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EFFECTS OF GENETICS ON THE NEURODYNAMICS OF ALZHEIMER'S DISEASE PATIENTS

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“To my grandparents“

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder and the leading cause of dementia. It has a significant impact on both the lives of patients and healthcare systems. AD is characterized by alterations in the electrical activity of the brain caused by multiple elements that ultimately lead to neuronal damage. Among these factors, disruptions in neurotransmission and synaptic modulation are especially significant. Cholinergic activity is known to be one of the most disturbed brain systems in AD, being the integrity of these neural populations essential in cognitive function. However, the genetic implications in cholinergic physiology during neurodegenerative states are still not clearly understood. For this reason, aiming to elucidate the relationship between cholinergic deficits and cortical rhythms is crucial to understand the disease from a fundamental level. The present study examined the effect of genetic alterations in neurotransmission systems to determine their impact on brain electrical activity. This work aims to characterize diverse features extracted from electroencephalogram (EEG) data depending on genetic variations associated with muscarinic and nicotinic acetylcholine receptors. To achieve this, the brain electrical activity of a cohort of 155 elderly subjects was analyzed, including 45 controls and 110 patients with Alzheimer's disease at different stages of severity. Multiple parameters derived from advanced signal analysis were calculated in both the time and frequency domains. The used metrics were relative power in classical bands (delta, theta, alpha, and beta), median frequency, spectral entropy, Lempel-Ziv complexity, and sample entropy. Subsequently, subjects with risk and protective variants of CHRNA3, CHRNA5, and CHRNA7 genes were compared. Specifically, five single nucleotide polymorphisms (SNPs) related to acetylcholine reception were studied: rs1044396, rs16969968, rs588765, rs8024987, and rs324650. Significant differences were found in relative power values within the alpha frequency band for the SNP rs1044396, between preclinical subjects with the risk variant (TT homozygote) and those with the protective variants (presence of the C allele). These findings align with previous research associating this genetic variant with reductions in alpha power and attention disorders. Considering the role of acetylcholine and the presence of attention impairment in AD, the results of the study suggest a relationship between the SNP rs1044396 and attention deficits, particularly in the preclinical stage. In addition, the results of the study suggest a relationship between the SNP rs1044396 and alterations in the electrical activity of the brain. Consequently, those modifications are proposed to be associated with mild cognitive impairment, or a higher probability to present dementia in

the future. On the other hand, variations in alpha power for AD were not observed. Consequently, it is suggested that, because of the previous impairment of the cholinergic system in the brains of AD patients, the genetic modification of only one subunit of a single receptor would not be meaningful. This study is an important step in expanding our knowledge about the implications of genetic factors in neurological disorders.

Resumen

La enfermedad de Alzheimer (EA) es un trastorno neurodegenerativo y la principal causa de demencia, con un impacto significativo tanto en la vida de los pacientes como en los sistemas de salud. La EA se caracteriza por alteraciones en la actividad eléctrica del cerebro causadas por múltiples elementos que finalmente conducen al daño neuronal. Entre estos factores, las interrupciones en la neurotransmisión y la modulación sináptica son especialmente relevantes. La actividad colinérgica se sabe que es uno de los sistemas cerebrales más afectados en la EA, siendo la integridad de estas poblaciones neuronales esencial para la función cognitiva. Sin embargo, las implicaciones genéticas en la fisiología colinérgica durante los estados neurodegenerativos aún no se comprenden claramente. Por esta razón, el objetivo de este estudio es dilucidar la relación entre los déficits colinérgicos y los ritmos corticales para comprender la enfermedad desde un nivel fundamental. El presente estudio examinó el efecto de las alteraciones genéticas en los sistemas de neurotransmisión para determinar su impacto en la actividad eléctrica del cerebro. Se caracterizaron diversas características extraídas de los datos del electroencefalograma (EEG) en función de las variaciones genéticas asociadas con los receptores de acetilcolina muscarínicos y nicotínicos. Para lograr esto, se analizó la actividad eléctrica cerebral de una cohorte de 155 sujetos ancianos, que incluía 45 controles y 110 pacientes con enfermedad de Alzheimer en diferentes etapas de gravedad. Se calcularon múltiples parámetros derivados del análisis avanzado de señales tanto en el dominio del tiempo como en el de la frecuencia. Las métricas utilizadas fueron la potencia relativa en las bandas clásicas (delta, theta, alfa y beta), la frecuencia mediana, la entropía espectral, la complejidad de Lempel-Ziv y la entropía muestral. Posteriormente, se compararon los sujetos con variantes de riesgo y protectoras de los genes CHRNA3, CHRNA5 y CHRNA7. Específicamente, se estudiaron cinco polimorfismos de nucleótido único (SNP) relacionados con la recepción de acetilcolina: rs1044396, rs16969968, rs588765, rs8024987 y rs324650. Se encontraron diferencias significativas en los valores de potencia relativa dentro de la banda de frecuencia alfa para el SNP rs1044396, entre los sujetos en etapa preclínica con la variante de riesgo (homocigoto TT) y aquellos con las variantes protectoras (presencia del alelo C). Estos hallazgos se alinean con investigaciones previas que relacionan esta variante genética con reducciones en la potencia alfa y trastornos de atención. Teniendo en cuenta el papel de la acetilcolina y la presencia de deterioro de la atención en la enfermedad de Alzheimer, los resultados del estudio sugieren una relación entre el SNP rs1044396 y déficits de atención, especial-

mente en la etapa preclínica. Además, los resultados del estudio sugieren una relación entre el SNP rs1044396 y alteraciones en la actividad eléctrica del cerebro. En consecuencia, se propone que estas modificaciones estén asociadas con un deterioro cognitivo leve o una mayor probabilidad de desarrollar demencia en el futuro. Por otro lado, no se observaron variaciones en la potencia alfa en la enfermedad de Alzheimer. Por lo tanto, se sugiere que, debido al deterioro previo del sistema colinérgico en los cerebros de los pacientes con enfermedad de Alzheimer, la modificación genética de solo una subunidad de un solo receptor no tendría un significado relevante. Este estudio es un paso importante para expandir nuestro conocimiento sobre las implicaciones de los factores genéticos en los trastornos neurológicos.

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Chapter 1

Introduction

1.1 Alzheimer's disease definition

Alzheimer's disease (AD) is a neurodegenerative disease primarily characterized by progressive cognitive deterioration, which also affects behavior and functioning [1]. Moreover, it is considered the most prevalent cause of dementia [2]. Two main subgroups can be distinguished based on the age of onset of clinical symptoms: early-onset familial AD, in which the onset age is below 65 years, and sporadic late-onset AD, whose onset age is over 65 years [2]. Although its clinical picture may vary among different patients, the most common symptom is the inability to recall recent information. Furthermore, depending on the stage of severity, AD can manifest additional symptoms, such as disorientation, apathy, impairment of attention, distrustfulness, behavioral changes, or difficulties in daily activities, including speaking or walking [3].

The most recent prevalence analysis indicates that the number of people affected by dementia in 2019 was 57.4 million. In fact, this number is projected to increase by more than 250 % by 2050 [4]. AD prevalence is expected to increase due to the aging in Western populations since global estimates suggest that by 2050, the population over 65 will increase from 18 to 38 %, compared to the year 2000 [5]. Actually, it has been estimated that by 2050, half of the patients in the United States of America (USA) with a diagnosis of AD will be over 85 years old [6]. AD also affects adults in their late working years, provoking early retirement or inability to work [5, 7]. Therefore, AD not only affects the physical aspects of the life of the patients but also modifies lifestyles. Since the risk of dementia increases with age, further studies are essential to address the potential burden caused by this fact [2]. Primarily, the estimated monetary cost of AD per case is taken into consideration in order to assess the disease's relevance. Basically, it includes healthcare, long-term care, and hospice costs, as well as possible social costs [2]. Excluding caregiving costs, the total payments for all individuals in the USA in 2022 have been estimated at $321 bil-

lion [2]. Medicare and Medicaid, two government-run health insurance programs in the USA [8], are expected to cover 64 % of the total care and long-term care payments for AD patients [2]. In addition, it is essential to note that the length of the disease before death affects not only economically but also socially. Mainly, it is due to the increasing time that patients spend in a dependent and disabled state which can lead to psychological problems related to anxiety or depression [4]. In addition, this not only affects patients but also their families and caregivers [2]. For the aforementioned reasons, research directed toward a better understanding of AD is crucial for the sake of public health. Since AD causes major disruptions in brain activity, the study of the associations between brain biochemical alterations and functions is of paramount importance.

From a histologic point of view, AD is mainly characterized by the appearance of senile plaques, together with neurofibrillary tangles in medial temporal lobe structures and cortical areas [1]. Senile plaques mainly present a variety of insoluble filamentous Aβ peptides, which accumulate between nerve cell gaps producing synapsis degeneration [9]. This Aβ peptide is a natural product of the amyloid precursor protein metabolism. On the other hand, neurofibrillary tangles are comprised of straight and paired helical filaments whose constitution is based on the microtubule-associated tau protein [10]. What differentiates these tangles is the fact that tau protein is abnormally phosphorylated, compromising neuronal function by interrupting normal intracellular transport and thus provoking cell death [10, 11]. Furthermore, tau is not found linked to the cytoskeleton, thus provoking a loss of stability and flexibility, leading to degradation and finally atrophy of the neuron [10].

There have been different lines of thought according to the cause of the disease, such as Aβ aggregation and deposition with plaque development, tau protein hyperphosphorylation with the tangle formation, neurovascular dysfunction, inflammatory processes or oxidative stress [12]. Although it has not been clarified whether these alterations are a cause or a consequence of the disease [13], it has been suggested that both, tau accumulation and Aβ peptide excess, represented by the imbalance between the production and clearance of Aβ [13], might be two of the most likely origins [14]. It has been observed a correlation between the appearance of these changes and the severity of dementia [4, 13]. This is due to the fact that these alterations are followed by damage and destruction of neurons [4, 13]. It has been suggested that senile plaques may interfere with the synapsis process while tau tangles are believed to block the transport of nutrients and molecules that are essential for neurons' normal function [4]. For the moment, although the sequence of events is not yet clear, it is thought that tau begins its accumulation after beta-amyloid and the increment in the first supposes an increment in the second [4]. Furthermore, additional changes related to inflammation and atrophy appear during AD [13]. On the one hand, tau proteins and toxic beta amyloids activate microglia immune cells, which sometimes are unable to maintain all the physiological needs, leading to chronic inflammation [4]. On the other hand, the loss of cells results in the atrophy process [4]. It is also characterized by a significant decline in the components of the cholinergic system [15, 16]. Research has

shown that the regions most impacted by this degeneration are the cortex, entorhinal area, hippocampus, ventral striatum, and basal part of the forebrain, where there can be found a serious degeneration of the cholinergic innervation [15–17].

The union of all these alterations entails an enormous problem for AD patients, representing the physical and psychological problems presented before. As well, it supposes a challenge to carry out studies in this regard in order to clarify those doubts that the disease presents, and whose number of patients continues to increase over the years.

1.2 Neurophysiology

It is important to consider the relevance of an accurate understanding of the disease, particularly in relation to neurophysiology. Neurophysiology is the science that studies the underlying processes that govern the functioning of the nervous system [18]. From a clinical perspective, neurophysiological studies aim to identify and characterize diseases affecting both the central and peripheral nervous systems, with the ultimate goal of comprehending their pathophysiology and providing appropriate treatment [19]. This procedure involves measuring and recording physiological changes manifested as alterations in electrical waveforms, electromagnetic fields, or secretory activities [19]. A perspective for studying neurophysiology involves investigating changes in electrical activity, which manifest as diverse physical phenomena.

On one hand, neuroimaging techniques primarily involve the functional representation of both anatomical structural and functional images, which provide valuable insights into the aforementioned collateral phenomena provoked by physiological causes [13]. Different imaging techniques have been proposed for AD analysis, including Computerized Tomography (CT), Positron Emission Tomography (PET), and Magnetic Resonance Imaging (MRI). CT scans provide more transparent images but are not considered a primary pillar of AD imaging analysis. Other methods offer higher accuracy and precision [9]. For instance, PET scans involve the administration of a radiotracer that emits positrons, which are captured by the scanning machine. This method provides effective accuracy, but it raises concerns about patient safety [9]. Similarly, PET scans pose challenges during processing stages [13]. Lastly, MRI scans utilize magnetic radiation bombardment to differentiate areas, but they suffer from significantly lower accuracy [13]. While these neuroimaging techniques offer several advantages and are commonly used, they also come with notable disadvantages related to radiation risks, economic and time costs, and processing difficulties [13]. Therefore, alternative non-neuroimaging options are also considered to analyze the functioning of the nervous system [13].

Following this line of thought, electroencephalography (EEG) has emerged as a widely accepted method for analyzing disruptions in brain functioning, allowing personalized

analysis at a relatively low cost [13]. It is a non-invasive technique that offers information about the behavior of the synaptic action, which is correlated with the brain state [20]. The synaptic activity occurring primarily in the cortex gives rise to rapid and dynamic electrical signals within the range of 10 to 100 milliseconds [20]. These signals are highly intricate and challenging to capture due to their fast nature. However, EEG stands out as one of the few techniques capable of effectively detecting and measuring these electrical changes, thanks to its high temporal resolution [20]. The scalp electrodes distributed along the cortical brain record the electrical activity at a very large scale, by recording the electric potentials that are generated by the cortical layer tissue and displaying posteriorly the graphical representation [13, 20]. These oscillations allow the understanding of certain aspects of brain functioning, such as a slowing of the electrical brain activity, with larger amplitude in deep sleep states [20]. In addition, it has been suggested that these disturbances may be related to both neurological and psychiatric disorders [21]. In the case of AD, a study suggested that EEG analysis might help the understanding of the association between synaptic plasticity and cognitive performance [22]. With the aforementioned high temporal resolution, EEG permits keeping track of typical AD abrupt variations of neuronal electrical activity, such as characteristics that give hints about slowing of the EEG, reduction in signal complexity, or perturbations in synchrony measures [13]. These changes are studied by calculating specific wave characteristics, including spectral, amplitude, coherence, and phase-based features [13]. As the use of EEG in AD may provide information regarding the development of the disease that other conventional techniques cannot provide, and given the rest of the advantages EEG offers over previously discussed techniques, its use in AD diagnosis and research is of paramount importance [13, 22].

1.3 Neurotransmission

Neurotransmission is the process of communication between neurons in order to carry information by means of nervous impulses [23]. Neurons in the human brain possess the remarkable capacity to communicate between themselves, a process that is known as synaptic transmission [23]. Synaptic communication can occur through two distinct mechanisms: electrical and chemical synapses. Electrical synapses, which are faster and simpler [24], rely on the direct ionic flow of currents through specialized channels that permit a connection between two neurons [23, 24]. Most electrical synapses are gap junctions, clusters of channels that are transcellular and are formed by protein subunits called connexins [24]. Their great advantage is based on bidirectionality, which allows almost symmetrical coupling strengths [24]. In contrast, chemical synapses, which are the most relevant and common mechanism for the signaling between neurons [23, 24], involve the release of specific chemicals, which induce a subsequent flow of currents in post-synaptic neurons [23]. The process of neurotransmission is divided into five phases. First of all, these chemicals are introduced into synaptic vesicles, which are the carriers [23]. The next step begins when there is an opening of voltage-gated calcium channels, which is produced

by the action potential arrival to the presynaptic membrane [23]. The calcium concentration in the presynaptic membrane rises, thus provoking the fusion between the vesicles with the membrane and therefore the liberation of the contents inside the vesicles to the synaptic cleft [23]. This process in which the neurotransmitters are released is called exocytosis. Posteriorly, there is a diffusion of the neurotransmitters through the cleft and arriving at the post-synaptic neuron, binding the specific receptors and causing them to either open or close, altering the flow of ions across the membrane, which leads to the conductance and action potential creation [23]. The process finishes with the removal of the neurotransmitters, terminating their action.

The effect produced by these neurotransmitters can be either excitatory or inhibitory [23]. Glutamate is considered the primary excitatory neurotransmitter [25]. It is involved in various brain functions such as cognition, memory, and learning. Furthermore, it plays a crucial role in the development of the Central Nervous System, contributing to synaptogenesis, cell differentiation, and cell death [25]. Conversely, γ-aminobutyric acid (GABA) serves as the principal inhibitory neurotransmitter [23, 26]. Alterations in GABA levels can lead to significant changes, as a decreased level of GABA in the brain can result in a loss of inhibition and trigger seizures [23]. As well, noradrenaline and adrenaline are neurotransmitters of significant importance [27]. Noradrenaline serves as the primary neurotransmitter of the sympathetic nervous system, primarily involved in modulating various physiological processes, including changes in cardiovascular tone [27]. On the other hand, adrenaline actively participates in maintaining homeostasis [27]. Collectively, these neurotransmitters contribute to the intricate regulatory mechanisms orchestrated by the brain to uphold the proper functioning of the body's internal systems [27]. Another relevant neurotransmitter is acetylcholine, which functions at the neuromuscular junctions, synapses in the visceral motor system, and various regions of the Central Nervous System [28]. Through its binding to specific receptors, it influences the transmission of signals and electrical information to subsequent neurons [29]. These receptors are divided between muscarinic and nicotinic [29]. The function of the receptor is not only defined by its type but also by its design and conformation, which hold significant importance. [29]. The relevance and detailed analysis of acetylcholine will be further explained in the next section of this document.

Importantly, abnormalities in neurotransmitters or their receptors have been implicated in the disruption of the transmission of information, and thus in the development of several neurological and psychiatric disorders [23]. Throughout history, different studies have stated the correlation between abnormalities in neurotransmission and the presence of genetic alterations [30]. Therefore, considering that existent bond, it is crucial to investigate how these genetic alterations affect the development of certain neurological disorders such as AD.

1.4 Genetics

It is an object of interest how genetic anomalies can lead to nervous alterations, which can posteriorly provoke neurodegeneration. First of all, the effect of genetics on the appearance and development of AD is under study, identifying the characteristics of the patients of interest. Early-onset AD is characterized by highly penetrant mutations in amyloid precursor protein (*APP*), presenilin-1 (*PSEN1*), and presenilin-2 (*PSEN2*) [31, 32].

Similar to other chronic diseases, sporadic late-onset AD is believed to develop as a result of multiple factors rather than a single cause. Hence, genetics is considered one of the risk factors to influence the disease, together with age or family history [2]. For instance, the apolipoprotein E gene (*APOE*) has been demonstrated to have a significant association with sporadic late-onset AD [33, 34]. Moreover, genome-wide association studies (GWAS) identified other genes, such as phosphatidylinositol binding clathrin assembly lymphoid-myeloid leukemia (*PICALM*), Complement C3b/C4b Receptor 1 (*CR1*), and clusterin (*CLU*), as genes with risk alleles [32, 35, 36]. Among the identified genes, *CLU* is one of the most consistent genes associated with risk alleles in terms of genetic association [31]. *CLU* is a gene found at the p21-p12 locus on chromosome 8, with an apparent role in altering Aβ aggregation and/or clearance [37]. Also, some of its variants have been linked to altered *CLU* expression at mRNA or protein level, affecting cognitive and memory function or brain structure [37]. In a study carried out three years ago [38], the (*PICALM*) gene was considered to be one of the top six most prevalent genetic risk factors for AD. *PICALM* is a gene located on chromosome 11q14 and encodes an accessory protein related to the absorption of extracellular molecules [39]. In addition, its relationship to AD pathogenesis through Aβ production impaired, tau protein clearance, and synaptic function has been suggested [40].

The genetic implications in the development of multiple diseases have been studied throughout the inspection of Single Nucleotide Polymorphisms (SNPs). SNPs are the most common type of genetic variation [41]. Basically, they are differences in a single specific base between two DNA sequences, which can be classified as either transition (C/T or G/A) or transversions (C/G, A/T, C/A, or T/G) [42]. Their importance lies in their evolutionary stability, which makes them useful markers in genetic mappings to track the inheritance of disease-associated genetic diseases and study human health [43].

1.5 Hypothesis

Based on the previously explained premise that alterations in neurotransmission can contribute to the onset of neurological disorders, and considering the established correlation between genetic variations and modifications in the normal neurotransmission pro-

cess, it is crucial to investigate the relationship between these genetic disruptions and the development of disorders such as AD. Therefore, a proposal is made suggesting that specific SNPs could potentially disrupt the proper expression of acetylcholine receptors. It is significant since alterations in the structure of these receptors provoke modifications in the binding process. It affects the synaptic connections between neurons, leading to disruptions in the correct transmission of information. Consequently, it is proposed that, by altering the transmission of information, these modifications may also affect the electrical activity, both in terms of frequency and temporal domain, potentially resulting in detectable disruptions in the normal EEG patterns of individuals with specific genetic alterations compared to those with normal expression of these genes. As a final hypothesis, it is suggested that these genetic alterations may play a role in the development of the disease.

The SNPs of particular interest in this study are related to alterations in the expression of the proteins that comprise the nicotinic and muscarinic acetylcholine receptors. The list of SNPs is formed by rs1044396 located on chromosome 20; rs16969968, rs588765, and rs8024987 located on chromosome 15; and rs324650 on chromosome 7.

1.6 Objectives

The objective of the presented project is to analyze and investigate genetic alterations in acetylcholine receptors to determine their impact on the electrical activity of the brain, which can be measured using EEG. The project aims to examine specific parameters such as relative power, median frequency, sample entropy, spectral entropy, and Lempel-Ziv complexity to identify consistent modifications in the EEG patterns of a significant number of subjects with altered genotypes compared to those without such alterations. By observing these consistent alterations, it can be inferred that the genetic variants under investigation are responsible for the changes in electrical activity. To confirm the hypothesis successfully, the following learning objectives need to be achieved:

i) Perform a review of the state of the art related to the effect of genetics on the proper functioning of neurotransmitters and their receptors, concretely in AD patients. By gaining an understanding of the neurophysiology of their brain, it is emphasized the novelty and significance of the study.

ii) Application of variated techniques that allow the extraction of the required information.

iii) Perform rigorous statistical analysis of the obtained results in order to identify differences within each differentiated group.

iv) Identify and enumerate the competencies acquired along the degree that have been applied during the project.

v) Draw conclusions from the developed study, considering limitations and potential future research.

Chapter 2

Neural Analysis

2.1 Introduction to Acetylcholine Biochemistry

In this section, the functioning of the cholinergic system will be explained, beginning with the neurotransmitter description, and continuing with the structure and design of its receptors, those which were the basis of the analysis of this study. Finally, the already-known genetic implications regarding both acetylcholine and its receptors were implemented as well. Proper knowledge of cholinergic system structure and mechanisms was of paramount interest in order to perform the analysis that this study required.

2.1.1 Synthesis and Function of Acetylcholine

Acetylcholine (ACh) is an endogenous neurotransmitter that exerts various effects in the body [44]. It plays a significant role in both the Central and Peripheral Nervous Systems, primarily affecting afferent neurons of the autonomic and somatic branches [28]. ACh is synthesized within the cytosol of nerve terminals by the enzyme choline acetyltransferase (ChAT) through a reaction involving choline and acetyl-coenzyme A (acetyl-CoA) as substrates [28, 29, 44]. Choline is not synthesized in the CNS but is transferred there because of the blood in free form [45]. It is present in high concentrations (10mM) in the plasma and is taken up by the high-affinity sodium-dependent choline transporter (ChT) [23, 46]. The storage process relies on a vesicular transporter (VAChT), which utilizes electrochemical gradients to accumulate approximately ten thousand ACh molecules per vesicle [23], facilitated by the exchange of two protons for each ACh molecule [47]. Subsequently, the release of ACh is predominantly initiated by the influx of Ca2+ ions through voltage-gated calcium channels [28]. Finally, ACh is rapidly hydrolyzed by acetylcholinesterase (AChE), resulting in transient action [44]. AChE exhibits high catalytic ac-

tivity, hydrolyzing approximately 5000 ACh molecules per second, leading to the breakdown of ACh into acetate and choline [23]. Notably, choline can be recycled, allowing for subsequent ACh synthesis. The effect produced by the binding of acetylcholine to its receptors is dependent on the specific type of receptor to which the molecules bind [29]. Therefore, there is no individual function of the neurotransmitter, but a dependent variety.

2.1.2 Receptors

Once it enters the synapse of the neurons, by definition, it binds two distinct types of receptors: muscarinic and nicotinic [29]. When it is released by the postganglionic parasympathetic nerves, it binds to muscarinic receptors [28]. Instead, in the ganglia, Ach has interactions with nicotinic receptors [28].

Muscarinic acetylcholine receptors (mAChRs) are metabotropic ligand-gated G protein-coupled receptors that can modulate neurotransmission either through inhibitory regulation or by simulating the action of nicotine [48]. These receptors have been stated as mediators of most of both the excitatory and inhibitory effects of ACh on CNS and peripheral nervous system (PNS) [49]. Their mechanisms of action are based on the activation of the receptors because of an agonist, which will provoke certain effects when it binds a G-protein [50]. When it happens, it triggers the activation of G subunits, that start the response. There exist five different subtypes: M1, M2, M3, M4 and M5, being M1, M2, and M3 the most important [28]. Each subtype is expressed in different regions of the nervous system, contributing to diverse physiological functions. M1 receptors count as the 60% of all the mAChRs in the CNS [51] and are mainly expressed in the cerebral cortex, hippocampus, and striatum, thus participating in synaptic plasticity, learning, and memory [29, 52]. As they are the most important type, studies regarding these receptors have been carried out. It has been observed how its expression is associated with both the formation of Aβ plaques and neurofibrillary tangles [51]. Therefore, the development of drugs that help the activation of these receptors is of paramount interest. For instance, activation of M1 receptors has been also related to improvement in cerebral blood flow [53]. Later, M2 receptors are highly located in the brainstem, thalamus, neocortex, and hippocampus [51]. Under the assumption that they are presynaptic autoreceptors that inhibit the ACh release [45, 54], they have been studied as potential targets for therapeutic interventions in disorders such as AD [29]. Then, M3 receptors are less expressed in the CNS than in the rest. Instead, there is a remarkably higher expression in smooth muscle tissues, creating a synergist relationship with M2 receptors [29]. M4 receptors are found in the caudate, putamen, and striatal projecting neurons, inhibiting dopaminergic activity. Because of that, these receptors are growing in their significance regarding behavioral deficits in neurodegenerative disorders [51]. Finally, M5 receptors are found in the substantia nigra and ventral tegmental areas of the brain, showing a probable role in dopamine transmission [29]. The mAChRs are divided into two classes. On the one hand, the first group (M1, M3, M5), preferentially coupled to heterotrimeric Gq/11 proteins [28]. After that, they stimulate phospholipase

C (PLC), thus provoking a release of calcium from Inositol trisphosphate (IP3)-sensitive intracellular stores by means of certain receptors on the cytoplasmatic surface. Furthermore, an increment in IP3 in the periphery has been observed in several tissues, such as the heart, gastrointestinal glands, or exocrine glands [50]. Instead, the second group (M2, M4) receptors show selectivity for Gi/o type of G-proteins, inhibiting the adenyl cyclase (AC), something that provokes a reduction in the accumulation of cytosolic cyclic adenosine monophosphate (cAMP), hence seeming to explain the presynaptic reduction in ACh release that has been related to the M2 receptor [45]. Therefore, it provokes a wide variety of effects with physiological significance both in the CNS and outside the CNS, such as in the heart or smooth muscle [50]. In fact, despite the fact that ACh does not have an excitatory role in the CNS, it has been observed an increment of neuronal excitability and responsiveness because of the activation of muscarinic receptors [45].

Nicotinic acetylcholine receptors (nAChRs) are fast ionotropic receptors [28], which are also known as Cys-loop receptors since all the family subunits have a sequence with a couple of cysteines separated by thirteen residues linked by a disulfide group [55]. They can be classified into two groups based on their subunit composition and function [56]. Muscle receptors, which participate in neuromuscular transmission, consist of five subunits arranged in a circular fashion around a central pore. The subunits $\alpha 1$, $\beta 1$, γ, δ, or ε are involved in muscle receptor formation [55–57]. In contrast, neuronal receptors are found in both the PNS and the CNS [58]. Twelve neuronal subunits have been identified in humans, ranging from $\alpha 2$ to $\alpha 7$, and $\alpha 9$-$\alpha 10$ and from $\beta 2$ to $\beta 4$ [55–57, 59]. The assembly of pentamers in neuronal receptors varies from muscle receptors as they can contain α or β subunits. While homomeric receptors can consist of a single type of α or β subunit, heteromeric receptors can include up to three different subunit types [56, 57]. Homomeric compounds are typically formed by $\alpha 7$ and $\alpha 9$ subunits [55, 60], while $\alpha 2$-$\alpha 6$ and $\alpha 10$ subunits require the presence of β subunits or other α-subunits for assembly [55, 61]. As allosteric receptors, nAChRs are oligomeric and possess multiple agonist-binding sites, non-competitive antagonist sites, and gates that interact at a distance [55]. According to a certain study on *Torpedo californica* species [62], muscle ACh nicotinic receptors have two binding sites per $(\alpha 1)2\beta 1\gamma\delta$ complex, one per α-subunit. In homomeric receptors, the amount of possible ACh-binding sites increases up to five, one per subunit. Instead, the heteromeric ones can only have two different binding sites. As the ligand-binding site is created at the region between the α subunit and its adjacent one, it has been predicted that by a different combination of subunits, the receptor presents different biophysical properties [63]. In fact, due to that variety in the assembly combinations, the diversity can be maximum [56].

As it has been stated previously in the document, nAChRs can be found either in the PNS or the CNS, exhibiting different compositions and distributions in each [56]. On the one hand, in the autonomic PNS, these receptors are involved in mediating ganglionic neurotransmission. Studies conducted on animals investigated the expression of mRNA encoding the subunits mentioned earlier in the olfactory bulb and trigeminal ganglion [56]. For instance, it was observed that only $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\beta 2$, and $\beta 4$ subunit mRNAs ap-

peared in the olfactory bulb of every animal subjected to the specific study [56]. As well, α2, α3, α6, α7, β2, and β4 subunit mRNAs made an appearance in trigeminal ganglions of every animal [64]. Instead, the rest of the subunits are only expressed at a certain percentage of animals. Later on, further analyses were performed on intracardiac ganglia [65] and bladder [66], leading to the conclusion that receptors containing α3, β2, and β4 subunits are of paramount importance in peripheral ganglia neurotransmission [56]. In ciliary ganglion neurons, α3 and α7 subunits were predominantly observed in the postsynaptic and perisynaptic membranes, respectively [61, 67]. That means that in case of an absence or blockade of neurons that contain those subunits, the neurotransmission can be inhibited [56]. In the CNS, nAChRs are primarily located on cell bodies, mediating postsynaptic effects, or on axon terminals, where they are thought to modulate synaptic transmission [56]. The α4 and β2 subunits are dominantly expressed in the CNS, being the main subtype α4β2 [68, 69]. In addition, it has been observed that most of the neurons express α7 subunit in the hippocampus, mainly in its homomeric form [68, 70], a region of the brain related to learning and memory [56]. These α7 nAChRs in the hippocampus modulate synaptic plasticity and may explain the effects of agonist drugs on learning and memory [71]. In addition, they participate in the proliferation, apoptosis, and clearance of aged neurons [69]. As well, as they have high calcium permeability, when they are activated presynaptically the calcium influx increases, thus depolarizing the membrane and liberating other neurotransmitters such as GABA, glutamate, or dopamine [69]. When they are activated post-synaptically, they both depolarize the postsynaptic membrane and regulate the release of GABA, thus participating in the cognitive function [69]. Later, α5 has been defined as appearing in heteromeric receptors together with α3 and α4 subtypes in the CNS [72, 73]. The expression of these subunits is related to brain development and injuries [74]. One example is the α3 nAChR domination in the prenatal brain or in injured neurons, whilst it is downregulated in healthy neurons and adults. And exactly the opposite occurs with α3 transcription [68]. Moreover, decreased hippocampal expression of α4 and α7 has been associated with age in various animal models [29].

Therefore, as described in this section, both muscarinic and nicotinic receptors are found in the PNS and CNS, and alterations in these receptors have been implicated in the pathogenesis of neurological disorders, such as AD. These receptors blockade has been associated with severe deficits related to attention, memory acquisition, and consolidation [75]. For instance, a reduction in mAChRs has been observed in the cortex and hippocampus of AD patients [45]. In fact, some studies have suggested the involvement of the M1 receptor in the development of AD, since the number of receptors decreases in the brains of AD patients [76]. Furthermore, M2 receptors in AD brains are reduced over hippocampal formation [76]. As well, there are diseases that disturb either the nAChR function or the number of receptors and consequently provoke an important impairment of the physiological processes, something evaluated in several studies [77–79]. In the case of AD, there appears to be a reduction in the number of nAChRs [56, 80], and cortical deficits of these receptors correlate with cognitive impairment [81]. Furthermore, the expression of complete compounds such as α4β2 receptors has been remarkably decreased in AD [82]. In

addition, both the homomeric α7 compound and a heteromer with β2 subunits have resulted in participation in AD pathology [59, 69, 83]. The intracellular Aβ accumulation of those neurons with α7 subunit compounds has been proved to alter several neurochemical processes such as calcium homeostasis or acetylcholine release, leading to the modulation of the physiological function in memory processes [69, 83, 84]. Considering that the genetic expression of these receptors, and particularly their subunits, has been studied and analyzed [56], it is possible to perform assessments with the objective of investigating how genetic variations might affect the functioning of the nervous system in AD patients.

2.1.3 Background on Genetic Implications

Different genetic associations, not only related to AD but to other disorders have been carried out, being of interest for this manuscript only those related to AD. Later, once the structure and mechanism of the receptors have been explained, the linkage between each type of receptor and each subunit protein, and the genes that encode them needs to be explained.

On the one hand, as it was previously explained, the alteration of these receptors, mainly M1, M2, and M3, has been related to impairment of cognition, attention, and memory. First of all, the M1 receptor coding gene (*CHRM1*) is found in chromosome 11, possessing a single long exon where the coding region is found [85, 86]. Until recently, no mutations had been demonstrated to be correlated to the development of a disease, but according to a study of 2021, some specific mutations in this gene have been associated with the onset of neurodevelopment disorders and epilepsy [87]. Later on, the M2 receptor gene (*CHRM2*) is located at chromosome 7 [88]. Genetic alterations of this gene have been studied widely over the years, demonstrating a certain association with neurological disorders such as depression [89], showing the role of the receptor in the cognition process and IQ [90], or participating in bipolar disorder by the provoking of the reduction in the binding capacity [91]. In addition, according to several studies [91, 92], there is certain evidence that alterations in these genes present a role in brain cognition and dynamics, because of the presentation of variations in the theta and delta band neuroelectric oscillations. Later, alterations in the M3 receptor gene (*CHRM3*), which is located at chromosome 1, have been related rather to visceral problems such as urinary bladder disease [93]. Some polymorphisms in the M4 receptor gene (*CHRM4*), in chromosome 11, have been associated with neurological disorders such as schizophrenia [94]. Finally, no significant neurological alterations regarding the M5 receptor gene (*CHRM5*) have been made.

On the other hand, the location of the chromosomes and specific genes that code the human neuronal nAChR subunits appears in figure 2.1. By measuring mRNA and the presence of receptors, the expression of nAChR genes in each tissue can be measured [56].

As was described in the previous section, in the CNS the main highly expressed re-

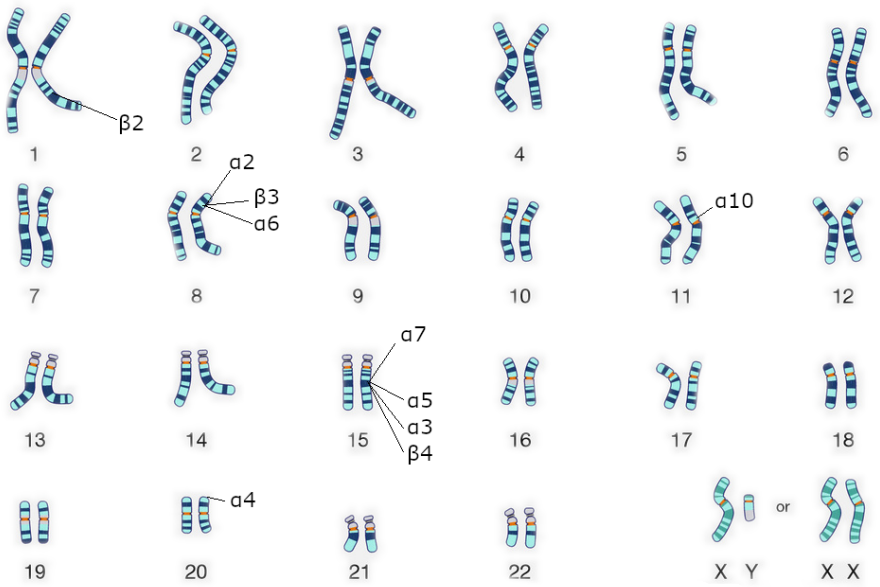

Figure 2.1: Representation of chromosomal locations of the genes coding for neuronal nAChR subunits, as was described in [56]. Among the most relevant subunits that are explained in the document, it is remarkable that α3, α5, α7, and β4 genes are located in chromosome 15, whereas the α3 subunit gene is found at chromosome 20

ceptor subtypes are the one composed of the heteromeric α4β2 compound, and the one formed by five α7 subunits, both being of certain importance in the analysis of AD [69]. Firstly, because of the aforementioned demonstrated relationship that the α4β2 compound has with AD risk, the nAChR α4 (*CHRNA4*) and β2 (*CHRNB2*) subunit genes have been also analyzed [95]. The *CHRNB2* gene is found in chromosome 1 as shown in the table 2.1 and approximately spans 9 kb of genomic DNA [96]. Firstly, mutations in this gene were associated with frontal lobe epilepsy and memory deficits [97, 98]. Later on, variations in the *CHRNB2* gene have shown a significantly important association with AD according to a previous study [95]. Later, as the table represents 2.1, the *CHRNA4* gene is found in chromosome 20. It consists of six exons which are distributed along 17 kb of genomic DNA [99]. The association between mutations in this gene was associated with epilepsy [100]. As well, the correlation between certain polymorphisms of the *CHRNA4* gene has been demonstrated to be linked to AD pathogenesis [101]. Alterations in the gene were also proposed as candidates to possess a relationship with the appearance of low-voltage EEG phenotype, which is characterized by the reduction of alpha activity in the EEG [99].

Next, the gene that encodes the α7 subunit is *CHRNA7* and is located in chromosome 15, as can be seen in the table 2.1. That gen has a total length of 75,000 bp, containing as well a 1509 bp cDNA, with 10 exons and 9 introns [69]. The gene is partially duplicated, from exon 5 to 10, and as the functioning and distribution are different in both parts, the analysis must be separated as two different genes, *CHRNA7* and *CHRFAM7A* in one [59].

Subunit	Location
α2	8p21.2
α3	15q24.3
α4	20q13.33
α5	15q24.3
α6	8p11.21
α7	15q13.1
α10	11p15.4
β2	1q23.1
β3	8p11.21
β4	15q24.3

Table 2.1: Representation of the loci in the gene for each subunit, as a specific complement of figure 2.1. It is an adaptation from [56]

An impaired expression of this gene has been directly linked to different neuropsychiatric disorders focusing on the effect produced in processes related to brain development, attention, behavior, cognition, and memory [59, 83]. Alterations in both parts of the gene have been studied to be related to schizophrenia [102], bipolar disorder [103], and autism [104]. Regarding AD, certain studies have been carried out, for instance, demonstrating the association of some polymorphisms to the appearance of delusions [105]. In contrast, a haplotype block in the intron 2 of *CHRNA7* showed a decreased risk of presenting the disease [106]. Furthermore, *CHRNA7* is not the only gene found in chromosome 15. As the figure represents, the genes that code the subunits α3, α5, and β4 are also found in the same chromosome. Respectively, *CHRNA3*, *CHRNA5*, and *CHRNB4* are linked forming a cluster, and hence polymorphisms in any of them have been thought to influence the rest of the genes [96]. So, according to several studies, several genetic alterations in those genes were thought to be related to nervous system disorders [107].

Hence, it was demonstrated that genetic alterations in both nicotinic and muscarinic receptor coding genes lead to the development of different neurological disorders. In this study, several SNPs were investigated and their implications are explained in the methodology chapter of the project. In addition, it was demonstrated in the literature the existent relationship between some genetic disruptions and the modification of electrical activity.

2.2 Introduction to Brain Electrical Activity

In this section, the functioning of the electrical activity along the brain was explained, including the bases of the communication between neurons, and providing information about EEG and its implications for analyzing neurological disorders such as AD.

2.2.1 Neural Communications

Despite the brief introduction explained in the first chapter regarding neurophysiology, a deeper understanding of the bases of the functioning of neural communication is necessary. Therefore, this section explains the whole process of communication between neurons from the minimal structural function. Neurons are specialized cells in the brain that allow the rapid communication of information, thus having a unique role as the central building block of the nervous system [108]. As in every cell, neurons are divided into different parts, each with a specific function. Firstly, the nucleus of the neuron is located in the soma, from which they grow branches called dendrites and axons [108]. The dendrites are in charge of offering a wide area for the signal inputs reception, which is then transmitted to the cell body [109]. Later, the signals are transmitted down to the axon [108, 110].

Neurons are considered excitable cells since they have ion channels in their surface membranes, which are opened when there is a change of voltage along the membrane [108]. This change depends on the difference in electrical charge between the ions dissolved in intra and extracellular fluids, thus leading to the so-called membrane potential [108, 110]. In the time passing between signals, the membrane potential is at a resting state in which the ions situate close to the membrane, with ions in high-concentration areas prepared to move towards low-concentration ones, and positive ions tending to move to negative zones [110]. In that phase, sodium is found at a higher concentration outside, tending to move inwards, and exactly the opposite happens with potassium [111]. In addition, the inner part of the cell is slightly more negative than the exterior, thus supporting sodium to cross the membrane inside [110]. As well as the influx of sodium increases, the internal part of the cell becomes more positive. Corresponding to stage 1 in the figure 2.2, if it reaches a certain threshold of excitation, the neuron activates, and therefore the action potential begins [110]. This process is called depolarization [111]. Instead, if the voltage does not reach the threshold, the depolarization is not triggered [111]. As the figure 2.2 shows, it continues up to the peak action potential when the sodium gates close and the potassium ones open, leading to the departure of potassium ions and hence a repolarization phase [111]. There is a point at which the potential becomes more negative than the resting potential, called the hyperpolarization phase, but it balances returning to the initial point [110]. Basically, these five phases constitute the action potential, in which the electrical signal moves from the soma up to the axon terminals, being propagated at every point of the axon, without dissipating [108, 110, 111]. After the action potential reaches the terminal axon, the synapsis is ready to occur. As was explained in the first chapter, the synapsis can be either electrical or chemical, transmitting the signal up to the dendrites of the following neuron. As it was explained, chemical neurotransmission is more relevant in humans. The process repeats sequentially neuron by neuron, thus producing the transmission of information [108, 110, 111]. In cortical neurons, action potentials and postsynaptic potentials are the main types of electrical activities [112]. Action potentials tend to induce short-time local currents in the axon, which presents a limited field [109]. Instead, the

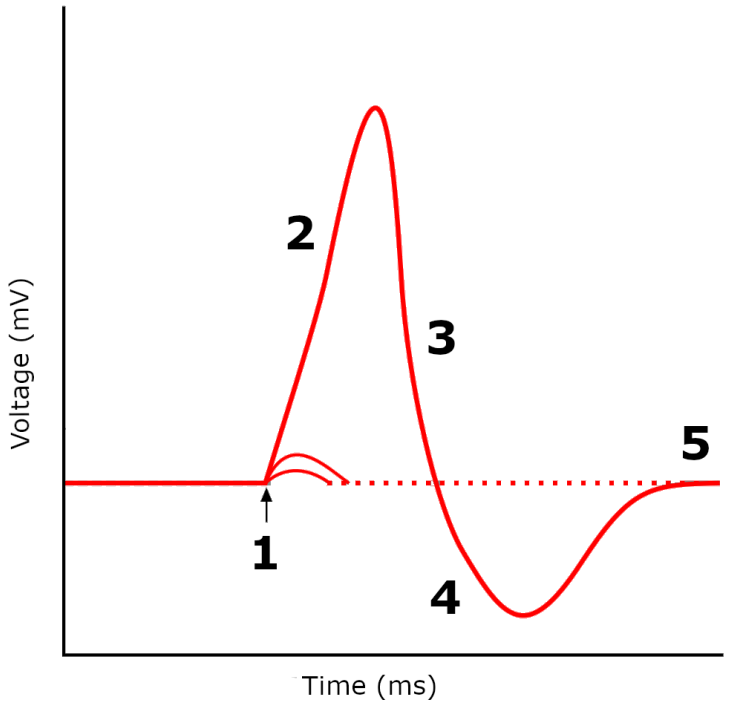

Figure 2.2: Representation of the action potential voltage (mV) curve over time in (ms). In the image, phase 1 corresponds to the initiation of the stimuli, and number 2 is the depolarization stage. After reaching the peak, it begins the third phase, repolarization. Later, it reaches a fourth state called hyperpolarization and, finally, it is set at the resting state. Adaptation from image explained in [111].

postsynaptic potentials, which are provoked because of the neurotransmitters binding to the post-synaptic receptors, present more duration and an increased potential field [109]. As well, these postsynaptic potentials have the ability to sum between themselves, thus providing a greater voltage, which can be measured by the EEG [109]. Because of these characteristics, the second group potentials are of greater interest in electrical analysis by applications such as EEG [113].

2.2.2 Electroencephalography

Electroencephalography is the name of the technique in charge of the recording of fluctuating electrical waveforms on the scalp [109, 114]. This technique was discovered in 1929 by Hans Berger, thus changing the history of neurologic analysis and diagnosis [115].

Nowadays, in order to obtain proper EEG signals, an appropriate system consists of a wide range of elements, such as electrodes with conductive gel, analog-to-digital converter, amplifiers with filters, and a recording device [109]. The electrodes are placed in the scalp with the objective of conducting the electrical activity from there [114]. There is

not a predefined number of electrodes to place on the scalp, since different theories have arisen over the years, with the important thought that an increment in the number increases the cost and also difficulties the detection [109, 116]. In 1958, a standard distribution was proposed by the International Federation in Electroencephalography and Clinical Neurophysiology [117]. Within that standard, called the International 10-20 System, the appropriate electrode placement, comprised of nineteen electrodes, was described, as it is shown in the figure 2.3. The conductive medium, either gel or saline, is usually applied to fill the space between the electrode and the scalp, thus allowing proper electric conductivity [109]. Later, the amplifiers are in charge of obtaining the electric voltage from the electrodes and amplifying it in order to be compatible with the converters, which transform the signal to digital form [109]. However, signals with aberrant amplitudes, frequencies, and shapes may appear in the recording, called artifacts [109]. The artifacts can be either physiological or non-physiological [109]. The first type of artifacts appears because the subject causes them, such as body and eye movements or sweating [109]. Later, the second type is related to technical problems related to the environment such as cable movements, broken wires, or low battery [109]. The interference of the electrical network is the most important non-physiological artifact [109]. Therefore, it is necessary to have a pre-processing state that consists of the removal of the artifacts without altering the raw data [109, 114]. Posteriorly, the data is also filtered, in order to remove noise at either very high or low frequencies [109]. Regarding that stage, there are four types of filters, as can be perceived in figure 2.4. Usually, in EEG studies, a band-pass filter is used to eliminate the artifacts that present low and high frequencies [109]. In addition, a notch filter is applied to remove the electrical network interference [109]. Before entering the following stage, extraction of the data epochs, while removing baseline values is performed, with a consecutive removal and interpolation of those channels that are unable to properly provide information of the brain activity [109]. When these measures are analyzed in a non-alterations state, it is called spontaneous EEG activity [109]. Instead, if the subjects are asked to perform any activity, or are subjected to any stimuli, it is called evoked activity [109]. These disturbances of spontaneous activity are usually related to sensory, cognitive, or motor events [109]. The name given to the stimuli is Event-Related Potentials (ERPs) [113], although other experts have called them evoked potentials (EP) [113]. They can be averaged to obtain more evidently the response to those stimuli [109].

Posteriorly, the extraction of characteristics can be performed, which can be spectral, non-linear, related to connectivity, etc [109]. In the case of the spectral estimation, it is based on frequency analysis since it allows the count of cycles of an oscillatory waveform per certain time [118]. The unit used for measuring the frequency is the hertz (Hz), meaning one cycle per second [109]. Basically, any EEG signal can be represented either in the time domain, measuring amplitude variation along time, or in the frequency domain, measuring power change along frequency [109]. In order to convert from the time domain to the frequency domain, spectral estimations can be applied [118]. Generally, for EEG signals, the use of non-parametric methods, which are based only on data, without needing any definition of the model, for spectral estimations are more widely accepted [109]. It is

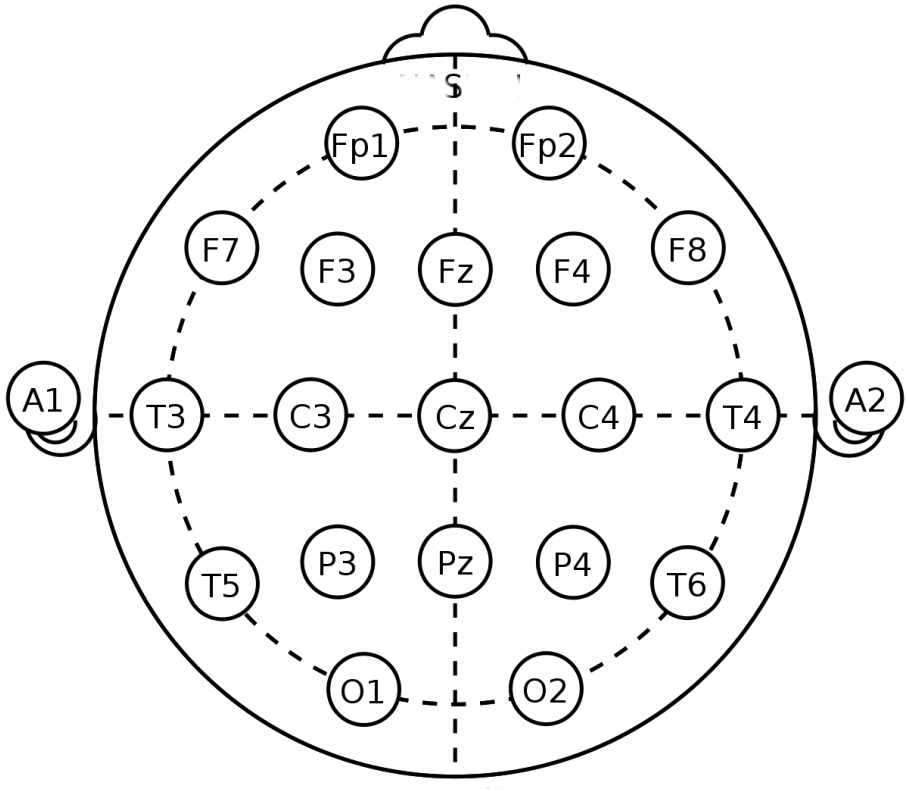

Figure 2.3: International 10-20 System for electrode placement, as was stated in 1958 [117]. In the image, the top part corresponds to the anterior part of the head, whereas the inferior part is related to the backward part of the head. The distribution of the nineteen electrodes is represented, in order to allow a proper recording

due to the width and smoothness of EEG signals spectra, as well as to the large amount of noise that these signals present, something that meets with the advantages that non-parametric methods present [109]. Different signal-processing techniques can be applied to convert from time to frequency domain, such as Fourier Transform, Wavelet, or Principal Component Analysis, being the first the most popular [119]. So, by applying the Fourier Transform, the power of the signals is computed by categorization into different frequency bands [109, 114], as it can be observed in the table 2.2. First, the delta stage comprises between 0.1 and 4 Hz, characterized by the highest amplitude (75-200μV) and the slowest wave [114, 120]. It is predominantly found in deep sleep stages [120]. Next, theta stage ranges from 4 to 8 Hz and is commonly related to subconscious activity, found in deep relaxation and meditation states [114]. It is rarely found in adult human brains, and rather normal in children, characterized by a relationship with the production of the human growth hormone, serotonin, and cortical hormones, which help memory and learning [114]. Later, the alpha band is associated with frequencies between 8 and 13 Hz, found mainly in adults in an awakened but relaxed state and with closed eyes [114, 120]. Instead, when there is certain mental activity or eyes are opened, this band is attenuated [109]. In this band, the production of serotonin is induced, a chemical related to relaxation increment and relief from pain [114]. Finally, the beta band is associated with frequencies

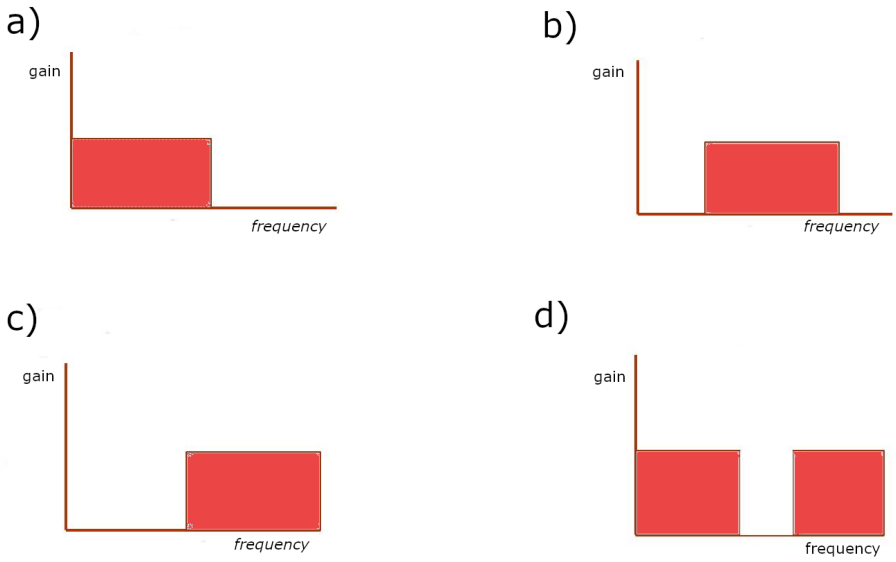

Figure 2.4: Representation of the distribution of the gain over frequency of different filters, being a) the High-pass filter, b) the Low-pass filter, c) the Band-pass filter, and d) the Band-stop or notch filter. It is an adaptation of the image given by the book [109]

between 13 and 30 Hz, related to states in which the alertness and attention are increased, as well as to the senses [114, 120]. Furthermore, the induction of cortisol production, the hormone that accelerates age in the brain, is also characteristic [114]. It also exists a gamma wave, ranging from more than 30 Hz, and characterized by states in which alertness is increased and there is an onset of voluntary movements [114, 120]. However, more features other than the distribution of the bands can be extracted through this process, such as the location, magnitude, or bandwidth of the spectral peaks, as well as statistical analysis to determine the most important frequencies. Traditionally, these spectral techniques have been applied to analyze EEG activity [121]. Their relevance is highly determinant in neurophysiology since a proper understanding of the electrical activity of the brain can be used for diagnosis or treatment [109]. These spectral parameters are commonly based on the analysis of the power spectral density (PSD) [122]. By extracting the PSD information, several parameters can be calculated, such as the relative power (RP), the median frequency (MF), or the spectral entropy (SE) [122].

On the other hand, since the mid-1990s, it was proposed that the human brain can be simulated as a non-linear dynamic system in order to capture neuronal activity [122, 123]. It provides complementary information to the spectral measures [123]. By Nonlinear Dynamic Analysis (NDA), EEG signal generation because of nonlinear deterministic processes is assumed [124]. It is due to the idea that neuronal interactions present certainly a nonlinear nature, behaving in the so-called deterministic chaos [124]. Different approaches have been designed over the years in order to evaluate cerebral activity, the most important being complexity and entropy [109]. Firstly, complexity evaluation in the EEG consists of a measurement of a signal fragment. Its capacity for information is firstly analyzed, poste-

Type	Frequency(Hz)	Psychological State
Delta	0-4	Deep rest
Theta	4-8	Deeply relaxed
Alpha	8-13	Calm state
Beta	13-30	Alert, active thinking
Gamma	>30	Excessive alertness

Table 2.2: Table showing the different frequency bands that exist in the brain, dividing them according to their limits. Information related to the psychological state manifestation is also provided. Adaptation of the table provided by [109]

riorly showing the degree of activeness of the neuron [109]. For instance, when a person has closed eyes, as the visual capacity of recording information is reduced, the neurons are less active and therefore the complexity is lower [109]. This complexity is commonly measured by a technique called Lempel-Ziv complexity (LZC), which was designed in 1976 [125]. LZC measurement enables analysis of several neurological alterations such as consciousness during anesthesia [126], Parkinson's Disease [127], schizophrenia [128], or AD [129]. Next, the assessment of the entropy is also included in EEG analysis [109]. Entropy is based on Shannon information theory and provides a measure of the distribution of characteristics of the signal [130]. Different entropy computations have been studied and applied to EEG over the years, such as sample entropy (SampEn) [131], fuzzy entropy (FuzzyEn) [131], or permutation entropy (PE) [132, 133]. All of them provide different estimations according to the regularity of the signal [109]. Finally, in order to evaluate the functional connectivity between different areas, coherence analysis can be used. When the functional connections between the different areas are reduced, then lower coherence values are observed [124].

2.2.3 Backkground on EEG Applications on AD

The usefulness of EEG is widely variated since it provides information regarding mental state, thought, or imagination [134]. Therefore, the range goes from Brain-Computer interfaces or biometrics to neuroscience and clinical applications or neuromarketing [134]. Regarding neurology, it is commonly used to analyze abnormal disorders and cognitive impairments such as epilepsy [135], Parkinson's disease [136], schizophrenia [137], anxiety [138], or AD [139], among others.

Regarding AD, as has been mentioned in the manuscript, several studies have demonstrated the existence of a slowing in the dominant posterior rhythm, and hence an increment in diffuse slow activity in AD patients [140, 141]. In fact, there is a strict correlation between the slowing of the EEG and the severity of the disease, which leads to cortical disorganization [142]. In addition, the relationship between abnormalities in EEG and

cognitive impairment has been demonstrated by several authors [140, 143, 144]. By measuring lower MF values in AD patients, it was observed the increment of slow rhythms in the spontaneous EEG activity [122]. Furthermore, it was observed a decrease in the mean frequency, with great correlation with the severity of dementia [145], and an increment of power in slow activity bands, delta, and theta, whereas a decrease in alpha and beta bands in AD patients compared to elderly subjects [145–147]. Actually, it is thought that the first changes that occur in the development of the disease are an increase in theta, and decrease in beta activities, and a consecutive alpha decrease [21]. Later, as the disease advances, the delta activity also increases [21]. More specifically, for patients whose dementia is rather mild, theta activity is increased and beta is decreased [148]. Later, in the case of severe patients, it has been observed mainly a decrease in alpha and an increase in delta activity [149], thus supporting the theory. Alpha reduction of the power is explained either because of an abnormal increment of cortical excitation or because of resting state disinhibition [147]. Considering the origin of the slowing of the EEG wave, several studies have been carried out regarding the influence of the existent deficit in the cholinergic system of AD patients [150, 151]. Therefore, as some studies have observed that ACh and the basal forebrain region are in charge of maintaining a desynchronized EEG activity, it would be reasonable to think that a loss in the innervation of the cholinergic system in the neocortex might affect the slowing in EEG [152, 153].

Furthermore, the use of NDA was also widely used in AD, showing that by using this technique, useful information could be extracted [21]. For instance, it has been analyzed a reduction in values of correlation dimension (D_2) in the occipital EEG of AD patients in comparison to healthy subjects [124]. Therefore, as D_2 is a measure of complexity, there is a relationship between AD brains and the decrease in the complexity of cerebral activity [124, 154]. In fact, it was found that a reduced D_2 value bears a certain correlation with an increment of the severity of dementia [129]. According to studies [122, 155], lower levels of LZC, FuzzyEn, and SampEn were observed in AD patients in comparison to control subjects, thus also reaffirming the reduced complexity in the brains of AD patients. As well, it showed that this brain electrical activity was more regular and less variable related to healthy controls [122, 155]. The decrease in the complexity was related to different ideas such as the death of the neurons or synapses, the acetylcholine deficit, and the loss of connectivity between the local neuronal networks [124].

In order to assess connectivity in the case of AD patients, coherence studies were used, permitting thus to discern if the appearing changes were related to the cognitive decline and to the state of dementia [124]. It was observed that there is an existent decrease in fast bands, both alpha and beta, which might suggest certain disconnection in the cortical regions [156, 157]. In fact, that decrease in coherence bears a certain correlation with the cognitive impairment previously mentioned [158]. Regarding ACh analysis, it has been estimated that when there is a loss of ChAT in the cortex, there is an increment of the power in slow bands in AD patients [159]. It was confirmed that deficits in the cholinergic system have an impairment effect on the synchronization mechanisms on which the alpha

power lies [147]. It was suggested that the decrease in EEG coherence might be caused by the anatomical disconnections in the cortex and the reduction in the cholinergic interactions among the cortical neurons [21]. Despite the advances discovered in this field, more extensive evaluations are required, related to the connectivity and to the acetylcholine alterations.

Chapter 3

Materials and Methods

3.1 Subjects

For this study, a database provided by the University of Valladolid was used, using information from Spanish and Portuguese patients provided thanks to the funding allowed by the Cooperation Programme Interreg V-A Spain-Portugal POCTEP 2014–2020. The subjects were categorized into different groups based on the severity of the disease. These groups included controls, patients with mild cognitive impairment (MCI), and patients with varying degrees of dementia, classified as mild, moderate, and severe. Consequently, there were five distinct groups, each comprising a different number of subjects. However, it was relevant to note that after data analysis, several subjects needed to be discarded, and, MCI subjects were not included either. Therefore, as it is shown in the table 3.1, in this study we analyzed 155 elderly patients, which were classified into two groups, controls, and AD patients. For each of the groups, the mean age and the Mini-Mental State Examination (MMSE) were computed. MMSE is a score based on 33 questions, which was described in 1975 in order to assess the cognitive state of each of the subjects [160]. It was mainly applied since it only required 30 minutes, a shorter time considering that elderly

Population	Control	AD Patients
Females/Males	22/23	52/58
Total	45	110
Mean age $\pm$ SD (y)	79.71 $\pm$ 7.27	80.56 $\pm$ 7.25
Mean MMSE $\pm$ SD	28.82 $\pm$ 1.12	12.83 $\pm$ 8.72

Table 3.1: Number of subjects that qualified for further analyses after genotyping quality control, and respective mean age and mean MMSE values with their corresponding standard deviation

and dementia patients are cooperative for short periods of time [160]. In the case of AD patients, the remarkably high standard deviation value appearing in the table 3.1 is due to the grouping of patients in any stage of the disease together. The patients were diagnosed after following criteria provided by the National Institute on Aging - Alzheimer's Association (NIA-AA) [161]. Individuals who met specific clinical criteria, including recent surgery, vascular pathology, hypercatabolic states, a clinical history of neoplasia, and chronic alcoholism, were excluded from the study. Informed consent was obtained from each participant, their relatives, or legal representatives in accordance with the guidelines set forth in the Code of Ethics of the World Medical Association. This study was conducted in adherence to the principles outlined in the Declaration of Helsinki, and its protocol received approval from the Ethics Committee of the University of Porto (Porto, Portugal, Report No. 38/CEUP/2018).

3.2 Genetic Analysis

In this section, an explanation of the genetic analysis performed during the development of this project was shown. Firstly, the basic theory required for the correct understanding of the differentiation between the allele and the reference allele, as well as the risk and protective alleles was explained. Later, the application of the theory to the database and study of interest was carried out.

3.2.1 Altered and Reference Allele

In order to describe the process of selecting and analyzing genetic modifications, it is necessary to understand key concepts related to chromosomes and gene variations. Homologous chromosomes exist in pairs, with each chromosome inherited from one parent [162]. Each chromatid has specific bands containing genes, separated by the centromere [162]. The different locations that genes can occupy along the chromosome are called loci [162]. Within each locus, there can be different versions of a gene, known as alleles [162]. As it can be seen in the figure 3.1, in any allele, at that specific locus, there can be two options, either the dominant or the recessive [162]. As it can be seen in the figure 3.1, when both alleles take the same representation, it is called a homozygous chromosome, whether it is BB or bb. Instead, if the alleles take a different representation, it is called a heterozygous chromosome (e.g., Bb) [162].

When analyzing genetic alterations such as polymorphisms, variations in specific nucleotides at a locus are observed [163]. For example, the usual base at a specific locus might be Thymine (T), which becomes the reference allele. However, if there is a change to Cytosine (C) at that point, it represents an alteration, making C the alternate allele [163]. In

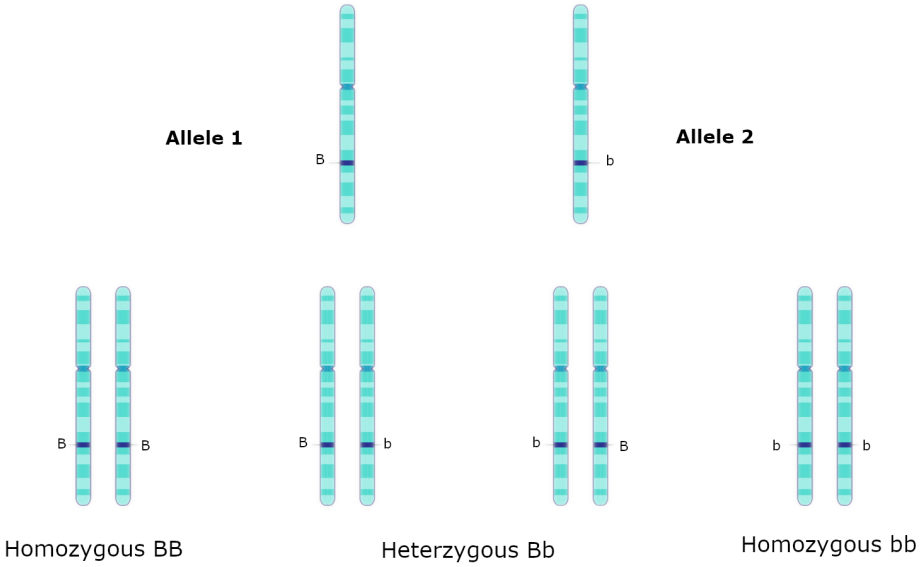

Figure 3.1: Representation of chromosomes conformation and different possibilities regarding the distribution of alleles for the same loci. In the top part, the two alleles are shown, each with a different type (B and b). When the combination is the same (e.g., BB or bb), it is called a homozygous chromosome. Instead, when it is different (e.g., Bb), then it is called a heterozygous chromosome. It is an adaptation of the image provided by [162]

autosomal chromosomes, with two alleles, the possibilities include TT, TC, and CC, with TT and CC representing homozygous alleles and TC representing heterozygous alleles [163]. Later, it is also important to explain how these modifications appear and are evaluated in genetic analyses of diseases. Different studies are carried out to assess which alteration of bases bears some relation with the development of the disease, and how the mechanism is produced [163]. The risk allele refers to the pair of bases associated with an increased risk of the disease, while the protective allele is not linked to disease risk [163]. For example, in the previous example, extensive research may reveal that the presence of TT and TC is associated with higher disease risk, while no association is observed with the CC allele.

Therefore, in order to carry out a proper study, it is crucial to gather reliable information from the literature, as subsequent procedures require accurate estimations to group subjects and compute relevant metrics.

3.2.2 SNPs Selection

As mentioned earlier, this study focuses on investigating different SNPs found in genes associated with the expression of subunits that form muscarinic and nicotinic acetylcholine

receptors. The selection of these SNPs was based on a thorough literature review to identify alterations that are either linked to disease progression through specific modifications in the cholinergic system or associated with disruptions in the normal EEG across different frequency bands. Following this comprehensive analysis, a group of SNPs was preselected, and all of them are included in the database provided by the University of Valladolid.

First of all, in chromosome 20, the SNPs rs1044396, rs1044393, rs1044394, and rs1044397 were included. All of them are alterations of the *CHRNA4* gene, and according to the literature they are associated with the N100 ERP, which is an electrophysiological endophenotype, also relating them to cognitive process affection [164]. Specifically, rs1044396 has also been linked to attention modulation [165] and has been investigated in the context of working memory [166]. In addition, it has also been related according to several studies to nicotine addiction [167], and anxiety because of Internet addiction [168]. Moving to chromosome 15, several SNPs from different genes have been studied. Firstly, variations in the *CHRNA5* gene, namely rs16969968 and rs588765 were included in the study. The SNP rs16969968 has been identified as a functionally compromising alteration and has been associated with a decrease in certain interneurons in the prefrontal cortex at high beta-amyloid levels [169]. In addition, it has been associated with the connectivity of the brain, with likely effects in smoking and schizophrenia [170], as well as with lung cancer [171]. On the other hand, rs588765 has been associated with Parkinson's disease [172]. As well, both of them have been recently evaluated in studies regarding the influence of these alterations in the oscillations in the theta band [173]. In the *CHRNA3* gene, the SNPs rs1051730 and rs578776 have been researched for analysis. In the first case, the SNP was used in certain analyses in which the association between tobacco and AD was investigated [174]. The second SNP has been studied in the same report that the SNPs of the *CHRNA5*, in association disruptions in theta band oscillation [173]. This SNP seems to bear such an important relationship with rs16969968. Finally, in *CHRNA7*, the SNP rs8024987 has shown a great response to some therapies in which AChE inhibitors are used in AD patients [175]. On the other hand, muscarinic receptor SNPs have also been taken into consideration (SNPs rs2350786 and rs324650, both located in the *CHRM2* gene. The first has been analyzed and linked to certain disequilibrium in the evoked oscillations of the EEG [92, 176], whereas the second has been associated with reduced binding of acetylcholine with the receptor in case of bipolar disorder [177], as well as with intelligence and performance IQ [178].

Once the candidate SNPs were identified, the next step was to assess their interconnection and determine if any redundancy existed. To accomplish this, the application LDlink was utilized. Linkage disequilibrium (LD) is the name given to the non-random association that regional variants have because of their low probability of meiotic recombination [179]. With it, the search for markers for risk alleles has been facilitated. Using the LDmatrix feature within LDlink, an interactive heatmap matrix of paired LD statistics between the SNPs is generated, providing the D' index, the R2 index, and information on allele correlation. In this study, SNPs were discarded based on R2 statistics analysis, which provides information about the strength or goodness of fit of a model [180]. Then

$$R^2 = r^2 \cdot q^2 = r^2 \cdot (2 \cdot MAF \cdot (1 - MAF) \cdot \beta^2) \tag{3.1}$$

where r^2 is the squared LD correlation between the causal variant and the genotyped one, *MAF* is the minimum allele frequency, and β is the effect size of an allele on the phenotype [181].

However, as in the case of this study, it is aimed to avoid higher R^2 values, as higher values indicate a stronger correlation in regression. Therefore, the threshold has been set at R^2 = 0.20, meaning that only those analyses of correlation lower than this threshold are kept for the development of the study, thus discarding every superior value. Hence, the redundancy was analyzed in each chromosome. Starting with chromosome 20, as shown in table 3.2, it was observed that certain redundancy appeared between rs1044396 and rs1044397, with an R^2 value of 0.87, as well as redundancy between rs10443944 and rs1044393, where the value exceeded the threshold. This redundancy was expected since these SNPs are located sequentially in the same locus on the chromosome. As a result, just rs1044396 and rs1044394 are retained, since the index values were below the threshold, thus discarding the other two redundant variants. Next, the process was repeated for those SNPs existent in chromosome 15. As it is shown in the table 3.3, it was observed an R^2 value of 0.85 between rs1051730 and rs16969968, leading to the exclusion of rs1051730. Subsequently, higher values were observed for the remaining SNPs, and hence the variant rs578776 was also discarded. Therefore, rs16969968, rs588765, and rs8024987 were retained. In the case of these SNPs, although they are associated with different genes (*CHRNA3*, *CHRNA5*, and *CHRNA7*), as was explained in the theoretical background, there is a certain linkage between the genes encoding α3 and α5 subunits. Finally, the process was repeated for those SNPs in chromosome 7, revealing an R^2 value of 0.08, indicating no correlation, and thus both SNPs were kept. Hence, after this first selection, the remaining SNPs were identified as rs1044396, rs1044394, rs16969968, rs588765, rs8024987, rs2350786, and rs324650.

The next step in SNP selection involved analyzing the distribution of patients in the provided database to ensure there were no significant differences between the number of individuals with the protective and risk allele variants. The reference allele was determined based on the information provided in the database while distinguishing between risk and protective alleles required an in-depth analysis of the relevant literature. Firstly, in the case of rs1044396, the existing literature [167] indicated that the reference allele was C, whereas T was the alternate. According to a study performed in 2009 [165], it was demonstrated that the presence of the homozygous TT was related to slowness in the performance of speed and attention tests, in contrast to TC or CC allele carriers. In addition, that effect was of paramount relevance in subjects between 70 and 79 years old [165].

Upon checking the database, an acceptable distribution of subjects was observed, as shown in the table 3.4: 26 controls with protective alleles, 19 controls with risk alleles, 54 patients with protective alleles, and 56 patients with risk alleles. Therefore, this SNP was

SNP	rs1044397	rs1044396	rs1044394	rs1044393
rs1044397	1.00	x	x	x
rs1044396	0.87	1.00	x	x
rs1044394	0.08	0.07	1.00	x
rs1044393	0.17	0.15	0.44	1.00

Table 3.2: Distribution of R^2 values between the four different SNPs tested on chromosome 20. For those values that are repeated, a cross (x) is represented. As well, for R^2 values between the same SNP, a value of exactly 1 appears, as it is logical. For the comparison of values, it can be observed that the R^2 values between rs1044396 and rs1044397, and between rs1044393 and rs1044394 overcome the threshold. Therefore, those values are marked in red color. The SNPs discarded are rs1044397 and rs1044393.

SNP	rs8024987	rs588765	rs16969968	rs578776	rs1051730
rs8024987	1.00	x	x	x	x
rs588765	0.00	1.00	x	x	x
rs16969968	0.01	0.07	1.00	x	x
rs578776	0.00	0.34	0.22	1.00	x
rs1051730	0.00	0.08	0.87	0.16	1.00

Table 3.3: Distribution of R^2 values between the five different SNPs tested on chromosome 14. Again, values repeated are represented with a cross (x). For the comparison of rs8024987 with the rest of the SNPs, the R^2 values are so small that in approximation to the centesimal value they seem to be zero, but they actually represent values easily approximated to millesimal, such as 0.003 or 0.004. Later, it is demonstrated how R^2 values between the SNP rs578776 with rs588765 and with rs16969968, as well as values between rs1051730 and rs16969968, are higher than the threshold. Hence, those values are represented in red color. Considering them, just rs578776 and rs1051730 are discarded.

deemed suitable for the study. Later, for the variant rs1044394, the database indicated G as the reference allele and A as the alternate allele. The literature [182] defined the risk allele as A, thus AA and GA are the risk variants. However, by performing the separation based on the database, a significant imbalance was observed, as shown in the table 3.4, with 38 protective controls versus only 7 risk ones. As well, there were 95 protective patients versus 15 patients with the risk variant. Therefore, due to this noticeable disequilibrium, this SNP was discarded. Moving on to rs16969968, the literature provided information in which the group GG was associated with protection, whereas GA and AA were related to risk alleles [169]. Later, upon checking the database of subjects, as shown in the table 3.4, it was observed that there is an appropriate equilibrium between protective and risk, as 17 control with protective and 28 with risk, whilst 46 AD patients with protective and 64 with risk were observed. Hence, this SNP was also included for analysis. For rs588765, C was identified as the reference allele, whereas T was the alternate one. According to the litera-

SNP	Genotype	Group	Count
rs1044396 (**TT**)	Control	R	19
		P	26
	AD Patients	R	56
		P	54
rs1044394 (**A carriers**)	Control	R	7
		P	38
	AD Patients	R	15
		P	95
rs169969968 (**A carriers**)	Control	R	28
		P	17
	AD Patients	R	64
		P	46
rs588765 (**CC**)	Control	R	18
		P	27
	AD Patients	R	42
		P	68
rs8024987 (**G carriers**)	Control	R	14
		P	31
	AD Patients	R	47
		P	63
rs2350786	Control	R	21
		P	24
	AD Patients	R	56
		P	54
rs324650 (**TT**)	Control	R	15
		P	30
	AD Patients	R	45
		P	65

Table 3.4: Representation of the count of the subjects that present the risk and protective variants, which are represented with their initials (R and P). That distribution appears for both controls and patients group and is repeated for each of the chromosomes. As well, the risk allele corresponding to each SNP is described in the first column, by marking it (**in bold**). The objective was to obtain a distribution between risk and protective that was similar, avoiding abnormal values. Those SNPs that presented abnormal distribution were discarded and represented in red color. In the case of rs2350786, it was discarded not because of an abnormal distribution but because of a lack of information in the literature. For the SNP rs1044394, it was remarkably abnormal, so it was clearly discarded. Therefore, the remaining SNPs continued the process and were analyzed.

ture, it was observed that in this specific case, the pair CC is the one related to higher risk, whereas CT and TT were regarded as protective [183]. Analysis of the database using the described script revealed, as shown in the table 3.4, that 27 control subjects had protective alleles, and 18 were at risk. Also, 68 AD patients had the protective variant, and 42 were at risk. Consequently, this SNP was also included in the study. Moving to rs8024987, with C as the reference allele, and G as the alternate, the literature described the pair of CC as the protective variant, whilst CG and GG were found as the risk ones [175]. Then, the database was checked and observed that there was a certain balance since, as shown in the table 3.4, 31 protective and 14 risk controls were found, whereas 63 protective and 47 risk AD subjects. Although the balance was not strictly perfect, this SNP was also selected for further analysis, since it does not show such an aberrant disbalance. Finally, for the case of the muscarinic receptors variants, it was impossible to find any information in the literature defining any variant as risk or protective alleles for SNP rs2350786, so, therefore, this SNP was discarded. For rs324650, as shown in the table 3.4, the database indicated that A was the reference and T was the alternate, thus being AA, AT, and TT the possibilities. According to the literature [177], it was described how those presenting the TT-homozygotes were associated with risk since their presence was related to a severe course of the disease [177]. Upon checking the database, a balanced distribution was observed, with 30 versus 15 in the case of the controls, and 65 versus 45 in AD patients. As well, the difference was not exaggeratedly wide, hence leading to the inclusion of this SNP in the analysis.

Therefore, after proceeding with this analysis and selection, the final SNPs that were included in the study were rs1044396, rs16969968, rs588765, rs8024987, and rs324650. Once the SNPs have been selected, a Matlab script that allows the analysis and metrics computation for each of the genetic variants was designed.

3.3 EEG Analysis

In this section, first, a brief explanation of the development of the EEG recording in this study was described. Later, the main metrics at which the EEG is subjected to analysis were described, allowing to interpret the results of the project.

3.3.1 EEG Recording

A 5-minute EEG recording was obtained from each subject using a 19-channel Nihon Kohden Neurofax JE-921A EEG system. The recordings were acquired at a sample frequency of 500 Hz and a common average reference was used. The EEG data were divided into 19 channels, corresponding to the electrode placement on the scalp, including those zones that followed the international 10-20 system Fp1, Fp2, F3, F4, C3, C4, P3, P4, O1,

O2, F7, F8, T3, T4, T5, T6, Fz, Cz, and Pz, of the international 10–20 system. EEG recordings were obtained from participants in a resting-state condition with their eyes closed, ensuring a relaxed and noise-free environment. The researchers carefully monitored the subjects' vigilance state to minimize drowsiness during the recordings. The recorded data were stored in ASCII format on a personal computer for further analysis. Subsequently, the EEG signals underwent a series of pre-processing steps, following the methodology described by previous studies [122, 184], which allow ensuring the quality and reliability of the EEG data for subsequent analysis.

Then, for this specific project, for each subject and channel, the data was divided into epochs to facilitate analysis and the application of metrics. Additionally, for each epoch, it was assigned a value indicating the presence of artifacts. The raw data had undergone filtering, but epochs containing artifacts were excluded from further analysis, leaving only artifact-free epochs. Once this pre-processing step was completed, relevant metrics of interest were computed to analyze disruptions in the EEG.

3.3.2 EEG Metrics

Once the EEG information relative to each subject was cleaned from artifacts, the different computation metrics were introduced. Spectral analysis of the EEG is considered one of the standard methods for characterizing the EEG activity [185]. In order to continue with the spectral analysis, different measures can be analyzed. One of these measures is the power spectral density (PSD), which provides information about the distribution of signal power across different frequencies [185]. In Figure 3.2, the distribution of power over healthy people is shown, something that is expected in control subjects in this study. As previously mentioned, the calculation of PSD requires the application of the Fourier Transform [186]. In this study, the method described by Welch was used, employing a 500ms window with no overlapping [186]. The computation of PSD subsequently allows for the calculation of other measures, including Relative Power, Median Frequency, and Shannon Spectral Entropy. Later, two nonlinear parameters were computed, Sample Entropy and Lempel-Ziv Complexity.

Relative Power

Indeed, the spectrum of power in EEG analysis is commonly examined by considering the sum of powers within specific frequency bands [185]. The absolute power of a particular frequency band is typically determined by calculating the area under the PSD curve, which can be obtained through integration methods [188]. In this study, the absolute power for each frequency band was computed by integrating the PSD curve. To facilitate the analysis and comparison of power across different frequency bands, the ab-

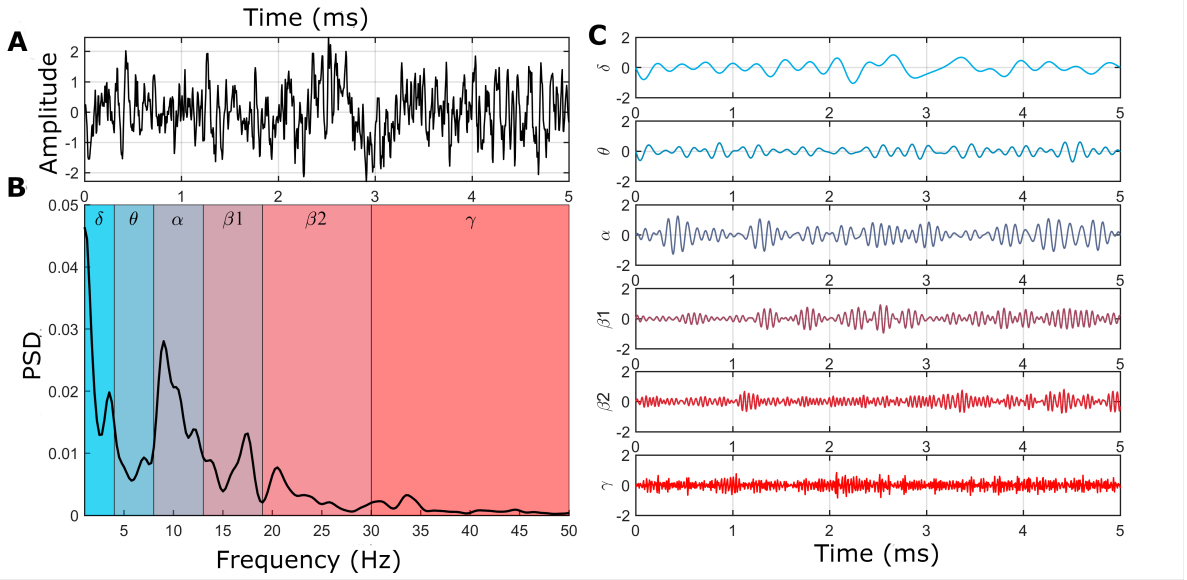

Figure 3.2: Scheme illustrating the typical spectral profile of the EEG of a healthy subject. In part A, it is shown the representation of the signal as a function of amplitude in microvolts over time in seconds. In part B, the PSD along the frequency appears, measured in $\mu V^2/Hz$. It divides the spectrum into the different possible bands, showing blue colors for those initial bands and red for the latest. Finally, part C represents the time distribution in each of the frequency bands. It is based on [187]

solute power values were normalized by dividing them by the sum of all spectral powers. This normalization process provides relative power values for each frequency band [189]. The relative power per frequency band is defined according to the article [122]

$$RP_{(f1,f2)} = \sum_{f_1}^{f_2} PSD_n(f) \tag{3.2}$$

being f_1 and f_2 the low and high cut-off frequencies of each of the bands subjected to analysis. As well, PSD_n is the normalized PSD in the frequency band of interest.

Therefore, five different relative powers were obtained: RP(delta), RP(theta), RP(alpha), RP(beta), and RP(gamma). However, it is important to note that the gamma band was not subjected to analysis in any of the subjects. It is due to the fact that its frequency band presents a lower amplitude, making it difficult to analyze, and, in addition, it might overlap with the electromyographic frequency band [190]. Hence, the analysis focused on the relative powers within the delta, theta, alpha, and beta frequency bands.

Median Frequency

The median frequency is defined as the frequency that divides the area under the previously described power curve by half and serves as a measure that characterizes the dis-

tribution of power across different frequency components in the EEG signal [191]. In fact, it was demonstrated that MF offers a better characterization of the electrical activity of the brain than the mean frequency [192]. In that way, half of the power is found at low frequencies and half at high frequencies. It is defined according to [122]

$$\sum_{f_i}^{MF} PSD_n(f) = 0.5 \sum_{f_i}^{f_m} PSD_n(f) \tag{3.3}$$

where f_i refers to the initial frequency set at 1Hz, f_m refers to the maximum frequency under analysis, set at 30 Hz since the gamma band is not included.

In general, studies have observed that individuals in an awake state tend to exhibit a median frequency of around 12 Hz, the value that can serve as a reference point and can help in assessing the level of alertness or wakefulness based on the MF [191]. In the case of AD patients, a certain study provided the information that MF is reduced in comparison to healthy controls, mainly in the occipital region [193].

Shannon Spectral Entropy

Spectral Entropy is found as a result of applying the Shannon function [194] to each of the values of the power spectrum [195]. By summing up these entropy values, the total spectral entropy is obtained, representing the overall homogeneity of the PSD [195]. Then the mathematical definition proposed by Shannon [194] was

$$H(f) = -\frac{1}{ln(N)} \sum_{i=1}^{N} p_i ln(p_i), \tag{3.4}$$

where p_i are considered the spectral amplitudes of the frequency bin *i* [196]. As well, *N* is considered the number of frequency bins [197]. It is important to note that p_i can be obtained as a normalized value of the PSD at each frequency bin [198].

It quantifies the irregularity or flatness of the frequency spectrum [196], so thus a high value indicates a broader and flatter spectrum, resembling the characteristics of white noise, a more complex and unpredictable signal [195, 197, 199]. On the other hand, low values suggest that the signal energy is concentrated in a few frequency bins, indicating less complex signals or specific frequencies such as sinusoids, hence suggesting a more regular and predictable pattern [195, 197, 199]. As well, it provides valuable information about the distribution of spectral energy [200]. Spectral Entropy is advantageous because it characterizes the EEG waveform without relying on specific frequency or amplitude information [195]. It has been used in order to detect subtle changes that can appear in EEG background activity, showing as well an abrupt decrease as the patient becomes unconscious during anesthesia [197]. This measure has shown promising results in different

studies of neurological disorders, including epilepsy [200]. Because of the definition, $H(f)$ can never be less than zero.

Sample Entropy

The use of sample entropy lies in the aim of estimating how random a series of data is, analyzing the regularity, without considering the source that generated this group of data [201]. It was first described by Richman and Moorman, in order to overcome the limitations of another entropy measure called approximate entropy (ApEn) [202]. Sample entropy is characterized by its relative consistency and robustness against transient interferences, and was defined by them [202] according to several steps. First, considering that N data points from a series $x(n) = x(1), x(2),...,x(N)$, it is necessary to follow some steps.

1. Create m vectors $X_m(1), ..., X_m(N - m + 1)$ counting as $1 \leq i \leq N - m + 1$. Those vectors try to represent m consecutive x values, beginning from the ith point.
2. Define the existent distance among the vectors $X_m(i)$ and $X_m(j)$, $d[X_m(i), X_m(j)]$, as the maximum absolute difference that exists among their scalar components:

$$d[X_m(i), X_m(j)] = max(|x(i + k) - x(j + k)|) \tag{3.5}$$

3. Count the number of j ($1 \leq i \leq N - m + 1, j \neq i$), defined as B_i such the distance is less or equal to r. So, for $1 \leq i \leq N - m + 1$,

$$B_i^m(r) = \frac{1}{N - m - 1} B_i \tag{3.6}$$

4. Set $B^m(r)$ as

$$B^m(r) = \frac{1}{N - m} \sum_{i=1}^{N-m} B_i^m(r). \tag{3.7}$$

5. Later, the dimension is increased to $m+1$, and A_i is computed, as for ranges of j from 1 to $N - m$ ($j \leq i$), defining $A_i^m(r)$ as

$$A^m(r) = \frac{1}{N - m - 1} A_i. \tag{3.8}$$

6. Set $A^m(r)$ as

$$A^m(r) = \frac{1}{N-m} \sum_{i=1}^{N-m} A_i^m(r). \tag{3.9}$$

Therefore, $B^m(r)$ is defined as the probability that two sequences match for m points, and instead, $A^m(r)$ is defined as the probability of a match for $m + 1$ point.

Finally, the sample entropy is defined as:

$$SampEn(m, r, N) = -ln[\frac{A^m(r)}{B^m(r)}]. \tag{3.10}$$

where m is the length of the sequences that are compared, r is the tolerance for accepting matches, and N is the length of the time series [196]. It is established as convenient to set the tolerance as r times the standard deviation of the data sequence [196, 202]. For this study, the values were set as $m = 1$ and $r = 0.25$ times the standard deviation, as used in [203].

It is designed to be independent of the length of the data series, which makes it a valuable technique for analyzing physiological signals [201, 204]. In the context of AD patients, it was first tested their electrical activity in comparison to control subjects, therefore it is a useful tool for analysis of AD patients [196]. By quantifying the irregularity of the EEG signals using sample entropy, researchers can gain insights into the differences in brain activity between AD patients and healthy individuals [196].

Lempel-Ziv Complexity

LZC is a metric that measures the complexity of the physiological signals [205]. It is a straightforward computational measure of complexity that is applicable to one-dimensional sequences of finite length [129]. It is based on the number of distinct substrings and the recurrence rate within the sequence, where higher values indicate greater complexity of the data [206]. Before calculating the complexity measure $c(n)$, the temporal signal needs to be converted into a binary sequence $P = s(1), s(2), ..., s(n)$ by comparing each sample of the signal with a threshold T_d [207]. The threshold value T_d is determined as the median of the signal, chosen for its robustness to outliers [207]. Each binary value $s(i)$ is defined as follows

$$s(i) = \begin{cases} 0 \; if \; x(i) \; < T_d \\ 1 \; if \; x(i) \; \geq T_d \end{cases} \tag{3.11}$$

where $x(i)$ is the i-th sample of the original temporal signal. The discrete sequence P is

checked from left to right and the complexity counter (n) is increased by one every time a new subsequence of consecutive elements is found [208]. After this study [208], the number of different subsequences in the sequence P is represented by $c(n)$. To obtain a complexity measure that is independent of the sequence length, the parameter $c(n)$ should be normalized. Later, in case the length of the original sequence is n, it has been demonstrated in a specific study [125], that the upper limit of $c(n)$, denoted as $b(n)$, can be expressed as:

$$b(n) = \frac{n}{log_2(n)} \tag{3.12}$$

and $c(n)$ can then be normalized by $b(n)$:

$$C(n) = \frac{c(n)}{b(n)} \tag{3.13}$$

where $C(n)$ is the final and normalized LZC.

It has been reported that AD patients show a decreased complexity of EEG signals [129]. This reduced complexity may reflect disruptions or abnormalities in the underlying neural processes associated with AD pathology [129].

3.4 Statistical Analysis

3.4.1 Mann-Whitney *U* test

After obtaining the metrics for each subject, it was important to assess the significance of these values. To visualize the distribution of values for each SNP and metric, violin plots were used, as described in the following section. The chosen statistical method in this study to analyze the data appropriately was the Mann-Whitney *U* Test. This test allows for the comparison of two groups and assesses whether there are significant differences between them. By applying this test, the study aims to determine if there are statistically significant variations in the metrics based on the genetic variants under investigation.

The Mann-Whitney *U* Test, also known as the Wilcoxon rank sum test, is a statistical method that analyzes differences between two groups of data, providing a single ordinal value that requires interpretation [209]. This test is considered non-parametric as it does not assume a specific distribution [210]. Its main objective is to determine whether two groups of data originate from the same population [210]. The null hypothesis assumes that both groups belong to the same population and needs to be tested [210]. In the test, observations from both groups are combined and ranked using a scoring system. Each score is then divided according to the respective group, resulting in sum scores for each

group [210]. The sample sizes of the groups are combined to calculate the U statistic, which follows a discrete distribution and allows for the determination of a specific probability, known as the p-value [210, 211]. These obtained p-values are used to test the null hypothesis. Typically, a significance threshold of 0.05 is chosen, although lower values such as 0.01 can be used for a more stringent analysis [211]. If the obtained p-values are lower than the chosen threshold (e.g., $p < 0.05$), the null hypothesis is rejected, indicating that the two samples are unlikely to come from the same population [210].

In this study, the Mann-Whitney U-test was conducted to compare subjects grouped as protective and risk alleles in both the control and AD patient groups. The aim was to determine if there were significant differences between these two groups based on the presence of specific genetic variants. The test results were evaluated by comparing the obtained values with a predetermined threshold. If the values were lower than the threshold, it indicated the presence of potentially significant differences. These findings are further analyzed, reviewed, and discussed in subsequent sections as they could contribute to important advancements in the research.

Chapter 4

Results

In this chapter, the results obtained after performing the previously described metrics and statistical analyses are presented. Initially, the control and AD patient groups were divided, including those with mild, moderate, and severe dementia, resulting in two subgroups for each genetic variation. Subsequently, the subjects were further divided into reference and alternate allele subgroups based on the previously described information. This led to a total of four subgroups displayed in each distribution plot.

The Mann-Whitney *U*-Test was then conducted between the reference and alternate allele subgroups within both the control and patient groups for each SNP. If the resulting *p*-value was below the predetermined threshold of 0.05, it was denoted with an asterisk (*) in the representation. Violin plots were chosen as the distribution visualization method to provide a clearer visual representation. The control group was depicted in blue, with different shades for the reference and alternate alleles, while the AD patient group was represented in red, also with varying shades. Additionally, the mean and median values were included in each distribution plot to facilitate visual comparisons.

Furthermore, several tables were generated to present the results. The tables represented exactly the same information as the figures, but it is provided in a more analytical view. Values lower than the threshold were represented in bold. A singular table for each metric analyzed was displayed, showing the mean values and standard deviations for the four groups discussed: control subjects with protective allele, control subjects with risk allele, patients with protective allele, and patients with risk allele.

For SNP rs1044396, as shown in Figure 1, none of the non-linear metrics present any significant difference. However, the *p*-values between AD patients with protective and risk variants in both LZC and SampEn metrics (tables 4.1 and 4.4) are very close to the set value of 0.05. In the case of spectral analysis, a significant result is observed in the alpha distribution between controls with the protective allele and controls with the risk allele (table

4.7, p-value = 0.017). However, no significant difference is observed in AD patients for RP(alpha). As well, as it appears in table 4.8, the p-value corresponding to the comparison between AD patients for RP(beta) also approximates closely to the set limit. In the case of SE, the p-value between controls with protective and risk alleles is close to the threshold but does not reach the desired value 4.3.

Regarding the non-linear metrics (LZC and SampEn), the distribution is observed to be quite similar, as shown in tables 4.1 and 4.4. In the case of SampEn, there is a slight decrease in AD patients in comparison to healthy subjects, without any significance, as appears in table 4.4. The values obtained in AD cases are reduced compared to controls for each SNP. For MF in AD cases, higher values are observed compared to controls. However, no significant difference is observed between subjects with the protective and risk variant for any SNP, as shown in table 4.2. In terms of SE, higher values were observed in controls compared to AD patients. However, no significant difference was found between the two groups, as indicated by the extremely high p-values for the SNPs other than rs1044396. Regarding the relative powers, RP(delta) showed lower values in healthy controls compared to patients. However, the obtained p-values (Table 4.5) were too high, indicating no significant difference in RP(delta) between the different genetic variants. For RP(theta), lower values were observed in healthy subjects compared to AD cases, aligning with the reduction of slow bands mentioned in previous chapters. The p-values for SNPs rs588765 and rs8024987 (Table 4.6) were relatively low, approaching the threshold but not surpassing it, indicating no statistical significance in this frequency band. In the fast activity bands, an increase in relative power was observed. However, for RP(alpha), apart from the significant p-value obtained in rs1044396, no other significant differences were observed (Table 4.7). In fact, for rs588765, the p-value obtained in the control subjects comparison was exactly 1.000, implying no statistical difference. Finally, for the beta band, as observed in table 4.8, apart from the mentioned close values that appeared in rs1044396, no more significant differences were appreciated, observing p-values extremely high for the rest of the SNPs.

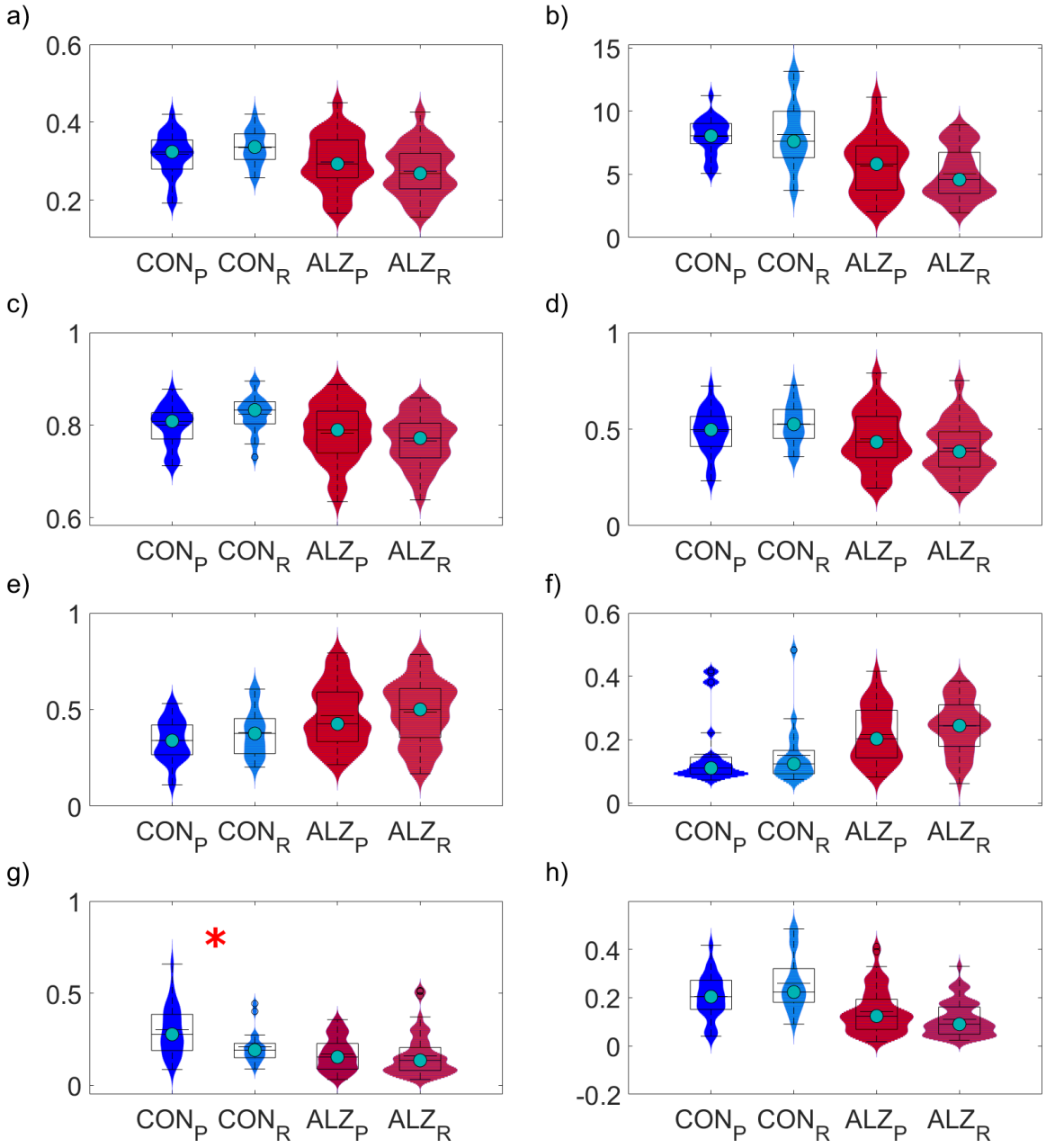

Figure 4.1: Representation of metrics of rs1044396. For each distribution, there is a separation between control subjects (CON) and AD patients (ALZ). As well, each of the groups is divided into two subgroups, with a protective allele (marked with "P") and a risk allele (marked with "R"). Therefore, four subgroups are represented, and differentiated by colors, being controls in different blue tones and AD patients in different red tones. Within the violin representation, the mean point is represented through a cyan circle, and the median is expressed through a black line. Figures represent a) LZC, b) MF c) SE, d) SampEn, and from e) to h) the relative powers in the corresponding order as RP(delta), RP(theta), RP(alpha), and RP(beta). Significant differences according to Mann Whitney *U*-Test are represented with an asterisk (*)

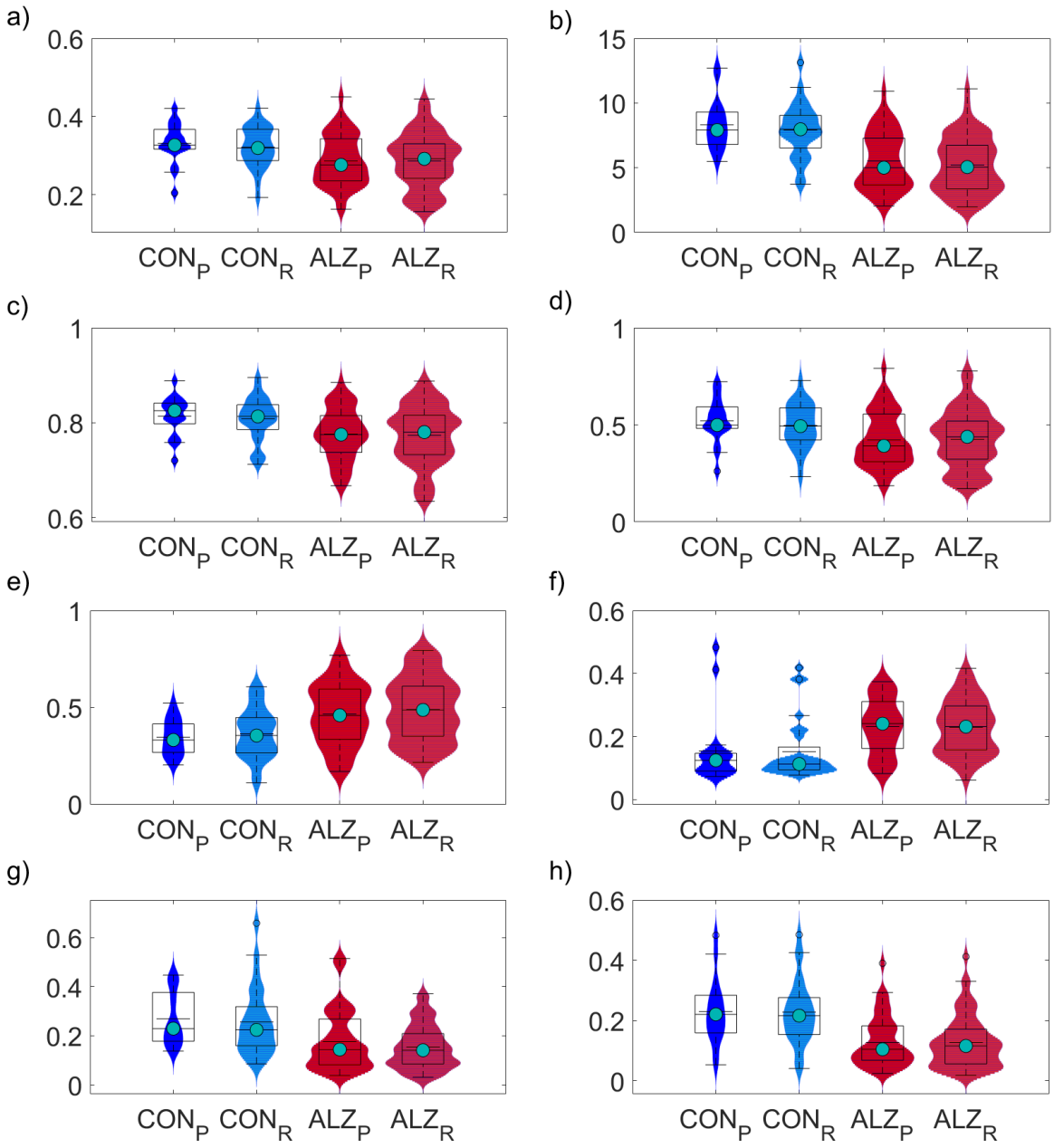

Figure 4.2: Representation of metrics of rs16969968. Again, each distribution plot displays a clear distinction between CON and ALZ groups, where controls are represented in blue and AD patients in red on the violin plot. Furthermore, both groups are further categorized into subgroups based on the presence of a protective allele (indicated as "P") or the risk variant (indicated as "R"). As well, in each violin plot, the mean value is represented by a cyan circle, while the median value is indicated by a black line. The figures showcase various metrics: a) LZC, b) MF, c) SE, d) SampEn, and e) to h) the relative powers, specifically RP(delta), RP(theta), RP(alpha), and RP(beta) respectively. As no significant difference has been obtained, there is no asterisk representing it

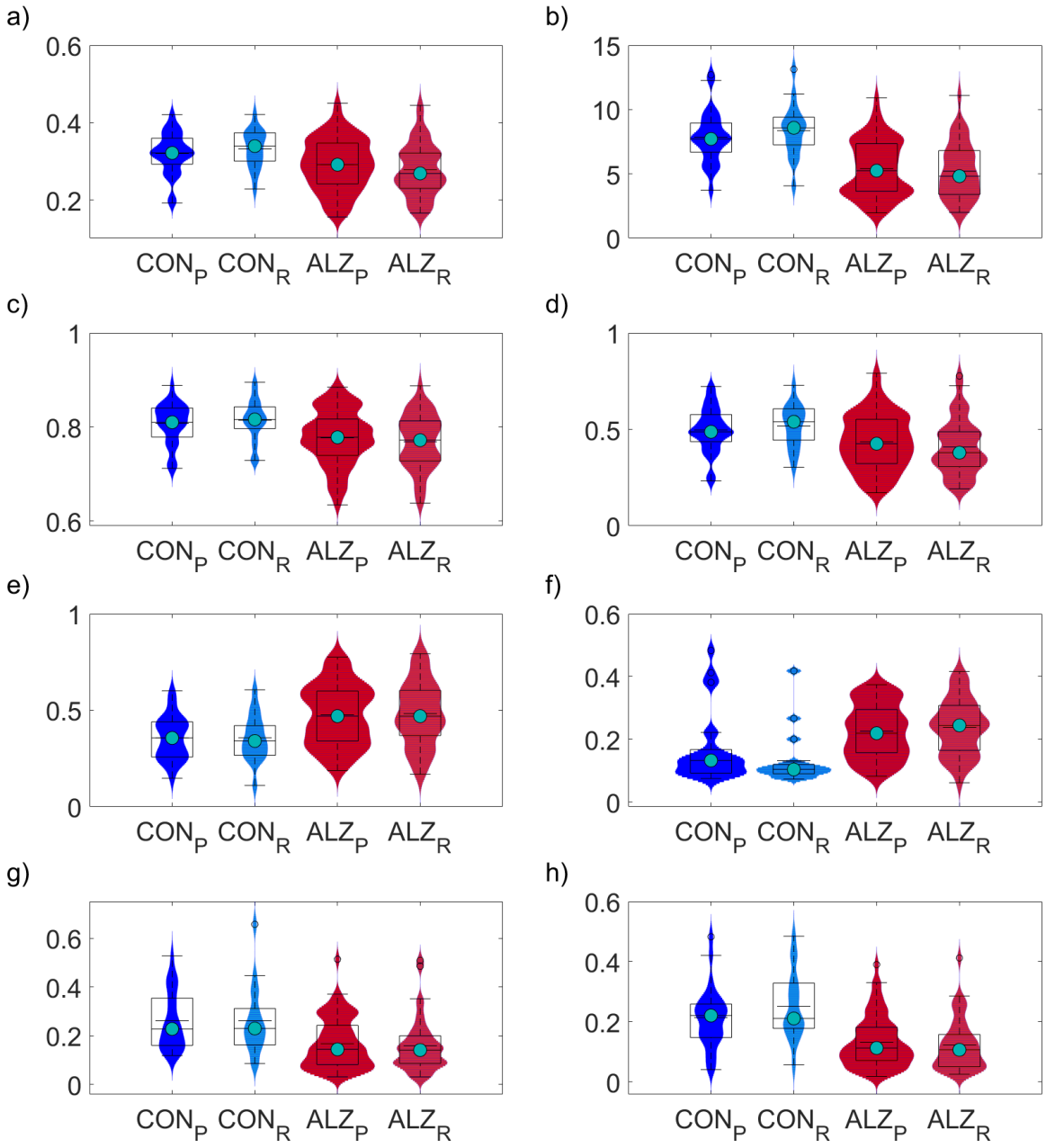

Figure 4.3: Representation of metrics of rs588765. Again the four different subgroups are represented, being comprised of controls with protective allele (CON_P), controls with risk allele (CON_R), AD patients with protective allele (ALZ_P), and AD patients with risk allele (ALZ_R). The representation is visually improved by means of different red tones for the ALZ group and blue ones for the CON group. The cyan circles represent the mean value, while the black lines indicate the median value. The figures display various metrics: a) LZC, b) MF, c) SE, d) SampEn, and e) to h) the relative powers, specifically RP(delta), RP(theta), RP(alpha), and RP(beta) respectively. It is important to note that no asterisks are present in the plots, indicating that no significant differences were observed.

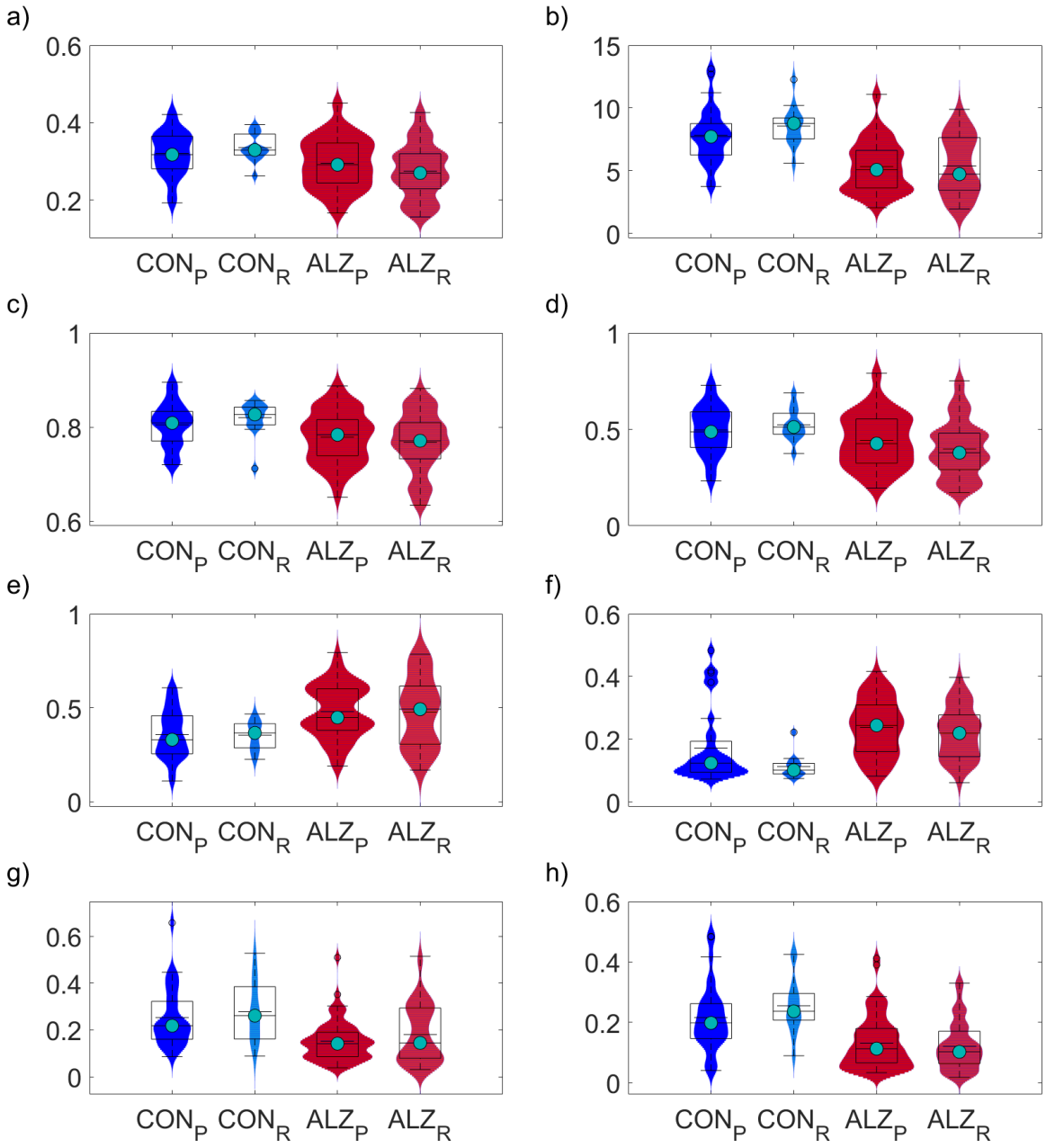

Figure 4.4: Representation of metrics of rs8024987. The four distinct subgroups are represented, including controls with a protective allele (CON_P), controls with a risk allele (CON_R), AD patients with a protective allele (ALZ_P), and AD patients with a risk allele (ALZ_R). The visualization is enhanced by using different shades of red for the ALZ group and shades of blue for the CON group. The cyan circles represent the mean value, while the black lines indicate the median value. The figures display various metrics: a) LZC, b) MF, c) SE, d) SampEn, and e) to h) the relative powers, specifically RP(delta), RP(theta), RP(alpha), and RP(beta) respectively. It is important to note that no asterisks are present in the plots, indicating that no significant differences were observed.

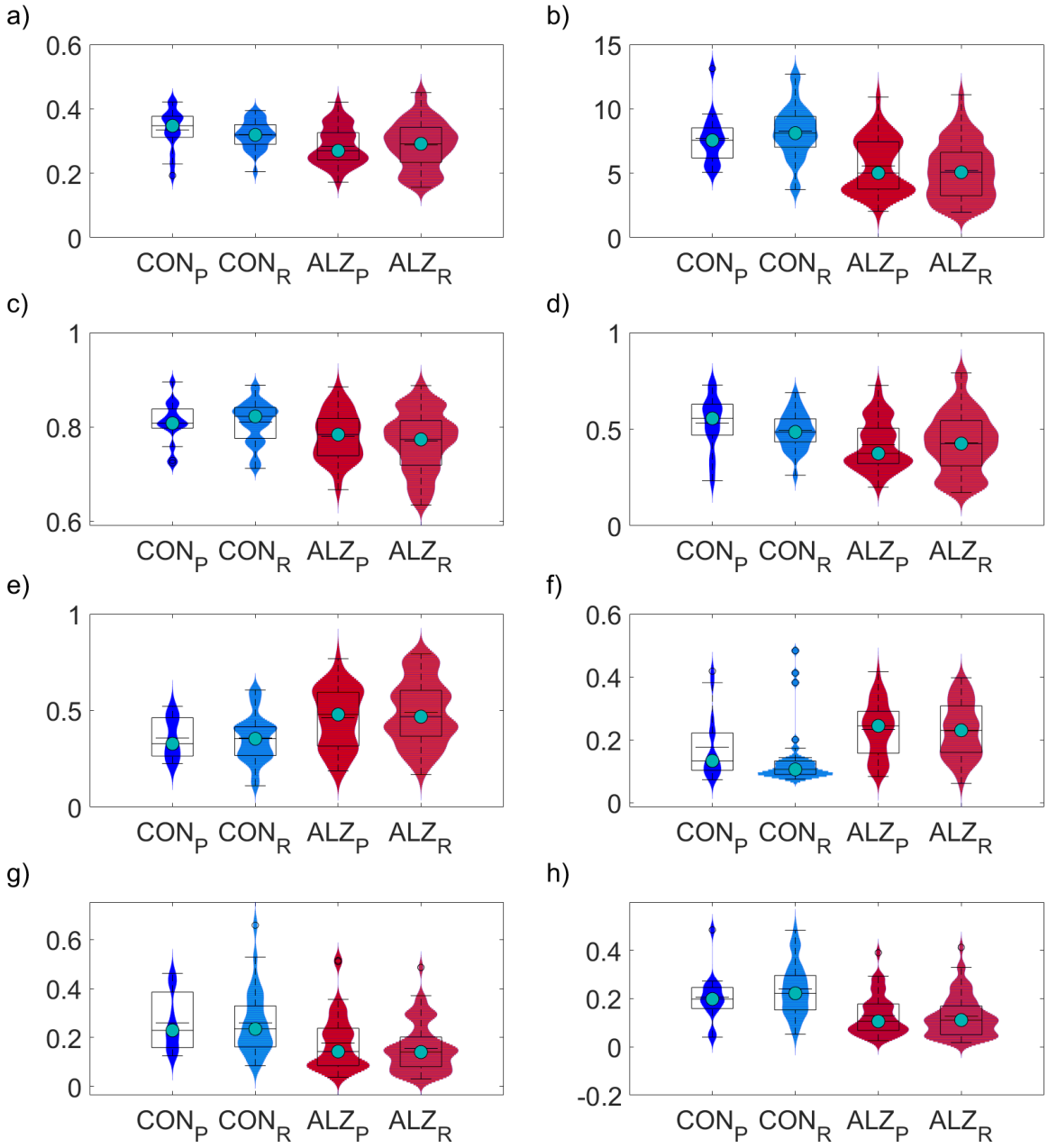

Figure 4.5: Representation of metrics of rs324650. For this polymorphism of the muscarinic receptors, equally to the others, the four distinct subgroups are represented, including CON_P, CON_R, ALZ_P, and ALZ_R. The visualization is enhanced by using different shades of red for the ALZ group and shades of blue for the CON group. The cyan circles represent the mean value, while the black lines indicate the median value of the distribution. The figures display various metrics: a) LZC, b) MF, c) SE, d) SampEn, and e) to h) the relative powers, specifically RP(delta), RP(theta), RP(alpha), and RP(beta) respectively. No asterisks are present in the plots, indicating that no significant differences were observed.

SNP	C_P	C_R	p-value	A_P	A_R	p-value
rs1044396	0.318 ± 0.056	0.335 ± 0.046	0.402	0.298 ± 0.070	0.275 ± 0.065	0.066
rs16969968	0.331 ± 0.053	0.321 ± 0.052	0.475	0.286 ± 0.065	0.287 ± 0.071	0.830
rs588765	0.320 ± 0.054	0.332 ± 0.049	0.452	0.292 ± 0.068	0.278 ± 0.068	0.267
rs8024987	0.320 ± 0.058	0.335 ± 0.036	0.398	0.296 ± 0.066	0.275 ± 0.070	0.123
rs324650	0.335 ± 0.066	0.320 ± 0.046	0.206	0.285 ± 0.059	0.288 ± 0.074	0.743

Table 4.1: LZC analysis for each of the SNPs under investigation. In the second and third columns, the mean and standard deviation value for controls with protective variant (C_P) and risk variant (C_R) is expressed. Continuously, the corresponding p-value is represented. Posteriorly, the same statistics are shown for AD patients with protective (A_P) and risk alleles (A_R), as well as the corresponding p-value.

SNP	C_P	C_R	p-value	A_P	A_R	p-value
rs1044396	7.996 ± 1.448	8.140 ± 2.675	0.899	5.646 ± 2.291	5.059 ± 2.020	0.238
rs16969968	8.338 ± 1.966	7.886 ± 2.086	0.665	5.523 ± 2.207	5.221 ± 2.146	0.465
rs588765	7.854 ± 2.017	8.361 ± 2.071	0.385	5.417 ± 2.180	5.234 ± 2.168	0.647
rs8024987	7.818 ± 2.182	8.585 ± 1.593	0.182	5.326 ± 2.040	5.375 ± 2.350	0.856
rs324650	7.695 ± 2.010	8.237 ± 2.051	0.198	5.566 ± 2.136	5.195 ± 2.192	0.359

Table 4.2: The table shows the analysis of the MF for each of the SNPs. The mean and standard deviation value for controls with protective variant (C_P) and risk variant (C_R) is expressed in columns 2 and 3. Later, the corresponding p-value is represented. Posteriorly, the same statistics are shown for AD patients with protective (A_P) and risk alleles (A_R), as well as the corresponding p-value.

SNP	C_P	C_R	p-value	A_P	A_R	p-value
rs1044396	0.800 ± 0.045	0.824 ± 0.041	0.096	0.783 ± 0.062	0.766 ± 0.055	0.122
rs16969968	0.814 ± 0.042	0.808 ± 0.046	0.615	0.776 ± 0.055	0.773 ± 0.062	0.983
rs588765	0.808 ± 0.044	0.814 ± 0.045	0.685	0.777 ± 0.059	0.769 ± 0.059	0.485
rs8024987	0.806 ± 0.047	0.821 ± 0.037	0.207	0.779 ± 0.055	0.768 ± 0.064	0.530
rs324650	0.809 ± 0.046	0.811 ± 0.044	0.639	0.781 ± 0.051	0.770 ± 0.064	0.576

Table 4.3: The table shows the spectral entropy analysis for each of the SNPs. The mean values for the comparison between controls with protective (C_P) and risk alleles (C_R) are displayed, with the corresponding p-value. Later, the same statistics are shown for AD patients with protective (A_P) and risk alleles (A_R), as well as the corresponding p-value.

SNP	C_P	C_R	p-value	A_P	A_R	p-value
rs1044396	0.489 ± 0.117	0.526 ± 0.108	0.441	0.448 ± 0.148	0.402 ± 0.137	0.081
rs16969968	0.520 ± 0.116	0.496 ± 0.113	0.447	0.423 ± 0.139	0.426 ± 0.148	0.816
rs588765	0.497 ± 0.117	0.517 ± 0.111	0.509	0.435 ± 0.144	0.408 ± 0.144	0.283
rs8024987	0.496 ± 0.126	0.524 ± 0.083	0.615	0.443 ± 0.139	0.400 ± 0.148	0.103
rs324650	0.531 ± 0.144	0.492 ± 0.095	0.166	0.418 ± 0.128	0.429 ± 0.154	0.648

Table 4.4: Sample Entropy analysis for each of the SNPs. The mean and standard deviation value for controls with protective variant (C_P) and risk variant (C_R) together with their corresponding *p*-value. Posteriorly, the same statistics are shown for AD patients with protective (A_P) and risk alleles (A_R), accompanied with their *p*-value.

SNP	C_P	C_R	p-value	A_P	A_R	p-value
rs1044396	0.340 ± 0.109	0.382 ± 0.123	0.364	0.470 ± 0.158	0.486 ± 0.163	0.509
rs16969968	0.346 ± 0.093	0.365 ± 0.128	0.631	0.463 ± 0.157	0.489 ± 0.163	0.499
rs588765	0.358 ± 0.115	0.357 ± 0.120	0.899	0.476 ± 0.158	0.482 ± 0.166	0.875
rs8024987	0.359 ± 0.130	0.354 ± 0.078	0.932	0.479 ± 0.141	0.477 ± 0.184	0.861
rs324650	0.359 ± 0.105	0.357 ± 0.122	0.933	0.462 ± 0.154	0.487 ± 0.165	0.539

Table 4.5: Delta band relative power comparative analysis for all the SNPs. It is represented the mean and standard deviation value for controls and AD patients with protective variant and risk variant, explained in the groups C_P, C_R, A_P, and A_R. The corresponding *p*-values are expressed.

SNP	C_P	C_R	p-value	A_P	A_R	p-value
rs1044396	0.156 ± 0.110	0.151 ± 0.095	0.722	0.217 ± 0.089	0.243 ± 0.083	0.107
rs16969968	0.154 ± 0.115	0.153 ± 0.097	0.972	0.232 ± 0.088	0.229 ± 0.085	0.868
rs588765	0.168 ± 0.112	0.132 ± 0.086	0.118	0.226 ± 0.086	0.238 ± 0.088	0.501
rs8024987	0.172 ± 0.117	0.112 ± 0.037	0.098	0.238 ± 0.089	0.221 ± 0.083	0.316
rs324650	0.176 ± 0.107	0.143 ± 0.101	0.152	0.232 ± 0.087	0.229 ± 0.087	0.798

Table 4.6: Theta band relative power analysis for each of the SNPs. In the second and third columns, the mean and standard deviation value for controls with protective variant (C_P) and risk variant (C_R) is expressed. Later, the corresponding *p*-value is represented. Posteriorly, in columns 5 and 6, the same statistics are shown for AD patients with protective (A_P) and risk alleles (A_R), as well as the corresponding *p*-value.

SNP	C_P	C_R	p-value	A_P	A_R	p-value
rs1044396	0.299 ± 0.137	0.207 ± 0.090	**0.017**	0.169 ± 0.092	0.160 ± 0.117	0.257
rs16969968	0.269 ± 0.110	0.255 ± 0.138	0.535	0.177 ± 0.124	0.155 ± 0.089	0.621
rs588765	0.260 ± 0.120	0.261 ± 0.140	1.000	0.168 ± 0.104	0.158 ± 0.109	0.599
rs8024987	0.252 ± 0.127	0.280 ± 0.130	0.358	0.151 ± 0.085	0.181 ± 0.126	0.518
rs324650	0.259 ± 0.119	0.261 ± 0.133	0.971	0.176 ± 0.114	0.156 ± 0.099	0.433

Table 4.7: Alpha band relative power analysis for each of the SNPs under investigation. The mean and standard deviation values for controls with protective variant (C_P) and risk variant (C_R) are expressed in the second and third columns per each SNP. Then, the corresponding *p*-value is represented in the fourth column. Next, the same statistics are shown for AD patients with protective (A_P) and risk alleles (A_R), as well as the corresponding *p*-value. The significant *p*-values obtained have been marked **in bold**.

SNP	C_P	C_R	p	A_P	A_R	p
rs1044396	0.205 ± 0.092	0.260 ± 0.118	0.172	0.144 ± 0.093	0.111 ± 0.072	0.055
rs16969968	0.231 ± 0.110	0.227 ± 0.106	0.972	0.128 ± 0.083	0.127 ± 0.086	0.920
rs588765	0.214 ± 0.100	0.251 ± 0.114	0.348	0.131 ± 0.084	0.122 ± 0.085	0.516
rs8024987	0.217 ± 0.111	0.254 ± 0.094	0.152	0.132 ± 0.087	0.122 ± 0.081	0.566
rs324650	0.206 ± 0.102	0.240 ± 0.108	0.329	0.130 ± 0.083	0.125 ± 0.085	0.697

Table 4.8: Beta band relative power analysis comparison for each of the SNPs under investigation. In the second and third columns, the mean and standard deviation values for controls with protective variant (C_P) and risk variant (C_R) is expressed. Continuously, the corresponding *p*-value is represented. In the fifth and sixth columns, the same statistics are shown for AD patients with protective (A_P) and risk alleles (A_R). Their corresponding *p*-value is expressed in the seventh column.

Chapter 5

Discussion and Conclusions

5.1 Introduction

In the final section of this study, the obtained results were analyzed, and based on the theoretical background provided in the initial chapters, interpretations, and discussions were presented to understand and explain the implications of the results. Additionally, the application of acquired knowledge and potential future directions that may stem from the analysis were proposed.

5.2 Competences Involved

First and foremost, a comprehensive explanation of the general and specific knowledge and skills acquired in the field of biomedical engineering was required. Regarding the general competencies outlined on the website, almost all of them were applied in the project's development. CG1, which involves analyzing, synthesizing, and effectively addressing problems in biomedical engineering, served as the foundation for the project. Additionally, CG2 and CG4 were utilized during the methodology and report writing stages, as they involve applying computational and experimental tools to analyze and quantify biomedical engineering problems, as well as employing project planning techniques and assessing associated risks. To meet these requirements, adherence to CG5 was necessary, which entailed understanding and adhering to current standards, regulations, and laws applicable to biomedical engineering projects. The practical sessions conducted at the University of Valladolid also provided an opportunity to demonstrate CG7, as effective collaboration in multidisciplinary teams comprising engineers and biomedical professionals was essential.

As for the specific competencies acquired and applied in the project, not all of the 36

ideas suggested on the website were directly relevant, as the focus of this thesis was on deep physiological analysis and the electrical functioning of the human body. Therefore, the specific skills applied and learned may differ from other projects. For example, CE1 and CE29, which revolve around understanding human organisms, were incorporated throughout the entire degree program and were applied in this project, although further investigation beyond the existing knowledge was required. Both competencies emphasize understanding the principles of organism functioning from the cellular level to the tissue level and up to the level of biological systems, as well as acquiring knowledge in bio-physics, anatomy, physiology, or pathophysiology to identify the most suitable processing technologies for a given problem in biomedical engineering. This knowledge was necessary to comprehend the functioning, morphology, and structure of acetylcholine receptors. CE15 and CE28, which deal with bioelectricity at the cellular level, were also relevant due to the emergence of problems related to bioelectricity that needed to be addressed. In addition, competencies related to genetics such as CE30, CE31, and CE32 were applied to understand the molecular basis of genetics, DNA functioning, and gene transmission in living organisms, as well as the structure and organization of genes. CE6 was implemented, as it focuses on designing systems to manage and analyze large datasets, similar to the database provided by the University of Valladolid, which contained a comprehensive description of thousands of SNPs per chromosome. CE7 and CE9 were necessary for the analysis of electrical signals. The first required an understanding of the frequency and power analysis of biomedical signals, as in the EEG. The latter involved the processing of physiological signals during EEG analysis. CE11 was applied to identify and adapt telecommunication techniques and metrics during the computational phase of the study. Programming skills, covered by CE19, were crucial throughout the project and improved with practice over time. CE2 and CE3 were also necessary for performing statistical analyses, utilizing state-of-the-art univariate and multivariate statistical techniques, and proposing new analyses rigorously for biomedical problems that cannot be addressed through standard methods of information analysis. In this study, statistical analysis was required to evaluate the correlation between different groups of data obtained in the results. CE35 was relevant for analyzing biotechnology applications in medicine and the pharmacology of AD. Lastly, effective communication in biomedical environments, both in the laboratory and during the development of the manuscript, was essential, fulfilling the requirements of CE27.

To summarize, the project represents a unique and individual undertaking that was presented and defended before a university panel. It focused on specific technologies in Biomedical Engineering, specifically exploring the effects of genetic alterations on the functioning of specific neurotransmitters and their receptors and their subsequent impact on the development of AD. This was achieved through the analysis of disruptions using EEG analysis. In essence, the project successfully integrated and synthesized the competencies acquired throughout the four-year program.

5.3 Discussion

In this study, multiple EEG-derived metrics were calculated for carriers of risk and protective alleles associated with acetylcholinergic receptors. The obtained results are discussed, with the objective of clarifying how the genetic alterations in the acetylcholine receptors might affect the electrical activity of the brain. The measurements analyzed in the study have the intention of showing if any significant variation appears in the EEG metrics for any of the genetic alterations proposed.

The only significant *p*-value appeared in the SNP rs1044396, and was associated with the altered alpha band relative power in the comparison between controls with the protective allelic variants and those with the risk TT-homozygous allele. For those control subjects that present the risk allele, the mean relative power in the alpha band is significantly lower than for those with the protective variants. The electrical activity of the alpha band was investigated regarding the role in cognitive [212] process. It was proposed that the alpha waves participate in a mechanism that controls cortical activation and excitability [213]. Furthermore, the study [214] concluded that alpha activity participates in the cognitive process and its self-regulation. Therefore, alterations in the alpha activity may lead to disruptions in the cognitive process [214]. Also, alpha activity was related to psychomotor [215], psycho-emotional [216], and physiological [217] aspects of human life. In addition, the alpha activity was analyzed in plenty of neurological disorders such as Multiple Sclerosis (MS), epilepsy, or AD [218]. In fact, the reduction of alpha power was observed in patients with dementia [219], thus reflecting cognitive and memory performance alterations. Analyses of other neurological disorders such as attention-deficit/hyperactivity disorder (ADHD) were carried out in order to assess the behavior of the different frequency bands, some of them offering unclear and variable analysis [220]. Recently, it was clarified that, in the case of the alpha band, differences between subjects and healthy patients were strong according to the decrease in alpha power [221]. Hence, that followed the analysis of the alterations in both motor and attentional processes that are observed during the development of the disease [221]. That theory confirmed many other studies that stated the reduction of the alpha power [222–224], suggesting the association between reductions of alpha power and ADHD [225–228]. Therefore, the relationship between reductions in alpha power and different neurological disorders was analyzed widely.

Additionally, several genetic factors that might affect those neurological disorders were investigated. The relationship between the genetic variations of the gene *CHRNA4* and EEG alterations was confirmed by [99]. According to the article [165], the presence of the homozygote group with two thymines in the SNP rs1044396 was associated with lower performance engaging in attention tasks, in subjects between 70 and 79 years old [75]. In addition, the article [165] proposed the future line of research that these attention impairments might be associated with cognitive decline. More studies were carried out according to the TT-homozygous variant of rs1044396, in which a correlation with slowing in the redirecting of the attention was observed [229]. The aforementioned study [229] proposed

that the mentioned alteration in rs1044396 had an effect on the modification of cognition, by affecting the understanding of new information. Further studies consisting of the joint implication of *CHRNA4* and *APOE* $\epsilon 4$ on healthy subjects reported a loss of cognitive function and decline [230], and slower reorientation as age increased [231], in the case that *APOE* $\epsilon 4$ was expressed together with TT-homozygous variant of rs1044396. Additionally, those publications [165, 229, 230], demonstrated a relationship between the genetic implication of TT-homozygote with the affection of cognition and attention procedures.

The relationship between modifications of *CHRNA4*, and particularly rs1044396, and alterations of the brain electrical activity was previously reported [229, 232]. According to Steinlein et al. [99], those alterations were demonstrated to be related to the appearance of the low-voltage EEG phenotype, which is characterized by a reduction in alpha activity. Therefore, the results obtained in this study are supported by the existing literature regarding the relationship between the reduction in alpha power and its impact on cognition, as well as the association between the TT-homozygote variant of rs1044396 and cognitive impairment. The connection between this specific genetic variant and the decrease in alpha power observed in this study leads to a significant finding, which could be explained by alterations in the cholinergic system. The role of acetylcholine was related to cognitive functions by means of the functional significance of the alpha oscillations [233]. The article remarked on the impact that oscillations in the alpha frequency have on the cognitive functions, that are dependent on acetylcholine [233]. In the literature, it was proposed [75] that alterations in the ACh-derived neurotransmission could be related to cognition and attention deficits. Posteriorly, Reinvang et al. [165] provided evidence regarding the impact of rs1044396 and the affection of the cholinergic system, which implied a worsening of attention and speed. Consequently, acetylcholine was one of the key points of the study, since it relates all the information provided in the discussion. Therefore, the appearance of a significant difference in relative alpha power in healthy controls has led to wide research on the mechanisms and possible relationships that it might imply. Hence, based on all the evidence provided, it could be inferred that modifications in the *CHRNA4* gene might lead to alterations in the electrical activity of the brain. Those alterations, despite not being pathological, might be associated with very mild cognitive impairment, or with a higher probability of presenting dementia in the future.

In the study, it was proved that, in contrast to healthy controls, AD patients carrying both risk and protective alleles did not exhibit significant differences. AD patients were associated with reductions in nonlinear metrics, SE, and MF; which align with the expected trends. Relative power increments in the slow bands and reductions in faster bands were observed in AD patients in comparison to controls, as it was previously stated [99]. As it has been described widely, that decrease worsens with the development of the disease, thus as the neurodegeneration worsens [145–147]. Among the typical characteristics of AD, it is of paramount importance to remark neuronal damage [4, 13], which eventually leads to neuronal atrophy [4]. Delbeuck et al. [234] proposed that some of the cognitive deficits that appear in AD might be explained by the disconnection between different cor-

tical areas. In addition, another relevant characteristic of AD brains is the appearance of abnormalities in the neurotransmission process [4]. More specifically, AD is related to deficits in the cholinergic system, which is manifested in elements such as ACh and AChE concentrations, or the muscarinic and nicotinic receptors [4]. Because of it, the cholinergic system is impaired in AD patients [4]. Therefore, it could be proposed that in AD cases the synaptic anomalies are so high that the effects produced by the alteration of a single subunit of an acetylcholine receptor would be eclipsed.

Remarkably, only the SNP rs1044396 reported statistically significant differences in a single metric (RP in alpha activity). This SNP is located at the *CHRNA4* gene, which belongs to chromosome 20. On the other hand, rs16969968, rs588765, and rs8024987 are located at chromosome 15, which codify alpha subunits joining together as a cluster [96]. Therefore, a genetic variation in any of these genes may affect the expression of the remaining genes in this cluster [96]. In that regard, the lack of significant statistical association in any of those three SNPs between controls and AD patients with different genotypes is consistent, so hence the theory proposed by Rempel et al. [96] cannot be discarded.

The SNP rs1044396 was defined as an alteration of the *CRNA4* gene, which encodes the α4 subunit among the nicotinic receptors, those who bind the acetylcholine neurotransmitter. It was demonstrated that polymorphisms were associated with alterations in the correct binding of acetylcholine to its receptors, thus provoking modifications in the neurotransmission process [23, 30]. Basically, considering the relevance of receptor structural configuration in neurotransmission, modifications in this structure lead to utter alteration of the process [235]. Therefore, according to the results obtained, the specific variation rs1044396 of the genetic expression of the α4 subunit would result in altered functioning of the cholinergic system. A previous study described a relationship between the genetic polymorphism and the acetylcholine mechanisms of action, by influencing the processing of information [236]. Therefore, the existence of this relationship reinforces the idea that a decrease in alpha relative power might imply a feasible association between the SNP rs1044396 and the appearance of early signs of cognitive impairment.

5.4 Limitations and Future Research Lines

This work has been carried out with several limitations that can be addressed in future endeavors. One significant limitation is the size of the databases used. To enhance the statistical power and achieve significant differences, it is recommended to use a larger database with a wider range of subjects. Additionally, it was observed that many SNPs mentioned in the literature were not present in the database. Therefore, expanding the database to include more genetic variants would enable parallel analyses of additional variations. Also, the MMSE score regarding AD patients presented a large standard deviation since the values corresponding to mild, moderate, and severe stages differed remarkably.

Hence, dividing the AD patients into different stages of severity could be of interest, not only for eliminating this bias, but also for allowing the study of the effect of genetic variations on the AD continuum [122].

Regarding the observed results, it would be valuable to conduct a focused analysis on the attention levels of pre-clinical subjects exhibiting the TT-homozygote variant of the SNP rs1044396. This analysis would serve to validate the proposed discussion. Furthermore, a longitudinal study of these subjects could be conducted to observe if they begin to develop any clinical stages, not only related to AD but also to other neurological disorders. Additionally, conducting a more extensive investigation of control subjects with protective variants would be highly beneficial, as the mean relative alpha power was found to be higher compared to other variants. Furthermore, it is worth noting that the original study that initially associated rs1044396 with attention impairment reported that the subjects displaying the most significant variations in alpha power were within the age range of 70 to 79 years old. In contrast, the mean age of the pre-clinical subjects in this study was 79.71, with a standard deviation of 7.27. Thus, it can be observed that several subjects exceeded the suggested age range. Considering this, any potential modifications to the database should also take into account the age of the subjects. It is crucial to have the most precise and comprehensive database possible in order to conduct further analyses aimed at examining the impact of the SNP on the development of attention disorders, which can ultimately lead to cognitive deficits and dementia.

In terms of methodology, it would be interesting to explore the subdivision of different frequency bands into their respective sub-bands, especially considering the statistical significance observed in the alpha band. Identifying the specific alpha sub-band where the alterations are evident could provide further insights into the effects caused by the specific SNP. Furthermore, a more comprehensive analysis of the alterations in the beta band would also be of interest. Additionally, given the observed significance in SNP rs1044396, exploring the other discarded variants present in chromosome 20, specifically in the *CHRNA4* gene, could be of potential interest. More concretely, the SNPs that might be of interest would be rs1044393 and rs1044397.

5.5 Conclusions

Based on the discussion presented in this chapter, several conclusions can be drawn. Firstly, a significant statistical difference was observed in the power of the alpha band between pre-clinical subjects with the risk variant (TT-homozygote) and the protective variant (C- present allelic variants) of the SNP rs1044396. The alpha band, which encompasses frequencies between 8 and 13 Hz, was found to be decreased in controls with the risk allele, supporting the notion that this genetic variant is associated with alpha band alterations. Furthermore, since the same genetic variant has been linked to attention deficits and reduc-

tions in alpha band power have been observed in individuals with attention impairment, the results of this study further support the relationship between the development of attention deficits and the influence of genetic factors, specifically alterations in the *CHRNA4* gene. Lastly, considering that attention and the reduction of alpha power are associated with cognitive impairment, it can be proposed that the observed reduction in alpha power in this study may provide genetic evidence for the implication of rs1044396 in the possibility of developing dementia in the future. This is further supported by the alterations observed in the cholinergic system, which is known to be affected in neurological disorders. However, no variations in alpha power were observed in AD. Therefore, it is suggested that due to the pre-existing impairment of the cholinergic system in the brains of AD patients, genetic modification of a single subunit in a solitary receptor would not have significant implications. However, it is important to note that further analysis is required to confirm the associations proposed in this study. Conducting additional evaluations with a wider database of subjects would improve the statistical power of the assessment. Despite this limitation, the present study demonstrates the novelty and significance of investigating the effect of genetics on the presence of neurological disorders, such as AD, through EEG analysis. It has the potential to advance our understanding of the genetic factors influencing neurological disorders and pave the way for future research in this field.

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