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The Effects of *L*-Cysteine on Alzheimer's Disease Pathology in *APOE2*, *APOE3*, and *APOE4* Homozygous Mice

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Master of Science

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ABSTRACT

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The *APOE* gene is of profound importance regarding the onset of Alzheimer's disease (AD). From the small physical differences among the protein products of the isoforms of this gene arises a profound difference in their physiologies. For example, the *APOE2* isoform confers resistance to AD, the *APOE3* isoform confers neutral susceptibility to AD, and the *APOE4* isoform confers proneness to AD. *L*-cysteine is an amino acid that has several anti-AD properties, among which are its ability to sequester iron and form glutathione – a powerful antioxidant – and therefore may be a promising potential dietary supplement for ameliorating AD pathology. In our experiment, we fed *Mus musculus* (mice) homozygous for *APOE2*, *APOE3*, and *APOE4* either a control diet or a diet high in *L*-cysteine. Using Western blotting analysis, we quantified Amyloid β (Aβ), hyper-phosphorylated Tau (HP-Tau), and the three APOE proteins that we extracted from post-mortem brains of *APOE2*, *APOE3*, and *APOE4* homozygous mice of 3-, 6-, 9-, and 12-month ages. We calculated a three-way ANOVA on a sample of 86 mice to examine the effect of age, genotype, and diet on protein quantities. We found that administration of *L*-cysteine trends towards lowering levels of Aβ in each cohort, but this effect is statistically insignificant. On the other hand, *L*-cysteine caused a significant decrease in APOE production with regard to diet [$F(1,62) = 6.17$, $p=0.02$], indicating that less APOE is produced due to the decrease in Aβ burden. Furthermore, administration of *L*-cysteine revealed no significant impact on or trends regarding HP-Tau deposition between diet types for each cohort. However, we observed that *L*-cysteine appeared to nullify the increasing trend in HP-Tau deposition between *APOE2* and *APOE4* cohorts. Thus, *L*-cysteine may be weakly affecting HP-Tau deposition via its ability to somewhat reduce Aβ burden and consequently prevent the shutdown of the proteosomes responsible for the degradation and clearance of HP-Tau. Taken together, these data suggest that *L*-cysteine should be considered as an intervention for AD pathology.

Keywords: alzheimer's disease, APOE4, APOE3, APOE2, amyloid β, tau, *L*-cysteine, diet

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INTRODUCTION

Functions of APOE and Aβ Proteins in Relation to AD Pathology

Several factors are involved in the onset and regulation of AD. Among these factors is the *APOE* gene, which encodes the APOE protein. One function of APOE is clearing Aβ from the brain, where it works by co-localizing with Aβ after it is secreted into the perivascular space (Roylan et al, 2011), thereby resulting in a decrease in oxidative stress and AD symptoms (Espiritu et al, 2008). A major role of Aβ is to sequester iron, which is toxic to the brain (Bousejra-Elgarah et al, 2011). Moreover, the role of APOE in the clearance of Aβ is important because Aβ can accumulate in the extracellular space and form plaques, which are toxic to neurons and thereby, facilitate the onset of AD.

There are three different alleles for *APOE*: ε_2, ε_3, and ε_4 – each of which codes for a protein that differs at only one or two amino acids from and in the same positions as the others (Tables 1 and 2) (Ghebranious et al, 2005). Consequently, there are six different genotypes of *APOE* that exist naturally: *APOE2*, *APOE2/3*, *APOE2/4*, *APOE3*, *APOE3/4*, and *APOE4*. Those with the *APOE2* genotype are at the most reduced risk for AD, because the ε_2 allele has protective effects against AD (Corder et al, 1994). *APOE2/3* is at a lesser risk for AD, *APOE2/4* and *APOE3* are at neutral risks for AD, *APOE3/4* is at an elevated risk for AD, and *APOE4* is at the greatest risk for AD (Razali et al, 2013).

Table 1: Amino Acid Differences Among APOE Isoforms.

APOE Isoform	Amino Acid Differences	Number of Tyrosines
APOE2	Cys-112; Cys-158	4
APOE3	Cys-112; Arg-158	4
APOE4	Arg-112; Arg-158	4

Table 2: Amino Acid Sequences of APOE2, APOE3, and APOE4.

Protein Isoform	Protein Sequence
APOE2	MKVEQAVETEPEPELRQQTEWQSGQRWELALGRFWDYLRWVQTLSEQVQ EELLSSQVTQELRALMDETMKELKAYKSELEEQLTPVAEETRARLSKELQA AQARLGADMEDVCGRLVQYRGEVQAMLGQSTEELRVRLASHLRKLRKRL LRDADDLQKCLAVYQAGAREGAERGLSAIRERLGPLVEQGRVRAATVGSL AGQPLQERAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEEQA QQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSD NH
APOE3	MKVEQAVETEPEPELRQQTEWQSGQRWELALGRFWDYLRWVQTLSEQVQ EELLSSQVTQELRALMDETMKELKAYKSELEEQLTPVAEETRARLSKELQA AQARLGADMEDVCGRLVQYRGEVQAMLGQSTEELRVRLASHLRKLRKRL LRDADDLQKRLAVYQAGAREGAERGLSAIRERLGPLVEQGRVRAATVGSL AGQPLQERAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEEQA QQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSD NH
APOE4	MKVEQAVETEPEPELRQQTEWQSGQRWELALGRFWDYLRWVQTLSEQVQ EELLSSQVTQELRALMDETMKELKAYKSELEEQLTPVAEETRARLSKELQA AQARLGADMEDVRGRLVQYRGEVQAMLGQSTEELRVRLASHLRKLRKRL LRDADDLQKRLAVYQAGAREGAERGLSAIRERLGPLVEQGRVRAATVGSL AGQPLQERAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEEQA QQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSD NH

In addition, a relationship exists between the clearance of Aβ and the isoformic identity of APOE. Aβ clearance decreases from APOE2 to APOE3 to APOE4 (Castellano, 2011). Moreover, due to the presence of the cysteine residue in APOE3, disulfide bridges can form between APOE3 molecules, allowing APOE3 to exist as monomers and homodimers (Aleshkov et al, 1999). Since APOE2 has two cysteine residues, it can exist not only as monomers and homodimers, but also as homopolymers via the formation of disulfide bridges (Aleshkov et al, 1999). However, the lack of cysteine residues in APOE4 does not allow for the formation of disulfide bonds between/among APOE4 molecules, which may contribute to the insufficiency of APOE4 at clearing Aβ from cells. Consequently, the buildup of Aβ increases oxidative stress and further facilitates the onset of AD in *APOE4* genotypes (Jofre-Monseny et al, 2008).

Palmitoylation of APOE Isoforms

Interestingly, a direct relationship was found between the ability of APOE proteins to form stable complexes with Aβ and their degrees of lipidation. APOE2 has the most lipidation and forms the most stable complex with Aβ, APOE3 has intermediate lipidation and forms a complex with Aβ of intermediate stability relative to APOE2 and APOE4, and APOE4 has the least lipidation and forms the least stable complex with Aβ (Gunzburg et al, 2007). Lipidation comes in three forms: prenylation (also known as farnesylation), myristoylation, and palmitoylation. Prenylation typically occurs at cysteine residues in the final four *C*-terminal amino acids of a protein (Yang, 2000), myristoylation typically occurs at *N*-terminal glycine residues (except in cases of caspase cleavage during apoptosis) (Podell et al, 2004), and palmitoylation only occurs at cysteine, tyrosine, serine, and threonine residues (Ji, 2013). Given that myristoylation and prenylation sites are not present in any of the APOE isoforms due to the lack of *N*-terminal glycines (and, for the purposes of this experiment, the lack of apoptotic caspase cleavage) and the lack of cysteines in the final four *C*-terminal amino acids, respectively, the most probable type of lipidation occurring in APOE isoforms is palmitoylation. Palmitoylation is most probable due to the abundance of serine, tyrosine, and threonine residues in all APOE isoforms, as well as the presence of two cysteines in APOE2 and one cysteine in APOE3.

Pathology of Aβ and Tau Proteins

Another factor that facilitates the onset of AD pathology is the protein Tau, which is necessary for the integrity of microtubules. Under degenerative conditions, mechanisms such as mitochondrial and metabolic dysfunction as well as loss of metal homeostasis result in the formation of reactive oxygen species (ROS) that cause hyper-phosphorylation of the Tau protein

(Mondragon-Rodriguez et al, 2013). Normally, HP-Tau proteins are degraded and cleared by proteosomes, but in AD patients, the high levels of Aβ inhibit these proteosomes, leading to the over-proliferation of HP-Tau (Tseng et al, 2008). The over-proliferation of HP-Tau contributes to the formation of neurofibrillary tangles, which in turn increases oxidative stress (Mondragon-Rodriguez et al, 2013). Thus, *APOE4*, Aβ plaques, and HP-Tau tangles facilitate the onset of AD pathology.

Diet and the *APOE4* Gene

Aside from the genetics of AD, there has also been research on how diet influences AD symptoms caused by *APOE4*. Specifically, researchers Chan et al (2008) tested a triple-combination diet consisting of *N*-acetylcysteine (NAC), acetyl-*L*-carnitine (ALCAR), and *S*-adenosylmethionine (SAMe) on the behavioral function of *APOE4* mice. The results showed a significant increase in behavioral function. For example, attack latency improved by approximately 90%, attack frequency decreased about 95%, and Y-maze testing showed that cognitive function increased by about 30% and then after one month, up to 70% (Chan et al, 2008). However, the increase in cognitive performance was reversible because the values reverted to those observed before the start of the treatment; within two weeks after withdraw of supplementation (Chan et al, 2008). When supplementation was given again, cognitive performance increased by approximately 70% (Chan et al, 2008). Thus, the extents of the individual roles of *L*-cysteine, ALCAR, and SAMe in treating AD in *APOE4* patients should be researched.

HYPOTHESES

I propose that a diet rich in *L*-cysteine will help to partially or completely alleviate the pathology of AD in mice carrying *APOE4* by inhibiting proliferation of Aβ plaques and HP-Tau tangles, with negligible side effects; furthermore, that a diet rich in *L*-cysteine will either generate AD pathology in *APOE3* and/or *APOE2* mice by enabling the proliferation of Aβ plaques and HP-Tau tangles, or will have no effect on *APOE3* and/or *APOE2* mice. I also propose that the arginine-to-cysteine changes among isoforms of APOE at positions 112 and 158 are important because in some instances they increase potential palmitoylation sites (via the presence of more cysteines) and in other instances they allow for the binding and clearing of more Aβ plaques (via the formation of homodimers and homopolymers of APOE isoforms); and because they allow for the palmitoylation of more tyrosines, serines, and threonines (due to the relieving of steric hindrance in the tertiary structures of APOE isoforms). Our objectives are outlined below:

Objective 1A

Confirm via Western blot analysis whether the administration of a diet rich in *L*-cysteine to *APOE4* mice helps to reduce or eliminate levels of oxidative stress and AD pathology by reducing or eliminating levels of Aβ plaques and HP-Tau tangles, with or without any negative side effects; furthermore, whether the administration of a diet rich in *L*-cysteine to *APOE3* and/or *APOE2* mice either increases levels of oxidative stress and AD pathology by enabling the proliferation of Aβ plaques and HP-Tau tangles, or has no effect (*ex vivo* analysis).

Objective 1B

Confirm the precise locations of all palmitoylation sites of APOE2, APOE3, and APOE4; the ratio of Aβ molecules in complex with monomers of APOE isoforms, homodimers of APOE

isoforms, and APOE2 homopolymers; and the clearance rates of APOE and Aβ per cohort, via mass spectrometry analysis (*ex vivo* analysis).

MATERIALS AND METHODS

Sources of Reagents, Antibodies, and Proteins

Protease inhibitor and rabbit polyclonal anti-phospho-MAPT ($pSer^{262}$) primary antibodies were purchased from Sigma-Aldrich. BCA assay kit was purchased from ThermoFisher Scientific (Pierce) and Spectra multicolor broad range protein ladder was purchased from ThermoFisher Scientific. Immunoprecipitation Kit – Dynabeads® Protein G (RID: 10007D) was purchased from Life Technologies (Novex). Precast Criterion Tris-HCl protein gels and nitrocellulose membranes were purchased from Bio-Rad. Rabbit polyclonal anti-APOE2/APOE3/APOE4 primary antibodies were purchased from BioVision. Rabbit polyclonal anti-beta Amyloid 1-42 primary antibodies were purchased from Bioss Antibodies. Rabbit monoclonal anti-GAPDH primary antibodies were purchased from Cell Signaling Technology. IRDye 800CW donkey anti-rabbit secondary antibodies were purchased from Li-Cor. Purified (>97%) mouse Amyloid 1-42 protein was purchased from rPeptide: Premiere Peptide Solutions. Purified (>95%) human MAPT protein was purchased from Syd Labs. Purified (>95%) human APOE3 and APOE4 (>90%) proteins were purchased from ProSpec Protein Specialists. Purified (>90%) human APOE2 was purchased from Creative BioMart.

Source of Transgenic Mice

APOE4-tg knockout mice (Tg(GFAP-APOE*4)1Hol) for endogenous mouse *APOE*, but expressing human *APOE4* under the direction of the human glial fibrillary acidic protein (GFAP) promoter, were purchased from Jackson Laboratory, USA. *APOE3*-tg knockout mice (Tg(GFAP-APOE*3)37Hol) for endogenous mouse *APOE*, but expressing human *APOE3* under the direction of the human GFAP promoter, were purchased from Jackson Laboratory, USA. These *APOE3* homozygous mice served as our wild type mice. *APOE2*-tg knockout mice (Tg(GFAP-

APOE*2)14Hol) for endogenous mouse *APOE*, but expressing human *APOE2* under the direction of the human GFAP promoter, were purchased from Jackson Laboratory, USA. *APOE* genotypes were not mixed when breeding these mice or any progeny mice in order to keep all mice homozygous with respect to *APOE* genotype. Breeders were kept separate from experimental mice, and both male and female mice were used in this experiment.

Sources of Control and Experimental Diets

The control diet was purchased from Envigo and consisted of standard laboratory chow, which contained 4g *L*-cysteine/kg total food weight. *L*-cysteine (97% purity) was purchased from Sigma-Aldrich. To make the experimental diet, 10g *L*-cysteine/kg total food weight was added to the control diet by Envigo, along with a blue-green dye for ease in identification. Both diets were isocaloric in relation to each other and were of equal palatability.

Care of Mice

Mice were divided into control and experimental diet groups. Prior to 2 months of age, the experimental diet group was fed the control diet. From 2 months of age until being sacrificed, the experimental diet group was fed only the experimental diet. The control diet group was always fed the control diet. All animals were housed under standard conditions (20℃ temperature, 12 hour light-dark cycle) with equal free access to air, food, and water. All procedures were performed in compliance with the USDA Animal Welfare Act and the NIH Public Health Service Policy on the Humane Care and Use of Animals.

Tissue Preparation

In compliance with institutional guidelines for the humane treatment of animals, mice were sacrificed using chambers containing isofluorane gas at 3-months, 6-months, 9-months, and 12-months of age. Mouse brains were carefully removed and cut in the mid-sagittal plane. For

each brain, one half was fixated in 4% formaldehyde solution for future immunohistochemistry analysis and the other half was snap frozen at -80℃ for Western blot analysis. Each frozen sample was thawed and homogenized in a mixture of protease inhibitor (1:100 dilution) and 25mM ammonium bicarbonate; following which, BCA analyses were performed.

SDS-PAGE and Western Blotting

Levels of APOE, HP-Tau, and Aβ proteins were examined by Western blotting. For each sample, ~30μg homogenate protein was separated using precast 4-15% and 4-20% gradient SDS-PAGE gels and transferred to nitrocellulose membranes (pore sizes 0.45μm). Each gel also contained ~2μg of a purified control protein based off of which primary antibody was to be used on its respective blot. All gel loading volumes were equal. Each membrane was incubated with its respective primary antibody (anti-APOE2/APOE3/APOE4 dilution = 1:3000; anti-phospho-MAPT ($pSer^{262}$) dilution = 1:1000; anti-beta Amyloid 1-42 dilution = 1:2000; anti-GAPDH dilution = 1:1000) in blocking buffer (3% nonfat dried milk in 1× tris-buffered saline with Tween 20 (TBST)) overnight at 4℃, washed three times with 1× TBST, and then incubated in blocking buffer for 1 hour at room temperature with Li-Cor IRDye 800CW secondary antibodies (donkey anti-rabbit IgG, 1:10000 dilution). Levels of proteins were estimated by densitometry analysis using the Li-Cor Image Studio 4.0 software. Anti-GAPDH immunoblots were used as loading controls.

In vivo D_2O Labeling and Clearance Analysis Preparation of APOE and Aβ

D_2O saline was injected into each mouse either one day (for APOE labeling) or eight days (for Aβ labeling) before sacrificing. The injection volume (in μL) was determined by multiplying the weight (in grams) of each mouse by a factor of 35. Upon sacrificing, the blood of

each mouse was collected and centrifuged to obtain its plasma, which plasma was then distilled to collect a mixture of D_2O/H_2O. Each D_2O/H_2O mixture was added to MΩ water (1:300 dilution) to prepare for trace gas spectrometry analysis.

Immunoprecipitation

The procedure from Immunoprecipitation Kit – Dynabeads® Protein G (RID: 10007D) from Life Technologies (Novex) was followed prior to mass spectrometry analysis.

Broad-Spectrum Mass Spectrometry Analysis Sample Preparation

Trypsin (1µg trypsin/100µg total protein in sample) digests (with and without subsequent HPLC preparation) and in-gel trypsin (concentration of trypsin prepared: 12.5ng/µL) digests (without subsequent HPLC preparation) were performed on tissue homogenate samples prior to running mass spectrometry analyses.

Image and Statistical Analysis

Western blot images were quantified using the Li-Cor Image Studio 4.0 software. Each image was cleaned three times using the noise removal tool. Selected bands were quantified based on their relative intensities and adjusted for background. Statistical tests were performed using SAS Version 9.4 (SAS Institute, Inc., Cary NC, USA). Following a logarithmic transformation of the mean intensities, relationships among diet type, *APOE* genotype, and age were analyzed using a three-way ANOVA with Tukey-Kramer *a posteriori* comparisons of significant main effects. To compare the palatability of each diet, differences in mouse weight between diet type and genotype cohorts were analyzed using a 2x2 factorial ANOVA statistical model. Statistical significance was defined at $\alpha = 0.05$. Statistical significance for trends was defined by non-overlapping standard error bars.

RESULTS

Comparison of Diet Palatability

The overall statistical model was insignificant [$F(5,106) = 1.919$, $n = 112$ mice, $p = 0.097$], as were the main effects of diet [$F(1,106) = 0.779$, $n = 112$ mice, $p = 0.379$], genotype [$F(2,106) = 1.521$, $n = 112$ mice, $p = 0.223$], and diet X genotype [$F(2,106) = 2.726$, $n = 112$ mice, $p = 0.070$].

Effect of *L*-Cysteine on Aβ Deposition

Three-way ANOVA analysis revealed significant mixed effects on Aβ deposition for the following: age [$F(3,62) = 5.97$, $n = 86$ mice, $p = 0.001$], *APOE* genotype [$F(2,62) = 3.82$, $n = 86$ mice, $p = 0.03$], age X *APOE* genotype [$F(6,62) = 5.91$, $n = 86$ mice, $p < 0.0001$], age X diet type [$F(3,62) = 4.20$, $n = 86$ mice, $p < 0.01$], and age X *APOE* genotype X diet type [$F(6,62) = 2.21$, $n = 86$ mice, $p = 0.05$] (Table 3). However, diet type [$F(1,62) = 0.56$, $n = 86$ mice, $p = 0.46$] and *APOE* genotype X diet type [$F(2,62) = 0.02$, $n = 86$ mice, $p = 0.98$] were not found to be significantly different (Table 3).

Table 3: Overall Statistics for Aβ Deposition.

Effect	Num DF	Den DF	*F* Value	*p* Value
Age	3	62	5.97	0.001
APOE Genotype	2	62	3.82	0.03
Diet Type	1	62	0.56	0.46
Age X *APOE* Genotype	6	62	5.91	<0.0001
Age X Diet Type	3	62	4.20	<0.01
APOE Genotype X Diet Type	2	62	0.02	0.98
Age X *APOE* Genotype X Diet Type	6	62	2.21	0.05

Follow-up *a posteriori* analyses for age showed significant decreases between 3-months and 6-months ($p = 0.002$), 3-months and 9-months ($p = 0.006$), and 3-months and 12-months ($p = 0.05$).

APOE genotype showed a significant increase between *APOE2* and *APOE4* genotypes ($p = 0.03$).

Age X *APOE* genotype revealed significant increases between 12-months X *APOE2* and *APOE3* ($p = 0.0001$), and between 12-months X *APOE2* and *APOE4* ($p = 0.0002$); and significant decreases between *APOE4* X 3-months and 6-months ($p = 0.02$), *APOE4* X 3-months and 9-months ($p = 0.03$), *APOE2* X 3-months and 12-months ($p = 0.006$), and *APOE2* X 9-months and 12-months ($p = 0.02$).

Age X diet type showed significant decreases between control diet X 3-months and 6-months ($p = 0.04$) and control diet X 3-months and 12-months ($p = 0.002$).

Age X *APOE* genotype X diet type revealed significant increases between 12-months X control diet X *APOE2* and *APOE3* ($p = 0.03$), and between 12-months X *L*-cysteine diet X *APOE2* and *APOE4* ($p = 0.04$); and significant decreases between *APOE2* X control diet X 3-months and 12-months ($p = 0.02$), *APOE2* X control diet X 6-months and 12-months ($p = 0.02$), and *APOE4* X control diet X 3-months and 6-months ($p = 0.01$).

Aβ plaques were found between 40kD and 50kD, at ~45kD (Figure 1). The bands at ~45kD typically represent chains of Aβ bound to a monomer of APOE (LaDu et al, 2012).

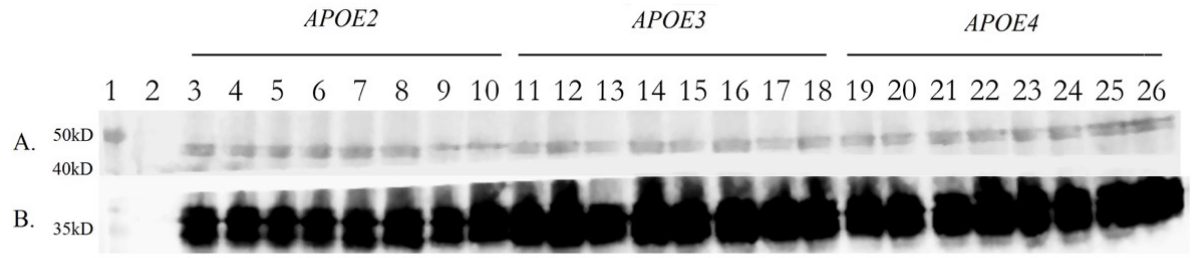

Figure 1: Western Blots Showing Aβ Plaques.
1A: Aβ (1-42) at ~45kD.
1B: GAPDH loading controls for Aβ (1-42) Western blot.
Lanes are listed in numerical order, from left to right.
Lane 1 = protein molecular weight ladder. Lane 2 = purified mouse Aβ protein (1-42). Lane 3 = *APOE2*/3-month/*L*-cysteine diet. Lane 4 = *APOE2*/3-month/control diet. Lane 5 = *APOE2*/6-month/*L*-cysteine diet. Lane 6 = *APOE2*/6-month/control diet. Lane 7 = *APOE2*/9-month/*L*-cysteine diet. Lane 8 = *APOE2*/9-month/control diet. Lane 9 = *APOE2*/12-month/*L*-cysteine diet. Lane 10 = *APOE2*/12-month/control diet. Lane 11 = *APOE3*/3-month/*L*-cysteine diet. Lane 12 = *APOE3*/3-month/control diet. Lane 13 = *APOE3*/6-month/*L*-cysteine diet. Lane 14 = *APOE3*/6-month/control diet. Lane 15 = *APOE3*/9-month/*L*-cysteine diet. Lane 16 = *APOE3*/9-month/control diet. Lane 17 = *APOE3*/12-month/*L*-cysteine diet. Lane 18 = *APOE3*/12-month/control diet. Lane 19 = *APOE4*/3-month/*L*-cysteine diet. Lane 20 = *APOE4*/3-month/control diet. Lane 21 = *APOE4*/6-month/*L*-cysteine diet. Lane 22 = *APOE4*/6-month/control diet. Lane 23= *APOE4*/9-month/*L*-cysteine diet. Lane 24 = *APOE4*/9-month/control diet. Lane 25 = *APOE4*/12-month/*L*-cysteine diet. Lane 26 = *APOE4*/12-month/control diet.

There were no statistically significant differences in Aβ deposition between the control and *L*-cysteine diets for any of the cohorts (Table 3), nor were there any statistically significant trends in protein amount (Figure 2). Nevertheless, we did observe a decrease in Aβ deposition for each cohort as a result of the *L*-cysteine diet (Figure 2). It is possible that increasing the number of subjects in the study would provide a better indication of the effect of diet.

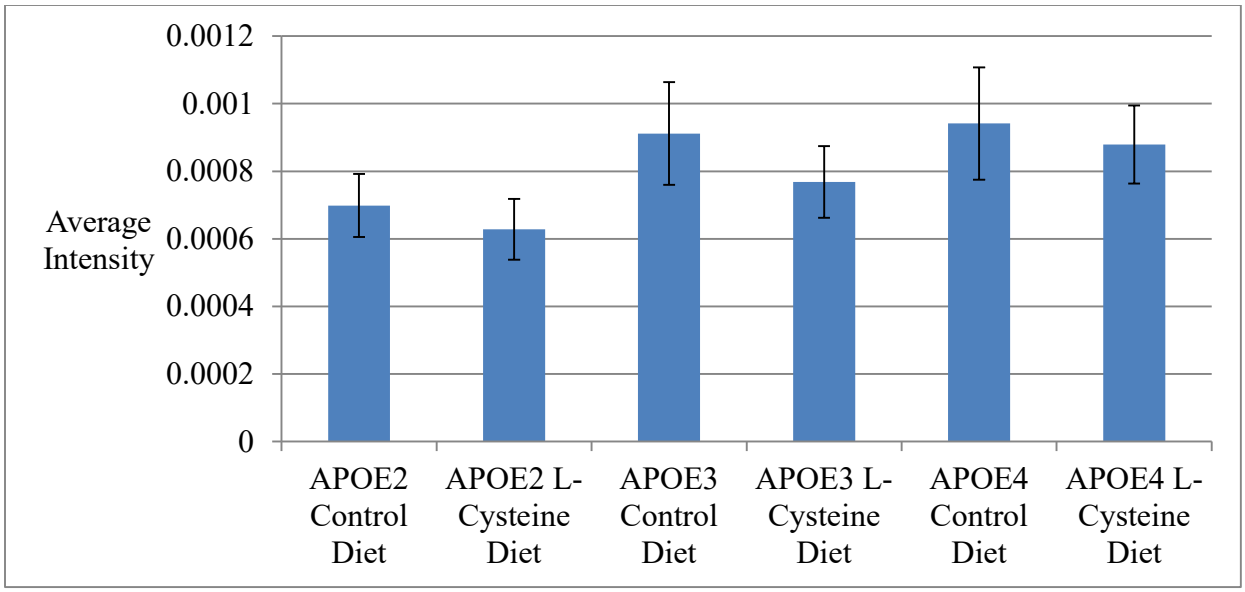

Figure 2: Trends in Aβ Deposition.

Effect of *L*-Cysteine on APOE Production

Three-way ANOVA analysis revealed significant mixed effects on APOE production for the following: age [$F(3,62) = 47.97$, $n = 86$ mice, $p < 0.0001$], *APOE* genotype [$F(2,62) = 17.42$, $n = 86$ mice, $p < 0.0001$], diet type [$F(1,62) = 6.17$, $n = 86$ mice, $p = 0.02$], and age X *APOE* genotype [$F(6,62) = 22.05$, $n = 86$ mice, $p < 0.0001$] (Table 4). However, age X diet type [$F(3,62) = 0.90$, $n = 86$ mice, $p = 0.45$], *APOE* genotype X diet type [$F(2,62) = 2.74$, $n = 86$ mice, $p = 0.07$], and age X *APOE* genotype X diet type [$F(6,62) = 0.43$, $n = 86$ mice, $p = 0.85$] were not found to be significantly different (Table 4).

Table 4: Overall Statistics for APOE Production.

Effect	Num DF	Den DF	*F* Value	*p* Value
Age	3	62	47.97	<0.0001
APOE Genotype	2	62	17.42	<0.0001
Diet Type	1	62	6.17	0.02
Age X *APOE* Genotype	6	62	22.05	<0.0001
Age X Diet Type	3	62	0.90	0.45
APOE Genotype X Diet Type	2	62	2.74	0.07
Age X *APOE* Genotype X Diet Type	6	62	0.43	0.85

Follow-up *a posteriori* analyses for age showed significant decreases between 3-months and 6-months ($p = 0.001$), and 9-months and 12-months ($p < 0.0001$); and significant increases between 3-months and 9-months ($p < 0.0001$), 6-months and 9-months ($p < 0.0001$), and 6-months and 12-months ($p = 0.0007$).

APOE genotype showed significant increases between *APOE2* and *APOE3* genotypes ($p < 0.0001$) and *APOE2* and *APOE4* genotypes ($p < 0.0001$).

Diet type revealed a significant decrease between control diet and *L*-cysteine diet ($p = 0.01$).

Age X *APOE* genotype revealed significant increases between 12-months X *APOE2* and *APOE3* ($p < 0.0001$), 12-months X *APOE2* and *APOE4* ($p < 0.0001$), 12-months X *APOE3* and *APOE4* ($p = 0.004$), *APOE2* X 3-months and 9-months ($p < 0.0001$), *APOE2* X 6-months and 9-months ($p < 0.0001$), *APOE3* X 6-months and 9-months ($p < 0.0001$), *APOE4* X 3-months and 9-months ($p < 0.0001$), *APOE4* X 3-months and 12-months ($p < 0.0001$), *APOE4* X 6-months and 9-months ($p < 0.0001$), and *APOE4* X 6-months and 12-months ($p < 0.0001$); and significant decreases between *APOE2* X 3-months and 12-months ($p < 0.0001$), *APOE2* X 6-months and 12-months ($p = 0.002$), and *APOE2* X 9-months and 12-months ($p < 0.0001$).

Although the weight of APOE is ~34kD per pure monomer, APOE2 is known to form monomers, dimers, and polymers of itself; whereas APOE3 is known to form monomers and dimers of itself (Aleshkov et al, 1997; Aleshkov et al, 1999). Furthermore, all APOE isoforms undergo intensive modifications of varying degrees, such as lipidation (Hanson et al, 2013), glycosylation (Wernette-Hammond et al, 1989, Ioannou et al, 1998; Aleshkov et al, 1999; Tams et al, 1999; Høiberg-Nielsen et al, 2006; Lee et al, 2010), nitrosylation (Abrams et al, 2011), and sialylation (Marmillot et al, 1999; Lee et al, 2010). Thus, modified and/or polymerized APOE was typically found at ~140kD in these blots, as well as various amounts at other weights (Figures 3.1 and 3.2).

Additionally, for all *APOE3* and *APOE4* subjects, we found a lesser amount of APOE at ~45kD (Figures 3.1 and 3.2), which typically signifies a monomer of APOE binding to chains of Aβ (LaDu et al, 2012). However, at the 12-month interval for the *APOE2* cohort, we saw no bands at ~45kD (Figure 3.1). Lastly, at all time intervals for all cohorts, we saw trace amounts of APOE at ~97kD (Figures 3.1 and 3.2), which typically signifies a dimer of APOE binding to chains of Aβ (LaDu et al, 2012).

Figure 3: Western Blots Showing APOE Levels.
3A: APOE at ~140kD.
3B: APOE at ~97kD.
3C: APOE at ~45kD.
3D: APOE monomers at ~35kD.
3E: GAPDH loading controls for APOE Western blot.
Lanes are listed in numerical order, from left to right.
Figure 3.1: Lane 1 = protein molecular weight ladder. Lane 2 = purified human APOE2. Lane 3 = *APOE2*/3-month/*L*-cysteine diet. Lane 4 = *APOE2*/3-month/control diet. Lane 5 = *APOE2*/6-month/*L*-cysteine diet. Lane 6 = *APOE2*/6-month/control diet. Lane 7 = *APOE2*/9-month/*L*-cysteine diet. Lane 8 = *APOE2*/9-month/control diet. Lane 9 = *APOE2*/12-month/*L*-cysteine diet. Lane 10 = *APOE2*/12-month/control diet. Lane 11 = purified human APOE3. Lane 12 = *APOE3*/3-month/*L*-cysteine diet. Lane 13 = *APOE3*/3-month/control diet. Lane 14 = *APOE3*/6-month/*L*-cysteine diet. Lane 15 = *APOE3*/6-month/control diet. Lane 16 = *APOE3*/9-month/*L*-cysteine diet. Lane 17 = *APOE3*/9-month/control diet. Lane 18 = *APOE3*/12-month/*L*-cysteine diet. Lane 19 = *APOE3*/12-month/control diet.
Figure 3.2: Lane 1 = protein molecular weight ladder. Lane 2 = purified human APOE4. Lane 3 = *APOE4*/3-month/*L*-cysteine diet. Lane 4 = *APOE4*/3-month/control diet. Lane 5 = *APOE4*/6-month/*L*-cysteine diet. Lane 6 = *APOE4*/6-month/control diet. Lane 7= *APOE4*/9-month/*L*-cysteine diet. Lane 8 = *APOE4*/9-month/control diet. Lane 9 = *APOE4*/12-month/*L*-cysteine diet. Lane 10 = *APOE4*/12-month/control diet.

Although there was an overall statistically significant decrease in APOE production between the control and *L*-cysteine diets, there were no significant differences in APOE production between each diet for any of the individual cohorts (Table 4). However, we did observe statistically significant trends. For example, there was a significant trend towards a decrease in APOE4 production between the control and *L*-cysteine diets (Figure 4). There was also a significant trend towards an increase in APOE production between *APOE2* and *APOE4* cohorts on the control diet; interestingly, this trend becomes insignificant with administration of the *L*-cysteine diet (Figure 4).

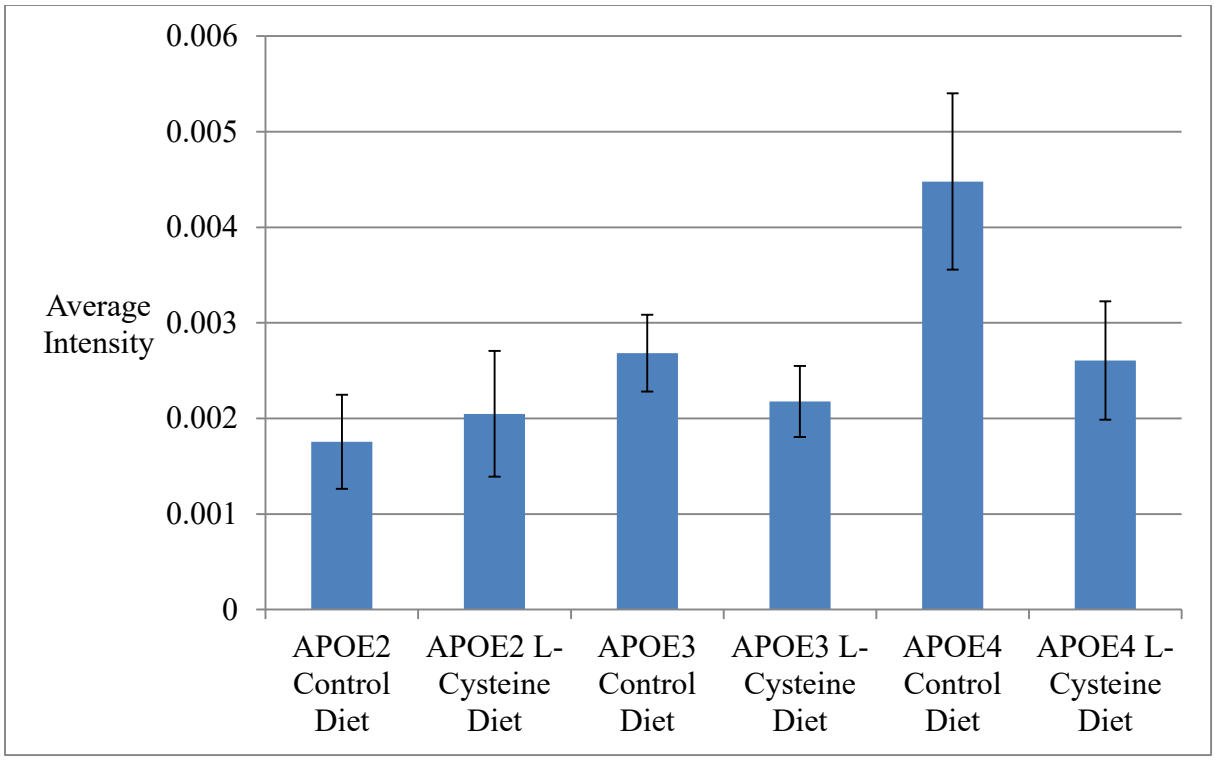

Figure 4: Trends in APOE Production.

Effect of *L*-Cysteine on HP-Tau Deposition

Three-way ANOVA analysis revealed significant mixed effects on HP-Tau deposition for the following: age [$F(3,62) = 9.64$, $n = 86$ mice, $p < 0.0001$], *APOE* genotype [$F(2,62) = 8.18$, $n = 86$ mice, $p = 0.0007$], and age X *APOE* genotype [$F(6,62) = 3.13$, $n = 86$ mice, $p = 0.01$] (Table 5). However, diet type [$F(1,62) = 0.98$, $n = 86$ mice, $p = 0.33$], age X diet type [$F(3,62) = 1.02$, $n = 86$ mice, $p = 0.39$], *APOE* genotype X diet type [$F(2,62) = 2.23$, $n = 86$ mice, $p = 0.12$], and age X *APOE* genotype X diet type [$F(6,62) = 1.54$, $n = 86$ mice, $p = 0.18$] were not found to be significantly different (Table 5).

Table 5: Overall Statistics for HP-Tau Deposition.

Effect	Num DF	Den DF	*F* Value	*p* Value
Age	3	62	9.64	<0.0001
APOE Genotype	2	62	8.18	0.0007
Diet Type	1	62	0.98	0.33
Age X *APOE* Genotype	6	62	3.13	0.01
Age X Diet Type	3	62	1.02	0.39
APOE Genotype X Diet Type	2	62	2.23	0.12
Age X *APOE* Genotype X Diet Type	6	62	1.54	0.18

Follow-up *a posteriori* analyses for age showed a significant decrease between 3-months and 6-months ($p < 0.0001$); and significant increases between 6-months and 9-months ($p = 0.03$), and 6-months and 12-months ($p = 0.001$).

APOE genotype showed significant increases between *APOE2* and *APOE3* genotypes ($p = 0.02$) and *APOE2* and *APOE4* genotypes ($p = 0.002$).

Age X *APOE* genotype revealed significant increases between 3-months X *APOE2* and *APOE3* ($p = 0.03$), 6-months X *APOE2* and *APOE4* ($p = 0.02$); and significant decreases between *APOE3* X 3-months and 6-months ($p = 0.0001$), and *APOE3* X 3-months and 9-months ($p = 0.04$).

Typically, we saw HP-Tau tangle fragments at ~20kD for all subjects (Figure 5). For the *APOE2* cohort at 3-months and for all *APOE4* subjects, we also found more developed tangles at ~48kD and ~62kD (Figure 5). Interestingly, the *APOE3* cohort from 3-9 months showed a similar amount of tangles at ~48kD as at ~20kD, but none at 62kD (Figure 5). By 12-months, all cohorts expressed these more developed HP-Tau tangles at both ~48kD and ~62kD, except for the *APOE2* cohort (Figure 5).

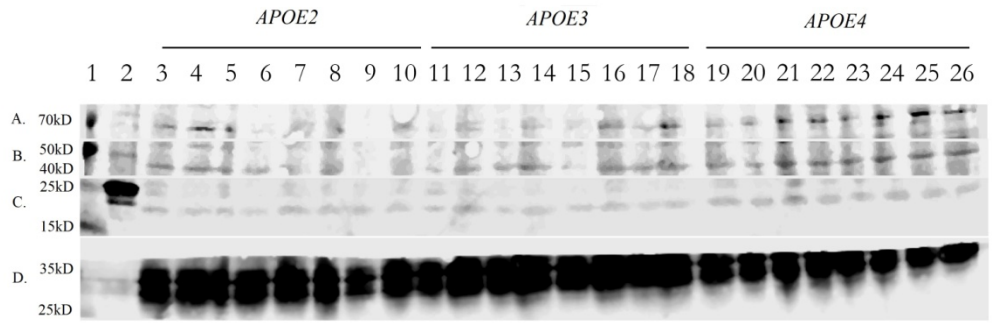

Figure 5: Western Blots Showing HP-Tau Tangles.
5A: HP-Tau at ~62kD.
5B: HP-Tau at ~48kD.
5C: HP-Tau at ~20kD.
5D: GAPDH loading controls for HP-Tau Western blot.
Lanes are listed in numerical order, from left to right.
Lane 1 = protein molecular weight ladder. Lane 2 = purified human MAPT protein. Lane 3 = *APOE2*/3-month/*L*-cysteine diet. Lane 4 = *APOE2*/3-month/control diet. Lane 5 = *APOE2*/6-month/*L*-cysteine diet. Lane 6 = *APOE2*/6-month/control diet. Lane 7 = *APOE2*/9-month/*L*-cysteine diet. Lane 8 = *APOE2*/9-month/control diet. Lane 9 = *APOE2*/12-month/*L*-cysteine diet. Lane 10 = *APOE2*/12-month/control diet. Lane 11 = *APOE3*/3-month/*L*-cysteine diet. Lane 12 = *APOE3*/3-month/control diet. Lane 13 = *APOE3*/6-month/*L*-cysteine diet. Lane 14 = *APOE3*/6-month/control diet. Lane 15 = *APOE3*/9-month/*L*-cysteine diet. Lane 16 = *APOE3*/9-month/control diet. Lane 17 = *APOE3*/12-month/*L*-cysteine diet. Lane 18 = *APOE3*/12-month/control diet. Lane 19 = *APOE4*/3-month/*L*-cysteine diet. Lane 20 = *APOE4*/3-month/control diet. Lane 21 = *APOE4*/6-month/*L*-cysteine diet. Lane 22 = *APOE4*/6-month/control diet. Lane 23= *APOE4*/9-month/*L*-cysteine diet. Lane 24 = *APOE4*/9-month/control diet. Lane 25 = *APOE4*/12-month/*L*-cysteine diet. Lane 26 = *APOE4*/12-month/control diet.

There were no statistically significant differences in HP-Tau deposition between the control and *L*-cysteine diets for any of the cohorts (Table 5). However, we did observe a statistically significant trend. Specifically, there was a significant trend towards an increase in HP-Tau deposition between *APOE2* and *APOE4* cohorts on the control diet; interestingly, this trend becomes insignificant with administration of the *L*-cysteine diet (Figure 6).

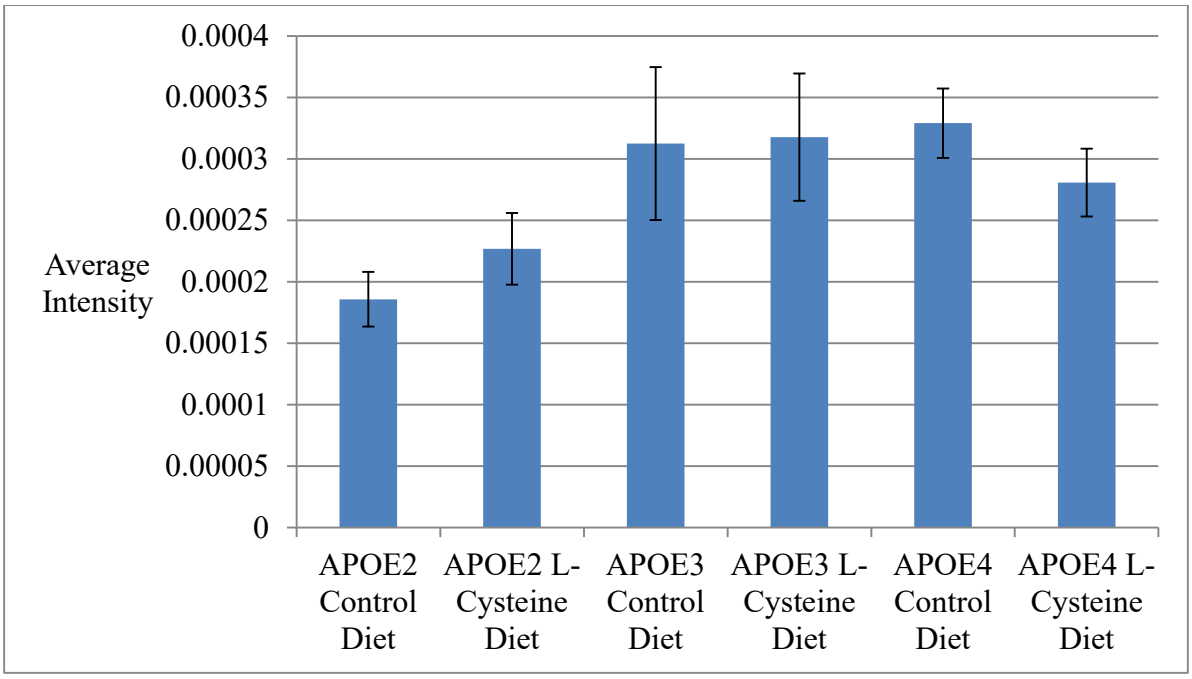

Figure 6: Trends in HP-Tau Deposition.

Palmitoylation Sites and Patterns of Different APOE Isoforms, and their Interactions with Aβ

Unfortunately, due to the low concentrations of APOE in murine brains, we were unable to analyze the palmitoylation sites and patterns of different APOE isoforms, their clearance rates, or their covalent molecular interactions with Aβ using the technology available to us at this time. Several attempts at mass spectrometry analysis that began with sample preparation involving immunoprecipitation, trypsin digest from total brain homogenate, in-gel trypsin digest, and HPLC mass spectrometry preparation all proved unfruitful. However, as we have recently obtained access to improved mass spectrometry technology, we will continue our experiments to reveal the palmitoylation sites and patterns of different APOE isoforms, as well as their clearance rates and interactions with Aβ.

DISCUSSION

Effects of *L*-Cysteine

The administration of *L*-cysteine to our mice resulted in no significant effect or trends concerning Aβ deposition, but nevertheless resulted in somewhat of a decrease in Aβ deposition for each cohort. Perhaps *L*-cysteine is indeed having an effect, but our statistical power was too low to confirm any effect.

However, *L*-cysteine did cause a statistically significant decrease in APOE production, but these results were considering diet alone, as interactions with age and/or *APOE* genotype were insignificant. Although interactions with age and/or *APOE* genotype were insignificant for APOE production, we did see a significant trend towards a decrease in APOE4 production with administration of *L*-cysteine as well as a nullifying effect of *L*-cysteine on the significance of a trend towards an increase in APOE production between *APOE2* and *APOE4* cohorts.

Furthermore, despite the fact that *L*-cysteine had no significant effect on HP-Tau deposition, we saw a significant trend towards an increase in HP-Tau deposition between *APOE2* and *APOE4* cohorts. Interestingly, the *L*-cysteine diet had nullified the significance of this trend.

Taken together, these results may indicate that the *L*-cysteine diet is helping with the clearance of Aβ as well as HP-Tau, which would be a novel find and worth pursuing in the future. For any future studies, increasing the number of subjects may give us a better indication of the effect of the *L*-cysteine diet.

Triple Combination Diet

It appears that consistent administration of *L*-cysteine trends towards lowering levels of Aβ in each cohort, but this effect is insignificant. On the other hand, *L*-cysteine caused a significant decrease in APOE production, but only with regard to diet. Thus, it is evident that

even though the trend of *L*-cysteine towards decreasing levels of Aβ plaques is profoundly weak, significantly less APOE is produced due to this slight decrease in Aβ burden, indicating that even small levels of Aβ invoke a mass production of APOE. Furthermore, administration of *L*-cysteine revealed no significant impact on or trends regarding HP-Tau deposition between diet types for each cohort. Nevertheless, *L*-cysteine did nullify the significance of a trend towards an increase in HP-Tau deposition between *APOE2* and *APOE4* cohorts. Thus, *L*-cysteine may be weakly affecting HP-Tau deposition via its ability to somewhat reduce Aβ burden and consequently prevent the shutdown of the proteosomes responsible for the degradation and clearance of HP-Tau (Tseng et al, 2008). However, our effect was not as profound as what Chan et al (2008) described. A possible explanation for this significance is because NAC was used instead of *L*-cysteine (Chan et al, 2008).

NAC is a prodrug to *L*-cysteine created by the attachment of an acetic acid group to enable the molecule to enter systemic circulation more easily after metabolism (Tirouvanziam et al, 2006). Therefore, a possible reason for the inefficiency (relative to NAC) of *L*-cysteine in relieving AD pathology could be because upon metabolism, too much is lost to have an effect on neurological function similar to NAC. Additionally, *L*-cysteine may only yield the degree of the effects seen by Chan et al (2008) when administered with ALCAR and/or SAMe. On the other hand, *L*-cysteine may alleviate AD pathology to the same extent regardless of how it is administered (as NAC or with ALCAR and/or SAMe in combination). Thus, further experiments will be needed to determine the extent of the role of *L*-cysteine vs. NAC, as well as the roles of ALCAR and SAMe in relation to *L*-cysteine/NAC.

Although the results of Chan et al (2008) show much success, there still remain problems with treatment application. For instance, both NAC and SAMe have negative adverse effects in

humans. Although NAC has not yet been proven to be carcinogenic, it is known to have cancer-exacerbating effects. For example, NAC increases proliferation of tumor cells by reducing ROS, DNA damage, and p53 expression in both mouse and human lung tumor cells (Holtzman et al, 2000; Bensaad et al, 2005; Chen et al, 2012). High levels of ROS up-regulate p53 to induce apoptosis of damaged and/or cancerous cells (Holtzman, 2000; Bensaad et al, 2005; Chen et al, 2012). Also, NAC has been reported (in some cases) to cause nausea, vomiting, fevers, and rashes when taken orally (DailyMed, 2014). Additionally, SAMe has been reported (in some cases) to cause dyspepsia, gastrointestinal disorders, and anxiety (Najm et al, 2004). SAMe is also a weak DNA-alkylating agent (Rydberg et al, 1982). Taken together, this triple-combination diet appears to be potentially unsafe for long-term use in humans. On the other hand, there are no known adverse effects of *L*-cysteine and ALCAR. *L*-cysteine is converted to alanine after donating its sulfide to sequester iron (Lill et al, 2006) and an increase in alanine levels leads to an increase in alanine aminotransferase (ALT) levels, which in turn increases the risk of developing type 2 diabetes mellitus (Sattar et al, 2004). However, the increase in alanine caused by the sequestering of iron by *L*-cysteine would likely not be enough to raise ALT to dangerous levels.

Possible Mechanisms of Treatment for *L*-Cysteine

As a treatment for AD, *L*-cysteine may act in a number of possible ways. For example, *L*-cysteine can sequester iron via its sulfide, making iron-sulfur clusters and alanine (Figure 7) (Lill et al, 2006). As a result, it is suggestive that Aβ will not be formed to sequester the iron, and thus Aβ plaque levels will decrease. Consequently, the inability of APOE4 to sufficiently clear Aβ plaques would be negated.

Additionally, free *L*-cysteine is oxidized in the body to form cystine (two *L*-cysteine molecules bound together via a disulfide bridge), which works as a substrate for the cystine-glutamate antiporter (Figure 7) (Lo et al, 2008). The cystine-glutamate antiporter pumps cystine into the cell and glutamate out of the cell (Lo et al, 2008). The cystine-glutamate antiporter is important in AD, because excitotoxicity due to excess glutamate release, as well as reduced uptake by impaired glutamate transporters, damages nerve cells and exacerbates AD via the binding of excess glutamate to the *N*-methyl-*D*-aspartate (NMDA) receptor (Liu et al, 2000; Hynd et al, 2004; Yi et al, 2006). So far, astrocytal excitatory amino acid transporter 2 (EAAT2) has been identified as an impaired glutamate transporter in AD (Yi et al, 2006), although the same discovery has not yet been made for neuronal EAAT2 (Furness et al, 2008). EAAT2 is uncommon in neurons (Furness et al, 2008), but common in astrocytes (Yi et al, 2006), whereas EAAT3 is the most common neuronal transporter of glutamate and cysteine, but is not found in astrocytes (Holmseth et al, 2012). Moreover, glycine serves as a necessary co-agonist to glutamate for the NMDA receptor (Liu et al, 2000). Interestingly, *L*-cysteine can combine with glutamate and glycine in cells to form the antioxidant glutathione (GSH) (Dringen et al, 1999; Aoyama et al, 2008), and by so doing, potentially bind excess intracellular glutamate before that glutamate can be pumped out of the astrocytes via the cystine-glutamate antiporters or the broken EAAT2 transporters (Figure 7). GSH is a powerful antioxidant (Dringen, 2000) that naturally reduces oxidative stress, which is a powerful contributing factor to AD.

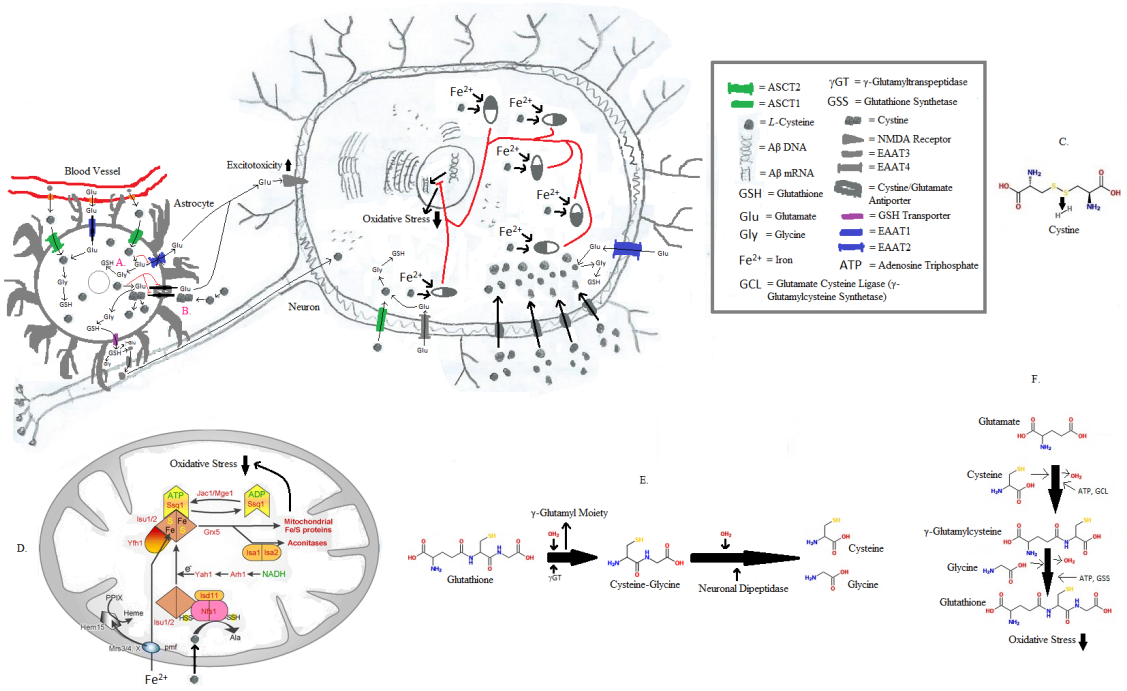

Figure 7: Possible Effects of *L*-Cysteine on Oxidative Stress.
7A. *L*-cysteine combining with glutamate and glycine in an astrocyte to form GSH, and thus binding excess intracellular glutamate before that glutamate can be pumped out of the astrocyte via the cystine-glutamate antiporters or the broken EAAT2 transporters.
7B. Free *L*-cysteine being oxidized in the body to form cystine, which works as a substrate for the cystine-glutamate antiporter.
7C. Cystine synthesis from two free cysteines.
7D. *L*-cysteine sequestering iron via its sulfide, making iron-sulfur clusters and alanine (occurs inside the mitochondria).
7E. Glutathione breakdown.
7F. Glutathione synthesis.

In addition, *L*-cysteine may form disulfide bridges with free cysteine residues in APOE3 and APOE2, thus preventing dimerization of APOE3 and dimerization/polymerization of APOE2, which might in turn reduce the number of Aβ molecules bound to these isoforms; however, it would not have these adverse effects on APOE4, because APOE4 has no cysteine residues (Figure 8).

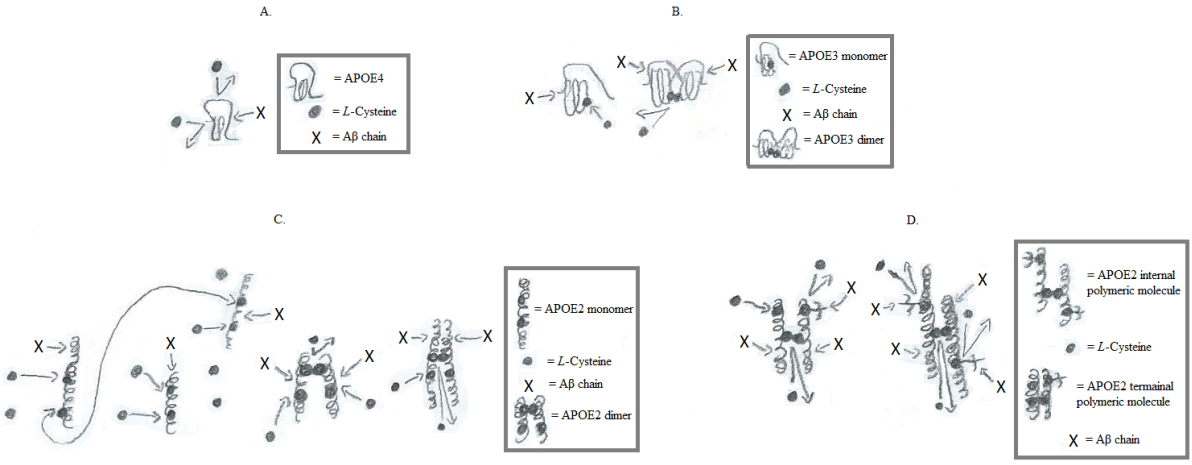

Figure 8: Potential Effects of *L*-Cysteine on the Number of Bound Aβ Molecules per APOE Molecule and on the Polymerization of APOE.
8A. APOE4 (only monomers). No effect.
8B. APOE3 (monomers and dimers). May prevent dimerization by binding to Cys-112, thus potentially allowing for the attachment of only one Aβ chain, for example. For dimers, there would be no effect.
8C. APOE2 (monomers and dimers). In monomers, may prevent polymerization by binding to Cys-112 or Cys-158, thus potentially allowing for the attachment of only one Aβ chain (if APOE2 remains monomeric) or two Aβ chains (if APOE2 becomes dimeric), for example. May also prevent polymerization by binding to both Cys-112 and Cys-158, thus potentially allowing for the attachment of only one Aβ chain, for instance. In dimers, may prevent further polymerization by binding to both free cysteine residues, thus potentially allowing for the attachment of only two Aβ chains, for example. If only one free cysteine residue is bound, then further polymerization of APOE2, and consequently, further binding of Aβ chains can occur.
8D. APOE2 (internal and terminal polymeric molecules). No effect on internal polymeric molecules. On terminal polymeric molecules, may prevent further polymerization by binding to Cys-112 or Cys-158, thus potentially allowing for the attachment of a number of Aβ chains equal to the maximum number of APOE2 molecules polymerized, for example.

Moreover, the binding of *L*-cysteine to free cysteine residues in APOE3 and APOE2 would prevent *S*-palmitoylation of those bound cysteine residues, which would in turn lessen the stability of APOE3/Aβ and APOE2/Aβ complexes; however, it would not have these adverse effects in APOE4 or in dimers of APOE3, because APOE4 lacks cysteine residues and APOE3 dimers lack free cysteine residues (Figure 9).

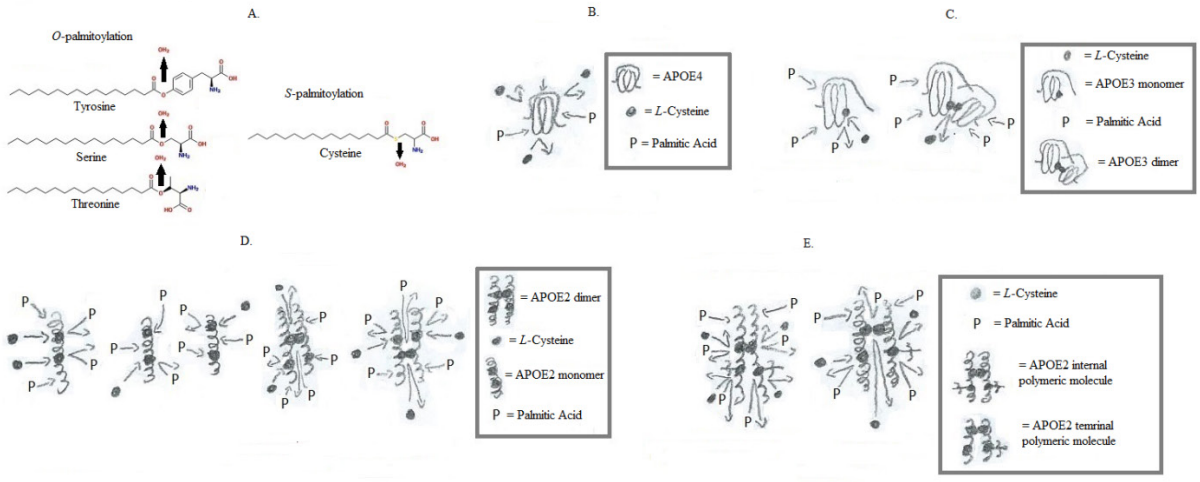

Figure 9: Potential Effects of *L*-Cysteine on Palmitoylation.
9A. *O*-palmitoylation vs. *S*-palmitoylation.
9B. APOE4 (only monomers). No effect; only *O*-palmitoylation is possible.
9C. APOE3 (monomers and dimers). May prevent *S*-palmitoylation at Cys-112; no effect on potential *O*-palmitoylation for monomers. For dimers, there would be no effect, as only *O*-palmitoylation is possible.
9D. APOE2 (monomers and dimers). For monomers, if *L*-cysteine binds both Cys-112 and Cys-158, *S*-palmitoylation is prevented, but there is no effect on *O*-palmitoylation. If *L*-cysteine binds either Cys-112 or Cys-158, one half of the possible *S*-palmitoylation scenarios are prevented, but there is no effect on *O*-palmitoylation. For dimers, if *L*-cysteine binds both free cysteine residues, *S*-palmitoylation is prevented, but there is no effect on *O*-palmitoylation. If *L*-cysteine binds either free cysteine residue, one half of the possible *S*-palmitoylation scenarios are prevented, but there is no effect on *O*-palmitoylation.
9E. APOE2 (internal and terminal polymeric molecules). No effect; only *O*-palmitoylation is possible for internal polymeric molecules. For terminal polymeric molecules, there would be no effect on *O*-palmitoylation; however, the binding of *L*-cysteine to Cys-112 or Cys-158 would prevent *S*-palmitoylation.

Additionally, *L*-cysteine can be converted to palmitic acid inside of neurons and other cells of the body (Figure 10). If made in cells other than neurons, the palmitic acid molecules could leave those cells and enter the bloodstream, which could carry them to astrocytes where they could cross the blood-brain barrier to enter neurons (Figure 10). If made in neurons (or after having entered neurons), the neuronal increase in palmitic acid might result in an increase in *O*-palmitoylation of APOE4, thus increasing the stability of APOE/Aβ complexes – potentially to the level of stability had with APOE2/Aβ complexes (Figure 10). *O*-palmitoylation and *S*-

palmitoylation of APOE3 and APOE2 might also increase, resulting in more stable APOE3/Aβ and APOE2/Aβ complexes, respectively (Figure 10). However, *S*-palmitoylation of APOE3 prevents its dimerization and binding of *L*-cysteine, and of APOE2, prevents its dimerization/polymerization and binding of *L*-cysteine; consequently, less Aβ molecules might be able to bind APOE3 and APOE2, and more *L*-cysteine would be available in these cases (Figure 10). Furthermore, free *L*-cysteine could potentially bind free palmitic acid, which would prevent the further use of either of those molecules, so long as they were bound to each other (Figure 10). Therefore, although the administration of *L*-cysteine to *APOE2* and *APOE3* genotypes potentially yields more risks than benefits, the administration of *L*-cysteine to *APOE4* genotypes would have no adverse effects and only has the potential to benefit those suffering from *APOE4*-induced AD.

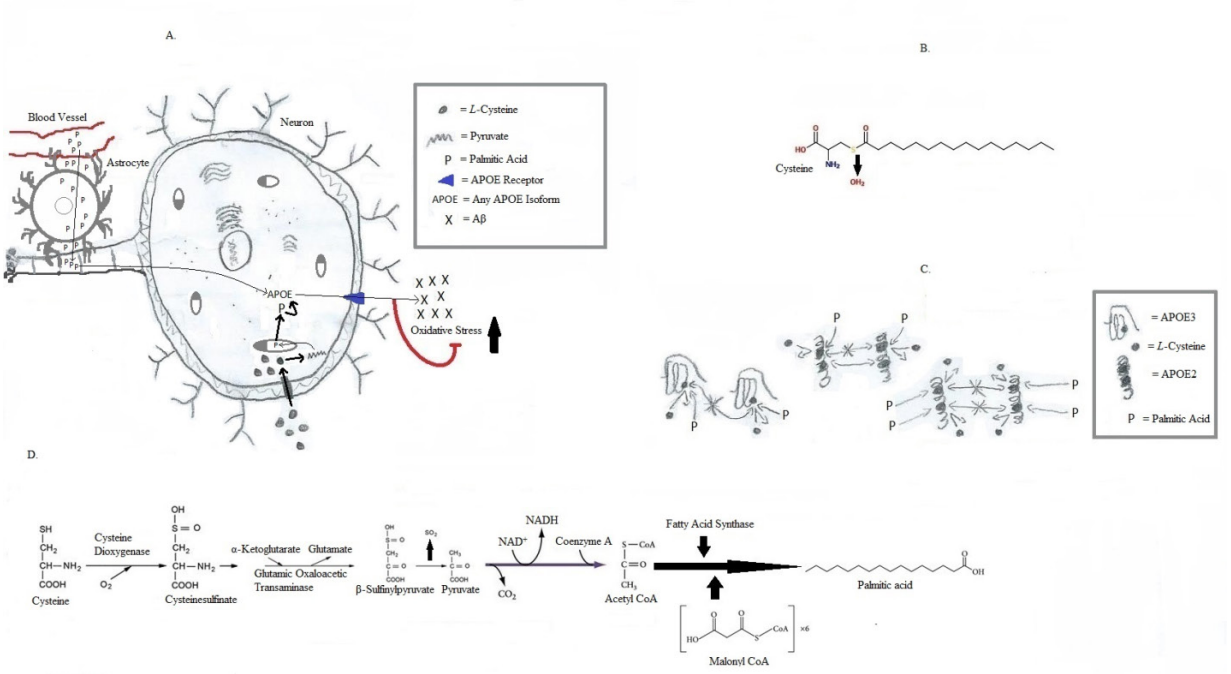

Figure 10: Other Possibilities.
10A. Palmitic acid molecules made from *L*-cysteine in other cells of the body can potentially leave those cells, enter the blood stream, travel to astrocytes, and then travel to neurons. The increase in free intracellular palmitic acid may in turn increase *O*-palmitoylation and *S*-palmitoylation, which would consequently increase the stability of APOE/Aβ complexes.
10B. Free *L*-cysteine can potentially bind palmitic acid (or any other hydroxyl- or thiol- containing molecule) and vice-versa, thus rendering the bound palmitic acid (or other hydroxyl- or thiol-containing molecule) and free *L*-cysteine molecule useless, so long as they were bound to each other.
10C. An increase in free palmitic acid may prevent the dimerization of APOE3 and any form of polymerization of APOE2 via *S*-palmitoylation of Cys-112 and of Cys-112 and/or Cys-158, respectively; such an increase may prevent the binding of free *L*-cysteine to APOE3 and APOE2 molecules. It is important to note that any compound containing a hydroxyl group (except water) or a thiol group would have the same potential effects as palmitic acid or *L*-cysteine, respectively, on APOE3 dimerization and any form of APOE2 polymerization.
10D. Conversion of Cysteine to Palmitic Acid. The mechanism of conversion of *L*-cysteine to palmitic acid is conserved throughout different cells of the body.

Possible Polymerization of APOE4

While performing this experiment, we made another interesting observation regarding the possible polymerization of APOE4. Western blot analyses revealed the majority of APOE4 to be at weights ≥140kD (Figure 3.1), at weights equal to that of APOE3 and APOE2 (Figure 3.2).

Moreover, APOE4 was found at ~97kD (although to a lesser degree than at ≥140kD), which typically signifies a dimer of APOE binding to chains of Aβ (Figure 3.2) (LaDu et al, 2012). APOE2 and APOE3 were also found at ~97kD at all time intervals (Figure 3.1). Since APOE4 lacks cysteine residues (Tables 1 and 2), these possible dimers and polymers of APOE4 cannot be forming via disulfide bonds. On the other hand, APOE4 could be forming dimers and polymers (and APOE3, polymers) via dityrosine bonds, as tyrosine residues are equally abundant in all APOE isoforms (Tables 1 and 2). Dityrosine bonds are formed via oxidation (Al-Hilaly et al, 2013) and an abundance of oxidative stress, as is found in AD (Al-Hilaly et al, 2013), may lead to the formation of dityrosine bonds between APOE4 molecules and also between APOE3 molecules, causing dimers and polymers of APOE4 to form and polymers of APOE3 to form.

Furthermore, Aβ polymerizes via dityrosine bonds (Al-Hilaly et al, 2013). Given that Aβ is produced in neurons (Hartmann et al, 1997) and that APOE is produced in microglia and astrocytes (Holtzman et al, 2012), and to a lesser degree, in neurons (albeit, to a greater degree in neurons when in response to excitotoxic injury, which is common in AD) (Liu et al, 2000; Hynd et al, 2004; Xu, 2006; Yi et al, 2006), it is plausible that APOE4 is forming dimers and polymers, and that APOE3 is forming polymers via the same mechanism as Aβ. We will perform further experiments in the near future with SDS-PAGE and mass spectrometry analysis to determine if APOE4 is able to form dimers and polymers, and if APOE3 is able to form polymers via dityrosine bonds or some other means. Therefore, since it is currently believed that APOE4 cannot dimerize, proving otherwise would significantly impact our perception of APOE4 and of its contribution to AD pathology.

Possible Interactions of APOE Isoforms with Aβ

A plausible explanation as to why APOE2 is the most efficient (and APOE4, the least efficient) at clearing Aβ is that homodimers of APOE isoforms can clear twice as many molecules of Aβ as monomers of APOE isoforms, and homopolymers of APOE2 can clear any number times as many molecules of Aβ as monomers of APOE isoforms (Figure 11).

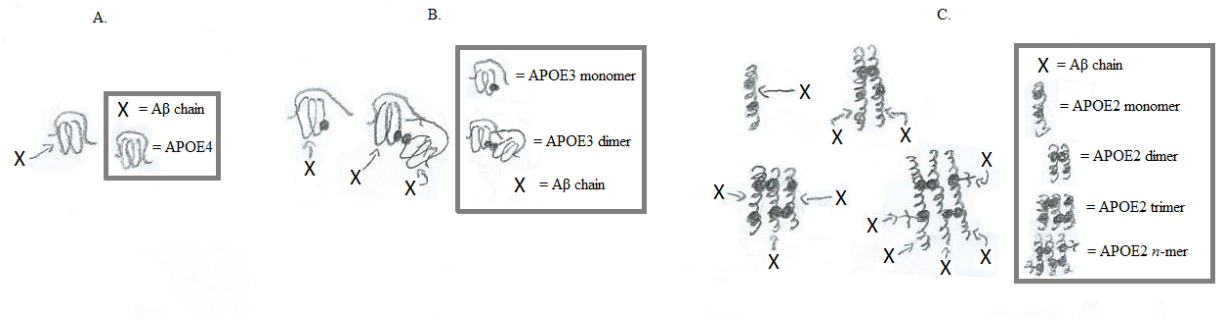

Figure 11: Potential Number of Bound Aβ Molecules to Monomers, Oligomers, and Polymers of Different APOE Isoforms.
11A. APOE4 (only monomers). For example, 1 Aβ chain/APOE4 molecule.
11B. APOE3 (monomers or dimers).For example, 1 Aβ chain/APOE3 monomer and 2 Aβ chains/APOE3 dimer.
11C. APOE2 (monomers, dimers, trimers, or polymers). For example, 1 Aβ chain/APOE2 monomer, 2 Aβ chains/APOE2 dimer, 3 Aβ chains/APOE2 trimer, and *n* Aβ chains/APOE2 *n*-mer.

Thus, the arginine-to-cysteine changes among APOE isoforms are significant. In addition, not only are the changes of arginine to cysteine at positions 112 and/or 158 important – these positions themselves are important. For example, the change of arginine to cysteine at position 112 causes the repulsion of positively-charged arginine-61 from arginine-112 to be lost, and consequently the attraction of arginine-61 to negatively-charged glutamate-255 is lost (Zhong et al, 2008) (Figure 12). As a result, much of the steric hindrance is relieved between the *C*-terminal helix and the central helices of the protein (Zhong et al, 2008) (Figure 12), revealing approximately 12-17 potential palmitoylation sites. Furthermore, although the change of arginine

to cysteine at position 158 causes the attraction of negatively-charged aspartate-154 to arginine-158 to be lost, it allows for the binding of arginine-150 to aspartate-154, which significantly changes the orientation of the protein from position 158 up to the *N*-terminus (Dong et al, 1996) (Figure 12).

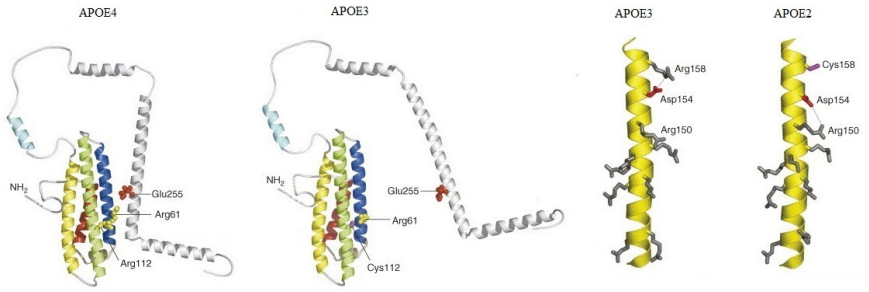

Figure 12: Crystal Structures and Intramolecular Ionic Interactions of Different APOE Isoforms.

Consequently, approximately 19 potential palmitoylation sites are revealed. For a detailed map of potential palmitoylation sites of each APOE isoform concerning each respective polymerization scenario, see Table 6.

Table 6: Differences in Potential Palmitoylation Sites Between APOE Isoforms and their Monomers, Oligomers, and Polymers.
Possible sites of *S*-palmitoylation in blue. Possible sites of *O*-palmitoylation in red. C = Cysteine. S = Serine. T = Threonine. Y = Tyrosine.

Protein Isoform	Protein Sequence
APOE4	MKVEQAVETEPEPELRQQTEWQSGQRWELALGRFWDYLRW VQTLSEQVQEELLSSQVTQELRALMDETMKELKAYKSELEEQ LTPVAEETRARLSKELQAAQARLGADMEDVRGRLVQYRGEV QAMLGQSTEELRVRLASHLRKLRKRLLRDADDLQKRLAVYQ AGAREGAERGLSAIRERLGPLVEQGRVRAATVGSLAGQPLQE RAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEE QAQQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQA AVGTSAAPVPSDNH
APOE3 Monomer	MKVEQAVETEPEPELRQQTEWQSGQRWELALGRFWDYLRW VQTLSEQVQEELLSSQVTQELRALMDETMKELKAYKSELEEQ LTPVAEETRARLSKELQAAQARLGADMEDVCGRLVQYRGEV QAMLGQSTEELRVRLASHLRKLRKRLLRDADDLQKRLAVYQ AGAREGAERGLSAIRERLGPLVEQGRVRAATVGSLAGQPLQE RAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEE QAQQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQA AVGTSAAPVPSDNH
APOE3 Dimer	MKVEQAVETEPEPELRQQTEWQSGQRWELALGRFWDYLRW VQTLSEQVQEELLSSQVTQELRALMDETMKELKAYKSELEEQ LTPVAEETRARLSKELQAAQARLGADMEDVCGRLVQYRGEV QAMLGQSTEELRVRLASHLRKLRKRLLRDADDLQKRLAVYQ AGAREGAERGLSAIRERLGPLVEQGRVRAATVGSLAGQPLQE RAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEE QAQQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQA AVGTSAAPVPSDNH
APOE2 Monomer	MKVEQAVETEPEPELRQQTEWQSGQRWELALGRFWDYLRW VQTLSEQVQEELLSSQVTQELRALMDETMKELKAYKSELEEQ LTPVAEETRARLSKELQAAQARLGADMEDVCGRLVQYRGEV QAMLGQSTEELRVRLASHLRKLRKRLLRDADDLQKCLAVYQ AGAREGAERGLSAIRERLGPLVEQGRVRAATVGSLAGQPLQE RAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEE QAQQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQA AVGTSAAPVPSDNH

APOE2 Homodimer/APOE2 Homotrimer (terminal APOE2 molecule)/APOE2 Homopolymer (terminal APOE2 molecule)	MKVEQAVETEPEPELRQQTEWQSGQRWELALGRFWDYLRW VQTLSEQVQEELLSSQVTQELRALMDETMKELKAYKSELEEQ LTPVAEETRARLSKELQAAQARLGADMEDVCGRLVQYRGEV QAMLGQSTEELRVRLASHLRKLRKRLLRDADDLQKCLAVYQ AGAREGAERGLSAIRERLGPLVEQGRVRAATVGSLAGQPLQE RAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEE QAQQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQA AVGTSAAPVPSDNH or MKVEQAVETEPEPELRQQTEWQSGQRWELALGRFWDYLRW VQTLSEQVQEELLSSQVTQELRALMDETMKELKAYKSELEEQ LTPVAEETRARLSKELQAAQARLGADMEDVCGRLVQYRGEV QAMLGQSTEELRVRLASHLRKLRKRLLRDADDLQKCLAVYQ AGAREGAERGLSAIRERLGPLVEQGRVRAATVGSLAGQPLQE RAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEE QAQQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQA AVGTSAAPVPSDNH
APOE2 Homotrimer (internal APOE2 molecule)/APOE2 Homopolymer (internal APOE2 molecule)	MKVEQAVETEPEPELRQQTEWQSGQRWELALGRFWDYLRW VQTLSEQVQEELLSSQVTQELRALMDETMKELKAYKSELEEQ LTPVAEETRARLSKELQAAQARLGADMEDVCGRLVQYRGEV QAMLGQSTEELRVRLASHLRKLRKRLLRDADDLQKCLAVYQ AGAREGAERGLSAIRERLGPLVEQGRVRAATVGSLAGQPLQE RAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEE QAQQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEKVQA AVGTSAAPVPSDNH

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CURRICULUM VITAE

Stephen Gerard Cieslak Jr.

Education

- BS (earned December 2013 from Brigham Young University in Provo, UT)
  - Genetics (Microbial Emphasis)
  - Biotechnology
  - German Studies
- MS (earned December 2016 from Brigham Young University in Provo, UT)
  - Physiology
  - Developmental biology
  - Graduate GPA of 3.89

Employment and Research Experience

- Researcher for Dr. Rick Jellen of Brigham Young University in Provo, UT performing cytology with *Avena sativa* during spring 2011 and fall 2011 (for employment), and with Quinoa during summer 2012
  - Prepared over 100 *A. sativa* seeds for karyotyping
  - Discovered that *A. sativa* genome is dynamic and that there has been non-homologous chromosomal exchange throughout its evolutionary history
  - Helped/supervised three students with work on cytology with quinoa during summer 2012
  - Prepared scores of Quinoa seeds for karyotyping
  - Discovered that Quinoa chromosomes are physically too small to karyotype
- Researched relationships between multidrug resistance and fitness in B079 strain of *E. coli* under Dr. Joel Griffitts of Brigham Young University in Provo, UT during fall 2012
  - Designed experiment
  - Engineered triple-drug-resistance in B079 strain of *E. coli*
  - Discovered that when streptomycin resistance is paired with rifampicin resistance, the mutations somehow cause the *E. coli* cells to proliferate rapidly in plain liquid LB media, but to rapidly die off after two hours of growth
- Worked on Immigrant Ancestors Project German Section under Professor Jill Crandell at Brigham Young University in Provo, UT from August 2013 to January 2014
  - Extracted, transcribed, and indexed German vital and immigration records from early to late 1800s
  - Verified correctness of work of other German team members
  - Completed Staatsarchiv Landhut Auswanderungen project

    - Translated biographies of other employees into German
- Since January 2014, researching Alzheimer's disease genetics (*APOE4* gene) with Dr. Jon Wisco and Dr. J.C. Price at Brigham Young University in Provo, UT
    - Designed experiment
    - Effects of high *L*-cysteine diet on amyloid β plaque and HP-Tau tangle formation in *APOE4*, *APOE3*, and *APOE2* homozygous mice
    - Bred and raised over 200 mice
    - Submitted paper with methods and results to Journal of Neuroscience for publication (see Submissions section)
- TA for cellular biology for Dr. Liz Bailey at Brigham Young University in Provo, UT during winter 2014
    - Helped grade assignments and tests
    - Help students learn course material
    - Helped teach the course
- Worked on Immigrant Ancestors Project German Section under Professor Jill Crandell at Brigham Young University in Provo, UT from April 2014 to April 2016
    - Extracted, transcribed, and indexed German vital and immigration records from early 1800s to early 1900s
    - Verified correctness of work of other German team members
    - Completed German Immigrants to Chile project
    - Completed Bremen ship records project
    - Worked on Fürth Auswanderungen project
    - Translated biographies of other employees into German
- TA for Dr. Alison Woods for human physiology lab during spring 2016
    - Taught the lab
    - Graded assignments
    - Helped students learn course material
- Worked under MS stipend with Dr. Jon Wisco at Brigham Young University in Provo, UT April 2016 – December 2016
    - Designed experiment
    - Researched effects of high *L*-cysteine diet on amyloid β plaque and HP-Tau tangle formation in *APOE4*, *APOE3*, and *APOE2* homozygous mice
    - Bred and raised over 200 mice
    - Submitted paper with methods and results to Journal of Neuroscience for publication

Scholarships

- TA for Cellular Biology during January 2014 – April 2014
- TA for Human Physiology Lab during April 2016 – June 2016

- RA and tuition during April 2016 – December 2016

Skills

- Sample preparation for mass spectrometry analysis
- Sample preparation for HPLC analysis
- Immunoprecipitation
- Plasma collection from blood samples
- SDS-PAGE
- Western blotting
- Protein analysis
- DNA analysis
- PCR
- Breeding laboratory mice
- BSL-1 and BSL-2 laboratory work
- Human cadaver dissection
- Microsoft Word, Microsoft Excel, and Microsoft PowerPoint
- Python programming (very basic)
- German language
- Bacterial culture and drug resistance
- Vital Record Indexing/Keeping
- Immigration Record Indexing/Keeping
- German Gothic Script

Submissions

- The Effects of *L*-Cysteine on Alzheimer's Disease Pathology in *APOE2*, *APOE3*, and *APOE4* Homozygous Mice
    - Stephen G. Cieslak, Dennis L. Eggett, Jonathan J. Wisco
    - Journal of Neuroscience – Submitted for publication November 2016

Test Scores

- German reading proficiency exam score of advanced-high, writing proficiency exam score of intermediate-high/advanced-low, and speaking proficiency exam score of intermediate-medium
- GRE verbal reasoning score of 155, quantitative reasoning score of 157, GRE written analysis score of 5.0, GRE total multiple choice score of 312.

Volunteer Work

- Served a family history service mission for the Church of Jesus Christ of Latter-day Saints from August 1, 2010 to August 1, 2012
    - Taught genealogy/family history
    - Indexed and interpreted vital records
    - Helped perform all manner of work in the temples of the Church
- Children's unit at Utah State Hospital from July 2015 – August 2015
    - Observed behavior of children
    - Assisted in character-building activities