Comparative Lipid Profiling of Human Stratum Corneum between Atopic Dermatitis and Healthy Subjects

by
Shelby L. Stewart

A THESIS

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biology
(Honors Associate)

Presented May 20, 2016
Commencement June 2016

AN ABSTRACT OF THE THESIS OF

Shelby L. Stewart for the degree of Honors Baccalaureate of Science in Biology presented on May 20, 2016. Title: Comparative Lipid Profiling of Human Stratum Corneum between Atopic Dermatitis and Healthy Subjects.

Abstract approved: _____

Arup Indra

Abstract Body:

Dermatological issues are a widespread problem in humans that are often overlooked. In our present study, we have isolated lipids from de-identified epidermal skin tape samples from both Atopic Dermatitis and healthy individuals using a high efficiency one-step extraction method, which were subsequently analyzed using modified Liquid chromatography–mass spectrometry (LC-MS). A study was performed on both an original set of samples and follow up samples from patients revisiting the clinic several months later. From our initial study, we have observed a general trend of increased triglycerides, CER[A], CER[N], and cholesterol-3-sulfate, decreased free fatty acids of specific chain length, and both increased and decreased CER[EO] subclass ceramides. In the follow up study, there was a general trend of unaltered cholesterol and triglyceride levels, normalized cholesterol-3-sulfate, decreased free fatty acids, CER[EO] and CER[N] subclass ceramides, and decreased/normalized CER[A] subclass ceramides. All of these changes in lipid composition are likely affecting the AD status of the individuals and influencing their barrier functions since lipids play a major role in the formation and maintenance of epidermal barrier of the skin. Altered lipid composition is likely a

contributing factor towards impaired skin barrier functions and the manifestation of symptoms related to skin problems that are likely life long issues in AD subjects.

Key Words: Stratum Corneum, Epidermal Barrier, Lipidomics, Mass Spectrometry, Ceramides, Cholesterol, Cholesterol-3-sulfate, Triglycerides, Free Fatty acids, Atopic Dermatitis

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Arup Indra, Mentor, representing Pharmacy

Gitali Indra, Committee Member, representing Pharmacy

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Toni Doolen, Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Shelby L. Stewart, Author

ACKNOWLEDGEMENTS

I would not have been able to complete my honors college undergraduate thesis without the help of many individuals. I would first like to thank Dr. Indra for giving me the opportunity to become a member of his research lab. Dr. Indra was very knowledgeable about the thesis process and was willing to meet and spend as much time as was needed with me to explain the details of the research I would perform. He helped ease me into research to allow me the time to adjust to the new experience before helping to guide me through the thesis research process. I am also grateful to the College of Pharmacy Undergraduate Research Fellowship committee for funding me during the summer so that I was able to continue my thesis project uninterrupted with no coursework distractions.

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I would lastly like to thank my family for encouraging me throughout my college experience and thesis project. They were always there to give advice or support whenever I needed it, listen patiently as I talked about all the classwork and research I had to complete, and spend many a night proofreading my numerous projects. My education would not have been possible without the help of my family.

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1. INTRODUCTION

1.1 Atopic Dermatitis

Atopic dermatitis is the most chronic inflammatory skin disease and is characterized by impaired epidermal barrier abilities [1,2]. Roughly 15-20% of children and 1-3% of adults are affected by atopic dermatitis [3,4]. Previous research has shown that one of the contributing factors of atopic dermatitis may be a faulty skin barrier due to altered skin lipid composition [5,6]. Furthermore, it is likely that the lipid composition is different within the uppermost layer, the stratum corneum, of the skin [5]. This faulty barrier could lead to dry skin that becomes irritated and itchy. If the affected area is then scratched, bacteria and allergens can also be introduced and lead to further allergic conditions [5] (Figure 1). As of now, most treatments can only help alleviate symptoms rather than cure the root cause of the disease. If the root cause of the illness can be found, there are numerous opportunities to create drug therapies that specifically target certain systems or regions of the body.

1.2 Skin Structure and Function

The skin is comprised of three layers: the hypodermis, dermis, and epidermis [7] (Figure 2). The epidermis is further split up into the stratum basal, stratum suprabasal, stratum granulosum, stratum lucidum, and the stratum corneum [7] (Figure 2). As the

outermost layer, the stratum corneum's main functions are to provide a waterproof barrier for the body and to protect the body against irritation and infection [5]. This layer of the skin has a brick and mortar-like structure that is composed of skins cells (corneocytes) and lipids [8] (Figure 2). The stratum corneum lipid content is mostly composed of ceramides, followed by cholesterol, free fatty acids, and other lipids including triglycerides [8].

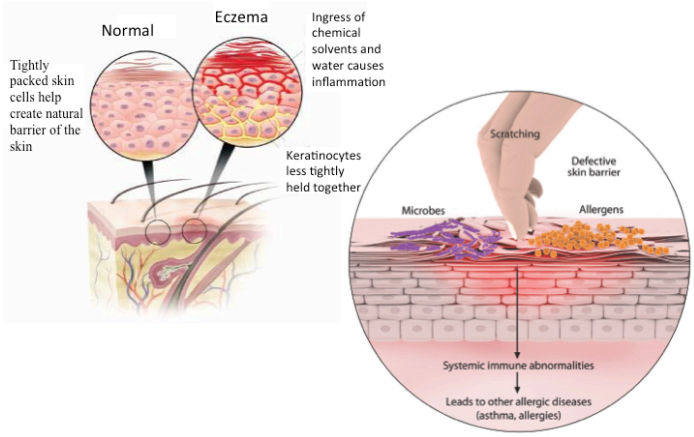

Figure 1: *Diagram of the skin showcasing the atopic dermatitis symptoms of red, irritated, and itchy skin that is prone to allergen and bacterial invasion, which then creates an immune response that often worsens symptoms (National Jewish Health, 2016).*

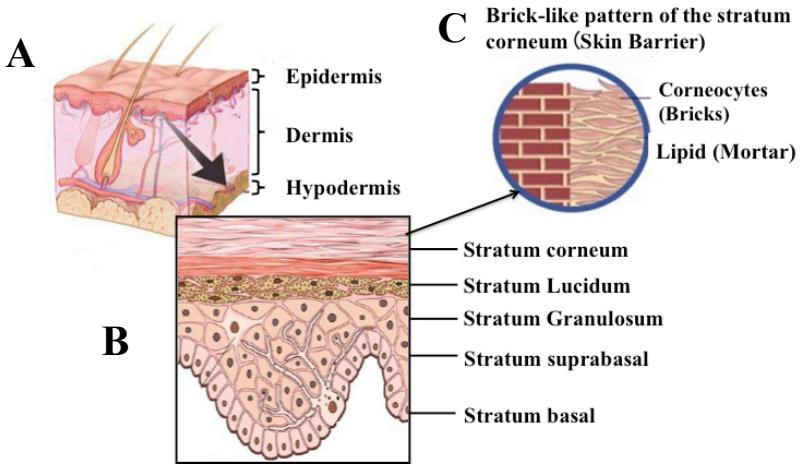

Figure 2: *Diagram of the skin detailing the (A) layers of the skin (epidermis, dermis, and hypodermis), (B) layers of the epidermis (stratum basal, stratum suprabasal, stratum granulosum, stratum lucidum, and stratum corneum), (C) and the brick and mortar pattern of the stratum corneum composed of corneocytes and lipids (Supplied by Shan Li, graduate student, OSU Indra Lab).*

1.3 Function of Ceramides in Human Skin

Ceramide, the most abundant lipid class in human SC (50%), is made of a sphingoid base linked through an amide bond to a fatty acid [11]. Variations in the fatty acid and sphingoid base give rise to a large number of ceramide subclasses [11]. To date, 12 ceramide subclasses including AS, AH, AP, ADS, NS, NDS, NP, NH, EOdS, EOP, EOH, and EOS have been identified in healthy human SC [13] (Table 1). It is also thought that ceramides play important functions in water retention roles of the skin [14]. Several reported studies have demonstrated that changes in ceramide composition may be connected to an impaired skin barrier, but details have not yet been discovered [11]. In our study, the comprehensive SC ceramide profile was compared between AD and healthy individuals.

Fatty acids / Sphingoid bases	**Non-hydroxy Fatty acid [N]**	**α -Hydroxy fatty acid [A]**	**Esterfied ω -Hydroxy fatty acid [EO]**
Dihydroxysphingosine [dS]	[NDS]	[ADS]	[EOdS]
Sphingosine [S]	[NS]	[AS]	[EOS]
Phytosphingosine [P]	[NP]	[AP]	[EOP]
6-hydroxy sphingosine [H]	[NH]	[AH]	[EOH]

Table 1: *The twelve ceramide subclasses in human SC. (Modified from Janessens et al., 2012, J. Lipid Res)*

1.4 Function of Cholesterol, Free Fatty Acids, and Triglycerides in Human Skin

Cholesterol, the second most abundant lipid in the SC, acts as a stabilizing force in lipid membranes and cholesterol sulfate can control serine proteases involved in skin epidermis cell linkage [15]. Cholesterol and cholesterol sulfate reduction would, therefore, likely decrease the stability of bilayers [15]. Cholesterol sulfate also

participates in keratinocyte differentiation, prompting genes that are engaged in development of the human skin barrier [15]. As the third most abundant lipid in human SC, fatty acids play a key role in the epidermal lipid matrix that adheres the epidermal corneocytes together [8]. Fatty acids also play a major part in the composition of ceramides and triglycerides, two of the other major lipids in the stratum corneum [8]. Epidermal triglyceride, a small fraction of the SC lipids, changes lead to incorrect formation of the cornified skin lipid envelope [16]. This epidermal structure usually provides an interface to uphold the permeability barrier in healthy subjects [16].

1.5 Use of Mass Spectrometry in Lipidomics Studies

Lipidomics has revealed a wide variety of ceramides, and other lipids, linked to human stratum corneum. Quantitative thin layer chromatography (TLC) was used to identify several ceramide subclasses in human stratum corneum in the late 1970s [9]. In 2003, a mixture of high performance thin layer chromatography (HPTLC) and nuclear magnetic resonance spectroscopy (NMR) was used to relate a reasonably complete account of the human epidermal ceramides [10]. Later, a total of twelve ceramide subclasses in human stratum corneum was recorded when liquid chromatography tandem mass spectrometry (LC-MS/MS) with an ion trap (IT) system, a Fourier transform-ion cyclotron resonance system and a triple quadruple system was used [11].
This progression of ceramide analysis also applies to the other lipids of the stratum corneum.

The research just mentioned above and several other studies mostly focus on one or two of the lipids within the SC [9,10, 11]. Therefore, these studies only provide a

partial lipid profile of their AD populations. In our study, a lipidomics analysis was performed to compare complete lipid profiles between healthy and AD individuals using LC-MS/MS methodology. We sought to determine if all the major lipids within the human SC were contributing to the development of a faculty skin barrier and consequently the development of AD. It was hypothesized that the lipid composition of AD individuals would be altered and/or possibly missing lipids compared to those of healthy individuals and that these differences in the AD subjects were promoting improper barrier structure and functions.

2. MATERIALS AND METHODS

2.1 Skin Tape Samples

De-identified, barcoded skin lipid tape strips were obtained from collaborators at Oregon Health and Sciences University and were analyzed for lipid composition. In total 42 individuals participated in the study, 27 of these subjects exhibit atopic dermatitis and 15 had a healthy phenotype. The only defining features of the samples we received were their skin disease status. Each subject had 20 tape strip samples and our study was performed using sample numbers five through eight, as these would provide a good lipid concentration from the stratum corneum. Another set of follow up visit tape strip samples from 11 of the original subjects was also received. Six were from healthy individuals and five from atopic dermatitis individuals. These samples were also subsequently analyzed in the same fashion as the original samples. In this current project we have compared the lipid profile of 11 subjects that were accepted for repeat visits.

2.2 Lipid Extraction and Mass Spectrometry Preparation

The lipids were extracted from the tape strips using a modified Bligh and Dyer extraction method [12]. A one step lipid extraction was performed from a modified method to increase efficiency and produce a higher yield of lipids (Figure 3). For the procedure, 1 ml extraction solvent ($CHCl_3$: CH_3OH: H_2O, 1:2:0.5) and 25 µl internal standard were added to the tubes with the tape strips, the tubes were then vortexed for two minutes and incubated at room temperature for one hour and subsequently centrifuged at 2,000 rpm for ten minutes. The chloroform phase with the dissolved lipids

was transferred to new vials pooling each individual's four tape strip chloroform phases into one vial. The samples were then dried under nitrogen and reconstituted using 300 µl of a methylene chloride: isopropanol: methanol (25:10:65) solution for mass spectrometry analysis.

Figure 3: *Schematic of one step tape strip lipid extraction from a modified Bligh and Dyer method. The extraction solvent and internal standard is added into tubes that contain the tape strips and the tubes are then incubated and centrifuged. The chloroform phase with the lipids is collected and pooled before being dried under nitrogen and then reconstituted for UPLC MS/MS analysis. This method was applied both for the original and follow up samples.*

2.3 Running the Mass Spectrometer (UPLC MS/MS)

Our samples were analyzed using ultra performance liquid chromatography tandem mass spectrometry with a triple quadruple system in collaboration with Jaewoo choi and Dr. Fred Stevens of the Linus Pauling Institute (Figure 4). Samples were first separated physically using liquid chromatography before flowing through the mass spectrometer. The ions flowed through the mass spectrometer according to mass and charge. The ions were then split into fragment ions and flowed through the final section of the spectrometer where they finally ended at a detector that recorded the data. Once the mass spectrometry was completed, samples were analyzed using PeakView computer software.

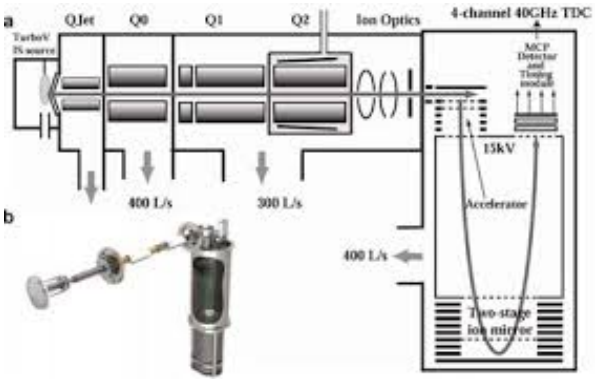

Figure 4: *Schematic of general mass spectrometry technique in which samples are injected into the mass spectrometer and ionized before flowing through the first chamber. The ions are then fragmented and flow through the last chamber before striking the detector plate. (Hopkins Medicine, 2012).*

3. SECTION 1: CERAMIDES

3.1 Original Study Ceramide Levels

3.1.1 CER[A] subclass

We first compared the level of CER[A] subclasses containing α-hydroxy fatty acid and different sphingoid bases between AD and healthy individuals. CER[ADS] (Figure 5A), CER[AP] (Figure 5B), CER[AS] (Figure 5C), and CER[AH] (Figure 5D) were significantly up-regulated in the AD individuals compared to healthy individuals, most noticeably the short chain CER and specifically the C34 chain length (e.g. CER[ADS], CER[AP], CER[AH]). The CER[ADS], CER[AP], CER[AH] C34 individual graphs also show the possibility of specific up-regulated AD subgroups within the AD population (Figure 6).

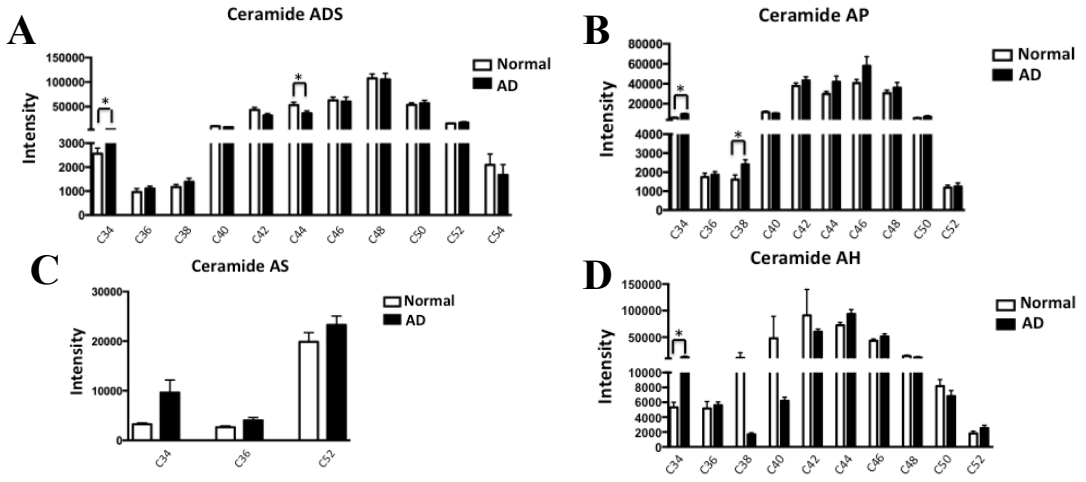

Figure 5: *Graphs from UPLC MS/MS analysis of the detected CER[A] subclasses with the white bars indicating CER[A] levels in healthy patients and the black bars showing levels in AD patients. (A) Up-regulated CER[ADS], composed of a dihydroxysphingosine base and an α-Hydroxy fatty acid, levels in AD subjects. (B) Up-regulated CER[AP], composed of a Phytosphingosine base and an α-Hydroxy fatty acid, levels in AD subjects. (C) Up-regulated CER[AS], composed of a sphingosine base and an α-Hydroxy fatty acid, levels in AD subjects. (D) Up-regulated CER[AH], composed of a 6-hydroxy sphingosine base and an α-Hydroxy fatty acid, levels in AD subjects. Statistical analyses were performed by student's unpaired t-test using Graphpad Prism software. *p<0.05.*

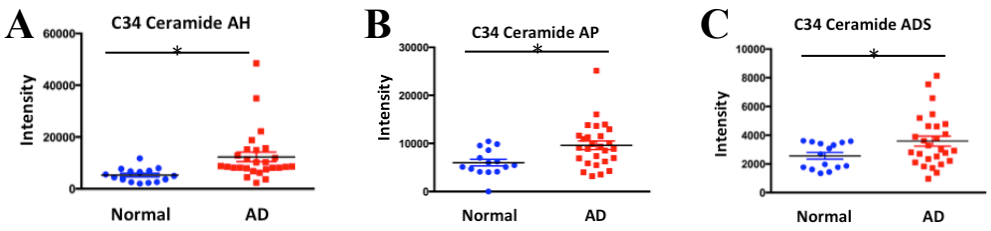

Figure 6: *The scatterplots from UPLC MS/MS analysis of the significantly up-regulated (A) CER[AH], (B) CER[AP], and (C) CER[ADS] C34 chain length with distinct up-regulated subgroups (significant difference = p value < 0.05). The blue circles indicate healthy subject CER[A] levels and the red squares indicate AD subject CER[A] levels. Statistical analyses were performed by student's unpaired t-test using Graphpad Prism software. *p<0.05.*

3.1.2 CER[N] subclass

The CER[N] subclasses contain non-hydroxy fatty acid and different sphingoid bases. Our results showed that short chain CER[NDS] (e.g C38,C40) (Figure 7A) and CER[NP] (e.g C34) (Figure 7C) were significantly up-regulated in AD subjects, while the long chain CER[NDS] were significantly down-regulated (Figure 7A). Both CER[NH] (Figure 7B) and CER[NS] (Figure 7D) were up-regulated in AD individuals at all chain lengths.

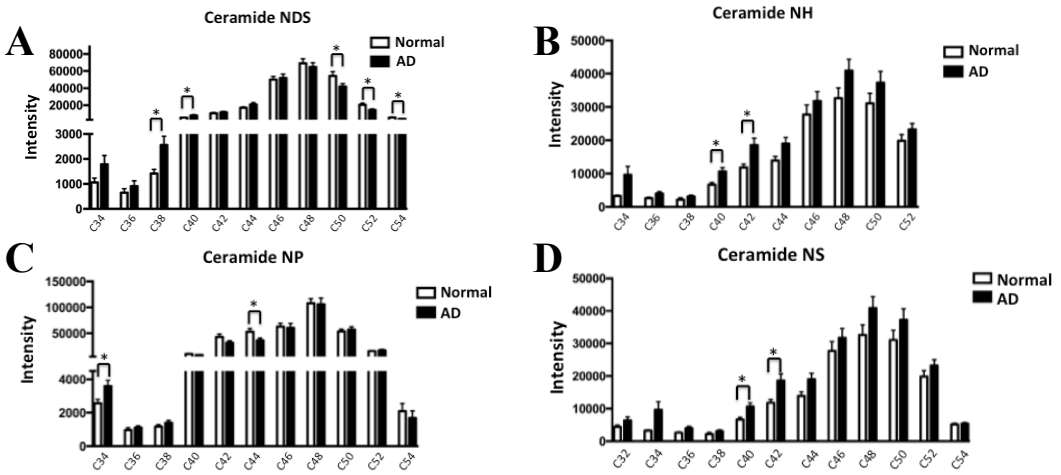

Figure 7: *Graphs from UPLC MS/MS analysis of the detected CER[N] subclasses with the white bars indicating CER[N] levels in healthy patients and the black bars showing levels in AD patients. (A) Up-regulated short chain CER[NDS] and down-regulated long chain CER[ADS], composed of a dihydroxysphingosine base and a non-hydroxy fatty acid, levels in AD subjects. (B) Up-regulated CER[NH], composed of a 6-hydroxy sphingosine base and a non-hydroxy fatty acid, levels in AD subjects. (C) Up-regulated C34 chain length CER[NP], composed of a Phytosphingosine base and a non-hydroxy fatty acid, in AD subjects. (D) Up-regulated CER[NS], composed of a sphingosine base and a non-hydroxy fatty acid, levels in AD subjects. Statistical analyses were performed by student's unpaired t-test using Graphpad Prism software. *p<0.05.*

3.1.3 CER[EO] subclass

Lastly, the CER[EO] subclasses contain esterfied ω-Hydroxy fatty acid with differing sphingoid bases. Our results showed the CER[EOdS] (e.g. C60, C62, C64, and C66) (Figure 8C) and CER[EOP] (e.g. C60) (Figure 8D) were significantly up-regulated in AD subjects, while the CER[EOdS] (e.g. C70 and C72) (Figure 8C), CER[EOP] (Figure 8D), CER[EOH] (Figure 8A), and CER[EOS] (Figure 8B) were down-regulated. The CER[EOP] C60 chain length individual graph interestingly showcases many ceramides that were at levels too low to detect (Figure 9).

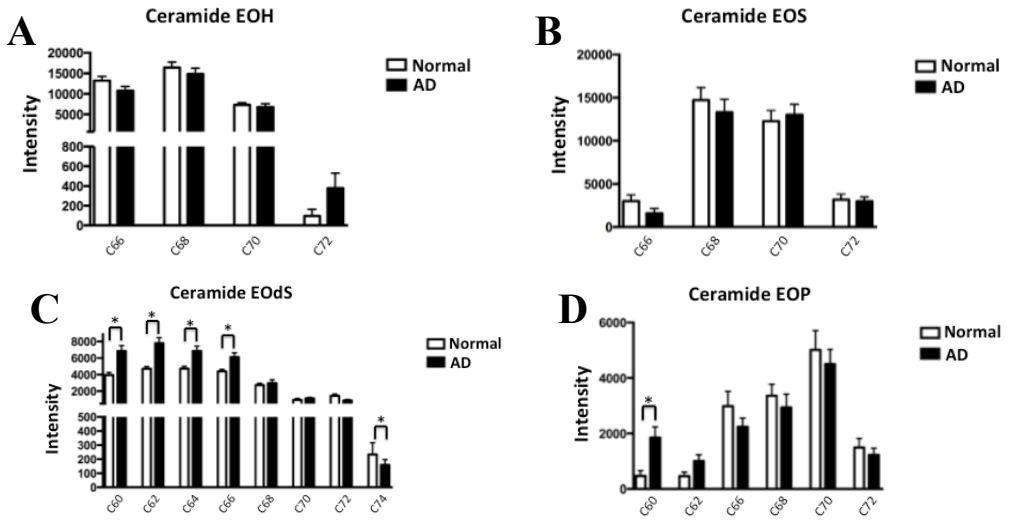

Figure 8: *Graphs from UPLC MS/MS analysis of the detected CER[EO] subclasses with the white bars indicating CER[EO] levels in healthy patients and the black bars showing levels in AD patients. (A) Down-regulated CER[EOH], composed of a 6-hydroxy sphingosine base and an esterfied ω-Hydroxy fatty acid, levels in AD subjects. (B) Down-regulated CER[EOS], composed of a sphingosine base and an esterfied ω-Hydroxy fatty acid, levels in AD subjects. (C) Down-regulated and up-regulated CER[EOdS], composed of a dihydroxysphingosine base and an esterfied ω-Hydroxy fatty acid, levels in AD subjects. (D) Down-regulated CER[EOP], composed of a Phytosphingosine base and an esterfied ω-Hydroxy fatty acid, levels in AD subjects. Statistical analyses were performed by student's unpaired t-test using Graphpad Prism software. *p<0.05.*

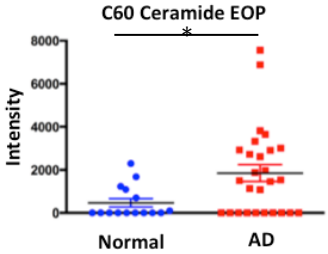

Figure 9: *Scatterplot from UPLC MS/MS analysis of the significantly up-regulated CER[EOP] C60 chain length showcasing ceramide levels too low to detect (significant difference = p value < 0.05). The blue circles indicate healthy subject CER[EOP] levels and the red squares AD subject CER[EOP] levels. Statistical analyses were performed by student's unpaired t-test using Graphpad Prism software. *p<0.05.*

3.2 Comparison Study Ceramide Levels

3.2.1 CER[A] subclass

CER[AS] (Figure 10A), CER[ADS] (Figure 10B), CER[AP] (Figure 10C), and CER[AH] (Figure 10D) all showed normalizations in the follow up study compared to the original study.

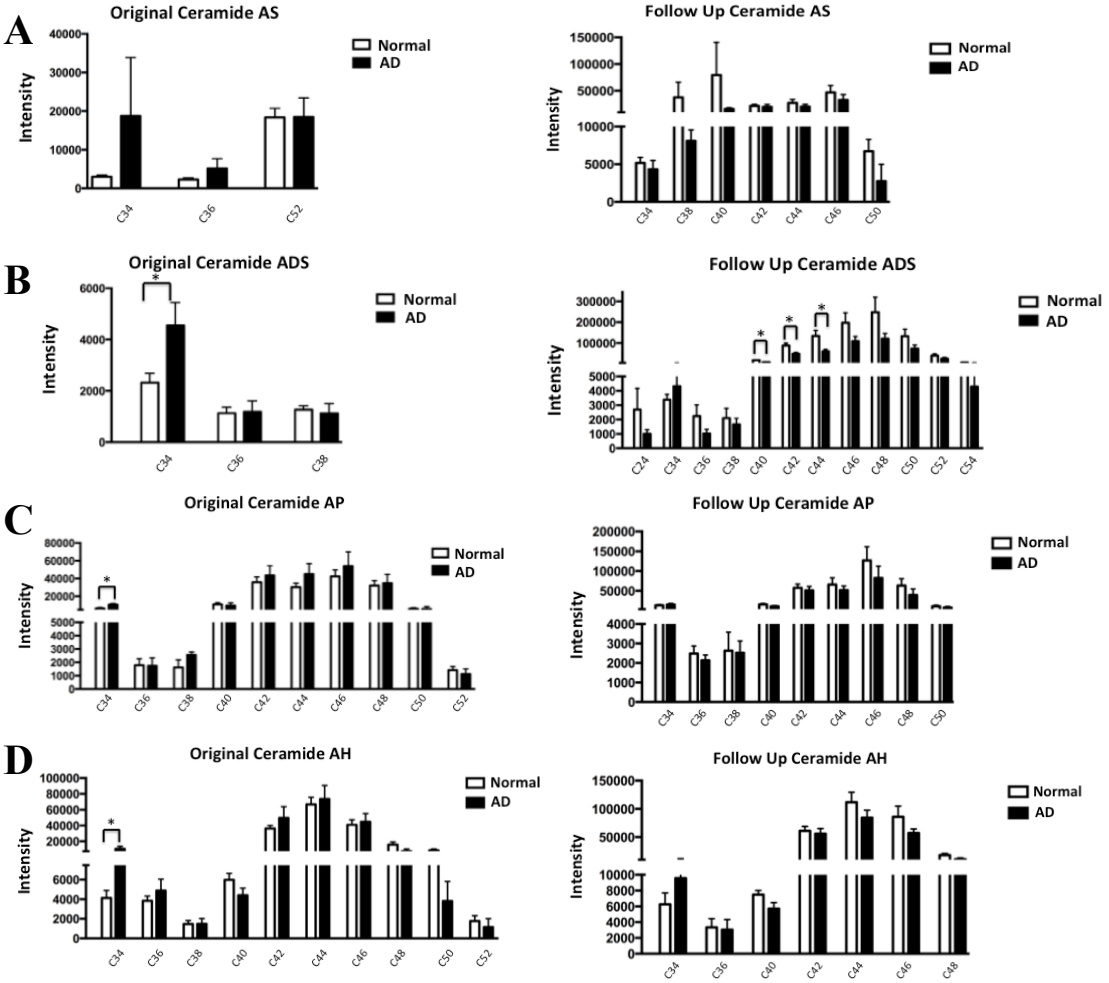

Figure 10: *Graphs from UPLC MS/MS analysis of the original and follow up detected CER[A] subclasses with the white bars indicating CER[A] levels in healthy patients and the black bars showing levels in AD patients. (A) Normalized CER[AS], composed of a sphingosine base and an α-Hydroxy fatty acid, levels in AD subjects in the follow up study compared to the original study. (B) Normalized CER[ADS], composed of a dihydroxysphingosine base and an α-Hydroxy fatty acid, levels in AD subjects in the follow up study compared to the original study. (C) Normalized CER[AP], composed of a Phytosphingosine base and an α-Hydroxy fatty acid, levels in AD subjects in the follow up study compared to the original study. (D) Normalized CER[AH], composed of a 6-hydroxy sphingosine base and an α-Hydroxy fatty acid, levels in AD subjects in the follow up study compared to the original study. Statistical analyses were performed by student's unpaired t-test using Graphpad Prism software. *p<0.05.*

3.2.2 CER[N] subclass

CER[NS] (Figure 11A), CER[NP] (Figure 11B), CER[NDS] (Figure 11C), and CER[NH] (Figure 11D) all showed normalizations in the follow up study compared to

the original study. CER[NP] (Figure 11B) and CER[NH] (Figure 11D) in particular showed over-normalizations, an example being CER[NH] C44 chain length in which the original CER[NH] was significantly up-regulated and the follow up CER[NH] is significantly down-regulated (Figure 12).

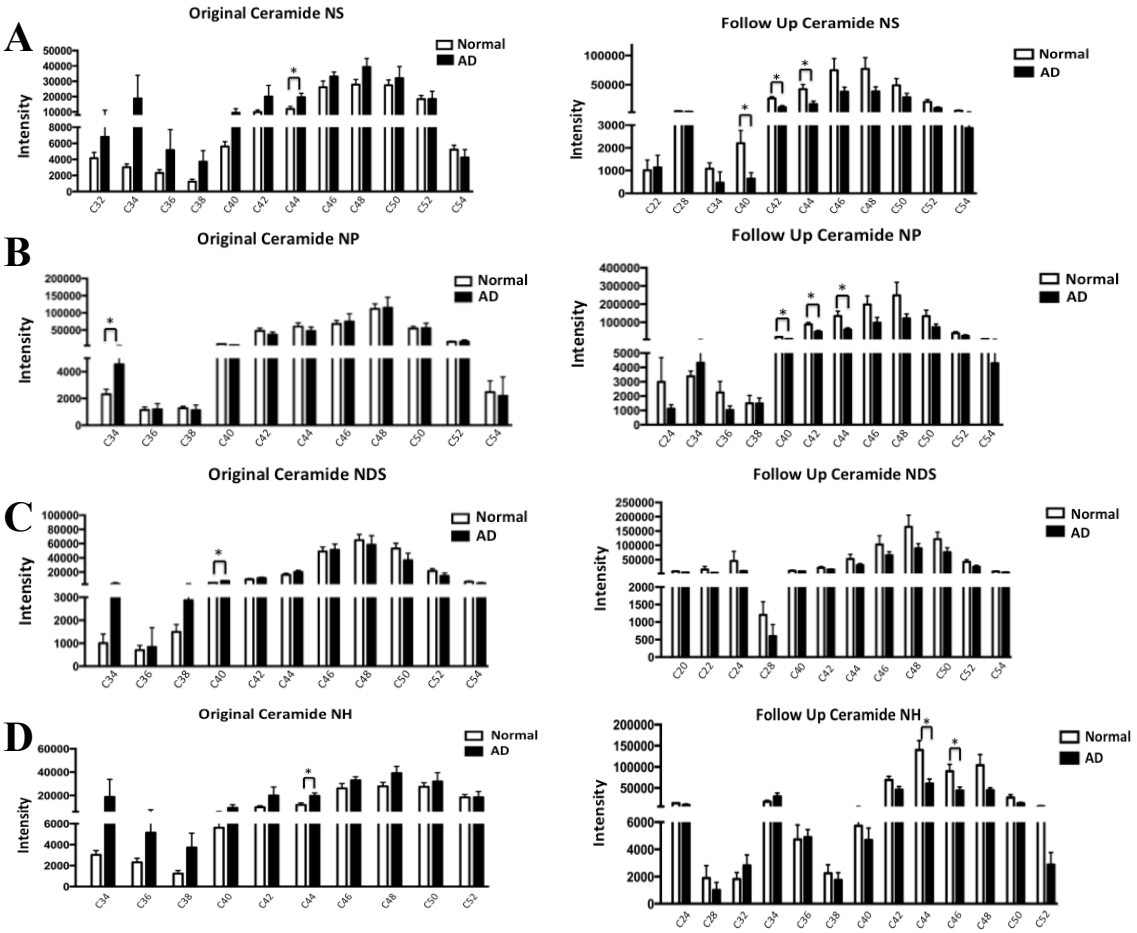

Figure 11: *Graphs from UPLC MS/MS analysis of the original and follow up detected CER[N] subclasses with the white bars indicating CER[N] levels in healthy patients and the black bars showing levels in AD patients. (A) Normalized CER[NS], composed of a sphingosine base and a non-Hydroxy fatty acid, levels in AD subjects in the follow up study compared to the original study. (B) Normalized CER[NP], composed of a Phytosphingosine base and a non-Hydroxy fatty acid, levels in AD subjects in the follow up study compared to the original study. (C) Normalized CER[NDS], composed of a dihydroxysphingosine base and a non-Hydroxy fatty acid, levels in AD subjects in the follow up study compared to the original study. (D) Normalized CER[NH], composed of a 6-hydroxy sphingosine base and a non-Hydroxy fatty acid, levels in AD subjects in the follow up study compared to the original study. Statistical analyses were performed by student's unpaired t-test using Graphpad Prism software. *$p<0.05$.*

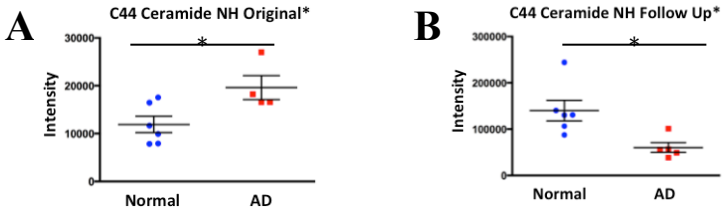

Figure 12: *Scatterplots from UPLC MS/MS analysis of the (A) significantly up-regulated original CER[NH] C44 chain length and (B) significantly down-regulated follow up CER[NH] C44 chain length (significant difference = p value < 0.05) showing an over-normalization in this ceramide in AD subjects. The blue circles indicate healthy subject CER[NH] levels and the red squares AD subject CER[NH] levels. Statistical analyses were performed by student's unpaired t-test using Graphpad Prism software. *p<0.05.*

3.2.3 CER[EO] subclass

CER[EOS] (Figure 13A), CER[EOP] (Figure 13B), and CER[EOdS] (Figure 13C) all showed normalizations in the follow up study compared to the original study. CER[EOS] (Figure 13A) and CER[EOP] (Figure 13B) in particular showed over-normalizations. CER[EOH], however, remained down-regulated between the original and follow up study (Figure 13D).

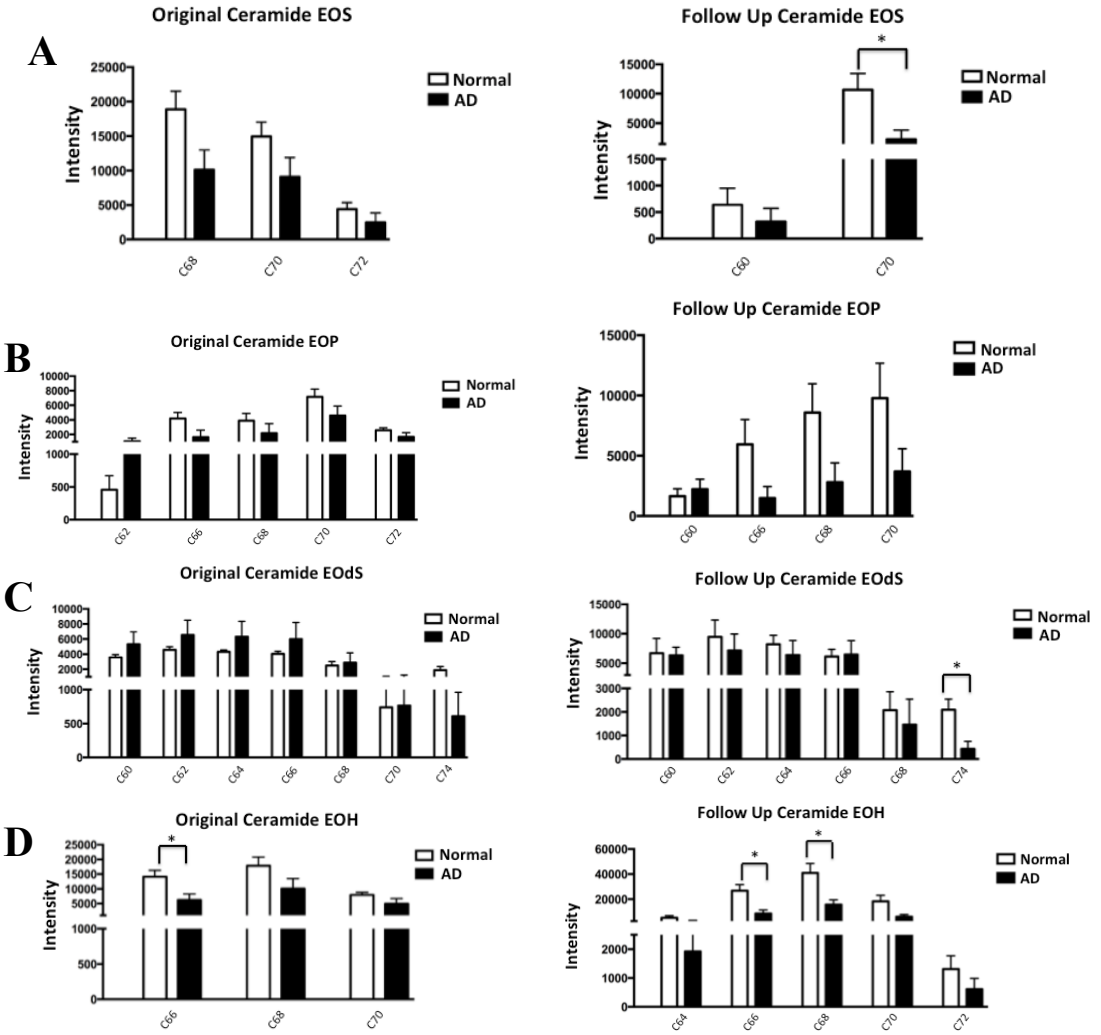

Figure 13: *Graphs from UPLC MS/MS analysis of the original and follow up detected CER[EO] subclasses with the white bars indicating CER[EO] levels in healthy patients and the black bars showing levels in AD patients. (A) Normalized CER[EOS], composed of a sphingosine base and an esterfied ω-Hydroxy fatty acid, levels in AD subjects in the follow up study compared to the original study. (B) Normalized CER[EOP], composed of a Phytosphingosine base and an esterfied ω-Hydroxy fatty acid, levels in AD subjects in the follow up study compared to the original study. (C) Normalized CER[EOdS], composed of a dihydroxysphingosine base and an esterfied ω-Hydroxy fatty acid, levels in AD subjects in the follow up study compared to the original study. (D) Normalized CER[EOH], composed of a 6-hydroxy sphingosine base and an esterfied ω-Hydroxy fatty acid, levels in AD subjects in the follow up study compared to the original study. Statistical analyses were performed by student's unpaired t-test using Graphpad Prism software. *p<0.05.*

4. SECTION 2: CHOLESTEROL, FREE FATTY ACIDS, AND TRIGLYCERIDES

4.1 Original Study Cholesterol, Free Fatty Acid, and Triglyceride Levels

There was no significant difference in cholesterol levels between healthy and AD individuals. Cholesterol-3-sulfate, however, was significantly up-regulated in AD subjects (Figure 14). Most of the FFA were down-regulated in AD individuals. Out of the nine free fatty acids detected, only two showed a significant down regulation, FFA 24:1 and 26:0 (Figure 15). Many of the triglycerides were up-regulated in AD individuals, excluding TAG 40:0 and 40:1 which were down regulated (Figure 16A). Three triglycerides showed significant up-regulation with possible up-regulated subgroups within the AD population (e.g. TAG 52:0, 52:3, 56:3) (Figure 16B, 16C, 16D).

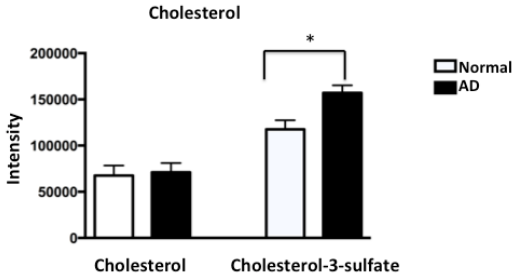

Figure 14: *Graph from UPLC MS/MS analysis of the unchanged cholesterol and significantly up-regulated cholesterol-3-sulfate in AD individuals compared to healthy individuals with the white bars indicating cholesterol and cholesterol-3-sulfate levels in healthy patients and the black bars showing levels in AD patients. Statistical analyses were performed by student's unpaired t-test using Graphpad Prism software. *p<0.05.*

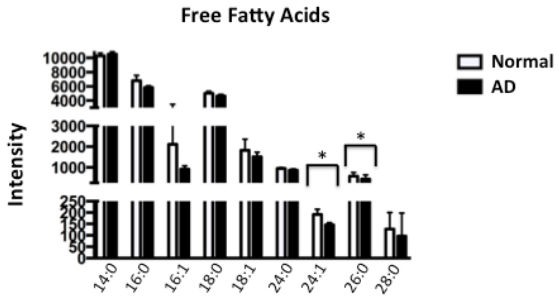

Figure 15: *Graph from UPLC MS/MS analysis of the down-regulated free fatty acids in AD subjects compared to the healthy subjects with the white bars indicating FFA levels in healthy patients and the black bars showing levels in AD patients. FFA 24:1 and 26:0 are significantly down-regulated in AD individuals (significant difference = p value < 0.05). Statistical analyses were performed by student's unpaired t-test using Graphpad Prism software. *p<0.05.*

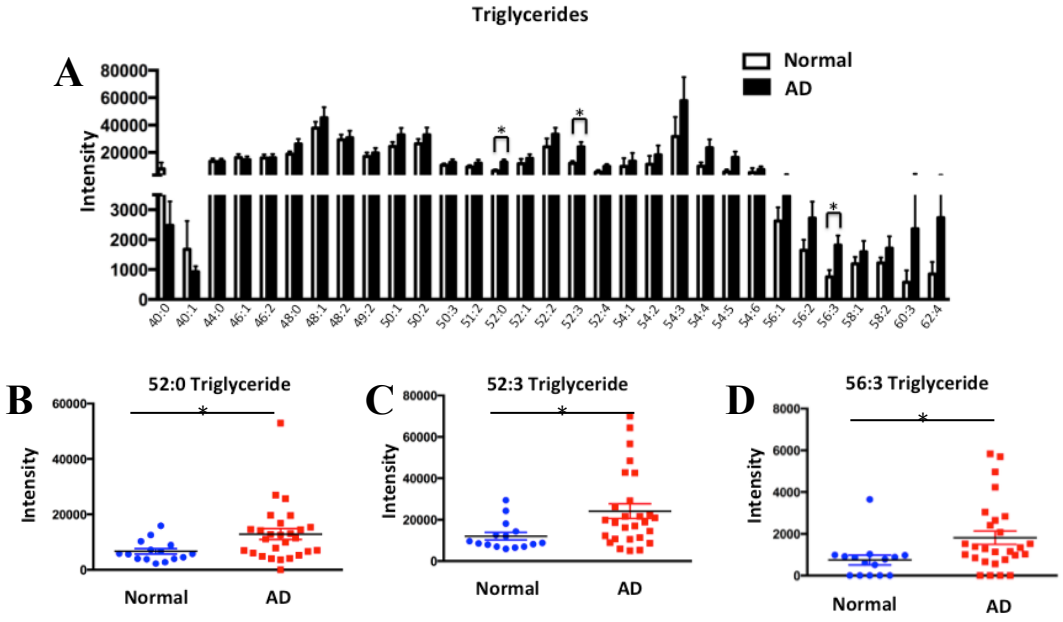

Figure 16: (A) *Graph from UPLC MS/MS analysis of the up-regulated triglycerides with the white bars indicating triglyceride levels in healthy patients and the black bars showing levels in AD patients. Scatterplots from UPLC MS/MS analysis of the significantly up-regulated (B) TAG 52:0, (C) TAG 52:3, and (D) TAG 56:3 with up-regulated subgroups (significant difference = p value < 0.05). The blue circles indicate healthy subject triglyceride levels and the red squares AD subject levels. Statistical analyses were performed by student's unpaired t-test using Graphpad Prism software. *p<0.05.*

4.2 Comparison Study Cholesterol, Free Fatty Acid, and Triglyceride Levels

The cholesterol showed no significant difference between the original and follow up research. Cholesterol-3-sulfate, however, was normalized in the follow up study (Figure 17). Some of the triglycerides (e.g. TAG 46:2, 48:0, 49:2, 52:0) (Figure 19) and

FFA 14:0 (Figure 18) were normalized in the follow up study compared to the original as well. The remaining triglycerides (e.g. TAG 40:0, 48:1, 48:2, 54:3, etc.) (Figure 19) and FFA (e.g FFA 16:0, 18:0, 18:1, etc.) (Figure 18), however, show relatively little change between the original and follow up research.

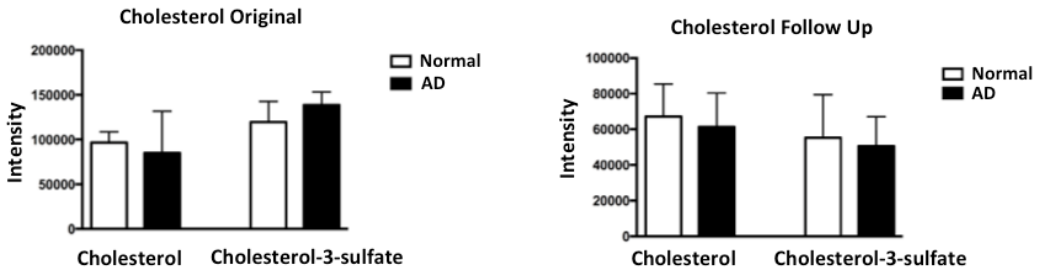

Figure 17: *Graphs from UPLC MS/MS analysis of the original and follow up detected cholesterol and cholesterol-3-sulfate with the white bars indicating cholesterol and cholesterol-3-sulfate levels in healthy patients and the black bars showing levels in AD patients. Cholesterol levels remain unchanged between the original and follow up study, while the cholesterol-3-sulfate was normalized in the follow up study compared to the original data. Statistical analyses were performed by student's unpaired t-test using Graphpad Prism software. *p<0.05.*

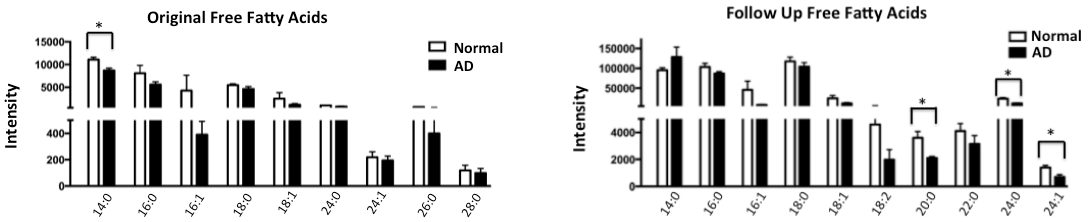

Figure 18: *Graphs from UPLC MS/MS analysis of the original and follow up detected free fatty acids with the white bars indicating FFA levels in healthy patients and the black bars showing levels in AD patients. Most of the FFA showed relatively little change between the original and follow up studies, except for FFA 14:0, which was normalized in the follow up study. Statistical analyses were performed by student's unpaired t-test using Graphpad Prism software. *p<0.05.*

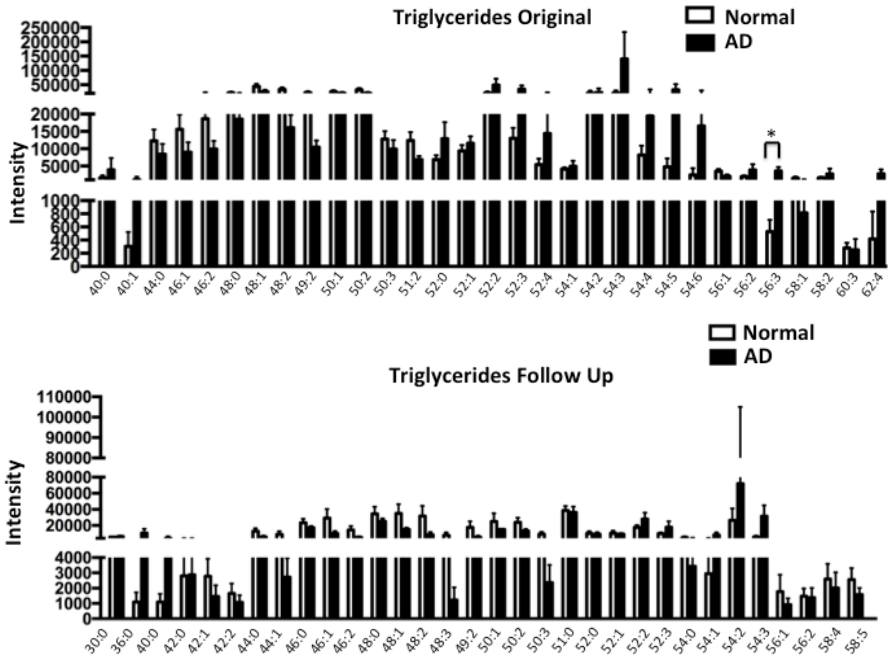

Figure 19: *Graphs from UPLC MS/MS analysis of the original and follow up detected triglycerides with the white bars indicating triglyceride levels in healthy patients and the black bars showing levels in AD patients. Some of the triglycerides (e.g. TAG 46:2, 48:0, 49:2, 52:0) were normalized in the follow up study compared to the original. The remaining triglycerides (e.g. TAG 40:0, 48:1, 48:2, 54:3, etc.) show relatively little change between the original and follow up research. Statistical analyses were performed by student's unpaired t-test using Graphpad Prism software. *$p<0.05$.*

5. DISCUSSION

5.1 Differences in Ceramide Levels in the Original Study

From the research reported by Masukawa et al., ceramides are known to have important functions in the water retention of the skin and, therefore, in the process of skin barrier formation and maintenance[14]. Increases in short chain CER and decreases in some long chain CER subclasses were also directly correlated to decreased SC barrier function in a TEWL study conducted by Janssens et al. [15]. The results of our study were consistent with the findings of these two previous studies. It is therefore likely that the changes seen in our AD patients are contributing to failure of their skin barriers and connected to the dry, itchy skin symptoms that many skin disease subjects experience [11].

Disparities were also found when comparing previous studies: several studies have previously detected significantly down regulated CER[NP] levels in AD individuals, while our research showed a mixture of both up regulated and down regulated CER[NP] levels [6, 15, 16]. In the study by Janssen et al., a down regulation of CER[NH] and CER[NDS] subclasses was also reported, whereas our study detected an up regulation in the CER[NH] and both up and down regulations in the CER[NDS] [16]. Their study also indicated an up regulation in the CER[AH] subclass, yet our study once again varied with both up regulations and down regulations within this subclass [16]. These differences could be attributed to the testing of different subpopulations of AD individuals in the two cases. Differing environmental and genetic conditions could be responsible for altering the lipid compositions of different AD populations to different

degrees leading to the variable results between studies. The relative amount of the bacteria *Staphylococcus aureus* on an AD subject's skin may also be affecting lipid composition as bacterial populations have been found to have an effect on the severity of AD symptoms [17].

5.2 Differences in Cholesterol, Free Fatty Acid, and Triglyceride Levels in the Original Study Group

As Cholesterol sulfate is important in keratinocyte differentiation that deals with the development of the skin barrier [18], an increase in cholesterol sulfate in AD subjects, as seen in our study, could lead to excess differentiation in keratinocytes disrupting the skin homeostasis. At this time, it appears that our study is one of the first to compare cholesterol and cholesterol-3-sulfate compositions in human stratum corneum between AD and healthy subjects using UPLC MS/MS. Moreover, increased levels of cholesterol-3-sulfate have been previously observed in X-linked ichthyosis with disrupted skin barrier function [19, 20] and unchanged levels of cholesterol were previously detected in AD individuals [21] using other techniques. Our results specifically suggest that increased levels of cholesterol-3-sulfate in AD subjects could be a risk factor for developing atopic dermatitis.

A down regulation of free fatty acids in AD patients, as seen in our research, would likely destabilize the lipid matrix of the epidermis [8]. A decrease in free fatty acids could also cause a drop in ceramide and triglyceride levels as free fatty acids are a critical component of their structures [8]. Decreases in FFA chain lengths in AD subjects were sited by Macheleidt et al. [22] and were directly correlated to decreased SC barrier

function in a TEWL study conducted by van Smeden et al. [6]. It is therefore likely that the decreases in FFA chain lengths seen in our AD patients are contributing to failure of their skin barriers.

The general up regulation in the triglycerides in AD individuals that our study showed could also be disturbing the proper balance of lipids in the skin that would then impair the skin barrier function, as triglyceride metabolism has been found to play a major role in the formation of a proper skin barrier from the research of Radner and Fischer [23]. Like cholesterol, our study appears to be one of the first to explore triglyceride composition differences between AD and healthy individuals using UPLC MS/MS. This is possibly due to the fact that triglycerides are only a small component of the lipid content of human SC.

5.3 Changes in Ceramide Levels in the Follow Up Subjects

The normalization of the CER[A] subclasses, the CER[NS] subclass, the CER[EOdS] subclass, the CER[NP] C34 chain length, and the CER[NDS] C40 chain length in the return patients possibly indicates that some type of treatment was performed on the AD individuals between their original visit and their follow up visit. We were not, however, informed if a treatment was in fact implemented during that time so we can only speculate on the relative ability of a treatment to effectively normalize the subject's ceramide values. These normalizations could have also been due to natural fluctuations in ceramide levels in the SC.

The enhanced down regulation in the follow up study of the CER[NP] subclass, CER[EOS] C70 chain length, and CER[EOP] subclass compared to the original study

may be an over-normalization to some treatment that the individuals received or it could also be due to the general fluctuation of these ceramide levels. The conversion from an up regulation in the original study to a down regulation in the follow up of the CER[NH] subclass may also signify an over-normalization. If treatments were utilized, these over-normalizations could indicate that the treatments were changing CER levels too drastically in AD subjects. With this knowledge, it could be possible to subsequently change the AD individual's treatment for producing normal CER levels. A few of the ceramides, the CER[NDS] and CER[EOH] subclasses, showed no changes, which could denote they did not respond to a certain treatment given or that no treatment was given and they had no fluctuations in these ceramide levels.

5.4 Changes in Cholesterol, Free Fatty Acid, and Triglyceride Levels in the Comparison Study of the Follow Up Subjects

The general normalization of several of the TAG, and the cholesterol-3-sulfate in the follow up study could suggest a response to some treatment or just show a general fluctuation in lipid content. Relatively no change in the cholesterol, remaining TAG, and most of the FFA levels, however, would signify these lipids did not possibly respond to treatment or simply did not fluctuate in their levels.

5.5 Research Benefits and Future Directions

The goal of this research was to determine the differences in SC skin lipid composition between AD and healthy subjects, likely one of the major factors contributing to skin disease. Understanding how lipid composition changes in AD

individuals could lead to the eventual development of more effective and specific treatments by supplementing those lipids back to the AD patients. Instead of just managing skin disease symptoms, we could be successful in mitigating disease progression and improving human health. The next steps in this research would be to determine the expression of genes involved in the skin barrier process and how they are regulating lipid synthesis and metabolism in the skin and also to further this study by identifying potential lipids that could be used for more effective treatment protocols.

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