elegant physiological

studies. Substitution of the essential fatty acid DHA with the similar n-6 fatty acids, DPA, does not rescue cognitive deficits in DHA deficient rat pups (Stark et al., 2007). Rats were artificially reared with a base n-3 deficient diet supplemented with either DHA, DPA or DHA+DPA. In Morris Water Maze experiments, rats reared with the base n-3 deficient diet with or without DPA performed poorer than DHA supplemented diet. Rats fed DHA+DPA performed similarly to DHA fed animals. It appears that a critical membrane fluidity is required for effective neuronal and synaptic function (Eldho et al., 2003). Loss of DHA from these membranes results in less fluid membranes, which cannot be compensated for by other (n-6) fatty acids (Lim et al., 2005).

The work by Lim et al. (2005) and Stark et al. (2007) suggest that DPA replaces DHA in the brain only when the supply of DHA is insufficient for requirements. When DHA is delivered to the brain in sufficient quantities, higher concentrations of DPA do not increase DPA or decrease DHA in the brain. The original hypothesis speculated that increased DPA in circulation might compete for DHA for incorporation into brain. This hypothesis is supported by Kim et al. (2011) based on graded dietary n-3 deficiency studies in rats. Lower dietary n-3 fatty acids lead to greater conversion of LNA/AA to DPA, which was inversely correlated with DHA in plasma and brain. A strength of this study was the measurement of fatty acids in both plasma and brain. Kim et al. suggested a 'threshold' concentration of n-3 fatty acids in diets at which cerebral lipid composition changes. Alterations in brain DHA concentration was observed when the proportion of dietary n-3 fatty acids were 0.8 % or below. This contrasts with plasma, where changes were observed when dietary n-3 fatty acid composition was reduced to 2.6 %. Extrapolating this threshold to the diet used in Chapter 3 and accounting for the higher fat content, these animals should be in the n-3 sufficient range for brain tissue. This suggests that interacting factors with other circulating lipids may increase the likelihood of changes in cerebral lipids. Kim et al. reported that the concentration of AA did not change in plasma or brain in any of the treatment groups investigated. This is a point of difference to the results of Chapter 3, where putative AA containing phospholipids were increased in both regions of the brain and plasma. It's possible that higher consumption of saturated fats and LNA could account for these differences. Indeed, Cintra et al. (2012) measured the fatty acid composition of blood and hypothalamus

in mice fed high fat diets. The AA concentration in blood was ~40 % higher in mice fed the high fat diet. However, this was not accompanied by a change in hypothalamic AA concentration. Rather, there was a subtle rearrangement in fatty acid composition, reflecting lower PUFA, high MUFA and increases in some very-long chain saturated fatty acids.

Prior to the work presented in this thesis, no studies have reported associations between intact plasma and cerebral lipids. Rather, scientific work has focused on the fatty acid composition of plasma and the brain. Cunnane et al. (2012) measured the fatty acid profile of plasma and brain from MCI and AD subjects by first hydrolysing fatty acids from intact lipids. The only statistically significant association was found between the percentage of DHA liberated from circulating lipids and the percentage of DHA from PE species from just one region of the brain and only in the control group. The results from this study suggest a negligible relationship between circulating lipids and cerebral lipid composition. However, collection of tissue samples during post-mortem autopsy involves a lengthy lag-phase which is known to significantly impact cerebral lipid composition (Lee & Hajra, 1991). Although not directly measuring cerebral fatty acid composition, Guest, Garg, Bilgin, and Grant (2013) examined the relationship between plasma NEFA and CSF NEFA in matching samples from human subjects. For each n-3 fatty acid analysed, there was no statistically significant association between circulating and CSF concentrations. However, an aggregate of circulating n-3 fatty acids was positively associated with CSF DHA concentration. Further, circulating AA was positively associated with CSF AA. Interestingly, Guest et al. found no significant associations with SFA and even observed a negative association between CSF and circulating oleic acid (18:1n-9). These observations are similar to those of Jumpertz et al. (2012). In overweight human subjects, NEFA concentrations were determined in plasma and CSF. In these subjects, only ALA and DHA were associated (positively) between plasma and CSF.

In Chapter 3, a multivariate analysis was performed to determine the plasma lipid species that are jointly and independently associated with changes in the cerebral lipidome. The results provide a measure of ‘importance’ of each plasma lipid species as a function of its ability to explain changes in the cerebral lipidome.

Strong positive associations were observed between highly unsaturated phospholipids (containing 6 or 7 double bonds) in plasma and highly unsaturated phospholipids in both regions of the brain. Similarly, higher abundance of phospholipids with 4 double bonds were associated with higher abundance of phospholipids with 4 double bonds in the brain. The multivariate model identifies a reciprocal relationship between highly unsaturated species with 6 or more double bonds and unsaturated species containing 4 double bonds. This relationship describes the competition between LA and ALA for the elongation and desaturation enzymes responsible for producing the very-long and highly unsaturated fatty acids ARA and DHA, respectively (Brenna, 2002). Interestingly, there are subsets of phospholipids with inverse relationships between blood and brain. That is, higher concentrations in blood are associated with lower concentrations in the brain. The multivariate model revealed these lipids to be phospholipids of shorter total chain length with fewer double bonds. Mice consuming the SFA diet had higher circulating levels of these lipids, while these lipid species were higher in brains of mice consuming the control diet. A possible explanation for this observation involves careful consideration of the relative importance of lipid supply versus selectivity of fatty acids. Due to the lower concentration of DHA in cerebral phospholipids of SFA fed mice, membrane fluidity would be decreased. To correct this reduced fluidity, changes in fatty acid selectivity may occur (Hulbert, Turner, Storlien, & Else, 2005). This process reduces the incorporation of shorter and less saturated fatty acids in favour of longer and more unsaturated species – AA/DPA in this instance. This hypothesis suggests that lipid composition of neuronal membranes is altered to achieve the desired membrane fluidity. Indeed, it is true neuronal membranes are continuously remodelled and some level of selectivity of this process has been observed (Rapoport, 2008; Rapoport, Rao, & Igarashi, 2007).

The strongest associations between plasma and brain lipidome were observed in phospholipids. However, the multivariate analysis considers all plasma lipids that jointly influence the brain lipidome, regardless of whether the same species was altered in the brain. This is a considerable point of difference to correlation analysis which is typically performed (Cunnane et al., 2012; Guest et al., 2013). Therefore, it is also possible to identify circulating lipid species that supply fatty acids to the brain. We observe that several CE and TG lipid species are ranked highly as plasma

lipids that explained changes in the brain lipidome. Neutral lipids containing the fatty acids C22:6, C22:5, and C20:5 were indicated as significant regulators of cerebral lipids, whereas those containing C20:4 contributed much less. Furthermore, TG species contained a variety of fatty acids, including saturated-, monounsaturated-, and polyunsaturated-fatty acids. These results suggest that multiple lipid classes are involved in regulating lipid metabolism in the brain, including those located within lipoproteins.

An interesting concept is to apply the findings of this study to predictive blood-based biomarkers for neurodegenerative diseases. Mapstone et al. (2014) described and validated a set of 10 lipids from a cohort of older aged individuals that predicted phenoconversion to mild cognitive impairment or Alzheimer's disease within the proceeding 2-3 years. Subjects who phenoconverted had lower levels of all lipids on the panel. The lipid panel consisted of six PC species, one LPC, one PC(O), an acyl-carnitine and a hydroxylated fatty acid. Of the lipid species in the panel proposed by Mapstone et al., all species that were also within our lipidomics analysis were positively correlated with an improved cerebral lipidome, except for LPC 18:2. This is not surprising because four lipids identified by Mapstone et al. putatively contain DHA, which was shown to have beneficial effects in our study. However, it is not clear whether our opposite observations of LPC 18:2 is due to differences in metabolism of LPCs between humans and mice, or if it represents a specific signature of progression to neurodegeneration. Mice have very high levels of circulating HDL compared to humans, which is enriched in lecithin cholesterol acyltransferase. During the esterification of cholesterol for reverse cholesterol transport, LPCs are produced by the hydrolysis action on PC lipids (Santamarina-Fojo, Lambert, Hoeg, & Brewer, 2000). It is possible the decrease in LPC 18:2 in Mapstone's study represents a reduction in reverse cholesterol transport, a risk factor for neurodegenerative and vascular diseases (Merched, Xia, Visvikis, Serot, & Siest, 2000; Rader, Alexander, Weibel, Billheimer, & Rothblat, 2009).

The results presented in Chapter 3 show for the first-time, the associated changes in the brain lipidome relative to plasma abundance. Central to the thesis hypothesis, specific classes of lipids in the hippocampus and cerebral cortex were strongly associated with plasma lipids. Despite acknowledging the importance of

circulating lipids in maintaining lipid composition of the brain, no studies had attempted to explore the associations between the two prior to this work. Developing an understanding of how circulating lipids, independently and collectively, modulate cerebral lipid composition will lead to better diagnostic tests, therapies and strategies for maintaining and improving neurological health.

5.1.3 Regulation of circulating and cerebral ceramides

Aberrant lipid metabolism has been associated with AD since Alois Alzheimer first described the neuropathological hallmarks of AD. Throughout the brain, Alois described ‘extraordinarily strong accumulation of lipoid material in the ganglion cells, glia and vascular wall cells’. These observations have largely been overlooked by the field until very recently. With the tremendous advancements in the field of lipidomics, the biochemical alterations that Alois Alzheimer first described in 1906 have been elucidated. Dysregulation of sphingolipids are the most commonly reported lipid disorder in the brains of AD patients. A range of observations have been described, including increased ceramides, decreased sphingomyelins, decreased S1P and decreases in glycosphingolipids. Indeed, numerous reviews have been dedicated to describing the involvement of sphingolipids in AD.

While there is convincing evidence for dysregulation of sphingolipids in the brain of AD patients, a growing number of studies are suggesting circulating sphingolipids may be involved in the initiation and progression of AD. Cross-sectional and prospective studies have suggested that plasma ceramides and sphingomyelins are associated with cognitive decline, AD risk and disordered white-matter microstructure (Gonzalez et al., 2016; Mielke et al., 2012; Mielke, Haughey, et al., 2010; Mielke et al., 2011; Mielke et al., 2016; Savica et al., 2016). Interestingly, several studies have suggested that circulating ceramides are predictive of future cognitive decline and hippocampal volume loss. These studies collectively suggest an involvement of circulating sphingolipids in the development and progression of cognitive decline and AD.

Despite the accumulating evidence suggesting a causal role for circulating ceramides in neurodegenerative disease risk, few studies have considered a lipid exchange axis. Several potential mechanisms have been proposed in the literature to explain the role of circulating sphingolipids: (1) circulating sphingolipids reflect changes in lipid metabolism that is occurring in the brain. This hypothesis suggests that measurement of plasma sphingolipids would be a surrogate marker for cerebral lipids – which cannot be measured in living humans. (2) Circulating sphingolipids are taken up by the brain where they accumulate and disrupt the cerebral lipidome. (3) Circulating sphingolipids indirectly influence the brain through causing peripheral insulin resistance or affecting the cerebrovasculature. However, to date, no studies have attempted to reconcile these alternate hypotheses.

Chapter 4 explores cerebral sphingolipid homeostasis in a physiological context, reflecting potential capillary exchange of plasma sphingolipids with the brain. Using an established model of peripheral ceramide dysregulation alongside several sphingolipid modulating agents, putative regulatory associations were determined. Mice consuming the SFA diet had large increases in dihydroceramide and ceramide in plasma, suggesting *de novo* synthesis is increased. Inhibition of sphingolipid *de novo* synthesis with myriocin confirmed *de novo* synthesis was upregulated, supporting previous observations by Holland et al. (2007). However, Boini, Zhang, Xia, Poklis, and Li (2010) suggested acidic sphingomyelinase is responsible for elevated ceramides in high fat fed mice. Results in Chapter 4 do not agree with their observations. Inhibiting acidic sphingomyelinase with amitriptyline had no effect on circulating ceramides. Lastly, activation of S1P receptors had no effect on circulating sphingolipids. This finding supports the observations of Ottenlinger et al. (2016), who found FTY720 had no effect on plasma sphingolipids in multiple sclerosis patients.

Contrary to the hypothesis indicated, higher circulating ceramides were not associated with elevated ceramides in the hippocampus or cerebral cortex. The results of this study are surprising in light of previous studies proposing the hepatic ceramide-brain axis, suggesting that peripheral ceramides are capable of increasing cerebral ceramides (Lyn-Cook et al., 2009a). de la Monte et al. (2010) examined this hypothesis by providing intraperitoneal injections of short-chain ceramides (N-

acetyl-D-sphingosine) to Long Evans rat pups. Intraperitoneal injection of short-chain ceramides led to increases in liver, blood and brain. However, the use of short chain ceramides requires the results of this study to be considered with caution. Short chain ceramides have a much higher water solubility than endogenous ceramides. Additionally, short chain ceramides are permeable of cellular membranes, allowing rapid movement between and within cells. Another reason the results of this study are surprising, are based on our understanding of the relationship between saturated fats and sphingolipid synthesis. The sphingolipid modulating effects of saturated fatty acids have been demonstrated in vitro and in vivo (Paumen, Ishida, Muramatsu, Yamamoto, & Honjo, 1997; Watt, Barnett, et al., 2012). Once palmitate enters a cell and is activated, it is a direct substrate for the rate limiting enzyme in de novo sphingolipid synthesis – serine-palmitoyltransferase (SPT). Serine palmitoyltransferase catalyses the irreversible condensation of L-serine and palmitoyl-CoA, forming a committed step to synthesis of sphingolipids. Indeed, it has been shown that infusion of palmitate is sufficient to increase de novo synthesis and consequently sphingolipid accumulation in blood and peripheral organs (Watt, Barnett, et al., 2012). Two studies have suggested saturated fatty acids can cause ceramide accumulation in the brain. Borg et al. (2012) fed C57BL/6 mice a high fat diet (59 % energy from fat) for 12 weeks. Total dihydroceramides and several ceramide species were elevated in the hypothalamus. Holland et al. (2011) intravenously infused Sprague-Dawley rats with a 20 % lard oil emulsion for 6 hours. Hypothalamic ceramides were measured by enzymatic assay and determined to have increased more than 30 % compared to saline infused rats. Both studies analysed ceramide accumulation in the hypothalamus, an area of the brain proposed to be more susceptible to lipid insults (Borg et al., 2012; Martinez de Morentin et al., 2010; McNay et al., 2010). However, the quantity of lipids the brain is exposed to in both studies might represent a supra-physiological amount for humans. Indeed, the six-hour infusion of lard oil utilised by Holland et al. represents an extraordinary exposure to circulating lipids. In the same study, Holland et al. utilised intraperitoneal injections of myriocin to prevent ceramide accrual in soleus muscle. However, the dose utilized was 3000-fold higher than typically used (Hojjati et al., 2005a; Park, Rosebury, Kindt, Kowala, & Panek, 2008; Yang et al., 2009) and approached the LD50 for an oral dose (Kluepfel, Bagli, Baker, Charest, & Kudelski, 1972).

A recent study by den Hoedt et al. (2016) investigated the effect of ApoE isoform and interaction with high fat/cholesterol diet (19 % butter, 0.5 % cholate and 1.25 % cholesterol) on cerebral ceramide concentrations. In wild-type mice and mice expressing the human isoform of apeE4, consumption of the high fat diet did not alter the ceramide levels in whole brain homogenates. This finding supports the results observed in Chapter 4, where consumption of the SFA diet did not alter cerebral ceramide concentration. In addition to ceramide levels, den Hoedt et al. reported mRNA expression of several sphingolipid metabolising enzymes. mRNA expression of SPT, aSMase, nSMase2, ceramide transporters and ceramide synthases were reduced in the brains of wild-type mice fed the high fat diet. Reduced expression of these sphingolipid metabolizing enzymes suggest a co-ordinated effort to prevent ceramide accumulation. Indeed, results of Chapter 4 suggest a small but non-significant decrease in nearly all ceramide species in both regions of the brain with consumption of the SFA diet. A change in the regulation of sphingolipid metabolism towards a phenotype observed by den Hoedt et al. could explain this minor decrease.

Results presented in Chapter 4 suggest a negative association between circulating ceramides and HPF ceramides. Presently, the physiological function of circulating ceramides is unclear, however lines of evidence suggest they may exert biological effects without overtly raising tissue concentrations. Boon et al. (2013) isolated the biological effects of lipoprotein-associated ceramide by infusing mice with LDL artificially enriched in ceramides. Three hours of infusion with LDL-enriched with ceramides induced insulin resistance and inflammation, compared to LDL. Despite a dramatic reduction in skeletal muscle insulin sensitivity, skeletal muscle ceramides remained unchanged. Compared to whole muscle, ceramides tended to increase in isolated plasma membranes. Multiple mechanisms connect elevated plasma membrane ceramides to insulin resistance (Chavez & Summers, 2012; Summers, 2006). Boon et al. suggested that circulating ceramides may mediate their actions by lipid-soluble exchange with the outer-leaflet of the plasma membrane. However, model membranes and molecular dynamic simulations suggest slow rates of transfer for ceramides (Simon, Holloway, & Gear, 1999). Certainly, the rate of transfer by any method is tissue, lipid and lipoprotein dependent

(Boyanovsky, Karakashian, King, Giltiy, & Nikolova-Karakashian, 2003a; Morita et al., 2004; Xu & Tabas, 1991; Zolnik et al., 2008).

If circulating ceramides enter the brain, their metabolic fate is not known. Circulating lipoproteins can be taken up into endothelial cells through internalisation of lipoprotein-lipoprotein receptor complexes. Once inside the cell, complexes can be targeted for lysosomal degradation where lipid contents are hydrolysed. Lysosomes are enriched with sphingolipid degrading enzymes, including ceramidases which hydrolyse ceramides to sphingosine. Once sphingolipids are broken down to sphingosine, they may be re-acylated to ceramides or phosphorylated to S1P (Kitatani, Idkowiak-Baldys, & Hannun, 2008). A recent finding by Gulbins et al. (2016) suggests cerebral endothelial cells can release ceramides into the extracellular space of the hippocampus. Using confocal microscopy, Gulbins et al. observed that endothelial cells of the hippocampus became enriched with ceramides when treating mice with glucocorticosterone. Extracellular extracts of the hippocampus of stressed mice were enriched in ceramides. As ceramides are insoluble in aqueous environments, it was speculated that ceramides were secreted as micro vesicles. Interestingly, these effects were abrogated by cotreatment with amitriptyline – suggesting an involvement of lysosomal sphingolipid metabolism. However, as acknowledged by Gulbins et al., glucocorticosterone is not known to activate acidic sphingomyelinase. Rather, glucocorticosterone causes large elevations in circulating ceramides, principally through de novo synthesis in the liver (Holland et al., 2007). It is possible the increase in ceramides observed were not generated *in situ*, but a result of increased uptake of circulating ceramide by hippocampal endothelial cells.

Chapter 4 explored cerebral sphingolipid homeostasis in a physiological context, reflecting potential capillary exchange of plasma and CSF lipids, degradation and efflux. Concomitant measures of plasma and cerebral lipids enabled exploration of putative regulatory associations. Opposite to the thesis hypothesis, cerebral ceramides were not positively associated with circulating ceramides. Alterations in the plasma lipidome are commonly indicated in neurodegenerative disorders and increasingly implicated in a causal context. However, there remains a paucity of studies which have interrogated the capillary axis central to lipid kinetics.

Understanding the mechanisms involved will lead to improved understanding of disease pathogenesis and diagnostic tests.

5.2 Limitations of the present studies

The genetically un-manipulated mice used in this study are commonly used rodent models for the study of lipid metabolism of blood and the brain. In these mice, the distribution of lipids between structures of the brain closely resemble that of humans (Bozek et al., 2015; Chan et al., 2012b; Park et al., 2017). However, several differences exist between mice and humans in the abundance and distribution of lipids in circulation (Gordon et al., 2015; Ishikawa et al., 2015; Yin et al., 2012). When considering the distribution of circulating lipoproteins, LDL is the most abundant lipoprotein class in blood, whereas in mice, HDL is the most abundant. This difference in abundance of lipoprotein classes is reflected in the difference in abundance of lipid classes – as lipid classes are not uniformly distributed among lipoprotein classes. These differences can be observed in the lipidomic datasets, such as the low abundance of TG and high abundance of LPC in mouse plasma (Ishikawa et al., 2015; Yin et al., 2012). Given this was the first-time associations between whole plasma and brain lipidomes were assessed, the significance of this difference is not known. Certainly, circulating TG is a major source of lipids for many peripheral organs and lipoprotein lipases are present on the BBB.

In both studies presented in this thesis, the abundance of lipids in plasma and brain were concomitantly determined and putative associations explored. Therefore, associations were assessed in the ‘steady-state’ and might not reflect dynamic associations between the two (Castro-Perez et al., 2011). Chapter 3 highlights the remarkable stability of the brain lipidome despite substantial changes in circulating lipids. The circulating ceramide hypothesis suggests that elevated levels of circulating ceramides accumulate in the brain. However, in physiologically relevant models, changes in plasma ceramides appear unlikely to induce changes in cerebral ceramides, perhaps because of compensatory mechanisms that ameliorate increased blood-to-brain kinetics (den Hoedt et al., 2016). These mechanisms may include

altered kinetics across the BBB, changes in abundance of regulatory enzymes, increased degradation or secretion of lipids. Since kinetic measurements were not explored in this candidacy, it is not possible to elucidate the mechanisms through which cerebral ceramide homeostasis is achieved.

The experiments in Chapters 3 and 4 utilised a targeted lipidomics platform using mass spectrometry (Weir et al., 2013b). Liquid chromatography electrospray ionisation tandem mass spectrometry allowed targeted analysis of lipids. Chromatographic separation and scheduled precursor/product transitions were optimized to simultaneously measure many lipid classes. While acyl chain composition information can be determined, a shortcoming of this approach is that double bond position cannot be determined. Differences in double bond position (n-3, n-6 and n-9) do not alter the precursor/product transitions in conventional LC-ESI-MS/MS platforms. Therefore, it is possible that some lipids may appear over-represented due to multiple contributions from different lipids. For instance, the lipid specie PE-40:5 may be a combination of PE-20:0/20:5n3 and PE-18:0/22:5n6, PE-20:1/20:4n6 or several other variants. With adequate chromatography, these species can be separated and elucidation of each specie determined in negative ESI mode. However, in the current configuration, isomeric n-3 and n-6 species were not chromatographically separated. Therefore, given the current instrumentation used, it is not possible to confidently designate a lipid as containing n-3, n-6 or n-9 fatty acids.

5.3 Future directions for research

The principal findings presented in this thesis suggest a highly-regulated axis through which circulating lipids regulate lipids in the brain. In a physiological system associated changes of the brain lipidome relative to plasma abundance may reflect changes in kinetics across the BBB, cerebral metabolic responses and/or changes in degradation and efflux through CSF transport. Potential mechanisms include via a consequence of altered blood-to-brain kinetics; via altered cerebral metabolism of

lipids; alteration of capillary architecture and function; or indirectly, for example via changes in redox homeostasis.

The results of Chapter 4 suggest an incomplete model for how circulating ceramides may influence neurodegenerative disease risk. Abundant evidence from cross sectional and longitudinal studies highly supports a causative role of ceramides. However, this mechanism of action is still not elucidated. For the first time, it was demonstrated that circulating ceramides did not contribute to ceramide accrual in the brain, nor reflect cerebral ceramide metabolism. Rather, specific negative associations were shown between plasma and HPF. Several lines of evidence suggest circulating ceramides may increase neurodegenerative disease risk through compromising the BBB: (1) The BBB is the interface between blood and brain, and therefore continuously exposed to circulating ceramides. It is possible that long-term exposure compromises the integrity of the BBB, opening the brain up to potentially neurotoxic plasma components (Symons & Abel, 2013; van Doorn, Nijland, Dekker, Witte, Lopes-Pinheiro, et al., 2012). (2) Ceramides are known to induce endothelial dysfunction in vivo (Bharath et al., 2015). Multiple pathways that induce ceramide accumulation in endothelial cells (obesity, high fat diets, corticosteroids) have been shown to prevent insulin signalling and nitric oxide generation of endothelial cells (Mehra et al., 2014; Smith, Visioli, Frei, & Hagen, 2006; Zhang et al., 2012a). These metabolic effects are also accompanied by oxidative stress, a well characterised effect of ceramide accumulation (Corda, Laplace, Vicaut, & Duranteau, 2001; Li et al., 2002). (3) Increased S1P is thought to restore BBB damage through action of S1P receptors and possibly through restoration of aberrant ceramide levels (Prager, Spampinato, & Ransohoff, 2015; Spampinato et al., 2015; van Doorn, Nijland, Dekker, Witte, Lopes-Pinheiro, et al., 2012). Clearly, delineating the mechanism through which circulating ceramides influence the brain is a key area for future studies to be considered.

Most of the studies examining physiological regulation of cerebral lipids have been completed in otherwise healthy animals. It is not unreasonable to expect the metabolic flux and metabolism of lipids to change considerably with cellular stress and inflammation. Indeed, cerebrovascular inflammation, associated with BBB disruption, has been shown to alter the rates of passage of molecules between blood

and the brain (Takechi, Pallegage-Gamarallage, et al., 2013a). In a healthy organism, the rate of vesicular mediated transcytosis across the BBB is low. However, during times of vascular stress, there is a considerable increase in the rate of non-specific vesicular transcytosis. Electron micrographs show increased caveolae mediated endocytosis of cerebral capillaries during stress (Lossinsky, Vorbrodt, & Wisniewski, 1983; Povlishock, Kontos, Wei, Rosenblum, & Becker, 1980). Caveolae have been suggested to carry circulating lipoproteins across endothelial cell layers (Schubert et al., 2001). Indeed, the perivascular abundance of apolipoprotein B, an exclusive marker of peripherally derived lipoproteins, increases considerably following inflammatory insults in mice (Takechi, Galloway, Pallegage-Gamarallage, Wellington, et al., 2010). Interestingly, ceramides have been identified as a bioactive lipid associated with caveolae (Czarny, Liu, Oh, & Schnitzer, 2003; Der, Cui, & Das, 2006; Hajduch et al., 2008; Kuebler, Yang, Samapati, & Uhlig, 2010). The increased delivery of lipids to the brain during times of stress do not appear to be exclusive to lipoproteins. A study by Karmi et al. (2010) used positron emission tomography to measure the rate of fatty acid uptake into the brain of patients with metabolic syndrome compared to healthy controls. Patients with metabolic syndrome showed 50-86 % greater uptake of fatty acids into their brain. The results of these studies suggest that during times of stress, alterations in lipid uptake kinetics occur, which may occur through an alteration of capillary architecture and function. Indeed, the endothelium of the BBB forms the sole exchange barrier between the brain and blood. As such, it is exposed to circulating lipids and lipoproteins to a high degree. Therefore, it is susceptible to many of the physiological and pathological effects of lipids and may represent a key component in regulation of the cerebral lipidome by circulating lipids.

Bibliography

- Abbott, N. J., Ronnback, L., & Hansson, E. (2006). Astrocyte-endothelial interactions at the blood-brain barrier. *Nat Rev Neurosci*, 7(1), 41-53. doi:10.1038/nrn1824
- Afanador, N. L., Tran, T. N., & Buydens, L. M. C. (2013). Use of the bootstrap and permutation methods for a more robust variable importance in the projection metric for partial least squares regression. *Analytica Chimica Acta*, 768, 49-56. doi:http://dx.doi.org/10.1016/j.aca.2013.01.004
- Agrawal, R., & Gomez-Pinilla, F. (2012). 'Metabolic syndrome' in the brain: deficiency in omega-3 fatty acid exacerbates dysfunctions in insulin receptor signalling and cognition. *J Physiol*, 590(10), 2485-2499. doi:10.1113/jphysiol.2012.230078
- Akbar, M., Calderon, F., Wen, Z., & Kim, H. Y. (2005). Docosahexaenoic acid: a positive modulator of Akt signaling in neuronal survival. *Proc Natl Acad Sci U S A*, 102(31), 10858-10863. doi:10.1073/pnas.0502903102
- Alashmali, S. M., Hopperton, K. E., & Bazinet, R. P. (2016). Lowering dietary n-6 polyunsaturated fatty acids: interaction with brain arachidonic and docosahexaenoic acids. *Curr Opin Lipidol*, 27(1), 54-66. doi:10.1097/MOL.0000000000000255
- Allen, J. A., Halverson-Tamboli, R. A., & Rasenick, M. M. (2007). Lipid raft microdomains and neurotransmitter signalling. *Nat Rev Neurosci*, 8(2), 128-140. doi:10.1038/nrn2059
- Alles, B., Samieri, C., Feart, C., Jutand, M. A., Laurin, D., & Barberger-Gateau, P. (2012). Dietary patterns: a novel approach to examine the link between nutrition and cognitive function in older individuals. *Nutr Res Rev*, 25(2), 207-222. doi:10.1017/S0954422412000133

- Alshehry, Z. H., Barlow, C. K., Weir, J. M., Zhou, Y., McConville, M. J., & Meikle, P. J. (2015). An Efficient Single Phase Method for the Extraction of Plasma Lipids. *Metabolites*, 5(2), 389-403. doi:10.3390/metabo5020389
- Ambroise, C., & McLachlan, G. J. (2002). Selection bias in gene extraction on the basis of microarray gene-expression data. *Proc Natl Acad Sci U S A*, 99(10), 6562-6566. doi:10.1073/pnas.102102699
- Anderson, G. J., & Connor, W. E. (1988). Uptake of fatty acids by the developing rat brain. *Lipids*, 23(4), 286-290.
- Anderson, G. J., & Connor, W. E. (1989). On the demonstration of omega-3 essential-fatty-acid deficiency in humans. *Am J Clin Nutr*, 49(4), 585-587.
- Anderson, G. J., Hohimer, A. R., & Willeke, G. B. (1993). Uptake of docosahexaenoic acid by microvessels from developing rat brain. *Life Sci*, 53(13), 1089-1098.
- Anderson, G. J., Tso, P. S., & Connor, W. E. (1994). Incorporation of chylomicron fatty acids into the developing rat brain. *J Clin Invest*, 93(6), 2764-2767. doi:10.1172/JCI117293
- Anderson, T. W., & Darling, D. A. (1954). A Test of Goodness of Fit. *Journal of the American Statistical Association*, 49(268), 765-769. doi:10.1080/01621459.1954.10501232
- Astarita, G., Jung, K. M., Berchtold, N. C., Nguyen, V. Q., Gillen, D. L., Head, E., . . . Piomelli, D. (2010). Deficient liver biosynthesis of docosahexaenoic acid correlates with cognitive impairment in Alzheimer's disease. *PLoS One*, 5(9), e12538. doi:10.1371/journal.pone.0012538
- Aung, H. H., Altman, R., Nyunt, T., Kim, J., Nuthikattu, S., Budamagunta, M., . . . Villablanca, A. C. (2016). Lipotoxic brain microvascular injury is mediated

by activating transcription factor 3-dependent inflammatory and oxidative stress pathways. *J Lipid Res*, 57(6), 955-968. doi:10.1194/jlr.M061853

Balazs, Z., Panzenboeck, U., Hammer, A., Sovic, A., Quehenberger, O., Malle, E., & Sattler, W. (2004). Uptake and transport of high-density lipoprotein (HDL) and HDL-associated alpha-tocopherol by an in vitro blood-brain barrier model. *J Neurochem*, 89(4), 939-950. doi:10.1111/j.1471-4159.2004.02373.x

Ballabh, P., Braun, A., & Nedergaard, M. (2004). The blood-brain barrier: an overview: structure, regulation, and clinical implications. *Neurobiol Dis*, 16(1), 1-13. doi:10.1016/j.nbd.2003.12.016

Bascoul-Colombo, C., Guschina, I. A., Maskrey, B. H., Good, M., O'Donnell, V. B., & Harwood, J. L. (2016). Dietary DHA supplementation causes selective changes in phospholipids from different brain regions in both wild type mice and the Tg2576 mouse model of Alzheimer's disease. *Biochim Biophys Acta*, 1861(6), 524-537. doi:10.1016/j.bbalip.2016.03.005

Battistin, L., Cesari, A., Galligioni, F., Marin, G., Massarotti, M., Paccagnella, D., . . . Tonin, P. (1985). Effects of GM1 ganglioside in cerebrovascular diseases: a double-blind trial in 40 cases. *Eur Neurol*, 24(5), 343-351.

Bazan, N. G. (2003a). Synaptic lipid signaling: significance of polyunsaturated fatty acids and platelet-activating factor. *J Lipid Res*, 44(12), 2221-2233. doi:10.1194/jlr.R300013-JLR200

Bazan, N. G. (2003b). Synaptic lipid signaling: significance of polyunsaturated fatty acids and platelet-activating factor. *Journal of Lipid Research*, 44(12), 2221-2233. doi:10.1194/jlr.R300013-JLR200

Bazinet, R. P., & Laye, S. (2014). Polyunsaturated fatty acids and their metabolites in brain function and disease. *Nat Rev Neurosci*, 15(12), 771-785. doi:10.1038/nrn3820

- Becker, K. A., Tummler, B., Gulbins, E., & Grassme, H. (2010). Accumulation of ceramide in the trachea and intestine of cystic fibrosis mice causes inflammation and cell death. *Biochem Biophys Res Commun*, *403*(3-4), 368-374. doi:10.1016/j.bbrc.2010.11.038
- Beermann, C., Mobius, M., Winterling, N., Schmitt, J. J., & Boehm, G. (2005). sn-position determination of phospholipid-linked fatty acids derived from erythrocytes by liquid chromatography electrospray ionization ion-trap mass spectrometry. *Lipids*, *40*(2), 211-218.
- Belkouch, M., Hachem, M., Elgot, A., Lo Van, A., Picq, M., Guichardant, M., . . . Bernoud-Hubac, N. (2016). The pleiotropic effects of omega-3 docosahexaenoic acid on the hallmarks of Alzheimer's disease. *J Nutr Biochem*, *38*, 1-11. doi:10.1016/j.jnutbio.2016.03.002
- Benjamini, Y., & Hochberg, Y. (1995a). Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B-Methodological*, *57*(1), 289-300.
- Benjamini, Y., & Hochberg, Y. (1995b). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, *57*(1), 289-300.
- Benoit, S. C., Kemp, C. J., Elias, C. F., Abplanalp, W., Herman, J. P., Migrenne, S., . . . Clegg, D. J. (2009). Palmitic acid mediates hypothalamic insulin resistance by altering PKC-theta subcellular localization in rodents. *J Clin Invest*, *119*(9), 2577-2589. doi:10.1172/JCI36714
- Berdyshev, E. V., Gorshkova, I., Skobeleva, A., Bittman, R., Lu, X., Dudek, S. M., . . . Natarajan, V. (2009). FTY720 inhibits ceramide synthases and up-regulates dihydrosphingosine 1-phosphate formation in human lung endothelial cells. *J Biol Chem*, *284*(9), 5467-5477. doi:10.1074/jbc.M805186200

- Bharath, L. P., Ruan, T., Li, Y., Ravindran, A., Wan, X., Nhan, J. K., . . . Symons, J. D. (2015). Ceramide initiated protein phosphatase 2A activation contributes to arterial dysfunction in vivo. *Diabetes*. doi:10.2337/db15-0244
- Bickel, P. J., Li, B., Tsybakov, A. B., van de Geer, S. A., Yu, B., Valdés, T., . . . van der Vaart, A. (2006). Regularization in statistics. *Test*, 15(2), 271-344. doi:10.1007/bf02607055
- . Biosynthesis of Plasmalogens in Brain. (2008) *Metabolism and Functions of Bioactive Ether Lipids in the Brain* (pp. 17-37). New York, NY: Springer New York.
- Bjerve, K. S., Daae, L. N. W., & Bremer, J. (1974). The selective loss of lysophospholipids in some commonly used lipid-extraction procedures. *Analytical Biochemistry*, 58(1), 238-245. doi:http://dx.doi.org/10.1016/0003-2697(74)90463-1
- Bjerve, K. S., Mostad, I. L., & Thoresen, L. (1987). Alpha-linolenic acid deficiency in patients on long-term gastric-tube feeding: estimation of linolenic acid and long-chain unsaturated n-3 fatty acid requirement in man. *Am J Clin Nutr*, 45(1), 66-77.
- Blanksby, S. J., & Mitchell, T. W. (2010). Advances in mass spectrometry for lipidomics. *Annu Rev Anal Chem (Palo Alto Calif)*, 3, 433-465. doi:10.1146/annurev.anchem.111808.073705
- Bleeker, S. E., Moll, H. A., Steyerberg, E. W., Donders, A. R. T., Derksen-Lubsen, G., Grobbee, D. E., & Moons, K. G. M. (2003). External validation is necessary in, prediction research: A clinical example. *Journal of Clinical Epidemiology*, 56(9), 826-832. doi:10.1016/S0895-4356(03)00207-5
- Bligh, E. G., & Dyer, W. J. (1959). A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. *Canadian Journal of Biochemistry and Physiology*, 37(1), 911-917. doi:10.1139/y59-099

- Boini, K. M., Zhang, C., Xia, M., Poklis, J. L., & Li, P. L. (2010). Role of sphingolipid mediator ceramide in obesity and renal injury in mice fed a high-fat diet. *J Pharmacol Exp Ther*, 334(3), 839-846. doi:10.1124/jpet.110.168815
- Boon, J., Hoy, A. J., Stark, R., Brown, R. D., Meex, R. C., Henstridge, D. C., . . . Watt, M. J. (2013). Ceramides contained in LDL are elevated in type 2 diabetes and promote inflammation and skeletal muscle insulin resistance. *Diabetes*, 62(2), 401-410. doi:10.2337/db12-0686
- Borg, M. L., Omran, S. F., Weir, J., Meikle, P. J., & Watt, M. J. (2012). Consumption of a high-fat diet, but not regular endurance exercise training, regulates hypothalamic lipid accumulation in mice. *J Physiol*, 590(17), 4377-4389. doi:10.1113/jphysiol.2012.233288
- Bou Khalil, M., Hou, W., Zhou, H., Elisma, F., Swayne, L. A., Blanchard, A. P., . . . Figeys, D. (2010). Lipidomics era: accomplishments and challenges. *Mass Spectrom Rev*, 29(6), 877-929. doi:10.1002/mas.20294
- Boyanovsky, B., Karakashian, A., King, K., Giltiy, N., & Nikolova-Karakashian, M. (2003a). Uptake and Metabolism of Low Density Lipoproteins with Elevated Ceramide Content by Human Microvascular Endothelial Cells: IMPLICATIONS FOR THE REGULATION OF APOPTOSIS. *Journal of Biological Chemistry*, 278(29), 26992-26999. doi:10.1074/jbc.M301536200
- Boyanovsky, B., Karakashian, A., King, K., Giltiy, N., & Nikolova-Karakashian, M. (2003b). Uptake and metabolism of low density lipoproteins with elevated ceramide content by human microvascular endothelial cells: implications for the regulation of apoptosis. *J Biol Chem*, 278(29), 26992-26999. doi:10.1074/jbc.M301536200
- Bozek, K., Wei, Y., Yan, Z., Liu, X., Xiong, J., Sugimoto, M., . . . Khaitovich, P. (2015). Organization and Evolution of Brain Lipidome Revealed by Large-

Scale Analysis of Human, Chimpanzee, Macaque, and Mouse Tissues.
Neuron, 85(4), 695-702. doi:http://dx.doi.org/10.1016/j.neuron.2015.01.003

Bradley-Whitman, M. A., & Lovell, M. A. (2015). Biomarkers of lipid peroxidation in Alzheimer disease (AD): an update. *Arch Toxicol*, 89(7), 1035-1044.
doi:10.1007/s00204-015-1517-6

Brankatschk, M., & Eaton, S. (2010). Lipoprotein particles cross the blood-brain barrier in *Drosophila*. *J Neurosci*, 30(31), 10441-10447.
doi:10.1523/JNEUROSCI.5943-09.2010

Breckenridge, W. C., Gombos, G., & Morgan, I. G. (1972). The lipid composition of adult rat brain synaptosomal plasma membranes. *Biochim Biophys Acta*, 266(3), 695-707.

Brenna, J. T. (2002). Efficiency of conversion of alpha-linolenic acid to long chain n-3 fatty acids in man. *Curr Opin Clin Nutr Metab Care*, 5(2), 127-132.

Breteler, M. M. (2000). Vascular risk factors for Alzheimer's disease: an epidemiologic perspective. *Neurobiol Aging*, 21(2), 153-160.

Brinkmann, V., Davis, M. D., Heise, C. E., Albert, R., Cottens, S., Hof, R., . . . Lynch, K. R. (2002). The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J Biol Chem*, 277(24), 21453-21457.
doi:10.1074/jbc.C200176200

Brites, P., Ferreira, A. S., da Silva, T. F., Sousa, V. F., Malheiro, A. R., Duran, M., . . . Wanders, R. J. (2011). Alkyl-glycerol rescues plasmalogen levels and pathology of ether-phospholipid deficient mice. *PLoS One*, 6(12), e28539.
doi:10.1371/journal.pone.0028539

Brites, P., Ferreira, A. S., Ferreira da Silva, T., Sousa, V. F., Malheiro, A. R., Duran, M., . . . Wanders, R. J. A. (2011). Alkyl-Glycerol Rescues Plasmalogen

Levels and Pathology of Ether-Phospholipid Deficient Mice. *PLoS One*, 6(12), e28539. doi:10.1371/journal.pone.0028539

Brites, P., Waterham, H. R., & Wanders, R. J. (2004). Functions and biosynthesis of plasmalogens in health and disease. *Biochim Biophys Acta*, 1636(2-3), 219-231. doi:10.1016/j.bbaliip.2003.12.010

Brouwers, J. F. (2011). Liquid chromatographic–mass spectrometric analysis of phospholipids. Chromatography, ionization and quantification. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1811(11), 763-775. doi:https://doi.org/10.1016/j.bbaliip.2011.08.001

Brown, M. B., & Forsythe, A. B. (1974). Robust Tests for the Equality of Variances. *Journal of the American Statistical Association*, 69(346), 364-367. doi:10.1080/01621459.1974.10482955

Brown, M. S., & Goldstein, J. L. (1979). Receptor-mediated endocytosis: insights from the lipoprotein receptor system. *Proc Natl Acad Sci U S A*, 76(7), 3330-3337.

Bruce, C. R., Risis, S., Babb, J. R., Yang, C., Lee-Young, R. S., Henstridge, D. C., & Febbraio, M. A. (2013). The sphingosine-1-phosphate analog FTY720 reduces muscle ceramide content and improves glucose tolerance in high fat-fed male mice. *Endocrinology*, 154(1), 65-76. doi:10.1210/en.2012-1847

Buccoliero, R., & Futerman, A. H. (2003). The roles of ceramide and complex sphingolipids in neuronal cell function. *Pharmacological Research*, 47(5), 409-419. doi:http://dx.doi.org/10.1016/S1043-6618(03)00049-5

Buettner, R., Parhofer, K. G., Woenckhaus, M., Wrede, C. E., Kunz-Schughart, L. A., Schölmerich, J., & Bollheimer, L. C. (2006). Defining high-fat-diet rat models: metabolic and molecular effects of different fat types. *Journal of Molecular Endocrinology*, 36(3), 485-501. doi:10.1677/jme.1.01909

- Burckhardt, M., Herke, M., Wustmann, T., Watzke, S., Langer, G., & Fink, A. (2016). Omega-3 fatty acids for the treatment of dementia. *Cochrane Database Syst Rev*, 4, CD009002. doi:10.1002/14651858.CD009002.pub3
- Cajka, T., & Fiehn, O. (2016). Increasing lipidomic coverage by selecting optimal mobile-phase modifiers in LC–MS of blood plasma. *Metabolomics*, 12(2), 34. doi:10.1007/s11306-015-0929-x
- Candela, P., Gosselet, F., Miller, F., Buee-Scherrer, V., Torpier, G., Cecchelli, R., & Fenart, L. (2008). Physiological pathway for low-density lipoproteins across the blood-brain barrier: transcytosis through brain capillary endothelial cells in vitro. *Endothelium*, 15(5-6), 254-264. doi:10.1080/10623320802487759
- Capel, F., Cheraiti, N., Acquaviva, C., Henique, C., Bertrand-Michel, J., Vianey-Saban, C., . . . Morio, B. (2016). Oleate dose-dependently regulates palmitate metabolism and insulin signaling in C2C12 myotubes. *Biochim Biophys Acta*, 1861(12 Pt A), 2000-2010. doi:10.1016/j.bbailip.2016.10.002
- Carrasco-Pancorbo, A., Navas-Iglesias, N., & Cuadros-Rodríguez, L. (2009). From lipid analysis towards lipidomics, a new challenge for the analytical chemistry of the 21st century. Part I: Modern lipid analysis. *TrAC Trends in Analytical Chemistry*, 28(3), 263-278. doi:http://dx.doi.org/10.1016/j.trac.2008.12.005
- Carrie, I., Clement, M., de Javel, D., Frances, H., & Bourre, J. M. (2000a). Phospholipid supplementation reverses behavioral and biochemical alterations induced by n-3 polyunsaturated fatty acid deficiency in mice. *J Lipid Res*, 41(3), 473-480.
- Carrie, I., Clement, M., de Javel, D., Frances, H., & Bourre, J. M. (2000b). Specific phospholipid fatty acid composition of brain regions in mice. Effects of n-3 polyunsaturated fatty acid deficiency and phospholipid supplementation. *J Lipid Res*, 41(3), 465-472.

- Casanova, R., Varma, S., Simpson, B., Kim, M., An, Y., Saldana, S., . . .
Thambisetty, M. (2016). Blood metabolite markers of preclinical Alzheimer's disease in two longitudinally followed cohorts of older individuals. *Alzheimers Dement*, *12*(7), 815-822. doi:10.1016/j.jalz.2015.12.008
- Castro-Perez, J., Previs, S. F., McLaren, D. G., Shah, V., Herath, K., Bhat, G., . . .
Hubbard, B. K. (2011). In vivo D2O labeling to quantify static and dynamic changes in cholesterol and cholesterol esters by high resolution LC/MS. *Journal of Lipid Research*, *52*(1), 159-169. doi:10.1194/jlr.D009787
- Cermenati, G., Mitro, N., Audano, M., Melcangi, R. C., Crestani, M., De Fabiani, E.,
& Caruso, D. (2015). Lipids in the nervous system: from biochemistry and molecular biology to patho-physiology. *Biochim Biophys Acta*, *1851*(1), 51-60. doi:10.1016/j.bbalip.2014.08.011
- Chan, R. B., Oliveira, T. G., Cortes, E. P., Honig, L. S., Duff, K. E., Small, S. A., . . .
Di Paolo, G. (2012a). Comparative lipidomic analysis of mouse and human brain with Alzheimer disease. *J Biol Chem*, *287*(4), 2678-2688. doi:10.1074/jbc.M111.274142
- Chan, R. B., Oliveira, T. G., Cortes, E. P., Honig, L. S., Duff, K. E., Small, S. A., . . .
Di Paolo, G. (2012b). Comparative Lipidomic Analysis of Mouse and Human Brain with Alzheimer Disease. *Journal of Biological Chemistry*, *287*(4), 2678-2688. doi:10.1074/jbc.M111.274142
- Chan, R. B., Perotte, A. J., Zhou, B., Liang, C., Shorr, E. J., Marder, K. S., . . .
Alcalay, R. N. (2017). Elevated GM3 plasma concentration in idiopathic Parkinson's disease: A lipidomic analysis. *PLoS One*, *12*(2), e0172348. doi:10.1371/journal.pone.0172348
- Chavez, Jose A., & Summers, Scott A. (2012). A Ceramide-Centric View of Insulin Resistance. *Cell Metabolism*, *15*(5), 585-594. doi:http://dx.doi.org/10.1016/j.cmet.2012.04.002

- Checa, A., Bedia, C., & Jaumot, J. (2015). Lipidomic data analysis: tutorial, practical guidelines and applications. *Anal Chim Acta*, 885, 1-16.
doi:10.1016/j.aca.2015.02.068
- Chen, C. T., Domenichiello, A. F., Trepanier, M. O., Liu, Z., Masoodi, M., & Bazinet, R. P. (2013). The low levels of eicosapentaenoic acid in rat brain phospholipids are maintained via multiple redundant mechanisms. *J Lipid Res*, 54(9), 2410-2422. doi:10.1194/jlr.M038505
- Chen, C. T., Green, J. T., Orr, S. K., & Bazinet, R. P. (2008). Regulation of brain polyunsaturated fatty acid uptake and turnover. *Prostaglandins Leukot Essent Fatty Acids*, 79(3-5), 85-91. doi:10.1016/j.plefa.2008.09.003
- Chen, C. T., Kitson, A. P., Hopperton, K. E., Domenichiello, A. F., Trepanier, M. O., Lin, L. E., . . . Bazinet, R. P. (2015). Plasma non-esterified docosahexaenoic acid is the major pool supplying the brain. *Sci Rep*, 5, 15791.
doi:10.1038/srep15791
- Chen, C. T., Ma, D. W., Kim, J. H., Mount, H. T., & Bazinet, R. P. (2008). The low density lipoprotein receptor is not necessary for maintaining mouse brain polyunsaturated fatty acid concentrations. *J Lipid Res*, 49(1), 147-152.
doi:10.1194/jlr.M700386-JLR200
- Chen, X., & Gross, R. W. (1994). Phospholipid subclass-specific alterations in the kinetics of ion transport across biologic membranes. *Biochemistry*, 33(46), 13769-13774.
- Cheng, H., Wang, M., Li, J. L., Cairns, N. J., & Han, X. (2013). Specific changes of sulfatide levels in individuals with pre-clinical Alzheimer's disease: an early event in disease pathogenesis. *J Neurochem*, 127(6), 733-738.
doi:10.1111/jnc.12368
- Cheng, H., Zhou, Y., Holtzman, D. M., & Han, X. (2010). Apolipoprotein E mediates sulfatide depletion in animal models of Alzheimer's disease.

Neurobiology of Aging, 31(7), 1188-1196.

doi:10.1016/j.neurobiolaging.2008.07.020

- Cheng, M. L., Chang, K. H., Wu, Y. R., & Chen, C. M. (2016). Metabolic disturbances in plasma as biomarkers for Huntington's disease. *J Nutr Biochem*, 31, 38-44. doi:10.1016/j.jnutbio.2015.12.001
- Chetelat, G., Desgranges, B., de la Sayette, V., Viader, F., Berkouk, K., Landeau, B., . . . Eustache, F. (2003). Dissociating atrophy and hypometabolism impact on episodic memory in mild cognitive impairment. *Brain*, 126(Pt 9), 1955-1967. doi:10.1093/brain/awg196
- Chong, I.-G., & Jun, C.-H. (2005). Performance of some variable selection methods when multicollinearity is present. *Chemometrics and Intelligent Laboratory Systems*, 78(1), 103-112. doi:http://dx.doi.org/10.1016/j.chemolab.2004.12.011
- Choquet, D., & Triller, A. (2003). The role of receptor diffusion in the organization of the postsynaptic membrane. *Nat Rev Neurosci*, 4(4), 251-265. doi:10.1038/nrn1077
- Cintra, D. E., Ropelle, E. R., Moraes, J. C., Pauli, J. R., Morari, J., Souza, C. T., . . . Velloso, L. A. (2012). Unsaturated fatty acids revert diet-induced hypothalamic inflammation in obesity. *PLoS One*, 7(1), e30571. doi:10.1371/journal.pone.0030571
- Coe, N. R., Smith, A. J., Frohnert, B. I., Watkins, P. A., & Bernlohr, D. A. (1999). The fatty acid transport protein (FATP1) is a very long chain acyl-CoA synthetase. *J Biol Chem*, 274(51), 36300-36304.
- Coetzee, T., Fujita, N., Dupree, J., Shi, R., Blight, A., Suzuki, K., . . . Popko, B. (1996). Myelination in the absence of galactocerebroside and sulfatide: normal structure with abnormal function and regional instability. *Cell*, 86(2), 209-219.

- Coll, T., Eyre, E., Rodriguez-Calvo, R., Palomer, X., Sanchez, R. M., Merlos, M., . . . Vazquez-Carrera, M. (2008). Oleate reverses palmitate-induced insulin resistance and inflammation in skeletal muscle cells. *J Biol Chem*, 283(17), 11107-11116. doi:10.1074/jbc.M708700200
- Contreras, M. A., Greiner, R. S., Chang, M. C., Myers, C. S., Salem, N., Jr., & Rapoport, S. I. (2000). Nutritional deprivation of alpha-linolenic acid decreases but does not abolish turnover and availability of unacylated docosahexaenoic acid and docosahexaenoyl-CoA in rat brain. *J Neurochem*, 75(6), 2392-2400.
- Cooney, G. J., Thompson, A. L., Furler, S. M., Ye, J., & Kraegen, E. W. (2002). Muscle Long-Chain Acyl CoA Esters and Insulin Resistance. *Annals of the New York Academy of Sciences*, 967(1), 196-207. doi:10.1111/j.1749-6632.2002.tb04276.x
- Corda, S., Laplace, C., Vicaut, E., & Duranteau, J. (2001). Rapid reactive oxygen species production by mitochondria in endothelial cells exposed to tumor necrosis factor-alpha is mediated by ceramide. *Am J Respir Cell Mol Biol*, 24(6), 762-768. doi:10.1165/ajrcmb.24.6.4228
- Cotman, C., Blank, M. L., Moehl, A., & Snyder, F. (1969). Lipid composition of synaptic plasma membranes isolated from rat brain by zonal centrifugation. *Biochemistry*, 8(11), 4606-4612.
- Couttas, T. A., Kain, N., Daniels, B., Lim, X. Y., Shepherd, C., Kril, J., . . . Don, A. S. (2014). Loss of the neuroprotective factor Sphingosine 1-phosphate early in Alzheimer's disease pathogenesis. *Acta Neuropathologica Communications*, 2(1), 9. doi:10.1186/2051-5960-2-9
- Couttas, T. A., Kain, N., Suchowerska, A. K., Quek, L. E., Turner, N., Fath, T., . . . Don, A. S. (2016). Loss of ceramide synthase 2 activity, necessary for myelin biosynthesis, precedes tau pathology in the cortical pathogenesis of

Alzheimer's disease. *Neurobiol Aging*, 43, 89-100.

doi:10.1016/j.neurobiolaging.2016.03.027

Cunnane, S. C., Chouinard-Watkins, R., Castellano, C. A., & Barberger-Gateau, P. (2013). Docosaehaenoic acid homeostasis, brain aging and Alzheimer's disease: Can we reconcile the evidence? *Prostaglandins Leukot Essent Fatty Acids*, 88(1), 61-70. doi:10.1016/j.plefa.2012.04.006

Cunnane, S. C., Schneider, J. A., Tangney, C., Tremblay-Mercier, J., Fortier, M., Bennett, D. A., & Morris, M. C. (2012). Plasma and brain fatty acid profiles in mild cognitive impairment and Alzheimer's disease. *J Alzheimers Dis*, 29(3), 691-697. doi:10.3233/JAD-2012-110629

Cutler, R. G., Kelly, J., Storie, K., Pedersen, W. A., Tammara, A., Hatanpaa, K., . . . Mattson, M. P. (2004). Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. *Proceedings of the National Academy of Sciences*, 101(7), 2070-2075. doi:10.1073/pnas.0305799101

Czarny, M., Liu, J., Oh, P., & Schnitzer, J. E. (2003). Transient Mechanoactivation of Neutral Sphingomyelinase in Caveolae to Generate Ceramide. *Journal of Biological Chemistry*, 278(7), 4424-4430. doi:10.1074/jbc.M210375200

Das, A. K., & Hajra, A. K. (1988). High incorporation of dietary 1-O-heptadecyl glycerol into tissue plasmalogens of young rats. *FEBS Letters*, 227(2), 187-190. doi:http://dx.doi.org/10.1016/0014-5793(88)80895-0

Das, A. K., Holmes, R. D., Wilson, G. N., & Hajra, A. K. (1992). Dietary ether lipid incorporation into tissue plasmalogens of humans and rodents. *Lipids*, 27(6), 401-405. doi:10.1007/bf02536379

Davis, J. E., Gabler, N. K., Walker-Daniels, J., & Spurlock, M. E. (2008). Tlr-4 Deficiency Selectively Protects Against Obesity Induced by Diets High in Saturated Fat. *Obesity*, 16(6), 1248-1255. doi:10.1038/oby.2008.210

- Dawson, G., & Qin, J. (2011). Gilenya (FTY720) inhibits acid sphingomyelinase by a mechanism similar to tricyclic antidepressants. *Biochem Biophys Res Commun*, 404(1), 321-323. doi:10.1016/j.bbrc.2010.11.115
- de la Monte, S. M., Tong, M., Nguyen, V., Setshedi, M., Longato, L., & Wands, J. R. (2010). Ceramide-mediated insulin resistance and impairment of cognitive-motor functions. *J Alzheimers Dis*, 21(3), 967-984. doi:10.3233/JAD-2010-091726
- de Urquiza, A. M., Liu, S., Sjoberg, M., Zetterstrom, R. H., Griffiths, W., Sjoval, J., & Perlmann, T. (2000). Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. *Science*, 290(5499), 2140-2144.
- DeGeorge, J. J., Noronha, J. G., Bell, J., Robinson, P., & Rapoport, S. I. (1989). Intravenous injection of [1-14C]arachidonate to examine regional brain lipid metabolism in unanesthetized rats. *Journal of Neuroscience Research*, 24(3), 413-423. doi:10.1002/jnr.490240311
- DeMar, J. C., Jr., Ma, K., Bell, J. M., & Rapoport, S. I. (2004a). Half-lives of docosahexaenoic acid in rat brain phospholipids are prolonged by 15 weeks of nutritional deprivation of n-3 polyunsaturated fatty acids. *J Neurochem*, 91(5), 1125-1137. doi:10.1111/j.1471-4159.2004.02789.x
- DeMar, J. C., Ma, K., Bell, J. M., & Rapoport, S. I. (2004b). Half-lives of docosahexaenoic acid in rat brain phospholipids are prolonged by 15 weeks of nutritional deprivation of n-3 polyunsaturated fatty acids. *Journal of Neurochemistry*, 91(5), 1125-1137. doi:10.1111/j.1471-4159.2004.02789.x
- den Hoedt, S., Janssen, C. I., Astarita, G., Piomelli, D., Leijten, F. P., Crivelli, S. M., . . . Mulder, M. T. (2016). Pleiotropic Effect of Human ApoE4 on Cerebral Ceramide and Saturated Fatty Acid Levels. *J Alzheimers Dis*. doi:10.3233/JAD-160739

- Der, P., Cui, J., & Das, D. K. (2006). Role of lipid rafts in ceramide and nitric oxide signaling in the ischemic and preconditioned hearts. *Journal of Molecular and Cellular Cardiology*, 40(2), 313-320.
doi:<http://dx.doi.org/10.1016/j.yjmcc.2005.10.005>
- Domenichiello, A. F., Kitson, A. P., & Bazinet, R. P. (2015). Is docosahexaenoic acid synthesis from alpha-linolenic acid sufficient to supply the adult brain? *Prog Lipid Res*, 59, 54-66. doi:[10.1016/j.plipres.2015.04.002](https://doi.org/10.1016/j.plipres.2015.04.002)
- Du, P., Stolovitzky, G., Horvatovich, P., Bischoff, R., Lim, J., & Suits, F. (2008). A noise model for mass spectrometry based proteomics. *Bioinformatics*, 24(8), 1070-1077. doi:[10.1093/bioinformatics/btn078](https://doi.org/10.1093/bioinformatics/btn078)
- Dufner, D., & Previs, S. F. (2003). Measuring in vivo metabolism using heavy water. *Current Opinion in Clinical Nutrition & Metabolic Care*, 6(5), 511-517.
- Dunn, O. J. (1961). Multiple Comparisons among Means. *Journal of the American Statistical Association*, 56(293), 52-64.
doi:[10.1080/01621459.1961.10482090](https://doi.org/10.1080/01621459.1961.10482090)
- Dunn, W. B., Erban, A., Weber, R. J. M., Creek, D. J., Brown, M., Breitling, R., . . . Viant, M. R. (2013). Mass appeal: metabolite identification in mass spectrometry-focused untargeted metabolomics. *Metabolomics*, 9(1), 44-66.
doi:[10.1007/s11306-012-0434-4](https://doi.org/10.1007/s11306-012-0434-4)
- Dunnett, C. W. (1955). A Multiple Comparison Procedure for Comparing Several Treatments with a Control. *Journal of the American Statistical Association*, 50(272), 1096-1121. doi:[10.1080/01621459.1955.10501294](https://doi.org/10.1080/01621459.1955.10501294)
- Edmond, J. (2001). Essential polyunsaturated fatty acids and the barrier to the brain: the components of a model for transport. *J Mol Neurosci*, 16(2-3), 181-193; discussion 215-121. doi:[10.1385/JMN:16:2-3:181](https://doi.org/10.1385/JMN:16:2-3:181)

- Edmond, J., Higa, T. A., Korsak, R. A., Bergner, E. A., & Lee, W. N. (1998). Fatty acid transport and utilization for the developing brain. *J Neurochem*, *70*(3), 1227-1234.
- Edmond, J., Korsak, R. A., Morrow, J. W., Torok-Both, G., & Catlin, D. H. (1991). Dietary cholesterol and the origin of cholesterol in the brain of developing rats. *J Nutr*, *121*(9), 1323-1330.
- Ekroos, K., Chernushevich, I. V., Simons, K., & Shevchenko, A. (2002). Quantitative profiling of phospholipids by multiple precursor ion scanning on a hybrid quadrupole time-of-flight mass spectrometer. *Anal Chem*, *74*(5), 941-949.
- Elahy, M., Jackaman, C., Mamo, J. C., Lam, V., Dhaliwal, S. S., Giles, C., . . . Takechi, R. (2015). Blood-brain barrier dysfunction developed during normal aging is associated with inflammation and loss of tight junctions but not with leukocyte recruitment. *Immun Ageing*, *12*, 2. doi:10.1186/s12979-015-0029-9
- Elahy, M., Lam, V., Pallegage-Gamarallage, M. M., Giles, C., Mamo, J. C., & Takechi, R. (2015). Nicotine Attenuates Disruption of Blood-Brain Barrier Induced by Saturated-Fat Feeding in Wild-Type Mice. *Nicotine Tob Res*, *17*(12), 1436-1441. doi:10.1093/ntr/ntv044
- Eldho, N. V., Feller, S. E., Tristram-Nagle, S., Polozov, I. V., & Gawrisch, K. (2003). Polyunsaturated docosahexaenoic vs docosapentaenoic acid-differences in lipid matrix properties from the loss of one double bond. *J Am Chem Soc*, *125*(21), 6409-6421. doi:10.1021/ja029029o
- Emmanuel, Y., Cochlin, L. E., Tyler, D. J., de Jager, C. A., Smith, A. D., & Clarke, K. (2013). Human hippocampal energy metabolism is impaired during cognitive activity in a lipid infusion model of insulin resistance. *Brain Behav*, *3*(2), 134-144. doi:10.1002/brb3.124

- Erion, D. M., & Shulman, G. I. (2010). Diacylglycerol-mediated insulin resistance. *Nature medicine*, *16*(4), 400-402. doi:10.1038/nm0410-400
- Falcon, A., Doege, H., Fluitt, A., Tsang, B., Watson, N., Kay, M. A., & Stahl, A. (2010). FATP2 is a hepatic fatty acid transporter and peroxisomal very long-chain acyl-CoA synthetase. *Am J Physiol Endocrinol Metab*, *299*(3), E384-393. doi:10.1152/ajpendo.00226.2010
- Farmer, K., Smith, C. A., Hayley, S., & Smith, J. (2015). Major Alterations of Phosphatidylcholine and Lysophosphatidylcholine Lipids in the Substantia Nigra Using an Early Stage Model of Parkinson's Disease. *Int J Mol Sci*, *16*(8), 18865-18877. doi:10.3390/ijms160818865
- Farooqui, A. A., Horrocks, L. A., & Farooqui, T. (2007). Modulation of inflammation in brain: a matter of fat. *J Neurochem*, *101*(3), 577-599. doi:10.1111/j.1471-4159.2006.04371.x
- Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., & Whitehouse, C. M. (1990). Electrospray ionization—principles and practice. *Mass Spectrometry Reviews*, *9*(1), 37-70. doi:10.1002/mas.1280090103
- Fernandez, C. A., Des Rosiers, C., Previs, S. F., David, F., & Brunengraber, H. (1996). Correction of ¹³C mass isotopomer distributions for natural stable isotope abundance. *J Mass Spectrom*, *31*(3), 255-262. doi:10.1002/(SICI)1096-9888(199603)31:3<255::AID-JMS290>3.0.CO;2-3
- Fhaner, C. J., Liu, S., Ji, H., Simpson, R. J., & Reid, G. E. (2012). Comprehensive Lipidome Profiling of Isogenic Primary and Metastatic Colon Adenocarcinoma Cell Lines. *Analytical Chemistry*, *84*(21), 8917-8926. doi:10.1021/ac302154g
- Fiandaca, M. S., Zhong, X., Cheema, A. K., Orquiza, M. H., Chidambaram, S., Tan, M. T., . . . Federoff, H. J. (2015). Plasma 24-metabolite Panel Predicts

Preclinical Transition to Clinical Stages of Alzheimer's Disease. *Front Neurol*, 6, 237. doi:10.3389/fneur.2015.00237

Figeys, D., & Aebersold, R. (1997). High sensitivity identification of proteins by electrospray ionization tandem mass spectrometry: Initial comparison between an ion trap mass spectrometer and a triple quadrupole mass spectrometer. *ELECTROPHORESIS*, 18(3-4), 360-368.
doi:10.1002/elps.1150180310

Filippov, V., Song, M. A., Zhang, K., Vinters, H. V., Tung, S., Kirsch, W. M., . . . Duerksen-Hughes, P. J. (2012). Increased ceramide in brains with Alzheimer's and other neurodegenerative diseases. *J Alzheimers Dis*, 29(3), 537-547. doi:10.3233/JAD-2011-111202

Floudas, C. A., & Gounaris, C. E. (2009). A review of recent advances in global optimization. *J. of Global Optimization*, 45(1), 3-38. doi:10.1007/s10898-008-9332-8

Folch-Fortuny, A., Arteaga, F., & Ferrer, A. (2015). PCA model building with missing data: New proposals and a comparative study. *Chemometrics and Intelligent Laboratory Systems*, 146, 77-88.
doi:10.1016/j.chemolab.2015.05.006

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*, 226(1), 497-509.

Fong, T. G., Neff, N. H., & Hadjiconstantinou, M. (1997). GM1 ganglioside improves spatial learning and memory of aged rats. *Behav Brain Res*, 85(2), 203-211.

Fonteh, A. N., Ormseth, C., Chiang, J., Cipolla, M., Arakaki, X., & Harrington, M. G. (2015). Sphingolipid metabolism correlates with cerebrospinal fluid Beta

amyloid levels in Alzheimer's disease. *PLoS One*, *10*(5), e0125597.
doi:10.1371/journal.pone.0125597

Foster, C. A., Howard, L. M., Schweitzer, A., Persohn, E., Hiestand, P. C., Balatoni, B., . . . Billich, A. (2007). Brain penetration of the oral immunomodulatory drug FTY720 and its phosphorylation in the central nervous system during experimental autoimmune encephalomyelitis: consequences for mode of action in multiple sclerosis. *J Pharmacol Exp Ther*, *323*(2), 469-475.
doi:10.1124/jpet.107.127183

Fraser, T., Tayler, H., & Love, S. (2010). Fatty acid composition of frontal, temporal and parietal neocortex in the normal human brain and in Alzheimer's disease. *Neurochem Res*, *35*(3), 503-513. doi:10.1007/s11064-009-0087-5

Freund-Levi, Y., Vedin, I., Hjorth, E., Basun, H., Faxen Irving, G., Schultzberg, M., . . . Basu, S. (2014). Effects of supplementation with omega-3 fatty acids on oxidative stress and inflammation in patients with Alzheimer's disease: the OmegAD study. *J Alzheimers Dis*, *42*(3), 823-831. doi:10.3233/JAD-132042

Fujino, T., Yamada, T., Asada, T., Tsuboi, Y., Wakana, C., Mawatari, S., & Kono, S. (2017). Efficacy and Blood Plasmalogen Changes by Oral Administration of Plasmalogen in Patients with Mild Alzheimer's Disease and Mild Cognitive Impairment: A Multicenter, Randomized, Double-blind, Placebo-controlled Trial. *EBioMedicine*. doi:10.1016/j.ebiom.2017.02.012

Furse, S., Egmond, M. R., & Killian, J. A. (2015). Isolation of lipids from biological samples. *Molecular Membrane Biology*, *32*(3), 55-64.
doi:10.3109/09687688.2015.1050468

Gao, S., Zhang, Z.-P., & Karnes, H. T. (2005). Sensitivity enhancement in liquid chromatography/atmospheric pressure ionization mass spectrometry using derivatization and mobile phase additives. *Journal of Chromatography B*, *825*(2), 98-110. doi:http://dx.doi.org/10.1016/j.jchromb.2005.04.021

- Geladi, P., & Kowalski, B. R. (1986). Partial least-squares regression: a tutorial. *Analytica Chimica Acta*, *185*, 1-17. doi:[http://dx.doi.org/10.1016/0003-2670\(86\)80028-9](http://dx.doi.org/10.1016/0003-2670(86)80028-9)
- Ghaste, M., Mistrik, R., & Shulaev, V. (2016). Applications of Fourier Transform Ion Cyclotron Resonance (FT-ICR) and Orbitrap Based High Resolution Mass Spectrometry in Metabolomics and Lipidomics. *Int J Mol Sci*, *17*(6). doi:[10.3390/ijms17060816](https://doi.org/10.3390/ijms17060816)
- Giles, C., Albrecht, M. A., Lam, V., Takechi, R., & Mamo, J. C. (2016). Biostatistical analysis of quantitative immunofluorescence microscopy images. *J Microsc*, *264*(3), 321-333. doi:[10.1111/jmi.12446](https://doi.org/10.1111/jmi.12446)
- Giles, C., Takechi, R., Mellett, N. A., Meikle, P. J., Dhaliwal, S., & Mamo, J. C. (2016). The Effects of Long-Term Saturated Fat Enriched Diets on the Brain Lipidome. *PLoS One*, *11*(12), e0166964. doi:[10.1371/journal.pone.0166964](https://doi.org/10.1371/journal.pone.0166964)
- Giles, C., Takechi, R., Mellett, N. A., Meikle, P. J., Dhaliwal, S., & Mamo, J. C. (2017). Differential regulation of sphingolipid metabolism in plasma, hippocampus, and cerebral cortex of mice administered sphingolipid modulating agents. *J Neurochem*. doi:[10.1111/jnc.13964](https://doi.org/10.1111/jnc.13964)
- Ginsberg, L., Rafique, S., Xuereb, J. H., Rapoport, S. I., & Gershfeld, N. L. (1995). Disease and anatomic specificity of ethanolamine plasmalogen deficiency in Alzheimer's disease brain. *Brain Res*, *698*(1-2), 223-226.
- Glaros, E. N., Kim, W. S., Quinn, C. M., Jessup, W., Rye, K. A., & Garner, B. (2008). Myriocin slows the progression of established atherosclerotic lesions in apolipoprotein E gene knockout mice. *J Lipid Res*, *49*(2), 324-331. doi:[10.1194/jlr.M700261-JLR200](https://doi.org/10.1194/jlr.M700261-JLR200)
- Glatz, J. F., Luiken, J. J., & Bonen, A. (2010). Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease. *Physiol Rev*, *90*(1), 367-417. doi:[10.1152/physrev.00003.2009](https://doi.org/10.1152/physrev.00003.2009)

- Gonzalez, C. E., Venkatraman, V. K., An, Y., Landman, B. A., Davatzikos, C., Ratnam Bandaru, V. V., . . . Resnick, S. M. (2016). Peripheral sphingolipids are associated with variation in white matter microstructure in older adults. *Neurobiol Aging*, *43*, 156-163. doi:10.1016/j.neurobiolaging.2016.04.008
- Goodenowe, D. B., Cook, L. L., Liu, J., Lu, Y., Jayasinghe, D. A., Ahiahonu, P. W., . . . Wood, P. L. (2007). Peripheral ethanolamine plasmalogen deficiency: a logical causative factor in Alzheimer's disease and dementia. *J Lipid Res*, *48*(11), 2485-2498. doi:10.1194/jlr.P700023-JLR200
- Gordon, S. M., Li, H., Zhu, X., Shah, A. S., Lu, L. J., & Davidson, W. S. (2015). A Comparison of the Mouse and Human Lipoproteome: Suitability of the Mouse Model for Studies of Human Lipoproteins. *Journal of proteome research*, *14*(6), 2686-2695. doi:10.1021/acs.jproteome.5b00213
- Goti, D., Hrzenjak, A., Levak-Frank, S., Frank, S., van der Westhuyzen, D. R., Malle, E., & Sattler, W. (2001). Scavenger receptor class B, type I is expressed in porcine brain capillary endothelial cells and contributes to selective uptake of HDL-associated vitamin E. *J Neurochem*, *76*(2), 498-508.
- Grange, E., Deutsch, J., Smith, Q. R., Chang, M., Rapoport, S. I., & Purdon, A. D. (1995). Specific activity of brain palmitoyl-CoA pool provides rates of incorporation of palmitate in brain phospholipids in awake rats. *J Neurochem*, *65*(5), 2290-2298.
- Greenwood, C. E., & Winocur, G. (1990). Learning and memory impairment in rats fed a high saturated fat diet. *Behav Neural Biol*, *53*(1), 74-87.
- Greenwood, C. E., & Winocur, G. (2001). Glucose treatment reduces memory deficits in young adult rats fed high-fat diets. *Neurobiol Learn Mem*, *75*(2), 179-189. doi:10.1006/nlme.2000.3964
- Grey, E. C. (1913). The Fatty Acids of the Human Brain. *Biochemical Journal*, *7*(2), 148-156.

- Grimm, M. O. W., Grösgen, S., Riemenschneider, M., Tanila, H., Grimm, H. S., & Hartmann, T. (2011). From brain to food: Analysis of phosphatidylcholins, lyso-phosphatidylcholins and phosphatidylcholin–plasmalogens derivatives in Alzheimer's disease human post mortem brains and mice model via mass spectrometry. *Journal of Chromatography A*, 1218(42), 7713-7722. doi:http://dx.doi.org/10.1016/j.chroma.2011.07.073
- Gromski, P. S., Muhamadali, H., Ellis, D. I., Xu, Y., Correa, E., Turner, M. L., & Goodacre, R. (2015). A tutorial review: Metabolomics and partial least squares-discriminant analysis--a marriage of convenience or a shotgun wedding. *Anal Chim Acta*, 879, 10-23. doi:10.1016/j.aca.2015.02.012
- Guan, Z. (2009). Discovering novel brain lipids by liquid chromatography/tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*, 877(26), 2814-2821. doi:10.1016/j.jchromb.2009.03.002
- Guan, Z., Wang, Y., Cairns, N. J., Lantos, P. L., Dallner, G., & Sindelar, P. J. (1999). Decrease and structural modifications of phosphatidylethanolamine plasmalogen in the brain with Alzheimer disease. *J Neuropathol Exp Neurol*, 58(7), 740-747.
- Guest, J., Garg, M., Bilgin, A., & Grant, R. (2013). Relationship between central and peripheral fatty acids in humans. *Lipids in Health and Disease*, 12(1), 79. doi:10.1186/1476-511x-12-79
- Guilhaus, M. (1995). Special feature: Tutorial. Principles and instrumentation in time-of-flight mass spectrometry. Physical and instrumental concepts. *Journal of Mass Spectrometry*, 30(11), 1519-1532. doi:10.1002/jms.1190301102
- Gulbins, A., Grassmé, H., Hoehn, R., Wilker, B., Soddemann, M., Kohnen, M., . . . Gulbins, E. (2016). Regulation of Neuronal Stem Cell Proliferation in the Hippocampus by Endothelial Ceramide. *Cellular Physiology and Biochemistry*, 39(2), 790-801.

- Gulbins, E., Palmada, M., Reichel, M., Luth, A., Bohmer, C., Amato, D., . . . Kornhuber, J. (2013). Acid sphingomyelinase-ceramide system mediates effects of antidepressant drugs. *Nat Med*, *19*(7), 934-938. doi:10.1038/nm.3214
- Hager, J. W., & Le Blanc, J. C. Y. (2003). High-performance liquid chromatography–tandem mass spectrometry with a new quadrupole/linear ion trap instrument. *Journal of Chromatography A*, *1020*(1), 3-9. doi:http://dx.doi.org/10.1016/S0021-9673(03)00426-6
- Hajdich, E., Turban, S., Le Liepvre, X., Le Lay, S., Lipina, C., Dimopoulos, N., . . . Hundal, Harinder S. (2008). Targeting of PKC ζ and PKB to caveolin-enriched microdomains represents a crucial step underpinning the disruption in PKB-directed signalling by ceramide. *Biochemical Journal*, *410*(2), 369-379. doi:10.1042/bj20070936
- Hajjar, T., Meng, G. Y., Rajion, M. A., Vidyadaran, S., Othman, F., Farjam, A. S., . . . Ebrahimi, M. (2012). Omega 3 polyunsaturated fatty acid improves spatial learning and hippocampal Peroxisome Proliferator Activated Receptors (PPAR α and PPAR γ) gene expression in rats. *BMC Neuroscience*, *13*, 109-109. doi:10.1186/1471-2202-13-109
- Hamilton, J. A. (2003). Fast flip-flop of cholesterol and fatty acids in membranes: implications for membrane transport proteins. *Curr Opin Lipidol*, *14*(3), 263-271. doi:10.1097/01.mol.0000073507.41685.9b
- Hammad, S. M., Pierce, J. S., Soodavar, F., Smith, K. J., Al Gadban, M. M., Rembiesa, B., . . . Bielawska, A. (2010). Blood sphingolipidomics in healthy humans: impact of sample collection methodology. *J Lipid Res*, *51*(10), 3074-3087. doi:10.1194/jlr.D008532
- Han, X. (2007). Potential mechanisms contributing to sulfatide depletion at the earliest clinically recognizable stage of Alzheimer's disease: a tale of shotgun

lipidomics. *J Neurochem*, 103 Suppl 1, 171-179. doi:10.1111/j.1471-4159.2007.04708.x

Han, X. (2010). Multi-dimensional mass spectrometry-based shotgun lipidomics and the altered lipids at the mild cognitive impairment stage of Alzheimer's disease. *Biochim Biophys Acta*, 1801(8), 774-783. doi:10.1016/j.bbaliip.2010.01.010

Han, X., Cheng, H., Fryer, J. D., Fagan, A. M., & Holtzman, D. M. (2003). Novel Role for Apolipoprotein E in the Central Nervous System: MODULATION OF SULFATIDE CONTENT. *Journal of Biological Chemistry*, 278(10), 8043-8051. doi:10.1074/jbc.M212340200

Han, X., D. M. H., McKeel, D. W., Jr., Kelley, J., & Morris, J. C. (2002). Substantial sulfatide deficiency and ceramide elevation in very early Alzheimer's disease: potential role in disease pathogenesis. *J Neurochem*, 82(4), 809-818.

Han, X., & Gross, R. W. (2005). Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples. *Mass Spectrom Rev*, 24. doi:10.1002/mas.20023

Han, X., Holtzman, D. M., & McKeel, D. W., Jr. (2001). Plasmalogen deficiency in early Alzheimer's disease subjects and in animal models: molecular characterization using electrospray ionization mass spectrometry. *J Neurochem*, 77(4), 1168-1180.

Han, X., M. Holtzman, D., W. McKeel, D., Kelley, J., & Morris, J. C. (2002). Substantial sulfatide deficiency and ceramide elevation in very early Alzheimer's disease: potential role in disease pathogenesis. *Journal of Neurochemistry*, 82(4), 809-818. doi:10.1046/j.1471-4159.2002.00997.x

Han, X., Rozen, S., Boyle, S. H., Hellegers, C., Cheng, H., Burke, J. R., . . . Kaddurah-Daouk, R. (2011). Metabolomics in early Alzheimer's disease:

identification of altered plasma sphingolipidome using shotgun lipidomics. *PLoS One*, 6(7), e21643. doi:10.1371/journal.pone.0021643

Han, X., Yang, K., & Gross, R. W. (2012). Multi-dimensional mass spectrometry-based shotgun lipidomics and novel strategies for lipidomic analyses. *Mass Spectrom Rev*, 31(1), 134-178. doi:10.1002/mas.20342

Han, X., Yang, K., Yang, J., Fikes, K. N., Cheng, H., & Gross, R. W. (2006). Factors influencing the electrospray intrasource separation and selective ionization of glycerophospholipids. *J Am Soc Mass Spectrom*, 17(2), 264-274. doi:10.1016/j.jasms.2005.11.003

Hanada, K. (2003). Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1632(1-3), 16-30. doi:https://doi.org/10.1016/S1388-1981(03)00059-3

Hannun, Y. A. (1996). Functions of ceramide in coordinating cellular responses to stress. *Science*, 274(5294), 1855-1859.

Harnack, K., Andersen, G., & Somoza, V. (2009). Quantitation of alpha-linolenic acid elongation to eicosapentaenoic and docosahexaenoic acid as affected by the ratio of n6/n3 fatty acids. *Nutr Metab (Lond)*, 6, 8. doi:10.1186/1743-7075-6-8

Harrell, F. (2015). *Regression modeling strategies: with applications to linear models, logistic and ordinal regression, and survival analysis*: Springer.

Haughey, N. J., Cutler, R. G., Tamara, A., McArthur, J. C., Vargas, D. L., Pardo, C. A., . . . Mattson, M. P. (2004). Perturbation of sphingolipid metabolism and ceramide production in HIV-dementia. *Ann Neurol*, 55(2), 257-267. doi:10.1002/ana.10828

- He, X., Huang, Y., Li, B., Gong, C.-X., & Schuchman, E. H. (2010). Deregulation of sphingolipid metabolism in Alzheimer's disease. *Neurobiology of Aging*, 31(3), 398-408. doi:10.1016/j.neurobiolaging.2008.05.010
- Heidari Nejad, S., Takechi, R., Mullins, B. J., Giles, C., Larcombe, A. N., Bertolatti, D., . . . Mamo, J. (2015). The effect of diesel exhaust exposure on blood-brain barrier integrity and function in a murine model. *J Appl Toxicol*, 35(1), 41-47. doi:10.1002/jat.2985
- Hennebelle, M., Plourde, M., Chouinard-Watkins, R., Castellano, C. A., Barberger-Gateau, P., & Cunnane, S. C. (2014). Ageing and apoE change DHA homeostasis: relevance to age-related cognitive decline. *Proc Nutr Soc*, 73(1), 80-86. doi:10.1017/S0029665113003625
- Heron, D. S., Shinitzky, M., Hershkowitz, M., & Samuel, D. (1980). Lipid fluidity markedly modulates the binding of serotonin to mouse brain membranes. *Proc Natl Acad Sci U S A*, 77(12), 7463-7467.
- Herrero, A., Portero-Otin, M., Bellmunt, M. J., Pamplona, R., & Barja, G. (2001). Effect of the degree of fatty acid unsaturation of rat heart mitochondria on their rates of H₂O₂ production and lipid and protein oxidative damage. *Mech Ageing Dev*, 122(4), 427-443.
- Higgins, C. F. (1994). Flip-flop: the transmembrane translocation of lipids. *Cell*, 79(3), 393-395.
- Hirabayashi, Y., & Furuya, S. (2008). Roles of l-serine and sphingolipid synthesis in brain development and neuronal survival. *Progress in Lipid Research*, 47(3), 188-203. doi:https://doi.org/10.1016/j.plipres.2008.01.003
- Hojjati, M. R., Li, Z., Zhou, H., Tang, S., Huan, C., Ooi, E., . . . Jiang, X.-C. (2005a). Effect of Myriocin on Plasma Sphingolipid Metabolism and Atherosclerosis in apoE-deficient Mice. *Journal of Biological Chemistry*, 280(11), 10284-10289. doi:10.1074/jbc.M412348200

- Hojjati, M. R., Li, Z., Zhou, H., Tang, S., Huan, C., Ooi, E., . . . Jiang, X. C. (2005b). Effect of myriocin on plasma sphingolipid metabolism and atherosclerosis in apoE-deficient mice. *J Biol Chem*, *280*(11), 10284-10289.
doi:10.1074/jbc.M412348200
- Holland, W. L., Bikman, B. T., Wang, L. P., Yuguang, G., Sargent, K. M., Bulchand, S., . . . Summers, S. A. (2011). Lipid-induced insulin resistance mediated by the proinflammatory receptor TLR4 requires saturated fatty acid-induced ceramide biosynthesis in mice. *J Clin Invest*, *121*(5), 1858-1870.
doi:10.1172/JCI43378
- Holland, W. L., Brozinick, J. T., Wang, L. P., Hawkins, E. D., Sargent, K. M., Liu, Y., . . . Summers, S. A. (2007). Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. *Cell Metab*, *5*(3), 167-179. doi:10.1016/j.cmet.2007.01.002
- Hong, S. H., Belayev, L., Khoutorova, L., Obenaus, A., & Bazan, N. G. (2014). Docosahexaenoic acid confers enduring neuroprotection in experimental stroke. *J Neurol Sci*, *338*(1-2), 135-141. doi:10.1016/j.jns.2013.12.033
- Horrocks, L. A., & Farooqui, A. A. (2004). Docosahexaenoic acid in the diet: its importance in maintenance and restoration of neural membrane function. *Prostaglandins Leukot Essent Fatty Acids*, *70*(4), 361-372.
doi:10.1016/j.plefa.2003.12.011
- Hsu, F.-F., & Turk, J. (2008). Structural Characterization of Unsaturated Glycerophospholipids by Multiple-Stage Linear Ion-Trap Mass Spectrometry with Electrospray Ionization. *Journal of the American Society for Mass Spectrometry*, *19*(11), 1681-1691.
doi:http://dx.doi.org/10.1016/j.jasms.2008.07.023
- Hsu, F.-F., & Turk, J. (2010). Electrospray Ionization Multiple-Stage Linear Ion-trap Mass Spectrometry for Structural Elucidation of Triacylglycerols: Assignment of Fatty Acyl Groups on the Glycerol Backbone and Location of

Double Bonds. *Journal of the American Society for Mass Spectrometry*, 21(4), 657-669. doi:<http://dx.doi.org/10.1016/j.jasms.2010.01.007>

Hsu, F.-F. u., & Turk, J. (2003). Electrospray ionization/tandem quadrupole mass spectrometric studies on phosphatidylcholines: the fragmentation processes. *Journal of the American Society for Mass Spectrometry*, 14(4), 352-363. doi:[http://dx.doi.org/10.1016/S1044-0305\(03\)00064-3](http://dx.doi.org/10.1016/S1044-0305(03)00064-3)

Hsu, F. F., & Turk, J. (2009). Electrospray ionization with low-energy collisionally activated dissociation tandem mass spectrometry of glycerophospholipids: mechanisms of fragmentation and structural characterization. *J Chromatogr B Analyt Technol Biomed Life Sci*, 877(26), 2673-2695. doi:10.1016/j.jchromb.2009.02.033

Hsu, T. M., & Kanoski, S. E. (2014). Blood-brain barrier disruption: mechanistic links between Western diet consumption and dementia. *Front Aging Neurosci*, 6, 88. doi:10.3389/fnagi.2014.00088

Hulbert, A. J., Turner, N., Storlien, L. H., & Else, P. L. (2005). Dietary fats and membrane function: implications for metabolism and disease. *Biol Rev Camb Philos Soc*, 80(1), 155-169.

Huynh, K., Martins, R. N., & Meikle, P. J. (2017). Lipidomic Profiles in Diabetes and Dementia. *J Alzheimers Dis*. doi:10.3233/JAD-161215

Huynh, K., Mundra, P. A., & Meikle, P. J. (2015). Sample Handling and Automation: Batch Effect. In M. R. Wenk (Ed.), *Encyclopedia of Lipidomics* (pp. 1-5). Dordrecht: Springer Netherlands.

Ifuku, M., Katafuchi, T., Mawatari, S., Noda, M., Miake, K., Sugiyama, M., & Fujino, T. (2012). Anti-inflammatory/anti-amyloidogenic effects of plasmalogens in lipopolysaccharide-induced neuroinflammation in adult mice. *J Neuroinflammation*, 9, 197. doi:10.1186/1742-2094-9-197

- Igarashi, M., Gao, F., Kim, H. W., Ma, K., Bell, J. M., & Rapoport, S. I. (2009). Dietary n-6 PUFA deprivation for 15 weeks reduces arachidonic acid concentrations while increasing n-3 PUFA concentrations in organs of post-weaning male rats. *Biochim Biophys Acta*, *1791*(2), 132-139. doi:10.1016/j.bbalip.2008.11.002
- Igarashi, M., Kim, H. W., Chang, L., Ma, K., & Rapoport, S. I. (2012). Dietary n-6 polyunsaturated fatty acid deprivation increases docosahexaenoic acid metabolism in rat brain. *J Neurochem*, *120*(6), 985-997. doi:10.1111/j.1471-4159.2011.07597.x
- Igarashi, M., Ma, K., Gao, F., Kim, H.-W., Rapoport, S. I., & Rao, J. S. (2011a). Disturbed choline plasmalogen and phospholipid fatty acid concentrations in Alzheimer disease prefrontal cortex. *Journal of Alzheimer's disease : JAD*, *24*(3), 507-517. doi:10.3233/JAD-2011-101608
- Igarashi, M., Ma, K., Gao, F., Kim, H. W., Rapoport, S. I., & Rao, J. S. (2011b). Disturbed choline plasmalogen and phospholipid fatty acid concentrations in Alzheimer's disease prefrontal cortex. *J Alzheimers Dis*, *24*(3), 507-517. doi:10.3233/JAD-2011-101608
- Ikemoto, A., Ohishi, M., Sato, Y., Hata, N., Misawa, Y., Fujii, Y., & Okuyama, H. (2001). Reversibility of n-3 fatty acid deficiency-induced alterations of learning behavior in the rat: level of n-6 fatty acids as another critical factor. *J Lipid Res*, *42*(10), 1655-1663.
- Imgrund, S., Hartmann, D., Farwanah, H., Eckhardt, M., Sandhoff, R., Degen, J., . . . Willecke, K. (2009). Adult ceramide synthase 2 (CERS2)-deficient mice exhibit myelin sheath defects, cerebellar degeneration, and hepatocarcinomas. *J Biol Chem*, *284*(48), 33549-33560. doi:10.1074/jbc.M109.031971
- Ishikawa, M., Saito, K., Urata, M., Kumagai, Y., Maekawa, K., & Saito, Y. (2015). Comparison of circulating lipid profiles between fasting humans and three

animal species used in preclinical studies: mice, rats and rabbits. *Lipids in Health and Disease*, 14(1), 104. doi:10.1186/s12944-015-0104-4

Ivanescu, A. E., Li, P., George, B., Brown, A. W., Keith, S. W., Raju, D., & Allison, D. B. (2016). The importance of prediction model validation and assessment in obesity and nutrition research. *Int J Obes (Lond)*, 40(6), 887-894. doi:10.1038/ijo.2015.214

Iverson, S. J., Lang, S. L. C., & Cooper, M. H. (2001). Comparison of the bligh and dyer and folch methods for total lipid determination in a broad range of marine tissue. *Lipids*, 36(11), 1283-1287. doi:10.1007/s11745-001-0843-0

Jana, A., Hogan, E. L., & Pahan, K. (2009). Ceramide and neurodegeneration: susceptibility of neurons and oligodendrocytes to cell damage and death. *J Neurol Sci*, 278(1-2), 5-15. doi:10.1016/j.jns.2008.12.010

Jicha, G. A., & Markesbery, W. R. (2010). Omega-3 fatty acids: potential role in the management of early Alzheimer's disease. *Clin Interv Aging*, 5, 45-61.

Jumpertz, R., Guijarro, A., Pratley, R. E., Mason, C. C., Piomelli, D., & Krakoff, J. (2012). Associations of fatty acids in cerebrospinal fluid with peripheral glucose concentrations and energy metabolism. *PLoS One*, 7(7), e41503. doi:10.1371/journal.pone.0041503

Kaduk, M., Hoefsloot, H. C., Vis, D. J., Reijmers, T., van der Greef, J., Smilde, A. K., & Hendriks, M. M. (2014). Correlated measurement error hampers association network inference. *J Chromatogr B Analyt Technol Biomed Life Sci*, 966, 93-99. doi:10.1016/j.jchromb.2014.04.048

Kalmijn, S., Launer, L. J., Ott, A., Witteman, J. C., Hofman, A., & Breteler, M. M. (1997). Dietary fat intake and the risk of incident dementia in the Rotterdam Study. *Ann Neurol*, 42(5), 776-782. doi:10.1002/ana.410420514

- Kamleh, M. A., Ebbels, T. M. D., Spagou, K., Masson, P., & Want, E. J. (2012). Optimizing the Use of Quality Control Samples for Signal Drift Correction in Large-Scale Urine Metabolic Profiling Studies. *Analytical Chemistry*, *84*(6), 2670-2677. doi:10.1021/ac202733q
- Kamp, F., Zakim, D., Zhang, F., Noy, N., & Hamilton, J. A. (1995). Fatty acid flip-flop in phospholipid bilayers is extremely fast. *Biochemistry*, *34*(37), 11928-11937.
- Kangas, L. J., Metz, T. O., Isaac, G., Schrom, B. T., Ginovska-Pangovska, B., Wang, L., . . . Miller, J. H. (2012). In silico identification software (ISIS): a machine learning approach to tandem mass spectral identification of lipids. *Bioinformatics*, *28*(13), 1705-1713. doi:10.1093/bioinformatics/bts194
- Kanoski, S. E., & Davidson, T. L. (2011). Western diet consumption and cognitive impairment: links to hippocampal dysfunction and obesity. *Physiol Behav*, *103*(1), 59-68. doi:10.1016/j.physbeh.2010.12.003
- Karmi, A., Iozzo, P., Viljanen, A., Hirvonen, J., Fielding, B. A., Virtanen, K., . . . Nuutila, P. (2010). Increased Brain Fatty Acid Uptake in Metabolic Syndrome. *Diabetes*, *59*(9), 2171-2177. doi:10.2337/db09-0138
- Katsel, P., Li, C., & Haroutunian, V. (2007). Gene Expression Alterations in the Sphingolipid Metabolism Pathways during Progression of Dementia and Alzheimer's Disease: A Shift Toward Ceramide Accumulation at the Earliest Recognizable Stages of Alzheimer's Disease? *Neurochemical Research*, *32*(4), 845-856. doi:10.1007/s11064-007-9297-x
- Kawakita, E., Hashimoto, M., & Shido, O. (2006). Docosahexaenoic acid promotes neurogenesis in vitro and in vivo. *Neuroscience*, *139*(3), 991-997. doi:10.1016/j.neuroscience.2006.01.021
- Kennedy, A., Martinez, K., Chuang, C.-C., LaPoint, K., & McIntosh, M. (2009). Saturated Fatty Acid-Mediated Inflammation and Insulin Resistance in

Adipose Tissue: Mechanisms of Action and Implications. *The Journal of Nutrition*, 139(1), 1-4. doi:10.3945/jn.108.098269

Khoury, S., El Banna, N., Tfaili, S., & Chaminade, P. (2016). A study of inter-species ion suppression in electrospray ionization-mass spectrometry of some phospholipid classes. *Anal Bioanal Chem*, 408(5), 1453-1465. doi:10.1007/s00216-015-9245-6

Kim, H.-Y., Wang, T.-C. L., & Ma, Y.-C. (1994). Liquid Chromatography/Mass Spectrometry of Phospholipids using Electrospray Ionization. *Analytical Chemistry*, 66(22), 3977-3982. doi:10.1021/ac00094a020

Kim, H. W., Rao, J. S., Rapoport, S. I., & Igarashi, M. (2011). Regulation of rat brain polyunsaturated fatty acid (PUFA) metabolism during graded dietary n-3 PUFA deprivation. *Prostaglandins Leukot Essent Fatty Acids*, 85(6), 361-368. doi:10.1016/j.plefa.2011.08.002

Kim, H. Y. (2014). Neuroprotection by docosahexaenoic acid in brain injury. *Mil Med*, 179(11 Suppl), 106-111. doi:10.7205/MILMED-D-14-00162

Kind, T., Liu, K.-H., Lee, D. Y., DeFelice, B., Meissen, J. K., & Fiehn, O. (2013). LipidBlast in silico tandem mass spectrometry database for lipid identification. *Nat Meth*, 10(8), 755-758. doi:10.1038/nmeth.2551

<http://www.nature.com/nmeth/journal/v10/n8/abs/nmeth.2551.html#supplementary-information>

Kind, T., Wohlgemuth, G., Lee, D. Y., Lu, Y., Palazoglu, M., Shahbaz, S., & Fiehn, O. (2009). FiehnLib: Mass Spectral and Retention Index Libraries for Metabolomics Based on Quadrupole and Time-of-Flight Gas Chromatography/Mass Spectrometry. *Analytical Chemistry*, 81(24), 10038-10048. doi:10.1021/ac9019522

- Kitatani, K., Idkowiak-Baldys, J., & Hannun, Y. A. (2008). The sphingolipid salvage pathway in ceramide metabolism and signaling. *Cellular signalling*, 20(6), 1010-1018. doi:10.1016/j.cellsig.2007.12.006
- Kitteringham, N. R., Jenkins, R. E., Lane, C. S., Elliott, V. L., & Park, B. K. (2009). Multiple reaction monitoring for quantitative biomarker analysis in proteomics and metabolomics. *Journal of Chromatography B*, 877(13), 1229-1239. doi:http://dx.doi.org/10.1016/j.jchromb.2008.11.013
- Kluepfel, D., Bagli, J., Baker, H., Charest, M. P., & Kudelski, A. (1972). Myriocin, a new antifungal antibiotic from *Myriococcum albomyces*. *J Antibiot (Tokyo)*, 25(2), 109-115.
- Kofeler, H. C., Fauland, A., Rechberger, G. N., & Trotsmuller, M. (2012). Mass spectrometry based lipidomics: an overview of technological platforms. *Metabolites*, 2(1), 19-38. doi:10.3390/metabo2010019
- Koivusalo, M., Haimi, P., Heikinheimo, L., Kostianen, R., & Somerharju, P. (2001). Quantitative determination of phospholipid compositions by ESI-MS: effects of acyl chain length, unsaturation, and lipid concentration on instrument response. *J Lipid Res*, 42(4), 663-672.
- Kou, J., Kovacs, G. G., Hoftberger, R., Kulik, W., Brodde, A., Forss-Petter, S., . . . Berger, J. (2011). Peroxisomal alterations in Alzheimer's disease. *Acta Neuropathol*, 122(3), 271-283. doi:10.1007/s00401-011-0836-9
- Koudinov, A. R., & Koudinova, N. V. (2001). Essential role for cholesterol in synaptic plasticity and neuronal degeneration. *FASEB J*, 15(10), 1858-1860.
- Kramer, J. H., Rosen, H. J., Du, A. T., Schuff, N., Hollnagel, C., Weiner, M. W., . . . Delis, D. C. (2005). Dissociations in hippocampal and frontal contributions to episodic memory performance. *Neuropsychology*, 19(6), 799-805. doi:10.1037/0894-4105.19.6.7999

- Kruskal, W. H., & Wallis, W. A. (1952). Use of ranks in one-criterion variance analysis. *Journal of the American Statistical Association*, 47(260), 583-621.
- Krzywinski, M., & Altman, N. (2014). Points of significance: Comparing samples[mdash]part II. *Nat Meth*, 11(4), 355-356. doi:10.1038/nmeth.2900
- <http://www.nature.com/nmeth/journal/v11/n4/abs/nmeth.2900.html#supplementary-information>
- Kuczynski, B., & Reo, N. V. (2006). Evidence that plasmalogen is protective against oxidative stress in the rat brain. *Neurochem Res*, 31(5), 639-656. doi:10.1007/s11064-006-9061-7
- Kuebler, W. M., Yang, Y., Samapati, R., & Uhlig, S. (2010). Vascular barrier regulation by PAF, ceramide, caveolae, and NO - an intricate signaling network with discrepant effects in the pulmonary and systemic vasculature. *Cell Physiol Biochem*, 26(1), 29-40. doi:10.1159/000315103
- Kuratko, C. N., Barrett, E. C., Nelson, E. B., & Salem, N., Jr. (2013). The relationship of docosahexaenoic acid (DHA) with learning and behavior in healthy children: a review. *Nutrients*, 5(7), 2777-2810. doi:10.3390/nu5072777
- Kurek, K., Piotrowska, D. M., Wiesiolek-Kurek, P., Lukaszuk, B., Chabowski, A., Gorski, J., & Zendzian-Piotrowska, M. (2014). Inhibition of ceramide de novo synthesis reduces liver lipid accumulation in rats with nonalcoholic fatty liver disease. *Liver Int*, 34(7), 1074-1083. doi:10.1111/liv.12331
- Kvalheim, O. M., & Karstang, T. V. (1989). Interpretation of latent-variable regression models. *Chemometrics and Intelligent Laboratory Systems*, 7(1), 39-51. doi:http://dx.doi.org/10.1016/0169-7439(89)80110-8
- Labrousse, V. F., Nadjar, A., Joffre, C., Costes, L., Aubert, A., Gregoire, S., . . . Laye, S. (2012). Short-term long chain omega3 diet protects from

neuroinflammatory processes and memory impairment in aged mice. *PLoS One*, 7(5), e36861. doi:10.1371/journal.pone.0036861

Lagarde, M., Bernoud, N., Brossard, N., Lemaitre-Delaunay, D., Thies, F., Croset, M., & Lecerf, J. (2001). Lysophosphatidylcholine as a preferred carrier form of docosahexaenoic acid to the brain. *J Mol Neurosci*, 16(2-3), 201-204; discussion 215-221. doi:10.1385/JMN:16:2-3:201

Laitinen, M. H., Ngandu, T., Rovio, S., Helkala, E. L., Uusitalo, U., Viitanen, M., . . . Kivipelto, M. (2006). Fat intake at midlife and risk of dementia and Alzheimer's disease: a population-based study. *Dement Geriatr Cogn Disord*, 22(1), 99-107. doi:10.1159/000093478

Lam, S. M., Tian, H., & Shui, G. (2017). Lipidomics, en route to accurate quantitation. *Biochim Biophys Acta*. doi:10.1016/j.bbaliip.2017.02.008

Lam, S. M., Wang, Y., Duan, X., Wenk, M. R., Kalaria, R. N., Chen, C. P., . . . Shui, G. (2014). Brain lipidomes of subcortical ischemic vascular dementia and mixed dementia. *Neurobiol Aging*, 35(10), 2369-2381. doi:10.1016/j.neurobiolaging.2014.02.025

Lam, V., Albrecht, M. A., Takechi, R., Giles, C., James, A. P., Foster, J. K., & Mamo, J. C. (2013). The Serum Concentration of the Calcium Binding Protein S100B is Positively Associated with Cognitive Performance in Older Adults. *Front Aging Neurosci*, 5, 61. doi:10.3389/fnagi.2013.00061

Lam, V., Takechi, R., Pallabage-Gamarallage, M., Giles, C., & Mamo, J. C. (2015). The vitamin D, ionised calcium and parathyroid hormone axis of cerebral capillary function: therapeutic considerations for vascular-based neurodegenerative disorders. *PLoS One*, 10(4), e0125504. doi:10.1371/journal.pone.0125504

- Le Cao, K. A., Gonzalez, I., & Dejean, S. (2015). mixOmics: Omics Data Integration Project (Version R package version 5.2.0). Retrieved from <https://CRAN.R-project.org/package=mixOmics>
- Le Cessie, S., & Van Houwelingen, J. C. (1992). Ridge Estimators in Logistic Regression. *Journal of the Royal Statistical Society. Series C (Applied Statistics)*, 41(1), 191-201. doi:10.2307/2347628
- Leamy, A. K., Egnatchik, R. A., & Young, J. D. (2013). Molecular Mechanisms and the Role of Saturated Fatty Acids in the Progression of Non-Alcoholic Fatty Liver Disease. *Progress in Lipid Research*, 52(1), 10.1016/j.plipres.2012.1010.1004. doi:10.1016/j.plipres.2012.10.004
- Lee, C. H., & Hajra, A. K. (1991). Molecular species of diacylglycerols and phosphoglycerides and the postmortem changes in the molecular species of diacylglycerols in rat brains. *J Neurochem*, 56(2), 370-379.
- Lee, D. Y., Kind, T., Yoon, Y.-R., Fiehn, O., & Liu, K.-H. (2014). Comparative evaluation of extraction methods for simultaneous mass-spectrometric analysis of complex lipids and primary metabolites from human blood plasma. *Analytical and Bioanalytical Chemistry*, 406(28), 7275-7286. doi:10.1007/s00216-014-8124-x
- Lepinay, A. L., Larrieu, T., Joffre, C., Acar, N., Garate, I., Castanon, N., . . . Darnaudery, M. (2015). Perinatal high-fat diet increases hippocampal vulnerability to the adverse effects of subsequent high-fat feeding. *Psychoneuroendocrinology*, 53, 82-93. doi:10.1016/j.psyneuen.2014.12.008
- Levene, H. (1960). Robust tests for equality of variances.
- Levy, M., & Futerman, A. H. (2010). Mammalian ceramide synthases. *IUBMB Life*, 62(5), 347-356. doi:10.1002/iub.319

- Li, D., Misialek, J. R., Boerwinkle, E., Gottesman, R. F., Sharrett, A. R., Mosley, T. H., . . . Alonso, A. (2017). Prospective associations of plasma phospholipids and mild cognitive impairment/dementia among African Americans in the ARIC Neurocognitive Study. *Alzheimers Dement (Amst)*, *6*, 1-10. doi:10.1016/j.dadm.2016.09.003
- Li, H., Junk, P., Huwiler, A., Burkhardt, C., Wallerath, T., Pfeilschifter, J., & Förstermann, U. (2002). Dual Effect of Ceramide on Human Endothelial Cells. *Induction of Oxidative Stress and Transcriptional Upregulation of Endothelial Nitric Oxide Synthase*, *106*(17), 2250-2256. doi:10.1161/01.cir.0000035650.05921.50
- Libby, A. E., Wang, H., Mittal, R., Sungelo, M., Potma, E., & Eckel, R. H. (2015). Lipoprotein lipase is an important modulator of lipid uptake and storage in hypothalamic neurons. *Biochem Biophys Res Commun*, *465*(2), 287-292. doi:10.1016/j.bbrc.2015.08.026
- Liebisch, G., Lieser, B., Rathenberg, J., Drobnik, W., & Schmitz, G. (2004). High-throughput quantification of phosphatidylcholine and sphingomyelin by electrospray ionization tandem mass spectrometry coupled with isotope correction algorithm. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, *1686*(1-2), 108-117. doi:https://doi.org/10.1016/j.bbali.2004.09.003
- Lightle, S., Tosheva, R., Lee, A., Queen-Baker, J., Boyanovsky, B., Shedlofsky, S., & Nikolova-Karakashian, M. (2003). Elevation of ceramide in serum lipoproteins during acute phase response in humans and mice: role of serine-palmitoyl transferase. *Archives of Biochemistry and Biophysics*, *419*(2), 120-128. doi:http://dx.doi.org/10.1016/j.abb.2003.08.031
- Lilliefors, H. W. (1967). On the Kolmogorov-Smirnov Test for Normality with Mean and Variance Unknown. *Journal of the American Statistical Association*, *62*(318), 399-402. doi:10.1080/01621459.1967.10482916

- Lim, S. Y., Hoshiba, J., & Salem, N., Jr. (2005). An extraordinary degree of structural specificity is required in neural phospholipids for optimal brain function: n-6 docosapentaenoic acid substitution for docosahexaenoic acid leads to a loss in spatial task performance. *J Neurochem*, *95*(3), 848-857. doi:10.1111/j.1471-4159.2005.03427.x
- Lim, S. Y., & Suzuki, H. (2000). Effect of dietary docosahexaenoic acid and phosphatidylcholine on maze behavior and fatty acid composition of plasma and brain lipids in mice. *Int J Vitam Nutr Res*, *70*(5), 251-259. doi:10.1024/0300-9831.70.5.251
- Lin, L. E., Chen, C. T., Hildebrand, K. D., Liu, Z., Hopperton, K. E., & Bazinet, R. P. (2015). Chronic dietary n-6 PUFA deprivation leads to conservation of arachidonic acid and more rapid loss of DHA in rat brain phospholipids. *J Lipid Res*, *56*(2), 390-402. doi:10.1194/jlr.M055590
- Lin, P. Y., Chiu, C. C., Huang, S. Y., & Su, K. P. (2012). A meta-analytic review of polyunsaturated fatty acid compositions in dementia. *J Clin Psychiatry*, *73*(9), 1245-1254. doi:10.4088/JCP.11r07546
- Liu, Z., Rochfort, S., & Cocks, B. G. (2016). Optimization of a single phase method for lipid extraction from milk. *Journal of Chromatography A*, *1458*, 145-149. doi:http://dx.doi.org/10.1016/j.chroma.2016.06.055
- Lofgren, L., Stahlman, M., Forsberg, G. B., Saarinen, S., Nilsson, R., & Hansson, G. I. (2012). The BUME method: a novel automated chloroform-free 96-well total lipid extraction method for blood plasma. *J Lipid Res*, *53*(8), 1690-1700. doi:10.1194/jlr.D023036
- Lossinsky, A. S., Vorbrodt, A. W., & Wisniewski, H. M. (1983). Ultracytochemical studies of vesicular and canalicular transport structures in the injured mammalian blood-brain barrier. *Acta Neuropathologica*, *61*(3), 239-245. doi:10.1007/bf00691992

- Lukiw, W. J., Cui, J. G., Marcheselli, V. L., Bodker, M., Botkjaer, A., Gotlinger, K., . . . Bazan, N. G. (2005). A role for docosahexaenoic acid-derived neuroprotectin D1 in neural cell survival and Alzheimer disease. *J Clin Invest*, *115*(10), 2774-2783. doi:10.1172/JCI25420
- Lyn-Cook, L. E., Jr., Lawton, M., Tong, M., Silbermann, E., Longato, L., Jiao, P., . . . de la Monte, S. M. (2009a). Hepatic ceramide may mediate brain insulin resistance and neurodegeneration in type 2 diabetes and non-alcoholic steatohepatitis. *J Alzheimers Dis*, *16*(4), 715-729. doi:10.3233/JAD-2009-0984
- Lyn-Cook, L. E., Lawton, M., Tong, M., Silbermann, E., Longato, L., Jiao, P., . . . de la Monte, S. M. (2009b). Hepatic Ceramide May Mediate Brain Insulin Resistance and Neurodegeneration in Type 2 Diabetes and Non-alcoholic Steatohepatitis. *Journal of Alzheimers Disease*, *16*(4), 715-729. doi:10.3233/Jad-2009-0984
- Macala, L. J., Yu, R. K., & Ando, S. (1983). Analysis of brain lipids by high performance thin-layer chromatography and densitometry. *J Lipid Res*, *24*(9), 1243-1250.
- Mamo, J., & Proctor, S. (1999). Chylomicron remnants and atherosclerosis. *Plasma lipids and their role in disease*. Harwood Academic Publishers, 109-137.
- Mamo, J. C., Lam, V., Giles, C., Coulson, S. H., Fimognari, N., Mooranian, A., . . . Takechi, R. (2017). Antihypertensive agents do not prevent blood-brain barrier dysfunction and cognitive deficits in dietary-induced obese mice. *Int J Obes (Lond)*. doi:10.1038/ijo.2017.57
- Mann, H. B., & Whitney, D. R. (1947). On a Test of Whether one of Two Random Variables is Stochastically Larger than the Other. *The Annals of Mathematical Statistics*, *18*(1), 50-60.

- Mapstone, M., Cheema, A. K., Fiandaca, M. S., Zhong, X., Mhyre, T. R., MacArthur, L. H., . . . Federoff, H. J. (2014). Plasma phospholipids identify antecedent memory impairment in older adults. *Nat Med*, *20*(4), 415-418. doi:10.1038/nm.3466
- Martin-Nizard, F., Meresse, S., Cecchelli, R., Fruchart, J. C., & Delbart, C. (1989). Interactions of high-density lipoprotein 3 with brain capillary endothelial cells. *Biochim Biophys Acta*, *1005*(3), 201-208.
- Martinez de Morentin, P. B., Varela, L., Ferno, J., Nogueiras, R., Dieguez, C., & Lopez, M. (2010). Hypothalamic lipotoxicity and the metabolic syndrome. *Biochim Biophys Acta*, *1801*(3), 350-361. doi:10.1016/j.bbaliip.2009.09.016
- Marwarha, G., Claycombe, K., Schommer, J., Collins, D., & Ghribi, O. (2016). Palmitate-induced Endoplasmic Reticulum stress and subsequent C/EBPalpha Homologous Protein activation attenuates leptin and Insulin-like growth factor 1 expression in the brain. *Cell Signal*, *28*(11), 1789-1805. doi:10.1016/j.cellsig.2016.08.012
- Mattson, F. H., & Grundy, S. M. (1985). Comparison of effects of dietary saturated, monounsaturated, and polyunsaturated fatty acids on plasma lipids and lipoproteins in man. *Journal of Lipid Research*, *26*(2), 194-202.
- Matyash, V., Liebisch, G., Kurzchalia, T. V., Shevchenko, A., & Schwudke, D. (2008). Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *J Lipid Res*, *49*(5), 1137-1146. doi:10.1194/jlr.D700041-JLR200
- Mayer, C. M., & Belsham, D. D. (2010). Palmitate attenuates insulin signaling and induces endoplasmic reticulum stress and apoptosis in hypothalamic neurons: rescue of resistance and apoptosis through adenosine 5' monophosphate-activated protein kinase activation. *Endocrinology*, *151*(2), 576-585. doi:10.1210/en.2009-1122

- McNay, E. C., Ong, C. T., McCrimmon, R. J., Cresswell, J., Bogan, J. S., & Sherwin, R. S. (2010). Hippocampal memory processes are modulated by insulin and high-fat-induced insulin resistance. *Neurobiol Learn Mem*, 93(4), 546-553. doi:10.1016/j.nlm.2010.02.002
- Mehmood, T., Liland, K. H., Snipen, L., & Sæbø, S. (2012). A review of variable selection methods in Partial Least Squares Regression. *Chemometrics and Intelligent Laboratory Systems*, 118, 62-69. doi:http://dx.doi.org/10.1016/j.chemolab.2012.07.010
- Mehra, V. C., Jackson, E., Zhang, X. M., Jiang, X.-C., Dobrucki, L. W., Yu, J., . . . Bender, J. R. (2014). Ceramide-Activated Phosphatase Mediates Fatty Acid-Induced Endothelial VEGF Resistance and Impaired Angiogenesis. *The American Journal of Pathology*, 184(5), 1562-1576. doi:http://dx.doi.org/10.1016/j.ajpath.2014.01.009
- Meija, J., Coplen, T. B., Berglund, M., Brand, W. A., De Bièvre, P., Gröning, M., . . . Prohaska, T. (2016). Isotopic compositions of the elements 2013 (IUPAC Technical Report). *Pure and Applied Chemistry*, 88(3), 293-306. doi:10.1515/pac-2015-0503
- Meikle, P. J., & Summers, S. A. (2017). Sphingolipids and phospholipids in insulin resistance and related metabolic disorders. *Nat Rev Endocrinol*, 13(2), 79-91. doi:10.1038/nrendo.2016.169
- Meikle, P. J., Wong, G., Barlow, C. K., Weir, J. M., Greeve, M. A., MacIntosh, G. L., . . . Shaw, J. (2013). Plasma lipid profiling shows similar associations with prediabetes and type 2 diabetes. *PLoS One*, 8(9), e74341. doi:10.1371/journal.pone.0074341
- Meikle, P. J., Wong, G., Tsorotes, D., Barlow, C. K., Weir, J. M., Christopher, M. J., . . . Kingwell, B. A. (2011). Plasma Lipidomic Analysis of Stable and Unstable Coronary Artery Disease. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 31(11), 2723-2732. doi:10.1161/atvbaha.111.234096

- Mencarelli, C., & Martinez-Martinez, P. (2013). Ceramide function in the brain: when a slight tilt is enough. *Cell Mol Life Sci*, 70(2), 181-203. doi:10.1007/s00018-012-1038-x
- Mencarelli, C., & Martinez-Martinez, P. (2013). Ceramide function in the brain: when a slight tilt is enough. *Cellular and Molecular Life Sciences*, 70(2), 181-203. doi:10.1007/s00018-012-1038-x
- Merched, A., Xia, Y., Visvikis, S., Serot, J. M., & Siest, G. (2000). Decreased high-density lipoprotein cholesterol and serum apolipoprotein AI concentrations are highly correlated with the severity of Alzheimer's disease☆. *Neurobiology of Aging*, 21(1), 27-30. doi:http://dx.doi.org/10.1016/S0197-4580(99)00103-7
- Meresse, S., Delbart, C., Fruchart, J. C., & Cecchelli, R. (1989). Low-density lipoprotein receptor on endothelium of brain capillaries. *J Neurochem*, 53(2), 340-345.
- Merlin, J. F., Gresti, J., Bellenger, S., & Narce, M. (2006). Fast high performance liquid chromatography analysis in lipidomics: Separation of radiolabelled fatty acids and phosphatidylcholine molecular species using a monolithic C18 silica column. *Analytica Chimica Acta*, 565(2), 163-167. doi:http://dx.doi.org/10.1016/j.aca.2006.02.037
- Merrill, A. H., Jr., Sullards, M. C., Allegood, J. C., Kelly, S., & Wang, E. (2005). Sphingolipidomics: high-throughput, structure-specific, and quantitative analysis of sphingolipids by liquid chromatography tandem mass spectrometry. *Methods*, 36(2), 207-224. doi:10.1016/j.ymeth.2005.01.009
- Merrill, A. H., Lingrell, S., Wang, E., Nikolova-Karakashian, M., Vales, T. R., & Vance, D. E. (1995). Sphingolipid Biosynthesis de Novo by Rat Hepatocytes in Culture.: CERAMIDE AND SPHINGOMYELIN ARE ASSOCIATED WITH, BUT NOT REQUIRED FOR, VERY LOW DENSITY

LIPOPROTEIN SECRETION. *Journal of Biological Chemistry*, 270(23), 13834-13841. doi:10.1074/jbc.270.23.13834

Messamore, E., & McNamara, R. K. (2016). Detection and treatment of omega-3 fatty acid deficiency in psychiatric practice: Rationale and implementation. *Lipids Health Dis*, 15, 25. doi:10.1186/s12944-016-0196-5

Miao, H., Chen, H., Pei, S., Bai, X., Vaziri, N. D., & Zhao, Y.-Y. (2015). Plasma lipidomics reveal profound perturbation of glycerophospholipids, fatty acids, and sphingolipids in diet-induced hyperlipidemia. *Chemico-Biological Interactions*, 228, 79-87. doi:http://dx.doi.org/10.1016/j.cbi.2015.01.023

Michalski, A., Damoc, E., Hauschild, J.-P., Lange, O., Wieghaus, A., Makarov, A., . . . Horning, S. (2011). Mass Spectrometry-based Proteomics Using Q Exactive, a High-performance Benchtop Quadrupole Orbitrap Mass Spectrometer. *Molecular & Cellular Proteomics*, 10(9). doi:10.1074/mcp.M111.011015

Mielke, M. M., Bandaru, V. V., Haughey, N. J., Rabins, P. V., Lyketsos, C. G., & Carlson, M. C. (2010). Serum sphingomyelins and ceramides are early predictors of memory impairment. *Neurobiol Aging*, 31(1), 17-24. doi:10.1016/j.neurobiolaging.2008.03.011

Mielke, M. M., Bandaru, V. V., Haughey, N. J., Xia, J., Fried, L. P., Yasar, S., . . . Carlson, M. C. (2012). Serum ceramides increase the risk of Alzheimer disease: the Women's Health and Aging Study II. *Neurology*, 79(7), 633-641. doi:10.1212/WNL.0b013e318264e380

Mielke, M. M., Haughey, N. J., Bandaru, V. V., Schech, S., Carrick, R., Carlson, M. C., . . . Lyketsos, C. G. (2010). Plasma ceramides are altered in mild cognitive impairment and predict cognitive decline and hippocampal volume loss. *Alzheimers Dement*, 6(5), 378-385. doi:10.1016/j.jalz.2010.03.014

- Mielke, M. M., Haughey, N. J., Bandaru, V. V., Weinberg, D. D., Darby, E., Zaidi, N., . . . Lyketsos, C. G. (2011). Plasma sphingomyelins are associated with cognitive progression in Alzheimer's disease. *J Alzheimers Dis*, *27*(2), 259-269. doi:10.3233/JAD-2011-110405
- Mielke, M. M., Haughey, N. J., Bandaru, V. V., Zetterberg, H., Blennow, K., Andreasson, U., . . . Carlsson, C. M. (2014). Cerebrospinal fluid sphingolipids, beta-amyloid, and tau in adults at risk for Alzheimer's disease. *Neurobiol Aging*, *35*(11), 2486-2494. doi:10.1016/j.neurobiolaging.2014.05.019
- Mielke, M. M., Haughey, N. J., Han, D., An, Y., Bandaru, V. V., Lyketsos, C. G., . . . Resnick, S. M. (2016). The Association Between Plasma Ceramides and Sphingomyelins and Risk of Alzheimer's Disease Differs by Sex and APOE in the Baltimore Longitudinal Study of Aging. *J Alzheimers Dis*. doi:10.3233/JAD-160925
- Mielke, M. M., & Lyketsos, C. G. (2010). Alterations of the sphingolipid pathway in Alzheimer's disease: new biomarkers and treatment targets? *Neuromolecular Med*, *12*(4), 331-340. doi:10.1007/s12017-010-8121-y
- Mielke, M. M., Maetzler, W., Haughey, N. J., Bandaru, V. V., Savica, R., Deuschle, C., . . . Liepelt-Scarfone, I. (2013). Plasma ceramide and glucosylceramide metabolism is altered in sporadic Parkinson's disease and associated with cognitive impairment: a pilot study. *PLoS One*, *8*(9), e73094. doi:10.1371/journal.pone.0073094
- Milanski, M., Degasperi, G., Coope, A., Morari, J., Denis, R., Cintra, D. E., . . . Velloso, L. A. (2009). Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: implications for the pathogenesis of obesity. *J Neurosci*, *29*(2), 359-370. doi:10.1523/JNEUROSCI.2760-08.2009

- Mitchell, R. W., & Hatch, G. M. (2011). Fatty acid transport into the brain: of fatty acid fables and lipid tails. *Prostaglandins Leukot Essent Fatty Acids*, 85(5), 293-302. doi:10.1016/j.plefa.2011.04.007
- Mitchell, R. W., On, N. H., Del Bigio, M. R., Miller, D. W., & Hatch, G. M. (2011). Fatty acid transport protein expression in human brain and potential role in fatty acid transport across human brain microvessel endothelial cells. *J Neurochem*, 117(4), 735-746. doi:10.1111/j.1471-4159.2011.07245.x
- Moraes, J. C., Coope, A., Morari, J., Cintra, D. E., Roman, E. A., Pauli, J. R., . . . Velloso, L. A. (2009). High-fat diet induces apoptosis of hypothalamic neurons. *PLoS One*, 4(4), e5045. doi:10.1371/journal.pone.0005045
- Moriguchi, T., Harauma, A., & Salem, N., Jr. (2013). Plasticity of mouse brain docosahexaenoic acid: modulation by diet and age. *Lipids*, 48(4), 343-355. doi:10.1007/s11745-013-3775-5
- Moriguchi, T., & Salem, N., Jr. (2003). Recovery of brain docosahexaenoate leads to recovery of spatial task performance. *J Neurochem*, 87(2), 297-309.
- Morita, S.-y., Kawabe, M., Sakurai, A., Okuhira, K., Vertut-Doi, A., Nakano, M., & Handa, T. (2004). Ceramide in Lipid Particles Enhances Heparan Sulfate Proteoglycan and Low Density Lipoprotein Receptor-related Protein-mediated Uptake by Macrophages. *Journal of Biological Chemistry*, 279(23), 24355-24361. doi:10.1074/jbc.M402035200
- Morris, M. C., Evans, D. A., Tangney, C. C., Bienias, J. L., Schneider, J. A., Wilson, R. S., & Scherr, P. A. (2006). Dietary copper and high saturated and trans fat intakes associated with cognitive decline. *Arch Neurol*, 63(8), 1085-1088. doi:10.1001/archneur.63.8.1085
- Morris, M. C., & Tangney, C. C. (2014). Dietary fat composition and dementia risk. *Neurobiol Aging*, 35 Suppl 2, S59-64. doi:10.1016/j.neurobiolaging.2014.03.038

- Moyano, A. L., Pituch, K., Li, G., Breemen, R., Mansson, J. E., & Givogri, M. I. (2013). Levels of plasma sulfatides C18:0 and C24:1 correlate with disease status in relapsing-remitting multiple sclerosis. *J Neurochem*, 127. doi:10.1111/jnc.12341
- Muller, C. P., Reichel, M., Muhle, C., Rhein, C., Gulbins, E., & Kornhuber, J. (2015). Brain membrane lipids in major depression and anxiety disorders. *Biochim Biophys Acta*, 1851(8), 1052-1065. doi:10.1016/j.bbalip.2014.12.014
- Murphy, E. J., Stephens, R., Jurkowitz-Alexander, M., & Horrocks, L. A. (1993). Acidic hydrolysis of plasmalogens followed by high-performance liquid chromatography. *Lipids*, 28(6), 565-568.
- Nariai, T., DeGeorge, J. J., Greig, N. H., Genka, S., Rapoport, S. I., & Purdon, A. D. (1994). Differences in rates of incorporation of intravenously injected radiolabeled fatty acids into phospholipids of intracerebrally implanted tumor and brain in awake rats. *Clinical & Experimental Metastasis*, 12(3), 213-225. doi:10.1007/bf01753889
- Naudi, A., Cabre, R., Jove, M., Ayala, V., Gonzalo, H., Portero-Otin, M., . . . Pamplona, R. (2015). Lipidomics of human brain aging and Alzheimer's disease pathology. *Int Rev Neurobiol*, 122, 133-189. doi:10.1016/bs.irn.2015.05.008
- Neuringer, M., Anderson, G. J., & Connor, W. E. (1988). The essentiality of n-3 fatty acids for the development and function of the retina and brain. *Annu Rev Nutr*, 8, 517-541. doi:10.1146/annurev.nu.08.070188.002505
- Nguyen, L. N., Ma, D., Shui, G., Wong, P., Cazenave-Gassiot, A., Zhang, X., . . . Silver, D. L. (2014). Mfsd2a is a transporter for the essential omega-3 fatty acid docosahexaenoic acid. *Nature*, 509(7501), 503-506. doi:10.1038/nature13241

- Norton, W. T., & Poduslo, S. E. (1973). MYELINATION IN RAT BRAIN: CHANGES IN MYELIN COMPOSITION DURING BRAIN MATURATION¹. *Journal of Neurochemistry*, *21*(4), 759-773.
doi:10.1111/j.1471-4159.1973.tb07520.x
- O'Brien, J. S., & Sampson, E. L. (1965a). Fatty acid and fatty aldehyde composition of the major brain lipids in normal human gray matter, white matter, and myelin. *J Lipid Res*, *6*(4), 545-551.
- O'Brien, J. S., & Sampson, E. L. (1965b). Lipid composition of the normal human brain: gray matter, white matter, and myelin. *J Lipid Res*, *6*(4), 537-544.
- Ogiso, H., Suzuki, T., & Taguchi, R. (2008). Development of a reverse-phase liquid chromatography electrospray ionization mass spectrometry method for lipidomics, improving detection of phosphatidic acid and phosphatidylserine. *Analytical Biochemistry*, *375*(1), 124-131.
doi:http://dx.doi.org/10.1016/j.ab.2007.12.027
- Oh, H., Boghossian, S., York, D. A., & Park-York, M. (2013). The effect of high fat diet and saturated fatty acids on insulin signaling in the amygdala and hypothalamus of rats. *Brain Res*, *1537*, 191-200.
doi:10.1016/j.brainres.2013.09.025
- Olazaran, J., Gil-de-Gomez, L., Rodriguez-Martin, A., Valenti-Soler, M., Frades-Payo, B., Marin-Munoz, J., . . . Calero, M. (2015). A blood-based, 7-metabolite signature for the early diagnosis of Alzheimer's disease. *J Alzheimers Dis*, *45*(4), 1157-1173. doi:10.3233/JAD-142925
- Ottenlinger, F. M., Mayer, C. A., Ferreiros, N., Schreiber, Y., Schwiebs, A., Schmidt, K. G., . . . Radeke, H. H. (2016). Interferon-Beta Increases Plasma Ceramides of Specific Chain Length in Multiple Sclerosis Patients, Unlike Fingolimod or Natalizumab. *Front Pharmacol*, *7*, 412.
doi:10.3389/fphar.2016.00412

- Ouellet, M., Emond, V., Chen, C. T., Julien, C., Bourasset, F., Oddo, S., . . . Calon, F. (2009). Diffusion of docosahexaenoic and eicosapentaenoic acids through the blood-brain barrier: An in situ cerebral perfusion study. *Neurochem Int*, 55(7), 476-482. doi:10.1016/j.neuint.2009.04.018
- Pardridge, W. M., & Mietus, L. J. (1980). Palmitate and cholesterol transport through the blood-brain barrier. *J Neurochem*, 34(2), 463-466.
- Park, H. R., Park, M., Choi, J., Park, K. Y., Chung, H. Y., & Lee, J. (2010). A high-fat diet impairs neurogenesis: involvement of lipid peroxidation and brain-derived neurotrophic factor. *Neurosci Lett*, 482(3), 235-239. doi:10.1016/j.neulet.2010.07.046
- Park, J. W., Park, W. J., & Futerman, A. H. (2014). Ceramide synthases as potential targets for therapeutic intervention in human diseases. *Biochim Biophys Acta*, 1841(5), 671-681. doi:10.1016/j.bbalip.2013.08.019
- Park, K.-T., Shon, J. C., Kim, J.-E., Park, G. H., Choi, H. J., & Liu, K.-H. (2017). Sulfatides Primarily Exist in the Substantia Nigra Region of Mouse Brain Tissue. *Lipids*, 52(2), 179-187. doi:10.1007/s11745-016-4224-z
- Park, T.-S., Rosebury, W., Kindt, E. K., Kowala, M. C., & Panek, R. L. (2008). Serine palmitoyltransferase inhibitor myriocin induces the regression of atherosclerotic plaques in hyperlipidemic ApoE-deficient mice. *Pharmacological Research*, 58(1), 45-51. doi:http://dx.doi.org/10.1016/j.phrs.2008.06.005
- Patterson, R. E., Ducrocq, A. J., McDougall, D. J., Garrett, T. J., & Yost, R. A. (2015). Comparison of blood plasma sample preparation methods for combined LC-MS lipidomics and metabolomics. *J Chromatogr B Analyt Technol Biomed Life Sci*, 1002, 260-266. doi:10.1016/j.jchromb.2015.08.018
- Paumen, M. B., Ishida, Y., Muramatsu, M., Yamamoto, M., & Honjo, T. (1997). Inhibition of Carnitine Palmitoyltransferase I Augments Sphingolipid

Synthesis and Palmitate-induced Apoptosis. *Journal of Biological Chemistry*, 272(6), 3324-3329. doi:10.1074/jbc.272.6.3324

Phillips, M. A., Childs, C. E., Calder, P. C., & Rogers, P. J. (2015). No Effect of Omega-3 Fatty Acid Supplementation on Cognition and Mood in Individuals with Cognitive Impairment and Probable Alzheimer's Disease: A Randomised Controlled Trial. *International Journal of Molecular Sciences*, 16(10), 24600-24613. doi:10.3390/ijms161024600

Piomelli, D., Astarita, G., & Rapaka, R. (2007). A neuroscientist's guide to lipidomics. *Nat Rev Neurosci*, 8(10), 743-754. doi:10.1038/nrn2233

Pipatpiboon, N., Pintana, H., Prachayasakul, W., Chattipakorn, N., & Chattipakorn, S. C. (2013). DPP4-inhibitor improves neuronal insulin receptor function, brain mitochondrial function and cognitive function in rats with insulin resistance induced by high-fat diet consumption. *Eur J Neurosci*, 37(5), 839-849. doi:10.1111/ejn.12088

Pocai, A., Lam, T. K., Obici, S., Gutierrez-Juarez, R., Muse, E. D., Arduini, A., & Rossetti, L. (2006). Restoration of hypothalamic lipid sensing normalizes energy and glucose homeostasis in overfed rats. *J Clin Invest*, 116(4), 1081-1091. doi:10.1172/JCI26640

Polo, A., Kirschner, G., Guidotti, A., & Costa, E. (1994). Brain content of glycosphingolipids after oral administration of monosialogangliosides GM1 and LIGA20 to rats. *Mol Chem Neuropathol*, 21(1), 41-53.

Posey, K. A., Clegg, D. J., Printz, R. L., Byun, J., Morton, G. J., Vivekanandan-Giri, A., . . . Niswender, K. D. (2009). Hypothalamic proinflammatory lipid accumulation, inflammation, and insulin resistance in rats fed a high-fat diet. *Am J Physiol Endocrinol Metab*, 296(5), E1003-1012. doi:10.1152/ajpendo.90377.2008

- Povlishock, J. T., Kontos, H. A., Wei, E. P., Rosenblum, W. I., & Becker, D. P. (1980). Changes in the Cerebral Vasculature after Hypertension and Trauma: A Combined Scanning and Transmission Electron Microscopic Analysis. In H. M. Eisenberg & R. L. Suddith (Eds.), *The Cerebral Microvasculature: Investigation of the Blood—Brain Barrier* (pp. 227-241). Boston, MA: Springer US.
- Prager, B., Spampinato, S. F., & Ransohoff, R. M. (2015). Sphingosine 1-phosphate signaling at the blood-brain barrier. *Trends Mol Med*, *21*(6), 354-363. doi:10.1016/j.molmed.2015.03.006
- Proitsi, P., Kim, M., Whiley, L., Pritchard, M., Leung, R., Soininen, H., . . . Legido-Quigley, C. (2015). Plasma lipidomics analysis finds long chain cholesteryl esters to be associated with Alzheimer's disease. *Translational Psychiatry*, *5*(1), e494. doi:10.1038/tp.2014.127
- Proitsi, P., Kim, M., Whiley, L., Simmons, A., Sattlecker, M., Velayudhan, L., . . . Legido-Quigley, C. (2017). Association of blood lipids with Alzheimer's disease: A comprehensive lipidomics analysis. *Alzheimers Dement*, *13*(2), 140-151. doi:10.1016/j.jalz.2016.08.003
- Prospective Studies, C., Lewington, S., Whitlock, G., Clarke, R., Sherliker, P., Emberson, J., . . . Collins, R. (2007). Blood cholesterol and vascular mortality by age, sex, and blood pressure: a meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths. *Lancet*, *370*(9602), 1829-1839. doi:10.1016/S0140-6736(07)61778-4
- Pulfer, M., & Murphy, R. C. (2003). Electrospray mass spectrometry of phospholipids. *Mass Spectrom Rev*, *22*(5), 332-364. doi:10.1002/mas.10061
- Purdon, D., Arai, T., & Rapoport, S. (1997). No evidence for direct incorporation of esterified palmitic acid from plasma into brain lipids of awake adult rat. *J Lipid Res*, *38*(3), 526-530.

- Quarles, R., & Folch-Pi, J. (1965). SOME EFFECTS OF PHYSIOLOGICAL CATIONS ON THE BEHAVIOUR OF GANGLIOSIDES IN A CHLOROFORM-METHANOL-WATER BIPHASIC SYSTEM*. *Journal of Neurochemistry*, *12*(7), 543-553. doi:10.1111/j.1471-4159.1965.tb04247.x
- Quehenberger, O., Armando, A. M., Brown, A. H., Milne, S. B., Myers, D. S., Merrill, A. H., . . . Dennis, E. A. (2010). Lipidomics reveals a remarkable diversity of lipids in human plasma. *J Lipid Res*, *51*(11), 3299-3305. doi:10.1194/jlr.M009449
- Quinn, J. F., Raman, R., Thomas, R. G., Yurko-Mauro, K., Nelson, E. B., Van Dyck, C., . . . Aisen, P. S. (2010). Docosahexaenoic Acid Supplementation and Cognitive Decline in Alzheimer Disease: A Randomized Trial. *Jama*, *304*(17), 1903-1911. doi:10.1001/jama.2010.1510
- R Core Team. (2015). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from <https://www.R-project.org/>
- Rabiei, Z., Bigdeli, M. R., & Rasoulilian, B. (2013). Neuroprotection of dietary virgin olive oil on brain lipidomics during stroke. *Curr Neurovasc Res*, *10*(3), 231-237.
- Rader, D. J., Alexander, E. T., Weibel, G. L., Billheimer, J., & Rothblat, G. H. (2009). The role of reverse cholesterol transport in animals and humans and relationship to atherosclerosis. *J Lipid Res*, *50* Suppl, S189-194. doi:10.1194/jlr.R800088-JLR200
- Rahman, T., Taha, A. Y., Song, B. J., Orr, S. K., Liu, Z., Chen, C. T., & Bazinet, R. P. (2010). The very low density lipoprotein receptor is not necessary for maintaining brain polyunsaturated fatty acid concentrations. *Prostaglandins Leukot Essent Fatty Acids*, *82*(2-3), 141-145. doi:10.1016/j.plefa.2009.11.003

- Rapoport, S. I. (2001). In vivo fatty acid incorporation into brain phospholipids in relation to plasma availability, signal transduction and membrane remodeling. *Journal of Molecular Neuroscience*, *16*(2), 243-261. doi:10.1385/jmn:16:2-3:243
- Rapoport, S. I. (2008). Brain arachidonic and docosahexaenoic acid cascades are selectively altered by drugs, diet and disease. *Prostaglandins Leukot Essent Fatty Acids*, *79*(3-5), 153-156. doi:10.1016/j.plefa.2008.09.010
- Rapoport, S. I., Chang, M. C., & Spector, A. A. (2001). Delivery and turnover of plasma-derived essential PUFAs in mammalian brain. *J Lipid Res*, *42*(5), 678-685.
- Rapoport, S. I., & Igarashi, M. (2009). Can the rat liver maintain normal brain DHA metabolism in the absence of dietary DHA? *Prostaglandins Leukot Essent Fatty Acids*, *81*(2-3), 119-123. doi:10.1016/j.plefa.2009.05.021
- Rapoport, S. I., Purdon, D., Shetty, H. U., Grange, E., Smith, Q., Jones, C., & Chang, M. C. (1997). In vivo imaging of fatty acid incorporation into brain to examine signal transduction and neuroplasticity involving phospholipids. *Ann N Y Acad Sci*, *820*, 56-73; discussion 73-54.
- Rapoport, S. I., Ramadan, E., & Basselin, M. (2011). Docosahexaenoic acid (DHA) incorporation into the brain from plasma, as an in vivo biomarker of brain DHA metabolism and neurotransmission. *Prostaglandins Other Lipid Mediat*, *96*(1-4), 109-113. doi:10.1016/j.prostaglandins.2011.06.003
- Rapoport, S. I., Rao, J. S., & Igarashi, M. (2007). Brain metabolism of nutritionally essential polyunsaturated fatty acids depends on both the diet and the liver. *Prostaglandins Leukot Essent Fatty Acids*, *77*(5-6), 251-261. doi:10.1016/j.plefa.2007.10.023
- Rasmiena, A. A., Barlow, C. K., Stefanovic, N., Huynh, K., Tan, R., Sharma, A., . . . Meikle, P. J. (2015). Plasmalogen modulation attenuates atherosclerosis in

- ApoE- and ApoE/GPx1-deficient mice. *Atherosclerosis*, 243(2), 598-608.
doi:10.1016/j.atherosclerosis.2015.10.096
- Reis, A., Rudnitskaya, A., Blackburn, G. J., Mohd Fauzi, N., Pitt, A. R., & Spickett, C. M. (2013). A comparison of five lipid extraction solvent systems for lipidomic studies of human LDL. *J Lipid Res*, 54(7), 1812-1824.
doi:10.1194/jlr.M034330
- Retra, K., Bleijerveld, O. B., van Gestel, R. A., Tielens, A. G., van Hellemond, J. J., & Brouwers, J. F. (2008). A simple and universal method for the separation and identification of phospholipid molecular species. *Rapid Commun Mass Spectrom*, 22(12), 1853-1862. doi:10.1002/rcm.3562
- Rohrbough, J., & Broadie, K. (2005). Lipid regulation of the synaptic vesicle cycle. *Nat Rev Neurosci*, 6(2), 139-150. doi:10.1038/nrn1608
- Rolim, A. E., Henrique-Araujo, R., Ferraz, E. G., de Araujo Alves Dultra, F. K., & Fernandez, L. G. (2015). Lipidomics in the study of lipid metabolism: Current perspectives in the omic sciences. *Gene*, 554(2), 131-139.
doi:10.1016/j.gene.2014.10.039
- Rosqvist, F., Iggman, D., Kullberg, J., Cedernaes, J., Johansson, H. E., Larsson, A., . . . Riserus, U. (2014). Overfeeding polyunsaturated and saturated fat causes distinct effects on liver and visceral fat accumulation in humans. *Diabetes*, 63(7), 2356-2368. doi:10.2337/db13-1622
- Rubingh, C. M., Bijlsma, S., Derks, E. P., Bobeldijk, I., Verheij, E. R., Kochhar, S., & Smilde, A. K. (2006). Assessing the performance of statistical validation tools for megavariate metabolomics data. *Metabolomics*, 2(2), 53-61.
doi:10.1007/s11306-006-0022-6
- Saito, M., Chakraborty, G., Hegde, M., Ohsie, J., Paik, S. M., Vadasz, C., & Saito, M. (2010). Involvement of ceramide in ethanol-induced apoptotic

neurodegeneration in the neonatal mouse brain. *J Neurochem*, 115(1), 168-177. doi:10.1111/j.1471-4159.2010.06913.x

Santamarina-Fojo, S., Lambert, G., Hoeg, J. M., & Brewer, H. B., Jr. (2000).

Lecithin-cholesterol acyltransferase: role in lipoprotein metabolism, reverse cholesterol transport and atherosclerosis. *Curr Opin Lipidol*, 11(3), 267-275.

Sathishkumar, S., Boyanovsky, B., Karakashian, A. A., Rozenova, K., Giltiy, N. V.,

Kudrimoti, M., . . . Nikolova-Karakashian, M. (2005). Elevated sphingomyelinase activity and ceramide concentration in serum of patients undergoing high dose spatially fractionated radiation treatment: implications for endothelial apoptosis. *Cancer Biol Ther*, 4(9), 979-986.

Savica, R., Murray, M. E., Persson, X. M., Kantarci, K., Parisi, J. E., Dickson, D. W.,

. . . Mielke, M. M. (2016). Plasma sphingolipid changes with autopsy-confirmed Lewy Body or Alzheimer's pathology. *Alzheimers Dement (Amst)*, 3, 43-50. doi:10.1016/j.dadm.2016.02.005

Sawilowsky, S. (2005). *Misconceptions Leading to Choosing the t Test Over the*

Wilcoxon Mann-Whitney Test for Shift in Location Parameter (Vol. 4).

Schacht, J. (1981). [52] Extraction and purification of polyphosphoinositides.

Methods in Enzymology, 72, 626-631. doi:http://dx.doi.org/10.1016/S0076-6879(81)72054-8

Schaefer, E. J., Bongard, V., Beiser, A. S., Lamon-Fava, S., Robins, S. J., Au, R., . . .

Wolf, P. A. (2006). Plasma phosphatidylcholine docosahexaenoic acid content and risk of dementia and Alzheimer disease: the Framingham Heart Study. *Arch Neurol*, 63(11), 1545-1550. doi:10.1001/archneur.63.11.1545

Schiffmann, S., Birod, K., Mannich, J., Eberle, M., Wegner, M. S., Wanger, R., . . .

Grosch, S. (2013). Ceramide metabolism in mouse tissue. *Int J Biochem Cell Biol*, 45(8), 1886-1894. doi:10.1016/j.biocel.2013.06.004

- Schissel, S. L., Jiang, X.-c., Tweedie-Hardman, J., Jeong, T.-s., Camejo, E. H., Najib, J., . . . Tabas, I. (1998). Secretory Sphingomyelinase, a Product of the Acid Sphingomyelinase Gene, Can Hydrolyze Atherogenic Lipoproteins at Neutral pH: IMPLICATIONS FOR ATHEROSCLEROTIC LESION DEVELOPMENT. *Journal of Biological Chemistry*, 273(5), 2738-2746. doi:10.1074/jbc.273.5.2738
- Schmelzer, K., Fahy, E., Subramaniam, S., & Dennis, E. A. (2007). The lipid maps initiative in lipidomics. *Methods Enzymol*, 432, 171-183. doi:10.1016/S0076-6879(07)32007-7
- Schnaar, R. L., Suzuki, A., & Stanley, P. (2009). Glycosphingolipids. In A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart, & M. E. Etzler (Eds.), *Essentials of Glycobiology* (2nd ed.). Cold Spring Harbor (NY).
- Schneider, J. S., Cambi, F., Gollomp, S. M., Kuwabara, H., Brasic, J. R., Leiby, B., . . . Wong, D. F. (2015). GM1 ganglioside in Parkinson's disease: Pilot study of effects on dopamine transporter binding. *J Neurol Sci*, 356(1-2), 118-123. doi:10.1016/j.jns.2015.06.028
- Schneider, J. S., Gollomp, S. M., Sendek, S., Colcher, A., Cambi, F., & Du, W. (2013). A randomized, controlled, delayed start trial of GM1 ganglioside in treated Parkinson's disease patients. *J Neurol Sci*, 324(1-2), 140-148. doi:10.1016/j.jns.2012.10.024
- Schubert, W., Frank, P. G., Razani, B., Park, D. S., Chow, C.-W., & Lisanti, M. P. (2001). Caveolae-deficient Endothelial Cells Show Defects in the Uptake and Transport of Albumin in Vivo. *Journal of Biological Chemistry*, 276(52), 48619-48622. doi:10.1074/jbc.C100613200
- Schuhmann, K., Almeida, R., Baumert, M., Herzog, R., Bornstein, S. R., & Shevchenko, A. (2012). Shotgun lipidomics on a LTQ Orbitrap mass

spectrometer by successive switching between acquisition polarity modes.
Journal of Mass Spectrometry, 47(1), 96-104. doi:10.1002/jms.2031

Schwarz, E., Prabakaran, S., Whitfield, P., Major, H., Leweke, F. M., Koethe, D., . . .
Bahn, S. (2008). High throughput lipidomic profiling of schizophrenia and
bipolar disorder brain tissue reveals alterations of free fatty acids,
phosphatidylcholines, and ceramides. *J Proteome Res*, 7(10), 4266-4277.
doi:10.1021/pr800188y

Schwenk, R. W., Holloway, G. P., Luiken, J. J., Bonen, A., & Glatz, J. F. (2010).
Fatty acid transport across the cell membrane: regulation by fatty acid
transporters. *Prostaglandins Leukot Essent Fatty Acids*, 82(4-6), 149-154.
doi:10.1016/j.plefa.2010.02.029

Schwingshackl, L., & Hoffmann, G. (2013). Comparison of Effects of Long-Term
Low-Fat vs High-Fat Diets on Blood Lipid Levels in Overweight or Obese
Patients: A Systematic Review and Meta-Analysis. *Journal of the Academy of
Nutrition and Dietetics*, 113(12), 1640-1661.
doi:http://dx.doi.org/10.1016/j.jand.2013.07.010

Senko, M. W., Beu, S. C., & McLafferty, F. W. (1995). Determination of
monoisotopic masses and ion populations for large biomolecules from
resolved isotopic distributions. *Journal of the American Society for Mass
Spectrometry*, 6(4), 229-233. doi:http://dx.doi.org/10.1016/1044-
0305(95)00017-8

Shaikh, N. A. (1994). Assessment of Various Techniques for the Quantitative
Extraction of Lysophospholipids from Myocardial Tissues. *Analytical
Biochemistry*, 216(2), 313-321. doi:http://dx.doi.org/10.1006/abio.1994.1047

Shaner, R. L., Allegood, J. C., Park, H., Wang, E., Kelly, S., Haynes, C. A., . . .
Merrill, A. H. (2009a). Quantitative analysis of sphingolipids for lipidomics
using triple quadrupole and quadrupole linear ion trap mass spectrometers.

Journal of Lipid Research, 50(8), 1692-1707. doi:10.1194/jlr.D800051-JLR200

Shaner, R. L., Allegood, J. C., Park, H., Wang, E., Kelly, S., Haynes, C. A., . . . Merrill, A. H., Jr. (2009b). Quantitative analysis of sphingolipids for lipidomics using triple quadrupole and quadrupole linear ion trap mass spectrometers. *J Lipid Res*, 50(8), 1692-1707. doi:10.1194/jlr.D800051-JLR200

Shapiro, S. S., & Wilk, M. B. (1965). An Analysis of Variance Test for Normality (Complete Samples). *Biometrika*, 52(3/4), 591-611. doi:10.2307/2333709

Shevchenko, A., & Simons, K. (2010). Lipidomics: coming to grips with lipid diversity. *Nat Rev Mol Cell Biol*, 11(8), 593-598. doi:10.1038/nrm2934

Shi, H., Kokoeva, M. V., Inouye, K., Tzameli, I., Yin, H., & Flier, J. S. (2006). TLR4 links innate immunity and fatty acid-induced insulin resistance. *Journal of Clinical Investigation*, 116(11), 3015-3025. doi:10.1172/JCI28898

Shmueli, G. (2010). To Explain or to Predict? *Statistical Science*, 25(3), 289-310. doi:10.1214/10-Sts330

Sidransky, E., Nalls, M. A., Aasly, J. O., Aharon-Peretz, J., Annesi, G., Barbosa, E. R., . . . Ziegler, S. G. (2009). Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. *N Engl J Med*, 361(17), 1651-1661. doi:10.1056/NEJMoa0901281

Simon, C. G., Holloway, P. W., & Gear, A. R. L. (1999). Exchange of C16-Ceramide between Phospholipid Vesicles. *Biochemistry*, 38(44), 14676-14682. doi:10.1021/bi991537w

Skorve, J., Hilvo, M., Vihervaara, T., Burri, L., Bohov, P., Tillander, V., . . . Alexson, S. E. (2015). Fish oil and krill oil differentially modify the liver and

brain lipidome when fed to mice. *Lipids Health Dis*, 14, 88.
doi:10.1186/s12944-015-0086-2

Smith, A. R., Visioli, F., Frei, B., & Hagen, T. M. (2006). Age-related changes in endothelial nitric oxide synthase phosphorylation and nitric oxide dependent vasodilation: evidence for a novel mechanism involving sphingomyelinase and ceramide-activated phosphatase 2A. *Aging Cell*, 5(5), 391-400.
doi:10.1111/j.1474-9726.2006.00232.x

Smith, C. A., O'Maille, G., Want, E. J., Qin, C., Trauger, S. A., Brandon, T. R., . . . Siuzdak, G. (2005). METLIN: a metabolite mass spectral database. *Ther Drug Monit*, 27(6), 747-751.

Smith, R. D., Loo, J. A., Edmonds, C. G., Barinaga, C. J., & Udseth, H. R. (1990). New developments in biochemical mass spectrometry: electrospray ionization. *Analytical Chemistry*, 62(9), 882-899. doi:10.1021/ac00208a002

Spampinato, S. F., Obermeier, B., Coteleur, A., Love, A., Takeshita, Y., Sano, Y., . . . Ransohoff, R. M. (2015). Sphingosine 1 Phosphate at the Blood Brain Barrier: Can the Modulation of S1P Receptor 1 Influence the Response of Endothelial Cells and Astrocytes to Inflammatory Stimuli? *PLoS One*, 10(7), e0133392. doi:10.1371/journal.pone.0133392

Sparks, D. L., Kuo, Y. M., Roher, A., Martin, T., & Lukas, R. J. (2000). Alterations of Alzheimer's disease in the cholesterol-fed rabbit, including vascular inflammation. Preliminary observations. *Ann N Y Acad Sci*, 903, 335-344.

Spector, A. A. (2001). Plasma free fatty acid and lipoproteins as sources of polyunsaturated fatty acid for the brain. *J Mol Neurosci*, 16(2-3), 159-165; discussion 215-121. doi:10.1385/JMN:16:2-3:159

Spector, A. A., & Yorek, M. A. (1985). Membrane lipid composition and cellular function. *Journal of Lipid Research*, 26(9), 1015-1035.

- Stadelmann-Ingrand, S., Favreliere, S., Fauconneau, B., Mauco, G., & Tallineau, C. (2001). Plasmalogen degradation by oxidative stress: production and disappearance of specific fatty aldehydes and fatty alpha-hydroxyaldehydes. *Free Radic Biol Med*, *31*(10), 1263-1271.
- Stark, K. D., Lim, S. Y., & Salem, N., Jr. (2007). Artificial rearing with docosahexaenoic acid and n-6 docosapentaenoic acid alters rat tissue fatty acid composition. *J Lipid Res*, *48*(11), 2471-2477. doi:10.1194/jlr.M700317-JLR200
- Stenvinkel, P., Diczfalusy, U., Lindholm, B., & Heimbürger, O. (2004). Phospholipid plasmalogen, a surrogate marker of oxidative stress, is associated with increased cardiovascular mortality in patients on renal replacement therapy. *Nephrol Dial Transplant*, *19*(4), 972-976. doi:10.1093/ndt/gfh035
- Steyerberg, E. W., Bleeker, S. E., Moll, H. A., Grobbee, D. E., & Moons, K. G. (2003a). Internal and external validation of predictive models: a simulation study of bias and precision in small samples. *J Clin Epidemiol*, *56*(5), 441-447.
- Steyerberg, E. W., Bleeker, S. E., Moll, H. A., Grobbee, D. E., & Moons, K. G. M. (2003b). Internal and external validation of predictive models: A simulation study of bias and precision in small samples. *Journal of Clinical Epidemiology*, *56*(5), 441-447. doi:http://dx.doi.org/10.1016/S0895-4356(03)00047-7
- Steyerberg, E. W., Eijkemans, M. J., Harrell, F. E., Jr., & Habbema, J. D. (2000). Prognostic modelling with logistic regression analysis: a comparison of selection and estimation methods in small data sets. *Stat Med*, *19*(8), 1059-1079.
- Steyerberg, E. W., Harrell, F. E., Borsboom, G. J. J. M., Eijkemans, M. J. C., Vergouwe, Y., & Habbema, J. D. F. (2001a). Internal validation of predictive

models. *Journal of Clinical Epidemiology*, 54(8), 774-781.
doi:[http://dx.doi.org/10.1016/S0895-4356\(01\)00341-9](http://dx.doi.org/10.1016/S0895-4356(01)00341-9)

Steyerberg, E. W., Harrell, F. E., Borsboom, G. J. J. M., Eijkemans, M. J. C., Vergouwe, Y., & Habbema, J. D. F. (2001b). Internal validation of predictive models: Efficiency of some procedures for logistic regression analysis. *Journal of Clinical Epidemiology*, 54(8), 774-781. doi:Doi 10.1016/S0895-4356(01)00341-9

Storey, J. D. (2011). False Discovery Rate. In M. Lovric (Ed.), *International Encyclopedia of Statistical Science* (pp. 504-508). Berlin, Heidelberg: Springer Berlin Heidelberg.

Stranahan, A. M., Cutler, R. G., Button, C., Telljohann, R., & Mattson, M. P. (2011). Diet-induced elevations in serum cholesterol are associated with alterations in hippocampal lipid metabolism and increased oxidative stress. *J Neurochem*, 118(4), 611-615. doi:10.1111/j.1471-4159.2011.07351.x

Summers, S. A. (2006). Ceramides in insulin resistance and lipotoxicity. *Progress in Lipid Research*, 45(1), 42-72.
doi:<https://doi.org/10.1016/j.plipres.2005.11.002>

Sun, G. Y., Xu, J., Jensen, M. D., & Simonyi, A. (2004). Phospholipase A2 in the central nervous system: implications for neurodegenerative diseases. *J Lipid Res*, 45(2), 205-213. doi:10.1194/jlr.R300016-JLR200

Suzuki, K., Vanier, M. T., Coetzee, T., & Popko, B. (1999). Drastically abnormal gluco- and galactosylceramide composition does not affect ganglioside metabolism in the brain of mice deficient in galactosylceramide synthase. *Neurochem Res*, 24(4), 471-474.

Svennerholm, L., & Fredman, P. (1980). A procedure for the quantitative isolation of brain gangliosides. *Biochim Biophys Acta*, 617(1), 97-109.

- Symons, J. D., & Abel, E. D. (2013). Lipotoxicity contributes to endothelial dysfunction : a focus on the contribution from ceramide. *Reviews in endocrine & metabolic disorders*, *14*(1), 59-68. doi:10.1007/s11154-012-9235-3
- Szymanska, E., Saccenti, E., Smilde, A. K., & Westerhuis, J. A. (2012). Double-check: validation of diagnostic statistics for PLS-DA models in metabolomics studies. *Metabolomics*, *8*(Suppl 1), 3-16. doi:10.1007/s11306-011-0330-3
- Taguchi, R., Houjou, T., Nakanishi, H., Yamazaki, T., Ishida, M., Imagawa, M., & Shimizu, T. (2005). Focused lipidomics by tandem mass spectrometry. *Journal of Chromatography B*, *823*(1), 26-36. doi:http://dx.doi.org/10.1016/j.jchromb.2005.06.005
- Taha, A. Y., Burnham, W. M., & Auvin, S. (2010). Polyunsaturated fatty acids and epilepsy. *Epilepsia*, *51*(8), 1348-1358. doi:10.1111/j.1528-1167.2010.02654.x
- Taha, A. Y., Chang, L., & Chen, M. (2016). Threshold changes in rat brain docosahexaenoic acid incorporation and concentration following graded reductions in dietary alpha-linolenic acid. *Prostaglandins Leukot Essent Fatty Acids*, *105*, 26-34. doi:10.1016/j.plefa.2015.12.002
- Takechi, R., Galloway, S., Pallegage-Gamarallage, M. M., Lam, V., Dhaliwal, S. S., & Mamo, J. C. (2013). Probucol prevents blood-brain barrier dysfunction in wild-type mice induced by saturated fat or cholesterol feeding. *Clin Exp Pharmacol Physiol*, *40*(1), 45-52. doi:10.1111/1440-1681.12032
- Takechi, R., Galloway, S., Pallegage-Gamarallage, M. M., Lam, V., & Mamo, J. C. (2010). Dietary fats, cerebrovasculature integrity and Alzheimer's disease risk. *Prog Lipid Res*, *49*(2), 159-170. doi:10.1016/j.plipres.2009.10.004
- Takechi, R., Galloway, S., Pallegage-Gamarallage, M. M., Wellington, C. L., Johnsen, R. D., Dhaliwal, S. S., & Mamo, J. C. (2010). Differential effects of

dietary fatty acids on the cerebral distribution of plasma-derived apo B lipoproteins with amyloid-beta. *Br J Nutr*, *103*(5), 652-662.
doi:10.1017/S0007114509992194

Takechi, R., Pallegage-Gamarallage, M. M., Lam, V., Giles, C., & Mamo, J. C. (2013a). Aging-related changes in blood-brain barrier integrity and the effect of dietary fat. *Neurodegener Dis*, *12*(3), 125-135. doi:10.1159/000343211

Takechi, R., Pallegage-Gamarallage, M. M., Lam, V., Giles, C., & Mamo, J. C. (2013b). Nutraceutical agents with anti-inflammatory properties prevent dietary saturated-fat induced disturbances in blood-brain barrier function in wild-type mice. *J Neuroinflammation*, *10*, 73. doi:10.1186/1742-2094-10-73

Takechi, R., Pallegage-Gamarallage, M. M., Lam, V., Giles, C., & Mamo, J. C. (2014). Long-term probucol therapy continues to suppress markers of neurovascular inflammation in a dietary induced model of cerebral capillary dysfunction. *Lipids Health Dis*, *13*, 91. doi:10.1186/1476-511X-13-91

Teichgraber, V., Ulrich, M., Endlich, N., Riethmuller, J., Wilker, B., De Oliveira-Munding, C. C., . . . Gulbins, E. (2008). Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis. *Nat Med*, *14*(4), 382-391. doi:10.1038/nm1748

Testai, F. D., Kilkus, J. P., Berdyshev, E., Gorshkova, I., Natarajan, V., & Dawson, G. (2014). Multiple sphingolipid abnormalities following cerebral microendothelial hypoxia. *J Neurochem*, *131*(4), 530-540.
doi:10.1111/jnc.12836

Thiès, F., Delachambre, M. C., Bentejac, M., Lagarde, M., & Lecerf, J. (1992). Unsaturated Fatty Acids Esterified in 2-Acyl-1-Lysophosphatidylcholine Bound to Albumin Are More Efficiently Taken up by the Young Rat Brain than the Unesterified Form. *Journal of Neurochemistry*, *59*(3), 1110-1116.
doi:10.1111/j.1471-4159.1992.tb08353.x

- Tibshirani, R. (1996). Regression Shrinkage and Selection via the Lasso. *Journal of the Royal Statistical Society. Series B (Methodological)*, 58(1), 267-288.
- Tillman, T. S., & Cascio, M. (2003). Effects of membrane lipids on ion channel structure and function. *Cell Biochem Biophys*, 38(2), 161-190.
doi:10.1385/CBB:38:2:161
- Torkelson, T. R., Oyen, F., & Rowe, V. K. (1976). The toxicity of chloroform as determined by single and repeated exposure of laboratory animals. *American Industrial Hygiene Association Journal*, 37(12), 697-705.
doi:10.1080/0002889768507551
- Tu, W. C., Muhlhausler, B. S., Yelland, L. N., & Gibson, R. A. (2013). Correlations between blood and tissue omega-3 LCPUFA status following dietary ALA intervention in rats. *Prostaglandins Leukot Essent Fatty Acids*, 88(1), 53-60.
doi:10.1016/j.plefa.2012.04.005
- Tukey, J. W. (1949). Comparing Individual Means in the Analysis of Variance. *Biometrics*, 5(2), 99-114. doi:10.2307/3001913
- Valkenburg, D., Mertens, I., Lemiere, F., Witters, E., & Burzykowski, T. (2012). The isotopic distribution conundrum. *Mass Spectrom Rev*, 31(1), 96-109.
doi:10.1002/mas.20339
- van Blitterswijk, W. J., van der Luit, A. H., Veldman, R. J., Verheij, M., & Borst, J. (2003). Ceramide: second messenger or modulator of membrane structure and dynamics? *Biochem J*, 369(Pt 2), 199-211. doi:10.1042/BJ20021528
- van de Haar, H. J., Burgmans, S., Jansen, J. F., van Osch, M. J., van Buchem, M. A., Muller, M., . . . Backes, W. H. (2016). Blood-Brain Barrier Leakage in Patients with Early Alzheimer Disease. *Radiology*, 152244.
doi:10.1148/radiol.2016152244

- van den Berg, R. A., Hoefsloot, H. C., Westerhuis, J. A., Smilde, A. K., & van der Werf, M. J. (2006). Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC Genomics*, *7*, 142. doi:10.1186/1471-2164-7-142
- van Doorn, R., Nijland, P. G., Dekker, N., Witte, M. E., Lopes-Pinheiro, M. A., van het Hof, B., . . . de Vries, H. E. (2012). Fingolimod attenuates ceramide-induced blood-brain barrier dysfunction in multiple sclerosis by targeting reactive astrocytes. *Acta Neuropathol*, *124*(3), 397-410. doi:10.1007/s00401-012-1014-4
- van Doorn, R., Nijland, P. G., Dekker, N., Witte, M. E., Lopes-Pinheiro, M. A., van het Hof, B., . . . de Vries, H. E. (2012). Fingolimod attenuates ceramide-induced blood-brain barrier dysfunction in multiple sclerosis by targeting reactive astrocytes. *Acta Neuropathologica*, *124*(3), 397-410. doi:10.1007/s00401-012-1014-4
- Vandal, M., Alata, W., Tremblay, C., Rioux-Perreault, C., Salem, N., Jr., Calon, F., & Plourde, M. (2014). Reduction in DHA transport to the brain of mice expressing human APOE4 compared to APOE2. *J Neurochem*, *129*(3), 516-526. doi:10.1111/jnc.12640
- Vidaurre, O. G., Haines, J. D., Katz Sand, I., Adula, K. P., Huynh, J. L., McGraw, C. A., . . . Casaccia, P. (2014). Cerebrospinal fluid ceramides from patients with multiple sclerosis impair neuronal bioenergetics. *Brain*, *137*(Pt 8), 2271-2286. doi:10.1093/brain/awu139
- Wallin, A., Gottfries, C. G., Karlsson, I., & Svennerholm, L. (1989). Decreased myelin lipids in Alzheimer's disease and vascular dementia. *Acta Neurologica Scandinavica*, *80*(4), 319-323. doi:10.1111/j.1600-0404.1989.tb03886.x
- Wang, G., Zhou, Y., Huang, F. J., Tang, H. D., Xu, X. H., Liu, J. J., . . . Jia, W. (2014). Plasma metabolite profiles of Alzheimer's disease and mild cognitive impairment. *J Proteome Res*, *13*(5), 2649-2658. doi:10.1021/pr5000895

- Wang, M., Wang, C., Han, R. H., & Han, X. (2016). Novel advances in shotgun lipidomics for biology and medicine. *Prog Lipid Res*, 61, 83-108. doi:10.1016/j.plipres.2015.12.002
- Wang, M., Wang, C., & Han, X. (2016). Selection of internal standards for accurate quantification of complex lipid species in biological extracts by electrospray ionization mass spectrometry-What, how and why? *Mass Spectrom Rev*. doi:10.1002/mas.21492
- Warden, C. H., & Fisler, J. S. (2008). Comparisons of diets used in animal models of high fat feeding. *Cell Metabolism*, 7(4), 277-277. doi:10.1016/j.cmet.2008.03.014
- Watt, M. J., Barnett, A. C., Bruce, C. R., Schenk, S., Horowitz, J. F., & Hoy, A. J. (2012). Regulation of plasma ceramide levels with fatty acid oversupply: evidence that the liver detects and secretes de novo synthesised ceramide. *Diabetologia*, 55(10), 2741-2746. doi:10.1007/s00125-012-2649-3
- Watt, M. J., Hoy, A. J., Muoio, D. M., & Coleman, R. A. (2012). Distinct roles of specific fatty acids in cellular processes: implications for interpreting and reporting experiments. *Am J Physiol Endocrinol Metab*, 302(1), E1-3. doi:10.1152/ajpendo.00418.2011
- Weir, J. M., Wong, G., Barlow, C. K., Greeve, M. A., Kowalczyk, A., Almasy, L., . . . Meikle, P. J. (2013a). Plasma lipid profiling in a large population-based cohort. *J Lipid Res*, 54(10), 2898-2908. doi:10.1194/jlr.P035808
- Weir, J. M., Wong, G., Barlow, C. K., Greeve, M. A., Kowalczyk, A., Almasy, L., . . . Meikle, P. J. (2013b). Plasma lipid profiling in a large population-based cohort. *Journal of Lipid Research*, 54(10), 2898-2908. doi:10.1194/jlr.P035808

- Welch, B. L. (1947). THE GENERALIZATION OF 'STUDENT'S' PROBLEM WHEN SEVERAL DIFFERENT POPULATION VARIANCES ARE INVOLVED. *Biometrika*, 34(1-2), 28-35. doi:10.1093/biomet/34.1-2.28
- Wenk, M. R. (2005). The emerging field of lipidomics. *Nat Rev Drug Discov*, 4(7), 594-610. doi:10.1038/nrd1776
- Westerhuis, J. A., van Velzen, E. J. J., Hoefsloot, H. C. J., & Smilde, A. K. (2010). Multivariate paired data analysis: multilevel PLS-DA versus OPLS-DA. *Metabolomics*, 6(1), 119-128. doi:10.1007/s11306-009-0185-z
- Wiesner, P., Leidl, K., Boettcher, A., Schmitz, G., & Liebisch, G. (2009). Lipid profiling of FPLC-separated lipoprotein fractions by electrospray ionization tandem mass spectrometry. *Journal of Lipid Research*, 50(3), 574-585. doi:10.1194/jlr.D800028-JLR200
- Wilson, I. D., Oresic, M., Dettmer, K., Smith, R. M., Soga, T., Sikanen, T., & Hirayama, A. (2013). *Chromatographic methods in metabolomics*: Royal Society of Chemistry.
- Winger, B. E., Hofstadler, S. A., Bruce, J. E., Udseth, H. R., & Smith, R. D. (1993). High-resolution accurate mass measurements of biomolecules using a new electrospray ionization ion cyclotron resonance mass spectrometer. *Journal of the American Society for Mass Spectrometry*, 4(7), 566-577. doi:http://dx.doi.org/10.1016/1044-0305(93)85018-S
- Winocur, G., & Greenwood, C. E. (2005). Studies of the effects of high fat diets on cognitive function in a rat model. *Neurobiol Aging*, 26 Suppl 1, 46-49. doi:10.1016/j.neurobiolaging.2005.09.003
- Witting, M., Ruttkies, C., Neumann, S., & Schmitt-Kopplin, P. (2017). LipidFrag: Improving reliability of in silico fragmentation of lipids and application to the *Caenorhabditis elegans* lipidome. *PLoS One*, 12(3), e0172311. doi:10.1371/journal.pone.0172311

- Wold, S., Sjöström, M., & Eriksson, L. (2001). PLS-regression: a basic tool of chemometrics. *Chemometrics and Intelligent Laboratory Systems*, 58(2), 109-130. doi:http://dx.doi.org/10.1016/S0169-7439(01)00155-1
- Wong, G., Chan, J., Kingwell, B. A., Leckie, C., & Meikle, P. J. (2014). LICRE : unsupervised feature correlation reduction for lipidomics. *Bioinformatics*, 30(19), 2832-2833. doi:10.1093/bioinformatics/btu381
- Wood, P. L., Barnette, B. L., Kaye, J. A., Quinn, J. F., & Woltjer, R. L. (2015). Non-targeted lipidomics of CSF and frontal cortex grey and white matter in control, mild cognitive impairment, and Alzheimer's disease subjects. *Acta Neuropsychiatr*, 27(5), 270-278. doi:10.1017/neu.2015.18
- Wood, P. L., Locke, V. A., Herling, P., Passaro, A., Vigna, G. B., Volpato, S., . . . Zuliani, G. (2016). Targeted lipidomics distinguishes patient subgroups in mild cognitive impairment (MCI) and late onset Alzheimer's disease (LOAD). *BBA Clin*, 5, 25-28. doi:10.1016/j.bbacli.2015.11.004
- Wood, P. L., Mankidy, R., Ritchie, S., Heath, D., Wood, J. A., Flax, J., & Goodenowe, D. B. (2010). Circulating plasmalogen levels and Alzheimer Disease Assessment Scale-Cognitive scores in Alzheimer patients. *J Psychiatry Neurosci*, 35(1), 59-62.
- Wu, S., Ding, Y., Wu, F., Li, R., Hou, J., & Mao, P. (2015). Omega-3 fatty acids intake and risks of dementia and Alzheimer's disease: a meta-analysis. *Neurosci Biobehav Rev*, 48, 1-9. doi:10.1016/j.neubiorev.2014.11.008
- Wyne, K. L., Pathak, K., Seabra, M. C., & Hobbs, H. H. (1996). Expression of the VLDL receptor in endothelial cells. *Arterioscler Thromb Vasc Biol*, 16(3), 407-415.
- Xing, Y., Tang, Y., Zhao, L., Wang, Q., Qin, W., Ji, X., . . . Jia, J. (2016). Associations between plasma ceramides and cognitive and neuropsychiatric

manifestations in Parkinson's disease dementia. *J Neurol Sci*, 370, 82-87.
doi:10.1016/j.jns.2016.09.028

Xu, X. X., & Tabas, I. (1991). Sphingomyelinase enhances low density lipoprotein uptake and ability to induce cholesteryl ester accumulation in macrophages. *Journal of Biological Chemistry*, 266(36), 24849-24858.

Yamashita, S., Kiko, T., Fujiwara, H., Hashimoto, M., Nakagawa, K., Kinoshita, M., . . . Miyazawa, T. (2016). Alterations in the Levels of Amyloid-beta, Phospholipid Hydroperoxide, and Plasmalogen in the Blood of Patients with Alzheimer's Disease: Possible Interactions between Amyloid-beta and These Lipids. *J Alzheimers Dis*, 50(2), 527-537. doi:10.3233/JAD-150640

Yang, G., Badeanlou, L., Bielawski, J., Roberts, A. J., Hannun, Y. A., & Samad, F. (2009). Central role of ceramide biosynthesis in body weight regulation, energy metabolism, and the metabolic syndrome. *American Journal of Physiology - Endocrinology And Metabolism*, 297(1), E211-E224.
doi:10.1152/ajpendo.91014.2008

Yang, K., & Han, X. (2016). Lipidomics: Techniques, Applications, and Outcomes Related to Biomedical Sciences. *Trends Biochem Sci*, 41(11), 954-969.
doi:10.1016/j.tibs.2016.08.010

Yaqoob, P., Sherrington, E. J., Jeffery, N. M., Sanderson, P., Harvey, D. J., Newsholme, E. A., & Calder, P. C. (1995). Comparison of the Effects of a Range of Dietary Lipids Upon Serum and Tissue Lipid-Composition in the Rat. *International Journal of Biochemistry & Cell Biology*, 27(3), 297-310.
doi:Doi 10.1016/1357-2725(94)00065-J

Yassine, H. N., Croteau, E., Rawat, V., Hibbeln, J. R., Rapoport, S. I., Cunnane, S. C., & Umhau, J. C. (2017). DHA brain uptake and APOE4 status: a PET study with [1-11C]-DHA. *Alzheimers Res Ther*, 9(1), 23.
doi:10.1186/s13195-017-0250-1

- Yergey, J. A. (1983). A general approach to calculating isotopic distributions for mass spectrometry. *International Journal of Mass Spectrometry and Ion Physics*, 52(2), 337-349. doi:http://dx.doi.org/10.1016/0020-7381(83)85053-0
- Yin, W., Carballo-Jane, E., McLaren, D. G., Mendoza, V. H., Gagen, K., Geoghagen, N. S., . . . Strack, A. M. (2012). Plasma lipid profiling across species for the identification of optimal animal models of human dyslipidemia. *Journal of Lipid Research*, 53(1), 51-65. doi:10.1194/jlr.M019927
- Yu, H., Bi, Y., Ma, W., He, L., Yuan, L., Feng, J., & Xiao, R. (2010). Long-term effects of high lipid and high energy diet on serum lipid, brain fatty acid composition, and memory and learning ability in mice. *Int J Dev Neurosci*, 28(3), 271-276. doi:10.1016/j.ijdevneu.2009.12.001
- Yurko-Mauro, K., Alexander, D. D., & Van Elswyk, M. E. (2015). Docosahexaenoic acid and adult memory: a systematic review and meta-analysis. *PLoS One*, 10(3), e0120391. doi:10.1371/journal.pone.0120391
- Zhang, M., Sun, S., Tang, N., Cai, W., & Qian, L. (2013). Oral administration of alkylglycerols differentially modulates high-fat diet-induced obesity and insulin resistance in mice. *Evid Based Complement Alternat Med*, 2013, 834027. doi:10.1155/2013/834027
- Zhang, Q.-J., Holland, W. L., Wilson, L., Tanner, J. M., Kearns, D., Cahoon, J. M., . . . Symons, J. D. (2012a). Ceramide Mediates Vascular Dysfunction in Diet-Induced Obesity by PP2A-Mediated Dephosphorylation of the eNOS-Akt Complex. *Diabetes*, 61(7), 1848-1859. doi:10.2337/db11-1399
- Zhang, Q. J., Holland, W. L., Wilson, L., Tanner, J. M., Kearns, D., Cahoon, J. M., . . . Symons, J. D. (2012b). Ceramide mediates vascular dysfunction in diet-induced obesity by PP2A-mediated dephosphorylation of the eNOS-Akt complex. *Diabetes*, 61(7), 1848-1859. doi:10.2337/db11-1399

- Zhang, Y., Chen, J., Qiu, J., Li, Y., Wang, J., & Jiao, J. (2016). Intakes of fish and polyunsaturated fatty acids and mild-to-severe cognitive impairment risks: a dose-response meta-analysis of 21 cohort studies. *Am J Clin Nutr*, *103*(2), 330-340. doi:10.3945/ajcn.115.124081
- Zhao, L., Spassieva, S. D., Jucius, T. J., Shultz, L. D., Shick, H. E., Macklin, W. B., . . . Ackerman, S. L. (2011). A deficiency of ceramide biosynthesis causes cerebellar purkinje cell neurodegeneration and lipofuscin accumulation. *PLoS Genet*, *7*(5), e1002063. doi:10.1371/journal.pgen.1002063
- Zolnik, B. S., Stern, S. T., Kaiser, J. M., Heakal, Y., Clogston, J. D., Kester, M., & McNeil, S. E. (2008). Rapid Distribution of Liposomal Short-Chain Ceramide in Vitro and in Vivo. *Drug Metabolism and Disposition*, *36*(8), 1709-1715. doi:10.1124/dmd.107.019679
- Zou, H., & Hastie, T. (2005). Regularization and variable selection via the elastic net. *Journal of the Royal Statistical Society: Series B (Statistical Methodology)*, *67*(2), 301-320. doi:10.1111/j.1467-9868.2005.00503.x

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I, Corey Giles, conceived the study design and contributed to experimental procedures, data collection, data analysis, interpretation, and manuscript preparation for the publication entitled 'The Effects of Long-Term Saturated Fat Enriched Diets on the Brain Lipidome'.

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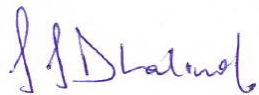
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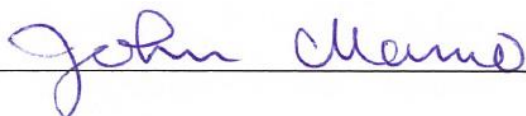
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Appendix II: R Code for isotope distribution, partial least squares, double cross validation and permutation tests

R code to calculate exact mass and isotope envelope.

```
## Generate molecular formula for lipids
FA<- c("12_0", "13_0", "14_0", "15_0",
"16_0", "16_1", "17_0", "17_1", "18_0", "18_1", "18_2", "18_3", "20_0", "20_1",
", "20_2", "20_3", "20_4", "20_5", "22_0", "22_1", "22_5", "22_6", "24_0", "24_1",
", "26_0", "26_1")
length<-c( 12,    13,    14,    15,    16,    16,    17,    17,
18,    18,    18,    18,    20,    20,    20,    20,    20,    20,
22,    22,    22,    22,    24,    24,    26,    26)
db<- c( 0,    0,    0,    0,    0,    1,    0,    1,
0,    1,    2,    3,    0,    1,    2,    3,    4,    5,
0,    1,    5,    6,    0,    1,    0,    1)
fattyAcids<-data.frame (FA=FA,length=length,db=db)

headGroup<-
c("PC","PE","PS","PI","OH","TG","Pi","Glu","Lac","Sulfatide")

## Coefficients
# Gly Chol Sph PC PE PS PI OH Pi Glu Lac Sul SN1 DB SN2 DB
SN3 DB (P) (O) sn1 sn2 sn3 offset
C<-c( 3, 27, 0, 5, 2, 3, 6, 0, 0, 6, 12, 6, 1, 0, 1, 0,
1, 0, 0, 0, 0, 0, 0, 0)
H<-c( 3, 41, 0, 12, 6, 6, 11, 0, 1, 10, 20, 10, 2, -2, 2, -2,
2, -2, 0, 2, -2, -2, -2, 5)
N<-c(-1, -1, 0, 1, 1, 1, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0,
0, 0, 0, 0, 0, 0, 1)
O<-c( 2, 0, 0, 3, 3, 5, 8, 0, 3, 5, 10, 8, 0, 0, 0, 0, 0,
0, 0, -1, -1, 1, 1, 1, 1)
P<-c( 0, 0, 0, 1, 1, 1, 1, 0, 1, 0, 0, 0, 0, 0, 0, 0, 0,
0, 0, 0, 0, 0, 0, 0)
S<-c( 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 1, 0, 0, 0, 0,
0, 0, 0, 0, 0, 0, 0)
elementalCoef<-matrix(c(C,H,N,O,P,S),ncol=6,dimnames =
list(c("Glycerol","Cholesterol","Sphingoid","PC","PE","PS","PI","OH"
```

```
, "Pi", "Glu", "Lac", "Sulfatide", "SN1-chain", "SN1-DB", "SN2-chain", "SN2-DB", "SN3-Chain", "SN3-DB", "(P)", "(O)", "sn1", "sn2", "sn3", "offset"), NULL))
```

```
## Generate lipid structure
```

```
generateLipid<-
```

```
function(backBone="Glycerol", headGroup="PC", acylSN1="18_0", acylSN2="18_1", acylSN3=NA, modification=NA) {
  composition<-numeric(24)
  if(backBone=="Glycerol") {
    composition[1:3]<-c(1,0,0)
  } else if(backBone=="Cholesterol") {
    composition[1:3]<-c(0,1,0)
    if(!is.na(acylSN1)) {
      headGroup=NA
    }
    acylSN2<-NA
    acylSN3<-NA
  } else if(backBone=="Sphingoid") {
    composition[1:3]<-c(0,0,1)
    acylSN3<-NA
  }
  if(!is.na(headGroup)) {
    if(headGroup=="PC") {
      composition[4:12]<-c(1,0,0,0,0,0,0,0,0)
      acylSN3<-NA
    } else if(headGroup=="PE") {
      composition[4:12]<-c(0,1,0,0,0,0,0,0,0)
      acylSN3<-NA
    } else if(headGroup=="PS") {
      composition[4:12]<-c(0,0,1,0,0,0,0,0,0)
      acylSN3<-NA
    } else if(headGroup=="PI") {
      composition[4:12]<-c(0,0,0,1,0,0,0,0,0)
      acylSN3<-NA
    } else if(headGroup=="OH") {
      composition[4:12]<-c(0,0,0,0,1,0,0,0,0)
      acylSN3<-NA
    } else if(headGroup=="Pi") {
      composition[4:12]<-c(0,0,0,0,0,1,0,0,0)
    }
  }
}
```

```

} else if(headGroup=="Glu") {
  composition[4:12]<-c(0,0,0,0,0,0,0,1,0,0)
  acylSN3<-NA
} else if(headGroup=="Lac") {
  composition[4:12]<-c(0,0,0,0,0,0,0,0,1,0)
  acylSN3<-NA
} else if(headGroup=="Sulfatide") {
  composition[4:12]<-c(0,0,0,0,0,0,0,0,0,1)
  acylSN3<-NA
}
} else {
  composition[4:12]<-c(0,0,0,0,0,0,0,0,0,0)
}

if(backBone=="Glycerol" || (backBone=="Cholesterol"&!is.na(acylSN1)))
{
  composition[13]<-
fattyAcids$length[which(fattyAcids$FA==acylSN1)]
  composition[14]<-fattyAcids$db[which(fattyAcids$FA==acylSN1)]
  if(!is.na(acylSN2)) {
    composition[15]<-
fattyAcids$length[which(fattyAcids$FA==acylSN2)]
    composition[16]<-fattyAcids$db[which(fattyAcids$FA==acylSN2)]
  }
  if(!is.na(acylSN3)) {
    composition[17]<-
fattyAcids$length[which(fattyAcids$FA==acylSN3)]
    composition[18]<-fattyAcids$db[which(fattyAcids$FA==acylSN3)]
  }
} else if(backBone=="Sphingoid") {
  composition[13]<-
fattyAcids$length[which(fattyAcids$FA==acylSN1)]
  composition[14]<-fattyAcids$db[which(fattyAcids$FA==acylSN1)]
  if(!is.na(acylSN2)) {
    composition[15]<-
fattyAcids$length[which(fattyAcids$FA==acylSN2)]
    composition[16]<-fattyAcids$db[which(fattyAcids$FA==acylSN2)]
  }
}
}
if(!is.na(modification)) {
  if(modification=="P") {

```

```

        composition[19:20]<-c(1,0)
    } else if(modification=="O") {
        composition[19:20]<-c(0,1)
    }
}
if(!is.na(acylSN1)) {
    composition[21]<-1
}
if(!is.na(acylSN2)) {
    composition[22]<-1
}
if(!is.na(acylSN3)) {
    composition[23]<-1
}
composition[24]<-1
return(composition)
}

## Calculate isotope envelope and exact mass
elements<- c("C",      "H",      "N",      "O",      "P",
"S")
mass1<-    c(12,      1.007825, 14.003074, 15.994915, 30.973762,
31.972071)
mass2<-    c(13.003355, 2.014102, 15.000109, 17.99916,  30.973762,
33.967867)
abundance<- c(0.9893,  0.999885, 0.996360,  0.99757,  1.0,
0.9499)

natural.isotopic.abundance<-
data.frame(elements=elements,mass1=mass1,mass2=mass2,abundance=abundance)

isotope.distribution<-function(compound) {
    output.combined<-matrix(c(0,1),nrow=1,ncol=2)
    for(p in 1:length(elements)) {
        n<-compound[p]
        mass1<-natural.isotopic.abundance[p,2]
        mass2<-natural.isotopic.abundance[p,3]
        abundance<-natural.isotopic.abundance[p,4]
        mass.dist<-matrix(0,nrow=n+1,ncol=2)
    }
}

```



```

    for(k in 0:n) {
      mass.dist[k+1,1]<-mass1*k+(n-k)*mass2
      mass.dist[k+1,2]<-dbinom(k,n,abundance)
    }
    eval(parse(text=paste("output.combined<-
rbind(",paste(rep("output.combined",nrow(mass.dist)),collapse=","),"
)",sep="")))
    output.combined[,1]<-
output.combined[,1]+rep(mass.dist[,1],each=nrow(output.combined)/nro
w(mass.dist))
    output.combined[,2]<-
output.combined[,2]*rep(mass.dist[,2],each=nrow(output.combined)/nro
w(mass.dist))
  }
  output.combined<-
output.combined[which(output.combined[,2]>0.0001),,drop=FALSE]
  output.combined[,2]<-output.combined[,2]
  output.combined<-output.combined[order(output.combined[,1]),]
  return(output.combined)
}

## Simulate the low resolution of a quadrupole mass filter
quadrupole<-function(compound) {
  isotope.envelope<-isotope.distribution(compound)
  mz<-seq(min(isotope.envelope[,1]) -
1,max(isotope.envelope[,1])+1,0.01)

  count<-numeric(length(mz))
  out<-numeric(length(mz))

  for(i in 1:nrow(isotope.envelope)) {
    position<-which.min(abs(mz-isotope.envelope[i,1]))
    count[position]<-count[position]+isotope.envelope[i,2]
  }

  conv<-dnorm(seq(-5,5,0.1),0,1.5)
  convolution<-convolve(count,conv/max(conv),type="o") [51:752]
  names(convolution)<-mz
  return(convolution)
}

```

```
# Generate PC(18:0/22:6)
PC_18_0_22_6<-
generateLipid("Glycerol",headGroup="PC",acylSN1="18_0",acylSN2 =
"22_6")*%*%elementalCoef
envelope<-quadrupole(PC_18_0_22_6)

# Plot isotope envelope
plot(names(envelope),envelope,type='l',xlab='m/z',ylab='isotope
abundance',main='PC(18:0/22:6)')
lines(isotope.distribution(PC_18_0_22_6),type='h',col='red')
```

R code to perform partial least squares, double cross-validation and permutation tests.

```
library(mixOmics)
## Mean centre and unit-variance scale datasets
Plasma<-scale(Plasma)
CTX<-scale(CTX)
HPF<-scale(HPF)

#=====
#
# Double cross validation for CTX/Plasma
#
#=====

n<-nrow(Plasma)
CV.pls.MSEP<-matrix(NA,nrow=n,ncol=ncol(CTX))
list.lv<-rep(0,nrow(Plasma))
for(i in 1:n) {
  CTX.train<-CTX[-i,]
  CTX.test<-CTX[i,,drop=FALSE]
  Plasma.train<-Plasma[-i,]
  Plasma.test<-Plasma[i,,drop=FALSE]

  train.pls<-pls(Plasma.train,CTX.train,ncomp=5,mode="regression")
  train.perf<-perf(train.pls,validation="loo")
  MSEP<-colMeans(train.perf$MSEP)
  optim.lv<-which(MSEP==min(MSEP))

  CTX.pls<-
pls(Plasma.train,CTX.train,ncomp=optim.lv,mode="regression")
  CTX.test.hat<-predict(CTX.pls,Plasma.test)
  CV.pls.MSEP[i,]<-(CTX.test.hat$predict[, , optim.lv]-CTX.test)^2
  list.lv[i]<-optim.lv
}

CTX.sig<-sum(colMeans(CV.pls.MSEP))
```

```

#=====
#
#  Permutation test for CTX/Plasma
#
#=====
B<-1000
out<-rep(0,B)
for(p in 1:B) {
  CV.pls.MSEP<-matrix(NA,nrow=n,ncol=ncol(CTX))
  list.lv<-rep(0,nrow(Plasma))
  perm<-sample(1:19,replace=FALSE)
  CTX.perm<-CTX[perm,]
  Plasma.perm<-Plasma
  for(i in 1:n) {
    CTX.train<-CTX.perm[-i,]
    CTX.test<-CTX.perm[i,,drop=FALSE]
    Plasma.train<-Plasma.perm[-i,]
    Plasma.test<-Plasma.perm[i,,drop=FALSE]

    train.pls<-pls(Plasma.train,CTX.train,ncomp=3,mode="regression")
    train.perf<-perf(train.pls,validation="loo")
    MSEP<-colMeans(train.perf$MSEP)
    optim.lv<-which(MSEP==min(MSEP))

    CTX.pls<-
pls(Plasma.train,CTX.train,ncomp=optim.lv,mode="regression")
    CTX.test.hat<-predict(CTX.pls,Plasma.test)
    CV.pls.MSEP[i,]<-(CTX.test.hat$predict[, , optim.lv]-CTX.test)^2
    list.lv[i]<-optim.lv
  }
  out[p]<-sum(colMeans(CV.pls.MSEP))
}

hist(out,breaks=50)
abline(v=CTX.sig)

write.table(out,file="lipidomics.pls.CTX.permutation.test.csv",sep="
,")
quantile(out,probs=c(0.025,0.975))

```

```

#=====
#
# Double cross validation for HPF/Plasma
#
#=====
n<-nrow(Plasma)
CV.pls.MSEP<-matrix(NA,nrow=n,ncol=ncol(HPF))
list.lv<-rep(0,nrow(Plasma))
for(i in 1:n) {
  HPF.train<-HPF[-i,]
  HPF.test<-HPF[i,,drop=FALSE]
  Plasma.train<-Plasma[-i,]
  Plasma.test<-Plasma[i,,drop=FALSE]

  train.pls<-pls(Plasma.train,HPF.train,ncomp=5,mode="regression")
  train.perf<-perf(train.pls,validation="loo")
  MSEP<-colMeans(train.perf$MSEP)
  optim.lv<-which(MSEP==min(MSEP))

  HPF.pls<-
pls(Plasma.train,HPF.train,ncomp=optim.lv,mode="regression")
  HPF.test.hat<-predict(HPF.pls,Plasma.test)
  CV.pls.MSEP[i,]<-(HPF.test.hat$predict[, , optim.lv]-HPF.test)^2
  list.lv[i]<-optim.lv
}

HPF.sig<-sum(colMeans(CV.pls.MSEP))

#=====
#
# Permutation test for HPF/Plasma
#
#=====
B<-1000
out<-rep(0,B)
for(p in 1:B) {
  CV.pls.MSEP<-matrix(NA,nrow=n,ncol=ncol(HPF))
  list.lv<-rep(0,nrow(Plasma))
  perm<-sample(1:19,replace=FALSE)
  HPF.perm<-HPF[perm,]
  Plasma.perm<-Plasma

```

```

for(i in 1:n) {
  HPF.train<-HPF.perm[-i,]
  HPF.test<-HPF.perm[i,,drop=FALSE]
  Plasma.train<-Plasma.perm[-i,]
  Plasma.test<-Plasma.perm[i,,drop=FALSE]

  train.pls<-pls(Plasma.train,HPF.train,ncomp=3,mode="regression")
  train.perf<-perf(train.pls,validation="loo")
  MSEP<-colMeans(train.perf$MSEP)
  optim.lv<-which(MSEP==min(MSEP))

  HPF.pls<-
pls(Plasma.train,HPF.train,ncomp=optim.lv,mode="regression")
  HPF.test.hat<-predict(HPF.pls,Plasma.test)
  CV.pls.MSEP[i,]<-(HPF.test.hat$predict[, ,optim.lv]-HPF.test)^2
  list.lv[i]<-optim.lv
}
out[p]<-sum(colMeans(CV.pls.MSEP))
}

hist(out,breaks=50)
abline(v=HPF.sig)

write.table(out,file="lipidomics.pls.HPF.permutation.test.csv",sep="
,")
quantile(out,probs=c(0.025,0.975))

```