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Sequence Analysis of APOE Gene Across Mammalian Species in Thailand

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Abstract-The current study utilized five mammalian species, including rhesus monkey *(Macaca mulatta*), Thai Bangkaew dog (*Canis lupus familiaris*), Thai domestic cat (*Felis catus*), tiger (*Panthera tigris*) and Thai domestic rabbit *(Oryctolagus cuniculus*) to survey the prevalence of apolipoprotein E isoform distribution. Screening for the three common apolipoprotein E (APOE) isoforms (E2, E3 and E4) was achieved using APOE gene sequencing analysis. The results clearly revealed the homology of APOE protein sequences in the analyzed mammals, ranging from 59.74-96.22% and 64.15-100% identity and similarity, respectively. Thai Bangkaew dog, Thai domestic cat, tiger, and rhesus monkey show arginine at the positions 112 and 158, which are found in APOE4 of human. This result demonstrates that for these five investigated mammals are apolipoprotein E4, and is associated with an increased genetic risk factor of both Alzheimer's disease (AD) and coronary heart disease (CHD). Remarkably, Thai domestic rabbit has cysteine at position 112 and arginine at position 158. This indicates that the APOE isoform of Thai domestic rabbit is identical to that of human APOE3, and is not associated with the risk for Alzheimer's disease. The evolutionary history inferred that Thai Bangkaew dog, Thai domestic cat and tiger are distinguishable as a group from Thai domestic rabbit, rhesus monkey and human by certain differences in amino acid sequences. This study provides valuable genetic resources for future study to identify potential biomarkers of neurodegenerative diseases and cardiovascular disease in mammals using apolipoprotein E gene expression profiles.

Keywords: Apolipoprotein E, mammals, polymorphism

1. Introduction

Apolipoprotein E (APOE) is a polymorphic lipoprotein that is a major cholesterol carrier in the mammalian brain. It involved in several functions, including lipid metabolism neuronal signaling, and neurodegenerative diseases (Muñoz *et al*., 2019 ; Herz and Beffert, 2000). Among mammalian species, it is clearly documented that humans have three different isoforms of APOE, termed E2, E3, and E4, with the E3 isoform being the most common, while other species have one APOE isoform (McIntosh *et al*., 2012). These three common isoforms (APOE2, APOE3, and APOE4) are encoded by a gene on chromosome19 (Frieden *et al*., 2012). Variants of the three APOE protein isoforms correspond to mutations in the coding sequence of the APOE gene resulting in amino acid substitutions (Arginine and Cysteine) at positions 112 and 158 of the protein. In non-human mammals, APOE genotype is (Threonine61/Arginine112/ Arginine158) while all human APOE alleles have an Arginine in position 61, 112 and 158 (Belloy *et al*., 2019). It has been reported that polymorphism in the APOE gene is a major risk for developing late onset alzheimer disease (LOAD) (Yamazaki *et al*., 2019).

According to studies of the structure of human and animal APOE isoforms, the structure differences influence their ability to bind lipids, receptors, and amyloid-β (Aβ), which accumulates in plaques within the brain. These clearly demonstrate that APOE4 regulate neuroinflammation, tau hyperphosphorylation, Aβ aggregation and clearance, which is clearly associated with an increased risk of developing late onset alzheimer disease (Tachibana *et al*., 2019 ; Kloske & Wilcock, 2020 ; Vasievskaya *et al*., 2020). Genetically, the ε4 allele of the APOE gene has been identified as the main risk factor in late onset alzheimer disease. Researchers have suggested that an important factor leading to an increased risk of developing late onset alzheimer disease through the inheritance of APOE4, resulting in the inability of this isoform to scavenge the neurotoxic lipid peroxidation product, 4-hydroxynonenal, because of the absence of the two critical Cysteine residues in the protein (Pedersen *et al*., 2000 ; Lauderback *et al*., 2002). Consequently, lipid peroxidation, nitration of tyrosine residues and oxidative damage could occur in human and other mammal APOE4 carriers, leading to neuronal death and Alzheimer's disease (Butterfield, 2019). Taken together, this evidence indicates that the potential existence of one or more additional genetic susceptibility factors in the APOE sequence predicts the APOE4-associated risk of developing Alzheimer's disease.

The importance of apolipoprotein E polymorphism in relation to Alzheimer's disease or other neurodegenerative diseases risk in mammals remains to be determined. Additionally, there have been no published reports on genetic polymorphism of mammal apolipoprotein E in Thailand. Therefore, in the present study, we aimed to investigate the genetic polymorphism of APOE gene of mammalian species in Thailand using polymerase chain reaction and nucleotide sequencing techniques. Our findings could serve as the primary genetic data to support the identification of APOE genotypes and the development of an important genetic biomarker for Alzheimer's disease pathophysiology or neurodegenerative diseases in mammals.

2. Materials and Methods

2.1 Sample Collection and DNA Extraction

Veterinarians took blood samples from animals using blood collection tubes (BD Vacutainer®, Becton Dickinson, USA) with K_2EDTA as an anticoagulant. The samples were obtained from of the rhesus monkey (*Macaca mulatta*), Thai Bangkaew dog (*Canis lupus familiaris*), Thai domestic cat (*Felis catus*), tiger (*Panthera tigris*) and Thai domestic rabbit (*Oryctolagus cuniculus*). Total genomic DNA was extracted from whole blood using a QIAamp DNA Blood Kits (Qiagen, Hilden, *Germany*), according to the manufacturer's instructions. DNA concentration was spectrophotometrically determined. The quality of the isolated DNA was verified by 0.8% agarose gel electrophoresis and then staining with GelRed® Nucleic Acid Stain 10000X DMSO (Biotium, Inc., USA) under UV light. The isolated DNA was stored at-20°C before analysis.

2.2 PCR Amplification of ApoE Gene from Genomic DNA

Polymerase chain reactions were performed using APOE primers to ensure the amplified gene is the gene of interest. The primers were design based on the conserved nucleotide sequences of APOE gene from GenBank. A polymerase chain reaction (PCR) amplified the partial APOE gene with an upstream primer, GAGCTGCAGGCGG CGCAGGC, and a downstream primer, -GAACCAGCTCTTGAGGCGGG-. In this study an APOE gene was amplified from genomic DNA of five mammals. A DNA polymerase enzyme with proof reading activity to remove misincorporated nucleotides (DynaZyme EXT™ DNA polymerase, Finazyme) was used for the amplification of particular target gene. The reaction of 25 µl total volume of PCR reaction consists of 20-40 ng of DNA template, 0.5 units of DNA polymerase, 2.5 pM primers, 1x buffer supplied with enzyme, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and sterile distilled water for adjusting volume. The PCR was done in Thermocycler (GeneAmp PCR system 2400, Perkin-Elmer), pre-heated at 94°C done for 3 minutes before starting with 30 cycles of the denature temperature of 94°C for 1 minutes, annealing temperature of 65°C for 1 minutes, and extension temperature of 72°C for 2 minutes. After 30 cycles of the reaction was completed, the final extension at 72°C was done for 8 minutes. The PCR products were electrophorized on a 2% agarose gel.

2.3 Cloning of APOE Gene

The PCR products were purified from gel by using QIAquick™ Gel Extraction kit (Qiagen®). The amplified DNA fragments were excised from agarose gel with a clean surgergical blade under long-wave UV lamp. The excised gels were then transferred to 1.5 ml microtube. DNA fragments were extracted from gel using the company's protocol. The concentration of purified product was determined. Purified bands were used for construction of APOE-pGEM®-T vector in the ligation reaction. Ligation of purified bands of APOE gene with the linear cloning vectors, pGEM®-T vector (Promega, USA) were done on ice box for 3 hours. Five ml of total volume of reaction contained 25 ng of pGEM®-T cloning vector, 2 µl of purified PCR product and 1x ligation buffer. The ligation products

were transformed to the competent cells (*DH*5α) to manipulate the APOE gene in the pGEM®-T plasmid. The successful clones were induced with IPTG combined with X-gal as the substrate analogues (white-blue screening method). The correct insertion of the gene was presented as white colonies with ampicillin resistance and was selected for further expression whereas blue colonies were used as a control. PCR was used to confirm that the clones carried the correct gene into the right position of the vector and to verify the DNA sequence of the positive clones.

2.4 DNA Sequencing

Cloned PCR fragments in pGEM®-T vector were sequenced on both strands using T7 promoter primer and SP6 promoter primer. The DNA sequencing reactions were carried out using ABI PRISM® BigDye™ Terminator Cycle V2.0 DNA Sequencing Ready Reaction Kit (Perkin-Elmer Biosystems). Sequencing reactions were performed in a GeneAmp® PCR system each reaction containing 200 ng of plasmid DNA, 2 µl of premixed reaction, 10 pmole primer and 1x sequencing buffer. The 25 cycles of denaturation (96°C for 10 sec), annealing (50 °C for 5 sec) and extension (60°C for 4 min) were done. The extension product was precipitated by ethanol-sodium acetate precipitation and was loaded to ABI PRISM™ 310 Sequencer (Perkin-Elmer, USA). This process was performed by Macrogen, the Republic of Korea.

2.5 Nucleotide Sequences Analysis

The sequencing data of individual clones was analysed by BLAST program (Altschul *et al*., 1990). This program showed the degree of similarity between unknown APOE clones and known APOE sequences from Genbank. The DNA and amino acid sequence were also analysed with SIAS program (Rech *et al*., 1990) to show the sequence homology between APOE sequence of five investigated mammals and published human APOE sequences from Genbank. Multiple sequence alignments were analyzed using the ClustalW.

2.6 Phylogenetic Tree Analysis

The phylogenetic tree of mammalian species was constructed in MEGA X based on APOE amino acid sequences (Kumar *et al*., 2018). The evolutionary history was inferred using the Maximum Likelihood method and JTT matrix-based model (Jones *et al*., 1992). The bootstrap analysis was performed with 2000 replicates.

3. Results

The amplification of APOE gene target sequence was performed using a pair of primers in an optimized PCR reaction. The molecular size of the PCR products was estimated to be 510 base pairs by comparing with DNA size marker (Figure 1). Screening for the three common APOE isoforms (E2, E3 and E4) in five mammalian species, including rhesus monkey, Thai Bangkaew dog, Thai domestic cat, tiger and Thai domestic rabbit was achieved using APOE gene sequencing analysis. The results revealed a very high homology of

APOE protein sequences in the examined mammals, ranging from 59.74-96.22% and 64.15-100% identity and similarity, respectively (Table 1, Table 2). Multiple sequence alignment of partial APOE gene sequence among seven mammals, including human APOE3 (GenBank accession no. NM_001302689.2) and human APOE4 (GenBank accession no. M10065.1) was carried out to study apolipoprotein E polymorphism. The alignment revealed Thai Bangkaew dog, Thai domestic cat, tiger, and rhesus monkey contain arginine at positions 112 and 158, which are found in APOE4 of human. Thai domestic rabbit has cysteine at the position 112 and arginine at the position 158, respectively (Figure 2). Our results also found the substitution (deletion) in the APOE sequence of Thai domestic cat and tiger (Figure 2). The phylogenetic tree was constructed using MEGA X software based on the partial APOE amino acid sequence alignment of seven selected mammals, including human APOE3 human APOE4 from the GenBank to show the relationship between the selected mammalian species and human APOE. The phylogenetic clustering in the APOE coding region of all seven mammalian species was distinctly divided into two lineages. Thai Bangkaew dog, Thai domestic cat and tiger belong to the same cluster while Thai domestic rabbit, rhesus monkey and human were grouped closely together (Figure 3).

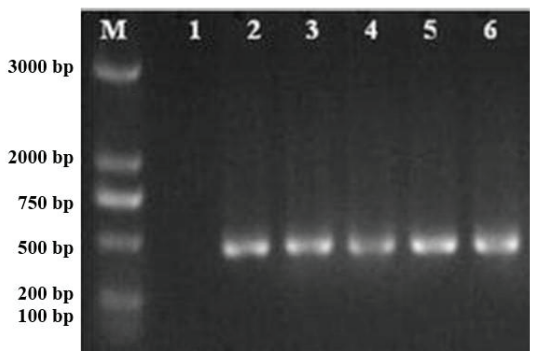

Figure 1. Polymerase chain reaction amplification results of APOE gene in five mammals. Lane 1 is negative control, lane 2, 3, 4, 5 and 6 are amplicon of APOE gene of Thai Bangkaew dog, Thai domestic cat, tiger, and rhesus monkey, respectively.

Table 1. Levels (%) of amino acid sequence identity among different mammalian species, including Thai Bangkaew dog (*Canis lupus familiaris*), Thai domestic cat (*Felis catus*), tiger (*Panthera tigris*), Thai domestic rabbit (*Oryctolagus cuniculus*), rhesus monkey (*Macaca mulatta* in the partial apolipoprotein E.

Monkey	100%				
Dog	73.52%	100%			
Cat	63.12%	67.5%	100%		
Tiger	61.63%	66.03%	96.22%	100%	
Rabbit	81.17%	74.7%	60.62%	59.74%	100%
	Monkey	**Dog**	**Cat**	**Tiger**	**Rabbit**

IDENTITY STATS

```
min      = 59.74
max      = 96.22
mean     = 70.425
stddev   = 10.8516618542968
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Table 2. Levels (%) of amino acid sequence similarity among different mammalian species, including Thai Bangkaew dog (*Canis lupus familiaris*), Thai domestic cat (*Felis catus*), tiger (*Panthera tigris*), Thai domestic rabbit (*Oryctolagus cuniculus*), rhesus monkey (*Macaca mulatta* in the partial apolipoprotein E.

Monkey	100%				
Dog	79.41%	100%			
Cat	66.25%	73.12%	100%		
Tiger	64.77%	71.69%	96.85%	100%	
Rabbit	85.29%	77.64%	65%	64.15%	100%
	Monkey	**Dog**	**Cat**	**Tiger**	**Rabbit**

SIMILARITY STATS

```
min      = 64.15
max      = 100
mean     = 79.5336
stddev   = 13.649127702531
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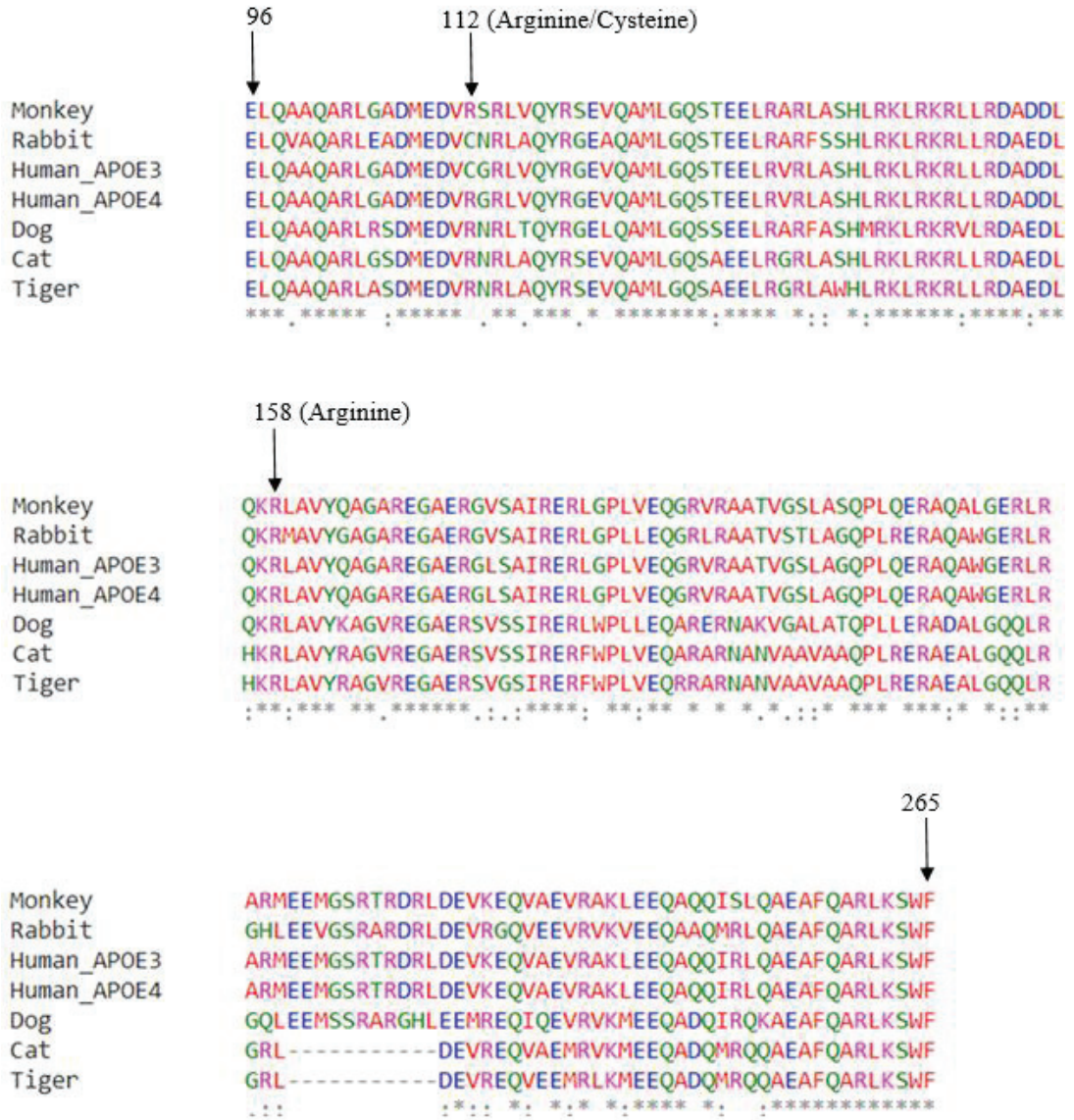

Figure 2. Multiple sequence alignment analysis. ClustalW was used to align amino acid sequences from site 96 to 265 of the partial apolipoprotein E gene in mammalian species, including Thai Bangkaew dog (*Canis lupus familiaris*), Thai domestic cat (*Felis catus*), tiger (*Panthera tigris*), Thai domestic rabbit (*Oryctolagus cuniculus*), rhesus monkey (*Macaca mulatta*), Human APOE3 (*Homo sapiens* Apolipoprotein E3 GenBank accession no. NM_001302689.2) and Human APOE4 (*Homo sapiens* Apolipoprotein E4 GenBank accession no. M10065.1)

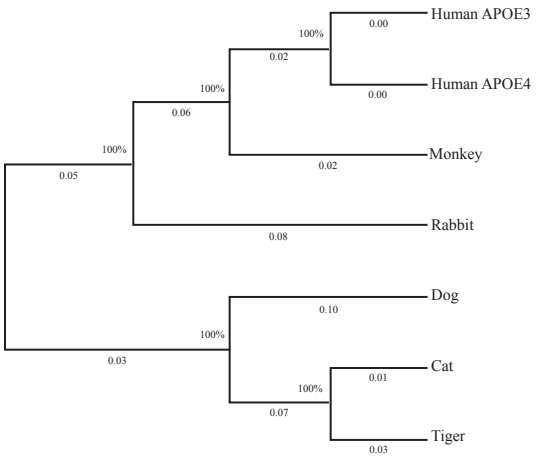

Figure 3. Phylogenetic tree of mammalian species, including Thai Bangkaew dog (*Canis lupus familiaris*), Thai domestic cat (*Felis catus*), tiger (*Panthera tigris*), Thai domestic rabbit (*Oryctolagus cuniculus*), rhesus monkey (*Macaca mulatta*), Human APOE3 (*Homo sapiens* Apolipoprotein E3 GenBank accession no. NM_001302689.2) and Human APOE4 (*Homo sapiens* Apolipoprotein E4 GenBank accession no. M10065.1) constructed by MEGA X based on the partial APOE amino acid sequences (Kumar *et al*., 2018). The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches.

4. Discussion and Conclusion

Sequence comparison of the APOE gene in mammalian species revealed that Thai Bangkaew dog, Thai domestic cat, tiger, and rhesus monkey show arginine codons that are identical to the human APOE4 sequence at positions homologous to amino acids 112 and 158. There were no polymorphisms at position 112 and 158 in the four mammals that were examined. Strikingly, only the Thai domestic rabbit has cysteine at the position 112 and arginine at the position 158. This finding indicates that the APOE isoform of Thai domestic rabbit is identical to that of human APOE3, which is not associated with the risk for Alzheimer's disease and other neurogenerative diseases. In addition, the results also suggest APOE isoforms of Thai Bangkaew dog, Thai domestic cat, tiger, and rhesus monkey correspond to human APOE4, which supports the finding that chimpanzee APOE genotypes, analyzed by PCR/restriction enzymes, have arginine codons which are similar to those of human APOE4. These mammal APOE4 carriers are associated with an increased risk of Alzheimer's disease (Gearing *et al*., 1994). The similarities in the APOE sequences between different species allows one to make certain deductions about the relatedness of the mammal species we have examined, which are related to the study of certain human apolipoprotein characteristics, and allows a comparison of human and primate APOE protein sequences. Furthermore, non-human primate apolipoprotein E have arginine at position 112 and 158, like human APOE4 (McIntosh, *et al*., 2012). Thai Bangkaew dog, Thai domestic cat and tiger are distinguishable as a group from Thai domestic rabbit, rhesus monkey and human by amino acid sequence differences at codons 173, 176, 182, 192, 197, 212, 213, 243, 248 and 252 (Figure 2), which correspond to the phylogenetic relationships between these seven mammalian species (Figure 3). Although the sequences were performed for 180 codons, the APOE in all of the examined species differed from the human APOE4 by at least one amino acid (Figure 2). Interestingly, it is possible that the substitution (deletion) found in the APOE sequence of Thai domestic cat and tiger could alter the structure of their apolipoprotein E protein so that they behave differently in lipid transport and in the nervous system. As previous report, APOE4 allele is found

to be major common risk factor for both Alzheimer's disease (AD) (Belloy *et al*., 2019 ; Qian, *et al*., 2021) and coronary heart disease (CHD) (Garcia *et al*., 1994), due to its role in lipid metabolism and associated inflammation (Huebbe and Rimbach, 2017). APOE4 carriers have been found to increase levels of total cholesterol, low-density lipoprotein (LDL), and oxidized LDL (Safieh *et al*., 2019 ; Yassine and Finch, 2020). Moreover, Fainman *et al* (2007) have analyzed the APOE gene sequences of 30 vervet monkeys (*Chlorocebus aethiops*) ranging in age from 15 to 28 years, all examined animals were found to be APOE4 homozygous. This study demonstrated that the vervet monkey APOE4 carriers are associated with the major neuropathology of Alzheimer's disease, senile (amyloid) plaques, and neurofibrillary tangles.

Collectively, the investigation of APOE in mammalian species from Thailand suggests an association with increased genetic risk factor for Alzheimer's disease, coronary heart disease, and other neurodegenerative disorders. Importantly, these findings provide the primary genetic data to support the identification of APOE genotypes and further study on apolipoprotein E expression in prospective clinical settings, in particular by predicting the risk of neurodegenerative diseases in mammals.

5. Acknowledgement

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6. References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool. *Journal of Molecular Biology*, *215*, 403-410.

Belloy, M.E., Napolioni, V. & Greicius, M.D. (2019). A Quarter Century of APOE and Alzheimer's Disease: Progress to Date and the Path Forward. *Neuron*, *101* (5), 820-838.

Butterfield, D.A. & Halliwell, B. (2019). Oxidative stress, glucose dysmetabolism and Alzheimer disease. *Nature Reviews Neuroscience, 20* (3), 148-160.

Fainman, J., Eid, M.D., Ervin, F.R. & Palmour, R.M. (2007). A primate model for Alzheimer's disease: Investigation of the apolipoprotein E profile of the vervet monkey of St. Kitts. *American Journal of Medical Genetics Part B Neuropsychiatric Genetics, 144B* (6), 818-819.

Frieden, C. & Garai, K. (2019). Concerning the structure of apoE. *ProteiScience*, *22* (12), 1820-1825.

Frieden, C. & Garai, K. (2012). Structural differences between apoE3 and apoE4 may be useful in developing therapeutic agents for Alzheimer's disease. *Proceedings of the National Academy of Sciences 109 (23)* (pp. 8913-8918). United states of America.

Gearing, M., Rebeck, G.W., Hyman, B. T., Tigges, J. & Mirra. S.S. (1994). Neuropathology and Apoliprotein E Profile of Aged Chimpanzees: Implications for Alzheimer Disease. *Proceedings of the National Academy of Sciences 91(20)* (pp. 9382-9386). United states of America.

Herz, J. & Beffert, U. (2021). Apolipoprotein E receptors: linking brain development and Alzheimer's *disease*. *Nature Reviews Neurosciences, 1*(1), 51-58.

Huebbe, P. & Rimbach, G. (2021). Evolution of human apolipoprotein e (APOE) isoforms: Gene structure, protein function and interaction with dietary factors. *Ageing Research Reviews*, *37*, 146-161.

Jones, D.T., Taylor, W.R. & Thornton, J.M. (1992). The rapid generation of mutation data matrices from protein sequences. *Computer Applications in the Biosciences*, *8*, 275-282.

Kloske, C.M. & Wilcock, D.M. (2020). The important interface between apolipoproteinE and neuroinflammation in Alzheimer's disease. *Frontier in Immunology, 11*, 754.

Kumar, S., Stecher, G., Li M., Knyaz C. & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution, 35*, 1547-1549.

Lauderback, C., Kanski, M.J., Hackett, J.M., Maeda, N., Kindy, M.S. & Butterf, D.A. (2002). Apolipoprotein E modulates Alzheimer's Ab (1-42)-induced oxidative damage to synaptosomes in an allele-specific manner. *Brain Research*, *924*, 90-97.

McIntosh A.M., Bennett C., Dickson D., Anestis S.F., Watts D.P., Webster T.H., Babette, M.F. & Bradley, B.J. (2012). The apolipoprotein E (APOE) gene appears functionally monomorphic in chimpanzees (*Pan troglodytes*). *PLoS ONE*, *7* (10).

Muñoz, S.S., Garner, B. & Ooi, L. (2019). Understanding the Role of ApoE Fragments in Alzheimer's Disease. *Neurochemical Research*, *44* (6), 1297-1305.

Pedersen, W.A., Chan, S.L. & Mattson, M.P. (2019). A mechanism for the neuroprotective effect of apolipoprotein E: isoform-specific modification by the lipid peroxidation product 4-hydroxynonenal. *Journal of Neurochemistry*, *74* (4), 1426-33.

Qian, J., Betensky, R.A., Hyman, B.T. & Pozo, A.-S. (2021). Association of APOE Genotype With Heterogeneity of Cognitive Decline Rate in Alzheimer Disease. *Neurology*, *96*, e2414-e2428

Rech, P. (2008). *SIAS: Sequence identities and similarities*. http://imed.med.ucm.es/Tools/sias.html.

Safieh, M., Korczyn, A.D. & Michaelson, D.M. (2019). ApoE4: an emerging therapeutic target for Alzheimer's disease. *BMC Medicine*, *17*, 64.

Tachibana, M., Holm, M.-L., Liu, C.-C., Shinohara, M., Aikawa, T., Oue, H., Yamazaki,Y., Martens, Y.A., Murray, M.E., Sullivan, P.M., Weyer, K., Glerup, S., Dickson, D.W., Bu, G. & Takahisa Kanekiyo. (2019). APOE4-mediated amyloid-β pathology depends on its neuronal receptor LRP1. *The Journal of Clinical Investigation, 129* (3), 1272-1277.

Vasilevskaya, A., Taghdiri, F., Burke, C., Tarazi, A., Naeimi, S.A., Khodadadi, M,. Goswami, R., Sato, C., Grinberg, M., Moreno, D., Wennberg, R., Mikulis, D., Green, R., Colella, B., Davis, K.D., Rusjan, P., Houle, S., Tator, C., Rogaeva. E. & Tartagliaa, M.C. (2019). Interaction of APOE4 alleles and PET tau imaging in former contact sport athletes. *NeuroImage*: *Clinical*, *26*, 102212.

Yamazaki, Y., Zhao, N., Caulfield, T.R., Liu, C.C. & Bu, G. (2019). Apolipoprotein E and Alzheimer disease: pathobiology and targeting strategies. *Nature Reviews*, *15* (9), 501-518.

Yassine, H.N. & Finch, C.E. (2019). APOE Alleles and Diet in Brain Aging and Alzheimer's Disease. *Frontiers in Aging Neuroscience*, *10*, 12-150.