Proteomics and Lipidomics to Study Racial Disparities in Alzheimer's Disease

By

Mostafa Jobaer Khan

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Chemistry

January 31, 2021

Nashville, Tennessee

Approved:

Renã AS Robinson, Ph.D.

John A. McLean, Ph.D.

Kevin L. Schey, Ph.D.

Ned A. Porter, Ph.D.

ACKNOWLEDGEMENTS

First and foremost, I would thank Allah for giving me the strength and patience during the last five years as a graduate student. Next, I would like to thank my advisor Dr. Renã AS Robinson, for taking me in as a student. Over the years, she has been the perfect mentor, who has provided guidance in my projects, has pushed me to think more deeply about the projects I have been involved in, and taught me how to think like a scientist. I loved how she always made me think of the big picture of every experiment. Her passion for the work we do is truly amazing and it has inspired me to give my best effort in every experiment. I know I could be hard to deal with at times, so I appreciate her for sticking with me till the end. She is also an amazing person, who cares a lot for every student associated with her, and always focused on the importance of family over everything else. For all of this and so much more that I haven't mentioned here, I would like to say to Dr. Robinson, thank you for everything.

In addition to my advisor, I would also like to thank my dissertation committee: Dr. John A. McLean, Dr. Kevin L. Schey and Dr. Ned A. Porter for taking time from their busy schedule to agree to be on this committee, and also for providing insight and direction. Their advice has been really significant in the progress I have made thus far.

My sincere gratitude goes to all the past and present members of the RASR laboratory. I would like to thank Dr. Bushra Amin for training me with proteomics and mass spectrometry (MS) instruments, and also for helping me with planning my first experiments. Special thanks to Dr. Christina King for helping me in developing MS methods as well as for thinking about my projects in general. I would also like to thank Dr. Albert Arul, Kaitlyn Stepler, David Vassallo, and Nadjali Chung for being there as a colleague and also as a friend, and also for providing

important feedback on the presentations to improve my work. I would also like to thank Ryan Dyer, for suggesting to me the RASR laboratory when I was looking for a new lab, and also being a good friend over the years.

A special thanks to all the collaborators I have been involved with, especially Dr. Codreanu and Dr. Sherrod for their help in developing the lipidomics workflow. I would also like to thank Dr. Wages for the sterol analysis. I would also like to thank all of the undergraduates that I have been involved with over the years. Special thanks to Dr. Pearson from Sciex for training me on the Sciex platform and also for providing important advice for instrument troubleshooting.

Last but not the least, I would like to thank my friends and family for supporting me through the years. My father's dream was for one of his sons to follow his footsteps and complete a PhD and I am a fulfillment of his dream. Being away from home for such a long time has been tough for my mother, who has been calling and caring for me every day for the past 5 years. I wish I could show them how much I love them. Thanks to my brother and sister-in-law for providing emotional support during this period. Finally, I would like to thank my loving wife, who ever since our marriage has provided more support than I could have imagined, she has truly been an amazing partner. I love you wifey.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I

Introduction

1.1. Alzheimer's Disease and Racial Disparities

Alzheimer's disease (AD) is a neurodegenerative disorder that causes memory loss and decreases cognitive function to the point that it disrupts daily activity. Recent studies report 5.8 million Americans are suffering from this disease and it is the 6th leading cause of death in the USA.[1] While deaths due to diseases such as heart disease, HIV and breast cancer have decreased significantly from 2000-2015, deaths due to AD increased by 146%.[1] To date, there is no cure available for this disease and the estimated cost per year to treat, provide care, and give support stands at $306 billion in 2020.[1]

One aspect of AD, which has been severely understudied in basic science research is the aspect of racial disparities. It is well reported that African American/Black adults are 2-3 times more likely to develop AD compared to non-Hispanic White adults.[1-2] AD is also the 4th leading cause of death among African American/Blacks.[3] The incidence of AD is higher in the population aged 65 and older and by 2060, African American/Black and other minorities aged 65 years and older will constitute 45% of the US population.[1, 4]

Despite the apparent disparity in incidence of AD among African American/Black adults, there is no difference in the initial manifestation of the disease among different racial groups.[2, 5] This disparity could be a consequence of the differences in quality of education, socioeconomic status, genetic factors and presence of comorbidities.[6-9] Recently, there has been evidence of differences in AD biomarkers in the African American/Black population. Multiple studies have

reported lower cerebrospinal fluid (CSF) concentrations of total tau, phosphorylated tau [10-11] and interleukin-9 [12] in African American/Black adults compared to non-Hispanic White adults. There have been few studies involving African American/Black samples in AD research [13-14] despite better diversity of samples in other diseases such as prostate cancer [15-16], bladder cancer [17-18], Wilms tumor [19], cardiovascular disease [20-21], and endometrial cancer.[22]

1.2. Blood-based Biomarkers in AD

According to the Food and Drug Administration (FDA)- National Institute of Health (NIH) Biomarker Working group, a biomarker is "A defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention".[23] An ideal biomarker for AD should then be- 1) able to detect a fundamental feature of AD neuropathology; 2) validated in neuropathologically-confirmed AD cases; 3) precise (able to detect AD early in its course and distinguish it from other dementias); 4) reliable; 5) non-invasive; 6) simple to perform; and 7) inexpensive.[24] Much effort has gone into AD biomarker discovery with varying degrees of success. To date, five biomarkers have been successfully established in AD- CSF measurements of amyloid beta 42 (Aβ42), total tau and phosphorylated tau concentration, and imaging biomarkers including Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET) imaging of amyloid plaques, and hyperphosphorylated tau.[25-26] Despite the high accuracy and specificity of these biomarkers for AD, the invasive nature of these biomarkers makes their application in all disease stages challenging. Furthermore, MRI and PET scanning are expensive and require specific training for administration. Thus, there is still an urgent need for blood-based biomarkers to be applied as first line diagnostic tools for AD.

Finding blood-based biomarkers has been very challenging due to the low concentrations of these biomarkers in blood. Levels of Aβ42 as a potential biomarker has been well studied in

human plasma with varying degrees of success.[27-28] A recent meta-analysis found the Aβ42 ratio between AD and cognitively normal individuals to be non-significant.[29] Comparatively, the Aβ42/Aβ40 ratio has had better success as a potential blood-based biomarker in AD. Reduced plasma levels of Aβ42/Aβ40 ratio have been reported in plasma [30-31], and have reported high diagnostic accuracy for the detection of amyloidosis.[32] Positive correlation of the Aβ42/Aβ40 ratio between plasma and CSF exists and is lower in AD, compared to mild cognitive impairment (MCI) and subjective cognitive decline (SCD) individuals.[33]

Other potential blood-based biomarkers include tau protein, which has been found to be increased in AD.[34] Increased tau protein has been reported to be associated with faster disease progression.[35] Similar increase in neurofilament light (NF-L) levels in plasma have high diagnostic accuracy in AD.[36] Variations in the levels of cytokines and chemokines have also been suggested as potential blood-based biomarkers in AD.[37] Another approach for blood-based biomarker discovery in AD is the use of 'omics techniques such as proteomics and lipidomics, which is discussed below in **Section 1.4** and **1.7**.

1.3. Plasma Proteomics

Plasma is one of the major blood components constituting 55% of the total volume of blood.[38] Plasma is composed of mostly water, with 7% attributed to proteins.[38] Plasma proteins contribute to blood viscosity, which in term contribute to maintaining blood pressure.[38] Besides the classical proteins (albumin, IgG) plasma contains circulation proteins from different organs in the body.[39] These characteristics make plasma an ideal candidate for disease study of protein alterations. Also, plasma samples are less invasive than CSF, easy to collect and the number of samples available in tissue banks are much higher compared to other tissue types. As a result, human plasma has received wide popularity for disease studies using proteomics.

Proteomics is defined as the analysis of the protein complement of a cell, tissue, or organism under a specific, defined set of conditions.[40] Proteomics studies have the ability to provide information on protein abundance, pathways involved, protein function as well as post translational modifications. In general, proteomics can be divided into three different methods- top-down [41], middle-down [42], and bottom-up [43] proteomics. In **Chapter II** of this dissertation, we applied bottom-up techniques for plasma proteomics analysis. In general, bottom-up proteomics involves the proteolytic digestion of proteins into their corresponding peptides before being analyzed using mass spectrometric analysis. A general overview of a plasma proteomics workflow is provided in **Figure 1.1**. Plasma proteomics involves the following steps- immunodepletion of crude plasma [44], digestion using trypsin-Lys-C enzymes [45], isobaric tagging [46], high pH fractionation [47], and liquid chromatography-mass spectrometry (LC-MS) MS/MS MS^3 analysis.

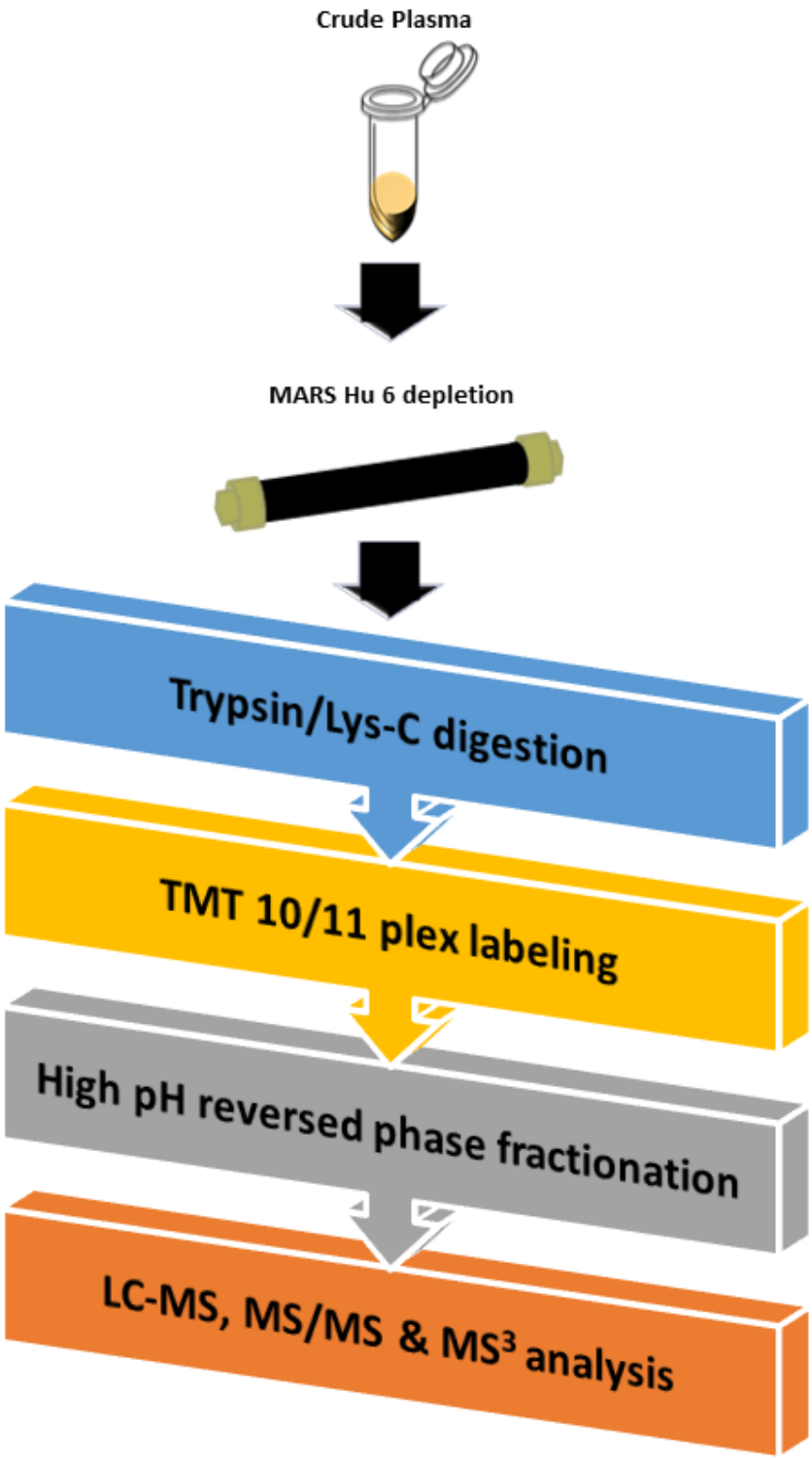

Figure 1.1. Plasma proteomics workflow. Crude plasma samples are first immunodepleted, followed by digestion and isobaric tagging. This is followed by high pH reversed phase fractionation of the samples followed by LC-MS, MS/MS, MS^3 analysis. Figure adopted from the recently submitted research article "Dataset of Why race matters in plasma proteomics biomarker discovery for Alzheimer's disease." (Under review in *data in brief*)

1.3.1. Immunodepletion of plasma

Despite its popularity in disease studies, human plasma is a very complicated fluid for protein analysis. Plasma has a high dynamic range (~10^{10}-10^{12}) in protein concentration [39], which makes detection of low abundant proteins highly challenging. Additionally, 22 proteins constitute almost 99% of the total plasma protein mass.[39] Various methods have been proposed to mitigate this issue. By far the most popular is the immunodepletion of high abundance proteins.[39] Different immunodepletion techniques are available commercially, which includes multiple affinity removal system (MARS) [48], ProteoPrep [49], and Proteominer.[50] MARS depletion has been found to be the most efficient and reproducible among all the available techniques.[51] MARS is a LC stationary phase column, which contains antibodies for the six most abundant proteins in plasma (albumin, IgG, IgA, transferrin, haptoglobin and anti-trypsin).[48] The crude plasma is loaded onto the column and, the six proteins are separated from plasma by an antigen-antibody interaction, while the remaining proteins pass through the column and the undepleted proteins are collected for further MS analysis. In **Chapter II** of this dissertation, we used a MARS-6 column for plasma depletion.

1.3.2. Isobaric tagging

In quantitative proteomics, sample multiplexing allows analysis of multiple samples in a single injection. This multiplexing reduces instrumentation acquisition time as well as reduces sample variation introduced due to sample preparation. One of the most popular sample multiplexing techniques is the use of isobaric tagging [46] such as isobaric tags for relative and absolute quantitation (iTRAQ) [52], tandem mass tags (TMT) [53], and N, N-dimethyl leucine (DiLeu).[54] TMT reagents have the capability to multiplex 6 [55], 11 [56], and 16 [57] samples in a single analysis. The TMT reagent has three groups in its structure- a reporter ion group, a balancer group and amine reactive group (**Figure 1.2a**). For the TMT-11 plex tag, heavy isotopes (^{13}C, ^{15}N) are

incorporated into the reporter ion and balancer groups in such a manner that the overall mass of the tag remains the same (229.16 Da), while the mass of the reporter ion groups vary from 126-131 Da (**Figure 1.2b**). During the tagging reaction, the N-terminus and the lysine residue react with the reactive group, leaving the mass reporter and balancer groups on the peptide. The tags are individually added to each sample and then pooled together into a single sample before injection into the mass spectrometer. Despite the incorporation of the heavy isotopes, the labeled peptides all elute simultaneously. Once the labeled peptide is eluted and selected for fragmentation, the individual reporter ion intensities are quantified based on their respective peptide abundances (**Figure 1.2c**).

1.3.3. MS^3 quantification using tribrid mass spectrometer

Quantitation of peptides labeled with TMT requires high resolution (>50000), due to the small mass differences (~6 mDa) between neutron coded isobaric tags.[58] Orbitrap instruments provide the necessary resolution to resolve this small mass difference and enables quantitation.[58] In spite of this, TMT reagents suffer from inaccurate quantitation due to ion interferences.[59] This issue can be resolved by the use of the more advanced tribrid Orbitrap instruments, which have better sensitivity for detecting low abundant proteins, as well as high resolution compared to previous hybrid models.[60] Also, the tribrid instruments have multi-notch MS^3 capability called synchronous precursor selection (SPS), which allows multiple MS/MS fragments to be selected for MS^3 quantification, which increases the number of quantifiable peptides as well as reduces distorted reporter ion ratios.[61] Performing MS^3 quantification has the advantage of providing more accurate quantitative information, which is necessary when studying various disease states and facilitating biomarker discovery.

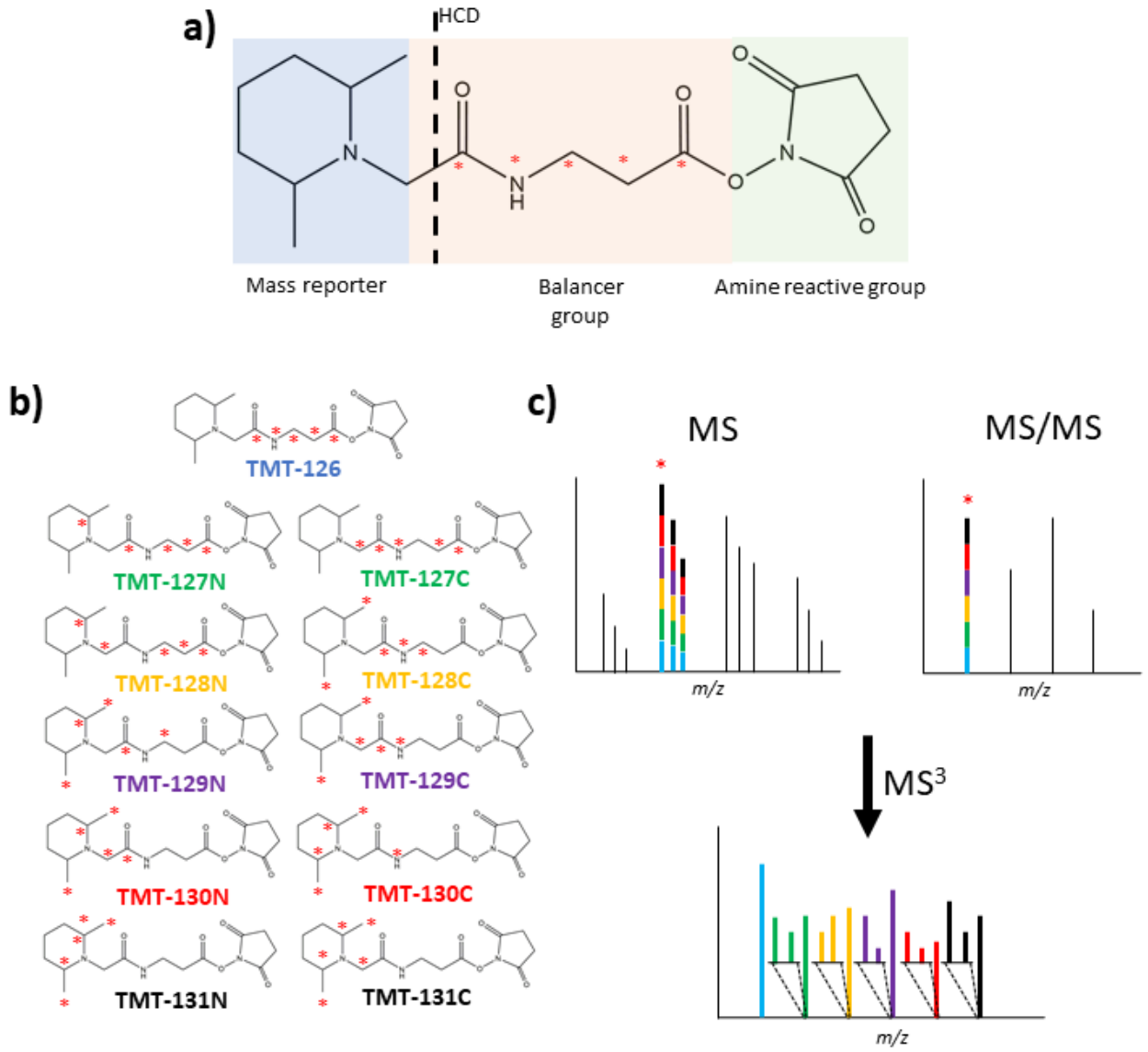

Figure 1.2. TMT-11 plex labeling strategy. a) Example structure of TMT-126; **b)** TMT-11plex reagent structures with corresponding isotope position denoted by *; **c)** Resulting spectra from TMT analysis.

1.4. Plasma Proteomics and AD

1.4.1. Protein dysregulation in AD

Proteomics studies have been performed using plasma/serum samples comparing AD and cognitively normal samples to identify dysregulated proteins in AD.[62-71] However, there has been inconsistency in findings, as seen in **Figure 1.3**. Alpha-2 macroglobulin [62, 67, 69, 71-72] reported to be genetically associated with AD [73] was increased in AD by five studies [62-63, 67, 71-72], while others have reported it to be decreased in AD.[69, 71] Other proteins which are dysregulated in AD include afamin [68-71, 74], several apolipoproteins such as apolipoprotein A1, apolipoprotein B-100, apolipoprotein A4, apolipoprotein E, apolipoprotein J, apolipoprotein C [62, 65-66, 68-71, 74, 75], and several complement proteins such as complement factor H, complement factor B, complement C4a precursor protein.[62-63, 66-67, 69-72, 74, 76-78] Pathways include lipid metabolism [79], inflammation response [80-81], and coagulation.[82]

1.4.2. Protein panels for blood-based biomarker discovery

Biomarker panels for classifying both AD and MCI samples have been proposed based on proteomic analyses. A panel of 18 signaling proteins classified AD with 90% accuracy.[83] Others tried, but failed to replicate such results in different sample cohorts.[84-85] On the other hand, a subset (5 of the 18 -IL-1α, IL-3, EGF, TNFα and G-CSF) of the protein panel have reported diagnostic accuracy of 96% in predicting clinical AD.[86] Similar attempts of using a subset of the protein panel did not replicate and had lower accuracy.[87-88] Despite the 18-protein panel lacking replicability in separate cohorts, it still is considered a benchmark for AD blood-based biomarker development.

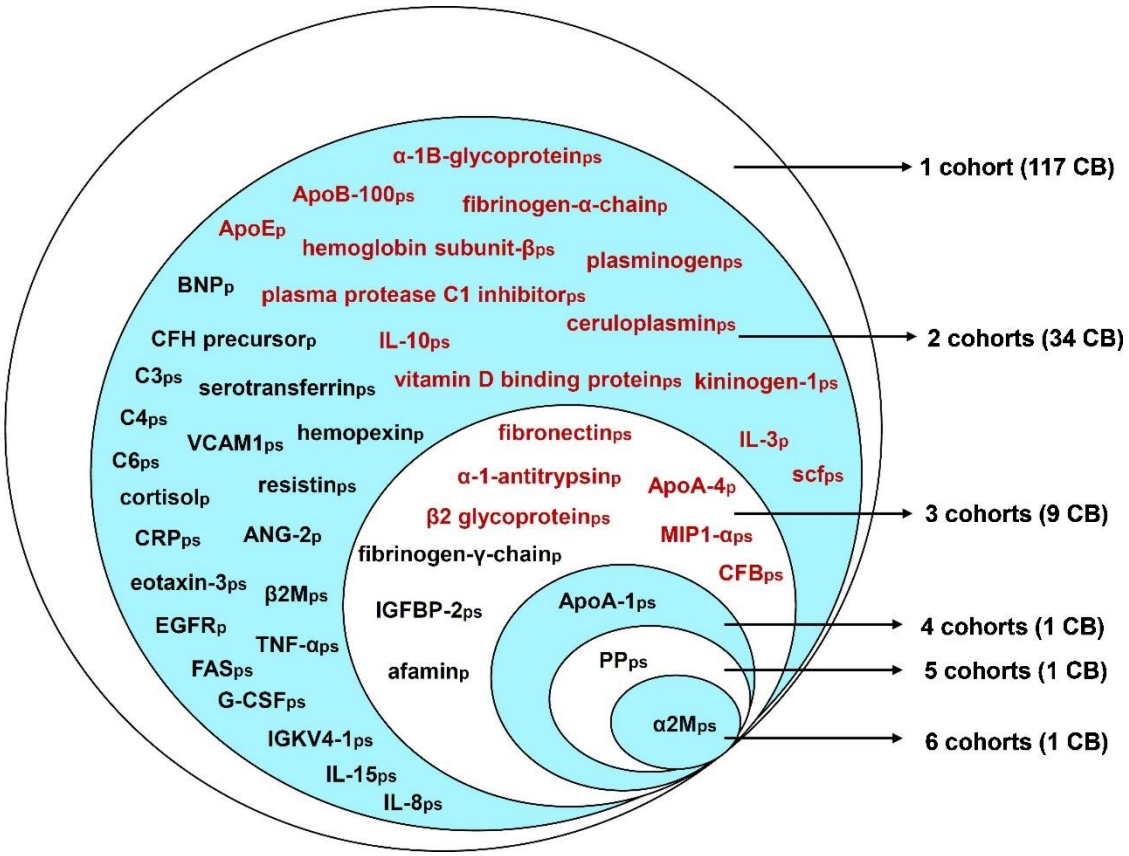

Figure 1.3. Reproducibility of candidate biomarkers across proteomics experiment. Here, the CB in red represent proteins reporting inconsistent changes, while the CB in black represents proteins with consistent changes across multiple studies. (Source: See ref [89])

Similar biomarker panels based on blood-based proteins [90-92] have been demonstrated with accuracies of 89% [84] and 91-95%.[93] These models improve when clinical variables (age, sex, education and APOE status) were added to the model.[93] A panel involving the most replicated proteins dysregulated in AD from proteomics studies (α-1 antitrypsin, α-2-macroglobulin, apolipoprotein E and complement C3) achieved an accuracy of 77%.[94]

1.4.3. Protein panels involving minority groups

The number of proteomics studies involving African American/Black or Hispanic population is very limited despite the higher incidence of AD. Attempts to establish protein panels involving Mexican American adults demonstrated accuracies from 88-96%.[95-97] To date, there are no proteomics experiments in AD that have focused on African American/Black adults in order to further understanding of racial disparities or ensure effective biomarkers for diagnosis are available. In **Chapter II** of this dissertation, we employed plasma proteomics to discover potential diagnostic biomarkers in AD in a cohort comprising both African American/Black and non-Hispanic White adults.

1.5. Plasma Lipidomics

According to the LipidMaps Consortium, lipids can be broadly defined as hydrophobic or amphipathic small molecules that originate entirely or in part by carbanion-based condensations of thioesters and/or by carbocation-based condensations of isoprene units.[98] Lipids can broadly be classified into eight categories- fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and ployketides.[98]

Lipidomics is the study of extracted lipids from biological samples on a large scale. Lipidomics has advanced steadily [99-102], however despite better instrumentation and platforms,

there is still a need for better global lipidomics approach. Broadly, lipidomics experiments can be divided into either untargeted or targeted lipidomics. Untargeted lipidomics involves the global analysis of all the lipid species present in a certain sample. In this method, instead of monitoring a specific lipid class of interest, all lipid classes are monitored in tandem. This approach is ideal for discovering altered lipid species in disease and as a screening technique for discovering potential disease biomarkers. In **Chapter IV** of this dissertation, we employed such an untargeted approach in the study of plasma samples in AD. Despite the apparent advantage of this approach, limitation lies in assigning confident annotations to lipid species, which are being addressed with ion mobility.[103] This is due to the presence of a high number of isomers in lipid species.[104] Also, the lack of established databases, specifically MS/MS fragmentation databases also makes assigning confident identifications complicated.

On the other hand, a targeted lipidomics approach involves monitoring either a specific lipid class or classes, or specific lipid species in a certain sample type. This approach involves targeting characteristic fragmentation patterns for a lipid class or lipid species for analysis. Common fragmentation patterns of lipid species are given in **Figure 1.4**. For example, in case of phosphatidylcholine species, characteristic phosphatidylcholine fragment product (*m/z* 184) is targeted for its identification. Similarly, loss of fragment ion characteristic of phosphatidylethanolamine (*m/z* 141) is targeted for its identification of phosphatidylethanolamine species. The targeted approach has the advantage of achieving confident identification of lipids. It also has the ability to provide absolute quantitative information.

Sciex introduced a novel multiple reaction monitoring (MRM) based targeted lipidomics method specific for plasma samples. This method has the capability to analyze ~1150 lipids from 19 lipid classes.[105] This approach uses hydrophilic interaction liquid chromatography (HILIC)

separation in conjunction with MRM to analyze lipid species. HILIC separates lipids by their respective lipid classes, as well as achieves molecular lipid identification upto its molecular species level, by targeting the loss of fatty acid chains instead of the loss of head groups. This approach has the capability to remove complexities due to isobaric lipid species by using HILIC separation on the front of MS analysis. This method has been established as a potential alternative to the untargeted method using high resolution mass spectrometry in **Chapter III**.

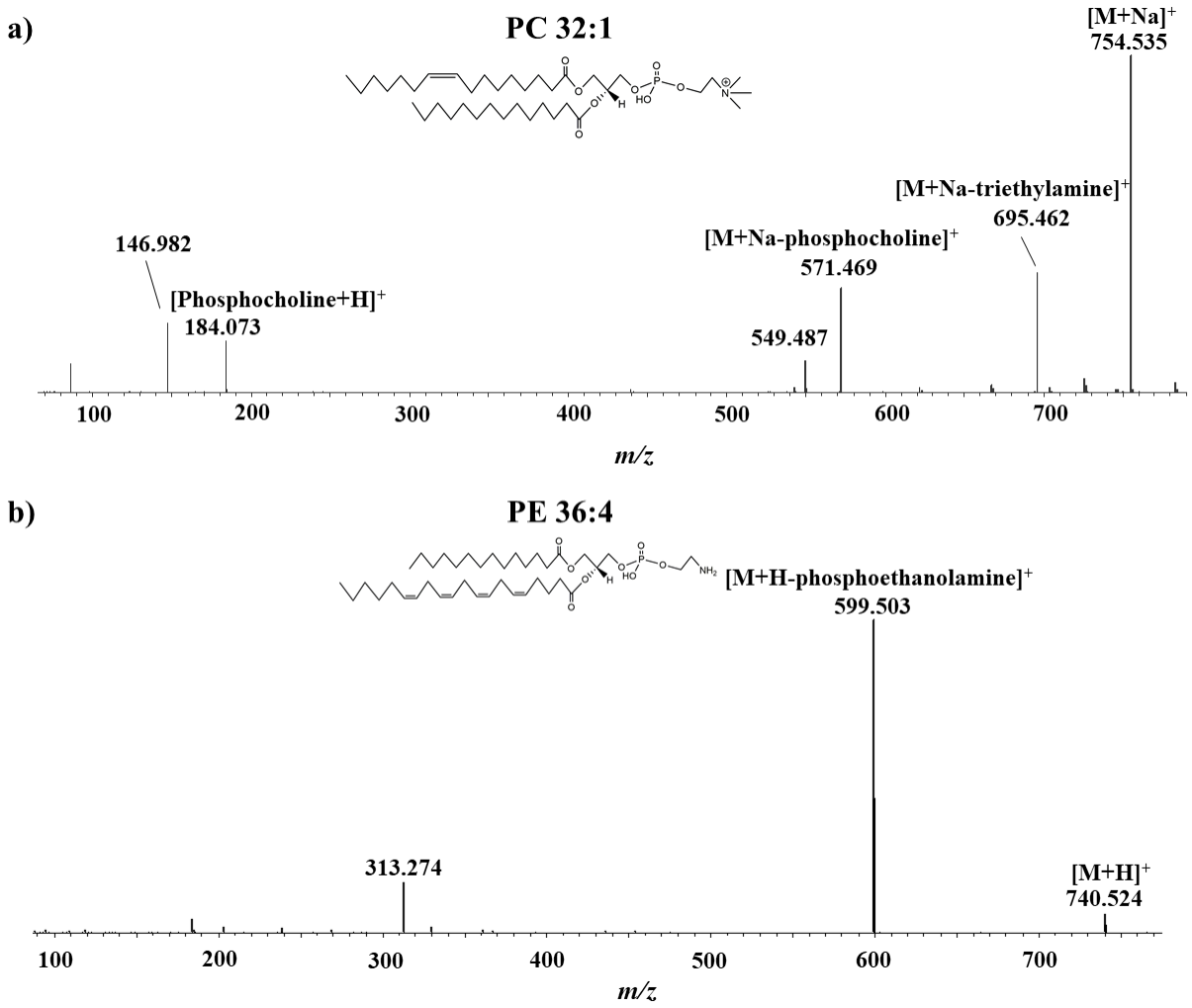

Figure 1.4. Example characteristic fragmentation pattern of **a)** phosphatidylcholine and **b)** phosphatidylethanolamine lipid species in LC-MS analysis**.** (Source: Modified from Ref [105])

1.6. Lipids and AD

Involvement of lipids and altered lipid metabolism are critical in the pathogenesis of AD.[79, 106-108] For example, cholesterol is actively involved in AD pathogenesis, as its been demonstrated in **Figure 1.5**. Brain contains the highest concentration of cholesterol, which is responsible for maintaining fluidity of the plasma membrane.[109] In the brain, a majority of free cholesterol is derived from *de novo* biosynthesis.[110] Excess free cholesterol is converted to cholesterol ester by the enzyme acyl-coenzymeA:cholesterol acyl-transferase 1 (ACAT1). Levels of cholesterol ester are correlated with the formation of Aβ.[111] Increase in the amount of cholesterol ester, increases the formation of Aβ, while the inhibition of ACAT reduces Aβ formation.[112] On the other hand, free cholesterol can be converted into 24(S)-hydroxycholesterol, which is capable of passing the blood brain barrier. Increased 24(S)-hydroxycholesterol levels in AD patients have been reported.[79, 107] Cholesterol efflux also has a role in Aβ formation. Increased levels of ATP-binding cassette sub-family A member 1 (ABCA1), which is responsible for regulating efflux of excess cholesterol to lipid acceptors such as APOE decrease Aβ formation in AD brain,[113] while poor APOE lipidation promotes Aβ formation. Additionally, cholesterol influences Aβ formation by modulating secretase activity.[114] Aβ formation is predominantly controlled by β-secretase 1 (BACE1) and γ-secretase, which in term is influenced by cholesterol levels in the membrane.[79, 107]

Glycerophospholipids have also been widely implicated to be involved in AD pathogenesis.[79, 106, 108] Glycerophospholipids are the main component of the cell membrane.[115] Two of the major glycerophospholipids are- phosphatidylethanolamines and phosphatidylcholines. These subclasses of glycerophospholipids along with phosphatidylinositol are reduced in AD, which impacts membrane fluidity, leading to oxidative stress.[107] In the human brain, it's been found that plasmalogens are reduced in frontal, parietal and temporal regions of

the brain at early stages of AD.[116] Similar reduction has also been reported in white matter of the brain.[116] Reduction of plasmalogens have been correlated with disease severity.[117] Similar reduction in phosphatidylcholines have been reported in the frontal, primary auditory and parietal cortices in brain of AD patients.[108]

Two of the major classes of sphingolipids are sphingomyelins and ceramides. Sphingomyelins are the most abundant sphingolipids in the brain, and are found mostly in the myelin sheaths.[118] Sphingomyelins are an important component of lipid raft, which is responsible for facilitating the formation and aggregation of Aβ.[119] Sphingomyelins act as inhibitors of γ-secretase activity, which in turn reduces the formation of Aβ.[120] On the other hand, ceramide is involved in sphingolipid metabolism, and has been reported to be elevated in AD brain.[75, 121] Ceramides regulate BACE1 activity, promoting the breakdown of amyloid precursor protein via amyloidogenic pathway.[108] Glycerolipids, such as monoacylglycerides and diacylglycerides have also been linked with AD.[122]

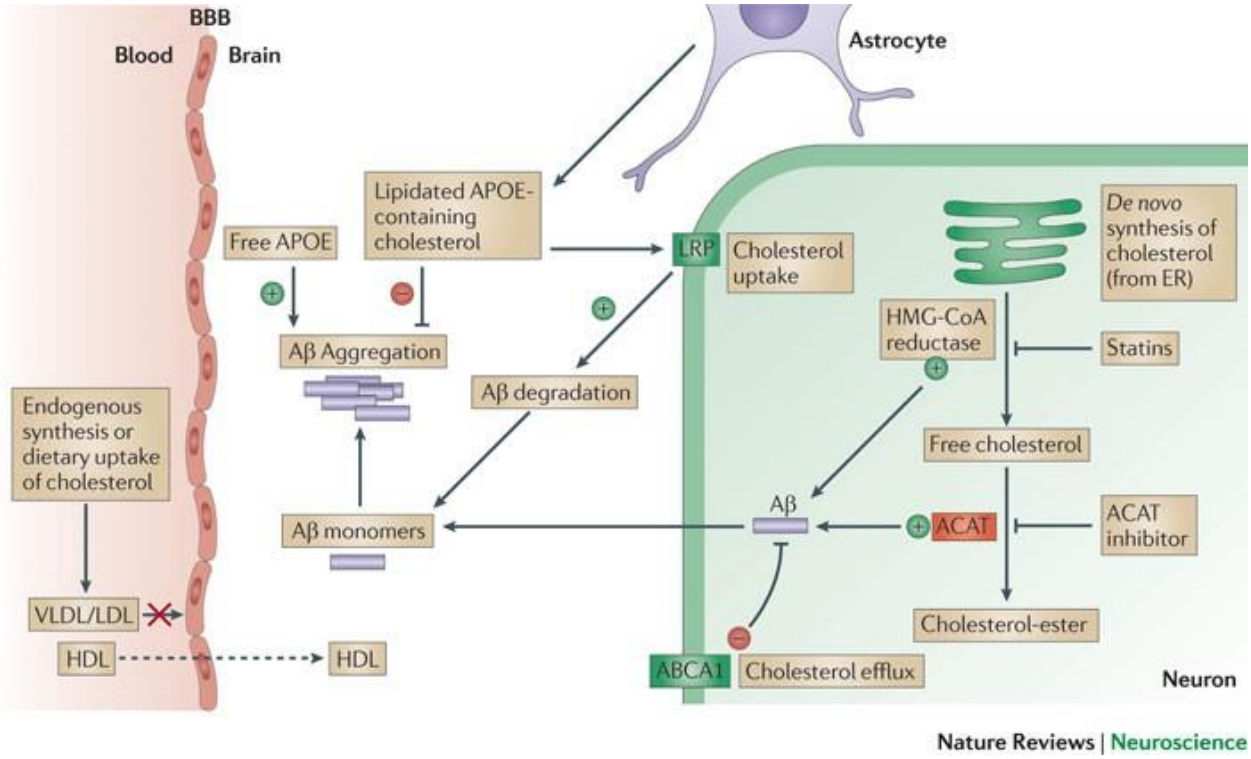

Figure 1.5. Role of cholesterol in Aβ formation in AD pathogenesis. Here the protein in green decrease Aβ formation, while the proteins in red increases Aβ formation. (Source: Ref [79])

1.7. Lipidomics and AD

1.7.1. Lipid dysregulation in AD

Comparison between cognitively normal and AD individuals involving lipidomics have revealed changes to various lipid classes. These include alterations to lysophosphatidylcholine and lysophosphatidylethanolamine species [123-127]; reduction of phosphatidylcholines [123, 125, 128-134], phosphatidylethanolamines [123], plasmalogens [123, 130, 135-136], sphingomyelins [125, 137], and increase of phosphatidylcholines [123], phosphatidylethanolamines [125], and ceramides.[137-140] Other lipid species reported to be altered in plasma of AD individuals includes triacylglyceride [125, 141], and diacylglyceride species [124, 130, 135, 138, 141-142], cholesterol ester [132, 141, 143], desmosterol [144], lanosterol and lathosterol.[145] Although individual lipid species may differ in the majority of cases, the alterations to lipid classes are reproducible across studies.

1.7.2. Lipid panels for blood-based biomarker discovery

With recent advancements in lipidomics, several studies have proposed lipid panels in blood as potential biomarkers in AD. One of the most cited lipid panels involving 8 phospholipids and 2 acylcarnitines achieved an AUC of 0.96, also validated in a separate cohort with an AUC of 0.827.[133] Attempts to validate this panel by others have been unsuccessful.[146] Similar panels including phospholipids have reported AUC ranging from 0.76 to 0.83.[131, 134, 147] The ratio of PC 34:4/ LysoPC 18:2 achieved an AUC of 0.823 for predicting AD.[131]

Similar panels involving cholesterol and its precursors reported good accuracy for predicting AD.[144] Other panels involving both metabolite and lipids reported an AUC of 0.792 with a specificity and sensitivity of 76.9% and 81.8% respectively.[132] Other mixed metabolite/lipid

panels reported an AUC of 0.918 with a 7 metabolite panel [125], accuracy of 71% with a 24 feature panel [143], and AUC of 0.886 with 9 features.[141]

Despite the increased number of panels, the number of studies involving African American/Black participants have been very limited. One such study attempted to replicate prior results [133] on a cohort of 221 participants, 97% of which were African American/Black adults, reported an AUC of 0.609 for predicting AD.[148] Although, it is suggested that this could be a result of different study conditions [149], this is a clear indication of the need for better AD biomarkers effective for African American/Black individuals. As a result, more studies focusing on African American/Black participants is necessary. Such a study has been described in **Chapter V** of this dissertation, indicating possible differences between the African American/Black and non-Hispanic White lipidomes in AD pathology.

1.8. Overview of Dissertation

This dissertation aimed to apply 'omics technologies to better understand health disparities in AD. To achieve this goal, in **Chapter II** we applied a robust plasma proteomics technology to plasma samples collected from African American/Black and non-Hispanic White adults, to validate the importance of inclusion in AD research. We combined the proteomics results from this study with machine learning to discover potential diagnostic biomarkers for AD. With the increasing number of studies focusing on lipids and their involvement in disease pathology, in **Chapter III** we evaluated a novel MRM based targeted lipidomics approach against an untargeted approach to find the best approach for plasma lipidomics analysis. Based on the involvement of lipid and altered lipid metabolism in AD, **Chapter IV** discusses the application of multi-lipidomics approaches to study AD. In **Chapter V**, a targeted MRM based approach was applied to plasma samples collected from African American/Black and non-Hispanic White adults to study

involvement of lipids in health disparities in AD. Finally, future directions of this work are discussed in **Chapter VI**.

1.9. References

1. 2020 Alzheimer's disease facts and figures. *Alzheimer's & Dementia* **2020,** *16* (3), 391-460.

2. Chin, A. L.; Negash, S.; Hamilton, R., Diversity and disparity in dementia: the impact of ethnoracial differences in Alzheimer disease. *Alzheimer disease and associated disorders* **2011,** *25* (3), 187-195.

3. Stephen, C. E.; Regina, L. E., Alzheimer's Disease in African Americans: A Preliminary Systematic Review. *Neurol Res Surg* **2020,** *3* (1), 1-3.

4. Matthews, K. A.; Xu, W.; Gaglioti, A. H.; Holt, J. B.; Croft, J. B.; Mack, D.; McGuire, L. C., Racial and ethnic estimates of Alzheimer's disease and related dementias in the United States (2015-2060) in adults aged ≥65 years. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2019,** *15* (1), 17-24.

5. Barnes, L. L.; Leurgans, S.; Aggarwal, N. T.; Shah, R. C.; Arvanitakis, Z.; James, B. D.; Buchman, A. S.; Bennett, D. A.; Schneider, J. A., Mixed pathology is more likely in black than white decedents with Alzheimer dementia. *Neurology* **2015,** *85* (6), 528-534.

6. Barnes, L. L.; Bennett, D. A., Alzheimer's disease in African Americans: risk factors and challenges for the future. *Health Aff (Millwood)* **2014,** *33* (4), 580-586.

7. Barnes, L. L.; Wilson, R. S.; Hebert, L. E.; Scherr, P. A.; Evans, D. A.; Mendes de Leon, C. F., Racial differences in the association of education with physical and cognitive function in older blacks and whites. *The journals of gerontology. Series B, Psychological sciences and social sciences* **2011,** *66* (3), 354-363.

8. Sisco, S.; Gross, A. L.; Shih, R. A.; Sachs, B. C.; Glymour, M. M.; Bangen, K. J.; Benitez, A.; Skinner, J.; Schneider, B. C.; Manly, J. J., The role of early-life educational quality and literacy in explaining racial disparities in cognition in late life. *The journals of gerontology. Series B, Psychological sciences and social sciences* **2015,** *70* (4), 557-67.

9. Stepler, K.; Robinson, R., The Potential of 'Omics to Link Lipid Metabolism and Genetic and Comorbidity Risk Factors of Alzheimer's Disease in African Americans. *Adv Exp Med Biol*. **2019**, *1118*, 1-28.

10. Garrett, S. L.; McDaniel, D.; Obideen, M.; Trammell, A. R.; Shaw, L. M.; Goldstein, F. C.; Hajjar, I., Racial Disparity in Cerebrospinal Fluid Amyloid and Tau Biomarkers and Associated Cutoffs for Mild Cognitive Impairment. *JAMA network open* **2019,** *2* (12), e1917363.

11. Morris, J. C.; Schindler, S. E.; McCue, L. M.; Moulder, K. L.; Benzinger, T. L. S.; Cruchaga, C.; Fagan, A. M.; Grant, E.; Gordon, B. A.; Holtzman, D. M.; Xiong, C., Assessment of Racial Disparities in Biomarkers for Alzheimer Disease. *JAMA neurology* **2019,** *76* (3), 264-273.

12. Wharton, W.; Kollhoff, A. L.; Gangishetti, U.; Verble, D. D.; Upadhya, S.; Zetterberg, H.; Kumar, V.; Watts, K. D.; Kippels, A. J.; Gearing, M.; Howell, J. C.; Parker, M. W.; Hu, W. T., Interleukin 9 alterations linked to alzheimer disease in african americans. *Ann Neurol* **2019,** *86* (3), 407-418.

13. Buford, T. W.; Manini, T. M.; Kairalla, J. A.; McDermott, M. M.; Vaz Fragoso, C. A.; Chen, H.; Fielding, R. A.; King, A. C.; Newman, A. B.; Tranah, G. J., Mitochondrial DNA Sequence Variants Associated With Blood Pressure Among 2 Cohorts of Older Adults. *J Am Heart Assoc* **2018,** *7* (18), e010009-e010009.

14. Grewal, R.; Haghighi, M.; Huang, S.; Smith, A. G.; Cao, C.; Lin, X.; Lee, D. C.; Teten, N.; Hill, A. M.; Selenica, M.-L. B., Identifying biomarkers of dementia prevalent among amnestic mild cognitively impaired ethnic female patients. *Alzheimer's research & therapy* **2016,** *8* (1), 43.

15. Panigrahi, G. K.; Praharaj, P. P.; Kittaka, H.; Mridha, A. R.; Black, O. M.; Singh, R.; Mercer, R.; van Bokhoven, A.; Torkko, K. C.; Agarwal, C.; Agarwal, R.; Abd Elmageed, Z. Y.; Yadav, H.; Mishra, S. K.; Deep, G., Exosome proteomic analyses identify inflammatory phenotype and novel biomarkers in African American prostate cancer patients. *Cancer medicine* **2019,** *8* (3), 1110-1123.

16. Zhou, X.; Mei, H.; Agee, J.; Brown, T.; Mao, J., Racial differences in distribution of fatty acids in prostate cancer and benign prostatic tissues. *Lipids in health and disease* **2019,** *18* (1), 189.

17. Vantaku, V.; Amara, C. S.; Piyarathna, D. W. B.; Donepudi, S. R.; Ambati, C. R.; Putluri, V.; Tang, W.; Rajapakshe, K.; Estecio, M. R.; Terris, M. K.; Castro, P. D.; Ittmann, M. M.; Williams, S. B.; Lerner, S. P.; Sreekumar, A.; Bollag, R.; Coarfa, C.; Kornberg, M. D.; Lotan, Y.; Ambs, S.; Putluri, N., DNA methylation patterns in bladder tumors of African American patients point to distinct alterations in xenobiotic metabolism. *Carcinogenesis* **2019,** *40* (11), 1332-1340.

18. Vantaku, V.; Donepudi, S. R.; Piyarathna, D. W. B.; Amara, C. S.; Ambati, C. R.; Tang, W.; Putluri, V.; Chandrashekar, D. S.; Varambally, S.; Terris, M. K.; Davies, K.; Ambs, S.; Bollag, R.; Apolo, A. B.; Sreekumar, A.; Putluri, N., Large-scale profiling of serum metabolites in African American and European American patients with bladder cancer reveals metabolic pathways associated with patient survival. *Cancer* **2019,** *125* (6), 921-932.

19. Axt, J.; Murphy, A. J.; Seeley, E. H.; Martin, C. A.; Taylor, C.; Pierce, J.; Caprioli, R. M.; Whiteside, M.; Lovvorn, H. N., 3rd, Race disparities in Wilms tumor incidence and biology. *J Surg Res* **2011,** *170* (1), 112-119.

20. Buie, J. N. J.; Hammad, S. M.; Nietert, P. J.; Magwood, G.; Adams, R. J.; Bonilha, L.; Sims-Robinson, C., Differences in plasma levels of long chain and very long chain ceramides

between African Americans and whites: An observational study. *PLoS One* **2019,** *14* (5), e0216213-e0216213.

21. Halade, G. V.; Kain, V.; Dillion, C.; Beasley, M.; Dudenbostel, T.; Oparil, S.; Limdi, N. A., Race-based and sex-based differences in bioactive lipid mediators after myocardial infarction. *ESC Heart Failure* **2020,** *7* (4), 1700-1710.

22. Bateman, N. W.; Dubil, E. A.; Wang, G.; Hood, B. L.; Oliver, J. M.; Litzi, T. A.; Gist, G. D.; Mitchell, D. A.; Blanton, B.; Phippen, N. T.; Tian, C.; Zahn, C. M.; Cohn, D. E.; Havrilesky, L. J.; Berchuck, A.; Shriver, C. D.; Darcy, K. M.; Hamilton, C. A.; Conrads, T. P.; Maxwell, G. L., Race-specific molecular alterations correlate with differential outcomes for black and white endometrioid endometrial cancer patients. *Cancer* **2017,** *123* (20), 4004-4012.

23. Group, F.-N. B. W., In *BEST (Biomarkers, EndpointS, and other Tools) Resource*, Food and Drug Administration (US) National Institutes of Health (US): Silver Spring (MD), Bethesda (MD), **2016**.

24. Consensus Report of the Working Group on: "Molecular and Biochemical Markers of Alzheimer's Disease" 11The names of the Working Group Members and the names of the Working Group Advisory Committee Members are listed in the Appendix A(section VI). 22The Reagan Institute Working Groups are planned and organized by Z. S. Khachaturian and T.S. Radebaugh; Fax: 301-879-2023; E-mail: zaven@idt.net. *Neurobiology of Aging* **1998,** *19* (2), 109-116.

25. Khoury, R.; Ghossoub, E., Diagnostic biomarkers of Alzheimer's disease: A state-of-the-art review. *Biomarkers in Neuropsychiatry* **2019,** *1*, 100005.

26. Robinson, R. A. S.; Amin, B.; Guest, P. C., Multiplexing Biomarker Methods, Proteomics and Considerations for Alzheimer's Disease. *Advances in experimental medicine and biology* **2017,** *974*, 21-48.

27. Hampel, H.; O'Bryant, S. E.; Molinuevo, J. L.; Zetterberg, H.; Masters, C. L.; Lista, S.; Kiddle, S. J.; Batrla, R.; Blennow, K., Blood-based biomarkers for Alzheimer disease: mapping the road to the clinic. *Nature Reviews Neurology* **2018,** *14* (11), 639-652.

28. Zetterberg, H.; Burnham, S. C., Blood-based molecular biomarkers for Alzheimer's disease. *Molecular Brain* **2019,** *12* (1), 26.

29. Olsson, B.; Lautner, R.; Andreasson, U.; Öhrfelt, A.; Portelius, E.; Bjerke, M.; Hölttä, M.; Rosén, C.; Olsson, C.; Strobel, G.; Wu, E.; Dakin, K.; Petzold, M.; Blennow, K.; Zetterberg, H., CSF and blood biomarkers for the diagnosis of Alzheimer's disease: a systematic review and meta-analysis. *The Lancet Neurology* **2016,** *15* (7), 673-684.

30. Nakamura, A.; Kaneko, N.; Villemagne, V. L.; Kato, T.; Doecke, J.; Doré, V.; Fowler, C.; Li, Q.-X.; Martins, R.; Rowe, C., High performance plasma amyloid-β biomarkers for Alzheimer's disease. *Nature* **2018,** *554* (7691), 249-254.

31. Pannee, J.; Törnqvist, U.; Westerlund, A.; Ingelsson, M.; Lannfelt, L.; Brinkmalm, G.; Persson, R.; Gobom, J.; Svensson, J.; Johansson, P.; Zetterberg, H.; Blennow, K.; Portelius, E., The amyloid-β degradation pattern in plasma--a possible tool for clinical trials in Alzheimer's disease. *Neurosci Lett* **2014,** *573*, 7-12.

32. Ovod, V.; Ramsey, K. N.; Mawuenyega, K. G.; Bollinger, J. G.; Hicks, T.; Schneider, T.; Sullivan, M.; Paumier, K.; Holtzman, D. M.; Morris, J. C.; Benzinger, T.; Fagan, A. M.; Patterson, B. W.; Bateman, R. J., Amyloid β concentrations and stable isotope labeling kinetics of human plasma specific to central nervous system amyloidosis. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2017,** *13* (8), 841-849.

33. Janelidze, S.; Stomrud, E.; Palmqvist, S.; Zetterberg, H.; van Westen, D.; Jeromin, A.; Song, L.; Hanlon, D.; Tan Hehir, C. A.; Baker, D.; Blennow, K.; Hansson, O., Plasma β-amyloid in Alzheimer's disease and vascular disease. *Scientific reports* **2016,** *6*, 26801.

34. Zetterberg, H.; Wilson, D.; Andreasson, U.; Minthon, L.; Blennow, K.; Randall, J.; Hansson, O., Plasma tau levels in Alzheimer's disease. *Alzheimers Res Ther* **2013,** *5* (2), 9-9.

35. Mielke, M. M.; Hagen, C. E.; Wennberg, A. M.; Airey, D. C.; Savica, R.; Knopman, D. S.; Machulda, M. M.; Roberts, R. O.; Jack, C. R.; Petersen, R. C., Association of plasma total tau level with cognitive decline and risk of mild cognitive impairment or dementia in the mayo clinic study on aging. *JAMA Neurol* **2017,** *74* (9), 1073-1080.

36. Mattsson, N.; Andreasson, U.; Zetterberg, H.; Blennow, K., Association of Plasma Neurofilament Light With Neurodegeneration in Patients With Alzheimer Disease. *JAMA Neurol* **2017,** *74* (5), 557-566.

37. Lee, K. S.; Chung, J. H.; Choi, T. K.; Suh, S. Y.; Oh, B. H.; Hong, C. H., Peripheral Cytokines and Chemokines in Alzheimer's Disease. *Dementia and geriatric cognitive disorders* **2009,** *28* (4), 281-287.

38. Farley, A.; Hendry, C.; McLafferty, E., Blood components. *Nursing standard (Royal College of Nursing (Great Britain) : 1987)* **2012,** *27* (13), 35-42.

39. Anderson, N. L.; Anderson, N. G., The Human Plasma Proteome. *History, Character, and Diagnostic Prospects* **2002,** *1* (11), 845-867.

40. Yu, L.-R.; Stewart, N. A.; Veenstra, T. D., Chapter 8 - Proteomics: The Deciphering of the Functional Genome. In *Essentials of Genomic and Personalized Medicine*, Ginsburg, G. S.; Willard, H. F., Eds. Academic Press: San Diego, **2010**, 89-96.

41. Sze, S. K.; Ge, Y.; Oh, H.; McLafferty, F. W., Top-down mass spectrometry of a 29-kDa protein for characterization of any posttranslational modification to within one residue. *Proc Natl Acad Sci U S A* **2002,** *99* (4), 1774-1779.

42. Wu, C.; Tran, J. C.; Zamdborg, L.; Durbin, K. R.; Li, M.; Ahlf, D. R.; Early, B. P.; Thomas, P. M.; Sweedler, J. V.; Kelleher, N. L., A protease for 'middle-down' proteomics. *Nature Methods* **2012,** *9* (8), 822-824.

43. Zhang, Y.; Fonslow, B. R.; Shan, B.; Baek, M.-C.; Yates III, J. R., Protein analysis by shotgun/bottom-up proteomics. *Chem Rev* **2013,** *113* (4), 2343-2394.

44. Pieper, R.; Su, Q.; Gatlin, C. L.; Huang, S. T.; Anderson, N. L.; Steiner, S., Multi-component immunoaffinity subtraction chromatography: an innovative step towards a comprehensive survey of the human plasma proteome. *Proteomics* **2003,** *3* (4), 422-32.

45. Saveliev, S.; Bratz, M.; Zubarev, R.; Szapacs, M.; Budamgunta, H.; Urh, M., Trypsin/Lys-C protease mix for enhanced protein mass spectrometry analysis. *Nature Methods* **2013,** *10* (11), i-ii.

46. Rauniyar, N.; Yates, J. R., 3rd, Isobaric labeling-based relative quantification in shotgun proteomics. *Journal of proteome research* **2014,** *13* (12), 5293-5309.

47. Cao, Z.; Tang, H.-Y.; Wang, H.; Liu, Q.; Speicher, D. W., Systematic comparison of fractionation methods for in-depth analysis of plasma proteomes. *Journal of proteome research* **2012,** *11* (6), 3090-3100.

48. Tu, C.; Rudnick, P. A.; Martinez, M. Y.; Cheek, K. L.; Stein, S. E.; Slebos, R. J. C.; Liebler, D. C., Depletion of abundant plasma proteins and limitations of plasma proteomics. *Journal of proteome research* **2010,** *9* (10), 4982-4991.

49. Crawford, A. S.; Schuchard, M. D.; Melm, C. D.; Chapman, H. A.; Wildsmith, J.; Ray, K. B.; Mehigh, R. J.; Chen, D. E.; Scott, G. B., Novel ProteoPrep® 20 Immunoaffinity Depletion Resin for Human Plasma. *Complement* **2006,** *1*, 1.

50. Li, S.; He, Y.; Lin, Z.; Xu, S.; Zhou, R.; Liang, F.; Wang, J.; Yang, H.; Liu, S.; Ren, Y., Digging More Missing Proteins Using an Enrichment Approach with ProteoMiner. *Journal of Proteome Research* **2017,** *16* (12), 4330-4339.

51. Björhall, K.; Miliotis, T.; Davidsson, P., Comparison of different depletion strategies for improved resolution in proteomic analysis of human serum samples. *Proteomics* **2005,** *5* (1), 307-17.

52. Ross, P. L.; Huang, Y. N.; Marchese, J. N.; Williamson, B.; Parker, K.; Hattan, S.; Khainovski, N.; Pillai, S.; Dey, S.; Daniels, S.; Purkayastha, S.; Juhasz, P.; Martin, S.; Bartlet-Jones, M.; He, F.; Jacobson, A.; Pappin, D. J., Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Molecular & cellular proteomics : MCP* **2004,** *3* (12), 1154-69.

53. Thompson, A.; Schäfer, J.; Kuhn, K.; Kienle, S.; Schwarz, J.; Schmidt, G.; Neumann, T.; Johnstone, R.; Mohammed, A. K.; Hamon, C., Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal Chem* **2003,** *75* (8), 1895-904.

54. Xiang, F.; Ye, H.; Chen, R.; Fu, Q.; Li, L., N,N-dimethyl leucines as novel isobaric tandem mass tags for quantitative proteomics and peptidomics. *Analytical chemistry* **2010,** *82* (7), 2817-2825.

55. Dayon, L.; Hainard, A.; Licker, V.; Turck, N.; Kuhn, K.; Hochstrasser, D. F.; Burkhard, P. R.; Sanchez, J. C., Relative quantification of proteins in human cerebrospinal fluids by MS/MS using 6-plex isobaric tags. *Anal Chem* **2008,** *80* (8), 2921-31.

56. O'Connell, J. D.; Paulo, J. A.; O'Brien, J. J.; Gygi, S. P., Proteome-wide evaluation of two common protein quantification methods. *Journal of proteome research* **2018,** *17* (5), 1934-1942.

57. Thompson, A.; Wölmer, N.; Koncarevic, S.; Selzer, S.; Böhm, G.; Legner, H.; Schmid, P.; Kienle, S.; Penning, P.; Höhle, C.; Berfelde, A.; Martinez-Pinna, R.; Farztdinov, V.; Jung, S.; Kuhn, K.; Pike, I., TMTpro: Design, Synthesis, and Initial Evaluation of a Proline-Based Isobaric 16-Plex Tandem Mass Tag Reagent Set. *Analytical Chemistry* **2019,** *91* (24), 15941-15950.

58. Werner, T.; Becher, I.; Sweetman, G.; Doce, C.; Savitski, M. M.; Bantscheff, M., High-resolution enabled TMT 8-plexing. *Anal Chem* **2012,** *84* (16), 7188-94.

59. Arul, A. B.; Robinson, R. A. S., Sample Multiplexing Strategies in Quantitative Proteomics. *Analytical chemistry* **2019,** *91* (1), 178-189.

60. Senko, M. W.; Remes, P. M.; Canterbury, J. D.; Mathur, R.; Song, Q.; Eliuk, S. M.; Mullen, C.; Earley, L.; Hardman, M.; Blethrow, J. D.; Bui, H.; Specht, A.; Lange, O.; Denisov, E.; Makarov, A.; Horning, S.; Zabrouskov, V., Novel Parallelized Quadrupole/Linear Ion Trap/Orbitrap Tribrid Mass Spectrometer Improving Proteome Coverage and Peptide Identification Rates. *Analytical Chemistry* **2013,** *85* (24), 11710-11714.

61. McAlister, G. C.; Nusinow, D. P.; Jedrychowski, M. P.; Wühr, M.; Huttlin, E. L.; Erickson, B. K.; Rad, R.; Haas, W.; Gygi, S. P., MultiNotch MS3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cell line proteomes. *Analytical chemistry* **2014,** *86* (14), 7150-7158.

62. Zhang, R.; Barker, L.; Pinchev, D.; Marshall, J.; Rasamoelisolo, M.; Smith, C.; Kupchak, P.; Kireeva, I.; Ingratta, L.; Jackowski, G., Mining biomarkers in human sera using proteomic tools. *Proteomics* **2004,** *4* (1), 244-56.

63. Hye, A.; Lynham, S.; Thambisetty, M.; Causevic, M.; Campbell, J.; Byers, H. L.; Hooper, C.; Rijsdijk, F.; Tabrizi, S. J.; Banner, S.; Shaw, C. E.; Foy, C.; Poppe, M.; Archer, N.; Hamilton, G.; Powell, J.; Brown, R. G.; Sham, P.; Ward, M.; Lovestone, S., Proteome-based plasma biomarkers for Alzheimer's disease. *Brain : a journal of neurology* **2006,** *129* (Pt 11), 3042-50.

64. Liu, H. C.; Hu, C. J.; Chang, J. G.; Sung, S. M.; Lee, L. S.; Yuan, R. Y.; Leu, S. J., Proteomic identification of lower apolipoprotein A-I in Alzheimer's disease. *Dementia and geriatric cognitive disorders* **2006,** *21* (3), 155-61.

65. Liao, P. C.; Yu, L.; Kuo, C. C.; Lin, C.; Kuo, Y. M., Proteomics analysis of plasma for potential biomarkers in the diagnosis of Alzheimer's disease. *Proteomics. Clinical applications* **2007,** *1* (5), 506-12.

66. Cutler, P.; Akuffo, E. L.; Bodnar, W. M.; Briggs, D. M.; Davis, J. B.; Debouck, C. M.; Fox, S. M.; Gibson, R. A.; Gormley, D. A.; Holbrook, J. D.; Hunter, A. J.; Kinsey, E. E.; Prinjha,

R.; Richardson, J. C.; Roses, A. D.; Smith, M. A.; Tsokanas, N.; Willé, D. R.; Wu, W.; Yates, J. W.; Gloger, I. S., Proteomic identification and early validation of complement 1 inhibitor and pigment epithelium-derived factor: Two novel biomarkers of Alzheimer's disease in human plasma. *Proteomics. Clinical applications* **2008,** *2* (4), 467-77.

67. Thambisetty, M.; Hye, A.; Foy, C.; Daly, E.; Glover, A.; Cooper, A.; Simmons, A.; Murphy, D.; Lovestone, S., Proteome-based identification of plasma proteins associated with hippocampal metabolism in early Alzheimer's disease. *Journal of neurology* **2008,** *255* (11), 1712-20.

68. Kitamura, Y.; Usami, R.; Ichihara, S.; Kida, H.; Satoh, M.; Tomimoto, H.; Murata, M.; Oikawa, S., Plasma protein profiling for potential biomarkers in the early diagnosis of Alzheimer's disease. *Neurological research* **2017,** *39* (3), 231-238.

69. Chen, M.; Xia, W., Proteomic Profiling of Plasma and Brain Tissue from Alzheimer's Disease Patients Reveals Candidate Network of Plasma Biomarkers. *Journal of Alzheimer's Disease* **2020,** *76*, 349-368.

70. Muenchhoff, J.; Poljak, A.; Song, F.; Raftery, M.; Brodaty, H.; Duncan, M.; McEvoy, M.; Attia, J.; Schofield, P. W.; Sachdev, P. S., Plasma protein profiling of mild cognitive impairment and Alzheimer's disease across two independent cohorts. *Journal of Alzheimer's disease : JAD* **2015,** *43* (4), 1355-73.

71. Song, F.; Poljak, A.; Kochan, N. A.; Raftery, M.; Brodaty, H.; Smythe, G. A.; Sachdev, P. S., Plasma protein profiling of Mild Cognitive Impairment and Alzheimer's disease using iTRAQ quantitative proteomics. *Proteome science* **2014,** *12* (1), 5.

72. Zabel, M.; Schrag, M.; Mueller, C.; Zhou, W.; Crofton, A.; Petersen, F.; Dickson, A.; Kirsch, W. M., Assessing candidate serum biomarkers for Alzheimer's disease: a longitudinal study. *Journal of Alzheimer's disease : JAD* **2012,** *30* (2), 311-21.

73. Blacker, D.; Wilcox, M. A.; Laird, N. M.; Rodes, L.; Horvath, S. M.; Go, R. C. P.; Perry, R.; Watson, B.; Bassett, S. S.; McInnis, M. G.; Albert, M. S.; Hyman, B. T.; Tanzi, R. E., Alpha-2 macroglobulin is genetically associated with Alzheimer disease. *Nature Genetics* **1998,** *19* (4), 357-360.

74. Shen, L.; Liao, L.; Chen, C.; Guo, Y.; Song, D.; Wang, Y.; Chen, Y.; Zhang, K.; Ying, M.; Li, S.; Liu, Q.; Ni, J., Proteomics Analysis of Blood Serums from Alzheimer's Disease Patients Using iTRAQ Labeling Technology. *Journal of Alzheimer's disease : JAD* **2017,** *56* (1), 361-378.

75. Thambisetty, M.; Simmons, A.; Velayudhan, L.; Hye, A.; Campbell, J.; Zhang, Y.; Wahlund, L.-O.; Westman, E.; Kinsey, A.; Güntert, A.; Proitsi, P.; Powell, J.; Causevic, M.; Killick, R.; Lunnon, K.; Lynham, S.; Broadstock, M.; Choudhry, F.; Howlett, D. R.; Williams, R. J.; Sharp, S. I.; Mitchelmore, C.; Tunnard, C.; Leung, R.; Foy, C.; O'Brien, D.; Breen, G.; Furney, S. J.; Ward, M.; Kloszewska, I.; Mecocci, P.; Soininen, H.; Tsolaki, M.; Vellas, B.; Hodges, A.; Murphy, D. G. M.; Parkins, S.; Richardson, J. C.; Resnick, S. M.; Ferrucci, L.; Wong, D. F.; Zhou, Y.; Muehlboeck, S.; Evans, A.; Francis, P. T.; Spenger, C.; Lovestone, S., Association of plasma

clusterin concentration with severity, pathology, and progression in Alzheimer disease. *Arch Gen Psychiatry* **2010,** *67* (7), 739-748.

76. Bennett, S.; Grant, M.; Creese, A. J.; Mangialasche, F.; Cecchetti, R.; Cooper, H. J.; Mecocci, P.; Aldred, S., Plasma levels of complement 4a protein are increased in Alzheimer's disease. *Alzheimer disease and associated disorders* **2012,** *26* (4), 329-34.

77. Henkel, A. W.; Müller, K.; Lewczuk, P.; Müller, T.; Marcus, K.; Kornhuber, J.; Wiltfang, J., Multidimensional plasma protein separation technique for identification of potential Alzheimer's disease plasma biomarkers: a pilot study. *Journal of Neural Transmission* **2012,** *119* (7), 779-788.

78. Yang, H.; Lyutvinskiy, Y.; Herukka, S. K.; Soininen, H.; Rutishauser, D.; Zubarev, R. A., Prognostic polypeptide blood plasma biomarkers of Alzheimer's disease progression. *Journal of Alzheimer's disease : JAD* **2014,** *40* (3), 659-66.

79. Di Paolo, G.; Kim, T.-W., Linking lipids to Alzheimer's disease: cholesterol and beyond. *Nature Reviews Neuroscience* **2011,** *12* (5), 284-296.

80. Akiyama, H., Inflammatory response in Alzheimer's disease. *The Tohoku journal of experimental medicine* **1994,** *174* (3), 295-303.

81. Rogers, J., The inflammatory response in Alzheimer's disease. *Journal of periodontology* **2008,** *79*, 1535-1543.

82. Cortes-Canteli, M.; Zamolodchikov, D.; Ahn, H. J.; Strickland, S.; Norris, E. H., Fibrinogen and Altered Hemostasis in Alzheimer's Disease. *Journal of Alzheimer's Disease* **2012,** *32*, 599-608.

83. Ray, S.; Britschgi, M.; Herbert, C.; Takeda-Uchimura, Y.; Boxer, A.; Blennow, K.; Friedman, L. F.; Galasko, D. R.; Jutel, M.; Karydas, A.; Kaye, J. A.; Leszek, J.; Miller, B. L.; Minthon, L.; Quinn, J. F.; Rabinovici, G. D.; Robinson, W. H.; Sabbagh, M. N.; So, Y. T.; Sparks, D. L.; Tabaton, M.; Tinklenberg, J.; Yesavage, J. A.; Tibshirani, R.; Wyss-Coray, T., Classification and prediction of clinical Alzheimer's diagnosis based on plasma signaling proteins. *Nature medicine* **2007,** *13* (11), 1359-62.

84. Soares, H. D.; Chen, Y.; Sabbagh, M.; Roher, A.; Schrijvers, E.; Breteler, M., Identifying early markers of Alzheimer's disease using quantitative multiplex proteomic immunoassay panels. *Annals of the New York Academy of Sciences* **2009,** *1180*, 56-67.

85. Björkqvist, M.; Ohlsson, M.; Minthon, L.; Hansson, O., Evaluation of a Previously Suggested Plasma Biomarker Panel to Identify Alzheimer's Disease. *PLoS One* **2012,** *7* (1), e29868.

86. Gómez Ravetti, M.; Moscato, P., Identification of a 5-protein biomarker molecular signature for predicting Alzheimer's disease. *PLoS One* **2008,** *3* (9), e3111-e3111.

87. Marksteiner, J.; Kemmler, G.; Weiss, E. M.; Knaus, G.; Ullrich, C.; Mechtcheriakov, S.; Oberbauer, H.; Auffinger, S.; Hinterhölzl, J.; Hinterhuber, H.; Humpel, C., Five out of 16 plasma signaling proteins are enhanced in plasma of patients with mild cognitive impairment and Alzheimer's disease. *Neurobiol Aging* **2011,** *32* (3), 539-40.

88. Biella, G.; Franceschi, M.; De Rino, F.; Davin, A.; Giacalone, G.; Brambilla, P.; Bountris, P.; Haritou, M.; Magnani, G.; Martinelli Boneschi, F.; Forloni, G.; Albani, D., Multiplex assessment of a panel of 16 serum molecules for the differential diagnosis of Alzheimer's disease. *Am J Neurodegener Dis* **2013,** *2* (1), 40-45.

89. Rehiman, S. H.; Lim, S. M.; Neoh, C. F.; Majeed, A. B. A.; Chin, A.-V.; Tan, M. P.; Kamaruzzaman, S. B.; Ramasamy, K., Proteomics as a reliable approach for discovery of blood-based Alzheimer's disease biomarkers: A systematic review and meta-analysis. *Ageing Research Reviews* **2020,** *60*, 101066.

90. Doecke, J. D.; Laws, S. M.; Faux, N. G.; Wilson, W.; Burnham, S. C.; Lam, C. P.; Mondal, A.; Bedo, J.; Bush, A. I.; Brown, B.; De Ruyck, K.; Ellis, K. A.; Fowler, C.; Gupta, V. B.; Head, R.; Macaulay, S. L.; Pertile, K.; Rowe, C. C.; Rembach, A.; Rodrigues, M.; Rumble, R.; Szoeke, C.; Taddei, K.; Taddei, T.; Trounson, B.; Ames, D.; Masters, C. L.; Martins, R. N., Blood-based protein biomarkers for diagnosis of Alzheimer disease. *Archives of neurology* **2012,** *69* (10), 1318-25.

91. O'Bryant, S. E.; Xiao, G.; Barber, R.; Huebinger, R.; Wilhelmsen, K.; Edwards, M.; Graff-Radford, N.; Doody, R.; Diaz-Arrastia, R., A blood-based screening tool for Alzheimer's disease that spans serum and plasma: findings from TARC and ADNI. *PLoS One* **2011,** *6* (12), e28092.

92. O'Bryant, S. E.; Xiao, G.; Barber, R.; Reisch, J.; Hall, J.; Cullum, C. M.; Doody, R.; Fairchild, T.; Adams, P.; Wilhelmsen, K.; Diaz-Arrastia, R., A blood-based algorithm for the detection of Alzheimer's disease. *Dementia and geriatric cognitive disorders* **2011,** *32* (1), 55-62.

93. O'Bryant, S. E.; Xiao, G.; Barber, R.; Reisch, J.; Doody, R.; Fairchild, T.; Adams, P.; Waring, S.; Diaz-Arrastia, R., A serum protein-based algorithm for the detection of Alzheimer disease. *Archives of neurology* **2010,** *67* (9), 1077-81.

94. Kiddle, S. J.; Sattlecker, M.; Proitsi, P.; Simmons, A.; Westman, E.; Bazenet, C.; Nelson, S. K.; Williams, S.; Hodges, A.; Johnston, C.; Soininen, H.; Kłoszewska, I.; Mecocci, P.; Tsolaki, M.; Vellas, B.; Newhouse, S.; Lovestone, S.; Dobson, R. J., Candidate blood proteome markers of Alzheimer's disease onset and progression: a systematic review and replication study. *Journal of Alzheimer's disease : JAD* **2014,** *38* (3), 515-31.

95. O'Bryant, S. E.; Xiao, G.; Edwards, M.; Devous, M.; Gupta, V. B.; Martins, R.; Zhang, F.; Barber, R.; Texas Alzheimer's, R.; Care, C., Biomarkers of Alzheimer's disease among Mexican Americans. *Journal of Alzheimer's disease : JAD* **2013,** *34* (4), 841-849.

96. Edwards, M.; Hall, J.; Williams, B.; Johnson, L.; O'Bryant, S., Molecular Markers of Amnestic Mild Cognitive Impairment among Mexican Americans. *Journal of Alzheimer's Disease* **2016,** *49*, 221-228.

97. Petersen, M.; Hall, J.; Parsons, T.; Johnson, L.; O'Bryant, S., Combining Select Blood-Based Biomarkers with Neuropsychological Assessment to Detect Mild Cognitive Impairment among Mexican Americans. *Journal of Alzheimer's disease : JAD* **2020,** *75* (3), 739-750.

98. Fahy, E.; Subramaniam, S.; Murphy, R. C.; Nishijima, M.; Raetz, C. R. H.; Shimizu, T.; Spener, F.; van Meer, G.; Wakelam, M. J. O.; Dennis, E. A., Update of the LIPID MAPS comprehensive classification system for lipids. *J Lipid Res* **2009,** *50 Suppl* (Suppl), S9-S14.

99. Blanksby, S. J.; Mitchell, T. W., Advances in mass spectrometry for lipidomics. *Annual Review of Analytical Chemistry* **2010,** *3*, 433-465.

100. Hsu, F.-F., Mass spectrometry-based shotgun lipidomics–a critical review from the technical point of view. *Analytical and bioanalytical chemistry* **2018,** *410* (25), 6387-6409.

101. Hu, T.; Zhang, J. L., Mass-spectrometry-based lipidomics. *Journal of separation science* **2018,** *41* (1), 351-372.

102. Jurowski, K.; Kochan, K.; Walczak, J.; Barańska, M.; Piekoszewski, W.; Buszewski, B., Comprehensive review of trends and analytical strategies applied for biological samples preparation and storage in modern medical lipidomics: State of the art. *TrAC Trends in Analytical Chemistry* **2017,** *86*, 276-289.

103. Picache, J. A.; Rose, B. S.; Balinski, A.; Leaptrot, Katrina L.; Sherrod, S. D.; May, J. C.; McLean, J. A., Collision cross section compendium to annotate and predict multi-omic compound identities. *Chemical Science* **2019,** *10* (4), 983-993.

104. Rustam, Y. H.; Reid, G. E., Analytical Challenges and Recent Advances in Mass Spectrometry Based Lipidomics. *Analytical Chemistry* **2018,** *90* (1), 374-397.

105. Khan, M. J.; Codreanu, S. G.; Goyal, S.; Wages, P. A.; Gorti, S. K. K.; Pearson, M. J.; Uribe, I.; Sherrod, S. D.; McLean, J. A.; Porter, N. A.; Robinson, R. A. S., Evaluating a targeted multiple reaction monitoring approach to global untargeted lipidomic analyses of human plasma. *Rapid Communications in Mass Spectrometry* **2020,** *34* (22), e8911.

106. Kao, Y.-C.; Ho, P.-C.; Tu, Y.-K.; Jou, I. M.; Tsai, K.-J., Lipids and Alzheimer's Disease. *Int J Mol Sci* **2020,** *21* (4), 1505.

107. Liu, Q.; Zhang, J., Lipid metabolism in Alzheimer's disease. *Neurosci Bull* **2014,** *30* (2), 331-345.

108. Touboul, D.; Gaudin, M., Lipidomics of Alzheimer's disease. *Bioanalysis* **2014,** *6* (4), 541-61.

109. Gamba, P.; Testa, G.; Sottero, B.; Gargiulo, S.; Poli, G.; Leonarduzzi, G., The link between altered cholesterol metabolism and Alzheimer's disease. *Annals of the New York Academy of Sciences* **2012,** *1259*, 54-64.

110. Dietschy, J. M.; Turley, S. D., Cholesterol metabolism in the brain. *Current opinion in lipidology* **2001,** *12* (2), 105-12.

111. Ledesma, M. D.; Dotti, C. G., Amyloid excess in Alzheimer's disease: What is cholesterol to be blamed for? *FEBS Letters* **2006,** *580* (23), 5525-5532.

112. Puglielli, L.; Konopka, G.; Pack-Chung, E.; Ingano, L. A.; Berezovska, O.; Hyman, B. T.; Chang, T. Y.; Tanzi, R. E.; Kovacs, D. M., Acyl-coenzyme A: cholesterol acyltransferase modulates the generation of the amyloid beta-peptide. *Nature cell biology* **2001,** *3* (10), 905-12.

113. Sun, Y.; Yao, J.; Kim, T. W.; Tall, A. R., Expression of liver X receptor target genes decreases cellular amyloid beta peptide secretion. *The Journal of biological chemistry* **2003,** *278* (30), 27688-94.

114. Liu, Q.; Zerbinatti, C. V.; Zhang, J.; Hoe, H.-S.; Wang, B.; Cole, S. L.; Herz, J.; Muglia, L.; Bu, G., Amyloid precursor protein regulates brain apolipoprotein E and cholesterol metabolism through lipoprotein receptor LRP1. *Neuron* **2007,** *56* (1), 66-78.

115. O'Brien, J. S.; Sampson, E. L., Fatty acid and fatty aldehyde composition of the major brain lipids in normal human gray matter, white matter, and myelin. *J Lipid Res* **1965,** *6* (4), 545-551.

116. Han, X.; Holtzman, D. M.; McKeel, D. W., Jr., Plasmalogen deficiency in early Alzheimer's disease subjects and in animal models: molecular characterization using electrospray ionization mass spectrometry. *Journal of neurochemistry* **2001,** *77* (4), 1168-80.

117. Wood, P. L.; Mankidy, R.; Ritchie, S.; Heath, D.; Wood, J. A.; Flax, J.; Goodenowe, D. B., Circulating plasmalogen levels and Alzheimer Disease Assessment Scale-Cognitive scores in Alzheimer patients. *Journal of psychiatry & neuroscience : JPN* **2010,** *35* (1), 59-62.

118. Gault, C. R.; Obeid, L. M.; Hannun, Y. A., An overview of sphingolipid metabolism: from synthesis to breakdown. *Advances in experimental medicine and biology* **2010,** *688*, 1-23.

119. Rushworth, J. V.; Hooper, N. M., Lipid Rafts: Linking Alzheimer's Amyloid-β Production, Aggregation, and Toxicity at Neuronal Membranes. *International Journal of Alzheimers Disease* **2011,** *2011*, 603052.

120. Grimm, M. O. W.; Grimm, H. S.; Pätzold, A. J.; Zinser, E. G.; Halonen, R.; Duering, M.; Tschäpe, J.-A.; Strooper, B. D.; Müller, U.; Shen, J.; Hartmann, T., Regulation of cholesterol and sphingomyelin metabolism by amyloid-β and presenilin. *Nature cell biology* **2005,** *7* (11), 1118-1123.

121. Mielke, M. M.; Lyketsos, C. G., Alterations of the sphingolipid pathway in Alzheimer's disease: new biomarkers and treatment targets? *Neuromolecular Med* **2010,** *12* (4), 331-340.

122. Wood, P. L.; Medicherla, S.; Sheikh, N.; Terry, B.; Phillipps, A.; Kaye, J. A.; Quinn, J. F.; Woltjer, R. L., Targeted Lipidomics of Fontal Cortex and Plasma Diacylglycerols (DAG) in Mild Cognitive Impairment and Alzheimer's Disease: Validation of DAG Accumulation Early in the

Pathophysiology of Alzheimer's Disease. *Journal of Alzheimer's disease : JAD* **2015,** *48* (2), 537-46.

123. González-Domínguez, R.; García-Barrera, T.; Gómez-Ariza, J. L., Combination of metabolomic and phospholipid-profiling approaches for the study of Alzheimer's disease. *Journal of Proteomics* **2014,** *104*, 37-47.

124. Wood, P.; Phillipps, A.; Woltjer, R. L.; Kaye, J.; Quinn, J., Increased lysophosphatidylethanolamine and diacylglycerol levels in Alzheimer's disease plasma. *JSM Alzheimer's Disease and Related Dementia* **2014,** *1*.

125. Olazarán, J.; Gil-de-Gómez, L.; Rodríguez-Martín, A.; Valentí-Soler, M.; Frades-Payo, B.; Marín-Muñoz, J.; Antúnez, C.; Frank-García, A.; Acedo-Jiménez, C.; Morlán-Gracia, L.; Petidier-Torregrossa, R.; Guisasola, M. C.; Bermejo-Pareja, F.; Sánchez-Ferro, Á.; Pérez-Martínez, D. A.; Manzano-Palomo, S.; Farquhar, R.; Rábano, A.; Calero, M., A blood-based, 7-metabolite signature for the early diagnosis of Alzheimer's disease. *Journal of Alzheimer's disease : JAD* **2015,** *45* (4), 1157-73.

126. Peña-bautista, C.; Roca, M.; López-cuevas, R.; Baquero, M.; Vento, M.; Cháfer-pericás, C., Metabolomics study to identify plasma biomarkers in alzheimer disease: ApoE genotype effect. *Journal of Pharmaceutical and Biomedical Analysis* **2020,** *180*, 113088.

127. Li, N.-j.; Liu, W.-t.; Li, W.; Li, S.-q.; Chen, X.-h.; Bi, K.-s.; He, P., Plasma metabolic profiling of Alzheimer's disease by liquid chromatography/mass spectrometry. *Clinical biochemistry* **2010,** *43* (12), 992-997.

128. Costa, A. C.; Joaquim, H. P. G.; Forlenza, O.; Talib, L. L.; Gattaz, W. F., Plasma lipids metabolism in mild cognitive impairment and Alzheimer's disease. *The World Journal of Biological Psychiatry* **2019,** *20* (3), 190-196.

129. Fiandaca, M. S.; Zhong, X.; Cheema, A. K.; Orquiza, M. H.; Chidambaram, S.; Tan, M. T.; Gresenz, C. R.; FitzGerald, K. T.; Nalls, M. A.; Singleton, A. B.; Mapstone, M.; Federoff, H. J., Plasma 24-metabolite Panel Predicts Preclinical Transition to Clinical Stages of Alzheimer's Disease. *Front Neurol* **2015,** *6*, 237-237.

130. González-Domínguez, R.; García-Barrera, T.; Gómez-Ariza, J. L., Metabolomic study of lipids in serum for biomarker discovery in Alzheimer's disease using direct infusion mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* **2014,** *98*, 321-326.

131. Klavins, K.; Koal, T.; Dallmann, G.; Marksteiner, J.; Kemmler, G.; Humpel, C., The ratio of phosphatidylcholines to lysophosphatidylcholines in plasma differentiates healthy controls from patients with Alzheimer's disease and mild cognitive impairment. *Alzheimers Dement (Amst)* **2015,** *1* (3), 295-302.

132. Proitsi, P.; Kim, M.; Whiley, L.; Pritchard, M.; Leung, R.; Soininen, H.; Kloszewska, I.; Mecocci, P.; Tsolaki, M.; Vellas, B.; Sham, P.; Lovestone, S.; Powell, J. F.; Dobson, R. J. B.; Legido-Quigley, C., Plasma lipidomics analysis finds long chain cholesteryl esters to be associated with Alzheimer's disease. *Transl Psychiatry* **2015,** *5* (1), e494-e494.

133. Mapstone, M.; Cheema, A. K.; Fiandaca, M. S.; Zhong, X.; Mhyre, T. R.; MacArthur, L. H.; Hall, W. J.; Fisher, S. G.; Peterson, D. R.; Haley, J. M.; Nazar, M. D.; Rich, S. A.; Berlau, D. J.; Peltz, C. B.; Tan, M. T.; Kawas, C. H.; Federoff, H. J., Plasma phospholipids identify antecedent memory impairment in older adults. *Nature medicine* **2014,** *20* (4), 415-8.

134. Whiley, L.; Sen, A.; Heaton, J.; Proitsi, P.; García-Gómez, D.; Leung, R.; Smith, N.; Thambisetty, M.; Kloszewska, I.; Mecocci, P.; Soininen, H.; Tsolaki, M.; Vellas, B.; Lovestone, S.; Legido-Quigley, C.; AddNeuroMed, C., Evidence of altered phosphatidylcholine metabolism in Alzheimer's disease. *Neurobiology of aging* **2014,** *35* (2), 271-278.

135. Wood, P. L.; Locke, V. A.; Herling, P.; Passaro, A.; Vigna, G. B.; Volpato, S.; Valacchi, G.; Cervellati, C.; Zuliani, G., Targeted lipidomics distinguishes patient subgroups in mild cognitive impairment (MCI) and late onset Alzheimer's disease (LOAD). *BBA Clinical* **2016,** *5*, 25-28.

136. Goodenowe, D. B.; Cook, L. L.; Liu, J.; Lu, Y.; Jayasinghe, D. A.; Ahiahonu, P. W.; Heath, D.; Yamazaki, Y.; Flax, J.; Krenitsky, K. F.; Sparks, D. L.; Lerner, A.; Friedland, R. P.; Kudo, T.; Kamino, K.; Morihara, T.; Takeda, M.; Wood, P. L., Peripheral ethanolamine plasmalogen deficiency: a logical causative factor in Alzheimer's disease and dementia. *J Lipid Res* **2007,** *48* (11), 2485-98.

137. Han, X.; Rozen, S.; Boyle, S. H.; Hellegers, C.; Cheng, H.; Burke, J. R.; Welsh-Bohmer, K. A.; Doraiswamy, P. M.; Kaddurah-Daouk, R., Metabolomics in early Alzheimer's disease: identification of altered plasma sphingolipidome using shotgun lipidomics. *PLoS One* **2011,** *6* (7), e21643.

138. González-Domínguez, R.; García-Barrera, T.; Gómez-Ariza, J. L., Application of a novel metabolomic approach based on atmospheric pressure photoionization mass spectrometry using flow injection analysis for the study of Alzheimer′s disease. *Talanta* **2015,** *131*, 480-489.

139. Kim, M.; Nevado-Holgado, A.; Whiley, L.; Snowden, S. G.; Soininen, H.; Kloszewska, I.; Mecocci, P.; Tsolaki, M.; Vellas, B.; Thambisetty, M.; Dobson, R. J. B.; Powell, J. F.; Lupton, M. K.; Simmons, A.; Velayudhan, L.; Lovestone, S.; Proitsi, P.; Legido-Quigley, C., Association between Plasma Ceramides and Phosphatidylcholines and Hippocampal Brain Volume in Late Onset Alzheimer's Disease. *Journal of Alzheimer's disease : JAD* **2017,** *60* (3), 809-817.

140. Mielke, M. M.; Bandaru, V. V. R.; Haughey, N. J.; Xia, J.; Fried, L. P.; Yasar, S.; Albert, M.; Varma, V.; Harris, G.; Schneider, E. B.; Rabins, P. V.; Bandeen-Roche, K.; Lyketsos, C. G.; Carlson, M. C., Serum ceramides increase the risk of Alzheimer disease: the Women's Health and Aging Study II. *Neurology* **2012,** *79* (7), 633-641.

141. Anand, S.; Barnes, J. M.; Young, S. A.; Garcia, D. M.; Tolley, H. D.; Kauwe, J. S. K.; Graves, S. W., Discovery and Confirmation of Diagnostic Serum Lipid Biomarkers for Alzheimer's Disease Using Direct Infusion Mass Spectrometry. *Journal of Alzheimer's disease : JAD* **2017,** *59* (1), 277-290.

142. Wood, P. L.; Medicherla, S.; Sheikh, N.; Terry, B.; Phillipps, A.; Kaye, J. A.; Quinn, J. F.; Woltjer, R. L., Targeted lipidomics of fontal cortex and plasma diacylglycerols (DAG) in mild

cognitive impairment and Alzheimer's disease: validation of DAG accumulation early in the pathophysiology of Alzheimer's disease. *Journal of Alzheimer's Disease* **2015,** *48* (2), 537-546.

143. Proitsi, P.; Kim, M.; Whiley, L.; Simmons, A.; Sattlecker, M.; Velayudhan, L.; Lupton, M. K.; Soininen, H.; Kloszewska, I.; Mecocci, P.; Tsolaki, M.; Vellas, B.; Lovestone, S.; Powell, J. F.; Dobson, R. J. B.; Legido-Quigley, C., Association of blood lipids with Alzheimer's disease: A comprehensive lipidomics analysis. *Alzheimer's & Dementia* **2017,** *13* (2), 140-151.

144. Sato, Y.; Suzuki, I.; Nakamura, T.; Bernier, F.; Aoshima, K.; Oda, Y., Identification of a new plasma biomarker of Alzheimer's disease using metabolomics technology. *J Lipid Res* **2012,** *53* (3), 567-576.

145. Kölsch, H.; Heun, R.; Jessen, F.; Popp, J.; Hentschel, F.; Maier, W.; Lütjohann, D., Alterations of cholesterol precursor levels in Alzheimer's disease. *Biochimica et biophysica acta* **2010,** *1801* (8), 945-50.

146. Casanova, R.; Varma, S.; Simpson, B.; Kim, M.; An, Y.; Saldana, S.; Riveros, C.; Moscato, P.; Griswold, M.; Sonntag, D.; Wahrheit, J.; Klavins, K.; Jonsson, P. V.; Eiriksdottir, G.; Aspelund, T.; Launer, L. J.; Gudnason, V.; Legido Quigley, C.; Thambisetty, M., Blood metabolite markers of preclinical Alzheimer's disease in two longitudinally followed cohorts of older individuals. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2016,** *12* (7), 815-822.

147. Oresic, M.; Hyotylainen, T.; Herukka, S.; Sysi-Aho, M.; Mattila, I.; Seppanan-Laakso, T.; Julkunen, V.; Gopalacharyulu, P.; Hallikainen, M.; Koikkalainen, J. Metabolome in progression to Alzheimer's disease. *Transl Psychiatry.* **2011**, *1* (12), e57.

148. Li, D.; Misialek, J. R.; Boerwinkle, E.; Gottesman, R. F.; Sharrett, A. R.; Mosley, T. H.; Coresh, J.; Wruck, L. M.; Knopman, D. S.; Alonso, A., Prospective associations of plasma phospholipids and mild cognitive impairment/dementia among African Americans in the ARIC Neurocognitive Study. *Alzheimers Dement (Amst)* **2016,** *6*, 1-10.

149. Oeckl, P.; Otto, M., A Review on MS-Based Blood Biomarkers for Alzheimer's Disease. *Neurology and therapy* **2019,** *8* (Suppl 2), 113-127.

CHAPTER II

Why Inclusion Matters for Alzheimer's Disease Biomarker Discovery in Plasma

"This chapter is adopted from the research articles: **Khan, MJ, Desaire, H, Lopez, OL, Kamboh, MI, Robinson, RAS, "Why race matters in plasma proteomics biomarker discovery for Alzheimer's disease." Accepted in JAD** and **Khan, MJ, Desaire, H, Lopez, OL, Kamboh, MI, Robinson, RAS, "Dataset of why race matters in plasma proteomics biomarker discovery for Alzheimer's disease." Under review**"

2.1. Introduction

One of the fastest growing populations in the United States are ethnic minorities, such that Hispanics and African American/Blacks (AA) are estimated to account for 40% of older adults in 2050.[1-3] This is critical for the field of Alzheimer's disease (AD) which disproportionately impacts these populations and accounts for higher incidence rates of 1.5 to 2x for African American/Black adults.[4-6] In addition to disparities in disease incidence, there is a disparate economic burden of AD costs and caregiving for African American/Black families.[7-8] Racial and ethnic disparities in AD are multi-factorial and can include contributions from socioeconomic status [1, 5, 9-10], quality and level of education [11-14], comorbidities [1, 15], genetic risk factors [15], environmental stressors [16-17], healthcare access [1, 5], and systemic racism.[18] Disease pathology is similar in African American/Black and Hispanic adults with regards to what we have learned for decades about AD in non-Hispanic White (NHW) populations.[5, 19-23] Although, understanding disease pathogenesis is complicated by higher frequency of mixed dementia cases and vascular comorbidities that are often found for example in African American/Black adults with AD.[19, 21-22]

Importantly, for better diagnosing AD in all communities and for developing effective therapies, better strategies are needed to increase research participation of African American/Black adults into AD and related dementia research.[24] Currently, African American/Black adults participate in clinical trials at a rate of <~5% and in AD research assessments at <~13%.[25-27] Recently, there have been reports that demonstrate that biomarker discovery efforts for AD need

to be inclusive of African American/Black adults as the standard cerebrospinal fluid (CSF) biomarkers, total-tau and phosphorylated (p)-tau, have different concentration levels in a cohort of African American/Black adults when compared to non-Hispanic White adults.[28-33] Other circulatory proteins in plasma also have been reported to have differences in African American/Black AD patients such as interleukin-9.[34] Examples of plasma biomarker studies that include or solely focus on African American/Black adults are few.[35-37] This is critical as plasma is a less-invasive biofluid to obtain than CSF, and more importantly, as diagnostic or prognostic biomarkers for AD need to be effective for all.

Plasma proteomics is a growing field within AD biomarker discovery.[38-45] Many studies have conducted plasma proteomics analyses across the AD spectrum from cognitively normal (CN) to mild cognitively impaired to confirmed early and late-onset AD.[46-64] The most widely used biomarkers in plasma are amyloid-beta 40 (Aβ40) and 42 (Aβ42) peptides.[38, 45, 65-67] Other potential plasma protein biomarkers, many of which have been validated within the same cohort [68] and few in independent cohorts [46, 48, 59, 69-70], also exist. A recent review of plasma biomarkers for AD [40] analyzed findings from 20 previous discovery-based proteomics studies and found a lack of reproducibility across those studies. For example, the review reported alpha-2 macroglobulin as a biomarker candidate in six different studies, while pancreatic polypeptide was reported in five studies.[40] The remaining proteins reported in only more than two studies included apolipoprotein A1, afamin, fibronectin, apolipoprotein A4, alpha -1- antitrypsin, fibrinogen-γ chain, insulin like growth factor binding protein-2, macrophage inflammatory protein 1-α, beta-2 glycoprotein and complement factor B.[40]

Few plasma biomarker studies utilized diverse cohorts that incorporated African American/Black adults into the study design.[35, 37, 71] Plasma biomarkers for an amnestic MCI

cohort of females from African American/Black, Hispanic, and non-Hispanic White backgrounds were identified as having levels unique to a given racial and ethnic group and highlight the importance of including diverse groups in biomarker studies.[37] Age was deemed a critical factor in mid-life plasma Aβ concentrations in a large cohort of European and African American/Black adults, whereby there were also different genes that had race-specific changes.[35] For example, cystathionine beta-synthase gene had genome-wide significant association with plasma homocysteine levels in African American/Blacks and Yoruba cohorts, that are associated with African ancestry.[71] Inclusion of African American/Black adults in study designs is critical to ensure that specific biomarkers, combinations of biomarkers in a panel, or biomarker levels that able to accurately distinguish and diagnose disease in all groups.

Herein, we conducted a pilot study with plasma samples available from the University of Pittsburgh Alzheimer's Disease Research Center (ADRC) that included self-reported African American/Black and non-Hispanic White adults that participated in ADRC research. Individual participants were either cognitively normal or had a clinically confirmed diagnosis of AD, and plasma was collected from a time point away from baseline that was coincident with clear disease pathology and clinical diagnosis. The samples (N=113) were randomly divided into two sets in order to accommodate a study design that would allow us to 1) conduct discovery-based proteomics to identify differentially-expressed proteins in a cohort that included African American/Black adults; 2) use the data generated from the discovery-based proteomics studies to determine the utility of the selected panel of proteins as diagnostic AD biomarkers using machine learning; and 3) determine the extent to which the model performance depended on the racial and ethnic background of the training set samples. Comprehensive plasma proteomics biomarker discovery was conducted using quantitative tandem mass tags of peptides from plasma

immunodepletion, liquid chromatography (LC), and mass spectrometry (MS) workflows. Processed proteomics data along with machine learning was used to test whether the racial and ethnic background of the training set samples impacts the accuracy of the biomarker panels for AD. In this approach, we divided the cohort into two sets: Set 1 (N = 73) was used to select the optimal protein features and Set 2 (N = 40) was used to validate the results.

2.2. Methods

2.2.1. Plasma sample collection

Human plasma samples (N=113) from African American/Black and non-Hispanic White individuals were obtained from the University of Pittsburgh Alzheimer's Disease Research Center (ADRC). Detailed characteristics of the individuals are provided in **Table 2.1**. Approval for the participation of human subjects were obtained by the Institutional Review Boards of the University of Pittsburgh and Vanderbilt University. The Mini-Mental State Examination (MMSE) was performed and disease individuals were clinically diagnosed with mild to moderate dementia at the time of blood draw according to the National Institute on Aging-Alzheimer's Association and National Alzheimer's Coordinating Center criteria. The samples were divided into two separate sample sets of N = 73 and N = 40, using a blinded study design. Plasma samples were obtained from participants at the most recent blood draw at the start of this study in December 2016, in order to analyze plasma that was close in chronological year and storage conditions and that corresponded to clear disease diagnosis.

Table 2.1. Demographics of plasma sample cohorts.

	African American/Black		***non-Hispanic White***		**p-value***
	Cognitively normal	**Alzheimer's disease**	**Cognitively normal**	**Alzheimer's disease**	
Sample size	26	30	28	29	
Sex (Male/Female)	8 / 18	8 / 22	9 /19	12 / 17	0.69
Mean age at draw (SD)	72.5 (7.4)	74.5 (7.8)	71.1 (9.1)	76.2 (8.7)	0.58
Mean year of most current blood draw (SD)	2006.4 (7.4)	2007.4 (6.6)	2008.8 (6.6)	2007.2 (6.0)	0.55
Mean years of education (SD)	13.3 (2.0)	13.4 (3.9)	16.1 (2.4)	13.2 (2.6)	0.0004
MMSE[a] Mean (SD)	27.1(2.4)	14.5(7.1)	27.8(4.5)	13.6(5.9)	$1.94E^{-22}$
Diabetes[b]	N=15	N=18	N=19	N=13	0.22
Hypercholesterolemia[b]	N=15	N=18	N=19	N=13	0.65
Hypertension[b]	N=15	N=18	N=19	N=13	0.046
***APOE*[c] genotype**					
2/3	4	4	5	1	
2/4	2	0	0	0	
3/3	14	10	18	11	
3/4	5	9	4	15	
4/4	1	7	1	2	

[a] MMSE- Mini-Mental State Exam,

[b] Presence of comorbidity prior to or during blood draw.

[c] *APOE*- Apolipoprotein E

2.2.2. Plasma depletion

Plasma samples were depleted of the top six most abundant proteins (albumin, IgG, IgA, α1-antitrypsin, transferrin and haptoglobin) using the Multiple Affinity Removal System (MARS) Column Human 6 (Agilent, Santa Clara) according to manufacturer's instructions. In brief, 30 μL of crude plasma sample was diluted 4 times using buffer A (Agilent, Santa Clara) and centrifuged at 16000 g for 1 min through a 0.22 μm spin filter to remove particulates. The sample was injected onto the MARS 6 column using a Waters Alliance 2695 Separation module LC system and the resulting fractions were collected. The flow through fractions were concentrated using a 5 kDa molecular weight cutoff concentrator at 4695 g for 1.5 hr followed by a bicinchoninic acid (BCA) assay to determine protein concentration. A pooled sample containing equal amounts of protein from each of the plasma samples was generated and used as quality control (QC) sample.

2.2.3. Digestion

Samples were randomized into eight and four batches respectively, for Set 1 and Set 2 with corresponding QC sample in each batch. In Set 1, in solution digestion was performed in 100 mM ammonium bicarbonate buffer. Proteins (100 μg) were reduced using 200 mM dithiothreitol (DTT) for 45 min at 55°C, while alkylation was performed using 200 mM iodoacetamide (IAM) in the dark for 30 min. Finally, proteins were digested using trypsin/Lys-C mix (Promega, Madison) overnight at 37 °C (1:50 enzyme:protein ratio). The digested samples were acidified with formic acid and desalted using HLB cartridges (Waters Corporation, Milford) per manufacturer's instructions. For Set 2, the digestion was carried out using the filter assisted sample preparation (FASP) protocol.[72] In brief, proteins (100 μg) were transferred onto a 10 kDa molecular weight cutoff filter (Sartorius, Gloucestershire, UK) and reduced for 15 min with 20 mM DTT in 100 mM Tris with 8 M urea. This was followed by centrifugation at 14000 rpm for 30 min and the resulting

filtrate was discarded. Proteins were then alkylated with 20 mM IAM in the dark for 15 min followed by centrifugation to remove the excess reagents. Samples were washed using 100 mM Tris in 1 M urea in a centrifuge at 14000 rpm and trypsin/Lys-C mix (Promega, Madison) was added and digested for 8 hr at 37 °C (1:50 enzyme:protein ratio). After digestion, the peptides were acidified with formic acid and desalted using HLB cartridges. TMT 10-plex or 11-plex labeling (ThermoFisher Scientific, Waltham) was performed following manufacturer's instruction with 25 μg of peptides. Labeled peptides were desalted and separated into 12 fraction using high pH (pH=10) reversed-phase fractionation with acetonitrile (ACN(%)-3,5,8,10,13,18,22,30,45,60,80,95) on an HLB cartridge. Fractions were dried down and reconstituted in water with 0.1% formic acid.

2.2.4. LC-MS/MS and MS^3 parameters

Peptides were analyzed using an Orbitrap Fusion Lumos (ThermoFisher Scientific, Waltham) in positive ionization mode. The samples were loaded onto a self-packed C18 (5 μm, 200Å, MICHROM Bioresources Inc.) trap column (100 μM ID x 2.5 cm, IntegraFrit Capillary), and separation was performed on an in-house packed C18 (2.5 μm, 100Å, XBridge BEH from Waters) capillary column (100 μM ID x 25 cm, Polymicro Technologies) at 300 nL/min using solvent A (water with 0.1 % formic acid) and solvent B (acetonitrile with 0.1% formic acid). The gradient was as follows: 0-7 min, 10% B; 7-67 min, 10-30% B; 67-75 min, 30-60% B; 75-77 min, 60-90% B; 77-82 min, 90% B; 82-83 min, 90-10% B; and 83-100 min, 10% B. Full MS scans were acquired over a mass range of *m/z* 375–1500 at a resolution of 120,000 with the automatic gain control (AGC) target set at 4×10^5 ions and maximum ion injection (IT) time of 50 ms. Data dependent acquisition (DDA) was used to acquire MS/MS spectra with a cycle time of 3 s. MS/MS fragmentation was performed using collision-induced dissociation (CID) with an NCE= 35%. The

AGC was set at 1×10^4 using an isolation width of 0.7 *m/z*, maximum injection time of 100 ms, and a dynamic exclusion of 20 s. Synchronous precursor selection (SPS) mode was used for collecting MS^3 spectra of the top 10 most intense ions from the MS/MS fragments. Higher-energy collisional dissociation (HCD) was used for MS^3 with the following Orbitrap parameters: NCE= 55%, scan range = 100-400 *m/z*, resolution = 60,000, AGC = 5×10^4, maximum injection time = 118 ms and isolation width = 2 *m/z*. Each fraction was injected in duplicate and the injection order was randomized for each batch.

2.2.5. Data analysis

Raw files were analyzed using Proteome Discoverer software (version 2.2) and searched against the Uniprot human reviewed protein database (07/17/2018, 20289 sequences) using SEQUEST-HT. The following parameters were used: maximum two trypsin miscleavages, precursor mass tolerance 10 ppm, fragment mass tolerance 0.6 Da; dynamic modification of methionine oxidation (+15.995 Da), protein N-termini acetyl (42.011 Da), TMT 10 (229.163 Da)/11 plex (229.169 Da) on peptide N-termini and lysine residue, static modification of cysteine carbamidomethyl (+57.02 Da). Decoy database searching was employed to generate high confidence peptides (FDR < 1%). TMT reporter ions (i.e. *m/z* 126 – 131) were identified with the following parameters: most confident centroid and 20 ppm for reporter ion mass tolerance. Technical replicates and fractions from each batch were combined into one result file. Finally, the individual batch data were combined together for further processing using a in-house Python script.

Protein filtering criteria included peptide spectral matches (PSMs) ≥ 2, and reporter ion intensity values above the minimum threshold in at least 75% of the TMT channels (i.e., present

in any 54 of 73 samples or 30 of 40 samples). The final list of proteins was normalized using a two-step internal reference scaling (IRS) method which has been described previously.[73] Briefly, in-batch normalization was performed by calculating a scaling factor (SF), which is the ratio of the sum of intensity of the pooled channel to the sum of each individual TMT channel, followed by multiplying the intensities by the SF for each individual batch. Next, across-batch normalization was applied by the use of SF from the geometric mean of the TMT intensity of pooled samples. Once normalized, differentially-expressed proteins (p-value<0.05) were determined by student's *t*-test's between AD and CN samples groups within each sample set. We did not use Bonferroni or other multiple hypothesis testing to keep a less stringent initial set of data to feed into the machine learning algorithm.[74-75] A fold change cut-off of 1.23 (Set 1) and 1.33 (Set 2) was established based on biological and technical variation in the data.[76] The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org/) via the PRIDE [77] partner repository with the dataset identifier PXD022265.

2.2.6. Machine learning

Data preparation. Supervised classification was performed using RStudio, R version 3.5.1. The data sets with at least 75% of the TMT channels filled were selected for further analysis. Models were built with two different approaches: 1) using only the protein data, and 2) using the protein data along with the variables of sex, age, years of education, and APOE status. In the models with combined data types, sex and *APOE* genotype status were converted to numeric variables. For *APOE* status, any patient with an 4/4genotype was coded as a 4. Genotypes of 3/4 and 3/3 were coded as 3 and 2, respectively. All remaining patients, who had at least one *APOE*2* allele (2/3, 2/4 genotypes), were coded as 1. Prior to classification, differentially-expressed

proteins were selected as the protein feature set. These were either combined or not combined with the clinical variables and then the matrix was scaled using the embedded scale function in R. After scaling, any missing values were replaced with the average value for the given feature in the dataset under consideration. Since the data had been scaled, the average value was zero in each case.

Classification. All supervised classification was performed with a Support Vector Machine (SVM) using the R package, e1071.[78] All area under the receiver-operator curve (AUC) values reported herein were calculated using the R package, pROC.[79] In order to ensure comparability from one experiment and one data set to the next, all of the SVM classifications were performed using identical parameters (i.e., leave-one-out cross-validation was performed in every case, and hyperparameters were not optimized). The reported accuracy was based on comparing the true class values to the probabilities generated in the model. The probabilities were also used in calculating the AUC.

To account for class imbalance and the fact that the data sets had different proportions of cases versus controls, a random undersampling and aggregation technique was employed. During undersampling, a random set of training samples was selected for model building, with the number for each class being equal and determined by subtracting one from the smallest class of samples. (For example, if there were 39 AD patients and 34 controls, the number of samples in each class in the training set would be 33.) The test sample was always excluded from consideration, and then training samples from each class were randomly selected, based on the pre-set number of samples to be used in the model. The SVM classification commenced, and the probability of the sample being assigned to Group 1 was recorded. This process was repeated 300 times for each sample. The 300 probabilities that resulted from the 300 classifications for each sample were averaged in order to determine a single probability for each sample. This algorithm maximally

leverages all available data while not imparting a bias in the results based on the relative sizes of the two classes in the training set.

2.3. Results

Plasma samples (N=113) from four study groups- African American/Black cognitively normal (African American/Black CN, N=26) and AD (African American/Black AD, N=30), non-Hispanic White cognitively normal (non-Hispanic White CN, N=28) and AD (non-Hispanic White AD, N=29) were obtained from the University of Pittsburgh ADRC. Generally, there were twice as many females in each group and no significant differences were found in CN compared to AD groups with regards to sex, age, year of blood draw and presence of other comorbidities (diabetes, hypercholesterolemia, hypertension). Each of the groups had an average 13 years of education, except the non-Hispanic White CN group which had ~16 years of education (**Table 2.1**). The average MMSE scores for the CN samples were above 27 while for the AD samples they were below 15. Each patient sample also had information about *APOE* genotypes, and we note that the African American/Black AD group had a higher percentage (i.e., 23%) of the 4/4 genotype compared to non-Hispanic White AD group. AD groups overall had higher percentages of individuals who carried a homozygous and/or heterozygous 3/4 or 4/4 genotypes compared to the CN group.

A general overview of the plasma proteomics workflow employed for both Set 1 and Set 2 is shown in **Figure 2.1**. For both experiments, all steps, except the digestion process were similar in order to simulate known variations in sample preparation study designs across proteomics laboratories. A correlation plot of average normalized TMT reporter ion intensities for all proteins between different batches for both experiments is given in **Figure B2.1**, demonstrating high

reproducibility among the batches in each experiment. The average R2 values for Set 1 and Set 2 were 0.99 and 0.9939, respectively. A total of 538 high confidence proteins (1% FDR, PSMs ≥ 2) were identified in Set 1, and 596 proteins were identified in Set 2 yielding a total of 740 proteins from both sets (**Figure 2.2**). Between Set 1 and Set 2, 395 proteins were identified in both sets. While this level of coverage is desirable, many of the proteins were identified in only a sub-population of the samples, so different filtering criteria were assessed to choose a data set size that balanced the competing needs of retaining many proteins in the data set while having quantitative data for as many samples as possible for each of the proteins. When considering only proteins with 50% of the TMT channels present, 285 and 380 proteins remained in Set 1 and Set 2, respectively. When considering only proteins with 75% of the TMT channels present, 249 and 314 proteins, respectively, were present in Set 1 and Set 2, with >95% of the proteins in Set 1 also appearing in Set 2. This filtering option results in significantly better coverage for each protein while only minimally shrinking the size of the data set. We also considered filtering the data to include only proteins with 100% of the TMT channels present, which resulted in 189 proteins in Set 1, 257 proteins in Set 2, and 183 proteins in common between the two sets. This filtering option would likely remove too many important but low-abundant proteins. We selected those proteins present in 75% of the patient samples for further analysis, as this data set best balanced the needs of retaining as many proteins as possible while providing quantitative data for as many samples as possible for each protein.

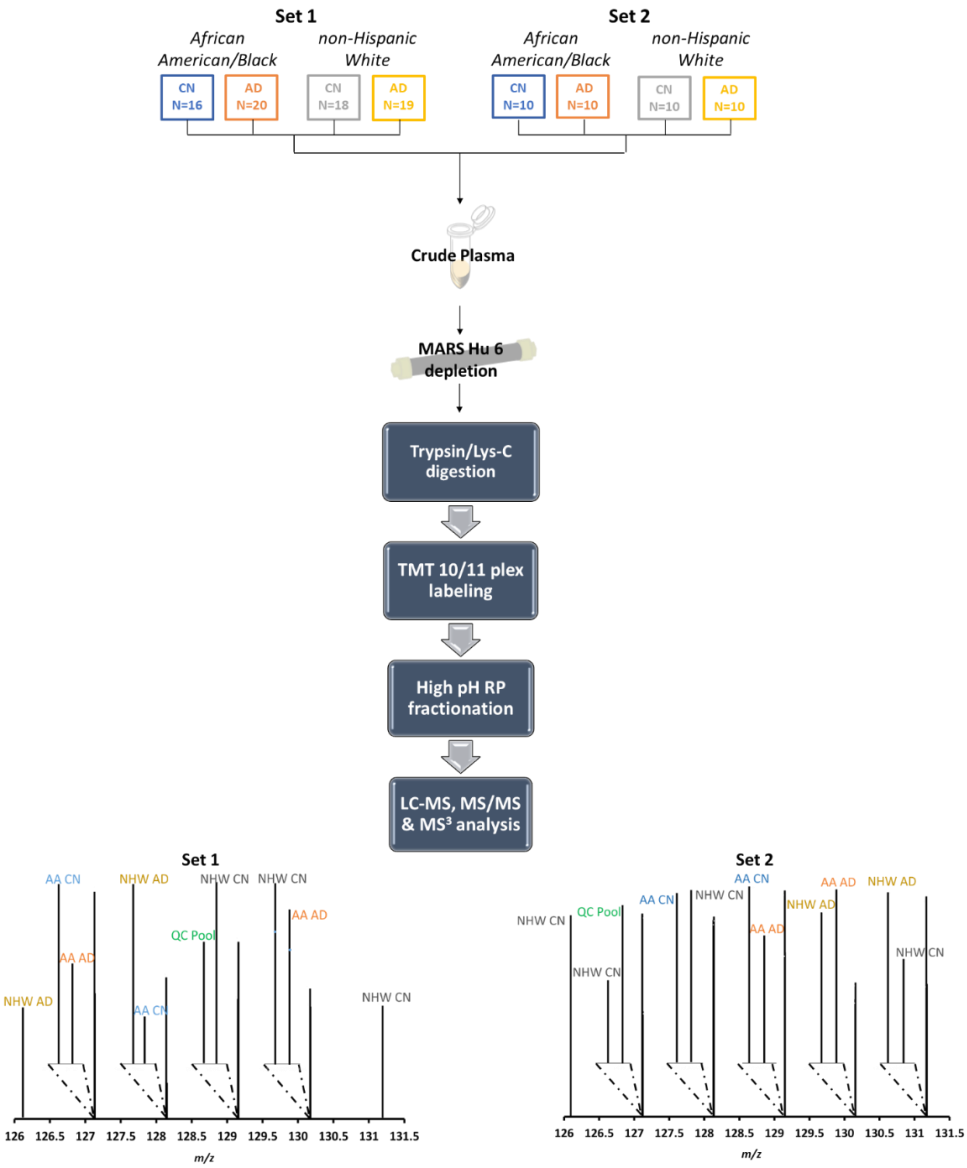

Figure 2.1. Overview of the plasma proteomics workflow. Samples from four study groups- African American/Black Alzheimer's disease (AD) and cognitively normal (CN), non-Hispanic White AD and CN-were obtained from the University of Pittsburgh ADRC. Samples were divided into Set 1 (N=73) and Set 2 (N=40) for this study. Samples were randomized into eight batches for Set 1 and four batches for Set 2. There was one QC pool sample in each batch and representation of one sample from each study group in each batch. The samples were randomly assigned TMT channels for both experiments. The experimental workflow was maintained the same except for the digestion step, where in solution digestion was used for Set 1, while FASP digestion was employed in Set 2. The plasma samples were immunodepleted of the six most abundant proteins, followed by proteolytic digestion. This was followed by isobaric tagging using either TMT 10/11 plex labels, followed by high pH reversed-phase fractionation. The resulting peptides were loaded into an Ultimate 3000 RPLC system coupled to an Orbitrap Fusion Lumos mass spectrometer for LC-MS, MS/MS and MS^3 analysis. Example representative MS^3 reporter ion spectra for TMT-10 plex sample (Set 1) and TMT-11 plex sample (Set 2) is also provided, demonstrating analysis of multiple samples using a single injection.

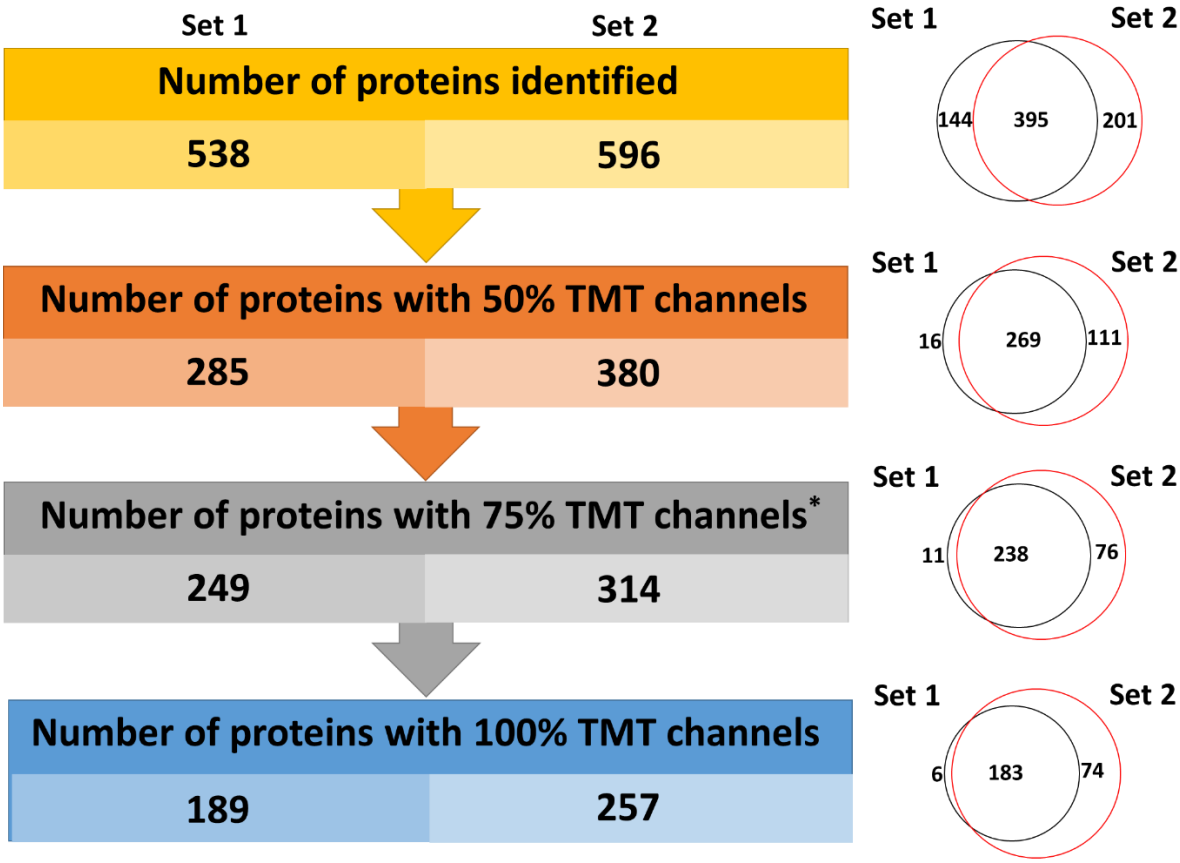

Figure 2.2. Summary of the number of identified proteins in both sample sets. On the left, are the number of high confidence identified proteins as a function of missing channels for TMT reporter ions. Values are provided for Set 1 and Set 2. On the right are Venn diagrams, displaying the overlap in common proteins at each level from Set 1 and Set 2.

2.3.1. Differentially-expressed proteins in AD

Since only a small fraction of plasma proteins were expected to be differentially expressed between AD and CN groups, a robust process to select the optimal proteins for machine learning was needed. Thus, we focused on identifying differentially-expressed proteins in Set 1 (N= 34 CN, N= 39 AD) which had a larger number of samples compared to Set 2 (N= 20 CN, N= 20 AD). For this initial analysis, we combined data from all of the CN individuals into one group and all with AD into a second group. **Figure 2.3a** displays a volcano plot distribution of protein TMT ratios of the AD compared to CN groups. Of the proteins with significant p-values in Set 1 ($p < 0.05$), four proteins had fold-changes that were >1.18: beta-ala-his dipeptidase (FC- 0.73, p-value 0.0001), keratin type I cytoskeletal 9 (FC- 0.71, p-value 0.049), apolipoprotein L1 (FC- 0.84, p-value 0.03), and adiponectin (FC- 1.40, p-value 0.02). Beta-Ala-His dipeptidase, keratin type I cytoskeletal 9, and apolipoprotein L1 were all lower in AD compared to CN, while adiponectin was higher in AD. These changes are consistent with literature reports.[80-82] Thus, these four proteins from Set 1 were selected as the protein feature to use in subsequent machine learning studies, and their utility for confirming AD was tested in both Set 1 and Set 2.

Using an SVM classifier and leave-one-out cross-validation, we determined the utility of the four selected proteins for confirming AD. Two models were tested: one included only the four proteins (beta-ala-his dipeptidase, keratin type I cytoskeletal 9, apolipoprotein L1, and adiponectin), and the second contained these proteins along with four additional variables: age, sex, years of education, and *APOE* status. These models were tested twice, using samples from either Set 1 or Set 2. The classification accuracies of the two models in Set 1 and Set 2, are shown in **Figure 2.4**.

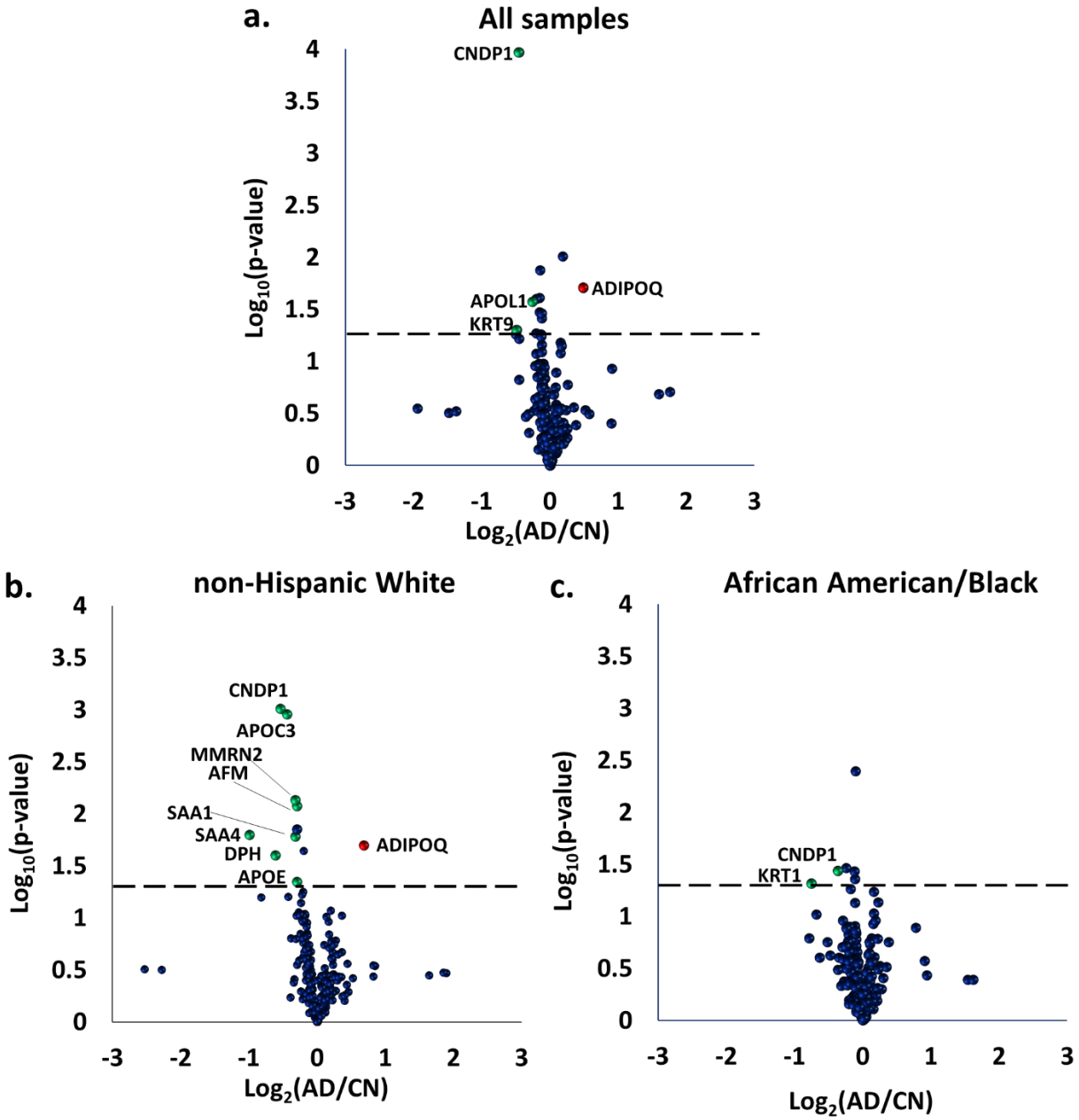

Figure 2.3. Volcano plots of differentially-expressed proteins between Alzheimer's disease (AD) and cognitively normal individuals (CN) for the entire set of samples in **a)** Set 1 (N=39 AD, N= 34 CN); **b)** data from the non-Hispanic White group only, Set 1 (N=19 AD, N= 18 CN); and **c)** data from the African American/Black group only, Set 1 (N=20 AD, N= 16 CN). Red circles coincide with proteins higher in AD compared to CN, while green circles coincide with proteins lower in AD. Abbreviations: CNDP1- Beta-Ala-His dipeptidase, KRT9- Keratin type I cytoskeletal 9, APOL1- Apolipoprotein L1, ADIPOQ- Adiponectin, KRT1- Keratin type II cytoskeletal 1, APOC3- Apolipoprotein C3, MMRN2- Multimerin-2, AFM- Afamin, SAA1- Serum amyloid A-1 protein , SAA4- Serum amyloid A-4 protein, DPH- Dopamine beta-hydroxylase, APOE- Apolipoprotein E.

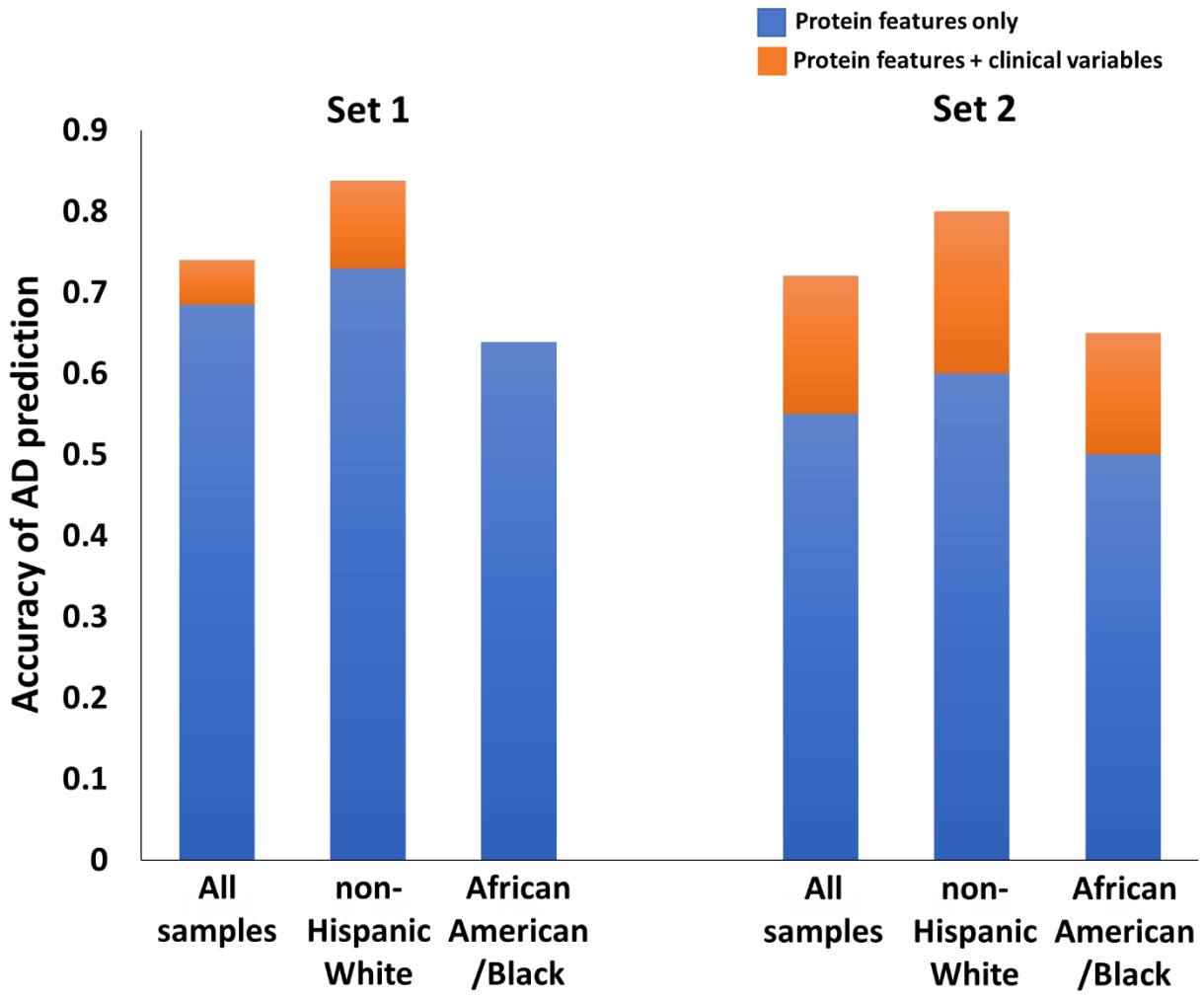

Figure 2.4. Histogram displaying classification accuracy for predicting AD in Set 1: N=73 samples and Set 2: N=40 samples. Blue bars: Accuracy determined when only the four differentially expressed proteins (beta-ala-his dipeptidase, keratin type I cytoskeletal 9, apolipoprotein L1, and adiponectin) are included in the model. Orange bars: Additional improvement in accuracy when clinical variables (age, sex, education, and APOE) are also included in the model.

Several significant outcomes are noted based on results in **Figure 2.4**. In all four classifications, the non-Hispanic White adult samples had higher accuracy than the African American/Blacks adult samples. Additionally, in both Set 1 and Set 2, a higher overall accuracy was obtained when the other variables were included. However, the variables of age, sex, years of education, and *APOE* status, provided a bigger boost to the accuracy of the samples from non-Hispanic White adults compared to African American/Black adults. The fact that the two tested models were not as effective for the samples from African American/Black adults as they were for the samples from non-Hispanic White adults caused us to consider race-stratification of proteomics data prior to machine learning.

2.3.2. Differentially-expressed proteins in AD in race-stratified groups

Differentially-expressed proteins between AD and CN groups for the African American/Black and non-Hispanic White samples in Set 1, are shown using volcano plots in **Figures 2.3b and 2.3c**. Nine proteins were differentially expressed for the non-Hispanic White samples. Most of these proteins were decreased in AD with serum amyloid A-1 protein having the largest change (FC = 0.5, p-value 0.02). The remaining proteins included: beta-ala-his dipeptidase (FC = 0.69, p-value 0.0009), dopamine beta-hydroxylase (FC = 0.69, p-value 0.03), apolipoprotein C3 (FC = 0.74, p-value 0.001), serum amyloid A-4 protein (FC = 0.8, p-value 0.02), multimerin-2 (FC = 0.8, p-value 0.01), apolipoprotein E (FC = 0.81, p-value 0.045) and afamin (FC = 0.81, p-value 0.01). Adiponectin (FC = 1.61, p-value 0.02) was the only proteins that increased in non-Hispanic White AD samples.

Two proteins were differentially-expressed in samples from African American/Black adults in Set 1 (**Figure 2.3c**): beta-ala-his dipeptidase (FC = 0.78, p-value 0.04) and keratin type

II cytoskeletal 1 (FC = 0.59, p-value 0.048). Only beta-ala-his dipeptidase was differentially-expressed in AD for both African American/Black and non-Hispanic White samples. A list of differentially-expressed proteins with corresponding p- and fold-change values are provided in **Table 2.2** and **Table B2.1**.

2.3.3. Supervised classification of differentially-expressed proteins

Eight unique classifications were performed using the differentially-expressed proteins selected from volcano plots in **Figures 2.3b** and **2.3c**. The first SVM classification used the set of nine proteins that were differentially expressed in the non-Hispanic White group from Set 1 (**Figure 2.3b**) as a feature set, and the non-Hispanic White group data from Set 1 for training. Similarly, a second classification was conducted in the same was with Set 2 samples. Two additional classifications were performed in which the nine proteins described above were combined with the four variables (age/sex/education/*APOE* status). In total, four classifications were performed using the proteins that were differentially expressed in the samples from non-Hispanic White AD adults in Set 1. A parallel set of four classifications was performed using the set of proteins that were differentially expressed in samples from African American/Black AD adults (**Figure 2.3c**). These proteins were tested in Set 1 and Set 2, either on their own or with the four clinical variables. In every case where the feature set included proteins that had been differentially expressed in samples from African American/Black adults, those samples from the set being tested, were used to train the model. Results for all eight classifications, separated by racial group, are shown in **Table 2.3**. Test samples were always left out when training the models (see 2.2 Methods).

Table 2.2. List of differentially-expressed proteins in race-stratified groups (Set 1).

Accession	Protein name	*African American*		*Non-Hispanic White*	
		Fold change#	**p-value***	**Fold change#**	**p-value***
Q96KN2	Beta-Ala-His dipeptidase	**0.78**	**0.04**	**0.69**	**9.78E-4**
P04264	Keratin type II cytoskeletal 1	**0.59**	**0.05**	0.85	0.5
Q15848	Adiponectin	1.19	0.3	**1.61**	**0.02**
P43652	Afamin	0.98	0.77	**0.81**	**0.01**
P02656	Apolipoprotein C3	1.04	0.72	**0.74**	**1.1E-3**
P02649	Apolipoprotein E	1.02	0.93	**0.81**	**0.05**
P09172	Dopamine beta-hydroxylase	1.17	0.32	**0.69**	**0.03**
Q9H8L6	Multimerin-2	1.02	0.86	**0.8**	**0.01**
P0DJI8	Serum amyloid A-1 protein	1.06	0.78	**0.5**	**0.02**
P35542	Serum amyloid A-4 protein	1.01	0.86	**0.8**	**0.02**

Fold change cut off 1.23; * p-value < 0.05

Bold denotes proteins that meet both fold-change cut-off and p-value < 0.05

Table 2.3. Summary of results from machine learning.

	non-Hispanic White (NHW) *				*African American/Black (AA)* *			
	No extra variables		**Age/Sex[a]/Edu[b]/*APOE*[c]**		**No extra variables**		**Age/Sex[a]/Edu[b]/*APOE*[c]**	
Set 1	**NHW**	**AA**	**NHW**	**AA**	**NHW**	**AA**	**NHW**	**AA**
AUC[d]	0.91	0.49	0.97	0.61	0.83	0.59	0.91	0.47
Accuracy	86%	47%	86%	56%	73%	67%	84%	47%
Set 2	**NHW**	**AA**	**NHW**	**AA**	**NHW**	**AA**	**NHW**	**AA**
AUC[d]	0.73	0.31	0.94	0.43	0.63	0.84	0.40	0.56
Accuracy	65%	40%	85%	45%	55%	70%	45%	50%

Abbreviations: a. Sex, b. Education, c. Apolipoprotein E4 genotype, d. Area under the curve

Set 1: N=73 samples, Set 2: N=40 samples

***** Differentially-expressed proteins from Set 1 were used for the SVM classification; The full list of differentially-expressed proteins is provided in **Table 2.2**

Overall, when differentially-expressed proteins from the non-Hispanic White group were used to classify AD in the samples, an effective model (i.e, AUC was 0.91 and the accuracy was 86%) was developed, but only for the samples from the non-Hispanic White adults. In contrast, the model performed noticeably worse (i.e., AUC of 0.49 and accuracy of 47%) when classifying the data from samples of African American/Black adults. When other (age, sex, education, *APOE* status) variables were also included in the model, the classification accuracy (i.e., AUC was 0.61 and the accuracy was 56%) of the samples from African American/Black adults improved, yet not to the level of accuracy what would be required for an effective biomarker assay. Incorporating (age, sex, education, *APOE* status) variables improved the accuracy from 0.91 to 0.97 for the non-Hispanic White group, demonstrating that these variables could be combined with protein data to generate a model that can effectively predict AD in this demographic. This desirable outcome, which did not happen when those variables were included for the African American/Black group, suggests that other variables are needed to improve the accuracy of a given model for the African American/Black adult samples in this study.

When SVM classifications were performed on Set 2, the overall findings from Set 1 were generally replicated. Using data from samples in only the African American/Black adult group did not result in accurate classification (i.e., maximum AUC was 0.43 and the accuracy was 45%), either with or without (age, sex, education, *APOE* status) variables included. In Set 2, samples from non-Hispanic White adults were best classified by combining protein data and (age, sex, education, *APOE*) variables (**Table 2.3**).

Differences between the two racial groups are readily apparent when the classification model is built using differentially-expressed proteins (N = 2, **Table 2.2**) from the African American/Black group and only samples from this group to train the model. The best overall

outcome for samples from non-Hispanic White adults occurred when Set 1 was classified using the clinical variables (sex/age/education/*APOE* status) and the protein features. In this case, an AUC of 0.91 and accuracy of 84% was obtained. Using similar data from samples in the African American/Black groups, resulted in worse classification performance (i.e., AUC of 0.47 and accuracy of 47%). The best overall outcome for samples from African American/Black adults was observed when no clinical variables were used and when testing data in Set 2. In that case, an improved classification was obtained (i.e, AUC of 0.84 and an accuracy of 70%) demonstrating how critical it was to evaluate various parameters and testing approaches in finding the best classification for both racial groups. However, it's clear that the performance outcomes of the machine learning models is also dependent on the samples and protein data obtained in training and test sets. In all the tests where samples from African American/Black adults were used for model training, more accurate classification without the age/sex/education/*APOE* status variables was obtained. By contrast, in three out of the four classifications where these variables were included for samples from non-Hispanic White adults, the samples from that group were more accurately classified.

2.4. Discussion

In this study we performed two independent plasma proteomics experiments between AD and CN samples collected from African American/Black and non-Hispanic White participants from the University of Pittsburgh ADRC. At the time of this study, there were only 56 African American/Black participants that had banked plasma sample meeting our criterion. We used the demographics of those participants, primarily age, sex, and AD diagnosis, to match a similar size of non-Hispanic White participants. The AD diagnosis was clinically confirmed, and we used the most recent blood draw for these plasma analyses. Most characteristics that we evaluated of the

participants were generally similar between African American/Black and non-Hispanic White groups.

We identified 740 proteins in total using TMT-based quantitative proteomics on an Orbitrap Fusion Lumos MS instrument, which is on par with recent plasma proteomics publications.[83-90] However, with substantial increases in high pH reversed-phase fractionation in the sample processing, higher numbers of plasma protein identifications could have been identified.[91] Additionally, we note that our MS data acquisition method used MS^3 for TMT measurements in order to increase the quantitative accuracy of differentially-expressed proteins. However, using this approach is known to increase instrument duty cycle and result in lesser protein identifications. After applying stringent criteria for identification, presence of TMT reporter ion channels across samples, and fold-change and p-value cutoffs, we identified a total of 27 differentially-expressed proteins (see Methods) in AD. These proteins were either different in a specific racial group or in comparisons of both racial groups together. Several of these proteins had changes in AD that were previously reported.[80-81, 90] Notable in this study was the inclusion of samples from African American/Black ADRC participants. Based on our analyses, there was only one differentially-expressed protein (beta-Ala-His dipeptidase) in AD that was significant in both African American/Black and non-Hispanic White groups. Lower expression of this protein in AD for both groups is consistent with previous reports.[90]

One of the proteins that was only differentially expressed in non-Hispanic White samples included ApoE, which has been widely reported as potential plasma biomarker in AD. There have been contradictory reports of ApoE being higher in AD [92], while others have reported it as lower in AD.[52] In our study, in samples from non-Hispanic White adults, ApoE was lower in adults in AD, whereas it did not have a change in AD vs CN in samples from African American/Black

adults. Other proteins that were lower in samples from non-Hispanic White AD included afamin, ApoC3, serum amyloid A1 protein and serum amyloid A4, which all have been reported to also change in previous studies. Direction of change in afamin, ApoC3, and adiponectin **(Table 2.2)** were also consistent with literature reports.[47, 49-50, 80, 90, 93-95] Some of the differentially-expressed proteins (i.e., serum amyloid A1 and serum amyloid A4 proteins) that we identified were observed in other studies, however the direction of change in AD vs CN was not consistent.[90] Two novel findings in this work were the differential expression of dopamine beta-hydroxylase and multimerin-2 which were only different in the non-Hispanic White group.

The main outcome from our machine learning analyses is that samples from the African American/Black and non-Hispanic White participants had notable differences in performance outcomes. Proteomics data from non-Hispanic White adults was classified substantially more accurately than data from African American/Black adults when both racial groups were combined in the training data (**Figure 2.4**) and when only the non-Hispanic White group was used as training data (**Table 2.3**). The other main difference observed is that samples from the non-Hispanic White group were typically best classified when (age, sex, education, and *APOE* status) variables were included in the model, while the classification of the African American/Black group benefitted less from including these variables. In fact, including these variables was detrimental in both cases where samples only from the African American/Black group were used to train the model and using proteins differentially expressed only in the African American/Black group. Even in the case where samples from both African American/Black and non-Hispanic White groups were used to identify differentially-expressed proteins and to train the model (**Figure 2.4**), including (age, sex, education, and *APOE* status) variables only improved the classification of the samples from African American/Black adults in test data sets.

Overall, these studies show how critical it is for biomarker discovery efforts to be inclusive of individuals from various racial and ethnic backgrounds as it can have a huge impact on effectiveness of machine learning models. These studies found overall that samples from the non-Hispanic White group were more accurately classified with SVM based on changes in plasma proteins from CN and AD adults. Also, the addition of age, sex, years of education, and *APOE* status as variables in the model disproportionally improved the classification accuracy of the non-Hispanic White group compared to the African American/Black group. It is not clear from our findings that the machine learning performance outcomes are simply just tied to the self-reported race as many factors such as life experiences, stress, age, education, mixed-dementia pathology are intermixed in the construct of race and can contribute to protein levels in plasma. These factors should be considered for the African American/Black group, however are not easy to evaluate in terms of contributions to differential protein expressions in AD, which ultimately was used to feed proteins into the machines learning model. Achieving satisfactory outcomes with machine learning for AD with samples from African American/Black adults was possible in this study for one set of plasma proteins and without the use of covariates of age, sex, years of education, and *APOE* status variables. However, these studies clearly point to a need for increased plasma proteomics studies and number of plasma samples from African American/Black adults that are cognitively normal and with clinical diagnoses of AD in order to facilitate training of machine learning models and importantly, ensure favorable outcomes for African American/Black adults in plasma proteomics biomarker discovery efforts.

2.4.1. Study strengths and limitations

One of the major strengths of our study is the inclusion of plasma samples from both African American/Black and non-Hispanic White individuals. The number of samples from each

group was evenly distributed, with no significant differences due to sex, age, or comorbidities. Also, the AD samples having higher percentage of *APOE*4* allele present, as reported by previous studies [1] was maintained by the samples in this study. This is the first study of its kind to compare these two racial groups in plasma proteomics experiments and directly evaluate in machine learning models for AD biomarker discovery. The majority of the proteins we found to be differentially expressed have been previously reported to change in AD; yet a few findings, with regard to AD-related protein expression, are novel, particularly when considering the protein expression data specific to samples from African American/Black adults. These findings coupled with the fact that the number of studies involving samples from African American/Black adults are very limited, puts emphasis on the need for conducting more AD research including African American/Black patient samples. This additional effort is necessary to both better understand disparities in disease incidence but also to ensure biomarker discovery efforts are effective for everyone.

We employed a randomized blinded study design and ensured samples from each study group were included in every TMT batch. This allowed us to minimize error due to sample preparation. Also, we performed MS^3 quantification, which provides highly accurate quantitative information and thus enhances confidence in the fold-changes observed for AD. This choice is potentially critical for facilitating biomarker discovery by focusing on robust protein changes.[96] Despite the use of MS^3, which requires a higher duty cycle, we were able to identify similar numbers of proteins compared to recent publications using MS/MS approaches.[83-90]

Another strength is that we had enough plasma samples to establish two independent data sets so that the results obtained from a training set (Set 1) could be validated with a test set (Set 2). There was a high degree of overlap in terms of protein identifications in the training and test

sets that could be considered for use in machine learning. Our study design allowed us to stratify our data post-analysis based on the self-reported racial and ethnic group of the participants. In this case, the machine learning model was able to differentiate AD samples with high accuracy for the non-Hispanic White group using a set of nine proteins that were selected based on volcano plot analysis. We believe it was a strength that our study design allowed us to test if self-reported race was a critical factor in the accuracy of potential biomarker candidates. The same protein set that produced high accuracy in the non-Hispanic White group performed poorly when applied to the African American/Black group in both data sets. This strongly indicates a need for more studies that have inclusive designs and for evaluation of whether self-reported race or other variables are critical for biomarker development.

A notable limitation of this study was that protein identifications were cut in half by filtering out proteins that were missing TMT values for at least 50% of the samples. We note that this loss is likely due to batch effects; for every additional TMT batch acquired, new proteins are observed while others can go undetected due to the stochastic nature of the data dependent acquisition.[97] Also, the use of MS^3 could result in proteins being missed, as they may not have been selected for fragmentation in one TMT batch but were in another. DIA or targeted MRM methods could avoid this issue by only focusing on known protein identifications throughout the entire run.

While our sample size was a total of N = 113, which was on par with our similar studies involving AD [58, 63-64, 90, 95, 98], but we note that for biomarker discovery efforts this sample size is still small. Obtaining samples from African American/Black participants is challenging with limited availability in ADRCs; however, we will need to work with other ADRCs and focused studies [99-100] to generate large sample sizes especially from available African American/Black

participants. Sample size is especially critical as any racial and ethnic group is not homogenous and existing knowledge in plasma proteomics for AD is based mostly on participants from non-Hispanic White and European backgrounds. We believe our moderate sample size also limited our ability to identify an adequate number and set of differentially-expressed proteins that could be effective for the machine learning classification of African American/Black adults. This is recognized by our study design to split samples into two sets for training and validation which reduced sample size in each group. However, our sample size was substantially higher than in other reported AD biomarker studies of African American/Black adults.[37]

Finally, we note another limitation to this study was not including additional variables other than age/sex/education/*APOE*4* status that may have improved machine learning outcomes for samples from the African American/Black group. For example, it is recognized that biases in mini-mental state examination and other cognitive tests [101-102] may result in inadequacies of generating similar types of participants and that quality of education may be a far more important factor than years of education to include in study designs.[103] Genetic ancestry could be included as an additional measure as well as the use of other types of genetic markers that have AD risk associated with African American/Black adults.[104] Stratification of groups based on self-reported race has limits also because it does not capture life-long experiences, such as discrimination and exposure to systemic racism [18], that have been shown to lead to inherent biases in healthcare and also impact plasma proteomic cytokine levels.[105-106]

2.5. Conclusions

Plasma proteomics analysis combined with classification by machine learning is a powerful strategy for identifying potential biomarker candidates that can be used for AD diagnosis. Plasma

proteomics biomarker discovery efforts have largely excluded samples from African American/Black adults, and this study sought to include samples from this group to help facilitate biomarker discovery efforts for everyone. Our analyses demonstrated that potential biomarker candidates for AD diagnosis could be identified with high accuracy in plasma samples from non-Hispanic White adults that were cognitively normal or clinically diagnosed with AD, and that these same candidates were not effective in samples from African American/Black adults. Further improved machine learning outcomes for AD biomarker discovery were possible with the addition of variables such as age, sex, years of education, and *APOE*4* status in the training model; however, these specific variables do not appear to be as effective for classifying samples from African American/Black adults with plasma proteomics data. Thus, the search is on for a better set of plasma proteins and/or combined use of clinical/demographic variables which can be used to ensure biomarker discovery efforts in AD are effective for everyone, including African American/Black adults.

2.6. Acknowledgements

This work has been supported by the funding from the Alzheimer's Association (AARGD-17-533405), pilot funds from the University of Pittsburgh Alzheimer Disease Research Center funded by the National Institutes of Health and National Institute on Aging (P50AG005133, RASR), NICHD (R01 HD064727, NAP), the Vanderbilt Institute of Chemical Biology (T32-GM06508), the National Institutes of Health (R35GM130354, HD) and the National Institute on Aging (AG041718, AG030653, AG064877, MIK).

2.7. References

1. Barnes, L. L.; Bennett, D. A., Alzheimer's disease in African Americans: risk factors and challenges for the future. *Health Aff (Millwood)* **2014,** *33* (4), 580-586.

2. Matthews, K. A.; Xu, W.; Gaglioti, A. H.; Holt, J. B.; Croft, J. B.; Mack, D.; McGuire, L. C., Racial and ethnic estimates of Alzheimer's disease and related dementias in the United States (2015-2060) in adults aged ≥65 years. *Alzheimers Dement* **2019,** *15* (1), 17-24.

3. Lines, L.; Sherif, N.; Wiener, J., Racial and ethnic disparities among individuals with Alzheimer's disease in the United States: A literature review. *RTI Press, RTI Press Publication*, **2014**, No. RR-0024-1412.

4. 2020 Alzheimer's disease facts and figures. *Alzheimer's & Dementia* **2020,** *16* (3), 391-460.

5. Chin, A. L.; Negash, S.; Hamilton, R., Diversity and disparity in dementia: the impact of ethnoracial differences in Alzheimer disease. *Alzheimer disease and associated disorders* **2011,** *25* (3), 187-195.

6. Rajan, K. B.; Weuve, J.; Barnes, L. L.; Wilson, R. S.; Evans, D. A., Prevalence and incidence of clinically diagnosed Alzheimer's disease dementia from 1994 to 2012 in a population study. *Alzheimer's & Dementia* **2019,** *15* (1), 1-7.

7. Gilligan, A. M.; Malone, D. C.; Warholak, T. L.; Armstrong, E. P., Health disparities in cost of care in patients with Alzheimer's disease: an analysis across 4 state Medicaid populations. *Am. J. Alzheimers Dis. Other Demen.* **2013,** *28* (1), 84-92.

8. Husaini, B. A.; Sherkat, D. E.; Moonis, M.; Levine, R.; Holzer, C.; Cain, V. A., Racial differences in the diagnosis of dementia and in its effects on the use and costs of health care services. *Psychiatr. Serv.* **2003,** *54* (1), 92-96.

9. Mehta, K. M.; Yeo, G. W., Systematic review of dementia prevalence and incidence in United States race/ethnic populations. *Alzheimer's & Dementia* **2017,** *13* (1), 72-83.

10. Yaffe, K.; Falvey, C.; Harris, T. B.; Newman, A.; Satterfield, S.; Koster, A.; Ayonayon, H.; Simonsick, E., Effect of socioeconomic disparities on incidence of dementia among biracial older adults: prospective study. *BMJ : British Medical Journal* **2013,** *347*, f7051.

11. Weuve, J.; Barnes, L. L.; Mendes de Leon, C. F.; Rajan, K. B.; Beck, T.; Aggarwal, N. T.; Hebert, L. E.; Bennett, D. A.; Wilson, R. S.; Evans, D. A., Cognitive Aging in Black and White Americans: Cognition, Cognitive Decline, and Incidence of Alzheimer Disease Dementia. *Epidemiology* **2018,** *29* (1), 151-159.

12. Barnes, L. L.; Wilson, R. S.; Hebert, L. E.; Scherr, P. A.; Evans, D. A.; Mendes de Leon, C. F., Racial differences in the association of education with physical and cognitive function in older blacks and whites. *J. Gerontol. B Psychol. Sci. Soc. Sci.* **2011,** *66* (3), 354-63.

13. Manly, J. J.; Schupf, N.; Tang, M.-X.; Stern, Y., Cognitive Decline and Literacy Among Ethnically Diverse Elders. *J. Geriatr. Psychiatry Neurol.* **2005,** *18* (4), 213-217.

14. Sisco, S.; Gross, A. L.; Shih, R. A.; Sachs, B. C.; Glymour, M. M.; Bangen, K. J.; Benitez, A.; Skinner, J.; Schneider, B. C.; Manly, J. J., The role of early-life educational quality and literacy in explaining racial disparities in cognition in late life. *The journals of gerontology. Series B, Psychological sciences and social sciences* **2015,** *70* (4), 557-67.

15. Stepler, K.; Robinson, R., The Potential of 'Omics to Link Lipid Metabolism and Genetic and Comorbidity Risk Factors of Alzheimer's Disease in African Americans. Adv Exp Med Biol. **2019**, *1118*, 1-28.

16. Turner, A. D.; James, B. D.; Capuano, A. W.; Aggarwal, N. T.; Barnes, L. L., Perceived Stress and Cognitive Decline in Different Cognitive Domains in a Cohort of Older African Americans. *The American Journal of Geriatric Psychiatry* **2017,** *25* (1), 25-34.

17. Campdelacreu, J., Parkinson's disease and Alzheimer disease: environmental risk factors. *Neurología (English Edition)* **2014,** *29* (9), 541-549.

18. Wilkins, C. H.; Schindler, S. E.; Morris, J. C., Addressing Health Disparities Among Minority Populations: Why Clinical Trial Recruitment Is Not Enough. *JAMA Neurology* **2020,** *77* (9), 1063-1064.

19. Filshtein, T. J.; Dugger, B. N.; Jin, L. W.; Olichney, J. M.; Farias, S. T.; Carvajal-Carmona, L.; Lott, P.; Mungas, D.; Reed, B.; Beckett, L. A.; DeCarli, C., Neuropathological Diagnoses of Demented Hispanic, Black, and Non-Hispanic White Decedents Seen at an Alzheimer's Disease Center. *J. Alzheimers Dis.* **2019,** *68* (1), 145-158.

20. Kamara, D. M.; Gangishetti, U.; Gearing, M.; Willis-Parker, M.; Zhao, L.; Hu, W. T.; Walker, L. C., Cerebral Amyloid Angiopathy: Similarity in African-Americans and Caucasians with Alzheimer's Disease. *Journal of Alzheimer's disease : JAD* **2018,** *62* (4), 1815-1826.

21. Graff-Radford, N. R.; Besser, L. M.; Crook, J. E.; Kukull, W. A.; Dickson, D. W., Neuropathologic differences by race from the National Alzheimer's Coordinating Center. *Alzheimers Dement* **2016,** *12* (6), 669-77.

22. Barnes, L. L.; Leurgans, S.; Aggarwal, N. T.; Shah, R. C.; Arvanitakis, Z.; James, B. D.; Buchman, A. S.; Bennett, D. A.; Schneider, J. A., Mixed pathology is more likely in black than white decedents with Alzheimer dementia. *Neurology* **2015,** *85* (6), 528-534.

23. Gavett, B. E.; Fletcher, E.; Harvey, D.; Farias, S. T.; Olichney, J.; Beckett, L.; DeCarli, C.; Mungas, D., Ethnoracial differences in brain structure change and cognitive change. *Neuropsychology* **2018,** *32* (5), 529-540.

24. Robinson, R. A. S.; Williams, I. C.; Cameron, J. L.; Ward, K.; Knox, M.; Terry, M.; Tamres, L.; Mbawuike, U.; Garrett, M.; Lingler, J. H., Framework for creating storytelling materials to promote African American/Black adult enrollment in research on Alzheimer's disease and related disorders. *Alzheimer's & Dementia: Translational Research & Clinical Interventions* **2020,** *6* (1), e12076.

25. Besser, L.; Kukull, W.; Knopman, D. S.; Chui, H.; Galasko, D.; Weintraub, S.; Jicha, G.; Carlsson, C.; Burns, J.; Quinn, J.; Sweet, R. A.; Rascovsky, K.; Teylan, M.; Beekly, D.; Thomas, G.; Bollenbeck, M.; Monsell, S.; Mock, C.; Zhou, X. H.; Thomas, N.; Robichaud, E.; Dean, M.; Hubbard, J.; Jacka, M.; Schwabe-Fry, K.; Wu, J.; Phelps, C.; Morris, J. C., Version 3 of the National Alzheimer's Coordinating Center's Uniform Data Set. *Alzheimer Dis Assoc Disord* **2018,** *32* (4), 351-358.

26. Besser, L. M.; Kukull, W. A.; Teylan, M. A.; Bigio, E. H.; Cairns, N. J.; Kofler, J. K.; Montine, T. J.; Schneider, J. A.; Nelson, P. T., The Revised National Alzheimer's Coordinating Center's Neuropathology Form-Available Data and New Analyses. *J. Neuropathol. Exp. Neurol.* **2018,** *77* (8), 717-726.

27. Doody, R. S.; Thomas, R. G.; Farlow, M.; Iwatsubo, T.; Vellas, B.; Joffe, S.; Kieburtz, K.; Raman, R.; Sun, X.; Aisen, P. S.; Siemers, E.; Liu-Seifert, H.; Mohs, R., Phase 3 trials of solanezumab for mild-to-moderate Alzheimer's disease. *N. Engl. J. Med.* **2014,** *370* (4), 311-21.

28. Rosenmann, H., CSF biomarkers for amyloid and tau pathology in Alzheimer's disease. *J. Mol. Neurosci.* **2012,** *47* (1), 1-14.

29. Howell, J. C.; Watts, K. D.; Parker, M. W.; Wu, J.; Kollhoff, A.; Wingo, T. S.; Dorbin, C. D.; Qiu, D.; Hu, W. T., Race modifies the relationship between cognition and Alzheimer's disease cerebrospinal fluid biomarkers. *Alzheimers Res. Ther.* **2017,** *9* (1), 88.

30. Morris, J. C.; Schindler, S. E.; McCue, L. M.; Moulder, K. L.; Benzinger, T. L. S.; Cruchaga, C.; Fagan, A. M.; Grant, E.; Gordon, B. A.; Holtzman, D. M.; Xiong, C., Assessment of racial disparities in biomarkers for Alzheimer disease. *JAMA Neurol.* **2019,** *76* (3), 264-273.

31. Blennow, K.; Vanmechelen, E.; Hampel, H., CSF total tau, Aβ42 and phosphorylated tau protein as biomarkers for Alzheimer's disease. *Mol. Neurobiol.* **2001,** *24* (1), 87-97.

32. Wallin, Å. K.; Blennow, K.; Andreasen, N.; Minthon, L., CSF biomarkers for Alzheimer's disease: levels of β-amyloid, tau, phosphorylated tau relate to clinical symptoms and survival. *Dement. Geriatr. Cogn. Disord.* **2006,** *21* (3), 131-138.

33. Garrett, S. L.; McDaniel, D.; Obideen, M.; Trammell, A. R.; Shaw, L. M.; Goldstein, F. C.; Hajjar, I. Racial Disparity in Cerebrospinal Fluid Amyloid and Tau Biomarkers and Associated Cutoffs for Mild Cognitive Impairment *JAMA Netw Open*, **2019**, *2* (12), e1917363.

34. Wharton, W.; Kollhoff, A. L.; Gangishetti, U.; Verble, D. D.; Upadhya, S.; Zetterberg, H.; Kumar, V.; Watts, K. D.; Kippels, A. J.; Gearing, M.; Howell, J. C.; Parker, M. W.; Hu, W. T., Interleukin 9 alterations linked to Alzheimer disease in African Americans. *Ann. Neurol.* **2019,** *86*, 407-418.

35. Simino, J.; Wang, Z.; Bressler, J.; Chouraki, V.; Yang, Q.; Younkin, S. G.; Seshadri, S.; Fornage, M.; Boerwinkle, E.; Mosley, T. H., Jr., Whole exome sequence-based association analyses of plasma amyloid-β in African and European Americans; the Atherosclerosis Risk in Communities-Neurocognitive Study. *PLoS One* **2017,** *12* (7), e0180046.

36. Tranah, G. J.; Yokoyama, J. S.; Katzman, S. M.; Nalls, M. A.; Newman, A. B.; Harris, T. B.; Cesari, M.; Manini, T. M.; Schork, N. J.; Cummings, S. R.; Liu, Y.; Yaffe, K.; Health, A.; Body Composition, S., Mitochondrial DNA sequence associations with dementia and amyloid-β in elderly African Americans. *Neurobiol. Aging* **2014,** *35* (2), 442.e1-442.e4428.

37. Grewal, R.; Haghighi, M.; Huang, S.; Smith, A. G.; Cao, C.; Lin, X.; Lee, D. C.; Teten, N.; Hill, A. M.; Selenica, M.-L. B., Identifying biomarkers of dementia prevalent among amnestic mild cognitively impaired ethnic female patients. *Alzheimers Res Ther* **2016,** *8* (1), 43.

38. Robinson, R. A.; Amin, B.; Guest, P. C., Multiplexing Biomarker Methods, Proteomics and Considerations for Alzheimer's Disease. *Advances in experimental medicine and biology* **2017,** *974*, 21-48.

39. Kiddle, S. J.; Sattlecker, M.; Proitsi, P.; Simmons, A.; Westman, E.; Bazenet, C.; Nelson, S. K.; Williams, S.; Hodges, A.; Johnston, C.; Soininen, H.; Kłoszewska, I.; Mecocci, P.; Tsolaki, M.; Vellas, B.; Newhouse, S.; Lovestone, S.; Dobson, R. J. B., Candidate Blood Proteome Markers of Alzheimer's Disease Onset and Progression: A Systematic Review and Replication Study. *J. Alzheimers Dis.* **2014,** *38*, 515-531.

40. Rehiman, S. H.; Lim, S. M.; Neoh, C. F.; Majeed, A. B. A.; Chin, A. V.; Tan, M. P.; Kamaruzzaman, S. B.; Ramasamy, K., Proteomics as a reliable approach for discovery of blood-based Alzheimer's disease biomarkers: A systematic review and meta-analysis. *Ageing Res Rev* **2020,** *60*, 101066.

41. Thygesen, C.; Larsen, M. R.; Finsen, B., Proteomic signatures of neuroinflammation in Alzheimer's disease, multiple sclerosis and ischemic stroke. *Expert review of proteomics* **2019,** *16* (7), 601-611.

42. Lista, S.; Faltraco, F.; Prvulovic, D.; Hampel, H., Blood and plasma-based proteomic biomarker research in Alzheimer's disease. *Progress in neurobiology* **2013,** *101-102*, 1-17.

43. Htike, T. T.; Mishra, S.; Kumar, S.; Padmanabhan, P.; Gulyás, B., Peripheral Biomarkers for Early Detection of Alzheimer's and Parkinson's Diseases. *Mol. Neurobiol.* **2019,** *56* (3), 2256-2277.

44. Shi, L.; Baird, A. L.; Westwood, S.; Hye, A.; Dobson, R.; Thambisetty, M.; Lovestone, S., A Decade of Blood Biomarkers for Alzheimer's Disease Research: An Evolving Field, Improving Study Designs, and the Challenge of Replication. *J. Alzheimers Dis.* **2018,** *62* (3), 1181-1198.

45. Oeckl, P.; Otto, M., A Review on MS-Based Blood Biomarkers for Alzheimer's Disease. *Neurol Ther* **2019,** *8* (Suppl 2), 113-127.

46. Shi, L.; Westwood, S.; Baird, A. L.; Winchester, L.; Dobricic, V.; Kilpert, F.; Hong, S.; Franke, A.; Hye, A.; Ashton, N. J.; Morgan, A. R.; Bos, I.; Vos, S. J. B.; Buckley, N. J.; Kate, M. T.; Scheltens, P.; Vandenberghe, R.; Gabel, S.; Meersmans, K.; Engelborghs, S.; De Roeck, E. E.; Sleegers, K.; Frisoni, G. B.; Blin, O.; Richardson, J. C.; Bordet, R.; Molinuevo, J. L.; Rami, L.; Wallin, A.; Kettunen, P.; Tsolaki, M.; Verhey, F.; Lleo, A.; Alcolea, D.; Popp, J.; Peyratout, G.; Martinez-Lage, P.; Tainta, M.; Johannsen, P.; Teunissen, C. E.; Freund-Levi, Y.; Frolich, L.;

Legido-Quigley, C.; Barkhof, F.; Blennow, K.; Zetterberg, H.; Baker, S.; Morgan, B. P.; Streffer, J.; Visser, P. J.; Bertram, L.; Lovestone, S.; Nevado-Holgado, A. J., Discovery and validation of plasma proteomic biomarkers relating to brain amyloid burden by SOMAscan assay. *Alzheimers Dement* **2019,** *15* (11), 1478-1488.

47. Kitamura, Y.; Usami, R.; Ichihara, S.; Kida, H.; Satoh, M.; Tomimoto, H.; Murata, M.; Oikawa, S., Plasma protein profiling for potential biomarkers in the early diagnosis of Alzheimer's disease. *Neurological research* **2017,** *39* (3), 231-238.

48. Zhao, X.; Lejnine, S.; Spond, J.; Zhang, C.; Ramaraj, T. C.; Holder, D. J.; Dai, H.; Weiner, R.; Laterza, O. F., A Candidate Plasma Protein Classifier to Identify Alzheimer's Disease. *J. Alzheimers Dis.* **2015,** *43*, 549-563.

49. Muenchhoff, J.; Poljak, A.; Song, F.; Raftery, M.; Brodaty, H.; Duncan, M.; McEvoy, M.; Attia, J.; Schofield, P. W.; Sachdev, P. S., Plasma Protein Profiling of Mild Cognitive Impairment and Alzheimer's Disease Across Two Independent Cohorts. *J. Alzheimers Dis.* **2015,** *43*, 1355-1373.

50. Song, F.; Poljak, A.; Kochan, N. A.; Raftery, M.; Brodaty, H.; Smythe, G. A.; Sachdev, P. S., Plasma protein profiling of Mild Cognitive Impairment and Alzheimer's disease using iTRAQ quantitative proteomics. *Proteome science* **2014,** *12* (1), 5-5.

51. Zabel, M.; Schrag, M.; Mueller, C.; Zhou, W.; Crofton, A.; Petersen, F.; Dickson, A.; Kirsch, W. M., Assessing candidate serum biomarkers for Alzheimer's disease: a longitudinal study. *Journal of Alzheimer's disease : JAD* **2012,** *30* (2), 311-321.

52. Hu, W. T.; Holtzman, D. M.; Fagan, A. M.; Shaw, L. M.; Perrin, R.; Arnold, S. E.; Grossman, M.; Xiong, C.; Craig-Schapiro, R.; Clark, C. M.; Pickering, E.; Kuhn, M.; Chen, Y.; Van Deerlin, V. M.; McCluskey, L.; Elman, L.; Karlawish, J.; Chen-Plotkin, A.; Hurtig, H. I.; Siderowf, A.; Swenson, F.; Lee, V. M. Y.; Morris, J. C.; Trojanowski, J. Q.; Soares, H.; Alzheimer's Disease Neuroimaging, I., Plasma multianalyte profiling in mild cognitive impairment and Alzheimer disease. *Neurology* **2012,** *79* (9), 897-905.

53. Henkel, A. W.; Muller, K.; Lewczuk, P.; Muller, T.; Marcus, K.; Kornhuber, J.; Wiltfang, J., Multidimensional plasma protein separation technique for identification of potential Alzheimer's disease plasma biomarkers: a pilot study. *J Neural Transm (Vienna)* **2012,** *119* (7), 779-88.

54. Bennett, S.; Grant, M.; Creese, A. J.; Mangialasche, F.; Cecchetti, R.; Cooper, H. J.; Mecocci, P.; Aldred, S., Plasma levels of complement 4a protein are increased in Alzheimer's disease. *Alzheimer disease and associated disorders* **2012,** *26* (4), 329-34.

55. Ijsselstijn, L.; Dekker, L. J. M.; Stingl, C.; van der Weiden, M. M.; Hofman, A.; Kros, J. M.; Koudstaal, P. J.; Sillevis Smitt, P. A. E.; Ikram, M. A.; Breteler, M. M. B.; Luider, T. M., Serum Levels of Pregnancy Zone Protein are Elevated in Presymptomatic Alzheimer's Disease. *Journal of Proteome Research* **2011,** *10* (11), 4902-4910.

56. O'Bryant, S. E.; Xiao, G.; Barber, R.; Reisch, J.; Doody, R.; Fairchild, T.; Adams, P.; Waring, S.; Diaz-Arrastia, R., A serum protein-based algorithm for the detection of Alzheimer disease. *Arch. Neurol.* **2010,** *67* (9), 1077-81.

57. Soares, H. D.; Chen, Y.; Sabbagh, M.; Roher, A.; Schrijvers, E.; Breteler, M., Identifying early markers of Alzheimer's disease using quantitative multiplex proteomic immunoassay panels. *Annals of the New York Academy of Sciences* **2009,** *1180*, 56-67.

58. Thambisetty, M.; Hye, A.; Foy, C.; Daly, E.; Glover, A.; Cooper, A.; Simmons, A.; Murphy, D.; Lovestone, S., Proteome-based identification of plasma proteins associated with hippocampal metabolism in early Alzheimer's disease. *Journal of neurology* **2008,** *255* (11), 1712-20.

59. Cutler, P.; Akuffo, E. L.; Bodnar, W. M.; Briggs, D. M.; Davis, J. B.; Debouck, C. M.; Fox, S. M.; Gibson, R. A.; Gormley, D. A.; Holbrook, J. D.; Hunter, A. J.; Kinsey, E. E.; Prinjha, R.; Richardson, J. C.; Roses, A. D.; Smith, M. A.; Tsokanas, N.; Willé, D. R.; Wu, W.; Yates, J. W.; Gloger, I. S., Proteomic identification and early validation of complement 1 inhibitor and pigment epithelium-derived factor: Two novel biomarkers of Alzheimer's disease in human plasma. *PROTEOMICS – Clinical Applications* **2008,** *2* (4), 467-477.

60. Ray, S.; Britschgi, M.; Herbert, C.; Takeda-Uchimura, Y.; Boxer, A.; Blennow, K.; Friedman, L. F.; Galasko, D. R.; Jutel, M.; Karydas, A.; Kaye, J. A.; Leszek, J.; Miller, B. L.; Minthon, L.; Quinn, J. F.; Rabinovici, G. D.; Robinson, W. H.; Sabbagh, M. N.; So, Y. T.; Sparks, D. L.; Tabaton, M.; Tinklenberg, J.; Yesavage, J. A.; Tibshirani, R.; Wyss-Coray, T., Classification and prediction of clinical Alzheimer's diagnosis based on plasma signaling proteins. *Nature medicine* **2007,** *13* (11), 1359-62.

61. Liao, P. C.; Yu, L.; Kuo, C. C.; Lin, C.; Kuo, Y. M., Proteomics analysis of plasma for potential biomarkers in the diagnosis of Alzheimer's disease. *Proteomics. Clinical applications* **2007,** *1* (5), 506-12.

62. German, D. C.; Gurnani, P.; Nandi, A.; Garner, H. R.; Fisher, W.; Diaz-Arrastia, R.; O'Suilleabhain, P.; Rosenblatt, K. P., Serum biomarkers for Alzheimer's disease: proteomic discovery. *Biomed. Pharmacother.* **2007,** *61* (7), 383-9.

63. Liu, H. C.; Hu, C. J.; Chang, J. G.; Sung, S. M.; Lee, L. S.; Yuan, R. Y.; Leu, S. J., Proteomic identification of lower apolipoprotein A-I in Alzheimer's disease. *Dementia and geriatric cognitive disorders* **2006,** *21* (3), 155-61.

64. Hye, A.; Lynham, S.; Thambisetty, M.; Causevic, M.; Campbell, J.; Byers, H. L.; Hooper, C.; Rijsdijk, F.; Tabrizi, S. J.; Banner, S.; Shaw, C. E.; Foy, C.; Poppe, M.; Archer, N.; Hamilton, G.; Powell, J.; Brown, R. G.; Sham, P.; Ward, M.; Lovestone, S., Proteome-based plasma biomarkers for Alzheimer's disease. *Brain : a journal of neurology* **2006,** *129* (Pt 11), 3042-50.

65. Generoso, J. S.; Morales, R.; Barichello, T., Biomarkers in Alzheimer disease: are we there yet? *Revista brasileira de psiquiatria (Sao Paulo, Brazil : 1999)* **2020,** *42* (4), 337-339.

66. El Kadmiri, N.; Said, N.; Slassi, I.; El Moutawakil, B.; Nadifi, S., Biomarkers for Alzheimer Disease: Classical and Novel Candidates' Review. *Neuroscience* **2018,** *370*, 181-190.

67. O'Bryant, S. E.; Mielke, M. M.; Rissman, R. A.; Lista, S.; Vanderstichele, H.; Zetterberg, H.; Lewczuk, P.; Posner, H.; Hall, J.; Johnson, L.; Fong, Y.-L.; Luthman, J.; Jeromin, A.; Batrla-Utermann, R.; Villarreal, A.; Britton, G.; Snyder, P. J.; Henriksen, K.; Grammas, P.; Gupta, V.; Martins, R.; Hampel, H., Blood-based biomarkers in Alzheimer disease: Current state of the science and a novel collaborative paradigm for advancing from discovery to clinic. *Alzheimer's & Dementia* **2017,** *13* (1), 45-58.

68. Guo, L. H.; Alexopoulos, P.; Wagenpfeil, S.; Kurz, A.; Perneczky, R., Plasma proteomics for the identification of Alzheimer disease. *Alzheimer disease and associated disorders* **2013,** *27* (4), 337-42.

69. Shah, D. J.; Rohlfing, F.; Anand, S.; Johnson, W. E.; Alvarez, M. T. B.; Cobell, J.; King, J.; Young, S. A.; Kauwe, J. S. K.; Graves, S. W., Discovery and Subsequent Confirmation of Novel Serum Biomarkers Diagnosing Alzheimer's Disease. *J. Alzheimers Dis.* **2016,** *49*, 317-327.

70. Doecke, J. D.; Laws, S. M.; Faux, N. G.; Wilson, W.; Burnham, S. C.; Lam, C. P.; Mondal, A.; Bedo, J.; Bush, A. I.; Brown, B.; De Ruyck, K.; Ellis, K. A.; Fowler, C.; Gupta, V. B.; Head, R.; Macaulay, S. L.; Pertile, K.; Rowe, C. C.; Rembach, A.; Rodrigues, M.; Rumble, R.; Szoeke, C.; Taddei, K.; Taddei, T.; Trounson, B.; Ames, D.; Masters, C. L.; Martins, R. N., Blood-based protein biomarkers for diagnosis of Alzheimer disease. *Archives of neurology* **2012,** *69* (10), 1318-25.

71. Kim, S.; Nho, K.; Ramanan, V. K.; Lai, D.; Foroud, T. M.; Lane, K.; Murrell, J. R.; Gao, S.; Hall, K. S.; Unverzagt, F. W.; Baiyewu, O.; Ogunniyi, A.; Gureje, O.; Kling, M. A.; Doraiswamy, P. M.; Kaddurah-Daouk, R.; Hendrie, H. C.; Saykin, A. J., Genetic Influences on Plasma Homocysteine Levels in African Americans and Yoruba Nigerians. *Journal of Alzheimer's disease : JAD* **2016,** *49* (4), 991-1003.

72. Wiśniewski, J. R.; Zougman, A.; Nagaraj, N.; Mann, M., Universal sample preparation method for proteome analysis. *Nat Methods* **2009,** *6* (5), 359-62.

73. Plubell, D. L.; Wilmarth, P. A.; Zhao, Y.; Fenton, A. M.; Minnier, J.; Reddy, A. P.; Klimek, J.; Yang, X.; David, L. L.; Pamir, N., Extended Multiplexing of Tandem Mass Tags (TMT) Labeling Reveals Age and High Fat Diet Specific Proteome Changes in Mouse Epididymal Adipose Tissue. *Mol. Cell. Proteomics* **2017,** *16* (5), 873-890.

74. Pascovici, D.; Handler, D. C.; Wu, J. X.; Haynes, P. A., Multiple testing corrections in quantitative proteomics: A useful but blunt tool. *Proteomics* **2016,** *16* (18), 2448-53.

75. Wang, W.; Sue, A. C. H.; Goh, W. W. B., Feature selection in clinical proteomics: with great power comes great reproducibility. *Drug discovery today* **2017,** *22* (6), 912-918.

76. Cao, Z.; Yende, S.; Kellum, J. A.; Robinson, R. A. S., Additions to the Human Plasma Proteome via a Tandem MARS Depletion iTRAQ-Based Workflow. *International journal of proteomics* **2013,** *2013*, 654356-654356.

77. Perez-Riverol, Y.; Csordas, A.; Bai, J.; Bernal-Llinares, M.; Hewapathirana, S.; Kundu, D. J.; Inuganti, A.; Griss, J.; Mayer, G.; Eisenacher, M.; Pérez, E.; Uszkoreit, J.; Pfeuffer, J.; Sachsenberg, T.; Yilmaz, S.; Tiwary, S.; Cox, J.; Audain, E.; Walzer, M.; Jarnuczak, A. F.; Ternent, T.; Brazma, A.; Vizcaíno, J. A., The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.* **2019,** *47* (D1), D442-d450.

78. Dimitriadou, E.; Hornik, K.; Leisch, F.; Meyer, D.; Weingessel, A., E1071: Misc Functions of the Department of Statistics (E1071), TU Wien. **2009**, *1*.

79. Robin, X.; Turck, N.; Hainard, A.; Tiberti, N.; Lisacek, F.; Sanchez, J.-C.; Müller, M., pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics* **2011,** *12* (1), 77.

80. Une, K.; Takei, Y. A.; Tomita, N.; Asamura, T.; Ohrui, T.; Furukawa, K.; Arai, H., Adiponectin in plasma and cerebrospinal fluid in MCI and Alzheimer's disease. *Eur. J. Neurol.* **2011,** *18* (7), 1006-1009.

81. Richens, J. L.; Spencer, H. L.; Butler, M.; Cantlay, F.; Vere, K.-A.; Bajaj, N.; Morgan, K.; O'Shea, P., Rationalising the role of Keratin 9 as a biomarker for Alzheimer's disease. *Sci. Rep.* **2016,** *6* (1), 22962.

82. Chen, M.; Xia, W., Proteomic Profiling of Plasma and Brain Tissue from Alzheimer's Disease Patients Reveals Candidate Network of Plasma Biomarkers. *Journal of Alzheimer's disease : JAD* **2020,** *76* (1), 349-368.

83. Wang, Z.; Liu, F.; Ye, S.; Jiang, P.; Yu, X.; Xu, J.; Du, X.; Ma, L.; Cao, H.; Yuan, C.; Shen, Y.; Lin, F.; Zhang, R.; Li, C., Plasma proteome profiling of high-altitude polycythemia using TMT-based quantitative proteomics approach. *J. Proteomics* **2019,** *194*, 60-69.

84. Wang, T.; Shen, H.; Deng, H.; Pan, H.; He, Q.; Ni, H.; Tao, J.; Liu, S.; Xu, L.; Yao, M., Quantitative proteomic analysis of human plasma using tandem mass tags to identify novel biomarkers for herpes zoster. *J. Proteomics* **2020,** *225*, 103879.

85. Kiebish, M. A.; Cullen, J.; Mishra, P.; Ali, A.; Milliman, E.; Rodrigues, L. O.; Chen, E. Y.; Tolstikov, V.; Zhang, L.; Panagopoulos, K.; Shah, P.; Chen, Y.; Petrovics, G.; Rosner, I. L.; Sesterhenn, I. A.; McLeod, D. G.; Granger, E.; Sarangarajan, R.; Akmaev, V.; Srinivasan, A.; Srivastava, S.; Narain, N. R.; Dobi, A., Multi-omic serum biomarkers for prognosis of disease progression in prostate cancer. *Journal of Translational Medicine* **2020,** *18* (1), 10.

86. Ye, S.; Ma, L.; Zhang, R.; Liu, F.; Jiang, P.; Xu, J.; Cao, H.; Du, X.; Lin, F.; Cheng, L.; Zhou, X.; Shi, Z.; Liu, Y.; Huang, Y.; Wang, Z.; Li, C., Plasma proteomic and autoantibody profiles reveal the proteomic characteristics involved in longevity families in Bama, China. *Clin. Proteomics* **2019,** *16* (1), 22.

87. Shen, N.; Chen, N.; Zhou, X.; Zhao, B.; Huang, R.; Liang, J.; Yang, X.; Chen, M.; Song, Y.; Du, Q., Alterations of the gut microbiome and plasma proteome in Chinese patients with adolescent idiopathic scoliosis. *Bone* **2019,** *120*, 364-370.

88. Lu, J.; Zhang, W.; Zhang, L.; Lou, Y.; Gu, P.; Nei, W.; Qian, J.; Xu, J.; Zhao, X.; Zhong, H.; Wang, H.; Han, B., Plasma Proteomics Analysis for Screening Anlotinib Responders in Non-small Cell Lung Cancer. *Research Square* **2020**.

89. Wang, Z.; Zhang, R.; Liu, F.; Jiang, P.; Xu, J.; Cao, H.; Du, X.; Ma, L.; Lin, F.; Cheng, L.; Zhou, X.; Shi, Z.; Liu, Y.; Huang, Y.; Ye, S.; Li, C., TMT-Based Quantitative Proteomic Analysis Reveals Proteomic Changes Involved in Longevity. *PROTEOMICS – Clinical Applications* **2019,** *13* (4), 1800024.

90. Chen, M.; Xia, W., Proteomic Profiling of Plasma and Brain Tissue from Alzheimer's Disease Patients Reveals Candidate Network of Plasma Biomarkers. *J. Alzheimers Dis.* **2020**.

91. Zhou, B.; Zhou, Z.; Chen, Y.; Deng, H.; Cai, Y.; Rao, X.; Yin, Y.; Rong, L., Plasma proteomics-based identification of novel biomarkers in early gastric cancer. *Clinical Biochemistry* **2020,** *76*, 5-10.

92. Nazeri, A.; Ganjgahi, H.; Roostaei, T.; Nichols, T.; Zarei, M.; Alzheimer's Disease Neuroimaging, I., Imaging proteomics for diagnosis, monitoring and prediction of Alzheimer's disease. *Neuroimage* **2014,** *102 Pt 2* (Pt 2), 657-665.

93. Moya-Alvarado, G.; Gershoni-Emek, N.; Perlson, E.; Bronfman, F. C., Neurodegeneration and Alzheimer's disease (AD). What Can Proteomics Tell Us About the Alzheimer's Brain? *Molecular & cellular proteomics : MCP* **2016,** *15* (2), 409-425.

94. Oeckl, P.; Halbgebauer, S.; Anderl-Straub, S.; Steinacker, P.; Huss, A. M.; Neugebauer, H.; von Arnim, C. A. F.; Diehl-Schmid, J.; Grimmer, T.; Kornhuber, J.; Lewczuk, P.; Danek, A.; Ludolph, A. C.; Otto, M., Glial Fibrillary Acidic Protein in Serum is Increased in Alzheimer's Disease and Correlates with Cognitive Impairment. *J. Alzheimers Dis.* **2019,** *67* (2), 481-488.

95. Zhang, R.; Barker, L.; Pinchev, D.; Marshall, J.; Rasamoelisolo, M.; Smith, C.; Kupchak, P.; Kireeva, I.; Ingratta, L.; Jackowski, G., Mining biomarkers in human sera using proteomic tools. *Proteomics* **2004,** *4* (1), 244-56.

96. McAlister, G. C.; Nusinow, D. P.; Jedrychowski, M. P.; Wühr, M.; Huttlin, E. L.; Erickson, B. K.; Rad, R.; Haas, W.; Gygi, S. P., MultiNotch MS3 Enables Accurate, Sensitive, and Multiplexed Detection of Differential Expression across Cancer Cell Line Proteomes. *Anal. Chem.* **2014,** *86* (14), 7150-7158.

97. Brenes, A.; Hukelmann, J.; Bensaddek, D.; Lamond, A. I., Multibatch TMT Reveals False Positives, Batch Effects and Missing Values. *Molecular & cellular proteomics : MCP* **2019,** *18* (10), 1967-1980.

98. Shen, L.; Liao, L.; Chen, C.; Guo, Y.; Song, D.; Wang, Y.; Chen, Y.; Zhang, K.; Ying, M.; Li, S.; Liu, Q.; Ni, J., Proteomics Analysis of Blood Serums from Alzheimer's Disease Patients Using iTRAQ Labeling Technology. *Journal of Alzheimer's disease : JAD* **2017,** *56* (1), 361-378.

99. Barnes, L. L.; Shah, R. C.; Aggarwal, N. T.; Bennett, D. A.; Schneider, J. A., The Minority Aging Research Study: ongoing efforts to obtain brain donation in African Americans without dementia. *Current Alzheimer research* **2012,** *9* (6), 734-745.

100. Albert, S. M.; Costa, R.; Merchant, C.; Small, S.; Jenders, R. A.; Stern, Y., Hospitalization and Alzheimer's Disease: Results From a Community-Based Study. *The Journals of Gerontology: Series A* **1999,** *54* (5), M267-M271.

101. Jones, R. N., Racial bias in the assessment of cognitive functioning of older adults. *Aging Ment. Health* **2003,** *7* (2), 83-102.

102. Manly, J. J., Advantages and disadvantages of separate norms for African Americans. *Clin. Neuropsychol.* **2005,** *19* (2), 270-5.

103. Manly, J. J.; Jacobs, D. M.; Touradji, P.; Small, S. A.; Stern, Y., Reading level attenuates differences in neuropsychological test performance between African American and White elders. *J. Int. Neuropsychol. Soc.* **2002,** *8* (3), 341-8.

104. Hohman, T. J.; Cooke-Bailey, J. N.; Reitz, C.; Jun, G.; Naj, A.; Beecham, G. W.; Liu, Z.; Carney, R. M.; Vance, J. M.; Cuccaro, M. L.; Rajbhandary, R.; Vardarajan, B. N.; Wang, L.-S.; Valladares, O.; Lin, C.-F.; Larson, E. B.; Graff-Radford, N. R.; Evans, D.; De Jager, P. L.; Crane, P. K.; Buxbaum, J. D.; Murrell, J. R.; Raj, T.; Ertekin-Taner, N.; Logue, M. W.; Baldwin, C. T.; Green, R. C.; Barnes, L. L.; Cantwell, L. B.; Fallin, M. D.; Go, R. C. P.; Griffith, P.; Obisesan, T. O.; Manly, J. J.; Lunetta, K. L.; Kamboh, M. I.; Lopez, O. L.; Bennett, D. A.; Hardy, J.; Hendrie, H. C.; Hall, K. S.; Goate, A. M.; Lang, R.; Byrd, G. S.; Kukull, W. A.; Foroud, T. M.; Farrer, L. A.; Martin, E. R.; Pericak-Vance, M. A.; Schellenberg, G. D.; Mayeux, R.; Haines, J. L.; Thornton-Wells, T. A.; Alzheimer Disease Genetics, C., Global and local ancestry in African-Americans: Implications for Alzheimer's disease risk. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2016,** *12* (3), 233-243.

105. Juster, R. P.; McEwen, B. S.; Lupien, S. J., Allostatic load biomarkers of chronic stress and impact on health and cognition. *Neurosci. Biobehav. Rev.* **2010,** *35* (1), 2-16.

106. Danese, A.; McEwen, B. S., Adverse childhood experiences, allostasis, allostatic load, and age-related disease. *Physiol. Behav.* **2012,** *106* (1), 29-39.

CHAPTER III

Evaluating a Targeted MRM Approach to a Global Untargeted Approach for Lipidomic Analyses of Human Plasma

"This chapter is adopted from published research article: **Khan, MJ, Codreanu, SG, Goyal, S, et al. Evaluating a targeted multiple reaction monitoring approach to global untargeted lipidomic analyses of human plasma. Rapid Commun Mass Spectrom. 2020; 34: e8911**"

3.1. Introduction

Lipidomics is one of the most rapidly developing branches of science over the last decade due to the potential of linking lipids to different human health issues. With the advancement in mass spectrometric instruments over the last few decades, more studies are being conducted focusing on lipids and their role in different disease pathologies, examples of which include diabetes [1-2], obesity [3], cystic fibrosis [4] and many types of cancers.[5-9] Due to the high content of lipids in the brain and central nervous system (CNS), Alzheimer's disease (AD) and many other neurological diseases such as multiple sclerosis, epilepsy, schizophrenia, and Parkinson's disease have been associated with faulty lipid metabolism.[10-14] Numerous lipidomics studies have established a correlation between altered lipid metabolism and AD.[15-19] Although several studies have been conducted over the years, there are still many areas in the field that require improvement. For example, there is a major need for a universal method for the analysis of lipids from various classes.

Targeted and untargeted lipidomics approaches can be utilized to study single or multiple lipid species. In targeted approaches, a known lipid molecule and/or lipid class of interest is selectively chosen for mass spectrometry (MS) analysis, while in an untargeted approach, all extracted lipid compounds from diverse classes are monitored simultaneously in a single MS assay. Relative and absolute quantification [20] can be performed using internal standards in both of these approaches. While targeted approaches have advantages of being highly specific, selective,

and accurate with regards to quantification, these approaches are biased on *a priori* selection of species, leaving many unknowns undetected. On the other hand, untargeted approaches monitor all species in a putatively unbiased manner and have the potential to discover new lipid species that may be indicative of a disease state.

Recently, SCIEX introduced a targeted lipidomics method using a combination of hydrophilic interaction liquid chromatography (HILIC) separation and a multiple reaction monitoring (MRM) based assay to analyze ~1150 different lipids from 19 different classes of lipids.[21] This method has the advantage of being highly specific, with the ability to identify a broad array of lipids with high accuracy and precision and, with streamlined data analysis.[21] The QTRAP 6500+ mass spectrometer offers fast polarity switching between positive and negative ionization modes and high sensitivity at higher acquisition rates. These features in conjunction with HILIC separation of the lipids into their individual classes, makes it easier to assign MRM measurements to individual lipid species within a narrow retention time window.[21]

Here we evaluated the performance of this targeted MRM method against a conventional untargeted approach, using a reversed phase separation in conjunction with the Q Exactive-HF mass spectrometer. The untargeted approach used two separate injections, for both the positive and negative ionization modes, unlike the targeted approach, which utilized polarity switching within the same run. In these studies, we focused our evaluations on (1) the number of lipids identified by both approaches, (2) classes of lipids individually identified by each approach, (3) relative quantification of lipid classes, and (4) the overall ease of data acquisition and data processing. We also benchmarked these findings against previously reported studies.

3.2. Methods

3.2.1. Plasma sample collection

Human plasma samples (N=5) from healthy individuals were collected from the University of Pittsburgh Alzheimer's Disease Research Center (ADRC). Approval for the participation of human subjects was obtained by the Institutional Review Boards of the University of Pittsburgh and Vanderbilt University. Samples were collected between 2000-2015 from cognitively normal individuals. The average age of all the patients at the time of draw was ~75 years and both male and female individuals were included in the study.

3.2.2. Untargeted lipidomics study

3.2.2.1. Lipid extraction

Lipids were extracted using a modified Bligh-Dyer extraction protocol.[22] Briefly, plasma samples (30 μL) were transferred into a borosilicate glass tube followed by addition of 4 mL of solvent A (chloroform/methanol solution (1:1, v/v)) and 2 mL of 50 mM LiCl. The tubes were vortexed for 20 s and centrifuged at 2,700 × g for 10 min. The bottom organic layer containing the lipids was carefully collected, and 2 mL of chloroform was added to the aqueous phase (upper layer) to re-extract the remaining lipids. The sample was vortexed and centrifuged again. The subsequent bottom layer was combined with the previously collected lipids and dried down using centrifugal evaporation. The whole procedure was repeated again with the dried down sample as above except 10 mM LiCl was used instead of 50mM LiCl solution. The lipid layer was collected and dried down before being reconstituted for injection into the instrument.

3.2.2.2. LC – MS/MS analyses

For the untargeted analysis, a reconstitution solution was prepared by adding internal standard solution (Splash Lipidomix® from Avanti), which constituted a mixture of 14 different isotopically labelled lipids (**Table C3.1**) at a 1:5 ratio of standard to solvent A

(chloroform:methanol solution (1:1, v/v).[23-25] The dried down lipids were reconstituted with 100 µL of the reconstitution solution and vortexed to dissolve all the lipids. A quality control (QC) sample was prepared by adding an equal amount of each sample to generate a QC pool mixture. LC-MS/MS analysis was performed on a Vanquish HPLC system (Thermo Fisher Scientific, Bremen, Germany) coupled to an Orbitrap Q Exactive-HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Both positive and negative ionization ESI modes were used for the analysis. In the positive mode, 4 µL of sample was loaded onto a Hypersil Gold C18 3 µm, 2.1 mm × 100 mm column (Thermo Fisher Scientific, Waltham, MA), while in the negative mode 6 µL of sample was injected. RPLC separation was performed at 250 µL/min using solvent A (water with 0.1 % formic acid) and solvent B (isopropanol:acetonitrile:water at 60:36:4 ratio with 0.1% formic acid) with the following gradient: 40-70% B over 5 min, 70-100% B for 10 min, 100% B for 7 min, 90-20% B for 1 min, 20% B for 3 min, 40% B for 4 min for a total gradient time of 30 min. Full MS scans were acquired over a mass range of m/z 100–1500 for both positive and negative ionization modes. The Q Exactive-HF has the capability of performing polarity switching which has been used for untargeted lipidomics experiments previously [26] and could have been used herein. However, in order to ensure comparable sampling across each lipid species, separate injections for positive and negative modes on Q Exactive-HF were preferred. The source parameters were as follows: spray voltage = 3 kV (both positive and negative mode); capillary temperature = 280°C; sheath gas = 30 (positive), 40 (negative); auxiliary gas = 5 (positive), 10 (negative); spare gas = 1; probe heater temperature = 300°C (positive), 400°C (negative); Slens = 40%. The resolution was set to 60,000 with the automatic gain control (AGC) target set at 1×10^6 ions and maximum ion injection (IT) time of 100 ms. The top two most intense precursors were selected for MS/MS. The MS/MS scans were acquired at 15,000 resolution using an isolation

width of 1.5 *m/z*, stepped collision energy (NCE 15, 20, 25), and a dynamic exclusion of 6 s. The AGC target was set at 2×10^5 ions and IT of 100 ms.

3.2.2.3. Data analysis

RAW files were analyzed using Progenesis QI (Non-linear Dynamics, Newcastle, UK) following a previously described process.[27] Briefly, all the data files (both sample and QC) were imported and aligned against a full MS QC pool reference and adduct ions ($[M+Na]^+$, $[M+K]^+$, $[M+Li]^+$, $[M+H]^+$, $[M+H-H_2O]^+$, $[M-H]^-$, $[M-H_2O-H]^-$, $[M+Cl]^-$) were selected for data processing and deconvolution. Peak picking was performed at a minimum threshold of 2.5×10^5 ion intensity. Unique ions (retention time and *m/z* pairs) were grouped (a sum of the abundances of unique ions) using both adduct and isotope deconvolution to generate unique "features" (retention time and *m/z* pairs) representative of each compound. Data were normalized using Progenesis QI to all compounds. Annotations were assigned within Progenesis QI using accurate mass measurements (<5 ppm error), isotope distribution similarity, and manual assessment of fragmentation spectral matching (when applicable) from LipidMaps [28], Lipidblast [29] and Human Metabolome Database (HMDB).[30]

3.2.3. MRM targeted study

3.2.3.1. LC-MS/MS analysis

Plasma samples were extracted using the same protocol as the untargeted study, but prepared on a separate day compared to the untargeted approach, using aliquots from the same sample set. The extracted lipids were reconstituted using ethanol with internal standards (Splash Lipidomix® mix and standard mix made by combining individual standards bought from Avanti) added at different concentrations (**Table C3.1**). The Amide based LC-MS/MS analysis was

performed using an ExionLC™ System, a high-performance (HPLC) system consisting of a binary high pressure mixing gradient pump with a degasser, a thermostated autosampler, and a column oven. Separation was achieved on a Waters XBridge Amide column (4.6 × 150 mm, 3.5 µm). The LC method details are listed below: column temperature was set at 35 ºC, flow rate of 0.7 mL/min, injection volume of 5 µL. The mobile phases were as follows: solvent A (water:acetonitrile) at 5:95 ratio with 1 mM ammonium acetate (adjusteded to pH 8.4) and solvent B (water:acetonitrile) at 50:50 ratio with 1 mM ammonium acetate, pH 8.2 with the following gradient: 0-6% B over 6 min, 6-25% B for 4 min, 25-98% B for 1 min, 98-100% B for 2 min, 100% B for 5.6 min, 100-0.1% B for 0.1 min, 0.1% B for 5.3 min for a total gradient time of 24 min. The SCIEX QTRAP 6500+ System was equipped with an IonDrive™ Turbo V source and was operated in low mass and MRM mode with ESI polarity switching. Source and gas setting were as follow: curtain gas = 35; CAD gas = medium for positive mode and low for negative mode, ion spray voltage = 5.2 kV in positive mode and −4.5 kV in negative mode; temperature= 550 °C, declustering potential (DP) = 60 (positive mode), -80 (negative mode); entrance potential = 10 (Positive mode), -10 (Negative mode). Data processing was performed using SCIEX OS™ software and Microsoft Excel for post data analysis.

3.3. Results and Discussion

3.3.1. Untargeted *vs* Targeted study

3.3.1.1 Lipid identification and class assignment

A general overview of the untargeted and targeted workflows is shown in **Figure 3.1**. To assign confident identification to the lipid classes in the untargeted study, the elution time profiles of the heavy-labeled lipid standard mixture was generated. The sample profile was referenced to

the standards to assign elution order of lipid classes (**Figure 3.2**). Negative ionization mode was observed to be favored by phosphatidylinositol (PI) species, while phosphatidylcholine (PC) and phosphatidylethanolamine (PE) species were more abundant in the positive ionization mode. Assigned annotations and lipid classes were confirmed by matching the MS/MS spectra of the lipid to the expected fragmentation patterns documented in the literature, when available, and also filtering based on the fragmentation score assigned by Progenesis QI for individual lipids.[31-34]

An example MS/MS spectrum for a PC 32:1 is shown in **Figure 3.3a**. For PCs, the most common adducts are either the protonated form $[M+H]^+$ or alkali metal adduct $[M+Na]^+$ or $[M+Li]^+$. Here, we observed the characteristic PC fragment ion peak at *m/z* 184.073, for the $[phosphocholine+H]^+$ species. There are also fragment ions present at *m/z* 695.462 $[M+Na-59]^+$ which corresponds to the loss of a trimethylamine and *m/z* 571.469, which corresponds to $[M+Na-183]^+$, loss of a phosphocholine. Similarly, PE species can form either a protonated adduct $[M+H]^+$ or alkali metal adduct $[M+Na]^+$ or $[M+Li]^+$ and a fragment species at *m/z* 599.503 $[M+H-141]^+$ was observed, corresponding to the loss of phosphoethanolamine (**Figure 3.3b**). For sphingomyelins (SM), we observed $[M+Na]^+$ species and fragment ion peaks owing to the neutral loss of trimethylamine at *m/z* 750.571 $[M+Na-59]^+$, neutral loss of phosphocholine at *m/z* 626.588 $[M+Na-183]^+$, and a fragment ion peak at *m/z* 184.073 for the $[phosphocholine+H]^+$ species (**Figure 3.3c**). Ceramides (Cer) have characteristic intact ion peaks for sphingoid base ions at *m/z* 264.268 and 282.279 and also fragment peaks at *m/z* 502.499 $[M+H-2H_2O]^+$ and *m/z* 520.508 $[M+H-H_2O]^+$, corresponding to neutral water losses (**Figure 3.3d**). Similar strategies for lipid characterization were applied across all detected lipid classes, these include: phosphatidylserine, lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and others. After removing redundant annotations, the most confident annotation is selected based on both the score output

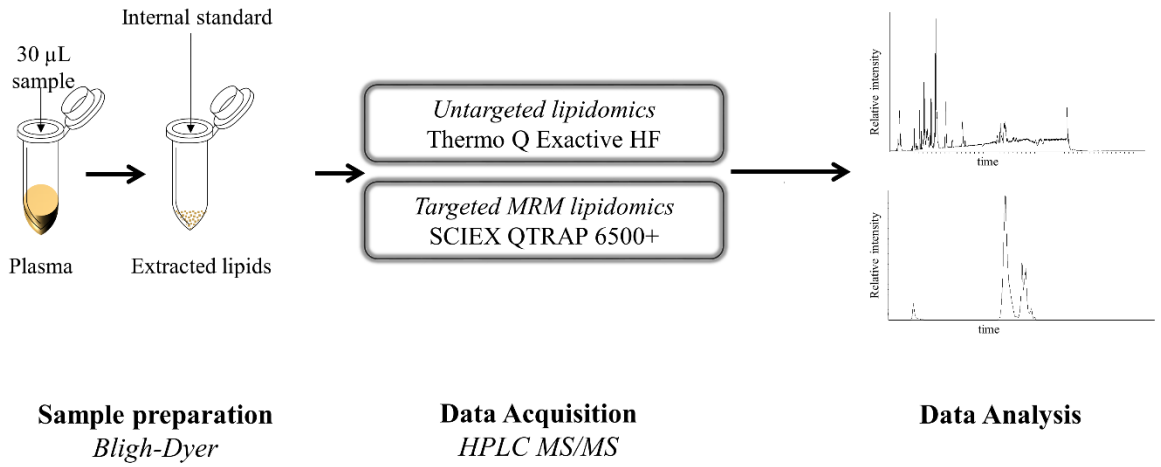

Figure 3.1. Experimental workflow. Lipids were extracted from 30 µL plasma and reconstituted using assay specific solvent with the addition of internal standards. Extracted lipids were analyzed using a Thermo Orbitrap Q Exactive HF and SCIEX QTRAP 6500+ mass spectrometers and the resulting raw files were analyzed using software specific to the approach.

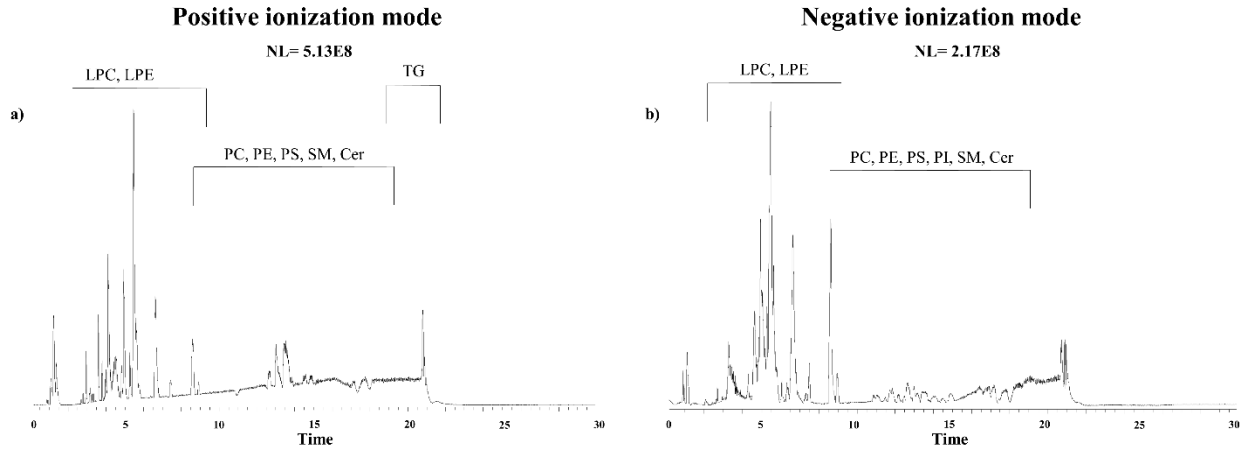

Figure 3.2. Total ion current chromatograms in positive and negative ionization mode showing regions where lipids of different classes elute in an untargeted approach. Lipid class elution in **(a)** positive and **(b)** negative ionization mode. Elution order determined by analyzing internal standard by itself and comparing the elution order to that of the actual samples. (Abbreviations: DG- Diaclyglycerol, LPC- Lysophosphatidylcholine, LPE- Lysophosphatidylethanolamine, PC- Phosphatidylcholine, PE- Phosphatidylethanolamine, PS- Phosphatidylserine, PI- Phosphatidylinositol, SM- Sphingomyelin, Cer- Ceramide, TG- Triacylglycerol)

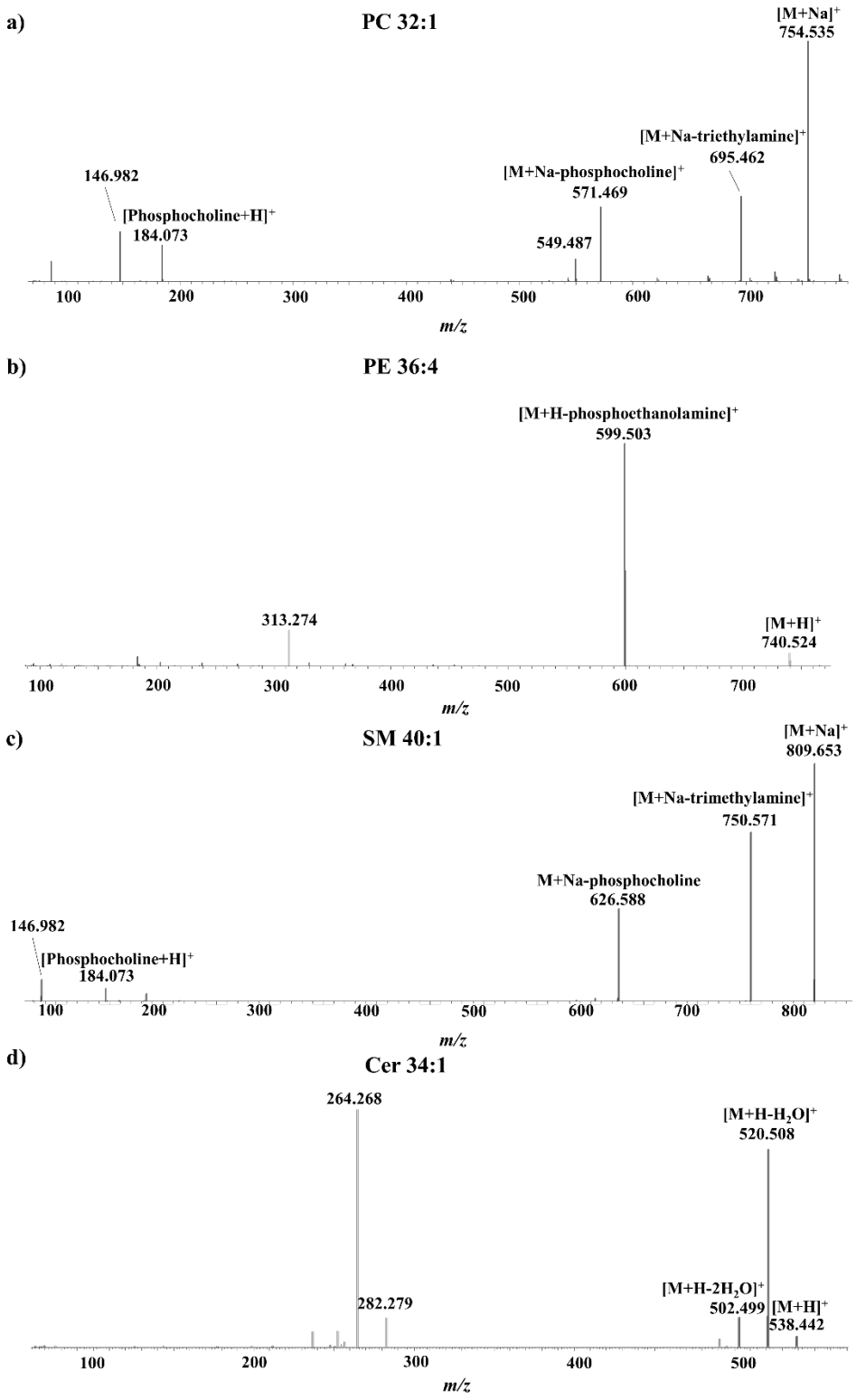

Figure 3.3. MS/MS fragmentation pattern of different lipids. Example MS/MS spectra from positive mode analysis of **(a)** PC 32:1, **(b)** PE 36:1, **(c)** SM 40:1, and **(d)** Cer 34:1 from the Thermo Orbitrap Q Exactive HF.

provided by Progenesis QI and manual curation and verification of the MS/MS spectra available in external databases. In this study, exact mass similarity, isotope similarity, and fragmentation score were used to determine annotation score.

In the targeted lipidomics workflow, a previously established MRM method was used to target a large set of ~1150 lipids using the SCIEX platform.[35-36] The targeted analysis was accomplished using a "Global MRM list" of ~1150 lipids which constitute the most commonly identified lipids in human plasma from 19 different classes of lipids. Details of the MRM list are provided in **Table C3.2**. These lipids were analyzed using both positive and negative ionization modes on the same run using the fast polarity switching mode of the QTRAP 6500+. This method has the advantage of identifying lipids at the molecular level, especially for phospholipids in which instead of using the loss of head group in the positive ionization mode for lipid identification, it uses the loss of fatty acid chains from lipids in the negative ion mode.[21] The samples were analyzed using a ScheduledMRM™ algorithm, which involves performing a series of unscheduled and scheduled analyses by injecting a QC sample, which represents similar complexity of all samples in the experiment. This is accomplished with a HILIC separation which separates the lipids on the basis of head group functionality (**Figure 3.4**) and has recently been shown having better utility in separating lipid classes than reversed-phase separation.[37] For example, TG, Cer and CE elute at t_R 2.3 min; PS, PE, and PC at t_R 9.8 min; LPS, LPI, SM, and LPG at t_R 12.0-13.5 min; LPC, PI, LPE at t_R 13.0 min (**Figure 3.4a**). One of the major benefits using HILIC separation is its high reproducibility in elution times across triplicate injections (3 days) of the individual sample (**Figure 3.4b**). Although, there are differences in the intensities across some injections, these are mostly due to the intensity differences of the patient samples. Also, as the endogenous lipids in the sample are co-eluting with its corresponding internal standard, differences in ionization

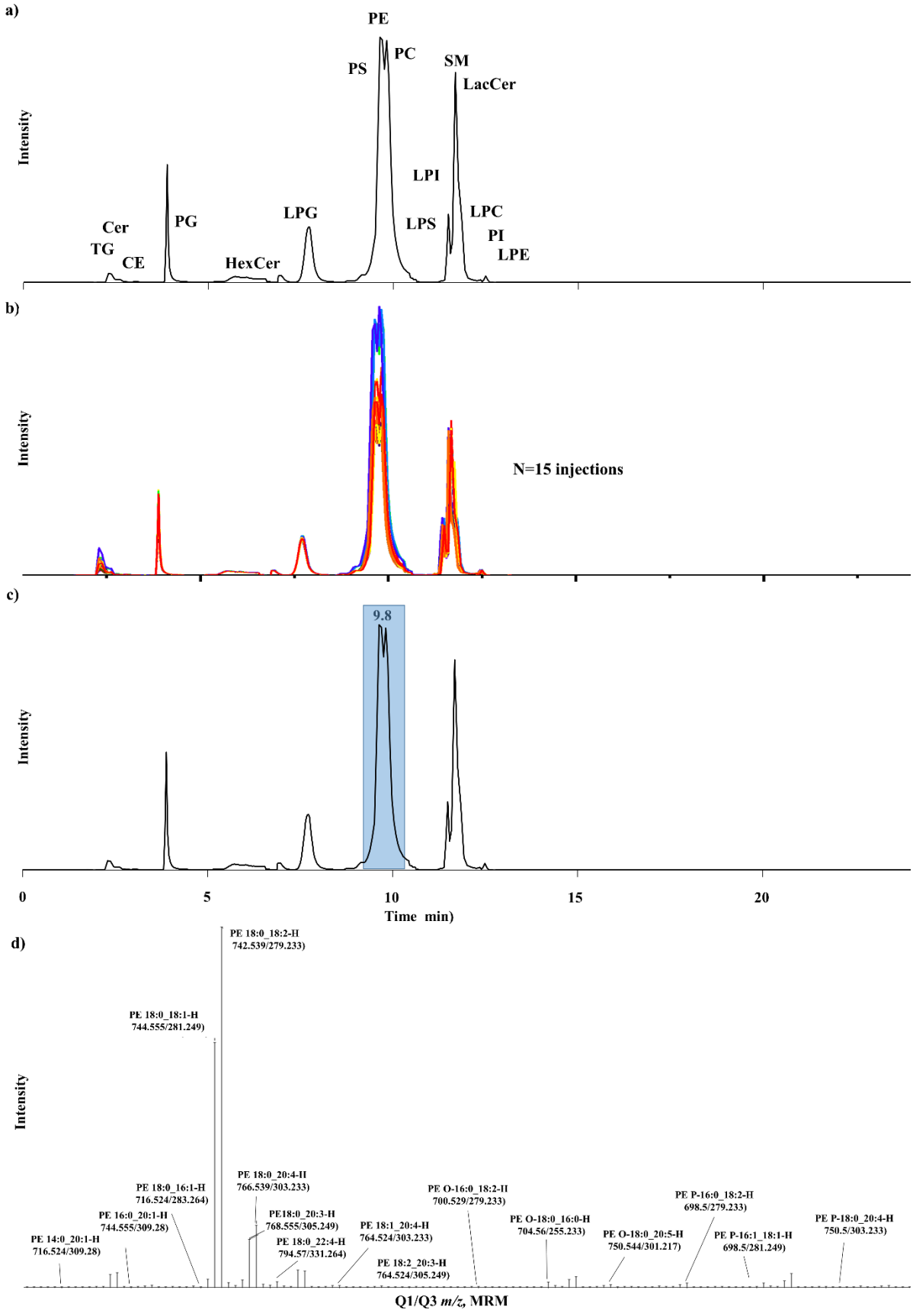
a)
Intensity
PE
PC
PS
SM
LacCer
LPI
LPS
LPC
PI
LPE
Cer
TG
CE
PG
HexCer
LPG
b)
Intensity
N=15 injections
c)
Intensity
9.8
0
5
10
15
20
Time min)
d)
Intensity
PE 18:0_18:2-H
742.539/279.233)
PE 18:0_18:1-H
744.555/281.249)
PE 18:0_20:4-H
766.539/303.233)
PE 18:0_16:1-H
716.524/283.264)
PE 16:0_20:1-H
744.555/309.28)
PE18:0_20:3-H
768.555/305.249)
PE 14:0_20:1-H
716.524/309.28)
PE 18:0_22:4-H
794.57/331.264)
PE 18:1_20:4-H
764.524/303.233)
PE 18:2_20:3-H
764.524/305.249)
PE O-16:0_18:2-H
700.529/279.233)
PE O-18:0_16:0-H
704.56/255.233)
PE O-18:0_20:5-H
750.544/301.217)
PE P-16:0_18:2-H
698.5/279.233)
PE P-16:1_18:1-H
698.5/281.249)
PE P-18:0_20:4-H
750.5/303.233)
Q1/Q3 m/z, MRM

Figure 3.4: Chromatographic profile of lipids from targeted MRM. (a) Total ion chromatogram of all the lipid classes detected, **(b)** overlayed chromatograms across 15 plasma injections, **(c)** total ion chromatograms for all lipid classes with the highlighted portion for PE species which eluted at t_R 9.8 min and **(d)** integrated MS spectra from **(c)** with peaks labeled according to annotations based on the fatty acid masses specific to the species matched from the MRM list with its corresponding Q1 and Q3 masses (listed as Q1/Q3); (Abbreviations: TG- Triacylglycerol, CE- Cholesterol Ester, Cer- Cermaide, HexCer- Hexosylceramide, LacCer- Lactosylceramide, PG- Phosphatidylglycerol, PS- Phosphatidylserine, PE- Phosphatidylethanolamine, PC- Phosphatidylcholine, LPI- Lysophosphatidylinositol, LPS- Lysophosphatidylserine, LPC- Lysophosphatidylcholine, PI- Phosphatidylinositol, LPE- Lysophosphatidylethanolamine).

efficiencies and ion suppression effects can be accounted. After ionization, the lipids are scanned in Q1, fragmented in Q2, and fatty acid specific scans are conducted in Q3. An example is shown in **Figure 3.4c**, in which PE species elute at t_R 9.8 min. Upon completion of fatty acid specific scans and data processing, the peaks at specific *m/z* values are annotated to PE species (**Figure 3.4d**). Similar strategies were used for annotating all the lipid classes in the targeted MRM method. A list of all identified lipids in both approaches is provided in **Table C3.3**. Fatty acid composition of lipids in the untargeted analysis for PC, PE, PI, and TG species is based on fragmentation scores provided by Progenesis QI software, while LipidMaps naming convention was used for lipid annotations.[38]

3.3.1.2. Overall performance evaluation

We evaluated the performance of the untargeted and targeted approaches by comparing the overall variation in number of lipids identified against previous studies performed on human plasma samples.[39-40] Specifically, we compared the coverage of the lipid classes common between the two approaches and also their overlap with previous studies.[39-40] Overall, 297 lipids were annotated using the untargeted approach, while 619 lipids were annotated using the targeted MRM approach from 11 classes of lipids (CE- Cholesterol Ester, Cer- Ceramide DG- Diacylglycerol, LPC- Lysophosphatidylcholine, LPE- Lysophosphatidylethanolamine, PC- Phosphatidylcholine, PE- Phosphatidylethanolamine, PI- Phosphatidylinositol, PS- Phosphatidylserine, SM- Sphingomyelin, TG- Triacylglycerol). In the targeted MRM approach the lipids were annotated based on their individual fatty acid chain information, while in the untargeted study, fatty acid chains were reported as a total number of carbons added together with the corresponding degree of unsaturation, identical to previous LipidMaps [39] and NIST [40] studies. To better compare the two approaches, the lipid annotations in the MRM approach were converted to the same annotations.

In doing so, the total number of annotated lipids was reduced from 619 to 327 lipids. In total, 465 lipids were identified in both approaches, 159 lipids were similar between the two approaches (**Figure 3.5a**), 168 lipids were unique to the targeted MRM approach, and 138 lipids were unique to the untargeted approach. **Figure 3.5b** shows the distribution of lipids classes in both the approaches. A majority of the lipids in the untargeted approach were from PC and PE lipid classes. On the other hand, TG species were the major lipid class in the targeted method as would be expected from plasma samples, along with high numbers of PC and PE species. The number of LPC and LPE species identified in each approach were similar. A comparison of the different lipid classes annotated between the two approaches is shown in **Figure 3.5c**. The targeted approach had a higher number of TG species identified, compared to the untargeted approach, due to lack of ammoniated buffer, while the number of SMs and PCs were higher in the untargeted approach. There were similar numbers of lipids from PE, PI, LPC, and LPE classes of lipids in both approaches, while there were higher numbers of lipids from Cer and PS lipid classes in the targeted approach. As for the common lipids identified between the two approaches, most identified species were from PE, TG, and PC classes (**Figure 3.5d**). PI and PE had similar numbers of unique lipids for both approaches. As for other classes, CE had no unique lipids in untargeted approach, and both LPE and LPC had a higher number of lipids that were common between the two approaches as opposed to those that were unique in each approach (**Figure 3.5d**).

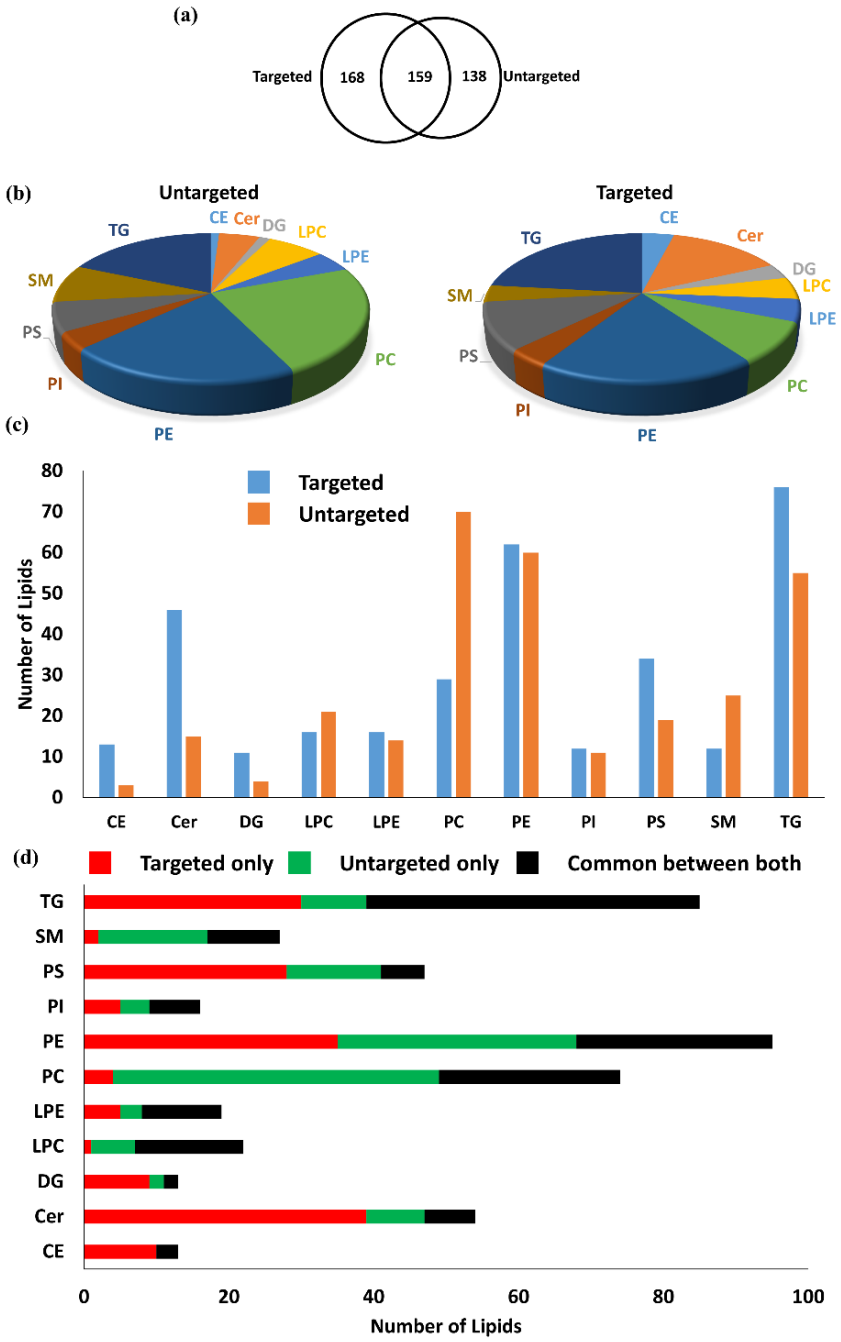

Figure 3.5. Distribution of lipid classes in both approaches. (a) Overlap of the lipid identified between the two approaches; **(b)** pie chart showing the different classes of lipids identified in both of the methods; **(c)** comparison of the number of lipids from different classes in both approaches; **(d)** total number of lipids identified per lipid class in both approaches. (Abbreviations: CE- Cholesterol Ester, Cer- Ceramide, DG- Diacylglycerol, LPC- Lysophosphatidylcholine, LPE- Lysophosphatidylethanolamine, PC- Phosphatidylcholine, PE- Phosphatidylethanolamine, PI- Phosphatidylinositol, PS- Phosphatidylserine, SM- Sphingomyelin, TG- Triacylglycerol).

Our results were compared against previous studies of human plasma and serum by LipidMaps [39] and NIST.[40] Both of these studies are considered benchmarks for estimating concentrations of lipid species from several lipid classes and also report the lipid species and lipid class complexity in these sample types. Although the total number of identified lipids in both of those studies were higher (1527 lipids), especially in the NIST study [40], we focused our comparisons against the most commonly observed lipids in human serum or plasma. Additionally, the lipid annotations herein were reformatted to match that of those studies. For example- in the LipidMaps study, Cer species were reported as a corresponding sphingoid base, this formatting is similar to our targeted approach, but in the NIST study, they were reported as the total number of carbons in the fatty acid chains, similar to the annotations in the untargeted study. Once the necessary conversion was complete, 142 and 191 lipids were common in the untargeted approach when compared to LipidMaps and NIST studies, respectively (**Figure 3.6a&c**). In the targeted MRM approach, 189 and 208 lipids were common with the LipidMaps and NIST study, respectively (**Figure 3.6b&d**). When comparing the numbers for individual lipid classes against the LipidMaps study, higher numbers of Cers, PSs, and DGs were common with the targeted MRM study compared to the untargeted study, while the number of SM species in common were higher in the untargeted study. The remaining classes had similar numbers for both approaches. On the other hand, higher numbers of PC and SM species were common with the NIST study for the untargeted approach, while there were higher number of TG, Cer and PE species common with the NIST study for the targeted MRM approach (**Figure 3.6e-h**).

We further compared the two approaches through evaluation of the inter-day relative abundances and concentrations of lipid species across three days for five individual patient plasma samples. The ratio of the MS signal for a given endogenous lipid to the corresponding spiked-in

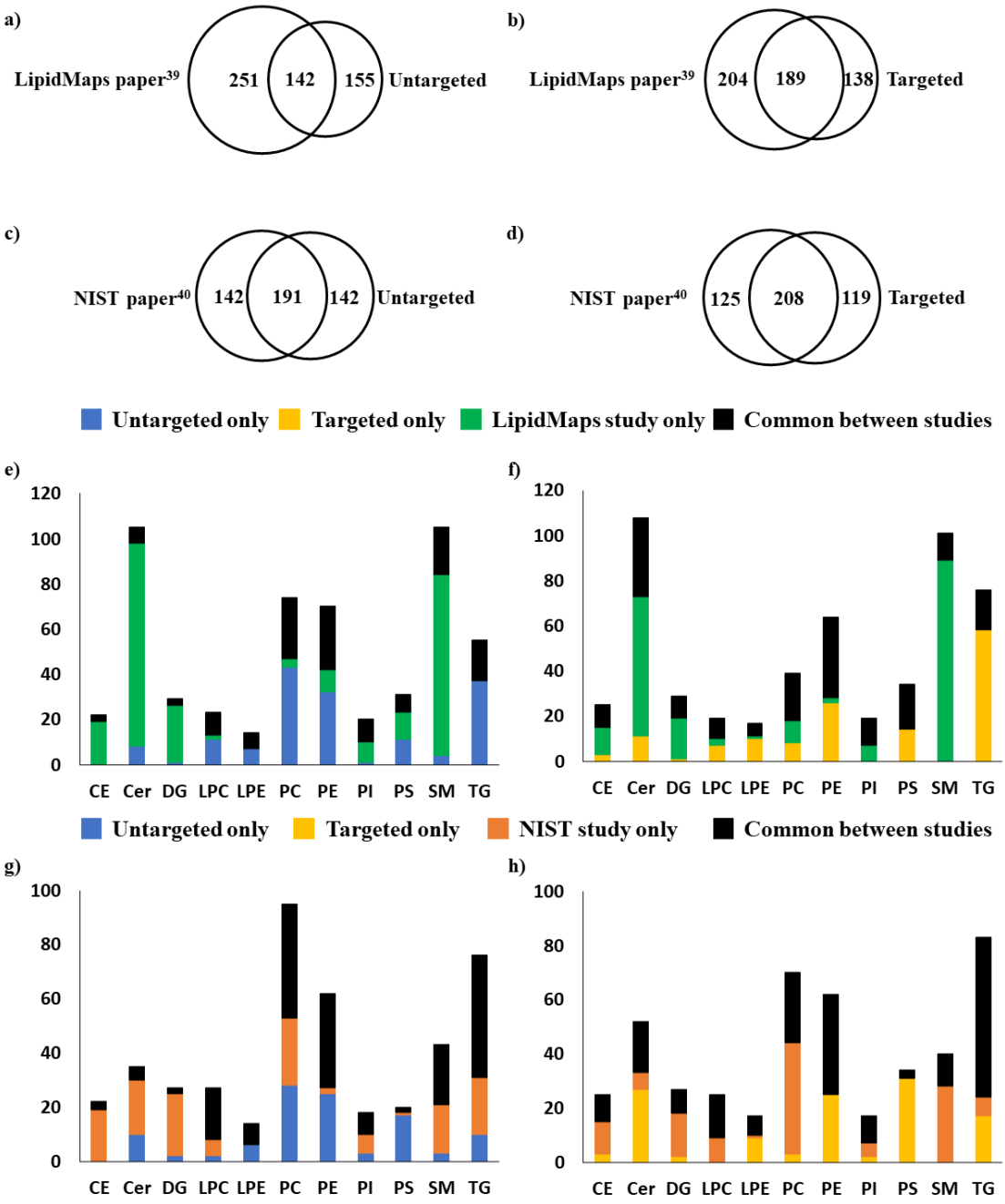

Figure 3.6. Comparison of the lipid classes identified for two approaches against previous studies. Overlap of the lipids identified between **(a)** LipidMaps study and untargeted study, **(b)** LipidMaps study and targeted study, **(c)** NIST study and untargeted study**, (d)** NIST study and targeted study, **(e) & (f)** comparison of lipids identified in the untargeted and targeted approach against the LipidMaps study; **(g) & (h)** comparison of lipids identified in the untargeted and targeted approach against the NIST study**.** (Abbreviations: CE- Cholesterol Ester, Cer- Ceramide, DG- Diacylglycerol, LPC- Lysophosphatidylcholine, LPE- Lysophosphatidylethanolamine, PC- Phosphatidylcholine, PE- Phosphatidylethanolamine, PI- Phosphatidylinositol, PS- Phosphatidylserine, SM- Sphingomyelin, TG- Triacylglycerol).

internal standard in the same lipid class was calculated. Because the sample internal standard mixture was used in both untargeted and targeted approaches, this can help to account for differences related to separation and ionization of lipids. However, we note that matrix effects are complex between the HILIC and RP separation methods and may require more complex normalization approaches. Due to the higher injection amount of the internal standards for some of the classes in the MRM approach, the final ratio values were much smaller, compared to the untargeted approach of the same species. Despite this, the ratios were more consistent for the MRM approach, compared to the untargeted approach. Overall, the lipid classes had a higher percent coefficient of variation (%CV) across the three-day injections for the untargeted approach, compared to the targeted MRM approach. For example, the LPC species had a 17% CV for the untargeted approach, while it was 6.4% CV for the targeted MRM approach. Similarly, the LPE species had a 16% CV and 3% CV for the untargeted and targeted MRM approaches, respectively. Overall, the %CV for the ratio of six most abundant lipid classes (PC, PE, LPC, LPE, TG and SM) was ~9% for the targeted MRM approach whereas, it was ~23% for the untargeted approach. For plasma samples, there is inherent biological variation across patients or in this study healthy volunteers. We assessed inter-day variation in plasma concentrations (nmol/mL of plasma) for both the untargeted and targeted approaches, and show results from four example lipid species across several classes. As the data was collected on patient samples, there was variation in the ratio values across the samples (as can be seen in **Figure 3.7**). This was evident in all the lipid species identified in both approaches. Generally, the plasma concentrations determined from the MRM analyses were more stable across the three days than those determined from the untargeted approach. For example, PC 38:3 had average concentrations of 52.06, 47.2 and 68.1 nmol/mL plasma for Days 1, 2, and 3, respectively, in the untargeted approach whereas for the MRM

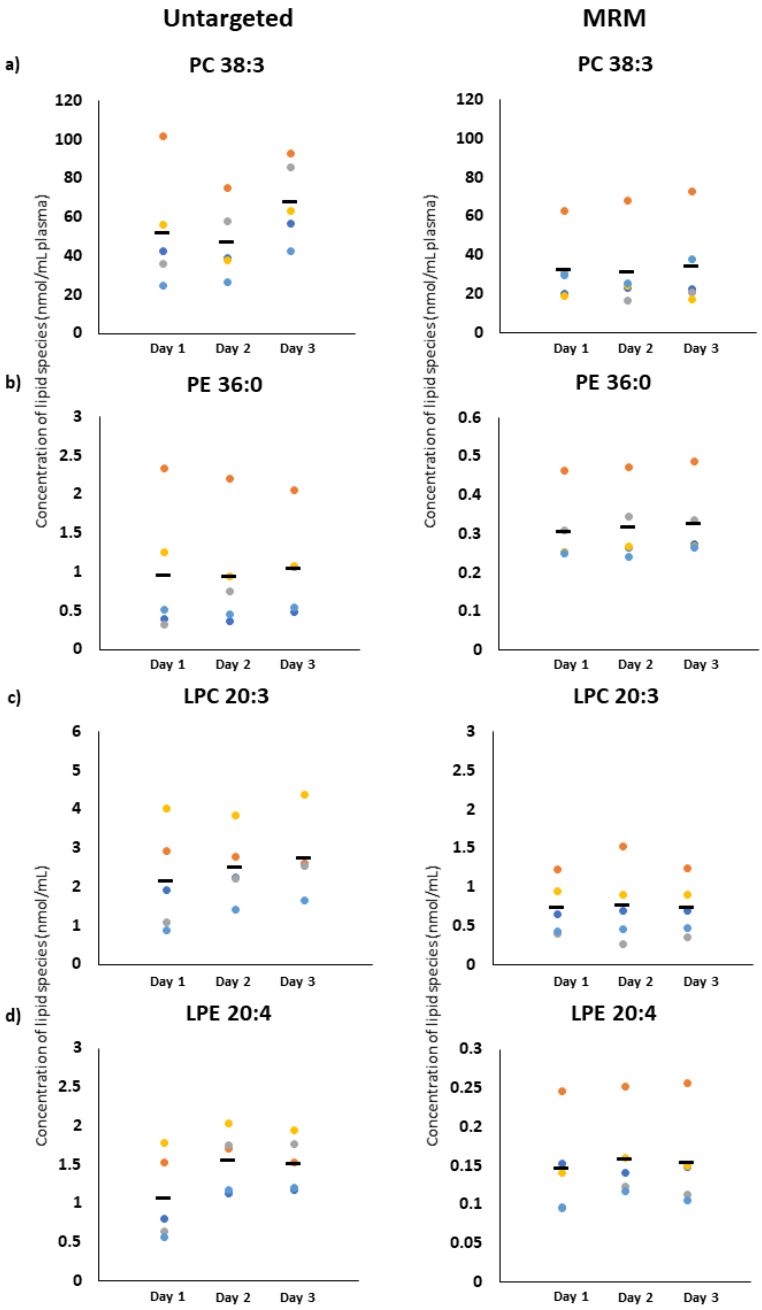

Figure 3.7. Concentrations of lipid species from different lipid classes demonstrating the inter-day variation of patient sample across the three days for both the targeted and untargeted platforms. Example of lipid species from **(a)** Phophotidylcholine (PC), **(b)** Phosphotidylethanolamine, **(c)** Lysophophotidylcholine and **(d)** Lysophosphotidylethanolamine lipid classes. The five different color points represent individual patient sample, while the black point represent the average concentration among all the patient samples for that lipid species for that day.

approach, the concentrations were 32.5, 31.4 and 34.2 nmoL/mL plasma for Days 1, 2, and 3, respectively (**Figure 3.7a**). It should be noted that one patient sample had PC 38:3 concentrations that were noticeably higher than the other four patient samples. Other species such as PE 36:0, LPC 20:4, and LPE 20:3, also had more consistent average concentrations with the MRM approach than the untargeted approach across the three days (**Figure 3.7b-d**). Thus, these results are consistent with the lower %CV values observed overall for the MRM approach.

We also compared the relative total quantities of the six most abundant lipid classes in plasma (**Figure 3.8a**), which was calculated by multiplying the ratio of the abundance of the endogenous species and its corresponding internal standard by the amount of standard injected. In most cases the amounts (ng) were similar in both approaches, except for SM species. In both approaches, LPE had the lowest amount (~1ng) of lipids, while TG had the highest amount (~500-700 ng). We also compared the sum of concentration of lipid species (nmol/mL plasma) in our study against that of the LipidMaps [39] & NIST [40] studies (**Figure 3.8b**) for PC, PE, LPC, LPE, SM, and TG classes. Only the lipid species that were common among all the studies were compared. For LPC and SM classes, all four studies had comparatively similar values. For the PC class the untargeted approach had similar values to the NIST study, while the targeted MRM approach and LipidMaps studies had similar outcomes. On the other hand, there were similarities between the untargeted and LipidMaps study for the PE class, while the targeted MRM approach and NIST study showed similar results. For the LPE class, LipidMaps had higher values. Relative concentrations of lipids common between the untargeted and MRM approach is given in **Table C3.4**.

While the untargeted and targeted MRM approach were able to identify lipids from similar classes and had similar total numbers of observed lipids, the lipid assignments were less specific

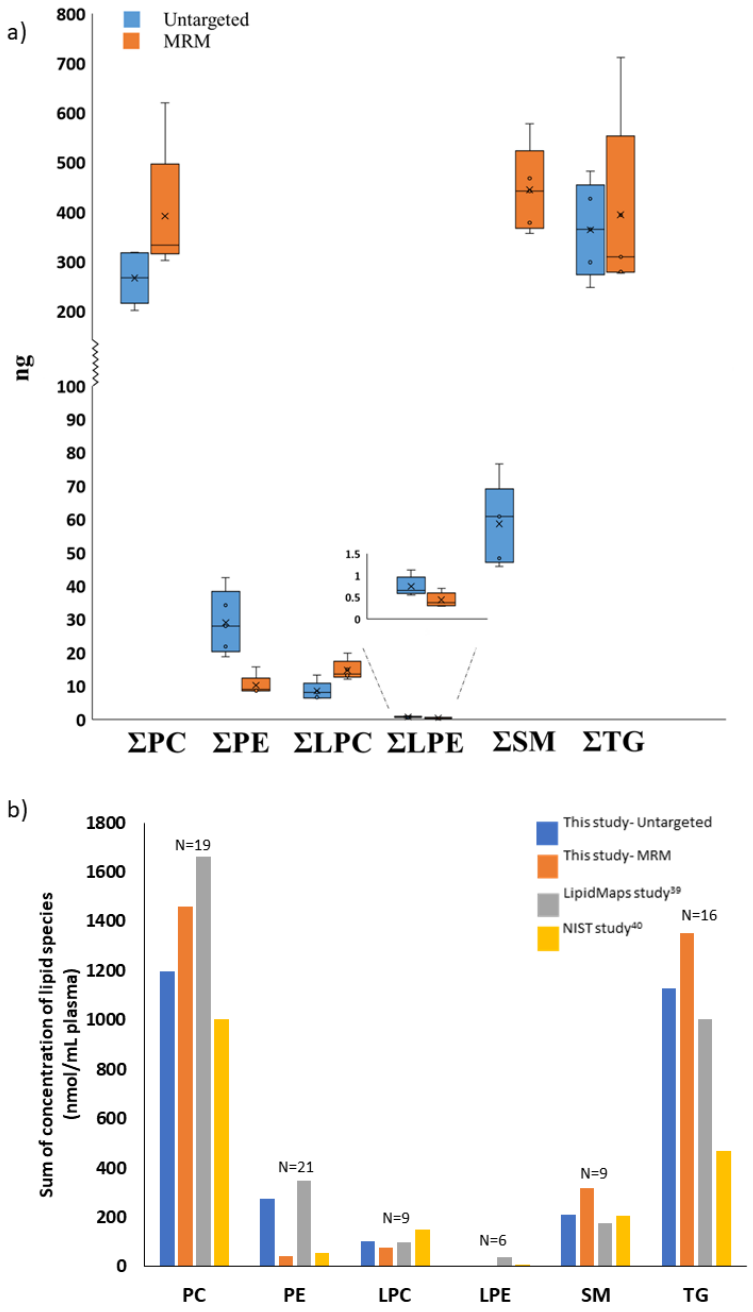

Figure 3.8. Comparison of relative concentration of lipid classes in both MRM and untargeted approaches. a) Box plot showing the sum of relative amount (ng) of lipids compared to the corresponding internal standard for phosphotidylcholine, phosphotidylethanolamine, lysophophotidylcholine, lysophosphotidylethanolamine, sphingomyelin and triacylglyceride classes in both the untargeted and targeted (MRM) approaches**; b)** Bar chart of comparison of the sum of lipid species concentration (nmol/mL plasma) of our study against LipidMaps and NIST studies (N= lipids species common among the studies being compared) for the classes mentioned above.

with the untargeted approach. In this untargeted approach, we were unable to distinguish the individual fatty acid chains. This was expected and could be in part due to limitations in database annotations and searching.[20] For example, in the targeted MRM approach, PC, PE, PI, and PS species were identified up to their individual fatty acid chains, which was unavailable in the case of the untargeted approach, which reports it as a sum of the total carbons in the fatty acid chains. Similarly, in the targeted MRM approach, SM species were assumed to have 18:1 sphingosine backbone due to its higher abundance in human plasma and reports the other fatty acid chain, however, the untargeted approach is able to identify both fatty acid chains. Similarly, TG species in the targeted MRM approach report only one fatty acid chain and the remaining chain as the total number of fatty acids, while the untargeted approach has the capability to identify all three fatty acid chains individually. We also acknowledge that the use of HILIC and reversed phase separation in different approaches could have also influenced the number of lipids identified, especially for the untargeted approach where the number of identified lipids could have increased with the use of longer gradient times. Buffer composition can have an impact on the species detected and we note that a lower number of TG species was identified in the untargeted study although they have been previously reported to be higher in plasma samples. The incorporation of an ammoniated buffer could increase the observation of this lipid class. Despite all the positives in the targeted MRM approach, it does not account for any isotope correction, which is a limitation of the method. Also, the targeted MRM approach has been developed for human plasma samples and its application for other samples require further method development. Furthermore, the untargeted approach can identify more lipid classes in addition to the 11 lipid classes included here. Despite these limitations, the streamlined nature of the targeted MRM data analysis makes this approach very attractive. The major advantage of the targeted MRM method is its capability of reporting

each lipid at its molecular species level, unlike the untargeted method, where the lipids were reported as the sum composition of their respective fatty acids. Additionally, each lipid in the MRM approach has been pre-verified with standards and fragmentation, resulting in higher confidence level for the identifications. Further development could include improvements in assays, such as use of the SelexION capabilities of the QTRAP 6500+ for identification and quantification of lipid species. Also, improvement in lipid annotations, validated identifications, and isotopic corrections are necessary. Data analysis in the targeted MRM approach is much simpler (albeit this is subjective), straight forward, and less time consuming. Overall, these considerations make the targeted MRM approach highly attractive for plasma lipid analysis.

While an ideal study would include a direct comparison of both HILIC and RP separation methods on each MS analyzer used in this study, our focus was to determine general pros and cons of these two entire platforms including different separation methods and MS analyzers. Others have recently reported direct comparisons of NIST human plasma standard on HILIC and RP on the same Q Exactive Plus MS instrument. Those studies provide further evidence that HILIC and RP can yield similar quantification for several lipid classes such as PE, LPE, and SM, however, overestimation of lipid concentrations for LPCs may occur with HILIC.[41] RP offers higher separation power than HILIC, and is the most commonly used method in untargeted lipidomics studies.[42-44] The separation observed using HILIC, having many species in a lipid class co-elute from the column is particularly helpful for the analysis of phospholipids and sphingomyelins [37] and is better for ensuring similar ionization and matrix effects occur when deuterated internal standards are used.[41]

3.4. Conclusions

Overall, both the untargeted Q Exactive-HF and targeted MRM SCIEX QTRAP 6500+ approaches identified similar numbers of lipids across 11 lipid classes from human plasma. The targeted MRM approach had the advantage of identifying lipids at the molecular level with confidence compared to the untargeted approach. Also, the targeted MRM approach had a much lower inter-day variability of lipid abundances and concentrations for the patient samples in comparison to the untargeted approach. Despite these positives, the targeted MRM approach is limited by the number of lipid transitions and lipid classes it can monitor to-date, as it focuses on 1150 lipid transitions. The targeted MRM approach is specific for human plasma samples and would require further method development for other sample types. On the other hand, the untargeted approach identified more unique lipids and also lipids from other classes outside the 11 lipid classes mentioned. This is likely due to the separation power of RP liquid chromatography. In conclusion, the targeted MRM assay developed by SCIEX on the QTRAP 6500+ seems promising for characterizing the lipidome of human plasma samples.

3.5. Acknowledgements

This work was supported by the funding from the Alzheimer's Association (AARGD-17-533405), the Vanderbilt University Start-Up Funds, pilot funds from the University of Pittsburgh Alzheimer Disease Research Center funded by the National Institutes of Health and National Institute on Aging (P50AG005133, RASR), NICHD (R01 HD064727, NAP) and the Vanderbilt Institute of Chemical Biology (fellowship, MJK). This work was supported in part using the resources of the Center for Innovative Technology at Vanderbilt University. Further acknowledgement to SCIEX for their partnership in the analysis of the targeted MRM samples.

3.6. References

1. Suarna, C.; Dean, R. T.; May, J.; Stocker, R., Human atherosclerotic plaque contains both oxidized lipids and relatively large amounts of alpha-tocopherol and ascorbate. *Arterioscler Thromb Vasc Biol* **1995,** *15* (10), 1616-24.

2. Han, X.; Yang, J.; Yang, K.; Zhao, Z.; Abendschein, D. R.; Gross, R. W., Alterations in myocardial cardiolipin content and composition occur at the very earliest stages of diabetes: a shotgun lipidomics study. *Biochemistry* **2007,** *46* (21), 6417-28.

3. Miyoshi, N.; Iwasaki, N.; Tomono, S.; Higashi, T.; Ohshima, H., Occurrence of cytotoxic 9-oxononanoyl secosterol aldehydes in human low-density lipoprotein. *Free Radic Biol Med* **2013,** *60*, 73-9.

4. Ollero, M.; Astarita, G.; Guerrera, I. C.; Sermet-Gaudelus, I.; Trudel, S.; Piomelli, D.; Edelman, A., Plasma lipidomics reveals potential prognostic signatures within a cohort of cystic fibrosis patients. *Journal of lipid research* **2011,** *52* (5), 1011-22.

5. Fhaner, C. J.; Liu, S.; Ji, H.; Simpson, R. J.; Reid, G. E., Comprehensive lipidome profiling of isogenic primary and metastatic colon adenocarcinoma cell lines. *Anal Chem* **2012,** *84* (21), 8917-26.

6. Gorke, R.; Meyer-Base, A.; Wagner, D.; He, H.; Emmett, M. R.; Conrad, C. A., Determining and interpreting correlations in lipidomic networks found in glioblastoma cells. *BMC Syst Biol* **2010,** *4*, 126.

7. Ishikawa, S.; Tateya, I.; Hayasaka, T.; Masaki, N.; Takizawa, Y.; Ohno, S.; Kojima, T.; Kitani, Y.; Kitamura, M.; Hirano, S.; Setou, M.; Ito, J., Increased expression of phosphatidylcholine (16:0/18:1) and (16:0/18:2) in thyroid papillary cancer. *PLoS One* **2012,** *7* (11), e48873.

8. Zhou, X.; Mao, J.; Ai, J.; Deng, Y.; Roth, M. R.; Pound, C.; Henegar, J.; Welti, R.; Bigler, S. A., Identification of plasma lipid biomarkers for prostate cancer by lipidomics and bioinformatics. *PLoS One* **2012,** *7* (11), e48889.

9. Yu, Z.; Chen, H.; Zhu, Y.; Ai, J.; Li, Y.; Gu, W.; Borgia, J. A.; Zhang, J.; Jiang, B.; Chen, W.; Deng, Y., Global lipidomics reveals two plasma lipids as novel biomarkers for the detection of squamous cell lung cancer: A pilot study. *Oncol Lett* **2018,** *16* (1), 761-768.

10. Adibhatla, R. M.; Hatcher, J. F., Role of Lipids in Brain Injury and Diseases. *Future Lipidol* **2007,** *2* (4), 403-422.

11. Di Paolo, G.; Kim, T.-W., Linking lipids to Alzheimer's disease: cholesterol and beyond. *Nature reviews. Neuroscience* **2011,** *12* (5), 284-296.

12. Adibhatla, R. M.; Hatcher, J. F., Altered lipid metabolism in brain injury and disorders. *Sub-cellular biochemistry* **2008,** *49*, 241-268.

13. Liu, Q.; Zhang, J., Lipid metabolism in Alzheimer's disease. *Neuroscience bulletin* **2014,** *30* (2), 331-345.

14. Kolsch, H.; Heun, R.; Jessen, F.; Popp, J.; Hentschel, F.; Maier, W.; Lutjohann, D., Alterations of cholesterol precursor levels in Alzheimer's disease. *Biochimica et biophysica acta* **2010,** *1801* (8), 945-50.

15. Xianlin, H., Lipid Alterations in the Earliest Clinically Recognizable Stage of Alzheimers Disease: Implication of the Role of Lipids in the Pathogenesis of Alzheimers Disease. *Current Alzheimer Research* **2005,** *2* (1), 65-77.

16. Wood, P. L.; Cebak, J. E.; Woltjer, R. L., Diacylglycerols as biomarkers of sustained immune activation in Proteinopathies associated with dementia. *Clinica Chimica Acta* **2018,** *476*, 107-110.

17. Wong, M. W.; Braidy, N.; Poljak, A.; Sachdev, P. S., The application of lipidomics to biomarker research and pathomechanisms in Alzheimer's disease. *Curr Opin Psychiatry* **2017,** *30* (2), 136-144.

18. Stepler, K. E.; Robinson, R. A. S., The potential of 'omics to link lipid metabolism and genetic and comorbidity risk factors of Alzheimer's disease in African Americans. In *Reviews on Biomarker Studies in Psychiatric and Neurodegenerative Disorders* **2019**, 1-28.

19. Wong, M. W.; Braidy, N.; Poljak, A.; Pickford, R.; Thambisetty, M.; Sachdev, P. S., Dysregulation of lipids in Alzheimer's disease and their role as potential biomarkers. *Alzheimer's & Dementia* **2017,** *13* (7), 810-827.

20. Bou Khalil, M.; Hou, W.; Zhou, H.; Elisma, F.; Swayne, L. A.; Blanchard, A. P.; Yao, Z.; Bennett, S. A. L.; Figeys, D., Lipidomics era: Accomplishments and challenges. *Mass Spectrometry Reviews* **2010,** *29* (6), 877-929.

21. Mackenzie Pearson, S. K. K.; Wu, S.; Baker, P. R., Achieve Broad Lipid Quantitation using a High-Throughput Targeted Lipidomics Method **2018**.

22. Wang, M.; Han, X., Advanced Shotgun Lipidomics for Characterization of Altered Lipid Patterns in Neurodegenerative Diseases and Brain Injury. *Methods in molecular biology (Clifton, N.J.)* **2016,** *1303*, 405-22.

23. Bilgin, M.; Markgraf, D. F.; Duchoslav, E.; Knudsen, J.; Jensen, O. N.; de Kroon, A. I.; Ejsing, C. S., Quantitative profiling of PE, MMPE, DMPE, and PC lipid species by multiple precursor ion scanning: a tool for monitoring PE metabolism. *Biochimica et biophysica acta* **2011,** *1811* (12), 1081-9.

24. Ejsing, C. S.; Sampaio, J. L.; Surendranath, V.; Duchoslav, E.; Ekroos, K.; Klemm, R. W.; Simons, K.; Shevchenko, A., Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. *Proceedings of the National Academy of Sciences of the United States of America* **2009,** *106* (7), 2136-41.

25. Reis, A.; Rudnitskaya, A.; Blackburn, G. J.; Mohd Fauzi, N.; Pitt, A. R.; Spickett, C. M., A comparison of five lipid extraction solvent systems for lipidomic studies of human LDL. *Journal of lipid research* **2013,** *54* (7), 1812-24.

26. Breitkopf, S. B.; Ricoult, S. J. H.; Yuan, M.; Xu, Y.; Peake, D. A.; Manning, B. D.; Asara, J. M., A relative quantitative positive/negative ion switching method for untargeted lipidomics via high resolution LC-MS/MS from any biological source. *Metabolomics : Official journal of the Metabolomic Society* **2017,** *13* (3), 30.

27. Gibson, C. L.; Codreanu, S. G.; Schrimpe-Rutledge, A. C.; Retzlaff, C. L.; Wright, J.; Mortlock, D. P.; Sherrod, S. D.; McLean, J. A.; Blakely, R. D., Global untargeted serum metabolomic analyses nominate metabolic pathways responsive to loss of expression of the orphan metallo beta-lactamase, MBLAC1. *Mol Omics* **2018,** *14* (3), 142-155.

28. Sud, M.; Fahy, E.; Cotter, D.; Brown, A.; Dennis, E. A.; Glass, C. K.; Merrill, J. A. H.; Murphy, R. C.; Raetz, C. R. H.; Russell, D. W.; Subramaniam, S., LMSD: LIPID MAPS structure database. *Nucleic Acids Research* **2007,** *35* (suppl_1), D527-D532.

29. Kind, T.; Liu, K.-H.; Lee, D. Y.; DeFelice, B.; Meissen, J. K.; Fiehn, O., LipidBlast in silico tandem mass spectrometry database for lipid identification. *Nature methods* **2013,** *10* (8), 755-758.

30. Wishart, D. S.; Tzur, D.; Knox, C.; Eisner, R.; Guo, A. C.; Young, N.; Cheng, D.; Jewell, K.; Arndt, D.; Sawhney, S.; Fung, C.; Nikolai, L.; Lewis, M.; Coutouly, M. A.; Forsythe, I.; Tang, P.; Shrivastava, S.; Jeroncic, K.; Stothard, P.; Amegbey, G.; Block, D.; Hau, D. D.; Wagner, J.; Miniaci, J.; Clements, M.; Gebremedhin, M.; Guo, N.; Zhang, Y.; Duggan, G. E.; Macinnis, G. D.; Weljie, A. M.; Dowlatabadi, R.; Bamforth, F.; Clive, D.; Greiner, R.; Li, L.; Marrie, T.; Sykes, B. D.; Vogel, H. J.; Querengesser, L., HMDB: the Human Metabolome Database. *Nucleic Acids Res* **2007,** *35* (Database issue), D521-6.

31. Pulfer, M.; Murphy, R. C., Electrospray mass spectrometry of phospholipids. *Mass Spectrometry Reviews* **2003,** *22* (5), 332-364.

32. Hsu, F.-F.; Turk, J., Electrospray ionization with low-energy collisionally activated dissociation tandem mass spectrometry of glycerophospholipids: mechanisms of fragmentation and structural characterization. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* **2009,** *877* (26), 2673-2695.

33. Han, X., Lipidomics: Comprehensive Mass Spectrometry of Lipids. In *Lipidomics: Comprehensive Mass Spectrometry of Lipids*, John Wiley & Sons, Inc, New York, **2016**.

34. Murphy, R. C., *Tandem mass spectrometry of lipids: molecular analysis of complex lipids*. Royal Society of Chemistry **2014**.

35. Wang, J.; Fu, L.; Gorti, S. K. K.; Pearson, M. J.; Wu, S.; Xiong, L.; Baker, P. R., Development of an Amide Column-Based LC-MS/MS Method for High-Throughput Targeted Lipidomics. In *ASMS Conference on Mass Spectrometry and Allied Topics*, San Diego, CA, **2018**.

36. Gorti, S. K. K.; Merkel, D.; Fu, L.; Pearson, M. J.; Ubhi, B. K.; Xiong, L.; Baker, P., HILIC LC-MSMS assay for high-throughput targeted lipdomics analysis. In *Lipidomics Forum*, Dortmund, Germany, **2018**.

37. Ritchie, M.; Mal, M.; Wong, S., UPLC BEH HILIC: The Preferred Separation Chemistry for Targeted Analysis of Phospholipids. *PEs* **2012,** *34*, 1.5-6.

38. Liebisch, G.; Vizcaino, J. A.; Kofeler, H.; Trotzmuller, M.; Griffiths, W. J.; Schmitz, G.; Spener, F.; Wakelam, M. J., Shorthand notation for lipid structures derived from mass spectrometry. *Journal of lipid research* **2013,** *54* (6), 1523-30.

39. Quehenberger, O.; Armando, A. M.; Brown, A. H.; Milne, S. B.; Myers, D. S.; Merrill, A. H.; Bandyopadhyay, S.; Jones, K. N.; Kelly, S.; Shaner, R. L.; Sullards, C. M.; Wang, E.; Murphy, R. C.; Barkley, R. M.; Leiker, T. J.; Raetz, C. R. H.; Guan, Z.; Laird, G. M.; Six, D. A.; Russell, D. W.; McDonald, J. G.; Subramaniam, S.; Fahy, E.; Dennis, E. A., Lipidomics reveals a remarkable diversity of lipids in human plasma. *Journal of lipid research* **2010,** *51* (11), 3299-3305.

40. Bowden, J. A.; Heckert, A.; Ulmer, C. Z.; Jones, C. M.; Koelmel, J. P.; Abdullah, L.; Ahonen, L.; Alnouti, Y.; Armando, A. M.; Asara, J. M.; Bamba, T.; Barr, J. R.; Bergquist, J.; Borchers, C. H.; Brandsma, J.; Breitkopf, S. B.; Cajka, T.; Cazenave-Gassiot, A.; Checa, A.; Cinel, M. A.; Colas, R. A.; Cremers, S.; Dennis, E. A.; Evans, J. E.; Fauland, A.; Fiehn, O.; Gardner, M. S.; Garrett, T. J.; Gotlinger, K. H.; Han, J.; Huang, Y.; Neo, A. H.; Hyötyläinen, T.; Izumi, Y.; Jiang, H.; Jiang, H.; Jiang, J.; Kachman, M.; Kiyonami, R.; Klavins, K.; Klose, C.; Köfeler, H. C.; Kolmert, J.; Koal, T.; Koster, G.; Kuklenyik, Z.; Kurland, I. J.; Leadley, M.; Lin, K.; Maddipati, K. R.; McDougall, D.; Meikle, P. J.; Mellett, N. A.; Monnin, C.; Moseley, M. A.; Nandakumar, R.; Oresic, M.; Patterson, R.; Peake, D.; Pierce, J. S.; Post, M.; Postle, A. D.; Pugh, R.; Qiu, Y.; Quehenberger, O.; Ramrup, P.; Rees, J.; Rembiesa, B.; Reynaud, D.; Roth, M. R.; Sales, S.; Schuhmann, K.; Schwartzman, M. L.; Serhan, C. N.; Shevchenko, A.; Somerville, S. E.; St. John-Williams, L.; Surma, M. A.; Takeda, H.; Thakare, R.; Thompson, J. W.; Torta, F.; Triebl, A.; Trötzmüller, M.; Ubhayasekera, S. J. K.; Vuckovic, D.; Weir, J. M.; Welti, R.; Wenk, M. R.; Wheelock, C. E.; Yao, L.; Yuan, M.; Zhao, X. H.; Zhou, S., Harmonizing lipidomics: NIST interlaboratory comparison exercise for lipidomics using SRM 1950–Metabolites in Frozen Human Plasma. *Journal of lipid research* **2017,** *58* (12), 2275-2288.

41. Lange, M.; Fedorova, M., Evaluation of lipid quantification accuracy using HILIC and RPLC MS on the example of NIST(R) SRM(R) 1950 metabolites in human plasma. *Analytical and bioanalytical chemistry* **2020**.

42. Contrepois, K.; Mahmoudi, S.; Ubhi, B. K.; Papsdorf, K.; Hornburg, D.; Brunet, A.; Snyder, M., Cross-Platform Comparison of Untargeted and Targeted Lipidomics Approaches on Aging Mouse Plasma. *Sci Rep* **2018,** *8* (1), 17747.

43. Cajka, T.; Smilowitz, J. T.; Fiehn, O., Validating Quantitative Untargeted Lipidomics Across Nine Liquid Chromatography–High-Resolution Mass Spectrometry Platforms. *Analytical Chemistry* **2017,** *89* (22), 12360-12368.

44. Breitkopf, S. B.; Ricoult, S. J. H.; Yuan, M.; Xu, Y.; Peake, D. A.; Manning, B. D.; Asara, J. M., A relative quantitative positive/negative ion switching method for untargeted lipidomics via high resolution LC-MS/MS from any biological source. *Metabolomics* **2017,** *13* (3), 30.

CHAPTER IV

Multi-Lipidomics Approaches to Study Alzheimer's Disease

4.1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that causes memory loss and decreases cognitive function to the point that it disrupts daily activity. AD is the sixth leading cause of death in the USA, with 5.8 million people suffering and the number is expected to triple by the year 2050.[1-2] While deaths due to diseases such as heart disease, HIV, and breast cancer have decreased significantly in the last decade, deaths due to AD have increased by 123%.[3] To date, there are no cures available for this disease and the estimated cost per year to treat and provide care-giver support stands at $277 billion.[3]

It is well known that the primary hallmarks of AD are the formation of amyloid beta (Aβ) plaques and hyperphosphorylated tau, leading to the development of neurofibrillary tangles within the brain.[4] The formation of Aβ peptides, either predominant Aβ40 or less abundant Aβ42, from the amyloid precursor protein (APP) by different secretases is influenced by lipids.[5-7] Along with Aβ and hyperphosphorylated tau, the breakdown of the cellular membrane in the brain and altered lipid metabolism in both the brain and the periphery have been demonstrated in AD[8] and are regarded as two of the primary characteristics of neurodegeneration.[9-12] In addition, there has been numerous evidence—both ante and post mortem—of the blood brain barrier being compromised in AD patients[13] which makes plasma well suited for biomarker discovery in AD.

Lipidomics analyses have been used to study various diseases such as diabetes,[14-15] obesity,[16] cystic fibrosis,[17] and many types of cancers.[18-22] Association between dysregulated lipid metabolism and different neurological diseases such as multiple sclerosis, epilepsy, schizophrenia

and Parkinson's disease have been well studied.[23-27] Similar association has been established between faulty lipid metabolism and AD by lipidomics studies.[8, 28-31] Among various phospholipids, the levels of phosphatidylethanolamines (PE), phosphatidylinositols (PI) and plasmalogens (PPE) were observed to decrease, while phosphatidylserine (PS) was significantly increased in AD compared to cognitively normal (CN) individuals.[32-35] However, contradictory results have been reported for phosphatidylcholine (PC) levels with its content reported: unchanged in white and grey matter, increased in temporal, parietal, caudate and cerebellar cortex, and decreased in plasma.[11, 33, 36-37] Despite the conflicting reports, PC deficiency has been considered as a potential biomarker for AD.

Diacylglycerol (DAG) levels were increased in brain, plasma, and serum studies of mild cognitive impairment and AD, and the early increase in DAG levels has also been considered as an early biomarker for AD.[32-33, 38] Several classes of sphingolipids are altered in AD[39-42] and have shown the importance of the role cholesterol and its precursors play in AD both in brain and the periphery.[6, 43] For example, plasma levels of the 24S-hydroxycholesterol and 27-hydroxycholesterol have been observed to be reduced in AD.[44] Several intermediates from the cholesterol biosynthesis pathway,[45] such as lanosterol and desmosterol, are lower in AD sample types.[27, 46-47] Many studies have adopted a targeted approach for a particular class of lipids in the context of AD.[32, 35, 37-38] In contrast, there are fewer reports using untargeted lipidomics in aging[48] and in AD,[33, 39, 49-50] especially for plasma. It is recognized that focusing on broad lipid classes potentially may not suffice to understand prevailing disease mechanisms whereas individual lipid identifications is often more insightful.[51]

Here we describe a multi-lipidomics platform, including both untargeted and targeted MS-based approaches, to obtain a more comprehensive view of peripheral lipid metabolism in AD. For

this, we used plasma samples from a small cohort of clinically diagnosed AD and CN individuals and performed three independent MS-based lipidomics analyses. First, we performed an untargeted lipidomics study and an evaluation of Bligh-Dyer and Folch lipid extractions for their ability to identify lipids across all classes. Secondly, we performed multiple reaction monitoring (MRM) of 1250 of the most common plasma lipids using an MRM approach that is an alternative to the Lipidyzer platform.[48] Advantages and disadvantages of these two approaches have been described in **Chapter III**. Lastly, we performed selected reaction monitoring (SRM) of cholesterol and its precursors. Here, the merits of this multi-lipidomics approach and the findings of plasma lipid and lipid classes that are altered in this AD cohort study will be discussed.

4.2. Methods

4.2.1. Plasma sample collection

Plasma samples (N=5 AD, N=5 CN) were collected from non-Hispanic White participants from the University of Pittsburgh Alzheimer's Disease Research Center (ADRC). Detailed characteristics of the individuals are given in **Table 4.1**. Approval for the participation of human subjects was obtained by the Institutional Review Boards of the University of Pittsburgh and Vanderbilt University. Samples were collected in 2000-2015. The average age of all the individuals at the time of draw was ~75 years and both male and female individuals were included in the study. The Mini-Mental State Examination was performed and disease individuals were clinically diagnosed with mild to moderate dementia at the time of draw according to the National Institute on Aging-Alzheimer's Association and National Alzheimer's Coordinating Center criteria.[52-55]

Methods applied in this study have been described in detail previously[56] and also in **Chapter III, section 3.2.** These are described in brief in the following sections.

Table 4.1. Characteristics of AD and cognitively normal participants.

	AD[b] (N=5)	**Cognitively Normal (N=5)**
Age[a]	76 (69-93)	75 (70-93)
Male	4	1
Female	1	4
Year of blood draw	2009 (2007-2011)	2009 (2000-2015)
MMSE[c], mean	19.2 (14-25)	26.8 (23-30)
APOE4 alleles	3/3 and 3/4	2/3, 3/3 and 3/4
BMI[d], kg/m^2	28.51	28.99
Diabetes, n	0	0[*]
Hypertension, n	1	1[*]
Hypercholesterolemia, n	3	2[*]

Range given in parentheses; Abbreviations: a- average age with range, b- Alzheimer's Disease (AD), c- Mini-mental state examination score (MMSE), d- Body mass index (BMI)
[*] Information not available for N=2 patient samples

4.2.2. Lipid extraction

Lipids were extracted using a modified Bligh-Dyer extraction protocol.[57] Plasma sample (30 µL) was transferred into a borosilicate glass tube followed by addition of 4 mL of solvent A (chloroform/methanol solution (1:1, v/v)) and 2 mL of 50 mM LiCl. The tubes were vortexed and centrifuged. The bottom layer was collected, followed by the addition of 2 mL of chloroform to re-extract any remaining lipids. The sample was vortexed and centrifuged again. The subsequent bottom layer was combined with the previously collected lipids and dried down. The whole procedure was repeated again.

4.2.3 LC – MS/MS analyses

For the untargeted analysis, a reconstitution solution was prepared by adding internal standard solution (Splash® from Avanti) at a 1:5 ratio of standard to solvent A (chloroform:methanol solution (1:1, v/v)) . A quality control (QC) sample was prepared by adding an equal amount of each sample to generate a pooled mixture. LC-MS/MS analysis was performed on a Vanquish UHPLC system (Thermo Fisher Scientific, Bremen, Germany) coupled to an Orbitrap QExactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Both positive and negative ionization ESI modes were used for the analysis. Samples were loaded (4 µL positive mode, 6 µL negative mode) onto a Hypersil Gold C18 3 µm, 2.1 mm × 100 mm column (Thermo Fisher Scientific, Waltham, MA). RPLC separation was performed at 250 µL/min. using solvent A (water with 0.1 % formic acid) and solvent B (isopropanol:acetonitrile:water at 60:36:4 ratio with 0.1% formic acid). The gradient used was as followed: 40-70% B over 5 min, 70-100% B for 10 min, 100% B for 7 min, 90-20% B for 1 min, 20% B for 3 min, 40% B for 4 min. for a total gradient time of 30 min. Full MS scans were acquired over a mass range of *m/z* 100–1500 for

both positive and negative ionization modes. Detailed LC-MS parameters have been provided in **Chapter III, section 3.2.2.2**.

For the targeted MRM analysis, the extracted lipids were reconstituted using ethanol with the Avanti Splash® mix at a 1:10 ratio. LC-MS/MS analysis was performed using an ExionLC™ System, coupled to an SCIEX QTRAP 6500+ mass spectrometer. Separation was achieved on a Waters XBridge Amide column (4.6 × 150 mm, 3.5 µm). at a flow rate of 0.7 mL/min. with an injection volume of 5 µL. The mobile phases were as follows: solvent A (water:acetonitrile) at 5:95 ratio with 1 mM ammonium acetate (adjusted to pH 8.2) and solvent B (water:acetonitrile) at 50:50 ratio with 1 mM ammonium acetate, pH 8.2 with the following gradient: 0-6% B over 6 min, 6-25% B for 4 min, 25-98% B for 1 min, 98-100% B for 2 min, 100% B for 5.6 min, 100-0.1% B for 0.1 min, 0.1% B for 5.3 min. for a total gradient time of 24 min. The SCIEX QTRAP 6500+ System was equipped with an IonDrive™ Turbo V source and was operated in low mass and MRM mode with ESI polarity switching. Detailed LC-MS parameters have been provided in **Chapter III section, 3.2.3.1**.

4.2.4. Data analysis

For the untargeted analysis, RAW files were analyzed using Progenesis QI (Non-linear Dynamics, Newcastle, UK) following a previously described process.[58] Briefly, all the data files (both sample and QC) were imported and aligned against a full MS QC referenced for data processing and deconvolution. Peak picking was performed at a minimum threshold of 2.5×10^5 ion intensity. Unique ions (retention time and *m/z* pairs) were grouped (a sum of the abundances of unique ions) using both adduct and isotope deconvolution to generate unique "features" (retention time and *m/z* pairs) representative of each compound. Data were normalized using Progenesis QI to all compounds and exported to EZ Info (Umetrics Software). Supervised (% of

mean) partial least square for discriminant analysis (PLS-DA) and principal component analysis (PCA) were used to visualize clustering of data groups (all features included) prior to ANOVA analysis. Significantly different lipids were considered those with a p value ≤ 0.05 and fold change of $\geq |1.5|$. Detailed description has been provided in **Chapter III section 3.2.2.3**.

In case of the targeted MRM method, data processing was performed using MultiQuant™ Software for post data acquisition and MarkerView™ Software for statistical analysis**.**

4.2.5. Sterol extraction and analysis

Cholesterol and four cholesterol precursors—7-dehydrocholesterol (7-DHC), 8-dehydrocholesterol (8-DHC), desmosterol (Des) and lanosterol (Lan)—were analyzed with a 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) derivatization method. Analysis of cholesterol and 7-DHC, Des and Lan have been described previously.[59-60] All sterol standards, natural and isotopically labeled, used in this study are available from Kerafast, Inc. (Boston, MA). To a 10 μL aliquot of plasma sample, 0.3 nmol d_7-cholesterol, 0.03 nmol d_7-7-dehydrocholesterol, 0.03 nmol d_7-8-dehydrocholesterol, 0.03 nmol $^{13}C_3$-desmosterol, and 0.03 nmol $^{13}C_3$-lanosterol was added. Folch solution (2:1 chloroform:methanol) was added (400 μL), vortexed, and an equal volume of 0.9% NaCl was added, vortexed, and centrifuged at max speed for 5 min. The organic layer was transferred to HPLC vials (Fisher Scientific 03377B) and dried under vacuum. PTAD solution in methanol (100 μL of 2 mg/mL) was added to each vial and shaken for 30 min. at room temperature. Samples were then analyzed by LC-MS/MS. Briefly, derivatized sterol samples (10 μL injections) were analyzed on a UPLC C18 column (Acquity UPLC BEH C18, 1.7 um, 2.1 mm × 50 mm) with 100% methanol (0.1% v/v acetic acid) mobile phase at a flow rate of 500 μL/min. and runtime of 1.2 min. A Thermo TSQ Quantum Ultra tandem mass spectrometer (ThermoFisher) was used for MS detection, and data were acquired with a Finnigan Xcalibur software package. SRMs of the

PTAD derivatives were acquired in the positive ion mode using atmospheric pressure chemical ionization (APCI). MS parameters were optimized for the 7-DHC-PTAD adduct and were as follows: auxiliary nitrogen gas pressure at 55 psi and sheath gas pressure at 60 psi; discharge current at 22 µA and vaporizer temperature at 342 °C. Collision induced dissociation (CID) was optimized at 12 eV under 1.0 mTorr of argon. The monitored transitions included: 7-DHC 560→365, d_7-7-DHC 567→372, 8-DHC 558→363, d_7-8-DHC 565→370, Des 592→365, Lan 634→602,$^{13}C_3$-Des 595→368, and $^{13}C_3$-Lan 637→605. Cholesterol and d7-cholesterol were monitored using pseudo-SRMs of 369→369 and 376→376, respectively, during the same analytical run. Sterol levels were analytically determined based on response to their respective isotopically labeled internal standard and normalized to plasma sample volume. Statistical analysis was performed using Microsoft Excel.

4.3. Results

4.3.1. Untargeted lipidomics study

4.3.1.1. Developing a robust untargeted workflow

For establishing a robust lipidomics workflow, we first determined the better extraction method between Bligh-Dyer[61] and Folch.[62] We evaluated the number of compounds identified, percent recovery of lipids using each method, and the reproducibility of the methods. We also used a commercial mix of lipids (Splash®, Avanti), which contained 14 deuterated lipids from various classes (**Table D4.1**), and tested for the optimal standard to sample volume ratio (1:5, 1:10, and 1:20) for both extraction methods. This was necessary to check for signal suppression of plasma lipids and to detect standards with appropriate signal. Based on the number of compounds identified, the Bligh-Dyer extraction showed a higher total number of compounds compared to

Folch (*i.e.*, 4336 and 3233 compounds, resulting in 1881 and 1757 lipid identifications respectively, (**Figure 4.1**). A similar number of lipid identifications were also observed in other lipidomic studies previously performed (**Table D4.2**). Folch and Bligh-Dyer methods were extremely reproducible (> 98%) with regards to detecting similar compounds across triplicate technical injections (**Figure 4.1a**). We also optimized the internal standard injection amount and determined that a 1:5 ratio was optimal for all the lipid classes (**Figure 4.1b**). For given individual lipids, the Folch extraction resulted in slightly higher abundances; the Bligh-Dyer resulted in more lipid identifications and was selected for further analyses. A general overview of the multi-lipidomics approach included untargeted and targeted workflows is shown in **Figure 4.2**. Lipid identifications for the untargeted approach were assigned based on the criteria described previously[56] and also in **Chapter III, section 3.3.1.1**.

4.3.1.2. Changes in lipids related to Alzheimer's disease

A small subset of plasma samples from AD and CN individuals were used in this study to monitor changes in lipids due to AD. We detected 3644 and 2258 compounds in positive and negative ionization ESI modes respectively, of which 1984 and 1305 were assigned an annotation (**Table 4.2**). Based on the classifications from the LipidMaps consortium[63], our study observed lipids from seven classes (**Figure 4.3**). Of all the observed lipids, 54% came from the glycerophospholipid (GP) class in positive ionization mode. A majority of the GPs were from phosphatidylcholine and phosphatidylethanolamine subclasses. Among other lipid classes, fatty acyls (FA, 19%) and sterols (ST, 8%) were also observed in the study (**Figure 4.3**). Similar distributions across lipid classes were observed in both ionization modes, however, there were

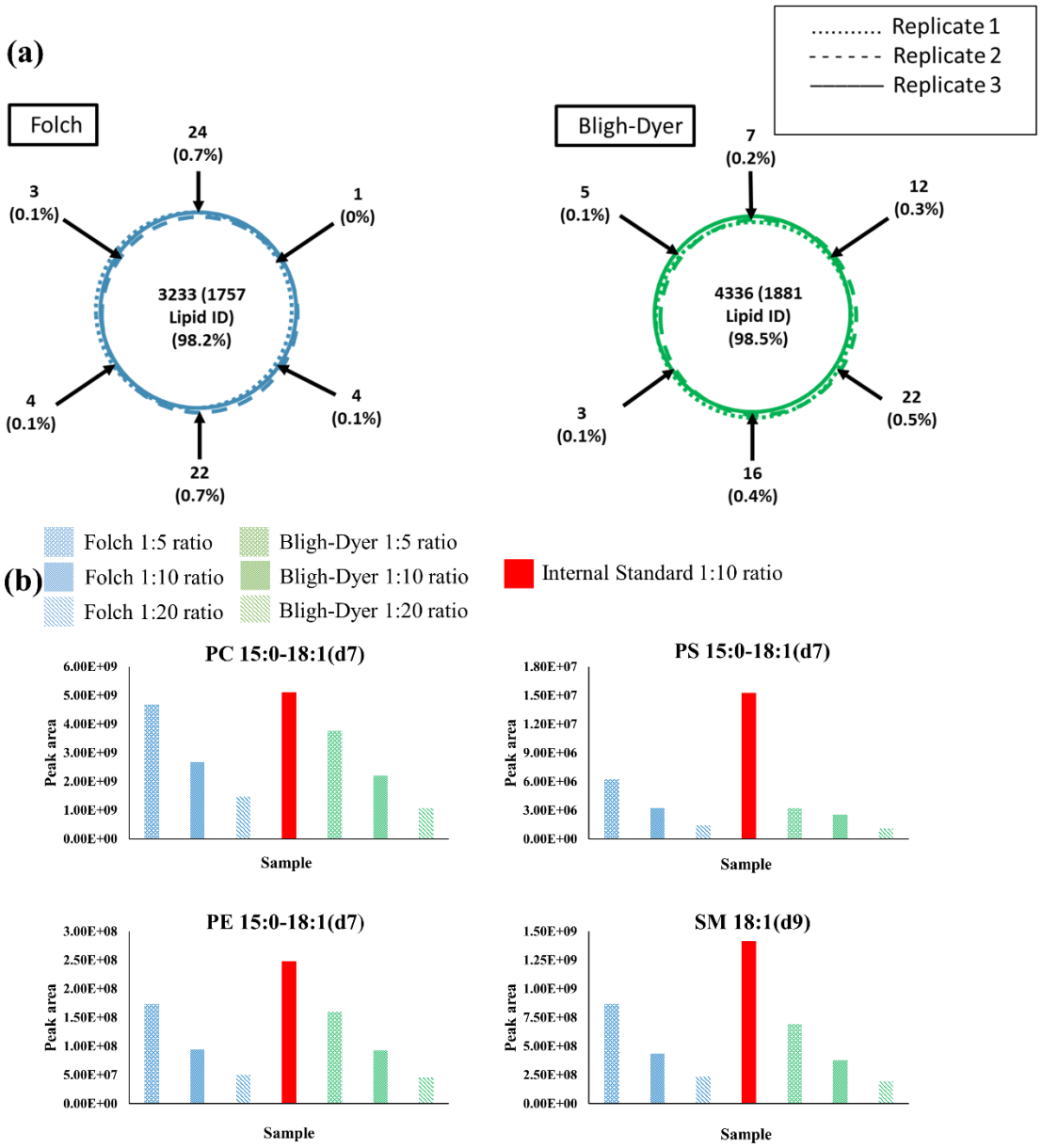

Figure 4.1. Comparison between extraction methods. (a) Lipids were extracted using Bligh-Dyer and Folch extraction methods using three workflow replicates each. The circles represent the number of lipid species detected for a given replicate injection with each method. (**b)** Histogram plots of peak areas for selected example lipids in the QC sample using different ratio of sample:internal standard. The internal standard signal injected alone at a volume similar to that of the 1:10 ratio is shown in red.

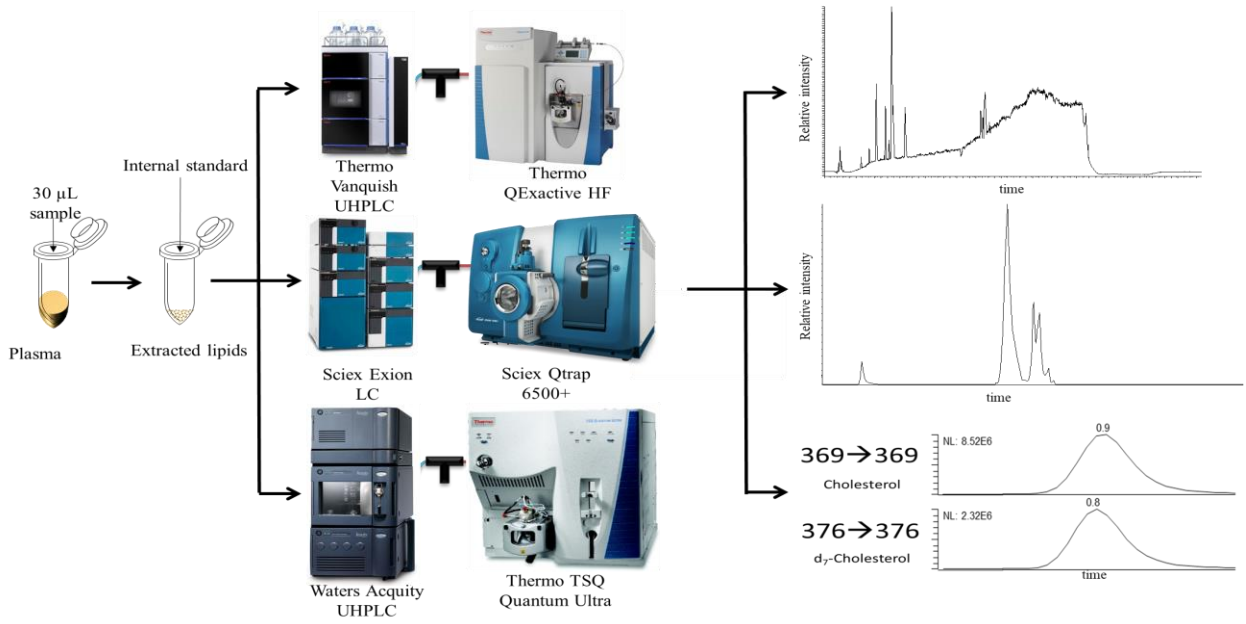

Figure 4.2. Experimental workflow. Lipids were extracted from 30 µL plasma and reconstituted using assay specific solvent with the addition of internal standards. Resulting lipids were analyzed using a Thermo Orbitrap QExactive HF, SCIEX QTRAP 6500+ and Thermo TSQ Quantum Ultra mass spectrometers and the resulting raw files were analyzed using software specific to each approach.

Table 4.2. Number of lipids annotated in untargeted lipidomics analysis.

Ionization mode	Total Compounds*	Assigned identifications
Positive	3644 (105)	1984 (88)
Negative	2258 (122)	1305 (105)

*Numbers given in parenthesis are significantly different lipids with $p \leq 0.05$ and fold change $\geq |1.5|$.

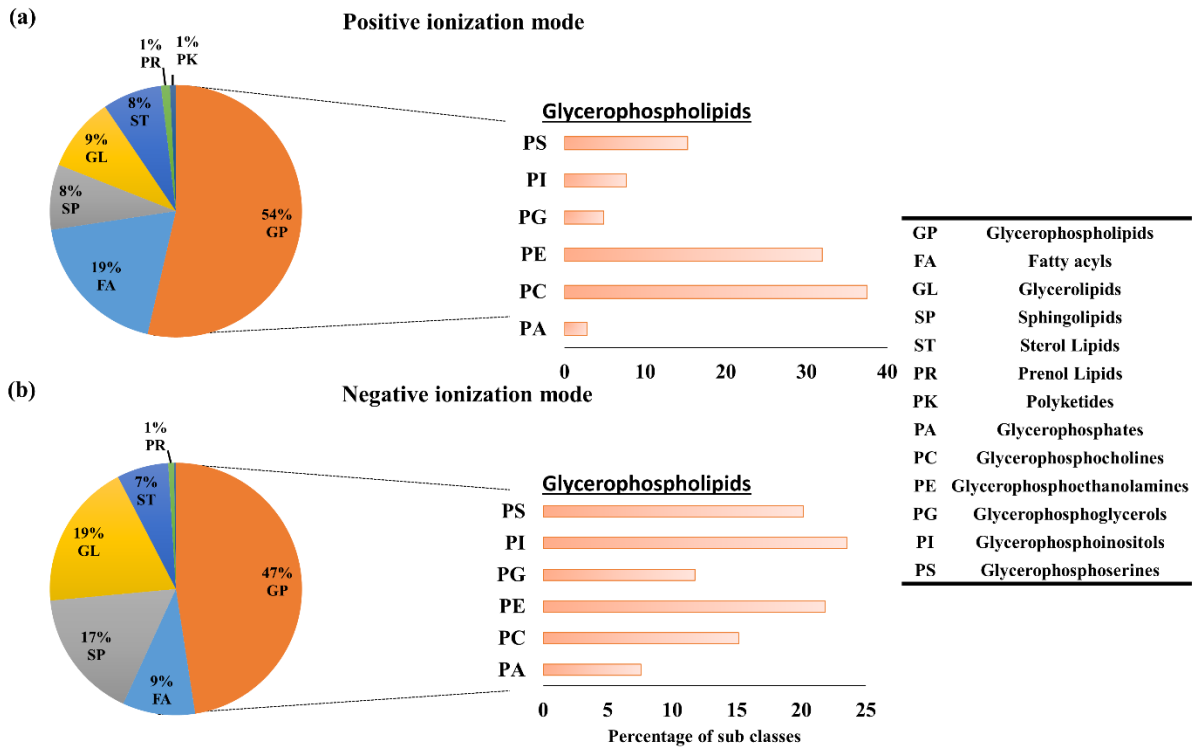

Figure 4.3. Lipid classes identified. Identified lipids were classified according to LipidMaps. Lipid identification was assigned to each lipid based on its score in Progenesis QI and corresponding theoretical fragmentation patterns and separated into classes. The pie chart shows representation of lipid classes for **(a)** positive and **(b)** negative ionization mode of the lipids identified and the bar chart shows percentages of different subclasses of glycerophospholipids. The legend provides definitions of abbreviations.

more sphingolipids (SP, 17%) and glycerolipids (GL, 19%) and less fatty acyls (FA, 9%) for negative ion mode. Higher abundances of glycerophosphoinositols (PI, 23%), glyerophosphoglycerols (PG, 12%), and glycerophosphates (GP, ~8%) were observed in negative compared to positive ionization modes.

The supervised PLS-DA of AD and CN groups shown in **Figures 4.4a & b** demonstrate two distinct study groups in both ionization modes. We note that triplicate injections of each sample were analyzed on three different days. The data reflects this clustering of the groups and indicates slight variations or differences in inter-day analyses. During these analyses, we observed solvent evaporation, which may affect measurement of lipids in the sample and has an impact on inter-day variations in lipid signal. Over 200 lipid compounds were statistically significant (*p* value ≤ 0.05 and fold change $\geq |1.5|$) in AD vs CN sample types (**Figure 4.4**). Lipids that are lower in AD, are 47 and 25 in positive and negative ionization mode respectively, while lipids that were higher in AD, are 58 and 97 positive and negative ionization modes, respectively. In this study, the GP lipid class was observed to have the most lipids with statistically significant changes in AD for both positive and negative ionization modes. There were also significant changes to several of the sphingolipids (SP), fatty acids (FA), and sterols (ST). Selected changes for individual lipids are provided in **Figure 4.5**.

4.3.1.3. Changes in glycerophospholipids

Among various GPs, PCs showed significant reduction in AD compared to CN in this study. For example, PC 32:1 (*p=0.01*), PC 32:2 (*p=0.03*), PC 34:4 (*p=0.04*) & PC 36:6 (*p=0.002*) were 1.8, 1.6, 1.5 and 2.2x lower in AD individuals (**Figure 4.5a**), respectively. On the other hand, plasmalogen and other PEs (e.g., PE 36:4 (*p= 0.01*), PE-P 36:2 (*p=0.02*), were increased in AD (**Figure 4.5b**), except for PE 26:1 (*p=0.003*, data not shown), which was reduced in AD.

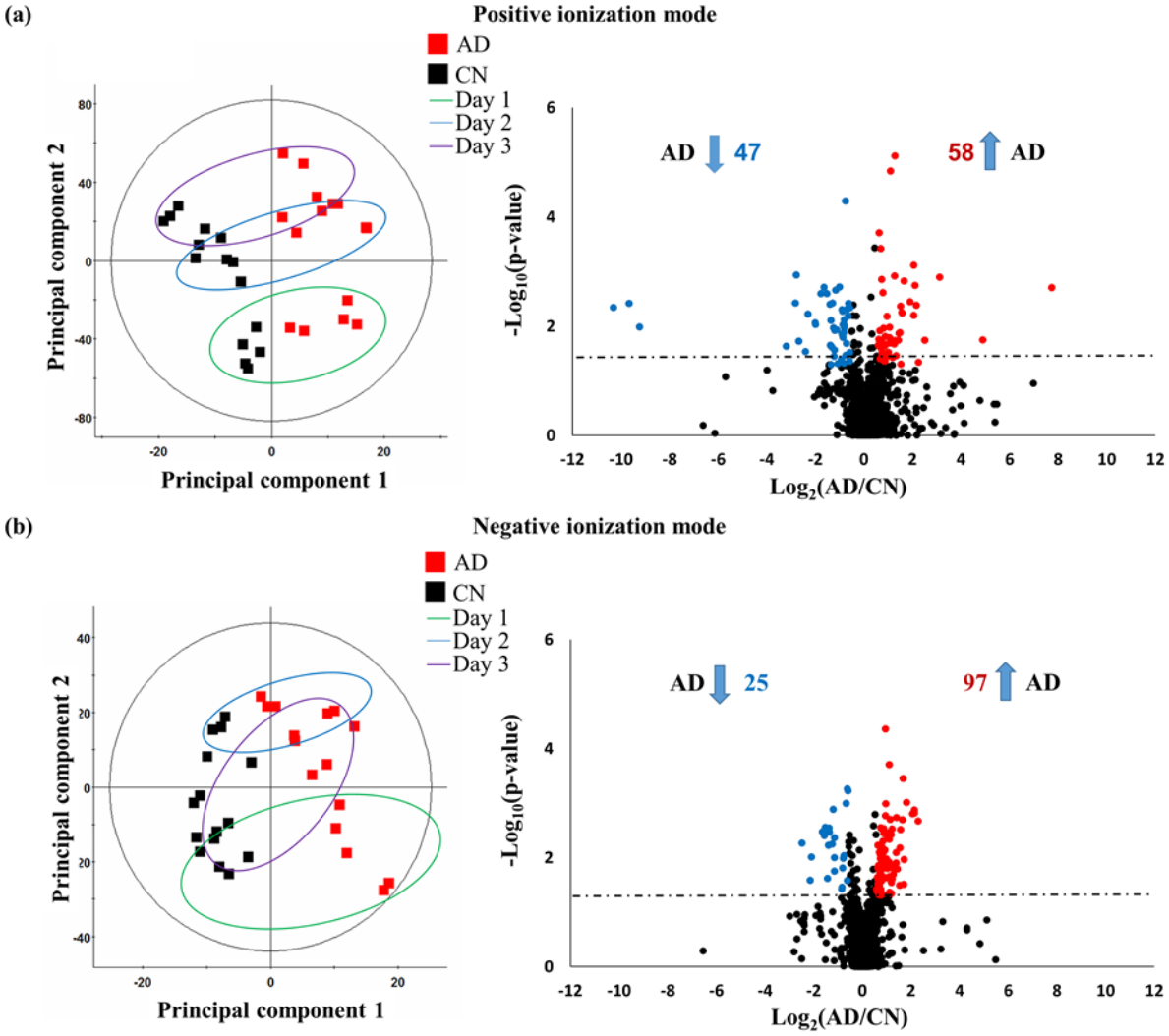

Figure 4.4: Statistical analyses of the findings from the untargeted approach. PLS-DA of lipids across CN and AD groups and volcano plots of lipids from **(a)** positive and **(b)** negative ionization mode. Each sample was run in triplicate across three separate days, with the days denoted by circles on the PLS-DA plots. On the volcano plots, lipids that were increased in AD are in red and those decreased in blue. A *p*-value of 0.05 is denoted by the dashed line.

A majority of PSs such as PS 38:5 (*p=0.01*), and different lyso variants (LPC, LPE, LPS), were also increased in AD. For example, LPC 18:1 (*p=0.04*) and LPE 22:4 (*p=0.03*) both were increased in AD by 3x, while LysoSM 18:1 (*p=0.001*) showed an approximate ninefold increase in AD compared to CN (**Figure 4.5c**).

4.3.1.4. Changes in sphingolipids and glycerolipids

Among SPs, Cer and SM levels were significantly increased and decreased in AD, respectively. For example, Cer d34:1 (*p=0.001*) and LacCer 34:1 (*p=0.01*) both were 1.6x increased in AD, while SM 42:1 (*p=0.009*) and SM 42:2 (*p=0.01*) were 4 and 1.5x reduced in AD, respectively (**Figure 4.5d**). Among glycerolipids (GL), both monoacylglycerides (MG) and triacylglyceride (TG) were increased in AD.

4.3.1.5. Changes in other lipid classes

Many other lipid classes such as fatty acids (octadecanoids), bile acids, and STs (*e.g.* secosteroids) had contradictory results for individual lipids although the overall class was generally higher in AD (**Figure 4.6**). The annotations for the lipids that were statistically significant between the sample types is given in **Table D4.3** along with the corresponding fold-change values of the lipids in AD compared to CN.

4.3.2. MRM targeted study

4.3.2.1. Identification of lipids

Here we used MRM to target a large set of lipids established previously with bovine heart extract on the SCIEX platform,[64-65] which has been described in detail previously.[56] (**Chapter III, section 3.3.1.1**.)

4.3.2.2. Changes in lipids related to AD

In the targeted MRM study, we identified 1098 molecular species from the list of 1250 lipids, which come from four separate lipid classes: GP, GL, SP and ST. Approximately, 47% and 46% of the total lipids were from GL and GP classes, respectively. We identified 155 lipids that were statistically significant in AD compared to CN plasma samples, of which 75 were higher and 80 were lower in AD, respectively (**Table D4.4**).

4.3.2.3. Changes in glycerophospholipids

PCs showed significant reduction in AD compared to CN, consistent with our untargeted analysis (**Figure D4.2**). There were mixed changes for other classes such as PE, PG, PS, and PI species when considering individual lipid species; however, as a lipid class (GP) these were overall increased in AD. Among the PE species, the plasmalogens were increased in AD, except for PE-P 18:1_20:5. The PGs were also higher in AD, except for PG 18:2_22:5 ($p=0.008$). Out of 13 PIs that were significantly different in AD, nine were reduced in AD, while PI 14:0_18:1 ($p=0.014$), PI 14:0_18:2 ($p=0.022$), PI 16:0_14:0 ($p=0.021$) and PI 16:0_16:0 ($p=0.05$) were increased in AD by 7.5, 2.4, 1.9, and 1.6x, respectively. Similar trends were also visible for PS species. Among the lyso variants, LPC, LPE, LPG were increased in AD, except for LPG 16:1 (p=0.008) decreased by 1.8x in AD. Lyso variants were generally increased in AD from the untargeted study, showing consistency between the approaches.

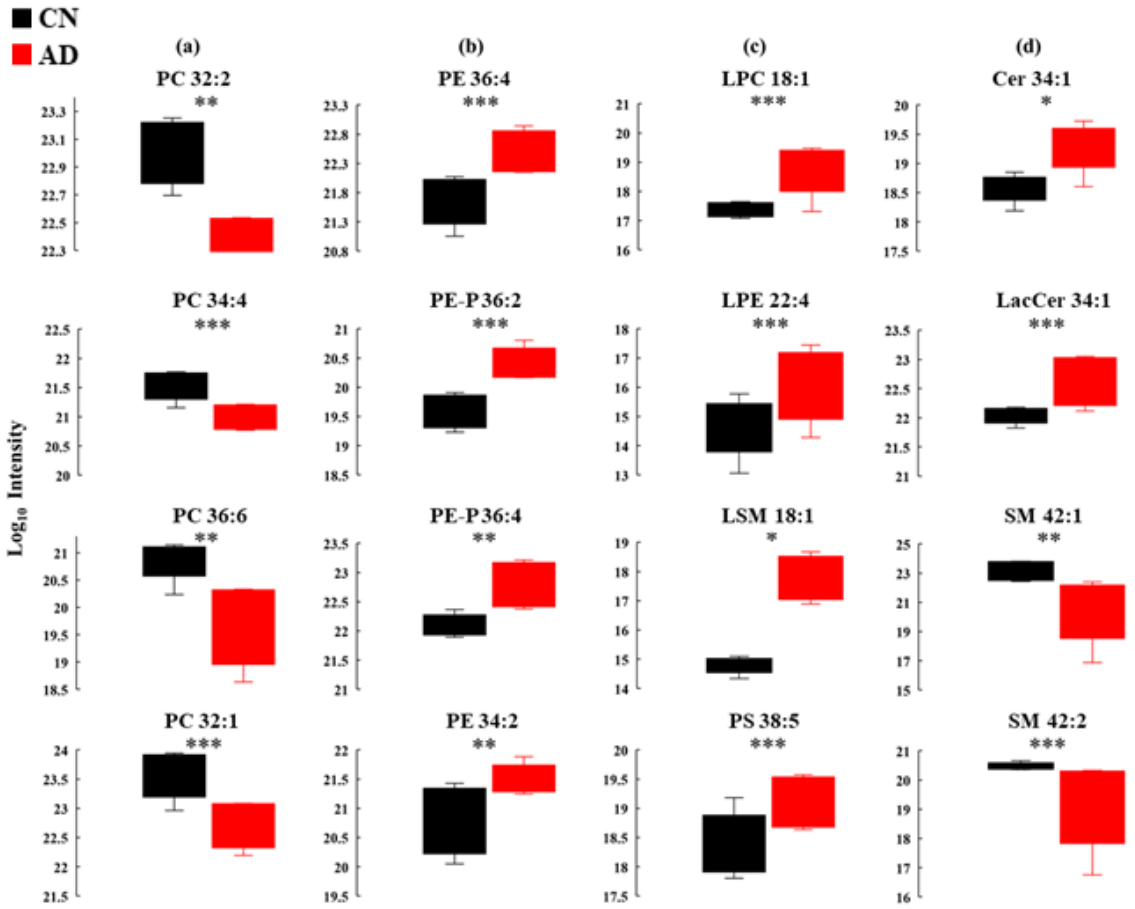

Figure 4.5: Box plots of selected lipids with assigned identifications. Changes from CN (black) and AD (red) in **(a)** phosphatidylcholine (PC), **(b)** phosphatidylethanolamine (PE), **(c)** lyso variants, and **(d)** sphingomyelins (SM) as noted in the figure headers. *p< 0.001, **p< 0.01, ***p< 0.05

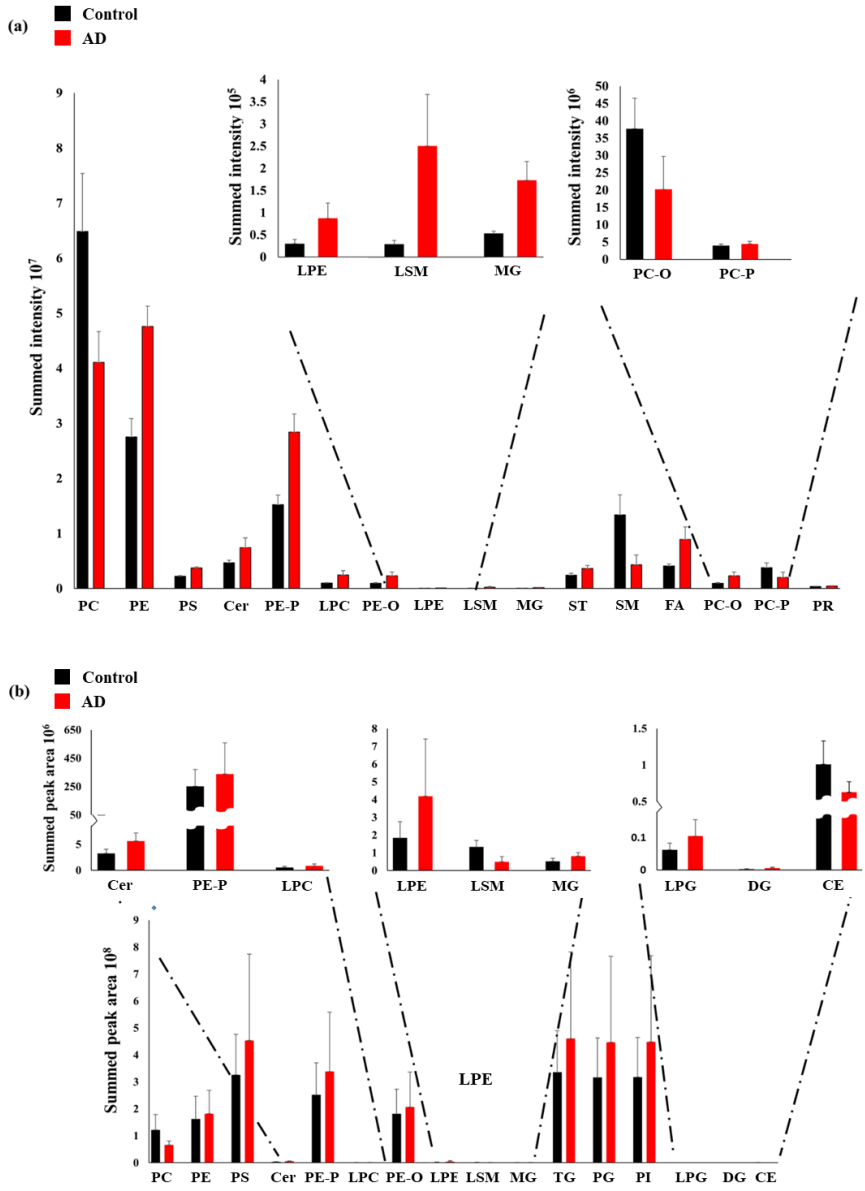

Figure 4.6. Total changes in lipid classes due to AD using both approaches. (a) Bar chart of summed intensities and **(b)** bar chart of summed total peak area of lipids in a given class across triplicate injections of N=5 per group for the untargeted and targeted MRM approach respectively. Zoomed regions are shown for lower intensity classes. CN are shown in black and AD are shown in red. Error bars represent standard error of the mean. (Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Cer, ceramide; PE-P, alkenylphosphatidylethanolamine (plasmalogen); LPC, lysophosphatidylcholine; PE-O, alkylphosphatidylethanolamine; LPE, lysophosphatidylethanolamine; LSM, lysosphingomyelin; MG, monoacylglycerol; TG, tricylglycerol; ST, sterol; SM, sphingomyelin; FA, fatty acyls; PC-O, alkylphosphatidylcholine; PC-P. alkenylphosphatidylcholine; PR, prenol; PG, phosphatidylglycerol; PI, phosphatidylinositol; LPG, lysophosphatidylglycerol; DG, diacylglycerol; CE, cholesterol ester)

4.3.2.4. Changes in other lipid classes

Among SPs, all the Cers were significantly increased in AD. GLs such as mono and diacylglycerides were higher in AD, while the TGs had mixed changes. Additionally, there were also three cholesterol esters, CE 20:2 (*p=0.0002*), CE 20:5 (*p=0.021*) and CE 22:5 (*p=0.02*), that were significantly decreased in AD. The identifications of the lipids that were significantly different in AD using the MRM assay are provided in **Table D4.4**.

4.3.3. SRM cholesterol targeted study

4.3.3.1. Changes in cholesterol and its precursors

In order to further assess the cholesterol biosynthesis pathway, we applied a targeted SRM cholesterol assay on plasma samples. Details on the sterol assay are explained elsewhere.[60] It should be noted that this assay is ~1 min. in total MS scan time. Here we used SRM to monitor cholesterol and its four precursors: 7-dehydrocholesterol (7-DHC), 8-dehydrocholesterol (8-DHC), desmosterol (Des) and lanosterol (Lan). Sterols were analyzed and quantified using an isotopically labeled standard. The elution of the standard to the endogenous species was compared. Typical extracted ion chromatograms are shown in **Figure 4.7**. Pseudo-SRMs for cholesterol and d7-cholesterol were observed at t_R 0.8 min. at *m/z* 369→369 and *m/z* 376→376, respectively (**Figures 4.7a & 8b**). Peaks for Des and $^{13}C_3$-Des adducts were observed at t_R 0.4 min. at *m/z* 592 →365 (**Figure 4.7c**) and t_R 0.4 min. at *m/z* 595→368 (**Figure 4.7d**), respectively. Similar peaks for 7-DHC and Lan (**Figures 4.7e-h**) were observed.

Plasma levels of cholesterol were significantly lower (*p<0.04)* in AD compared to CN samples even after considering characteristic ratios such as Des/Chol, Des/Lan, and Lan/Chol (**Table 4.3**). None of the other precursors were found to be significantly different in our study.

Table 4.3. Plasma levels of cholesterol and its precursors in plasma using SRM.

	CN (Mean±SD)	AD(Mean±SD)	*p value*
Cholesterol (ng/µL)	**47.43 ± 11.28**	**31.25 ± 9.28**	**0.04**
7-DHC (pg/µL)	145.22 ± 76.55	151.44 ± 95.77	0.91
8-DHC (pg/µL)	525.54 ± 407.03	597.09 ± 488.38	0.81
Desmosterol (pg/µL)	221.33 ± 112.65	149.60 ± 40.33	0.22
Lanosterol (pg/µL)	733.06 ± 1212.66	174.32 ± 34.29	0.33
Desmosterol/Cholesterol	0.01 ± 0.00	0.01 ± 0.00	0.83
Desmosterol/Lanosterol	0.80 ± 0.55	0.90 ± 0.38	0.74
Lanosterol/Cholesterol	0.01 ± 0.02	0.01 ± 0.00	0.36

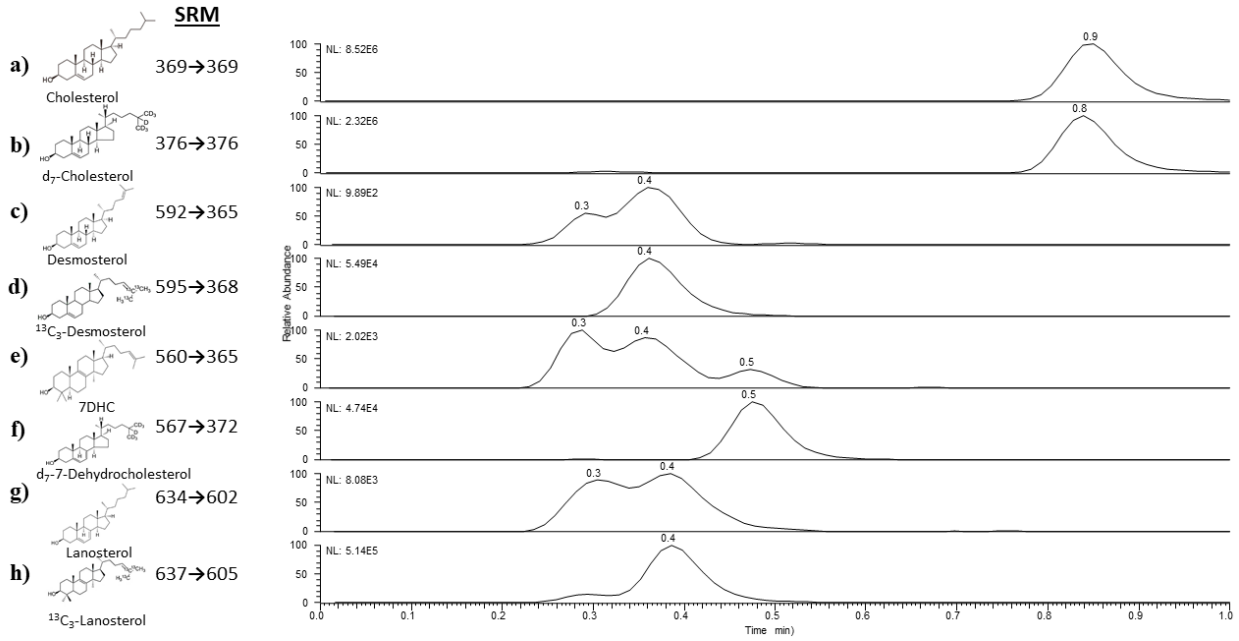

Figure 4.7. UPLC chromatogram of cholesterol and its precursors using SRM targeted assay. Chemical structures, ion chromatograms, and SRM transitions of **(a)** cholesterol, **(b)** d_7-cholesterol, **(c)** desmosterol, **(d)** $^{13}C_3$- Des, **(e)** 7-DHC, **(f)** d_7-7-DHC, **(g)** lanosterol, and **(h)** $^{13}C_3$-Lan species.

4.4 Discussion

Several reports have linked lipid changes to AD pathology[34, 39, 41, 66] especially for phospholipids.[35, 67-68] Phospholipids are an integral part of the cell membrane. It has been reported that Aβ interacts with and binds phospholipids within the membrane and changes the lipid bilayer[69], which leads to the disruption of the cell membrane. Multiple studies report decreased levels of phosphatidylcholines,[36, 67] phosphatidylethanolamines,[70-71] phosphatidylinositols,[68, 72] and plasmalogens,[73] whereas phosphatidylserines are increased[74-75] in AD. In this study, our data show similar reduced levels of phosphatidylcholines and increased levels of phosphatidylserines. However, data from our study conflicts with current literature as the untargeted approach detected higher plasmalogen and phosphatidylethanolamine levels in AD. Herein, similar results were observed with the untargeted and targeted MRM analyses of our subset of plasma samples. In the MRM study, there was a mixed response in the changes to individual phosphatidylserine and phosphatidylinositol lipid species, where there were species that were both increased and decreased in AD. This highlights the power of using the untargeted and targeted MRM approaches: detailed insight to biochemical pathways is gained by monitoring individual lipids whereas such information may be lost in only monitoring global changes of a given lipid class.[51]

Sphingolipids, specifically sphingomyelins and ceramides, are another important class associated with AD. Altered sphingolipid metabolism is related to neurodegenerative disease, especially AD.[76-77] Sphingolipids are one of the major constituents of lipid rafts, and many studies have suggested that the amyloidogenic processing of the APP protein using BACE1 and γ secretase happens in the lipid raft region.[6, 78] It has been found that increasing levels of ceramides promote the formation of Aβ by targeting the BACE1 secretase towards the lipid rafts.[6] Recent studies using human samples have found elevated levels of ceramides and decreased levels of sphingomyelins

in AD.[39-41] Our findings using the multi-lipidomics approaches, were consistent. For glycerolipid species, mono and diacylglycerol species were also observed with similar trends to previous reports [29, 32-33], however this cohort data showed mixed results for the triglyceride species.

One of the most important lipid species implicated in AD is cholesterol and its metabolic precursors. Several studies have reported that desmosterol levels are low in AD brain,[79] while the desmosterol:cholesterol ratio has been found to be low in AD plasma samples.[47] Others report low levels of lanosterol in AD.[27] In this study, only cholesterol was observed to be significantly altered in AD, and none of the other precursors showed any statistical changes. This could be due to the small sample size of the pilot study, potentially sex specific effects, or a true reflection of the dynamic changes in AD and CN individuals. To validate this, we performed another sterol analysis using a cohort of 40 samples (AD= 20, CN= 20; data not shown). Neither cholesterol or any of the other precursors were found to be significantly different in this study, indicating a need for much larger sample cohort sizes.

The multi-lipidomic approach utilized here, using untargeted and targeted MRM and SRM assays, has allowed a comprehensive analysis of plasma lipid changes in AD. We do acknowledge that the low number of samples per study group (N=5) may be considered a limitation in this pilot study, but the goal of this work was to evaluate the comprehensive depth of lipid identification that could be obtained across three lipidomic methods in AD. It should be noted that for most individual lipids and lipid classes the changes observed in AD were consistent across approaches, but there were cases in which they differed, especially for plasmalogens and triglycerides. Although there were some inconsistencies, mostly between the untargeted and targeted approaches, further development related to improvements in assays, data annotations, validated identifications, and additions to the global MRM list are necessary. As for the targeted SRM

cholesterol approach, this was a highly selective and rapid assay. We identified and quantified four cholesterol precursors; we however were unable to find any significant changes in AD using a larger cohort of samples.

4.5. Conclusions

The multi-lipidomics approach described here determined significant changes in lipid profiles between CN and AD individuals for a small cohort of plasma samples. Our lipid results were mostly consistent with previous literature reports indicating confidence in the comprehensive multi-lipidomics approach. This multi-lipidomics approach will be helpful for other plasma lipidomics studies and especially in early stages of study design devoted to discovery whereby comprehensive lipid coverage is desired. Focused strategies on lipids of interest may be more suitable using targeted MRM and SRM approaches for larger sample sizes. We applied a targeted MRM lipid assay to a larger cohort of samples (**Chapter V**) in order to reveal more robust evidence and deeper insight to complex lipid metabolism changes in AD.

4.6. Acknowledgements

This work has been supported by the funding from the Alzheimer's Association (AARGD-17-533405), the Vanderbilt University Start-Up Funds, pilot funds from the University of Pittsburgh Alzheimer Disease Research Center funded by the National Institutes of Health and National Institute on Aging (P50AG005133, RASR), NICHD (R01 HD064727, NAP) and the Vanderbilt Institute of Chemical Biology (fellowship, MJK). This work was supported in part using the resources of the Center for Innovative Technology at Vanderbilt University. We would like to thank Dr. Ned Porter and Dr. Philip Wages for conducting targeted SRM analysis of sterols.

We would also like to acknowledge SCIEX for the academic partnership in the analysis of the targeted MRM samples.

4.7. References

1. 2020 Alzheimer's disease facts and figures. *Alzheimer's & Dementia* **2020,** *16* (3), 391-460.

2. Hebert, L. E.; Weuve, J.; Scherr, P. A.; Evans, D. A., Alzheimer disease in the United States (2010-2050) estimated using the 2010 census. *Neurology* **2013,** *80* (19), 1778-83.

3. 2018 Alzheimer's disease facts and figures. *Alzheimer's & Dementia: The Journal of the Alzheimer's Association* **2018,** *14* (3), 367-429.

4. Xiang, Y.; Lam, S. M.; Shui, G., What can lipidomics tell us about the pathogenesis of Alzheimer disease? *Biological chemistry* **2015,** *396* (12), 1281-91.

5. Tanzi, R. E.; Bertram, L., Twenty Years of the Alzheimer's Disease Amyloid Hypothesis: A Genetic Perspective. *Cell* **2005,** *120* (4), 545-555.

6. Di Paolo, G.; Kim, T. W., Linking lipids to Alzheimer's disease: cholesterol and beyond. *Nat Rev Neurosci* **2011,** *12* (5), 284-96.

7. Nagy, Z.; Esiri, M. M.; Jobst, K. A.; Morris, J. H.; King, E. M.; McDonald, B.; Litchfield, S.; Smith, A.; Barnetson, L.; Smith, A. D., Relative roles of plaques and tangles in the dementia of Alzheimer's disease: correlations using three sets of neuropathological criteria. *Dementia* **1995,** *6* (1), 21-31.

8. Stepler, K. E.; Robinson, R. A. S., The potential of 'omics to link lipid metabolism and genetic and comorbidity risk factors of Alzheimer's disease in African Americans. In Reviews on Biomarker Studies in Psychiatric and Neurodegenerative Disorders **2019**, 1-28.

9. Klein, J., Membrane breakdown in acute and chronic neurodegeneration: focus on choline-containing phospholipids. *J Neural Transm (Vienna)* **2000,** *107* (8-9), 1027-63.

10. Gottfries, C. G.; Karlsson, I.; Svennerholm, L., Membrane components separate early-onset Alzheimer's disease from senile dementia of the Alzheimer type. *Int Psychogeriatr* **1996,** *8* (3), 365-72.

11. Blusztajn, J. K.; Lopez Gonzalez-Coviella, I.; Logue, M.; Growdon, J. H.; Wurtman, R. J., Levels of phospholipid catabolic intermediates, glycerophosphocholine and glycerophosphoethanolamine, are elevated in brains of Alzheimer's disease but not of Down's syndrome patients. *Brain Res* **1990,** *536* (1-2), 240-4.

12. Zipser, B. D.; Johanson, C. E.; Gonzalez, L.; Berzin, T. M.; Tavares, R.; Hulette, C. M.; Vitek, M. P.; Hovanesian, V.; Stopa, E. G., Microvascular injury and blood-brain barrier leakage in Alzheimer's disease. *Neurobiol Aging* **2007,** *28* (7), 977-86.

13. Sweeney, M. D.; Sagare, A. P.; Zlokovic, B. V., Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. *Nature reviews. Neurology* **2018,** *14* (3), 133-150.

14. Suarna, C.; Dean, R. T.; May, J.; Stocker, R., Human atherosclerotic plaque contains both oxidized lipids and relatively large amounts of alpha-tocopherol and ascorbate. *Arterioscler Thromb Vasc Biol* **1995,** *15* (10), 1616-24.

15. Han, X.; Yang, J.; Yang, K.; Zhao, Z.; Abendschein, D. R.; Gross, R. W., Alterations in myocardial cardiolipin content and composition occur at the very earliest stages of diabetes: a shotgun lipidomics study. *Biochemistry* **2007,** *46* (21), 6417-28.

16. Miyoshi, N.; Iwasaki, N.; Tomono, S.; Higashi, T.; Ohshima, H., Occurrence of cytotoxic 9-oxononanoyl secosterol aldehydes in human low-density lipoprotein. *Free Radic Biol Med* **2013,** *60*, 73-9.

17. Ollero, M.; Astarita, G.; Guerrera, I. C.; Sermet-Gaudelus, I.; Trudel, S.; Piomelli, D.; Edelman, A., Plasma lipidomics reveals potential prognostic signatures within a cohort of cystic fibrosis patients. *Journal of lipid research* **2011,** *52* (5), 1011-22.

18. Fhaner, C. J.; Liu, S.; Ji, H.; Simpson, R. J.; Reid, G. E., Comprehensive lipidome profiling of isogenic primary and metastatic colon adenocarcinoma cell lines. *Anal Chem* **2012,** *84* (21), 8917-26.

19. Gorke, R.; Meyer-Base, A.; Wagner, D.; He, H.; Emmett, M. R.; Conrad, C. A., Determining and interpreting correlations in lipidomic networks found in glioblastoma cells. *BMC Syst Biol* **2010,** *4*, 126.

20. Ishikawa, S.; Tateya, I.; Hayasaka, T.; Masaki, N.; Takizawa, Y.; Ohno, S.; Kojima, T.; Kitani, Y.; Kitamura, M.; Hirano, S.; Setou, M.; Ito, J., Increased expression of phosphatidylcholine (16:0/18:1) and (16:0/18:2) in thyroid papillary cancer. *PLoS One* **2012,** *7* (11), e48873.

21. Zhou, X.; Mao, J.; Ai, J.; Deng, Y.; Roth, M. R.; Pound, C.; Henegar, J.; Welti, R.; Bigler, S. A., Identification of plasma lipid biomarkers for prostate cancer by lipidomics and bioinformatics. *PLoS One* **2012,** *7* (11), e48889.

22. Yu, Z.; Chen, H.; Zhu, Y.; Ai, J.; Li, Y.; Gu, W.; Borgia, J. A.; Zhang, J.; Jiang, B.; Chen, W.; Deng, Y., Global lipidomics reveals two plasma lipids as novel biomarkers for the detection of squamous cell lung cancer: A pilot study. *Oncol Lett* **2018,** *16* (1), 761-768.

23. Adibhatla, R. M.; Hatcher, J. F., Role of Lipids in Brain Injury and Diseases. *Future Lipidol* **2007,** *2* (4), 403-422.

24. Di Paolo, G.; Kim, T.-W., Linking lipids to Alzheimer's disease: cholesterol and beyond. *Nature reviews. Neuroscience* **2011,** *12* (5), 284-296.

25. Adibhatla, R. M.; Hatcher, J. F., Altered lipid metabolism in brain injury and disorders. *Sub-cellular biochemistry* **2008,** *49*, 241-268.

26. Liu, Q.; Zhang, J., Lipid metabolism in Alzheimer's disease. *Neurosci Bull* **2014,** *30* (2), 331-345.

27. Kölsch, H.; Heun, R.; Jessen, F.; Popp, J.; Hentschel, F.; Maier, W.; Lütjohann, D., Alterations of cholesterol precursor levels in Alzheimer's disease. *Biochimica et biophysica acta* **2010,** *1801* (8), 945-50.

28. Xianlin, H., Lipid Alterations in the Earliest Clinically Recognizable Stage of Alzheimers Disease: Implication of the Role of Lipids in the Pathogenesis of Alzheimers Disease. *Current Alzheimer Research* **2005,** *2* (1), 65-77.

29. Wood, P. L.; Cebak, J. E.; Woltjer, R. L., Diacylglycerols as biomarkers of sustained immune activation in Proteinopathies associated with dementia. *Clinica Chimica Acta* **2018,** *476*, 107-110.

30. Wong, M. W.; Braidy, N.; Poljak, A.; Sachdev, P. S., The application of lipidomics to biomarker research and pathomechanisms in Alzheimer's disease. *Curr Opin Psychiatry* **2017,** *30* (2), 136-144.

31. Wong, M. W.; Braidy, N.; Poljak, A.; Pickford, R.; Thambisetty, M.; Sachdev, P. S., Dysregulation of lipids in Alzheimer's disease and their role as potential biomarkers. *Alzheimer's & Dementia* **2017,** *13* (7), 810-827.

32. Wood, P. L.; Locke, V. A.; Herling, P.; Passaro, A.; Vigna, G. B.; Volpato, S.; Valacchi, G.; Cervellati, C.; Zuliani, G., Targeted lipidomics distinguishes patient subgroups in mild cognitive impairment (MCI) and late onset Alzheimer's disease (LOAD). *BBA Clin* **2016,** *5*, 25-8.

33. Wood, P. L.; Barnette, B. L.; Kaye, J. A.; Quinn, J. F.; Woltjer, R. L., Non-targeted lipidomics of CSF and frontal cortex grey and white matter in control, mild cognitive impairment, and Alzheimer's disease subjects. *Acta Neuropsychiatr* **2015,** *27* (5), 270-8.

34. Han, X., Multi-dimensional mass spectrometry-based shotgun lipidomics and the altered lipids at the mild cognitive impairment stage of Alzheimer's disease. *Biochimica et biophysica acta* **2010,** *1801* (8), 774-83.

35. Gonzalez-Dominguez, R.; Garcia-Barrera, T.; Gomez-Ariza, J. L., Combination of metabolomic and phospholipid-profiling approaches for the study of Alzheimer's disease. *J Proteomics* **2014,** *104*, 37-47.

36. Whiley, L.; Sen, A.; Heaton, J.; Proitsi, P.; Garcia-Gomez, D.; Leung, R.; Smith, N.; Thambisetty, M.; Kloszewska, I.; Mecocci, P.; Soininen, H.; Tsolaki, M.; Vellas, B.; Lovestone,

S.; Legido-Quigley, C.; AddNeuroMed, C., Evidence of altered phosphatidylcholine metabolism in Alzheimer's disease. *Neurobiol Aging* **2014,** *35* (2), 271-8.

37. Kim, M.; Nevado-Holgado, A.; Whiley, L.; Snowden, S. G.; Soininen, H.; Kloszewska, I.; Mecocci, P.; Tsolaki, M.; Vellas, B.; Thambisetty, M.; Dobson, R. J. B.; Powell, J. F.; Lupton, M. K.; Simmons, A.; Velayudhan, L.; Lovestone, S.; Proitsi, P.; Legido-Quigley, C., Association between Plasma Ceramides and Phosphatidylcholines and Hippocampal Brain Volume in Late Onset Alzheimer's Disease. *Journal of Alzheimer's disease : JAD* **2017,** *60* (3), 809-817.

38. Wood, P. L.; Medicherla, S.; Sheikh, N.; Terry, B.; Phillipps, A.; Kaye, J. A.; Quinn, J. F.; Woltjer, R. L., Targeted Lipidomics of Fontal Cortex and Plasma Diacylglycerols (DAG) in Mild Cognitive Impairment and Alzheimer's Disease: Validation of DAG Accumulation Early in the Pathophysiology of Alzheimer's Disease. *Journal of Alzheimer's disease : JAD* **2015,** *48* (2), 537-46.

39. Han, X.; Rozen, S.; Boyle, S. H.; Hellegers, C.; Cheng, H.; Burke, J. R.; Welsh-Bohmer, K. A.; Doraiswamy, P. M.; Kaddurah-Daouk, R., Metabolomics in Early Alzheimer's Disease: Identification of Altered Plasma Sphingolipidome Using Shotgun Lipidomics. *PLOS ONE* **2011,** *6* (7), e21643.

40. Panchal, M.; Gaudin, M.; Lazar, A. N.; Salvati, E.; Rivals, I.; Ayciriex, S.; Dauphinot, L.; Dargere, D.; Auzeil, N.; Masserini, M.; Laprevote, O.; Duyckaerts, C., Ceramides and sphingomyelinases in senile plaques. *Neurobiol Dis* **2014,** *65*, 193-201.

41. Han, X.; D, M. H.; McKeel, D. W., Jr.; Kelley, J.; Morris, J. C., Substantial sulfatide deficiency and ceramide elevation in very early Alzheimer's disease: potential role in disease pathogenesis. *J Neurochem* **2002,** *82* (4), 809-18.

42. Cutler, R. G.; Kelly, J.; Storie, K.; Pedersen, W. A.; Tammara, A.; Hatanpaa, K.; Troncoso, J. C.; Mattson, M. P., Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. *Proceedings of the National Academy of Sciences of the United States of America* **2004,** *101* (7), 2070-5.

43. Gamba, P.; Testa, G.; Sottero, B.; Gargiulo, S.; Poli, G.; Leonarduzzi, G., The link between altered cholesterol metabolism and Alzheimer's disease. *Annals of the New York Academy of Sciences* **2012,** *1259*, 54-64.

44. Kolsch, H.; Heun, R.; Kerksiek, A.; Bergmann, K. V.; Maier, W.; Lutjohann, D., Altered levels of plasma 24S- and 27-hydroxycholesterol in demented patients. *Neuroscience letters* **2004,** *368* (3), 303-8.

45. Wages, P. A.; Kim, H. H.; Korade, Z.; Porter, N. A., Identification and characterization of prescription drugs that change levels of 7-dehydrocholesterol and desmosterol. *Journal of lipid research* **2018,** *59* (10), 1916-1926.

46. Popp, J.; Meichsner, S.; Kolsch, H.; Lewczuk, P.; Maier, W.; Kornhuber, J.; Jessen, F.; Lutjohann, D., Cerebral and extracerebral cholesterol metabolism and CSF markers of Alzheimer's disease. *Biochem Pharmacol* **2013,** *86* (1), 37-42.

47. Sato, Y.; Bernier, F.; Yamanaka, Y.; Aoshima, K.; Oda, Y.; Ingelsson, M.; Lannfelt, L.; Miyashita, A.; Kuwano, R.; Ikeuchi, T., Reduced plasma desmosterol-to-cholesterol ratio and longitudinal cognitive decline in Alzheimer's disease. *Alzheimer's & dementia (Amsterdam, Netherlands)* **2015,** *1* (1), 67-74.

48. Contrepois, K.; Mahmoudi, S.; Ubhi, B. K.; Papsdorf, K.; Hornburg, D.; Brunet, A.; Snyder, M., Cross-Platform Comparison of Untargeted and Targeted Lipidomics Approaches on Aging Mouse Plasma. *Sci Rep* **2018,** *8* (1), 17747.

49. Barupal, D. K.; Fan, S.; Wancewicz, B.; Cajka, T.; Sa, M.; Showalter, M. R.; Baillie, R.; Tenenbaum, J. D.; Louie, G.; Alzheimer's Disease Neuroimaging, I.; Alzheimer's Disease Metabolomics, C.; Kaddurah-Daouk, R.; Fiehn, O., Generation and quality control of lipidomics data for the alzheimer's disease neuroimaging initiative cohort. *Scientific Data* **2018,** *5*, 180263.

50. Anand, S.; Barnes, J. M.; Young, S. A.; Garcia, D. M.; Tolley, H. D.; Kauwe, J. S.; Graves, S. W., Discovery and confirmation of diagnostic serum lipid biomarkers for Alzheimer's disease using direct infusion mass spectrometry. *Journal of Alzheimer's Disease* **2017,** *59* (1), 277-290.

51. Kliman, M.; Vijayakrishnan, N.; Wang, L.; Tapp, J. T.; Broadie, K.; McLean, J. A., Structural mass spectrometry analysis of lipid changes in a Drosophila epilepsy model brain. *Molecular BioSystems* **2010,** *6* (6), 958-966.

52. Albert, M. S.; DeKosky, S. T.; Dickson, D.; Dubois, B.; Feldman, H. H.; Fox, N. C.; Gamst, A.; Holtzman, D. M.; Jagust, W. J.; Petersen, R. C.; Snyder, P. J.; Carrillo, M. C.; Thies, B.; Phelps, C. H., The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2011,** *7* (3), 270-9.

53. Jack, C. R., Jr.; Albert, M. S.; Knopman, D. S.; McKhann, G. M.; Sperling, R. A.; Carrillo, M. C.; Thies, B.; Phelps, C. H., Introduction to the recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2011,** *7* (3), 257-62.

54. McKhann, G. M.; Knopman, D. S.; Chertkow, H.; Hyman, B. T.; Jack, C. R., Jr.; Kawas, C. H.; Klunk, W. E.; Koroshetz, W. J.; Manly, J. J.; Mayeux, R.; Mohs, R. C.; Morris, J. C.; Rossor, M. N.; Scheltens, P.; Carrillo, M. C.; Thies, B.; Weintraub, S.; Phelps, C. H., The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2011,** *7* (3), 263-9.

55. Sperling, R. A.; Aisen, P. S.; Beckett, L. A.; Bennett, D. A.; Craft, S.; Fagan, A. M.; Iwatsubo, T.; Jack, C. R., Jr.; Kaye, J.; Montine, T. J.; Park, D. C.; Reiman, E. M.; Rowe, C. C.; Siemers, E.; Stern, Y.; Yaffe, K.; Carrillo, M. C.; Thies, B.; Morrison-Bogorad, M.; Wagster, M. V.; Phelps, C. H., Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2011,** *7* (3), 280-92.

56. Khan, M. J.; Codreanu, S. G.; Goyal, S.; Wages, P. A.; Gorti, S. K. K.; Pearson, M. J.; Uribe, I.; Sherrod, S. D.; McLean, J. A.; Porter, N. A.; Robinson, R. A. S., Evaluating a targeted multiple reaction monitoring approach to global untargeted lipidomic analyses of human plasma. *Rapid Communications in Mass Spectrometry* **2020,** *34* (22), e8911.

57. Wang, M.; Han, X., Advanced Shotgun Lipidomics for Characterization of Altered Lipid Patterns in Neurodegenerative Diseases and Brain Injury. *Methods in molecular biology (Clifton, N.J.)* **2016,** *1303*, 405-22.

58. Gibson, C. L.; Codreanu, S. G.; Schrimpe-Rutledge, A. C.; Retzlaff, C. L.; Wright, J.; Mortlock, D. P.; Sherrod, S. D.; McLean, J. A.; Blakely, R. D., Global untargeted serum metabolomic analyses nominate metabolic pathways responsive to loss of expression of the orphan metallo beta-lactamase, MBLAC1. *Mol Omics* **2018,** *14* (3), 142-155.

59. Liu, W.; Xu, L.; Lamberson, C.; Haas, D.; Korade, Z.; Porter, N. A., A highly sensitive method for analysis of 7-dehydrocholesterol for the study of Smith-Lemli-Opitz syndrome. *Journal of lipid research* **2014,** *55* (2), 329-337.

60. Korade, Z.; Kim, H. Y.; Tallman, K. A.; Liu, W.; Koczok, K.; Balogh, I.; Xu, L.; Mirnics, K.; Porter, N. A., The Effect of Small Molecules on Sterol Homeostasis: Measuring 7-Dehydrocholesterol in Dhcr7-Deficient Neuro2a Cells and Human Fibroblasts. *J Med Chem* **2016,** *59* (3), 1102-15.

61. Bligh, E. G.; Dyer, W. J., A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. *Canadian Journal of Biochemistry and Physiology* **1959,** *37* (8), 911-917.

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

63. Fahy, E.; Subramaniam, S.; Murphy, R. C.; Nishijima, M.; Raetz, C. R.; Shimizu, T.; Spener, F.; van Meer, G.; Wakelam, M. J.; Dennis, E. A., Update of the LIPID MAPS comprehensive classification system for lipids. *Journal of lipid research* **2009,** *50 Suppl*, S9-14.

64. Wang, J.; Fu, L.; Gorti, S. K. K.; Pearson, M. J.; Wu, S.; Xiong, L.; Baker, P. R., Development of an Amide Column-Based LC-MS/MS Method for High-Throughput Targeted Lipidomics. In *ASMS Conference on Mass Spectrometry and Allied Topics*, San Diego, CA, **2018**.

65. Gorti, S. K. K.; Merkel, D.; Fu, L.; Pearson, M. J.; Ubhi, B. K.; Xiong, L.; Baker, P., HILIC LC-MSMS assay for high-throughput targeted lipdomics analysis. In *Lipidomics Forum*, Dortmund, Germany, **2018**.

66. Han, X., Lipid alterations in the earliest clinically recognizable stage of Alzheimer's disease: implication of the role of lipids in the pathogenesis of Alzheimer's disease. *Curr Alzheimer Res* **2005,** *2* (1), 65-77.

67. Igarashi, M.; Ma, K.; Gao, F.; Kim, H.-W.; Rapoport, S. I.; Rao, J. S., Disturbed choline plasmalogen and phospholipid fatty acid concentrations in Alzheimer's disease prefrontal cortex. *Journal of Alzheimer's Disease* **2011,** *24* (3), 507-517.

68. Pettegrew, J. W.; Panchalingam, K.; Hamilton, R. L.; McClure, R. J., Brain membrane phospholipid alterations in Alzheimer's disease. *Neurochem Res* **2001,** *26* (7), 771-82.

69. Haughey, N. J.; Bandaru, V. V. R.; Bae, M.; Mattson, M. P., Roles for dysfunctional sphingolipid metabolism in Alzheimer's disease neuropathogenesis. *Biochimica et biophysica acta* **2010,** *1801* (8), 878-886.

70. Nitsch, R. M.; Blusztajn, J. K.; Pittas, A. G.; Slack, B. E.; Growdon, J. H.; Wurtman, R. J., Evidence for a membrane defect in Alzheimer disease brain. *Proceedings of the National Academy of Sciences* **1992,** *89* (5), 1671-1675.

71. Prasad, M. R.; Lovell, M. A.; Yatin, M.; Dhillon, H.; Markesbery, W. R., Regional membrane phospholipid alterations in Alzheimer's disease. *Neurochem Res* **1998,** *23* (1), 81-8.

72. Stokes, C. E.; Hawthorne, J. N., Reduced phosphoinositide concentrations in anterior temporal cortex of Alzheimer-diseased brains. *Journal of neurochemistry* **1987,** *48* (4), 1018-1021.

73. Han, X.; Holtzman, D. M.; McKeel, D. W., Jr., Plasmalogen deficiency in early Alzheimer's disease subjects and in animal models: molecular characterization using electrospray ionization mass spectrometry. *Journal of neurochemistry* **2001,** *77* (4), 1168-80.

74. Farooqui, A. A.; Rapoport, S. I.; Horrocks, L. A., Membrane Phospholipid Alterations In Alzheimer's Disease: Deficiency of Ethanolamine Plasmalogens. *Neurochemical Research* **1997,** *22* (4), 523-527.

75. Wells, K.; Farooqui, A. A.; Liss, L.; Horrocks, L. A., Neural membrane phospholipids in alzheimer disease. *Neurochemical Research* **1995,** *20* (11), 1329-1333.

76. Katsel, P.; Li, C.; Haroutunian, V., Gene expression alterations in the sphingolipid metabolism pathways during progression of dementia and Alzheimer’s disease: a shift toward ceramide accumulation at the earliest recognizable stages of Alzheimer’s disease? *Neurochemical research* **2007,** *32* (4-5), 845-856.

77. Haughey, N. J.; Cutler, R. G.; Tamara, A.; McArthur, J. C.; Vargas, D. L.; Pardo, C. A.; Turchan, J.; Nath, A.; Mattson, M. P., Perturbation of sphingolipid metabolism and ceramide production in HIV-dementia. *Ann Neurol* **2004,** *55* (2), 257-67.

78. Ehehalt, R.; Keller, P.; Haass, C.; Thiele, C.; Simons, K., Amyloidogenic processing of the Alzheimer beta-amyloid precursor protein depends on lipid rafts. *J Cell Biol* **2003,** *160* (1), 113-23.

79. Wisniewski, T.; Newman, K.; Javitt, N. B., Alzheimer's disease: brain desmosterol levels. *Journal of Alzheimer's Disease* **2013,** *33* (3), 881-888.

CHAPTER V

Targeted Lipidomics to Understand Health Disparities in Alzheimer's Disease

"This chapter is adopted from the recently submitted research articles: **Khan, MJ, Desaire, Chung, NC, Lopez, OL, Kamboh, MI, Robinson, RAS, "Targeted lipidomics to understand health disparities in Alzheimer's disease." Under review**

5.1. Introduction

Alzheimer's disease (AD) is one of the fastest growing diseases with currently 5.8 million Americans suffering from it.[1] One aspect of this disease that is severely understudied is the disproportionate impact of AD among the African American/Black population. AD is the 4th leading cause of death among African American/Black adults.[2] African American/Black individuals are also more likely to develop AD compared to their non-Hispanic White counterparts.[1, 3-4] It is estimated that, by 2050, of the number of people age 65 and older, 40% will be African American/Black and Hispanics.[5] This is extremely concerning as age is the largest risk factor for AD.[1] The initial manifestation of AD is similar for both African American/Black and non-Hispanic White individuals [3,6], however mixed-dementia is more prominent among African American/Blacks.[6] Disparities in disease incidence can be attributed to systemic racism [7], education [8-10], presence of comorbidities [11-12], healthcare access [11], and genetics.[12]

Recent studies have found evidence of differences among African American/Black and non-Hispanic White individuals when it comes to established AD biomarkers. For example, the concentration levels of tau and phosphorylated tau in cerebrospinal fluid (CSF) [13-14] and Interleukin-9 in plasma from African American/Black individuals were lower than in non-Hispanic Whites.[15] Our laboratory recently identified race specific differentially-expressed proteins in AD in post mortem brain tissues from African American/Black and non-Hispanic White adults.[16] Whether or not these molecular differences are casual or the result of "weathering" [17-20] in combination with social, environmental, and genetic factors is not clear.

Numerous studies have demonstrated a link between AD and altered lipid metabolism making lipids key in the pathogenesis of AD.[12, 21-24] Advances in mass spectrometry (MS) instrumentation have further accelerated lipidomics applications in AD research.[25-30] Reduction in the plasma levels of glycerophospholipids, especially phosphatidylcholines have been reported.[31-36] Several studies have proposed lipid biomarker panels for AD discovery including phosphatidylcholines.[35-37] Other glycerophospholipids altered in AD plasma include phophatidylethanolamines, lysophophatidylcholines, lysophosphatidylethanolamines and plasmalogens.[32-34, 38-41] Sphingolipids change in plasma from AD adults such that sphingomyelins decrease and ceramide levels increase in AD.[42-45] Diacylglycerols and triacylglycerols also change in AD [42, 46-48] and together these studies support the role of lipids in AD and their potential use as diagnostic biomarkers.

Despite the large number of studies involving lipids in AD, very few have focused on or included samples from African American/Black adults. A recent study of the lipid panel proposed by Mapstone et al. [35] failed to replicate in a cohort of African American/Black adults.[49] Similar lipidomics evidence of racial disparities in cancer and myocardial infarction [50-53], cardiometabolic diseases [54], as well lupus [55] have been reported. Distinct metabolite profiles were observed in African American/Black adults with bladder cancer.[52] Together, these studies point to potential molecular differences in lipids that warrant further studies in the context of AD.

Here, we conducted a lipidomics study using plasma samples from cognitively normal (CN) and AD individuals of African American/Black and non-Hispanic White backgrounds. Plasma samples were analyzed using a multiple reaction monitoring (MRM) MS targeted lipidomics analysis of ~1150 lipids, recently evaluated in our laboratory.[56] The goal was to determine if there were any differences in the plasma lipidome in African American/Black and

non-Hispanic White adults due to AD. Our findings signify the importance of inclusion in AD research, as our results demonstrate some differences in lipid expression in AD based on racial background.

5.2. Methods

5.2.1. Plasma demographics

Plasma samples (N=113) from African American/Black and non-Hispanic White adults were obtained from the University of Pittsburgh Alzheimer's Disease Research Center (ADRC). Detailed characteristics are given in **Table 5.1**. Approval for the participation of human subjects was obtained by the Institutional Review Boards of the University of Pittsburgh and Vanderbilt University. The disease individuals were clinically diagnosed with mild to moderate dementia at the time of draw according to the National Institute on Aging-Alzheimer's Association and National Alzheimer's Coordinating Center criteria.[57-60] The AD diagnosis for all the participants was clinically confirmed and blood from the most recent draw was used for this analysis.

Table 5.1. Demographics of participant cohort**.**

	African American/Black		***non-Hispanic White***		**p-value***
	Cognitively normal	**Alzheimer's disease**	**Cognitively normal**	**Alzheimer's disease**	
Sample size	26	30	28	29	
Sex (Male/Female)	8 / 18	8 / 22	9 /19	12 / 17	0.69
Mean age at draw (SD)	72.5 (7.4)	74.5 (7.8)	71.1 (9.1)	76.2 (8.7)	0.58
MMSE[a] Mean (SD)	27.1(2.4)	14.5(7.1)	27.8(4.5)	13.6(5.9)	$1.94E^{-22}$
Diabetes[b]	15	18	19	13	0.22
Hypercholesterolemia[b]	15	18	19	13	0.65
Hypertension[b]	15	18	19	13	0.046

[a] MMSE- Mini-Mental State Exam,
[b] Number of individuals with the presence of comorbidity prior to or during blood draw.

5.2.2. Lipid extraction

Plasma samples were divided into four batches in such a way that there were no differences among the batches in terms of the number of samples from each study group, and also other variables such as age, sex and presence of comorbidities. Each sample batch was prepared simultaneously and ran sequentially. Plasma sample (25 µL) was transferred into a borosilicate glass tube followed by addition of internal standards (Splash® Lipidomix, Avanti Polar, Alabaster, AL) at 1:1 ratio of internal standard (IS):plasma. The complete list of IS and their corresponding concentrations have been described previously.[56] Water, methanol and chloroform at a 1:2:0.9 ratio was added to the glass tube. The mixture was vortexed and left to sit at room temperature for 30 min. Next, water and chloroform were added at a 1:0.9 ratio and the sample tube was inverted several times. The tubes were centrifuged at 3500 rpm for 30 min. The bottom organic layer containing the lipids was carefully collected, and 2 mL of chloroform was added to the aqueous phase (upper layer) to re-extract the lipids. Tubes were vortexed and centrifuged again. The subsequent bottom layer was combined with the previously collected lipids and dried down using a nitrogen stream.

5.2.3. LC – MS/MS analyses

Extracted lipids were reconstituted using 9:1 methanol:chloroform solvent. The LC-MS/MS method used in this study has been described previously.[56] In short, the LC-MS/MS analysis was performed using an ExionLC™ System coupled to an Sciex QTRAP 6500+ mass spectrometer. The lipids were loaded onto a Waters XBridge Amide column (4.6 × 150 mm, 3.5 µm) using a thermostated autosampler. The LC method details are as follows: column temperature = 35 ºC, flow rate of 0.7 mL/min, injection volume = 5 µL. The mobile phases were as follows:

solvent A (water:acetonitrile) at a 5:95 ratio with 10 mM ammonium acetate (adjusted to pH 8.2) and solvent B (water:acetonitrile) at a 50:50 ratio with 10 mM ammonium acetate, pH 8.2. Chromatographic separation was achieved with the following gradient: 0-6% B over 6 min, 6-25% B for 4 min, 25-98% B for 1 min, 98-100% B for 2 min, 100% B for 5.6 min, 100-0.1% B for 0.1 min, 0.1% B for 5.3 min for a total gradient time of 24 min. The source and gas settings were as follows: curtain gas (N_2) pressure, 35 a.u.; CAD gas (N_2) pressure, medium (positive mode) low (negative mode); ion spray voltage, 5.5 kV (positive mode) and −4.5 kV (negative mode); temperature, 550°C; ion source gas 1, 50 a.u.; ion source gas 2, 60 a.u. The compound settings were as follows: declustering potential, 60 V (positive mode) and −200 V (negative mode); entrance potential, 10 V (positive mode) and −10 V (negative mode); collision energy, 43 V (positive mode) and -50 V (negative mode); collision cell exit potential, 15 V (positive mode) and -12 V (negative mode). In order to check the quality of the data, a quality control sample containing an equimolar pool of all patient samples was injected every 12 hours.

5.2.4. Data analysis

Data processing was performed using SCIEX OS™ software for peak integration and post data acquisition analysis. For peak integration the following parameters were used: minimum peak width, 2 points; S/N integration threshold, 2; noise percentage, 80%; baseline subtract window, 2 min; peak splitting, 2 points. Analyte concentrations were calculated as follows:

$$\text{Analyte concentration} = [\text{Analyte area}] / [\text{IS area}] \times [\text{IS concentration}]$$

where IS denotes the internal standard for a given lipid class.[56] Batch correction and partial least squares discriminant analysis (PLS-DA) were performed using Metaboanalyst.[61] Differentially-expressed lipids (p-value <0.05) were determined by student's *t*-test's. Multiple hypothesis testing

using Bonferroni correction [62] was applied to find differentially expressed lipids using an in-house R script.

5.3. Results

Plasma samples (N=113) were obtained from the University of Pittsburgh Alzheimer's Disease Research Center from African American/Black (AD=30, CN=26) and non-Hispanic White (AD=29, CN=28) adults. A detailed description of the sample demographics is provided in **Table 5.1**. The samples had no significant differences in terms of sex, age, and presence of comorbidities such as diabetes, hypertension, and hypercholesterolemia. The average age was 73.6 ± 8.5 years for the four study groups. The mean mini mental state examination (MMSE) scores for the AD groups were lower for the non-Hispanic White samples, compared to the African American/Black samples.

A general overview of the lipidomics workflow is given in **Figure 5.1a**. Internal standards were added to the crude plasma (25 µL) and lipid extraction was performed. Extracted lipids were analyzed using an MRM-based LC-MS/MS analysis, details of which have been described previously.[63-64] In total, the MRM assay contains 1214 lipids from 19 classes specific for human plasma and internal standards (**Figure 5.1b**). In the first step, an unscheduled analysis was performed for each batch to determine the retention times of the lipid species, and also to eliminate any non-detectable lipids from the assay. The unscheduled assay of 1160 lipid species was reduced to 733 based on detection of lipids in all four sample batches. Next, we filtered the data to only include lipids from glycerophospholipid and sphingolipid (sphingomyelin only) classes (i.e. 355 lipids). Finally, we removed lipids with an average %CV >20% across all the samples to generate a final robust set of 329 lipid species (**Figure 5.1b**) for further analysis. Distribution of the lipid

species based on their respective classes is given in **Figure 5.1c**. The majority of these lipids (56%) were phosphatidylethanolamine (PE) and phosphatidylcholine (PC) species. The remaining lipid classes were phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), lysophosphatidycholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylglycerol (LPG), and sphingomyelins (SM) (**Figure 5.1c**).

Next, to eliminate any possible issues due to multiple batch sample preparation, we performed batch correction and assessed the variation of the data (**Figure E5.1**). The internal standards had lower %CV variation (3.5-15.2%) for LPG, PE, PG, PS and SM lipid classes, while PS had the highest variation across the four batches (**Figure E5.1a**). Overall, across all the lipid classes the average %CV was <20%, with LPE, LPG, PE, PG and SM species having the lowest %CV values (3.3-10.8%) among all the lipid classes (**Figure E5.1b**). These CV values demonstrate the robustness of this dataset and gave us a confident set of lipids with which to evaluate changes in AD.

5.3.1. Differentially-expressed lipids in AD

We performed PLS-DA in order to determine if lipids could distinguish AD and CN sample groups (**Figure 5.2a**). The PLS-DA plot shows a clear separation between CN and AD study groups using the lipidomics profile. PLS-DA achieved positive scores for both Q2 and R2 scores (*data not shown*). Next, we compared lipid species concentrations of lipid classes in AD and CN groups (**Figure E5.2**). There was a significant decrease (p-value < 0.05) between AD and CN sample groups for the overall total lipid concentration, as well as for the PC and PS lipid classes. Other classes did not have significant changes in AD.

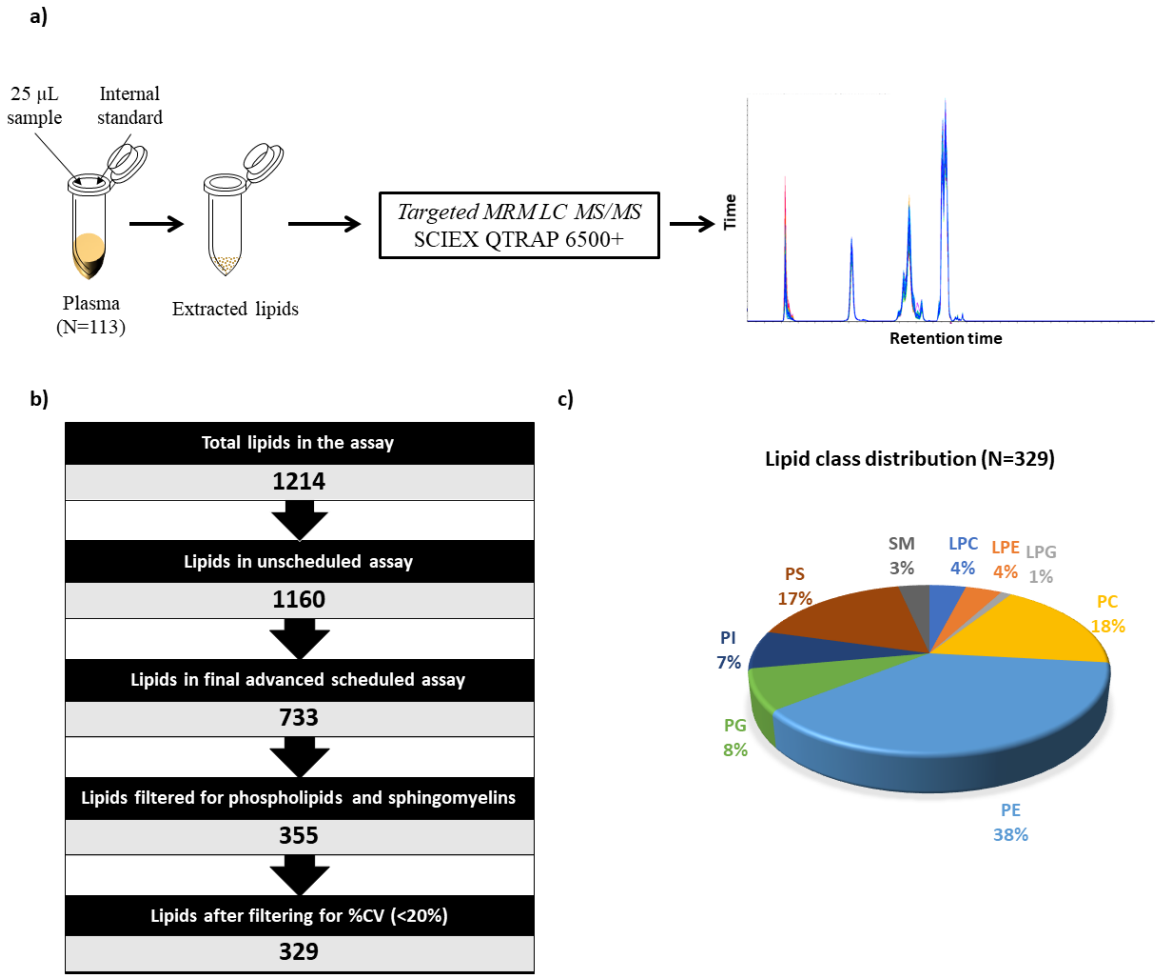

Figure 5.1. Experimental workflow and distribution of lipid classes in lipidomics experiment. **a)** General workflow for plasma lipidomics analysis; lipids are extracted from crude plasma samples and injected into an Exion HPLC system coupled to an Sciex QTRAP 6500+ mass spectrometer for MRM based LC-MS, MS/MS analysis. **b)** Distribution of lipids analyzed in different stages of method development and statistical analysis; **c)** Pie chart showing the distribution of lipid classes in final set of lipids (N=329). Abbreviations: PG- Phosphatidylglycerol, PI- Phosphatidylinositol PS- Phosphatidylserine, PE- Phosphatidylethanolamine, PC- Phosphatidylcholine, LPC- Lysophosphatidylcholine, LPE- Lysophosphatidylethanolamine, LPG- Lysophosphatidyglycerol, SM- Sphingomyelin)

Next, we determined differentially-expressed lipids based on their p- and fold-change values. Initially, 42 lipids were found to be differentially-expressed (p-value < 0.05 and fold-change cut off 1.2) between AD and CN samples. Upon multiple-hypothesis testing, five lipids were differentially-expressed between AD and CN, all of which were decreased in AD samples (**Figure 5.2b**). Example box plots of differentially-expressed lipids in AD are shown in **Figures 5.2c-f**, with their respective structures. These lipid species were PS 18:0_18:0, PS 18:0_20:0, PC 16:0_22:6, PC 18:0_22:6 and PS 18:1_22:6 (**Table 5.2**).

5.3.2. Differentially-expressed lipids in race-stratified AD groups

Lipid data was reanalyzed by PLS-DA, based on racial background stratification of CN and AD groups (**Figure 5.3**). There is a strong overlap between CN racial groups and yet a clear separation between CN and AD groups in general. One strikingly noticeable feature here was the separation between the African American/Black and non-Hispanic White AD groups. In contrast to the CN groups, none of the lipid features overlapped for any African American/Black and non-Hispanic White AD adults. Next, we determined the differentially-expressed lipids between African American/Black CN & AD sample groups, where we started by performing no multiple-hypothesis correction. We found 8 lipids differentially-expressed whereas, there were 33 lipids differentially-expressed for the non-Hispanic White samples in similar comparisons only (**Figure 5.4a**). A detailed list of differentially-expressed lipids specific to both African American/Black and non-Hispanic White groups is given in **Table E5.1**. There were four differentially-expressed lipids in common between the African American/Black and non-Hispanic White groups and they each had similar changes in AD for both African American/Black and non-Hispanic White AD.

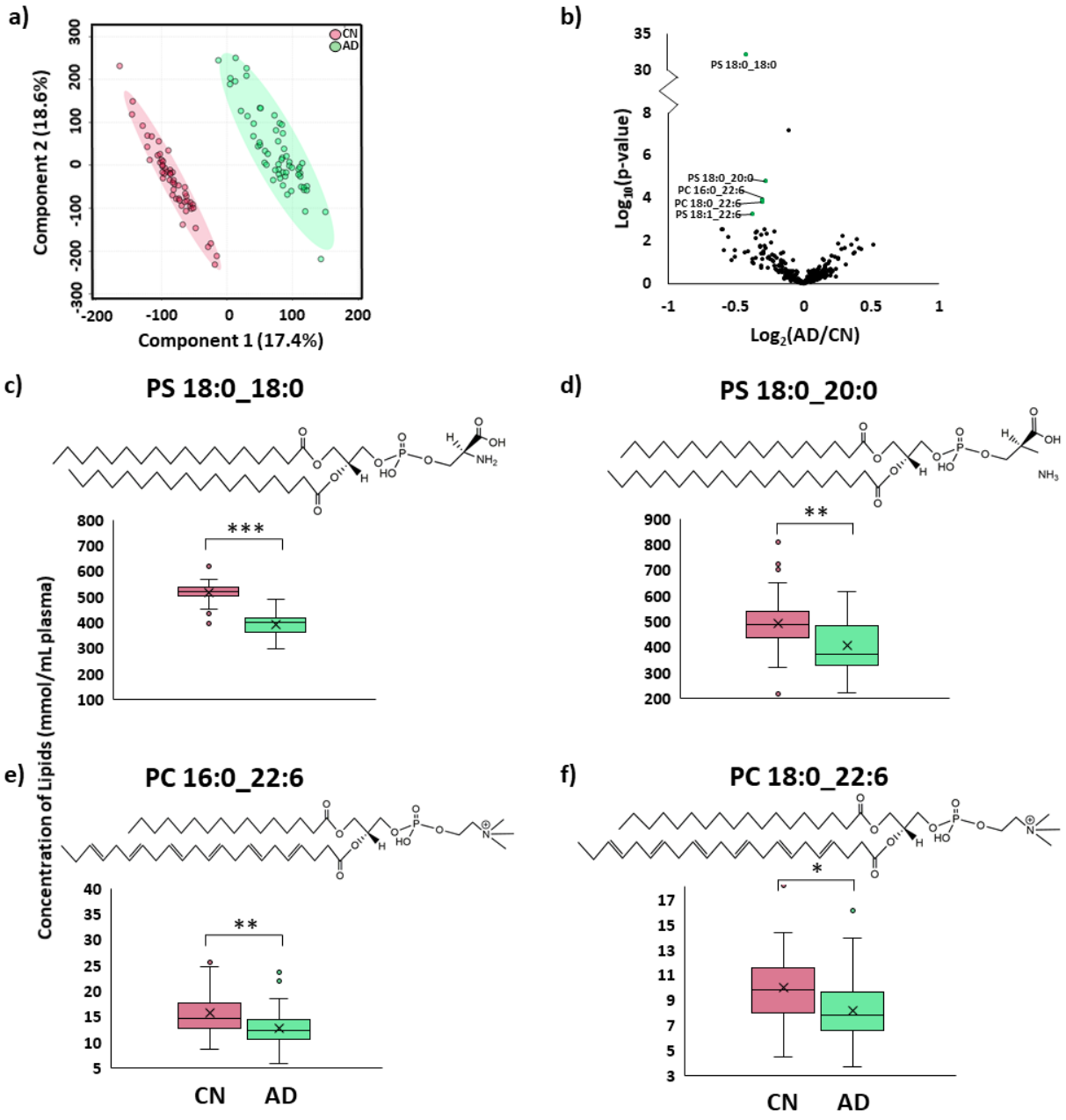

Figure 5.2. **Summary of results from comparison between Alzheimer's disease cognitively normal individuals. a)** PLS-DA plot for the 329 lipids in CN (Red) and AD (Green) groups; **b)** Volcano plot of lipid ratios in AD/CN groups. Significant lipids after multiple hypothesis testing are shown in green and labeled accordingly; **c-f)** Example box plots of differentially-expressed lipids and their corresponding lipid structure.

Table 5.2. Differentially-expressed lipids after multiple hypothesis testing.

Lipid	p-value*	Adjusted p-value**	Fold change#
PS 18:0_18:0	6.92E-33	2.28E-30	0.76
PS 18:0_20:0	1.54E-5	1.69E-3	0.83
PC 16:0_22:6	1.2E-4	9.87E-3	0.81
PC 18:0_22:6	1.57E-4	0.01	0.81
PS 18:1_22:6	5.44E-4	0.03	0.77

Fold change cut off 1.2
* p-value < 0.05
**p-value adjusted using Bonferroni correction

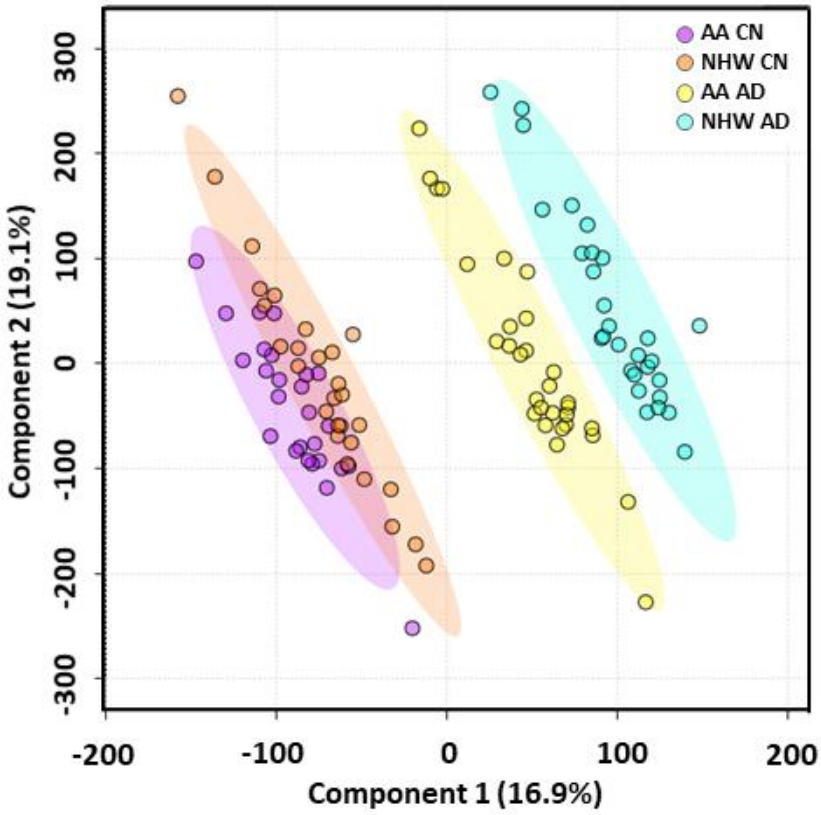

Figure 5.3. PLS-DA plot for the 329 lipids obtained from lipidomics analysis. The plot is demonstrating the separation of the four study groups (African American/Black disease and cognitively normal, non-Hispanic White disease and cognitively normal) in the lipidomics analysis. (Abbreviations: AA CN (Purple)- African American/Black cognitively normal, NHW CN (Orange)- non-Hispanic White cognitively normal, AA AD (Yellow)- African American/Black Alzheimer's disease, NHW CN (Blue)- non-Hispanic White Alzheimer's disease)

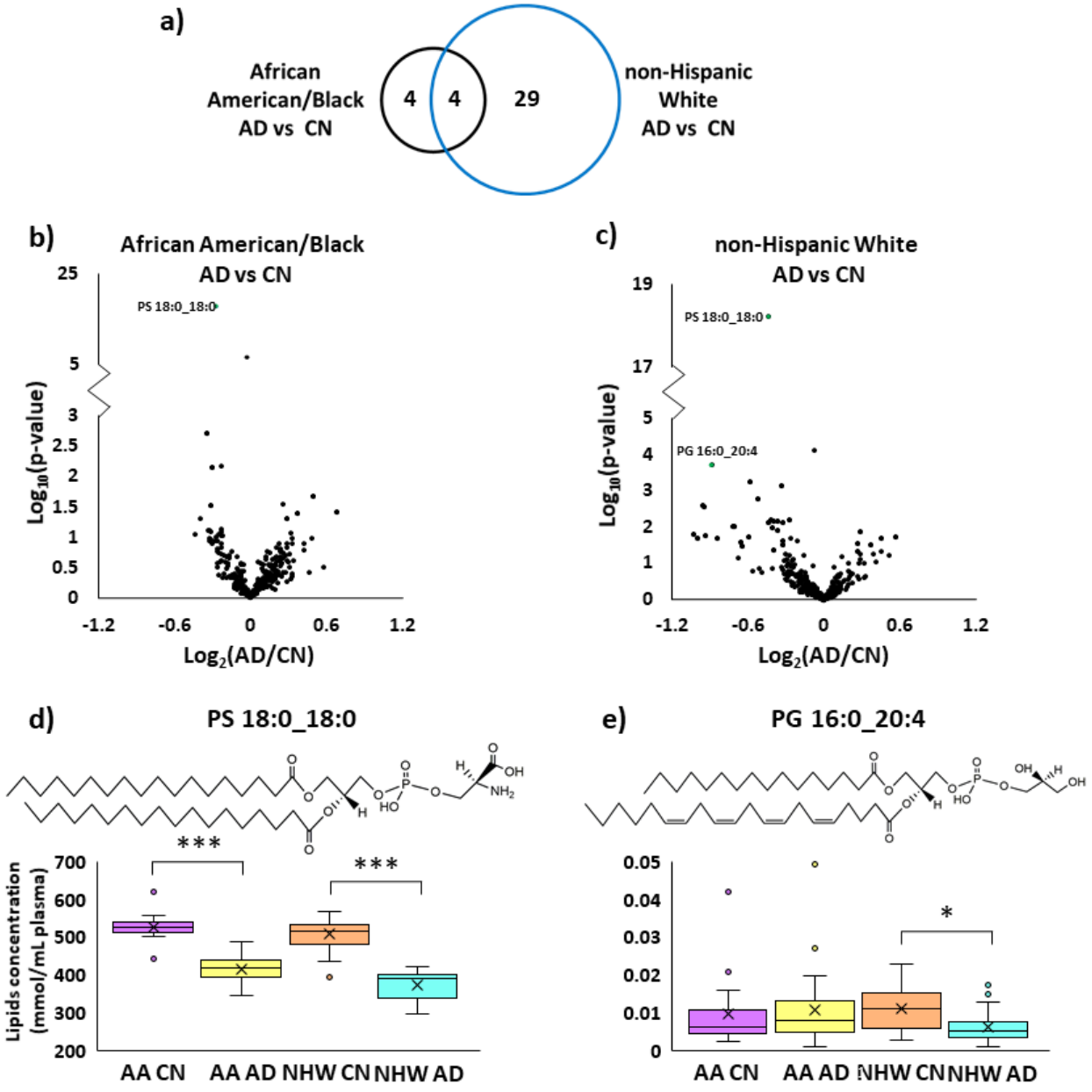

Figure 5.4. a) Venn diagram of differentially-expressed lipids common between African American/Black and non-Hispanic White sample groups after race stratified comparisons; **b & c)** Volcano plot of lipid ratios in AD/CN groups for African American/Black adults only (N= 30 AD, N= 26 CN) and non-Hispanic White adults. Significant lipids after multiple hypothesis testing are shown in green and labeled accordingly; **d & e)** Box plots of lipids after race stratified comparisons

After consideration of adjusted p-values, for the African American/Black sample group, only PS 18:0_18:0 met the criteria for differentially-expressed lipids, while PS 18:0_18:0 and PG 16:0_20:4 were differentially-expressed in the non-Hispanic White groups (**Table 5.3 and Figure 5.4b-e**). These lipids were all reduced in AD.

Table 5.3. Differentially-expressed lipids in race-stratified groups after multiple hypothesis testing.

	African American/Black			***non-Hispanic White***		
	p value*	**Adjusted p value****	**Fold Change#**	**p value***	**Adjusted p value****	**Fold Change#**
PS 18:0_18:0	**2.03E-18**	**6.68E-16**	**0.78**	**6.27E-19**	**2.06E-16**	**0.74**
PG 16:0_20:4	0.66	0.99	1.11	**0.05**	**0.022**	**0.54**

Fold change cut off 1.2
* p-value < 0.05
**p-value adjusted using Bonferroni correction

5.4. Discussion

Here, we conducted an MRM-based targeted lipidomics study on plasma samples obtained from African American/Black and non-Hispanic White adults that were CN or clinically diagnosed with AD. Samples were carefully selected by matching for age, sex and AD diagnosis and as a result there were no significant differences amongst these variables.

We acquired MS data on 1160 lipid species from 19 different lipid classes, and reduced our analysis to 329 lipid species from glycerophospholipid and sphingolipid classes. These classes represent a majority of the reported changes in lipid species in AD such as PC, PE, PG, PS, PI, LPC, LPE, LPG and SM lipid classes, with 38% of the lipids constituting PE species.[32-36, 38-40, 43-44, 46, 65-67] PLS-DA of the lipid species revealed clear separation between CN and AD sample groups, indicating the ability of lipids to distinguish diagnosed cases of AD. For individual lipid classes, PC showed an overall decrease in AD, findings similar to previous studies.[32-33, 35-36, 40, 44, 65-67] In this study, we measured alteration in PS in AD, which has not been previously reported in plasma. After application of strict criteria, we identified 5 lipid species that were decreased in AD. Among them, PS 18:0_18:0 had the largest significance (adjusted p-value = $2.28E10^{-30}$, fold change = 0.76) and represents a novel finding. PC 16:0_22:6 and PC 18:0_22:6 were decreased in our study and supported by others.[32, 36, 66] PS 18:0_20:0 and PC 18:1_22:6 also represent novel findings in this work.

Alteration of phospholipid metabolism in AD has been previously reported.[29] PC accounts for 32.8% of human brain [68] and loss of PC content has been reported in early and late stages of AD [35-36], indicating its critical role in AD progression. Both PC 16:0_22:6 and PC 18:0_22:6 have been correlated to poorer memory performance in nondemented individuals.[69-70] These two lipids along with PC 16:0_20:5 have been well evaluated as potential AD biomarkers in plasma.[36] On

the other hand, involvement of PS in AD has not been as well studied compared to other lipid classes, despite it being a major component of plasma membrane.[27] Reduced PS in the brain cortex from AD mouse model, and increase of PS in brain of AD patients have been reported.[71-73]

A critical design of this study was the inclusion of samples from both African American/Black and non-Hispanic White study groups. It is generally regarded that African American/Black adults have a much healthier lipid profile compared to their non-Hispanic White counterparts.[74] Despite this, African American/Black adults have a higher incidence of AD and other lipid relevant diseases such as diabetes, cardiovascular disease, and hypertension. In this study, the lipidome of African American/Black and non-Hispanic White adults with AD were highly distinguishable whereas CN adults were more similar in both racial backgrounds. It is possible that the distinction is a result of disease heterogeneity, or other underlying factors unique in the racial groups or samples selected for this study. Comparatively lower triglyceride concentration in African American/Black adults has been well reported.[74-75] Additionally, the inability to replicate the lipidomics results of a non-Hispanic White cohort [35], in a cohort of African American/Black participants [49], supports the notion that potential differences in lipidome profiles exist. Knowledge of such differences and environmental, genetic and physiological factors that may contribute to this disparity in lipids [74] is crucial for designing effective biomarker therapies and furthering disease understanding.

In total, there were 37 lipids that were differentially-expressed between AD and CN samples in either African American/Black (N=56) or non-Hispanic White adults (N=57). Among these, four lipids were common between African American/Black and non-Hispanic White adults, including PC 18:0_22:6 reported by others.[32, 36, 66] After multiple hypothesis testing, only PS 18:0_18:0 and PG 16:0_20:4 met the criteria for being significant and only PS 18:0_18:0 was

common between two racial groups. Neither of these lipids has been previously reported to change in AD, and are novel findings. PS 18:0_18:0 has drastically different profiles in CN and AD samples for both racial groups, increasing its potential as a future biomarker in AD.

One of the major strengths of this study is the inclusion of plasma samples from African American/Black adults. General patient demographics were matched within groups. However, improvements could be made to examine social and other factors that may influence lipid profiles in future studies. Despite the challenges associated with obtaining plasma samples from African American/Black adults, our sample size was large enough to monitor changes with statistical confidence.

Another major strength of our study is that we were able to identify differentially-expressed lipids despite using strict criterion for significance. We performed batch correction to account for any variation in sample preparation and data acquisition. Also, we added internal standards specific to each of the lipid classes being studied and to endogenous lipids before extraction to account for any differences due to the extraction procedure. Despite this, further validation of these findings in a larger cohort is necessary to establish any of the lipids as potential biomarkers in AD.

A limitation of this study could be our focus on a subset of lipid classes that have been previously implicated in AD. Although, we added internal standards specific to each lipid class, there were instances such as plasmalogens, where other lipid species specific internal standards would have provided more accurate quantitative information. One possible solution to this could be the use of Lipidyzer^{TM} standards [76], that have over 50 labeled molecular species from 13 different classes. Additionally, some of the lipid classes had higher %CV values and were filtered from our final data analysis steps. Recently, the targeted MRM method has been updated to include

more lipid classes and lipid species.[77] Future studies could focus on ceramides, diacylglycerols, triacylglycerols and cholesterol esters, which were not the subject of this work.

5.5. Conclusions

A limited number of 'omics AD studies involving plasma samples from African American/Black adults exists. This study is one of the first to measure lipidome profiles to better understand change in racial and ethnic disparities in AD. We identified lipid species previously reported by other studies in mostly non-Hispanic White adults, but also identified novel lipid species changing in AD. Plasma lipidome profiles in individuals diagnosed with AD were overall distinct in African American/Black and non-Hispanic White adults. Few differentially-expressed lipids were in common between African American/Black and non-Hispanic White adults but those that were, had a consistent direction of change in AD. Overall, with the increase in incidence of AD among minority groups, this study provides evidence that there is an urgent need for more 'omics research that includes African American/Black and other underrepresented minority population. Additionally, it highlights the potential of lipids for AD biomarker discovery efforts.

5.6. Acknowledgements

This work has been supported by the funding from the Alzheimer's Association (AARGD-17-533405), pilot funds from the University of Pittsburgh Alzheimer Disease Research Center funded by the National Institutes of Health and National Institute on Aging (P50AG005133, RASR), NICHD (R01 HD064727, NAP), the Vanderbilt Institute of Chemical Biology (T32-GM06508), and the National Institute on Aging (AG041718, AG030653, AG064877, MIK). The authors also acknowledge Sciex for an academic partnership award.

5.7. References

1. 2020 Alzheimer's disease facts and figures. *Alzheimer's & Dementia* **2020,** *16* (3), 391-460.

2. Stephen, C. E.; Regina, L. E., Alzheimer's Disease in African Americans: A Preliminary Systematic Review. *Neurol Res Surg* **2020,** *3* (1), 1-3.

3. Chin, A. L.; Negash, S.; Hamilton, R., Diversity and disparity in dementia: the impact of ethnoracial differences in Alzheimer disease. *Alzheimer disease and associated disorders* **2011,** *25* (3), 187-195.

4. Rajan, K. B.; Weuve, J.; Barnes, L. L.; Wilson, R. S.; Evans, D. A., Prevalence and incidence of clinically diagnosed Alzheimer's disease dementia from 1994 to 2012 in a population study. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2019,** *15* (1), 1-7.

5. Matthews, K. A.; Xu, W.; Gaglioti, A. H.; Holt, J. B.; Croft, J. B.; Mack, D.; McGuire, L. C., Racial and ethnic estimates of Alzheimer's disease and related dementias in the United States (2015-2060) in adults aged ≥65 years. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2019,** *15* (1), 17-24.

6. Barnes, L. L.; Leurgans, S.; Aggarwal, N. T.; Shah, R. C.; Arvanitakis, Z.; James, B. D.; Buchman, A. S.; Bennett, D. A.; Schneider, J. A., Mixed pathology is more likely in black than white decedents with Alzheimer dementia. *Neurology* **2015,** *85* (6), 528-534.

7. Wilkins, C. H.; Schindler, S. E.; Morris, J. C., Addressing Health Disparities Among Minority Populations: Why Clinical Trial Recruitment Is Not Enough. *JAMA Neurology* **2020**. *77* (9), 1063-1064.

8. Barnes, L. L.; Wilson, R. S.; Hebert, L. E.; Scherr, P. A.; Evans, D. A.; Mendes de Leon, C. F., Racial differences in the association of education with physical and cognitive function in older blacks and whites. *The journals of gerontology. Series B, Psychological sciences and social sciences* **2011,** *66* (3), 354-363.

9. Manly, J. J.; Schupf, N.; Tang, M. X.; Stern, Y., Cognitive decline and literacy among ethnically diverse elders. *Journal of geriatric psychiatry and neurology* **2005,** *18* (4), 213-7.

10. Sisco, S.; Gross, A. L.; Shih, R. A.; Sachs, B. C.; Glymour, M. M.; Bangen, K. J.; Benitez, A.; Skinner, J.; Schneider, B. C.; Manly, J. J., The role of early-life educational quality and literacy in explaining racial disparities in cognition in late life. *The journals of gerontology. Series B, Psychological sciences and social sciences* **2015,** *70* (4), 557-67.

11. Barnes, L. L.; Bennett, D. A., Alzheimer's disease in African Americans: risk factors and challenges for the future. *Health Aff (Millwood)* **2014,** *33* (4), 580-586.

12. Stepler, K.; Robinson, R., The Potential of 'Omics to Link Lipid Metabolism and Genetic and Comorbidity Risk Factors of Alzheimer's Disease in African Americans. Adv Exp Med Biol. **2019**, *1118*, 1-28.

13. Garrett, S. L.; McDaniel, D.; Obideen, M.; Trammell, A. R.; Shaw, L. M.; Goldstein, F. C.; Hajjar, I., Racial Disparity in Cerebrospinal Fluid Amyloid and Tau Biomarkers and Associated Cutoffs for Mild Cognitive Impairment. *JAMA network open* **2019,** *2* (12), e1917363.

14. Morris, J. C.; Schindler, S. E.; McCue, L. M.; Moulder, K. L.; Benzinger, T. L. S.; Cruchaga, C.; Fagan, A. M.; Grant, E.; Gordon, B. A.; Holtzman, D. M.; Xiong, C., Assessment of Racial Disparities in Biomarkers for Alzheimer Disease. *JAMA neurology* **2019,** *76* (3), 264-273.

15. Wharton, W.; Kollhoff, A. L.; Gangishetti, U.; Verble, D. D.; Upadhya, S.; Zetterberg, H.; Kumar, V.; Watts, K. D.; Kippels, A. J.; Gearing, M.; Howell, J. C.; Parker, M. W.; Hu, W. T., Interleukin 9 alterations linked to alzheimer disease in african americans. *Ann Neurol* **2019,** *86* (3), 407-418.

16. Stepler, K. E.; Mahoney, E. R.; Kofler, J.; Hohman, T. J.; Lopez, O. L.; Robinson, R. A. S., Inclusion of African American/Black adults in a pilot brain proteomics study of Alzheimer's disease. *Neurobiology of Disease* **2020,** *146*, 105129.

17. Geronimus, A. T., The weathering hypothesis and the health of African-American women and infants: evidence and speculations. *Ethnicity & disease* **1992,** *2* (3), 207-21.

18. Forde, A. T.; Crookes, D. M.; Suglia, S. F.; Demmer, R. T., The weathering hypothesis as an explanation for racial disparities in health: a systematic review. *Annals of Epidemiology* **2019,** *33*, 1-18.e3.

19. Forrester, S.; Jacobs, D.; Zmora, R.; Schreiner, P.; Roger, V.; Kiefe, C. I., Racial differences in weathering and its associations with psychosocial stress: The CARDIA study. *SSM - Population Health* **2019,** *7*, 100319.

20. Simons, R. L.; Lei, M.-K.; Klopack, E.; Beach, S. R. H.; Gibbons, F. X.; Philibert, R. A., The effects of social adversity, discrimination, and health risk behaviors on the accelerated aging of African Americans: Further support for the weathering hypothesis. *Social Science & Medicine* **2020**, 113169.

21. Di Paolo, G.; Kim, T.-W., Linking lipids to Alzheimer's disease: cholesterol and beyond. *Nature Reviews Neuroscience* **2011,** *12* (5), 284-296.

22. Hooijmans, C. R.; Kiliaan, A. J., Fatty acids, lipid metabolism and Alzheimer pathology. *European Journal of Pharmacology* **2008,** *585* (1), 176-196.

23. Jones, L.; Harold, D.; Williams, J., Genetic evidence for the involvement of lipid metabolism in Alzheimer's disease. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **2010,** *1801* (8), 754-761.

24. Zhu, T.-B.; Zhang, Z.; Luo, P.; Wang, S.-S.; Peng, Y.; Chu, S.-F.; Chen, N.-H., Lipid metabolism in Alzheimer’s disease. *Brain Research Bulletin* **2019,** *144*, 68-74.

25. Wood, P. L., Lipidomics of Alzheimer's disease: current status. *Alzheimers Res Ther* **2012,** *4* (1), 5.

26. Wong, M. W.; Braidy, N.; Poljak, A.; Pickford, R.; Thambisetty, M.; Sachdev, P. S., Dysregulation of lipids in Alzheimer's disease and their role as potential biomarkers. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2017,** *13* (7), 810-827.

27. Touboul, D.; Gaudin, M., Lipidomics of Alzheimer's disease. *Bioanalysis* **2014,** *6* (4), 541-61.

28. Lim, W. L.; Martins, I. J.; Martins, R. N., The involvement of lipids in Alzheimer's disease. *Journal of genetics and genomics = Yi chuan xue bao* **2014,** *41* (5), 261-74.

29. Kosicek, M.; Hecimovic, S., Phospholipids and Alzheimer's disease: alterations, mechanisms and potential biomarkers. *Int J Mol Sci* **2013,** *14* (1), 1310-1322.

30. Han, X., *Lipidomics: Comprehensive mass spectrometry of lipids*. John Wiley & Sons: **2016**.

31. Costa, A. C.; Joaquim, H. P. G.; Forlenza, O.; Talib, L. L.; Gattaz, W. F., Plasma lipids metabolism in mild cognitive impairment and Alzheimer's disease. *The World Journal of Biological Psychiatry* **2019,** *20* (3), 190-196.

32. González-Domínguez, R.; García-Barrera, T.; Gómez-Ariza, J. L., Combination of metabolomic and phospholipid-profiling approaches for the study of Alzheimer's disease. *Journal of Proteomics* **2014,** *104*, 37-47.

33. González-Domínguez, R.; García-Barrera, T.; Gómez-Ariza, J. L., Metabolomic study of lipids in serum for biomarker discovery in Alzheimer's disease using direct infusion mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* **2014,** *98*, 321-326.

34. Klavins, K.; Koal, T.; Dallmann, G.; Marksteiner, J.; Kemmler, G.; Humpel, C., The ratio of phosphatidylcholines to lysophosphatidylcholines in plasma differentiates healthy controls from patients with Alzheimer's disease and mild cognitive impairment. *Alzheimers Dement (Amst)* **2015,** *1* (3), 295-302.

35. Mapstone, M.; Cheema, A. K.; Fiandaca, M. S.; Zhong, X.; Mhyre, T. R.; MacArthur, L. H.; Hall, W. J.; Fisher, S. G.; Peterson, D. R.; Haley, J. M.; Nazar, M. D.; Rich, S. A.; Berlau, D. J.; Peltz, C. B.; Tan, M. T.; Kawas, C. H.; Federoff, H. J., Plasma phospholipids identify antecedent memory impairment in older adults. *Nature medicine* **2014,** *20* (4), 415-8.

36. Whiley, L.; Sen, A.; Heaton, J.; Proitsi, P.; García-Gómez, D.; Leung, R.; Smith, N.; Thambisetty, M.; Kloszewska, I.; Mecocci, P.; Soininen, H.; Tsolaki, M.; Vellas, B.; Lovestone, S.; Legido-Quigley, C.; AddNeuroMed, C., Evidence of altered phosphatidylcholine metabolism in Alzheimer's disease. *Neurobiology of aging* **2014,** *35* (2), 271-278.

37. Oresic, M.; Hyotylainen, T.; Herukka, S.; Sysi-Aho, M.; Mattila, I.; Seppanan-Laakso, T.; Julkunen, V.; Gopalacharyulu, P.; Hallikainen, M.; Koikkalainen, J. *Metabolome in progression to Alzheimer's disease. Transl Psychiatry.* **2011,** *1*, e57.

38. Goodenowe, D. B.; Cook, L. L.; Liu, J.; Lu, Y.; Jayasinghe, D. A.; Ahiahonu, P. W.; Heath, D.; Yamazaki, Y.; Flax, J.; Krenitsky, K. F.; Sparks, D. L.; Lerner, A.; Friedland, R. P.; Kudo, T.; Kamino, K.; Morihara, T.; Takeda, M.; Wood, P. L., Peripheral ethanolamine plasmalogen deficiency: a logical causative factor in Alzheimer's disease and dementia. *J Lipid Res* **2007,** *48* (11), 2485-98.

39. Li, N.-j.; Liu, W.-t.; Li, W.; Li, S.-q.; Chen, X.-h.; Bi, K.-s.; He, P., Plasma metabolic profiling of Alzheimer's disease by liquid chromatography/mass spectrometry. *Clinical biochemistry* **2010,** *43* (12), 992-997.

40. Olazarán, J.; Gil-de-Gómez, L.; Rodríguez-Martín, A.; Valentí-Soler, M.; Frades-Payo, B.; Marín-Muñoz, J.; Antúnez, C.; Frank-García, A.; Acedo-Jiménez, C.; Morlán-Gracia, L.; Petidier-Torregrossa, R.; Guisasola, M. C.; Bermejo-Pareja, F.; Sánchez-Ferro, Á.; Pérez-Martínez, D. A.; Manzano-Palomo, S.; Farquhar, R.; Rábano, A.; Calero, M., A blood-based, 7-metabolite signature for the early diagnosis of Alzheimer's disease. *Journal of Alzheimer's disease : JAD* **2015,** *45* (4), 1157-73.

41. Wood, P.; Phillipps, A.; Woltjer, R. L.; Kaye, J.; Quinn, J., Increased lysophosphatidylethanolamine and diacylglycerol levels in Alzheimer's disease plasma. *JSM Alzheimer's Disease and Related Dementia* **2014,** *1*.

42. González-Domínguez, R.; García-Barrera, T.; Gómez-Ariza, J. L., Application of a novel metabolomic approach based on atmospheric pressure photoionization mass spectrometry using flow injection analysis for the study of Alzheimer′s disease. *Talanta* **2015,** *131*, 480-489.

43. Han, X.; Rozen, S.; Boyle, S. H.; Hellegers, C.; Cheng, H.; Burke, J. R.; Welsh-Bohmer, K. A.; Doraiswamy, P. M.; Kaddurah-Daouk, R., Metabolomics in early Alzheimer's disease: identification of altered plasma sphingolipidome using shotgun lipidomics. *PLoS One* **2011,** *6* (7), e21643.

44. Kim, M.; Nevado-Holgado, A.; Whiley, L.; Snowden, S. G.; Soininen, H.; Kloszewska, I.; Mecocci, P.; Tsolaki, M.; Vellas, B.; Thambisetty, M.; Dobson, R. J. B.; Powell, J. F.; Lupton, M. K.; Simmons, A.; Velayudhan, L.; Lovestone, S.; Proitsi, P.; Legido-Quigley, C., Association between Plasma Ceramides and Phosphatidylcholines and Hippocampal Brain Volume in Late Onset Alzheimer's Disease. *Journal of Alzheimer's disease : JAD* **2017,** *60* (3), 809-817.

45. Mielke, M. M.; Haughey, N. J.; Bandaru, V. V. R.; Schech, S.; Carrick, R.; Carlson, M. C.; Mori, S.; Miller, M. I.; Ceritoglu, C.; Brown, T.; Albert, M.; Lyketsos, C. G., Plasma ceramides are altered in mild cognitive impairment and predict cognitive decline and hippocampal volume loss. *Alzheimer's & Dementia* **2010,** *6* (5), 378-385.

46. Anand, S.; Barnes, J. M.; Young, S. A.; Garcia, D. M.; Tolley, H. D.; Kauwe, J. S. K.; Graves, S. W., Discovery and Confirmation of Diagnostic Serum Lipid Biomarkers for

Alzheimer's Disease Using Direct Infusion Mass Spectrometry. *Journal of Alzheimer's disease : JAD* **2017,** *59* (1), 277-290.

47. Wood, P. L.; Locke, V. A.; Herling, P.; Passaro, A.; Vigna, G. B.; Volpato, S.; Valacchi, G.; Cervellati, C.; Zuliani, G., Targeted lipidomics distinguishes patient subgroups in mild cognitive impairment (MCI) and late onset Alzheimer's disease (LOAD). *BBA Clinical* **2016,** *5*, 25-28.

48. Wood, P. L.; Medicherla, S.; Sheikh, N.; Terry, B.; Phillipps, A.; Kaye, J. A.; Quinn, J. F.; Woltjer, R. L., Targeted lipidomics of fontal cortex and plasma diacylglycerols (DAG) in mild cognitive impairment and Alzheimer's disease: validation of DAG accumulation early in the pathophysiology of Alzheimer's disease. *Journal of Alzheimer's Disease* **2015,** *48* (2), 537-546.

49. Li, D.; Misialek, J. R.; Boerwinkle, E.; Gottesman, R. F.; Sharrett, A. R.; Mosley, T. H.; Coresh, J.; Wruck, L. M.; Knopman, D. S.; Alonso, A., Prospective associations of plasma phospholipids and mild cognitive impairment/dementia among African Americans in the ARIC Neurocognitive Study. *Alzheimers Dement (Amst)* **2016,** *6*, 1-10.

50. Buie, J. N. J.; Hammad, S. M.; Nietert, P. J.; Magwood, G.; Adams, R. J.; Bonilha, L.; Sims-Robinson, C., Differences in plasma levels of long chain and very long chain ceramides between African Americans and whites: An observational study. *PLoS One* **2019,** *14* (5), e0216213-e0216213.

51. Halade, G. V.; Kain, V.; Dillion, C.; Beasley, M.; Dudenbostel, T.; Oparil, S.; Limdi, N. A., Race-based and sex-based differences in bioactive lipid mediators after myocardial infarction. *ESC Heart Failure* **2020,** *7* (4), 1700-1710.

52. Vantaku, V.; Donepudi, S. R.; Piyarathna, D. W. B.; Amara, C. S.; Ambati, C. R.; Tang, W.; Putluri, V.; Chandrashekar, D. S.; Varambally, S.; Terris, M. K.; Davies, K.; Ambs, S.; Bollag, R.; Apolo, A. B.; Sreekumar, A.; Putluri, N., Large-scale profiling of serum metabolites in African American and European American patients with bladder cancer reveals metabolic pathways associated with patient survival. *Cancer* **2019,** *125* (6), 921-932.

53. Zhou, X.; Mei, H.; Agee, J.; Brown, T.; Mao, J., Racial differences in distribution of fatty acids in prostate cancer and benign prostatic tissues. *Lipids in health and disease* **2019,** *18* (1), 189.

54. Bentley, A. R.; Rotimi, C. N., Interethnic Differences in Serum Lipids and Implications for Cardiometabolic Disease Risk in African Ancestry Populations. *Global heart* **2017,** *12* (2), 141-150.

55. Hammad, S. M.; Hardin, J. R.; Wilson, D. A.; Twal, W. O.; Nietert, P. J.; Oates, J. C., Race disparity in blood sphingolipidomics associated with lupus cardiovascular comorbidity. *PLoS One* **2019,** *14* (11), e0224496.

56. Khan, M. J.; Codreanu, S. G.; Goyal, S.; Wages, P. A.; Gorti, S. K. K.; Pearson, M. J.; Uribe, I.; Sherrod, S. D.; McLean, J. A.; Porter, N. A.; Robinson, R. A. S., Evaluating a targeted

multiple reaction monitoring approach to global untargeted lipidomic analyses of human plasma. *Rapid Communications in Mass Spectrometry* **2020,** *34* (22), e8911.

57. Jack, C. R., Jr.; Albert, M. S.; Knopman, D. S.; McKhann, G. M.; Sperling, R. A.; Carrillo, M. C.; Thies, B.; Phelps, C. H., Introduction to the recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2011,** *7* (3), 257-62.

58. Albert, M. S.; DeKosky, S. T.; Dickson, D.; Dubois, B.; Feldman, H. H.; Fox, N. C.; Gamst, A.; Holtzman, D. M.; Jagust, W. J.; Petersen, R. C.; Snyder, P. J.; Carrillo, M. C.; Thies, B.; Phelps, C. H., The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2011,** *7* (3), 270-9.

59. McKhann, G. M.; Knopman, D. S.; Chertkow, H.; Hyman, B. T.; Jack, C. R., Jr.; Kawas, C. H.; Klunk, W. E.; Koroshetz, W. J.; Manly, J. J.; Mayeux, R.; Mohs, R. C.; Morris, J. C.; Rossor, M. N.; Scheltens, P.; Carrillo, M. C.; Thies, B.; Weintraub, S.; Phelps, C. H., The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2011,** *7* (3), 263-9.

60. Sperling, R. A.; Aisen, P. S.; Beckett, L. A.; Bennett, D. A.; Craft, S.; Fagan, A. M.; Iwatsubo, T.; Jack, C. R., Jr.; Kaye, J.; Montine, T. J.; Park, D. C.; Reiman, E. M.; Rowe, C. C.; Siemers, E.; Stern, Y.; Yaffe, K.; Carrillo, M. C.; Thies, B.; Morrison-Bogorad, M.; Wagster, M. V.; Phelps, C. H., Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & dementia : the journal of the Alzheimer's Association* **2011,** *7* (3), 280-92.

61. Chong, J.; Wishart, D. S.; Xia, J., Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. *Current Protocols in Bioinformatics* **2019,** *68* (1), e86.

62. Haynes, W., Bonferroni Correction. In *Encyclopedia of Systems Biology*, Dubitzky, W.; Wolkenhauer, O.; Cho, K.-H.; Yokota, H., Eds. Springer New York: New York, NY, **2013**; pp 154-154.

63. Gorti SKK, M. D., Fu L In *HILIC LC-MSMS assay for high-throughput targeted lipdomics analysis. Lipidomics Forum*, Lipidomics Forum, Dortmund, Germany, Dortmund, Germany, **2018**.

64. Wang J, F. L., Gorti SKK In *Development of an Amide Column-Based LC-MS/MS Method for High-Throughput Targeted Lipidomics*, ASMS Conference on Mass Spectrometry and Allied Topics, San Diego, CA, San Diego, CA, **2018**.

65. Fiandaca, M. S.; Zhong, X.; Cheema, A. K.; Orquiza, M. H.; Chidambaram, S.; Tan, M. T.; Gresenz, C. R.; FitzGerald, K. T.; Nalls, M. A.; Singleton, A. B.; Mapstone, M.; Federoff, H.

J., Plasma 24-metabolite Panel Predicts Preclinical Transition to Clinical Stages of Alzheimer's Disease. *Front Neurol* **2015,** *6*, 237-237.

66. Proitsi, P.; Kim, M.; Whiley, L.; Pritchard, M.; Leung, R.; Soininen, H.; Kloszewska, I.; Mecocci, P.; Tsolaki, M.; Vellas, B.; Sham, P.; Lovestone, S.; Powell, J. F.; Dobson, R. J. B.; Legido-Quigley, C., Plasma lipidomics analysis finds long chain cholesteryl esters to be associated with Alzheimer's disease. *Transl Psychiatry* **2015,** *5* (1), e494-e494.

67. Proitsi, P.; Kim, M.; Whiley, L.; Simmons, A.; Sattlecker, M.; Velayudhan, L.; Lupton, M. K.; Soininen, H.; Kloszewska, I.; Mecocci, P.; Tsolaki, M.; Vellas, B.; Lovestone, S.; Powell, J. F.; Dobson, R. J. B.; Legido-Quigley, C., Association of blood lipids with Alzheimer's disease: A comprehensive lipidomics analysis. *Alzheimer's & Dementia* **2017,** *13* (2), 140-151.

68. O'Brien, J. S.; Sampson, E. L., Fatty acid and fatty aldehyde composition of the major brain lipids in normal human gray matter, white matter, and myelin. *J Lipid Res* **1965,** *6* (4), 545-551.

69. Simpson, B. N.; Kim, M.; Chuang, Y. F.; Beason-Held, L.; Kitner-Triolo, M.; Kraut, M.; Lirette, S. T.; Windham, B. G.; Griswold, M. E.; Legido-Quigley, C.; Thambisetty, M., Blood metabolite markers of cognitive performance and brain function in aging. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* **2016,** *36* (7), 1212-23.

70. Wong, M. W.; Braidy, N.; Poljak, A.; Sachdev, P. S., The application of lipidomics to biomarker research and pathomechanisms in Alzheimer's disease. *Current Opinion in Psychiatry* **2017,** *30* (2), 136-144.

71. Farooqui, A. A.; Rapoport, S. I.; Horrocks, L. A., Membrane phospholipid alterations in Alzheimer's disease: deficiency of ethanolamine plasmalogens. *Neurochem Res* **1997,** *22* (4), 523-7.

72. Wells, K.; Farooqui, A. A.; Liss, L.; Horrocks, L. A., Neural membrane phospholipids in alzheimer disease. *Neurochemical Research* **1995,** *20* (11), 1329-1333.

73. Yao, J. K.; Wengenack, T. M.; Curran, G. L.; Poduslo, J. F., Reduced membrane lipids in the cortex of Alzheimer's disease transgenic mice. *Neurochem Res* **2009,** *34* (1), 102-8.

74. Bentley, A. R.; Rotimi, C. N., Interethnic Variation in Lipid Profiles: Implications for Underidentification of African-Americans at risk for Metabolic Disorders. *Expert Rev Endocrinol Metab* **2012,** *7* (6), 659-667.

75. Chang, M. H.; Ned, R. M.; Hong, Y.; Yesupriya, A.; Yang, Q.; Liu, T.; Janssens, A. C.; Dowling, N. F., Racial/ethnic variation in the association of lipid-related genetic variants with blood lipids in the US adult population. *Circulation. Cardiovascular genetics* **2011,** *4* (5), 523-33.

76. Contrepois, K.; Mahmoudi, S.; Ubhi, B. K.; Papsdorf, K.; Hornburg, D.; Brunet, A.; Snyder, M., Cross-Platform Comparison of Untargeted and Targeted Lipidomics Approaches on Aging Mouse Plasma. *Scientific reports* **2018,** *8* (1), 17747.

77. Pearson, M.; Kapil, S. K.; Wu, S.; Baker, P., Achieve broad lipid quantitation using a high-throughput targeted lipidomics method. *SCIEX* **2018**.

CHAPTER VI

Conclusions and Future Directions

Here, ‘omics techniques were employed on human plasma samples to study Alzheimer’s disease, and discover potential biomarkers of Alzheimer’s disease (AD). In **Chapter II**, quantitative proteomics was used to discover biomarkers for AD with the use of multiplexing technologies. We also discovered proteins, that were specifically dysregulated in AD and tested their utility as potential biomarker candidates for disease diagnosis. Our analysis revealed that the protein panels achieved a higher accuracy as biomarkers for AD for non-Hispanic White sample groups, compared to African American/Black sample groups. The accuracy of the biomarkers improved considerably when coupled with clinical variables (age, sex, years of education and *APOE* status) for the non-Hispanic White samples only, which points to the need for inclusion of other variables that may be more relevant for the African American/Black sample group. Furthermore, these studies highlight the importance of diversity in the make-up of future sample cohorts in AD research. In order to study lipidomic changes in AD, a comparison between an untargeted approach and a novel targeted multiple reaction monitoring (MRM) based approach has been discussed in **Chapter III**. With its high reproducibility and molecular level identification of lipids, the targeted MRM based approach was demonstrated as a highly robust method, and an ideal alternative to the untargeted lipidomics approach for the analysis of human plasma. This method and an untargeted lipidomics approach were applied in a pilot study of AD in **Chapter IV**, along with a targeted selective reaction monitoring (SRM) approach. Overall, there was a decrease in phosphatidylcholine (PC) and sphingomyelins (SM), while ceramides and plasmalogens increased in AD samples compared to cognitively normal (CN) samples using the

untargeted and targeted MRM approaches. Also, there was a significant decrease in plasma cholesterol levels in AD, although that result did not replicate in a larger sample cohort which highlights the necessity for large sample sizes in these 'omics studies. Overall, the results in **Chapter IV** had good overlap with previous studies in AD and demonstrated the benefits of lipidomics in the discovery stage of AD research. In order to study these changes in depth, a larger cohort of plasma samples were studied using a targeted MRM approach in **Chapter V**. The study was able to identify previously reported lipids (PC 16:0_22:6 and PC 18:0_22:6), as well novel lipid class (phosphatidylserine-PS) and species (PS 18:0_18:0, PS 18:0_20:0, PS 18:1_22:6) changing in AD plasma. Specifically, changes to phosphatidylserine species in plasma have not been reported previously. We examined the effect of race in determining differential expression of lipids in AD, and found lipids whose expression in AD was unique to non-Hispanic White or African American/Black racial groups. These findings were similar to protein results in **Chapter II**, and point to possible molecular differences between African American/Black and non-Hispanic White AD cases that can impact discovery efforts. This dissertation work points to the need for more inclusion in AD research, especially more studies focusing on African American/Black and other minority groups.

6.2. Future Directions

Throughout this dissertation, omics' approaches have been applied to plasma samples to identify differentially-expressed proteins (**Chapter II**) or lipids (**Chapter V**) in AD to be used as potential diagnostic biomarkers. Further analysis is required to replicate and validate these as potential biomarkers in AD. In the case of differentially-expressed proteins, we have proposed a panel of proteins having good accuracy in determining AD in non-Hispanic White individuals. Immunoassays targeting these proteins or even a targeted mass spectrometric analysis in a larger

cohort is necessary to validate these findings. Although our study demonstrated lower accuracy of these proteins in African American/Black AD individuals, one possible reason for this could have been related to the number of differentially-expressed proteins that we applied in the machine learning algorithm which was lower, compared to the number of proteins identified. This issue could be resolved by modifying the existing plasma proteomics workflow to include liquid chromatography (LC) based high pH fractionation instead of a solid-phase extraction fractionation approach. Recent publications have reported over 2000 proteins being identified from plasma proteins using a combination of immunodepletion, higher number of fractions in high pH fractionation step, and MS^2 quantification.[1] Improvements in protein coverage and high reproducibility of those measurements should result in higher numbers of statistically significant proteins that would change in AD and be fed into the machine learning models for determining diagnostic accuracy of biomarker panels. Additionally, analysis of larger sample sizes and samples from different cohorts in each group would help to determine how generalizable these results are in the groups. Another possible path could be the implementation of data independent acquisition in the mass spectrometry (MS) analysis, which has shown promise in plasma proteomics.[2-3] As for biomarker panels specific to African American/Black adults, very little is known about how basic science contributes to or is impacted by racial disparities in AD amongst African American/Black adults. So, more such studies focusing on African American/Black and other underrepresented minority populations is necessary to better understand the heterogeneity of disease pathology. Also, one aspect that was clear from our study was that common variables (age/sex/education/*APOE* status) did not improve biomarker accuracy in samples from African American/Black adults in AD. So, inclusion of other variables such as genetic markers, quality of

education, environmental stress, and measures of discrimination could be helpful in achieving a better biomarker panel for African American/Black adults and likely other groups as well.

We have reported in **Chapter V** a list of lipids that are differentially-expressed in AD from a targeted lipidomics analysis. One such lipid, PS 18:0_18:0 had drastically different profiles in AD and CN individuals. Also, we identified other phosphatidylserines as potential biomarkers in AD, yet little is known about the mechanisms of this lipid class in AD pathogenesis. Thus, a targeted study involving phosphatidylserines on a larger cohort sample could be beneficial, to understand the effects of this class in AD pathogenesis. To accomplish this, the same targeted lipidomics approach employed in this dissertation can be used to target the phosphatidylserine species in a larger sample cohort, with more class specific internal standards having varying fatty acid compositions being employed in order to achieve more accurate quantitative information from the analysis. Assessment of phosphatidylserine species, specifically PS 18:0_18:0 as a potential biomarker for AD can be accomplished by constructing ROC curves in order to calculate their diagnostic accuracies either as a single biomarker or as a combination of multiple species. In addition to this, pathway analysis could also reveal specific pathways the lipids of interest are involved in. Phosphatidylserines are known to reduce with age, affecting memory and cognition ability. There has been evidence of phosphatidylserines improving cognition, specifically providing phosphatidylserine supplements have shown to improve cognition in early stages of AD patients.[4-6] The underlying mechanisms of these actions are still unknown and requires further research. Pathway analysis involving lipid is still improving, and all the known pathway mapping software's only provide information at the class level.[7] Further research is ongoing to include specific lipid species in pathway analysis.[8] The targeted experiment proposed above can be used

as a pilot study to understand the involvement of phosphatidylserines in improving cognition, until more in-depth pathway analysis in available.

Other lipid species of interest in this study are PC 16:0_22:6 and PC 18:0_22:6, which have been reported to be reduced in AD by others. As a result, these two lipid species have potential to be considered as AD biomarker candidates. Further follow up could involve validating the findings from this study on a larger sample cohort. At the same time, machine learning analyses similar to those reported in **Chapter II**, would demonstrate the effectiveness of the lipids as potential diagnostic biomarkers. Integration of the proteomics and lipidomics network analysis has revealed involvement of lipid and immunity pathways with AD in a recent study [9], similar analysis using the findings from the proteomics and lipidomics analysis can be employed to further study AD pathogenesis.

Although the targeted MRM based lipidomics method is highly reproducible for measuring glycerophospholipids as demonstrated in **Chapters IV & V**, other classes had higher levels of variation in their measurements. This is due to the inadequate separation of the glycerolipids using amide based hydrophilic interaction liquid chromatography (HILIC). To eliminate this issue, Sciex recently updated the existing method, to include a new NH_2 based column [10], which also separates the glycerolipids, as well as the remaining lipid classes with similar reproducibility as the previous method had achieved. This improved approach could be employed for future studies.

The work completed in this dissertation has been focused to plasma. AD is a neurodegenerative disease, therefore understanding the link between changes being monitored in plasma and brain would be ideal and further the search for potential AD biomarkers. For example, analysis of cerebrospinal fluid (CSF) and plasma samples from the same patients could be used to monitor possible changes due to AD. Recent studies on CSF have indicated possible racial

disparities in CSF biomarkers for AD, indicating the need to understand the complexities of AD in diverse groups.[11-12] One possible study of direction could be a targeted experiment for proteins related to lipid metabolism. Previous studies have targeted lipid metabolism-related proteins in either CSF or plasma using targeted MS analysis.[13-15] Most of these studies focused on primarily apolipoproteins, as these proteins have been consistently implicated in AD pathogenesis. One approach could be to target the entire lipid metabolism pathway in a longitudinal study, instead of focusing on a few proteins at a time, which would provide a more comprehensive picture of AD. With the increasing evidence of involvement of altered lipid metabolism in AD, this study would be able to reveal specific protein changes due to disease progression. Very few studies to date have evaluated the relationship between changes in CSF and plasma using the same cohort. Monitoring changes happening in both plasma and CSF, we would be able to link the changes together, and uncover more mechanisms about the pathogenesis that might help with the fight against AD.

Despite all the biomarker studies involving plasma, the biggest obstacle to date has been the lack in reproducibility across studies from different research groups, which is true for both proteins and lipids. Possible explanations to this could be the apparent differences in plasma itself, as well differences in conditions of blood draw and sample collection protocols. Also, standardized analytical protocols across research groups do not exist, and as we showed in **Chapter II**, slight differences to the digestion protocols could impact protein detection and replication. Also, the disease stages and presence of other dementias or comorbidities can have a major effect on the findings. As a result, more standardized protocols from the collection of blood in study cohorts to the MS detection methods are necessary for advancing future biomarker efforts in AD.

6.3. References

1. Zhou, B.; Zhou, Z.; Chen, Y.; Deng, H.; Cai, Y.; Rao, X.; Yin, Y.; Rong, L., Plasma proteomics-based identification of novel biomarkers in early gastric cancer. *Clinical Biochemistry* **2020,** *76*, 5-10.

2. Nigjeh, E. N.; Chen, R.; Brand, R. E.; Petersen, G. M.; Chari, S. T.; von Haller, P. D.; Eng, J. K.; Feng, Z.; Yan, Q.; Brentnall, T. A.; Pan, S., Quantitative Proteomics Based on Optimized Data-Independent Acquisition in Plasma Analysis. *Journal of proteome research* **2017,** *16* (2), 665-676.

3. Zhang, S.; Raedschelders, K.; Venkatraman, V.; Huang, L.; Holewinski, R.; Fu, Q.; Van Eyk, J. E., A Dual Workflow to Improve the Proteomic Coverage in Plasma Using Data-Independent Acquisition-MS. *Journal of Proteome Research* **2020,** *19* (7), 2828-2837.

4. Crook, T.; Petrie, W., Effects of Phosphatidylserine in. *Psychopharmacology Bulletin* **1966**, 61.

5. Heiss, W. D.; Kessler, J.; Mielke, R.; Szelies, B.; Herholz, K., Long-Term Effects of Phosphatidylserine, Pyritinol, and Cognitive Training in Alzheimer's Disease. *Dementia and geriatric cognitive disorders* **1994,** *5* (2), 88-98.

6. Zhang, Y.; Yang, L.; Guo, L., Effect of phosphatidylserine on memory in patients and rats with Alzheimer's disease. *Genet Mol Res* **2015,** *14* (3), 9325-9333.

7. Züllig, T.; Trötzmüller, M.; Köfeler, H. C., Lipidomics from sample preparation to data analysis: a primer. *Analytical and Bioanalytical Chemistry* **2020,** *412* (10), 2191-2209.

8. Han, X., *Lipidomics: Comprehensive mass spectrometry of lipids*. John Wiley & Sons: 2016.

9. Xu, J.; Bankov, G.; Kim, M.; Wretlind, A.; Lord, J.; Green, R.; Hodges, A.; Hye, A.; Aarsland, D.; Velayudhan, L.; Dobson, R. J. B.; Proitsi, P.; Legido-Quigley, C.; on behalf of the AddNeuroMed, C., Integrated lipidomics and proteomics network analysis highlights lipid and immunity pathways associated with Alzheimer's disease. *Translational Neurodegeneration* **2020,** *9* (1), 36.

10. Pearson, M.; Kapil, S. K.; Wu, S.; Baker, P., Achieve broad lipid quantitation using a high-throughput targeted lipidomics method. *SCIEX* **2018**.

11. Garrett, S. L.; McDaniel, D.; Obideen, M.; Trammell, A. R.; Shaw, L. M.; Goldstein, F. C.; Hajjar, I., Racial Disparity in Cerebrospinal Fluid Amyloid and Tau Biomarkers and Associated Cutoffs for Mild Cognitive Impairment. *JAMA network open* **2019,** *2* (12), e1917363-e1917363.

12. Morris, J. C.; Schindler, S. E.; McCue, L. M.; Moulder, K. L.; Benzinger, T. L. S.; Cruchaga, C.; Fagan, A. M.; Grant, E.; Gordon, B. A.; Holtzman, D. M.; Xiong, C., Assessment of Racial Disparities in Biomarkers for Alzheimer Disease. *JAMA Neurol* **2019,** *76* (3), 264-273.

13. Begcevic, I.; Brinc, D.; Brown, M.; Martinez-Morillo, E.; Goldhardt, O.; Grimmer, T.; Magdolen, V.; Batruch, I.; Diamandis, E. P., Brain-related proteins as potential CSF biomarkers of Alzheimer's disease: A targeted mass spectrometry approach. *J Proteomics* **2018,** *182*, 12-20.

14. Brinkmalm, G.; Sjödin, S.; Simonsen, A. H.; Hasselbalch, S. G.; Zetterberg, H.; Brinkmalm, A.; Blennow, K., A Parallel Reaction Monitoring Mass Spectrometric Method for Analysis of Potential CSF Biomarkers for Alzheimer's Disease. *Proteomics. Clinical applications* **2018,** *12* (1).

15. Koch, M.; DeKosky, S. T.; Fitzpatrick, A. L.; Furtado, J. D.; Lopez, O. L.; Kuller, L. H.; Mackey, R. H.; Hughes, T. M.; Mukamal, K. J.; Jensen, M. K., Apolipoproteins and Alzheimer's pathophysiology. *Alzheimers Dement (Amst)* **2018,** *10*, 545-553.

APPENDIX A

References of adaptation of chapters

Chapter II. **Khan, MJ**, Desaire, H, Lopez, OL, Kamboh, MI, Robinson, RAS, "Why race matters in plasma proteomics biomarker discovery for Alzheimer's disease." Accepted in JAD

Khan, MJ, Desaire, H, Lopez, OL, Kamboh, MI, Robinson, RAS, "Dataset of why race matters in plasma proteomics biomarker discovery for Alzheimer's disease." Under review

Chapter III. **Khan, M. J**.; Codreanu, S. G.; Goyal, S.; Wages, P. A.; Gorti, S. K. K.; Pearson, M. J.; Uribe, I.; Sherrod, S. D.; McLean, J. A.; Porter, N. A.; Robinson, R. A. S., Evaluating a targeted multiple reaction monitoring approach to global untargeted lipidomic analyses of human plasma. Rapid Communications in Mass Spectrometry 2020, 34 (22), e8911.

Chapter V. **Khan, MJ**, Desaire, Chung, NC, Lopez, OL, Kamboh, MI, Robinson, RAS, "Targeted lipidomics to understand health disparities in Alzheimer's disease." Under review

APPENDIX B

SUPPLEMENTAL INFORMATION FOR CHAPTER II

Table B2.1. List of differentially-expressed proteins in race-stratified groups (Set 2).

Accession	Protein name	*African American/Black*		*non-Hispanic White*	
		Fold change#	**p-value***	**Fold change#**	**p-value***
Q01518	Adenylyl cyclase-associated protein 1	**1.71**	**0.03**	1.12	0.73
P12814	Alpha actinin-1	**1.78**	**4.87E-02**	1.00	0.99
P06733	Alpha-enolase	**1.56**	**0.01**	1.04	0.85
P11226	Mannose-binding protein C	**0.56**	**0.04**	0.78	0.18
P02775	Platelet basic protein	**1.63**	**0.03**	0.85	0.59
P07737	Profilin 1	**1.94**	**0.02**	0.96	0.91
P14618	Pyruvate kinase PKM	**1.58**	**0.04**	1.03	0.92
Q9Y490	Talin 1	**1.72**	**0.04**	1.06	0.87
P62328	Thymosin beta-4	**2.27**	**0.03**	1.00	0.99
P06753	Tropomyosin alpha-3 chain,	**1.79**	**0.02**	0.93	0.71
P67936	Tropomyosin alpha-4 chain	**2.08**	**0.03**	1.11	0.76
P18206	Vinculin	**1.67**	**0.04**	1.08	0.82
P62258	14-3-3 protein epsilon	1.07	0.63	**1.55**	**0.04**
P14136	Glial fibrillary acidic protein	0.87	0.66	**2.64**	**1.07E-03**
P00738	Haptoglobin	1.10	0.64	**1.71**	**0.03**

Fold change cut off 1.33; * p-value < 0.05

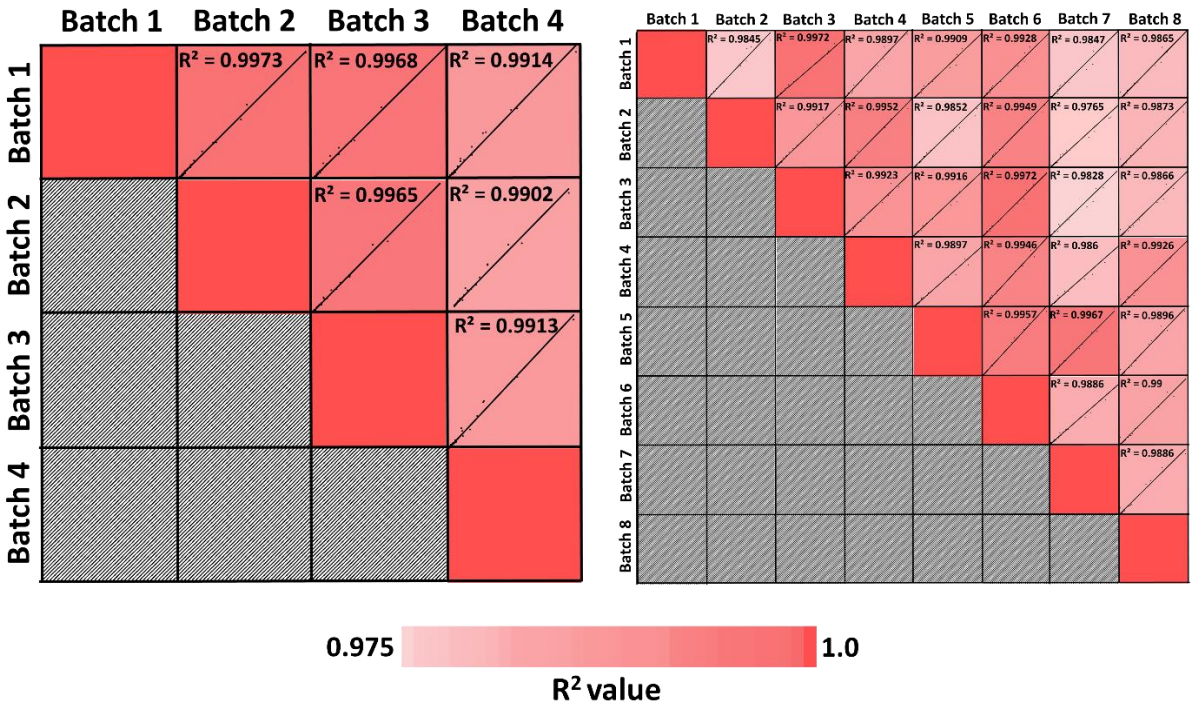

Figure B2.1. Correlation plot of average normalized TMT reporter ion intensities for all proteins between different batches for both Set 1 and Set 2. Both displayed positive correlation among all the batches. In case of Set 1, batch 3 and batch 6 showed the best correlation with an R2 value of 0.9972, while batch 2 and batch 7 showed the lowest co-relation with an R2 value of 0.9765. On average Set 1 had an R2 value of 0.99. On the other hand, for Set 2, batch 1 and batch 2 had the best co-relation with an R2 value of 0.9973, and with an R2 value of 0.9902, batch 2 and batch 4 had the lowest co-relation. On average Set 1 had an R2 value of 0.9939.

APPENDIX C

SUPPLEMENTAL INFORMATION FOR CHAPTER III

Table C3.1. Internal standard lipids and their concentration.

Name	Formula	Target Conc. (µg/mL)
PC 15:0-18:1(d7)	C41H73 D7 NO8 P	160
PE 15:0-18:1(d7)	C38H67D7NO8P	5
PS 15:0-18:1(d7)	C39H66 D7 NNaO10 P	5
PG 15:0-18:1(d7)	C39H67D7NaO10 P	30
PI 15:0-18:1(d7)	C42H75D7NO13P	10
PA 15:0-18:1(d7)	C36H61D7NaO8P	7
LysoPC 18:1(d7)	C26H45D7NO7P	25
LysoPE 18:1(d7)	C23H39D7NO7P	5
LysoPI 17:1*	C26H52NO12P	0.0005
LysoPG 17:1*	C23H44NaO9P	0.025
LysoPS 17:1*	C23H43NNaO9P	0.025
Chol Ester18:1(d7)	C45H71D7O2	350
MG 18:1(d7)	C21H33 D7O4	2
DG 15:0-18:1(d7)	C36H61D7O5	10
TG 15:0-18:1(d7)-15:0	C51H89 D7O6	55
SM 18:1(d9)	C41H72 D9N2O6P	30
Cholesterol (d7)	C27H39OD7	100
Ceramide C12*	C30H59NO3	0.025
C8 Dihydroceramide (d18:0/8:0) *	C26H53NO3	0.025
C12 Glucosyl(ß) Ceramide (d18:1/12:0) *	C36H69NO8	0.025
C12 Lactosyl(ß) Ceramide (d18:1/12:0) *	C42H79NO13	0.025

*MRM approach only

Table C3.2. Number of transitions monitored per lipid class*.

Lipid class	Ionization mode	Number of transitions per class
CE	+	22
Cer	+	12
DG	+	51
DCer	+	12
HexCer	+	19
LacCer	+	19
LPC	-	17
LPE	-	17
LPG	-	17
LPI	-	17
LPS	-	17
MG	+	18
PC	-	80
PE	-	143
PG	-	79
PI	-	78
PS	-	79
SM	+	13
TG	+	446

*Full assay is available at https://sciex.com/x115304

Table C3.3. Lipids identified in both approaches.

Untargeted		Targeted	
Lipid class	Lipid ID [*]	Lipid class	Lipid ID
CE	CE 18:2	CE	CE 16:0
CE	CE 20:4	CE	CE 18:1
CE	CE 20:5	CE	CE 18:2
Cer	Cer 34:1	CE	CE 18:3
Cer	Cer 40:1	CE	CE 20:0
Cer	Cer 41;1	CE	CE 20:3
Cer	Cer 42:0	CE	CE 20:4
Cer	Cer 42:1	CE	CE 20:5
Cer	Cer 42:2	CE	CE 22:1
Cer	Cer 43:1	CE	CE 22:2
Cer	HexCer 32:2	CE	CE 22:4
Cer	HexCer 34:1	CE	CE 22:5
Cer	LacCer 32:1	CE	CE 22:6
Cer	LacCer 34:1	Cer	Cer d18:1_14:0
Cer	LacCer 42:2	Cer	Cer d18:1_16:0
DG	DG 36:2	Cer	Cer d18:1_18:0
DG	DG 36:4	Cer	Cer d18:1_18:1
DG	DG 38:5	Cer	Cer d18:1_20:0
DG	DG 38:7	Cer	Cer d18:1_20:1
DG	DG 46:5	Cer	Cer d18:1_22:0
LPC	LPC 14:0	Cer	Cer d18:1_22:1
LPC	LPC 15:0	Cer	Cer d18:1_24:0
LPC	LPC 16:0	Cer	Cer d18:1_24:1
LPC	LPC 16:1	Cer	Cer d18:1_26:1
LPC	LPC 17:0	Cer	Cer d18:0_16:0
LPC	LPC 18:0	Cer	Cer d18:0_18:0
LPC	LPC 18:1	Cer	Cer d18:0_18:1
LPC	LPC 18:2	Cer	Cer d18:0_20:0
LPC	LPC 18:3	Cer	Cer d18:0_20:1
LPC	LPC 20:0	Cer	Cer d18:0_22:0
LPC	LPC 20:1	Cer	Cer d18:0_22:1
LPC	LPC 20:2	Cer	Cer d18:0_24:0
LPC	LPC 20:3	Cer	Cer d18:0_24:1
LPC	LPC 20:4	Cer	Cer d18:0_26:0
LPC	LPC 20:5	Cer	Cer d18:0_26:1
LPC	LPC 22:4	Cer	HexCer d18:1_14:0
LPC	LPC 22:5	Cer	HexCer d18:1_16:0
LPC	LPC O-18:0	Cer	HexCer d18:0_18:0
LPC	LPC P-16:0	Cer	HexCer d18:1_18:0

Table C3.3 cont.

LPC	LPC P-18:0	Cer	HexCer d18:1_18:1
LPC	LPC P-18:1	Cer	HexCer d18:0_20:0
LPE	LPE 16:0	Cer	HexCer d18:1_20:0
LPE	LPE 16:1	Cer	HexCer d18:1_20:1
LPE	LPE 18:0	Cer	HexCer d18:1_22:0
LPE	LPE 18:1	Cer	HexCer d18:1_22:1
LPE	LPE 18:2	Cer	HexCer d18:0_24:0
LPE	LPE 20:0	Cer	HexCer d18:1_24:0
LPE	LPE 20:1	Cer	HexCer d18:0_24:1
LPE	LPE 20:2	Cer	HexCer d18:1_24:1
LPE	LPE 20:3	Cer	HexCer d18:0_26:0
LPE	LPE 20:4	Cer	HexCer d18:1_26:0
LPE	LPE 20:5	Cer	HexCer d18:0_26:1
LPE	LPE 22:0	Cer	LacCer d18:1_14:0
LPE	LPE 22:1	Cer	LacCer d18:1_16:0
LPE	LPE 22:4	Cer	LacCer d18:0_18:0
LPE	LPE 22:5	Cer	LacCer d18:1_18:0
LPE	LPE 22:6	Cer	LacCer d18:1_22:1
PC	PC 28:0 [PC 14:0_14:0]	Cer	LacCer d18:1_24:0
PC	PC 29:1	Cer	LacCer d18:0_24:1
PC	PC 30:0 [PC 16:0_14:0]	Cer	LacCer d18:1_24:1
PC	PC 30:1 [PC 16:1_14:0]	Cer	LacCer d18:1_26:0
PC	PC 31:0	Cer	LacCer d18:0_26:1
PC	PC 31:1	DG	DG 14:0_18:1
PC	PC 32:0 [PC 16:0_16:0]	DG	DG 16:0_16:1
PC	PC 32:1 [PC 16:0_16:1]	DG	DG 16:0_18:1
PC	PC 32:2 [PC 16:1_16:1]	DG	DG 16:1_18:0
PC	PC 33:1	DG	DG 16:0_18:2
PC	PC 33:3	DG	DG 16:1_18:1
PC	PC 33:4	DG	DG 16:0_18:3
PC	PC 34:0 [PC 16:0_18:0]	DG	DG 16:1_18:2
PC	PC 34:1 [PC 16:0_18:1]	DG	DG 14:0_20:4
PC	PC 34:2 [PC 16:0_18:2]	DG	DG 16:1_18:3
PC	PC 34:3 [PC 16:1_18:2]	DG	DG 16:1_20:0
PC	PC 34:4 [PC 16:1_18:3]	DG	DG 18:0_18:1
PC	PC 34:5 [PC 14:0_20:5]	DG	DG 18:0_18:2
PC	PC 35:2	DG	DG 18:1_18:1
PC	PC 35:3	DG	DG 16:0_20:3
PC	PC 35:4	DG	DG 16:1_20:2
PC	PC 35:6	DG	DG 18:0_18:3
PC	PC 36:1 [PC 18:0_18:1]	DG	DG 18:1_18:2

Table C3.3 cont.

PC	PC 36:2 [PC 18:0_18:2]	DG	DG 16:1_22:6
PC	PC 36:3 [PC 18:0_18:3]	DG	DG 18:2_20:5
PC	PC 36:4 [PC 18:2_18:2]	DG	DG 18:0_22:6
PC	PC 36:5 [PC 16:0_20:5]	DG	DG 18:1_22:5
PC	PC 36:6 [PC 16:1_20:5]	DG	DG 18:2_22:4
PC	PC 37:7	LPC	LPC 14:0
PC	PC 38:1 [PC 18:1_20:0]	LPC	LPC 16:0
PC	PC 38:3 [PC 18:1_20:2]	LPC	LPC 16:1
PC	PC 38:4 [PC 18:0_20:4]	LPC	LPC 18:0
PC	PC 38:5 [PC 18:0_20:5]	LPC	LPC 18:1
PC	PC 38:6 [PC 18:1_20:5]	LPC	LPC 18:2
PC	PC 38:7 [PC 18:2_20:5]	LPC	LPC 18:3
PC	PC 40:10 [PC 20:5_20:5]	LPC	LPC 20:0
PC	PC 40:4 [PC 20:0_20:4]	LPC	LPC 20:1
PC	PC 40:5 [PC 20:0_20:5]	LPC	LPC 20:2
PC	PC 40:6 [PC 18:0_22:6]	LPC	LPC 20:3
PC	PC 40:7 [PC 18:1_22:6]	LPC	LPC 20:4
PC	PC 40:8 [PC 18:2_22:6]	LPC	LPC 20:5
PC	PC 42:10	LPC	LPC 22:4
PC	PC 42:7	LPC	LPC 22:5
PC	PC 42:8	LPC	LPC 22:6
PC	PC O-32:0 [PC O-16:0_16:0]	LPE	LPE 14:0
PC	PC O-32:1 [PC O-16:0_16:1]	LPE	LPE 16:0
PC	PC O-34:1	LPE	LPE 16:1
PC	PC O-34:2	LPE	LPE 18:0
PC	PC O-34:3	LPE	LPE 18:1
PC	PC O-35:4	LPE	LPE 18:2
PC	PC O-36:1	LPE	LPE 18:3
PC	PC O-36:3	LPE	LPE 20:0
PC	PC O-36:4 [PC O-16:0_20:4]	LPE	LPE 20:1
PC	PC O-36:5	LPE	LPE 20:2
PC	PC O-38:4	LPE	LPE 20:3
PC	PC O-38:5	LPE	LPE 20:4
PC	PC O-42:1 [PC O-20:0_22:1]	LPE	LPE 20:5
PC	PC O-44:3	LPE	LPE 22:4
PC	PC P-32:0 [PC P-16:0_16:0]	LPE	LPE 22:5
PC	PC P-34:0 [PC P-18:0_16:0]	LPE	LPE 22:6
PC	PC P-34:2 [PC P-16:0_18:2]	PC	PC 14:0_14:0
PC	PC P-36:0 [PC P-18:0_18:0]	PC	PC 16:0_14:0
PC	PC P-36:2 [PC P-18:0_18:2]	PC	PC 16:0_16:0
PC	PC P-36:3 [PC P-16:0_20:3]	PC	PC 18:0_14:0

Table C3.3 cont.			
PC	PC P-36:4 [PC P-16:0_20:4]	PC	PC 14:0_18:2
PC	PC P-38:3 [PC P-18:0_20:3]	PC	PC 18:0_16:1
PC	PC P-38:4 [PC P-18:4_20:0]	PC	PC 14:0_20:2
PC	PC P-40:6	PC	PC 16:0_18:2
PC	PC P-42:0 [PC P-20:0_22:0]	PC	PC 16:1_18:1
PC	PC P-44:4 [PC P-20:2_24:4]	PC	PC 18:1_16:1
PE	PE 32:1 [PE 16:0_16:0]	PC	PC 14:0_20:3
PE	PE 34:0 [PE 16:0_18:0]	PC	PC 16:0_18:3
PE	PE 34:1 [PE 16:0_18:1]	PC	PC 16:1_18:2
PE	PE 34:2 [PE 16:0_18:2]	PC	PC 18:2_16:1
PE	PE 35:0	PC	PC 14:0_20:4
PE	PE 36:0 [PE 18:0_18:0]	PC	PC 18:0_18:0
PE	PE 36:1 [PE 18:0_18:1]	PC	PC 16:0_20:1
PE	PE 36:2 [PE 18:0_18:2]	PC	PC 18:0_18:1
PE	PE 36:3 [PE 18:1_18:2]	PC	PC 20:0_16:1
PE	PE 36:4 [PE 18:1_18:3]	PC	PC 16:0_20:2
PE	PE 36:5 [PE 18:2_18:3]	PC	PC 18:0_18:2
PE	PE 38:1 [PE 18:1_20:0]	PC	PC 18:1_18:1
PE	PE 38:3 [PE 18:2_20:1]	PC	PC 16:0_20:3
PE	PE 38:4 [PE 18:1_20:3]	PC	PC 18:0_18:3
PE	PE 38:5 [PE 16:0_22:5]	PC	PC 18:1_18:2
PE	PE 38:6 [PE 18:0_20:6]	PC	PC 14:0_22:4
PE	PE 38:7 [PE 18:2_20:5]	PC	PC 16:0_20:4
PE	PE 39:2	PC	PC 18:1_18:3
PE	PE 40:4 [PE 18:0_22:4]	PC	PC 18:2_18:2
PE	PE 40:5 [PE 18:0_22:5]	PC	PC 16:0_20:5
PE	PE 40:6 [PE 18:0_22:6]	PC	PC 18:2_18:3
PE	PE 40:7 [PE 18:1_22:6]	PC	PC 14:0_22:6
PE	PE 41:2	PC	PC 18:0_20:0
PE	PE 41:3	PC	PC 18:0_20:1
PE	PE 41:5	PC	PC 20:0_18:1
PE	PE 42:9	PC	PC 18:0_20:2
PE	PE O-18:1 [PE O-18:1_0:0]	PC	PC 18:1_20:1
PE	PE O-32:0 [PE O-16:0_16:0]	PC	PC 18:0_20:3
PE	PE O-34:0 [PE O-18:0_16:0]	PC	PC 18:1_20:2
PE	PE O-34:1 [PE O-18:1_16:0]	PC	PC 18:2_20:1
PE	PE O-34:2 [PE O-18:2_16:0]	PC	PC 16:0_22:4
PE	PE O-34:3 [PE O-18:3_16:0]	PC	PC 18:0_20:4
PE	PE O-36:5	PC	PC 18:1_20:3
PE	PE O-38:0 [PE O-16:0_22:0]	PC	PC 18:2_20:2
PE	PE O-38:4 [PE O-18:0_20:4]	PC	PC 16:0_22:5

Table C3.3 cont.			
PE	PE O-38:5 [PE O-18:1_20:4]	PC	PC 18:0_20:5
PE	PE O-38:6 [PE O-16:0_22:6]	PC	PC 18:1_20:4
PE	PE O-40:6 [PE O-18:0_22:6]	PC	PC 18:2_20:3
PE	PE P-16:1 [PE P-16:1_0:0]	PC	PC 16:0_22:6
PE	PE P-18:0 [PE P-18:0_0:0]	PC	PC 18:1_20:5
PE	PE P-22:0 [PE P-22:0_0:0]	PC	PC 18:2_20:5
PE	PE P-22:4 [PE P-22:4_0:0]	PC	PC 18:0_22:4
PE	PE P-24:0 [PE P-22:0_2:0]	PC	PC 20:0_20:4
PE	PE P-34:1 [PE P-18:1_16:0]	PC	PC 18:0_22:6
PE	PE P-34:2 [PE P-16:0_18:2]	PC	PC 18:1_22:5
PE	PE P-36:2 [PE P-18:0_18:2]	PC	PC 18:1_22:6
PE	PE P-36:4 [PE P-16:0_20:4]	PE	PE 14:0_16:1
PE	PE P-36:5 [PE P-16:0_20:5]	PE	PE 16:0_16:0
PE	PE P-38:1 [PE P-18:1_20:0]	PE	PE 18:0_14:0
PE	PE P-38:3 [PE P-18:0_20:3]	PE	PE 14:0_18:1
PE	PE P-38:4 [PE P-18:0_20:4]	PE	PE 16:0_16:1
PE	PE P-38:5 [PE P-18:1_20:4]	PE	PE 14:0_18:2
PE	PE P-38:6 [PE P-18:1_20:5]	PE	PE 18:1_16:1
PE	PE P-38:7 [PE P-18:1_20:6]	PE	PE 14:0_20:3
PE	PE P-40:4 [PE P-18:0_22:4]	PE	PE 16:0_18:3
PE	PE P-40:5 [PE P-18:0_22:5]	PE	PE 18:2_16:1
PE	PE P-40:6 [PE P-18:1_22:5]	PE	PE 14:0_20:4
PE	PE P-40:7 [PE P-18:1_22:6]	PE	PE 18:0_18:0
PI	PI 34:1 [PI 16:0_18:1]	PE	PE 16:0_20:1
PI	PI 34:2 [PI 16:0_18:2]	PE	PE 18:0_18:1
PI	PI 36:1 [PI 18:0_18:1]	PE	PE 16:0_20:2
PI	PI 36:2 [PI 18:1_18:1]	PE	PE 18:0_18:2
PI	PI 36:3 [PI 18:1_18:2]	PE	PE 18:1_18:1
PI	PI 36:4 [PI 16:0_20:4]	PE	PE 16:0_20:3
PI	PI 38:3 [PI 18:0_20:3]	PE	PE 18:0_18:3
PI	PI 38:4 [PI 16:0_20:4]	PE	PE 18:1_18:2
PI	PI 38:5 [PI 18:1_20:4]	PE	PE 14:0_22:4
PI	PI 40:3	PE	PE 16:0_20:4
PI	PI 40:6 [PI 18:0_22:6]	PE	PE 18:1_18:3
PI	PI 42:6	PE	PE 18:2_18:2
PS	PS 18:1	PE	PE 14:0_22:5
PS	PS 28:0	PE	PE 16:0_20:5
PS	PS 28:1	PE	PE 18:2_18:3
PS	PS 30:1	PE	PE 18:0_20:1
PS	PS 30:3	PE	PE 18:0_20:2
PS	PS 34:1	PE	PE 18:1_20:1

Table C3.3 cont.			
PS	PS 36:0	PE	PE 18:0_20:3
PS	PS 36:1	PE	PE 18:1_20:2
PS	PS 36:3	PE	PE 18:2_20:1
PS	PS 38:4	PE	PE 16:0_22:4
PS	PS 38:5	PE	PE 18:0_20:4
PS	PS 40:3	PE	PE 18:1_20:3
PS	PS 40:4	PE	PE 18:2_20:2
PS	PS 40:5	PE	PE 16:0_22:5
PS	PS 44:7	PE	PE 18:0_20:5
PS	PS O-36:4	PE	PE 18:1_20:4
PS	PS O-38:6	PE	PE 18:2_20:3
PS	PS P-36:3	PE	PE 16:0_22:6
PS	PS P-38:5	PE	PE 18:1_20:5
SM	SM 30:1	PE	PE 18:2_20:4
SM	SM 31:1	PE	PE 18:0_22:4
SM	SM 32:0	PE	PE 18:0_22:5
SM	SM 32:1	PE	PE 18:1_22:4
SM	SM 32:2	PE	PE 18:0_22:6
SM	SM 33:1	PE	PE 18:1_22:5
SM	SM 33:2	PE	PE 18:2_22:4
SM	SM 34:0	PE	PE 18:1_22:6
SM	SM 34:1	PE	PE 18:2_22:5
SM	SM 34:2	PE	PE O-16:0_16:0
SM	SM 35:1	PE	PE O-16:0_16:1
SM	SM 35:2	PE	PE O-18:0_16:0
SM	SM 36:0	PE	PE O-16:0_18:2
SM	SM 36:1	PE	PE O-16:0_20:1
SM	SM 36:2	PE	PE O-18:0_18:1
SM	SM 38:1	PE	PE O-16:0_20:2
SM	SM 38:2	PE	PE O-18:0_18:2
SM	SM 39:1	PE	PE O-16:0_20:3
SM	SM 40:1	PE	PE O-18:0_18:3
SM	SM 40:2	PE	PE O-16:0_20:4
SM	SM 41:1	PE	PE O-16:0_20:5
SM	SM 41:2	PE	PE O-18:0_20:1
SM	SM 42:1	PE	PE O-18:0_20:2
SM	SM 42:2	PE	PE O-18:0_20:3
SM	SM 42:3	PE	PE O-16:0_22:4
TG	TG 42:0	PE	PE O-18:0_20:4
TG	TG 44:1	PE	PE O-16:0_22:5
TG	TG 46:1 [TG 16:0_14:0_16:1]	PE	PE O-18:0_20:5

Table C3.3 cont.			
TG	TG 46:2	PE	PE O-16:0_22:6
TG	TG 48:0 [TG 16:0_16:0_16:0]	PE	PE O-18:0_22:4
TG	TG 48:1 [TG 16:0_14:0_18:1]	PE	PE O-18:0_22:5
TG	TG 48:2 [TG 18:2_14:0_16:0]	PE	PE O-18:0_22:6
TG	TG 48:3 [TG 14:0_16:1_18:2]	PE	PE P-16:0_18:0
TG	TG 49:1 [TG 15:0_18:1_16:0]	PE	PE P-18:0_16:0
TG	TG 50:0 [TG 16:0_16:0_18:0]	PE	PE P-16:0_18:1
TG	TG 50:1 [TG 16:0_16:0_18:1]	PE	PE P-18:0_16:1
TG	TG 50:2 [TG 16:1_16:0_18:1]	PE	PE P-18:1_16:0
TG	TG 50:3 [TG 16:0_18:2_16:1]	PE	PE P-16:0_18:2
TG	TG 50:4 [TG 16:0_14:0_20:4]	PE	PE P-16:1_18:1
TG	TG 50:5 [TG 18:2_14:0_18:3]	PE	PE P-18:1_16:1
TG	TG 50:6	PE	PE P-16:0_18:3
TG	TG 50:7	PE	PE P-18:0_18:0
TG	TG 51:1 [TG 15:0_18:1_18:0]	PE	PE P-16:0_20:1
TG	TG 51:2 [TG 15:0_18:1_18:1]	PE	PE P-18:0_18:1
TG	TG 51:3 [TG 15:0_18:1_18:2]	PE	PE P-18:1_18:0
TG	TG 51:4 [TG 15:0_18:1_18:3]	PE	PE P-16:0_20:2
TG	TG 52:1 [TG 16:0_18:0_18:1]	PE	PE P-18:0_18:2
TG	TG 52:2 [TG 16:0_18:1_18:1]	PE	PE P-18:1_18:1
TG	TG 52:3 [TG 18:0_16:0_18:3]	PE	PE P-16:0_20:3
TG	TG 52:4 [TG 18:1_16:0_18:3]	PE	PE P-18:0_18:3
TG	TG 52:5 [TG 16:0_18:2_18:2]	PE	PE P-18:1_18:2
TG	TG 52:6	PE	PE P-16:0_20:4
TG	TG 52:8	PE	PE P-18:1_18:3
TG	TG 53:2 [TG 17:0_18:1_18:1]	PE	PE P-18:2_18:2
TG	TG 53:3 [TG 17:0_18:1_18:1]	PE	PE P-16:0_20:5
TG	TG 53:6	PE	PE P-16:0_22:4
TG	TG 54:1 [TG 18:0_18:0_18:1]	PE	PE P-18:0_20:4
TG	TG 54:2 [TG 18:0_18:1_18:1]	PE	PE P-18:1_20:3
TG	TG 54:3 [TG 18:0_18:1_18:2]	PE	PE P-18:2_20:4
TG	TG 54:4 [TG 18:1_18:1_18:2]	PE	PE P-16:0_22:5
TG	TG 54:5 [TG 18:1_16:0_20:4]	PE	PE P-18:0_20:5
TG	TG 54:6 [TG 18:0_16:0_20:5]	PE	PE P-18:1_20:4
TG	TG 54:7 [TG 16:0_18:2_20:5]	PE	PE P-16:0_22:6
TG	TG 54:8	PE	PE P-18:1_20:5
TG	TG 54:9	PI	PI 16:0_16:1
TG	TG 55:5 [TG 17:0_18:1_20:4]	PI	PI 16:0_18:0
TG	TG 55:6 [TG 17:0_18:1_20:5]	PI	PI 16:0_18:1
TG	TG 56:10	PI	PI 18:0_16:1
TG	TG 56:3 [TG 18:0_18:2_20:1]	PI	PI 16:0_18:2

Table C3.3 cont.			
TG	TG 56:4 [TG 18:0_18:1_20:3]	PI	PI 18:1_16:1
TG	TG 56:5 [TG 18:0_18:1_20:4]	PI	PI 16:0_18:3
TG	TG 56:6 [TG 16:0_18:1_22:5]	PI	PI 18:2_16:1
TG	TG 56:7 [TG 18:1_16:0_22:5]	PI	PI 16:0_20:3
TG	TG 56:8 [TG 18:2_18:2_20:4]	PI	PI 18:0_18:3
TG	TG 58:10	PI	PI 18:1_18:2
TG	TG 58:11	PI	PI 16:0_20:4
TG	TG 58:6	PI	PI 18:1_18:3
TG	TG 58:8	PI	PI 18:2_18:2
TG	TG 58:9	PI	PI 16:0_22:4
		PI	PI 18:0_20:4
		PI	PI 18:1_20:3
		PI	PI 16:0_22:5
		PI	PI 18:1_20:4
		PI	PI 18:0_22:5
		PI	PI 18:0_22:6
		PS	PS 16:0_16:0
		PS	PS 18:0_14:0
		PS	PS 14:0_18:1
		PS	PS 16:0_16:1
		PS	PS 14:0_18:2
		PS	PS 16:0_18:1
		PS	PS 18:0_16:1
		PS	PS 16:0_18:2
		PS	PS 18:1_16:1
		PS	PS 14:0_20:3
		PS	PS 18:2_16:1
		PS	PS 14:0_20:4
		PS	PS 14:0_20:5
		PS	PS 18:0_18:0
		PS	PS 18:0_18:1
		PS	PS 20:0_16:1
		PS	PS 16:0_20:2
		PS	PS 18:0_18:2
		PS	PS 18:1_18:1
		PS	PS 16:0_20:3
		PS	PS 18:1_18:2
		PS	PS 14:0_22:4
		PS	PS 16:0_20:4
		PS	PS 18:1_18:3
		PS	PS 18:2_18:2

Table C3.3 cont.		
	PS	PS 14:0_22:5
	PS	PS 18:0_20:0
	PS	PS 18:0_20:1
	PS	PS 20:0_18:1
	PS	PS 20:0_18:2
	PS	PS 18:0_20:3
	PS	PS 18:1_20:2
	PS	PS 16:0_22:4
	PS	PS 18:0_20:4
	PS	PS 18:1_20:3
	PS	PS 16:0_22:5
	PS	PS 18:0_20:5
	PS	PS 18:1_20:4
	PS	PS 18:2_20:3
	PS	PS 16:0_22:6
	PS	PS 18:1_20:5
	PS	PS 18:2_20:4
	PS	PS 18:2_20:5
	PS	PS 20:0_20:1
	PS	PS 20:0_20:2
	PS	PS 20:0_20:3
	PS	PS 20:0_20:4
	PS	PS 18:0_22:5
	PS	PS 18:1_22:4
	PS	PS 18:0_22:6
	PS	PS 18:2_22:4
	PS	PS 18:1_22:6
	PS	PS 18:2_22:5
	PS	PS 18:2_22:6
	PS	PS 20:0_22:5
	PS	PS 20:0_22:6
	SM	SM 32:1
	SM	SM 34:1
	SM	SM 36:1
	SM	SM 36:2
	SM	SM 38:1
	SM	SM 38:2
	SM	SM 40:1
	SM	SM 40:2
	SM	SM 42:1
	SM	SM 42:2

Table C3.3 cont.		
	SM	SM 44:1
	SM	SM 44:2
	TG	TG 40:0_FA14:0+NH4
	TG	TG 40:0_FA16:0+NH4
	TG	TG 42:0_FA16:0+NH4
	TG	TG 42:1_FA14:0+NH4
	TG	TG 42:1_FA18:1+NH4
	TG	TG 44:0_FA14:0+NH4
	TG	TG 44:0_FA16:0+NH4
	TG	TG 44:1_FA14:0+NH4
	TG	TG 44:1_FA16:0+NH4
	TG	TG 44:1_FA18:1+NH4
	TG	TG 44:2_FA16:0+NH4
	TG	TG 44:2_FA18:2+NH4
	TG	TG 46:0_FA16:0+NH4
	TG	TG 46:0_FA18:0+NH4
	TG	TG 46:1_FA14:0+NH4
	TG	TG 46:1_FA16:0+NH4
	TG	TG 46:1_FA16:1+NH4
	TG	TG 46:1_FA18:1+NH4
	TG	TG 46:2_FA14:0+NH4
	TG	TG 46:2_FA16:0+NH4
	TG	TG 46:2_FA18:1+NH4
	TG	TG 46:2_FA18:2+NH4
	TG	TG 46:3_FA18:1+NH4
	TG	TG 46:3_FA18:2+NH4
	TG	TG 48:0_FA14:0+NH4
	TG	TG 48:0_FA16:0+NH4
	TG	TG 48:0_FA18:0+NH4
	TG	TG 48:1_FA14:0+NH4
	TG	TG 48:1_FA16:0+NH4
	TG	TG 48:1_FA16:1+NH4
	TG	TG 48:1_FA18:0+NH4
	TG	TG 48:1_FA18:1+NH4
	TG	TG 48:2_FA14:0+NH4
	TG	TG 48:2_FA16:0+NH4
	TG	TG 48:2_FA16:1+NH4
	TG	TG 48:2_FA18:1+NH4
	TG	TG 48:2_FA18:2+NH4
	TG	TG 48:3_FA14:0+NH4
	TG	TG 48:3_FA16:0+NH4

Table C3.3 cont.		
	TG	TG 48:3_FA16:1+NH4
	TG	TG 48:3_FA18:1+NH4
	TG	TG 48:3_FA18:2+NH4
	TG	TG 48:4_FA18:2+NH4
	TG	TG 48:4_FA18:3+NH4
	TG	TG 49:1_FA16:0+NH4
	TG	TG 49:1_FA17:0+NH4
	TG	TG 49:1_FA18:1+NH4
	TG	TG 49:2_FA16:0+NH4
	TG	TG 49:2_FA16:1+NH4
	TG	TG 49:2_FA18:2+NH4
	TG	TG 50:0_FA16:0+NH4
	TG	TG 50:0_FA18:0+NH4
	TG	TG 50:1_FA14:0+NH4
	TG	TG 50:1_FA16:0+NH4
	TG	TG 50:1_FA16:1+NH4
	TG	TG 50:1_FA18:0+NH4
	TG	TG 50:1_FA18:1+NH4
	TG	TG 50:2_FA14:0+NH4
	TG	TG 50:2_FA16:0+NH4
	TG	TG 50:2_FA16:1+NH4
	TG	TG 50:2_FA18:0+NH4
	TG	TG 50:2_FA18:1+NH4
	TG	TG 50:2_FA18:2+NH4
	TG	TG 50:3_FA14:0+NH4
	TG	TG 50:3_FA16:0+NH4
	TG	TG 50:3_FA16:1+NH4
	TG	TG 50:3_FA18:1+NH4
	TG	TG 50:3_FA18:2+NH4
	TG	TG 50:3_FA18:3+NH4
	TG	TG 50:4_FA14:0+NH4
	TG	TG 50:4_FA16:1+NH4
	TG	TG 50:4_FA18:1+NH4
	TG	TG 50:4_FA18:2+NH4
	TG	TG 50:4_FA18:3+NH4
	TG	TG 50:5_FA18:3+NH4
	TG	TG 51:1_FA16:0+NH4
	TG	TG 51:1_FA17:0+NH4
	TG	TG 51:1_FA18:1+NH4
	TG	TG 51:2_FA16:0+NH4
	TG	TG 51:2_FA17:0+NH4

Table C3.3 cont.		
	TG	TG 51:2_FA18:1+NH4
	TG	TG 51:2_FA18:2+NH4
	TG	TG 51:3_FA18:2+NH4
	TG	TG 51:4_FA18:2+NH4
	TG	TG 52:0_FA16:0+NH4
	TG	TG 52:0_FA18:0+NH4
	TG	TG 52:1_FA16:0+NH4
	TG	TG 52:1_FA18:0+NH4
	TG	TG 52:1_FA18:1+NH4
	TG	TG 52:2_FA16:0+NH4
	TG	TG 52:2_FA16:1+NH4
	TG	TG 52:2_FA18:0+NH4
	TG	TG 52:2_FA18:1+NH4
	TG	TG 52:2_FA18:2+NH4
	TG	TG 52:3_FA16:0+NH4
	TG	TG 52:3_FA16:1+NH4
	TG	TG 52:3_FA18:0+NH4
	TG	TG 52:3_FA18:1+NH4
	TG	TG 52:3_FA18:2+NH4
	TG	TG 52:3_FA18:3+NH4
	TG	TG 52:4_FA16:0+NH4
	TG	TG 52:4_FA16:1+NH4
	TG	TG 52:4_FA18:1+NH4
	TG	TG 52:4_FA18:2+NH4
	TG	TG 52:4_FA18:3+NH4
	TG	TG 52:4_FA20:0+NH4
	TG	TG 52:4_FA20:3+NH4
	TG	TG 52:4_FA20:4+NH4
	TG	TG 52:5_FA16:0+NH4
	TG	TG 52:5_FA16:1+NH4
	TG	TG 52:5_FA18:1+NH4
	TG	TG 52:5_FA18:2+NH4
	TG	TG 52:5_FA18:3+NH4
	TG	TG 52:5_FA20:4+NH4
	TG	TG 52:5_FA22:5+NH4
	TG	TG 52:6_FA16:0+NH4
	TG	TG 52:6_FA16:1+NH4
	TG	TG 52:6_FA18:2+NH4
	TG	TG 52:6_FA18:3+NH4
	TG	TG 52:6_FA20:4+NH4
	TG	TG 53:0_FA16:0+NH4

Table C3.3 cont.		
	TG	TG 53:1_FA18:1+NH4
	TG	TG 53:2_FA16:0+NH4
	TG	TG 53:2_FA17:0+NH4
	TG	TG 53:2_FA18:1+NH4
	TG	TG 53:2_FA18:2+NH4
	TG	TG 53:3_FA16:0+NH4
	TG	TG 53:3_FA17:0+NH4
	TG	TG 53:3_FA18:2+NH4
	TG	TG 53:4_FA18:2+NH4
	TG	TG 54:1_FA18:0+NH4
	TG	TG 54:1_FA18:1+NH4
	TG	TG 54:1_FA20:0+NH4
	TG	TG 54:2_FA16:0+NH4
	TG	TG 54:2_FA18:0+NH4
	TG	TG 54:2_FA18:1+NH4
	TG	TG 54:2_FA18:2+NH4
	TG	TG 54:2_FA20:0+NH4
	TG	TG 54:2_FA20:1+NH4
	TG	TG 54:3_FA16:0+NH4
	TG	TG 54:3_FA18:0+NH4
	TG	TG 54:3_FA18:1+NH4
	TG	TG 54:3_FA18:2+NH4
	TG	TG 54:3_FA20:1+NH4
	TG	TG 54:3_FA20:2+NH4
	TG	TG 54:4_FA16:0+NH4
	TG	TG 54:4_FA16:1+NH4
	TG	TG 54:4_FA18:0+NH4
	TG	TG 54:4_FA18:1+NH4
	TG	TG 54:4_FA18:2+NH4
	TG	TG 54:4_FA18:3+NH4
	TG	TG 54:4_FA20:2+NH4
	TG	TG 54:4_FA20:3+NH4
	TG	TG 54:4_FA20:4+NH4
	TG	TG 54:5_FA16:0+NH4
	TG	TG 54:5_FA16:1+NH4
	TG	TG 54:5_FA18:0+NH4
	TG	TG 54:5_FA18:1+NH4
	TG	TG 54:5_FA18:2+NH4
	TG	TG 54:5_FA18:3+NH4
	TG	TG 54:5_FA20:3+NH4
	TG	TG 54:5_FA20:4+NH4

Table C3.3 cont.		
	TG	TG 54:5_FA22:5+NH4
	TG	TG 54:6_FA16:0+NH4
	TG	TG 54:6_FA16:1+NH4
	TG	TG 54:6_FA18:1+NH4
	TG	TG 54:6_FA18:2+NH4
	TG	TG 54:6_FA18:3+NH4
	TG	TG 54:6_FA20:3+NH4
	TG	TG 54:6_FA20:4+NH4
	TG	TG 54:6_FA20:5+NH4
	TG	TG 54:6_FA22:5+NH4
	TG	TG 54:6_FA22:6+NH4
	TG	TG 54:7_FA16:1+NH4
	TG	TG 54:7_FA18:2+NH4
	TG	TG 54:7_FA18:3+NH4
	TG	TG 54:7_FA20:4+NH4
	TG	TG 54:7_FA20:5+NH4
	TG	TG 54:7_FA22:6+NH4
	TG	TG 54:8_FA22:6+NH4
	TG	TG 55:1_FA16:0+NH4
	TG	TG 55:1_FA18:1+NH4
	TG	TG 55:2_FA18:2+NH4
	TG	TG 55:3_FA18:1+NH4
	TG	TG 55:3_FA18:2+NH4
	TG	TG 56:2_FA20:0+NH4
	TG	TG 56:3_FA18:1+NH4
	TG	TG 56:3_FA20:0+NH4
	TG	TG 56:3_FA20:1+NH4
	TG	TG 56:4_FA18:0+NH4
	TG	TG 56:4_FA18:1+NH4
	TG	TG 56:4_FA18:2+NH4
	TG	TG 56:4_FA20:1+NH4
	TG	TG 56:4_FA20:2+NH4
	TG	TG 56:4_FA20:3+NH4
	TG	TG 56:5_FA16:0+NH4
	TG	TG 56:5_FA18:0+NH4
	TG	TG 56:5_FA18:1+NH4
	TG	TG 56:5_FA18:2+NH4
	TG	TG 56:5_FA20:2+NH4
	TG	TG 56:5_FA20:3+NH4
	TG	TG 56:5_FA20:4+NH4
	TG	TG 56:5_FA22:4+NH4

Table C3.3 cont.		
	TG	TG 56:5_FA22:5+NH4
	TG	TG 56:6_FA16:0+NH4
	TG	TG 56:6_FA18:1+NH4
	TG	TG 56:6_FA18:2+NH4
	TG	TG 56:6_FA20:3+NH4
	TG	TG 56:6_FA20:4+NH4
	TG	TG 56:6_FA22:4+NH4
	TG	TG 56:6_FA22:5+NH4
	TG	TG 56:6_FA22:6+NH4
	TG	TG 56:7_FA16:0+NH4
	TG	TG 56:7_FA18:0+NH4
	TG	TG 56:7_FA18:1+NH4
	TG	TG 56:7_FA18:2+NH4
	TG	TG 56:7_FA18:3+NH4
	TG	TG 56:7_FA20:3+NH4
	TG	TG 56:7_FA20:4+NH4
	TG	TG 56:7_FA20:5+NH4
	TG	TG 56:7_FA22:5+NH4
	TG	TG 56:7_FA22:6+NH4
	TG	TG 56:8_FA16:0+NH4
	TG	TG 56:8_FA18:1+NH4
	TG	TG 56:8_FA18:2+NH4
	TG	TG 56:8_FA20:4+NH4
	TG	TG 56:8_FA20:5+NH4
	TG	TG 56:8_FA22:5+NH4
	TG	TG 56:8_FA22:6+NH4
	TG	TG 56:9_FA20:4+NH4
	TG	TG 56:9_FA20:5+NH4
	TG	TG 56:9_FA22:6+NH4
	TG	TG 57:2_FA18:1+NH4
	TG	TG 58:10_FA20:4+NH4
	TG	TG 58:10_FA22:6+NH4
	TG	TG 58:6_FA18:1+NH4
	TG	TG 58:7_FA18:1+NH4
	TG	TG 58:7_FA18:2+NH4
	TG	TG 58:7_FA22:5+NH4
	TG	TG 58:7_FA22:6+NH4
	TG	TG 58:8_FA18:1+NH4
	TG	TG 58:8_FA18:2+NH4
	TG	TG 58:8_FA22:5+NH4
	TG	TG 58:8_FA22:6+NH4

Table C3.3 cont.		
	TG	TG 58:9_FA18:1+NH4
	TG	TG 58:9_FA18:2+NH4
	TG	TG 58:9_FA20:4+NH4
	TG	TG 58:9_FA22:5+NH4
	TG	TG 58:9_FA22:6+NH4
	TG	TG 60:12_FA22:6+NH4

*****Fatty acid composition based on fragmentation score provided by Progenesis QI software

Table C3.4. Concentrations (nmol/mL plasma) for lipids common between the two approaches.

Lipid ID	Untargeted	MRM
LysoPC(16:0)	7.45	36.14
LysoPC(16:1)	2.95	1.17
LysoPC(18:0)	35.18	15.77
LysoPC(18:1)	17.41	10.74
LysoPC(18:2)	28.55	11.91
LysoPC(18:3)	0.46	0.10
LysoPC(20:0)	0.11	0.08
LysoPC(20:1)	0.27	0.13
LysoPC(20:2)	0.32	0.17
LysoPC(20:3)	2.47	0.74
LysoPC(20:4)	7.21	0.54
LysoPC(20:5)	0.78	0.03
LysoPC(22:4)	0.07	0.04
LysoPC(22:5)	0.34	0.04
LysoPC(22:6)	1.71	0.02
LysoPE(16:0)	0.74	0.37
LysoPE(18:0)	0.89	0.68
LysoPE(18:1)	0.50	0.56
LysoPE(18:2)	0.23	0.65
LysoPE(20:0)	2.23	0.01
LysoPE(20:1)	0.28	0.02
LysoPE(20:2)	0.09	0.01
LysoPE(20:3)	0.22	0.07
LysoPE(20:4)	1.38	0.15
LysoPE(22:5)	0.06	0.02
LysoPE(22:6)	1.24	0.01
PC 28:0	0.13	0.56
PC 30:0	1.26	5.66
PC 32:0	7.71	186.34
PC 32:1	10.99	28.27
PC 32:2	7.47	7.38
PC 34:0	0.93	87.11
PC 34:1	148.72	280.49
PC 34:2	248.22	513.73
PC 34:3	6.20	7.53
PC 34:4	2.76	1.45
PC 36:1	27.16	1.03
PC 36:2	220.27	47.12
PC 36:3	117.06	63.58
PC 36:4	134.74	111.26
PC 36:5	40.99	5.78
PC 36:6	0.74	0.33

Table C3.4 cont.		
PC 38:1	2.99	2.69
PC 38:3	55.80	32.73
PC 38:4	125.07	69.86
PC 38:5	5.02	16.53
PC 38:6	19.44	10.21
PC 38:7	0.22	0.19
PC 40:4	0.51	1.55
PC 40:5	0.92	3.39
PC 40:6	21.66	0.90
PC 40:7	4.81	1.18
PE 34:0	1.64	0.03
PE 34:1	0.23	0.73
PE 34:2	1.81	1.15
PE 36:0	0.98	0.32
PE 36:1	2.09	11.25
PE 36:2	207.04	19.06
PE 36:3	26.17	0.25
PE 36:4	0.35	0.25
PE 36:5	0.18	0.01
PE 38:1	105.00	0.06
PE 38:2	0.22	0.01
PE 38:3	3.12	2.84
PE 38:4	2.13	3.82
PE 38:5	2.52	0.21
PE 38:6	5.02	0.05
PE 40:4	0.55	0.17
PE 40:5	9.27	0.35
PE 40:6	3.08	0.26
PE 40:7	1.17	0.01
PE O- 32:0	0.30	0.01
PE O-36:4	2.60	0.03
PE P-34:0	0.41	0.14
PE P-34:1	3.58	0.13
PE P-34:2	0.74	0.35
PE P-36:1	2.28	0.37
PE P-36:2	0.81	0.92
PE P-36:4	0.83	0.22
PE p-36:5	20.80	0.01
PE P-38:3	0.41	0.12
PE P-38:4	2.24	0.46
PE P-38:5	1.39	0.19
PE P-38:6	0.45	0.02
SM 32:1	23.55	2.80

Table C3.4 cont.

SM 34:1	102.76	29.31
SM 36:1	19.92	5.11
SM 36:2	10.93	3.48
SM 38:1	0.78	107.41
SM 40:1	17.03	87.98
SM 40:2	1.94	56.43
SM 41:2	9.68	7.91
SM 42:1	23.45	17.91
TG 44:0	7.95	2.94
TG 44:1	9.74	2.60
TG 46:1	4.13	4.81
TG 46:2	13.55	3.29
TG 48:0	8.81	4.21
TG 48:1	16.09	11.40
TG 48:2	20.40	12.73
TG 48:3	24.64	10.42
TG 49:1	9.74	2.92
TG 50:0	65.12	6.27
TG 50:1	62.77	4.34
TG 50:2	53.17	27.15
TG 50:3	39.90	50.68
TG 50:4	15.92	10.62
TG 50:5	13.48	1.45
TG 51:1	26.73	2.63
TG 51:2	8.50	10.68
TG 52:2	193.65	255.90
TG 52:3	161.07	277.16
TG 52:4	131.12	171.04
TG 52:5	68.90	41.03
TG 52:6	26.22	4.40
TG 53:2	18.86	4.24
TG 54:1	6.95	3.61
TG 54:2	77.18	39.89
TG 54:3	73.73	142.73
TG 54:4	63.84	159.54
TG 54:5	42.68	100.59
TG 54:6	40.48	41.49
TG 54:7	44.79	10.97
TG 56:3	5.84	2.16
TG 56:5	20.23	10.83
TG 56:6	27.11	12.83
TG 56:7	36.68	15.19

APPENDIX D

SUPPLEMENTAL INFORMATION FOR CHAPTER IV

Table D4.1. Internal standard lipids and their concentration.

Name	Formula	Target Conc. (µg/mL)
PC 15:0-18:1(d7)	C41H73 D7 NO8 P	160
PE 15:0-18:1(d7)	C38H67D7NO8P	5
PS 15:0-18:1(d7)	C39H66 D7 NNaO10 P	5
PG 15:0-18:1(d7)	C39H67D7NaO10 P	30
PI 15:0-18:1(d7)	C42H75D7NO13P	10
PA 15:0-18:1(d7)	C36H61D7NaO8P	7
LysoPC 18:1(d7)	C26H45D7NO7P	25
LysoPE 18:1(d7)	C23H39D7NO7P	5
Chol Ester18:1(d7)	C45H71D7O2	350
MG 18:1(d7)	C21H33 D7O4	2
DG 15:0-18:1(d7)	C36H61D7O5	10
TG 15:0-18:1(d7)-15:0	C51H89 D7O6	55
SM 18:1(d9)	C41H72 D9N2O6P	30
Cholesterol (d7)	C27H39OD7	100

Table D4.2. Number of lipids identified.

Extraction method	Ionization mode	Total Compounds	Assigned Identifications	MS/MS spectra
Folch	Positive	2140	941	817
	Negative	1495	582	469
Bligh-Dyer	Positive	2588	1197	863
	Negative	1214	549	224

Table D4.3. Assigned IDs of altered lipids in AD using the untargeted approach.

Tentative Identification	Anova p value	Fold Change value	Annotation Score
LSM 18:1	0.001	8.66	42.1
Stearoylcarnitine	0.018	5.68	37.3
10S-HOME	0.002	4.92	38.5
3-hydroxylinoleoylcarnitine	0.004	4.44	40.6
PE-O-16:0	0.001	4.42	38
11Z-Octadecenylcarnitine	0.002	4.31	44.4
PE-O-18:0	0.002	4.19	39.8
PE 34:1	0.001	4.14	55.6
Octadec-9-enoic Acid	0.011	3.26	37.6
MG 18:1	0.031	3.24	39.3
Palmitoylcarnitine	0.001	3.14	49.6
Oleoyl L-carnitine	0.006	2.99	56.4
PE-P-38:1	0.007	2.92	42.8
LPE 22:4	0.032	2.90	52.3
LPC 18:1	0.050	2.86	53.1
PE-P 18:0	0.013	2.80	52.3
LPC-O 18:0	0.013	2.72	44.6
PS-O 18:0	0.016	2.71	52.4
LPC-P 18:0	0.034	2.51	36.2
PE-P 38:4	0.000	2.42	52.4
PE-P 34:1	0.020	2.42	50.3
PC-O 16:0	0.018	2.42	43.3
PE 38:7	0.001	2.40	40.4
PE 36:1	0.003	2.34	55.7
1-Stearoylglycerophosphoserine	0.024	2.33	39.9
(1alpha,3beta,20S,22R,24S,25S)-Pubescenin	0.045	2.29	37.3
3-Methyl-5-propyl-2-furanundecanoic acid	0.037	2.26	41.4
PE-P 20:0	0.022	2.18	50.1
PE-P 36:1	0.000	2.14	43.7
PE-P 38:3	0.000	2.14	47
O-tetradecanoylcarnitine	0.030	2.01	41.8
1alpha-hydroxy-2beta-(5-hydroxypentoxy) cholecalciferol	0.001	1.95	41.9
PE-P 40:4	0.007	1.93	38.6
PE-P 40:6	0.000	1.93	40.8
7,10-Octadecadienoic acid	0.023	1.92	43.6
PC-O 20:0	0.007	1.90	37.3
PE-P 40:5	0.015	1.87	43.9
1-(6-[3]-ladderane-hexanoyl)-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanolamine	0.021	1.87	36.1

Table D4.3 cont.			
9-HODE	0.010	1.84	49.9
(1R,2R)-3-oxo-2-pentyl-cyclopentanehexanoic acid	0.003	1.83	48.4
hexadecadienoylcarnitine	0.023	1.80	37.5
7beta-acetoxy-gorgostan-3beta,5alpha,6beta-triol	0.010	1.79	46.3
20,24-Epoxy-25,26-dihydroxydammaran-3-one	0.012	1.77	49.9
PS 22:0	0.047	1.77	37.9
(25R)-3alpha,7alpha,12alpha-trihydroxy-5alpha-cholestan-26-oic acid	0.026	1.74	42.3
3b,12a-Dihydroxy-5a-cholanoic acid	0.026	1.73	53
PE 34:0	0.002	1.72	51.8
5Z,9Z-hexadecadienoic acid	0.003	1.72	48.9
PE 36:4	0.011	1.72	52
1alpha,25-dihydroxy-2beta-(3-hydroxypropoxy) cholecalciferol	0.027	1.71	42.3
PE-P 34:2	0.040	1.70	51.6
PS 20:3	0.007	1.69	41.1
Cer 34:1	0.022	1.69	50.2
2alpha-methyl-1beta,25-dihydroxycholecalciferol	0.032	1.68	42.7
PE 38:5	0.032	1.67	54.7
3alpha,7alpha,12alpha-Trihydroxy-24-methyl-5beta-cholest-23-en-26-oic acid	0.018	1.66	51.3
PE 36:3	0.018	1.64	36.1
3S,7,11-Trimethyl-6,10-dodecadienoic acid	0.008	1.63	47.2
1-(8-[5]-ladderane-octanoyl)-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanolamine	0.000	1.61	51.5
(24S)-1alpha,24-dihydroxy-26,27-dimethyl-22-oxacholecalciferol	0.025	1.61	40.2
PS 38:5	0.014	1.59	38.7
LacCer 34:1	0.012	1.57	53.3
PE-P 36:2	0.025	1.56	51.4
6alpha-Hydroxycastasterone	0.030	1.56	40
PE P-36:4	0.006	1.55	54.3
PS 36:3	0.042	1.55	50.7
PE 38:4	0.000	1.55	48.4
PE 36:2	0.017	1.53	53.3
PE 34:2	0.022	1.50	52.6
PC 34:4	0.044	-1.50	45.70
PC 32:2	0.004	-1.54	52.60
SM 42:4	0.006	-1.56	37.90
PC 36:5	0.005	-1.61	46.90
SM 42:2	0.011	-1.76	51.20
PC 32:1	0.013	-1.77	46.90

Table D4.3 cont			
GlcCer 36:2	0.035	-1.79	53.30
3-O-(2-O-(2E-decenoyl)-alpha-L-rhamnopyranosyl)-3-hydroxydecanoic acid	0.037	-1.80	39.10
SM 40:1	0.009	-1.82	51.40
1-Stearoyl-2-linoleoyl-sn-glycero-3-phosphocholine	0.005	-1.88	46.40
PC 34:1	0.012	-2.18	54.20
TG 50:5	0.010	-2.23	37.20
PC 36:6	0.002	-2.25	54.70
26-Glucosyl-1,3,11,22-tetrahydroxyergosta-5,24-dien-26-oate	0.001	-2.31	48.40
PC 30:1	0.027	-2.31	41.80
PC 34:5	0.011	-2.39	41.20
3alpha-Hydroxy-1,7-dioxo-5beta-cholan-24-oic Acid	0.024	-2.52	46.00
PE 26:1	0.003	-2.56	47.70
3beta-(3-methyl-butanoyloxy)-villanovane-13alpha,17-diol	0.003	-2.57	50.50
Notoginsenoside T1	0.006	-2.63	55.30
2-Hydroxyfelbamate	0.024	-2.70	37.90
PC-P 44:4	0.002	-3.11	53.50
SM 42:1	0.009	-3.97	53.80
9K,12,13-diHODE	0.009	-4.03	37.00
11,12,13-TriHOME	0.005	-5.59	41.00
2S-aminohexadecanoic acid	0.001	-6.90	52.40

p-value < 0.05

Table D4.4. Significantly altered lipids in AD using the targeted MRM approach.

Lipid ID	*p-value*	AD/CN
PS 16:0_22:4	0.013	15.03
PI 14:0_18:1	0.014	7.48
PE 14:0_22:4	0.026	6.86
PE 18:0_22:4	0.007	5.46
PS 18:0_20:3	0.016	4.17
PS 18:1_20:5	0.044	3.85
PE 18:2_22:4	0.006	3.83
PS 14:0_22:4	0.018	3.72
PE-O 18:0_22:4	0.003	3.68
TG 50:5-FA14:0	0.029	3.67
LPG 22:4	0.001	3.24
PE 16:0_22:4	0.014	3.10
DG 18:1_22:5	0.020	2.94
PG 16:0_22:4	0.007	2.63
PE 18:1_22:4	0.020	2.61
PE 18:2_18:3	0.010	2.52
TG 48:4-FA20:4	0.033	2.50
PE 18:0_16:0	0.011	2.46
PE 18:2_22:5	0.029	2.44
PI 14:0_18:2	0.022	2.43
TG 54:5-FA18:0	0.020	2.43
PE-P 16:0_18:0	0.012	2.42
PE 18:2_18:2	0.000	2.41
PE P-18:0_22:4	0.008	2.35
PG 18:1_22:4	0.025	2.29
LPE 22:4	0.008	2.27
LPG 20:4	0.001	2.22
PE-O 16:0_18:1	0.009	2.20
PE-O 16:0_20:1	0.012	2.20
TG 54:4-FA18:0	0.039	2.19
PG 18:2_20:2	0.003	2.18
PS 14:0_22:5	0.002	2.16
PE 16:0_20:1	0.022	2.12
PG 14:0_20:4	0.007	2.11
PE 14:0_22:5	0.033	2.07
PG 18:1_18:3	0.002	2.06
PE 14:0_20:1	0.036	2.04
TG 54:8-FA18:3	0.014	2.00
PE-P 16:0_22:4	0.013	1.99
PE 18:2_22:6	0.017	1.95

Table D4.4 cont.

PE-O 16:0_22:4	0.008	1.94
PI 16:0_14:0	0.021	1.93
PE 16:0_18:1	0.046	1.92
LPG 22:5	0.019	1.91
PG 18:1_18:2	0.004	1.87
PE-P 18:0_20:1	0.006	1.81
TG 54:8-FA18:2	0.018	1.81
PG 20:0_20:5	0.044	1.80
HCer 22:1	0.000	1.78
PG 16:0_16:0	0.049	1.77
PE 18:1_20:1	0.010	1.76
PE 18:2_20:4	0.026	1.76
PE-O 16:0_20:2	0.034	1.76
PE-P16:0_18:1	0.014	1.76
PS 14:0_20:4	0.002	1.74
HCer 24:1	0.002	1.70
PG 16:0_22:6	0.032	1.70
TG45:0-FA16:0	0.045	1.66
TG54:2-FA18:2	0.037	1.65
PE-O 18:0_20:4	0.000	1.63
DCer 18:0	0.007	1.63
PE-O 18:0_20:1	0.042	1.62
PI 16:0_16:0	0.050	1.62
LPS 18:1	0.039	1.59
MG 22:5	0.003	1.59
LPG 14:0	0.004	1.58
PE 18:1_16:1	0.028	1.57
LPG 20:5	0.011	1.56
HCer d18:0_20:0	0.010	1.55
PE-P 18:0_22:5	0.024	1.54
MG 18:0	0.005	1.51
PE-P 16:0_20:1	0.050	1.51
LCer d18:0_18:0	0.033	1.51
LPC 20:1	0.037	1.51
PS 14:0_20:5	0.001	1.51
PE 18:1_20:5	0.044	0.67
TG 58:10-FA22:5	0.013	0.66
PE 18:0_20:2	0.001	0.65
CE 20:5	0.021	0.65
PG 18:2_22:5	0.008	0.65
TG 56:7-FA16:1	0.015	0.64

Table D4.4 cont.

PC 18:0_20:4	0.020	0.64
PC 18:0_20:0	0.014	0.64
PE-P 18:1_20:5	0.028	0.64
PC 14:0_22:5	0.032	0.64
PI 16:0_20:3	0.043	0.63
PE 18:0_20:3	0.022	0.63
PI 18:0_22:6	0.009	0.62
PC 16:0_20:1	0.013	0.62
PI 20:0_18:1	0.027	0.62
TG58:8-FA22:6	0.021	0.62
CE 20:2	0.000	0.61
PI 18:1_20:5	0.013	0.61
PE O-18:0_16:1	0.000	0.60
PS 16:0_20:3	0.003	0.60
PI 16:0_20:5	0.027	0.60
PC 16:0_22:5	0.002	0.60
PS 14:0_18:1	0.000	0.59
PC 16:0_18:1	0.003	0.59
PC 20:0_18:1	0.046	0.59
PS 18:1_20:2	0.000	0.58
PS 18:1_18:3	0.003	0.58
CE 22:5	0.018	0.58
PC 16:0_20:3	0.023	0.58
PE 18:0_18:3	0.006	0.58
PI 18:0_18:3	0.003	0.57
PI 18:0_20:5	0.001	0.57
PC 18:0_18:0	0.001	0.56
LPG 16:1	0.008	0.55
TG 56:7-FA22:6	0.015	0.54
PC 18:2_20:2	0.048	0.54
TG 56:7-FA22:5	0.012	0.54
PI 18:0_16:1	0.040	0.53
PC 18:0_20:2	0.013	0.53
PI 18:1_22:6	0.015	0.53
PC 16:0_22:4	0.011	0.52
PS 18:1_20:3	0.000	0.51
TG 54:3-FA16:1	0.007	0.51
PC 14:0_14:0	0.005	0.51
PS 20:0_18:2	0.005	0.51
PC 16:0_20:2	0.002	0.51
PC 18:0_22:5	0.020	0.51

Table D4.4 cont.		
PS 20:0_20:2	0.012	0.51
PS 18:2_20:5	0.007	0.50
PC 16:1_18:2	0.002	0.50
PS 18:2_20:3	0.002	0.49
PE 14:0_16:1	0.002	0.49
PS 16:0_16:1	0.012	0.49
PE 18:0_16:1	0.035	0.48
PC 18:2_20:3	0.049	0.48
PS 20:0_20:5	0.047	0.48
PC 18:0_18:3	0.022	0.48
PC 18:1_18:1	0.001	0.47
PS 20:0_16:1	0.000	0.47
PC 18:2_20:5	0.000	0.44
PC 20:0_20:3	0.025	0.43
PC 18:0_20:3	0.023	0.43
TG 56:7-FA20:5	0.008	0.43
PC 18:0_16:1	0.034	0.43
PC 18:1_18:3	0.015	0.42
PC 18:1_20:2	0.000	0.41
PC 16:0_18:3	0.004	0.41
PS 20:0_18:3	0.001	0.40
PC 20:0_20:4	0.009	0.40
PS 20:0_20:3	0.013	0.40
PC 16:0_16:1	0.007	0.39
PC 18:1_20:5	0.005	0.35
LPS 20:0	0.000	0.34
PC 18:0_20:5	0.000	0.33
PC 18:2_16:1	0.000	0.31
PC 18:1_20:3	0.005	0.31
PC 16:0_20:5	0.000	0.28
PC 18:1_16:1	0.006	0.28
PE 18:0_20:5	0.027	0.20
PC 18:1_22:5	0.025	0.07

*p-value < 0.05

APPENDIX E

SUPPLEMENTAL INFORMATION FOR CHAPTER V

Table E5.1. Differentially-expressed lipids in race-stratified groups.

	African American/Black		***non-Hispanic White***	
Lipids	**p value***	**Fold Change#**	**p value***	**Fold Change#**
PS 18:0_18:0	**2.03E-18**	**0.78**	**6.27E-19**	**0.74**
PC 16:0_22:6	**1.96E-03**	**0.79**	0.02	0.84
PC 18:0_22:6	**0.01**	**0.82**	**0.01**	**0.80**
LPC 22:4	**0.02**	**1.41**	0.20	1.17
PS 18:1_22:6	**0.03**	**0.81**	**0.01**	**0.74**
PE 16:0_22:4	**0.04**	**1.60**	0.22	1.21
PC 18:0_22:4	**0.04**	**1.30**	0.33	1.11
PC 16:0_22:4	**0.05**	**1.22**	**0.05**	**1.21**
PG 16:0_20:4	0.66	1.11	**1.97E-04**	**0.54**
PS 18:0_20:1	0.58	0.93	**6.10E-04**	**0.67**
PS 18:0_20:0	0.01	0.86	**7.89E-04**	**0.79**
PS 20:0_20:1	0.67	0.96	**1.83E-03**	**0.70**
PC 18:0_20:5	0.49	0.90	**2.62E-03**	**0.52**
PC 16:0_20:5	0.47	0.89	**2.99E-03**	**0.52**
PE-P 18:2_22:6	0.27	0.85	**0.01**	**0.75**
PS 16:0_18:0	0.18	0.90	**0.01**	**0.83**
PE-P 16:0_22:6	0.08	0.81	**0.01**	**0.76**
PE-P 18:1_16:0	0.62	0.94	**0.01**	**0.78**
PE 18:0_20:5	0.85	0.96	**0.01**	**0.61**
PE 16:0_20:5	0.89	1.03	**0.01**	**0.61**
PE-P 18:1_22:6	0.08	0.80	**0.01**	**0.76**
PS 18:2_22:6	0.10	0.86	**0.01**	**0.78**
PC 18:2_16:1	0.55	0.95	**0.01**	**1.22**
PS 18:2_20:5	0.81	1.04	**0.02**	**0.49**
PE-P 18:1_20:5	0.99	1.00	**0.02**	**0.52**
PE 18:1_20:5	0.88	0.97	**0.02**	**0.66**
PE-P 18:1_22:4	0.45	1.15	**0.02**	**1.49**
PE-P 18:0_20:5	0.71	0.93	**0.02**	**0.56**
PC 18:1_22:4	0.22	1.20	**0.02**	**1.38**
PE-P 16:0_20:5	0.98	1.01	**0.02**	**0.50**
PE 16:0_22:6	0.09	0.86	**0.03**	**0.80**
PC 18:1_20:5	0.63	0.93	**0.03**	**0.64**
PC 16:1_18:2	0.52	0.95	**0.03**	**1.21**

Table E5.1 cont.				
PC 16:0_14:0	0.96	1.01	**0.03**	**0.80**
PS 14:0_22:4	0.49	1.10	**0.03**	**1.30**
PS 16:0_16:0	0.09	0.74	**0.04**	**0.64**
PE-O 16:0_22:6	0.32	0.84	**0.04**	**0.76**

Fold change cut off 1.2; * p-value < 0.05 (no multiple hypothesis correction testing)

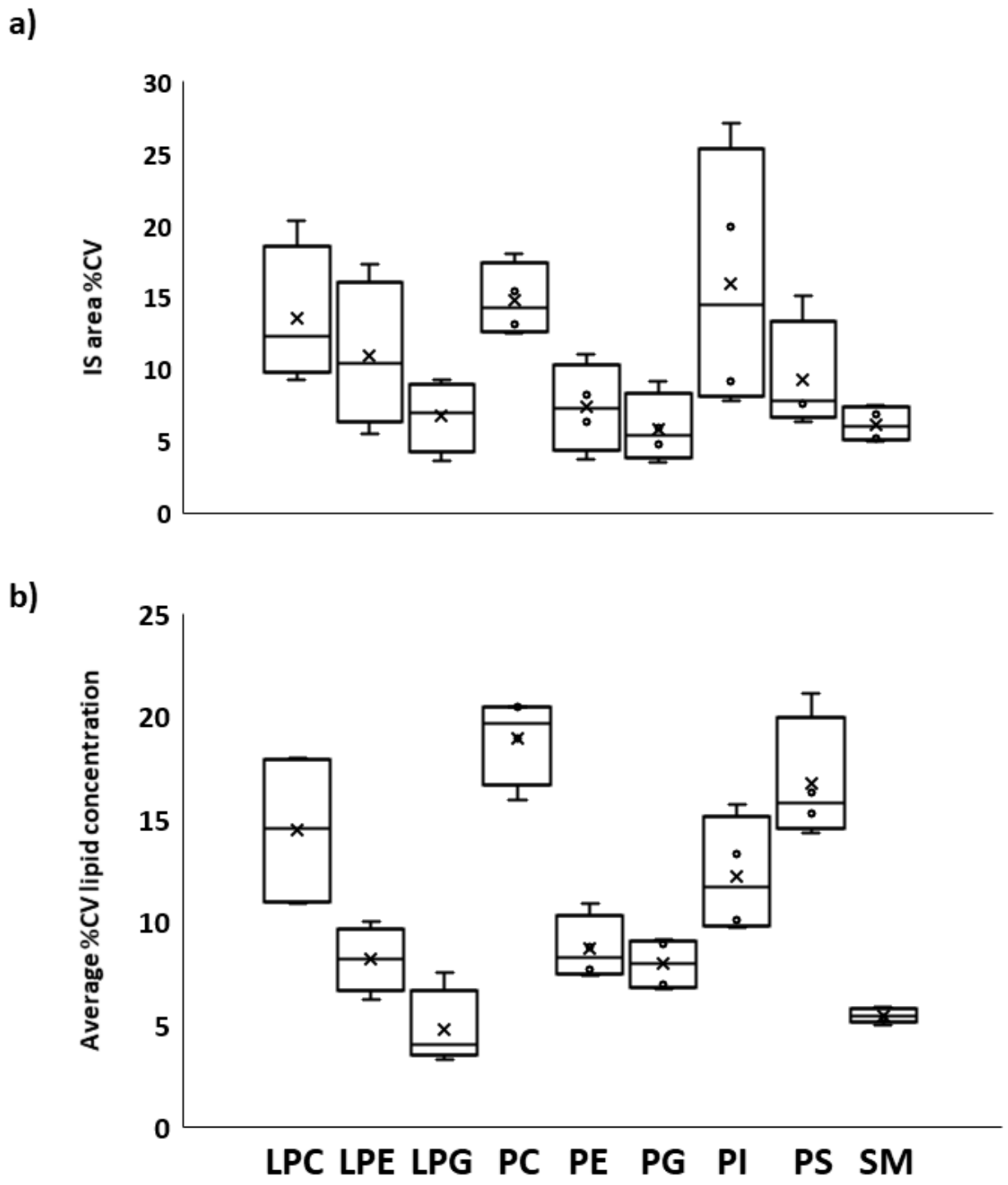

Figure E5.1. Box plot comparison of a) area of internal standards and **b**) average %CV of relative concentrations of endogenous lipids across all four sample batches, with the median value representing average median of all the samples analyzed, and the error bars representing standard deviation of the lipid of the values.

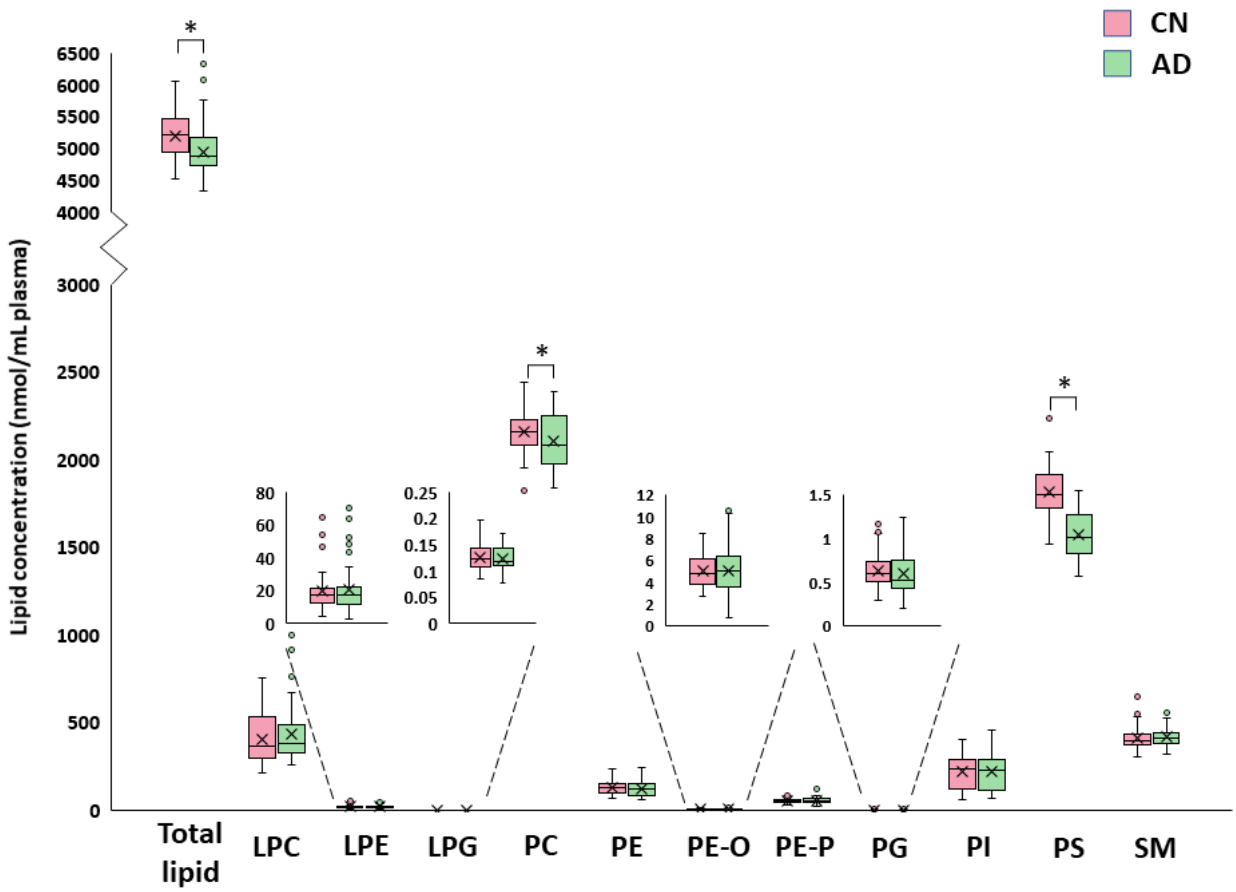

Figure E5.2. Box plot of total lipid concentrations in each lipid class. (For CN (red), N= 54, AD (Green), N= 59)

APPENDIX F

CURRICULAM VITAE

Mostafa J. Khan

www.linkedin.com/in/mostafa-khan | (310)-561-0782 | mostafa.khan@vanderbilt.edu

SUMMARY

- Analytical chemist with more than four years of experience in bottom-up proteomics and lipidomics research with focus on the discovery of diagnostic biomarkers for Alzheimer's disease.
- Development and subsequent application of 'omics technologies for the study of human plasma samples.
- Expertise in proteomic and lipidomic sample preparation from both body fluid and tissue source using established protocols.
- Extensive hands-on experience operating and maintaining mass spectrometry instrumentation's such as Thermo Orbitrap Fusion Lumos, QExactive HF, Elite, Sciex QTRAP 6500+.

EDUCATION

Ph.D., Analytical Chemistry (Expected graduation- January 2021)
Vanderbilt University, Nashville, TN — July 2017 - Present
University of Pittsburgh, Pittsburgh, PA (Transferred) — August 2015 - July 2017
B.S., Applied Chemistry & Chemical Engineering
University of Dhaka, Dhaka, Bangladesh — September 2014

RESEARCH EXPERIENCE

Vanderbilt University, University of Pittsburgh, Graduate Student Researcher May 2016- Present
Advisor: Renã A. S. Robinson, Ph.D.

- Established robust quantitative proteomics workflow for the analysis of human plasma samples.
- Investigated the effect of race on the discovery of biomarkers for Alzheimer's disease using high resolution mass spectrometry instrument.
- Developed both discovery-based and MRM based targeted lipidomics methods to study human plasma samples.
- Conducted MRM based targeted lipidomics experiments to understand racial disparities in Alzheimer's disease using a triple quadrupole mass spectrometer.
- Operate, maintain and calibrate mass spec instrumentation's such as Thermo Orbitrap Fusion Lumos, QExactive HF, Orbitrap Elite, Sciex QTRAP 6500+.

TEACHING EXPERIENCE

University of Pittsburgh, Pittsburgh, PA

Teaching Assistant/Fellow - General Chemistry 1 & 2 Lab Instructor Aug. 2015 – Dec.2016

- Prepare and teach general chemistry laboratory classes for 25 students.
- Grade lab notebooks and proctor exams.

TECHNICAL SKILLS

Laboratory Techniques: Bottom-up Proteomics, Lipidomics, Liquid chromatography, Mass spectrometry, LC-MS, Western blotting, Gel Electrophoresis

Software: Xcalibur, Proteome Discoverer, Progenesis, Sciex OS, Analyst, Empower

PUBLICATIONS

1. **Khan, MJ**, Codreanu, SG, Goyal, S, et al. Evaluating a targeted multiple reaction monitoring approach to global untargeted lipidomic analyses of human plasma. *Rapid Commun Mass Spectrom*. **2020**, 34; e8911.
2. **Khan, MJ**, Desaire, H, Lopez, OL, Kamboh, MI, Robinson, RAS, "Why race matters in plasma proteomics biomarker discovery for Alzheimer's disease." [*Accepted in JAD*]
3. **Khan, MJ**, Desaire, H, Lopez, OL, Kamboh, MI, Robinson, RAS, "Dataset of why race matters in plasma proteomics biomarker discovery for Alzheimer's disease." [*Under review in Data in brief*]
4. **Khan, MJ**, Chung, NC, Lopez, OL, Kamboh, MI, Robinson, RAS, "Targeted lipidomics to understand health disparities in Alzheimer's disease." [*Under review in Alzheimer's & dementia*]
5. **Khan, MJ**, Arul, A, Robinson, RAS, Review article in preparation.

POSTER PRESENTATIONS

1. **Khan, M.J.**; Robinson, R.A.S; Effect of Lipid Metabolism on Racial Disparities in Alzheimer's Disease, Presentation at the Alzheimer's Association International Conference, Los Angeles, CA, July 17, **2019**.
2. **Khan, M.J.**; Robinson, R.A.S; Application of plasma proteomics in Alzheimer's Disease, Presentation at the 67th ASMS conference on Mass Spectrometry and Allied Topics, Atlanta, GA, June 3, **2019**.
3. **Khan, M.J.**; Codreanu, S.G.; Sherrod, S.D.; McLean, J.A.; Robinson, R.A.S; 'Omics Approaches to Understand Health Disparities in Alzheimer's Disease, Presentation at the Alzheimer's Association International Conference, Chicago, IL, July 23, **2018**.
4. **Khan, M.J.;** Codreanu, S.G.; Sherrod, S.D.; McLean, J.A.; Robinson, R.A.S; Lipidomics to study health disparities in Alzheimer's disease, Presentation at the 66th ASMS conference on Mass Spectrometry and Allied Topics, San Diego, CA, June 4, **2018**.
5. **Khan, M.J.**; Stepler, K.E.; Robinson, R.A.S; Plasma Proteomics to Understand Health Disparities in Alzheimer's Disease, Presentation at the 65th ASMS conference on Mass Spectrometry and Allied Topics, Indianapolis, IN, June 8, **2017**.

ORAL PRESENTATIONS

1. **Khan, M.J.**; Robinson, R.A.S**;** Plasma Proteomics to Study Health Disparities in Alzheimer's disease**,** Presentation at the National Organization for the Professional Advancement of Black Chemist and Chemical Engineers (NOBCCHE) annual conference, Orlando, FL, September 20, **2018**.
2. **Khan, M.J.**; Codreanu, S.G.; Sherrod, S.D.; McLean, J.A.; Robinson, R.A.S; Establishing a Shotgun Lipidomics Workflow for Human Plasma Analysis in Alzheimer's Disease, Presentation at the Metabolomics in Translational Research, Nashville, TN, October 31, **2017**.

AWARDS/HONORS

- University of Pittsburgh Excellence Award (August 2015)
- ASMS Student Travel Stipend (June 2017, 2019)
- NOBCCHE Advancing Science Conference Grant, Orlando, FL (September 2018)
- Alzheimer's Association International Conference, ISTAART Student Volunteer award, CA (July 2019)
- Vanderbilt University Graduate Student Travel Award (June 2018, June 2019)