A Correlational Study Between Serum Cytokine Measures, Volumetric MR Measures and Global Cognitive Changes in Alzheimer's Disease

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ABSTRACT

A Correlational Study Between Serum Cytokine Measures, Volumetric MR Measures and Global Cognitive Changes in Alzheimer's Disease

Earlier detection and diagnosis of Alzheimer's disease (AD) would permit earlier intervention, which conceivably could delay progression of this dementing disorder. In order to accomplish this goal, reliable and specific biomarkers are needed. Unfortunately, there is no yet such a universally accepted biomarker. In this study, we aimed to analyze the association between volumetric MR measurements and possible AD related serum cytokine biomarkers and to determine biological and clinical predictors for patients at high risk to develop AD. 28 AD patients and 16 healthy controls were participated to the study. For this study biochemical markers (IL-1α, IL-1β, IL-10, TNF-α) which were considered to play a pivotal role in the inflammation process during AD were chosen. Additionally, volumetric MR measurements were done to determine atrophic regions in the brain of AD patients. For this purpose, a fully automated software (FreeSurfer) was used. First of all, our ELISA measurements indicated that patients with AD produce increased quantities of pro-inflammatory cytokines (IL-1β and TNF-α) than normal subjects and these results supporting the hypothesis that a pro-inflammatory phenotype contributes to AD. ROC curve analysis showed that IL-1β and TNF-α serum levels could not be used as a diagnostic test tool. However, serum IL-1α level might be a better candidate to make a better diagnostic decision. Secondly, regression analysis revealed that serum IL-1β level had a significant linear relation with the volume changes of cerebral white matter and amygdala/hippocampus. Additionally, the Mini-Mental State Examination (MMSE) score was used as a scale of AD severity. Regression analysis emphasized that serum cytokine levels did not have a significant relation with the severity of cognitive impairment.

Keywords: Alzheimer's Disease, Biomarker, Serum, Inflammation, IL-1α, IL-1β, IL-10, TNF-α, Volumetric MR, FreeSurfer, Mini-Mental State Examination

ÖZET

Alzheimer Hastalığında Serum Sitokin Ölçümleri, Hacimsel MR Ölçümleri ve Global Bilişsel Değişimler Arasındaki İlişkinin İncelenmesine Dayalı Bir Çalışma

Alzheimer hastalığının erken tanı ve teşhisi bize erken müdahale olanağı sağlayacağı için hastalığın ilerlemesi makul düzeyde geciktirilebilir. Erken tanı koyabilmek içinde belirli ve güvenilir biyomarkerlere ihtiyaç duyulmaktadır. Fakat henüz herkesçe kabul görmüş bir marker yoktur. Bu çalışmada biz hacimsel MR ölçümleri ve serum sitokin markerleri arasındaki ilişkiyi incelemeyi hedefledik ayrıca bu ilişkiyi kullanarak Alzheimer hastalığına yakalanma riski yüksek olan insanlar için biyolojik ve klinik belirleyiciler bulmayı da amaçladık. Çalışmaya 28 Alzheimer hastası ve 16 sağlıklı kontrol katılmıştır. Bu çalışma için, Alzheimer da inflamasyon sürecinde rol aldığı düşünülen markerler (IL-1α, IL-1β, IL-10, TNF-α) seçilmiştir. Ek olarak, Alzheimer hastalarının beyinlerindeki atrofik bölgeleri belirleyebilmek içinde hacimsel MR ölçümleri yapılmıştır. Bu hacimsel ölçümler otomatik bir yazılım olan FreeSurfer ile hesaplanmıştır. ELISA sonuçları bize Alzheimer hastalarının kontrollerden daha yüksek miktarda pro-inflamatuar sitokin (IL-1β ve TNF-α) ürettiğini göstermiştir ve bu sonuç Alzheimer hastalığında etkisi olduğu düşünülen pro-inflamatuar hipotezini desteklemektedir. ROC eğrisi analizleri IL-1β ve TNF-α serum seviyelerinin teşhis aracı olarak kullanılamayacağını göstermektedir. Fakat, teşhis aracı olarak serum IL-1α seviyesinin daha iyi bir aday olabileceği belirtilmiştir. Yapılan regresyon analizi, serum IL-1β seviyesi ile beyin beyaz maddesi ve hipokampüs/amigdala hacimlerinin değişimi arasında anlamlı lineer bir ilişki olduğunu göstermektedir. Bunların yanı sıra, Alzheimer hastalığının şiddetinin derecelendirilmesinde Mini-Mental Durum Muayenesi (MMDM) puanlaması kullanılmıştır. Yapılan regresyon analizi sonucunda serum sitokin seviyeleri ile bilişsel bozukluğun şiddeti arasında anlamlı bir ilişki olmadığı gösterilmiştir.

Anahtar Sözcükler: Alzheimer Hastalığı, Serum, Biyomarker, İnflamasyon, IL-1α, IL-1β, IL-10, TNF-α, Hacimsel MR, FreeSurfer, Mini-Mental Durum Muayenesi

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

α	alpha
β	beta
γ	gamma
μ	micro

LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
ELISA	Enzyme-Linked Immunosorbent Assay
NFTs	Neurofibrillary Tangles
SPs	Senile Plaques
APP	Amyloid Precursor Protein
sAPP	Ectodomain of APP
PS1	Presenilin Gene 1
PS2	Presenilin Gene 2
MAPs	Microtubule-Associated Proteins
CNS	Central Nervous System
PHF	Paired Helical Filament
MHC II	Major Histocompatibility Complex Type II
ATP	Adenosine Triphosphate
BBB	Blood-Brain Barrier
PKC	Protein Kinase C
NMDA	N-methyl D-aspartate
MCI	Mild Cognitive Impairment
CSF	Cerebrospinal Fluid
RPM	Round Per Minute
RBCs	Red Blood Cells
HRP	Horse Radish Peroxidase
TMB	Tetramethylbenzidine
ROC	Receiver Operating Characteristic
CC	Corpus Callosum
LV	Lateral Ventricle
OD	Optical Density
MR	Magnetic Resonance
M	Mean

nm	Nanometer
cdk5	Cyclin-Dependent Kinase 5
IL-1α	Interleukin-1alpha
IL-1β	Interleukin-1beta
IL-10	Interleukin-10
TNF-α	Tumor Necrosis Factor-alpha
Aβ	Amyloid Beta
Aβ40	40 Amino Acids Length Amyloid Beta
Aβ42	42 Amino Acids Length Amyloid Beta

1. INTRODUCTION

1.1 Background

Phytagoras (7th century B.C.) was known as the first person who mentioned about dementia. He described old age as a period of decline and decay of the human body and regression of mental capacities [8]. After Phytagoras, respectively Hippocrates, Plato and his student Aristotle had some observations on older people and they both commented in their writings on mental failure in old age. They concluded that old age is inseparable from mental failure, which means dementia with the onset of advancing age was inevitable, just as the passage of time was inevitable [9]. Main point in their observations was that dementia is a common process which comes with older age and it is not a medical situation or medical concept. On the other hand, Roman philosopher Cicero (2nd century B.C.) realized that senile dementia was not only the characteristic of all old men. It might be intrinsic to only those who were weak in will [8].

When we came to the Medieval age, Roger Bacon (1214 -1294) was one of the very few scientists who studied on dementia [9]. He wrote the work "Methods of Preventing the Appearance of Senility". In his studies, he tried to figure out there might be a relation between brain parts and our mental actions such as memory [10]. After Bacon's studies, some Renaissance scientists tried to understand the origins of dementia and its clinical consequences [11]. But the most promising progress was the observations and findings of Thomas Willis (1672). He firstly observed a correlation between brain atrophy and cognitive impairment [11].

In 1907 Alois Alzheimer, a German psychiatrist and scientist, observed the brain of a 51 year old woman. She was suffering from progressive cognitive decline during her life course. At autopsy, Alzheimer observed plaques and tangles (Figure 1.1) in her brain [1]. According to Alzheimer, these structural changes in brain was concomitant

with infiltration by an undetermined metabolic product.

Nowadays, amyloid plaques and neurofibrillary tangles are considered the main neuropathological hallmarks of Alzheimer's Disease (AD). It is a neurodegenerative disease and it is the most common form of irreversible dementia. Symptoms of the disease include memory loss, confusion, impaired judgment, personality changes, disorientation, and loss of language skills. Alzheimer's patients get worse over time, hence it is a fatal disease.

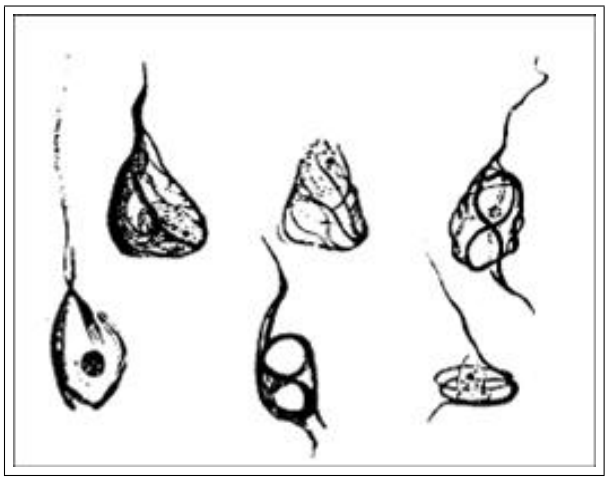

Figure 1.1 Neurofibrillary tangles from Auguste D, drawn by Alzheimer [1]

Several objective, measurable indicators of pre-clinical and clinical characteristics of AD are currently available or in development. Candidate proteins which are potential biomarkers for AD and which are reported to be associated with inflammatory cascade are studied in serum of AD patients with enzyme-linked immunosorbent assay (ELISA) method.

Some molecules in the inflammatory cascade are of great interest in Alzheimer researches, because it is considered that inflammation is associated with the neurodegenerative process characteristic of the AD brain [12, 13, 14, 15, 16]. Over-expression of cytokines and other inflammatory molecules is a common feature of AD brain pathology [9, 15, 17]. Thus, reactive astrocytosis is observed in the cortex and hippocampus of these patients and microglia cells are also activated within or near the amyloid plaques.

To date, little is known on the prevalence and the nature of the AD in Turkey. Gurvit *et al.*, 2008, published recently a study of 1019 persons over age of 70 years living in Istanbul [18]. He showed that the prevalence rates of dementia and AD in Istanbul, Turkey, are comparable with those seen in the Western world. A very important distinction is that Turkey is a country where the rate of consanguinity can, according to the area, rise up to %42, hence forms of AD compatible with a recessive autosomal or pseudo-dominant transmission will be more widespread [19]. Although Turkey has still got the young population, the age range of elder people is increasing everyday which means to have more patients with Alzheimer's disease in the next years. Therefore the number of studies on AD must be increased for the upcoming threat.

1.2 Objective

The overall aim of this study is to analyze the association between volumetric MR measurements of different regions of brain and possible AD related cytokine biomarkers and to determine biological and clinical predictors for patients at high risk to develop AD. My thesis consists in analyzing and measuring the levels of four cytokines (interleukin-1α, interleukin-1β, interleukin-10, tumor necrosis factor-α) by using ELISA method in the serum of 28 AD patients and 16 healthy control subjects. These biomarkers are promising because they promote identification of individuals at risk for AD onset and disease progression. Moreover, MR images of these patients and controls are taken to measure total brain volume and segmented brain regions volume.

1.3 Outline of the Study

This study consists of six chapters. The first chapter reviews a brief background and objectives of our study. Chapter 2 explains the pathogenesis of AD, various hypothesis about AD, role of inflammation during AD and atrophic brain regions in AD patients. Chapter 3 covers the information about blood sampling, ELISA proce-

dure, volumetric MR measurement, Mini-Mental State Examination (MMSE) test and statistical analysis methods. In the Chapter 4, results of ELISA, volumetric MR measurements and MMSE scores are given and statistical analysis were done. The results are discussed in Chapter 5. Conclusions and further recommendations are given in the sixth chapter of this study.

2. PATHOGENESIS OF ALZHEIMER'S DISEASE

Several hypotheses are suggested to explain the cause of AD. These hypothesis are cholinergic hypothesis, amyloid cascade hypothesis and the amyloid cascade/ neuroinflammation hypothesis. The oldest hypothesis which is proposed by the scientists is cholingergic hypothesis and most currently available drug therapies is based on this hypothesis. It postulates that AD is caused by reduced synthesis of the neurotransmitter acetylcholine. However, the cholinergic hypothesis is not satisfactory and it has not maintained widespread support, because the medications intended to treat acetylcholine deficiency has not been very effective [20].

2.1 Amyloid Cascade Hypothesis

Second hypothesis is the amyloid cascade hypothesis. It has recieved much support and has been under investigation for many years [4]. According to this hypothesis, AD is driven by two processes: extracellular deposition of amyloid beta (Aβ) and intracellular accumulation of tau protein. Both these compounds are insoluble. Aβ is the main component of senile plaques and tau is the component of neurofibrillary tangles (NFT). Aβ deposition is specific for AD and is thought to be primary deposit and deposits of Aβ are responsible for causing tau phosphorylation and NFT formation (secondary deposits) leading to neuronal death and dementia [21, 22].

2.1.1 Amyloid Beta Deposition

One of the hallmarks of AD is the accumulation of amyloid plaques between neurons in the brain. Amyloid Beta is a 39 to 43 amino acid peptide, which is part of a larger protein, the Amyloid Precursor Protein (APP). APP is a transmembrane protein, made by neurons and other brain cells. Its primary function is not known, though it has been implicated as a regulator of synapse formation [23] and neural

plasticity [24].

APP extends from the inside to the outside of brain cells by passing through a fatty membrane around the cell. When APP is "activated" to do its normal job, it is cut by other proteins into smaller sections that stay inside and outside cells. There are several different ways APP can be cut. Under some circumstances, one of the pieces produced is amyloid beta. Defective clearance of Aβ from aberrant cleavage of APP and other mechanisms results in its accumulation.

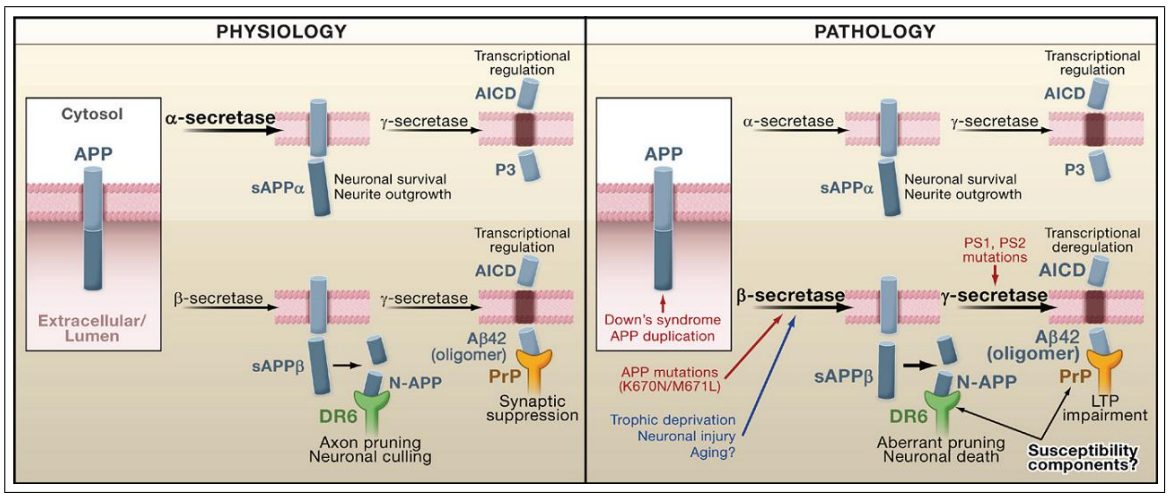

Figure 2.1 APP Processing in Physiology and Pathology [2]

As it is mentioned above, Aβ is a product of sequential cleavage of the APP. The ectodomain of APP (sAPPα or sAPPβ) is cut by either α- or β-secretase enzymes, and the remaining transmembrane domain is then cleaved by γ-secretase (Figure 2.1). α-secretase cleavage produces nontoxic or neurotrophic products while β-secretase initiates the amyloidogenic and pathogenic branch of APP processing. Further processing of the β-secretase cleavage product by γ-secretase releases Aβ peptides of varying length from the plasma membrane, depending on the site of cleavage. The most common isoforms are Aβ40 and Aβ42 [25]. Aβ42 has a great tendency to form the fibrillar amyloid aggregates that are found in the brains of AD patients and therefore Aβ42 is widely accepted as the main pathogenic species causing AD. If some genetic defects occur such

as trisomy 21 in Down's syndrome or mutations on APP gene then Aβ42 isoform is going to be produced more than other Aβ isoforms [2]. Moreover, mutations linked to AD are also found in two presenilin genes (PS1 and PS2). The exact function of the presenilins is not clear but the mutant forms appear to cause to the production of increased quantities of amyloid Aβ42 [26].

2.1.2 Tau Protein and Neurofibrillary Tangles

Tau is a low molecular weight component of cytoskeletal structures and is known as one of the major microtubule-associated proteins (MAPs) in the brain. Its main known biological function is to promote microtubule assembly and to stabilize microtubule's structure [27, 28]. Moreover, some studies show that Tau probably plays a key role in the regulation of neurite extension [29] and regulation of axonal transport [30, 31]. In AD, abnormal tau phosphorylation and/or processing are thought to occur as a result of elevated brain Aβ [32].

Tau proteins are abundant in neurons in the central nervous system (CNS) and its function is regulated by phosphorylation. Their hyperphosphorylation plays a critical role in AD because hyper phosphorylation negatively regulates its ability to stimulate microtubule assembly [33]. Microtubule associated protein tau is abnormally hyperphosphorylated in AD brain and in this form, it is the major protein subunit of the paired helical filaments (PHF) and straight filaments forming neurofibrillary tangles, neutrophil threads, and plaque dystrophic neurites in AD [34, 35].

In the early stages of tau pathology (pretangle), the predominant phosphorylation events are considered to cause a decrease in the ability of tau to bind microtubules rather than an increase in the ability of tau to self-associate; this might be caused by an imbalance in the activity of specific protein kinases or phosphatases [3]. In a study, pretangle neurons are labelled with antibodies that recognize phospho-Threonine231 and phospho-Serine262 sites and it is observed that phosphorylation of both of these sites significantly decreases interactions of tau with microtubules [36, 37]. Moreover,

cleavage of tau by caspase at additional sites such as Ser396/404 increases the propensity of tau to oligomerize and eventually form filamentous aggregates (Figure 2.2).

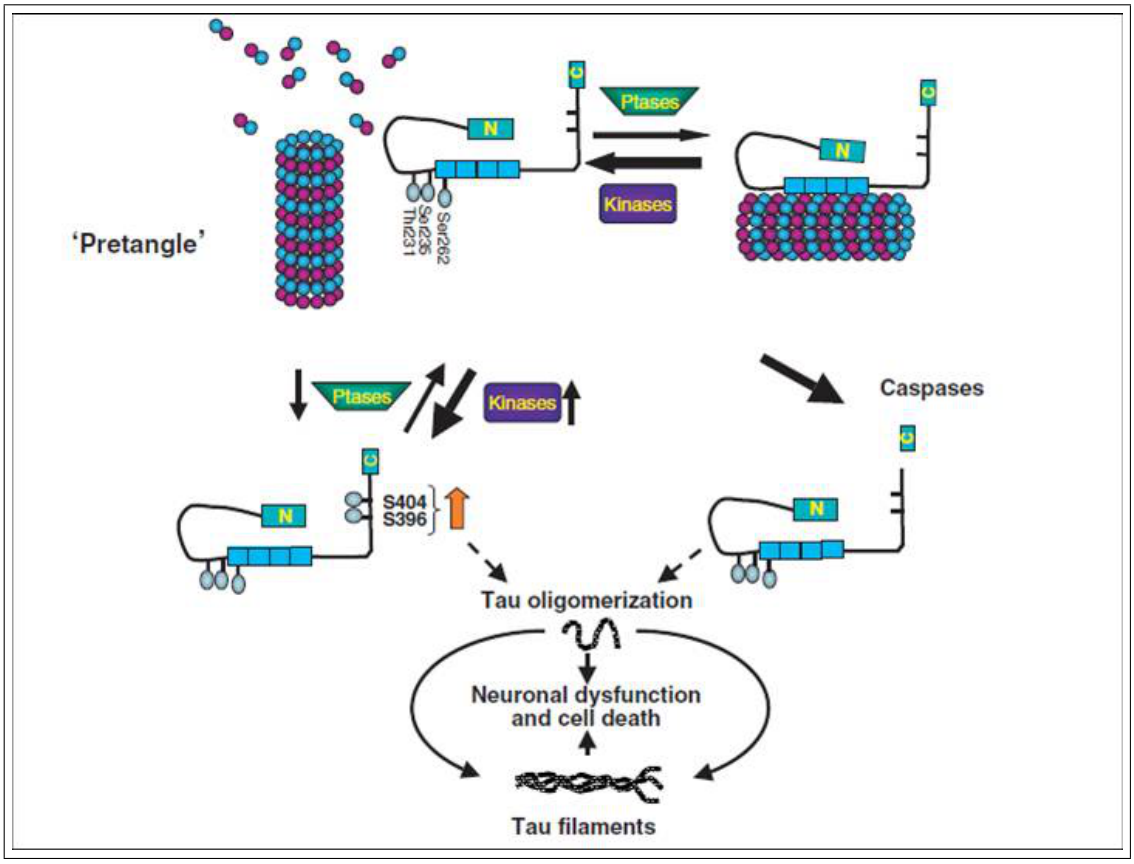

Figure 2.2 Development of Tau Pathology [3]

In a normal neuron, the biological function is dependent on an intact microtubule network through which much of the axoplasmic transport is supported. Tau has a pivotal role during microtubule assembly but if it is hyperphosphorylated then pathological conditions, such as NFT formation, will occur. In neurons with NFTs the normal cytoskeleton is disrupted and replaced by bundles of PHF [38]. The disruption of the microtubule network probably compromises the axonal transport and starts retrograde degeneration of the affected neurons [39]. These neurons eventually die, leaving behind the extracellular tombstones (Figure 2.3). As a result both amyloid plaque deposition and NFT formation are the main components which trigger the neurodegeneration process in AD.

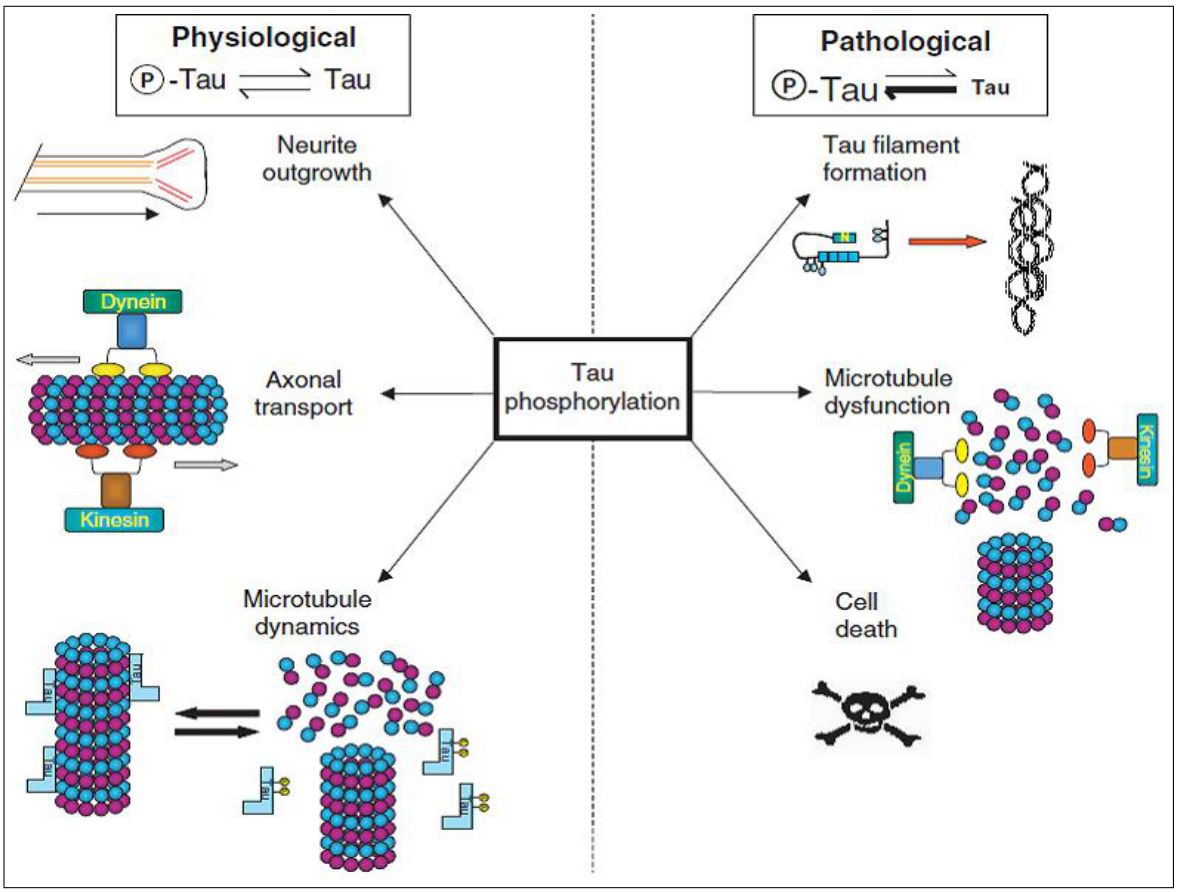

Figure 2.3 Tau Phosphorylation in Physiology and Pathology [3]

2.2 Neuroinflammation Hypothesis

My thesis study is based on amyloid cascade/neuroinflammation hypothesis. This hypothesis is a modified and extended version of the amyloid cascade hypothesis. In this version, microglial (M) cells enter the cascade (Figure 2.4) and play a crucial role in the disease process. According to neuroinflammation hypothesis, acute-phase proteins are elevated in serum [40, 41, 42] and deposited in senile plaques (SPs); microglial cells accumulate around SPs; and complement components are present in SPs. These activated microglial cells produce potentially neurotoxic substances, such as reactive oxygen and nitrogen species, cytokines and other inflammatory mediators, that trigger the neurodegenerative changes [16, 17, 43].

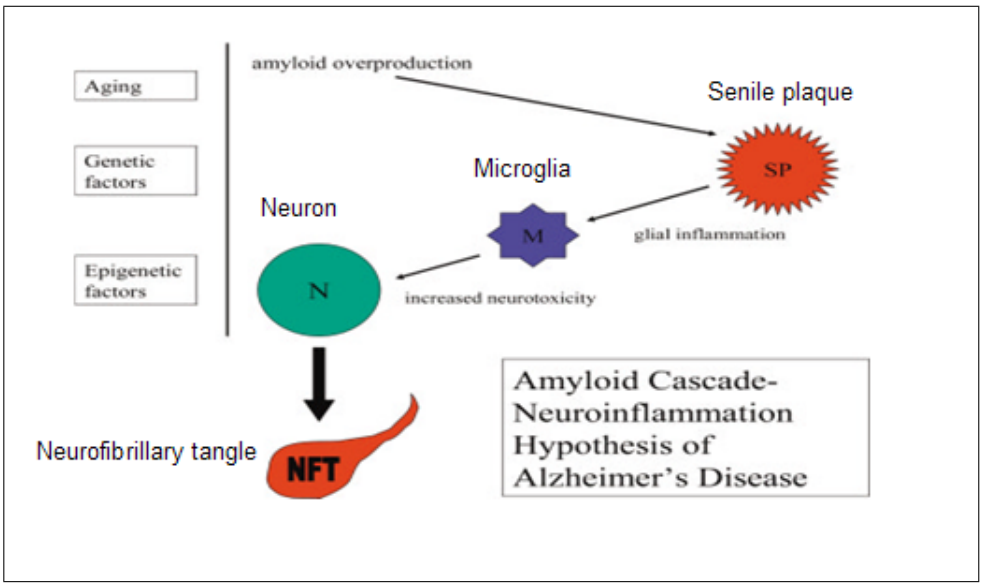

Figure 2.4 Amyloid Cascade/Neuroinflammation Hypothesis [4]

2.2.1 Neuroinflammation

Inflammation is defined as the reaction of living tissues to injury [44]. There are two types of inflammation: acute and chronic inflammation. Although the exact cause of AD is unclear, increasing evidence continues to support the involvement of inflammation in the development of AD [12, 13, 15, 16]. This is supported by the observation that anti-inflammatory medication delays the onset and slows down the progression of AD [45, 46].

The concept of chronic inflammation is more relevant in the context of understanding CNS disease. Therefore, the word neuroinflammation has come to stand for chronic inflammation, like glial responses, that may produce neurodegenerative symptoms such as Aβ plaque formation, dystrophic neurite growth, and excessive tau phosphorylation [47, 48]. Just as damaged tissue and the chronic presence of highly inert abnormal materials are classical stimulants of inflammation in the periphery, so also Aβ, tangles, and neurodegeneration are the most likely sources for inflammation in the AD brain [16].

Most AD research has concentrated on the activity of neuronal cells, however increasing evidence suggests a crucial role for glial cells and changes in their function in the process leading to neuronal degeneration [49, 50]. A current hypothesis is that an extracellular insult to neurons could increase the amount of inflammatory cytokines by astrocytes and microglia [51]. These cytokines are IL-1β, TNF-α and IL-6 that can affect the normal behavior of neuronal cells [5]. It is considered that dysfunction at this core level may cause some abnormalities such as neurofibrillary degeneration in AD [49]. Moreover, an abnormal release of IL-1, TNF-α and IL-6 [52] affects tau phosphorylation patterns and other intracellular events linked with neuronal degeneration (Figure 2.5). Therefore, a direct correlation has been established between Aβ -induced neurotoxicity in neurodegenerative conditions and cytokine production.

cdk5/p35 complex is one of the main protein kinases involved in tau hyperphosphorylation (Figure 2.5) and it is upregulated by the effects of both IL-1 and IL-6. p35 is a neuronal-specific Cyclin-dependent kinase 5 (cdk5) regulator that activates cdk5 kinase activity upon association [53].

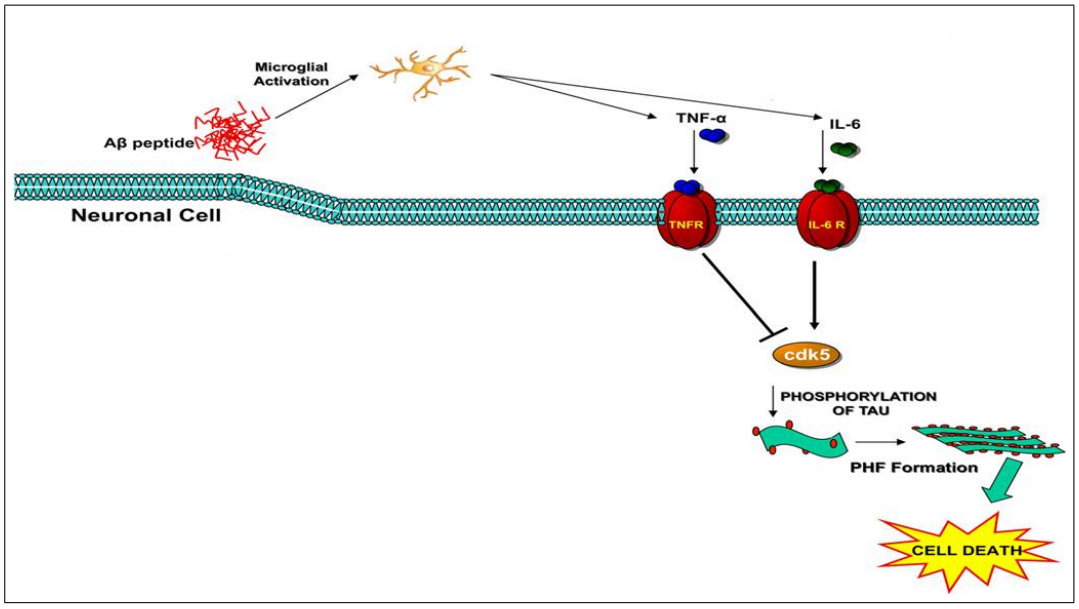

Figure 2.5 A Consequence of Microglial Activation: Tau hyperphosphorylation [5]

2.2.2 Microglia

The microglia is composed of cells that support and protect the neurons and their functions in the CNS and they play an important role to orchestrate the endogenous immune response of the CNS like the immunocompetent defense cells [54]. The microglia are formed mostly of mesodermally derived macrophages [55] and they are able to express major histocompatibility complex type II (MHC II), pro-inflammatory cytokines, chemokines, reactive oxygen species, and complement proteins [7, 54, 56].

The macrophage concept of microglia implies that these cells share essential features [57]. Microglial activation similar to that which occurs in peripheral macrophages during inflammatory attack was first demonstrated in the AD brain two decades ago [56]. Microglia has phagocytic and scavenger properties like macrophages and depending on the conditions that activate the microglia they can exercise both neuroprotective and neurotoxic functions in the brain [58].

The microglia plays a special role in the cellular response to pathological lesions, such as Aβ and neuritic plaques [48]. Aβ can attract and activate microglia leading to clustering of microglia around Aβ deposits sites in the brain. According to an in vitro study [6], microglial migration to Aβ deposit site is observed which is followed by phagocytosis. Microglia clustered at the site of an Aβ deposit are scavenging the Aβ (Figure 2.6). While microglia are phagocytosing the plaques, they express cytotoxic factors that kill neighboring neurons and neurites.

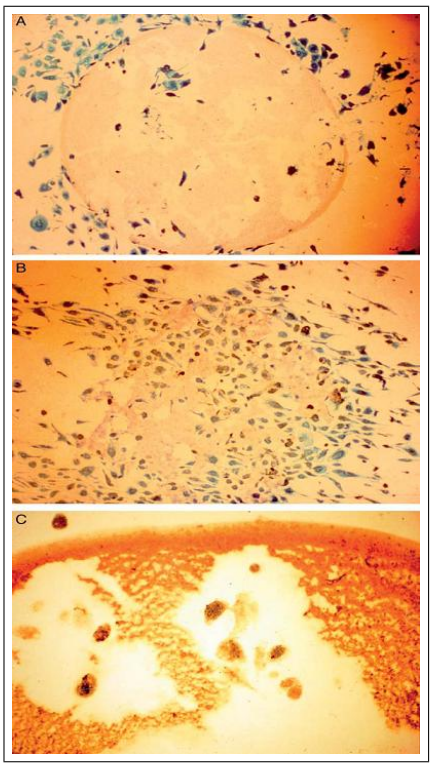

Figure 2.6 In Vitro Phagocytic Response of Activated Microglia to Aβ Deposits [6]: Picture C shows the result of phagocytic response.

2.2.3 Microglial Activation

Neurons may inform microglia about their situation whether they are damaged or healthy by releasing activity-associated cotransmitters, such as adenosine triphosphate (ATP), or fractalkine [7]. If there is a dangerous situation such as damaged neurons, disturbed fractalkine signaling or massive release of ATP then resting microglial cells go into transformation and they become activated [7]. Activated microglia produce cytokines (IL-1, IL-6, TNF-α) and other factors (proteases) with potential toxicity for neurons [5, 7, 16, 54]. Proteases secreted by microglia catabolise specific proteins causing direct cellular damage, while cytokines like IL-1 promote demyelination of neuronal axons [59]. Moreover, microglia can injure neurons through N-methyl D-aspartate (NMDA) receptor-mediated processes by secreting glutamate [7]. This process causes calcium ions to enter cells via NMDA receptor channels, leading to neuronal damage and eventual cell death.

Activated microglia activate or aggravate potential for astrocyte involvement and further microglial recruitment. Highly activated microglia aggregate around the senile plaques and extracellular tangles of AD-affected brain. Their purpose is to phagocytose the lesions, but they fail to do so. Instead, the attack spills over, destroying neighboring neurons and their processes (Figure 2.7). As a result, chronic inflammatory response can result in large scale neural damage as the microglia ravage the brain in an attempt to destroy the amyloid plaques.

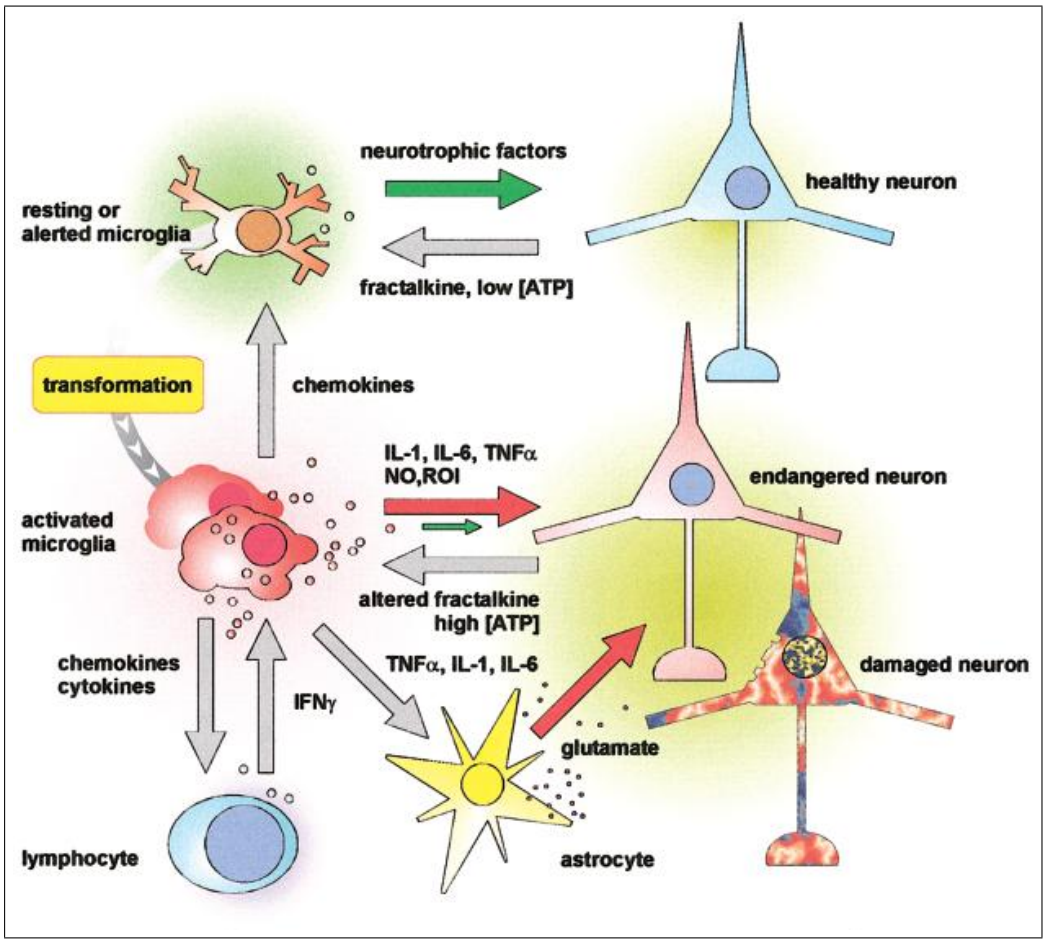

Figure 2.7 Activation of Microglia [7]

2.2.4 Cytokine Production

Cytokines are proteins secreted by the cells of innate and adaptive immunity that mediate many functions of these cells [60]. They are a category of signaling molecules

that are used extensively in cellular communication. They are heterogeneous protein mixtures of low molecular weight (8-80 kDa) and they regulate cell growth, survival, differentiation, and activities [7]. Moreover, they are mainly molecules with a fundamental role in the regulation of inflammatory processes. Under normal circumstances, the level of cytokines in CNS is strictly regulated and very low [40]. Recent studies indicate that the range of effects of cytokines and other brain neuroimmune modulators oscillates between the opposing actions of neuroprotection and neurodegeneration [5].

Inflammatory molecules, including cytokines, are produced in the brain of patients with dementia. The source of cytokines in the plasma has not been explained yet. They might in fact be produced by blood or endothelial cells, or they may originate from the brain. Although the blood-brain barrier (BBB) blocks peptides, such as cytokines, from travelling to the periphery, there are effective communication pathways where humoral, neuroendocrine, and sympathetic connections participate [40]. For instance, T-cells can cross the BBB so it is possible that they can liberate inflammatory mediators in brain during neurodegenerating diseases [61]. According to Chabot et al., interactions between activated T-cells and microglia induce the production of inflammatory cytokines [62].

2.2.4.1 Interleukin-1. Human IL-1 is a key mediator of the host response to various infectious, inflammatory and immunologic challenges. Two distinct polypeptides, IL-1α and IL-1β, mediate the biological activities and bind to the same cell surface receptors. They mediate acute-phase response and stimulate hepatic production of several acute-phase proteins [63]. IL-1 is known to regulate the synthesis of the amyloid protein precursor (APP) as well as processing of APP [64] and secretion of APP fragments by increasing the activity of Protein kinase C (PKC) [63]. Moreover, it upregulates the cdk5/p35 complex which is one of the main protein kinases involved in tau hyperphosphorylation [53].

IL-1 is an immunoregulatory cytokine that is overexpressed within affected cerebral cortical regions of the AD brain, as a result of activated microglia associated with

AD plaques [12, 65]. Griffin *et al*, 1989, first proposed that microglial and astrocytic activation would be accompanied by excessive expression of two cytokines, IL-1 and S100 protein, in brains of AD patients [66].

A study revealed that an acute inflammatory stimulus (such as IL-1), when administered to the brain, it induced the production of inflammatory cytokines in the periphery [40]. This study proposed that a peripheral immune alteration in AD patients may be triggered by brain immune activation, given the existence of inflammatory signaling pathway from the brain to the periphery. According to another study, endotoxins were given to rats to mimic a peripheral infection that was resulted in increased secretion of IL-1β within the central nervous system [67]. The authors of this study claimed that a systemic infection may act as a potent secondary stimulus to the "brain microglia" and initiated a positive feedback cycle that gave rise to an increasing accumulation of pathological changes and cognitive decline.

Most studies that have investigated serum or plasma levels of IL-1β have not detected any difference between AD patients and controls [68, 69, 70]. On the other hand, some studies found that AD patients had higher serum concentrations of IL-1β than controls [41, 71, 72]. There were really a few studies on IL-1α: according to these studies, serum IL-1α level did not show any significant differences between AD patients and healthy elderly subjects [73, 74].

2.2.4.2 Tumor Necrosis Factor-α. TNF-α is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. It is considered to increase the inflammation [75] and it manifests synergistic effects with IL-1 in inflammatory processes [76]. It initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection [77]. In addition to its pro-inflammatory functions, TNF-α has recently been recognized to be a gliotransmitter that regulates synaptic function in neural networks [78]. Gliotransmitters are chemicals released from glial cells that facilitate neuronal communication between neurons and other glial cells [79].

In the pathogenesis of AD, TNF-α is produced by activated microglia, mainly in response to the Aβ40 and Aβ42 peptides as well as to oxidative stress [51]. Increased amount of TNF-α play a crucial role in NFT formation [5]. One study demonstrated that TNF-α significantly increased fractalkine mRNA (>100 fold) and protein expression, which was associated with increased shedding of fractalkine from the cell [74]. As it is mentioned before, increased fractalkine acts as an anti-inflammatory response that prevents further microglial recruitment.

It is considered that serum concentration of TNF-α increase with age [80]. But, results regarding concentration of TNF-α in the serum of AD patients is not consistent. In several studies, serum TNF-α level has been found to be decreased [83,85] or unchanged [41, 81] in AD. In contrast to these findings, higher serum TNF-α concentration has been reported for AD patients [42, 80, 82, 83].

2.2.4.3 Interleukin-10. IL-10 is a potent anti-inflammatory cytokine in the CNS that plays an important role in the process of reducing inflammation during AD. Moreover, the function of IL-10 is to limit inflammation by decreasing the synthesis of proinflammatory cytokines, suppressing cytokine receptor expression and inhibiting markers of activation [51]. Furthermore, in the inflammatory response, TNF-α and IL-10 have opposing roles. While TNF-α is generally proinflammatory, IL-10 terminates inflammatory reactions [84]. It is a potent suppressor of TNF-α, IL-1α, IL-1β, and IL-6, all of which have been investigated for their potential association with AD [85]. It is produced primarily by monocytes and to a lesser extent by lymphocytes.

IL-10 serum levels in AD patients are reported to be unchanged [41, 81, 61] when compared with controls.

2.3 Brain Atrophy and Alzheimer's Disease

Characteristic degenerative pathology in Alzheimer's disease consists of atrophy and the increased presence of neurofibrillary tangles and amyloid plaques. This degenerative pathology gradually accumulates over a period of years, long before clinical symptoms become manifest [86]. Herein, atrophy may be considered as a better marker for impaired functioning of brain regions than the deposits of insoluble proteins in plaques and tangles. Moreover, atrophy measurements in some key brain regions, obtained from structural magnetic resonance (MR) images, are currently the most established biomarkers for studying disease progression, and assessing new therapies [87, 88].

Early detection of AD is seen as important because treatment may be most efficacious if introduced as early as possible. Several studies demonstrated that using MRI to evaluate atrophy of temporal lobe structures (hippocampus) can contribute to diagnostic accuracy [89, 90]. In pathological studies, a strong correlation between a decrease in the volume of hippocampus and a decrease in the total number of neurons in Alzheimer's disease has been reported [91]. Moreover, studies of autopsied cases of advanced AD have emphasized that amygdalar atrophy is related to the pathology of the disease [92, 93]. They showed that the amygdalar atrophy in Alzheimer's disease was due to the loss of neuronal somata and processes to the accumulation of neuritic plaques and neurofibrillary tangles. According to another study, atrophy was also observed at corpus callosum (cc) and this atrophy was mainly located in the anterior and posterior subregions [94]. As it is known, the corpus callosum connects the two cerebral hemispheres and it is the largest white matter fiber bundle in the human brain.

Direct measurement of the caudate nucleus in AD patients revealed decreased volume of this structure [95]. In addition, De Jong *et al.*, 2008, has reported that putaminal and thalamic atrophy play pivotal roles in cognitive decline in patients with AD [86]. Furthermore, Hayashi *et al.*, 2008, has noted that whole brain, cerebrum and cerebellum-brain stem volumes in patients with AD were significantly lower than those in normal volunteers [96].

Beside these different atrophies in AD brain, lateral ventricle (LV) enlargement is thought to be another indicator of AD. Creasey *et al.*, 1986, claimed that it was possible to differentiate Alzheimer's patients from normal subjects by measuring ventricular size and the progressive enlargement of LVs [97]. Moreover, another neuroimaging finding was that cerebrospinal fluid (CSF) volume was increased in AD patients [98].

3. MATERIALS AND METHODS

3.1 Recruitment

The recruitment of the patients was done by the Department of Neurology in Istanbul University, Istanbul Faculty of Medicine, Behavioral Neurology and Movement Disorders Unit. A neurologist and neuropsychologist, examined all patients. In order to characterize the families on clinical grounds, each patient was assessed according to a standardized protocol. 28 AD patients and 16 healthy controls participated to the study. All patients fulfilled the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for AD. Participants with evidence of systemic inflammation on clinical examination or serum biochemical tests (increased number of white blood cells, elevated C-reactive protein, elevated erythrocyte sedimentation rate) were excluded from the study. Written consent form was required from all participants before sampling. The project was approved by the local ethical committee of Istanbul University Faculty of Medicine. Exclusion criteria for controls were:

1. Being younger than 50 years old.
2. Evidence of progressive dementia
3. Having a history of stroke, repeated head injuries, encephalitis

3.2 Mini-Mental State Examination

Mini-Mental State Examination (MMSE) is a widely used, well-validated screening tool for cognitive impairment. It briefly measures orientation to time and place, immediate recall, short-term verbal memory, calculation, language, and construct ability. Each area tested has a designated point value, with the maximum possible score

on the MMSE being 30/30. In general, scores fall into four categories: 24 - 30 is "normal, " 20 - 23 points correspond to mild cognitive impairment or possible early-stage/mild Alzheimer's, 10 - 19 points is middle-stage/moderate Alzheimer's and 0- 9 is late-stage/severe Alzheimer's disease. Although, it is used to estimate the severity of cognitive impairment, MMSE should not be used all by itself to make a diagnosis. Mini-Mental State Examination was applied to 28 AD patients and 16 controls.

3.3 Blood Sampling and Serum Storage

Before sampling all participants received a personal code containing a number and the first three letters of their family names. Then, 10 ml blood was collected from patients at hungry state. The interior of the tubes were coated with clot activator (silica particles) to accelerate clotting. 30 minutes after sampling the clotting process was completed. In order to isolate serum, blood samples were centrifuged at 3500 rpm (Round Per Minute) for 15 minutes. Next, serum was pippetted into Eppendorf tubes and stored at -80°C until the ELISA analysis were performed.

3.4 Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

Sandwich ELISA is a biochemical technique used mainly in immunology to detect the presence of antibody or antigen concentration in unknown samples. It measures the amount of antigen between two layers of antibodies (i.e. capture and detection antibody). The antigen to be measured must contain at least two antigenic sites capable of binding to antibody, since at least two antibodies act in the sandwich. So sandwich assays are restricted to the quantitation of multivalent antigens such as proteins or polysaccharides. Sandwich ELISAs for quantitation of antigens are especially valuable when the concentration of antigens is low and/or they are contained in high concentrations of contaminating protein. The ELISA has been used as a diagnostic tool in medicine and plant pathology.

The sample with an unknown amount of antigen was immobilized on a polystyrene microtiter plate. After the antigen was immobilized, the detection antibody was added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody (Streptavidin - HRP) which was linked to an enzyme through bioconjugation. Between each step the plate was typically washed with a mild detergent solution to remove any proteins or antibodies that were not specifically bound. After the final wash step the plate was developed by adding an enzymatic substrate, tetramethylbenzemidine (TMB), to produce a visible signal, which indicates the quantity of antigen in the sample (ELISA procedure,(Figure 3.1)). Moreover, stop solution was used to terminate the peroxidase/TMB reaction for ELISA applications. The TMB substrate reacted with immobilized horseradish peroxidase (HRP) conjugated secondary antibodies to produce a blue solution. Color intensity is an indication of analyte level. After attaining the desired intensity, the reaction was terminated by addition of STOP Solution. Upon addition of STOP Solution the color turned from blue to yellow. In the end, absorbance was read at 450 nm with a microtitter plate reader in order to measure and calculate the amount of cytokines in the samples. The advantage of Sandwich ELISA is that the sample does not have to be purified before analysis, and the assay can be very sensitive.

ELISA was performed using commercial kits, which were specific to each cytokine, according to manufacturer's instructions (Invitrogen Elisa kits). All performances regarding the mentioned biomarkers were done in duplicate.

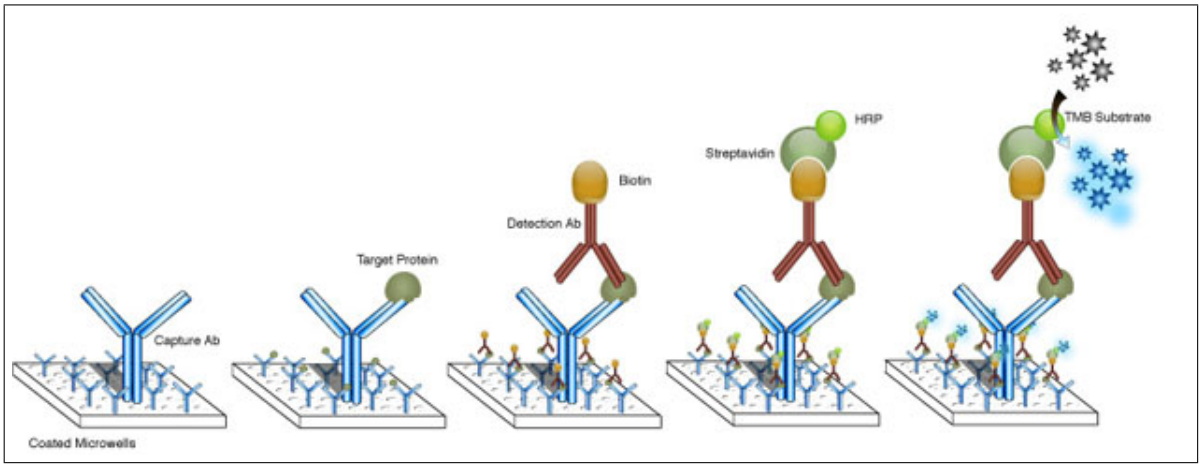

Figure 3.1 Sandwich Elisa Procedure (www.epitomics.com)

3.4.1 ELISA Procedure for Interleukin-1α

Interleukin -1 alpha ELISA kit (Invitrogen, catalog no. KAC1191) contains Hu IL-1α Standard, Standard Diluent Buffer, Hu IL-1α Antibody-Coated Wells, Hu IL-1α Biotin Conjugate, Incubation Buffer, Streptavidin-Peroxidase (HRP), Streptavidin-Peroxidase (HRP) Diluent, Wash Buffer, Stabilized Chromogen (Tetramethylbenzidine - TMB), Stop Solution, Plate Covers.

The procedure was conducted according to the instructions manuel as follows:

3.4.1.1 Preparation of Reagents. i) All standard solutions were prepared.

1. Standard was reconstituted to 10,000 pg/mL with Standard Diluent Buffer.
2. 0.010 mL of the 10,000 pg/mL standard was added to a tube containing 0.390 mL Standard Diluent Buffer and this tube was labeled as 250 pg/mL Hu IL-1α Mix.
3. 0.200 mL of Standard Diluent Buffer was added to each of 6 tubes labeled 125,

62.5, 31.3, 15.6, 7.8, and 3.9 pg/mL Hu IL-1α.

4. Serial dilutions of the standards were made.

ii) This step was same for all other kits. Streptavidin - HRP Preparation and Wash Buffer Dilution were performed as follows:

1. 120 μL of 100x Streptavidin - HRP concentrated solution was diluted with 12 mL of Streptavidin-HRP Diluent and it was labeled as Streptavidin-HRP Working Solution.
2. 25x Wash Buffer concentrate was allowed to reach room temperature and it was mixed gently to ensure that any precipitated salts have redissolved.
3. 1 volume of the 25x Wash Buffer concentrate was diluted with 24 volumes of deionized water (25ml - 600ml).

3.4.1.2 Assay Procedure.

1. 50 μL of the Standard Diluent Buffer was added to the zero standard wells. Wells reserved for chromogen blanks were left empty.
2. 50 μL of standards and serum samples were added to the appropriate microtiter wells.
3. 50 μL of Incubation Buffer was added into each well except the chromogen blank wells.
4. 50 μL of biotinylated anti-IL-1α (Biotin Conjugate) solution was pipetted into each well except the chromogen blanks.
5. Plate was covered with plate cover and was incubated for 2 hours at room temperature.

6. Solution from wells was thoroughly aspirated and liquid was discarded automatically by using a microtitter plate washer. Wells were washed 4 times.

7. 100 μL Streptavidin-HRP Working Solution was added to each well except the chromogen blanks.

8. Plate was covered with plate cover and was incubated for 30 minutes at room temperature.

9. Solution from wells was thoroughly aspirated and liquid was discarded automatically by using a microtitter plate washer. Wells were washed 4 times.

10. 100 μL of Stabilized Chromogen was added to each well. The liquid in the wells started to turn blue.

11. Plate was incubated for 30 minutes at room temperature and in the dark.

12. 100 μL of Stop Solution was added to each well. Side of plate was tapped gently to mix. In the end, the solution in the wells started to change from blue to yellow.

13. The absorbance of each well was read at 450 nm having blanked the plate reader against a chromogen blank composed of 100 μL each of Stabilized Chromogen and Stop Solution.

14. A curve fitting software was used to generate the standard curve.

15. The concentrations for unknown samples and controls were read from the standard curve.

3.4.2 ELISA Procedure for Interleukin-1β

Interleukin -1 beta ELISA kit (Invitrogen, catalog no. KHC0011) contains Hu IL-1β Standard, Standard Diluent Buffer, Hu IL-1β Antibody-Coated Wells, Hu IL-1β Biotin Conjugate, Streptavidin-Peroxidase (HRP), Streptavidin-Peroxidase (HRP) Diluent, Wash Buffer, Stabilized Chromogen (Tetramethylbenzidine - TMB), Stop Solution, Plate Covers.

The procedure was conducted according to the instructions manuel as follows:

3.4.2.1 Preparation of Reagents. i) Standard solutions were prepared.

1. Standard was reconstituted to 2500 pg/mL with Standard Diluent Buffer.
2. 0.100 mL of the 2500 pg/mL standard was added to a tube containing 0.900 mL Standard Diluent Buffer and this tube was labeled as 250 pg/mL Hu IL-1β Mix.
3. 0.250 mL of Standard Diluent Buffer was added to each of 6 tubes labeled 125, 62.5, 31.3, 15.6, 7.8, and 3.9 pg/mL Hu IL-1β.
4. Serial dilutions of the standard were made.

ii) Streptavidin - HRP preparation and Wash Buffer Dilution was same with IL-1α.

3.4.2.2 Assay Procedure.

1. 50 μL of the Standard Diluent Buffer was added to the zero standard wells. Wells reserved for chromogen blanks were left empty.
2. 50 μL of standards and serum samples were added to the appropriate microtiter wells.
3. 100 μL of biotinylated anti-IL-1β (Biotin Conjugate) solution was pipetted into each well except the chromogen blanks.
4. Plate was covered with plate cover and was incubated for 2 hours at room temperature.
5. Solution from wells was thoroughly aspirated and liquid was discarded automatically by using a microtitter plate washer. Wells were washed 4 times.

6. 100 μL Streptavidin-HRP Working Solution was added to each well except the chromogen blanks.

7. Plate was covered with plate cover and was incubated for 30 minutes at room temperature.

8. Solution from wells was thoroughly aspirated and liquid was discarded automatically by using a microtitter plate washer. Wells were washed 4 times.

9. 100 μL of Stabilized Chromogen was added to each well. The liquid in the wells started to turn blue.

10. Plate was incubated for 25 minutes at room temperature and in the dark.

11. 100 μL of Stop Solution was added to each well. Side of plate was tapped gently to mix. In the end, the solution in the wells started to change from blue to yellow.

12. The absorbance of each well was read at 450 nm having blanked the plate reader against a chromogen blank composed of 100 μL each of Stabilized Chromogen and Stop Solution.

13. A curve fitting software was used to generate the standard curve.

14. The concentrations for unknown samples and controls were read from the standard curve.

3.4.3 ELISA Procedure for Interleukin-10

Interleukin -10 ELISA kit (Invitrogen, catalog no. KHC0102) contains Hu IL-10 Standard, Standard Diluent Buffer, Incubation Buffer, Hu IL-10 Antibody-Coated Wells, Hu IL-10 Biotin Conjugate, Streptavidin-Peroxidase (HRP), Streptavidin-Peroxidase (HRP) Diluent, Wash Buffer, Stabilized Chromogen (Tetramethylbenzidine - TMB), Stop Solution, Plate Covers.

The procedure was conducted according to the instructions manuel as follows:

3.4.3.1 Preparation of Reagents. i) Standard solutions were prepared.

1. Standard was reconstituted to 5000 pg/mL with Standard Diluent Buffer.
2. 0.050 mL of the 5000 pg/mL standard was added to a tube containing 0.450 mL Standard Diluent Buffer and this tube was labeled as 500 pg/mL Hu IL-10 Mix.
3. 0.200 mL of Standard Diluent Buffer was added to each of 6 tubes labeled 250, 125, 62.5, 31.2, 15.6 and 7.8 pg/mL Hu IL-10.
4. Serial dilutions of the standard were made.

ii) Streptavidin - HRP preparation and Wash Buffer Dilution was same with IL-1α.

3.4.3.2 Assay Procedure.

1. 50 μL of the Standard Diluent Buffer was added to the zero standard wells. Wells reserved for chromogen blanks were left empty.
2. 50 μL of standards and samples were added to the appropriate microtiter wells.
3. 50 μL of Incubation Buffer was added into each well except the chromogen blank wells.
4. Plate was covered with plate cover and was incubated for 2 hours at room temperature.
5. Solution from wells was thoroughly aspirated and liquid was discarded automatically by using a microtitter plate washer. Wells were washed 4 times.
6. 100 μL of biotinylated anti-IL-10 (Biotin Conjugate) solution was pipetted into each well except the chromogen blanks.

7. Plate was covered with plate cover and was incubated for 2 hours at room temperature.

8. Solution from wells was thoroughly aspirated and liquid was discarded automatically by using a microtitter plate washer. Wells were washed 4 times.

9. 100 μL Streptavidin-HRP Working Solution was added to each well except the chromogen blanks.

10. Plate was covered with plate cover and was incubated for 30 minutes at room temperature.

11. Solution from wells was thoroughly aspirated and liquid was discarded automatically by using a microtitter plate washer. Wells were washed 4 times.

12. 100 μL of Stabilized Chromogen was added to each well. The liquid in the wells started to turn blue.

13. Plate was incubated for 30 minutes at room temperature and in the dark.

14. 100 μL of Stop Solution was added to each well. Side of plate was tapped gently to mix. In the end, the solution in the wells started to change from blue to yellow.

15. The absorbance of each well was read at 450 nm having blanked the plate reader against a chromogen blank composed of 100 μL each of Stabilized Chromogen and Stop Solution.

16. A curve fitting software was used to generate the standard curve.

17. The concentrations for unknown samples and controls were read from the standard curve.

3.4.4 ELISA Procedure for Tumor Necrosis Factor-α

Tumor Necrosis Factor (TNF-α) ELISA kit (Invitrogen, catalog no. KHC3012) contains Hu TNF-α Standard, Standard Diluent Buffer, Incubation Buffer, Hu TNF-α

Antibody-Coated Wells, Hu TNF-α Biotin Conjugate, Streptavidin-Peroxidase (HRP), Streptavidin-Peroxidase (HRP) Diluent, Wash Buffer, Stabilized Chromogen (Tetramethylbenzidine - TMB), Stop Solution, Plate Covers.

The procedure was conducted according to the instructions manuel as follows:

3.4.4.1 Preparation of Reagents. i) Standard solutions were prepared.

1. Standard was reconstituted to 2000 pg/mL with Standard Diluent Buffer.
2. 0.300 mL of the 2000 pg/mL standard was added to a tube containing 0.300 mL Standard Diluent Buffer and this tube was labeled as 1000 pg/mL Hu TNF-α Mix.
3. 0.300 mL of Standard Diluent Buffer was added to each of 6 tubes labeled 500, 250, 125, 62.5, 31.2 and 15.6 pg/mL Hu TNF-α.
4. Serial dilutions of the standard were made.

ii) Streptavidin - HRP preparation and Wash Buffer Dilution was same with IL-1α.

3.4.4.2 Assay Procedure.

1. 50 μL of Incubation Buffer was added into each well except the chromogen blank wells.
2. 100 μL of the Standard Diluent Buffer was added to the zero standard wells. Wells reserved for chromogen blanks were left empty.
3. 100 μL of standards and samples were added to the appropriate microtiter wells.

4. Plate was covered with plate cover and was incubated for 2 hours at room temperature.

5. Solution from wells was thoroughly aspirated and liquid was discarded automatically by using a microtitter plate washer. Wells were washed 4 times.

6. 100 μL of biotinylated anti-TNF-α (Biotin Conjugate) solution was pipetted into each well except the chromogen blanks.

7. Plate was covered with plate cover and was incubated for 1 hour at room temperature.

8. Solution from wells was thoroughly aspirated and liquid was discarded automatically by using a microtitter plate washer. Wells were washed 4 times.

9. 100 μL Streptavidin-HRP Working Solution was added to each well except the chromogen blanks.

10. Plate was covered with plate cover and was incubated for 30 minutes at room temperature.

11. Solution from wells was thoroughly aspirated and liquid was discarded automatically by using a microtitter plate washer. Wells were washed 4 times.

12. 100 μL of Stabilized Chromogen was added to each well. The liquid in the wells started to turn blue.

13. Plate was incubated for 30 minutes at room temperature and in the dark.

14. 100 μL of Stop Solution was added to each well. Side of plate was tapped gently to mix. In the end, the solution in the wells started to change from blue to yellow.

15. The absorbance of each well was read at 450 nm having blanked the plate reader against a chromogen blank composed of 100 μL each of Stabilized Chromogen and Stop Solution.

16. A curve fitting software was used to generate the standard curve.

17. The concentrations for unknown samples and controls were read from the standard curve.

3.5 MR Imaging and Volumetric Measurement

MR images were acquired on a 1.5 T imaging system (Philips Medical Systems, Eindhoven, Netherlands) at the NPI Neuropsychiatry Hospital, Istanbul. Two acquisitions of T1-weighted T1 TFE (MPRAGE) sequence (repetition time [TR] 8.6 ms, echo time [TE] 4 ms, flip angle [FA] 80 , inversion time [TI] 1000 ms, FOV 240 mm, Matrix 192/256 r, 150 coronal slices, slice thickness 1.2 mm without gaps, time per acquisition 7.23 min.) were planned to be acquired from each subject together in order to optimize signal-to-noise ratio for the use of gray/white contrast for segmentation. Volumetric segmentation was performed with the FreeSurfer image analysis suite (version 4.5.0), which was documented and freely available for download online (http://surfer.nmr.mgh.harvard.edu/fswiki). The technique has previously been shown to be comparable in accuracy to manual labeling [99].

FreeSurfer software can automatically parcellate the entire brain into anatomic regions and quantify the tissue properties in these regions for a single individual. Briefly, this processing includes removal of non-brain tissue using a hybrid watershed/surface deformation procedure, automated Talairach transformation, segmentation of the subcortical white matter and deep gray matter volumetric structures (including hippocampus, amygdala, caudate, putamen, ventricles), intensity normalization, tessellation of the gray matter white matter boundary, automated topology correction, and surface deformation following intensity gradients to optimally place the gray/white and gray/CSF borders at the location where the greatest shift in intensity defines the transition to the other tissue class [100].

3.6 Statistical Analysis

Independent samples t-test, receiver operating characteristic (ROC) analysis, factor and regression analysis were done with SPSS (version 15.0). Independent samples t-test was used to test for the difference between AD patients and controls on the means

of MMSE scores, volumetric MR measurements and cytokine levels in serum.

In order to observe the type of relation between serum cytokine levels and volumetric MR measurements in AD patients, regression analysis curve estimation method was used. Before regression analysis, a factor analysis was applied to our volumetric MR data. For factor analysis, 16 anatomical region was used to extract factor components. Then, each cytokine level was chosen as a dependent variable for regression analysis with factor analysis components.

A commonly known method was used to determine a cut-off value for IL-1α, IL-1β and TNF-α. The receiver operating characteristic (ROC) analysis was carried out to determine and calculate the cut-off points. ROC curve is related in a direct and natural way to cost/benefit analysis of diagnostic decision making. A ROC curve shows the characteristics of a diagnostic test by graphing the false-positive rate (1-specificity) and the true-positive rate (sensitivity) for various cut-off values. We determined best cut-off point and this cut-off value was tested for the accuracy of diagnostic test with GraphPad InStat (version 3.1) statistical software. Sensitivity, specificity, positive and negative predictive values for IL-1α, IL-1β and TNF-α were calculated. In order to find the type of relation between the MMSE scores (severity of AD) and ELISA cytokine measurements regression analysis curve estimation method was applied.

4. RESULTS

The mean (SD) age of the 28 AD patients followed up in the study was 69 (±7.8) years and the mean age of the 16 controls was 64 (±7,6) years.

4.1 ELISA Measurements

All ELISA performances were done in duplicate. 2 sets of kits were used for all samples since each kit had a limited number of sample capacity.

4.1.1 IL-1α Level

Optical density (OD) values of standards with known concentration were used to generate a standard curve and by using this standard curve's equation, serum IL-1α concentration of AD patients and controls were calculated (Figure 4.1).

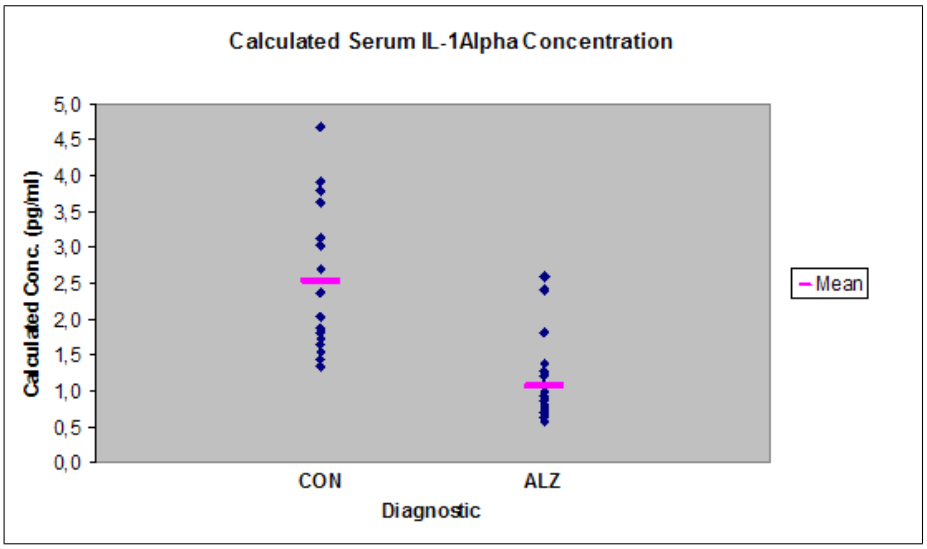

Figure 4.1 Calculated Serum IL-1α Concentration of AD Patients and Controls

According to independent samples t-test result (Table 4.1), mean of IL-1α con-

centration was found lower in AD patients (M=1,07 pg/ml) compared to controls (M=2,54 pg/ml) and this difference was significant; $t(42) = 5.33$, $p=0.000 < 0.05$.

Table 4.1
Mean IL-1α concentration and independent samples t-test result

Cytokine	Diagnostic	Number	Mean(pg/ml)	Std.Dev	t value	df	p value
IL-1α	Control	16	2.54	1.04	5.33	42	**0.000 ***
	Alzheimer	28	1.07	0.50			

After independent samples t-test, by using ROC curve best cut-off value (Figure 4.2) was determined for IL-1α. This determined cut-off value was 1.35 pg/ml. Sensitivity, specificity, positive and negative predictive values for IL-1α were calculated for this cut-off point. Statistical analysis was done with GraphPad InStat statistical software. The two-sided P value was $0.000 < 0.01$, considered extremely significant (Table 4.2). Sensitivity value (0.85) and negative predictive value (0.78) were high enough to use serum IL-1α level for a diagnostic test.

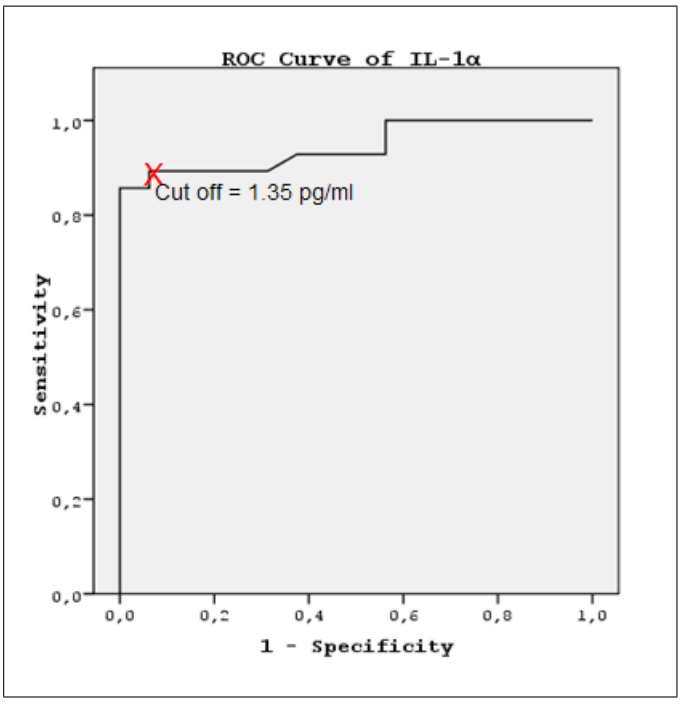

Figure 4.2 ROC Curve and Cut-off Value for IL-1α

Table 4.2
IL-1α Cut-off value of AD patients

Group	Cut-off Value (pg/ml)	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	P Value
AD vs Controls	1.35	0.85	0.93	0.96	0.78	**0.000 ****

4.1.2 IL-1β Level

OD values of standards with known concentration were used to generate a standard curve and by using this standard curve's equation, serum IL-1β concentration of AD patients and controls were calculated (Figure 4.3).

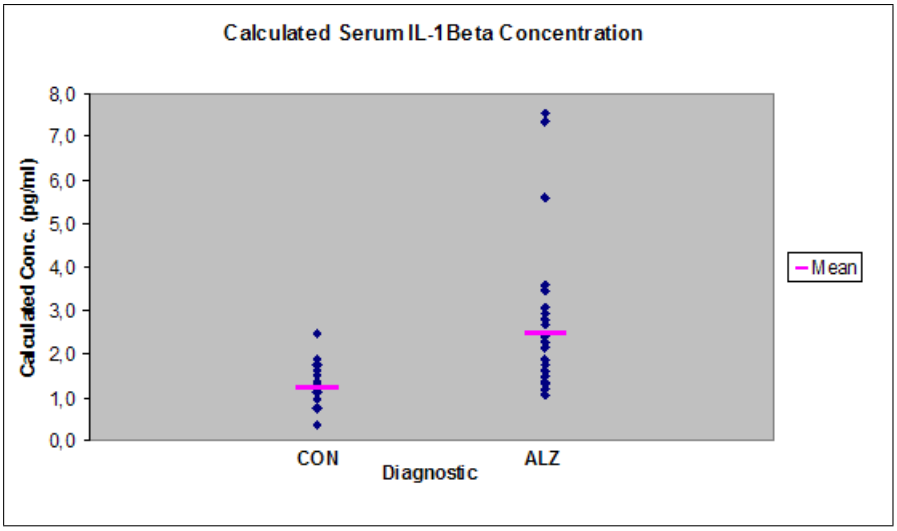

Figure 4.3 Calculated Serum IL-1β Concentration of AD Patients and Controls

According to independent samples t-test result (Table 4.3), mean of IL-1β concentration was found higher in AD patients (M=2,47 pg/ml) compared to controls (M=1,22 pg/ml) and this difference was significant; t(42) = -2.81 , p=0.007 $<$ 0.05.

Table 4.3
Mean IL-1β concentration and independent samples t-test result

Cytokine	Diagnostic	Number	Mean(pg/ml)	Std.Dev	t value	df	p value
IL-1β	Control	16	1.22	0.52	-2.81	42	**0.007 ***
	Alzheimer	28	2.47	1.72			

After independent samples t-test, by using ROC curve best cut-off value (Figure 4.4) was determined for IL-1β. This determined cut-off value was 1.8 pg/ml. Sensitivity, specificity, positive and negative predictive values for IL-1β were calculated for this cut-off point. Statistical analysis was done with GraphPad InStat statistical software. The two-sided P value was $0.021 < 0.05$, considered significant (Table 4.4). Sensitivity value (0.50) and negative predictive value (0.50) were low hence sample size should be increased to make a better diagnostic test decision.

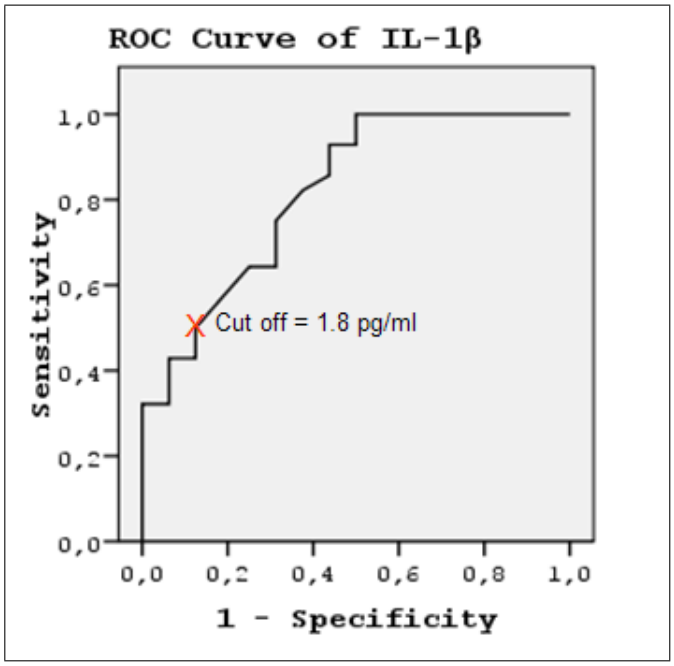

Figure 4.4 ROC Curve and Cut-off Value for IL-1β

Table 4.4
IL-1β Cut-off value of AD patients

Group	Cut-off Value (pg/ml)	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	P Value
AD vs Controls	1.8	0.50	0.87	0.87	0.50	**0.021 ***

4.1.3 IL-10 Level

OD values of standards with known concentration were used to generate a standard curve and by using this standard curve's equation, serum IL-10 concentration of AD patients and controls were calculated (Figure 4.5).

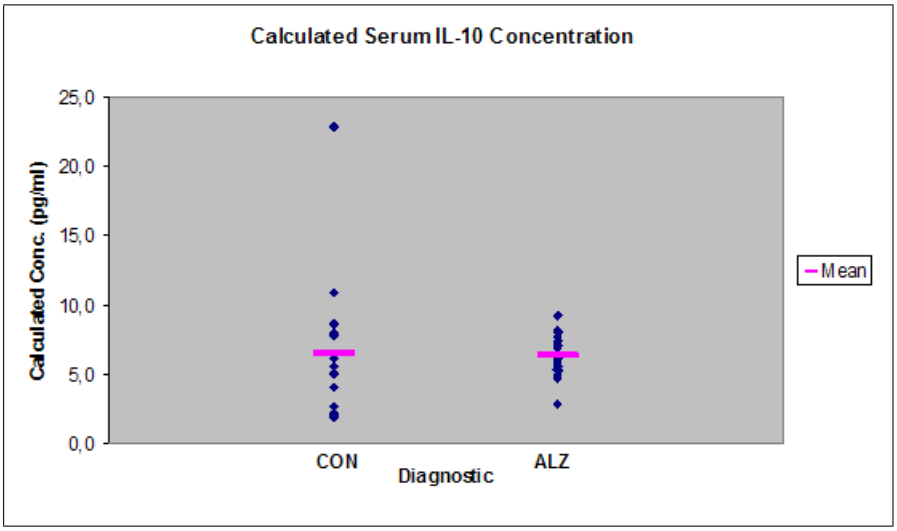

Figure 4.5 Calculated Serum IL-10 Concentration of AD Patients and Controls

According to independent samples t-test result (Table 4.5), mean of IL-10 concentration was not found significantly different in AD patients (M=6,42 pg/ml) compared to controls (M=6,46 pg/ml). $t(42) = 0.039$, $p=0.969 > 0.05$.

Table 4.5
Mean IL-10 concentration and independent samples t-test result

Cytokine	Diagnostic	Number	Mean(pg/ml)	Std.Dev	t value	df	p value
IL-10	Control	16	6.46	5.08	0.039	42	0.969
	Alzheimer	28	6.42	1.35			

4.1.4 TNF-α Level

OD values of standards with known concentration were used to generate a standard curve and by using this standard curve's equation, serum TNF-α concentration of AD patients and controls were calculated (Figure 4.6).

According to independent samples t-test result (Table 4.6), mean of serum TNF-α concentration was found higher in AD patients (M=28,50 pg/ml) compared to controls (M=18,49 pg/ml) and this difference was significant; t(42) = -3.43 , p=0.001 $<$ 0.05 .

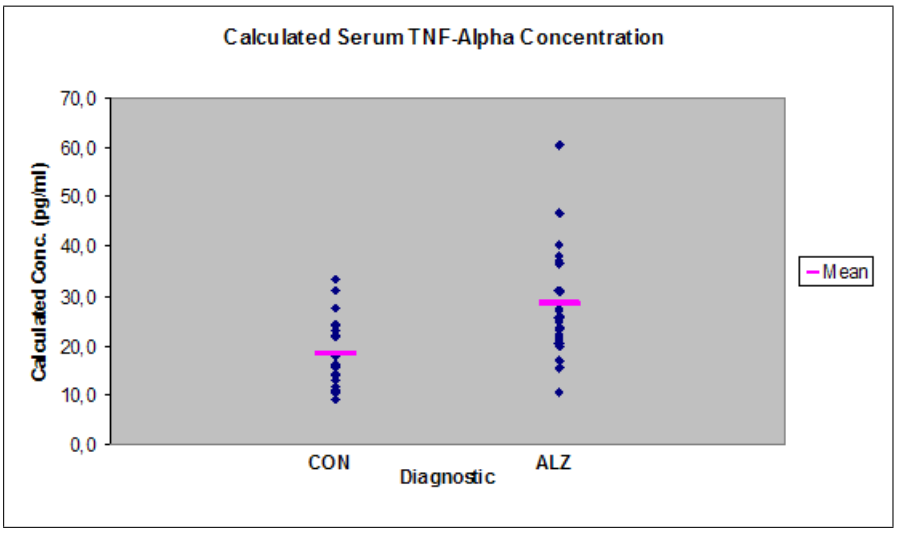

Figure 4.6 Calculated Serum TNF-α Concentration of AD Patients and Controls

Table 4.6
Mean TNF-α concentration and independent samples t-test result

Cytokine	Diagnostic	Number	Mean(pg/ml)	Std.Dev	t value	df	p value
TNF-α	Control	16	18.49	7.54	-3.43	42	**0.001 ***
	Alzheimer	28	28.50	10.18			

After independent samples t-test, by using ROC curve best cut-off value (Figure 4.7) was determined for TNF-α. This determined cut-off value was 25.18 pg/ml. Sensitivity, specificity, positive and negative predictive values for TNF-α were calculated for this cut-off point. Statistical analysis was done with GraphPad InStat statistical software. The two-sided P value was $0.011 < 0.05$, considered significant (Table 4.7). Sensitivity value (0.60) and negative predictive value (0.54) were low hence sample size should be increased to make a better diagnostic test decision.

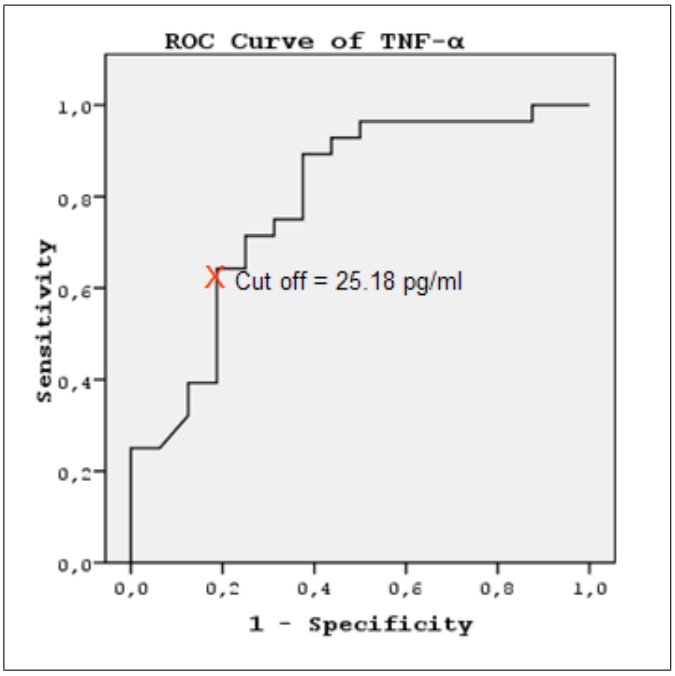

Figure 4.7 ROC Curve and Cut-off Value for TNF-α

Table 4.7
TNF-α Cut-off value of AD patients

Group	Cut-off Value (pg/ml)	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	P Value
AD vs Controls	25.18	0.60	0.81	0.85	0.54	**0.011 ***

4.2 Volumetric MR Measurements

MR images of subjects were taken at the NPI Neuropsychiatry Hospital, Istanbul. After taking MR images, volumetric segmentation was performed with the FreeSurfer image analysis suite (version 4.5.0). This software segmented brain into two hemispheres (right and left) and then gave the each segmented regions volume in terms of mm^3.

Total volume of each segmented region was analyzed in this study and each segmented region was divided by intracranial volume of each subject to correct the individual variance in brain size. According to independent samples t-test result (Table 4.8) the ratios of the cerebral white matter/cerebellum white matter/whole brain/thalamus proper/putamen/brain stem/hippocampus/amygdala/accumbens area and central/anterior regions of corpus callosum (CC) to the intracranial volume in AD patients were significantly lower than those in normal volunteers. On the other hand, the ratios of lateral ventricle and CSF to the intracranial volume in AD patients were significantly higher than those in controls. Other brain regions (caudate, pallidum and posterior CC) did not show any significant difference in AD patients compared to controls.

Table 4.8
Independent samples t-test result and mean ratios of each segmented region in AD patients and controls

Brain Region	Diagnostic	Number	Mean	Std.Dev	t value	df	p value
Cerebral White Matter	Control	16	0.30	0.018	8.023	42	**0.000 ***
	Alzheimer	28	0.26	0.018			
Lateral Ventricle	Control	16	0.013	0.011	-6.234	42	**0.000 ***
	Alzheimer	28	0.032	0.002			
Cerebellum White Matter	Control	16	0.019	0.002	3.01	42	**0.004 ***
	Alzheimer	28	0.017	0.002			
Thalamus Proper	Control	16	0.0086	0.0005	4.49	42	**0.000 ***
	Alzheimer	28	0.0078	0.0006			
Caudate	Control	16	0.0044	0.0006	0.02	42	0.984
	Alzheimer	28	0.0044	0.0008			
Putamen	Control	16	0.00688	0.00086	3.696	42	**0.001 ***
	Alzheimer	28	0.00590	0.00084			
Pallidum	Control	16	0.00204	0.00018	1.314	42	0.196
	Alzheimer	28	0.00196	0.00020			
Brain Stem	Control	16	0.01403	0.00104	2.029	42	**0.0491 ***
	Alzheimer	28	0.01326	0.00129			
Hippocampus	Control	16	0.00525	0.00054	8.895	42	**0.000 ***
	Alzheimer	28	0.00373	0.00054			
Amygdala	Control	16	0.00193	0.00025	7.481	42	**0.000 ***
	Alzheimer	28	0.00144	0.00018			
Cerebrospinal fluid (CSF)	Control	16	0.00086	0.00019	-3.391	42	**0.002 ***
	Alzheimer	28	0.00115	0.00032			
Accumbens Area	Control	16	0.00073	0.00009	7.204	42	**0.000 ***
	Alzheimer	28	0.00053	0.00009			
Corpus Callosum Posterior	Control	16	0.00062	0.00010	1.003	42	0.322
	Alzheimer	28	0.00059	0.00012			
Corpus Callosum Central	Control	16	0.00027	0.00005	3.537	42	**0.001 ***
	Alzheimer	28	0.00022	0.00004			
Corpus Callosum Anterior	Control	16	0.00055	0.00007	2.499	42	**0.016 ***
	Alzheimer	28	0.00049	0.00009			
Brain Segmented	Control	16	0.73648	0.03112	8.864	42	**0.000 ***
	Alzheimer	28	0.65337	0.02923			

4.2.1 Factor and Regression Analysis

First of all, factor analysis was performed with all volumetric MR parameters (16 defined anatomical region). After factor analysis, 5 factor components were extracted. After that, regression analysis curve estimation method was used to show the type of relation between each factor component and our serum cytokine measurements in AD patients (28 patients). We applied linear, logarithmic, inverse, quadratic, cubic, power and exponential regression models. We chose each cytokine levels as a dependent variable. TNF-α and IL-1α serum levels did not show a significant relation with our factor components. On the other hand, serum IL-1β level showed a significant linear relation with component 1 and component 2 (Table 4.9). By interpreting the rotated component matrix values, cerebral white matter was found highly loaded in component 1 while amygdala and hippocampus were found highly loaded in component 2.

Table 4.9
Linear regression analysis results (IL-1β dependent variable)

Model	R	R-square	df	t value	Sig. (p)
Component 1 (Cerebral White Matter)	0.381	0.145	26	-2.09	**0.046 ***
Component 2 (Amygdala and Hippocampus)	0.434	0.189	26	-2.46	**0.021 ***

4.3 Mini-Mental State Examination Scores

MMSE scores are accepted as a scale of severity in AD by clinicians. When the severity of the disease increases, a decrease in MMSE score is expected. In other words, there is a negative correlation between the severity of AD and MMSE score. Regression analysis curve estimation method was performed to observe the type of relation between the MMSE scores and cytokine ELISA measurements in AD patients

(28 patients). We applied linear, logarithmic, inverse, quadratic, cubic, power and exponential statistical regression models. MMSE was chosen as a dependent variable. Regression analysis curve estimation method revealed that there was not a significant relation between any cytokine level and MMSE score.

5. DISCUSSION

The overall aim of this study was to analyze the association between volumetric MR measurements of different regions of brain and possible AD related cytokine biomarkers in serum and to determine biological and clinical predictors for patients at high risk to develop AD. Biomarkers have many advantages for managing and predicting AD. Early diagnosis will allow clinicians in the community to successfully identify patients at high risk and this will shift the dynamic of treatment from slowing neurodegeneration to potential prevention, so that the patient would be more likely to die of natural causes and perhaps other factors, rather than AD. It is also likely that overall treatment effectiveness will be maximized when the treatment is started as early in the pathological process as possible.

In this study, ELISA method was used to detect cytokines level (IL-1α, IL-1β, IL-10, TNF-α) in the serum of both AD patients and control subjects. Blood samples and volumetric MR imaging data were collected from 28 AD patients and 16 control subjects. Mean (M) IL-1β concentration in AD patients was 2,47 pg/ml while it was 1,22 pg/ml in controls. This difference between control subjects and AD patients was significant according to independent samples t-test analysis (p= 0,007 $<$ 0,05). In the literature, some studies also found increased serum concentrations of IL-1β [41, 71, 72] in AD patients. Moreover, most studies could not detect any difference between AD patients and controls [68, 69, 70]. A study claimed that neuronal injury or insults including amyloid deposition may trigger a self-propagating cytokine cycle, which when chronically induced initiates a vicious feedback loop of continuing IL-1β elevation promoting further neuronal and synaptic dysfunction and Aβ plaque accumulation [101].

On the other hand, another pro-inflammatory cytokine IL-1α level, which is a member of IL-1 family like IL-1β, was found significantly decreased (p=0,000 $<$ 0,05) in AD patients (M=1,07 pg/ml) compared to controls (M=2,54 pg/ml). We

found only two studies about IL-1α level in serum of AD patients and they reported unchanged IL-1α level in AD patients [73, 74]. As we search the literature, this was the first study which shows a decrease in the serum IL-1α level of AD patients. To date most studies have focused on the CNS actions of IL-1β, rather than IL-1α, based on its more rapid induction following injury and Aβ deposition [102]. In our study, IL-1β level increased while IL-α level decreased in AD patients. They are encoded by separate genes sharing some sequence homology but they elicit similar biological actions. Although these results seem controversial it may indicate that somehow IL-1β synthesis during neuroinflammation down regulates the synthesis of IL-1α in the periphery. This was a really interesting result and CSF IL-1α level should be checked to see whether IL-1α decrease was specific to periphery or not. Even though the periphery cannot be regarded as a mirror to what is happening in the brain, the presence of an inflammatory situation in the brain results in an inflammatory response in the periphery.

In the pathogenesis of AD, TNF-α is produced by activated microglia, mainly in response to the Aβ40 and Aβ42 peptides as well as to oxidative stress [51]. It is one of the most investigated immune markers in AD where its role is considered to be equivocal either neurotrophic, or neurotoxic. In this study, TNF-α level in the serum of AD patients (M=28,50 pg/ml) was found significantly increased (p=0,001 $<$ 0,05) than controls (M=18,49 pg/ml). Many studies showed that TNF-α level in serum is not consistent. Some studies found higher TNF-α levels [42, 80, 82, 83] while some found lower levels [103, 104]. In other studies, it was reported that TNF-α level did not change [41, 81]. Additionally, TNF-α level was found increased in other neurodegenerative disease such as Parkinson [105]. However, we know that TNF-α manifests synergistic effects with IL-1 in inflammatory processes [76] and TNF-α may be trigger a positive feed-back cycle which increases the IL-1β secretion in the periphery or vice versa.

IL-10 is a potent anti-inflammatory cytokine in the CNS that plays an important role in the process of reducing inflammation (suppressor of TNF-α, IL-1α, IL-1β, and IL-6) during AD [90]. Several studies have reported unchanged serum IL-10 level in

AD patients [41, 61, 81]. In our study we also could not find any significant (p=0,969 $>$ 0,05) change between AD patients (M=6,42 pg/ml) and controls (M=6,46 pg/ml). This unchanged IL-10 level may explain the increased level of IL-1β and TNF-α in serum. Sufficient IL-10 is needed to suppress these pro-inflammatory cytokines (IL-1β and TNF-α) secretion otherwise their concentration will increase in the periphery. Our findings showed that IL-10 up-regulation was somehow inhibited in the periphery.

Low sensitivity and low negative predictive values of IL-1β and TNF-α indicated that sample size in our study might not be sufficient enough to make a better diagnostic decision and to identify individual patients which carry high risk. It can not be concluded that TNF-α and IL-1β levels in serum did not have enough power to be used as a diagnostic test in AD. A larger sample size is needed to make a better diagnostic decision. On the other hand, serum IL-1α can be used as a diagnostic test because we found a fine cut-off value (1.35 pg/ml) with a high sensitivity (0.85) and high negative predictive values (0.78). This finding was important and it should be strengthened with a larger sample size in a longitudinal study.

Hippocampus atrophy as the most common known atrophy in AD brain was extensively studied. Moreover, atrophy in amygdala [92, 93], in anterior and posterior subregions of corpus callosum [94], in caudate [95], in putamen [86], in thalamus [86], in whole brain and brain stem have been reported in some studies. Creasey *et al.*, 1986, reported lateral ventricle enlargement [97]. Furthermore, another neuroimaging finding was the increased CSF volume in AD patients [98].

When we come to our volumetric MR results, volume of cerebral white matter, cerebellum white matter, whole brain, thalamus proper, putamen, brain stem, hippocampus, amygdala, accumbens area and central and anterior regions of corpus callosum (CC) in AD patients were significantly lower than those in healthy controls. According to our results, these regions were obvious atrophic regions in brain of AD patients. In these findings, the main point is that whether these atrophic regions are specific to AD brain or not. Because in other neurodegenerative disease some of these regions may also be atrophic. On the other hand, lateral ventricle and cerebrospinal

fluid CSF volume in AD patients were found significantly increased than in controls. Different from these findings, we did not observe any significant volumetric change in caudate, pallidum and posterior CC between controls and AD patients. Volume of caudate, pallidum and posterior CC might be decreased at the severe stages of the disease. In order to determine AD specific atrophy regions a longitudinal study should be done with a patient control group such as mild cognitive impairment (MCI) patients. After observing quantitative morphological and functional brain changes in these subjects then more accurate interpretations can be made for the progression of atrophy regions in the brain of AD patients.

In this study, we studied biochemical and imaging biomarkers. While each of the candidate biomarkers has the potential to identify the presence of AD-related neurodegenerative pathology, it is unlikely any single one (imaging or biochemical) will have adequate positive or negative predictive power to be sufficient as the only test needed to make a definitive diagnosis in an individual patient. Many different parameters can be effective during AD progression. Therefore, we tried to observe the type of relation between serum cytokine levels and volumetric MR measurements in AD patients by using regression analysis curve estimation method. Before regression analysis, a factor analysis was applied to our volumetric MR data for 16 parameters. Regression analysis revealed that only serum IL-1β level had a significant linear relation with the volume changes of cerebral white matter and amygdala/hippocampus. This relation may explain the pivotal role of IL-1β in the neurodegeneration process. Decreased white matter volume in brain is a consequence of this neurodegeneration process in AD. We found a relation between the volume change of limbic system (amygdala/hippocampus) components and serum IL-1β level. However, it is really hard to explain the type of relation between IL-1β and amygdala/hippocampus.

Additionally, a regression analysis curve estimation method was applied to show the type of relation between our cytokine measurements and MMSE scores (severity of AD). No relation was observed between the severity of the cognitive impairment and serum cytokine measurements in AD patients. Cytokine production in the periphery may be accepted as the manifestation of Alzheimer disease. However, it can not be

concluded that cytokine production increases with the severity of the disease. Aβ levels in plasma and CSF may provide positive contribution to our finding. For instance, previous studies did not confirm an increase of Aβ levels in plasma or CSF of patients with AD [106, 107, 108]. Moreover, a study reported that they could not observe any relation between serum Aβ level and the severity of the disease [109]. These findings may indicate that Aβ load in brain is critical for the manifestation of AD. After passing a threshold Aβ amount in the brain serum cytokine and Aβ levels start to increase in the periphery. However, their level become stable during the disease progression.

6. CONCLUSIONS AND FURTHER RECOMMENDATIONS

The ELISA data from this study indicated that patients with AD produce increased quantities of pro-inflammatory cytokines (IL-1β and TNF-α) than normal subjects and these results supporting the hypothesis that a pro-inflammatory phenotype contributes to Alzheimer's disease. Consideration of the role of central neuroinflammatory cytokines in the modulation of peripheral immune responses, together with previous findings will allow for a more thoughtful and thorough analysis of the potential for serum biomarkers as predictors of mental status. As a result of this inflammatory response in AD, nonsteroidal anti-inflammatory drugs (NSAID) may protect against Alzheimer's disease (AD) risk. Secondly, volumetric MR data revealed that volume of different anatomical regions in AD brain showed significant differences than in controls. Moreover, a significant relation found between serum IL-1β level and cerebral white matter and amygdala/hippocampus. This relation may explain the crucial role of IL-1β in neurodegeneration process and makes it a strong candidate for further analysis. ROC analysis indicated that serum IL-1α level can be used as a diagnostic test. On the other hand,for our sample size TNF-α and IL-1β levels in serum were not sufficient for the diagnosis of AD. However, it does not mean that TNF-α and IL-1β levels in serum do not have enough power to be used as a diagnostic test. In order to make a better diagnostic decision, a larger sample size is required. At the end, we could not observe any significant relation between the peripheral cytokine production and the MMSE scores.

Our findings were promising but further analysis should be done in a larger sample size. Moreover, cytokine levels should also be measured in cerebrospinal fluid (CSF) to make stronger predictions. Because, combining CSF biomarker results with serum biomarker results will yield better diagnosis. Applying linear regression between CSF cytokine levels and volumetric MR measurements may provide more accurate results than our results. Moreover, mild cognitive impairment (MCI) patients should

be included into these type of studies. Because, mild cognitive impairment is conceived of as a transitional state between normal aging and Alzheimer's disease. Observing MCI patients cognitive state, volumetric MR measurements and cytokine level changes in a longitudinal study may enlighten our knowledge about the progression of AD. Beside imaging and biochemical markers, genetic analysis should also be included into extensive researches for the early diagnosis of AD.

As it is mentioned before, Turkey has a young population but the age range of elder people is increasing everyday which means to have more patients with Alzheimer's disease in the next years. Against the upcoming threat, the number of these studies must be increased.

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