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Section 1. General Biology

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IDENTIFICATION OF NEW DIFFERENTIAL EXPRESSED GENES OF ESOPHAGEAL CANCER

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Abstract

Esophageal cancer (ESCA) is a type of gastrointestinal malignancy. It has one of the lowest survival rates among all types of cancer. Exploring ESCA related genes can help reveal the mechanism of ESCA occurrence and development and develop new diagnostic biomarkers and therapeutic targets. In the current study, three genes including ADCY3, CAB39L and TCOF1 were identified by bioinformatics analysis to show differential expression in ESCA and the adjacent normal tissues. Among the three genes, ADCY3 and TCOF1 were up-regulated, whereas CAB39L was down-regulated in the cancerous tissues. Survival analysis suggests that the expression levels of these genes have no impact on overall survival when analyzed individually. However, in combination analysis, patients with low expression of ADCY3 but high expression of TCOF1 show severe adverse outcomes with the lowest survival level. Therefore, the result suggests the combination of these genes could act as biomarkers for prognostic evaluation.

Keywords: *Esophageal cancer; ADCY3; CAB39L; TCOF1; differential expression*

1. Background

Esophageal cancer (ESCA) is a common gastrointestinal malignancy including *esophageal* squamous cell *carcinoma* (ESCC) and *esophageal* adenocarcinoma (EAC) (Watanabe, et al., 2020). ESCA affecting people from all over the world. There are many factors that will lead to ESCA. The known factors are people' gender (male are more likely to get ESCA than female), ages (older than 50 years old), ethnicity, past diseases (Gastro-esophageal Reflux Disease and obesity), and living habits (alcohol consumption, smoking, and diet) (Huang & Yu, 2018).

ESCA only has a 5-year survival rate of about 20% and can be ranged from 12 to 39% (Lewis & Lukovic, 2022). Currently, ESCA is primarily diagnosed during its advanced stages because of lack of early clinical symptoms and prognosis of ESCA for scientists to identified (Domper Arnal, et al., 2015). Therefore, the lack of prognosis and rising incidence of ESCA shows that detection and prediction methods for ESCA is vitally essential for

researchers to do, so as to improve the survival rate of ESCA patient (Huang & Yu, 2018).

Currently, scientists have already identified multiple genes in human body that are responsible for the cause and development of ESCA (Lewis & Lukovic, 2022). Studies have shown that genes that control the cell cycle are often overexpressed, such as CDKN2A, RB1, and NFE2L2 et al (somatic mutation in TP53) (Huang & Yu, 2018). Other factors leading to ESCA including epidermal growth factor receptor, receptor tyrosine kinase, epigenetic factors, and vascular endothelial growth factor signal pathway (Huang & Yu, 2018). There are still a large proportional of mutated genes and biological process in human body stay unknown for scientists. Identification of novel genes involved in ESCA occurrence and development will contribute to the development of new diagnostic biomarkers and therapeutic targets. Therefore, the current study aimed to find new genes which are not being previously published relating to ESCA, and three genes were successfully identified in the final.

2. Methods

2.1 Finding Differential Expressed Genes (DEGs)

The gene expression data of 23 patient cases of ESCA tissue and the paired adjacent normal tissues were obtained from Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo) with the accession number of GSE130078 (You et al., 2019). Differentially expressed genes (DEGs) were analyzed by the online tool GEO2R (Barrett, et al., 2013). Significantly up-regulated genes and down-regulated genes were selected to be DEGs with the cutoffs of |log2(foldchange)|>=1 & p value <= 0.05. The Cancer Genome Atlas (TCGA) esophageal carcinoma datasets were also used for the comparison between normal and cancerous gene expression of DEGs. The data of cancer and para-cancer expression of ESCA for genes were obtained from University of California Santa Cruz Xena (UCSC Xena) (Goldman, et al., 2020). Selected DEGs were obtained based on the intersection of GEO differentially expressed genes and TCGA esophageal carcinoma database meeting the result of statistical significance.

2.2 Functional Enrichment Analysis

A functional enrichment test was performed to DEGs by The Database for Annotation, Visualization, and Integrated Discovery (DAVID) Functional Annotation Bioinformatics Microarray Analysis (Sherman et al., 2022). DEGs associated with biological process (BP) of gene ontology (GO) and KEGG pathway categories having a p-value lower than 0.05 were selected.

2.3 Statistical analysis

Paired Student's t-tests were performed by GraphPad Prism v8.0 to compare the expressional difference between the paired cancerous and adjacent normal tissues of multiple individuals. Level of significance was set as $p < 0.05$.

2.4 Survival analysis

The UALCAN (University of ALabama at Birmingham Cancer) data analysis portal (https://ualcan.path.uab.edu/) was used for TCGA data analysis (Chandrashekar, et al., 2022). Genes in comparison that have a p-value lower than 0.05 are referred as having a statistically significance and genes in comparison that have a p-value lower than 0.01 are referred as having a highly statistically significance.

The effects of multiple gene combination of expression on patient survival were analyzed online using ToPP database (http://www.biostatistics.online/topp/index.php). Median was used to group gene expressions.

3. Results

3.1 Result of Differential Expressed Genes

The aim of the current study is to discover new genes associated with the development of ESCA. Firstly, I identified the interested ESCA datasets from the GEO database and used the online tool GEO2R to identify differentially expressed genes (DEGs) between the cancerous and the adjacent normal tissues. Totally, 182 DEGs, including 112 up-regulated and 70 down-regulated DEGs were successfully identified. Because the DEGs were derived only from one dataset, in order to increase the reliability, these genes were further checked using another dataset from the TCGA project. Therefore, the intersected up- and down-regulated genes were identified.

Totally, 78 genes including 70 up-regulated and 8 down-regulated genes, which had a statistically significance in both datasets, were successfully identified (Table 1).

Table 1. – *Differentially expressed genes of ESCA supported by both GEO and TCGA datasets* *

Type	Gene	Log2 (fold-change)	P value (GEO)	P value (TCGA)	Type	Gene	Log2 (fold-change)	P value (GEO)	P value (TCGA)
Up	INHBA	3.12	7.06E-46	7.88E-05	Up	DNMT3B	1.75	1.11E-25	1.62E-12
Up	SERPINE1	2.51	4.16E-43	2.21E-07	Up	HOMER3	5.111	1.18e-25	2.00E-04
Up	STC2	2.485	1.27E-41	5.60E-11	Up	ADAM12	3.082	1.62E-25	2.11E-15
Up	MAPK12	2.711	2.83E-40	2.70E-09	Up	CHN1	2.715	2.06E-25	3.40E-02
Up	SNX10	3.41	2.26E-38	1.62E-12	Up	PPFIA1	2.815	8.45E-25	1.62E-12
Up	FADS1	1.781	7.00E-38	2.01E-02	Up	FADS2	2.214	1.50E-24	2.34E-02
Up	SPP1	1.522	1.24E-37	1.45E-10	Up	DNM1	3.536	1.63E-24	5.64E-03
Up	MMP3	1.762	2.93E-36	1.58E-06	Up	GPR176	2.067	2.15E-24	2.78E-12
Up	PLAU	3.739	7.36e-36	1.62E-12	Up	COL5A2	2.217	3.47E-24	4.95E-04
Up	C10ORF55	2.582	6.41E-34	1.63E-12	Up	CD276	4.029	3.63E-24	1.35E-06
Up	TUBB3	1.857	6.37E-34	5.76E-03	Up	HOXD10	3.591	4.06E-24	1.11E-16
Up	LRP12	2.614	4.15E-33	2.78E-04	Up	SERPINH1	4.046	4.06E-24	3.55E-05
Up	PLXNA1	2.281	1.82E-32	2.56E-06	Up	ZFP64	1.925	4.60E-24	9.48E-08
Up	B4GALNT1	1.664	2.24E-32	3.38E-09	Up	TCOF1	2.541	5.59E-24	2.13E-08
Up	IL24	2.443	6.43E-32	5.84E-05	Up	STIL	4.401	7.33E-24	1.62E-12
Up	COL1A1	1.945	7.68E-31	2.39E-07	Up	MFAP2	2.399	9.32E-24	3.61E-13
Up	TGFBI	2.037	1.35E-30	6.20E-08	Up	APLN	4.296	1.70E-23	8.67E-09
Up	BCL7A	2.315	3.99E-30	9.37E-08	Up	TDO2	4.206	2.01E-23	1.67E-12
Up	UCHL1	3.301	6.66E-30	3.83E-04	Up	CHST1	2.215	2.62E-23	2.01E-11
Up	ARTN	5.896	1.42E-29	4.21E-02	Up	HOXC13	5.05	3.62E-23	3.73E-09
Up	BGN	1.741	3.29E-29	2.98E-02	Up	PGF	2.699	6.38E-23	3.16E-08
Up	STK3	1.165	4.01E-29	3.62E-04	Up	COL1A2	4.419	4.40E-22	1.76E-02
Up	FADS3	1.623	8.73E-29	2.36E-03	Up	ANO1	2.264	5.51E-22	7.47E-09
Up	TNC	1.837	1.16E-28	7.93E-08	Up	MAD2L2	2.376	6.00E-22	1.54E-12
Up	ADAMTS12	1.672	1.41E-28	5.21E-12	Up	SH2D5	2.193	6.07E-22	1.80E-03
Up	MMP13	3.435	1.81E-28	1.42E-04	Up	PYCR2	4.741	6.34E-22	5.44E-11
Up	MMP11	4.269	4.28E-28	1.62E-12	Up	CTHRC1	4.347	2.25E-30	<1E-12
Up	LAMC2	4.48	6.00E-28	6.99E-15	Up	CDC25B	2.392	4.16E-37	<1E-12
Up	MMP10	2.501	7.07E-28	2.13E-05	Up	MCM2	2.523	3.52E-27	<1E-12
Up	OSM	2.703	7.30E-28	2.20E-06	Up	KIF26B	3.511	2.60E-24	<1E-12
Up	ADCY3	5.062	9.28E-28	1.14E-02	Up	ECT2	2.516	1.17E-23	<1E-12
Up	FAP	1.801	1.25E-27	2.48E-03	Down	KAT2B	-7.185	1.49E-41	1.02E-02
Up	FCGR2A	1.893	2.94E-27	1.36E-04	Down	UBL3	-5.361	4.54E-38	1.43E-04
Up	UAP1L1	2.312	3.40E-27	1.63E-12	Down	NUCB2	-4.728	1.76E-34	2.23E-02
Up	TNFAIP6	3.426	6.36E-27	2.90E-02	Down	CAB39L	-4.833	1.10E-31	1.65E-02
Up	ABL2	1.846	1.43E-26	2.98E-02	Down	PAIP2B	-6.092	5.96E-24	3.30E-03

Type	Gene	Log2 (fold-change)	P value (GEO)	P value (TCGA)	Type	Gene	Log2 (fold-change)	P value (GEO)	P value (TCGA)
Up	SHOX2	2.895	1.93E-26	1.51E-08	Down	GCOM1	-2.019	2.90E-23	1.39E-02
Up	MMP12	1.612	2.25E-26	1.70E-10	Down	HPGD	-2.293	6.77E-23	3.03E-02
Up	LIMK1	2.181	3.68E-26	7.53E-10	Down	LGALS3	-6.284	2.42E-22	1.37E-02

** The columns of 'Type' indicates up-regulated (Up) and down-regulated (Down) gene expression. The column 'Log2(foldchange)' indicates the result of log2(mean(cancerous group)/ mean(adjacent normal group)) from the GEO dataset. The p values indicate the statistical significance of t-tests in the GEO and TCGA datasets, respectively*

3.2 Result of Functional Enrichment Analysis

To investigate what functions, such as biological processes (BPs) based on GO (Gene Ontology) and signaling pathway based on KEGG (Kyoto Encyclopedia of Genes and Genomes), these DEGs were mainly involved in, I performed a functional enrichment analysis. As shown in Table 2, then significantly enriched functions included extracellular matrix organization, collagen fibril organization, cell adhesion and signaling transduction, and so on. Several KEGG pathway was also significantly enriched in the DEGs, such as extracellular matrix (ECM)-receptor interaction, focal adhesion and PI3K-Akt signaling pathway, and so on (Table 2).

Table 2. – *Result of DAVID functional enrichment analysis*

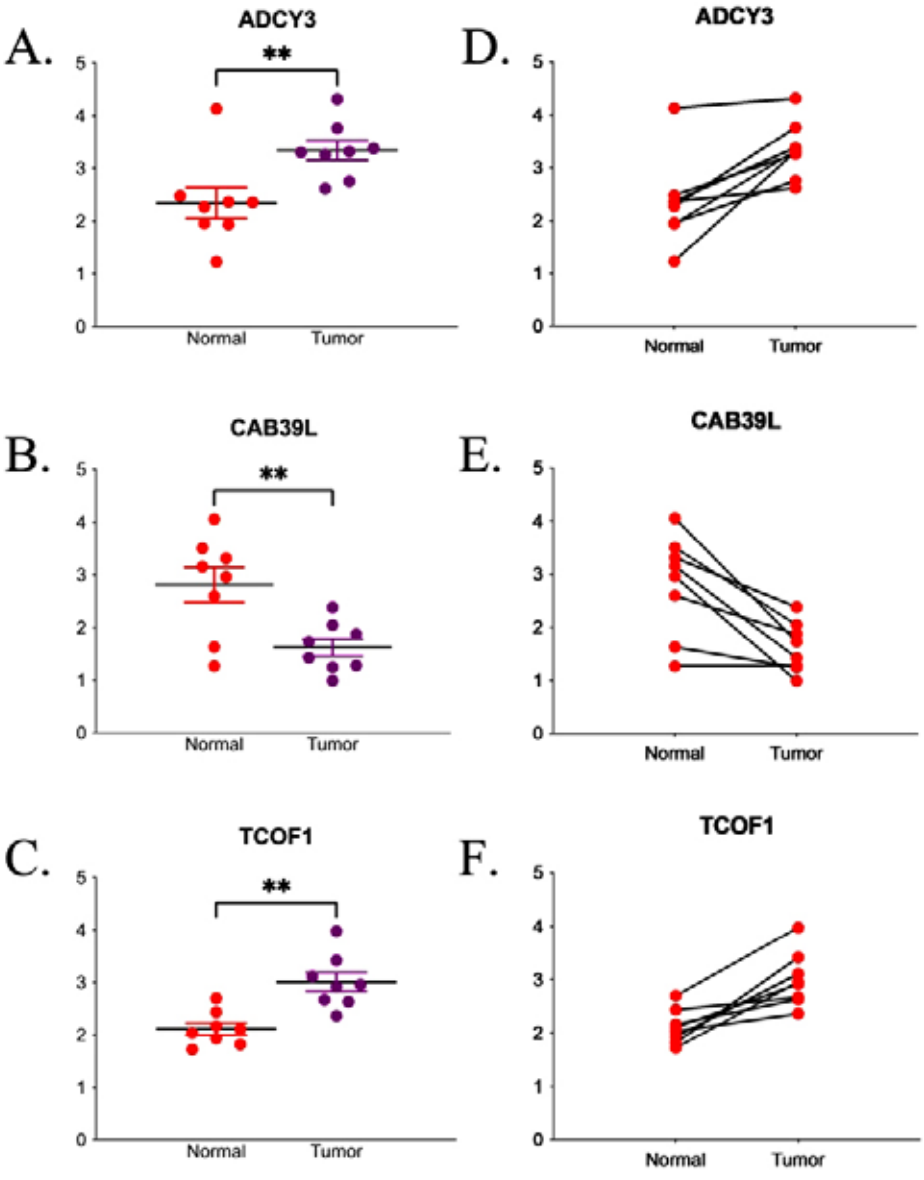
Term	P value	Gene
GO:0030198~extracellular matrix organization	3.62E-08	MMP12, COL1A1, MMP11, MMP13, COL1A2, COL5A2, MMP3, TGFBI, ADAMTS12, MMP10
GO:0030199~collagen fibril organization	1.05E-05	COL1A1, MMP11, COL1A2, COL5A2, SERPINH1, ADAMTS12
GO:0030574~collagen catabolic process	1.77E-05	MMP12, MMP11, MMP13, MMP3, MMP10
GO:0022617~extracellular matrix disassembly	2.81E-05	MMP12, MMP11, MMP13, MMP3, MMP10
GO:0006508~proteolysis	2.01E-04	MMP12, MMP11, MMP13, FAP, PLAU, ADAM12, MMP3, ADAMTS12, MMP10
GO:0007165~signal transduction	3.05E-04	TNFAIP6, IL24, LIMK1, ADCY3, PGF, MAPK12, STK3, APLN, PLAU, ARTN, CHN1, SPP1, PPFIA1, ABL2, LRP12
GO:1904645~response to beta-amyloid	1.27E-03	MMP12, MMP13, MMP3
GO:0007155~cell adhesion	1.34E-03	COL1A1, TNFAIP6, FAP, ADAM12, SPP1, TNC, ABL2, LAMC2, TGFBI
GO:0001649~osteoblast differentiation	1.41E-03	COL1A1, SHOX2, SPP1, TNC, CTHRC1
GO:0006636~unsaturated fatty acid biosynthetic process	1.46E-03	FADS3, FADS2, FADS1
GO:0001501~skeletal system development	1.72E-03	COL1A1, COL1A2, TCOF1, COL5A2, SHOX2
GO:0035556~intracellular signal transduction	8.09E-03	CAB39L, CHN1, ADCY3, ECT2, MAPK12, STK3, GCOM1

Term	P value	Gene
hsa04512: ECM-receptor interaction	1.12E-03	COL1A1, COL1A2, SPP1, TNC, LAMC2
hsa04611: Platelet activation	3.79E-03	COL1A1, FCGR2A, COL1A2, ADCY3, MAPK12
hsa04510: Focal adhesion	3.83E-03	COL1A1, COL1A2, SPP1, TNC, LAMC2, PGF
hsa04926: Relaxin signaling pathway	4.36E-03	COL1A1, MMP13, COL1A2, ADCY3, MAPK12
hsa04151: PI3K-Akt signaling pathway	9.44E-03	COL1A1, COL1A2, OSM, SPP1, TNC, LAMC2, PGF

3.3 Paired cancer and para-cancer analysis reveals new ESCA related genes

The GEO dataset mentioned above includes 46 paired cancerous and non-cancerous (or para-cancerous) tissues from 23 patients, whereas the TCGA dataset was derived from cancerous tissues of 184 samples and only 11 non-cancerous tissues of 11 samples.

Figure 1. *Differential expression of the three genes between the normal and cancerous tissues of ESCA*



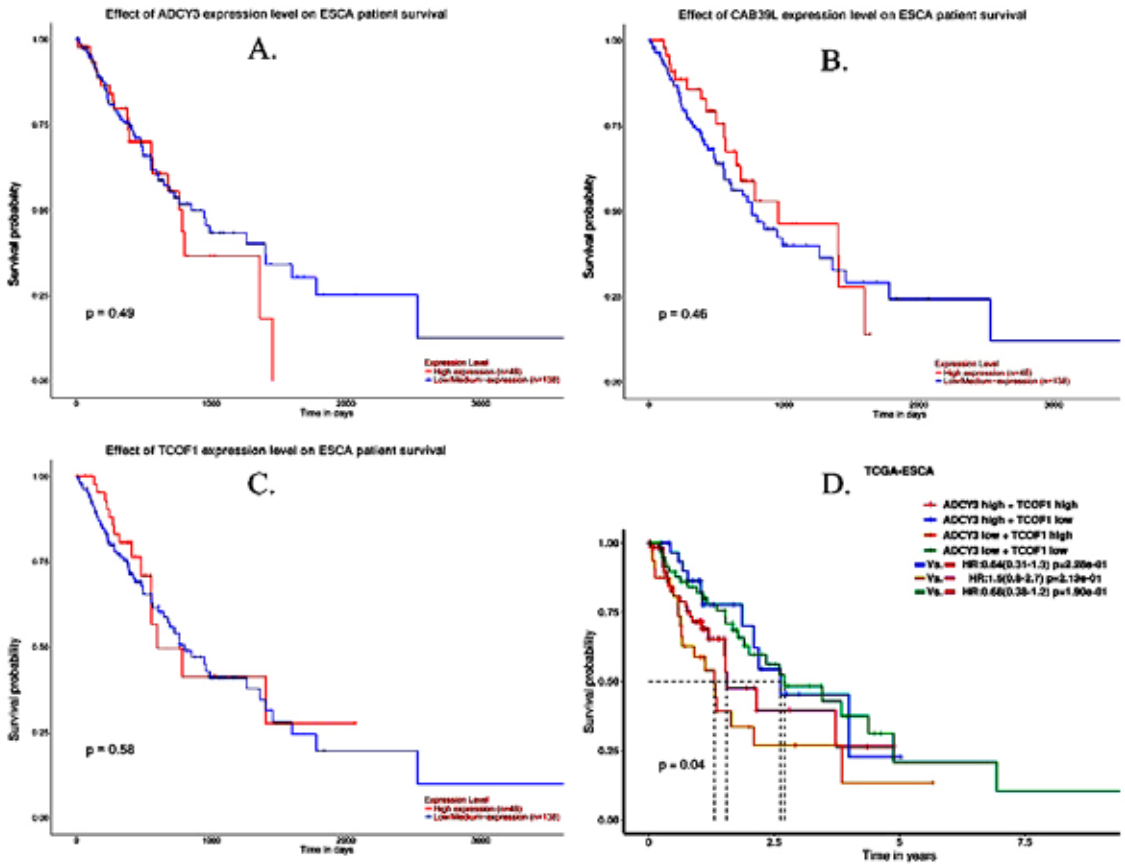
For the TCGA dataset, there were a total of 16 paired cancerous and non-cancerous tissues from 8 patients. Therefore, the total 42 genes involved in the enriched functions in Table 2 were further checked for the differential expression using the paired TCGA data. As a result, 32 genes were found to be significantly different comparing esophageal cancerous and the adjacent normal gene expression after *t*-test on the 42 genes we got from functional enrichment analysis. Between 32 genes, three new genes including adenylate cyclase 3 (ADCY3), calcium-binding protein 39 (CAB39L), and treacle ribosome biogenesis factor 1 (TCOF1) have not been published related to ESCA. Therefore, we focused on the three genes for the next analysis. Among the genes, ADCY3 and TCOF1 were up-regulated, whereas CAB39L was down-regulated (Figure 1).

The x-axes indicate groups of categories, while the y-axes indicate relative expression levels. The stars indicate statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Differential expression of three genes including ADCY3 (A, D), CAB39L (B, E), TCOF1 (C, F) between the normal and cancerous tissues of each individual ESCA patients are shown. The lines in D, E and F indicate the paired cancerous and adjacent normal tissues of the same patients.

3.4 Survival Analysis

Next, I investigated the clinical significance of the three genes and performed the survival analysis based on the overall survival (OS) of patients with different expression levels of the genes. The survival analysis model was built on those three genes (ADCY3, CAB39L, TCOF1) individually at first (Figure 2). However, there was no impact of individual gene on the survival probability (Figure 2, A, B & C). Therefore, the survival probability of combinations of pairing those three genes with each other was analyzed using the online ToPP server. The results revealed that the OS based on the combination of ADCY3 and TCOF1 showed a statistical significance (p-value lower than 0.05) in the survival probability impact on ESCA (Figure 2, D). However, other combinations did not show any significant difference of OS among different combined groups (data not shown).

Figure 2. *Survival probability model for the indicated three genes*



The results of survival analysis based on the single gene of ADCY3 (A), CAB39L (B) and TCOF1 (C), as well as the combination of ADCY3 and TCOF1, are shown. Combination of ADCY3 and TCOF1 (D) is the Survival probability model for the combination of expression of ADCY3 and TCOF1 over 2.5-, 5-, 7.5-, 10-years.

4. Discussion

Three new genes (ADCY3, CAB39L, TCOF1) associated with development ESCA are identified in the research. Based on current study, ADCY3 is associated with obesity (Saeed, et al., 2018). Mutated ADCY3 will increase the risk of type two diabetes and mutated ADCY3 is a type of genetic disease (Grarup, et al., 2018). According to previous studies, CAB39L often possessed diagnostic and prognostic values and tumor suppressor in several types of cancer, including kidney renal clear cell carcinoma (KIRC), gastric cancer (GC), and colorectal cancer (CRC) (Wu, et al., 2023; Li et al., 2018; Choi, et al., 2016). Existing studies shows that TCOF1 is a nucleolar factor regulated ribosomal DNA (rDNA) transcription in nucleolus (Wu, et al., 2022). TCOF1 can leads to disorder like Treacher Collins Syndrome (TCS), hepatocellular carcinoma (HCC) (Marszałek-Kruk, et al., 2021).

Though three new genes may contribute to the development of esophageal tumor, there is potential drawback in my research. In my study, the simple size of my experiment is not very big, though two different datasets were used. In addition, there is inadequate in research in my experiment. The current analysis is mainly based on bioinformatics analysis, the specific molecular mechanism of the three genes needs further investigation. Moreover, differential expression can be detected at protein level using clinical samples to confirm the DEGs identified at mRNA level.

5. Conclusions

Three new DEGs (ADCY3, CAB39L, TCOF1) of ESCA were identified, providing novel insight for potential mechanism of the oncogenesis and development of ESCA. They could be served as candidates for drug targets and/or biomarker for prognostic analysis and provide new attention for medical workers to make better clinical decisions, treatment strategies for ESCA patients and for researcher to conduct further experiment.

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Section 2. Life science

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INCIDENCE AND PREVALENCE OF GLAUCOMA IN GEORGIA

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Abstract

Glaucoma is the leading cause of irreversible blindness worldwide and is associated with a reduced quality of life. Patients with arterial hypertension, diabetes, family history, age more than 50 years are at an increased risk of glaucoma.

The current number of persons (aged 40–80 years) with glaucoma worldwide is 64.3 million and is expected to increase to 111.8 million in 2040. Asia accounts for the largest number of glaucoma cases despite having the lower prevalence of glaucoma.

The aim of our investigation was to study the incidence and prevalence of Glaucoma in Georgia, its specific share compared to other eye diseases. The obtained epidemiologic data were adjusted by age, sex and regions.

Keywords: *glaucoma, incidence, prevalence, epidemiology*

Introduction

Glaucoma is one of the serious, chronic, multifocal eye diseases, which is characterized by constant or periodic increase of intraocular pressure, characteristic changes in the field of vision **(**Tham, et al, 2014; Thomas, et al, 2015**).** Typically glaucoma remains asymptomatic until very severe. During glaucoma, damage to the optic disc and retinal ganglion cells, characteristic of glaucoma, develops – glaucomatous optic neuropathy. These changes lead to vision loss and even to complete blindness. Glaucoma is the leading cause of irreversible blindness worldwide. Most common type of Glaucoma is Primary Open Angle Glaucoma (POAG)

Risk factors

Among the risk factors, I would single out the family history, the genetic factor and predisposition are very important, which increases the risk of developing this disease tenfold. Around half of all primary OAG patients have a positive family history, and their first degree relatives (parents, siblings or children) have an approximately 9-fold increased risk of developing glaucoma (Awadalla, et al., 2015).

Risk factors include age and frailty, gender, myopia, genetics, smoking, race, systemic hypertension and hypertension, vasospasm, use of systemic or topical steroids,

migraine, obstructive sleep apnea syndrome, and most significantly, increased IOP (McMonnies, CW, 2017).

Other risk factors also include: eye trauma, narrow front cell, other eye diseases, pseudoexfoliation, etc.

Statistics

Staropoli et al. (2021) indicated that 2.4 million people in the United States alone are at risk of glaucoma, and worldwide the number of glaucoma patients will increase to 111 million by 2040. According to WHO estimates, by 2010 glaucoma accounts for 8% of global blindness, its number is increasing, by 2020 it reached – 13% (Staropoli, et al., 2021). In the United States, blindness is the third most dangerous health problem, after cancer and heart attack.

The prevalence of glaucoma increases with age and can thus be associated with age-related diseases like macular degeneration, vascular diseases, and obstructive sleep apnea. POAG is strongly correlated with age: its prevalence is highest among older Hispanic or Latino (18%), black (15%), white (7%), and Asian individuals (5%). Finally, there are gender differences in glaucoma as well. There was a reported 36% higher prevalence of glaucoma in males than females (Mc Monnies, 2017).

Prevalence of Glaucoma

2015 Nigeria Blindness Survey is so far the largest national population-based survey of eye disease in an ethnically diverse, indigenous black African population. They have described precise estimates of the prevalence of glaucoma in this region. The study sample was nationally representative by age, gender, ethnicity, rural/urban residence, and socioeconomic status with a high response rate. The results were generalizable to the whole country and people of the West African diaspora around the world (Kyari, et al., 2015).

The most common type of glaucoma in the UK is POAG, affecting 2% of individuals older than 40 years and 10% of individuals older than 75 years, particularly African-Caribbean population; PACG is not as prevalent and only affects 0.17% of individuals younger than 40 years, particularly East Asians (Imrie, C., Tatham, A.J., 2016). 7.8 million Persons were affected by POAG in Europe and the total prevalence is 2.51% (Allison, et al., 2020).

Prevalence of Glaucoma in Georgian

We collected the data from National Center For Disease Control and Public Health of Georgia.

For both sexes crude rate of the glaucoma incidence per 100,000 person was 61.2; age-standardized rate (ASR) – 35.6; age-adjusted rate (AAR) – 61.2; cumulative risk up to 69 years (CR0+69) – 1.44; cumulative risk up to 79 years (CR0+79) – 2.86; cumulative risk above 80 years (CR0+80) – 3.93. As we can see it from Figure 1 incidence of Glaucoma is rising depending on the age.

Figure 1. *The age-specific incidence of glaucoma for both sexes in Georgia, 2020–2022*

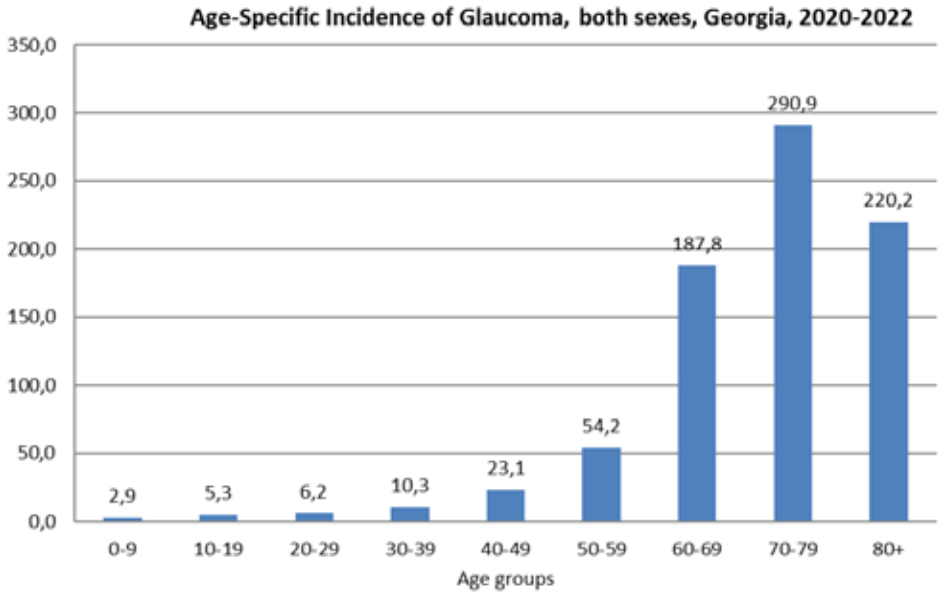
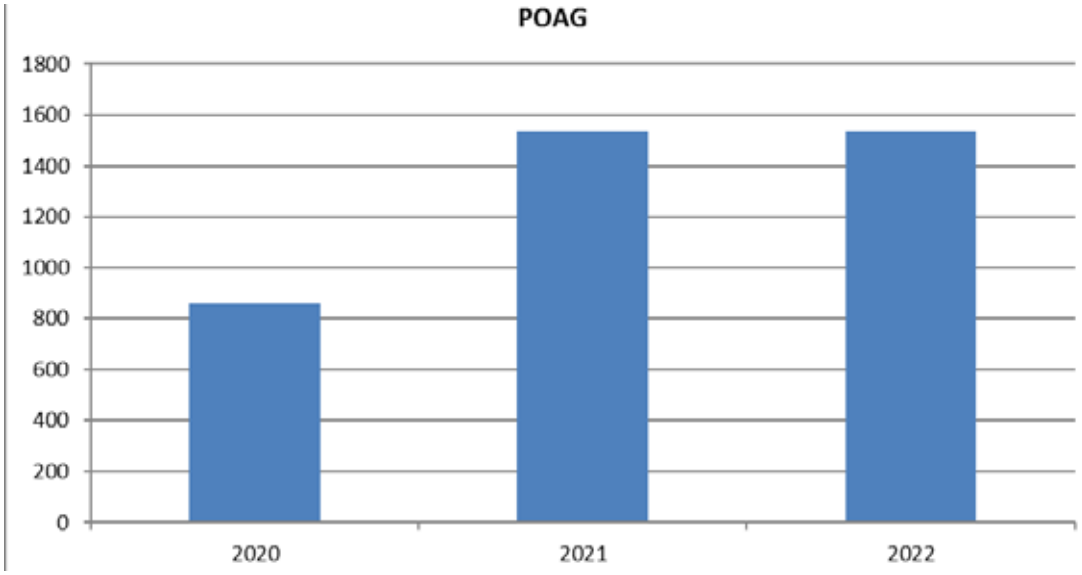


Figure 2. *The incidence of POAG for both sexes in Georgia, 2020–2022*



POAG is most common type of glaucoma in Georgia. As we can see it from Figure 2, from 2020 it was rising from 861 cases to 1536. It was as high as 1536 in 2022, the same as in 2021.

As we can see it from the Figure 3 the incidence of Glaucoma was higher in Males then in Females and it was rising with the age. Crude rate per 100,000 person at the age of 50–59 there were 41.7 cases in female population, and 68.1 cases in male population. In the age of 60–69 this number is 3.5-fold more compared to the previous age (50–59) in both sexes. The amount of glaucoma cases continued to increase in the next age group 70–79; glaucoma cases per 100,000 population in female population increased 1.4-times, and for male population it was higher by 1.7 times in age group 70–79 then in age group 60–69. As for the next age group, the amount of glaucoma was decreased as the population of this age group itself was also decreased.

The share of Glaucoma in Georgia for both sexes in 2007 was 10.0% out of all eye diseases, in 2013 it was the highest – 10.4% of all eye diseases and in 2022 it was dropped to 4.0% (see Figure 4).

Figure 3. *Age- and sex-specific distribution of patients (per 100,000 person) with Glaucoma in Georgia 2020–2022*

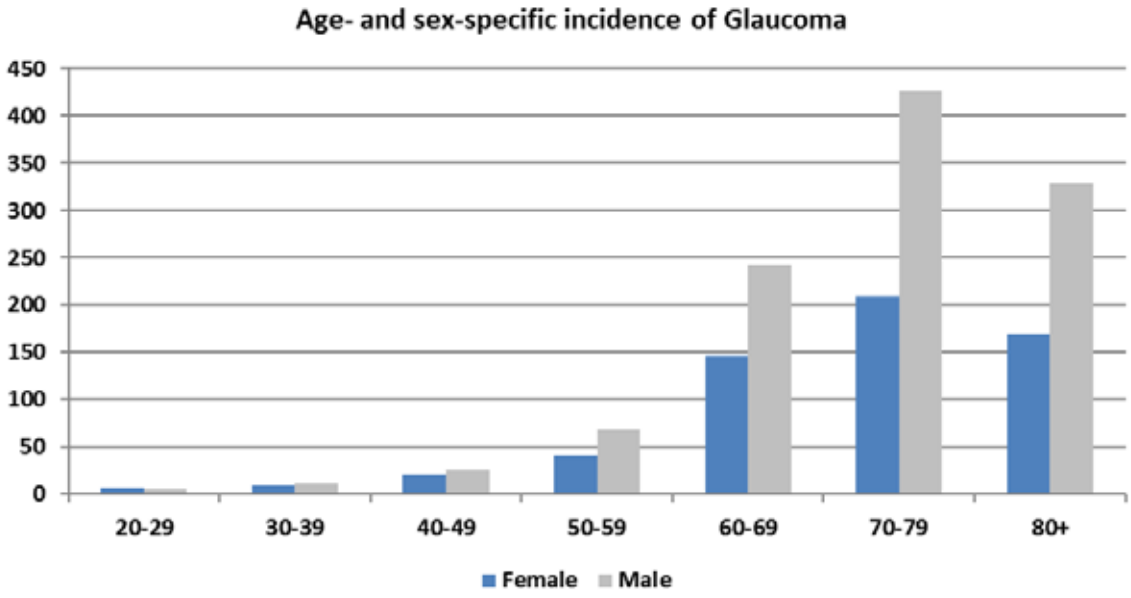
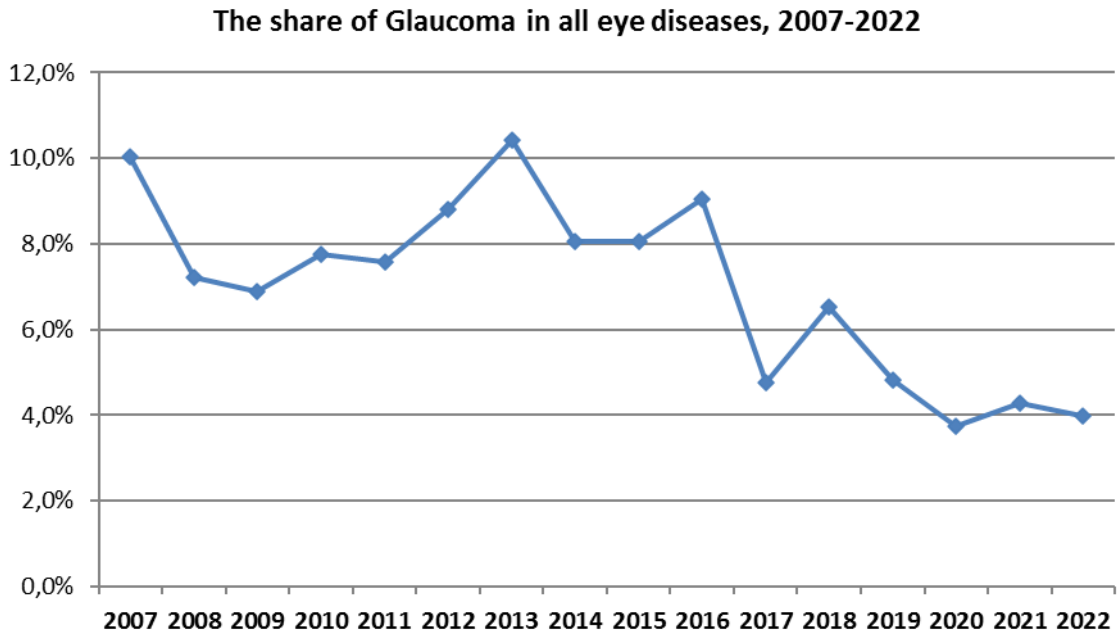


Figure 4. *The share of Glaucoma in all eye diseases, both sexes, Georgia, 2007–2022.*



Discussion

Prevention of blindness caused by glaucoma

Despite modern advances in medicine, glaucoma remains a major public health problem worldwide.

For the prevention of glaucoma, it is necessary to prevent it, which means timely screening visits to an ophthalmologist.

It is also a very noteworthy fact that the awareness of glaucoma is quite low. The data of studies performed in developed countries showed that the awareness of glaucoma reaches 50%; while in developing countries 90% of population did not know that they are sick with glaucoma. One of the reasons for this fact is that the disease goes almost silently to the late stage and the patient does not even realize that he is losing his sight (Allison, et al., 2020).

Since glaucoma is one of the leading causes of vision loss, glaucoma can affect many aspects, first of all, the quality of life of the patients and disrupt the ability to perform such daily activities as reading, walking, driving, moving.

The main goal of glaucoma treatment and management is to preserve the patient's vision and improve the quality of life.

Delayed diagnosis was associated with an increased risk of glaucoma-related disability and significantly reduced the quality of life.

Conclusion

The incidence of Glaucoma in Georgia is higher in Male then in Female and it is rising with the age. In the age of 60–69 number of glaucoma cases was 3.5 times more compared to the previous age (50–59) in both sexes. The amount of glaucoma cases continues to increase in the next age group 70–79. The study was conducted with data from2007 to 2022 which was accompanied by changes in the health care system and the Covid-19 pandemic, so it requires the further assessment.

Recommendations

It is necessary to make global changes to reduce the incidence of glaucoma and stop it from being a bigger public health threat. Some recommendations include the regular glaucoma screenings, and the stronger education. People with a high-risk factor for glaucoma – a family history of glaucoma – should be educated about it and screened for it as soon as possible.

Glaucoma awareness needs to be raised. January is Glaucoma Awareness Month. Public awareness campaigns, whether seminars, flyers, websites or posters, can reach the public and show them the importance of early diagnosis and early treatment to prevent blindness from glaucoma.

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Section 3. Pharmaceutical science

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A COMPUTATIONAL STUDY ON THE EFFICACY OF SMALL MOLECULES AS DUAL INHIBITORS FOR β-SECRETASE 1 AND ACETYLCHOLINESTERASE AS ALZHEIMER'S DISEASE THERAPEUTICS

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Abstract

Alzheimer's disease is characterized by the progressive decline of many cognitive functions that involve numerous parts of the brain. Due to the complex nature and multi-faceted pathogenesis of neurodegenerative diseases, AD's pathology has been correlated with a loss of cholinergic function as well as the overproduction of Aβ42. Because of this, multitargeted ligands have great potential as therapeutics. In this paper, we assessed the "bindability" of the proteins using various computational methods and online software. Then, we virtually screened through millions of potential small molecules using pharmacophore maps to find potential small molecule candidates. We then used software developed by the Swiss Institute of Bioinformatics (SIB) to both assess the binding energy of the small molecules to the compound (of which all compounds had a $\Delta G < -7.00$ kcal/mol) as well as assess the druggability of the small compounds through their ADME profiles. By the end, we were left with 4 organic compounds that showed the most promise as dual inhibitors. Ranked from most to least promising, they are: ZINC68569271, ZINC41367268, ZINC67202317, ZINC05611209. They all show strong binding affinities for both AChE and BACE1, with the majority of each compound having a Gibbs free energy value of $\Delta G < -7.00$ kcal/mol.

Keywords: *Alzheimer's Disease, Acetylcholinesterase, β-secretase 1, Molecular Docking, ADME*

1. Introduction

1.1. Alzheimer's disease

Alzheimer's disease (AD) is characterized by the progressive onset of cognitive, functional, and behavioral impairment. Currently, AD is the sixth leading cause of death and is one of the only top ten causes of deaths that is continually increasing in prevalence (Atri, 2019). AD is fatal due to a multitude of reasons, however one of the biggest prob-

lems with AD is identifying its existence. Unlike other diseases, AD's symptoms can often be attributed to regular aging. This causes symptoms to be left undiagnosed, untreated, and ignored, losing valuable time, and causing harmful delays in receiving appropriate therapeutics. For example, while lapses in memory and judgment are frequent symptoms among aging, complete irreversible loss of memory is a common side effect of AD (Atri, 2019). This demonstrates how AD often accentuates the symptoms of normal aging, often making it more severe.

As it stands right now it is estimated that by 2030, the number of Americans with AD would increase by 35%, with the potential to skyrocket up to tripling in prevalence by 2050. Current projections and estimates show that risk of dementia doubles roughly every 5 years from the age of 65 to 85. At 65 years old, the risk of developing AD is roughly 1% – 2%; by 85, the risk of developing neurodegenerative disease is 30% – 50% (Atri, 2019; Alzheimer's association, 2019).

1.2. AD Risk factors

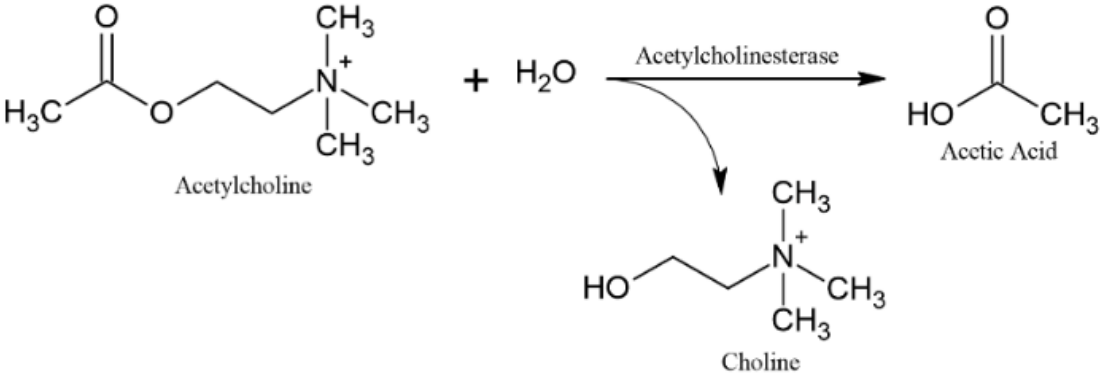
Unlike other diseases, AD is thought to be multi-faceted, meaning its pathogenesis requires a combination of factors. So far, the greatest known risk factor for AD is age, followed closely by family history and one's genotype (Alzheimer's disease, 2019). Previous twin and family studies indicate that genetic factors play a major role in roughly 80% of AD cases (Tanzi, R. E., 2012; Gatz, M. et al., 2006).

There are two categories of genes that can influence the pathogenesis of a disease: risk genes and deterministic genes. A risk gene simply increases the risk of developing a disease, while a deterministic gene all but guarantees the development of a disease. Rare mutations in the APP, PSEN1 and PSEN2 genes have been shown to be deterministic genes for AD, whereas common gene polymorphisms for the APOE gene such as ε4 and ε2 have been shown to influence but not cause the pathogenesis of AD (Tanzi, R. E., 2012).

1.3. Acetylcholine/Acetylcholinesterase

Neurons are the fundamental component of communication within an organic system. They communicate through electrochemical signals that are driven by charged particles. Communication occurs at tiny gaps between the neuron receptors called synapses, where the presynaptic neuron sends a neurotransmitter to the postsynaptic neuron which binds to receptor proteins on its surface (Lovinger, D. M., 2008). One of the most important neurotransmitters is called acetylcholine (ACh) (Kondziella, D., 2016), which is associated with AD and is one of the two proteins of focus of this study. Though AD has no cure, therapeutics can help delay symptoms or even completely halt the onset of dementia, one of the many symptoms of AD. Currently, the main class of drugs used to treat AD are acetylcholinesterase / cholinesterase inhibitors (ChEIs).

Figure 1. *Catalyzed hydrolysis of ACh into HAc and Choline (Cavalcante, et al., 2020; Trang, A. et al., 2020)*



Maintaining a healthy equilibrium of neurotransmitter systems such as ACh is essential to cognitive function. Although the cholinergic nervous system isn't the only neurological system affected by cognitive disorders such as AD, a lacking cholinergic

system has been attributed to the progressive impairment in learning and memory (Marucci, G. et al., 2020). For example, a premature loss of cholinergic neurons in the *basal forebrain* was observed for those afflicted with AD. Acetylcholinesterase is an enzyme with the capability to catalyze the hydrolysis of acetylcholine into acetate and choline (see fig. 1), thus reducing its concentration at the synapse and consequently, its ability to help conduct a synapse (Trang, A. et al., 2020).

1.4. β-secretase

β-secretase (BACE1) is responsible for the aggregation of amyloid-β (Aβ) and is the second of the two proteins that we are studying (Ma, W.-H. et al., 2021). BACE1 is responsible for the aggregation of $A\beta_{42}$, which has been correlated to increased toxicity in AD (Hampel, H., 2020). BACE1 has been measured in higher concentration and heightened activity in AD patients' brains and body fluids; corroborating the idea that BACE1 plays a critical role in the pathogenesis of AD (Hampel, H., 2020; Breijyeh, Z et al., 2020;).

Familial AD (FAD) cases are linked to mutations in the gene for amyloid precursor protein (APP) production. Patients who exhibit "increased dosage" of the APP gene, which is located on chromosome 21, are at higher risk to develop early-onset AD and overproduce the $A\beta_{42}$ peptide (Vassar, R. et al., 2009). Thus, the BACE1 enzyme is a prime drug target to develop inhibitors that inhibit the amyloid aggregation of $A\beta_{42}$.

1.5. Tau and Amyloid Aggregation

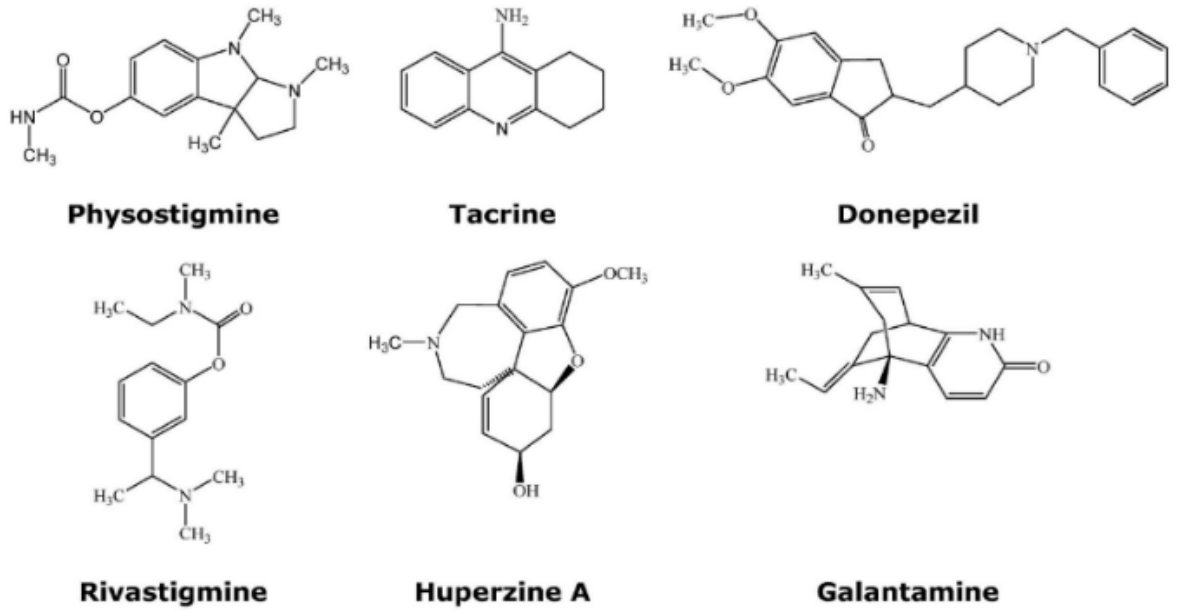
Another field of AD therapeutic research is the inhibition of tau and amyloid aggregation. Amyloid aggregation is the aggregation of the protein called amyloid in organs which can cause organ failure. As mentioned before, patients with FAD have a genetic mutation on their 21st chromosome which allows the overproduction of $A\beta_{42}$ (Ma W.-H. et al., 2021; Hardy, J., 2006; Breijyeh, Z et al., 2020;).

Under normal physiological conditions, the tau (τ) protein monitors and regulates the structural stability of microtubules and microfibers. However, in a diseased brain such as an Alzheimer's brain, tau becomes abnormally hyperphosphorylated. Then, the tau proteins aggregate together to form paired helical filaments, which then form neurofibrillary tangles which get in the way of the nervous system (Medeiros, R. et al.,)

1.6. FDA-approved therapeutics:

AChE has proven itself to be a viable target for therapeutic drugs and symptomatic improvement against the development of AD. Inhibition of AChE has real clinical results because of its proven efficacy as a treatment for previous ailments such as myasthenia gravis (Mehta M. et al., 2012).

Figure 2. *Chemical diagrams of known AChEIs. Note physostigmine is no longer in use (Marucci, G. et al., 2020)*



Currently, AChEIs are the main class of drugs to treat AD, with other drugs making it onto the list (Marucci, G. et al., 2020). Currently, the FDA-approved drugs available to treat AD are galantamine, rivastigmine, donepezil, and memantine (Medications for Memory, Cognition and Dementia-Related Behaviors, 2021). Note that while the first three drugs are AChEIs, memantine is not, rather than inhibiting acetylcholinesterase, it inhibits glutamate, a neurotransmitter that brains which are afflicted with AD produce too much of; which can cause harmful side effects (NHS, 2021).

1.7. Dual inhibitors of BACE1 and AChE

Both the amyloidal aggregation caused by the BACE1 protein and lack of cholinergic function caused by AChE has made research into finding dual inhibitors for both enzymes a hot spot for scientists in the field. This process of targeting two or more biomolecular targets with a single molecule is called the "multi-target directed ligands" (MTDL) process.

The MTDL approach is more well-received when attempting to therapize patients suffering from multi-faceted diseases such as AD. Reportedly, the MTDL approach is suggested to have seen better efficacy and safety compared to the original "STDL" approach (Stern, N. et al., 2022). Previous works by researchers discovered only 107 already FDA-approved drugs that can bind to AChE. Of those 107 drugs, 33 of them can also bind to BACE1 (Stern, N. et al., 2022).

2. Methods

2.1. Preparation of screening process and tools

Proteins and enzymes operate via binding sites; to properly inhibit their function, small molecules must preferentially bond to these binding sites to obstruct other objects from doing so. The approach we will use for this computational study is first, we identify readily available binding sites on both enzymes using freely available software online. Next, we aim to utilize pharmacophore maps to map out the interaction between our preferred enzyme and a receptor as the base pharmacophore map to compare other compounds too.

2.2. Identification of binding sites on AChE and BACE 1

Computationally, we can identify binding sites on our enzymes of choice using readily available free software online. There are two processes by which we can operate: Firstly, we can preliminarily detect potential binding sites on the protein by using a geometric method to identify the sizes of these binding sites. Note that if the binding site is too large, there is no proper "cup" that can hold the small molecule in place. Conversely, if the binding site is too small, then the small molecule cannot even fit in the binding site. For our experiments, we will be looking at a range of 100–550 Å, preferably looking for sites with volumes around 300 Å. This will be done using online software developed by the University of Hamburg called Proteins Plus (Schöning-Stierand, K. et al., 2022; Schöning-Stierand, K. et al., 2020; Fährrolfes, R. et al., 2017).

The second process of experimentation we will use is an energy-based method. The potential energy of the interaction between our small molecule and the binding site is as critical to our assessment of the binding site as the geometry of it. If there is no favorable energetic interaction between the binding site and small molecule, then nothing will happen. We will assess the energy of our interactions using online software developed by the VAJDA lab at Boston University called FT Site. Our last process of experimentation is a machine learning-based method. Instead of looking at the geometry or the energy of the interactions, machine learning is used to predict the quality of the ligand binding site. We will utilize online software called PrankWeb, which builds upon P2Rank, a stand-alone command line program that predicts ligand binding sites from its structure (Prankweb, 2017; Jackubec, D. et al., 2022; Jendele, L. et al, 2019; Krivák, R., 2018; Cusbg, 2020;).

2.3. Virtual screening of small molecules

Though sources dispute on the exact value, it is commonly agreed that only under 10% of the human proteome is druggable (Kana, O., et al., 2019). This means that potentially either of our enzymes of interest are not druggable, i.e., they do not have any suitable binding sites. After having

identified whether or not BACE1 and AChE are druggable, we will move on to our next phase of experimentation, where we will virtually screen millions of compounds to identify those that show promise when binding to either protein.

We will be accomplishing this through utilizing pharmacophore maps, a pharmacophore is a map of the interaction between two compounds which aims to identify important features for receptor binding. When looking at an interaction, there are three necessary points of information that are need-to-know. Firstly, we need to know the quantity of interactions; we ask, "how many interactions are there?" Secondly, we look at the location of the interactions; we ask, "where do these compounds interact?" Lastly, we must also identify the type of interactions; we ask, "what kind of intermolecular forces are making this interaction happen?" A pharmacophore map answers all these questions graphically.

Computationally, we will be utilizing an online software called PocketQuery (Koes D. R. et al., 2011; Koes, D. R., 2023). Developed by the University of Pittsburgh, Pocket Query looks at the interaction between a protein and a small molecule and generates a pharmacophore map for it based on the conditions we set. These pharmacophore maps is the main tool that we will be using to virtually screen through millions of FDA-approved compounds. If the pharmacophore map of the original interaction overlaps with the pharmacophore map of another compound, then the molecule of interest can bind very similarly to the original overlap and thus is a potential inhibitor of it.

In this study, we will first construct the pharmacophore map of the interaction between BACE1 and a peptide inhibitor, as peptide inhibitors are already rather small; the peptide we will be looking at is OM99–2. The reason we are looking at BACE1 first is because, in comparison to AChE, BACE1 is much less readily druggable. Thus, it is the more challenging target; and, if compounds that can inhibit it are identified, there is a much higher chance that it can also bind AChE. Conversely, had we identified compounds that could inhibit AChE first, there would be a much lower chance that it would have the dual function to also inhibit BACE1.

2.4 Molecular docking

Up to this point, while we can determine how well compounds can match the pharmacophore of OM99–2 with BACE1, with our current methods we cannot determine how well these compounds can actually bind to BACE1 and AChE. This is where we will utilize molecular docking; molecular docking is a key tool in structural molecular biology and computer-assisted drug design (Morris G. M. et al., 2008).

Molecular docking is a process where the energy of an interaction between a ligand and a biomolecular target is estimated. In this computational study, we will be utilizing an online docking software called SwissDock (Grosdidier, A. et al, 2011a; Grosdidier, A. et al., 2011b). Since some of the compounds potentially are not within SwissDock's database, we generated mol2 files detailing the composition of the ligands and small molecules using the ZINC IDs we found from Pocket Query and ZINCPharmarcy.

2.5 Pharmacokinetics, and ADME

Pharmacokinetics is a subbranch of pharmacology where the interaction between an administered drug and the human body is studied. Generally, there are four main parameters to be examined in this field, which includes absorption, distribution, metabolism, and excretion (aptly named ADME) (Grogan, S. et al., 2023).

Absorption is the process by which a drug, either in tablet or capsule form, enters the circulation system of the human body. This parameter determines how quickly the drug is able to enter the bloodstream and reach its desired location. Absorption itself has a sub-parameter, called liberation; liberation is the process by which the drug is released from its pharmaceutical form (i.e. tablet/capsule) (Grogan, S. et al., 2023).

Distribution is a parameter that describes how a substance can distribute itself throughout the human body. This parameter varies from substance to substance as substances have differing biochemical properties. Put simply, the process of distribution has two main factors: diffusion, and convection. These can be affected by the size, polarity, or binding ability of the substance (Grogan, S. et al., 2023).

Metabolism is the process by which the body processes and decomposes the drug. If

a drug is metabolized by the body too quickly before it reaches its designated compartmental destination, it is ineffective and therefore has an inadequate ADME profile; however, if the drug is difficult to metabolize, it has the potential to have potently toxic effects from being in the body for too long. Most of the metabolism done by the body onto drugs is through the phase I (CYP450) and PHASE II (UGT) reactions in the liver. The phase I reaction oxidizes the molecules and converts them into polar metabolites that can then be bound to polymers in the phase II reaction through glucuronidation (Miyauchi, Y. et al., 2021). Most importantly, the combined efforts of these processes generally inactivate the medical capabilities of the drugs processed. This demonstrates that a balance between being metabolized quickly or slowly is an essential part of the ADME profile (Grogan, S. et al., 2023; Miyauchi, M. et al., 2021).

Excretion is the process by which the drug is flushed and eliminated from the body. Generally, this process is done by the kidneys, however, excretion can also occur via the lungs, skin, or the gastrointestinal tract. In the kidney, drugs can be excreted by the passive glomerular filtration or the active tubular secretion (Grogan, S. et al., 2023; Madison J. E. et al., 2008).

2.6 Lipinski's Rule of 5

There are many biochemical properties that predict whether a substance would have an adequate ADME profile for draggability. For example, the number of hydrogen bond donors and acceptors, molecular weight, calculated LogP (CLogP), etc. The number of hydrogen bond donors and acceptors dictates how easily the drug can bond to receptors. A higher amount of bond donors/acceptors means that the drug will attempt to bind to every available protein on its way to the designated compartmental destination and reduce its effectiveness. Additionally, the molecular weight of the drug must be relatively light to allow skin absorption, as larger molecules cannot pass the corneal layer (Bos J. D. et al., 2020).

For Lipinski's set of rules, aptly named *Lipinski's rule of 5*, it requires that the compound has no more than 5 hydrogen bond donors and no more than 10 hydrogen bond acceptors. It also requires that the molecular weight of the molecule stays under 500 Daltons, which is equivalent to one atomic mass unit. And finally, a CLogP no more than 5 (Benet L. Z. et al., 2016). The calculated LogP is a measure of how hydrophobic or hydrophilic a compound is. If hydrophobic, then the compound is lipophilic, and vice versa; if hydrophilic, then the compound is lipophobic.

For our purposes, we will utilize an online software called SwissADME, utilizing the top five compounds that we determined from our data from SwissDock (Daina, A. et al., 2017). [41,] We can reconstruct their chemical structure in the software and the software will run an algorithm to determine many factors, namely the hydrogen bond donors and acceptors, molecular weight, CLogP, as well as any other important factors, such as gastrointestinal (GI) absorption, and blood-brain-barrier (BBB) permeant.

3. Results

3.1 Identifying binding sites on AChE and BACE1 geometrically

Using the geometric based method, we are given both the volume of the binding site as well as the drug score, which indicates the quality of the binding site and how druggable it is. On the tables below, the volumes and drug scores are detailed for the top five binding sites on both AChE and BACE1

Table 1. – *Top 5 binding sites on AChE filtered for size*

Pocket	me (Å3)	g Score
P_2	399.60	0.81
P_3	355.84	0.74
P_4	352.42	0.70
P_5	285.17	0.63
P_6	284.94	0.61

From table 1, we can infer that AChE is a very promising drug candidate. With an average volume of 335.59 $Å^3$, the average binding site sits well within the range decided earlier. Furthermore, the average drug score of the 5 pockets is 0.698. In figure 3, we can see a cartoon visualization of AChE as well as the 5 binding sites identified.

Figure 3. *Top 5 identified binding sites on AChE through the geometric method*

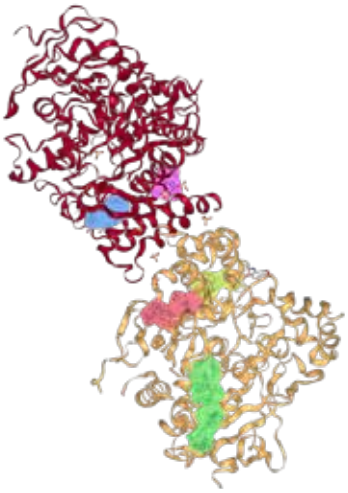
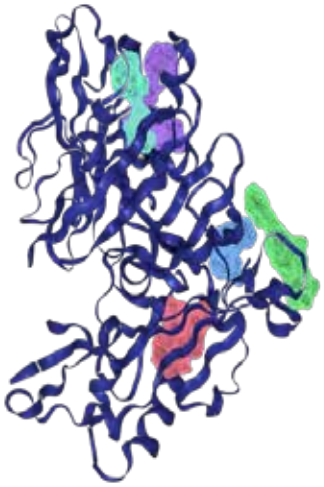


Table 2. *Top 5 binding sites on BACE1 filtered for size.*

Pocket	Volume (Å3)	Drug Score
P_4	305.39	0.73
P_5	291.68	0.66
P_6	225.62	0.66
P_7	292.61	0.51
P_8	171.87	0.50

Figure 4. *Top 5 identified binding sites on BACE1 through the geometric method*



From table 2, we can see that ftsite predicts that BACE1 is also druggable. There are also multiple binding sites all within the volume range earlier determined. However, note that the average binding site and drug score, with an average volume of 257.43 Å^3, and an average drug score of 61.2, is generally lower than that of AChE. From these results, we can reasonably infer that AChE is a more viable binding target than BACE1 is.

3.3 Identifying binding sites on AChE and BACE1 based off machine learning.

While the previous method predicted the presence of binding sites solely based off the geometry and the 3D structure of the protein. PrankWeb predicts the presence of binding sites using a machine learning algorithm that has become well adapted to predicting the presence of binding sites. PrankWeb is an extension of the machine learning algorithm P2Rank, which is a ligand binding site

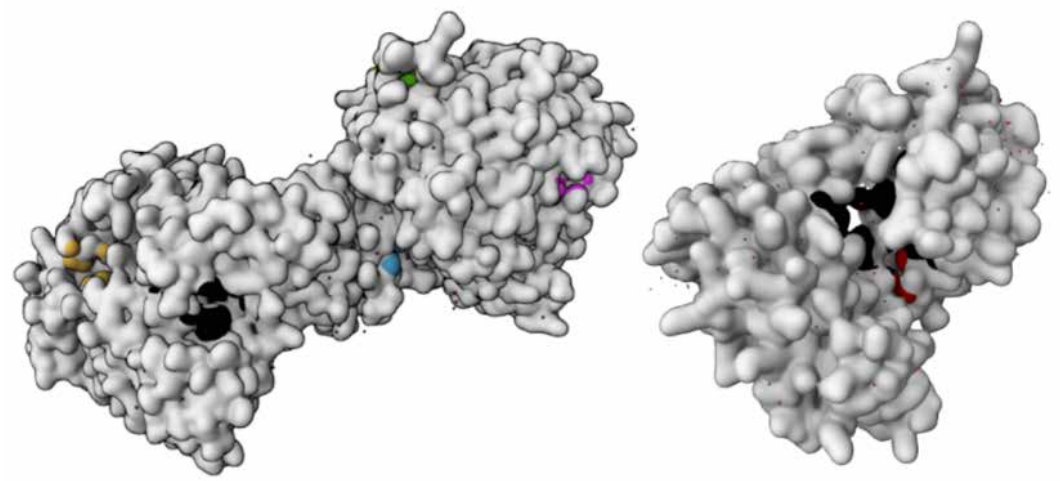
Figure 5. *The black binding site pocket profiles for AChE (top) and BACE1 (bottom)*

Figure 5 was generated by the machine learning-based algorithm multiple binding sites were identified. The machine learning algorithm detected only 2 pockets on BACE1 while it detected 12 pockets for AChE, further supporting our claim that BACE1 is less readily bindable relative to AChE.

In figure 5, the colored amino acids represent the detected binding sites on the protein. With the black colored amino acids representing the binding site that is ranked the highest. By being ranked the highest, the algorithm predicts that these binding sites are the most promising binding sites in terms of druggability. Figure 6 displays the pocket profiles of both pockets; the pocket profile is a quick summary of important parameters of the pocket such as its rank, the pocket score, its probability score, etc. We can see that these pockets have scores

of 29.65 and 28.47 for AChE and BACE1 respectively, which is indicative of their capability as binding sites. Overall, this shows us that while AChE is more readily bindable than BACE1 is, both protein targets do show promise of being drug targets.

Figure 6. *Images of AChE (left) and BACE1 (right) as generated from the machine learning-based method*

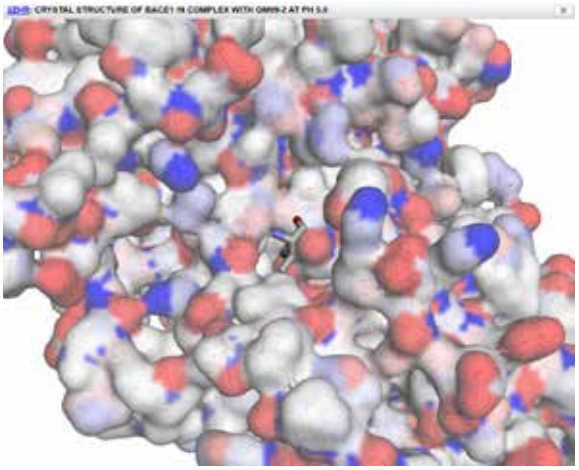


3.4 Pharmacophore maps

Now that AChE and BACE1 have both been assessed to be viable inhibitor targets, we must identify various compounds that have the potential to act as a ligand and inhibitor for these proteins. Since AChE has a "binding advantage," – being more capable of being bound – over BACE1, we will examine BACE1 first. Utilizing the base pharmacophore map of the interaction between BACE1 and OM99–2, we selected the top 5 interactions between the protein and small molecule. Listed below in table 3 is summative data on the information given about the interactions.

Table 3. *– Data on 5 different interactions between BACE1 and OM99–2*

PDB	Chain	Size	Distance	Avg ΔG	Residues	Score
2ZHR	C	1	0	–5.86	VAL	0.8654
2ZHR	C	2	4.4547	–3.02	VAL, ASN	0.8118
2ZHR	C	3	5.925	–3.15333	GLU, VAL, ASN	0.7919
2ZHR	C	2	7.3035	–3.565	ALA, PHE	0.7849
2ZHR	C	3	7.3035	–3.13	ALA, GLU, PHE	0.7627

Residue	Figure
VAL	

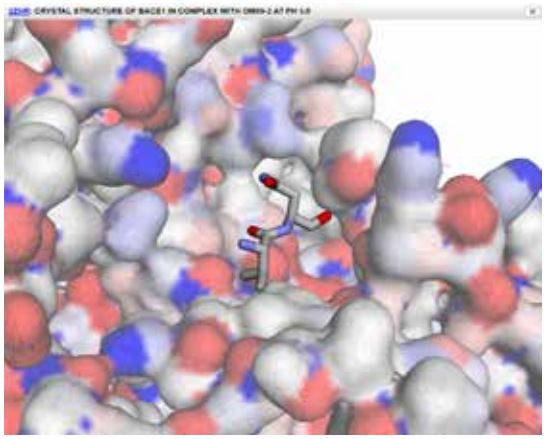
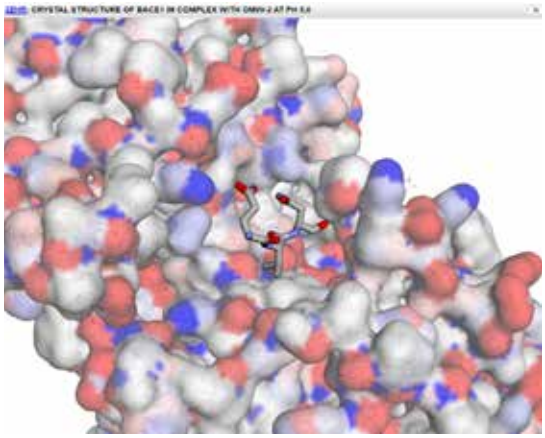
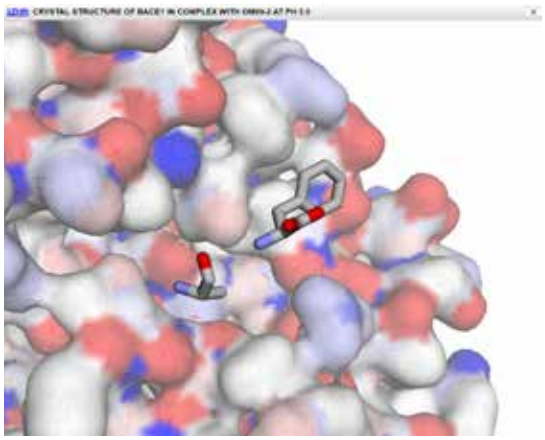
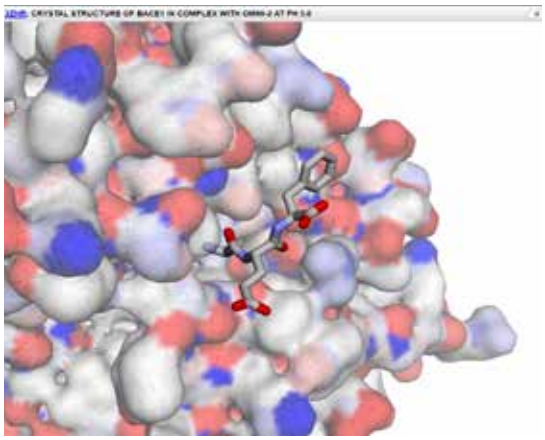
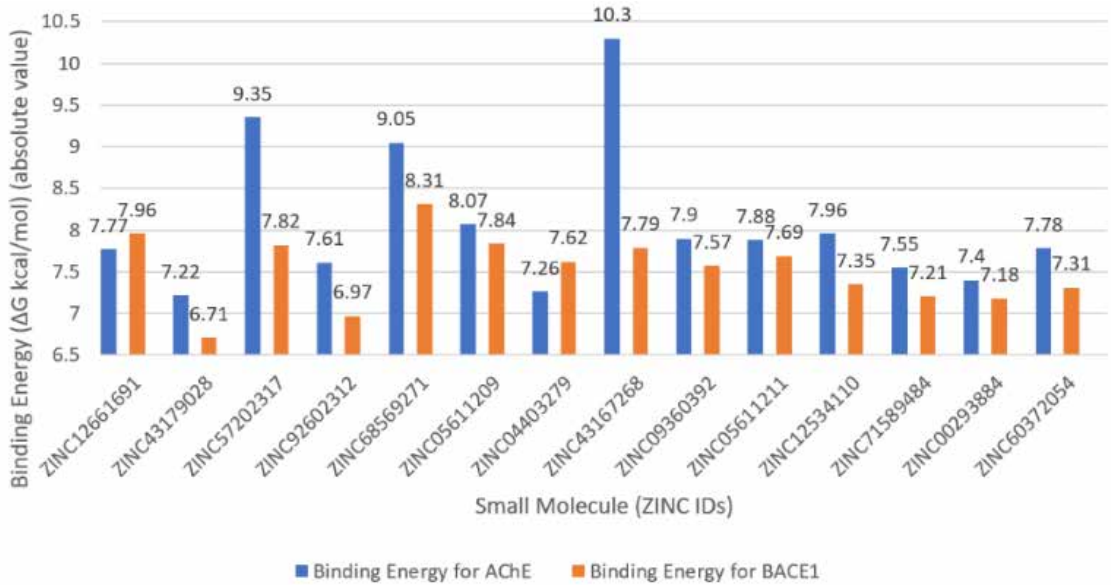
Residue	Figure
VAL, ASN	
GLU, VAL, ASN	
ALA, PHE	
ALA, GLU, PHE	

Table 4. – *The top 3 compounds that matched the pharmacophore map of its specific interaction the closest for all 5 interactions selected*

	1 st Choice	**2 nd Choice**	**3 rd Choice**
AA Residue: VAL	ZINC12661691 RMSD: 0.026	ZINC05611209 RMSD: 0.027	ZINC05611211 RMSD: 0.027
AA Residue: VAL ASN	ZINC43179028 RMSD: 0.036	ZINC04403279 RMSD: 0.037	ZINC12534110 RMSD: 0.046
AA Residue: GLU VAL ASN	ZINC57202317 RMSD: 0.045	ZINC43167268 RMSD: 0.049	ZINC71589484 RMSD: 0.052
AA Residue: ALA PHE	ZINC92602312 RMSD: 0.061	ZINC09360392 RMSD: 0.062	ZINC00293884 RMSD: 0.068
AA Residue: ALA GLU PHE	ZINC68569271 RMSD: 0.089	ZINC78555142 RMSD: 0.089	ZINC60372054 RMSD: 0.091

When selecting compounds to use for this study, PocketQuery provides us with a parameter called the root mean squared deviation (RMSD) value. The RMSD value is a quantitative measure of the similarity between superimposed atomic coordinates (Kufareva, I. et al., 2012). For our purposes, the RMSD value is used to determine how closely the chosen compound's pharmacophore map matches the overlay of the original pharmacophore map (the one between BACE1 and OM99–2). To ensure accuracy that we choose compounds with the highest likelihood to properly bind BACE1 (and hopefully AChE), we reject any compounds with values of RMSD > 0.10.

Figure 7. *Binding energies of identified small molecules with the enzymes AChE and BACE1*



3.5 Molecular Docking

From the data gathered and summarized in figure 7, we can see a few clear candidates as our "best" compound. For example, we can see that 43167268 shows promise as a candidate with its ΔG for AChE of 10.3. For our other compounds, we can determine which are the top five candidates by looking at the largest minimum ΔG value for each pair of values. Using this method, we will rank the top 5 compounds by ranking them from the largest minimum value to the smallest minimum value. This yields the ranking: 1. ZINC68569271, 2. ZINC41367268, 3. ZINC12661691, 4. ZINC67202317, 5. ZINC05611209.

3.6 ADME Profiles

In table 5, we can see the respective ADME profiles of all the 5 compounds chosen from the docking procedures. Of the 5 compounds examined, 4 successfully passed Lipinski's Rules, while ZINC12661691, with a molar mass (MM) = 518.52 g/mol, exceeded the MM limit of 500 g/mol.

Table 5. – *ADME profiles of the top 5 identified compounds from SwissDock*

	H-bond Donors	H-bond acceptors	LogP	Molecular Weight	GI Absorption	BBB permeant	Lipinski's Rules?
68569271	3	3	3.69	375.49 g/mol	High	No	Yes
41367268	1	7	2.03	337.31 g/mol	High	No	Yes
12661691	2	4	4.92	518.52 g/mol	High	No	No
67202317	2	2	3.75	379.56 g/mol	High	No	Yes
05611209	1	5	3.08	409.25 g/mol	High	No	Yes

Though SwissADME deems these compounds incapable of crossing the blood brain barrier (BBB), chemical modifications can be made to these compounds in the lab to allow them to cross the BBB. These findings act as a good starting point for the next series of trials and experiments needed to further validate the work of this paper.

4. Conclusion

Alzheimer's disease is a disease that afflicts millions throughout the world and has a heavy prevalence in our modern day. Alzheimer's is believed to be a multi-faceted disease, the facets we chose to attack in this study is by inhibiting the functions of the proteins AChE and BACE1. AChE's function in the brain is to catalyze the hydrolysis of ACh, a vital neurotransmitter, thereby reducing its concentration and therefore effectiveness at the synapse. BACE1's function is to cleave the APP gene, which causes the overproduction of Aβ42 peptides, which form long fibrils that interfere with neuronic communication and can lead the pathogenesis of AD.

We applied the computational methods listed above to search for dual inhibitors that could effectively inhibit the functions of both AChE and BACE1, which prevents the hydrolysis of ACh and the production of Aβ42 peptides, respectively. Because different binding targets have varying levels of druggability, we first assessed how capable AChE and BACE1 were of being bound through varying computational methods. Through these methods, we aimed to identify potential binding sites and utilize the data gathered to determine the general druggability of both proteins.

We first looked utilized a method called ProteinsPlus that uses a Difference of Gaussian (DoG) filter to detect potential binding pockets purely based on the physical structure of the protein. The method then predicts various parameters that describe the size, shape, and chemical properties of the pocket detected. We used this method to give a surface-level indication to the druggability of the proteins. Next, we utilized an online software called PrankWeb to further assess the druggability of potential binding sites on AChE and BACE1. PrankWeb utilizes machine learning to predict the location and druggability of binding sites on proteins. From these methods, we were able to determine that BACE1 was much less readily bindable than AChE is.

Due to the fact that AChE has a "binding advantage" over BACE1, it seemed more practical to first identify compounds that could inhibit the function of BACE1 first, then see how well these identified compounds could inhibit AChE. To accomplish this, we utilized pharmacophore maps. Using the interaction between BACE1 and OM99–2 as a base, we identified compounds that overlayed well with the base pharmacophore map to virtually screen through millions of compounds. Then, we chose the top fourteen compounds that bound to BACE1 the most similarly to OM99–2. We then utilized docking software to predict the binding energies (measured in ΔG°) of the interaction of these fourteen compounds with both AChE and BACE1. From the data collected, we chose the top 5 most appealing compounds that showed the capability to bind both BACE1 and AChE. Finally, we assessed the ADME profiles of the top 5 chosen compounds using online methods to determine how effective these compounds could be as a drug. Of our top 5 compounds identified, all but ZINC12661691 had adequate ADME profiles. All 5 compounds bound to both protein targets with binding energies less than −7.00 kJ/mol, with ZINC43167268 binding to AChE with a binding affinity of ΔG° = −10.30 kJ/mol. Thus, of the millions of compounds initially screened, we are left with only 4 compounds (from most to least promising): ZINC68569271, ZINC41367268, ZINC67202317, ZINC05611209. Note, SwissADME assessed that all 4 small molecules were incapable of crossing the BBB, this is a crucial part of drug discovery for neurodegenerative diseases, as being incapable of crossing the BBB effectively makes the drug useless; however, simple chemical modifications to the compounds can render them BBB-permeable.

Due to the nature of computational studies like this paper, there are several limitations that this paper has. For example, the computational methods used in this paper such as ftsite, PrankWeb, SwissDock, and SwissADME are all based upon prediction and have not been physically observed. The predicted binding affinities of these compounds potentially do

not reflect the real-world binding affinity. To further validate the work of this paper, there are numerous next steps that this line of work can take. Firstly, to alleviate the risk of using such an uncertain and small sample size, this computational experiment can be conducted several more times to identify new compounds that are able to bind to the protein targets as readily or more readily than the currently identified compounds. Once this is accomplished, the binding affinities of these compounds should be experimentally determined within the lab. Then pre-clinical *in vitro* trials can be conducted to determine the efficacy these drugs are within an organic system; note that during these *In vitro* trials, chemical modifications are to be made that can render the small molecules BBB permeable. Finally, clinical *in vivo* trails can be conducted to determine the efficacy of these drugs in the human body.

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