

Lipidomic Signature of Alcohol-Related Brain Injury

Caine Christopher Smith



A thesis submitted to fulfil requirements for the degree of
Doctor of Philosophy

The University of Sydney
Faculty of Medicine and Health

2023

Statement of originality

This is to certify that, to the best of my knowledge; the content of this thesis is my work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Caine Christopher Smith

28th February 2023

Abstract

Alcohol use disorder (AUD) is a diagnosis characterised by psychosocial behaviours manifesting from chronic alcohol misuse. The effects of chronic alcohol misuse on the brain are widespread and are often described as alcohol-related brain injury (ARBI). Lipid-rich brain structures are particularly vulnerable to alcohol toxicity, reflected by decreased white matter volume and integrity and localised neuron loss in heavy alcohol users. This thesis aims to understand the effect AUD has on lipids in the brain. Using brain samples from a cohort of 20 donors with AUD and 20 controls, the first study utilises liquid chromatography/mass spectrometry (LC/MS) techniques to describe which lipid classes are particularly affected in ARBI across the white and grey matter of three brain regions (prefrontal cortex, middle temporal gyrus, and the primary visual cortex). A significant finding was that sphingolipids and some glycerophospholipids decreased in both prefrontal and primary visual cortices in AUD cases. In addition, fatty acids were generally shorter in carbon chain length and had reduced double bonds. To continue this characterisation, enzymes that synthesise sphingolipids were assayed by western blotting, which revealed an increase in ceramide transport (CERT) protein levels, a protein that shuttles ceramide between organelles to be converted into other sphingolipids. Next, neuron loss has been previously described in the prefrontal but not the visual cortex from donors with AUD, so immunohistochemistry and cell counting software was used to quantify neurons in both cortices of this cohort.

There was a significant decrease in both regions, consistent with more remarkable lipidome changes in visual grey matter.

To explore the interaction of liver pathology and the pathomechanisms of ARBI, the next part of this thesis describes combining the three publicly available RNA-Seq datasets from AUD case-control comparisons and specifically looking at differentially expressed genes, whose products are involved in lipid metabolism. The findings revealed significantly enriched pathways relating to lipid metabolism and peroxisome proliferator-activated receptor (PPAR) signalling in AUD cases with liver cirrhosis but not steatosis or in the absence of pathology.

As neuroinflammation is known to contribute to ARBI, the final part of the thesis explored a potential role for statins in reducing the severity of ARBI. Statins are the most prescribed drug in Australia due to their major mechanism of action in reducing cholesterol, and so had been used by a subset of AUD donors. Statins also exhibit anti-inflammatory and antioxidant properties, and it was hypothesised that they would reduce lipid peroxidation in heavy alcohol users. This chapter used enzyme-linked immunosorbent assay (ELISA) to describe how fewer reactive aldehyde by-products (4-hydroxynonenal) are seen in AUD subjects who used statins compared to those with no history of statin use. This point is further explored in the discussion of how antioxidant therapies may ameliorate some of the damage to the brain lipidome caused by chronic alcohol misuse. In conclusion, the neurolipidome is dysregulated in ARBI

through a likely mechanism involving oxidative stress and is exacerbated by liver pathology.

Acknowledgements

I would first like to acknowledge the brain donors and their families, whose invaluable contributions made this work possible. The Using Our Brains donor program works in concert with the NSW Brain Tissue Resource Centre at the University of Sydney to provide high-quality post-mortem tissue to researchers.

My primary supervisor, Associate Professor Greg Sutherland, who has guided and been supportive of me, and has given me a lot of breadth in the lab to test out new ideas, thank you.

During my candidature, our group has seen leadership changes, notably the retirement of our former NSW BTRC manager, Mrs. Donna Sheedy, who has certainly helped nurture my research career and had opened as many doors as she could for me; and our former lab head, Emeritus Professor Jillian Kril, who has always given me sound advice. Present and other past group members have been supportive of me over the past several years, including Hamish, Mario, Ali, Julia S., Dr. Jie, Toni, Dr. Shelley, Dr. Fiona, Dr. Andrew (who also gave me a crash course in bioinformatics!), Julia L., Dr. Dhiraj, Clair, Marloes, Vanessa, and Dr. Patrick.

The Charles Perkins Centre community has been outstanding, and I would like to give special thanks to Associate Professor Anthony Don, who has been instrumental in my lipidomics work, along with his group members Holly and Oana who have been of great help. On the topic of mass spec, other acknowledgements must go out to Dr. Matt O'Rourke, who has been really enthusiastic about MALDI-IMS, and in the early part of my PhD we had good long chats; and the rest of the mass spec team including Dr. Ben Crossett and Dr. David Fuentes who provided facility support. Other CPC members who have really helped me get through my candidature include Dr. Alice H, Dr. Courtney W, and Rachael I, who has always been encouraging and great to have conversations with.

And of course, finally, I'd like to acknowledge family and friends, especially my parents Julie and Barry, and brothers Kim and Shaun, who have always supported my pursuit of higher education; as well as the friends I have made along the way, including Aaron, Melisa, Dr. Mike, Mahashweta, Sam, Dr. Jared, and Ashleigh who are all quite special to me.

Funding acknowledgements

I am grateful to have been awarded the *Reginald Maney Lake and Amy Laura Bonamy Scholarship*, awarded by the then Discipline of Pathology to provide stipend support to undertake research in pathology. I also wish to acknowledge the Research Training Program Fees Offset funded by the Australian government and administered by the University of Sydney to offset tuition fees for higher degree by research students.

Other funding sources that have enabled this research include the NIH-NIAAA R28AA012725, which supports the NSW Brain Tissue Resource Centre, which provided brain tissue and metadata for all research completed in this thesis, and internal funding, awarded to A/Prof. Sutherland which funded consumables for these projects.

Conferences and Presentations

2018	SOMS HDR presentations, USYD	Introductory HDR talk	Oral
2018	19 th International Society for Biomedical Research on Alcoholism symposium, Kyoto JP	Lipid Imaging of Alcohol-Related Brain Damage in Human Brain Tissue	Poster
2019	SOMS Pathology HDR presentations, USYD	Progress presentation	Oral
2019	5 th Charles Perkins Centre Early- to Mid-Career Researcher Symposium, USYD	Lipidomics of Alcoholism in the Human Brain	Oral
2020	SOMS Pathology HDR presentations, USYD (online)	Progress presentation	Oral
2021	Neuropathology Lab Retreat (online)	Lipidomics of ARBD	Oral
2021	7 th Charles Perkins Centre Early- to Mid-Career Researcher Symposium, USYD (online)	Lipidome Changes in Alcohol-Related Brain Damage	Oral
2022	SOMS Neuroscience Theme Meeting, USYD (online)	Lipidome Changes in Alcohol-Related Brain Damage	Oral
2022	Research Society for Alcoholism Conference, Orlando, FL	Reduced Lipid Peroxidation in the Post-Mortem Brain of Statin Users in Alcohol-Related Brain Injury	Poster
2022	Sydney Brainomics Retreat	Homeostasis in Alcohol-Related Brain Damage?	Oral

Committees and Positions

2019-2021	Charles Perkins Centre Early- to Mid-Career Researchers Committee	Newsletter & Communications / Committee Co-Chair
2019-2020	HDR Liaison Committee	FMH HDR Liaison

Publications

Pertaining to this thesis:

Smith, C.C., Sheedy, D.L., McEwen, H.P., Don, A.D., Kril, J.J., Sutherland, G.T. (2022) *Lipidome Changes in Alcohol-Related Brain Damage*. Journal of Neurochemistry.

Other publications contributed to during candidature

O'Rourke, M.B., Viengkhou, B., Smith, C.C., Sonderegger, L., Padula, M.P., Sutherland, G.T., Hofer, M.J., Crossett, B. (2021) *Matrix phase fractionation: Investigating the compromise between dynamic range of analyte extraction and spatial resolution in mass spectrometry imaging*. Rapid Communications in Mass Spectrometry, 35(13), e9106. DOI: 10.1002/rcm.9106

Kaul, D., Smith, C.C., Stevens, J., Fröhlich, A.S., Binder, E.B., Mechawar, N., Schwab, S.G., Matosin, N. (2020) *Severe childhood and adulthood stress associates with neocortical layer-specific reductions of mature spines in psychiatric disorders*. Neurobiology of Stress, 13. DOI: 10.1016/j.ynstr.2020.100270

O'Rourke, M.B., Smith, C.C., de la Monte, S.M., Sutherland, G.T., Padula, M.P. (2019) *Higher Mass Accuracy MALDI-TOF/TOF Lipid Imaging of Human Brain Tissue in Alzheimer's Disease*. Current Protocols in Molecular Biology e86. DOI: 10.1002/cpmb.86.

O'Rourke, M.B., Smith, C.C., Tse, B., Sutherland, G.T., Crossett, B., Padula, M.P. (2019) *'What did I do wrong?' An empirical evaluation of sample preparation methodologies in matrix-assisted laser desorption/ionization-mass spectrometry imaging*. Future Science OA, (5)4. DOI: 10.4155/fsoa-2018-0095

O'Rourke, M.B., Padula, M.P., Smith, C., Youssef, P., Cordwell, S., Witting, P., Sutherland, G., Crossett, B. (2018) *Optimal Preparation of Formalin Fixed Samples for Peptide Based Matrix Assisted Laser Desorption/Ionization Mass Spectrometry Imaging Workflows*. Journal of Visualized Experiments.

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Abbreviations

4-HNE	4-hydroxynonenal
ACAT	acyl-CoA:cholesterol acyltransferase
ADH	Alcohol dehydrogenase
ALD	Alcoholic liver disease
ALDH2	Aldehyde dehydrogenase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ARBI	Alcohol-related brain injury
AUD	Alcohol use disorder
BBB	Blood-brain barrier
BCA	Bicinchoninic acid assay
BMI	Body mass index
BSA	Bovine serum albumin
C	Carbon
cDNA	Complementary DNA
CDP-DAG	Cytidine diphosphate diacylglycerol
CDS	CTP:phosphatidate cytidyltransferase
CE	Cholesteryl ester
Cer	Ceramide
CerS	Ceramide synthase
CERT	Ceramide transfer protein
CSF	Cerebrospinal fluid
CYP2E1	Cytochrome P450 2E1
d18:2	Sphingadiene
DAB	3,3'-Diaminobenzidine
DAG	Diacylglycerol
DEG	Differentially expressed gene
DSM	Diagnostic and Statistical Manual of Mental Disorders
ER	Endoplasmic reticulum
FADS	Fatty acid desaturase
FDR	False discovery rate
FFPE	Formalin-fixed paraffin-embedded
GalCer	Galactosylceramide
GEO	Gene expression omnibus
GL	Glycerophospholipid

GlcCer	Glucosylceramide
GO	Gene ontology
H ₂ O ₂	Hydrogen peroxide
HE	Hepatic encephalopathy
HLA	Human leukocyte antigen
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
iNOS	Inducible nitric oxide synthase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LacCer	Lactosylceramide
LC/MS	Liquid chromatography/Mass spectrometry
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LSI	Lipidomics standards initiative
MALDI-IMS	Matrix Assisted Laser Desorption/Ionisation – Imaging Mass Spectrometry
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MTG	Middle temporal gyrus
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Next-generation sequencing
NIH	National Institutes of Health
NSW BTRC	New South Wales Brain Tissue Resource Centre
OPC	Oligodendrocyte precursor cell
OTC	Over-the-counter
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine N-methyltransferase
PFC	Prefrontal cortex
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PLD	Phospholipase D
PMI	Post-mortem interval

PPAR	Peroxisome proliferator-activated receptor
PS	Phosphatidylserine
PSS	Phosphatidylserine synthase
PUFA	Polyunsaturated fatty acid
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SM	Sphingomyelin
SMS	Sphingomyelin synthase
SPHK2	Sphingosine kinase 2
TBS-T	Tris-buffered saline with Tween20
TMM	Trimmed mean of M-values
TNF- α	Tumour Necrosis Factor-alpha
VIS	Visual cortex
VLDL	Very low-density lipoprotein
WKS	Wernicke-Korsakoff Syndrome
WMH	White matter hyperintensities

Terminology

Notes on terminology

Abstinent/abstinence refers to persons who used to drink alcohol but now abstain.

Alcohol refers to beverages containing ethanol or ethyl alcohol with the chemical formula C_2H_6O .

Alcoholic, when used to describe a person with an alcohol use disorder is a stigmatising term that has been superseded by person-centric syntax, however, is still currently used in this thesis in the disease context of ‘Alcoholic Liver Disease’ and in reference to the peer-support group ‘Alcoholics Anonymous’.

Alcoholism refers to the condition of people who meet the criteria for a DSM-V alcohol use disorder diagnosis or related conditions from previous DSM versions (e.g., DSM-III alcohol dependence syndrome), or equivalent ICD-11 or 10 alcohol dependence or harmful alcohol use or related conditions from previous ICD versions, or people who chronically misuse alcohol.

C, followed by a number, refers to the carbon number on a fatty acid chain, e.g., C22 refers to carbon number 22.

Neurolipidome refers to the lipid environment of the brain and nervous system, and *lipidome* refers to the lipid environment more generally.

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Chapter 1. Introduction

1.1. Alcohol

Alcohol drinking has been a cultural norm in most societies, except where prohibition is embedded within religious rules (Morris, 1998). Complex inter-related factors such as policy setting, community and family attitudes, local availability of alcohol, and social networks influence individual behaviours to drink (or abstain from) alcohol (Sudhinaraset et al., 2016). Worldwide, an estimated 57% of persons over the age of 15 consumed alcohol in 2015-16, and consumption per capita is expected to increase by 2025 in the Americas, Southeast Asia, and the Western Pacific regions (World Health Organization, 2018). In Australia, the National Drug Strategy Household Survey 2019 revealed that 77% of persons over the age of 14 consumed alcohol in the preceding 12 months, and 9.9% of this group were at risk of becoming dependent on alcohol (Australian Institute of Health and Welfare, 2020). The number of deaths due to alcohol in Australia rose by 7.4% between 2020 and 2021 from 1452 to 1559 (Australian Bureau of Statistics, 2021). The World Health Organization attributes 5.1% of the global burden of disease to alcohol consumption, which includes cardiovascular and infectious diseases, cancers, liver cirrhosis, and

acute injury (World Health Organization, 2018). Chronic heavy drinking may result in a neuropsychiatric disease pertinent to this introduction, alcohol use disorder (AUD).

Alcohol misuse is a significant public health burden where 6.2% of American adults (over 18 years old) had an AUD in 2015 (Substance Abuse and Mental Health Services Administration (SAMHSA), 2016). AUDs are psychosocial behaviours characterised by problem drinking and are classified by criteria listed in the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (American Psychiatric Association, 2013). The DSM-V criteria amalgamated the prior *alcohol abuse* and *alcohol dependence* diagnoses into the current *alcohol use disorder* diagnosis. Criteria to meet this diagnosis stems from a list of 10 behavioural, social, or physical symptoms and is sub-classified into mild (presence of 2-3 symptoms), moderate (presence of 4-5 symptoms), and severe (presence of 6 or more symptoms) (Higgins-Biddle and Babor, 2018). The odds ratio for the number of days drinking per month and self-reported problem drinking increases as the classification of AUD diagnosis increases from mild to severe (Mannes et al., 2021). Although the diagnosis of an AUD does not explicitly consider pathology associated with sustained and chronic heavy drinking, such as ARBI or alcoholic liver disease (ALD), the pathogenesis of these alcohol-related diseases is dose-dependent (Becker et al., 1996, Daviet et al., 2022, Kril et al., 1997).

1.2. Alcohol-related brain damage

The effects of chronic heavy drinking on the nervous system are widespread, with some neural structures more vulnerable to the effects of alcohol than others. Brain white matter is a significant target of chronic alcohol toxicity. White matter hyperintensities (WMH), represented by high-signal areas seen on T2-weighted magnetic resonance imaging (MRI) images and are white matter lesions thought to be due to incomplete infarction, are also observed. Moderate drinkers have less WMH than heavy drinkers and abstainers (den Heijer et al., 2004, Mukamal et al., 2001). Heavy drinkers who go on to then abstain have persisting WMH later in life (Fein et al., 2009). MRI studies have demonstrated white matter deficits in the frontal lobe, enlarged ventricles, and thinning of the corpus callosum (Kapogiannis et al., 2012, Pfefferbaum et al., 1996, Pfefferbaum et al., 1997). Similarly, post-mortem studies have found widespread white matter volume reductions in the brains of those with an AUD (de la Monte, 1988, McCorkindale et al., 2016), with substantial white matter atrophy observed in the frontal lobes (Kril et al., 1997). The cortical grey matter seems less affected, although there is some neuronal loss in the frontal lobe (Kril et al., 1997, Kril and Harper, 1989). In the hippocampus, there is white matter atrophy in the absence of grey matter loss (Harding et al., 1997), drawing attention to the specificity of alcohol toxicity to white matter. Oligodendrocytes produce and maintain the lipid-rich myelin sheathes that make up approximately 80% of white matter (O'Brien and Sampson, 1965), and so a

shift to understanding how alcohol affects oligodendrocyte function is important. Single-cell transcriptomics in the AUD post-mortem brain has shown enrichment in pathways related to neuroinflammation specifically in astrocytes, microglia, and oligodendrocytes (Brenner et al., 2020).

Behaviourally and clinically, these structural changes relating to ARBI present as impairments in episodic memory (Fama et al., 2021), problem-solving skills (Sullivan et al., 1993), and impulsivity control (Bjork et al., 2004); as well as impaired motor control (Parsons et al., 1972) including impaired upper limb coordination (Johnson-Greene et al., 1997) and cerebellar ataxia (Fitzpatrick et al., 2012), as well as impaired visuospatial abilities (Beatty et al., 1996).

1.2.1. Wernicke-Korsakoff syndrome

ARBI is due to both the direct effects of alcohol on the brain and indirect effects including malnutrition and liver damage (Butterworth, 1995, Kril, 1995). Wernicke-Korsakoff syndrome (WKS) comprises two neuropsychiatric disorders that often occur together in some AUD patients. The first disorder is considered the acute phase of the syndrome: Wernicke’s encephalopathy, which results from thiamine [vitamin B1] deficiency (Sechi and Serra, 2007). This leads to brain damage, including atrophy of the mammillary bodies involved in memory processing, the cerebellar vermis, and the mediodorsal thalamic nucleus (Victor et al., 1971). Untreated, Wernicke’s encephalopathy can progress to the second disorder of WKS, Korsakoff’s syndrome, which is considered to be the chronic

phase, and characterised by anterograde and retrograde amnesia (Arts et al., 2017). The prevalence of WKS is not well established due to underdiagnosis; however, prior to mandating thiamine supplementation in baking flour, an Australian prospective autopsy study suggested a prevalence of up to 2.1% in people over 15 years old (Harper et al., 1989). Since the introduction of thiamine supplementation to baking flour the prevalence of WKS in the context of AUD appears to be uncommon in Australia, but thiamine supplementation to those with an AUD is still recommended (Harper, 2006).

Thiamine is essential in neurons and glial cells for biochemical processes such as lipid (Volpe and Marasa, 1978) and carbohydrate metabolism (Gralak et al., 2019). Important factors leading up to the deficiency in thiamine in AUD are poor nutrition, decreased absorption in the small intestine, and a lack of adequately functioning enzymes that use thiamine (due to a concurrent magnesium deficiency) (Martin et al., 2003). Depending on the severity of the syndrome, cessation of alcohol consumption, and treatment with thiamine supplementation can improve the cognitive status in some individuals and stop the progression of brain damage (Bilici et al., 2015); however, this is often limited and requires additional rehabilitation (Oudman et al., 2022).

1.2.2. Hepatic encephalopathy

Hepatic encephalopathy (HE) is a neuropsychiatric disease resulting from liver dysfunction, which can be due to either acute or chronic liver failure, the latter

resulting from liver cirrhosis (Ferenci et al., 2002). Clinically, there are four stages of neurological or psychological presentations, from mild stage one symptoms, which include attention deficits, sleep disturbances, and confusion, to the most severe stage four, which presents as a comatose state and then death (Ferenci, 2017).

The pathogenesis of HE implicates multiple factors, the most characterised being hyperammonemia, which arises due to impaired liver function. In individuals with liver cirrhosis, the liver has a significantly reduced capacity to convert ammonia produced by the gastrointestinal tract (enterocytes and colonic bacteria) into urea (Romero-Gomez et al., 2015). Systemic ammonia, as gaseous NH_3 , can readily cross the blood-brain barrier (BBB) (Lockwood et al., 1980). In the brain, ammonia is taken up by astrocytes, where it is converted into glutamine via glutamine synthetase (Suarez et al., 2002). Astrocytes take up excess ammonia in hyperammonemia, resulting in excessive glutamine generation. This causes changes in osmolarity; the astrocyte swells and becomes what is known as an *Alzheimer type II astrocyte* (Swaminathan et al., 2018). The increased glutamine also results in oxidative stress due to mitochondrial dysfunction (Jayakumar et al., 2004). Other important factors for the pathogenesis of HE include systemic inflammation, which progresses HE to the severe stages of the disease (Vaquero et al., 2003, Shawcross et al., 2011). These pathological hallmarks appear similar irrespective of alcoholic or non-alcoholic liver causes of end-stage liver disease (Kril and

Butterworth, 1997), and differentiation between the two require further clinical investigation (Toshikuni et al., 2014).

1.2.3. Inflammation and neuro-immune responses

Acetaldehyde, a by-product of ethanol metabolism, increases gut permeability by disrupting the tight junctions of the gut epithelium, which allows gut-derived lipopolysaccharide (LPS) to enter the circulatory system (Rao et al., 2004). In healthy individuals, LPS is cleared by several non-immune mechanisms, including uptake into the liver (Yao et al., 2016). There is a sustained increase in plasma LPS levels in patients with ALD compared to non-alcoholic cirrhotic patients (Fukui et al., 1991). LPS stimulates the upregulation of proinflammatory cytokines such as Tumour Necrosis Factor- α (TNF- α) and interleukin-1 (IL-1) in the liver (Rao et al., 2004), serum and brain (Qin et al., 2008). These proinflammatory cytokines, along with reactive oxygen species (ROS) (a by-product of ethanol metabolism), activate ‘nuclear factor kappa-light-chain-enhancer of activated B cells’ (NF- κ B), a first-response transcription factor that coordinates innate and adaptive immunity (Gloire et al., 2006). There is growing evidence that these neuro-immune processes contribute to the development of addiction itself (Crews et al., 2011, Coleman and Crews, 2018) via the role of NF- κ B in mediating the rewarding properties of alcohol and its role in long-term potentiation (Nennig and Schank, 2017). Additionally, NF- κ B upregulates the activity of nicotinamide adenine dinucleotide phosphate

(NADPH) oxidases in microglia (Qin and Crews, 2012) and subsequent ROS production, creating a possible positive feedback loop (Anrather et al., 2006).

Microglial activation is also a feature of neuroinflammation in the neuropathology of ARBI (Kalinin et al., 2018). Microglia can be activated through Toll-like receptor 4 by LPS, pro-inflammatory cytokines, and directly by alcohol (Alfonso-Loeches et al., 2010). This, in turn, activates NF- κ B signalling pathways, where many genes, including genes for pro-inflammatory cytokines, are transcribed, translated, and released, perpetuating the inflammatory cycle.

1.3. Alcoholic liver disease

ALD is a spectrum of liver injury that results from chronic alcohol misuse. ALD can be grouped histologically (Sakhuja, 2014, Theise, 2013) into alcoholic steatosis, where lipid droplets accumulate in hepatocytes; alcoholic hepatitis, where liver injury is also observed, such as neutrophil-rich inflammation, hepatocyte ballooning, or apoptosis; alcoholic cirrhosis, where fibrous tissue continues to replace damaged tissue and nodules are formed; and hepatocellular carcinoma, where the incidence is 8% in patients with alcoholic cirrhosis and without hepatitis B or C (Fattovich et al., 2004).

Alcoholic steatosis results from multiple factors, including increased hepatic lipogenesis (You et al., 2002), reduced lipid catabolism (Osna et al., 2017), and defective lipid export (described in Section 1.3.1). Lipid droplets in

hepatocytes mainly comprise neutral lipids surrounded by a glycerophospholipid monolayer (DiAugustine et al., 1973), which are continually remodelled in response to cellular energy demands (Olzmann and Carvalho, 2019). The ratio of surface phosphatidylcholine (PC) to phosphatidylethanolamine (PE) decreases in rat models of alcoholic steatosis, which allows lipid droplets to fuse to form larger lipid droplets (Listenberger et al., 2018). The clinical significance of larger lipid droplets is currently unknown but has been shown to induce mechanic stress on the cell nucleus, potentially altering gene expression (Chin et al., 2020).

Systemic inflammation is important for the progression of steatosis to cirrhosis, which is driven by a complex interplay of various factors, including the activation of stellate cells (Fujita and Narumiya, 2016, Gao and Tsukamoto, 2016), oxidative stress (Vairappan, 2015), infiltrating macrophages, and angiogenesis (Ramirez-Pedraza and Fernandez, 2019). These factors support the development of fibrosis, which replaces healthy liver tissue, thereby reducing liver function (Louvet and Mathurin, 2015). The effects of liver cirrhosis on the brain was described previously in Section 1.2.2.

1.3.1. Lipid metabolism in alcoholic liver disease

The liver is central to lipid metabolism. Fatty acids are accreted in the liver partly via *de novo* synthesis and more significantly through uptake from the blood and chylomicron remnants via fatty acid transport proteins (Mashek,

2013). In chronic alcohol consumption, the supply of fatty acids to the liver is increased due to lipolysis of adipose tissue (Leggio et al., 2009, Zhong et al., 2012a) and chylomicron remnants from enterocytes (Lieber and Savolainen, 1984). This, along with CD36 expression, which also increases, promotes hepatic steatosis (Zhou et al., 2008, Clugston et al., 2014). Further exacerbating hepatic lipid accumulation, the capacity of fatty acid catabolism is decreased, most significantly due to the inhibition of mitochondrial β -oxidation (Blomstrand et al., 1973). Neutral lipids (e.g., cholesterol and triacylglycerol) are exported from the liver by very low-density lipoproteins (VLDL); however, the secretion (Venkatesan et al., 1988) and production (Kharbanda et al., 2007) of VLDL is impaired when the liver is chronically exposed to ethanol. This is due to ethanol's effect of reducing S-adenosyl methionine availability for phosphatidylethanolamine methyltransferase (PEMT), which is required for VLDL synthesis (Noga et al., 2002). Additionally, PEMT is involved in one of the PC synthesis pathways (by conversion of PE to PC), which is also therefore reduced (Kharbanda et al., 2007). Lastly, *de novo* synthesis of ceramide is increased in the liver in ALD (Longato et al., 2012), which promotes oxidative stress and disrupts β -oxidation of fatty acids, exacerbating ALD.

1.3.2. Gene expression related to lipid metabolism

Rodent models have shown that alcohol consumption can affect gene expression related to lipid metabolism. Justice et al. (2019) demonstrated in a 'moderate', intermittent ethanol exposure model an association with a decrease in genes

associated with cholesterol synthesis and an increase in paraoxonase-1, an inhibitor of LDL-cholesterol oxidation. On the other hand, in chronically ethanol-fed rodents, there is an observed increase in hepatic accumulation of fatty acids and fatty acid ethyl esters, a hallmark in hepatic steatosis (Clugston et al., 2011). This was associated with a decrease in gene expression related to fatty acid oxidation (Cpt1a and Acsl1), demonstrating that chronic ethanol exposure disrupts normal fatty acid metabolism in the liver, which is important for developing hepatic steatosis.

1.4. Biological lipids

Lipids are essential to life. In biology, lipids play roles as integral structural building blocks, such as those in the cell membrane and sub-cellular organelles (Piomelli et al., 2007), as energy storage, as signalling molecules (Bazan, 2005), and they contribute to protective mechanisms such as mitigating oxidative damage (Achanta and Rae, 2017). In modern eukaryotic cells, chemical and compositional diversity depend on the cell type, their location, and function – attributed to billions of years of evolution (Gould, 2018). Chemical diversity refers to the characteristics of each lipid molecule's net charge, lipid backbone and fatty acid chain(s) composition. These factors affect lipid-lipid and lipid-protein interactions. Compositional diversity refers to the collective proportions of types of lipids that make up a membrane. This affects membrane curvature, fluidity, and membrane order/disorder (Harayama and Riezman, 2018). The

compartmentalisation that lipid membranes provide is crucial for the survival of the cell and organism. In disease states where this diversity is disrupted, membrane integrity is compromised, and cell survival threatened. Lipids are generally defined as molecules that are soluble in nonpolar solvents (McNaught and Wilkinson, 1997). There are several classes of biological lipids: e.g., fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides; and lipids may belong to multiple classes. Table 1.1 provides examples of lipids from each category and an attributable function.

Table 1.1. Lipid classes and examples

Class	Example lipid	Example function*
Fatty acyls	Docosahexaenoic acid	Cell membrane structure
Glycerolipids	Triglyceride	Energy storage
Glycerophospholipids	Phosphatidylcholine	Cell membrane structure
Sphingolipids	Ceramide	Apoptosis
Sterol lipids	Cholesterol	Cell membrane fluidity
Prenol lipids	Squalene	Steroid hormone precursor
Saccharolipids	Ganglioside	Cell signalling
Polyketides	Coenzyme Q10	Electron transport chain

**Lipids can have numerous functions, e.g., ceramide has structural and signalling functions.*

Lipids are synthesized through a series of organelle-specific steps. Enzymes that synthesise lipid precursor molecules, such as fatty acid synthase and acetyl-CoA carboxylase, are found in the cytosol. In contrast, most complex lipid synthases are in the endoplasmic reticulum and, to a lesser extent, the mitochondria, Golgi apparatus, and peroxisomes (Bell et al., 1981). Most polyunsaturated fatty acids (PUFAs) used in the brain are transported from

the gut (dietary intake), via lipoproteins or albumin that are synthesized in the liver (Horrocks and Yeo, 1999). In the brain, astrocytes can synthesise long-chain polyunsaturated fatty acids *de novo*, however, their capacity is extremely limited and of insufficient quantity to meet demands (Williard et al., 2001, Bazinet and Laye, 2014). Monounsaturated and saturated fatty acids primarily come from dietary sources, however, the liver and brain are also capable of *de novo* synthesis (Alves-Bezerra and Cohen, 2017). Other lipid precursors such as glycerol are obtained from diet and *de novo* glycolysis and glyceroneogenesis pathways, and serine can also come from both dietary and *de novo* sources (Holecek, 2022). Choline and ethanolamine come primarily from the diet (*de novo* synthesis is via catabolism of phospholipids) (Zeisel and da Costa, 2009, Gibellini and Smith, 2010).

1.4.1. Glycerophospholipids

Glycerophospholipids are a class of lipids with a polar phosphate head group attached to two nonpolar fatty acid (acyl) chains via a glycerol backbone (Figure 1.1). These lipids constitute the bulk of cell membranes, including neurons. The net charge of the phosphate head group influences water solubility, and the variable length of the fatty acid chains and degree of unsaturation (i.e., double bonds) are significant determinants of the membrane physiochemical properties. The glycerol-3-phosphate backbone is produced either by phosphorylation of glycerol, by glycerol kinase, or by reduction of

dihydroxyacetone-3-phosphate by glycerol-3-phosphate dehydrogenase. Fatty acid chains and the phosphate head group are linked to the glycerol backbone via ester bonds; however, as in the case of plasmalogens, the *sn*-1 fatty acid chain is linked to the glycerol backbone via an ether bond, with the *sn*-2 and the head group linked via ester bonds. The glycerophospholipids are PC, PE, phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidic acid (PA).

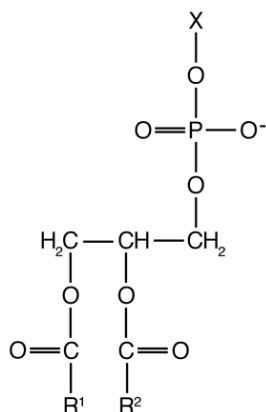


Figure 1.1. Generalised glycerophospholipid structure, with a phosphate headgroup and two fatty acid chains linked to the glycerol backbone

The main intermediary precursor to all glycerophospholipids is PA, which is formed by either 1) phospholipase D, which removes the headgroup from PC via hydrolysis; 2) phosphorylation of diacylglycerol (DAG); or 3) acylation of lysophosphatidic acid. This third method is most common: one of four glycerol-3-phosphate acyltransferases acylates the *sn*-1 position to form lysophosphatidic acid, followed by one of five acyl-CoA:lysophosphatidic acid

acyltransferases that acylates the *sn*-2 position of the glycerol backbone. The resulting PA acts as both a signalling molecule to recruit, activate, and inhibit proteins (Tanguy et al., 2019); but also as a precursor for GL synthesis. PA is either converted by phosphatidic phosphatase into DAG as a substrate in the Kennedy pathway for PC, PE, and subsequent PS synthesis; or cytidine diphosphate diacylglycerol (CDP-DAG) by phosphatidate cytidyltransferase 1 (CDS1) for PI and PG synthesis (Figure 1.2).

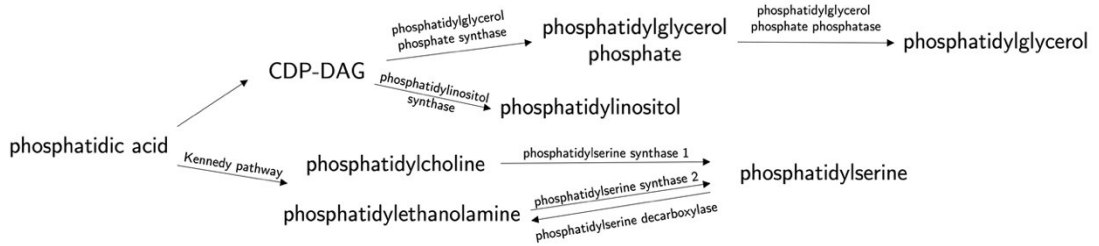


Figure 1.2. Glycerophospholipid synthesis pathways

The Kennedy pathway describes the synthesis of PC and PE (Kennedy and Weiss, 1956) – two highly abundant glycerophospholipids that account for approximately 18% of the total dry weight of both white and grey matter in the brain (O'Brien and Sampson, 1965). There are two branches of the Kennedy pathway, known as the CDP-choline pathway and the CDP-ethanolamine pathway. These branches are a series of three analogous steps. The first step phosphorylates choline or ethanolamine to phosphocholine or phosphoethanolamine, respectively. The second step uses cytidine triphosphate

to create cytidine-diphosphocholine (or cytidine-diphosphoethanolamine), catalysed by CTP:phosphocholine (or phosphoethanolamine) cytidyltransferase. The third is conversion to their final products (PC or PE) by adding diacylglycerol, catalysed by DAG:CDP-choline cholinephosphotransferase (or ethanolamine equivalent), summarised in Figure 1.3. PC can also be converted from PE via PEMT.

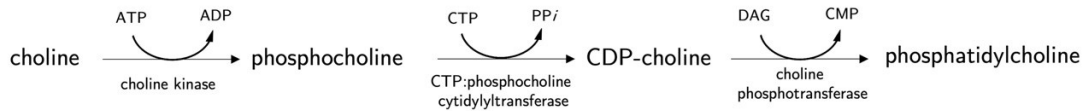


Figure 1.3. Kennedy pathway. An enzymatic three-step process from choline to phosphatidylcholine (or equivalent ethanolamine).

The CDP-DAG pathway involves PA being converted to CDP-DAG by CTP:phosphatidate cytidyltransferase (CDS) in the endoplasmic reticulum (ER) for PI and PG synthesis (there are two isoforms of this enzyme, its specificity relates to the acyl chain composition of the PA molecule) (Blunsom and Cockcroft, 2020), or TAMM41 in the mitochondria for PG synthesis. Following conversion to CDP-DAG, for PI synthesis, phosphatidylinositol synthase exchanges the CMP for an inositol molecule resulting in PI. For PG synthesis, which occurs in the inner mitochondrial membrane, CDP-DAG is converted to phosphatidylglycerol phosphate via phosphatidylglycerolphosphate synthase and then to PG via protein tyrosine phosphatase mitochondrial 1

enzyme. PG can be further metabolised to cardiolipin via cardiolipin synthase (Blunsom and Cockcroft, 2020).

PS synthesis in the brain occurs in microsomes by replacement of the choline or ethanolamine headgroups with serine from PC and PE by phosphatidylserine synthases (PSS1 for PC to PS; PSS2 for PE to PS). Serine comes either from the circulation as it can cross the BBB (O'Kane et al., 2004) or it can be derived by glycolysis in astrocytes (Hirabayashi and Furuya, 2008). Deletion of both PSS1 and PSS2 is embryonically lethal in mice, but deletion of either one alone does not affect PS levels in the brain (or other tissue organs) (Arikketh et al., 2008, Steenbergen et al., 2006). PS can also be converted back into PE by phosphatidylserine decarboxylase.

Phospholipases hydrolyse GL bonds at specific sites. There are four groups: phospholipase A, B, C, and D, which are ubiquitously expressed, and each has many subtypes (Joensuu et al., 2020). Phospholipase A1 and A2 cleave fatty acid chains *sn*-1 and *sn*-2 from the glycerol backbone, whereas phospholipase B cleaves both chains. Phospholipase C cleaves between the glycerol backbone and the phosphate headgroup, and phospholipase D cleaves after the phosphate (Figure 1.4).

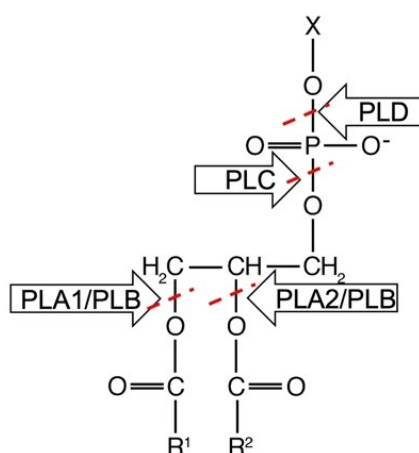


Figure 1.4. Sites of phospholipase action. The arrows indicate which bond site each phospholipase cleave.

1.4.2. Sphingolipids

In the human brain, sphingolipids typically consist of an 18-carbon dihydroxy-sphingoid base with the two hydroxyl groups attached at carbon 1 (C1) and C3 positions. This backbone can be saturated (sphinganine, *d18:0*), mono-unsaturated (sphingosine, *d18:1*), or have two double bonds (sphingadiene, *d18:2*). Ceramides consist of a sphingoid base and a fatty acid chain linked at an amine group at C2. In addition to a ceramide structure, sphingomyelin (SM) has a phosphocholine or ethanolamine group attached at C1. Whereas glycosphingolipids instead have a sugar residue (glucose or galactose) in place of the phosphor-headgroup attached at C1. A major glycosphingolipid is sulfatide, which contains a sulphated galactose.

De novo synthesis of ceramide (and ultimately other sphingolipids) begins in the ER with the condensation of palmitoyl CoA and serine via serine palmitoyltransferase to yield 3-dehydrosphinganine. 3-dehydrosphinganine is

then reduced to form dihydrosphingosine (also called sphinganine, d18:0). This sphingoid backbone is then *N*-acylated via one of six ceramide synthases, each specific to varying lengths of fatty acid chains (Table 1.2), forming dihydroceramide. Dihydroceramide is then desaturated between carbons 4 and 5 on the sphingoid backbone (to convert the base from sphinganine, d18:0, to sphingosine, d18:1) by dihydroceramide desaturase to form ceramide. These ceramides (containing a sphingosine (d18:1) backbone) can undergo further desaturation between C14 and C15 by fatty acid desaturase type 3 (FADS3) to form a sphingadiene backbone (d18:2) (Jojima et al., 2020, Karsai et al., 2020).

The amount of ceramide present in cells is tightly regulated, as ceramide can induce various inflammatory pathways (Lee et al., 2020), and too much accumulation fragments the ER and collapses the Golgi apparatus, leading to cell death (Vacaru et al., 2009). One of several things can now happen to the ceramide molecule. Ceramide can be converted to galactosylceramide (GalCer) within the ER by the addition of galactose to ceramide by UDP-galactose:ceramide galactosyltransferase (Holthuis et al., 2001). In the brain, this enzyme is primarily found in oligodendrocytes and Schwann cells (Vos et al., 1994). GalCer can then be transported to the Golgi apparatus for sulfatide synthesis (Compostella et al., 2012). For the synthesis of SM, ceramide-1-phosphate, and other glycosphingolipids, ceramide is transported from the cytosolic side of the ER to the *trans*-region of the Golgi apparatus by ceramide transfer protein (CERT) (Hanada et al., 2003), and additionally to the *cis*-

region of the Golgi apparatus via vesicles for glucosylceramide (GlcCer) synthesis (Liu et al., 2013). An isoform of sphingomyelin synthase (SMS1) is present in the Golgi and utilises phosphatidylcholine and ceramide to form sphingomyelin and diacylglycerol. SM is packaged into secretory vesicles for transport from the Golgi apparatus to the plasma membrane (Deng et al., 2016). Ceramide that reaches the *cis*-Golgi apparatus is glucosylated to produce GlcCer (Ichikawa and Hirabayashi, 1998), which in turn is galactosylated to produce lactosylceramide (LacCer). LacCer is the common precursor lipid for much more complex glycolipid synthesis (D'Angelo et al., 2013), not further discussed here. Lastly, ceramide can be phosphorylated by ceramide kinase in the Golgi apparatus to form ceramide-1-phosphate, an anti-apoptotic bioactive lipid.

Sphingolipids have a rapid turnover and can be catabolised in lysosomes by sphingomyelinases and glycosidases to revert to ceramide. Ceramidases remove the fatty acid chain to form sphingosine (Blom et al., 2012). This sphingoid backbone can then be phosphorylated by sphingosine kinase 1 (SPHK1) in the cytosol, phosphorylated by sphingosine kinase 2 (SPHK2) in the nucleus (both of which form sphingosine-1-phosphate, S1P), or recycled back into ceramide in the ER (Maceyka and Spiegel, 2014).

Table 1.2. Fatty acid chain lengths that each ceramide synthase acts upon

Ceramide synthase	Acyl chain length
CerS1	C18
CerS2	C22 C24
CerS3	$\geq$ C26
CerS4	C18 C20
CerS5	C14 C16
CerS6	C14 C16

1.4.3. Sterol lipids

Unesterified cholesterol in the brain accounts for 20% of the body's total cholesterol, the majority of which is present in myelin sheaths, and contributing approximately a quarter to the total myelin lipid pool (Norton and Poduslo, 1973). The remaining brain cholesterol is a constituent of other glial and neuronal membranes. Structurally, cholesterol comprises four hydrocarbon rings, a hydroxyl group, two methyl groups, and a hydrocarbon tail. The esterified cholesterol (cholesteryl ester, CE) portion makes up about 1% of total cholesterol in the brain, which are cholesterol molecules with a fatty acid chain esterified to a hydroxyl group.

Almost all brain cholesterol comes from *de novo* synthesis due to the inability of lipoproteins to cross the BBB. Cholesterol synthesis is at its highest during brain development when most myelination occurs. There is significantly less production in the adult brain due to the low turnover rate of brain

cholesterol, as the half-life for brain cholesterol is estimated to be five years, compared to a few days in blood plasma (Bjorkhem et al., 1998). There are two pathways of cholesterol synthesis: the Kandutsch-Russel pathway and the Bloch pathway. Both pathways begin with the eight-step mevalonate pathway, from acetyl-CoA to dimethylallyl pyrophosphate, via HMG-CoA. There are six further steps until we reach lanosterol, where the pathway diverges into the Kandutsch-Russel and Bloch pathways. These pathways have several more steps before converging at the cholesterol product (Singh et al., 2013). There is evidence that neuronal cholesterol synthesis favours the Kandutsch-Russel pathway, and astrocytes favour the Bloch pathway as those cell types predominantly contain intermediaries of those respective pathways (Nieweg et al., 2009). Neurons require more cholesterol than they can produce to maintain their long axons, extensive dendritic arbours and synapses, and therefore rely on (over)production from astrocytes (Nieweg et al., 2009). Excess cholesterol is stored as CE by adding a fatty acid chain to cholesterol via acyl-CoA:cholesterol acyltransferase (ACAT1) activity, located in the ER. CEs coalesce in the cytosol and are hydrolysed as needed to maintain homeostasis (Petrov et al., 2016).

The amount of cholesterol in a cell is tightly regulated, with mechanisms existing to remove excess cholesterol. These are esterification by ACAT1, previously mentioned; excretion by ABC transporters; and conversion to oxysterol, a bioactive molecule with signalling functions implicated in both oxidative stress and neuroinflammation (Weigel et al., 2019). ABC transporters

expressed in neurons transport cholesterol out of neurons and onto APOA1-containing lipoproteins in the cerebrospinal fluid (CSF), where two receptors in the endothelium of the CSF-blood-barrier – LRP1 and scavenger receptor class B1 – remove these lipoproteins (Zhang and Liu, 2015). Conversion of cholesterol to 24-hydroxysterol via cholesterol 24-hydroxylase is a major excretory pathway, as unlike cholesterol, it can readily cross the BBB by diffusion to leave the brain. The activity of 24-hydroxylase is mostly in neurons, not glia. Some cells, such as cortical pyramidal cells, cerebellar Purkinje cells, and some thalamic neurons, have higher activity of these cholesterol removal mechanisms, suggesting that they are particularly sensitive to overaccumulation of cholesterol (Lund et al., 2003). Experiments that reduce or deplete cholesterol in neuronal cell culture or mouse forebrains result in impaired vesicular exocytosis at synapses onto dendritic spines, and eventual synapse degeneration (Liu et al., 2010, Linetti et al., 2010).

Alcohol intake affects the levels of lipoproteins, which transport cholesterol (and triglycerides) in the blood. Specifically, high-density lipoprotein (HDL) transports cholesterol from tissues to the liver for excretion, and low-density lipoproteins (LDL) transport cholesterol from the liver to tissues (Feingold, 2022). Moderate alcohol intake increases HDL levels (De Oliveira et al., 2000), which is associated with being cardioprotective (Minzer et al., 2020). Conversely, higher alcohol intake is linked to unfavourable lipoprotein profiles, characterised by increased LDL and decreased HDL levels (Capurso and

Petrakis, 2016, Hao et al., 2015). However, it is important to consider the broader health implications instead of changes in individual factors such as increased HDL. A recent study using data from the UK biobank study showed that even low drinking may increase the overall risk of cardiovascular events (Schutte et al., 2022). So any health benefit may be outweighed by the risks (Chiva-Blanch and Badimon, 2019).

In the context of managing lipoprotein levels, statins, explored in Chapter 5 for their anti-oxidant properties, are commonly prescribed to lower LDL (Cholesterol Treatment Trialists et al., 2012). Statins increase HDL (McTaggart and Jones, 2008) and thereby restore a more favourable lipoprotein ratio. A key regulator of LDL levels is proprotein convertase subtilisin/kexin type 9 (PCSK9), which mediates the degradation of LDL receptors in the liver, thereby reducing the liver's ability to sense and clear LDL cholesterol (Horton et al., 2007, Lagace, 2014). Alcohol intake has been shown to methylate the PCSK9 gene, increasing gene expression and plasma PCSK9 protein levels (Lohoff et al., 2018). This exacerbates liver inflammation as well as neuroinflammation (Lee et al., 2021). PCSK9 inhibitors reduce neuroinflammation (Apaijai et al., 2019) and, therefore, may be a therapeutic target in AUD (Lee et al., 2021).

1.5. Brain lipids and alcoholism

The overall lipid content of white and grey matter is approximately 60% and 40% by dry weight, respectively (O'Brien and Sampson, 1965). Ethanol exerts direct and indirect effects on lipids in the brain. The effect ethanol has on cell membranes changes over time, depending on whether ethanol exposure is acute or chronic. Ethanol is a small amphipathic molecule that can freely cross the BBB (Wong et al., 2013). In the brain, ethanol interacts with cell membranes by forming hydrogen bonds inside the lipid bilayer (Patra et al., 2006). Acutely, this increases membrane fluidity as phospholipids are less tightly packed. Over time, cell membranes exposed to ethanol have less fluidity (Toth et al., 2014). This is due to indirect mechanisms changing the lipids' chemical composition, with a higher proportion of saturated fatty acids (Goldstein, 1986).

Myelin are specialised ensheathments that extend from oligodendrocytes to insulate axons. It comprises approximately 80% lipid by dry weight and lends its colour to white matter (O'Brien and Sampson, 1965). The number of oligodendrocytes in the adult brain appears to be stable (Yeung et al., 2014), although there is evidence in the adult brain that oligodendrocyte precursor cells (OPC) continue to divide and mature into oligodendrocytes (Young et al., 2013, Roy et al., 1999), and that exercise and learning can remodel white matter (Scholz et al., 2009). In rodent models of chronic alcohol exposure, there was a significant decrease in fluorescent labelling of new myelin but no change in OPC density or apoptosis (Guo et al., 2021).

1.5.1. Lipid peroxidation

In addition to the direct interactions between ethanol and lipids in the brain, products of ethanol metabolism are known to interact with lipids. Alcohol dehydrogenase (ADH) activity is low in the brain, and ethanol here is metabolised to acetaldehyde via both catalases in peroxisomes and through cytochrome P450 2E1 (CYP2E1), also known as the microsomal ethanol oxidising system (Zakhari, 2006). CYP2E1 associates with NADPH-cytochrome P450 reductase to catalyse the two-electron oxidation of ethanol to acetaldehyde or the one-electron oxidation to both acetaldehyde and 1-hydroxyethyl free radical metabolites. CYP2E1 has NADPH oxidase activity which transfers one electron from NADPH to oxygen to form NADP^+ and water (Figure 1.5a). Ethanol treatment in neuronal and glial cultures increased CYP2E1 activity (Kapoor et al., 2006), as well as in the brain of rodent models (Zhong et al., 2012b). However, catalysis by this isoform of cytochrome P450 is not usually coordinated and therefore creates a superoxide radical (Figure 1.5b):

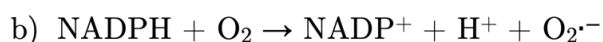
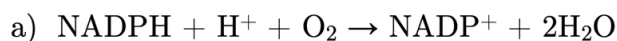


Figure 1.5. Production of a superoxide radical.

Superoxide dismutase can reduce superoxide ($\text{O}_2^{\cdot-}$) to form peroxide ($\text{O}_2^{\cdot 2-}$), which can readily accept two hydrogens to form hydrogen peroxide (H_2O_2).

Excess superoxide radicals can also donate their electron to a metal ion, which catalyses the conversion of hydrogen peroxide to hydroxyl radicals ($\text{HO}\cdot^-$) (Koop, 2006). These free radicals initiate lipid peroxidation by taking an electron from fatty acids, forming fatty acid radicals. Specifically, polyunsaturated fatty acids contain double bonds, and the hydrogen atoms between two double bonds are highly reactive and susceptible to free radical attack (Hernandez et al., 2016). Fatty acid radicals are unstable, so a chain reaction of fatty acid radical production begins. Lipid peroxides are also produced in the process. The by-products of lipid peroxidation are reactive aldehydes: malondialdehyde and 4-hydroxynonenal (4-HNE). For example, cardiolipin is a phospholipid with four fatty acid chains and is a major constituent of the inner mitochondrial membrane. Alcohol-induced oxidative stress reduces cardiolipin (Reddy et al., 2013) and increases 4-HNE, both of which are associated with neuronal cell death (Liu et al., 2011, Abarikwu et al., 2012). Additionally, oxidative stress in OPCs disrupts differentiation into mature oligodendrocytes (Spaas et al., 2021, French et al., 2009) and in primary culture, can lead to oligodendrocyte death (Jana and Pahan, 2007).

Other sources of oxidative stress that can result in lipid peroxidation are the neuroinflammatory responses that are activated in response to chronic alcohol intake. In particular, the activation of microglia and astrocytes produce ROS through NADPH oxidase upregulation (Qin and Crews, 2012).

There have been several candidate anti-oxidant compounds as potential therapeutic interventions to treat alcohol-induced oxidative stress (Tsermpini et al., 2022), including N-acetyl cysteine (Tomko et al., 2018), cannabidiol (De Ternay et al., 2019), and lobeline (da Costa et al., 2018). Statins have also been shown to possess anti-oxidant activity (Davignon et al., 2004). This effect, previously not explored in ARBI, will be investigated in Chapter 5.

1.5.2. White matter recovery

The cessation of alcohol consumption leads to the recovery of certain cognitive domains, including attention, memory, and reaction times. However, it does not appear to have the same restorative effect on spatial processing (Fein et al., 2006). Imaging studies have shown white matter microstructural improvements and volume recovery from one month of cessation of drinking, which continues over the long-term, and fractional anisotropy measurements approaching normalcy (Gazdzinski et al., 2010, Pfefferbaum et al., 2014). Rodent models of alcohol dependence show that abstinence following chronic ethanol exposure results in increased dendritic arborisation of pyramidal neurons and increased myelin basic protein in the prefrontal cortex (Navarro and Mandyam, 2015), and that after cessation of drinking in the short-term, partial recovery of altered lipid expression is seen (Yalcin et al., 2017).

Tobacco smoking is often seen in people with an AUD (Bobo and Husten, 2000). This behaviour interacts with white matter atrophy and recovery. In a

chronic alcohol exposure rodent model, it was found that nicotine-derived nitrosamine ketones in tobacco smoke reduced myelin sheath thickness. In comparison, alcohol-only treated rodents showed a decrease in unmyelinated fibre density (Papp-Peka et al., 2017). However, this doesn't appear to contribute to the overall white matter atrophy (McCorkindale et al., 2016). Smoking does however alter the pattern of white matter recovery (Gazdzinski et al., 2010).

The exact mechanism for white matter recovery during abstinence is unclear, however, it is probable that by removing oxidative stress, OPCs can differentiate into mature oligodendrocytes, and normal lipid synthesis is restored.

1.5.3. Lipidomics

Despite knowing that lipid-rich white matter is a significant target of chronic alcohol exposure, few studies have investigated how this changes lipid profiles more broadly. Lipidomics is the characterisation of the lipidome within a particular system. It includes the quantitation of different lipid species and classes, their associated molecular pathways in which they are synthesised and degraded, and mechanisms through which they carry out their functions or interact with other cellular components. Lipidomics research typically uses analytical techniques, such as liquid chromatography/mass spectrometry (LC/MS) or matrix-assisted laser desorption/ionisation imaging mass

spectrometry (MALDI-IMS). MALDI-IMS is a useful technique that combines spatial information from a tissue section with mass spectrometry. This thesis was originally going to use MALDI-IMS as the primary lipidomic technique, where I had contributed to method development in four publications (O'Rourke et al., 2018, O'Rourke et al., 2019, O'Rourke et al., 2021, O'Rourke et al., 2019). However, I decided to switch to LC/MS, as this would provide better resolution data, where individual fatty acid chains are able to be resolved, and the spatial information gained from MALDI-IMS beyond grey and white matter portions would be of limited value. Figure 1.6 shows early results obtained from this thesis (but not included in this thesis), which depicts where each ion (m/z value) is represented spatially on the orbitofrontal cortex section from a control female. Databases such as LIPID MAPS^{®1} are useful for the identification of each ion but are not definitive, and quantitation still remains a challenge (Tobias and Hummon, 2020).

¹ <https://www.lipidmaps.org>

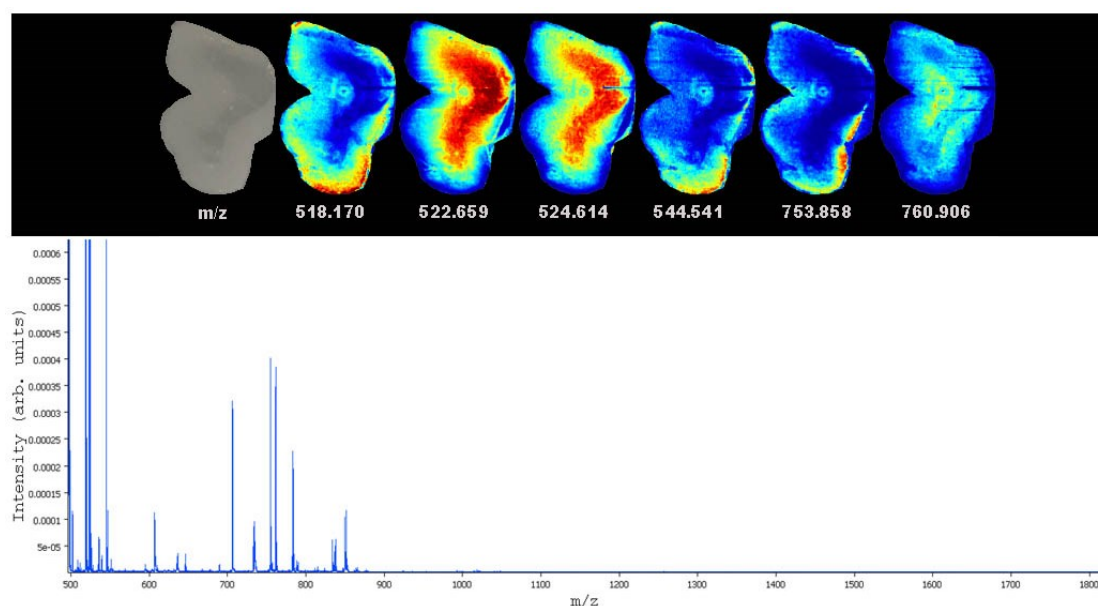


Figure 1.6. MALDI-IMS results output of lipid-related ions in an orbitofrontal section.

LC/MS separates complex mixtures of lipids via liquid chromatography, and then via electrospray ionisation, the eluant from the liquid chromatography column is analysed via mass spectrometry.

Earlier lipidomic studies in AUD models focused on the effect alcohol has on the fluidity of the cell membrane (Harris et al., 1984, Harris and Schroeder, 1981b, Johnson et al., 1979). More recently, comprehensive mass spectrometry analyses have measured how specific lipid classes are altered in chronic ethanol models. In rodents chronically exposed to ethanol for less than two months, whole-brain extracts showed that all measured ceramides were increased (Roux et al., 2015), but this did not correspond to any downstream change in GlcCer, LacCer, or sulfatide levels (Roux et al., 2016). This might suggest early neuroinflammatory responses in the aetiology of ARBI. Wang et al. (2019) looked at lipid extracts from the PFC and striatum separately after a year of

ethanol exposure in rodents. They concluded that many lipids were changed with increases in their unsaturation [i.e., more double bonds in fatty acid chains] and fatty acid chain length. There were differences between the two regions, and these changes were mostly due to altered synthesis pathways in the endoplasmic reticulum. In humans, a post-mortem MALDI-IMS study using motor and prefrontal cortex tissue from AUD and controls showed a decrease in ceramides, sulfatides, and phospholipids, in complete contrast to rodent models (de la Monte et al., 2018).

1.6. Summary

Chronic alcohol consumption is a significant health burden that has wide-ranging effects on multiple organ systems. The gut-liver-brain axis is especially affected. Systemic inflammation in response to gut bacteria is important for progressing liver pathology from steatosis to cirrhosis, through the upregulation of fibroblasts, and fibrosis replacing functional liver capacity. This, in turn, reduces the liver's ability to clear endotoxin, and metabolise alcohol and ammonia, which ultimately impairs astrocyte function in the brain. Concurrently, and in combination with the direct effects of alcohol, neurons and oligodendrocytes are affected, with the latter leading to demyelination and white matter atrophy. Studies investigating how lipids are affected in this system are conflicting. Advanced liver pathology is important for the aetiology of ARBI,

but this is rarely mentioned or addressed in chronic ethanol models and may be responsible for the discrepancy between animal and human lipidomic analyses.

1.7. Aims and hypotheses

This thesis aims to better understand the importance of lipid dysregulation in the brain in ARBI and the relative importance of liver pathology.

Specifically:

- 1) Determine if and how the brain lipidome is affected in ARBI of AUD donors with liver cirrhosis;
- 2) Investigate whether there are lipid-related gene expression changes in ARBI of AUD donors with or without cirrhosis and
- 3) Investigate the effect of statins, a potential anti-oxidant, in mitigating the degree of lipid peroxidation in ARBI.

I hypothesise that lipids will be differentially affected in different brain regions in ARBI, and that these effects are exacerbated by liver cirrhosis.

Chapter 2. Lipidome Changes in Alcohol-Related Brain Injury

The following employs mass spectrometry to investigate changes in the lipid profile associated with ARBI. While lipid-rich white matter is a prominent target of ARBI, existing studies on the specific lipid alterations have yielded conflicting results. To address this issue, we focused on three brain regions were chosen: the prefrontal cortex (PFC), middle temporal gyrus (MTG), and the visual cortex (VIS).

The selection of the PFC was based on its well-established susceptibility to ARBI. Additionally, the inclusion of the MTG region was influenced by previous findings from McCorkindale et al. (2016), which suggested trends towards volume reductions in this area. The VIS region was chosen as a comparison region because McCorkindale et al. (2016) did not report significant volume changes in this particular region.

Each of these regions was separated into white and grey matter portions. The aforementioned volumetric studies showed white matter atrophy in the PFC and trends towards white matter volume reduction in the MTG in brains with ARBI. While the PFC was known to show neuronal loss in people with an uncomplicated AUD (Krill et al., 1997, Krill and Harper, 1989), no studies have

documented pathological changes to the VIS region to date, so this region was included in this study as a comparison.

Prior studies using human tissue for lipidomic analysis in ARBI utilised MALDI-IMS, which provided spatial information on how lipids are abnormally distributed. However, mass accuracy is lower in MALDI-IMS than LC/MS methods, acyl chain lengths are unable to be determined, and relative (rather than absolute) changes in lipid quantities can be reported only. Therefore, LC/MS-MS was used here as the primary technique and using a deuterated internal standard per lipid class.

All AUD cases in this study also had liver cirrhosis, a common feature of alcoholism. The study adhered to guidelines from the Lipidomics Standards Initiative (LSI), a collaborative resource that provides minimum requirements for high-quality methods, allowing consistency across lipidomic studies.

2.1. Author attribution statement and contributions

This chapter contains the following published material:

Smith, C.C., Sheedy, D.L., McEwen, H.P., Don, A.D., Kril, J.J.,
Sutherland, G.T. (2022) *Lipidome Changes in Alcohol-Related Brain Damage*.
Journal of Neurochemistry.

CCS conceptualised, planned, and carried out all experiments, performed all data analyses, and wrote this manuscript. DLS was the NSW BTRC manager who organised cases into cohorts used in this study. HPM gave guidance on mass spectrometry operation and data analysis. ADD developed mass spectrometry methods that were used and assisted with revision reviews. JJK assisted with funding this project and reviewed manuscript drafts. GTS was the senior and corresponding author who administered and supervised this project.

Attestation

In addition to the statement above, permission to include the published material has been granted by the corresponding author.

Caine C. Smith, HDR candidate
February 2023

Greg T. Sutherland, Corresponding author
February 2023

2.2. Publication: Lipidome changes in alcohol-related brain damage

<next page>

ORIGINAL ARTICLE

Lipidome changes in alcohol-related brain damage

Caine C. Smith¹  | Donna L. Sheedy¹ | Holly P. McEwen² | Anthony S. Don^{1,2}  |
Jillian J. Kril¹ | Greg T. Sutherland¹ ¹Faculty of Medicine and Health, School of Medical Sciences and Charles Perkins Centre, The University of Sydney, Sydney, New South Wales, Australia²Centenary Institute, The University of Sydney, Sydney, New South Wales, Australia

Correspondence

Greg T. Sutherland, Faculty of Medicine and Health, School of Medical Sciences and Charles Perkins Centre, The University of Sydney, Sydney, NSW, Australia.
Email: g.sutherland@sydney.edu.au

Funding information

National Institute of Alcohol Abuse and Alcoholism, Grant/Award Number: R28AA012725.

Abstract

Alcohol-related brain injury is characterized by cognitive deficits and brain atrophy with the prefrontal cortex particularly susceptible. White matter in the human brain is lipid rich and a major target of damage from chronic alcohol abuse; yet, there is sparse information on how these lipids are affected. Here, we used untargeted lipidomics as a discovery tool to describe these changes in the prefrontal, middle temporal, and visual cortices of human subjects with alcohol use disorder and controls. Significant changes to the lipidome, predominantly in the prefrontal and visual cortices, and differences between the white and grey matter of each brain region were identified. These effects include broad decreases to phospholipids and ceramide, decreased polyunsaturated fatty acids, decreased sphingadiene backbones, and selective decreases in cholesteryl ester fatty acid chains. Our findings show that chronic alcohol abuse results in selective changes to the neurolipidome, which likely reflects both the direct effects on the brain and concurrent effects on the liver.

KEYWORDS

alcohol use disorder, alcohol-related brain injury, LCMS, lipidomics, post-mortem brain

1 | INTRODUCTION

Alcohol use disorder (AUD) is a diagnosis characterized by psychosocial behaviors, which manifest from chronic alcohol consumption. The effects of chronic alcohol consumption, often described as alcohol-related brain damage or injury, are widespread in the brain through both direct and indirect mechanisms (de la Monte & Kril, 2014; Sutherland et al., 2014). Alcohol (ethanol) is a small amphipathic molecule that readily crosses the blood-brain barrier and distributes throughout the brain by passive diffusion. Imaging, volumetric, and autopsy studies suggest that brain white matter is a major target of alcohol toxicity. This is reflected by significant atrophy or complete agenesis of white matter structures in the alcohol-exposed fetal brain (Jones & Smith, 1973; Moore et al., 2014), and decreased white matter volume in adult alcoholics, especially within the frontal lobes (Harper et al., 1985; Kril et al., 1997; McCorkindale

et al., 2016) where neuronal loss also occurs (Kril et al., 1997; Kril & Harper, 1989). In addition to the frontal cortex, volume reduction trends have been noted in the temporal pole and occipital white matter (McCorkindale et al., 2016) and imaging studies have also demonstrated white matter volume reductions in alcoholics (Zahr & Pfefferbaum, 2017). In the hippocampus, volume loss is exclusively within the white matter with no neuronal loss seen in any hippocampal subregion (Harding et al., 1997) suggesting that white matter atrophy is not secondary to neuronal loss. *In vivo* animal studies modelling alcohol-related brain damage suggests that white matter atrophy is mediated by demyelination, dysmyelination, and axonal degeneration (He et al., 2007; Papp-Peka et al., 2017). The deleterious effects of alcohol on the gross macrostructure of the brain are exacerbated by, and probably interact with, other factors that often accompany problem drinking, such as thiamine deficiency and liver damage (Kril, 1995).

Abbreviations: AUD, alcohol use disorder; Cer, ceramide; ChE, cholesteryl ester; DHA, docosahexaenoic acid; GM, grey matter; LC-PUFAs, long-chain polyunsaturated fatty acids; MTG, middle temporal gyrus; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PFC, prefrontal cortex; PI, phosphatidylinositol; PS, phosphatidylserine; RRID, Research Resource Identifier; SM, sphingomyelin; VIS, visual cortex; WM, white matter.

The human brain is a lipid-rich organ: the dry weight of grey and white matter lipid is approximately 40% and 65%, respectively (O'Brien & Sampson, 1965). *In vitro* experiments show that ethanol can form hydrogen bonds with lipids, and therefore accumulate in lipid bilayers, increasing membrane fluidity (Patra et al., 2006; Weber & de Bont, 1996). Ethanol accumulates specifically in the synaptic membrane, rather than the myelin membrane or axolemma (Harris & Schroeder, 1981). This, combined with changes in glycerophospholipid polyunsaturated fatty acids and cholesterol concentrations, contributes to synaptic fluidity (Harris et al., 1984; Patra et al., 2006).

Different lipids in the brain are synthesized through different enzymatic pathways that could be targeted therapeutically. However, there is sparse information about which lipid species are affected in the human brain because of chronic alcohol consumption and currently this is limited to imaging mass spectrometry studies (Yalcin & de la Monte, 2015; 2017; de la Monte et al., 2018) which note broad decreases in sphingolipid and glycerophospholipid species – two classes of lipids that have cell structural and signaling functions. On the contrary, rodent studies of chronic alcohol exposure suggest that glycerophospholipids are specifically increased in the prefrontal cortex and striatum (Wang et al., 2019), and that ceramides are increased and sphingomyelin is either unchanged or decreased in whole brain extracts (Roux et al., 2015, 2016).

This study aims to use untargeted lipidomics to thoroughly describe these lipid changes using post-mortem brain tissue from human subjects with AUD and controls across regions that are seemingly differentially affected by alcohol: the prefrontal cortex (PFC), middle temporal gyrus (MTG), and visual cortex (VIS).

2 | METHODS

2.1 | Human subjects

The use of human tissue for this study was approved by the University of Sydney Human Ethics Review Committee (approval number 2015/022). The NSW Brain Tissue Resource Centre obtains informed consent from each brain donor and operates in compliance

with the University of Sydney Human Research Ethics Committee (approval number 2019/531). Post-mortem unfixed, frozen brain tissue samples from 20 alcoholics that met DSM-V AUD criteria, and 20 matched controls (that were neurologically normal and had unremarkable neuropathology) were obtained from the NSW Brain Tissue Resource Centre which were prepared according to standard protocols (Sutherland et al., 2016) following approval from their Scientific Advisory Committee. G*Power 3.1 was used to calculate sample size using a two-tailed, normally distributed Wilcoxon signed-rank test for matched pairs, with a 0.5 effect size, type I error probability (α -level) 0.05, and power ($1-\beta$) of 0.85. Each tissue sample was blinded by the NSW Brain Tissue Resource Centre using a unique identification number. The mean age, gender, brain weight and volume, body mass index (BMI), post-mortem interval, brain pH, and extent of liver pathology are reported in Table 1. This study was not pre-registered.

2.2 | Tissue preparation

Unfixed, frozen human brain tissue from the prefrontal (PFC), temporal (MTG), and visual (VIS) cortices were separated into white (WM) and grey (GM) matter portions by an experienced technician at the NSW Brain Tissue Resource Centre and stored at -80°C until required for lipid extractions.

2.3 | Chemicals and internal standards

All chemicals used for LCMS were of HPLC quality. *For lipid extraction*: Ammonium acetate (Sigma-Aldrich, RRID:SCR_008988; cat. no. 73594), methanol (Merck, RRID:SCR_001287; cat. no. 1.06018), water (Merck, RRID:SCR_001287; cat. no. 1.15333), methyl-*tert*-butyl ether (Sigma-Aldrich, RRID:SCR_008988; cat. no. 34875). EquiSPLASH™ LIPIDOMIX® deuterated internal standards, a mix of internal standards used, were purchased from Avanti® Polar Lipids (RRID:SCR_016391, cat. no. 330731). *For liquid chromatography*: Isopropanol (Merck, RRID:SCR_001287; cat. no. 1.01040) and acetonitrile (AJAX FineChem™, cat. no. AJA2315).

Characteristic	Control	Alcohol use disorder	p value
Number of cases (n)	20	20	—
Age (mean $\pm$ SEM; range)	60.85 $\pm$ 2.35; 46–81	60 $\pm$ 2.54; 45–82	n.s.
Male/female	10/10	10/10	—
Brain weight (grams) (mean $\pm$ SEM)	1364 $\pm$ 29	1300 $\pm$ 32	n.s.
Brain volume (cm ³) (mean $\pm$ SEM)	1353 $\pm$ 28	1285 $\pm$ 30	n.s.
BMI (mean $\pm$ SEM)	30.5 $\pm$ 1.22	26.13 $\pm$ 1.76	n.s.
Post-mortem interval (hours)	25.98 $\pm$ 2.51	32.45 $\pm$ 3.74	n.s.
Brain pH	6.447 $\pm$ 0.07	6.439 $\pm$ 0.07	n.s.
Liver cirrhosis	0	20	—

Abbreviation: n.s., not significant.

TABLE 1 Brain donor characteristics summary

2.4 | Lipid extraction

Twenty milligrams of tissue sample underwent a biphasic lipid extraction process using methyl-*tert*-butyl ether, as described and validated in Matyash et al. (2008). Briefly, tissue was minced in 0.1% ammonium acetate in glass tubes, and then internal standards mixed with methanol were added, vortexed briefly, and then methyl-*tert*-butyl ether (MtBE) was added using a glass micropipette tip. Samples were then sonicated in an ice-cold water bath for 1 h. Water was added to separate phases, and samples were centrifuged at 1000 g for 10 min at room temperature. The top phase was kept (lipid containing) and dried in a vacuum centrifuge. Pellets were re-suspended in 300 μ L of methanol in autosampler vials for chromatographic separation.

2.5 | LCMS

Lipids were separated by liquid chromatography using a Vanquish™ UHPLC system (ThermoFisher Scientific) with an Accucore™ Vanquish™ C18+ UHPLC column 1.5 μ m, 2.1 $\times$ 100 mm (ThermoFisher Scientific; cat. 27101–102130). The mobile phase A contained 60:40 acetonitrile/water mix with 10 mM ammonium formate and 0.1% formic acid. The mobile phase B contained 90:10 isopropanol/acetonitrile mix with 10 mM ammonium formate and 0.1% formic acid. Samples were analyzed in a Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap™ mass spectrometer (ThermoFisher Scientific).

2.6 | Data analysis

Data were searched, peaks integrated, and aligned with LipidSearch™ 4.2 software (ThermoFisher Scientific). Chromatographic peak areas were normalized to tissue weight and deuterated internal standards. Deuterated internal standards of matching lipid class were of a known amount and used for the calculation of lipid concentrations. Lipids that were identified, but for which sn1 and sn2 fatty acid chains could not be resolved, were excluded from analysis. False discovery rates were calculated to determine significant differences between normalized lipid concentrations between AUD and control subjects. Volcano plots and bar graphs were constructed in GraphPad Prism 8. Regression analysis was performed to determine the relationship of level of unsaturation, and chain length with lipid concentration in AUD. Sparse partial least squares discriminant analysis (sPLS-DA) was performed on all data using MetaboAnalyst 5.0.

3 | RESULTS

This study investigated three regions known to be differentially affected by alcohol-related brain damage were in 20 AUD and 20 controls: prefrontal cortex (PFC), middle temporal gyrus (MTG), and visual cortex (VIS). These regions were further separated into white matter (WM) and grey matter (GM) components. Three general classes of lipids were quantified: glycerophospholipids (e.g., phosphatidylcholine), sphingolipids (e.g., ceramides), and sterol lipids

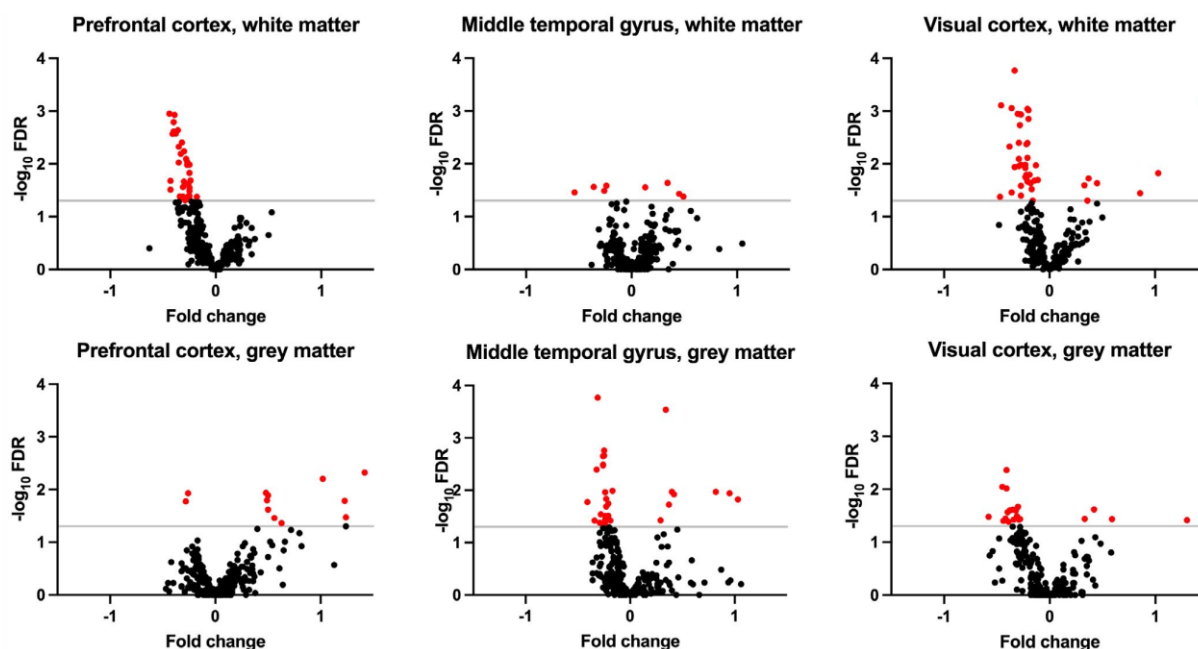


FIGURE 1 Volcano plots representing relative changes in glycerophospholipid and sphingolipid species across white and grey matter in the PFC, MTG, and VIS regions. Each point represents an individual lipid species. Points above the grey line indicate significance. Fold change is AUD relative to controls. n (subjects) = 20 per group

(e.g., cholesteryl esters). Full data of all lipid species identified in each region and their absolute concentrations are available (File S1).

The major effects of chronic alcohol consumption on lipid species in the brain were broad decreases in their concentrations. This was predominantly observed in the PFC WM, VIS WM, and VIS GM regions (Figure 1). A supervised clustering analysis, sPLS-DA, showed mostly good separation between control and AUD subjects, with minimal overlap (Figure 2). SM had the most differentiating effect in the PFC regions, PE in the MTG region, and PC in the VIS WM and Cer in VIS GM (File S2). Loadings data for the top 10 species from these analyses are also available (File S2).

3.1 | Glycerophospholipids

There were 470 unique glycerophospholipid species identified and measured across the three brain regions. Phosphatidylethanolamines (PE) were the most abundant glycerophospholipids across all WM and GM regions, followed by phosphatidylserines (PS), phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylglycerol (PG), the latter accounting for less than 2%. Graphed proportions of the

glycerophospholipids measured are available as File S3. Lysolipids and phosphatidic acid were not measured in this study. The number of glycerophospholipids affected in AUD varied according to headgroup with nearly 70% of individual PC species (PC + PC(O)) affected in the VIS GM (Table 2).

3.2 | Decreased glycerophospholipid concentrations in AUD

The total concentration of measured PCs (including those with an alkyl bond at the *sn*1 position, denoted as PC(O)) was significantly reduced in the VIS WM (−14.87%, $p = 0.0002$), and GM (−12.54%, $p = 0.044$) regions in AUD. The total concentration of measured PEs (including plasmalogens) was also significantly reduced in the VIS WM (−12.40%, $p = 0.011$) and GM (−18.59%, $p = 0.019$) in AUD (Figure 3). Additionally, total measured PI was reduced in the VIS WM (−10.27%, $p = 0.027$), PI(O) in the VIS GM (−27.14%, $p = 0.021$), PC(O) in the PFC WM (−12.77%, $p = 0.048$), and PE in the MTG GM (−16.34%, $p = 0.01$). File S1 contains a list of all lipid species detected and quantified and is highlighted where concentration significantly decreased.

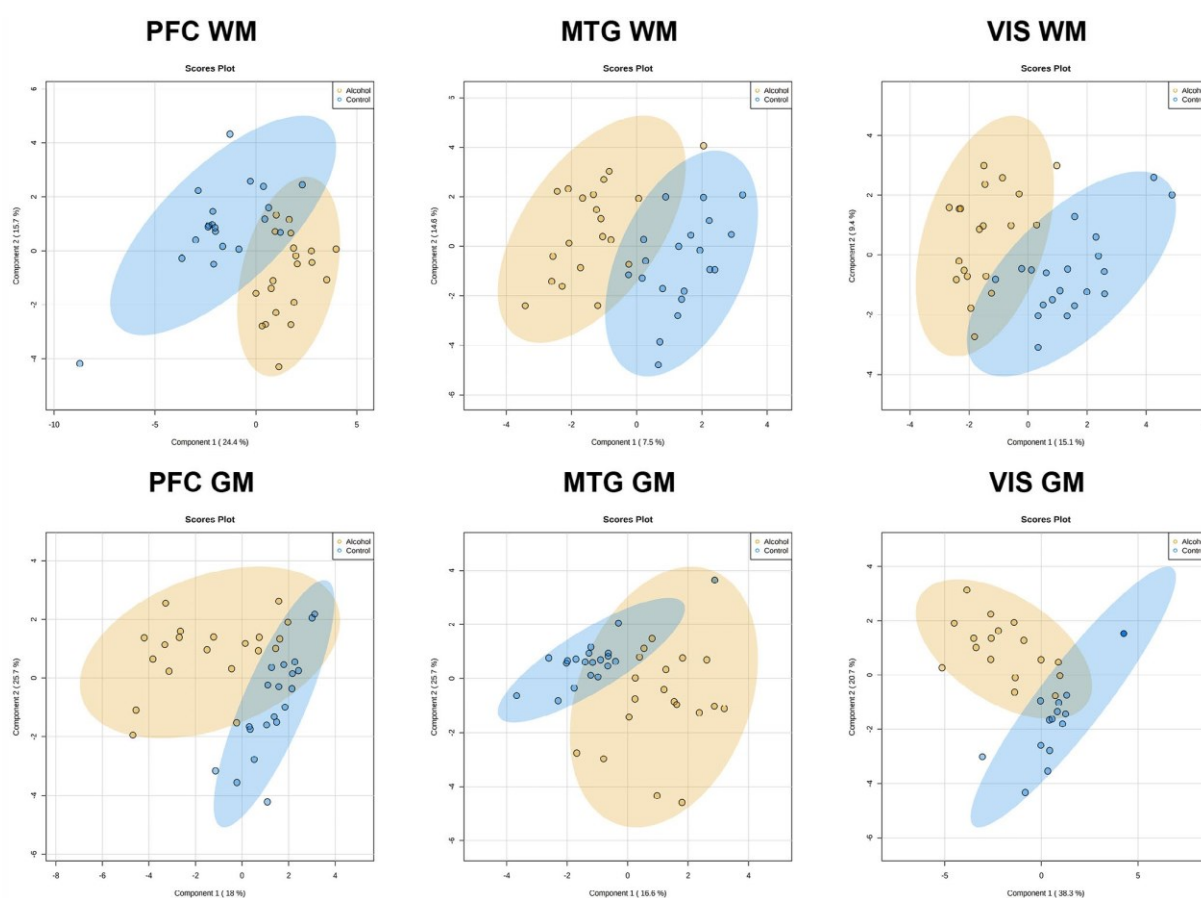


FIGURE 2 sPLS-DA of each subject. Blue coding represents control and orange represents AUD subjects. n (subjects) = 20 controls, 20 AUD)

3.3 | Significant decreases in polyunsaturated fatty acids and fatty acid chain length in phospholipids

The concentration of measured PC in AUD decreased as fatty acid chains became more unsaturated in the PFC WM ($R^2 = 0.70$, $p = 0.0003$), PFC GM ($R^2 = 0.61$, $p = 0.002$), and VIS WM ($R^2 = 0.75$, $p = 0.0003$). (Figure 4). This correlation was not observed in other headgroups or regions.

Similarly, the concentration of longer chain PCs decreased in AUD in both the PFC WM ($R^2 = 0.64$, $p < 0.001$) and VIS WM ($R^2 = 0.74$, $p < 0.0007$), and additionally longer chain PEs in MTG GM ($R^2 = 0.87$, $p < 0.0008$) and VIS GM ($R^2 = 0.84$, $p < 0.004$), and PE(O) for VIS WM ($R^2 = 0.85$, $p < 0.002$) (Figure 5). This correlation was not observed in other headgroups or region.

3.4 | Significant increases in glycerophospholipids containing 16:1 fatty acid

In contrast, approximately one third (58/150) of the measured glycerophospholipid species containing one or more 16:1 fatty acid chains increased in AUD. Glycerophospholipids that had two 16:1 fatty acids were more likely to be increased (10/14, 71%) than glycerophospholipids that had one 16:1 fatty acid (48/136, 28%) ($OR = 46.29$, $p < 0.0001$, 95% CI [16.67, 122.1]). By comparison, out of the 1544 lipid species across all regions that did not contain a 16:1 fatty acid, only 112 (7%) significantly increased.

3.5 | Sphingolipids

There were 37 unique ceramide (Cer) and 35 unique sphingomyelin (SM) species measured across all brain regions studied. Other sphingolipids were not measured in this study. The major effect of chronic alcohol consumption on sphingolipid species measured in the brain are broad reductions in concentrations of individual lipid species, however, with the exception of Cer in PFC WM and VIS GM, did not result in any net reductions.

The total concentration of Cer was significantly reduced in the PFC WM (-21.06% , $p = 0.0229$) and VIS GM (-26.85% , $p = 0.009$) in subjects with AUD (Figure 6). A full list of individual Cer and SM species that were quantified are available (File S1).

3.6 | Decreased sphingoid backbones

The most noticeable effect to sphingolipids were significant decreases in the sphingadiene (d18:2) backbone (Figure 7). This was observed in the PFC WM, MTG GM, and the VIS GM only. Sphingolipids containing sphingosine (d18:1) were lower in the PFC WM and VIS GM, while sphingolipids containing sphinganine (d18:0) were not decreased.

TABLE 2 Number of glycerophospholipid species detected within brain regions analyzed and number of these glycerophospholipids that are significantly changed in subjects with AUD

	PFC WM		PFC GM		MTG WM		MTG GM		VIS WM		VIS GM	
	Lipids detected	Lipids changed	Lipids detected	Lipids changed	Lipids detected	Lipids changed	Lipids detected	Lipids changed	Lipids detected	Lipids changed	Lipids detected	Lipids changed
PC	59	28	66	11	48	6	67	28	43	21	61	8
PC(O)	24	10	16	0	15	5	17	2	17	5	15	2
PE	34	7	42	10	36	5	41	17	30	8	36	18
PE(O)	28	7	17	2	36	7	30	2	23	7	13	5
PE(P)	35	6	21	2	40	5	22	3	20	6	17	8
PS	29	9	18	4	17	3	16	1	13	3	16	4
PG	20	2	29	6	24	5	20	1	18	2	18	3
PI	30	5	25	6	36	9	30	5	31	10	32	3
PI(O)	12	1	7	2	17	2	7	3	15	2	12	5
Total	271	75 (27.68%)	241	43 (17.84%)	269	47 (17.47%)	250	62 (24.80%)	210	64 (30.48%)	220	56 (25.45%)

Note: Bold type indicates where the overall lipid group has significantly changed.

Abbreviations: GM, grey matter; MTG, middle temporal gyrus; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PFC, prefrontal cortex; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; VIS, visual cortex; WM, white matter.

3.7 | Decrease in cholesteryl esters and perturbed esterification

The total concentration of cholesteryl esters was significantly decreased in the PFC GM (-45.51% , $p = 0.009$), MTG WM (-40.61% , $p = 0.0018$), and MTG GM only (-44.45% , $p = 0.0216$) (Figure 8). There were specific decreases in ChE(20:5) and ChE(18:2) in PFC WM and GM, MTG GM, and VIS GM (Figure 9).

4 | DISCUSSION

The current study has described changes to the lipidome in the post-mortem human brain of donors that met DSM-V criteria for AUD. The previously described lipidome changes in the PFC were confirmed (de la Monte et al., 2018), while a novel finding here were the similar effects also observed in the visual cortex, a region previously thought to be unaffected by alcohol-related brain damage (McCorkindale et al., 2016). The latter suggests a disturbance to

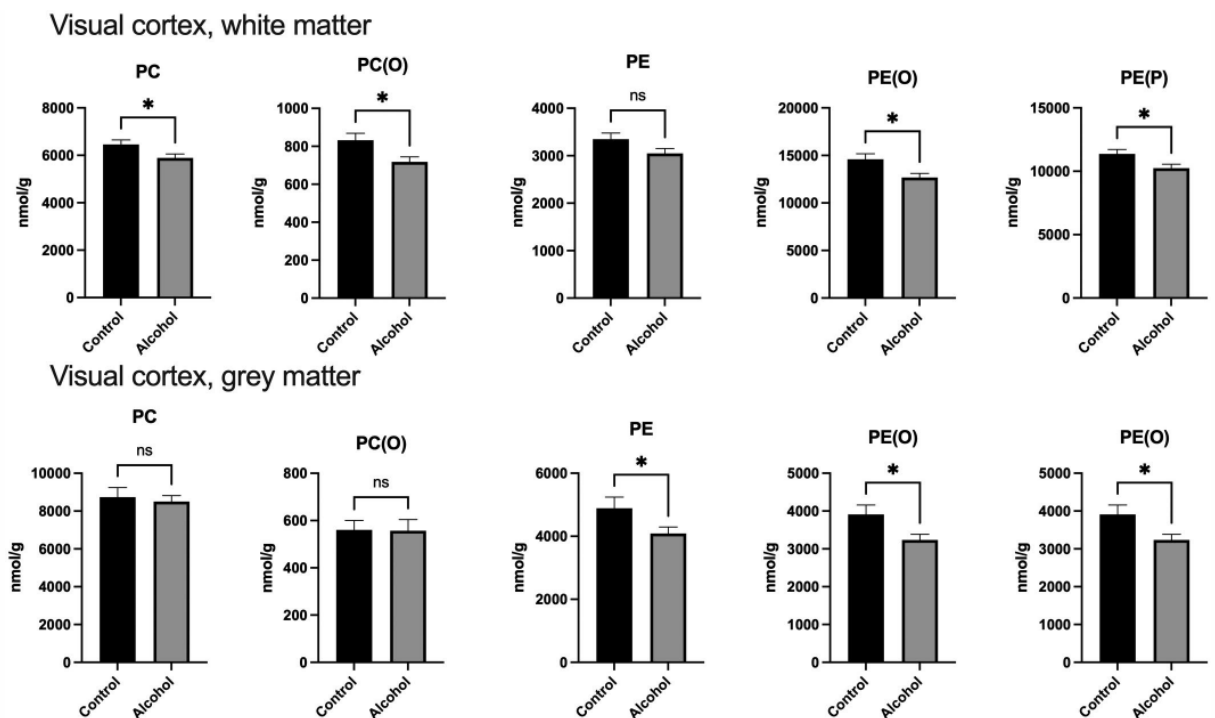


FIGURE 3 Changes in lipid concentration in the visual cortex white and grey matter. Each graph represents total lipid concentration for that headgroup, expressed as nmol per g of tissue. PC and PE indicate ester-linkages, PC(O) and PE(O) indicate plasmanyl, and PE(P) indicates plasmanyl linkages. * $p < 0.05$. n (subjects) = VISWM: 20 controls, 20 AUD, VISGM: 13 controls, 17 AUD

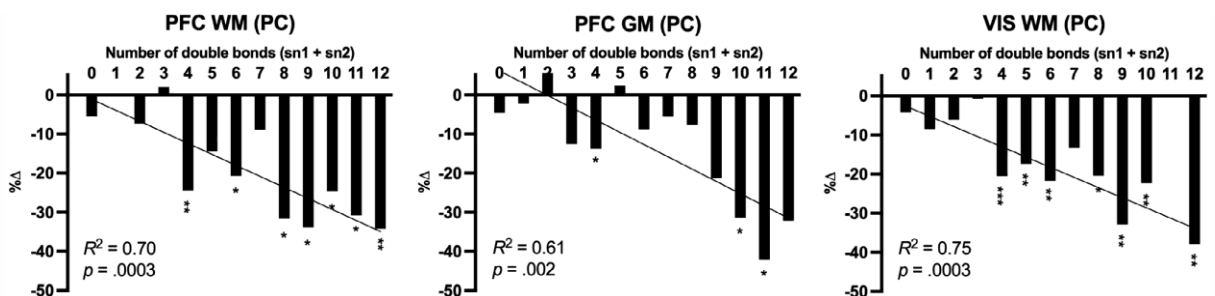


FIGURE 4 Percent change in the concentration of phosphatidylcholines by number of double bonds in the white matter of the prefrontal and visual cortices and grey matter of visual cortex, in AUD compared to controls. Number of double bonds are the total number of double bonds across both sn1 and sn2 fatty acid chains. Solid grey line indicates regression. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n (subjects) = 20 controls, 20 AUD

lipid homeostasis in the absence of, or prior to, gross morphological changes.

Under normal conditions, essential fatty acids, linoleic acid, and alpha-linolenic acid undergo alternating desaturation and elongation steps in the liver to form long-chain polyunsaturated fatty acids (LC-PUFAs), such as docosahexaenoic acid (DHA) (Jump, 2011). The liver also has considerable compensatory capacity to deal with any dietary shortfalls in fatty acids (Igarashi et al., 2007). These are transported to the blood–brain barrier as complexes with either albumin or lipoproteins, where they dissociate, and then cross the blood–brain barrier either by passive diffusion (Hamilton & Brunaldi, 2007) or facilitated by transporters, such as fatty acid transport protein (FATP) (Ochiai et al., 2017). The subsequent incorporation of plasma polyunsaturated fatty acids into brain phospholipids has been demonstrated using radiolabeled fatty acids in rodent experiments (Rapoport et al., 2001).

Experimentally, hepatocytes exposed to high ethanol concentrations over a prolonged period showed reductions in desaturase activity (Narce et al., 2001). Given all AUD subjects in this study had liver cirrhosis, the decrease in chain length and unsaturation in fatty acids esterified to phosphatidylcholine or phosphatidylethanolamine here probably results largely from decreased synthesis of longer chain n-3 fatty acids in the liver (Fernando et al., 2010).

The brain also has some capacity for *de novo* synthesis of LC-PUFAs (Bazinet & Laye, 2014; Williard et al., 2001), but this is also likely to be severely impaired in chronic alcoholics through a combination of hepatic encephalopathy (as seen by the presence of Alzheimer type II

astrocytes in most AUD subjects here (data not shown) (Dennis et al., 2014)), lipid peroxidation (Haorah et al., 2008), and the decrease in dietary provision of adequate essential fatty acids, generally observed with sustained alcohol abuse (Kim et al., 2007).

A potentially attenuating effect is the increase in brain phospholipid fatty acid 16:1 (palmitoleic acid) observed in AUD compared to controls. In serum (not measured in this study), increased palmitoleic acid is characteristic of alcohol consumption, even at low levels, and suggests stimulation of liver biosynthesis rather than changes in dietary fat intake (Simon et al., 1996; Warnet et al., 1985). The implications of increased palmitoleic acid in the brain have not yet been studied. Experiments in human adipocytes showed that the activation of PPAR γ by palmitoleic acid had metabolic benefits by increasing insulin sensitivity (Sauma et al., 2006), improving HDL profiles and lowering fasting glucose (Mozaffarian et al., 2010). Palmitoleic acid has also been shown to activate PPAR α in the liver, thus activating 5'AMP-activated protein kinase (AMPK) and decreasing sterol regulatory element-binding protein 1 (SREBP-1) (de Souza et al., 2017), a transcription factor which modulates lipogenic gene expression. All these factors combined would increase β -oxidation of fatty acids, inhibit lipid synthesis, and may contribute to the broad decrease in brain glycerophospholipids observed in our study.

The decrease in cholesteryl eicosapentaenoate (ChE(20:5), a cholesterol molecule esterified to omega-3 fatty acid EPA), has also been observed in other brain disorders, such as Alzheimer's disease

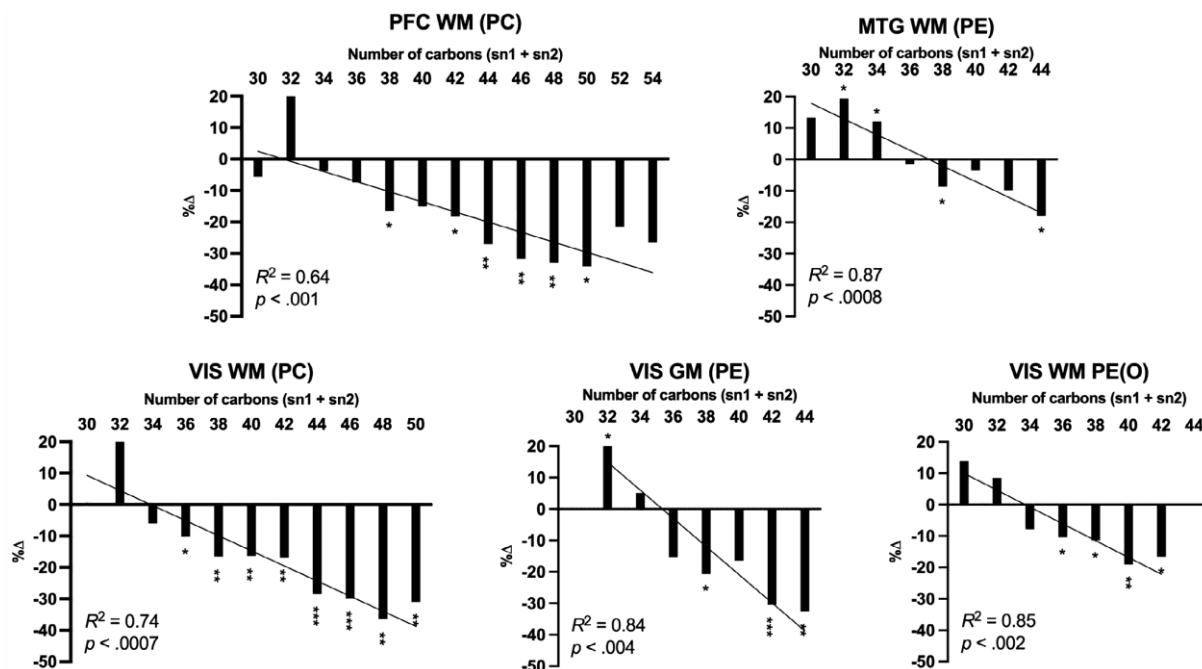


FIGURE 5 Percent change in the concentration of phosphatidylcholines by the number of carbons in the white matter of the prefrontal and visual cortices and grey matter of the visual cortex, in AUD compared to controls. Number of carbons are the total number of carbons across both sn1 and sn2 fatty acid chains. Solid grey line indicates regression. *p < 0.05, **p < 0.01, ***p < 0.001. n (subjects) = 20 controls, 20 AUD

(Tully et al., 2003). The only study linking EPA anomalies and ethanol metabolism to date has been in the model organism *Caenorhabditis elegans* where EPA was involved in acute functional tolerance (Raabe

et al., 2014). No changes in cholesteryl esters containing docosahexaenoic acid were observed in this study. Dietary intake of the subjects included in this study are not known, and so it remains unclear whether the decrease in EPA seen was due to changes in diet and/or polyunsaturated fatty acid metabolism, such as dysregulation of the *FADS1* gene that encodes delta-5 fatty acid desaturase, responsible for EPA synthesis. Generally, people with AUD have poorer nutrition status (Fawehinmi et al., 2012; Valencia-Martin et al., 2011), and as indicated above, hepatocyte desaturase activity is decreased in the presence of ethanol. Interestingly, heavy alcohol drinkers with no or mild liver injury had increased plasma EPA (Vatsalya et al., 2016), again pointing to the importance of liver pathology in the brain lipid findings. Reductions in brain cholesteryl linoleate (ChE(18:2)), as observed in this study, have been previously reported in plasma with alcohol use (Rosseneu et al., 1994). The implications of these cholesteryl ester changes are not currently known and deserve further examination.

This study reported decreases in the sphingoid base d18:2 (sphingadiene) in response to alcohol misuse in humans. The implications of a reduction in this understudied backbone remain unknown. A recent study suggests that sphingosine kinase 2 plays a role in the catabolism in sphingolipids (Couttas et al., 2020), which

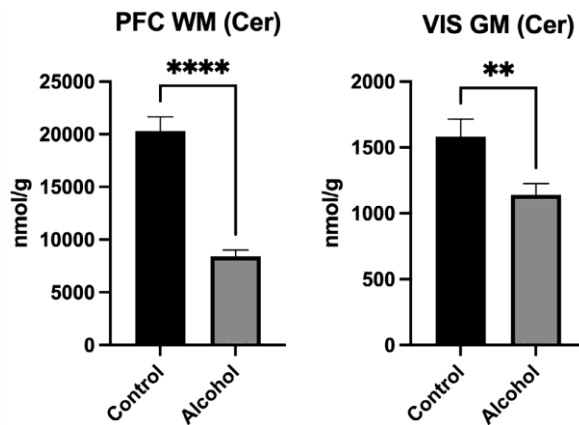


FIGURE 6 Total measured Cer in the grey matter of the prefrontal and visual cortices. Data are expressed as nmol per g of tissue. ** $p < 0.01$, **** $p < 0.0001$. n (subjects) = 20 controls, 20 AUD

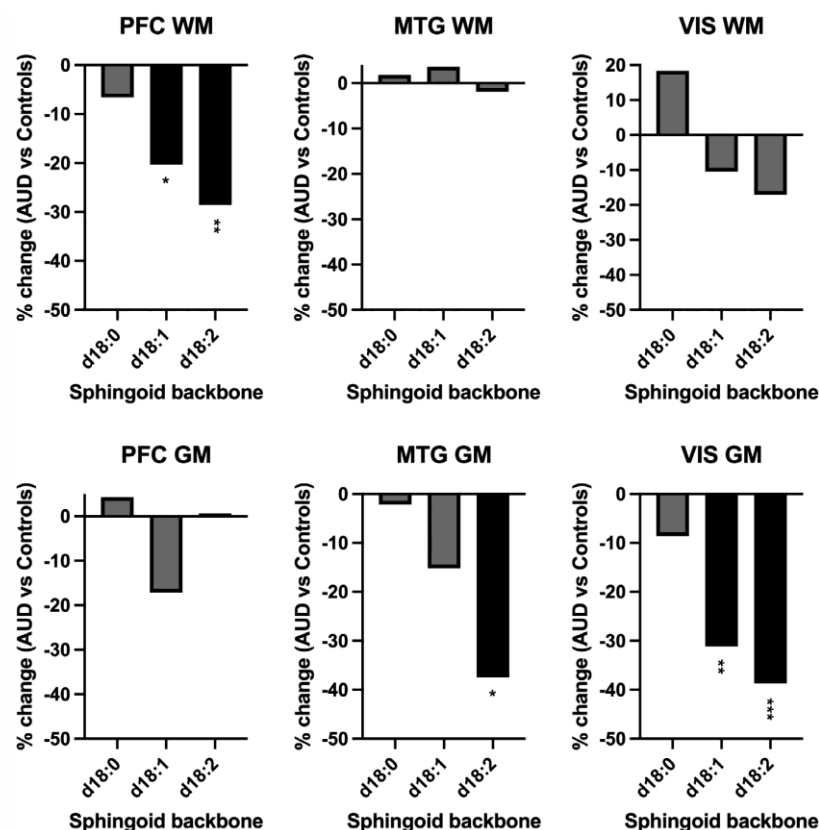


FIGURE 7 Changes to sphingoid backbones, expressed as percent change of AUD relative to controls. Abbreviations: Sphinganine (d18:0), Sphingosine (d18:1), Sphingadiene (d18:2), prefrontal cortex (PFC), middle temporal gyrus (MTG), visual cortex (VIS), white matter (WM), grey matter (GM). Grey bars indicate non-significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n (subjects) = 20 controls, 20 AUD

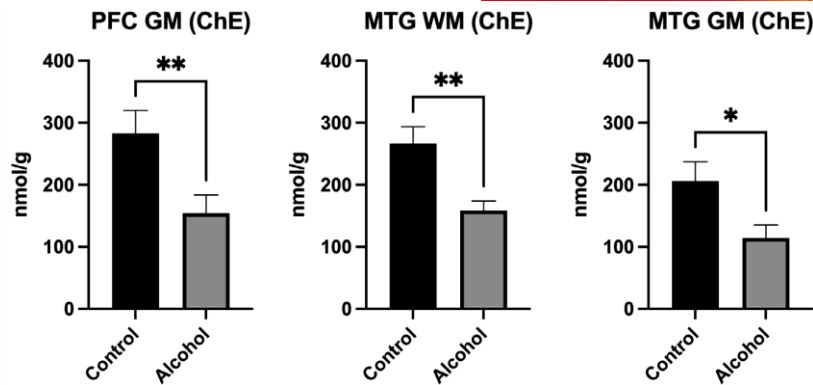


FIGURE 8 Total cholesteryl esters measured in the grey matter of the prefrontal cortex and in the white and grey matter of the middle temporal gyrus. Data expressed as nmol per g of tissue. * $p < 0.05$, ** $p < 0.01$. n (subjects) = 20 controls, 20 AUD

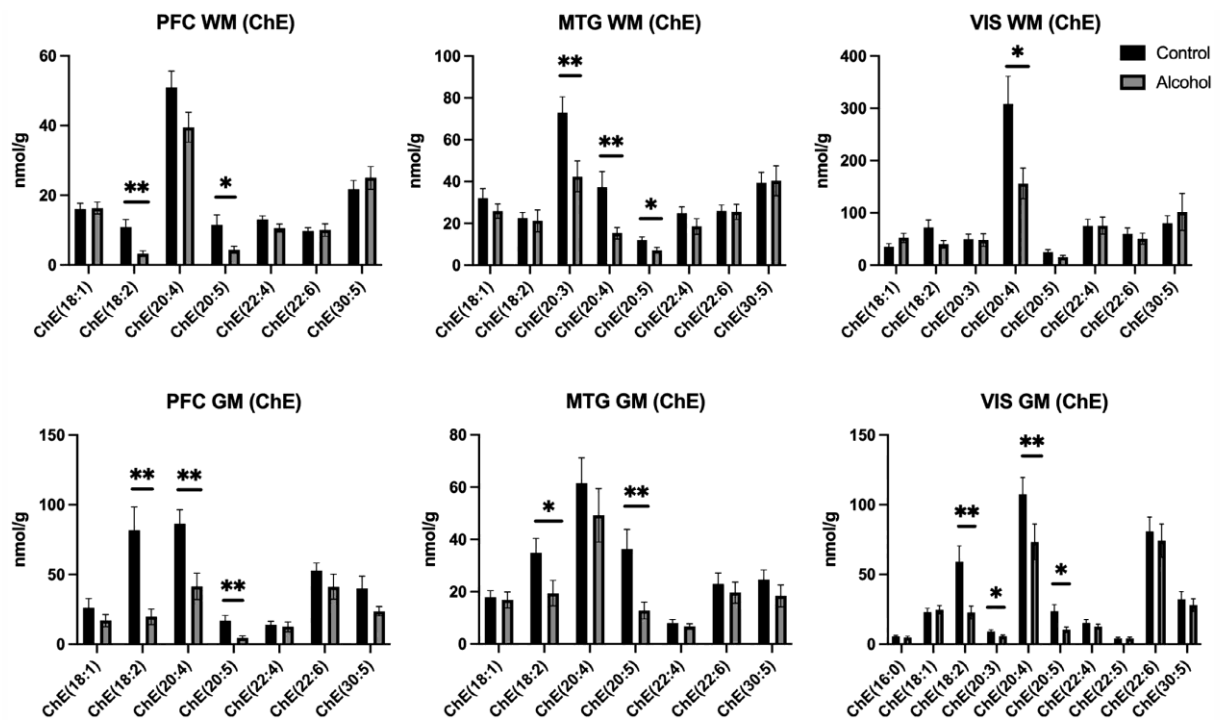


FIGURE 9 Amount of ChE species detected in each brain region. Data are expressed as nmol per g of tissue. * $p < 0.05$, ** $p < 0.01$. n (subjects) = 20 controls, 20 AUD

warrants investigation into metabolic kinase activity in this cohort. Other studies have shown that, by contrast, an increase of sphingadiene inhibits both colon cancer development in mice (Kumar et al., 2012), and the growth of neuroblastoma xenograft tumors similarly via the inhibition of the AKT-dependent mechanisms (Zhao et al., 2018). However, heavy alcohol use has not been associated with an increased risk of brain cancer (Galeone et al., 2013).

An important consideration of the data is the post-mortem interval (the time elapsed between death and when the brain was bio-banked). Whilst the post-mortem interval was not different between groups, it

is important to address the effect any delay may have on brain lipids. While RNA, microRNA, and proteins are stable for at least 24 h post mortem (Nagy et al., 2015), it has been reported that reductions in lipid concentration can occur in some lipid groups, as shown in macaques (Bozek et al., 2015). Another study looking at metabolome changes in the human brain showed negligible effect on palmitic acid (fatty acid 16:0) concentration after 3 days (Scholefield et al., 2020). A Pearson's correlation analysis (data not shown) showed no association between PMI and concentrations of any lipid groups reported. A further consideration is the MtBE lipid extraction protocol used here, which has been previously

validated in Matyash et al. (2008). A comparison of the MtBE protocol with chloroform extraction showed chloroform extraction yielded greater plasmalogen than with MtBE (Lopez-Bascon et al., 2018), which may explain lack of ether-linked alkene choline phospholipids (PC(P)) detected in our samples.

The changes to the AUD neurolipidome reported here are in concordance with previous imaging mass spectrometry studies using human post-mortem brain tissue (de la Monte et al., 2018; Nunez et al., 2016). However, some rodent studies have reported the opposite results (Roux et al., 2015, 2016; Wang et al., 2019). This could be because of several factors. One technical advantage of the current human tissue study over previous rodent studies was that white and grey matter portions of the brain were analyzed separately. As this delineation represents different cell types and proportions, the sensitivity to detect lipidome changes is amplified.

Our findings show that chronic alcohol abuse results in selective changes to the neurolipidome, which likely reflects a multifactorial etiology resulting from the diverse effects of chronic alcohol intoxication the diet, gut, liver as well as direct effects on the brain. Future research should investigate lipid metabolic pathways in the liver and brain, to assess whether these changes reflect anomalies in lipid synthesis or degradation, as well as why similar changes in the visual cortex appear to be non-symptomatic. This question could be addressed in future studies, including alcoholics with lesser degrees of liver pathology, for example, steatosis.

ACKNOWLEDGMENTS

Tissues were received from the New South Wales Brain Tissue Resource Centre at the University of Sydney which is supported by the University of Sydney. Research reported in this publication was supported by the National Institute of Alcohol Abuse and Alcoholism of the National Institutes of Health under Award Number R28AA012725. The content is solely the responsibility of the authors and does not represent the official views of the National Institutes of Health. We would also like to acknowledge subsidized access to Sydney Mass Spectrometry, a core facility at the University of Sydney.

All experiments were conducted in compliance with the ARRIVE guidelines.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTION

Caine Smith: Conceptualization, investigation, methodology, data analysis, writing: original draft, revision 1, 2, 3; Donna Sheedy: funding acquisition, resource procurement, original draft review; Holly McEwen: Method development and guidance; Jillian Kril: funding acquisition, resource procurement, original draft review; Anthony Don: Method development and guidance, discussion input, revision reviews; Greg Sutherland: Conceptualization, methodology, data analysis, writing: original draft, revision 1, 2, 3, funding acquisition, project administration, and supervision.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ORCID

Caine C. Smith  <https://orcid.org/0000-0003-4476-390X>

Anthony S. Don  <https://orcid.org/0000-0003-1655-1184>

Greg T. Sutherland  <https://orcid.org/0000-0003-2493-9736>

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SUPPORTING INFORMATION

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How to cite this article: Smith, C. C., Sheedy, D. L., McEwen, H. P., Don, A. S., Kril, J. J., & Sutherland, G. T. (2021). Lipidome changes in alcohol-related brain damage. *Journal of Neurochemistry*, 00, 1–12. <https://doi.org/10.1111/jnc.15530>

2.3. Postscript

This study provided the most comprehensive description of changes to the neurolipidome in human ARBI to date. Changes to the PFC agree with the prior de la Monte et al. (2018) MALDI-IMS study, that showed a broad reduction in phospholipid and sphingolipid species. The reduction in the MTG lipid species was expected due to the trend towards a reduced volume previously described (McCorkindale et al., 2016). An unexpected finding was the significant changes reported in lipid species of the VIS, despite the absence of gross volume changes. Despite no gross or cytoarchitectural alterations reported in the visual cortex in ARBI so far, diffusor tensor imaging has shown decreased microstructural integrity to the inferior and superior fronto-occipital fasciculi (Bagga et al., 2014b), and metabolic changes have been reported in proton magnetic resonance spectroscopy, suggestive of neuronal loss (Bagga et al., 2014a).

Another finding was that fatty acid chains on PC and PE lipids were less unsaturated (i.e., fewer double bonds) and that the length of the fatty acids was shorter. This finding is likely due to how double bonds are more reactive to ROS damage (Hernandez et al., 2016), which agrees with earlier membrane studies on how chronic ethanol exposure increases the amount of saturated lipids (Harris and Schroeder, 1981a). This result, however, contrasts with the lipidomic analysis by Wang et al. (2019), which showed an increase in unsaturation and longer chain lengths following a year of alcohol exposure.

Their publication did not report on whether their rodent model had any other pathology associated with chronic ethanol exposure and in particular, the condition of the liver. This is a limitation of rodent models when investigating the broad effects (e.g., alcohol exposure) on whole systems (such as the neurolipidome), as there is a failure to recognise the importance of factors extrinsic to the brain, such as the liver in this example, which is wholly important to the human condition that they are modelling.

Although liver status was reported in this publication, hepatic encephalopathy was not. Data provided by the NSW BTRC reported that of the cases used in this cohort, five had no evidence of hepatic encephalopathy, 12 had mild, two had moderate, and one had severe hepatic encephalopathy. No data was available for the control cases, however, in the absence of any liver pathology, hepatic encephalopathy is unlikely.

In summary, in ARBI there were decreased ceramides and phospholipids, as well as changes in fatty acid chain and level of unsaturation. Chapter 3 will address sphingolipid metabolism and neuronal numbers (phospholipids make up a significant portion of the cell membrane).

Note: Reference to access supplementary material is detailed in Appendix A.

Chapter 3. Changes in sphingolipid metabolism and neuronal loss

3.1. Introduction, aim, and hypothesis

Ceramide is a sphingolipid that is a precursor to other complex sphingolipids, but it also has other roles in the cell. As a secondary messenger, ceramide regulates pathways involved in apoptosis and inflammation (Mencarelli and Martinez-Martinez, 2013). It is involved in lipid raft formation and membrane trafficking (Bieberich, 2018) and brain energy metabolism (Cruciani-Guglielmacci et al., 2017). Ceramide is a component in mitochondrial membranes, and excess accumulation impairs mitochondrial function, resulting in ROS generation (Gaggini et al., 2022).

Very long-chain ceramides (C22-C24 ceramides) are generated by ceramide synthase 2 (CerS2) and are precursors for sulfatide, galactosylceramide, and SM generation, which are important constituents of myelin. Ceramide transfer protein (CERT) is a lipid transport protein, which transports ceramide from the ER to the Golgi apparatus for conversion to these more complex lipids (Hanada et al., 2009). CerS2 knockout mice have reduced myelin thickness and the proportion of myelinated axons, demonstrating its importance for myelin integrity (Teo et al., 2022). Additionally, CerS2 knockout

mice show increased C16 ceramide content in mitochondrial fractions, which results in chronic oxidative stress due to ROS generation (Zigdon et al., 2013). Overexpressing CERT decreases the C16 ceramide content in the mouse cortex (Crivelli et al., 2021). Sphingosine kinase 2 (SPHK2) is the dominant sphingosine kinase in the brain and acts to phosphorylate the ceramide backbone when separated from the fatty acid (i.e., when ceramide is catabolised), creating sphingosine-1-phosphate (Diaz Escarcega et al., 2021). In a study investigating the role of SPHK2 in oligodendrocytes, SPHK2 enzyme deficiency led to sphingadiene (d18:2) accumulation, including sphingadiene-backbone ceramides (Couttas et al., 2020). In the LC/MS study presented in Chapter 2, sphingadiene-backbone ceramides significantly decreased, suggesting a possible increase in SPHK2 activity. Specifically, ceramide content was reduced in the PFC white matter of AUD cases compared to controls (Smith et al., 2022).

Another aspect of the previous Chapter that requires follow-up is to see whether there was any neuronal cell loss in the PFC and VIS regions, as these exhibited significant GL declines. Earlier cell counting studies suggested no neuronal loss in the cortex in ARBI (Jensen and Pakkenberg, 1993), however, the general consensus now is that neuronal loss does occur in ARBI but is restricted to the frontal cortex (Harper et al., 1987, Kril et al., 1997, Kril and Harper, 1989), and not seen in other brain regions such as the hippocampus, cingulate and temporal cortices, although neuron soma size is globally reduced

(Harding et al., 1997, Kril and Harper, 1989). Neurons in many regions, including the visual cortex have not been quantified to date in ARBI.

Therefore, in this Chapter, the aim is to follow up on changes to sphingolipid metabolism and additionally quantify neuronal numbers. To achieve this, I specifically aimed to:

- 1) Extrapolate ceramide data to quantify C22 and C24 ceramides
- 2) Measure CerS2, SPHK2, and CERT expression via western blot
- 3) Quantify neuronal numbers in the PFC and VIS regions

It was hypothesised that C22 and C24 ceramides are the most abundant ceramide that similarly decreases in AUD compared to controls as total ceramide did in the PFC of Chapter 2. It was also hypothesised that this decrease was due to a decrease in CerS2 expression and that the decrease in d18:2-ceramides was due to the increase in SPHK2 expression. And thirdly, given the results in Chapter 2 demonstrating significant phospholipid decreases in the visual cortex grey matter, it was hypothesized that there would be a reduction in neuronal cell counts in the PFC and VIS regions. Due to there being minimal changes to the MTG region in Chapter 2, and given the previous Kril and Harper (1989) study that found no neuronal loss in the temporal cortex, this region was not included for neuronal counting.

3.2. Methods

3.2.1. Human subjects

The use of human tissue for this study was approved by the University of Sydney Human Ethics Review Committee (approval number 2015/022). The NSW Brain Tissue Resource Centre (NSW BTRC) obtains informed consent from each brain donor and complies with the University of Sydney Human Research Ethics Committee (approval number 2019/531). The same cohort from Chapter 2 was used in this study. For the western blotting study, a subset of seven AUD and seven age- and sex-matched control cases were used. This is so that case comparisons could be performed on one blot to reduce experimental variability (Ghosh et al., 2014). For the neuronal counts, all cases were included for counting.

3.2.2. Tissue samples

For the western blot analysis, additional white matter from the PFC was requested from the NSW BTRC. This region was chosen given that it had the most significant atrophy in ARBI (McCorkindale et al., 2016). For neuronal counts, formalin-fixed paraffin-embedded blocks from the prefrontal cortex (PFC) and primary visual cortex (VIS) were requested from the NSW BTRC, which were sectioned at 7 μ m onto Superfrost microscope slides. Sections were dried overnight at 37°C and kept in a desiccator cabinet under vacuum until required for immunostaining.

3.2.3. Western blotting protein extraction and sample preparation

Twenty milligrams of tissue was homogenised in a 1.5 mL microcentrifuge tube with a pestle and 60 μ L of extraction buffer containing: 20 mM Tris-Cl, 1 mM EDTA, 2 mM DTT, 0.1 mM PMSF (phenyl methyl sulfonyl fluoride), 10 mM CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate), 25 mM NaF, NaPP_i, 1 mM Na₃VO₄, protease cocktail inhibitor (Complete; Roche, Penzberg, Germany), and 0.5% SDS (Appendix B). Homogenates were sonicated for five min and then centrifuged for 20 min at 14000 $\times$ g and 4°C. The supernatant was retained, and the total protein concentration was measured using a Pierce™ BCA Protein Assay Kit (23225, ThermoFisher Scientific™) according to their protocol. Protein was frozen in 20 μ g aliquots, each made up to 7.8 μ L total volume with MilliQ water, until required.

The total amount of NuPAGE™ LDS Sample Buffer (4X) (NP0007, ThermoFisher™ Scientific) and 10X Bolt™ Sample Reducing Agent (B0009, ThermoFisher™ Scientific) required per experiment was prepared by mixing 3 μ L of the sample buffer with 1.2 μ L reducing agent per sample in a tube. When samples thawed on ice, 4.2 μ L of this mixture was added to each sample, vortexed, and then denatured at 95°C for five min. Samples were briefly spun down on a benchtop centrifuge, and then loaded into 15-well pre-cast gels (NW04125BOX, ThermoFisher™ Scientific), along with 10 μ m of Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards (1610375, Bio-Rad).

3.2.4. Western blotting

Gels were run with 1X NuPAGE™ MES SDS Running Buffer (NP0002, ThermoFisher™ Scientific) at 150 V for 50 min. While the gel ran, thick filter paper and transfer sponges were soaked in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). After the gel finished running, a transfer sandwich was assembled, from bottom to top: sponge, thick filter paper, gel, methanol-soaked PVDF membrane, thick filter paper, sponge, and put into a XCell II Blot Module (ThermoFisher™ Scientific). This was put into a transfer tank with chilled transfer buffer and ice pack and ran at 20 V for 60 min.

3.2.5. Western blotting antibody incubation

Blots were briefly stained (one second) with Ponceau S to confirm that the transfer was successful, and then rinsed with distilled water to remove the stain. Blots were blocked with 5% skim milk in tris-buffered saline with tween-20 (TBS-T) for one hour. Blots were washed three times in TBS-T for five min each. Blots were then incubated in rabbit polyclonal primary antibodies (Table 3) containing TBS-T and 5% bovine serum albumin (BSA) overnight at 4°C on a rocking platform. This was followed by another two washes in TBS-T for five min each. This process was repeated with another antibody for the housekeeping protein GAPDH (Table 3), as a loading control. The secondary antibody (Table 3) was prepared in 5% skim milk and incubated on the blot for 1.5 hours at

room temperature. The blot was washed a final three times in TBS-T for five min each. The antibodies and the concentrations used are in Table 3.1.

Table 3.1. Antibodies and their concentrations used for western blotting experiments

Antibody	Concentration	Catalogue number
SPHK2	1:1000	Abcam, Ab215750
CerS2	1:500	Abcam, Ab176709
CERT	1:500	Abcam, Ab72536
GAPDH	1:5000	Cell signalling, 2118
Anti-Rabbit, HRP-linked	1:2000	Cell signalling, 7074

3.2.6. Western blotting imaging and analysis

Chemiluminescent substrate (WBKLS0500, Merck) was prepared according to the manufacturer's instructions and added on top of the blot. Blots were imaged immediately using a ChemiDoc MP Imaging System (Bio-Rad) using both the colorimetric and Chemiluminescent blot imaging feature on the inbuilt Image Lab Touch software. Exposure time was set to Optimal auto-exposure. Images were exported from the ChemiDoc MP Imaging System and imported into Image Lab (Bio-Rad) software on a desktop computer. Each pre-stained band on the protein standard ladder was identified using the colorimetric image per blot. Pixel intensity for each protein band of interest, including the housekeeping protein bands, on the chemiluminescent image was quantified using Image Lab software (Bio-Rad) and exported into MS Excel. Intensity values for the protein of interest were normalised by dividing by the loading control (GADPH) values. Normalised values were separated into their

respective AUD and control groups and entered into GraphPad Prism 9 for statistical analysis, where T-tests were performed.

3.2.1. Neuronal immunohistochemistry

Sections were baked at 63°C for 40 min, briefly cooled to room temperature and then deparaffinised in three changes of xylene for ten min each. Sections were rehydrated in decreasing ethanol concentrations for three min each: 100% three times, 95%, 70%, 50%, and then distilled water.

Heat-induced epitope retrieval was performed using a Decloaking Chamber™ NxGen, for 30 min at 110°C in a pH 6 citrate buffer containing 0.05% Tween-20. Sections were cooled to room temperature in buffer and then washed briefly in tris-buffered saline (TBS). Endogenous peroxidase activity was blocked using a solution of 3% H₂O₂ in methanol for 20 min. Sections were rinsed twice in distilled water and then washed for five min in TBS three times. Tissue sections were drawn around using a PAP pen and then blocked in a solution of 10% normal goat serum in TBS for 30 min. The goat serum was then removed, and slides were incubated in a humidity chamber.

Rabbit polyclonal NeuN antibody (1:500; ABN78, Merck) was prepared in TBS with 0.05% Tween-20 (TBS-T) and pipetted onto each section for overnight incubation at 4°C. NeuN is a neuronal marker protein, which has been used for detecting cortical neurons (Wolf et al., 1996, Kelly and Hawken, 2017, Miller et al., 2014).

Sections were washed in three TBS-T changes for five min each on an agitator. For immuno-detection, Dako EnVision HRP/DAB+ kit (K5007, Agilent, Mulgrave, Victoria) was used according to the manufacturer's instructions. This kit contains a mixture of goat anti-mouse and goat anti-rabbit HRP-linked secondary antibodies, as well as 3,3'-Diaminobenzidine (DAB) and necessary reagents for detection. Sections were washed for five min in TBS-T three times and then dehydrated in increasing ethanol concentrations and then cleared in xylene three times for ten min each before mounting in DPX and cover slipped. Two additional sections were used in each immunohistochemistry (IHC) run: a positive control which was another prefrontal cortex section from an formalin-fixed paraffin-embedded (FFPE) block where we previously had NeuN-positive staining success, and a negative control, where the primary antibody was omitted. The experimental group each case belonged to was blinded throughout staining and counting.

3.2.2. Neuronal count imaging and analysis

Slides were scanned at 20X magnification using an Aperio XT slide scanner (Leica Biosystems), provided by the Sydney Microscopy and Microanalysis core facility located at the University of Sydney. Image files were kept in their original .svs file format and opened in Orbit Image Analysis, an open-source whole slide image analysis tool (Stritt et al., 2020). Orbit uses context-based structure classification, but with manual attribution of regions of interest (ROI).

Generally, a few NeuN-positive cells were selected within each cortical layer to cover the breadth of size and shape diversity of neurons to train the algorithm. Several background regions of interest were also included in the model, as background staining was not always homogenous within a section (Figure 3.1). Next, a large ROI was drawn within the grey matter, from the white-grey matter boundary to the cortical surface, and Orbit was run to count NeuN-positive features and calculate the ROI area (μm^2). Checks were made after each count to ensure that results were consistent with observed findings. If an area appeared undercounted, the model was retrained with more NeuN-positive cells added. Occasionally, overcounting resulted when one model was used to count another slide where the background pixel intensity approached the NeuN-positive cells from the previous slide. In these cases, a new model was created. IBM SPSS Statistics 28 was used to perform ANCOVA on NeuN-positive cell counts per mm^2 between control and AUD subjects in both the PFC and VIS regions to adjust for potential brain volume effects. GraphPad Prism 9 was used to graph results.

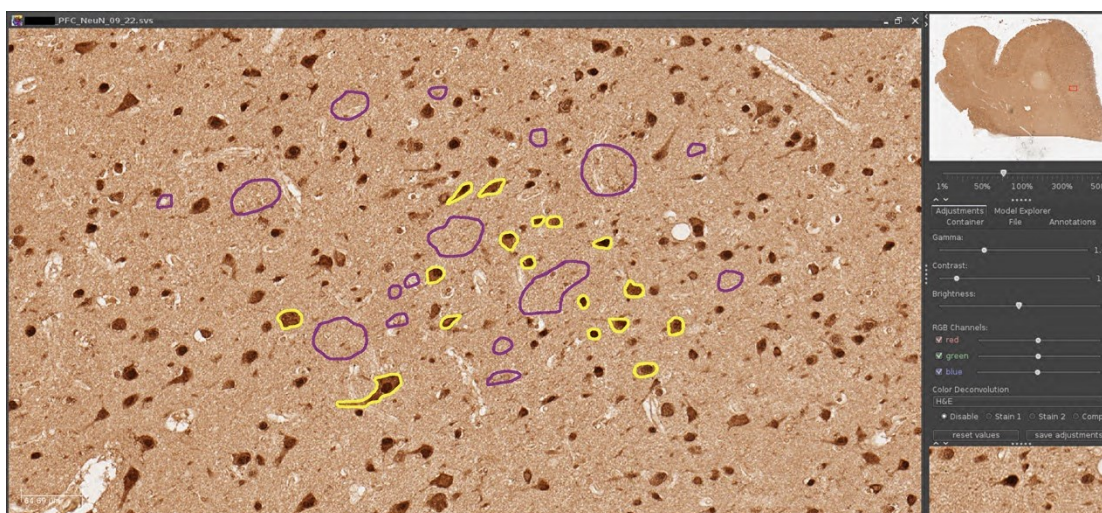


Figure 3.1. Optimisation of automated neuron quantification. The Orbit software algorithm was trained through iterative manual selections of both NeuN immunopositive cells (highlighted in yellow), and background regions (purple circles).

3.3. Results

3.3.1. C22 and C24 ceramides

The data pertaining to only C22 and C24 ceramides was extracted from the results generated in Chapter 2, which showed that approximately 80% of the total ceramide measured in PFC white matter were very long-chain ceramides (C22 and C24). The C22 and C24 content measured significantly decreased from $8.84 \pm 0.62 \mu\text{mol/g}$ of tissue in control cases and $6.94 \pm 0.50 \mu\text{mol/g}$ in AUD tissue ($F(19, 19) = 1.493$, $p = 0.02$). In addition to C22 and C24 ceramides, the total amount of Cer and SM are also shown in Figure 3.2.

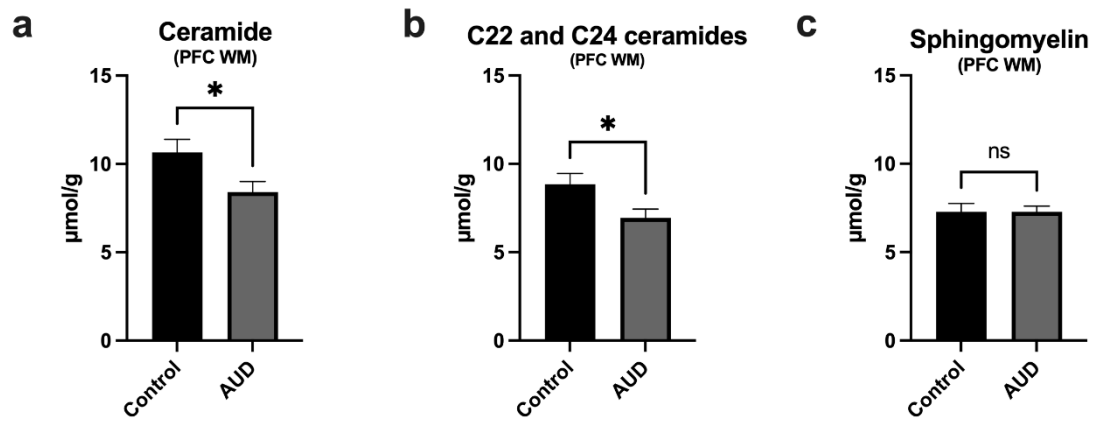


Figure 3.2. Total ceramide, C22 and C24 ceramides, and sphingomyelin content in the prefrontal cortex white matter.

3.3.2. CerS2 and SPHK2

There were no significant differences detected in CerS2 and SPHK2 protein levels between AUD and control cases (Figure 3.3). The full western blot images are attached in Appendix C.

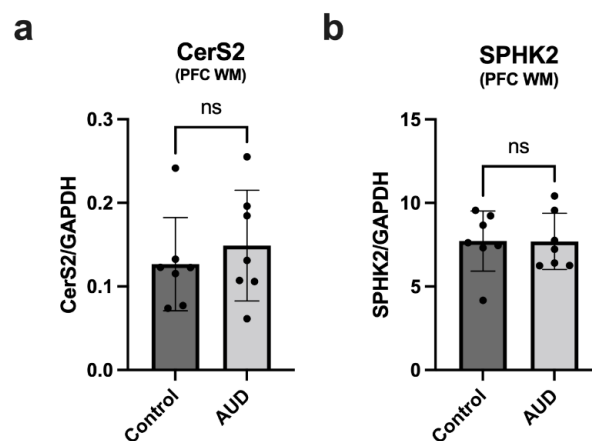


Figure 3.3. CerS2 and SPHK2 expression relative to GAPDH (loading control) in control and AUD cases in the PFC white matter

3.3.3. CERT

CERT protein expression was significantly increased in the AUD group compared to controls ($F(6, 6) = 2.229$, $p = 0.028$) (Figure 3.4). The full western blot image is attached in Appendix C.

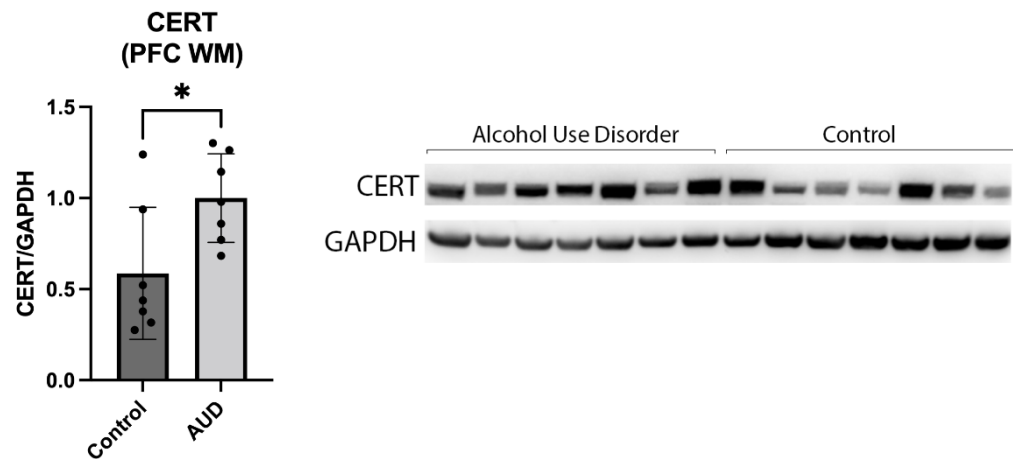


Figure 3.4. *CERT* expression relative to *GAPDH* (loading control) in control and AUD cases in the PFC white matter

3.3.4. Neuronal counts

NeuN-positive cells were quantified in two brain regions, PFC, and VIS, from brain donors that met DSM-V criteria for AUD and matched controls. Tissue sections where staining was overall poor quality were excluded from the analysis. Tissue with poor staining tended to be from FFPE blocks made from tissue that was in formalin for years, as opposed to the NSW BTRC standard three-week fixation. The total number of cases counted for the PFC was 37, and for the VIS was 30.

3.3.5. Neuronal counts in the prefrontal cortex

Sections from 19 controls and 18 AUD from the PFC were included here for quantification. The mean NeuN-positive cells per mm² in the PFC of the AUD group was significantly lower (346.5 ± 16.9 [mean $\pm$ SEM]) than in the PFC of the control group (410.4 ± 23.0 ; $F(18, 17) = 1.957$, $p = 0.0328$) (Figure 3.5). Brain volume had no significant effect size.

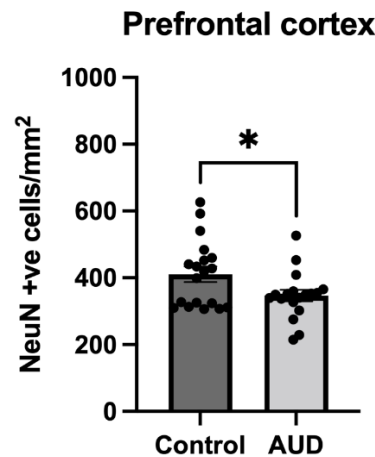


Figure 3.5. NeuN-positive cells per mm² in the prefrontal cortex

3.3.6. Neuronal counts in the visual cortex

Sections from 15 control and 18 AUD cases were included here for quantification. The mean NeuN-positive cells per mm² in the VIS of the AUD group was significantly lower (677.4 ± 19.8 [mean $\pm$ SEM]) than in the VIS of the control group (807.1 ± 20.0 ; $F(14, 14) = 1.014$, $p < 0.0001$) (Figure 3.6).

Brain volume had no significant effect size.

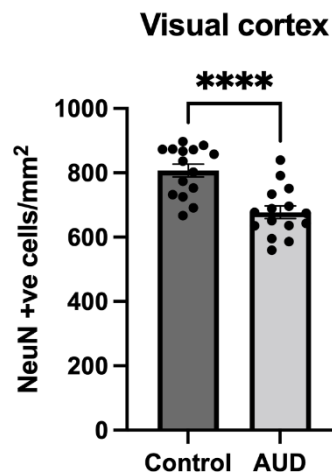


Figure 3.6. NeuN-positive cells per mm² in the visual cortex.

3.4. Discussion

The present study measured CerS2, SPHK2, and CERT protein levels in the PFC white matter of AUD and control cases. There were no measured changes in CerS2 and SPHK2 levels, but there was a significant increase in the amount of CERT, suggesting an increase in ceramide trafficking from the ER to the Golgi apparatus. In addition, the present study used automated cell counting software to demonstrate the loss of NeuN-positive staining neurons in both the PFC and VIS regions in AUD cases. The reduction in neurons in the PFC region agrees with the findings of Kril et al. (1997). A previously unreported finding

here was the significant reduction in NeuN-positive cells in the visual cortex of AUD.

The mass spectrometry measurements of total ceramide, including C22 and C24 ceramides, were decreased in AUD cases. The amount of CerS2 expression did not change, suggesting that the ceramide decrease was not due to a decrease in synthesizing enzymes. Other possibilities that may explain the decreased ceramide could be due to either increased catabolism of ceramide and/or, increased lipid peroxidation due to chronic alcohol exposure and/or ceramide conversion to SM, as consistent with the increase of CERT protein expression. Ceramidase activity was not assayed directly here, however, sphingosine/sphingadiene (a by-product of ceramidase activity), is phosphorylated by sphingosine kinase (SPHK2), and no change in SPHK2 expression were seen. Another possibility is that there was an increase in ceramide being trafficked to the Golgi apparatus to be converted to sphingomyelin to preserve myelin integrity, which might explain why there was only a decrease in Cer and not SM (Figure 3.2). Experiments increasing CERT activity decrease Cer and increase SM content in HeLa cells (Bosello Travain et al., 2020).

The cohort used in the present study was the same as that in Chapter 2 (Smith et al., 2022), which reported significant changes to the neurolipidome in the visual cortex in these cases. None of the AUD cases had Wernicke-Korsakoff syndrome, but all had liver cirrhosis. None of the control cases had liver

cirrhosis. A limitation of this study was that the cohort used did not include any AUD cases without liver cirrhosis, so we were unable to investigate whether liver cirrhosis exacerbates neuronal loss.

Chapter 4. Transcriptomics in ARBI: Meta-analysis

4.1. Introduction

Transcriptomics is the study of the transcriptome, or the complete set of transcripts (i.e., any RNA molecule transcribed from DNA, such as messenger RNA) in a cell, tissue, organ, or organism, including their quantity (Wang et al., 2009). Understanding the transcriptome in alcohol use disorders is important because it gives insight into which biological pathways or processes might be implicated in the disease and its progression and thereby provide targets for potential therapeutics.

Earlier transcriptomics techniques relied on northern blotting, developed in the 1970s, where RNA was electrophoretically separated on an agarose gel, transferred, probed on a membrane, and semi-quantified to give a relative level of gene expression (Alwine et al., 1977). The next decade saw the development of the reverse transcription polymerase chain reaction (RT-PCR), which involves the reverse transcription of complementary DNA (cDNA) and then amplification of the cDNA using PCR. This was much more sensitive and quantitative than blotting techniques, however, throughput was low (Saiki et al., 1985, Peake, 1989). In the 1990s, microarrays were first used. This technique

utilised custom slides printed with thousands of unique DNA sequence probes that when hybridized to fluorescently labelled sample RNA, emitted a fluorescent signal from each probe, that was directly proportional to the amount of mRNA present (Fodor et al., 1991). Microarray studies of the human post-mortem prefrontal cortex have shown a reduction in myelin-related genes in AUD compared to controls (Lewohl et al., 2000), enrichment in immune and cell survival pathways (Liu et al., 2004), and downregulation of mitochondrial energy metabolic pathways (Sutherland et al., 2014). Although still useful, this technique has been superseded by RNA sequencing (RNA-Seq), which first appeared in the late 2000s and has since become the gold standard, particularly as RNA-Seq can detect differentially expressed genes (DEG) with lower expression than microarrays (Wang et al., 2014, Li et al., 2016, Liu et al., 2015).

RNA-Seq is a high-throughput technique, where specific types of extracted RNA are selected (often mRNA via poly-A selection or ribo-depletion) and undergo reverse transcription to generate cDNA which is then fragmented. Adaptors (that facilitate attachment to the flow cell) are ligated to each end of each fragment (for paired-end reads) and then sequenced using next-generation sequencing (NGS) technology. The fragments are attached to a flow cell, denatured, and hybridised to complementary sequences on the flow cell, forming clusters. These clusters then undergo sequencing, in which nucleotides are incorporated into the DNA strand via a polymerase, such as phi29 DNA polymerase. Each of the four nucleotides (A, C, G, and T) are tagged with a

different fluorescent probe, and sequentially detected and recorded each time they are incorporated onto each DNA strand. Millions of reactions are recorded simultaneously, and this process is repeated for approximately 100 cycles, resulting typically in 30–50 million 100 bp reads. These reads are then aligned to a reference genome, quantified, the raw counts normalised (typically for sequencing depth, gene length, and relative RNA composition between samples), filtered, and DEGs statistically determined (Kukurba and Montgomery, 2015, Wang et al., 2009, Wilhelm and Landry, 2009).

Differential expression analysis is a method used to identify the transcripts that have altered expression (higher or lower) between groups of interest. However, DEG lists are typically large (hundreds or thousands of transcripts) and contain transcripts with varying degrees of annotation and in themselves offer little in the way of coordinated biological information. Since microarrays have been in use, various databases have been developed that use known biological relationships to combine genes into pathways/groups for DEG lists to be interrogated against (known as gene enrichment analysis). If a significant number of DEGs are from specific pathways/groups then those pathways/gene groups could be relevant to the disease. Two commonly used enrichment databases are the Gene Ontology (GO) database, which focuses on biological, cellular, and molecular processes (Ashburner et al., 2000, Gene Ontology, 2021), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, which focuses on metabolic and signalling pathways

(Kanehisa and Goto, 2000, Kanehisa, 2019). One caveat is that enrichment databases usually incorporate genes across the spectrum of biology, and information may not be organ-specific – a scenario pertinent to the human brain, with the most unique and varied transcriptome of any organ known (Kang et al., 2011).

RNA-Seq studies using post-mortem brain samples in alcohol research have revealed DEGs that have roles in neuronal adaptations such as NMDA receptor regulation in the hippocampus (Zhou et al., 2011), a negative correlation of a spliced transcript for a sodium channel subunit and lifetime drinking in the PFC (Shiosaka et al., 1989), and a positive association between MAPT expression and drinks per week in the PFC (Kapoor et al., 2021). Enriched pathways relating to immune signalling and lipid metabolism (Kapoor et al., 2021), neurological and neurodegenerative disorders (Rao et al., 2021), CREB and IL-8 signalling, and neuronal guidance (Lim et al., 2021) have also been found to be relevant to ARBI.

The National Institutes of Health (NIH) has a genomic data-sharing policy that requires researchers whose work is funded by the NIH to be made publicly available. This includes the previously mentioned RNA-Seq datasets from Lim et al. (2021), Kapoor et al. (2021), and Rao et al. (2021). The aim of this chapter is to combine and reprocess these publicly available datasets to discriminate the effect that liver pathology has on the progression of ARBI with respect to the lipidome, using differential expression analysis. It is hypothesized

that progression from no liver pathology to steatosis to cirrhosis is an important contributor to lipidome changes in ARBI.

4.2. Methods

This study uses publicly available RNA-Seq data from three previously published studies, available from the NCBI Gene Expression Omnibus (GEO) database, with GEO accession numbers: GSE189139 (Kapoor et al., 2021), GSE181520 (Lim et al., 2021), and GSE131470 (Rao et al., 2021). Only data generated from the PFC was used. The tissue provided for these studies was obtained from the NSW Brain Tissue Resource Centre (NSW BTRC). Additional metadata relating to these studies such as liver pathology classification was requested from the NSW BTRC to allow for targeted analyses and group comparisons.

4.2.1. Demographics

Raw data from 160 RNA-seq data samples were downloaded onto the high-performance computer Artemis, a computing cluster maintained by the Sydney Informatics Hub at the University of Sydney. Seven cases were excluded as they were not able to be unequivocally identified by the NSW BTRC, leaving 71 AUD and 82 control cases from the three combined studies. Demographic information on sex, age, post-mortem interval (PMI), brain pH, body mass index (BMI), and liver classification (normal, steatosis, or cirrhosis) was provided by the NSW BTRC (Table 4.1). Additionally, there was considerable

overlap between cases from these three studies and the lipidomics study in Chapter 2, with 15 out of 20 controls and 10 out of 20 AUD with liver cirrhosis cases from Smith et al. (2022) included here.

Table 4.1. Demographics of each of the three studies used in this analysis

	Control	AUD
<u>Kapoor et al. (2021):</u>		
Sex	5 female / 35 male	6 female / 23 male
Age (years $\pm$ SEM)	55.20 $\pm$ 2.03	53.69 $\pm$ 2.58
PMI (hours)	23.05 $\pm$ 2.11	34.17 $\pm$ 3.02*
Brain pH $\pm$ SEM	6.52 $\pm$ 0.06	6.56 $\pm$ 0.04
BMI $\pm$ SEM	28.11 $\pm$ 0.95	23.54 $\pm$ 1.20*
Liver classification	20 normal 17 steatotic 3 unknown	6 normal 10 steatotic 13 cirrhotic
<u>Lim et al. (2021):</u>		
Sex	6 female / 6 male	6 female / 6 male
Age (years $\pm$ SEM)	48.25 $\pm$ 2.20	48.58 $\pm$ 1.95
PMI (hours)	28.04 $\pm$ 4.06	40.54 $\pm$ 3.88*
Brain pH $\pm$ SEM	6.57 $\pm$ 0.09	6.60 $\pm$ 0.07
BMI $\pm$ SEM	30.69 $\pm$ 1.70	23.01 $\pm$ 1.09*
Liver classification	10 normal 1 steatotic 1 unknown	3 normal 4 steatotic 5 cirrhotic
<u>Rao et al. (2021):</u>		
Sex	7 female / 23 male	7 female / 23 male
Age (years $\pm$ SEM)	57.47 $\pm$ 1.63	57.47 $\pm$ 1.66
PMI (hours)	30.67 $\pm$ 2.25	34.57 $\pm$ 2.70
Brain pH $\pm$ SEM	6.63 $\pm$ 0.05	6.59 $\pm$ 0.04
BMI $\pm$ SEM	30.77 $\pm$ 1.08	26.42 $\pm$ 1.12*
Liver classification	10 normal 16 steatotic 4 unknown	11 normal 18 steatotic 1 cirrhotic

*significantly different to controls

4.2.2. Data mapping and quality control

The raw input data files (*fastq*) from each of the three studies were downloaded from Sequence Read Archive². Each subject had paired end read files. The quality of the raw data was assessed using *FastQC* (version 0.11.9)³, which is an open-source program that summarises quality metrics such as base composition, GC content, and percentage of duplicate reads (higher percentages result in over-represented transcripts). Data were trimmed using *fastp* (version 0.20.0)⁴ which removed regions with low-quality reads and adaptor sequences (Chen et al., 2018). Reads were then mapped to the GRCh38.p13 *homo sapiens* reference genome using *kallisto index* (version 0.46.0) and quantified using *kallisto quant* (version 0.46.0) with 100 bootstraps. Kallisto is a program developed by the Pachter Lab⁵ (California Institute of Technology), which maps and quantifies RNA transcripts from bulk RNA-Seq data. It uses a pseudoalignment algorithm to map reads to a reference genome. This is done using ‘k-mers’, which are positive integers that summarise genomic sequences (rather than aligning entire reads to the genome) and is purported to be faster than other alignment methods and more accurate in reporting abundances (Bray et al., 2016). Kallisto count data for each gene and sample were then imported into R (version 4.2.2) for further data processing and statistical analysis.

² <https://www.ncbi.nlm.nih.gov/sra>

³ <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>

⁴ <https://github.com/OpenGene/fastp>

⁵ <https://pachterlab.github.io/kallisto/about>

4.2.3. edgeR, outlier removal, case exclusions, and filtering

edgeR is a software package used in R to process and examine differential expression analysis data (Robinson et al., 2010). It is the most common tool used to interpret differential expression analysis results (McDermaid et al., 2019) and outperforms other tools such as Cuffdiff and DESeq2 in uncovering true positives (Zhang et al., 2014). Hierarchical clustering identified outlier samples for each group comparison [described next paragraph, 4.2.2]. These were considered to be technical outliers as they still clustered with other samples on the PCA plot, and therefore were removed as this can be detrimental to the edgeR model (or any parametric differential expression model) (Li and Tibshirani, 2013). Data were then normalised using the edgeR package using the *trimmed mean of M-values* (TMM) method. This method corrects for differences in library size and composition per sample [especially important as sample data from different studies are included here], which in effect increases the accuracy of results (Robinson et al., 2010, Robinson and Oshlack, 2010). Eleven samples were used in two studies (Figure 7), and in these cases, one sample of the duplicated samples was excluded. The excluded sample chosen was the one that had a high percentage of duplicate reads, as this can artificially inflate the abundance of gene transcripts. Additionally, only cases with complete liver information were included in the comparisons 2–5. Gene transcript counts were summed across all samples, and any genes that had less than ten counts

per million were filtered out. This reduces noise and improves the statistical power of the differential expression analysis (Sha et al., 2015).

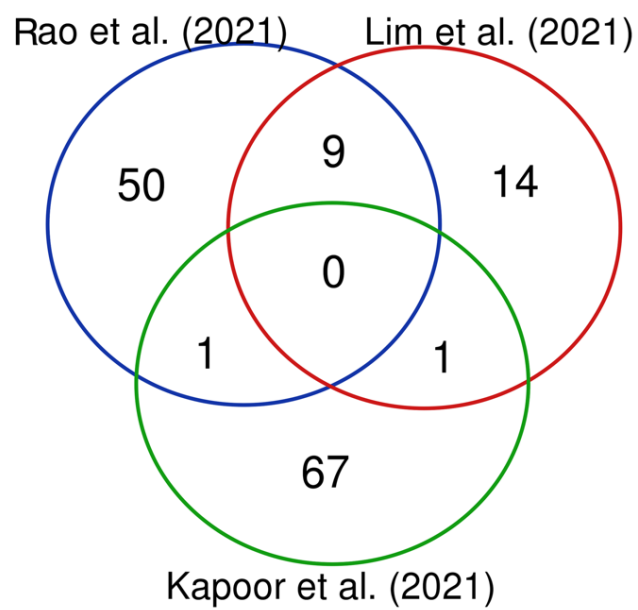


Figure 7. Venn diagram showing the sample overlap between the three studies used in this analysis. Rao et al (2021) in blue, Lim et al. (2021) in red, and Kapoor et al. (2021) in green.

4.2.4. Group comparisons

To look at differentially expressed genes (DEGs) more broadly, and then to focus on the effect liver pathology has on ARBI, five group comparisons were made (Table) and the metadata associated with each group comparison is represented in Table . These group comparisons allow us to examine specific gene expression changes associated with liver cirrhosis in individuals with AUD, while also considering the impact of alcohol consumption.

Table 4.2. Group comparisons for differential expression analysis.

Comparison	Reference Group	Disease group
1	All control cases	All AUD cases
2	Control cases without liver pathology	AUD cases without liver pathology
3	Control cases with liver steatosis	AUD cases with liver steatosis
4	Control cases without liver pathology	AUD cases with liver cirrhosis
5	AUD cases without liver pathology	AUD cases with liver cirrhosis

4.2.5. Covariates

Using the edgeR software package described in Section 4.2.5, an exploratory ANOVA was run for each group comparison to see which covariates to include in the differential expression model (Appendix D). The covariates included in the ANOVA were group (AUD or control), age, sex (male or female), brain pH, PMI, liver group (normal, steatosis, cirrhosis), and BMI. Age, sex, PMI, and BMI were included as covariates for all five group comparisons (Table).

Additionally, a principal components analysis was run with each of the three studies designated as groups. The samples from each study were clearly segregated together, and therefore the study of origin was also added as a covariate to account for any inter-study effects (Figure 8.2). Adding study as a covariate is explicit and therefore a better approach than using batch correction tools such as ComBat to account for batch effects, as these tools treat batch effects as random effects to be corrected for (Nygaard et al., 2016).

4.2.1. Generalised linear model

After the pre-processing steps for identifying and removing outliers, normalising, and filtering the data, the edgeR function `'glmLRT'` was used to perform the likelihood ratio test for the generalised linear model (Zhou et al., 2014). This compares the significance of gene expression between two groups, which in this case were each of the five group comparisons detailed in Table . False discovery rates (FDR) were controlled for by the Benjamini-Hochberg procedure.

Table 4.3. Demographics of each group comparison

	Control	AUD
<u>Group comparison 1:</u>		
Sex	17 female / 54 male	18 female / 52 male
Age (years $\pm$ SEM)	53.20 $\pm$ 1.13	54.13 $\pm$ 1.33
PMI (hours)	26.95 $\pm$ 1.56	35.79 $\pm$ 1.84*
Brain pH $\pm$ SEM	6.58 $\pm$ 0.04	6.58 $\pm$ 0.03
BMI $\pm$ SEM	29.95 $\pm$ 0.67	24.68 $\pm$ 0.66*
Liver classification	39 no pathology 32 steatotic	20 no pathology 32 steatotic 18 cirrhotic
<u>Group comparison 2:</u>		
Sex	12 female / 27 male	7 female / 13 male
Age (years $\pm$ SEM)	51.74 $\pm$ 1.71	58.15 $\pm$ 2.29*
PMI (hours)	27.77 $\pm$ 1.92	37.35 $\pm$ 3.33*
Brain pH $\pm$ SEM	6.63 $\pm$ 0.04	6.55 $\pm$ 0.04
BMI $\pm$ SEM	28.23 $\pm$ 0.75	25.28 $\pm$ 0.88*
Liver classification	39 no pathology	20 no pathology
<u>Group comparison 3:</u>		
Sex	5 female / 27 male	5 female / 27 male
Age (years $\pm$ SEM)	54.92 $\pm$ 1.31	51.88 $\pm$ 1.75
PMI (hours)	28.66 $\pm$ 2.82	36.55 $\pm$ 2.78*
Brain pH $\pm$ SEM	6.57 $\pm$ 0.06	6.62 $\pm$ 0.05
BMI $\pm$ SEM	30.72 $\pm$ 1.17	24.79 $\pm$ 1.27*
Liver classification	32 steatotic	32 steatotic
<u>Group comparison 4:</u>		
Sex	10 female / 24 male	7 female / 12 male
Age (years $\pm$ SEM)	52.69 $\pm$ 1.97	54.79 $\pm$ 2.56
PMI (hours)	26.71 $\pm$ 1.93	31.47 $\pm$ 3.88
Brain pH $\pm$ SEM	6.60 $\pm$ 0.05	6.52 $\pm$ 0.06
BMI $\pm$ SEM	28.19 $\pm$ 0.81	23.84 $\pm$ 1.17*
Liver classification	20 no pathology	19 cirrhotic
<u>Group comparison 5:</u>		
	AUD cases only	
	<u>No liver pathology</u>	<u>Liver cirrhosis</u>
Sex	7 female / 13 male	7 female / 12 male
Age (years $\pm$ SEM)	58.15 $\pm$ 2.29	54.79 $\pm$ 2.56
PMI (hours)	31.35 $\pm$ 3.33	31.47 $\pm$ 3.88
Brain pH $\pm$ SEM	6.55 $\pm$ 0.04	6.52 $\pm$ 0.06
BMI $\pm$ SEM	25.28 $\pm$ 0.88	23.84 $\pm$ 1.17

*significantly different $p < .0001$

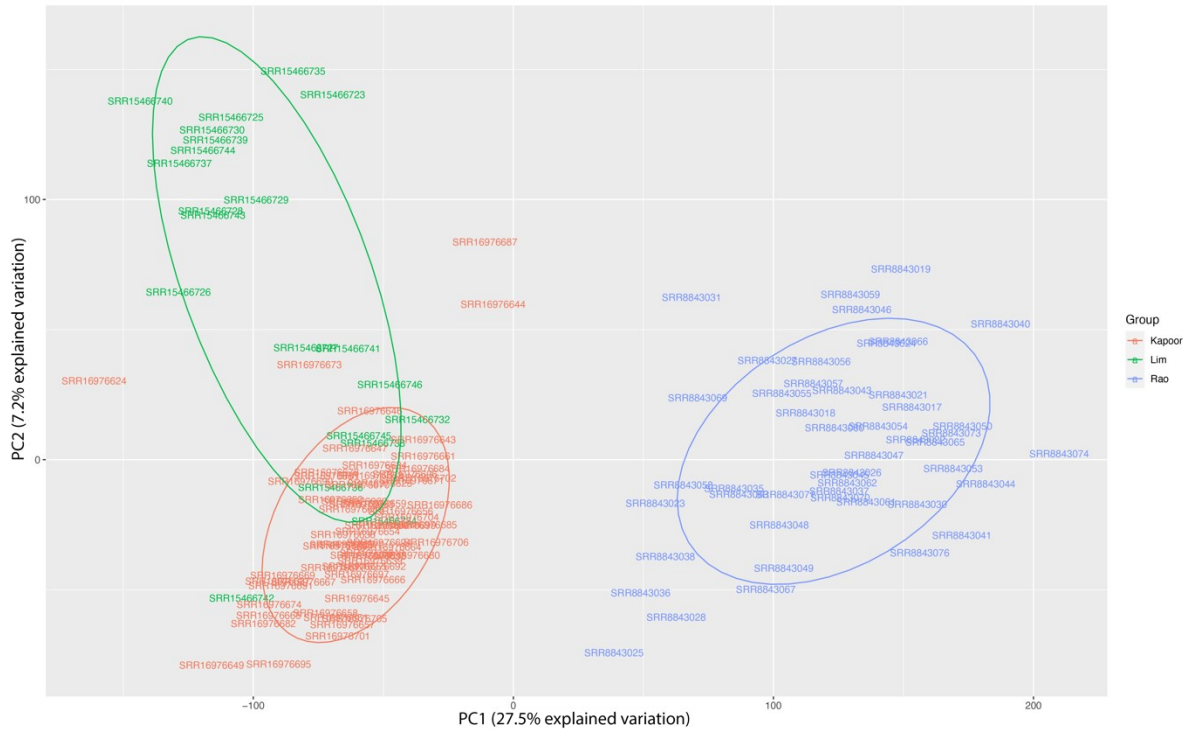


Figure 8.2. Principal component analysis to explore study-specific differences. A PCA plot shows definite clustering of samples on study of origin. Kapoor et al. (2021) (red); Lim et al. (2021) (green); and Rao et al. (2021) (blue).

4.3. Results

4.3.1. Number of differentially expressed genes

The total number of DEGs detected at both <0.05 and <0.1 FDR thresholds for each group comparison are shown in Table 4.4. In total there were 709 DEGs (FDR <0.1) detected across all groups, including 111 DEGs shared between two or more comparisons, and 90 of which were between comparisons 4 and 5, shown in Figure 4.3 as a Venn diagram created with InteractiVenn (Heberle et al., 2015). In general, there was a 10-fold increase in comparisons between cirrhotic and non-cirrhotic individuals versus individuals without severe liver pathology or when all cases and controls were compared.

Table 4.4. Number of DEGs for each group comparison, with both <0.05 and <0.1 FDR thresholds.

Group comparison	FDR <0.05	FDR <0.1
1: AUD (all) vs control (all)	8	24
2: AUD (normal liver) vs control (normal liver)	19	32
3: AUD (steatosis) vs control (steatosis)	19	29
4: AUD (liver cirrhosis) vs control (normal liver)	154	347
5: AUD (liver cirrhosis) vs AUD (normal liver)	152	277

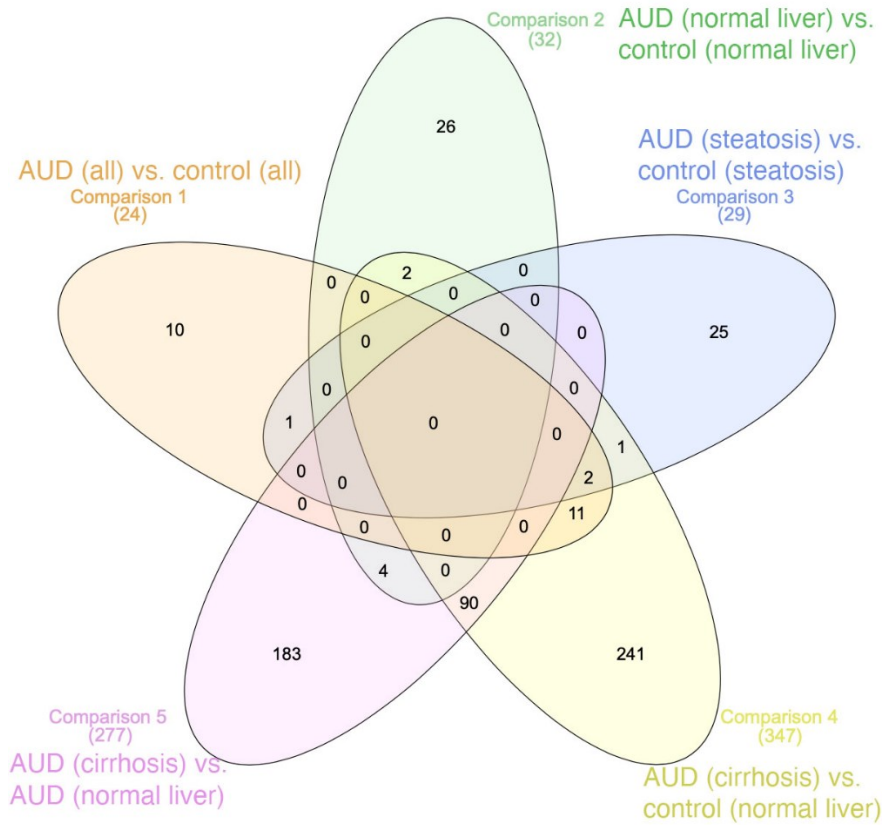


Figure 4.3. Five-way Venn diagram showing the number of DEGs ($FDR < 0.1$) expressed per group comparison and their overlap with other group comparisons.

4.3.2. Differential expression analysis of group comparison one (all AUD versus all controls)

The top ten DEGs from all AUD versus controls (group comparison one) are shown in Table . The highest-ranked DEG is *GOLGA8T*, which has a role in maintaining the Golgi apparatus architecture. There were no significantly enriched pathways, however, pathways relevant to the DEG list include stimulus detection, cardiac responses, lipid synthesis and degradation, and drug metabolism.

Table 4.5. Group comparison 1. Top DEGs of all AUD cases versus all control cases.

Rank	HGNC symbol	Transcript biotype	logFC*	FDR
1	<i>GOLGA8T</i>	Protein coding	3.71	1.77E-03
2	<i>MTCO3P22</i>	Processed pseudogene	-1.58	1.90E-03
3	<i>GUCY2D</i>	Protein coding	0.63	2.70E-03
4	<i>MTCO1P12</i>	Unprocessed pseudogene	-1.11	4.67E-03
5	<i>ENSG00000253881</i>	Processed pseudogene	2.37	6.25E-03
6	<i>ENSG00000284972</i>	Processed pseudogene	2.37	6.25E-03
7	<i>MESTP3</i>	Processed pseudogene	-0.77	7.11E-03
8	<i>USP17L3</i>	Protein coding	2.78	4.88E-02
9	<i>FUT5</i>	Protein coding	1.79	5.29E-02
10	<i>OTOS</i>	Protein coding	0.8	6.47E-02

* logFC represents the log fold change of the number of transcripts, relative to controls

4.3.3. Differential expression analysis of group comparison two (AUD with normal liver versus controls with normal liver)

The top ten DEGs for group comparison two (AUD v. controls, neither with any liver pathology) are shown in Table . The top two DEGs were pseudogenes, which do not translate into a functional protein. The most significantly changed protein-coding gene is *HLA-DRA* which is decreased relative to controls. *HLA-DRA* is a component of the major histocompatibility complex that is critical for normal immune functioning, allowing T cells to respond to pathogens. There were no significantly enriched pathways, however the highest-ranked GO analyses were related to translational fidelity (Table 4.7).

Table 4.6. Group comparison 2. Top DEGs of AUD cases versus control cases, neither with any liver pathology.

Rank	HGNC symbol	Transcript biotype	logFC*	FDR
1	<i>OR4G1P</i>	Unprocessed pseudogene	-2.46	9.07E-06
2	<i>NPM1P7</i>	Processed pseudogene	-0.37	3.57E-05
3	<i>HLA-DRA</i>	Protein coding	-2.94	4.35E-04
4	<i>ENSG00000281757</i>	Protein coding	-3.25	9.67E-04
5	<i>TMEM183AP1</i>	Processed pseudogene	3.21	2.86E-03
6	<i>MTND5P42</i>	Processed pseudogene	1.84	1.02E-02
7	<i>GTF2H4</i>	Protein coding	3.07	1.23E-02
8	<i>MDC1</i>	Protein coding	1.98	2.28E-02
9	<i>MICD</i>	Unprocessed pseudogene	4.48	2.28E-02
10	<i>VAR52</i>	Protein coding	2.89	2.28E-02

* logFC represents the log fold change of the number of transcripts, relative to controls

Table 4.7. Top five enrichment results for AUD versus controls, no liver pathology (group comparison two) using Gene Ontology and KEGG pathway gene sets.

Gene set	Description	Enrichment ratio	FDR
<u>Gene Ontology, biological process:</u>			
GO:0106074	aminoacyl-tRNA metabolism involved in translational fidelity	252.52	0.24
GO:0006450	regulation of translational fidelity	163.39	0.29

4.3.4. Differential expression analysis of group comparison three (AUD with steatosis versus control with steatosis)

The top ten DEGs for group comparison 3 are shown in Table 4.8. The top DEG was *MAGEL2*, which has roles in appetite and metabolism regulation.

There were no significantly enriched pathways.

Table 4.8. Group comparison 3. Top DEGs of AUD cases versus control cases, both groups with liver steatosis only.

Rank	HGNC symbol	Transcript biotype	logFC*	FDR
1	<i>MAGEL2</i>	Protein coding	-6.93	2.85E-32
2	<i>POLR1H</i>	Protein coding	-6.85	1.23E-19
3	<i>MS4A3</i>	Protein coding	-6.85	1.23E-19
4	<i>VASN</i>	Protein coding	6.15	7.44E-04
5	<i>LRRC10B</i>	Protein coding	4.55	1.28E-03
6	<i>OR7E47P</i>	Unprocessed pseudogene	4.04	1.45E-03
7	<i>GOLGA8T</i>	Protein coding	3.63	2.26E-03
8	<i>HSPA8P17</i>	Processed pseudogene	3.63	2.26E-03
9	<i>CARTPT</i>	Protein coding	-2.76	6.61E-03
10	<i>ENSG00000254851</i>	Unprocessed pseudogene	1.69	1.70E-02

* logFC represents the log fold change of the number of transcripts, relative to controls

4.3.5. Differential expression analysis of group comparison four (AUD with liver cirrhosis versus control with normal liver)

The top 10 out of 347 DEGs for group comparison 4 are shown in Table 4.9.

The top two highest ranked DEGs are *CA12* and *PLIN1*. In the brain, *CA12* expression is thought to be involved in the regulation of CSF pH, and *PLIN1* is primarily involved with lipid storage and metabolism in adipose tissue, but also synaptic plasticity and cognition in the cortex. Lipid metabolism and PPAR signalling pathways were significantly enriched (Table 4.10).

Table 4.9. Group comparison 4. Top DEGs of AUD cases with liver cirrhosis versus control cases without any liver pathology.

Rank	HGNC symbol	Transcript biotype	logFC*	FDR
1	<i>CA12</i>	Protein coding	1.43	2.33E-04
2	<i>PLIN1</i>	Protein coding	1.16	2.33E-04
3	<i>ARRDC3</i>	Protein coding	0.86	2.40E-04
4	<i>HMGB2</i>	Protein coding	0.75	2.40E-04
5	<i>ERBB2</i>	Protein coding	0.71	6.00E-04
6	<i>GNA14</i>	Protein coding	0.73	6.00E-04
7	<i>SDR42E1P5</i>	Processed pseudogene	2.95	3.09E-03
8	<i>SLC44A2</i>	Protein coding	0.53	3.43E-03
9	<i>TXNIP</i>	Protein coding	1.27	3.43E-03
10	<i>GUCY2D</i>	Protein coding	0.86	4.33E-03

* logFC represents the log fold change of the number of transcripts, relative to controls

Table 4.10. Top ten enrichment results for AUD with liver cirrhosis versus controls with no liver pathology (group comparison four) using Gene Ontology and KEGG pathway gene sets.

Gene set	Description	Enrichment ratio	FDR
<u>Gene Ontology, biological process:</u>			
GO:0006629	lipid metabolic process	2.28	2.13E-03
GO:0014070	response to organic cyclic compound	2.37	0.05
GO:0044255	cellular lipid metabolic process	2.23	0.05
GO:0051459	regulation of corticotropin secretion	37.04	0.11
GO:0009968	negative regulation of signal transduction	2.03	0.11
GO:0023057	negative regulation of signaling	1.98	0.11
GO:0051458	corticotropin secretion	31.75	0.11
GO:0007610	behavior	2.55	0.14
GO:0010648	negative regulation of cell communication	1.93	0.16
GO:0007614	short-term memory	24.69	0.17
<u>KEGG pathway analysis:</u>			
hsa03320	PPAR signaling pathway	6.93	0.02
hsa05213	Endometrial cancer	6.31	0.18

hsa05215	Prostate cancer	4.53	0.22
hsa04934	Cushing syndrome	3.33	0.41

Note: These databases use US English.

4.3.6. Differential expression analysis of group comparison five (AUD with liver cirrhosis versus AUD with normal livers)

The top 10 out of 277 DEGs for group comparison 5 are shown in Table 4.11.

The most significant DEG was *PBX2*, which is a transcription factor important for haematopoiesis and immune system function. Unlike previous group comparisons, here the top ten GO enriched pathways were all significant with themes relating to lipid metabolism and responses to copper, and four of the top ten KEGG pathways were significant and related to fatty acid degradation, PPAR signalling, cholesterol metabolism, and mineral absorption (Table 4.12).

Table 4.11. Group comparison 5. Top DEGs of AUD cases with liver cirrhosis versus AUD cases without any liver pathology.

Rank	HGNC symbol	Transcript biotype	logFC*	FDR
1	<i>PBX2</i>	Protein coding	4.12	1.24E-06
2	<i>HCG4B</i>	Unprocessed pseudogene	-4.83	1.41E-04
3	<i>VSIR</i>	Protein coding	1.24	1.76E-04
4	<i>MDK</i>	Protein coding	1.24	8.74E-04
5	<i>TFCP2L1</i>	Protein coding	1.18	8.74E-04
6	<i>ENSG00000272295</i>	Protein coding	4.65	1.07E-03
7	<i>ENSG00000288634</i>	Protein coding	3.48	1.07E-03
8	<i>MTA2</i>	Protein coding	0.57	1.34E-03
9	<i>CA12</i>	Protein coding	1.55	1.35E-03
10	<i>HMGB2</i>	Protein coding	0.87	1.35E-03

* logFC represents the log fold change of the number of transcripts, relative to controls

Table 4.12. Top ten enrichment results for AUD with liver cirrhosis versus AUD with normal liver gene enrichment analysis results (group comparison five) using Gene Ontology and KEGG pathway gene sets.

Gene set	Description	Enrichment ratio	FDR
<u>Gene Ontology, biological process:</u>			
GO:0006629	lipid metabolic process	2.47	2.73E-03
GO:0044255	cellular lipid metabolic process	2.61	8.25E-03
GO:0016054	organic acid catabolic process	4.72	9.44E-03
GO:0046395	carboxylic acid catabolic process	4.72	9.44E-03
GO:0015711	organic anion transport	3.59	9.77E-03
GO:0071280	cellular response to copper ion	18.42	9.77E-03
GO:0010273	detoxification of copper ion	27.37	1.18E-02
GO:1990169	stress response to copper ion	27.37	1.18E-02
GO:0044282	small molecule catabolic process	3.56	1.18E-02
GO:0061687	detoxification of inorganic compound	25.54	1.30E-02
<u>KEGG pathway analysis:</u>			
hsa00071	Fatty acid degradation	6.06	4.57E-02
hsa03320	PPAR signaling pathway	7.32	4.57E-02
hsa04979	Cholesterol metabolism	7.47	4.57E-02
hsa04978	Mineral absorption	6.79	4.57E-02
hsa04216	Ferroptosis	6.22	1.25E-01
hsa01212	Fatty acid metabolism	4.73	2.04E-01
hsa05150	Staphylococcus aureus infection	5.98	3.04E-01
hsa00061	Fatty acid biosynthesis	5.34	5.12E-01
hsa00380	Tryptophan metabolism	3.85	5.82E-01
hsa04610	Complement and coagulation cascades	11.49	6.90E-01

Note: These databases use US English.

4.4. Discussion

This study combined three publicly available RNA-Seq datasets to explore the effect of liver pathology in ARBI. All samples were from the PFC and all sourced from the same brain bank with overlapping samples between studies removed. Specifically, it was hypothesized that the PFC lipidome would become increasingly abnormal as liver pathology progressed from no pathology to steatosis to cirrhosis. Or, in other terms of transcriptomics, there would be greater fold changes of increased magnitude compared to controls and a broader range of changes in transcripts that subserve the lipidome. In support of this hypothesis, more DEGs were detected in the PFC as the severity of liver pathology worsened, and there were 90 DEGs in common when comparing AUD cases with liver cirrhosis to controls and AUD cases with and without any liver pathology respectively. Additionally, enriched pathways in the latter two comparisons were similar with ‘lipid metabolic process’ and cellular lipid metabolic process’ being two out of the three pathways significantly enriched in both. In contrast, there were insufficient DEGs to employ enrichment analysis in comparisons that featured groups without liver cirrhosis (group comparisons 2 and 3) or all AUD versus controls (group comparison 1). The top two significantly enriched GO analyses were ‘lipid metabolic process’ and ‘cellular lipid metabolic process’ and are consistent with the significant alterations to the lipidome in the PFC in Chapter 2 (Smith et al., 2022).

PPAR signalling was another enriched pathway identified in group 4 and 5 comparisons which implies that in AUD cases with liver cirrhosis, genes relating to PPAR signalling are over-represented compared to cases with normal liver pathology. Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that act as transcription factors and regulate gene expression. There are three subtypes: α , γ , and β/δ , each with different functions. PPARs have roles in preventing excess lipid accumulation in cells, suppressing pro-inflammatory cytokines, inhibiting microglial activation, and better insulin sensitivity and glucose metabolism (Tyagi et al., 2011). Chronic alcohol intake in mice reduces PPAR gene expression in multiple organ systems including the liver, heart, and lung (Matyas et al., 2016, Yeligar et al., 2016, Meng et al., 2020), but little is known about alcohol affects PPAR expression in the brain. Administration of PPAR γ and PPAR α agonists has been shown to reduce ethanol drinking in rodents (Stopponi et al., 2011, Ferguson et al., 2014). PPAR β/δ is the most abundantly expressed PPAR in the brain and specifically in neurons, astrocytes, and oligodendrocytes (Hall et al., 2008). Lipid turnover in myelin is essential for myelin homeostasis (Ando et al., 2003), and PPAR β/δ is required for unsaturated and long-chain fatty acid synthesis (Zhou et al., 2020). Ethanol treatment of B12 oligodendrocyte-like cells (Schubert et al., 1974) increases PPAR β/δ mRNA degradation, which may, directly and indirectly, interfere with normal myelination (Leisewitz et al., 2003).

Human leukocyte antigens (HLA) are expressed on immune cells such as macrophages and microglia. They recognise and respond to foreign debris but are also known to regulate the expression of other immune system molecules. This study showed increased *HLA-DP1* gene expression in AUD cirrhosis cases compared to AUD normal liver cases but decreased *HLA-DRA* expression in AUD cases with normal livers compared to control cases. Other RNA-Seq studies have shown similar trend increases in the expression of other HLA genes such as *HLA-DRA* and *HLA-DRB* in AUD cirrhosis cases compared to healthy controls (Yang et al., 2022).

In conclusion, genes were enriched in pathways relating to PPAR signalling and lipid metabolism, only when comparing AUD with liver cirrhosis, but not those with milder steatosis or controls. This suggests that, from a transcriptomics perspective, that liver cirrhosis is important for perturbations to the lipidome in ARBI. Although, this does not rule out the possibility of subtler effects on the lipidome with lesser or no liver pathology. If these transcription signals exist, they might be dwarfed by enrichment pathways of co-morbidities such as cancers, infections, and diabetes which dominate the transcription landscape of these non-cirrhotic cases.

Chapter 5. Statin use in ARBI

5.1. Introduction

There are many aspects to the standard treatment of AUDs, which often include a combination of psychosocial interventions such as cognitive behaviour therapy and motivational interviewing, peer support groups such as *Alcoholics Anonymous*, medications, nutritional support, and rehabilitation (Haber et al., 2021). Drug therapies that are used to treat AUD are to help individuals overcome addiction, such as naltrexone, an opioid antagonist that blocks the pleasurable effects of alcohol and reduces cravings (O'Brien et al., 1996), disulfiram, which produces nausea and vomiting when taken with alcohol to deter drinking behaviour (Fuller et al., 1986), and acamprosate, which restores neurotransmitter balance, reducing alcohol craving and improving abstinence success (Scott et al., 2005).

On the other hand, treatments for ARBI often focus on nutritional support, such as a high-calorie and protein diet to improve cognition and thiamine supplementation, which may prevent further damage to brain structures (Shakory, 2020). However, although these treatments are effective in managing AUD and subsequent ARBI, improving function, and slowing the

progression of the disease to prevent further damage, they do not specifically target the underlying neurological damage associated with ARBI, such as white matter atrophy.

There have been several drug trials that target ARBI. One such treatment is N-acetylcysteine (NAC) which is derived from cysteine and has antioxidant and anti-inflammatory properties. It is a precursor to glutathione, which is also an antioxidant. Current prescribed uses of NAC are to treat paracetamol overdose and to thin mucous in lung conditions such as chronic obstructive pulmonary disease (Burnham et al., 2021, Sadowska et al., 2006). Animal studies of NAC treatment in models of AUD suggest a reduction in alcohol-seeking behaviour (Lebourgeois et al., 2018), improved maintenance of abstinence (Fredriksson et al., 2023), and decreased hepatic inflammation and improved alcohol-related steatohepatitis (Setshedi et al., 2011). There is a phase four randomised controlled trial that is currently recruiting, which aims to evaluate the efficacy of NAC in treating AUD (ClinicalTrials.gov Identifier: NCT05408247).

Atorvastatin, followed by rosuvastatin, are the most prescribed drugs in Australia (NPS, 2021). These are both type 2 statins, which are prescribed to lower blood cholesterol levels in individuals with dyslipidaemia. Statins work by inhibiting HMG-CoA reductase, the rate-limiting enzyme in the mevalonate (cholesterol synthesis) pathway. Usage reduces cholesterol produced in the liver, and therefore less VLDL is required to export cholesterol, resulting in lower

blood plasma low-density lipoprotein (LDL). This lowers the risk of heart disease (Ramkumar et al., 2016). Statins have also been shown to exhibit antioxidant and anti-inflammatory properties (Davignon et al., 2004, Diamantis et al., 2017). In the brain, statins have been shown to inhibit iNOS (Morandi et al., 2011) and NADPH oxidase as well as reduce NF- κ B, which is thought to protect dopaminergic neurons in Parkinson's disease (Tong et al., 2018), decrease dementia risk, and reduce brain atrophy in patients with multiple sclerosis (Chataway et al., 2014, Torrandell-Haro et al., 2020, Zhang et al., 2018).

A large population-based study in Taiwan looked at the effect of statin use on outcomes in patients with AUD and ALD. The study used data from Taiwan's National Health Insurance Research Database. It examined claims from approximately 112,000 patients with an AUD to look at the incidence of decompensated liver cirrhosis and hepatocellular carcinoma [decompensated liver cirrhosis refers to late-stage cirrhosis where the liver is unable to function properly]. The study concluded that statin use decreased the risk of decompensated liver cirrhosis and hepatocellular carcinoma in people with AUD and ALD (Chiu et al., 2021).

In the current study, by-products of lipid peroxidation were measured in a post-mortem cohort of 18 males with an AUD, nine of whom were also prescribed atorvastatin. The aim was to measure the accumulation of 4-HNE in the PFC of AUD cases that were prescribed atorvastatin and those that were

not. This study also assayed iNOS via western blotting, which is known to be upregulated in chronic ethanol models (Valles et al., 2004) where the subsequent nitrosative stress combines with oxidative stress to induce neuroinflammation (Justo and Suemoto, 2022). Given the antioxidant effects of statins, it is hypothesised that there would be less lipid peroxidation in statin users demonstrated by a decrease in 4-HNE and iNOS in their post-mortem brain.

5.2. Methods

5.2.1. Demographics

This study was approved by the University of Sydney Human Research Ethics Committee (HREC 2015/022). The total number of cases requested from the NSW BTRC following their medication history review was 18. The study power was calculated by G*Power (version 3.1.9) to be 76%. Following approval from the scientific advisory board, cases were obtained that met the DSM-V criteria for AUD, nine of which were prescribed atorvastatin at the time of death and nine age-matched AUD without statin use. There were an insufficient number of females to include in this study. The demographics of the subjects used in this study are described in Table 5.1. Statin users and non-users were matched except for brain volume, which was lower in the atorvastatin group ($p = 0.046$).

Table 5.1. Demographics of cases used in this study.

	AUD-no statin	AUD-atorvastatin
Age	53.00 ± 1.94	52.78 ± 2.09
Sex	9 males	9 males
Brain weight	1441.78 ± 28.56	1373.56 ± 20.35
Brain volume	1414.16 ± 28.33	$1341.49 \pm 18.01^*$
BMI	25.30 ± 2.19	29.13 ± 2.43
PMI	36.22 ± 4.57	33.83 ± 5.54
Brain pH	7.08 ± 0.31	6.91 ± 0.38
Prescribed atorvastatin dosage (mg/day)	0	37.71 ± 7.66

$*p < 0.05$

5.2.2. ELISA and western blot

For both the ELISA and the western blot, frozen PFC grey matter was homogenised and extracted following the same method as described in Section 3.2.3, and total protein content was determined by a bicinchoninic acid (BCA) assay (ab207003, Abcam, Melbourne), according to their protocols.

For the ELISA, 4-HNE concentrations in the brain homogenates were measured using a 4-HNE lipid peroxidation competitive ELISA kit (ab238538, Abcam, Melbourne) to measure 4-HNE concentration. The kit included a 96-well plate that was pre-coated with 4-HNE conjugate. A dilution series of 4-HNE-BSA standards was set up in the well plate, including a blank, and protein samples from each case were added to the other wells in triplicate and incubated for ten min before adding anti-4-HNE antibody for an hour. The plate was washed with the kit wash buffer, and a secondary HRP antibody was added to each well and incubated. Following a further wash step, substrate solution was added until the colour was developed. A stop solution was added to stop the

enzymatic colour development, and absorbance was read using a Tecan microplate reader at 450 nm.

For iNOS western blotting, the same extraction and western blotting protocol was followed as in Sections 3.2.3–3.2.6. The primary antibody used for this experiment was rabbit monoclonal anti-iNOS antibody (ab178945, Abcam, Melbourne), at 1:625 dilution with 3% BSA in TBS-T, and the secondary antibody used was a HRP-linked anti-rabbit antibody, used at 1:2000 dilution in TBS-T (7074, Cell Signalling). GAPDH was used as a loading control, also described in Section 3.2.5. Other antibodies of interest failed optimisation, including C3b (ab231080) and PPAR α (ab215270). The blot was imaged using the ChemiDoc MP Imaging System and analysed using Image Lab software as described in Section 3.2.6. Data was normalised by dividing by the loading control and then data was imported into GraphPad Prism 9, where a t-test was performed and data graphed.

5.2.3. ELISA data analysis and statistics

For the ELISA, the absorbance values were obtained using Magellan 7.2 software, which interpreted the absorbance values from the microplate reader and calculated a standard curve from known concentrations for the dilution series of standards. The software interpolates unknown sample concentrations from the measured absorbances using this standard curve, representing 4-HNE

concentrations. Data were exported into MS Excel, where data was normalised to the total protein amount, determined earlier by BCA assay in Section 5.2.2.

ANCOVA was performed using IBM SPSS (version 25) software with brain volume as a covariate, as this was significantly different between groups. Adjusted concentrations were input into GraphPad Prism (version 9), where a t-test was performed, and graphed.

5.3. Results

The total amount of 4-HNE was significantly lower in the atorvastatin group compared to the no statins group ($F(8, 8) = 1.673$, $p = 0.009$). The total adjusted amount of 4-HNE in the AUD no statins group (17.74 ng/ μ g total protein) was greater than the AUD atorvastatin group (7.84 ng/ μ g total protein) (Figure 5.1).

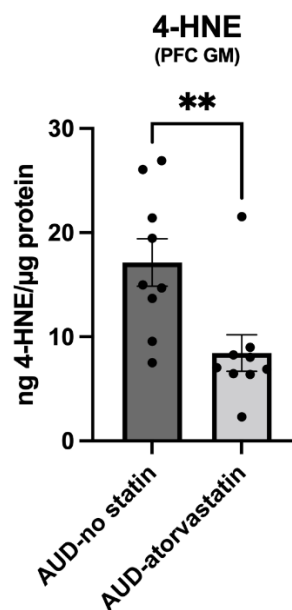


Figure 5.1. ELISA results of 4-HNE normalised to protein in AUD cases of statin users compared to AUD cases who had no record of statins being prescribed. $p = 0.009$.

The relative amount of iNOS was not significantly different between the atorvastatin and no statin group ($F(7, 7) = 3.874$, $p = 0.333$) (Figure 5.2).

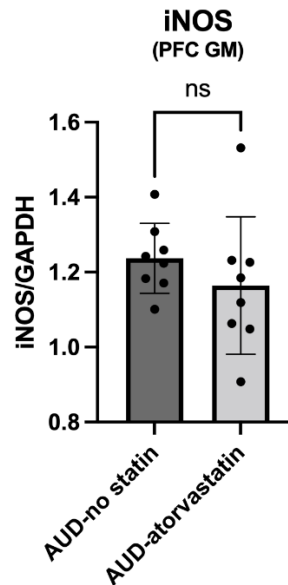


Figure 5.2. Western blot results of iNOS normalised to GAPDH (loading control) in AUD cases of statin users compared to AUD cases with no statins prescribed. $p = 0.33$.

5.4. Discussion

This study used a post-mortem cohort of 18 males with AUD, nine of whom were prescribed atorvastatin, to measure 4-HNE levels in the PFC. As per the hypothesis, total amount of 4-HNE significantly decreased in the atorvastatin group. iNOS was also measured as an indirect determinant of nitrosative stress, however, this was not different between the two groups.

4-hydroxynonenal is the major by-product of lipid peroxidation and is a hallmark feature of oxidative stress-induced cell damage. Along with iNOS, it

is upregulated in chronic alcohol models (Haorah et al., 2008). In addition to 4-HNE being a marker of lipid peroxidation, 4-HNE is a reactive aldehyde that covalently modifies proteins and DNA (Zhong and Yin, 2015). Protein modification by 4-HNE results in structural changes, impairing catalytic activity, as well as generating ‘neoantigens’ on proteins, triggering immune and inflammatory responses (Kurien and Scofield, 2008). 4-HNE can also cross-link DNA and introduce mutations to DNA sequences (Feng et al., 2003, Huang et al., 2010).

The mechanisms of how statin use decreases 4-HNE are not well understood, but probably due to both indirect and direct effects. In cardiac studies, statin use decreases ROS formation (Piechota-Polanczyk et al., 2012), and in neuronal cell cultures, atorvastatin has been shown to scavenge free radicals, promote neuronal viability by increasing pro-survival proteins PI3K and ERK, and decrease pro-apoptotic proteins cytochrome C, Bax, and cleaved caspases 3 and 9 (Lee et al., 2016).

Although iNOS expression was not changed in this study, atorvastatin treatment in traumatic brain injury in mouse models significantly decreased iNOS as well as CD11b, a microglia marker (Xu et al., 2017). While C3b is not a specific microglial marker, it is involved in immune responses to inflammation and can directly activate microglia (Crehan et al., 2012). An attempt to assay C3b via western blot was attempted here but unfortunately, not successful.

In summary, AUD cases who were prescribed atorvastatin had less 4-HNE accumulation in the PFC compared to AUD cases who were not prescribed any statin, which suggests less lipid peroxidation has occurred. Future studies should therefore look at whether this coincides with fatty acid chain length and double bond number in statin users, to see whether statin use contributes to the preservation of the lipidome.

Chapter 6. General Discussion

6.1. Summary of findings

This thesis explored the neurolipidome in ARBI and the importance of liver pathology in progressing ARBI. Specifically, the aims of this thesis were to 1) determine if and how the brain lipidome is affected in ARBI of AUD donors with and without liver cirrhosis; 2) investigate whether there are gene expression changes in the lipid metabolism machinery of ARBI of AUD donors with or without cirrhosis; and 3) to see how statin use might affect lipid peroxidation.

To address the first aim, Chapter 2 described using LC/MS was used to quantify PC, PE, PS, PG, PI, Cer, SM, and CEs in the PFC, MTG, and VIS white and grey matter of the human brain in AUD and control cases. This identified significant changes in glycerophospholipids and Cer, predominantly in the PFC and VIS regions. Using this information to address the second aim, Chapter 3 described assays for three proteins, CerS2, SPHK2, and CERT, which are involved in sphingolipid metabolism. This revealed that the decrease in ceramides is unlikely due to a change in CerS2 expression. However, there was an increase in CERT, which shuttles ceramide from the ER to the Golgi apparatus for conversion to sphingomyelin and other sphingolipids. One

interesting finding in Chapter 2 was that the lipidome was affected in the VIS as regions where ARBI-related pathology had been previously described, like the PFC. Quantification of the pan-neuronal marker, NeuN, showed similar and significant decreases in neurons in the PFC and VIS, the latter not previously examined.

In Chapter 3, western blotting was used to target three proteins associated with lipid metabolism.

To explore this question more broadly, Chapter 5 examined all the RNA-seq data publicly available that compared the AUD and control post-mortem PFC. By combining the three studies, additional covariates, such as liver pathology, could be introduced into the analysis. Lipid-related pathways were only enriched when comparing reference groups to AUD cases with liver cirrhosis, indicating that central lipid dysregulation is highly impacted by liver cirrhosis but not with lesser/no liver pathology.

To address the final aim, Chapter 5 describes 4-HNE accumulation in AUD cases prescribed atorvastatin to AUD cases not prescribed statins, using a different post-mortem cohort to the work above. The finding here was that less 4-HNE accumulated in the PFC in cases prescribed atorvastatin, suggesting a beneficial effect through reducing oxidative stress.

6.2. Results in context

The LC/MS study in Chapter 2 is the most comprehensive characterisation of the lipidome in ARBI to date. Prior studies characterising lipidomes in animal models had conflicting results, likely due to different ethanol exposure paradigms (e.g., intragastric injection versus *ad libitum* access), different disease aetiologies (e.g., limited information on liver pathology), lower mass spectrometry resolution (e.g., the Wang et al. (2019) and the de la Monte et al. (2018) studies were not able to resolve the composition of each fatty acid chain), and brain region(s) studied, which is important as there is region-specific susceptibility to chronic alcohol intake. MALDI-IMS methods were worked up for this project before deciding that an LC/MS-based approach would provide more sensitive data.

Constituent fatty acids are derived mainly from the diet, especially for PUFAs, although the brain can synthesise most of its required saturated and monounsaturated fatty acids (Moore, 2001). Other lipid precursor molecules such as glycerol and serine are derived from both dietary and hepatic *de novo* sources, and choline and ethanolamine are primarily derived from the diet. Using these molecules, the brain has a great capacity to synthesise most of its phospholipids, cholesterol, and ceramide (Chowdhury et al., 2022, Tracey et al., 2018, Zhang and Liu, 2015).

Astrocytes produce the majority of cholesterol in the brain, and neuronal cholesterol synthesis alone is insufficient (Zhang and Liu, 2015). Therefore,

disruptions to astrocytic cholesterol synthesis impair neuronal function (Ferris et al., 2017). Cholesteryl esters are the storage form of cholesterol and represent approximately 1% of total cholesterol in the brain (Koivuniemi et al., 2009, Zhang and Liu, 2015). Total CEs were significantly decreased in the PFC grey matter and the MTG white and grey matter in AUD cases, which might suggest that neurons or other cells are required to convert these CE stores to unesterified cholesterol. Astrocytic cholesterol synthesis is also increased in HE (three-quarters of AUD cases had some level of hepatic encephalopathy) (McMillin et al., 2018). There may be an increased cholesterol requirement in cell membranes to rebalance membrane fluidity (Hac-Wydro and Wydro, 2007) or as a protective mechanism against oxidative stress (Jove et al., 2023).

Ceramides were significantly decreased in AUD brains compared to control brains in Chapter 2, which agrees with the MALDI-IMS study from de la Monte et al. (2018). The expression levels of CerS2, which synthesises the most abundant type of ceramides in the brain (C22 and C24), were not changed between AUD and controls. However, it is important to mention that although protein levels of synthesis-related enzyme levels were constant between AUD and control brains, this does not necessarily mean there were no changes in enzyme activity. Similarly, ceramide synthesis could be unchanged between AUD and controls, but the increase in CERT expression, which transports ceramide to be converted into other sphingolipids, could (at least in part)

explain the decrease in ceramide. This would also contribute to the maintenance of SM levels we observed in Chapter 2.

The change in some phospholipids, but not others, is more difficult to explain. Free radicals are highly reactive with carbon-carbon double bonds (Ayala et al., 2014). In general, there was a decrease in PUFAs and fatty acid chain length, which seems reasonable as these would be preferentially targeted by peroxidation from oxidative stress in the brain caused by chronic alcohol misuse (Crews and Vetreno, 2014), which would be exacerbated in ALD (Tan et al., 2020). Lipid constituents that rely on dietary sources may also be affected. For example, choline is an essential nutrient (Zeisel and da Costa, 2009) and is required in PC synthesis. Selective PC decreases could be exacerbated by dietary deficiencies in choline (Lieber, 2003, Idrus et al., 2017, Klatskin and Krehl, 1954). Similarly, ethanolamine, another essential nutrient required for PE synthesis, may also be affected by dietary deficiencies (Zhou et al., 2017). PEMT is decreased in chronic alcohol consumption (explained in section 1.3.1), and although it is not a significant pathway for PE and subsequent PC synthesis in the brain, combined with lipid peroxidation, it might exacerbate the significant decreases of these select phospholipids.

Nearly 60% of neuronal membranes are composed of phospholipids, particularly PCs and PEs (Calderon et al., 1995), and so it is no surprise that changes in the lipidome reflect the neuronal loss seen in Chapter 3 in the PFC and VIS regions. A significant amount of focus of neuropathological research

into ARBI has been largely focused on the PFC, often neglecting other regions like the VIS region (Harding et al., 1997, Harper et al., 1987, Kril et al., 1997, Kril and Harper, 1989). Clinical symptoms that implicate the visual cortex have been previously described, such as impaired visuospatial processing (Gao et al., 2021), as well as metabolic aberrations (Bagga et al., 2014a), and less cortical activation in fMRI studies (Hermann et al., 2007), however neuronal counts in the VIS region have never been previously reported.

Alcohol consumption increases the level of palmitoleic acid (16:1) (Simon et al., 1996), with a similar increase observed in Chapter 2. PPAR α can be activated by palmitoleic acid (16:1), which also increases following moderate alcohol consumption (Simon et al., 1996). PPAR α was an enriched KEGG pathway in Chapter 4 when comparing groups of AUD donors with and without liver cirrhosis. Activation of PPAR α is generally beneficial as this phosphorylates AMPK, promoting fatty acid oxidation and glucose uptake, which is important for cellular energy balance (de Souza et al., 2017). However, during periods of chronic alcohol intake, ethanol decreases AMPK activation due to less availability of adenosine monophosphate, contributing to the accumulation of hepatic lipids and the development of steatosis (Sid et al., 2013). Additionally, acetaldehyde from ethanol metabolism inhibits PPAR α activation in the liver (Galli et al., 2001, Venkata et al., 2008), which increases oxidative stress (Nan et al., 2014). Although it is unclear whether PPAR α

activation is inhibited in the brain, it is decreased in Alzheimer's disease, which decreases antioxidant defences (Wojtowicz et al., 2020).

Oxidative stress occurs when more ROS are produced at a greater rate than the cell's ability to remove them, resulting in ROS accumulation. ROS interact with proteins, lipids, and nucleic acids. As described in section 1.5.1, hydroxyl radicals interact with double bonds on fatty acids, leading to the formation of lipid radicals and subsequent lipid peroxyl radicals, which interact with other lipid molecules, creating a chain reaction of lipid peroxidation, until this process is stopped by antioxidants. 4-HNE is a secondary product formed during lipid peroxidation (Ayala et al., 2014) and, thus, a proxy to indicate the amount of lipid peroxidation that has occurred in a tissue. 4-HNE is also a reactive aldehyde that forms adducts with cysteine, histidine, and lysine within proteins (Vila et al., 2008). This leads to conformational changes to protein structure and, therefore, function across multiple tissues, including the brain and liver (Zheng et al., 2014). The goal of Chapter 5 was to see if drugs that exhibit antioxidant activity, specifically atorvastatin, reduced 4-HNE in human brain protein extracts from cases with an AUD. Atorvastatin did this quite profoundly and would provide the basis for future studies to explore whether atorvastatin treatment can protect against lipid loss and white matter atrophy.

6.3. Liver pathology and the lipidome in ARBI

The basis by which ALD impacts the lipidome in ARBI is not fully understood, but this thesis provides evidence that the lipidome is affected in AUD cases with liver cirrhosis and that transcriptomic lipid metabolism pathways are implicated in the brain in AUD cases with liver cirrhosis, but not with steatosis or without any liver pathology.

In individuals with decompensated liver cirrhosis, there is less alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH2) activity (Vidal et al., 1990, Pares et al., 1987), and the microsomal ethanol-oxidising pathway is the predominant way to metabolise ethanol (described in section 1.5.1), and in theory acetaldehyde too, however not in the presence of ethanol (Wu et al., 1998, Harjumaki et al., 2021). Therefore, an accumulation of acetaldehyde occurs in ALD. Acetaldehyde exacerbates liver damage (Peters and Ward, 1988, Barry, 1988) and increases cancer risk (Seitz and Becker, 2007). At elevated levels, acetaldehyde can cross the BBB (Heap et al., 1995), and along with the small amounts of acetaldehyde produced by ethanol metabolism in the brain itself (Zimatkin et al., 2006), result in the accumulation of acetaldehyde in the brain. Acetaldehyde has been implicated in mediating an increase in NADPH oxidase expression in the brain, which results in ROS generation (Haorah et al., 2008). Additionally, acetaldehyde has been shown to bond covalently to proteins, largely in the white matter, disrupting protein function (Nakamura et al., 2003, Rintala et al., 2000).

Furthermore, hepatic encephalopathy arising from hyperammonemia, increased glutamine metabolism creates free radicals (Jayakumar et al., 2004, Murthy et al., 2001), activation of microglia and increased astrocyte reactivity (Claeys et al., 2022), and a microarray study by Song et al. (2002) showed that there was an upregulation of nNOS and heme oxygenase (both of which can contribute to oxidative stress) gene expression, and a decrease in superoxide dismutase gene expression, which catalyses the conversion of superoxide free radical, into less harmful hydrogen peroxide and oxygen.

6.4. Study strengths and limitations

All studies in this thesis utilised post-mortem tissue, which has advantages and disadvantages. The advantages of post-mortem tissue in AUD/ARBI research are that when tissue is characterised, such as the lipidome in Chapter 2, the characterisation considers the complete direct and indirect effects of a lifetime of chronic alcohol misuse, including how AUD has impacted the gut and liver and how these effects flow on to affect the brain. The reality is that a magnitude of variations exists between people, such as their nutritional intake, their pattern of alcohol consumption, their genetics, and their immune responses, and any random sample of the population may underestimate this variation and reach incomplete conclusions. Animal models are a great tool to specifically investigate specific effects of chronic alcohol intake on the brain and manipulate

different experimental conditions; however, these concentrate on single factors and neglect the complexity of human lives.

An advantage of the methodology used in this thesis includes the separation of white and grey matter tissue for LC/MS analysis in Chapter 2. This provided an opportunity to explore white and grey matter pathology separately but with higher mass resolution and accuracy than MALDI-IMS, which our colleagues had previously used. A big advantage of the Chapter 4 transcriptomic analysis was that data from three separate studies was reanalysed. Not only could all data be aligned to the current release of the human reference genome and be analysed in the same manner, but there was sufficient power to explore the impact of liver pathology within the AUD donors.

As mentioned above, a downside to post-mortem tissue is that although each case is generally well characterised from medical histories, toxicology reports, surveys, and neuropathological examinations, not every facet of the brain donor's life is able to be accounted for, which creates unknown confounders. There are blunt tools to deal with this issue, for example, including principal components as a covariate to account for some unknown influences on the data; however, the best way would be to use a larger cohort. This is often not a possibility; for example, in Chapter 5, only 18 cases from the NSW BTRC (the world's largest collection of AUD donors) met the inclusion criteria for that study, and so power is reduced, increasing the chance of spurious results. Another downside is that although liver diagnostic data was available, there

was no liver tissue from any of these cases for any laboratory assays, so investigating how the transcriptome of the liver changed in these cases was not possible.

6.5. Future directions

This thesis has provided evidence of changes to the neurolipidome in ARBI with a focus on liver cirrhosis.

Future human and animal models of chronic alcohol intake should pay attention to brain pathology in conjunction with liver pathology progression. To continue lipidomic characterisations, research should investigate the neurolipidome in AUD cases without liver pathology, which may strengthen our understanding of how liver pathology modulates the lipidome in ARBI, as suggested in the transcriptomics study in Chapter 4.

Other future research should characterise the neurolipidome in cases or models that were treated with antioxidant compounds, such as statins, but also other antioxidant drug therapies, including NAC, to measure real pathological implications, including the lipidome, transcriptome, neuronal loss, and brain volume changes. Antioxidant therapies hold great promise in reducing the severity of ARBI (Forman and Zhang, 2021).

6.6. Final conclusions

ARBI is complex. There is no single biomarker or pathological hallmark that is indicative of the disease, although white matter atrophy is a prominent feature.

ARBI severity is due to several factors, including dietary, genetic, the number of years drinking, and the amount of alcohol consumed over their lifetime. There appears to be a domino effect spanning multiple organ systems. Metabolism of alcohol creates oxidative stress, and the products of alcohol metabolism disrupt gut-tight junctions. This leads to a leaky gut and increases LPS in the circulatory system, resulting in systemic inflammation. Lipolysis of adipocytes and ethanol metabolism contribute to liver steatosis development. Systemic inflammation is essential to progress liver pathology from steatosis to cirrhosis. Patients with liver cirrhosis have a reduced capacity to metabolise alcohol and metabolise ammonia (important for the development of hepatic encephalopathy), which increases oxidative stress and neuroinflammation to damaging levels. The brain may be attempting to maintain homeostasis by converting ceramides to other sphingolipids to preserve and repair myelin, albeit in an environment where alcohol intake is incessant, causing the brain and existing fatty acids to be under free-radical attack. Second, to abstinence, antioxidant therapies targeting the brain and liver may provide significant therapeutic benefits.

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Appendices

Appendix A

All supplementary files for Chapter 2 are available under supporting information on the journal website at <https://doi.org/10.1111/jnc.15530>

Supplementary File 1

Tables containing all lipid species identified and quantified in this manuscript, ordered by region and head group. Highlighted cells indicate significance at $p < 0.05$.

Supplementary File 2

sPLS-DA loadings for components 1 and 2 for each region.

Supplementary File 3

Proportions of glycerophospholipids that were identified and included in this manuscript in controls and alcohol use disorder group, ordered by brain region.

Appendix B

Protein extraction buffer recipe

Chemical	Amount for 1 mL
Tris, 2 M, pH 7.6	10 μ L
PMSF, 1 M	1 μ L
cOmplete, 1 tablet dissolved in 2 mL of MQ	40 μ L
CHAPS	6 mg
EDTA, 650 mM, pH 8	2 μ L
NaF, 2.5 M	50 μ L
Na ₃ VO ₄ ,	10 μ L
DTT	2 μ L
NaPP _i	89.3 μ L
10% SDS	50 μ L
milliQ	Make up to 1 mL

To prepare each chemical component:

Tris: Dissolve 12.11g Tris into 40 mL MQ. Adjust to pH 7.6 with HCl. Adjust with MQ to final volume of 50 mL

PMSF: Dissolve 17.42 mg in 1 mL of isoproteranol. Store at -20°C.

cOmplete: Dissolve 1 tablet in 2 mL of MQ. Store at -20°C.

CHAPS: Store at 4°C

EDTA: Dissolve 1.861 g of EDTA in 5 mL of MQ, adjust to pH 8 with NaOH and make up to 10 mL with MQ.

NaF: Dissolve 1.0498 g of NaF in 10 mL of MQ.

Na₃VO₄: Dissolve 0.185 g into 10 mL of MQ. Adjust to pH 10 (solution becomes yellow). Boil until solution turns colourless, cool to RT, readjust to pH 10, repeat steps until solution remains colourless and pH stabilises at pH 10. Store at -20°C in 300 μ L aliquots.

DTT: Dissolve 38.6 mg of DTT in 250 μ L MQ. Store at -20°C.

NaPP_i: Dissolve 2.4979 g of NaPP_i in 50 mL of MQ.

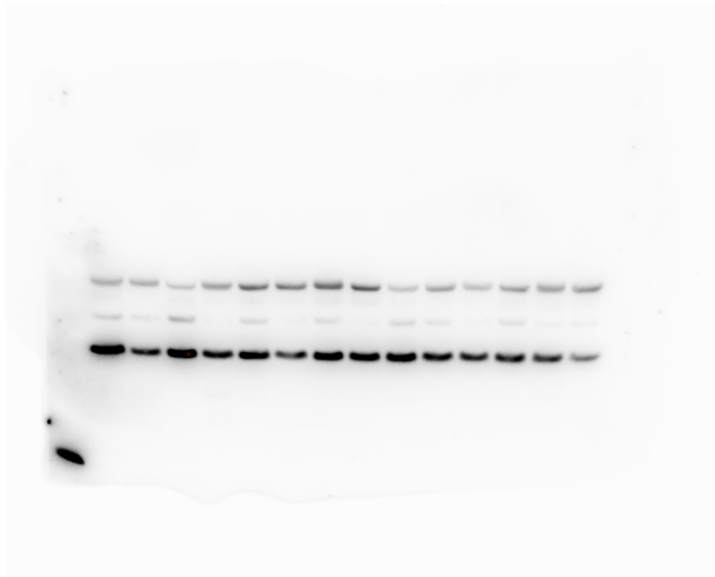
Appendix C

Western blot images

CerS2

Above: CerS2. Below: GAPDH (loading control).

ladder (lane 1), AUD cases (lanes 2-8), control cases (lanes 9-15)



SPHK2

Above: SPHK2. Below: GAPDH (loading control).

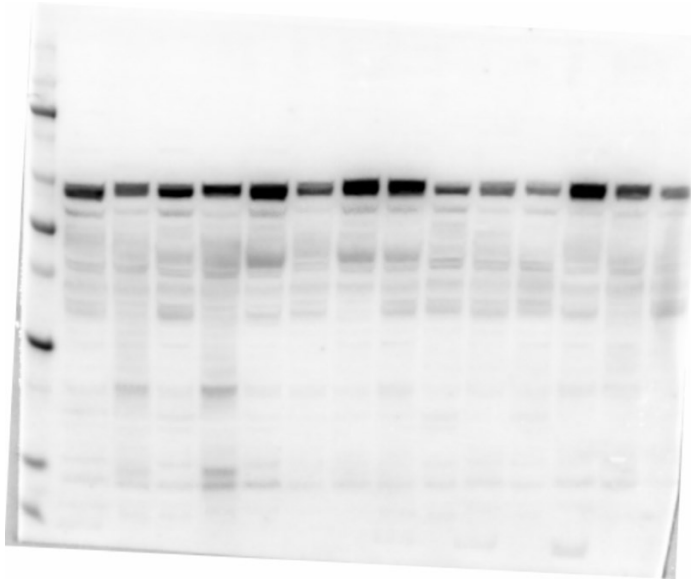
ladder (lane 1), AUD cases (lanes 2-8), control cases (lanes 9-15)



CERT

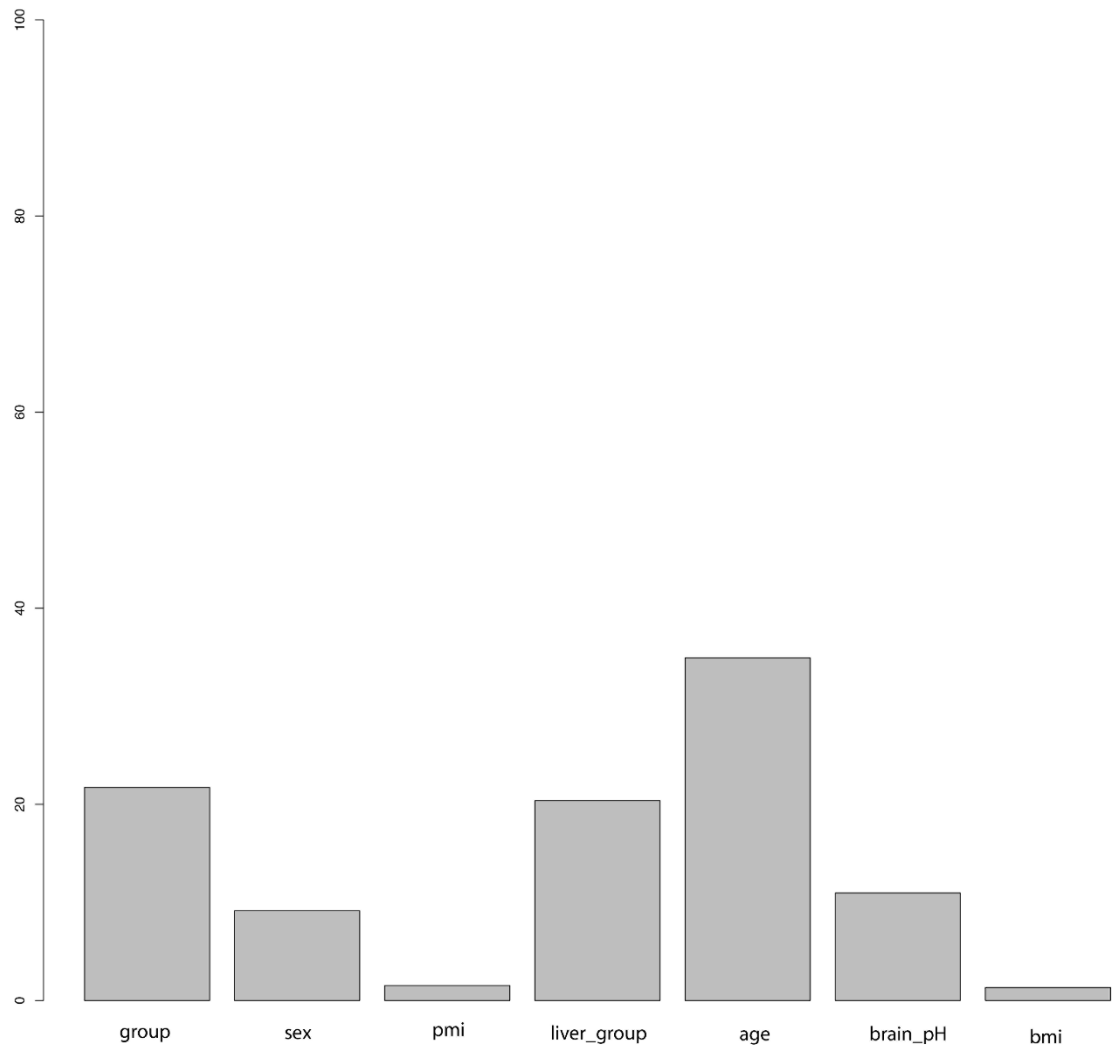
Above: CERT (chemiluminescent image merged with colorimetric image to show ladder). Below: GAPDH (loading control).

ladder (lane 1), AUD cases (lanes 2-8), control cases (lanes 9-15)



Appendix D

ANOVA for all AUD and all control group comparisons



R code used for this ANOVA run:

```
anova_vars <-  
  c("aud_status", "sex", "brain_pH", "age", "pmi", "liver_group",  
    "bmi")  
covar_anova <- covar[,names(covar) %in% anova_vars]  
covar_anova <- covar_anova[complete.cases(covar_anova),]  
reads_fil_anova <- reads_fil[,names(reads_fil) %in%  
  covar_anova$SRA_run]  
reads_norm <- data.frame(reads_norm)  
reads_norm_anova <- reads_norm[,names(reads_norm) %in%  
  covar_anova$SRA_run]  
z=reads_fil_anova  
z=reads_norm_anova  
m=melt(z)  
colnames(m) <- c("sample_ID", "counts")
```

```

group <- rep(as.numeric(covar_anova$aud_status, each=nrow(z)))
sex <- rep(as.numeric(covar_anova$sex, each=nrow(z)))
rin <- rep(as.numeric(covar_anova$rin, each=nrow(z)))
brain_pH <- rep(as.numeric(covar_anova$brain_pH, each=nrow(z)))
age <- rep(as.numeric(covar_anova$age, each=nrow(z)))
pmi <- rep(as.numeric(covar_anova$pmi, each=nrow(z)))
liver_group <- rep(as.numeric(covar_anova$liver_group,
                             each=nrow(z)))
bmi <- rep(as.numeric(covar_anova$bmi, each=nrow(z)))
matrix <-
  data.frame(m, group=group, sex=sex, pmi=pmi, age=age, brain_pH=br
ain_pH, liver_group=liver_group, bmi=bmi)
fit1 <- lm(counts ~ group + sex + pmi + liver_group + age +
  brain_pH + bmi, data=matrix)
a <- anova(fit1)
nfac <- length(a[,1])-1
maxval = 100
pdf(file=paste(study, "anova_caine_10_reads_norm_all.pdf", sep =
  "_"), height = 15, width = 15)
par(mar = c(10,4,4,2))
nfac <- length(a[,1])-1
barplot(100*a$"Sum Sq"[1:nfac]/sum(a$"Sum
  Sq"[1:nfac]), names.arg=rownames(a[1:nfac,]), ylim=c(0,maxval)
  , las=3)
dev.off()

```