Oaxaca, K. Celeste, Investigating the Effect of 17β-Estradiol on 24(S)-hydroxycholesterol Concentration in Brain Tissue. Master of Science (Biomedical Sciences), May 2021, 30 pp, 1 table, 11 illustrations, bibliography, 35 titles.

Estrogen, more specifically 17β-estradiol (E2), has been found to have neuroprotective effects in the brain. With Alzheimer's patients being comprised mainly of women, it is worthwhile to investigate the role that reproductive senescence plays in the development and progression of the disease due to decreased levels of estrogen. The purpose of this research was to develop a method for analyzing how estrogen affects the concentration of 24(S)-hydroxycholesterol (24S-OHC) in brain tissue. Ovariectomized (OVX) CD1 mice we subjected to daily injections of E2 for 5 days. Cholesterol and 24S-OHC from hippocampal tissue were analyzed by gas chromatography–mass spectrometry (GC-MS) assay developed for the purpose, and their concentrations were compared between the OVX E2 treated and OVX control groups. No significant difference was found between the two groups; nevertheless, the assay developed during this study will be beneficial for further investigation of E2 as a potential modulator of cholesterol metabolism in future studies.

INVESTIGATING THE EFFECT OF 17β-ESTRADIOL ON 24(S)-HYDROXYCHOLESTEROL CONCENTRATION IN BRAIN TISSUE

THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

K. Celeste Oaxaca, BA

Fort Worth, TX

May 2021

ACKNOWLEDGEMENTS

I would like to thank my major professor and PI, Dr. Laszlo Prokai, PhD, DSC, for laying the foundation for my research project, and for training me to operate the GC-MS, optimizing the method we used, and how to navigate through XCalibur. I would also like to thank my other advisory committee members, Dr. Katalin Prokai-Tatrai, PhD, Dr. Andras Lacko, PhD, and Dr. August Woerner, PhD, for your insights and for providing me with invaluable feedback throughout the development of this project.

I would also like to thank the other lab members Dr. Khadiza Zaman, Dr. Vien Nguyen, PhD, and Daniel de la Cruz, PhD candidate, who have helped me to carry out the experiment, oversee my work, and provided me with feedback in order to improve my technique. I sincerely appreciate you all.

TABLE OF CONTENTS

Page

LIST OF TABLES AND ILLUSTRATIONS

Page

CHAPTER I

INTRODUCTION

Estrogen as a neuroprotective agent

Estrogen, specifically 17β-estradiol (E2), has shown to be a neuroprotective hormone. There is evidence that suggest E2's role as a neuroprotective antioxidant [1,2,3] and show its ability to ameliorate certain effects of oxidative stress-related injury and redox dysregulation, both of which are involved in the initiation of neurodegenerative diseases [4,5].

Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis are developed and progressed to advanced stages of disease by neuroinflammation. These neurodegenerative diseases are associated with activation of microglia, which are involved in the immune and inflammatory response within the brain. Estrogens have been found to have anti-inflammatory effects, as they inhibit neuroinflammation and microglia activation [6]. For this reason, estrogens have been suggested as a potential therapeutic agent for delaying onset and progression of neurodegenerative diseases [6].

Evidence from prior epidemiological studies has shown that post-menopausal women are at greater risk for developing dementia than men in the same age group. It has been demonstrated that women's brain post-menopause tend to atrophy at an increased rate compared to men [7,8]. It is postulated that the marked decrease in estrogen levels accounts for the gender differences we see between men and women regarding prevalence of dementia.

Previous studies have shown that premature menopause is associated with a greater risk of dementia and cognitive impairment, and risk is even greater increased with a younger age for onset of menopause. Additionally, it has been reported that ovariectomy is associated with early-onset of dementia [7,9,10]. Because Alzheimer's disease is seen in females at a higher rate than in males, the use of ovariectomized (OVX) animals is a popular choice for studying the disease because of the correlation between diminished endogenous estrogens and increased occurrence of development of neurodegenerative disease in women [4,11].

A previous study from the lab utilized a double-transgenic Alzheimer's disease and OVX mouse model compared the use of E2 with the prodrug DHED that is converted to E2 in the brain by reductase in the brain [4,12]. The study showed that both E2 and DHED were successful at decreasing amyloid precursor protein (APP) and amyloid beta (Aβ) peptides in the brain tissue, while also improving outcomes of mice subjected to the radial arm water maze. This study illustrates the potential of E2 and E2-like compounds to slow the progression of AD.

Role of 24S-OHC as a biomarker for brain health

Oxysterols are cholesterol metabolites and intermediates of pregnenolone and steroid hormone synthesis [13, 14]. The oxidoreductase 24-hydroxylase (CYP461A) is responsible for the conversion of cholesterol to 24S-hydroxycholesterol (24S-OHC) in the brain. This enzyme is present in various regions of the brain including the hippocampus (especially the CA1 region), cerebellum, and the cortex, all of which are important in learning and memory. CYP461A appears to be expressed in a subset of neurons, which suggests that cholesterol synthesis and turnover are important to the function of these neurons. There is a constant turnover of cholesterol in the brain, which is metabolized to 24S-OHC and excreted into circulation. This

occurs at a rate equivalent to de novo synthesis of cholesterol in the brain [15] and is the brain's primary mechanism of replacing cholesterol to maintain steady-state sterol levels. One of the functions in question is long term potentiation (LTP) of synaptic transmission at Schaffer collateral-CA1 synapses. An experiment, which used CYP461A knockout mice, showed a deficit in LTP production at these specific synapses, which would impair learning and memory [16,17].

Additionally, a previous study utilizing CYP461A knockout mice models showed that overall cholesterol levels were unchanged between CYP461A knockout and wild type; however, there was a decrease in cholesterol metabolism in the knockout group. This may point to a compensatory mechanism to balance the decreased efflux of cholesterol metabolites from the CNS into the periphery in knockout mice. This compensatory mechanism was also illustrated in a prior study, which utilized 24-hydroxylase knockdown mice. In the mutant mice, there was nearly a 50% reduction in cholesterol breakdown that was balanced by a reduction of de novo cholesterol synthesis in order to maintain levels of cholesterol levels that are typical for wild type mice [16,18]. CYP461A knock-down in the striatum resulted in neurodegeneration and motor deficits that were similar to those seen in Huntington's disease [13,19].

On the other hand, upregulation of CYP461A resulted in reduction of both neurodegeneration and motor deficits. In a similar study that used an Alzheimer's disease mice model (APP23), overexpression of CYP461A resulted in mice that had decreased β-amyloid deposits, decreased astrogliosis and microgliosis, as well as improved spatial memory [13,20]. These findings suggest that 24S-OHC might play a role in neuroprotection and may be used as an indicator for brain health.

In human patients with Alzheimer's disease, there have been conflicting data regarding 24S-OHC levels in plasma. There are studies that have found AD patients to have increase in plasma 24S-OHC, while other studies have found decreased levels [15,21,22,23,24]. The reason for the variance between AD patients could be in part because of a link between increased 24S-OHC levels with early onset or mild cases of the disease, and decreased levels 24S-OHC with more advanced cases of the disease [15,25,26]. The role of estrogen on 24S-OHC levels in the brain has not been investigated, and the goal for this study is to understand how estrogen affects the concentration of 24S-OHC in brain tissue of OVX CD1 mice as a model. We expect E2 to result in an increase of 24S-OHC in the brain, considering the neuroprotective abilities of E2.

CHAPTER II

METHODS

Animals

Ovariectomized (OVX) CD1 mice (30 ± 4 g body weight) were obtained from Harlan Laboratories (Indianapolis, IN). The animals were housed in pairs under a 12 h light/dark cycle with the room conditioned to 21–23°C. Food and water were available ad libitum. Treatment groups were subjected to subcutaneous injections of E2 every day for 5 days before sacrifice, while control groups received control injections. Each experimental group consists of n=5, for a total of n=10. Treatment was a single daily dose selected based on dose-response studies (100 µg/kg body weight). Animals were sacrificed by cervical dislocation and decapitation as outlined by American Veterinary Medical Association (AVMA) Guidelines for Euthanasia of Animals. All treatments reviewed and approved by the Institutional Animal Care and Use Committee at UNTHSC.

Chemicals & Reagents

Cholesterol was purchased from Sigma-Aldrich (St. Louis, MO) and was prepared in a 1 µg/µL concentration in methanol, which was used during calibration along with 24(S)-hydroxycholesterol. Cholesterol-d_7 (CH-d_7), 24(S)-hydroxycholesterol (24S-OHC), and 24(S)-

hydroxycholesterol-d_7 (24S-OHC-d_7) were sourced from Cayman Chemical Company (Ann Arbor, MI) and were prepared in methanol in a 1 µg/µL, 5 ng/µL, and 5 ng/µL concentrations, respectively. Cholestenoic acid was sourced from Avanti Polar Lipids, Inc. (Alabaster, AL) and was prepared in a 1 µg/µL solution in methanol. 27-Hydroxycholesterol was sourced from Santa Cruz Biotechnology (Dallas, TX) and was prepared in a 1 µg/µL solution in methanol. Both cholestenoic acid and 27-hydroxycholesterol were used to generate total ion currents and both compounds were monitored but not quantified in our samples. Potassium hydroxide (KOH) was sourced from Thermo Fisher Scientific (Waltham, MA) and was prepared in a 2 M concentration for saponification of the analytes from the tissue. Butylated hydroxytoluene (BHT) was sourced from MP Biomedicals (Aurora, OH) and was prepared in a 200 µM concentration. Addition of BHT protects our analytes from autoxidation [27,28]. BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) + TMSC (trimethylchlorosilane) 99:1 was purchased from Cerilliant (Round Rock, TX) and was used to derivatize the analytes and internal standards in order to increase assay sensitivity. Hexane from Sigma-Aldrich (St. Louis, MO) was used for liquid-liquid extraction. Methanol (MeOH), acetonitrile (ACN), and de-ionized water (DI H_2O) were sourced from Fischer Scientific (Waltham, MA) and were of highly pure analytical grade for gas chromatography.

Solid Phase Extraction

During method development, solid phase extraction (SPE) was carried out using Supelclean™ LC-18 SPE tubes sourced from Supelco (Bellefonte, PA). The column was activated with 2 mL methanol. The first mL was gravity filtered, and the second mL was pushed

down the column with a syringe. The loading solvent was prepared and was comprised of MeOH, ACN, and DI H_2O in a 40:40:20 (v/v), respectively. The column was equilibrated by the addition of 2mL of the loading solvent. The sample was dissolved in 400 µL ACN, 400 µL MeOH, and 200 µL DI H_2O, and was vortexed in between each addition. The sample was loaded into the column and washed down with 1 mL of wash solvent, which was comprised of MeOH, ACN, DI H_2O in a 82:12:6 (v/v), and was collected as the oxysterol fraction. Next, 1 mL of MeOH was added to the column and the eluted portion was collected as the cholesterol fraction.

Instrumentation

Analyses were done on a TRACE 1300 gas chromatograph equipped with an AI 1310 Autoinjector and interfaced to an ISQ 7000 quadrupole mass spectrometer (Thermo Scientific, San Jose, CA). A 30 m × 0.25 mm i.d. column with 0.25 µm thick 5SILMS stationary phase (5% phenylsiloxane-containing methylsiloxane; TraceGold™, Thermo Scientific) was used for the separation at 1.5 mL/min flow rate of the helium carrier gas.

Assay Conditions

The column oven temperature was held isothermal at 150 °C for 1 min, then raised to 340 °C at 20 °C/min rate and held at 340 °C for 9 min. Samples (1 µL aliquots) were injected in splitless mode at injector temperature of 200 °C. The mass spectrometer was operated with electron ionization (EI) at electron energy set to 70 eV. Ion source and transfer line temperatures

were set to 250 °C and 300 °C, respectively. Full-scan mass spectra were acquired in the *m/z* 50–800 range with cycle time of 0.2 s. These parameters were the same for split mode, with the addition split flow set at 150 mL/min at a split ratio of 100. The system was controlled by Thermo Scientific's XCalibur software (version 4.0), and data was processed with the Qual Browser module of the program to determine ions of the analytes and internal standards (ISs) for quantitation by selected-ion monitoring (SIM). SIM-based quantitation was performed through the Quan Browser module of XCalibur [27]. SIM scans were acquired from 8 –12.24 minutes for the quantifier and qualifier ions listed in Table 1.

	Quantifier	**Qualifier**	**Retention Time**
	m/z	*m/z*	min
Cholesterol	368	329	10.41
Cholesterol-d_7	375	336	10.31
24S-OHC	145	456	11.18
24S-OHC-d_7	152	463	11.15
27-OHC	456	546	11.48
Cholestenoic acid	431	470	11.81

Table 1. Quantifier ions, qualifier ions, and retention times used for SIM acquisition for each compound.

Sample Preparation

The hippocampus was isolated for analysis, as both estrogen receptors and CYP461A are highly expressed in this brain region [16,29,30]. To prepare the samples, 10 mg hippocampal tissue of each animal was transferred to homogenizer tubes and homogenized in 500 µL de-ionized water. Then, 10 µL each of CH-d_7 and 24S-OHC-d_7 were added to the homogenizer tube as internal standards, vortexed for 1 min and each sample was transferred to a scintillation vial. In order to ensure complete transfer of sample, the homogenizer tube was washed with 250 µL KOH solution, which was then transferred into the scintillation vial. The same was done with 250 µL BHT solution. The samples were then incubated at 40 °C for 1 h to allow saponification to occur, so that the cholesterol and oxysterols become hydrolyzed and free from their ester form. DI H_2O (250 µl) was added to each sample, followed by 2 mL hexane. Samples were vortexed for 1 min, and then centrifuged for 15 min at 3000 rpm. The organic phase was collected, then an additional 2 mL hexane was added, and samples centrifuged again for a total of three extractions by hexane. The combined extracts were dried by Speedvac. The residue was derivatized by trimethylsilylation. The silylation reaction was done by addition of 200 µL BSTFA (containing 1% v/v TMCS as a catalyst) into the sample vial. Samples were then vortexed for 1 min and incubated at 60 °C for 30 minutes for derivatization to occur [27]. Samples were then transferred to autosampler vials for analysis by GC-MS.

CHAPTER III

RESULTS AND DISCUSSION

Assay Development and Implementation

Separate aliquots of the analytes and internal standards were derivatized with BSTFA and analyzed individually by GC-MS in order to generate total ion currents and observe fragmentation patterns for each compound. Derivatization with BSTFA is important because it favors detection and identification of oxysterols. Spectra and chromatograms for the analytes and internal standards can be found in the Appendix. Retention times, quantifier and qualifier ions are listed in Table 1. The isotope dilution principle was applied in this experiment through the use of deuterated internal standards. This method is ideal because of the identical structure and chemical properties of the internal standards to their corresponding analytes, which allows for precise quantification [28,31]. The analytes and their corresponding internal standards elute near the same retention time. The different mass-to-charge (*m/z*) values ensure total analyte separation from the internal standard when measuring their signals [28].

A mock extraction was done to create the calibration curve. The calibration curve plotted 5 calibration points of IS:analyte ratios of 1:0.1, 1:1, 1:3, 1:10, and 1:30. The calibration points were plotted to represent the ratio under the curve for cholesterol:cholesterol-d_7 and 24S-OHC:24S-OHC-d_7 as a function of absolute analyte concentration. Linear regression tests showed linearity for both calibration curves, with determination coefficient (R^2) of 0.985 for cholesterol, and 0.999 for 24S-OHC.

There is a possibility of signal overlap from the deuterated internal standards to the analyte because the standards elute near the same time as the analytes. To account for this potential overlap, both cholesterol-d_7 and 24S-OHC-d_7 were mixed together at the same volume and concentration used for tissue analysis. The mix was dried by vacuum (Speedvac) and derivatized with 200 µL BSTFA. The solution was analyzed with GC-MS three times and selected-ion monitoring (SIM) was used to measure signals for analytes and ISs. The ion-current ratio of analyte/IS was calculated for the three analyses and averaged. The average was then subtracted from the area of 24S-OHC in the calculation for the ion-current ratio of 24S-OHC to 24S-OHC-d7. The calibration curves continue to show linearity even after this correction in signal overlap (Figures 1 and 2).

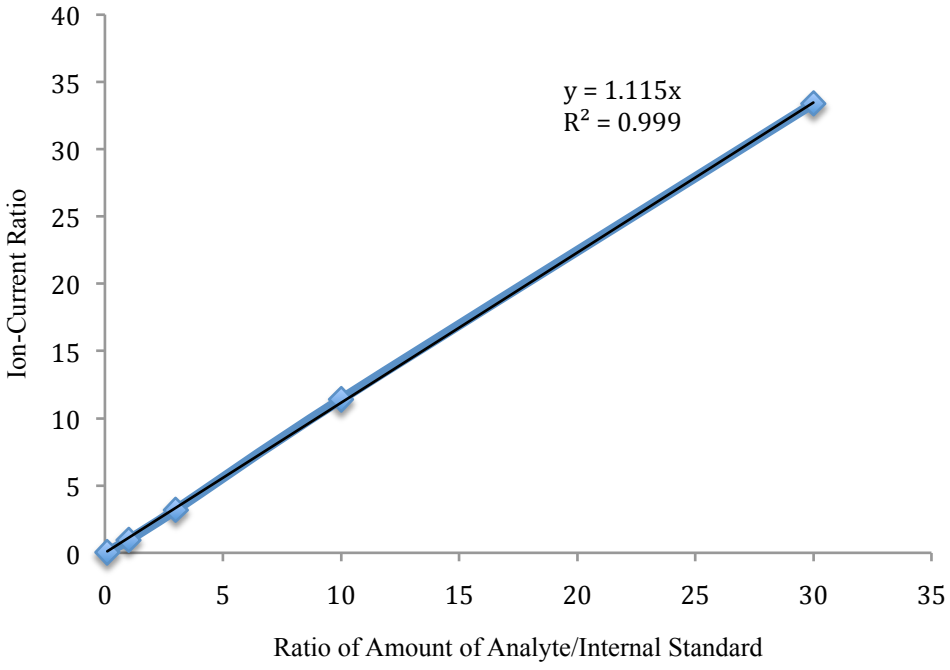

Figure 1. Calibration curve for 24S-OHC. Ion-current ratio versus amount ratio of analyte/IS.

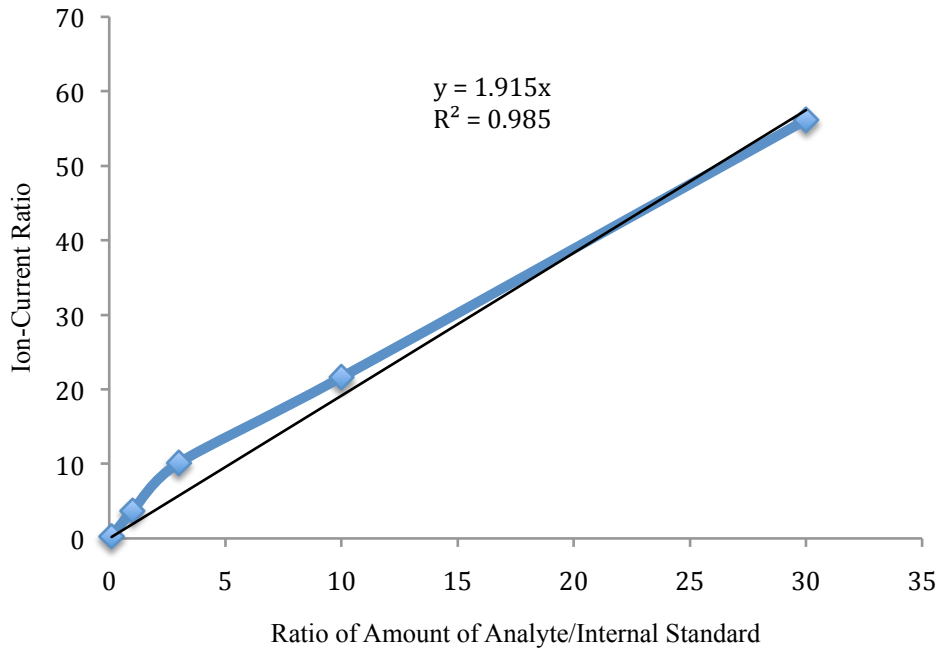

Figure 2. Calibration curve for cholesterol. Ion-current ratio versus amount ratio of analyte/IS.

To prepare for tissue processing, two mock tissue extractions were done in order to verify if SPE was an essential step in the protocol. One sample was subjected to SPE to fraction out the cholesterol and oxysterols. The second sample was not subjected to solid phase extraction and was derivatized by BSTFA just after drying from liquid-liquid extraction. Analysis from these separate extraction methods showed that while SPE did indeed enrich the 24S-OHC fraction, the recovery of the analytes and internal standards was not affected from forgoing SPE. Therefore, SPE was deemed an unnecessary step and was excluded from the protocol.

After preparing the tissue samples, the aliquots were run using the splitless injection method. Splitless injection is required for analysis of 24S-OHC because it is present in small amounts in the brain compared to cholesterol. The cholesterol was over-abundant in the samples,

and it overloaded the column showing chromatographic peaks with a fronting effect. The split injection method was used for a second run of each sample in the sequence to ameliorate this issue [32]. Ion-current ratios for 24S-OHC were taken from SIM chromatograms obtained by the splitless method, while ratios for cholesterol were taken from the SIM chromatograms recorded by using split injections. The absolute amount of analytes in the tissue was calculated with the equation from Prokai et al. [33]. The equation was rearranged to solve for the amount of analyte in the samples. The analyte/IS area ratio ($R_{\mathrm{Anal/IS}}$) was multiplied by the known concentration of the internal standard (n_{IS}), then divided the product by the k value, which is derived from the calibration curve. Analyte concentrations were expressed as ng/mg wet tissue (24S-OHC) and μg/mg wet tissue (cholesterol) based on the measured analyte amounts and tissue wet weight processed (10 mg).

Statistical Analysis

Linear regression analyses were used to confirm linear dependence between the ratio of analytes to internal standards and the concentration of the samples. Both the student's *t*-test and the Welch *t*-test were used to determine whether statistically significant differences ($p<0.05$) were obtained between the control and E2-treated groups when comparing concentration of 24S-OHC, cholesterol, and the ratio of 24S-OHC to cholesterol.

Assay Results

The difference in concentration of 24S-OHC and cholesterol were compared between the control group and the E2-treated group. The average concentration of both 24S-OHC and cholesterol were slightly elevated in the E2-treated group, with the treated group showing an average concentrations of 64.3 ng 24S-OHC/mg wet tissue and 70.0 µg cholesterol /mg wet tissue, compared to the control group which had averaged concentrations of 61.7 ng 24S-OHC/mg wet tissue and 62.4 µg cholesterol /mg wet tissue. The data was analyzed using the student's *t*-tests to determine if there was a significant change between the groups. Despite the slight elevation in the E2-treated group, no statistically significant difference ($p<0.05$) was found between the two groups. Representing the data in a box and whisker plot shows the variability among the data within each set, especially the control group. The coefficient of variation (CV) for both cholesterol and 24S-OHC of the control group was greater than 20. Interestingly, the E2 treated group showed data points that were close together (Figures 3 and 4) and both cholesterol and 24S-OHC had CV of 10% or less. The ratio of 24S-OHC to cholesterol was also analyzed and compared between the control and E2 treated group. The ratio of 24S-OHC to cholesterol between the two groups remained fairly consistent, and both groups had CV less than 10%. The control group had a slightly higher 24S-OHC/cholesterol ratio than the E2-treated group (Figure 5). The difference between the two groups was not statistically significant.

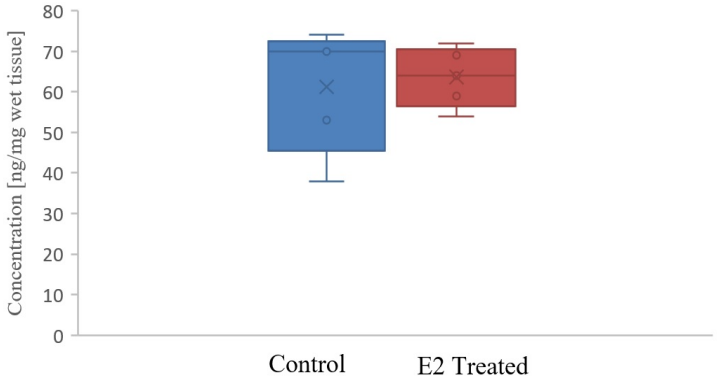

Figure 3. Box-and-whisker plot displaying the measured 24S)-OHC concentration in hippocampal tissue as ng/mg wet tissue (n=5/group). The horizontal line across each box represents the median, while the X represents the mean. No statistically significant difference is seen between the two groups ($p = 0.74$).

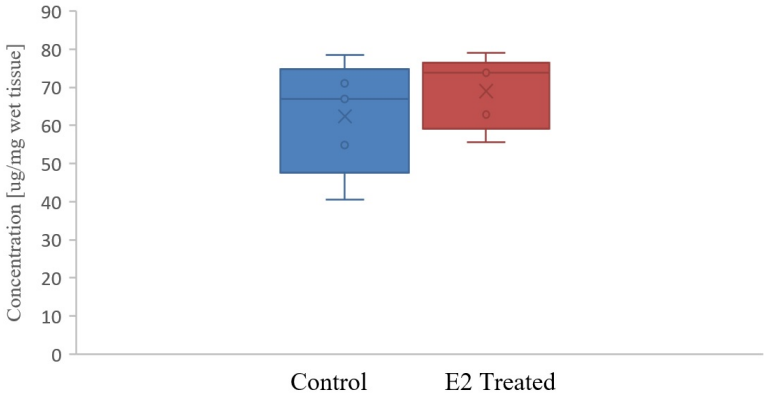

Figure 4. Box-and-whisker plot displaying the measured cholesterol concentration in hippocampal tissue as µg/mg wet tissue (n=5/group). The horizontal line across each box represents the median, while the X represents the mean. No statistically significant difference is seen between the two groups ($p = 0.38$).

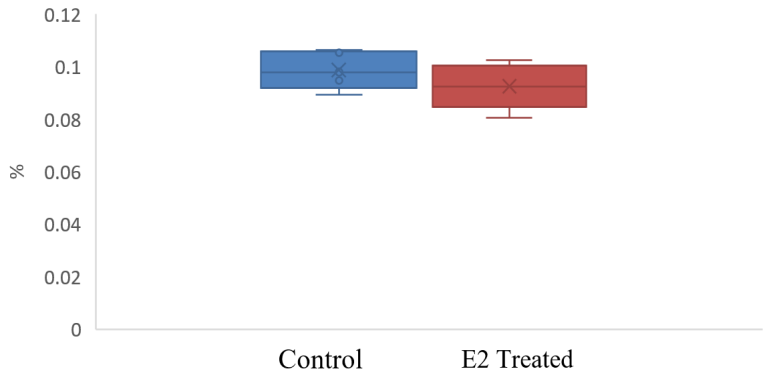

Figure 5. Box-and-whisker plot displaying the ratio of 24S-OHC to cholesterol expressed as a percent between the control and E2 treated group. This data represents n=5/group. The horizontal line across each box represents the median, while the X represents the mean. No statistically significant difference is seen between the two groups ($p = 0.35$)

Interpretation of the Results in Neurobiological Context

Cholesterol is highly concentrated in brain tissue because it is a major structural component of both myelin sheaths and neuronal cell membranes [15]. It is expected for cholesterol content to be in 10^3–10^6-fold greater than oxysterol content [34]. We expected for the E2-treated group to show an increase in 24S-OHC in the brain; however, this was not observed in this study. One possible reason why no difference was seen in 24S-OHC levels may be due to the length of treatment time. The mice in this study were subjected to injections of E2 once per day over a 5-day period. Five days of treatment may not be a sufficient amount of time to cause a change in the oxysterol profile of these animals. Perhaps a treatment regimen of 30 days or longer would show a difference in 24S-OHC between the experimental groups. Another possible reason why no significant differences were observed between cholesterol and 24S-OHC may be because

conversion of cholesterol to 24S-OHC by way of CYP46A1 is the main mechanism of action for cholesterol turnover and clearance from the brain [15]. It is possible that any increase in 24S-OHC concentration may have fluxed out of the CNS and into peripheral circulation to be metabolized by the liver [13].

The presence of both 27-hydroxycholesterol (27-OHC) and cholestenoic acid were monitored in the tissue as controls, but these compounds were not found within the samples, with any signal being indistinguishable from background noise. It was not expected for 27-OHC to be abundant in these tissues, as 27-OHC is synthesized only in minute quantities by neuronal and glial cells compared to 24S-OHC, which is the primary oxysterol derived from the brain [34]. 27-OHC is mainly synthesized in the periphery, where it gets taken up by the liver and converted to bile acids. Likewise, cholestenoic acid was not expected in great quantities in the brain tissue. While it is present in the brain in minute amounts as a cholesterol metabolite, it is found in the peripheral circulation in larger amounts. Nevertheless, it has been suggested that limited amounts of cholestenoic acid from the periphery cross over into the central nervous system [35].

CHAPTER IV

CONCLUSION

Future Studies and Perspectives

This study aimed to investigate if CD1 mice treated with E2 injections would have altered levels of oxysterol content in hippocampal tissue. While there was no significant change in 24S-OHC levels in this study, the method developed from this experiment will be useful for further studies moving forward. There are additional studies that should be considered in order to rule out estrogen as a moderator of cholesterol metabolism in the brain. It may be worthwhile to increase the length of E2 treatment in mice, as 5 days may not be long enough to significantly impact cholesterol metabolism. An extended treatment regimen would require changes to the treatment protocol, such as implantation of a mini-osmotic pump device such as the one used in Tschiffely et al. [12]. It is important to note that the ratio of 24S-OHC to cholesterol should be evaluated in studies moving forward, as this value seems to be more consistent than the concentrations themselves.

The serum of these animals has yet to be analyzed and has the potential to show a significant difference in the level of 24S-OHC between treated and control groups. 24S-OHC is synthesized from cholesterol as a means to clear cholesterol from the brain to make way for cholesterol synthesized de novo [15]. A possible increase may be hidden within the serum; however, a longer treatment duration may be required to see this change as well.

Additionally, the analysis of hippocampal tissue from male mice treated with E2 will be done to investigate any sex differences for the same analytes. Estrogen is an important hormone even to the male brain and has been shown to decrease accumulation of amyloid-beta plaques in an Alzheimer's disease mouse model of male mice [4]. However, there still may be no appreciable difference between treated and control groups in males due to the aforementioned reasons of treatment duration and flux of 24S-OHC to the periphery. Nevertheless, it is important to compare both sexes to conduct a thorough investigation.

APPENDIX

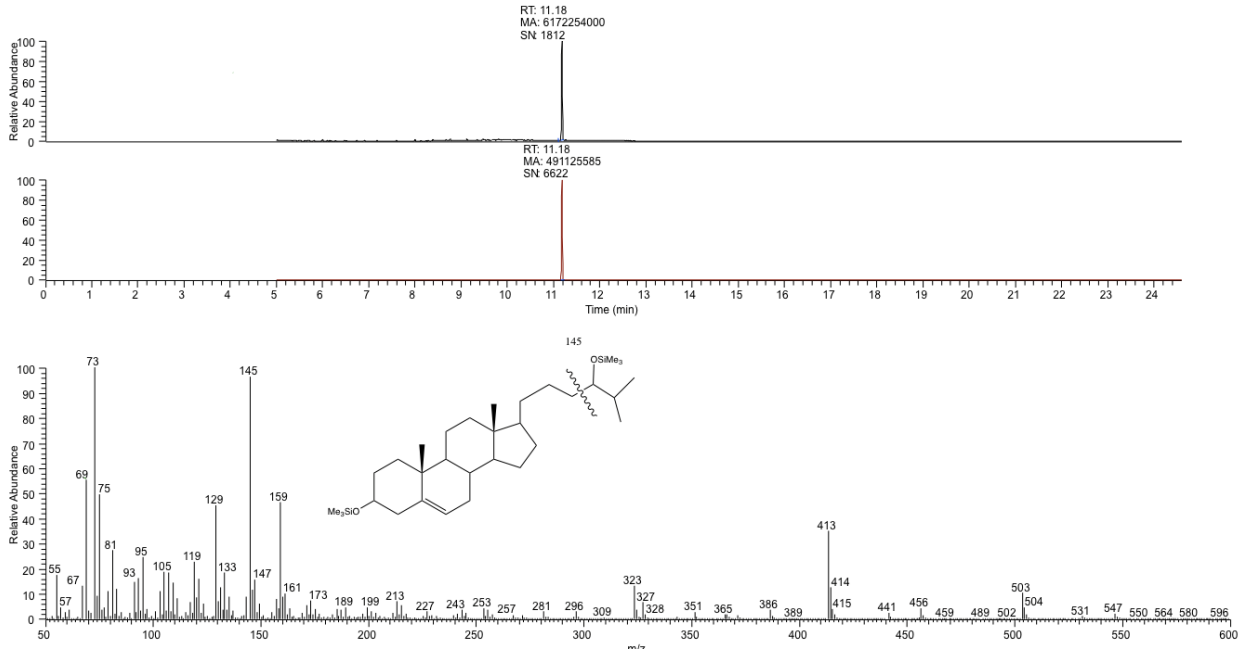

FIGURE S1. Chromatogram, full-scan EI mass spectrum, and chemical structure of silylated 24(S)-hydroxycholesterol, showing the fragmented quantifier ion at *m/z* 145.

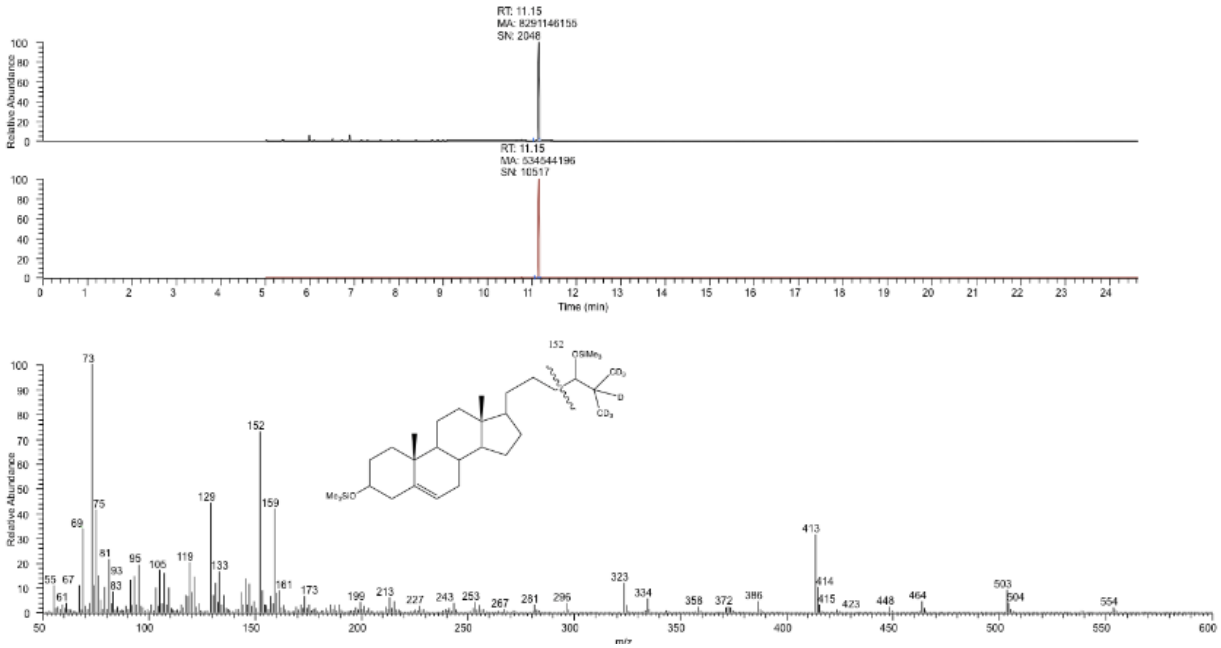

FIGURE S2. Chromatogram, full-scan EI mass spectrum, and chemical structure of silylated 24(S)-hydroxycholesterol-d_7, showing the fragmented quantifier ion at *m/z* 152.

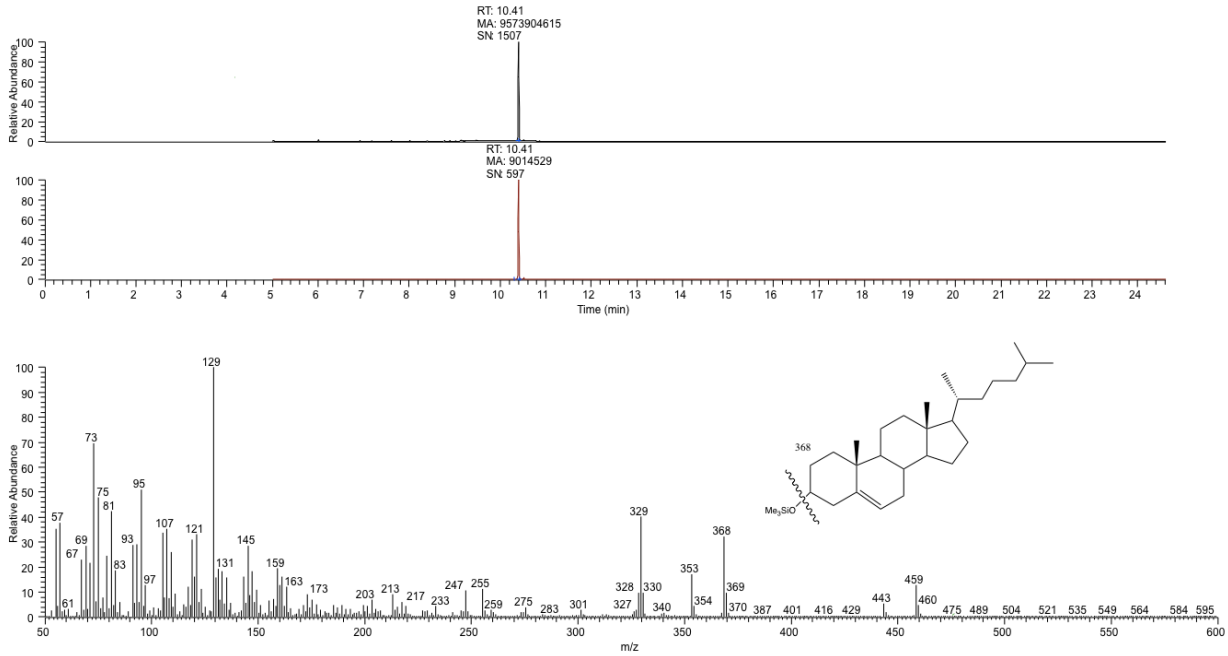

FIGURE S3. Chromatogram, full-scan EI mass spectrum, and chemical structure of silylated cholesterol, showing the fragmented quantifier ion at *m/z* 368.

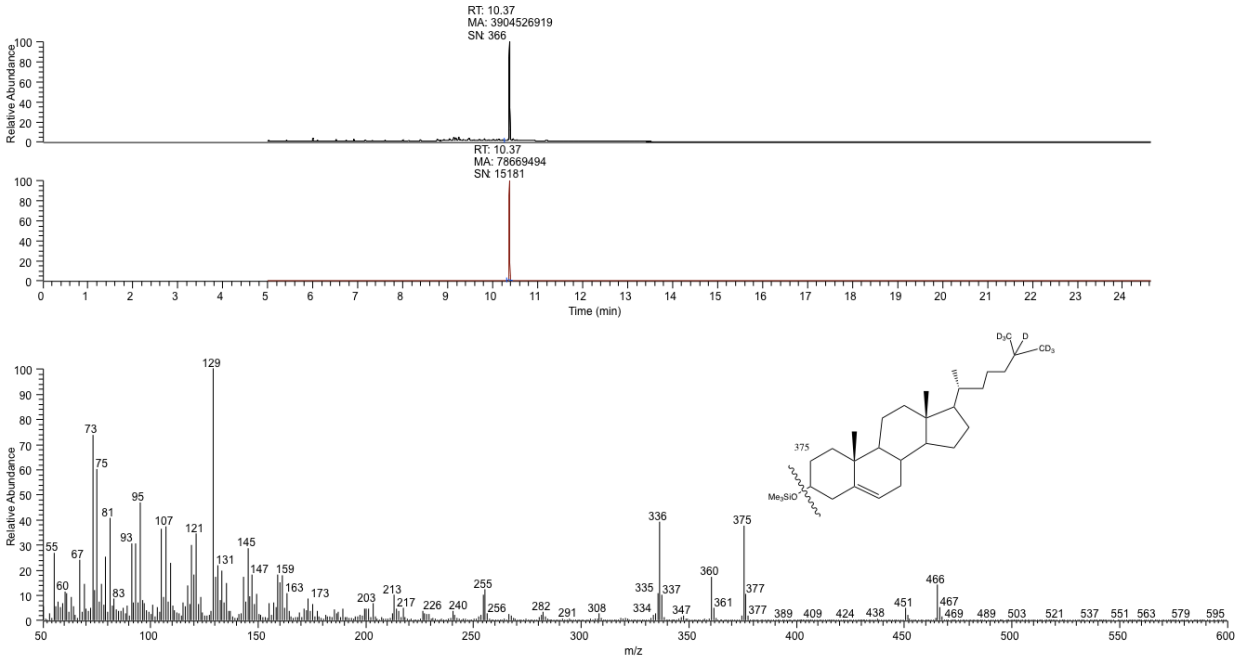

FIGURE S4. Chromatogram, full-scan EI mass spectrum, and chemical structure of silylated cholesterol-d_7, showing the fragmented quantifier ion at *m/z* 375.

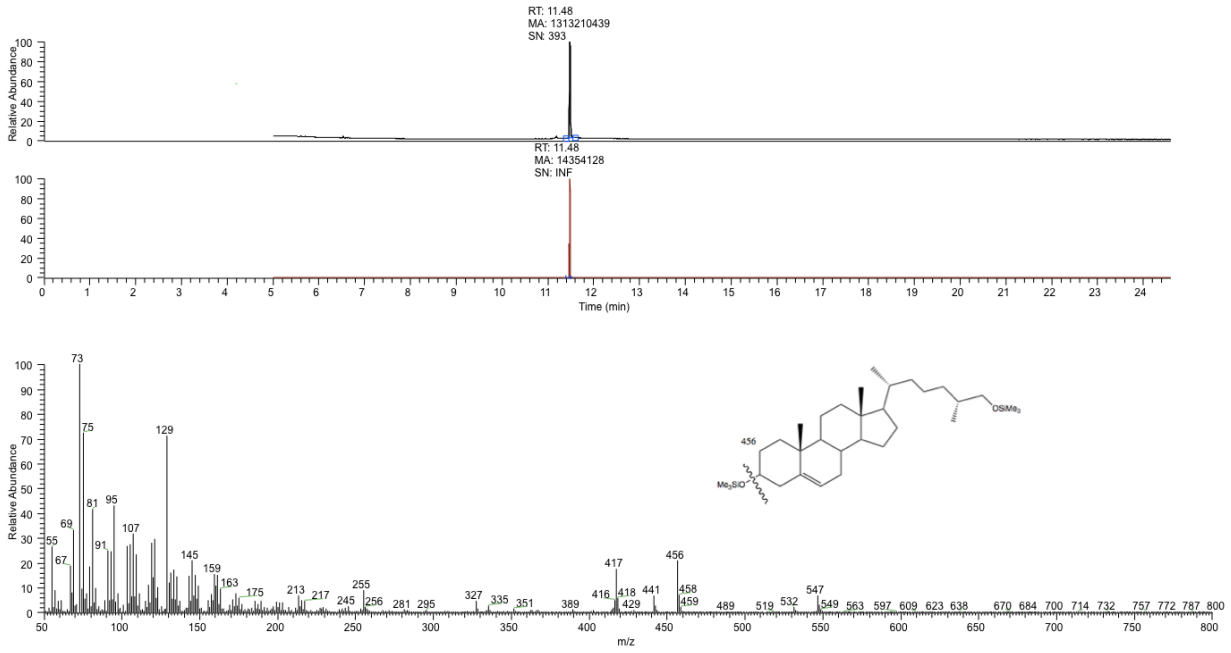

FIGURE S5. Chromatogram, full-scan EI mass spectrum, and chemical structure of silylated 27-hydroxycholesterol, showing the fragmented quantifier ion at *m/z* 456.

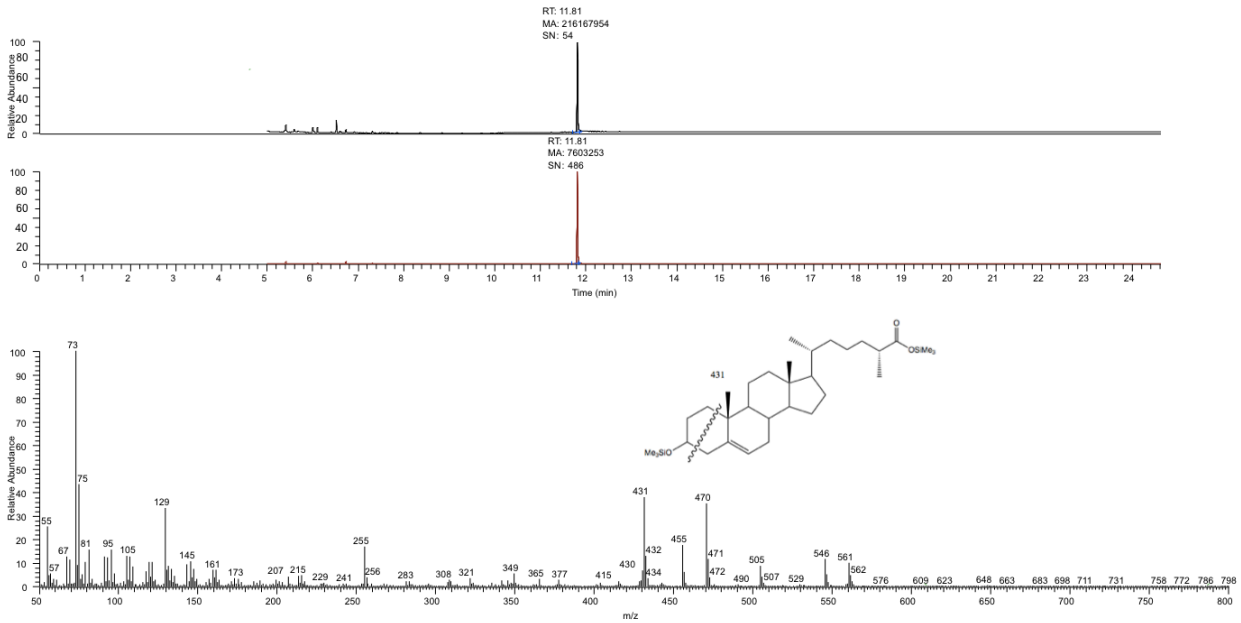

FIGURE S6. Chromatogram, full-scan EI mass spectrum, and chemical structure of silylated cholestenoic acid, showing the fragmented quantifier ion at *m/z* 431.

REFERENCES

1. Prokai, L., Prokai-Tatrai, K., Perjesi, P., Zharikova, A.D., Perez, E.J., Liu, R., Simpkins, J.W. (2003). Quinol-based cyclic antioxidant mechanism in estrogen neuroprotection. Proc. Natl. Acad. Sci. U. S. A. 100, 11741–11746.
2. Prokai-Tatrai, K., Perjesi, P., Rivera-Portalatin, N.M., Simpkins, J.W., Prokai, L. (2008). Mechanistic investigations on the antioxidant action of a neuroprotective estrogen derivative. Steroids 73, 280–288.
3. Prokai-Tatrai, K., Xin, H., Nguyen, V., Szarka, S., Blazics, B., Prokai, L., Koulen, P. (2013). 17β-estradiol eye drops protect the retinal ganglion cell layer and preserve visual function in an in vivo model of glaucoma. Mol. Pharm. 10, 3253–3261.
4. Tschiffely, A.E., Schuh, R.A., Prokai-Tatrai, K.; Ottinger, M.A., Prokai, L. (2018). An exploratory investigation of brain-selective estrogen treatment in males using a mouse model of Alzheimer's disease, Horm. Behav. 98, 16–21.
5. Von Bernhardi, R., Eugenin, J. (2012). Alzheimer's disease: redox dysregulation as a common denominator for diverse pathogenic mechanisms. Antioxid. Redox Signal. 16, 974–1031.
6. Vegeto, E., Benedusi, V., Maggi, A. (2008). Estrogen anti-inflammatory activity in brain: A therapeutic opportunity for menopause and neurodegenerative diseases. Frontiers in Neuroendocrinology 29, 507–519.

7. Yoo, J.E., Shin, D.W., Han, K., Kim, D., Won, H.-S., Lee, J., Kim, S.Y., Nam, G.E. and Park, H.S. (2020). Female reproductive factors and the risk of dementia: a nationwide cohort study. Eur. J. Neurol. 27, 1448–1458.
8. Birge, S.J. (1997). The role of estrogen in the treatment and prevention of dementia: introduction. Am. J. Med. 103: 1s–2s.
9. Ryan, J., Scali, J., Carriere, I., *et al.* (2014). Impact of a premature menopause on cognitive function in later life. BJOG 121, 1729–1739.
10. Rocca, W., Bower, J., Maraganore, D., et al. (2007). Increased risk of cognitive impairment or dementia in women who underwent oophorectomy before menopause. Neurology 69: 1074–1083.
11. Baum, L.W. (2005). Sex, hormones, and Alzheimer's disease. J. Gerontol. A Biol. Sci. Med. Sci. 60, 736–743.
12. Tschiffely, A.E,. Schuh, R.A., Prokai-Tatrai, K., Prokai, L., Ottinger, M.A. (2016). A comparative evaluation of treatments with 17β-estradiol and its brain-selective prodrug in a double-transgenic mouse model of Alzheimer's disease. Horm. Behav. 83, 39–44.
13. Mutemberezi, V., Guillemot-Legris, O., Muccioli, G. G. (2016). Oxysterols: From cholesterol metabolites to key mediators. Prog. Lipid Res. 64, 152–169.
14. Luu-The V. (2013). Assessment of steroidogenesis and steroidogenic enzyme functions. J Steroid Biochem. Mol. Biol. 137:176–82.
15. Chiappelli, J., Quinton, M.S., Volfson, D. *et al.* (2020). Assessment of brain cholesterol metabolism biomarker 24S-hydroxycholesterol in schizophrenia. NPJ Schizophr. 6, 34.

16. Ramirez, D.M., Andersson, S., Russell, D.W. (2008). Neuronal expression and subcellular localization of cholesterol 24-hydroxylase in the mouse brain. J. Comp. Neurol. 507, 1676–1693.

17. Lund, E.G., Guileyardo, J.M., Russell, D.W. (1999). cDNA cloning of cholesterol 24-hydroxylase, a regulator of cholesterol homeostasis in the brain. Proc. Natl. Acad. Sci. USA 96, 7238–7243.

18. Lund, E.G., Xie, C., Kotti, T., Turley, S.D., Dietschy, J.M., Russell, D.W. (2003). Knockout of the cholesterol 24-hydroxylase gene in mice reveals a brain-specific mechanism of cholesterol turnover. J. Biol. Chem. 278, 22980–22988.

19. Boussicault, L., Alves, S., Lamaziere, A., *et al.* (2016). CYP46A1, the rate-limiting enzyme for cholesterol degradation, is neuroprotective in Huntington's disease. Brain 139, 953–70.

20. Hudry, E., Van, D.D., Kulik, W., *et al.* (2010). Adeno-associated virus gene therapy with cholesterol 24-hydroxylase reduces the amyloid pathology before or after the onset of amyloid plaques in mouse models of Alzheimer's disease. Mol. Ther. 18, 44–53.

21. Lütjohann, D., *et al.* (2000). Plasma 24S-hydroxycholesterol (cerebrosterol) is increased in Alzheimer and vascular demented patients. J. Lipid Res. 41, 195–198.

22. Zuliani, G., *et al.* (2011). Plasma 24S-hydroxycholesterol levels in elderly subjects with late onset Alzheimer’s disease or vascular dementia: a case-control study. BMC Neurol. 11, 121.

23. Bretillon, L., *et al.* (2000). Plasma levels of 24S-hydroxycholesterol reflect the balance between cerebral production and hepatic metabolism and are inversely related to body surface. J. Lipid Res. 41, 840–845.

24. Solomon, A., *et al.* (2009). Plasma levels of 24S-hydroxycholesterol reflect brain volumes in patients without objective cognitive impairment but not in those with Alzheimer's disease. Neurosci. Lett. 462, 89–93.

25. Papassotiropoulos, A., *et al.* (2000). Plasma 24S-hydroxycholesterol: a peripheral indicator of neuronal degeneration and potential state marker for Alzheimer's disease. Neuroreport 11, 1959–1962.

26. Hughes, T. M., *et al.* (2012). Markers of cholesterol metabolism in the brain show stronger associations with cerebrovascular disease than Alzheimer's disease. J. Alzheimers Dis. 30, 53–61.

27. Shichiri, M., *et al.* (2011). α-Tocopherol suppresses lipid peroxidation and behavioral and cognitive impairments in the Ts65Dn mouse model of Down syndrome. Free Rad. Biol. Med. 50, 1801–1811.

28. Schött, H.F., Lütjohann,D. (2015). Validation of an isotope dilution gas chromatography–mass spectrometry method for combined analysis of oxysterols and oxyphytosterols in serum samples. Steroids 99(B), 139–150.

29. Uddin, M.S., Rahman, M.M., Jakaria, M. *et al.* (2020). Estrogen signaling in alzheimer's disease: molecular insights and therapeutic targets for Alzheimer's disease. Mol. Neurobiol. 57, 2654–2670.

30. Martin, M.G., Trovò,L., Perga, S., Sadowska, A., Rasola, A., Chiara, F., Dotti, C.G. (2011). CYP46-mediated cholesterol loss promotes survival in stressed hippocampal neurons Neurobiol. Aging 32, 933–943.

31. Dzeletovic, S., Breuer, O., Lund,E., Diczfalusy,U. (1995). Determination of cholesterol oxidation products in human plasma by isotope dilution-mass spectrometry. Anal. Biochem. 225, 73–80.

32. Snow, N.H. (2018). Split, splitless, and beyond—Getting the most from your inlet. LC-GC North America 36, 448–454.

33. Prokai, L., Fryčák, P., Stevens, S. M., Jr., Nguyen, V. (2008). Measurement of acetylcholine in rat brain microdialysates by LC - isotope dilution tandem MS. Chromatographia 68 (Suppl 1), S101–S105.

34. Björkhem, I., Heverin, M., Leoni, V., Meaney, S., Diczfalusy, U. (2006). Oxysterols and Alzheimer's disease. Acta. Neurol. Scand. Suppl. 185, 43–49.

35. Jung, J.I., Price, A.R., Ladd, T.B., *et al.* (2015). Cholestenoic acid, an endogenous cholesterol metabolite, is a potent γ-secretase modulator. Mol. Neurodegeneration 10, 29.