TREM 2 AND IT'S VARIANTS ANALYSIS IN NEURODEGENERATIVE DISORDERS LIKE ALZHEIMER'S DISEASE

Project report submitted in partial fulfillment of the requirement for the degree of Bachelor of Technology
In

Bio Informatics

By

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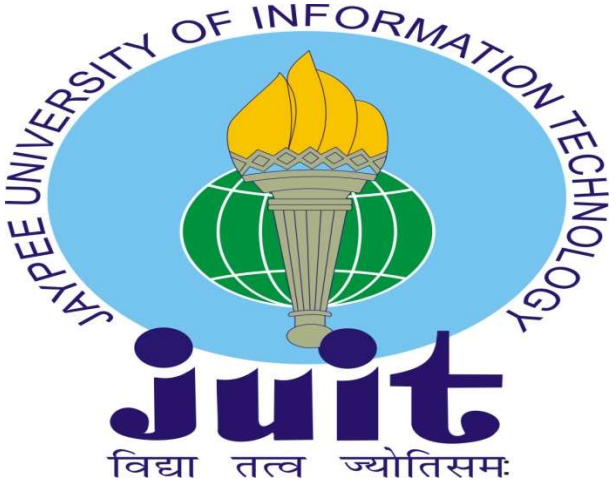
Under the supervision of

Dr. Tiratha Raj Singh

To



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Candidate's Declaration

I hereby declare that the work presented in this report entitled **"TREM 2 AND IT'S VARIANTS ANALYSIS IN NEURODEGENERATIVE DISORDERS LIKE ALZHEIMER'S DISEASE"** in partial fulfillment of the requirements for the award of the degree of Bachelor of Technology in Bioinformatics submitted in the department of Biotechnology and Bioinformatics and Information Technology, Jaypee University of Information Technology Waknaghat is an authentic record of my own work carried out over a period from July 2021 to May 22, 2022 under the supervision of **Dr. Tiratha Raj Singh**, Associate Professor (Senior Grade), Department of Biotechnology and Bioinformatics JUIT, Waknaghat Solan, H.P., India.

The matter embodied in the report has not been submitted for the award of any other degree or diploma.

Kushal Chakraborty (181507)

This is to certify that the above statement made by the candidate is true to the best of my knowledge.

Dr. Tiratha Raj Singh
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Dated:

ACKNOWLEDGEMENT

I would like to dedicate this work to my Grandparents, without whom nothing would ever have been possible. I would like to extend my sincerest and most heartfelt gratitude to my supervisor Dr. Tiratha Raj Singh for his unconditional support and tutelage throughout the working of this project report. Through his guidance and thoughtful input, this work received cogent reviews and constructive criticism that enabled it to be completed. Without his direction, any attempt to complete this report in the view of the vast nature of the subject would have been a near impossible task to finish. For his guidance and insights, I shall remain ever so grateful.

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Abstract:

Alzheimer's disease is a debilitating Neurodegenerative disorder (NDD) that impairs cognitive functions, language processing, memory, attention and comprehension abilities of people affected by it. This decline in cognitive performance, is in particular a huge risk for the elderly who are 65 and above, and requires intense medical care and attention to aid even the basic necessities of the patients to function for day-to-day life. The etiology of this disease was conventionally thought to have been predicated on two core pathologies namely Amyloid Beta plaque formation and Neurofibrillary tangles generation due to tau hyperphosphorylation. Decades of research and study for the aforementioned pathologies yielded often times conflicting and unsatisfactory results in explaining the exact mechanism and causation of Alzheimer's disease. Many studies that sought to investigate the underlying genetic cause, implicated the Apopolyprotein gene ApoE4 as a gene of significant interest conferring a very high likelihood of developing Alzheimer's disease. These lines of approaches still didn't answer a lot of the mystery surrounding AD, with a growing number of studies pointing towards a more complex picture. In recent years neuroinflammation and its role in NDD's have shed vital insight into the mechanism of various diseases, and how different systems and interacting entities contribute to the overall pathology of the particular disease in hand. Neuroinflammation can particularly be damaging in case of disease progression of cognitive dysfunctions like Alzheimer's and other CNS diseases. The role of Microglia, astrocytes, regulatory and receptive proteins and cascade of reactions involving chemokine and cytokine presents an ever more expansive field of research to understand the full picture of NDD's holistically. In recent years TREM 2 has been implicated as a gene of significant risk/interest in the underlying indicators, and or marks for Late Onset Alzheimer's diseases (LOAD), which was before uniquely characterized for ApoE4, as "The" gene of interest. TREM 2 since then has been deemed as a gene of high risk/significance for the underlying cause of LOAD. Various whole exome and genome studies has identified, rare variants of the TREM2 gene, that confers additional risk for the onset of Alzheimer's. Variants of specific interest are mostly naturally occurring missense SNP mutations, some of which may possibly confer an increased likelihood for the occurrence of LOAD (Late-Onset Alzheimer disease). The purpose of this project in particular is to evaluate and analyze TREM2 across various parameters, variants, different species, structurally and sequentially to get a deeper understanding of the role TREM2 plays in NDD's like AD.

CHAPTER-1 INTRODUCTION

Overview of Alzheimer disease:

Alzheimer's disease is an irreversible, degenerative brain illness that gradually erodes memory and thinking skills, as well as the ability to do even the most basic tasks. Symptoms of late-onset type occur in the mid-60s in the majority of patients with the condition. Alzheimer's disease that develops between the ages of 30 and 60 is extremely unusual. The most common cause of dementia in elderly people is Alzheimer's disease. AD now affects over 5 million Americans and is anticipated to grow more common as life expectancy increases.[1]

Total healthcare expenditures for Alzheimer's disease treatment are predicted to reach $305 billion in 2020, with prices expected to rise to more than $1 trillion as the population ages. Skilled nursing care, home healthcare, and hospice care account for the majority of the direct expenditures of Alzheimer's disease care. Indirect care expenses, such as quality of life and informal caregiving, are likely undervalued and are linked to considerable social and personal stress. [2] Given the clinical and financial costs of Alzheimer's disease, finding new mechanisms of etiology and treatment targets is critical.

Memory loss is usually one of the early indicators of Alzheimer's disease, however it varies from person to person. Other areas of thinking, such as finding the proper words, vision/spatial difficulties, and impaired reasoning or judgement, may also indicate Alzheimer's disease in its early stages. Mild cognitive impairment (MCI) can be a precursor to Alzheimer's disease, however not everyone with MCI will get the illness.

The injury or destruction of nerve cells (neurons) in areas of the brain involved in thinking, learning, and remembering (cognitive function) causes symptoms. Neurons in other sections of the brain are injured or killed as the disease advances. Nerve cells in regions of the brain that allow a person to perform fundamental physical activities like walking and eating are eventually impaired. Individuals become bedridden and demand 24-hour care. Alzheimer's disease is deadly in the end.

Two of the brain abnormalities linked with Alzheimer's are the buildup of the protein fragment beta-amyloid (called beta-amyloid plaques) outside neurons and the formation of an aberrant version of the protein tau (called tau tangles) inside neurons.

By interfering with neuron-to-neuron contact at synapses, plaques and smaller accumulations of beta-amyloid termed oligomers may lead to the damage and death of neurons (neurodegeneration). Tau tangles prevent nutrition and other critical chemicals from reaching neurons. Although the exact order of events is unknown, beta-amyloid may begin to accumulate before aberrant tau, and increased beta-amyloid buildup is linked to higher tau levels. [3,4]

Inflammation and atrophy are two other brain alterations. The presence of toxic beta-amyloid and tau proteins in the brain is thought to activate immune cells called microglia. Microglia attempt to eliminate harmful proteins and other waste from dead and dying cells. When the microglia can't keep up with everything that needs to be removed, chronic inflammation might develop. The loss of cells causes atrophy, or shrinking, of the brain. Decreases in the brain's capacity to metabolise glucose, its essential fuel, exacerbate normal brain function in Alzheimer's disease.

The development of a prolonged immunological response in the brain has emerged as a third key pathology in Alzheimer's disease during the last decade. The continuous stimulation of the brain's resident macrophages (microglia) and other immune cells has been shown to worsen both amyloid and taupathology, suggesting that it may play a role in the disorder's development.

Core pathologies in AD:

The neurofibrillary tangle and senile plaque are the two basic cardinal lesions linked with Alzheimer's disease. The neurofibrillary tangle is made up of improperly phosphorylated tau that accumulates in the perikaryal cytoplasm of some neurons. A central core of beta-amyloid, a 4-kD peptide, is surrounded by improperly structured neuronal processes or neurites in the senile plaque. Other neuropathological lesions are seen in Alzheimer's disease, but these two cardinal abnormalities identify and distinguish the condition. [5]

Ab Plaques:

The incorrect cleavage of the amyloid precursor protein (APP) results in Ab monomers that combine to create oligomeric Ab (Amyloid beta), which then aggregates to form Ab fibrils and plaques [6]. APP's function is uncertain; however, it is thought to have a role in cell health and growth [7]. Knowing the processes of Ab monomer production, clearance, and aggregation into oligomeric Ab are critical parts of understanding the start of Ab disease. Nonamyloidogenic proteolysis of APP by α-secretase and γ-secretase produces soluble fragments in normal processing of the APP sequence [8]. When APP is cleaved by γ-secretase and erroneous β-secretase, insoluble amyloid β peptides form insoluble β-amyloid plaques in the brain. [9]

NFT (Neurofibrillary tangles):

The hyperphosphorylation of tau, a microtubule-associated protein that stabilises microtubules, causes the second core pathology, NFT [10]. Phosphorylation of tau is required for intracellular trafficking because it allows tau to be removed from microtubules and transported, followed by dephosphorylation to restore tau to the microtubule [11]. In Alzheimer's disease, tau protein is phosphorylated at many locations, resulting in tau removal from the microtubule and disruption of a variety of cellular functions ranging from protein trafficking to overall cellular shape [12-14]. Furthermore, hyperphosphorylated tau (ptau) forms paired helical fragments, which eventually form neurofibrillary tangles [15-17]. The buildup of ptau tangles and cellular dysfunction results in neuronal dysfunction and, eventually, apoptosis [18].

A number of studies showed that, in addition to Ab plaques and NFT, people with Alzheimer's disease had a prolonged inflammatory response in their brains. The inflammatory response has now been seen in several investigations of postmortem tissues from AD patients, and it is commonly detected in preclinical model systems of AD.

Inflammation in AD:

Acute inflammation in the brain is a well-known defense against infection, toxins, and damage, but chronic inflammation (neuroinflammation) emerges when the balance of anti-inflammatory

and pro-inflammatory signals is disrupted, as shown in Alzheimer's disease [19-22]. Activated microglia cells and the production of many cytokines are to blame for this persistent neuroinflammation.

The pathophysiology of Alzheimer's disease is not limited to the neuronal compartment, but also involves immune systems in the brain. Misfolded and aggregated proteins link to pattern recognition receptors on micro- and astroglial cells, triggering an innate immune response that includes the release of inflammatory mediators, which contributes to disease development and severity. According to a genome-wide investigation, some genes linked to sporadic Alzheimer's disease code for components that control glial clearance of misfolded proteins and the inflammatory response. External variables such as systemic inflammation and obesity are likely to wreak havoc on the brain's immunological systems, hastening illness progression [23].

A large amount of evidence currently shows that a chronic immunological response in the brain not only causes neurodegeneration, but also promotes and exacerbates both Ab and NFT diseases. Furthermore, the inflammatory response has been hypothesized as a possible connection between the early Ab disease and the subsequent development of NFT [24-28]. Neuroinflammation is often assumed to be a consequence of one or more of the major AD pathologies or risk factors associated with AD, and it helps to exacerbate the illness by aggravating the β-amyloid and tau pathologies [29,30].

Inflammation in the brain appears to have a dual function, acting as a neuroprotective factor during an acute reaction but becoming deleterious when a chronic response develops [31]. Reactive oxygen species, nitric oxide, and cytokines are among the proinflammatory and toxic chemicals released by chronically activated microglia. There is an increase in cerebral Ab deposits 1–3 weeks after head trauma in dead individuals, and it has been established that raised levels of interleukin 1 (IL-1) are responsible for the increased APP synthesis and Ab burden [32,33]. Furthermore, increased levels of IL-1b have been demonstrated to promote the production of other cytokines, such as IL-6, which has been shown to trigger the activation of CDK5, a kinase that has been linked to tau hyperphosphorylation [34].

The neuroinflammation seen in Alzheimer's disease appears to have a key role in worsening Ab load and tau hyperphosphorylation, implying that this dual function might be a key connection between two seemingly unrelated basic AD diseases. The heightened immune response mediated by the brain's resident macrophages (microglia) is now a prominent theme in Alzheimer's disease research.

Neurological immune cell activity (of Microglia):

Microglia are immunological cells that live in the CNS. Microglia are defined morphologically as ramified cells with little somas in a healthy brain and are in an inactive, "resting" condition. The cell somas remain immobile in this condition, but the cell processes stretch and retract, assessing their surroundings and interacting with neurons and other glia cells [38]. A significant number of signalling systems are used to maintain overall monitoring of the surrounding neuronal environment [39]. This includes receptors for conventional neurotransmitters [40], receptors for many cytokines and chemokines [41], and receptors for ligands that are constitutively produced in healthy neuronal settings, such as fractalkine (CX3CR1) [42]. When microglia detect a danger to the CNS, such as invasion, damage, or illness, they become activated, resulting in morphological changes such as process retraction, cell expansion, and migration [43]. Changes in any of the aforementioned monitoring systems might cause an active state to emerge.

Activated microglia phagocytose Ab, according to several studies [44], however these microglia grow and lose their ability to process Ab over a lengthy period of time [45]. The mounting immunological response results in Ab clearance early in AD pathogenesis and has been shown to have beneficial effects on AD-related diseases in animal models' systems [46]. However, persistent immunological activation has been shown to exacerbate AD pathology, most likely as a result of chronic activation of microglia in a feed forward loop, a condition known as reactive microgliosis. This causes a buildup of Ab and long-term pro-inflammatory cytokine singling, which causes neurons to be damaged [47].

Microglia effectiveness for binding and phagocytosing Ab declines as a result of continuous stimulation, as does Ab degrading enzyme activity in microglia, resulting in a diminished

capacity to break down Ab plaques [48].

The ability of microglia to produce pro-inflammatory cytokines appears to be unaltered [45]. These findings reveal a unique characteristic of pathogenesis in which total Ab clearance is reduced but immune activation remains. Microglia's continual production of pro-inflammatory cytokines and neurotoxins exacerbates neuroinflammation and contributes to neurodegeneration, resulting in the activation of even more microglia.

Because microglia are engaged in Ab clearance, they generate a number of proinflammatory cytokines that attract more microglia to plaques [49], resulting in a halo of active microglia around plaques [50]. Recent research suggests that when microglia's ability to clear Ab declines, peripheral macrophages may be attracted to Ab plaque formation in an effort to remove Ab. The migration of peripheral macrophages into the brain is thought to worsen the consequences of chronic inflammation and consequently the pathogenesis of Alzheimer's disease.

Neurological immune cell activity (of Astroglia):

Reactive astrogliosis, a complicated, multi-stage, and pathology-specific reaction with astrocyte remodeling aiming at neuroprotection and repair of damaged brain tissue, is a pathological response of astrocytes. Hypertrophic reactive astrocytes, like activated microglia, aggregate around senile plaques and are widely seen in post-mortem human AD tissue and AD animal models. Glial cell activation can happen early in Alzheimer's disease, even before A deposits. Increased expression of glial fibrillary acidic protein (GFAP) and evidence of functional impairment define reactive astrocytes. When astrocytes are exposed to Ab, they produce cytokines, interleukins, nitric oxide, and other potentially lethal chemicals, similar to microglia, increasing the neuroinflammatory response.

Some key drivers in the neuroinflammation in Alzheimer’s disease:

Neuroinflammation begins long before Alzheimer's disease (AD) manifests clinically, and it is one of the first processes that determines the disease's pathophysiology. Microglia and

astrocytes are important regulators and drivers of neuroinflammation. They are vital for neurotransmission and synaptic homeostasis in normal physiological settings. In the pathophysiological development of Alzheimer's disease, glial cells maintain an overexpressed inflammatory response that combines with amyloid-β and tau buildup and causes neurotoxicity and neurodegeneration in a self-contained way.

Neuroinflammation is hypothesized to be associated to genetic variations such as TREM2, CD33, PILRA, CR1, MS4A, CLU, ABCA7, EPHA1, and HLA-DRB5-HLA-DRB1. Proinflammatory intracellular signaling, cytokine turnover, synaptic activity, lipid metabolism, and vesicle trafficking are all implicated in the majority of them. TNF-, TGF-, IL-1, and the receptor protein TREM2, all of which are implicated in neuroinflammation, have been shown to initiate and worsen a large range of interrelated aberrant molecular disorders.

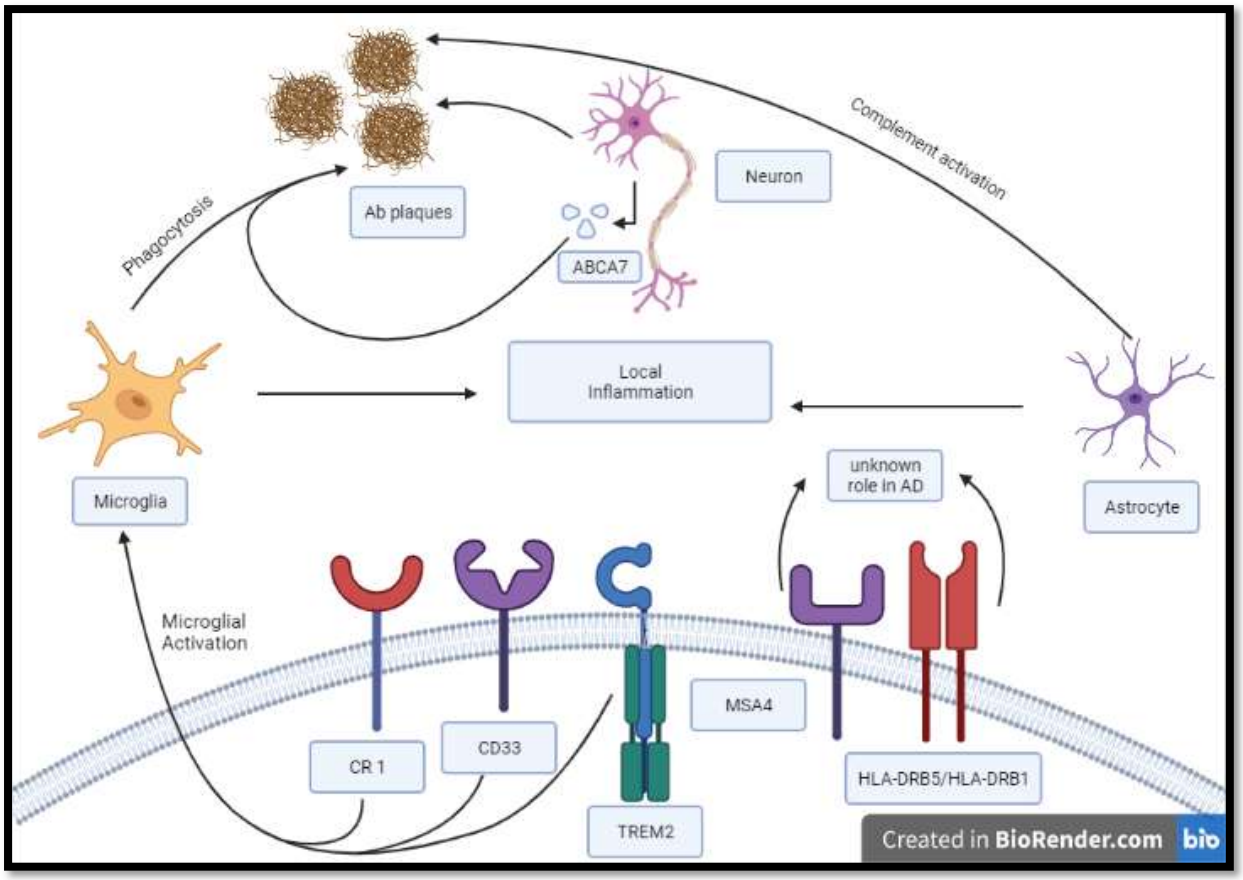

Figure 1: Cascade of reaction/activation for local inflammation

TREM2 structure:

TREM2, which stands for Triggering receptors expressed on myeloid cells 2, is a transmembrane glycoprotein found on the cell surface of myeloid cells. TREM2, which is found on human chromosome 6p21, codes for a 230-amino-acid protein. It is made up of an extracellular domain of V-immunoglobulin and a cytoplasmic tail. DNA, Bacterial lipopolysaccharides, phospholoipids, and sulfatides are some of the anionic ligands it interacts to.

TREM2 Mechanism of Action:

TREM2 expression on the cell surface is dependent on the adaptor proteins DAP12 and DAP10 (also known as Tyrosine kinase binding protein). The binding of the tyrosine kinase protein SNK transmits intracellular signals. DAP 10 stimulates PI3K recruitment in tandem (phatidylinositol 3-kinase). The signaling TREM2-DAP12-DAP10 complex actuates the triggering of small protein molecules and cascading of lipid phosphorylation, resulting in a variety of activities such as integrin activation, cytoskeleton rearrangement, Ca2+ mobilization, mTOR (mechanistic target of rapamycin) MAPK (mitogen activated protein kinase) signaling, and metabolism energetics.

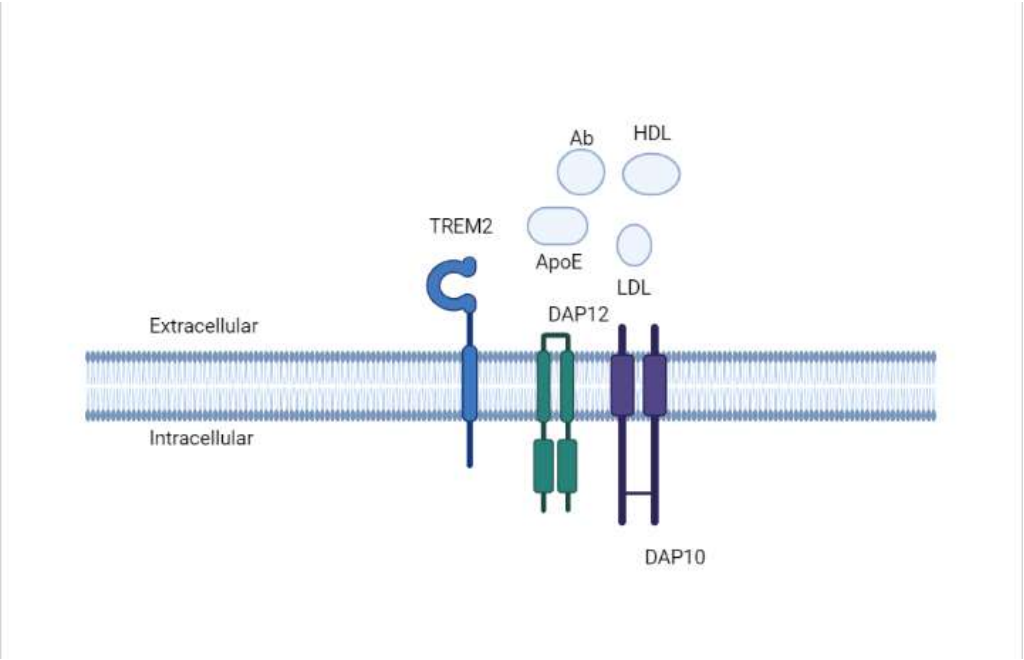

Figure 2: TREM2-DAP10-DAP12 complex

TREM2 expression:

TREM2 expression has been found to be elevated in pro-inflammatory pathological processes and situations, including as Alzheimer's disease. In vitro models, on the other hand, anti-inflammatory responses boosted TREM2 expression whereas pro-inflammatory responses decreased it. TREM2 expression is thought to be reduced in acute inflammation, such as those investigated in vitro, however it is exacerbated in pathological chronic situations like Alzheimer's disease.

TREM2 functions:

Microglial proliferation and response are one of TREM2's most important tasks. Phagocytosis of cellular debris, pathogens, and apoptotic neurons was dramatically hindered in TREM2 knockout mouse models, whereas enhanced TREM2 expression was shown to improve neuronal immune cell phagocytosis. TREM2 has also been found to have a function in anti-inflammatory

response and regulation, while research on its role in pro- or anti-inflammatory response are mixed. TNFa (Tumor Necrosis Factor alpha) and IL6 (Interleukin 6) levels were shown to be higher in myeloid cells that did not have TREM2 or DAP12 (adapter protein for TREM2). TREM2 overexpression, on the other hand, resulted in a reduction in TNFa, interleukins, and NO production. TREM2 appears to have a more sophisticated architecture and reaction to inflammation, according to research.

TREM2 and its role in microglial action in amyloid pathology:

TREM2 has been implicated in the function of microglia and their actions in a variety of diseases, including amyloid AD pathology, tau pathology, inflammation, and cytokine and chemokine responses, according to a number of research published in recent years. Reduced TREM2 expression has been linked to plaque clearance problems in Amyloid diseases. Furthermore, increased plaques have been seen in mouse models with TREM2 deletion, and the shape of amyloid plaques has been changed in certain results, such as that of R47H. In studies using mouse models such as APPPS1-21 and 5xFAd, TREM2 impairment was associated with a decrease in amyloid aggregation in the early stages, but an increase in plaque deposition in the later stages. As a result, TREM2's effect appears to be stage-dependent, with its helpful or detrimental effects based on the advancement of the disorder. TREM2 can also bind to Ab (amyloid beta), causing a proinflammatory reaction and microglial activity to help remove the plaque (but it binds specifically to Ab oligomers and not monomers).

CHAPTER-2 LITERATURE REVIEW

Microglia can protect Alzheimer's patients by promoting phagocytosis, breakdown, and eventually clearance of Aβ, the harmful protein accumulated in their brains. Microglia, on the other hand, become dysfunctional as illness progresses, releasing neurotoxins, losing their capacity to remove Aβ, and producing pro-inflammatory cytokines that encourage Aβ formation and accumulation.

The number of accumulating microglia/mononuclear phagocytes grows as Alzheimer's disease progresses. As a result, continuing to accumulate Aβ and allowing AD pathogenesis to persist despite the buildup of neuroimmune cells is counterproductive. The inability of these cells to arrest AD development is thought to be owing to an Aβ-induced phenotypic change that makes them more pro-inflammatory and less able to remove Aβ, resulting in decreased Aβ uptake and degradation, as well as increased Aβ buildup.
The interaction of Aβ with a receptor complex consisting of CD36 and the Toll Like Receptors (TLR) TLR4 and TLR6 expressed on microglia/mononuclear phagocytes causes these cells to become activated and generate proinflammatory cytokines, chemokines, and neurotoxins. In neuroimmune cells, these cytokines downregulate Aβ phagocytic receptors and Aβ degradation enzymes. In vitro, a lack of CD36, TLR4, or TLR6 lowers Aβ's neurotoxic effects and cytokine production.

The successive activities of β and γ secretases result in the proteolysis of APP, resulting in Aβ. Proinflammatory substances released by Aβ-stimulated microglia, including as TNFα, IL-1β, and interferon (IFN)-γ, activate the action of secretase and increase Aβ production, in addition to activating microglia and attracting more mononuclear phagocytes/microglia. Furthermore, secretase expression is stimulated by IFN-γIL-6 or TGF-β, resulting in enhanced Aβ synthesis. In AD animals, focal glial stimulation is associated with enhanced secretase activation and occurs before neuritic plaque development, indicating that cytokine-mediated control of Aβ synthesis occurs in vivo prior to plaque formation, potentially contributing to AD pathogenesis.

Inflammatory reactions mediated by microglia increase AD development in two ways. First, they cause neurotoxicity by producing reactive oxygen and nitrogen species, as well as other neurotoxins. Second, they promote Aβ accumulation by upregulating the levels and activity of the Aβ-generating enzymes γ secretase complex and β secretase.

TREM2 has been demonstrated to control myeloid cells' phagocytic capacity as well as their inflammatory response.

Several genetic risk factors for familial AD have been uncovered throughout the years of research, including mutations in the amyloid precursor proteins (APP), presenilin 1 and 2, and others (PS1 and PS2). These mutations appear to be fully penetrant, causing sickness to develop before the age of 60. The discovery of these mutations was crucial for patients who had them, and also led to the development of various mouse models for Alzheimer's disease. These mutations also revealed mechanisms that control APP processing, Aβ generation and clearance, and neuroinflammation in Alzheimer's disease.

Alzheimer's disease (AD) and other neurodegenerative disorders (NDDs) have both been linked to TREM2 variations. Because TREM2 codes for a receptor that is only expressed on immune cells, the discovery of these variations proves that the immune system can play a role in the etiology of NDDs. TREM2 mutations also have the highest probability of acquiring Alzheimer's disease of any known risk factor.

TREM2 is one of the most highly expressed receptors in microglia, with presence in microglia being >300 times more abundant than astrocytes.

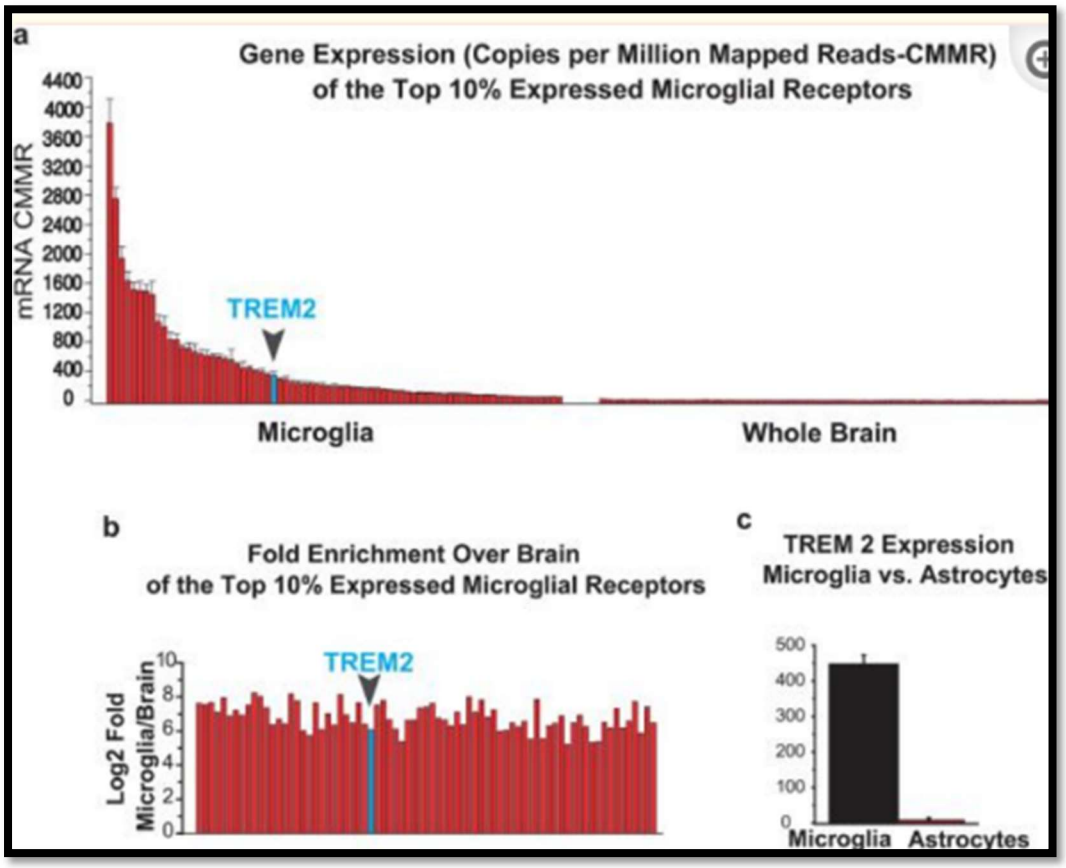

Figure 3: TREM2 is highly expressed in microglia (a) and highly enriched in microglia compared to whole brain (b), and to purified astrocytes

In the typical human brain, TREM2 is strongly expressed in white matter, with transcripts found in all brain areas. In the human AD brain, it is also strongly expressed in plaque-associated microglia. TREM2 expression has consistently increased with ageing and disease development in animal models. TREM2 regulates extracellular A pathology, tau hyperphosphorylation and aggregation, microgliosis, and inflammation in Alzheimer's disease, according to an increasing body of research. TREM2 has been shown to be cleaved by the β-secretase, which is essential for the production of Aβ peptides. TREM2 has also been discovered to interact with PSEN1, a major component of the β-secretase complex, in a way that affects TREM2-mediated phagocytic capability in microglia in a way that is independent of β-secretase activity.

TREM2 R47H, R62H, and H157Y heterozygous uncommon variations are linked to an increased risk of developing Alzheimer's disease in Europeans, African Americans, and Asians. TREM2 variations have also been associated to various neurodegenerative disorders such as

ALS, Parkinson's disease, and frontotemporal dementia. AD animals heterozygous for the Trem2 R47H gene were found to be equivalent to AD mice missing one copy of Trem2.

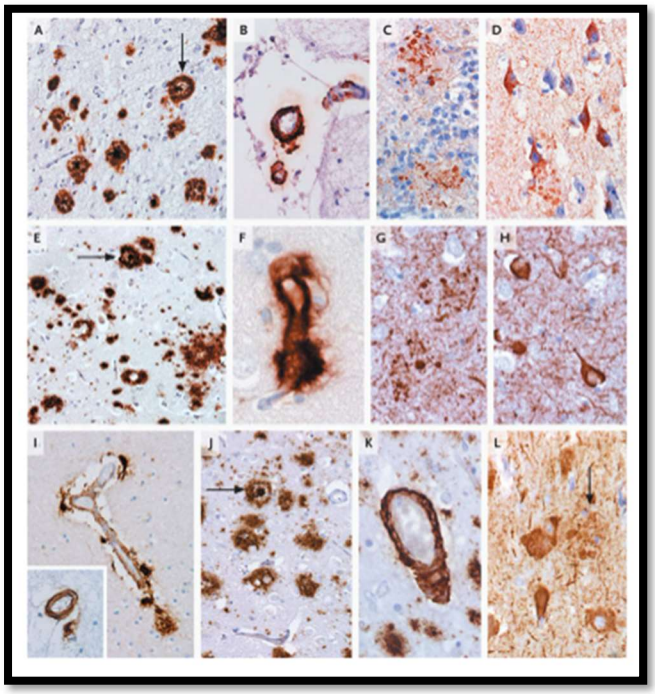

Figure 4: Pathological hallmarks in carriers with TREM2 variants

TREM2-mediated microglia activation can take several forms, including cell survival and proliferation, cytokine production regulation, and phagocytosis. TREM2 activation by these ligands is also critical for microglial activity in response to infections and other cell-damaging agents. TREM2 activation is required for Aβ-induced microglial depolarization, stimulation of the K+ inward current, cytokine production and secretion, migration, proliferation, death, and morphological alterations.

Autophagy (derived from the Greek words auto: self and phagos: eating) refers to a cell elimination and removal system that is self-sacrificing. At the most fundamental level, autophagy is responsible for maintaining cell homeostasis by digesting defective organelles and proteins. Autophagy pathways that are defective or autophagy-related genes that are altered have been discovered in a variety of human diseases, including neurodegenerative illnesses and microglia dysfunction.

Autophagy may impact microglial function throughout the evolution of age-related neurodegenerative disorders, according to research. TREM2 recycling in microglia is controlled by Beclin-1, an autophagy-related protein, and TREM2 levels are decreased in AD brain microglia, suggesting a possible relationship between TREM2 and autophagy. TREM2-mediated autophagy was found to be impaired in human AD brain and a mouse AD model in recent research, supporting this assumption. Autophagy is shown by the lipidation of microtubule-associated light chain 3 (LC3), which is an essential marker and effector. TREM2-deficient microglia have a higher amount of autophagic vesicles. These findings suggest that TREM2 is involved in microglial autophagy.

CHAPTER-3 Materials and Methodology

From The UniprotKB Database, the Amino Acid sequence of TREM2 was retrieved- (Q9NZC2) for analyses and various quantifications.

Uniprot Knowledgebase (UniprotKB) is a comprehensive repository for functional knowledge on proteins, complete with accurate, consistent, and detailed annotation. In addition to the necessary data for each UniProtKB entry (namely, the amino acid sequence, protein name or description, taxonomy data, and citation information), as much annotation information as feasible is provided. This contains generally established biological ontologies, classifications, and cross-references, as well as explicit indicators of annotation quality in the form of experimental and computational data evidence attribution.

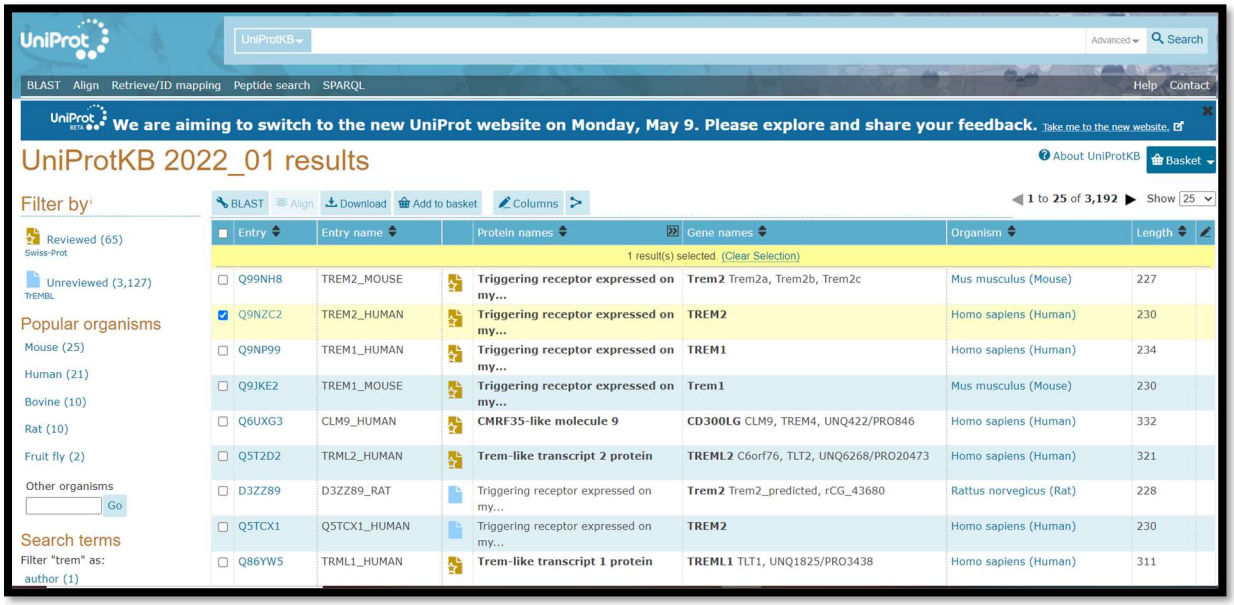

Figure 5: UniprotKB database

The results queried posits list of entries for the particular gene from a host of organisms, isoform or variants from which the gene of interest can be selected for further evaluation.

The repository contains detailed outline of the gene's functionalities and cited sources, addended for review of relevant literature on the topic.

Information related to databases for protein-protein interaction, gene expression, phylogenomic databases, family and domain databases, proteomic, enzyme, pathway and various other are provided for the gene of interest.

The query hit provides for variants (SNP's and frameshift mutations) from which relevant Variants of interest were extrapolated.

The database also provides for the 3d structure of the protein, that links to the RSCB PDB (Protein databank for the visualization of the protein structure, along with the composition Of Its secondary Structure (PDB I.D – 5ELI)

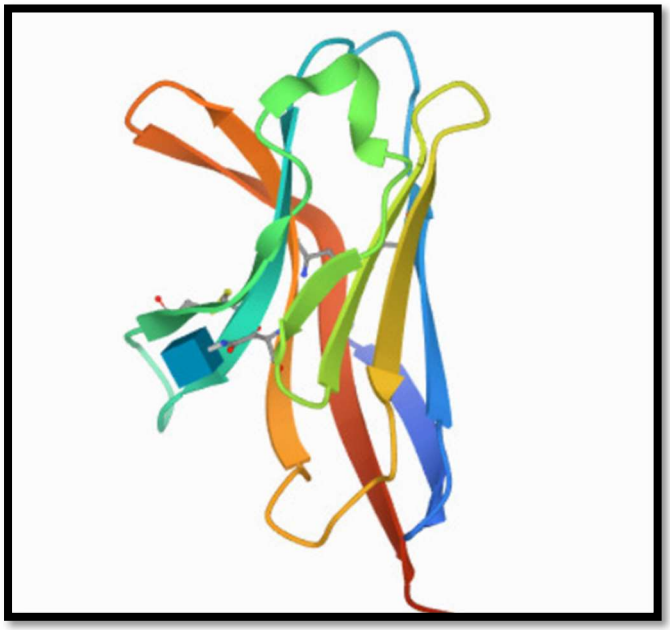

Figure 6: PDB I.D: – 5ELI (Chains A, B; Sequence Length 126)

Figure 7: Secondary Structure confirmation for Amino Acid Sequence of TREM2

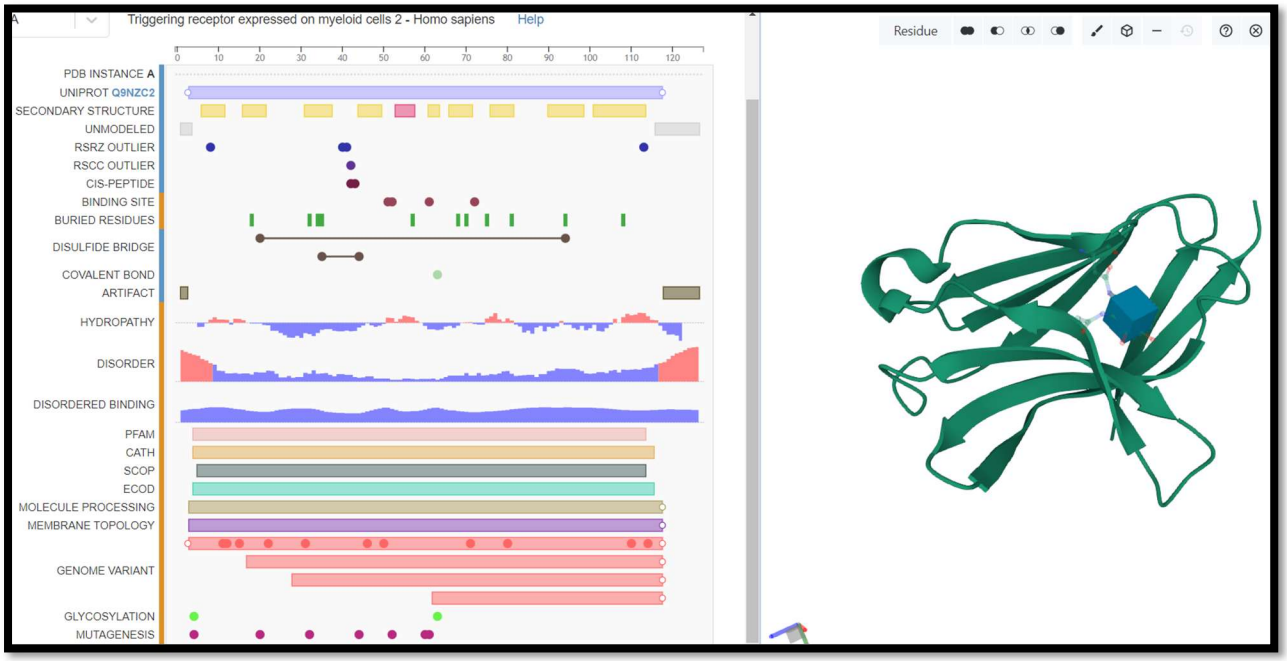

Figure 8: Protein structure feature view (mapping 1D positions of concern onto 3D structure of protein for assembly and visualization (Chain A of 5ELI).

Consurf:

The ConSurf server is a bioinformatics tool that uses phylogenetic relationships between homologous sequences to estimate the evolutionary conservation of amino/nucleic acid locations in a protein/DNA/RNA molecule. The evolutionary rate of an amino (or nucleic) acid position is highly influenced by its structural and functional relevance. As a result, conservation study of locations across members of the same family may frequently indicate the significance of each position for the structure or function of the protein (or nucleic acid). The evolutionary rate in ConSurf is calculated using the evolutionary relatedness of the protein (DNA/RNA) and its homologues, as well as the similarity of amino (nucleic) acids as represented in the substitutions matrix.

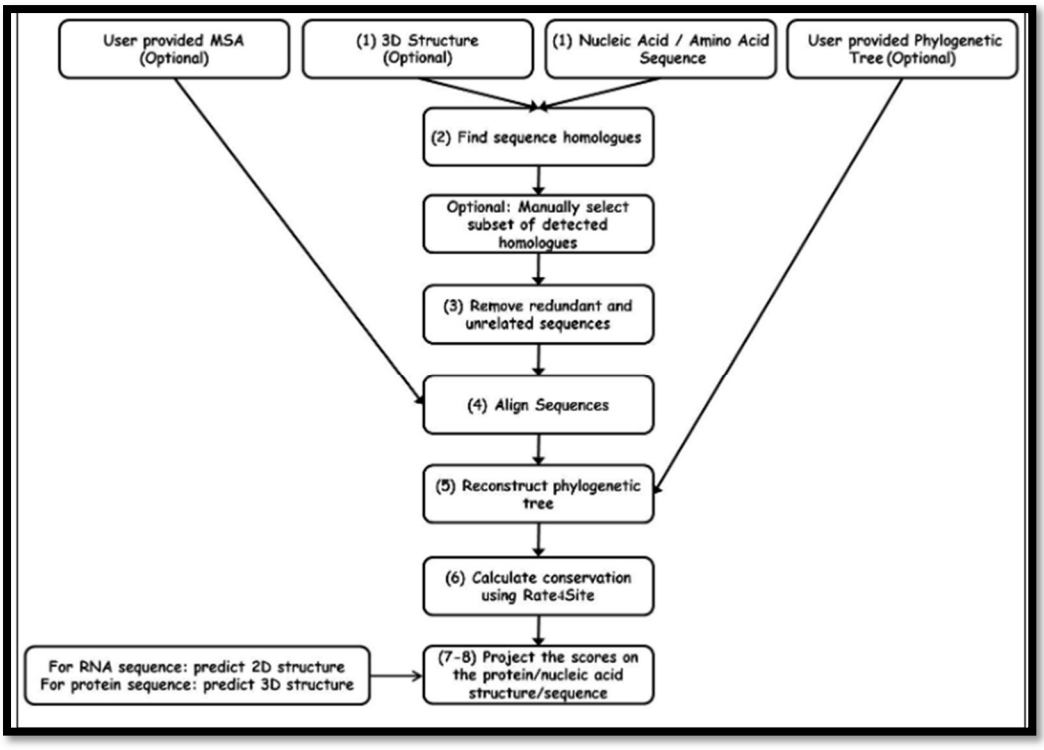

Figure 9: Consurf Protocol Flowchart

PDB structure of TREM2 (PDB Id: - 5ELI) was used to find regions of conservations in the two chains (Chain A and Chain B) which were denoted visually in a colour scheme to highlight the extent of conservation or variability in the different regions of the strucutres.

Clustal Omega:

Clustal Omega by European Bioinformatics Institute (EMBL-EBI), is a multiple sequence alignment tool that inputs multiple sequence queries and aligns them together in a computationally sound method, that reveals important insight and evolutionary relationships between multiple sequences that can be represented as phylogram or cladogram.

From the Uniprot database Multiple sequence Alignment was done on TREM2 (Q9NZC2) and its variants (Single Nucleotide Variants) and between TREM2 in Several Different Organisms: Q9NZC2 (human), Q99NH8 (mouse), D3ZZ89 (rat), H2QSZ0 (chimp), Q2YHU4 (chicken) and E2RP46 (dog) and cladogram was generated.

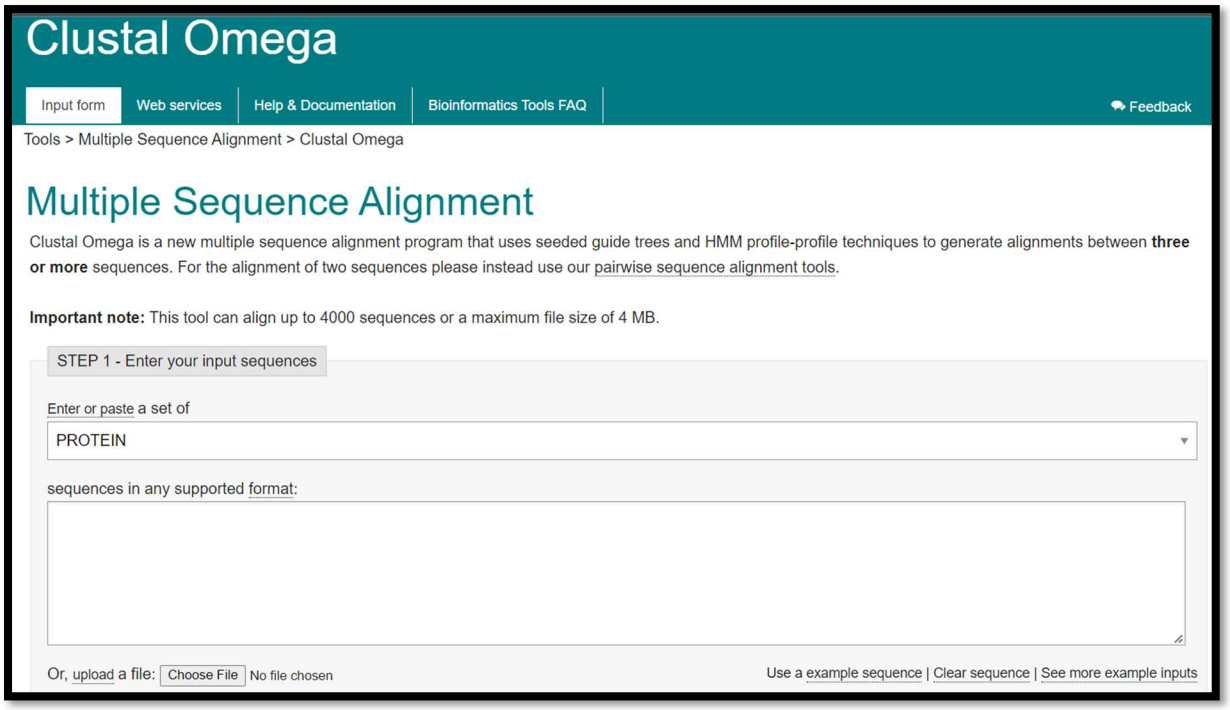

Figure 10: Clustal Omega for MSA

Shift:

Shift is an open-source web server by bioinfoindia.org that can detect buried stop codons in genomic DNA. It looks for hidden stops in both the +1 and -1 frame-shift genetic code systems. Stop codons that exist in a genetic sequence in the +1 or -1 frame are known as hidden stops. The frameshift mutation is caused by indels, which are single nucleotide insertions or deletions. Such indels may cause frame disruptions and concealed pauses.
The server calculates several codon types and their contributions to concealed stops. In the supplied sequence, it will also calculate the association between codon use frequencies and contribution of codons to hidden stops in off frame context (s). To get the t-values for statistically significant correlations, one-tailed t-tests are also used. The service aids studying frame-shifted translation in coding genomic sequences, as well as its evolutionary consequences and applications.

The frameshift analyses of TREM2 in the various aforementioned organisms were carried out and the correlation coefficient between codon usage frequency and codons conferring possible hidden stops was generated in graph format.

NCBI Conserved Domain Database (CDD):

CDD is a database of well-annotated multiple sequence alignment models for ancient domains and full-length proteins. These are accessible as position-specific score matrices (PSSMs), which may be used with RPS-BLAST to quickly identify conserved domains in protein sequences. CDD material comprises domain models imported from a variety of external source databases, as well as NCBI-curated domains that leverage 3D-structure information to clearly define domain borders and give insights into sequence/structure/function correlations.

Domain Identification of TREM2 on query returned the protein superfamily for the given Amino Acid Sequence.

Codon Usage bias:

Codon bias is the likelihood of the codon utilized for an amino acid over another codon that codes for the same amino acid. Synonym codons are different codons that code for the same amino acid. Even though synonymous codons encode the same amino acid, it has been discovered that distinct synonymous codons are employed at varied frequency in a wide range of species. Codon bias is the name given to this phenomenon.

Genetic drift, mutational pressure, and natural selection are all processes that impact changes in codon usage patterns, and these mechanisms are largely responsible for disparities in codon usage patterns between species.

GC, GC1, GC2, GC3s, A3s, T3s, C3, and G3s are codon usage indices denote the position at which the nucleotide occurs.

The ratio of observed codon frequency to predicted frequency is known as Relative synonymous codon usage (RSCU). If the RSCU value is 1, the codon is not biased; if the RSCU value is more than 1, the codon is often utilized.

Effective number of codons (ENC) is a measurement of non-uniform use among synonymous codon groupings. The ENC values range from 20 to 61 (severe bias, in which only one codon is utilized for one amino acid) to (random bias).

The Codon Adaptation Index (CAI) is a metric that assesses a gene's ability to adjust its codon use to those of highly expressed genes. The CAI scale is 0 to 1. The greater the value, the higher the gene expression and codon bias presented.

The above Codon usage bias metrics and evaluations were done using COUSIN and CAIcal for TREM2 in humans, along with a few other organismal entries.

PROVEAN:

Protein Variation Effect Analyzer (PROVEAN) is a bioinformatics tool that predicts the likelihood of sequence variants in conferring deleterious/damaging effects to the protein sequence/functionality.

PROVEAN can analyze and predict estimates as to whether a variant is damaging or not in a Sequence, which can be in forms of:

Single or multiple nucleotide insertions
Single or multiple nucleotide deletions
Single or multiple nucleotide substitution

A PROVEAN score of equal to or less than a predetermined threshold (e.g., -2.5) indicates the chance of the protein variation having a "deleterious" impact. The variation is anticipated to have a "neutral" effect if the PROVEAN score is over the threshold.

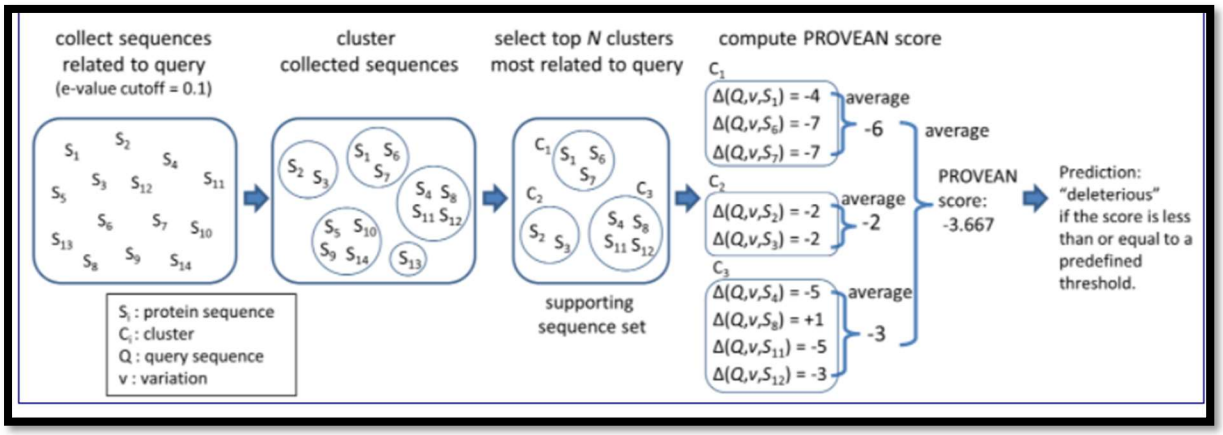

Figure 11: Computation Approach of PROVEAN's "Score".

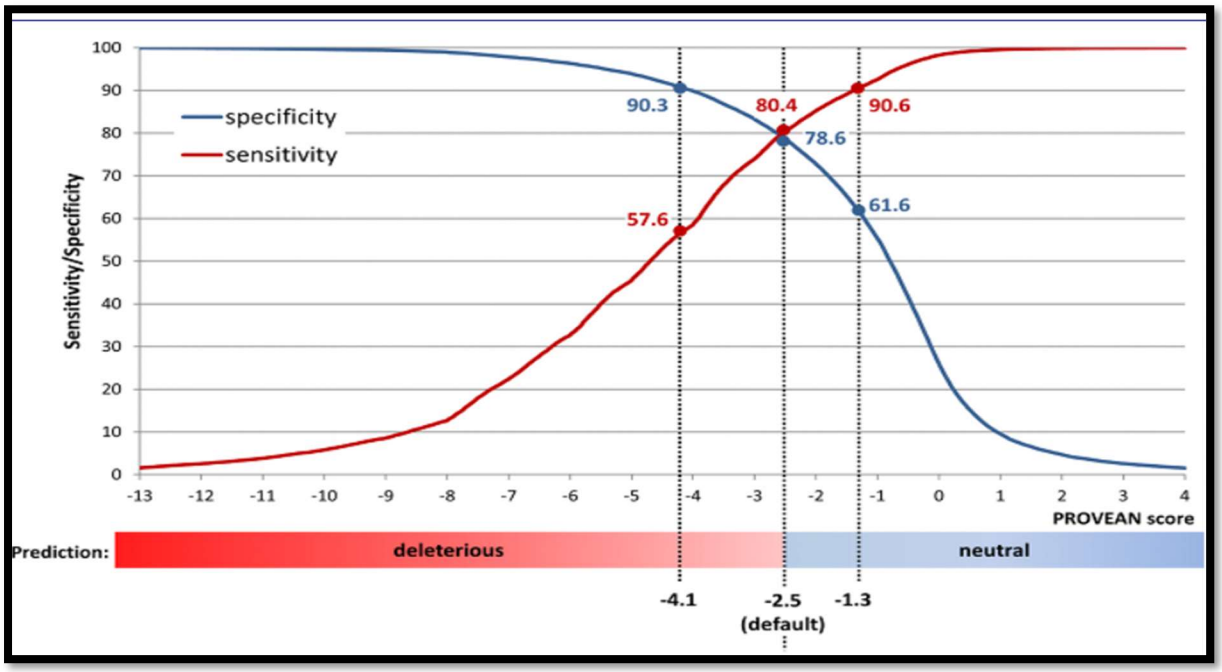

Figure 12: Plot of Sensitivity/Specificity Vs Deleterious/Neutral nature at various PROVEAN score Cutoffs

TREM2 Variants (SNP's) were selected for evaluating hypothetical polymorphisms that can be deleterious in nature Using PROVEAN.

Chapter-4 Results and Observations

The TREM2(Uniprot ID: Q9NZC2) isoform 1 is present at chromosome 6 (6p21) is of length 230 AA, presents 5 exomic region for protein translation.

Variants Y38C, R47H, R62H, T66M, N68K, D87N, T96K, R98W, R136Q, H157Y and L211P are highly conserved in mammalian genomes and are primarily expressed on the extracellular domain of the protein (Y38C, R47H, R62H, T66M, N68K, D87N, T96K, R98W, R136Q, H157Y).

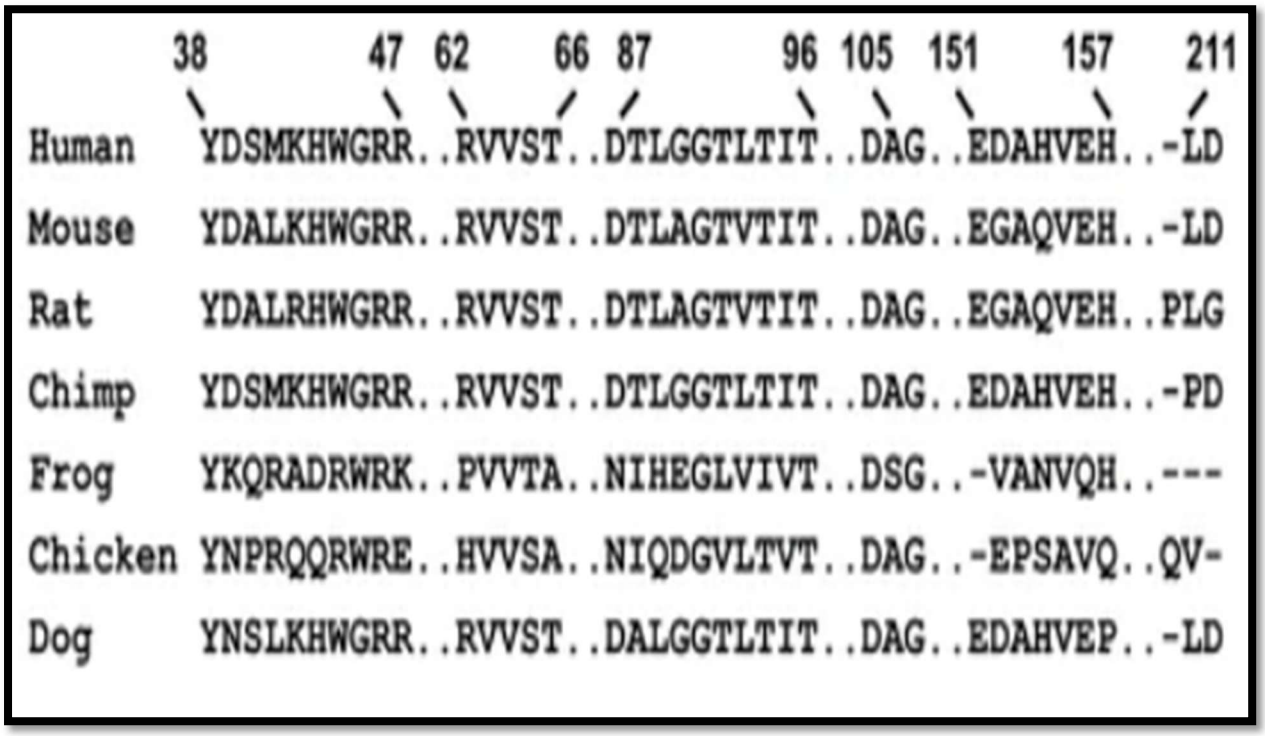

Figure 13: High levels of Conservation of Amino Acids Sequences in Aligned mammalian species

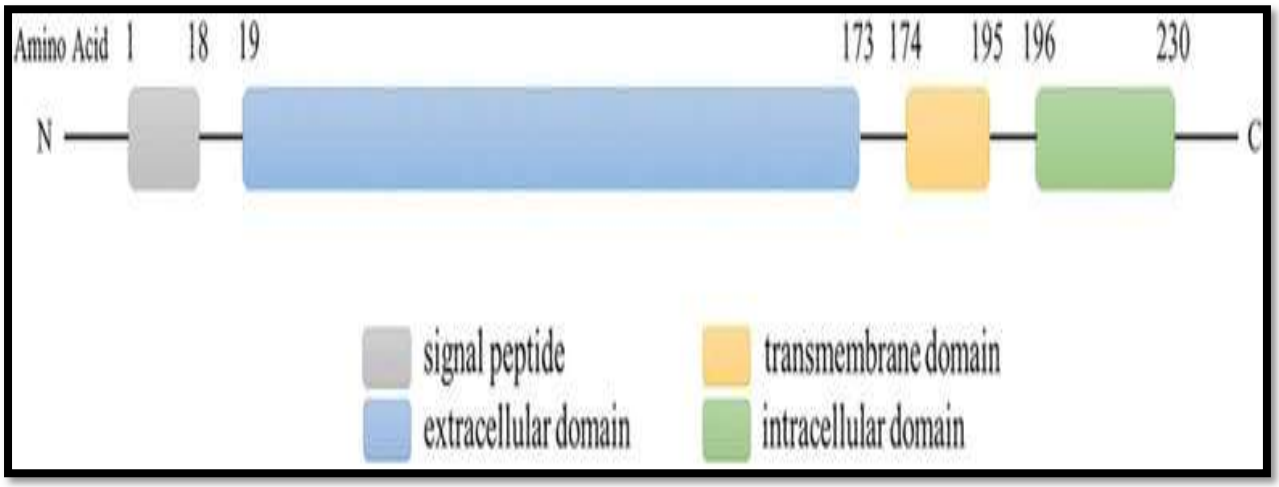

Figure 14: Domains encoded By particular regions of TREM2 sequence

Most of the reported variants that are thought to have an increased likelihood of causing Alzheimer's disease are present on the extracellular domain. The extracellular domain acts as the apical binding site, binding with anionic ligands such as DNA, Bacterial lipopolysaccharides, phospholoipids, and sulfatides.

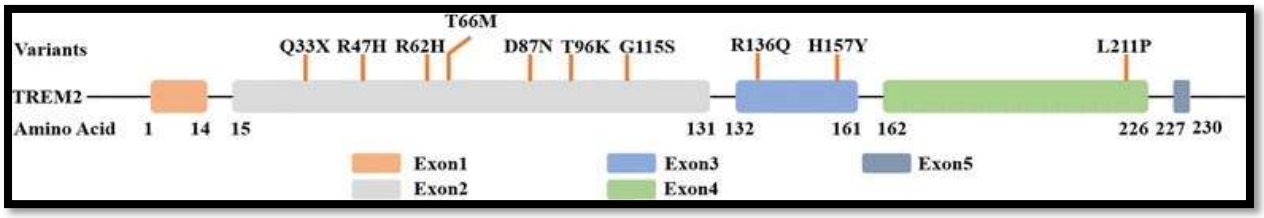

Figure 15: Variants present in the coding region (Exons) of TREM2

The Variants of Interests are mostly encoded in the exon region 2,3 and 4.

Using 5ELI as the as the input PDB.ID, The two chains A and B highlight the regions of high and variable conservation for the 3D TREM2 Structure.

Figure 16: Chain A

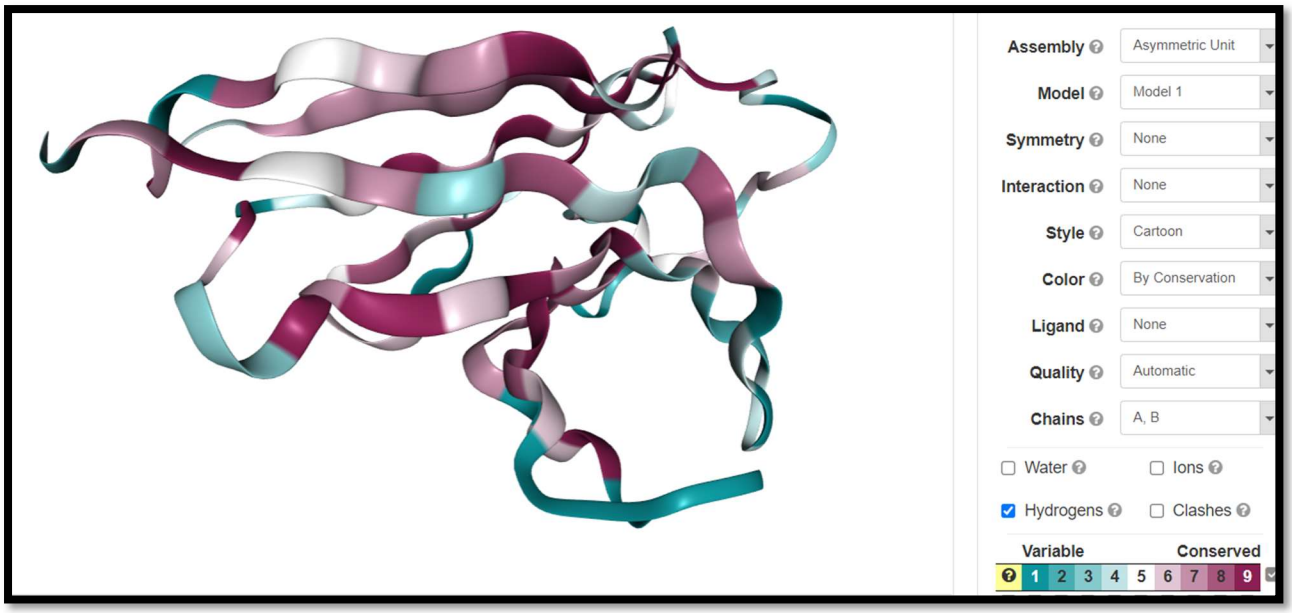

Figure 17: Chain B

The Multiple Sequence Alignment done On TREM2 in Several Different Organisms: Q9NZC2 (human), Q99NH8 (mouse), D3ZZ89 (rat), H2QSZ0 (chimp), Q2YHU4 (chicken) and E2RP46 (dog) and 24 natural variant after running generated the following Output:

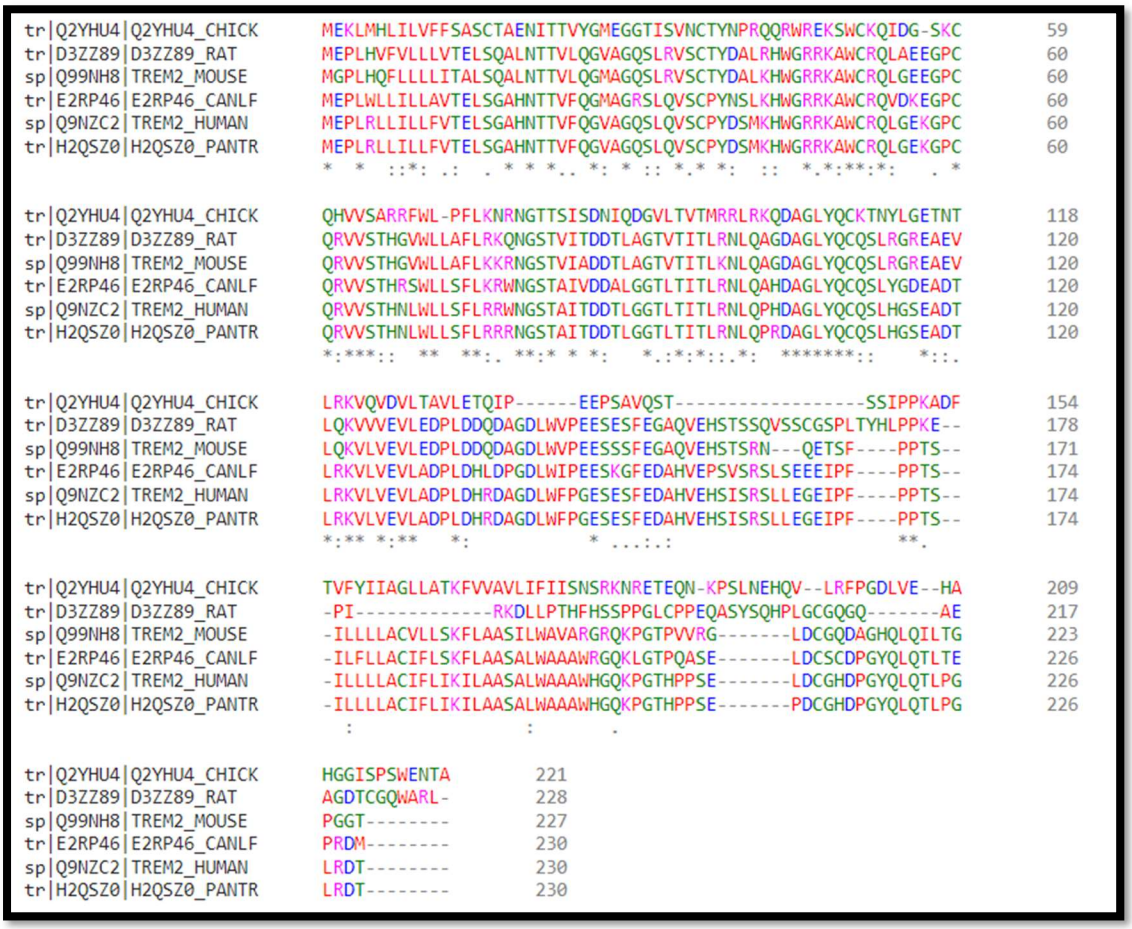

Figure 18: MSA of TREM2 sequence in different Organisms

From the MSA a phylogenetic tree and a phylogram were generated for the Aligned sequences.

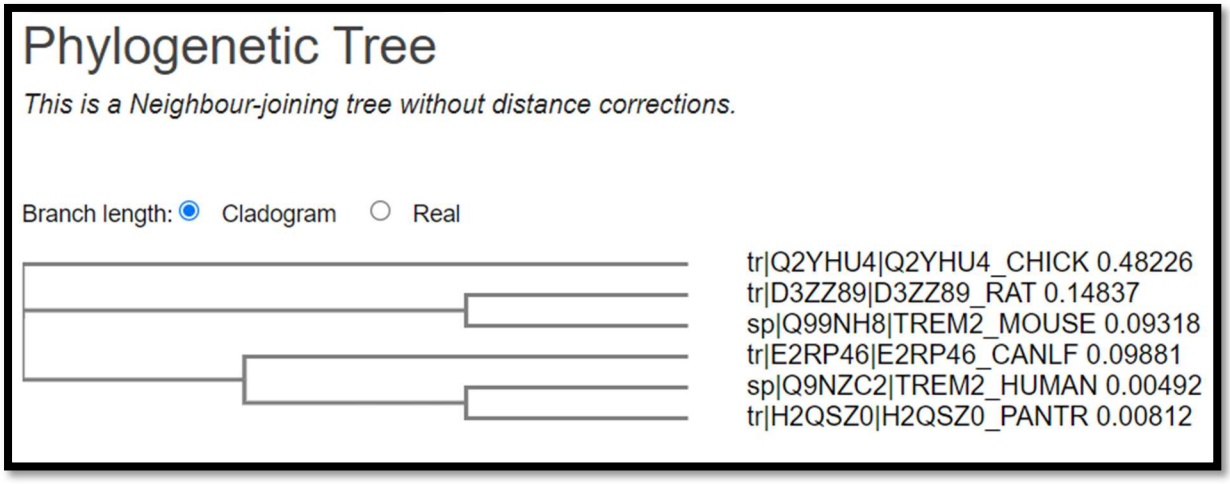

Figure 19: Phylogenetic Tree For TREM2 in different Organisms

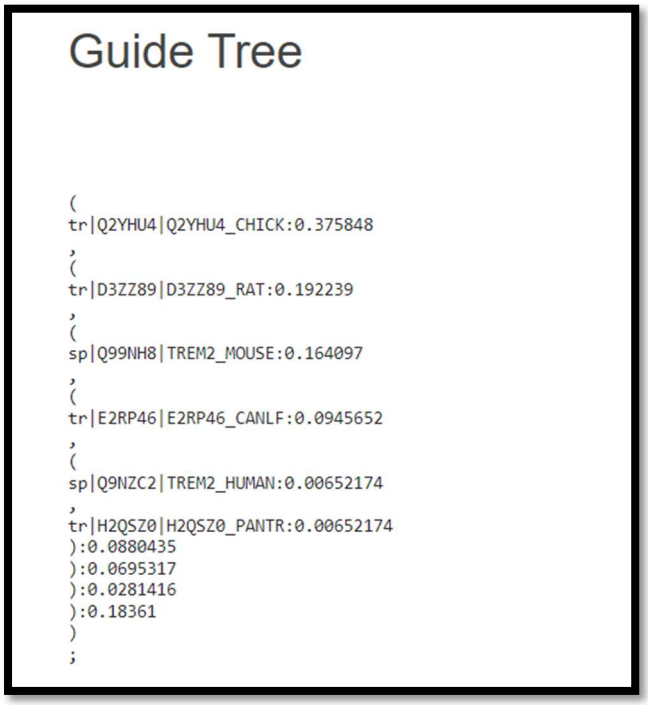

Figure 20: Guide Tree of TREM2

Figure 21: Phylogram of TREM2 in different Organisms

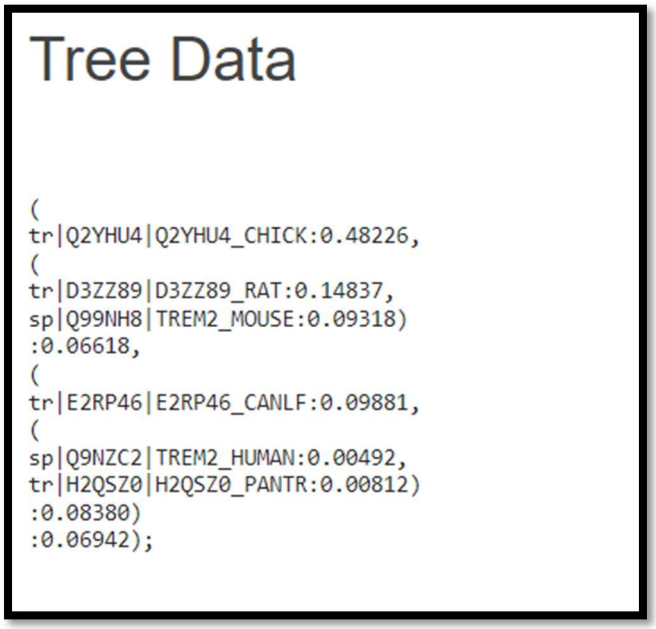

Figure 22: Tree Data

MSA for the variants V27M, A28V, S31F, Y38C, R47C, R47H, R62H, T66M, D87N, T96K, T96R, V126G, A130S, D134G, R136Q, R136W, E151K, H157Y, S162R, K186N, A192T, L211P, T223I generated the following phylogenetic tree:

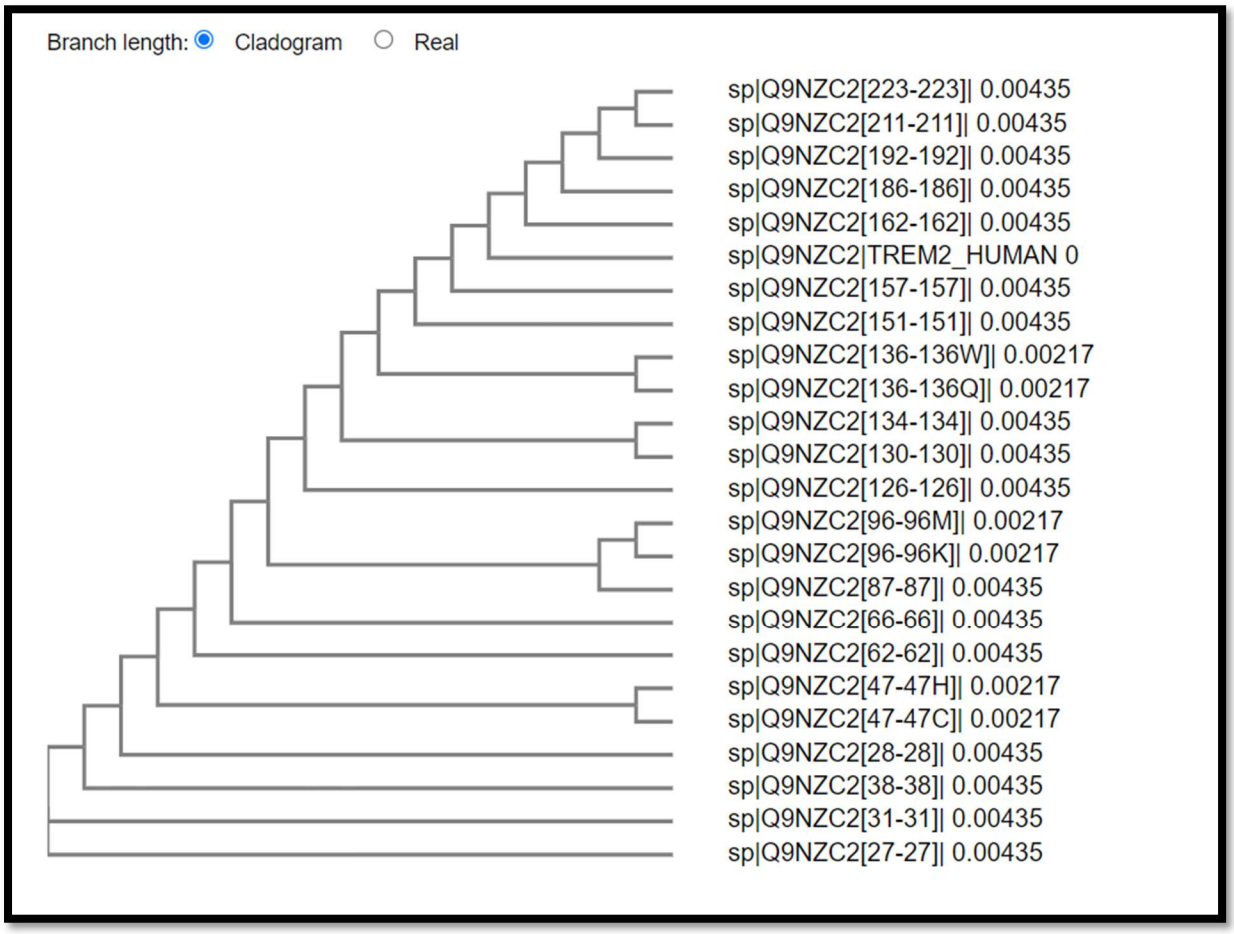

Figure 23: Phylogram for TREM2 variants in Human

For frameshift stop codon analyses, Using Shift webserver, the positions of the in-frame mutation for +1 and -1 was obtained along with correlation coefficient values for the graphs plotted for Codon usage frequency verses the category of Codon for the species groups Q9NZC2 (human), Q99NH8 (mouse), D3ZZ89 (rat), H2QSZ0 (chimp), Q2YHU4 (chicken) and E2RP46 (dog).

>Human

GC Count: 9.845% | **Length:** 693 | **CAI:** 0.757

Codon	# of hits	positions in +1 frame	positions in -1 frame
TAA (*)	0		
TAG (*)	1	563	
TGA (*)	10	122, 224, 680	114, 309, 348, 354, 441, 627, 645

Correlation Coeffcient(*r*): -0.616
negative t value

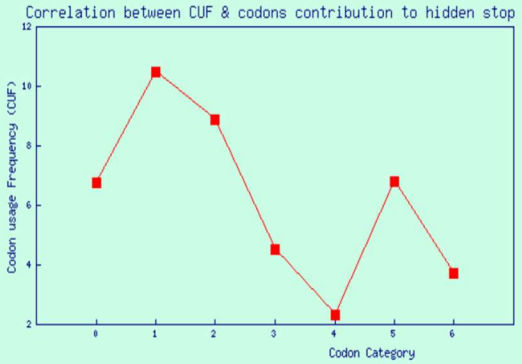

Figure 24: Frameshift codon analyses for Human

>Mouse

GC Count: 8.763% | **Length:** 684 | **CAI:** 0.730

Codon	# of hits	positions in +1 frame	positions in -1 frame
TAA (*)	0		
TAG (*)	1	398	
TGA (*)	13	32, 95, 122, 191, 224, 290, 542	114, 165, 258, 309, 354, 402

Correlation Coeffcient(*r*): -0.641
negative t value

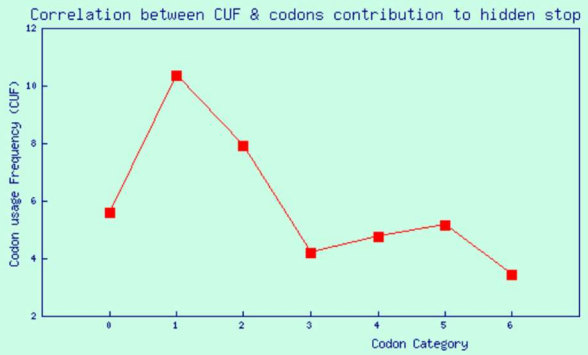

Figure 25: Frameshift codon analyses for Mouse

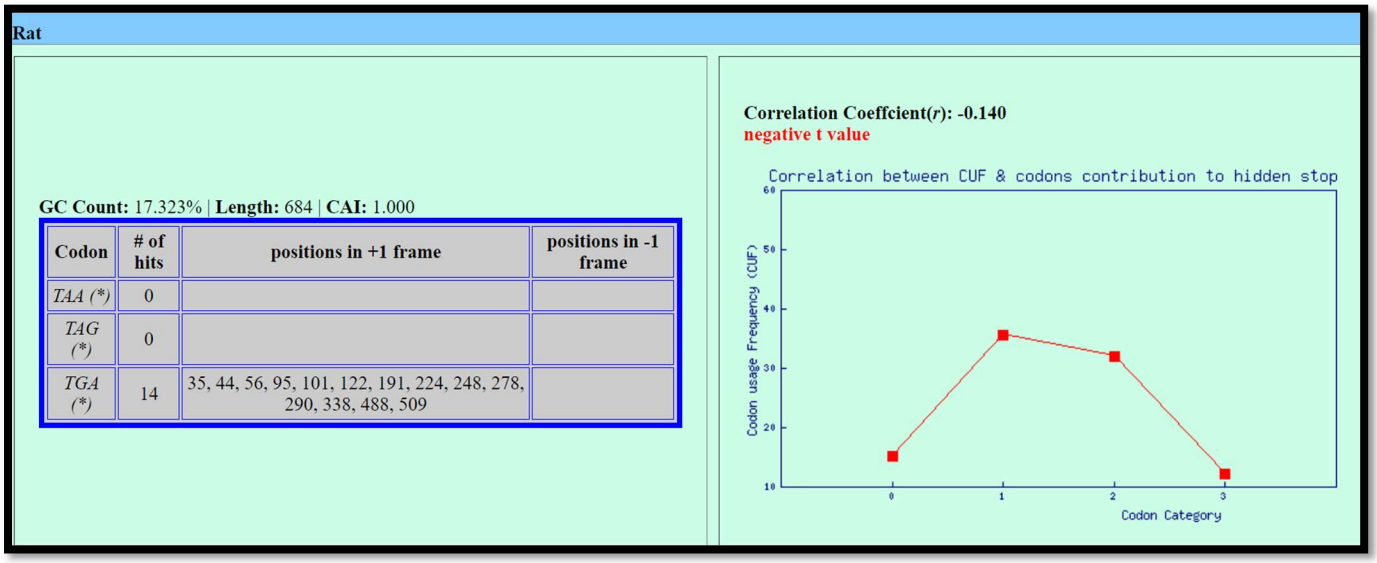

Figure 26: Frameshift codon Analyses for Rat

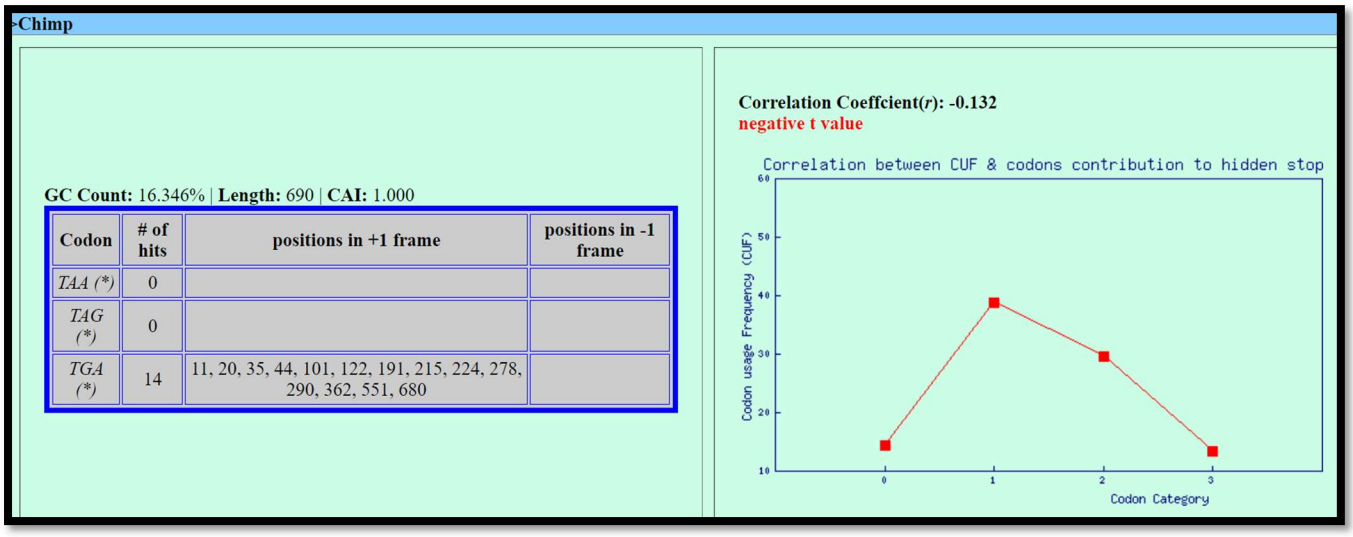

Figure 27: Frameshift codon analyses for Chimp

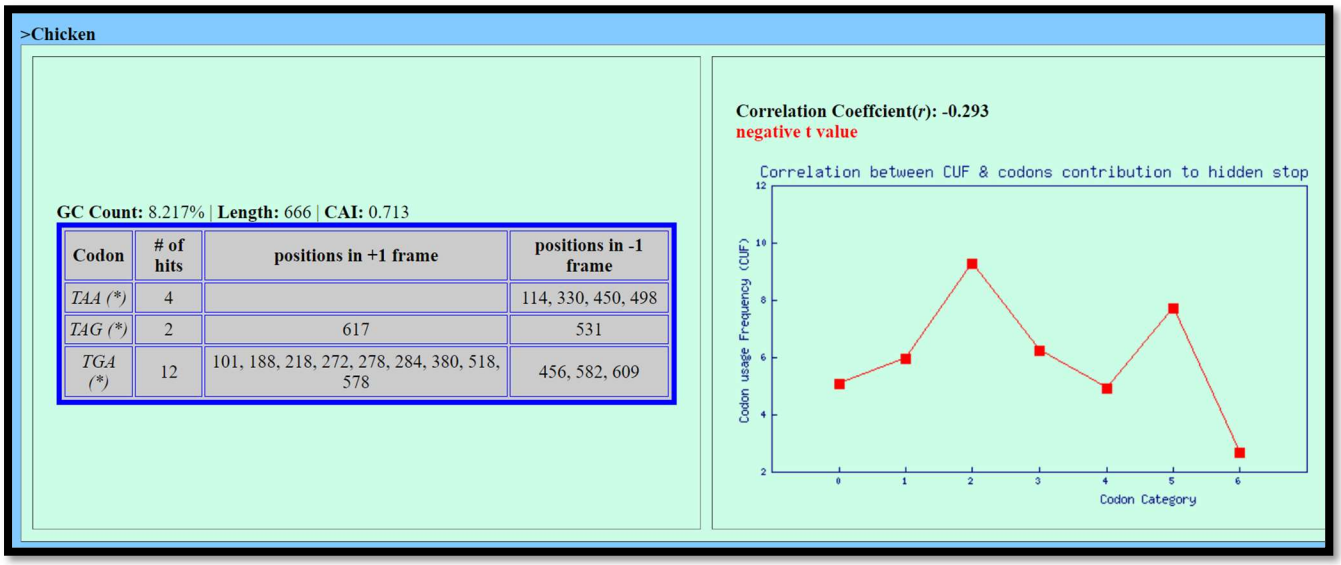

Figure 28: Frameshift codon analyses for chicken

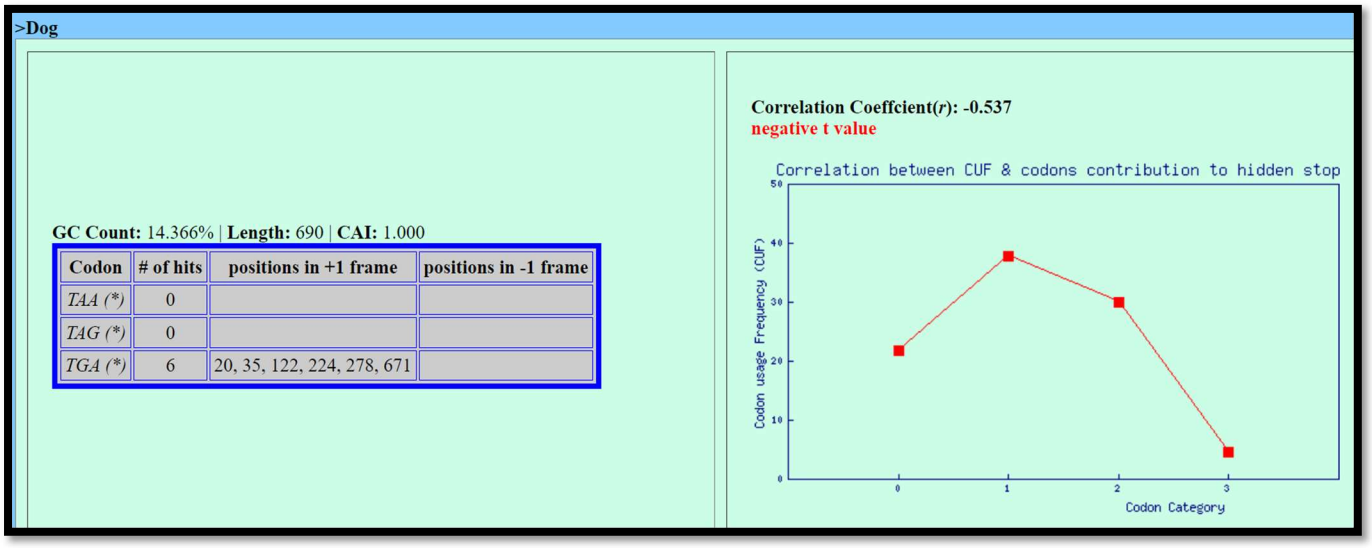

Figure 29: Frameshift codon analyses for Dog

The correlation coefficient "r" was negative for each nucleotide sequence of TREM2 suggesting an inverse relationship between the frequency of the codons used and the type of codons encoding for the amino acid. A summary table for the batch of sequences was created alongside, elucidating further features of the sequences in hand.

SHIFT: A Web Server for Hidden Stops analysis In Frameshifted Translation

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Summary Table

Seq #	Identifier	Length	CAI	GC %	Total # of HSC	CUF and Codon contribution Correlation	t Value
Seq1	>Human	693	0.757	9.845 %	11	-0.616	Negative T value
Seq2	>Mouse	684	0.730	8.763 %	14	-0.641	Negative T value
Seq3	>Rat	684	1.000	17.323 %	14	-0.140	Negative T value
Seq4	>Chimp	690	1.000	16.346 %	14	-0.132	Negative T value
Seq5	>Chicken	666	0.713	8.217 %	18	-0.293	Negative T value
Seq6	>Dog	690	1.000	14.366 %	6	-0.537	Negative T value

Figure 30: Comparative index table for different Organisms

Codon Adaptation Index (CAI) values assesses a gene's ability to adjust its codon use to those of highly expressed genes. The CAI scale ranges from 0 to 1. The greater the value, the higher the gene expression and codon bias presented. The above examples from the various organism, indicates a high level of CAI value for the TREM2 nucleotide sequence, suggesting the presence for higher codon bias for the encoding of amino acids by synonymous codons.

Using the CDD database, domain similarity search unearthed that the position 21-128 AA is a member of the Ig (Immunoglobin) superfamily, which includes immune system cell surface antigen receptors, co-receptors, and co-stimulatory molecules, antigen presentation to lymphocytes molecules, cell adhesion molecules, and some cytokine receptors.

Figure 31: CDD domain similarity Ig (Immunoglobin) Superfamily

Query sequence for TREM2 protein reveals its place in the Ig (Immunoglobin) superfamily of proteins.

Using CAIcal, the compositional information of Human TREM2 sequence was retrieved along with the RSCU values for the different codons involved in protein translation, delineating preferential bias of some synonymous codons over others. The data extrapolated is presented in tabular form elucidating a few interesting insights with regards to the nucleotide composition and synonymous codon bias.

NUCLEOTIDE COMPOSITION

SEQUENCES \ PARAMETERS	length	A	C	T	G	%A	%C	%T	%G	%G+C	%G+A	%G+T	%A+T	%A+C	%C+T	A1	C1	T1	G1	%A1	%C1	%T1	%G1
Human	693	140	217	135	201	20.20	31.31	19.48	29.00	60.32	49.21	48.48	39.68	51.52	50.79	51	75	35	70	22.08	32.47	15.15	30.30

TREM2 in Humans shows high G+C% content with the aforementioned nucleotides having a higher occupancy of the 1st codon position.

%G1+C1	%G1+A1	%G1+T1	%A1+T1	%A1+C1	%C1+T1	A2	C2	T2	G2
62.77	52.38	45.45	37.23	54.55	47.62	60	53	64	54

For the Second position in the codon lineup, A and T nucleotides have a slightly high occurance as compared to G and C, with nearly 53.68% percent of the 2nd position codon occurance being that of A or T

%A2	%C2	%T2	%G2	%G2+C2	%G2+A2	%G2+T2	%A2+T2	%A2+C2	%C2+T2	A3	C3	T3	G3
25.97	22.94	27.71	23.38	46.32	49.35	51.08	53.68	48.92	50.65	29	89	36	77

The 3rd codon position shows a high difference of occurrence with G and C nucleotides appearing 2.5-3 times higher than A and T nucleotides.

%A3	%C3	%T3	%G3	%G3+C3	%G3+A3	%G3+T3	%A3+T3	%A3+C3	%C3+T3	%G3s+C3s
12.55	38.53	15.58	33.33	71.86	45.89	48.92	28.14	51.08	54.11	71.04

%G3 + C3 is nearly 2.55 times higher than, %A3 and T3 in the occurrence of nucleotide at the 3rd position of the codon.

Following output is for the number of amino acids encoded by a particular synonymous codon, illustrating a clear bias or tendency for certain synonymous codon to be preferred over others.

CODON USAGE

CODONS	TTT	TTC	TTA	TTG	CTT	CTC	CTA	CTG	ATT	ATC	ATA	GTT	GTC	GTA	GTG	TCT	TCC	TCA	TCG	AGT	AGC	CCT	CCC	CCA	CCG	ACT	ACC	ACA	ACG
Aminoacids	F	F	L	L	L	L	L	L	I	I	I	V	V	V	V	S	S	S	S	S	S	P	P	P	P	T	T	T	T
Human	2	5	1	2	1	14	2	16	2	7	0	0	3	0	7	2	6	0	0	2	7	1	7	6	0	3	4	5	3

GCT	GCC	GCA	GCG	TAT	TAC	CAT	CAC	CAA	CAG	AAT	AAC	AAA	AAG	GAT	GAC
A	A	A	A	Y	Y	H	H	Q	Q	N	N	K	K	D	D
3	8	3	2	2	1	6	5	2	9	2	2	0	6	6	7

GAA	GAG	TGT	TGC	CGT	CGC	CGA	CGG	AGA	AGG	GGT	GGC	GGA	GGG	ATG	TGG
E	E	C	C	R	R	R	R	R	R	G	G	G	G	M	W
3	9	1	5	1	2	0	3	1	5	2	6	5	6	2	7

The Output presented below, highlights the RSCU values for the different codons occurring, which encodes for a particular amino Acid. RSCU values or Relative synonymous codon usage is the ratio of observed codon frequency to predicted codon frequency. If the RSCU value is 1, the codon is not biased; if the RSCU value is more than 1, the codon is often utilized.

Relative Synonimous Codon Usage (RSCU)

CODONS	TTT	TTC	TTA	TTG	CTT	CTC	CTA	CTG	ATT	ATC	ATA	GTT	GTC	GTA	GTG	TCT	TCC	TCA	TCG	AGT	AGC	CCT	CCC	CCA	CCG	ACT	ACC	ACA	ACG
Aminoacids	F	F	L	L	L	L	L	L	I	I	I	V	V	V	V	S	S	S	S	S	S	P	P	P	P	T	T	T	T
Human	0.57	1.43	0.17	0.33	0.17	2.33	0.33	2.67	0.67	2.33	0.00	0.00	1.20	0.00	2.80	0.71	2.12	0.00	0.00	0.71	2.47	0.29	2.00	1.71	0.00	0.80	1.07	1.33	0.80

The values for the synonymous codons generated, display and obvious bias or tendency for certain codons to be preferred over the others. The CAI value generated from the Shift tool further solidifies the observation, that there is a high level of bias for preferential codon usage when it comes to synonymous codon encoding for the same amino acid.

GCT	GCC	GCA	GCG	TAT	TAC	CAT	CAC	CAA	CAG	AAT	AAC	AAA	AAG	GAT	GAC
A	A	A	A	Y	Y	H	H	Q	Q	N	N	K	K	D	D
0.75	2.00	0.75	0.50	1.33	0.67	1.09	0.91	0.36	1.64	1.00	1.00	0.00	2.00	0.92	1.08

GAA	GAG	TGT	TGC	CGT	CGC	CGA	CGG	AGA	AGG	GGT	GGC	GGA	GGG
E	E	C	C	R	R	R	R	R	R	G	G	G	G
0.50	1.50	0.33	1.67	0.50	1.00	0.00	1.50	0.50	2.50	0.42	1.26	1.05	1.26

A follow up data table was generated for the comparison of the TREM2 nucleotide sequence between Human (Q9NZC2), chicken (Q2YHU4) and mouse (Q99NH8) Using COUSIN to compare the nucleotide 3rd position % occurrence, GC content and CAI and ENC values between the different homologous sequences. A similar comparison between the different TREM2 variants and the natural isoform 1 of TREM2 highlighted awfully little disparity to be considered significant for any further examinations.

Organism	Length	A3(%)	T3(%)	G3(%)	C3(%)	%GC(all)	CAI_59	ENC
Human	693	12.55	15.58	33.333	38.582	60.317	0.804	45.537
Chicken	666	14.414	16.667	32.432	36.486	53.303	0.847	45.42
Mouse	684	14.035	17.10	33.772	35.088	60.234	0.782	47.548

Values generated Using COUSIN.

Effective number of codons (ENC) is a measurement of non-uniform use among synonymous codon groupings. The ENC values range from 20 to 61 (severe bias, in which only one codon is utilized for one amino acid) to (random bias). The above examples highlight a tendency for the synonymous codons to have a relative bias that isn't extremely severe, for the various degenerate codons. The data trends for the vertebrate examples for TREM2 nucleotide sequences are highly similar, perhaps highlighting a good amount of conservation for the nucleotide sequence present in the natural isoforms over various species.

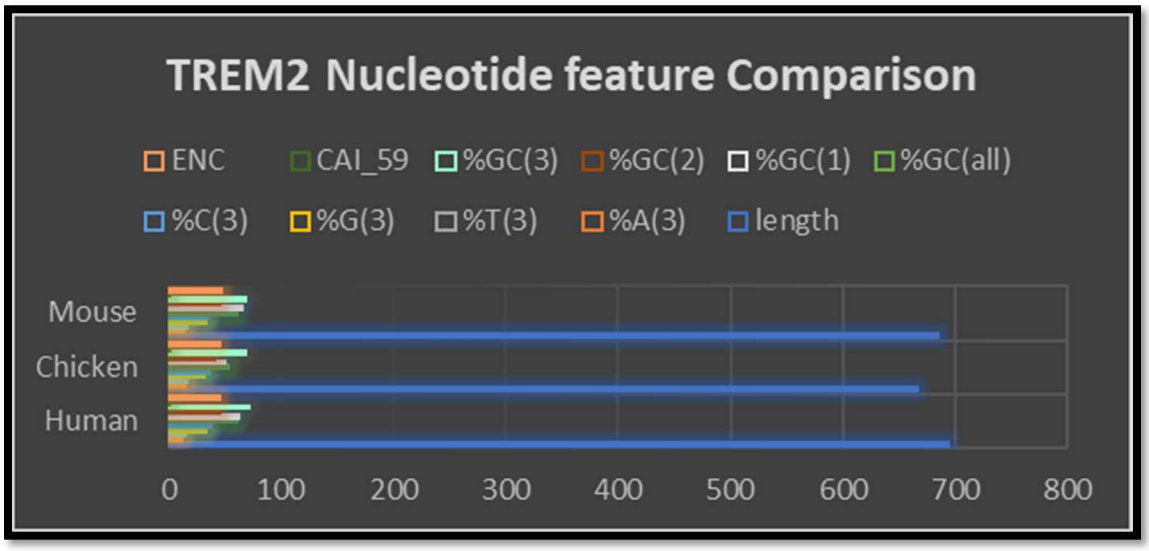

Figure 32: Comparative Codon Usage parameters between Human, Mouse and Chicken

Bar chart highlighting the various parameters for comparison between the three organisms selected.

Finally, the nature of the TREM2 variants occurring in humans was evaluated for their possible deleterious/neutral theoretical affect using PROVEAN for the 24 SNPs of particular interest. The following outputs their effect hypothesised under the particular cut-off following a specificity vs sensitivity comparison.

Variant	PROVEAN score	Prediction (cutoff= -2.5)
V27M	-0.569	Neutral
A28V	-0.879	Neutral
S31F	-4.441	Deleterious
Y38C	-8.803	Deleterious
R47C	-4.086	Deleterious
R47H	-2.661	Deleterious
R62H	0.031	Neutral
T66M	-3.578	Deleterious
D87N	-2.076	Neutral
T96K	-4.531	Deleterious
T96R	-4.448	Deleterious
V126G	-5.259	Deleterious
A130S	-0.181	Neutral
D134G	-1.021	Neutral
R136Q	1.053	Neutral
R136W	-1.257	Neutral
E151K	-1.141	Neutral
H157Y	-1.918	Neutral
S162R	-1.474	Neutral
K186N	-3.042	Deleterious
A192T	-0.287	Neutral
L211P	-1.041	Neutral
T223I	-2.166	Neutral

Figure 33: PROVEAN prediction for variants being possibly neutral or Deleterious.

Using the cut-off predictor, the variants at hand evaluated as deleterious exceed the threshold of -2.5. Out of the 23 variants selected for examination, 9 were deemed computationally significant enough to be deemed as variants of particular interest for deleterious damaging effect particularly variants S31F, Y38C, R47C, R47H, T66M, T96K, T96R, V126G, K186N.

Chapter-5 CONCLUSIONS

Discussion:

The above conducted analysis on various fronts attempts to extrapolate trends or features for the sequence of TREM2 in relation to its various variants that occur in the naturally presenting isoform in humans and when comparing it to various organisms that are vertebrates. Comparatively the mammalian examples of dog, mouse, rat and humans show a relatively higher conservation with reference to the various SNP variants reported as of significant interest for Alzheimer's disease. Consequently, the variants reported in various studies and via computation evaluations using tools such as PROVEAN and Poly-phen2 highlights the debilitating effect imparted by the SNP TREM2 in various NDD's encoded mainly by the exons 2, 3 and 4 of the proteomes. These Single nucleotide variations occur mostly in the extracellular domain of the protein, which structurally presents the V immunoglobin binding site, that binds to anionic ligands such as Aβ. A decrease in binding efficiency perhaps hampers the functionality of TREM2, that likely imparts the increased likelihood of AD being developed by those who express the TREM2 variants present in their systems. A further understanding of the binding disruption, efficiency and structural integrity of the ligand-receptor complex for the different variants can shed new light into the understanding of TREM2's functions and workings and its contribution or lack of in the pathologies of NDD's like Alzheimer's disease.

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