Syrian Arab Republic

Ministry of Higher Education and Scientific Research

Syrian Virtual University

الجمهورية العربية السورية

وزارة التعليم العالي والبحث العلمي

الجامعة الافتراضية السورية

In-Silico Identification of Single Nucleotide Polymorphisms (SNPs) Associated with Alzheimer's Disease

A project submitted for the Master's degree in Bioinformatics

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2023

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Aβ …….……..……. Amyloid beta

AD …….…… Alzheimer's Disease

AICD …….…… APP Intracellular Domain

AP-1 …….…… Activator Protein-1

APCs …… Antigen Presenting Cells

APP ……...… Amyloid Precursor Protein

APP-CTF …… Amyloid Precursor Protein C-terminal fragment

BACE1 ……..… Beta-site Amyloid Precursor Protein Cleaving Enzyme-1

BBB ……. Blood Brain Barrier

CD40L …… Cluster of differentiation 40 Ligand

c-Myb ……... Cellular Myelocytomatosis

CTCF …….…… CCCTC-binding Factor

CTLA4 …..…… Cytotoxic T-lymphocyte Associated Protein-4

DM1/2 …….. Diabetes Mellitus Type 1/2

Fasl …… Fas Ligand

FOXP3 …….… Forkhead Box P3

HEK ……..…… Human Embryonic Kidney cells

HLA …… Human Leukocyte Antigens

ICOS ……. Inducible Co-Stimulator

IFN-γ ……..… Interferon Gamma

IKK-γ …… Inhibitor of Nuclear Factor kappa-B kinase subunit Gamma

IKZF2 …… Ikaros Family Zinc Finger-2

IL-1 ……..… Interleukin-1

IL-1β ……...… Interleukin-1 beta

IL-2 ……..… Interleukin-2

IL-2R ……...… Interleukin-2 Receptor

IL-2RA ……..… Interleukin-2 Receptor Subunit alpha

IL-4 ……. Interleukin-4

IL-6 ……. Interleukin-6

IL-8 ……. Interleukin-8

IL-10 ……. Interleukin-10

IL-12 ……..… Interleukin-12

IL-17 ……..…… Interleukin-17

IL-17A ……...… Interleukin-17A

IPEX .. Immune Dysregulation Polyendocrinopathy Enteropathy X-linked

JNK ……..… c-Jun N-terminal Kinases

LAG3 ……...… Lymphocyte Activating 3

LD ……. Linkage Disequilibrium

LTA ……...… Lymphotoxin alpha

NFAT …… Nuclear Factor of Activated T cells

NF-κB ……...… Nuclear Factor kappa-B

NFKBIA/z ……..… Nuclear Factor kappa-B Inhibitor alpha/zeta

NFTs ……... Neurofibrillary Tangles

NK …… Natural Killer cells

MAPKs ……... Mitogen-Activated Protein Kinases

MHC …… Major Histocompatibility Complex

PAM ……...… Plaque-Associated Microglia

PRR …… Pattern Recognition Receptors

RA ……. Rheumatoid Arthritis

RIPK1 ……..… Receptor Interacting Serine/threonine-Protein Kinase 1

SLE …… Systemic Lupus Erythematosus

SNP ……...… Single Nucleotide Polymorphisms

SP1 ……..… Specificity Protein 1

Tau …… Tubulin Associated Unit

Teff ……..… T Effector cells

TGF-β …… Transforming Growth Factor beta

Th1/2 ……... T Helper cells 1/2

TNF-α/β …… Tumor Necrosis Factor-alpha/beta

TNFAIP3 ……..… TNF Alpha Induced Protein 3

TNFR1/2 ……..… Tumor Necrosis Factor Receptors 1/2

TNFRSF1B …….. TNF Receptor Superfamily Member 1B

Tmem ……. T Memory cells

TRAF2/6 ……...… TNF Receptor-Associated Factor 2/6

TRAIL …… TNF-Related Apoptosis-Inducing Ligand

Treg …… Regulatory T cells

TREM2 ……... Triggering Receptor Expressed on Myeloid cells 2

tTreg/pTreg ……..… Thymic/Peripheral Regulatory T cells

TYROBP …… Tyrosine Kinase-Binding Protein

YY1 ……..… Ying-Yang 1

Summary:

Background: With the increase in the aging population, the risk of age-related conditions such as AD has also grown. With AD being the most common neurodegenerative disease the need for further and detailed understanding of it also grew. One of the most important contributors to AD is neuroinflammation. As Aβ plaques aggregate in the brain, it triggers microglia to start phagocytosis but also triggers the production of cytokines and interleukins. In AD TNF-α is chronically released which causes chronic inflammation that stimulates Aβ production and aggregation contributing to the dysregulation of the immune system and the aggravation of neuroinflammation and neurodegeneration in Alzheimer's disease.

Aim of the study: in this project, we aim to identify genes contributing to neuroinflammation present in Alzheimer's disease, identify SNPs located in these genes, and test if they are in linkage equilibrium thus, they could be inherited as a haplotype. Also identifying a haplotype that could possibly be frequent in populations and associated with AD.

Methods: We used in silico approaches to identify SNPs related to TNFα and other immune factors affecting Alzheimer's disease whether directly or indirectly. We used various databases to search for genes that regulate inflammation through cytokines, interleukins, and immune cells**.**

Results: We identified 5 genes on chromosome 6 that are linked to inflammation, TNF-α, and Treg cells. We tested various SNP pairs to check if they are linked, and found 49 pairs to be in LD out of 84 pairs tested using LDpair. SNPs in high LD were selected and tested with LDhap to generate possible haplotypes. One haplotype with a frequency of 1.72% containing 4 significant SNPs was selected, which could be close to AD frequency in the elder (above 65 yrs) population (10.7%).

Conclusion: We searched several online databases and bioinformatics tools to find SNPs on chromosome 6 that have been shown to be in linkage disequilibrium, and a haplotype has been shown to have genetic variations significant to the role of the immune system in AD pathology, and a frequency of 0.0172 close to that of AD in the elderly population. Further studies should be done to investigate the association of these SNPs, and the actual frequency and importance of this haplotype among different populations, and if it can be used to predict AD in individuals and investigate other genes and SNPs that could belong to the same haplotype.

Introduction:

Alzheimer's disease (AD) is the most common diagnosis when it comes to neurodegenerative diseases (1). The increase in the aging population has increased the risk of age-related conditions, leading to the prediction that AD will become more and more widespread in society (2). AD is known to have characteristic extracellular amyloid-β (Aβ) deposition and intracellular accumulation of hyperphosphorylated aggregated tau. (1)

Aβ is cleaved from amyloid precursor protein (APP) by β and γ-secretase, producing Aβ of different lengths (although typically 40 and 42 amino acids in length) (Figure 1), and these monomers aggregate into oligomers and fibrils, ultimately forming Aβ-containing plaques that are 200–650 μm^2 in size. Aβ can also undergo posttranslational modifications such as phosphorylation and nitration, which accelerate this process (3). Tau is a protein found in neurons that are involved in microtubule stabilization, however, in AD tau becomes hyperphosphorylated and accumulates forming neurofibrillary tangles (NFTs). These deposits vary considerably in size from 10 to 500 μm^2. The severity of pathological changes (particularly the NFTs) correlates with neuronal loss and increased cognitive decline in those with AD (2).

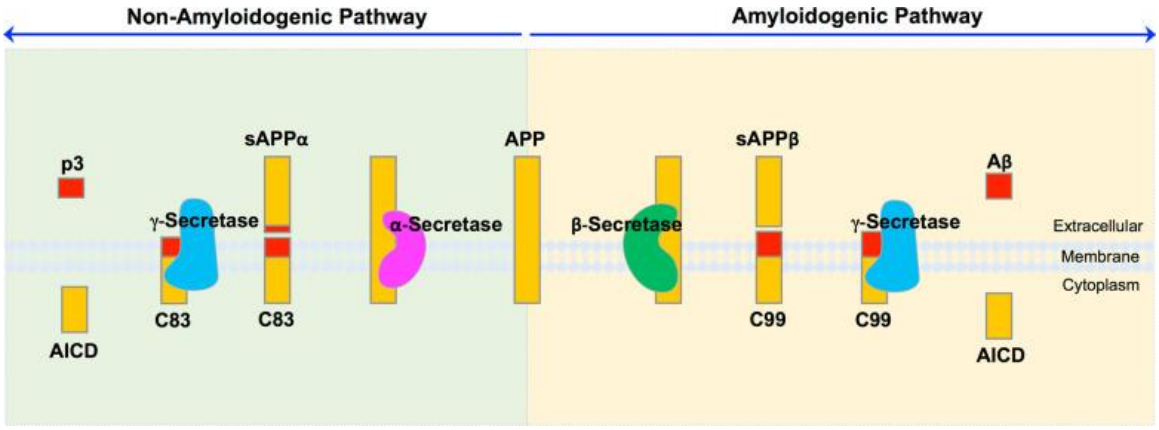

Figure 1: APP processing. In the amyloidogenic pathway, β-secretase cleaves APP extracellularly to release sAPPβ and a membrane-bound APP-CTF (C99). C99 is subsequently cleaved by γ-secretase to release Aβ and the APP intracellular domain (AICD). In the non-amyloidogenic pathway, APP is cleaved by α-secretase to release sAPPα and a membrane-bound APP-CTF (C83). C83 is cleaved further by γ-secretase to release p3 and AICD. Adopted from ref (10).

It has been found that several key regulators of innate immune pathways are genetic risk factors for AD. Emerging evidence points to the adaptive immune response having a role in disease pathogenesis as well (1). Microglia form the innate immune system in the brain, playing a central

role in protecting the brain during infections that trigger encephalitis (2). Furthermore, scientists have displayed a clear role for microglia-mediated neuroinflammation in the progression of AD (2). Alois Alzheimer himself noted the first signs of microglial activation when he described AD. Additionally, many risk genes identified in genome studies are highly expressed in microglia such as TREM2 or CD33 (3). In the early stages of Alzheimer's disease, Hong et al. demonstrated the possible role of dysregulated microglia in damage to brain synapses. Which plays a key role in the progression of neurodegenerative diseases (4). It is also important to mention that the peptides found in the AD brain trigger the activation of the microglial innate immune system. As the amount of these peptides continues to build instead of being removed from the environment of the microglia, the result is developing a case of chronic microglial activation (2). (Figure 2).

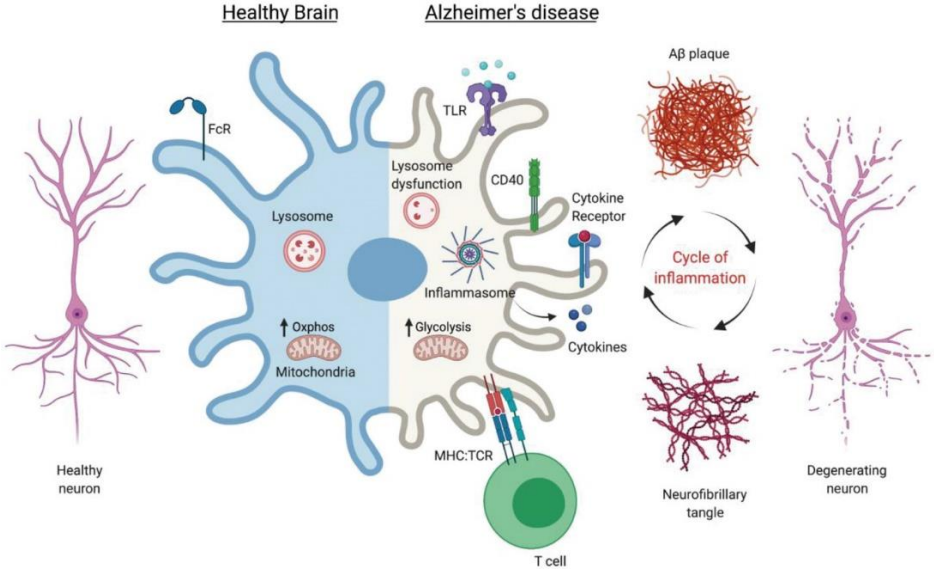

Figure 2: Healthy vs AD brain. During healthy aging, the microglia provide homeostatic support to neurons, they are anti-inflammatory and efficient at phagocytosis. In AD, the microglia become chronically activated due to the growing presence of Aβ or tau. In addition, there is increased infiltration of T cells in the AD brain, which contributes to the inflammatory environment. Together this creates an endless cycle of inflammation leading to neuronal death. Adopted from ref. (2).

Furthermore, Insoluble Amyloid β (Aβ) plaques in the brain of patients with AD attract a distinct population of myeloid cells known as plaque-associated microglia (PAM). These PAM cells form clusters that are two to five times denser than normal brain tissue (4). On binding, PRR receptors can mediate phagocytosis but they also trigger downstream signaling and activation of transcription factors to produce inflammatory cytokines and chemokines (such as TNF-α, IL-1β, and IL-6) that, initially, can help with

the detection and removal of pathogens (5). In AD, the same process can be initiated by Aβ itself both in vitro and in vivo (5).

In a recent study, human TNF-α cDNA flanked with a human cytomegalovirus promoter was delivered to the hippocampal CA1 region via an adeno-associated virus in 2-month-old 3xTg-AD mice (pre-pathological stage) for specific overexpression of TNF-α in neurons (6). This manipulation induced the activation of microglia and neuronal death, revealing that TNF-α-driven inflammation may have a deleterious effect on neurons (6). Combined with studies that reported activated microglia surrounding amyloid oligomers and senile plaques in acute and genetic rodent AD models (7), as well as in human brains (8) the data strongly suggest that TNF-α is chronically released during the course of AD, likely by activated microglia, neurons, and astrocytes stimulated by increased levels of extracellular Aβ (9). TNF-α increases the production of other pro-inflammatory cytokines, such as IL-1, IL-6, IL-17, and IL-8 (10), that can participate in the development of chronic inflammation when not counterbalanced by anti-inflammatory cytokines (e.g., IL-10). Furthermore, TNF-α was shown to stimulate the expression of APP and BACE1 in primary cultures of mouse astrocytes, as well as stimulate γ-secretase activity in HEK cells, which results in the release of Aβ peptides in large amounts (11-13). Neurodegenerative disorders are associated with chronic central inflammation (14) once chronic brain inflammation is engaged, a detrimental, auto-amplified upward spiral maintains excessive levels of TNF-α, which could stimulate Aβ synthesis and neuronal loss, as well as inhibit microglia phagocytosis of Aβ (15). TNF-α and IL-1β also inhibit the ability of microglia to phagocytose Aβ, leading to an increase in production alongside a reduction in the removal of Aβ, eventually facilitating the aggregation and deposition of Aβ in AD (16). On the other hand, TNF-α can impair the function of transporters and receptors that mediate the efflux of Aβ across BBB thus aiding in the accumulation of Aβ (17).

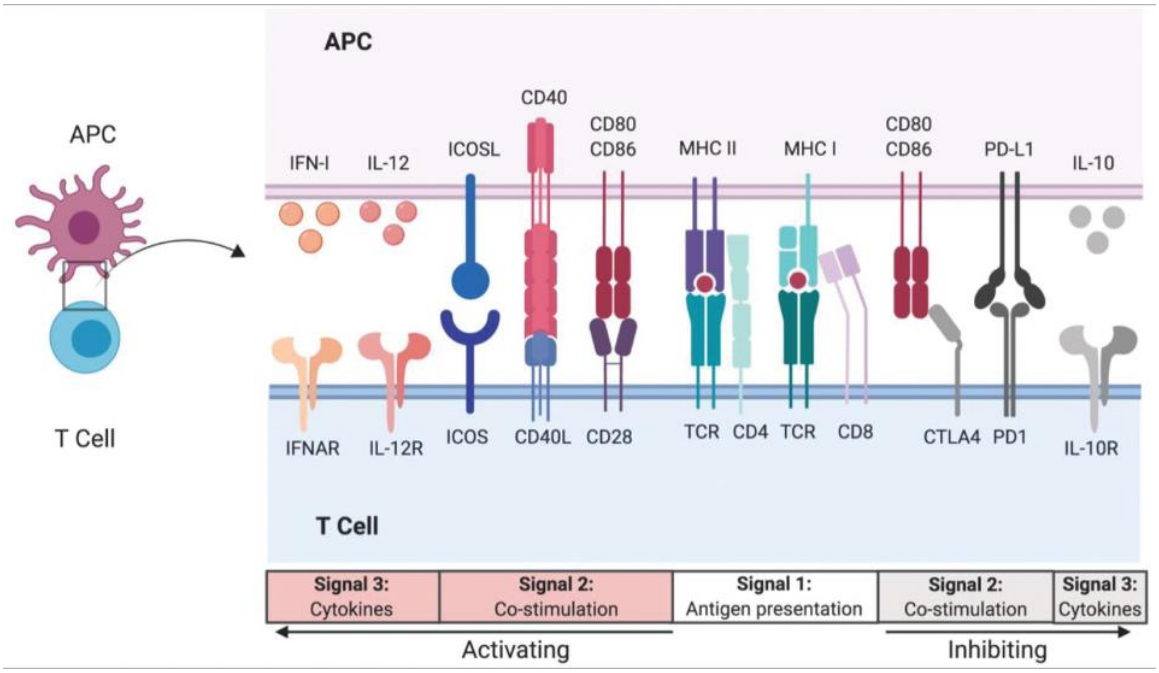

Figure 3: T cells activation and inhibition. APCs engage with T cells via MHC 1 and 2 molecules, then either a) APCs activate T cells via CD40 and cytokines like IL-12 and IFN-1, or b) inhibit T cells via CD80/CTLA4 and cytokines like IL-10. Adopted from ref (2).

Whenever the role of immunity is talked about, Regulatory T (Treg) Cells should be mentioned. As they are essential for maintaining peripheral tolerance, preventing autoimmune diseases, and limiting chronic inflammatory diseases (18). Tregs are a subtype of T cells that express $CD4^{+}$ $CD25^{hi}$ $FOXP3^{+}$ on their surface; it plays a crucial role in maintaining immune balance by preventing autoimmunity and limiting chronic inflammation. The various potential suppression mechanisms used by Treg cells can be grouped into four basic modes of action: 1} suppression by inhibitory cytokines such as IL-10 and TGFβ (19,20), 2} suppression by cytolysis mediated through the secretion of granzymes A and B (21-23), 3} suppression by metabolic disruption by the high expression level of CD25 empowers Treg cells to 'consume' local IL-2 and therefore starve actively dividing effector T cells by depleting the IL-2 they need to survive (24,25) or the expression of the ectoenzymes CD39 and CD73 was shown to generate pericellular adenosine, which suppressed effector T-cell function through activation of the adenosine receptor 2A (26,27), and 4} suppression by modulation of dendritic-cell (DC) maturation or function by utilizing CTLA-4 and/or LAG3 (28).(Figure 4).

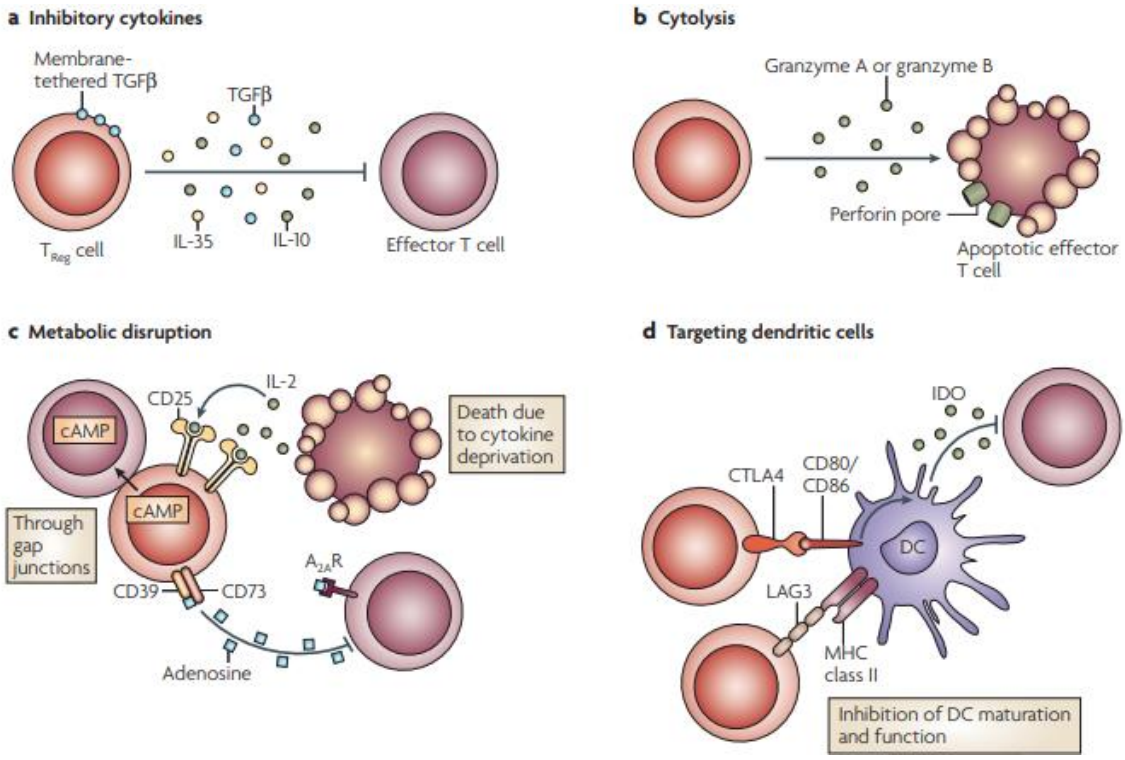

Figure 4: basic mechanisms used by T reg cells. a) Inhibitory cytokines such as IL-10, IL-35, TGFβ. b) cytolysis by granzymes A & B. c) metabolic disruption. d) targeting dendritic cells via CTLA4 & LAG3. Adopted from ref (28).

Several studies have shown that Treg cells are impaired in number, phenotype, and function in patients with Alzheimer's disease or animal models of the disease (29-31). This impairment may contribute to the dysregulation of the immune system and the aggravation of neuroinflammation and neurodegeneration in Alzheimer's disease (29,30).

Since AD is associated with neuroinflammation, it would be logical to assume that SNPs linked to autoimmune diseases and high expression of cytokines could possibly increase the risk of AD in patients or non-symptomatic people carrying these SNPs.

Methodology:

we started by searching databases such as Genebank (https://www.ncbi.nlm.nih.gov/genbank/), SNPedia (https://www.snpedia.com/index.php/SNPedia), OMIM (https://www.omim.org/), Alzgene (http://www.alzgene.org/), and dbSNP (https://www.ncbi.nlm.nih.gov/snp/), looking at chromosomes and genes expressing or regulating TNF-α, t reg cells, different cytokines, and

interleukins, and some single nucleotide polymorphisms (SNPs) that affect them.

Chromosome 7

IL6: a cytokine that could be anti- or pro-inflammatory, it enhances the differentiation and activation of b cells, t cells, and macrophages, it can also induce TNF-α expression in macrophages through the activation of NF-κB and AP-1 transcription factors (32,33). It can inhibit the differentiation and function of t-reg cells and could induce the conversion of t-reg cells into th17 cells which are pro-inflammatory, therefore it contributes to autoimmune diseases such as RA (34-36).

An increase in IL-6 levels due to SNPs in the promoter region like rs1800796 (572 G>C) also rs1800795 (-714 C>G) especially the G allele which is associated with higher levels of this interleukin and thus autoimmune diseases like DM typ2 and Crohn's, could cause an increase of TNF-α via certain pathways which could contribute to the cycle of TNF-α, induced immune response and AB aggregation (37,38).

Chromosome 10

IL2RA: This gene encodes a protein called interleukin 2 receptor subunit α (CD25), a part of the interleukin 2 receptor IL-2R. IL-2R mediates the function of IL2 in the regulation of the immune system by controlling Treg cells, IL-2Rα is selectively expressed by Treg cells, it enhances the binding affinity of IL-2 to IL-2R complex and increases the responsiveness of Treg cells to IL-2. (39,40).

This protein interacts with another protein called TYROBP forming the IL-2RA-TYROBP complex that is found in different immune cells such as microglia and dendritic cells and transmits signals to activate the immune system (40). IL2 promotes the development of Treg cells in the thymus and their homeostasis and expansion in the periphery, it also maintains the stability and suppressive function of Treg cells by inducing the FOXP3 and other genes involved in T reg identity and activity (41).

Mutations or variants in the IL2RA gene can affect the expression or function of IL-2Rα and impair the development, survival, or function of Tregs. This can result in reduced numbers or impaired activity of Tregs, leading to increased susceptibility to autoimmune diseases, such as type 1 diabetes mellitus, multiple sclerosis, and immunodeficiency 41 with lymphoproliferation and autoimmunity (41). rs2104286: This SNP is located in the promoter region of the IL2RA gene and affects the binding

of transcription factors, such as NFAT and AP-1, that regulate the expression of IL-2Rα. This SNP is associated with lower levels of IL-2Rα on T cells and reduced responsiveness to IL-2. This SNP is also associated with an increased risk of developing multiple sclerosis and type 1 diabetes mellitus (42). rs12722489: This SNP is located in the second intron of the IL2RA gene and affects the binding of transcription factors, such as CTCF and YY1, that regulate the expression of IL-2Rα. This SNP is associated with lower levels of IL-2Rα on T cells and reduced responsiveness to IL-2. This SNP is also associated with an increased risk of developing multiple sclerosis and type 1 diabetes mellitus (43). rs3118470: This SNP is located in the third exon of the IL2RA gene and causes a synonymous mutation that does not change the amino acid sequence of IL-2Rα. However, this SNP affects the splicing efficiency and stability of the IL2RA mRNA, leading to lower levels of IL-2Rα protein. This SNP is also associated with an increased risk of developing multiple sclerosis and type 1 diabetes mellitus (43).

Chromosome 4:

IL2: The IL2 gene is a gene that encodes a protein called interleukin 2 (IL-2). IL-2 is a cytokine that is produced by activated T cells and regulates the growth and function of immune cells, especially T cells. As we mentioned above, IL-2 binds to a receptor complex composed of three subunits: IL-2Rα (also called CD25), IL-2Rβ (also called CD122), and IL-2Rγ (also called CD132) (44). IL-2 signaling through this receptor complex promotes the development, survival, and function of various types of T cells, such as helper T cells, cytotoxic T cells, and regulatory T cells (Tregs). IL-2 is important for maintaining immune tolerance and preventing autoimmune diseases (44). IL-2 promotes the development of Treg cells in the thymus and their homeostasis and expansion in the periphery, it also maintains the stability and suppressive function of Treg cells by inducing the FOXP3 and other genes involved in T reg identity and activity (40,44). SNPs in the IL-2 gene causing a lower expression of this cytokine could impair or lose function in Tregs and their role as immune regulators. rs2069762: This SNP is located in the promoter region of IL-2 and has been found to decrease IL-2 expression, the T allele of rs2069762 is associated with decreased IL-2 expression. This mutation reduces the stability and secretion of IL-2 and decreases its biological activity (45,46).

Chromosome 2

CTLA4: CTLA4 is a protein receptor that functions as an immune checkpoint and downregulates immune responses. It is expressed by regulatory T cells and some activated conventional T cells. It binds to CD80 or CD86 on the surface of antigen-presenting cells and provides negative feedback for T-cell activation (47,48). CTLA4 is constitutively expressed by Tregs and can further upregulate upon T cell receptor (TCR) engagement (49). CTLA4 binds to CD80 or CD86 on antigen-presenting cells (APCs) and inhibits the costimulatory signal that is required for T-cell activation. CTLA4 also enhances Tregs suppressive activity (50). CTLA-4 engagement inhibits IL-2 synthesis and causes a T-cell cycle arrest (51).

Studies have shown that in AD lowered expression of CTLA4 was observed (52). And therefore, it's only logical to assume that SNPs that alter the expression of CTLA4 could have a possible association with AD. rs17268364: located in the intergenic region between CTLA4 and ICOS has been found to lower the gene expression, especially the G allele, this SNP was associated with lupus (53). rs231775 +49 A>G: has been linked to decreased expression and individuals carrying the G allele are more susceptible to autoimmune diseases such as Graves' disease, Hashimoto's thyroiditis, type 1 diabetes, celiac and systemic lupus (54,55). rs3087243 +6230 G>: The risk allele is (A), which reduces the expression of CTLA4 and impairs the negative regulation of T cells which leads to overstimulated T cells, e (A) allele abolished the binding site for SP1, a transcription factor that activates CTLA4 expression. (56). Another study found that the (A) allele altered the chromatin interactions between the CTLA4 locus and other regulatory elements. Thus, this SNP has been associated with various autoimmune diseases, such as type 1 diabetes, rheumatoid arthritis, lupus, and thyroid disease (57-60).

IKZF2: encodes a protein called Helios, which belongs to the Ikaros family of zinc-finger transcription factors. These factors are involved in the regulation of lymphocyte development and function, especially T cells. Helios can form homo- or hetero-dimers with other Ikaros family members and modulate their transcriptional activity. This protein is expressed in various types of T cells, such as regulatory T cells (Tregs), effector T cells (Teffs), and memory T cells (Tmems) and it plays a role in maintaining the stability and suppressive function of Tregs, as well as the differentiation and activation of Teffs and Tmems (61,62).

c.600C>A p.Y200X is a genetic variant that causes a premature stop codon in the IKZF2 gene, which encodes the transcription factor Helios. This variant introduces a truncation in the fourth DNA-binding zinc finger of Helios, which is essential for its dimerization and interaction with other proteins (62). This variant leads to reduced Helios expression and impaired function in both regulatory and effector T cells, resulting in chronic T cell activation, increased production of proinflammatory cytokines, and impaired immune homeostasis leading to an overstimulated immune system (62).

Chromosome 1

TNFRSF18: This gene encodes a protein called TNFRSF18/ CD357/GITR which is a member of the TNF receptor superfamily (63). GITR provides regulatory functions in peripheral and thymic Treg cells. It promotes the proliferation and differentiation of Tregs (64) and it increases the production of IL-10 (65).

The reduced expression of this protein due to SNPs or mutation in its gene could lead to impaired Treg cells and impaired suppression of the overstimulated immune system. Mutations in this gene could also affect the expression of other genes such as FOXP3, CTLA-4, and IL-10 (66).

rs3753344 -358G>A: was associated with lowered expression by affecting the binding of transcription factors such as NFAT and AP-1 to the promoter region of TNFRSF18 gene (67).

TNFRSF1B: this gene encodes TNFR2, a type 2 transmembrane TNF-α receptor that mediates some anti-inflammatory and protective effects of TNF-α in Tregs (68). It can promote the activation, survival, and proliferation of Tregs through the NF-κB pathway and modulate the balance between Treg and Teff cells by influencing their migration and cytokine production (69).

Mutations or SNPs in this gene could affect TNFR2 and its anti-inflammatory role in activating Tregs leading to chronic stimulation of the immune system. rs1061622 +676 T>G: which results in a non-synonymous amino acid change (M196R) in the extracellular domain of TNFR2 (70). This SNP was associated with a reduced binding affinity of TNF alpha to TNFR2 and impaired TNF alpha-induced apoptosis in vitro. This SNP was also associated with increased susceptibility to rheumatoid arthritis, systemic lupus erythematosus, ankylosing spondylitis, and systemic sclerosis (71,72). rs3397: is a SNP in the exon 9 of TNFRSF1B. This SNP

results in a non-synonymous amino acid change (T587I) in the intracellular domain of TNFR2 (73). This SNP has been associated with various clinical outcomes, such as Reduced TNF-alpha-induced NF-kappa B activation and apoptosis in vitro, Increased susceptibility to rheumatoid arthritis and systemic lupus erythematosus, and Increased risk of osteoporosis in rheumatoid arthritis (74).

IL-10: interleukin 10, which is a cytokine that has anti-inflammatory properties. IL-10 is produced by various cells of the immune system, such as T cells, B cells, macrophages, dendritic cells, and mast cells (75). It can inhibit the production and function of pro-inflammatory cytokines, such as TNF alpha, IL-1, IL-6, and IL-12, by various immune cells. IL-10 can also suppress the activation and proliferation of T helper 1 (Th1) cells, natural killer (NK) cells, and macrophages, which are involved in cell-mediated immunity and inflammation (75). IL-10 can also enhance the survival and function of regulatory T cells (Tregs), which are involved in maintaining immune tolerance and preventing autoimmunity. IL-10 can also modulate the expression of major histocompatibility complex (MHC) class II molecules and co-stimulatory molecules on antigen-presenting cells, which affect the activation of T cells (75).

rs1800871 -819 C>T: in the promoter region of IL-10, which is associated with lower transcriptional activity and lower IL-10 production by monocytes. This SNP was associated with AD in the Italian population and with several autoimmune diseases like Crohn's disease (76,77). rs1800896 -1082 A>G in the promoter region of IL10, which is associated with lower transcriptional activity and lower IL-10 production by monocytes and T cells. This SNP has been associated with various inflammatory and autoimmune diseases, such as rheumatoid arthritis, Crohn's disease, ulcerative colitis, and ankylosing spondylitis (78,79).

A study showed that rs1800871, rs1800872, rs3024490, and rs1554286 in IL-10 gene have led to lower IL-10 expression and higher TNF gene expression resulting in a pro-inflammatory response that could cause auto-immune diseases (80).

Chromosome X:

FOXP3: This gene encodes a transcription factor called Forkhead box P3 that controls the function and development of Tregs (81,82). FOXP3 controls and prevents transcription factors like NFAT and NF-κB from interacting with the genes responsible for inflammatory responses such as

IL-4, IL-2, and IFN-γ. But it favors the expression of regulatory genes such as CTLA-4, CD25 (IL-2RA), and GITR (TNFRSF18) in order to activate Treg lines (83-85).

FOXP3 expression is a specific marker of Tregs, thus mutations and SNPs in this gene would impact Tregs and their suppressive function and would lead to autoimmune syndrome IPEX and autoimmune diseases. rs3761549 -2383 C>T: the *C allele alters the binding of Ying Yang1 (YY1) transcription factor, thus decreasing the regulatory function of Tregs causing severe thyroid tissue destruction in patients with Hashimoto's disease, and autoimmune diseases like systemic lupus and psoriasis (86-89). rs3761548 -3279C>A: alters the FOXP3 expression pathway. The *A allele changes the E47 and c-Myb transcription factor binding sites, leading to modifications in the gene expression that predispose the individual to autoimmune disease development (90,91). rs2232365 -924A>G: is located in a FOXP3 gene region equivalent to the GATA3 transcription factor binding site, essential for differentiating the Th2 profile. The *G allele decreases the expression of the FOXP3 gene and causes an immunological imbalance, predisposing an individual to develop autoimmune diseases, including an increased risk of developing psoriasis and vitiligo (82,88,92).

CD40LG: encodes a transmembrane protein CD40L (CD154), which is a member of the tumor necrosis factor (TNF) superfamily It is mostly expressed on activated CD4+ T-cells and stimulated platelets. The costimulatory molecule CD40L and its receptor CD40 have essential roles in adaptive immunity; it also plays a role in inflammatory responses, which are important in innate immunity (93-96). CD40LG stimulates B cell proliferation, differentiation, class switching, and antibody production CD40LG also enhances the activation and survival of T cells. CD40LG can induce the expression of TNF-α and its receptors on B cells, which can then amplify their activation and survival, and induces the production of TNF-α by dendritic cells. TNF-α can upregulate the expression of CD40LG on T cells, which can then enhance their co-stimulation of B cells (97).

Polymorphisms in this ligand have been associated with various pathologies such as autoimmune and infectious diseases (98,99). rs3092952 -3459A>G: the *G allele in this SNP was linked to increased expression of CD40L and thereby leading to enhanced CD40 interactions and upregulated TNF-α production (100). rs1126535 220T>C: also, was

linked to increased expression, therefore more T cell activation and higher cytokines production (101).

Chromosome 6:

TNF: This gene encodes Tumor necrosis factor a (TNF-α, also known as cachectin) a strong pro-inflammatory cytokine that plays an important role in the immune system during inflammation, cell proliferation, differentiation, and apoptosis (102**).** TNF-α can bind to two types of receptors: TNFR1 and TNFR2, which have different cellular distribution and functions. TNFR1 is expressed by most cell types and mediates most of the pro-inflammatory and cytotoxic effects of TNF-α. TNFR2 is mainly expressed by immune and endothelial cells and mediates most of the anti-inflammatory and cytoprotective effects of TNF (103).TNF can activate various signaling pathways, such as nuclear factor-kappa B (NF-kB), mitogen-activated protein kinases (MAPKs), caspases, and c-Jun N-terminal kinases (JNKs), which can regulate the expression of genes involved in inflammation, apoptosis, cell survival, proliferation, differentiation, and migration**.** TNF-α can enhance the innate immune response by stimulating the production of other pro-inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), and interferon-gamma (IFN-γ); activating phagocytes, NK cells, and dendritic cells; inducing the expression of adhesion molecules and chemokines; and increasing the permeability of blood vessels (10,104). TNF-α can also modulate the adaptive immune response by promoting the activation, proliferation, differentiation, and survival of T cells and B cells; regulating the balance between Th1 and Th2 cells; enhancing the cytotoxicity of T cells and NK cells; inducing the expression of costimulatory molecules and major histocompatibility complex (MHC) molecules; and influencing the generation and function of Tregs (10,16). TNF-α can bind to TNFR1, which is expressed by most cell types, including Tregs, and activate its signaling pathways, such as caspases, JNKs, and p38. These pathways can induce the expression of pro-apoptotic molecules, such as FasL and TRAIL, and trigger cell death. TNF-α can also reduce the expression of Foxp3 and IL-10, which are essential for Treg suppressive activity (105). As mentioned above, TNFα has a major role in the pathology of AD because it enhances the production and aggregation of Aβ in neurons and impairs its clearance by microglia and across BBB (15-17).

Mutations and SNPs in the TNF gene can lead to enhanced expression of this gene and elevated levels of TNF-α in the system, therefore disturbing immune homeostasis and overstimulating the immune system leading to various autoimmune diseases and further aggravating AD. rs1800629 -

308G>A: This SNP is located in the promoter region and leads to increased transcriptional activity of the gene thus increased production of TNF-α, this SNP has been linked to autoimmune diseases like RA, Crohn's and psoriasis (106,108,110). Also, rs361525 -238G>A: located in the promoter region increases the transcriptional activity and therefore increases TNF-α in response to viral infection, psoriasis, and DM1 (106,107,110). rs1799724 -857C>T: also located in the promoter, increases the expression of the gene and thus increases the levels of TNF-α (106). This SNP has been associated with Alzheimer's (111), and various types of cancer such as prostate cancer, and non-Hodgkin lymphoma (112,113). rs1800630 -863C>A: this SNP also located in the promoter increases the transcription of this gene leading to higher production of TNF-α (106), it is associated with autoimmune diseases such as systemic lupus erythematosus (SLE) (114), and Grave's disease (115). rs1799964 -1031G>A: also, in the promoter region and leads to increased expression and therefore increased production of TNF-α, this SNP is linked to Grave's disease (115) and DM1 (107). rs3093662 +851A>G: this SNP located in intron 1 of the gene has been associated with higher expression and TNF-α production, and also is associated with DM1 (116) and RA (117).

TNFAIP3: A gene that encodes a protein called tumor necrosis factor alpha-induced protein 3 or A20**.** A protein that has both ubiquitin ligase and deubiquitinase activities and is involved in the regulation of inflammation and immune responses. This protein Inhibits TNF-mediated apoptosis**:** This protein can prevent TNF-induced cell death by removing K63-linked polyubiquitin chains from RIPK1, a key mediator of TNF signaling, and replacing them with K48-linked polyubiquitin chains, which target RIPK1 for proteasomal degradation. And terminate NF-kappa-B activation**:** This protein can terminate the activation of NF-κB, a transcription factor that regulates the expression of many inflammatory genes, by editing the ubiquitination status of various proteins involved in TNF signaling pathways, such as TRAF2, TRAF6, NEMO, and IKK-gamma. Also, it reduces TNF production**:** This protein can reduce the production of TNF by macrophages and dendritic cells in response to Toll-like receptor ligands or other stimuli, by inhibiting the expression of genes involved in TNF synthesis, such as NFKBIA, NFKBIZ, and MAP3K8 (118,119).

rs2230926 380T>G: this SNP may impair the deubiquitinating activity of A20 and enhances TNFα-induced NF-κB activation, also it was linked to RA (119), SLE (120), Crohn's, and psoriasis (121). Rs5029939 This SNP is located in intron 2 of the gene, it affects the splicing or the expression of the gene and reduces the inhibition effect of A20 on TNF-α-induced NF-

κB. This gene was associated with lupus nephritis (122) and systemic sclerosis (123).

HLA: The HLA complex encodes two main classes of molecules: class I and class II. Class I molecules are present on all nucleated cells and present peptides from inside the cell to CD8+ T cells, which can destroy infected or abnormal cells. Class II molecules are present on professional antigen-presenting cells (such as B cells, macrophages, and dendritic cells) and present peptides from outside the cell to CD4+ T cells, which can help activate other immune cells (124). The HLA gene is involved in the development and function of Tregs, which are a subset of CD4+ T cells that suppress excessive or harmful immune responses and prevent autoimmunity. The HLA gene can influence Treg cells' generation, stability, and plasticity by affecting their expression of FOXP3, a transcription factor essential for Treg identity and function. The HLA gene can also modulate the interaction between Treg cells and other immune cells by affecting their expression of costimulatory molecules and cytokines (125).

SNPs in this gene could affect a lot of aspects of the immune system. rs9271366: located near HLA-DRB1/HLA-DQA1, it may affect the function of Tregs by altering the expression of HLA-DQA1 (126). This SNP is associated with SLE (126), and DM1 (127). rs3135388: located in intron 1 of HLA-DRB1, it affects the function of both tTregs and pTregs by altering the binding affinity of HLA-DRB1. This SNP is associated with multiple sclerosis (128), and SLE (129). rs3131379: located in HLA region 1 near MSH5, and it is associated with lung cancer, and SLE (130,131). Rs9271192: located in HLA-DRB1, the *C allele of this SNP was associated with AD (132,133). rs2395182: this SNP is located near the HLA-DRA region, it was associated with multiple sclerosis (134) and RA (135).

LTA: This gene encodes a cytokine from the TNF family called TNF-β or lymphotoxin A (LTA). Like TNF-α it is produced from T cells and macrophages, and it induces the production of pro-inflammatory cytokines which enforces the inflammation and causes tissue damage. It mediates the differentiation of Tregs and could inhibit it by reducing the expression of FOXP3, also it could impair the suppressive function of Tregs by reducing the expression of CTLA4, GITR, and IL10 (136).

rs909253 252A>G: located in intron 1 of the gene, the *G allele is associated with higher TNFα and TNF-β/LTA, and is associated with SLE, multiple sclerosis, and vitiligo (136-139). rs1041981 804C>A: located in

exon 3, it was associated with higher LTA expression, and linked to lung cancer, and DM1 (140,141). rs2239704 +80A>C: located in 5'UTR in this gene, has been linked to enhancing the transcriptional level of LTA (141), and was associated with types of cancer such as non-Hodgkin lymphoma and gastric cancer (142,143).

IL-17A: encodes a protein called interleukin-17A. This protein is a proinflammatory cytokine that is produced by activated CD4+ T cells. IL-17A levels are significantly increased in the CSF of AD patients (144). IL-17 induces and activates glial cells such as microglia and induces cytokines secretion from it such as TNF-α and IL-6. It triggers the activation of NF-κB but also with it triggers the deubiquitination to restrain the activity of NF-κB. Therefore, SNP increasing the levels of IL-17A could be of utmost importance in mediating inflammation (144).

rs2275913 +197G>A: this SNP may increase the stability of mRNA and the secretion of IL-17A leading to increased levels of IL-17A and overstimulating the immune system, leading to autoimmune diseases like RA, Crohn's, and lupus (145-147).

We have found that chromosome 6 has various genes affecting TNFα, other cytokines and interleukins, and Treg cells. All these genes could affect Alzheimer's disease in a direct or non-direct pattern through the over-stimulation of the immune system and would be suitable to investigate for Linkage Disequilibrium, which is a measure of non-random association of alleles at different loci, and haplotype frequencies.

We used LDpair (https://ldlink.nih.gov/?tab=ldpair), which is one of the applications of LDlink. LDpair allows users to investigate potentially correlated alleles for a pair of variants in high LD. Users can input two variants by their RS numbers or genomic coordinates, and select one or more populations to query.

SNPs in the TNF gene (table 2) and in other genes on chromosome 6 (table 1) have been studied using LDpair to investigate whether these SNPs were in LD or not.

After demonstrating SNPs that are in LD, we selected high LD SNPs and used another bioinformatics tool called LDhap to Calculate population-specific haplotype frequencies of all haplotypes observed in the population (table 3).

LDhap (https://ldlink.nih.gov/?tab=ldhap) is another application of LDlink, which allows users to calculate population-specific haplotype frequencies of all haplotypes observed for a list of query variants.it will output a table displaying the haplotype frequencies and counts as well as the overall frequencies and count.

Results:

After searching for genes that have a role in inflammation, whether affecting cytokines, interleukins, or regulatory T cells in databases like Alzgen and Genebank, we identified 15 genes on 7 chromosomes. Then we looked into how these genes affect inflammation and what SNPs in these genes could cause overstimulation to the immune system.

In order to establish if these SNPs could be inherited together as a haplotype we needed to determine if they are in linkage disequilibrium, and therefore we used LDpair, a bioinformatics tool that investigates if variants are in LD or not using parameters such as D' (ranges from 0 to 1, where 0 means no linkage and 1 means complete linkage), chi-sq (a measure of how much the observed haplotype frequencies differ from the expected frequencies), and p-value.

Results in Table (1) show a variety of linked and not liked SNPs along the 6^{th} chromosome. 49 pairs of SNPs were in LD out of 84 pairs tested.

Table (1): SNPs LD results from LDpair .

	Rs1800629	Rs361525	Rs1799724	Rs1800630	Rs1799964	Rs3093662
Rs2230926	D'(0.051) χ^2(8.02) P(0.004)	D'(0.295) χ^2(4.59) P(0.032)	D'(0.5089) χ^2(23.126) P(<0.0001)	D'(0.294) χ^2(12.85) P(0.0003)	D'(0.288) χ^2(18.91) P(<0.0001)	D'(0.006) χ^2(0.10) P(0.744)
Rs5029939	D'(0.05) χ^2(8.02) P(0.004)	D'(0.29) χ^2(4.59) P(0.0321)	D'(0.5089) χ^2(23.12) P(<0.0001)	D'(0.2947) χ^2(12.85) P(0.0003)	D'(0.2881) χ^2(18.916) P(<0.0001)	D'(0.006) χ^2(0.106) P(0.744)
Rs6920220	D'(0.003) χ^2(0.048) P(0.825)	D'(0.0007) χ^2(0.001) P(0.968)	D'(0.103) χ^2(0.614) P(0.4331)	D'(0.0152) χ^2(0.6631) P(0.4155)	D'(0.022) χ^2(0.960) P(0.327)	D'(0.006) χ^2(0.156) P(0.692)
Rs2275913	D'(0.032) χ^2(0.218) P(0.640)	D'(0.115) χ^2(1.7831) P(0.181)	D'(0.1049) χ^2(14.641) P(<0.0001)	D'(0.047) χ^2(5.004) P(0.025)	D'(0.016) χ^2(0.934) P(0.333)	D'(0.171) χ^2(5.298) P(0.021)
Rs9271366	D'(0.335) χ^2(9.8342) P(0.0017)	D'(0.562) χ^2(18.077) P(<0.0001)	D'(0.407) χ^2(16.114) P(<0.0001)	D'(0.014) χ^2(1.059) P(0.303)	D'(0.099) χ^2(2.431) P(0.118)	D'(0.115) χ^2(1.017) P(0.313)
Rs3135388	D'(0.3816) χ^2(3.2454) P(0.0716)	D'(0.694) χ^2(7.0273) P(0.008)	D'(0.295) χ^2(2.157) P(0.1419)	D'(0.517) χ^2(10.953) P(0.0009)	D'(0.5753) χ^2(20.857) P(<0.0001)	D'(0.359) χ^2(2.5194) P(0.112)
Rs9277534	D'(0.030) χ^2(0.388) P(0.533)	D'(0.0158) χ^2(0.07) P(0.791)	D'(0.164) χ^2(12.776) P(0.0004)	D'(0.1244) χ^2(12.186) P(0.0005)	D'(0.088) χ^2(9.496) P(0.002)	D'(0.082) χ^2(2.532) P(0.1115)
Rs3131379	D'(0.5707) χ^2(574.23) P(<0.0001)	D'(1.0) χ^2(11.342) P(0.0008)	D'(1.0) χ^2(19.22) P(<0.0001)	D'(0.462) χ^2(6.821) P(0.009)	D'(0.6218) χ^2(18.96) P(<0.0001)	D'(0.777) χ^2(9.183) P(0.0024)
Rs9272346	D'(0.058) χ^2(1.533) P(0.215)	D'(0.5372) χ^2(84.67) P(<0.0001)	D'(0.125) χ^2(7.768) P(0.0053)	D'(0.050) χ^2(2.6005) P(0.1068)	D'(0.110) χ^2(15.574) P(<0.0001)	D'(0.341) χ^2(45.832) P(<0.0001)
Rs9271192	D'(0.319) χ^2(15.815) P(<0.0001)	D'(0.530) χ^2(28.45) P(<0.0001)	D'(0.354) χ^2(21.55) P(<0.0001)	D'(0.0742) χ^2(16.161) P(<0.0001)	D'(0.005) χ^2(0.133) P(0.7149)	D'(0.294) χ^2(11.738) P(0.0006)
Rs2395182	D'(0.011) χ^2(0.225) P(0.6349)	D'(0.451) χ^2(18.825) P(<0.0001)	D'(0.334) χ^2(17.529) P(<0.0001)	D'(0.110) χ^2(3.142) P(0.0763)	D'(0.1883) χ^2(14.1533) P(0.0002)	D'(0.3333) χ^2(13.721) P(0.0002)
Rs2239704	D'(1.0) χ^2(268.04) P(<0.0001)	D'(1.0) χ^2(175.21) P(<0.0001)	D'(0.993) χ^2(1007.8) P(<0.0001)	D"(0.918) χ^2(415.5) P(<0.0001)	D'(0.940) χ^2(6669.86) P(<0.0001)	D'(0.992) χ^2(231.19) P(<0.0001)
Rs909253	D'(1.0) χ^2(777.84) P(<0.0001)	D'(1.0) χ^2(207.45) P(<0.0001)	D'(0.994) χ^2(348.01) P(<0.0001)	D'(0.996) χ^2(579.1) P(<0.0001)	D'(0.997) χ^2(893.05) P(<0.0001)	D'(1.0) χ^2(277.6) P(<0.0001)
Rs1041981	D'(1.0) χ^2(778.49) P(<0.0001)	D'(1.0) χ^2(207.27) P(<0.0001)	D'(0.9896) χ^2(344.11) P(<0.0001)	D'(1.0) χ^2(582.4) P(<0.0001)	D'(1.0) χ^2(896.48) P(<0.0001)	D'(0.993) χ^2(273.89) P(<0.0001)

After Identifying SNPs that are in LD in genes on chromosome 6, we also tested SNPs in TNF gene alone to see which ones are in LD and if they could be inherited together. Table (2).

Table (2): SNPs in TNF gene LD results from LDpair.

	Rs1800629	Rs361525	Rs1799724	Rs1800630	Rs1799964	Rs3093662
Rs1800629		D'(1.0) χ^2(32.221) P(<0.0001)	D'(1.0) χ^2 (54.617) P(<0.0001)	D'(1.0) χ^2 (90.548) P(<0.0001)	D'(1.0) χ^2 (139.359) P(<0.0001)	D'(1.0) χ^2 (43.128) P(<0.0001)
Rs361525			D'(1.0) χ^2 (35.702) P(<0.0001)	D'(1.0) χ^2 (59.190) P(<0.0001)	D'(1.0) χ^2 (1157.89) P(<0.0001)	D'(1.0) χ^2 (3741.46) P(<0.0001)
Rs1799724				D'(1.0) χ^2 (100.33) P(<0.0001)	D'(1.0) χ^2 (154.41) P(<0.0001)	D'(1.0) χ^2 (47.788) P(<0.0001)
Rs31800630					D'(1.0) χ^2 (3253.9) P(<0.0001)	D'(1.0) χ^2 (79.22) P(<0.0001)
Rs1799964						D'(0.695) χ^2 (750.5264) P(<0.0001)
Rs3093662						

Then we picked SNPs that could affect AD both in a direct and indirect way and are in high LD, and we used another tool called LDhap which calculates population-specific haplotype frequencies of all haplotypes observed.

Figure (5) obtained from LDhap, shows these SNPs, their positions, allele frequencies, also haplotypes, their counts, and the haplotypes frequencies. We also searched for allele frequencies in 3 different populations in dbSNP: Europeans, Africans, and Asians. In Table (3) we demonstrate these frequencies along with frequencies in the total population, for reference when looking at haplotypes and their frequencies.

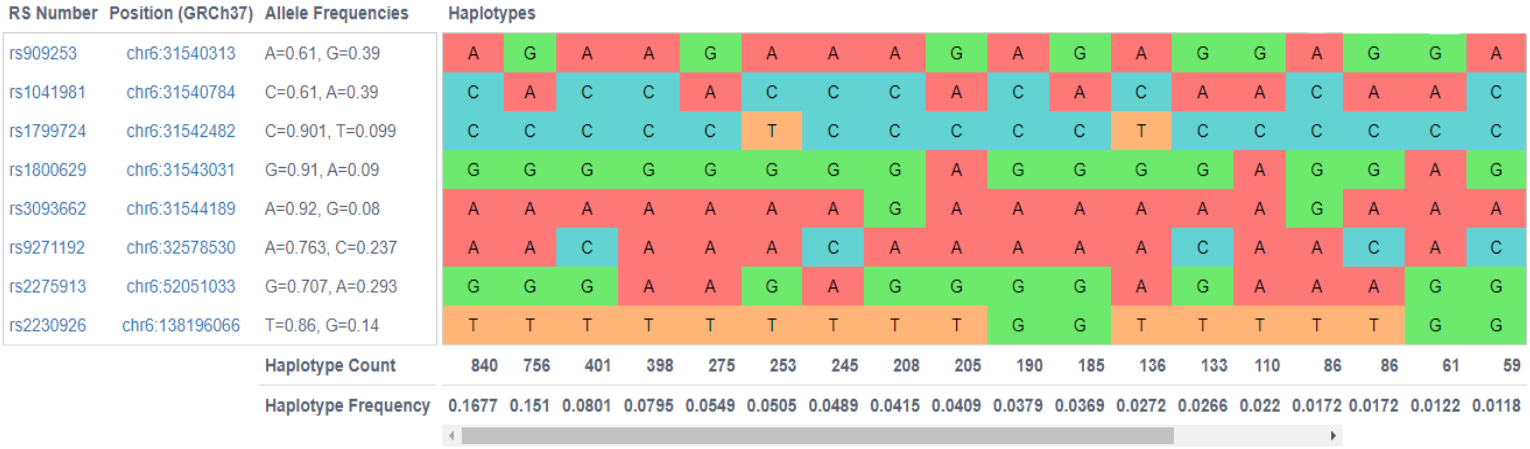

Figure (5): haplotypes frequencies from LDhap

Table (3): alleles frequencies across populations

RSID	alleles	European population	African population	Asian population	Total population
rs1799724	C	0.87791	0.9716	0.895	0.878652
	T	0.121348	0.12209	0.105	0.121348
rs3093662	A	0.920195	0.9242	0.9613	0.922002
	G	0.079805	0.0758	0.0387	0.077998
rs1800629	G	0.840730	0.8761	0.9275	0.847933
	A	0.159270	0.1239	0.0725	0.152067
rs2230926	T	0.965381	0.65441	0.9563	0.953373
	G	0.034619	0.34559	0.0437	0.046627
rs2275913	G	0.65146	0.9094	0.542	0.665743
	A	0.34854	0.0906	0.458	0.33427
rs9271192	C	0.27476	0.2333	0.259	0.27092
	A	0.72524	0.7667	0.741	0.72908
rs909253	A	0.680281	0.6233	0.5480	0.674698
	G	0.319719	0.3767	0.4520	0.325302
rs1041981	C	0.673234	0.5044	0.5482	0.665213
	A	0.326766	0.4956	0.4518	0.334787

Discussion:

With the increase in the aging population, the risk of age-related conditions such as AD has also grown (2). With AD being the most common neurodegenerative disease (1) the need for further and detailed

understanding of it also grew. One of the most important contributors to AD is neuroinflammation, and microglia cells play an important role in its pathology and progression (2,4). As Aβ plaques aggregate in the brain, it triggers microglia to start phagocytosis but also triggers transcription factors to produce cytokines and interleukins such as TNF-α and IL_6 (5). In AD TNF-α is chronically released which causes chronic inflammation that stimulates Aβ production and aggregation as well as inhibiting microglia (15), also affecting Tregs and their suppressive activity, contributing to the dysregulation of the immune system and the aggravation of neuroinflammation and neurodegeneration in Alzheimer's disease (30).

Starting from the importance of identifying key players in these mechanisms, we searched for genes and their location on the chromosomes, and identified: IL-6 on chromosome 7. IL-RA on chromosome 10. IL-2 on chromosome 4. CTLA4, and IKZF2 on chromosome 2. TNFRSF18, TNFRSF1B, and IL-10 on chromosome 1. FOXP3, and CD40LG on chromosome X. TNF, TNFAIP3, HLA, LTA, and IL-17A on chromosome 6. SNPs in these genes have various effects on the inflammatory process and immune cells. Some of these SNPs were associated with AD directly, such as rs1799724 and rs9271192 (111,133,134). And some could be indirectly linked to AD by causing affecting Tregs activation and differentiation or increasing the levels of pro-inflammation cytokines and interleukins, or decreasing the levels of anti-inflammation interleukins, such as rs361525, rs2275913, rs2230926, rs909253, rs3135388. (106,147,120,137,129).

Because most of the genes that affect TNF-α in different ways are located on the 6^{th} chromosome, studying the linkage between the SNPs in these genes was important in order to conclude if they could be inherited together on a haplotype. Using LDpair we obtained the results in Table (1). Rs2230926 and rs1799724 are in linkage because D'=0.5089 means that there is some LD between the two loci, chi-sq=23.126 indicates that LD is unlikely to be due to chance, and p-value=0.0001 indicating that there's significant LD between these two SNPs.

Rs2275913 and rs1799724 are also in linkage, D'=0.1049 meaning that there's low LD between the two loci, chi-sq=14.641 indicates that LD is unlikely to be due to chance, and p-value <0.0001 which indicates that this LD is significant.

On the other hand, rs6920220 and rs179924 are not in linkage, a very low D'=0.103 means a very low LD between them, coupled with chi-sq=0.0614 and p-value=0.4331 meaning that this LD is not significant and could be due to chance.

Rs2395182 and rs361525 are in LD, D'=0.4517 this indicates that there's some LD between them, chi-sq=18.825, and p-value <0.0001 which means that LD is significant and is unlikely to be due to chance.

Rs9271192 and rs361525 are also in LD, D'=0.530 which means that there's some LD between these two loci, chi-sq=28.45, and p-value <0.0001 indicating that this LD is significant and unlikely to be due to chance.

As seen above in (Table.1) all SNPs studied in the LTA gene (rs2239704, rs909253, rs1041981) are in high linkage with all SNPs from the TNF gene, with a high chi-square value, a very significant p-value, and a high D' that comes from them being close to each other on chromosome 6.

Rs9271192 and rs1800629 are in LD, D'=0.319 which shows some LD between these two SNPs and this result could be because the two loci are far apart on the chromosome but with chi-sq=15.815 and p-value <0.0001 this indicates that the linkage is significant and not due to chance.

Rs3131379 and rs1799724 are in LD with D'=1.0 being very high, chi-sq=19.22, and p-value <0.0001 which indicates that this linkage is very strong and significant and not due to chance.

Rs9272346 and rs361525 are also in LD, D'=0.5372 which shows a moderate linkage, chi-sq=84.67, and p-value <0.0001 meaning that this linkage is not due to chance and it is significant.

And because TNF-α is a key player in an overstimulated immune system, studying SNPs in this gene was important to show if they are in LD and if SNPs in different locations in the gene could be inherited together. In Table (2), rs3093662 and rs1799724 are in high LD, D'=1.0 as they are in the same gene, chi-sq=47.788, and p-value <0.0001, which indicates that this linkage is significant and not due to chance.

Rs1800629 and rs1799724 are in high LD, D'=1.0 as they are in the same gene, chi-sq=54.617, and p-value <0.0001, meaning that this linkage is significant and not due to chance.

Also, rs1800630 and rs1799724 are in high LD, D'= 1.0, chi-sq=100.33 with a p-value <0.0001 which indicates this linkage is significant and not due to chance.

rs3093662 and rs361525 are in high LD, D'=1.0, chi-sq=3741.46 and p-value is <0.0001. This linkage is significant and due to chance.

Rs1799964 and rs1800630 are in high LD, D'=1.0, chi-sq=3253.9, the p-value is <0.0001 which is significant and this link is not due to chance. All SNPs in TNF gene are in high LD which is not surprising given that these SNPs are all on one gene and very close to each other.

We picked high LD SNPs and ran them through LDhap to discover what haplotypes we could encounter in the populations and their frequencies. Alleles frequencies in 3 populations: European, African, and Asian were demonstrated in Table (3) for comparison with alleles frequencies in all populations and haplotype frequencies. After calculating haplotypes frequencies (Figure 5) and comparing them to AD frequency in elder (over 65 yrs) populations (10.7%), we found haplotypes with the higher number of SNPs to be haplotype 14 with a frequency of (2.2%) has 4 SNPs that we identified to affect Alzheimer's whether directly or indirectly (rs909253, rs1041981, rs1800629, rs2275913), also, haplotype 16 with a frequency of (1.7%) has 4 SNPs that we identified to affect Alzheimer's whether directly or indirectly (rs909253, rs1041981, rs9271192, rs2275913). We concluded that haplotype 16 is more significant because it has rs9271192 *C allele which has been proven to be associated with AD with 2 SNPs in LTA gene that increase its expression and thus stimulate inflammation, and a SNP in IL-17A gene that increases its secretion and thus activates microglia and induces cytokines secretion like TNF-α.

Conclusion:

We used in silico approaches to identify SNPs related to TNFα and other immune factors affecting Alzheimer's disease whether directly or indirectly. We searched several online databases and bioinformatics tools to find SNPs on chromosome 6 that have been shown to be in linkage disequilibrium, and a haplotype has been shown to have genetic variations significant to the role of the immune system in AD pathology, and a frequency of 0.0172 close to the prevalence of AD in the population.

Further statistical and clinical studies should be done to investigate the association of these SNPs on this haplotype with AD, and the actual frequency and importance of this haplotype among different populations, and if it can be used to predict AD in individuals, and investigate other genes and SNPs that could belong to the same haplotype.

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