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Unravelling the molecular basis of Alzheimer's disease and Frontotemporal dementia: genetic and epigenetic approach through Next Generation Sequencing and OpenArray technologies

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Abstract

Alzheimer's disease (AD) and Frontotemporal dementia (FTD) are complex heterogeneous disorders with a strong genetic background, but the identification of a genetic cause is difficult given the multifactorial aetiology of the disorders. Epigenetic and environmental factors interplay to influence this complexity. The aim of the present project was to explore the two most common forms of dementia using genetic and epigenetic approaches.

The first part focused on the genetic screening of 188 patients using a Next Generation Sequencing methodology. Different custom panels were designed to screen the most common genes involved in AD and FTD, common genetic risk factors and genes related to other neurodegenerative diseases causing dementia. Patients negative for the genetic screening of common causative genes but with strong family history for dementia and/or young onset of the symptoms were further investigated applying the SureSelect Custom Constitutional Panel 17Mb (CCP17), comprising more than 5000 genes associated to inherited diseases.

The second part of the projects was based on the study of expression profile of miRNAs in genetic FTD patients, aiming to find a signature able to distinguish the genetic subgroups. The study was performed to test the expression levels of 754 miRNAs in 30 patients carrying mutations in *C9ORF72*, *GRN* and *MAPT* genes, and 10 control subjects, using OpenArray technology.

Following the genetic study, a total of 35 variants were found in 36 over 188 patients screened. Some of these variants occurred in causative genes or in genetic risk factors associated to AD and FTD; other genes were classically associated to other phenotypes. Moreover, variants in genes unexpected in the clinical setting provide links to biological processes that need to be further explored.

As resulted from the epigenetic study, a specific signature of miRNAs has been found for each group, which can distinguish patients from healthy subjects with high sensitivity and specificity and predict the underlying pathology.

Targeted NGS confirms to be a highly efficient, cost-effective method able to unravel rare genetic variants whose significance need to be further explored. Studying epigenetic factors such as miRNA and their target genes would be helpful in gaining new insights into the pathogenic processes characteristic of dementias. This makes miRNAs interesting potential therapeutic targets.

Riassunto

La malattia di Alzheimer (AD) e la Demenza frontotemporale (FTD) sono malattie eterogenee complesse con una forte componente genetica, ma l'identificazione di una causa genetica è difficile data l'eziologia multifattoriale delle due patologie. Fattori epigenetici e ambientali interagiscono per influenzare ulteriormente questa complessità. Lo scopo di questo progetto è stato quello di esplorare le due forme più comuni di demenza utilizzando approcci genetici ed epigenetici.

La prima parte si è concentrata sullo screening genetico di 188 pazienti utilizzando la metodologia Next Generation Sequencing (NGS). Diversi pannelli sono stati disegnati per esaminare i geni più comuni coinvolti in AD e FTD, fattori di rischio genetici comuni e geni correlati ad altre malattie neurodegenerative che causano demenza. I pazienti risultati negativi allo screening genetico ma con una forte storia familiare per demenza e/o insorgenza precoce dei sintomi sono stati ulteriormente studiati applicando SureSelect Custom Constitutional Panel 17Mb (CCP17), che comprende più di 5000 geni associati a malattie ereditarie.

La seconda parte del progetto si è basata sullo studio del profilo di espressione dei miRNA in pazienti FTD genetici, con l'obiettivo di trovare una firma epigenetica in grado di distinguere il sottogruppo genetico. Lo studio è stato condotto per testare i livelli di espressione di 754 miRNA in 30 pazienti portatori di mutazioni nei geni *C9ORF72*, *GRN* e *MAPT* e 10 soggetti di controllo, utilizzando la tecnologia OpenArray.

A seguito dello screening genetico, sono state trovate in totale 35 varianti in 36 su 188 pazienti sottoposti a screening. Alcune di queste varianti sono state individuate in geni causativi o in fattori di rischio genetici associati ad AD e FTD; altre in geni classicamente associati ad altri fenotipi. Inoltre, altre varianti identificate in geni inaspettati in ambito clinico forniscono collegamenti a processi biologici che devono essere ulteriormente esplorati.

Come risultato dello studio epigenetico, è stata trovata una firma specifica di miRNA per ciascun gruppo, che può distinguere i pazienti da soggetti sani con elevata sensibilità e specificità e prevedere la patologia sottostante.

L'NGS si conferma un metodo altamente efficiente ed economico in grado di svelare rare varianti genomiche il cui significato deve essere ulteriormente esplorato. Studiare fattori epigenetici come i miRNA e i loro geni target sarebbe utile per ottenere nuove informazioni sui processi patogeni caratteristici delle demenze. Questo fa dei miRNA interessanti potenziali target terapeutici.

INDEX

1.1 INTRODUCTION

1.1 Dementia

Dementia is a heterogeneous group of disorders leading to a progressive decline in cognitive functions, which range from memory, thinking, orientation to language and changes in behavior [1].

As stated by the World Health Organization (WHO), worldwide about 55 million people suffer from dementia in 2019, with nearly ten million new cases every year, and this number is expected to reach 139 million by 2050 [2]**.** As the population ages, dementia is becoming a major global health problem; a disease-modifying treatment is still lacking, therefore there is increasing focus on risk reduction, timely diagnosis, and early intervention. The umbrella term dementia group several entities with different incidence on the population. Alzheimer's disease is the first cause of dementia, accounting for 60-70% of cases [3]. 10-20% of cases are Vascular dementia, caused by damage to brain blood vessels. Often coexist with AD. 10% of cases are caused by Frontotemporal dementia, mostly affecting people under the age of 65 years. 5% are Lewy body dementia, a disease characterized by abnormal inclusions of α-synuclein into neural cells [4].

Alzheimer's disease and frontotemporal dementia, the two main cause of dementia in senile and pre-senile population, are complex, multifactorial entities, since only a small percentage of the cases is familial with an autosomal dominant pattern of transmission. Sporadic cases are likely caused by a combination of risk factors including age, lifestyle and environment, interplaying with genetic and epigenetic factors [5].

1.2 Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia (60% of cases) [3].

It is a progressive and irreversible neurodegenerative disease affecting hippocampal and cortical regions of the CNS. Selective and localized neuronal loss causes memory impairments and disturbance of additional cognitive functions, namely word-finding, spatial cognition, reasoning, judgment and problem solving. It was firstly described by Alois Alzheimer in 1906 which identified the pathological hallmarks. AD is mostly sporadic, affecting people over 65 years (late-onset AD, LOAD), but there can be also an early onset presentation (early-onset AD, EOAD, <65 years, about 10% of cases). EOAD and LOAD patients are clinically and pathologically similar and both occur in familial and sporadic patients [6].

The incidence of AD is three new cases over 100.000 people under 60 years of age and 125/100.000 people over 60 years. The prevalence is estimated as 300/100.000 people between 60 and 69 years old, 3.200/100.000 in people between 70 and 79 years and 10.800/100.000 in subjects over 80 years of age [7].

1.2.1 Clinical characteristics

AD has an insidious onset, often hard to identify. Usually, the earliest symptoms are impairment of episodic memory and inability to store new information. Then, other cognitive deficits become evident. Language is impaired since the patient cannot remember words. Progressively, the patient became unable to recognize faces (prosopagnosia); ability of calculation is lost; visuospatial disorientation appears [8]. As the disease progresses, motor functions are impaired too. The patient is unable to dress himself, to use any tools. This situation is made difficult by the onset of psychiatric symptoms, mostly depression, psychosis, behavioral issues. In the final stages, the patients lose their self-sufficiency. The course of the disease ranges from 7 to 12 years and varies from patient to patient [8].

Currently, AD diagnosis is made in accordance with Dubois et al. 2014 [9] which revised and proposed advances to international Working Group (IWG) and the US National Institute on Aging–Alzheimer's Association criteria. AD diagnosis can be made in the presence of an amnestic syndrome, that can be associated to various cognitive or behavioral changes, and at least one of the changes indicative of in-vivo Alzheimer's pathology, namely a CSF profile consisting of decreased $A\beta_{1–42}$ levels together with increased T-tau or P-tau concentrations, or an increased retention on amyloid tracer PET.

1.2.2 AD neuropathological hallmarks

At macroscopic level, AD is characterized by reduced brain convolution and expanded ventricles, as a result of atrophy of frontal, temporal and parietal lobes, with main involvement of hippocampus [10].

At microscopic level, neuronal cell loss is observed in the cortices, particular hippocampus, entorhinal cortex, parahippocampal gyrus. Anterior nucleus of thalamus, amygdala and basal nucleus of Meynert are involved as well. Remaining neurons show reduced volume, with less dendrites and axons, and therefore synaptic loss. Neuronal alteration is coupled with astrocytic proliferation, as a compensatory and reparative mechanism [10]. Two are the neuropathological hallmarks of AD: extracellular accumulation of diffuse and neuritic amyloid plaques and the intraneuronal accumulation of neurofibrillary tangles (NFTs) [11] (Figure 1).

Amyloid plaques are made of fibrillary peptides arranged as β sheets [12]. They are surrounded by altered axons and dendrites, which are in turn associated to activated astrocytes and microglia. Amyloid-β (Aβ) protein is the principal component of amyloid plaques. Aβ presents with two main forms: a peptide made of 40 amino acids ($A\beta_{1-40}$), more common and less prone to aggregation, and $A\beta_{1-42}$ form which is more hydrophobic and highly prone to aggregate. Amyloid plaques are spread throughout the cortex, also in areas not involved in the AD symptomatology (as putamen, cerebellum) [13]. Remaining neurons present cytoskeleton abnormality, mainly neurofibrillary tangles. They consist in double helical filaments made of aggregates of tau protein and can be located in the soma, axons and dendrites. In NFT tau is hyperphosphorylated in several sites [14–16] .

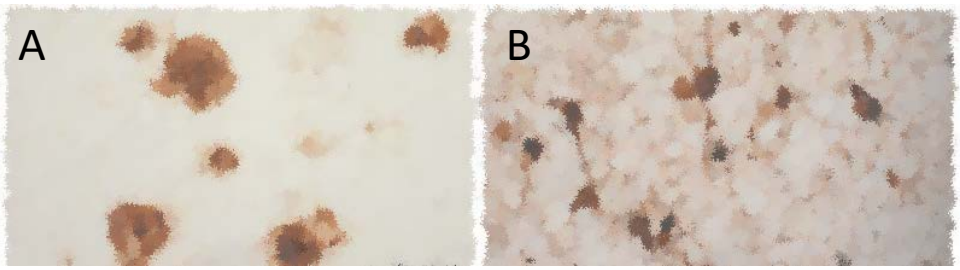


Figure 1. AD neuropathological hallmarks. A: senile plaques formed mainly by aggregated of Amyloid-β. B: tangles composed by aggregates of hyperphosphorylated tau protein.

1.2.3 AD pathogenesis

1.2.3 .1 Role of Aβ

Aβ comes from the amyloid precursor protein (APP) as consequence of sequential proteolytic cuts by several enzymes. APP is a transmembrane glycoprotein present in dendrites, axons and cell body of neurons but it is also present in non-neuronal cells (astrocytes, microglia, smooth endothelial cells) [17].

So far, several physiological roles of APP have been proposed. The extracellular domain of APP mediates cell-to-cell adhesion to support synaptic connection, mediate neuronal signalling and neurotransmitter release through the activation of calcium channels [18,19]. APP is cleaved by three proteolytic factors: α-, β-and γ-secretase. The sequence of this cleavage determines the amyloidogenic or the non-amyloidogenic processing of APP [20] (figure 2). α-secretase (ADAM9, 10 and 17) cleavage creates a soluble fragment (sAPPα) released in the extracellular space [21,22] and a transmembrane fragment of 83 amino acids (Carboxyterminal fragment-α, CTFα). γ-secretase [with presenilins (PSEN1-2) as major components] cleaves the CTFα creating the p3 peptide (non-amyloidogenic pathway) [17]. In the amyloidogenic pathway, β-secretase (whose major component is β-site APP cleaving enzyme 1, BACE1) cuts a soluble fragment of reduced size (sAPPβ), leaving a transmembrane fragment of 99 residues (CTFβ) [23,24]. The BACE product can then be transferred to the cell surface where it becomes a substrate for γ-secretase [26]. The subsequent action of γ-secretase creates the Aβ peptide.

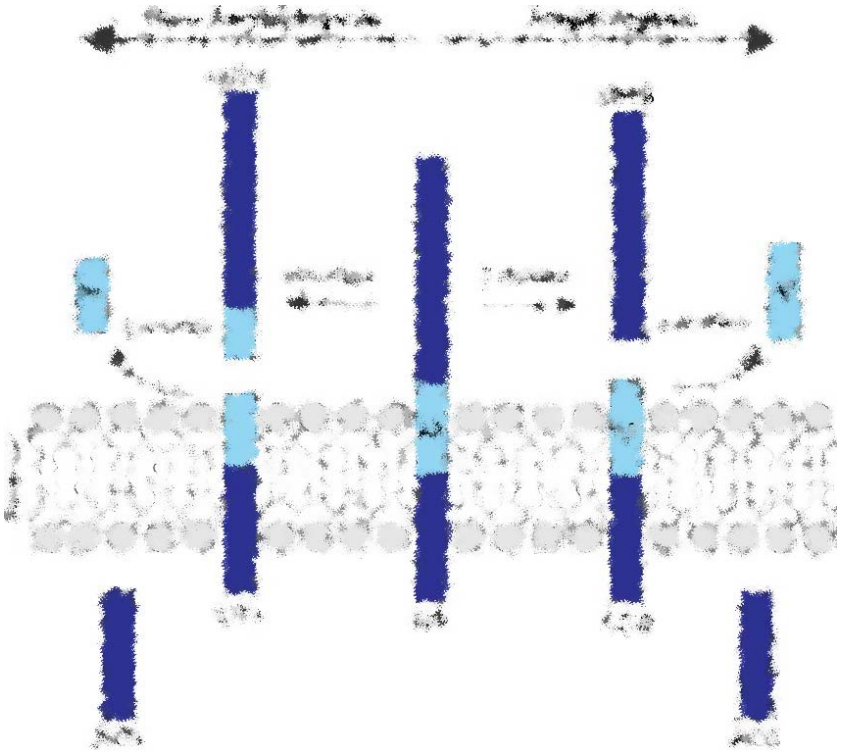


Figure 2. Amyloidogenic and non-amyloidogenic pathway. APP: amyloid precursor protein; CTFα: Carboxyterminal fragment-α; CTFβ: Carboxyterminal APP fragment-β. soluble APP fragment-α (sAPPα); soluble APP fragment- β (sAPPβ); AICD: amyloid precursor protein intracellular domain; p3: p3 fragment.

Peptide Aβ is constitutively produced and released by cells under normal conditions [27], and is measurable in cerebrospinal fluid (CSF) and plasma of normal subjects throughout life. Both the $Aβ_{1-40}$ and $Aβ_{1-42}$ forms can be identified in CSF and plasma. Other cell types in brain tissue also express variable amounts of APP (astrocytes, microglia, smooth endothelial cells) and could contribute to the process of secretion and deposition of Aβ [18].

Dysregulated APP processing may contribute to AD pathogenesis by elevating Aβ production, and reducing the $Aβ_{40}/Aβ_{42}$ ratio. Mutations in PS1 and PS2 primarily alter APP γ-cleavage, thereby resulting in a decreased $Aβ_{40}/Aβ_{42}$ ratio. Most FAD mutations in APP are located nearby the γ-secretase cleavage site, also altering the ratio [28,29]. During AD pathogenesis, Aβ aggregates are assembled from Aβ monomers into a variety of unstable oligomeric species. Oligomeric Aβ then further aggregates to protofibrils, which ultimately elongate into insoluble fibrillar assemblies comprising β-strand repeats. Extracellular Aβ aggregates in their fibrillar form are resistant to hydrolytic degradation. [30,31]. Evidence suggest that mitochondrial dysfunction is involved in AD pathogenesis [32]. Aβ has been observed in mitochondria in the brain of AD patients and AD mouse models. Abnormal accumulation in mitochondria, causes altered mitochondrial structure, decreased respiratory function and ATP production, impaired mitochondrial dynamics. This leads to elevated mitochondria associated oxidative stress, another important contributor to AD [33].

1.2.3.2 Role of tau protein

The tau protein is fundamental in stabilizing the microtubules. It is encoded by *MAPT* gene, located on chromosome 17 and including 16 exons. The tau protein exists in 6 different isoforms composed of 352-441 amino acids, due to alternative splicing at the level of exons 2,3 and 10 [29,34]. Tau is highly expressed in neurons in the mammalian brain, and normally localizes predominantly to axons as an important regulator of axonal transport [35]. Recent studies demonstrate that tau is also present in dendrites and postsynaptic compartments, possibly playing a role in regulating synaptic plasticity [36]. Tau is also moderately expressed in oligodendrocytes, where it plays a role in process outgrowth and myelination [37], and astrocytes, where its role is still unclear [38].

The pathological tau form is abnormally phosphorylated, and this hyperphosphorylation may be an early event of AD pathogenesis. Tau phosphorylation is regulated by multiple protein kinases, such as GSK-3β and CDK5, and phosphatases, including PPA1, PP2A, PP2b and PP5. The balance between phosphorylation by kinases and dephosphorylation by phosphatases appears to be crucial. For instance, in brains affected by AD there is a reduction in phosphatase activity; in particular, pyrophosphatase 2A (PP-2A) is reduced by 20%-30% in AD [38]. Phosphorylation affects tau microtubule binding, whereas concurrent tau hyperphosphorylation at numerus sites results in tau dissociation from microtubules and enhances tau aggregation [20]. Aggregation is increased also by mutations in *MAPT* gene and the presence of pathological tau seeds [20]. Pathogenic tau may impair microtubule assembly, disrupt axonal transport, impair pre- and postsynaptic functions, and induce neuronal cell death. Evidences suggest that tau overexpression and hyperphosphorylation can damage mitochondrial axonal transport, dynamics and function to impair neuronal viability [39]. However, it should be noted that no MAPT mutations have been associated with AD so far. Therefore, mechanisms underlying tau aggregation in AD may be different from those involved in tauopathies caused by the MAPT mutations [20].

1.2.3.3 Neuroinflammation and glial involvement

In the last decade, the discovery of increased levels of inflammatory markers in patients with AD and the identification of AD risk genes associated with innate immune functions [40–42] led to the acknowledgement of neuroinflammation as an important player in the pathogenesis of AD. Inflammation within the CNS can be caused by various pathological insults (infection, trauma, ischaemia and toxins). During the inflammatory response, pro-inflammatory cytokines, including interleukin (IL) 1β, IL-6, IL-18 and tumour necrosis factor (TNF), chemokines such as C-C motif chemokine ligand 1 (CCL1), CCL5 and C-X-C motif chemokine ligand 1 (CXCL1) are produced; moreover, small-molecule messengers, including prostaglandins and nitric oxide (NO), and reactive oxygen species are released by innate immune cells in the CNS [43,44]. The innate immune cells involved in this process are primarily microglia and astrocytes, but capillary endothelial cells and infiltrating

blood cells also contribute to neuroinflammation, especially when the blood–brain barrier (BBB) is disrupted [43,44]. The release of pro-inflammatory molecules can lead to synaptic dysfunction, neuronal death and inhibition of neurogenesis [45]. IL-1β induces synaptic loss by increasing prostaglandin E2 production, which leads to presynaptic glutamate release and postsynaptic *N*-methyl-D-aspartate (NMDA) receptor activation [46], and TNF causes neuronal death by activating TNF receptor 1 (TNFR1) and recruiting caspase 8 when the nuclear factor-κB (NF-κB) pathway is inhibited [47]. In addition, the complement system can be activated, promoting the phagocytic function of microglia. This could result in inappropriate pruning of synapses [48].
Anti-inflammatory cytokines, such as IL-1 receptor antagonist, IL-4, IL-10 and IL-11, are also produced during the neuroinflammatory process and could be part of a sophisticated mechanism to prevent excessive neuroinflammation. In the context of neurodegenerative disease, this compensative mechanism fail and neuroinflammation became a chronic process able to drive the disease [44].

1.2.3.3.1 Astrocytes

Astrocytes are specialized glial cells that play a role in regulation of cerebral blood flow [49], maintenance of fluid and neurotransmitter homeostasis [50]. Moreover they induce synapse formation and provide metabolic and neurotrophic support for synapses [51,52]. They also form unique perivascular channels in the CNS, being part of the glymphatic system, which eliminate potentially neurotoxic waste products, including amyloid and tau species [53].
Astrocytes respond to pathological insults through reactive gliosis, which is part of the neuroinflammatory process [54]. Reactive astrocytes exhibit hypertrophic processes and upregulation of glial fibrillary acidic protein (GFAP). However, various phenotypes have been observed in this population. Inflammatory insult has been proposed to induce the A1 astrocyte phenotype through the NF-κB pathway, which is characterized by the expression of inflammatory mediators, whereas ischaemia induces the A2 phenotype, marked by the expression of neurotrophic factors [55].
Amyloid-containing granules have been observed in astrocytes near amyloid plaques in human brains, suggesting an attempt by astrocytes to clear amyloid deposition during the disease process [56]. Further experiments demonstrated that astrocytes migrate towards amyloid plaques and degrade Aβ in vitro and in vivo [57]. However, A1 astrocytes, which are toxic to the CNS, are found in abundance in post-mortem brain tissue from people with AD, implying a detrimental role of these cells [58]. In an animal model of AD, reactive astrocytes were found to release excessive GABA and glutamate, leading to impaired memory and synaptic loss [59]. Moreover, these cells contribute to dysregulated microcirculation and disruption of the BBB, which facilitates the accumulation of Aβ and progression of the disease [60].

1.2.3.3.2 Microglia

Microglia are innate immune cells of the myeloid lineage that reside in the CNS. Microglial progenitors have been shown to arise from primitive haematopoiesis early in embryonic development and later migrate to the developing brain [61]. Microglia has important roles in developmental synaptic pruning, neuronal apoptosis, maintenance of synaptic plasticity and immune surveillance [62,63]. In the presence of an endogenous or exogenous pathological insult, microglial surface receptors can recognize pathogens, cell debris or abnormal proteins, including Aβ species, and induce a microglial response [44]. In such situation, microglia change its morphology from highly ramified to an amoeboid form [64,65]. Based on this morphology change and immunochemical markers, classically (and simplifying) microglia have been categorized as M1, representing a pro-inflammatory phenotype, and M2, representing an anti-inflammatory phenotype [66]. The transition to disease-associated microglia (DAM) is associated with the downregulation of homeostatic genes and the upregulation of genes known to be associated with AD, including apolipoprotein E (*APOE*), triggering receptor expressed on myeloid cells 2 (*TREM2*) and TYRO protein tyrosine kinase-binding protein (*TYROBP*)[67].

Reactive microglia have been established to associate with amyloid plaques [68] and pathological tau [69]. It can indirectly facilitate the production of Aβ producing cytokines, which can upregulate β-secretase production [70]. Moreover, it can also promote the seeding of amyloid plaques [71]. In vitro studies have found that tau oligomers and fibrils provide sufficient stimulus to induce microglial morphological change and expression of interleukins [72]. Moreover, analogous to their interaction with Aβ, microglia seem to actively spread tau through phagocytosis and exosome secretion [73].

A microglial receptor of particular interest is coded by *TREM2* gene, of which Arg47 and His62 variants are genetic risk factors for AD [74]. The enhanced expression of *TREM2* has been found on plaque-associated microglia in patients with AD as well as in a mouse model of AD [75]. TREM2 signalling has been suggested to promote microglial proliferation, phagocytosis and cytokine secretion and to regulate microglial metabolism and survival [76].

Current evidences generally support a model of neuroinflammation in which the chronic background inflammation that occurs with ageing provides an initial mild stimulus that results in microglial priming. It is followed by a possible protective wave of microglial activation in the preclinical stage of AD, along with the emergence of Aβ deposition. Ineffective clearance of Aβ combined with tau aggregation impair microglial defence functions and elicit an ongoing detrimental microglial activation process in late-stage AD [77].

1.2.4 Genetics of AD

Advances in genomic technologies unrevealed AD strong genetic background. AD can be divided into a rare familial form, accounting for 2-3% of patients, presenting with autosomal dominant inheritance, and a multifactorial sporadic form due to interplay between genes and environment.

Genetically inherited AD usually develops before 65 years of age (early onset AD, EOAD), whereas the sporadic forms often occur later in life in individuals older than 65 years and is referred to as late onset AD (LOAD). EO cases has a nearly entire genetic etiology, with 92-100% heritability. 35-60% of EOAD patients have at least an affected first-degree relative, and the mode of transmission is autosomal dominant in 10-15% of cases. Conversely, LOAD is a complex, heterogeneous disorder, presenting mostly spodadic [78,79].

From the advent of genetic studies, several approaches contributed to clarify the etiology of AD [80,81].

The first strategy developed was linkage analysis [82,83], which is a method to map loci responsible for a disease, observing related individuals. It led to the identification of the three main genes responsible for dominantly inherited AD: *APP, PSEN1* and *PSEN2* [84]. Thanks to candidate gene approach [85], consisting in the evaluation of the frequency of variants in patients compared to a control population, the major genetic risk factor for LOAD was identified, *APOE* ε4 allele [86]. By late 2000's, with the advent of high-throughput approaches, several risk factors were characterized. Genome wide association studies (GWASs) enabled researchers to test millions of genetic variants on huge cohort of individuals, using a hypothesis-free approach [87]. Finally, with the advent of Next generation sequencing (NGS) technologies, panels of genes (targeted sequencing), the protein coding regions (whole exome sequencing, WES) up to the entire genome (whole genome sequencing) can be massively sequenced [88,89]. These became cost-effective methods which recently reached the clinical setting.

1.2.4.1 familial AD

EOAD forms accounts for about 5-10% of cases. 35-60% of these patients have at least an affected first-degree relative, and the mode of transmission is autosomal dominant in 10-15% of cases. The pattern of transmission of the pathology is autosomal dominant, due to highly penetrant mutations. The identification of familial AD causative genes was due to Down syndrome: in this condition, which is caused by trisomy of chromosome 21, patients present AD brain pathology [90]. Linkage studies in AD families provide evidence for a defect on chromosome 21 [91]; the cloning of the gene coding for the amyloid β precursor protein (*APP*) and its mapping on chromosome 21 [92,93] paved the way to further studies in genetics of EOAD.

APP gene is located on the long arm of chromosome 21 (21q21.22) and encodes the transmembrane protein that is processed, giving rise to the Aβ fragments. To date, 32 pathogenic mutations have been reported [94,95]. Most of these mutations (Figure 3) are non-synonymous determining an amino acid substitution at the level of cleavage sites for secretases, thus altering the processing and favoring the clear prevalence of toxic amyloidogenic products [96]. Some of these mutations are present in Amyloid β region (p.A692G, p.E693Q, p.E693K, p.E693G, p.D694N, p.A713T, p.T714I). They can exacerbate Aβ peptide, increase the $Aβ_{42}/Aβ_{40}$ ratio and affect stability of APP CTFs [97–99]. Other two (p.K670N and p.M671L) are in the N-terminal region and increase absolute level of $Aβ_{42}$. [98,100]. Moreover, other mutations located in the transmembrane or in the C-terminal region (p.V715M, p.I716V p.V717L, p.I716T, p.L723P) increase $Aβ_{42}/Aβ_{40}$ ratio, affect stability of APP CTFs and reduce the efficiency of the γ-secretase cleavage [99,101,102].

As reviewed by Hooli and colleagues [103] several genomic duplications containing APP have been identified co-segregating with AD in as many autosomal dominant families, mimicking trisomy 21. Two mutations have been discovered in the *APP* gene, able to cause the pathology only in the state of homozygosis (p.A673V and p.E693del), while heterozygous carriers are not affected by FAD, suggesting a Mendelian inheritance also of recessive type [104,105]. Pathogenic mutations in APP genes account for 1% of EOAD patients [79]. Disease onset of *APP* mutation carriers ranged between 45 and 60 years [106,107]

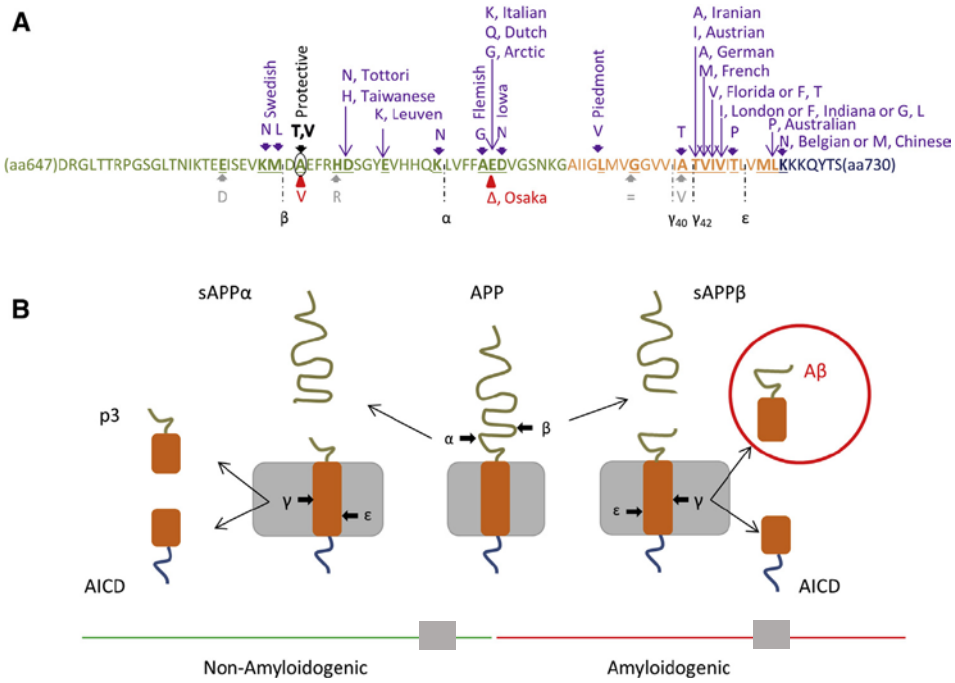


Figure 3. *APP* mutations and structure. A) APP protein sequence. Sequence of extracellular domain is presented in green the transmembrane domain in orange, in dark blue is the intracellular domain. Known pathogenic mutations are reported in purple, in red are the two recessive pathogenic mutations, in grey the non-pathogenic mutations. B) Schematic presentation of the APP proteolytic processes. APP: amyloid precursor protein; α: α-secretase; β: β-secretase; γ: γ-secretase; soluble APP fragment-α (sAPPα); soluble APP fragment- β (sAPPβ); AICD: amyloid precursor protein intracellular domain; p3: p3 fragment.

Segregation studies in EOAD families which were negative for *APP* mutations led to the identification of a new locus of chromosome 14 [108,109]: *Presenilin 1* (*PSEN1*) gene. Based on protein homology, another

presenilin protein was mapped on chromosome 1 [110,111]: *PSEN2.* As mentioned, presenilins are fundamental components of γ-secretase and their mutations seem to be able to unbalance the enzymatic pathway of *APP* in the amyloidogenic sense.

To date, 221 mutations have been described for *PSEN1* [95,112], accounting for 70% of EOAD patients. These mutations (figure 4) can be both single nucleotide variants and small insertions/deletions; in addition, a deletion able to cause *PSEN1* exon 9 skipping has been described [113]. As in the case of *APP*, the *PSEN1* mutation p.E280A in homozygous status has been reported; also, in this case, the severity of the disease was not influenced by the homozygosity of the mutation [114]. *PSEN1* mutations result in the most aggressive forms of AD and an early onset of the symptoms (30-50 years) [107]. *PSEN1* mutations are commonly inherited in an autosomal dominant manner, but de novo mutations in *PSEN1* have been described in EOAD patients with disease onset as early as 28 years [115,116].

19 mutations have been described in *PSEN2* [95,117] which account for less than 1% of cases [79]. *PSEN2* mutation (Figure 3) carriers have generally a wider onset age range, from 40 to 70 years. Disease penetrance is more difficult to establish because far less families have been reported and the onset age range is much wider.

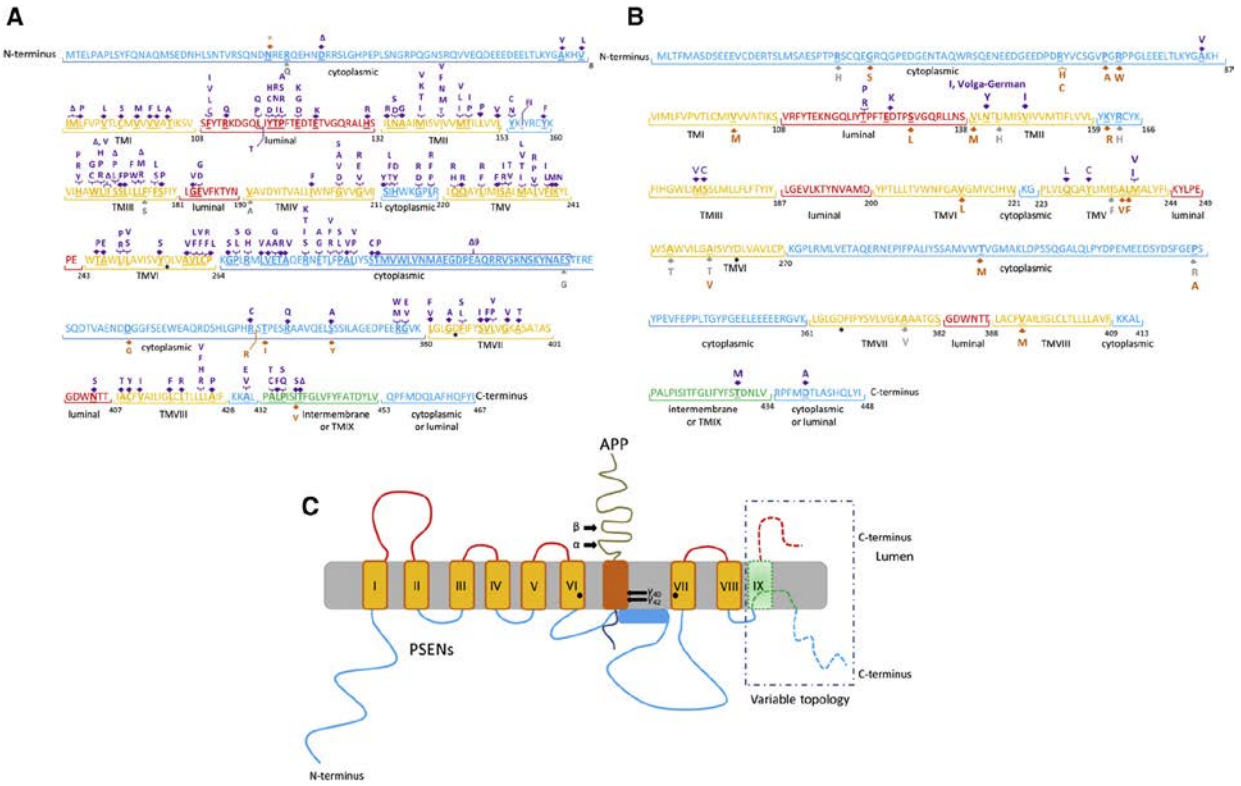


Figure 4. Presenilins (PSENs) mutations and protein structure. A: APP: amyloid precursor protein; *PSEN1*; B: *PSEN2*. α: α-secretase; β: β-secretase; γ: γ-secretase. PSENs protein mutations and structure. (A) PSEN1 and (B) PSEN2 protein sequences. In blue are marked the cytoplasmic domains, in yellow the transmembrane domains, in red the luminal domains, and in green the one intermembrane/IX transmembrane domain. Pathogenic or predicted pathogenic mutations are in purple. In orange are mutations with unclear pathogenicity and in grey are reported non-pathogenic mutations. (C) Schematic presentation of APP and PSEN complexes. For PSENs, alternative predicted protein conformations are shown.

Mutations in *APP*, *PSEN1* and *PSEN2* account only for a small percentage of fAD cases (5-10% of cases) [6]. Novel candidate genes have been associated to familial AD. Thanks to advances in NGS, variants in *NOTCH*, *SORL1*, *TREM*, *and ACAB7* genes were found[95]. In 2012, Guerreiro et al. [118] identified in a Turkish family a pathogenic mutation in *NOTCH3* gene. It is located on chromosome 19 and encodes for a transmembrane receptor involved in cell signalling and embryogenic development [119]. *NOTCH3* is implicated in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) [120]. Mutations in *SORL1* gene, encoding for the receptor of *APOE*, were found by two groups in familial AD [121–123] and in EOAD cases [124,125]. Variants in this gene have been shown to alter Aβ levels by interfering with APP trafficking [126]. Pottier and colleagues [127] identified some cases of familial AD carrying mutations in *TREM2* gene. It encodes the triggering receptor expressed on myeloid cells 2 protein, involved in immune response activation. Homozygous mutations cause Nasu-Hakola disease [128]. TREM2 is able to bind APOE, and some mutation are shown to reduce this affinity, thus reducing Aβ clearance [129]. Moreover, Coyvers group [130] detected a frameshift mutation in *ABCA7* gene in several AD families. This

gene encodes for a lipid transporter, and its inhibition seems to increase β-secretase cleavage of APP, therefore increasing the production of Aβ peptide [131].

1.2.4.2 Sporadic AD

Most AD cases (90-95%) are late onset (higher than 65 years) sporadic forms, with no obvious family segregation. Sporadic AD is a complex disorder, resulting from the interactions between genes and environment, i.e., predisposing susceptibility factors, including low schooling, brain trauma, cardiovascular diseases, high cholesterol levels and smoking [5].

Among the predisposing genetic factors, several candidate genes have been proposed. To date, the most well-established risk factor is the *Ɛ4* allele of the *APOE* gene. This gene encodes a 299-aminoacid protein involved in the transport of cholesterol and phospholipids. APOE is abundantly expressed in the periphery, mainly produced by hepatocytes and macrophages in the liver [132]. It does not cross the blood-brain barrier (BBB) but is abundantly expressed in the central nervous system (CNS), mainly by astrocytes, activated microglia and choroid plexus cells, and to a lesser extent in stressed neurons [132,133]. APOE-mediated cholesterol and lipid transport plays a critical role in synapse formation and tissue repair and neurite outgrowth after injury [134,135].

The *APOE* gene is located on chromosome 19 and is a polymorphic gene. In humans there are three different forms or alleles, due to two single nucleotide polymorphisms (SNPs): Cys112Arg (rs429358) and Arg158Cys (rs7412). The combination of the two gives rise to three alleles: *ε2 (Cys-130, Cys-176), ε3 (Cys-130, Arg-176), and ε4 (Arg-130, Arg-176)*; and six genotypes: *ε2/ε2, ε2/ε3, ε3/ε3, ε2/ε4, ε3/ε4, ε4/ε4;* each affect the individual's predisposition to AD differently [136].

The relationship between AD and *APOE* has been confirmed by several studies conducted in different population groups [137–139]. The *APOE ε*3 allele is the most common in all populations (69-85%); *APOE ε*4 has a variable frequency following an apparent north-to south reducing gradient. *APOE ε*2 allele is the least common, it has a frequency up to 7% [136]. Having one or two copies of the *APOE ε*4 allele increases late-onset AD risk approximately 3- or 12-fold, respectively. *APOE ε*4 influence AD onset: in late-onset AD, one or two copies shifts the age of onset earlier by approximately one to two decades compared to non-carriers. Other evidence recognizes the *ε2* allele as a protective factor for AD [140].

The mechanisms by which *APOE i*nfluences AD risk is not completely clear. It is well established that ApoE co-deposits with Aβ in amyloid plaques [141]. In amyloid model mice, knocking out endogenous *APOE* makes Aβ plaques from compact to diffused [142], suggesting that ApoE may play a major role in Aβ fibrilization and amyloid deposition. Importantly, the effect of ApoE on amyloid pathology is shown to be isoform-dependent (ApoE4 > ApoE3 > ApoE2*)* [143]. Postmortem and PET studies revealed that individuals carrying *APOE*4 have increased Aβ plaque deposition and earlier onset of amyloid pathology [144–146]. Conversely, *APOE2* carriers showed delayed onset of Aβ deposition, less severe pathology, and protected cognitive function

[147]. Studies of Aβ kinetics in the presence of APOE suggests that ApoE *ε*4 stabilizes soluble, cytotoxic, oligomeric Aβ fragments and enhances fibrillogenesis [148]. Besides the promoting of Aβ plaque formation, *APOE* is also involved in the clearance of Aβ via various mechanisms, such as receptor-mediated clearance and proteolytic degradation. LRP1 receptor in neurons is shown to mediate Aβ clearance via the uptake of Aβ/ApoE complexes [149,150]. Due to the reduced stability of the complex between APOE *ε*4 and Aβ [151], this uptake process is impaired in *APOE ε4* carriers. *APOE ε4* has been shown to increase tau phosphorylation compared to *APOE ε2* and *APOE ε3* in the presence of Aβ oligomers [152]. Human studies using PET imaging revealed that *APOE ε4* carriers show an increased tau deposition both in the presence and absence of Aβ plaques [153]. Interestingly, studies have demonstrated that APOE-deficient mice show reduced microglial reactivity to plaques [154], suggesting that APOE may be necessary for the microglial response to amyloid aggregation. The *APOE* is not only implicated in LOAD. *APOE ε4* allele also increased risk for EOAD in carriers of at least one e4 allele and was highest in those with a positive family history [79].

The advent of microarray technology has revolutionized genetics research, and it is now possible to assess several hundreds of thousands of SNPs in one experiment, to perform genome-wide association studies (GWAS). Thanks to this high-throughput approach, several gene/loci have been identified up to now.

In 2009, two seminal works pave the way to this approach, setting a milestone in GWAS field. Harold and colleagues [155] and Lambert and colleagues [156], with their large-scale GWASs, identified three genetic risk factors for AD: *CLU*, *CR1* and *PICALM* genes. *CLU* gene (or *APOJ*) present on chromosome 8, encodes for clusterin (or apolipoprotein j), the most abundantly expressed apolipoprotein in the brain together with *APOE*. It is present in amyloid plaques and can bind to Aβ [157], and its levels appear to be increased in CFS of AD patient [158]. The clearance of A-beta can be mediated by *CLU* enhancing endocytosis [159] and/or through transport across the blood–brain barrier [160]. *CR1* gene, located on chromosome 1, encodes for the main receptor of complement 3b protein. It plays an important role in the regulation of the complement cascade and the clearance of immune complexes [161]. It is also involved in A-beta clearance [162]. The *PICALM* gene encodes the ubiquitous protein involved in clathrin-mediated endocytosis (CME) [163]. It is essential in intracellular trafficking of molecules. It is indispensable for neurotransmitter release at the presynaptic membrane, which is important for memory formation and neuronal function, and it may play a role in A-beta clearance from the brain [164]

After the two seminal studies in 2009, GWASs in individuals of European ancestry [155,165–167] unrevealed additional loci associated to LOAD. Among them, *BIN1* (bridging integrator 1, chr 2) already identified by Harold and colleagues[155], get significance in the study of Seshadri in 2010 [165]. As PICALM, is implicated in CME, which allows the internalization and transport of lipids, and is a critical component of synaptic vesicle recycling [168]. BIN1 may influence the formation of the other main pathological features of AD brains, the NFTs, linking the microtubule cytoskeleton with the cellular membrane [169]. *ABCA7* [166] encodes an ATP-

binding cassette (ABC) transporter, which plays a role in transporting a wide range of substrates across cell membranes [170]. It is highly expressed in brain, particularly in the hippocampal CA1 neurons and microglia [171,172]. *ABCA7* is involved in the regulation of APP processing and inhibition of beta-amyloid secretion [173]. *EPHA1* [167] is a member of the ephrin receptor subfamily, which plays roles in cell and axon guidance and in synaptic development and plasticity [174,175].

The development of international GWAS consortia enabled large-scale studies with higher statistical power. For instance, a meta-analysis on 74.046 individuals led to the identification of eleven new susceptibility loci for AD with strong association [176]. Besides already known *ABCA7*, *APOE*, *BIN1*, *CLU*, *CR1*, *CD2AP*, *EPHA1*, *MS4A6A–MS4A4E* and *PICALM* genes, new loci confirm previous identified pathways: APP and in pathology tau (*SORL1, FERMT and CASS4*); immune response and inflammation (*HLA-DRB5–DRB1*, *INPP5D* and *MEF2C*). Moreover, new pathways have been proposed, including hippocampal synaptic function (*MEF2C* and *PTK2B*), cytoskeletal function and axonal transport (*CELF1*, *NME8* and *CASS4*), regulation of gene expression and posttranslational modification of proteins, and microglial and myeloid cell function (*INPP5D*).

One of the latest GWAS, 75 risk loci were found (42 new at the time of the analysis); 31 genes were identified suggestive of new genetically associated processes [177].

The advent of NGS and the recent fall in their costs allowed to gain further insight into genetics of multifactorial diseases, enabling the detection of rare variants in a large sample [81,178,179]. Of great interest are variants in *TREM2* gene [128], whose heterozygous missense mutations have been shown to increase AD risk by 3-fold [180]. *SORL1* [123] and *ABCA7* [181] were confirmed major risk of AD too**.** Recently, genes involved in Niemann-Pick type C disease (NPC) have been proposed as risk factor for AD [182]. In the adult form, NPC mimics dementia clinic; moreover, the two pathology share neuropathological features, such as the presence of neurofibrillary tangles [183] and increased amyloidogenic processing [184]. *NPC1* and *NPC2* genes are involved in lipid metabolism, a further intriguing link with the major AD risk factor, APOE.

Recently, large sequencing effort as the AD sequencing project (ADSP) [185] confirmed previously identified AD genes and led to the identification of novel genes. Despite the great genetic effort in assessing AD risk, it has been estimated that not all the genetic component of AD has been clarified [186]. The genetic landscape of AD genetics appears complicated, as shown in the chart in figure 5, by Dourlen and colleagues [187].

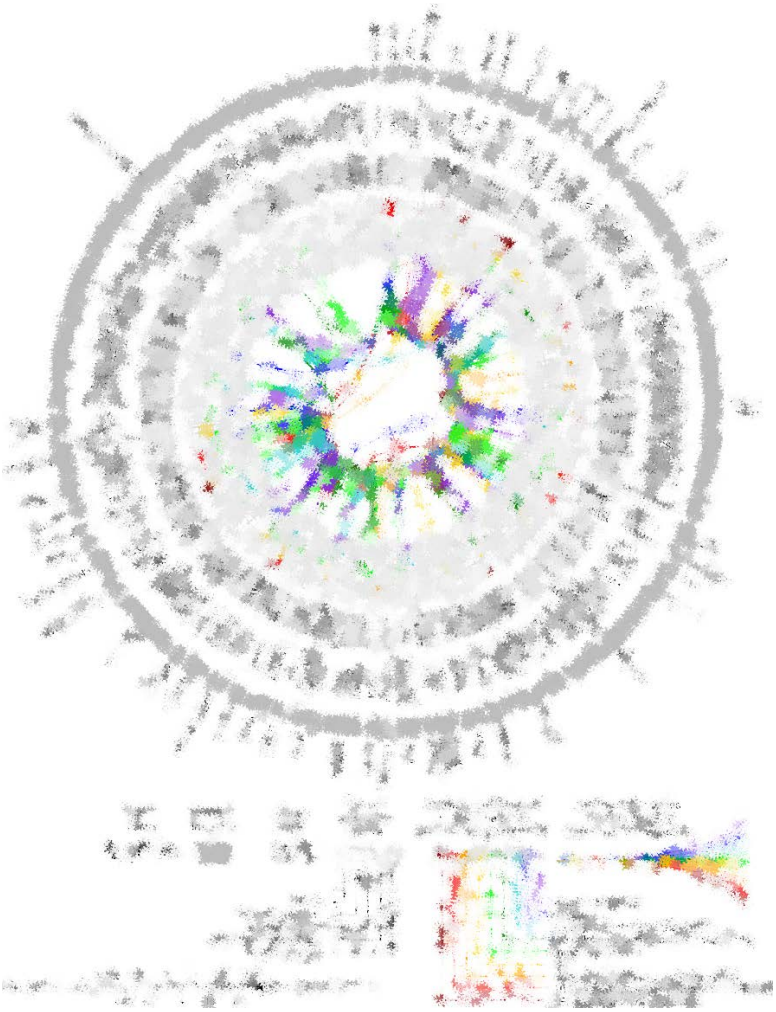


Figure 5. Diagram of AD genetic risk factors. From outside to inside, the diagram shows genomic loci in alphabetical order and genes therein; expression profiles of these genes in different cell types of the brain and the pathways/processes/proteins to which these genes have been functionally linked, identified by different colours.

1.3 Frontotemporal dementia

Frontotemporal dementia is an umbrella term encompassing a group of clinical syndromes, characterised by progressive changes in behaviour, executive function, or language [188]. These include behavioural variant frontotemporal dementia (bvFTD), and the two variants of primary progressive aphasia (PPA), namely non-fluent (nfPPA) and semantic variants (svPPA). Besides the typical forms characterized by behavioural or language impairment, atypical FTD forms can present overlap with motor neuron disease (FTD-MND) or parkinsonian features [189].

Frontotemporal lobar degeneration [190] is the pathological term for a group of neurodegenerative disorders which involve one or more proteinopathies and are typically associated with progressive degeneration, particularly in the frontotemporal neural networks. The major groups of proteinopathies include the tauopathies (e.g., Pick's disease, progressive supranuclear palsy, and corticobasal degeneration), the TDP43 proteinopathies, and the FET-related proteins [191].

FTD is the second most frequent cause of early-onset dementia after Alzheimer's disease, affecting people from 40 to 70 years old. FTD has a prevalence of 15-22/100,000 and an incidence of 3-15/100,000 individuals [192]. Among the FTD subtypes, bvFTD is the most common.

The clinical criteria for the diagnosis of FTD were originally developed in 1994 [174]. Even though the clinical aspects examined showed a good ability to exclude the presence of AD, these criteria gave no indication of the number of clinical features necessary to make a diagnosis. Subsequently, in 1998, Neary and collaborators proposed criteria [193], through which it became possible to distinguish between the three main types of FTD. Further updates of the criteria for the diagnosis of bvFTD were developed (first in 2007 [194], and then in 2011 [195]) and for PPA and its variants [196].

1.3.1 Clinical characteristics of FTD main subtypes

1.3.1.1 bvFTD

The behavioral variant is the most common subtype of FTD**,** occurring in 50% of cases. It is characterized by an early and insidious change in behavior and personality. The bvFTD is associated with focal atrophy of the orbital and mesial frontal lobes and anterior temporal lobes[197,198] . Change in personality manifests by apathy with social withdrawal, loss of empathy, loss of spontaneity, abulia, disinhibited outbursts, emotional bluntness, and change in eating patterns[199], inability to adhere to routines, inflexibility, and loss of attention span [200] . As the disease progresses, the dorsolateral prefrontal systems are also affected, and neurocognitive deficits, such as impairment of executive functions, problem solving, judgment, organization,

and planning emerge [201,202]. Altered speech pattern, with stereotypy, echolalia, lack of spontaneity, and in later stages mutism, although not very common in patients with FTD, is also seldom observed [203].
Histologically, at post-mortem of patients with bvFTD, bilateral frontotemporal atrophy with neuronal loss, micro vacuolation, and a variable degree of gliosis is observed [204]. Initially, mesial and orbital frontal regions are affected, followed by the temporal lobe, hippocampal formation, dorsolateral frontal cortex, and the basal ganglia with prominent sparing of the posterior cortical regions and visuospatial function [205]
Symmetrical frontal lobe atrophy in patients with bvFTD is associated with *C9ORF72* and *MAPT* gene mutations, whereas the asymmetrical pattern is associated with *GRN* gene mutations [206]. Patients with ALS who exhibit an FTD syndrome (ALS-FTD) show atrophy in the frontal and temporal regions [207]

1.3.1.2 PPA

The first description of a progressive pathology characterized by early involvement of linguistic abilities and atrophy of the frontal and temporal regions of the hemisphere was attributed to Pick and Serieux in the 90s of the nineteenth century. In the last century, Mesulam described a series of cases of "slowly progressive aphasia", a term later replaced by "primary progressive aphasia" (PPA) [208,209]. The semantic dementia variant was first described by Warrington in 1975 and fully defined in the 90s by Hodges and colleagues [210]. Subsequently, Grossmann and colleagues described an alternative form of language disorder, later renamed non-fluent aphasia[211].
We currently distinguish three different clinical forms of primary progressive aphasia: non-fluent or agrammatic aphasia, semantic dementia and the logopenic variant [196]. The three forms are characterized by an early and prominent involvement of language skills that determines an important ecological impact when verbal communication is required; the other cognitive functions prove to be relatively well preserved at least in the first part of the disease [212].
The non-fluent or agrammatic variant is characterized by important agrammatism resulting in disrupted language and errors in constructing a sentence; alterations in prosody and rhythm of speech are evident; comprehension is involved early and is evidently influenced by the grammatical complexity of the sentence (apraxia of language) [213]. Patients with PNFA show left frontal and perisylvian atrophy on structural MRI, with hypoperfusion and hypometabolism demonstrated in the same regions on functional imaging [214]
Patients suffering from semantic dementia, on the other hand, demonstrate an early important impairment of the understanding of individual words, an expression of a widespread deficit of semantic memory that involves the recognition of shapes, objects, faces and therefore people even when they are presented through different sensory inputs [215]. In structural MRI, SD is characterized by temporal lobe atrophy, which is more pronounced anteriorly, involving polar, anterior parahippocampal, and fusiform regions including the perirhinal cortex. The atrophy is bilateral, but typically asymmetric and often more severe on the left side [216].

Difficulties in recalling names and in the repetition of sentences are the main features of the logopenic variant; in these patients, spontaneous speech is poor, difficult and continuously interrupted. Unlike patients suffering from the non-fluent variant, there is no clear agrammatism and, unlike patients suffering from the semantic variant, phonological errors and a saving of the understanding of single words are observed above all [217].

1.3.1.3 FTD-ALS

Since the beginning of the twentieth century, a comorbidity of ALS with behavior alterations, cognitive decline and dementia has been documented. FTD may coincide with or emerge after the onset of motor symptoms [218]. In a large number of studies, it has been reported that about 47% of ALS patients have a degeneration of the frontal lobes; in contrast, 40% of patients with a clinical diagnosis of FTD show motor neuron dysfunction. Patients suffering from FLD-ALS have a very negative prognosis with a survival expectancy about 2-3 years from the onset of the first symptoms and the presence of a positive family history in about 50% of cases [219].

1.3.2 Neuropathological Features

FTD is highly heterogeneous from a pathological point of view: based on its intraneuronal inclusions, it is possible to distinguish pathological subgroups.

About 40% of patients show tau-positive inclusions; these include those cases of disease associated with mutations in *MAPT* gene [220]; patients with predominance of 3R tau and patients with predominance 4R tau can be distinguished [221]. Patients with these histological characteristics fall into the defined category of FTLD-T (FTLD associated with tau deposits). The deposits of cytoplasmic filaments composed of the abnormal hyperphosphorylated tau protein characterize a percentage of FTLD cases that also include Pick's disease (Pick's bodies) and other tauopathies [190].

About 50% of FTLD cases are tau-negative and are characterized by the presence of ubiquitin deposits, so they are called FTLD-U. Ubiquitin is a peptide of 76 amino acids that acts as a marker of proteins to be degraded in the proteasomal pathway [200].

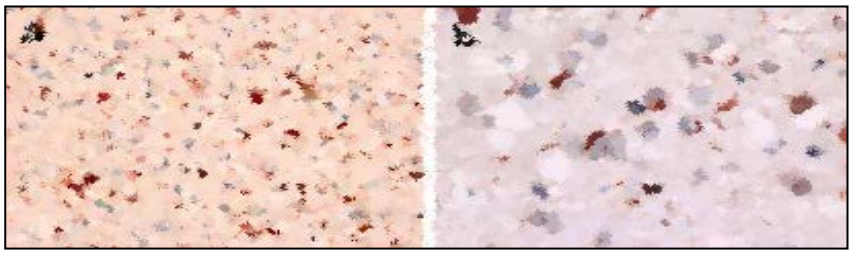


Figure 6. Ubiquitin inclusions in immunoreactive neurites (a) and neuronal cytoplasmic inclusions (b)

Histological examination shows protein aggregates consisting of *the TDP-43* protein (*TAR DNA-binding protein 43*) and therefore TDP-43 positive [222]. The immunohistochemical signal is localized in the cytoplasm of neurons belonging to the areas normally affected by the disease (figure 6). The neurons of the dentate gyrus of the hippocampus are also affected [222]. FTLD-TDP group comprises a subset of patients carries mutations in *C9ORF72* and *GRN* genes, rarely in *VCP* and *TARDBP can* be found [223]. Cases of disease with positivity towards FUS (Fused in sarcoma, FTD-FET) protein deposits have also been described in FTD patients, associated to mutations in *FUS* gene [224]. Both TDP-43 and FUS are proteins involved in the regulation of RNA processing, by binding nucleic acids.

Other inclusion bodies such as intermediate filament immunoreactive inclusions, basophilic inclusions, and P62-positive inclusions are also found in some patients. [190,222,224]

There is not preferential association between a given neuropathological picture and a specific spectrum of clinical manifestations of the behavioral variant. Patients with bvFTD mostly show tau-positive or TDP-43-positive inclusions, while a small percentage of patients show FUS-positive inclusions [225]. The non-fluent variant shows a significant association with tau-positive inclusions [226]. Semantic dementia is associated with TDP-43 positive inclusions [227]. Finally, patients with the logopenic form would appear to have a neuropathological profile compatible with AD [212].

Patients with ALS exhibiting FTD symptoms or the ALS-FTD has ubiquitin-positive, α-synuclein, and tau-negative inclusion bodies in the frontal cortex and hippocampus, and spongiform change in the first 2 layers of frontal cortex, along with degeneration of motor neurons in the brainstem and anterior horn of the spinal cord [207].

1.3.3 Genetics of FTD

FTD is a highly heritable disease. A positive family history for dementia was found in 30%-40% of subjects with FTD [228–230] which is transmitted as an autosomal dominant disease. In bvFTD, a positive family history has been documented in 48% of cases, while in PPA patients it accounts only for 12% of cases. Even more variable heritability is present in FTD-ALS cases, ranging from 10 to >40% [231].

Most of the heritability of FTD is due to autosomal dominant mutations in three genes: *MAPT* (Microtubule Associated Protein Tau) gene, *GRN (progranulin) gene, C9ORF72 (chromosome 9 open reading frame 72).* Each gene causes 5-10% of FTD cases. In recent years, rare mutations in other genes have been discovered [*VCP-1* (Valosin-Containing Protein), *CHMP2B* (Chromatin-Modifying 2B), *TARBDP* (TAR-DNA binding protein 43 encoding gene) and TBK1 (TANK binding kinase 1) are examples] accounting collectively for less than 5% of autosomal dominant cases [231]. Sporadic forms account for 60-70% of all FTD cases [232]. Besides few *MAPT, GRN* and *C9ORF72* mutation reported, they are caused by genetic risk markers, likely modulated by both genetic and environmental factors [233]

1.3.3.1 *MAPT*

The first evidence of the presence of a genetic cause for familial FTD forms came from the demonstration of a link between chromosome 17q21.2 and autosomal dominant forms of FTD associated with Parkinsonism [234–236], from which the name "Frontotemporal Dementia and Parkinsonism linked to chromosome 17" (FTDP-17) was derived. The gene responsible for this association, *MAPT* encodes the tau protein associated with microtubules, whose function is to stabilize them and promote their assembly. In the human brain, six distinct isoforms of tau exist based on the alternative splicing of exons 2, 3, and 10 [237]. Alternative splicing of exons 2 and 3 yields isoforms with 0, 1, or 2 N-terminal repeats (0N, 1N,2N), while alternative splicing of exon 10 results in tau with three or four repeats in the microtubule-binding domain (3R or 4R).

To date, about 55 mutations have been described [238]. Mutations in *MAPT* (figure 7) can be missense, deletions or intronic mutations. These mutations affect the splicing process, leading to the production of different protein isoforms and an alteration of their ratios (3R/4R). Several reside in exon and intron 10 and increase R4 isoform [237,239,240]. Other mutations alter binding to and stability of microtubules [241,242]. Moreover, some mutations are proven to enhance aggregation in cell cultures [243].

MAPT mutations cause 10-20% of familial FTD and up to 3% of sporadic FTD [244]. They are associated to variable cognitive, behavioural, and motor deficits. The age of onset is 49 years on average and the duration of disease of 8.5 years [245]. As already stated, ppatients with *MAPT* mutations may show tau-positive inclusions [246].

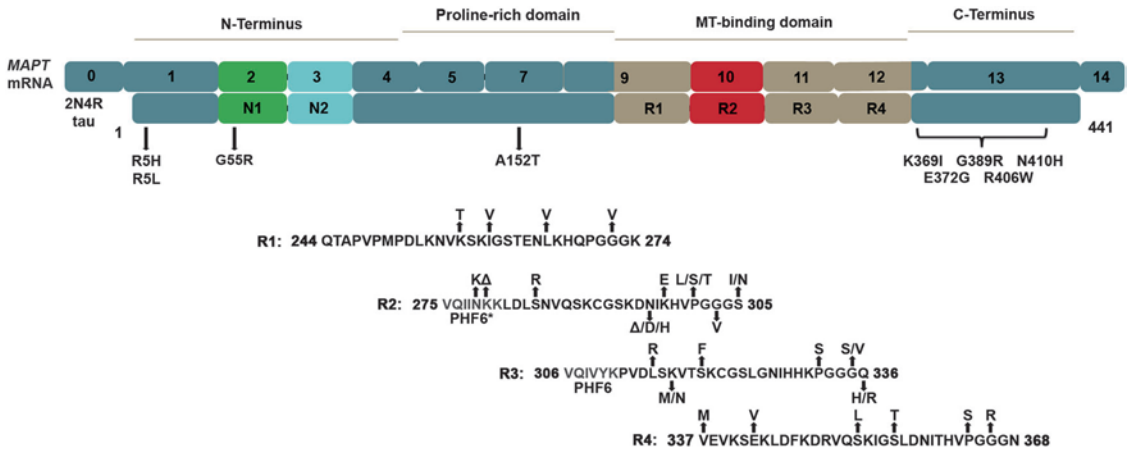


Figure 7. The longest tau isoform found in human brain, with its corresponding mRNA and known pathogenic mutations. The distinct colours highlight the regions of the protein that are alternatively spliced as well as the MT-binding domain. The N- terminal, proline-rich, MT-binding, and C-terminal regions are indicated above. Below the protein, known pathogenic missense mutations are indicated. MT: microtubule

1.3.3.2 *GRN*

After the discovery of *MAPT* as the causal gene for FTLD-17, there were still numerous families affected by autosomal dominant FTD genetically related to the chromosomal region 17q21, but which did not have any pathogenic mutations on the *MAPT* gene. In 2006, two groups independently identified *GRN* gene [247,248]. Progranulin is a growth factor that belongs to a family of proteins involved in numerous biological functions including development, wound repair and inflammation (by activating specific signal cascades), cell cycle progression and cell motility [249]. It is expressed not only in neurons, but also in activated microglia [247], during many neurodegenerative diseases.

Since the first mutation detected to date, about 80 different mutations (figure 8) have been described [250]. They accounts for 5-20% of familial and 1-5% of sporadic cases [251]. Mutations can create non-functional alleles that, in most cases, lead to premature termination of gene transcription, following the formation of a stop codon. The mRNA produced is therefore aberrant, undergoes to degradation, thus determining a phenomenon of haploinsufficiency [252]. An important contribution to obtain a correct independent diagnosis of the often variable phenotypic presentation is the demonstration that plasma levels of progranulin are extremely low in carriers of the *GRN* mutation and also in asymptomatic subjects [253].

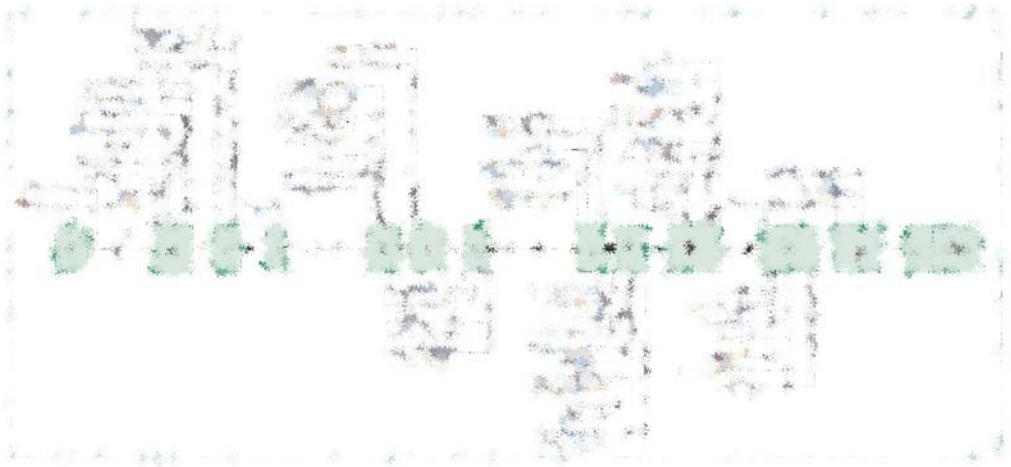


Figure 8. Overview of GRN mutations in exonic and intronic regions. del= deletion; fs= frameshift; ins= insertion; utr=untranslated region; X=stop codon.

From a clinical point of view, mutations in *GRN* are associated with extremely heterogeneous phenotypes [253]. The age of onset of the disease is extremely wide, even within the same family, and ranges from 47 to 79 years [244].

In *GRN* mutation carriers, the neuropathological examination shows intranuclear and cytoplasmic inclusions immunoreactive for ubiquitin, with TDP-43 protein as the most present [254].

1.3.3.3 *C9ORF72*

One of the most interesting discoveries concerning the genetics of FTD came from a linkage study of families affected by FTD-MND and led to the identification of a potential susceptibility locus on chromosome 9p21-22. The first scientific evidence of linkage with this genetic locus emerged in 2000 from a study published by Hosler and collaborators conducted on families affected by FTD-MND [255]. After this, numerous works have confirmed this linkage on chromosome 9p21-22. In 2011, two research groups independently identified the presence of an expansion of a hexanucleotide repeat (*GGGGCC)* present in a non-coding portion of the *C9ORF72* gene that is precisely positioned on chromosome 9p

21 [206,207]. It is the most common cause of both familial (20.30%) and sporadic (6%) FTD [256].

In the normal population, the size of the repetition varies between 3 and 25 units, while expansion of hundreds to thousands of repeats occurs in affected subjects. To date, it is not yet clear whether there is a minimum critical number of repetitions that can determine the onset of the disease. In vitro studies show inverse correlation between the repeat size (starting with 9 repeats) and gene expression, with 24 repeats providing 50% reduction in gene expression [257].

Three mechanisms have been proposed underlying the GGGGCC hexanucleotide repeat expansion pathogenicity (figure 9).

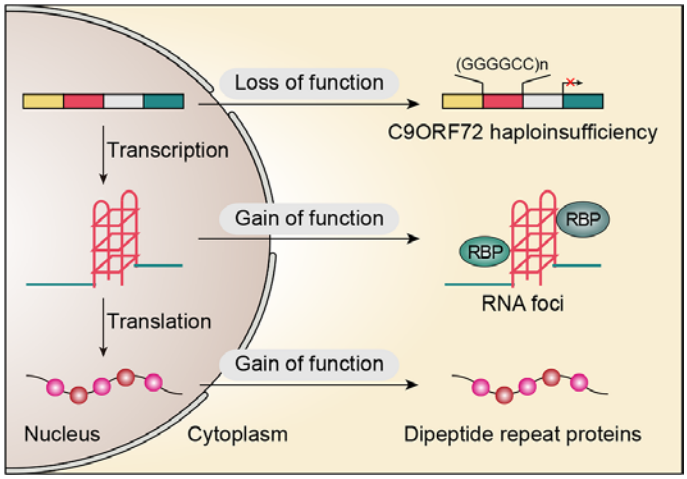


Figure 9. Mechanisms for *C9ORF72* hexanucleotide expansions pathogenicity. RBP: RNA binding protein.

One is loss-of-function [258] based on decreased expression of *C9ORF72* mRNA found in the frontal cortex of individuals with *C9ORF72* mutation: haploinsufficiency of *C9ORF72* may participate in neuronal degeneration. The second is gain-of-function caused by RNA foci [259]: the GGGGCC repeat expansion is transcribed into repeat RNA that can interact with DNA to fold into a G-quadruplex structure. The repeat RNA forms RNA foci that sequester RNA-binding proteins in the nucleus of vulnerable neurons. Neurotoxic foci have been identified in 25% of the nerve cell nuclei of both the frontal cortical region and in the spinal cord, belonging to patients carrying the expansion. Thirdly, gain-of-function caused by dipeptide repeat proteins (DPRs) [260]: In a repeat-associated non-ATG-initiated manner, the repeat RNA is translated into DPRs that form toxic aggregates in residual neurons. In recent years, several studies have implicated *C9ORF72* in cellular protein transport and that loss of C9ORF72 impairs autophagy and lysosome biogenesis [261].

The average age of onset is around 55-58 years. There is a great variability in the age of onset even between individuals of the same family. Most of the patients develop bvFTD with or without ALS [257].

1.3.3.4 *VCP*

Autosomal dominant mutations in *the VCP gene,* located on the long arm of chromosome 9p21-12, have been identified through linkage analysis in families with a rare familial body myopathy syndrome (IBM), Paget's disease as well as FTD [262]. The *VCP* gene encodes a ubiquitously expressed protein that functions as a molecular chaperone in a variety of cellular activities, including protein degradation, apoptosis, cell cycle, DNA repair, and cell membrane remodeling [262]

To date, 19 causal disease mutations have been described, with a frequency of 1.6% [257]. A phenomenon of incomplete penetrance was observed for all three clinical manifestations. The most accredited hypothesis of the pathogenetic mechanism of mutations in *VCP* lies in the ability to interfere with the ubiquitin-linked proteasome system that leads to a decrease in protein degradation and damage to the autophagic system.

Mean age at onset is 40 [244]. Patients with mutations in this gene also have inclusions of the TDP-43 protein especially in neocortical regions [263] mutations in VCP were found in less than 1% of familial cases of FTD, and more frequently in bvFTD and SD [218].

1.3.3.5 *CHMP2B*

A linkage analysis on a very large Danish family affected by FTD identified a mutation in the *CHMP2B* gene that is located on chromosome 3p11.2 [264].

In literature only 4 different mutations have been reported, all identified between or within exons 5 and 6 and described in 5 total families [218]. *CHMP2B* encodes a protein belonging to the ESCRT III complex, which is involved in the formation of the late endosome [265]. This gene is expressed in neurons present in most of the brain, and the mutations responsible for the pathology have all been identified in the part of the gene that codes for the C-terminal portion of the protein, leading to an aberrant splicing process. This leads to neurons with huge vacuoles that prevent the fusion of the endosome with the lysosome. Neuropathologically, patients with these mutations present themselves as FTD-U i.e. positive for ubiquitin, but negative for *TDP-43* inclusions [266]. Clinically, however, patients present a diagnosis of bvFTD in which there are very early personality changes. The presence of aphasia has also often been observed: it is characterized mainly by a drastic reduction in spontaneous speech that even leads to mutism. The average age of onset in affected patients is about 58 years with an age range ranging from 45 to 65 years [218].

1.3.3.6 *TARDBP*

Mutations in *TARDBP* account for 2–3% of ALS and are rare in FTD patients [267]. Among them, the most common phenotypes were bvFTD and SD [268]. Onset is usually between age 29 and 77 years. It encodes for TDP-43, RNA-binding protein that forms heterogeneous nuclear ribonucleoprotein complexes (hnRNP). As already stated, TDP-43 has a role in transcription, RNA splicing and microRNA processing [218].

1.3.3.7 FUS

FUS gene is located on chromosome 16q11.22 and is also a member of hnRNP family [218]. Mutations in FUS are predominantly found in ALS patients. 4 mutations have been described in FTD-MND patients, with an additional 2 cases with pure FTD with no overlap with MND [269,270]. FUS protein participates in DNA repair and RNA splicing regulation and contains 526 amino acids [244].

1.3.3.8 *TBK1*

TBK1 gene encodes TANK-binding kinase and was recently linked to FTD and ALS as well as pathologically confirmed FTLD-TDP cases [271–273]. Findings from European FTD cohorts indicate that pathogenic *TBK1* mutations may be a relatively common genetic cause of disease (accounting for 1–5% of FTD and FTD-ALS) [274]. Interestingly, some pathogenic *TBK1* mutations appear to impair the ability of *TBK1* to bind optineurin, thus implicating these mutations in dysfunctional autophagy, similar to pathogenic *SQSTM1* and *OPTN* mutations [272]. *TBK1* was very recently suggested to be a key regulator of inflammation in the brain by acting as a negative regulator of RIPK1 kinase activity [275].

1.3.4 *Genetic risk factors identified though GWAS*

GWAS led to the identification of several risk loci for FTD and thus helped to unravel new genes involved and related biological processes [276,277]**.** A GWA study published in 2010 which involved a series of 515 patients suffering from FTLD-TDP, led to the identification of the *TMEM106B* gene located on chromosome 7p21 [278]. The SNPs encompassing this gene were correlated with an increased risk of FTLD-TDP. Moreover, they appeared enriched in patients carrying mutations in *GRN* probably by modulating the levels of secretion of this protein [279]. TMEM106B is an integral membrane protein localized in late lysosomes and endosomes and modulate pgrn protein levels [280].

In 2014, Ferrari et al., published a large GWAS analysing bvFTD, PPA, and FTLD-MND [281]. The study unrevealed two novel susceptibility loci: one mapping *Ras-related protein Rab-38* and *Cathepsin C* (*RAB38-CTSC*) suggesting the involvement of lysosomal and autophagic pathways, and one encompassing *butyrophilin-like 2 (BTNL2)* and *major histocompatibility complex, class II, DRs (HLA-DRAeHLA-DRB5)*, implying alterations in immune system. A GWAS performed on a multicentre Italian cohort led to the identification of two new potential loci: SNPs encompassing *Centrosomal protein 131 (CEP131)* and *ENTH domain containing 2 (ENTHD2)* resulted significantly associated. These genes are involved in neuronal development, differentiation, and maturation processes, whose impairment might drive FTLD pathogenesis in the Italian population [282].

In 2018, Pottier and colleagues performed a GWAS on a selected cohort of patients carrying loss-of-function mutations in *GRN* gene [283], and identified significant signals in *TMEM106B* and in *GDNF family receptor alpha 2* (*GFRA2) loci.*

In a very exhaustive study in which a systematic investigation of genetic overlap between immune-mediated diseases and the spectrum of FTD-related disorders was performed, an immune-related genetic enrichment in FTD was also described. Moreover, the authors identified novel susceptibility loci within the *Leucine rich repeat kinase 2 (LRRK2)*, the *TBK1 binding protein 1 (TBKBP1)*, and the *PiggyBac transposable element derived (5PGBD5)* genes, involved in cell survival, immunity processes and genomic rearrangements, respectively [284]. Additionally, Mishra et al. (2017) reported an association of *APOE* and the *Translocase of outer*

mitochondrial membrane 40 (TOMM40) genes with bvFTD, and the *Rho GTPase activating protein 35 (ARHGAP35)* and the *Serpin family A member 1 (SERPINA1)* genes with progressive nonfluent aphasia [285].

1.3.5 Rare variants identified with NGS approaches

Advent in sequencing technologies allowed the identification of rare variants associated to FTD [277]

By gene targeted sequencing (TS), rare variants within the Sortilin 1 (*SORT1*) gene were identified in a Belgian FTD cohort. A subsequent study of cohorts sampled in Spain, Italy and Portugal revealed additional non-synonymous variants in European patients. *SORT1* encodes a neuronal receptor involved in intracellular protein transport and cellular signal transduction [286].

In sporadic FTD patients negative for *MAPT, GRN*, and *C9ORF72* mutations, novel variants were identified in two dementia-related genes, the *Colony stimulating factor 1 receptor (CSF1R)* and the *Mitochondrial alanyltRNA synthetase 2 (AARS2*), suggesting new genes to be considered for a genetic FTD diagnosis. *CSF1R* shows important role in innate immunity and inflammatory processes, while *AARS2* is involved in mitochondrial functions [287]. Recently, a TS of 12 FTD-associated genes was performed: this study revealed a rare variant in the *Triggering receptor expressed on myeloid cells 2* (*TREM2*) and two nonsense *GRN* mutations [288]. In Giannoccaro et al. (2017), a panel of dementia-associated genes was explored in an Italian group of ALS/FTD pedigrees by using a TS approach: genetic variants in additional ALS and dementia-related genes were found in four pedigrees, including a rare variant in the *Tyrosine kinase binding protein (TYROBP*) gene. The *TYROBP* protein, which interacts with several other proteins like TREM2, is specifically involved in immune pathway and inflammatory response [289]. In addition, the *TBK1 binding protein 1 (TBKBP1*) was screened in a wide cohort of FTD, ALS, FTD-ALS subjects, identifying deletions and missense mutations in this gene involved in immune response [290]. In van der Zee et al. 2014, rare variants in the *Sequestosome 1 (SQSTM1)* gene were identified in a cohort of FTD patients, suggesting a role of this gene in the etiology of disease [291]. Interestingly, a rare variant in *α-synuclein (SNCA)* gene, causing autosomal dominant Parkinson's disease (PD), was found in a patient diagnosed with bvFTD, suggesting a possible alteration of mitochondrial processes in FTD too.

1.4 Epigenetics in Alzheimer's disease and Frontotemporal dementia

Epigenetics represents the study of changes in gene expression and/or chromatin structure and cell function, caused by mechanisms other than changes in the underlying DNA sequence. This field is becoming an important area of investigation, since epigenetic modifications may account for complexity of AD and FTD, explaining differential regulation of risk genes and genomic regions, without changes to their DNA sequence and therefore undetected in genetic studies [292]. Epigenetic mechanisms comprise DNA methylation and histone modifications: together they regulate chromatin structure, which, in turn, regulates gene expression by facilitating access to DNA regulatory elements. Recently, noncoding RNAs (ncRNAs), such as long noncoding RNAs (lncRNAs) and microRNAs (miRNAs), among other regulatory RNA molecules, have been shown to play an important role in the regulation of genes by various mechanisms. Several studies have shown that epigenetic modifications are dynamic in post-natal brain and throughout the aging process [293,294]. Several post-translational modifications of histones, mainly histone methylation and acetylation, drift with age and are associated with age-related decline in cognitive and memory related process [295,296].

1.4.1 DNA Methylation

DNA methylation is the most studied and understood epigenetic mechanism. It consists in the addition of a methyl group on a cysteine residue [297] from S-adenosyl methionine [298], and is catalysed by DNA methyltransferase (DNMT) enzyme. In mammals, DNA methylation occurs in CpG sites (where cytosine is followed by a guanine). CpG-rich sites, called CpG Island, are frequent in regulatory regions. For this reason, DNA methylation affects gene expression [299,300]. The presence of a methyl group hampers the binding for transcriptional activators [301] or serves as anchor for methyl-CpG binding domain proteins (MBDs) which bind proteins that decrease gene expression [302]. DNA methylation is known to have a pivotal role in normal development, cell proliferation, and genome stability [303].

1.4.1.1 Methylation in AD

DNA methylation of several AD related genes has been investigated using candidate gene approach. So far, inconsistent data have been reported, with DNA methylation being decreased in AD [304–307], while some studies found no differences in DNA methylation levels [308,309]. On a genome-wide basis, contradictory results have been obtained, maybe due to the use of various tissue samples. Significant increase in DNA methylation has been reported in hippocampus, entorhinal cortex, dorsolateral prefrontal cortex [310], temporal cortex [311], and the temporal gyrus [312]. In contrast, decreased DNA methylation was observed in the prefrontal cortex and locus coeruleus [313,314]. Similarly, a decrease in DNA methylation was observed in blood samples [315]. The methylation of *ANK1* was increased in AD patients in four studies [310,316–318]. It is an integral membrane protein, important in cell proliferation, activation, and mobility

[319]. Interestingly, methylation of miRNA targeting genes involved in the AD pathology, such as *APP, BACE1, and sirtuin 1 (SIRT1)* appeared differentially regulated [311].

1.4.1.2 Methylation in FTD

Increased GRN promoter methylation was reported in FTD subjects, negatively impacting on GRN mRNA levels [320,321]. A further study, aiming to analyse genome-wide DNA methylation patterns in the peripheral blood of FTD patients, revealed a specific methylation signature associated with FTLD-tau. This signature could, therefore, be considered a risk factor for neurodegeneration [322]. Recently, the role of methylation at Pin1 gene promoter have been investigated in peripheral blood mononuclear cells (PBMC) of FTD [323]. A decreased expression of Pin1 with a higher DNA methylation was shown in FTD. Several studies extensively investigated the methylation of C9ORF72, trying to understand if this modification may play a role in C9ORF72 loss of function. Hypomethylation of CpG island located in the *C9ORF72* promoter region has been shown by different groups to be present in about 10–30% of *C9ORF72* FTD/ALS patients, likely leading to reduced *C9ORF72* expression levels [324].

1.4.2 Histone modifications

Histone proteins (H1, H2A, H2B, H3, and H4) interact with DNA to form nucleosomes, which are fundamental units of chromatin, and they represent an essential part of eukaryotic transcription regulation [325,326]. Specifically, histone modifications influence nucleosome stability, chromatin-mediated processes and histone-histone interactions, regulating repression or activation of gene expression [325,327]. Several post-translational modifications occur on specific residues of the histone N-terminal “tail” domain [325,328,329]. Among them, acetylation has been the most studied. Histone acetylation at lysine residues, catalysed by histone acetyltransferases (HATs), has been associated with “open” chromatin conformation and therefore transcriptional activation, whereas histone deacetylation, regulated by histone deacetylases (HDACs), has been involved in “closed” chromatin structure and transcriptional repression [326]. Histone acetylation plays an important role in the regulation of DNA replication, transcription, and various other cellular functions [325].

Histone methylation is one of the most complex post-translational modifications [325,330]. It consists in the addition or removal of methyl groups from lysine, arginine or histidine residues by methyl-transferases and demethylases, respectively. Histone methylation takes part in numerous cell processes, such as mitosis, meiosis, DNA repair, transcription, differentiation, response to stress, and aging [325].

Another type of histone modification is histone phosphorylation at serine, threonine, or tyrosine residues, regulated by protein kinases and protein phosphatases [326]. It is also involved in various cellular processes, including mitosis, gene transcription, and chromatin condensation [325].

Moreover, ubiquitination is an enzymatic process that starts with the activation by conjugation of ubiquitin to a cysteine residue. The role of ubiquitination, especially H2A and H2B histones, has been identified in DNA

repair, silencing, initiation, and elongation of transcription [331]. In addition to covalent histone modifications, histones can be reversibly modified by ADP-ribosylation, which is regulated by specific enzymes. Mono- and poly-ADP ribosilated histones play an important role in chromatin structure regulation, DNA repair, cell cycle, replication, and transcription [332,333].

1.4.2.1 Histon modifications in AD

Physiologically, histone modifications play an important role in neuronal development. They are also involved in aging brain, as well as in AD pathogenesis [334,335]. Widespread loss of heterochromatin has been observed and is suggested to promote tau-mediated neurodegeneration and aberrant gene expression in AD [336]. Acetylation dysregulation has been associated with impairments in signalling, proliferation, inflammation, immunity, apoptosis, and neuronal plasticity [337]. Marzi et al. identified thousands of differentially acetylated peaks, many located in AD causative genes or genetic risk variants (*APP, PSEN1, PSEN2,* and *MAPT*) [338]. Klein et al. profiled H3K9 histone acetylation in the dorsolateral prefrontal cortex [339]. Decreased histone acetylation has been observed in temporal lobe of subjects with AD [340], specifically on histone H4 (H4K16ac), which is involved in DNA damage and aging [335,341]. In addition to acetylation, alternations in histone methylation have been observed in AD. It is known that balance between histone methyltransferases and demethylases is important for brain integrity and memory in AD [332]. Increased trimethylation of lysine on histone H3 (H3K9), a marker of gene silencing and condensation of heterochromatin structure [342], as well as higher levels of histone methyltransferase EHMT1 mRNA were found in the post-mortem brain of subjects with AD [343]. HMT G9a, the enzyme specific for H3K9 di-methylation, is linked to cognitive performance in mice, whereas H3K4 demethylase is associated with memory deficits in humans [332]. Moreover, increased phosphorylation of serine on histone H3 [344], as well as increased phosphorylation of H2AX at Ser139, as evidence of DNA damage [345], have been observed respectively in AD hippocampal neurons and astrocytes. In addition, ADP-ribosylation of histone H1 has been found in different AD brain regions [345].

1.4.2.2 Histon modification in FTD

Histone modification at the C9ORF72 locus were found to reduce the expression of the gene in FTD and ALS but not in ALS patients without C9ORF72 expansion [346]. Recently, HDAC inhibitors have been identified as enhancers of progranulin expression. Multi-HDAC targeting compounds have been shown to increase GRN gene expression in animal and cell model and in a FTD patient [347,348]. These findings underline the role of epigenetic regulation of GRN and highlight the potential of HDAC inhibitors as a potential therapeutic approach to treat progranulin deficient FTD.

1.4.3 Non coding RNA

Advances in the study of human genome revealed that only 2% of the genome is translated into proteins. In the past it was believed that the remaining part was non-functional DNA. Now it is known that it is mostly transcribed into functional non-coding RNA (ncRNA) which exert pivotal regulatory functions. NcRNA comprise several types of RNA of different length: small non-coding RNA (sRNA), of less than 200 nucleotides and long non coding RNA (lncRNA) of more than 200 nucleotides.

1.4.3.1 MicroRNA

sRNAs are further subdivided as micro (mi)RNAs, short interfering (si)RNAs, and PIWI-associated (pi)RNAs Among them, microRNA (miRNA) recently raised great interest. miRNA are small noncoding molecules of ~22 nucleotides (nt) that regulate gene expression. MiRNAs originate from hairpin structured precursors, called primary miRNAs (pri-miRNAs). The pri-miRNAs are processed by two RNase III-type enzymes, Drosha and Dicer [349,350], and converted into precursor miRNAs (pre-miRNAs) and, finally, into small miRNAs [351,352]. One strand from the miRNA duplex is removed and the other one becomes a part of RNA-induced silencing complex (RISC) [353] and serves for targeted recognition of specific mRNAs [354]. In animals, they exert their effect by suppressing translation of mRNA (through the binding to the 3'-untranslated region (UTR) of target mRNA) or by degrading it (Figure n) [355,356].

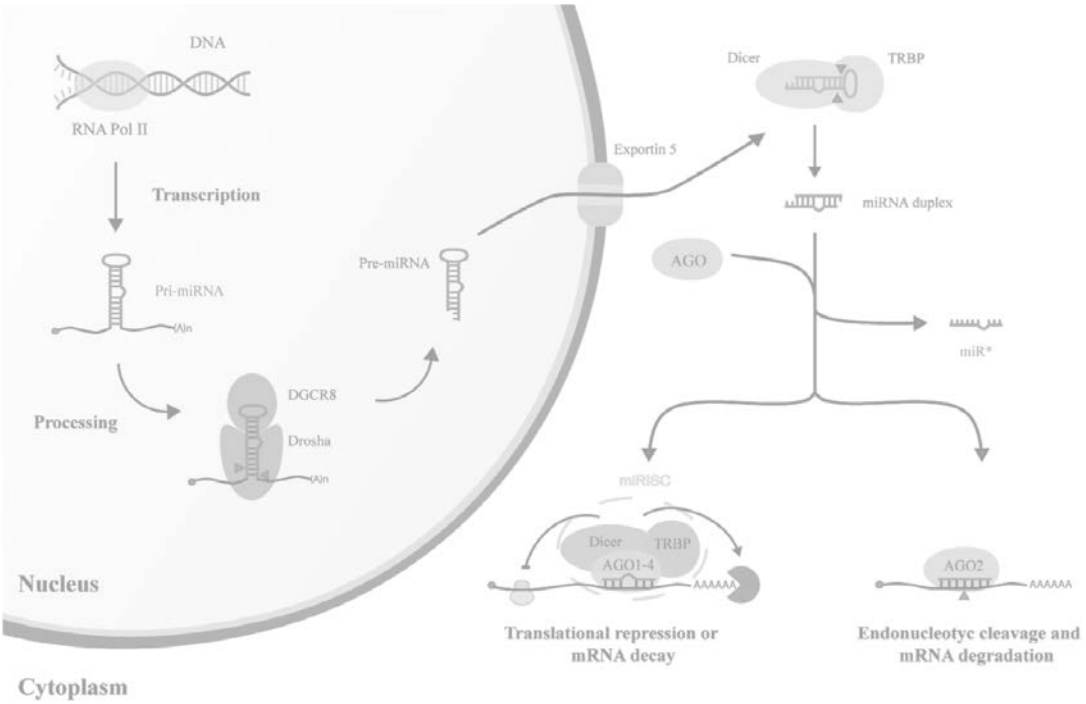


Figure 10. MiRNA biogenesis and modes of action. MiRNA are mostly transcribed by the RNA polymerase II into pri-miRNA that are processed into pre-miRNA. In the cytoplasm, pre-miRNA is further processed into a miRNA duplex). Following processing, miRNA are assembled into miRNA-induced silencing complexes (miRISC complex). Mostly in plants, miRNA exert their function through endonucleolytic cleavage and mRNA degradation. In animals, the miRNA act though translational repression and/or mRNA decay. DGCR8: DiGeorge Syndrome Critical Region 8; miRISC: miRNA-induced silencing complexes; TRBP: transactivation-response RNA-binding protein; AGO 1–4: Argonaut protein family.

Regulating gene expression, miRNA are pivotal actors in cellular processes such as proliferation, differentiation and apoptosis [357]. Circulating miRNAs can be detected in the peripheral circulation (serum, plasma, exosomes, whole blood, peripheral blood mononuclear cells) as well as in the CSF. The miRNAs are quite stable in different biological fluids, compared to mRNA [358]. Therefore, have potential as biomarkers and therapeutic targets. MiRNA have been found to be deregulated in neurodegenerative diseases.

1.4.3.1.1 miRNAs in AD

Many of the investigated miRNAs target genes directly involved in the pathophysiology of AD. Different miRNAs have been found to modulate the expression of BACE1, including miR-15b, miR-29c, miR-124, miR-135b, miR-195, and miR-339-5p [358–361] Some miRNAs, like miR-219, target microtubule associated protein tau (MAPT) gene [362] or they regulate the activity of different protein kinases responsible for the phosphorylation of tau protein, such as miR-124-3p and miR-125b [363–365]. BDNF, a key regulator of synaptic plasticity and transmission, has been suggested to induce the expression of miR-132 [366]. The let-7 miRNA family regulates neural stem cell proliferation and differentiation and exhibits pro-apoptotic activity in the central nervous system [367–370]. Independent replicate studies discovered that expression of both let7g-5p and let7d-5p are significantly increased in blood samples from multiple AD cohorts [371]. In addition to the let-7 family, other miRNAs which influence neuroprotection and regeneration are associated with dementia. A study by Fu et al. reported that miR-142-5p promotes neuronal synaptotoxicity both in vivo and in vitro [372]. Significant dysregulation of miR-142-5p expression, as well as that of miR-590-5p and miR-194-5p, was discovered in blood samples from a cohort of AD patients compared to healthy controls. Previous reports indicate that miR-342 plays a key role in proliferation and differentiation of neural stem cells as well as in neurotoxicity [373,374]. A recent study suggests that levels of miR-342-5p in the plasma may predict the rate of cognitive decline in AD [375]. Several studies investigated the association of miR-146 with the development of AD [376,377]; however, the results of the studies are contradictory demonstrating both downregulation and upregulation of this miRNA in serum, plasma, CSF, and CNS of AD subjects. The evidence suggests that transcription of miR-146a is regulated by nuclear factor kappa-B (NF-kB) [378]. By promoting miR-146 transcription, NF-kB suppresses translation of complement factor H (CFH) and affects inflammatory response in the CNS [378]. Dysregulation of this system in AD leads to increased inflammation and neurodegeneration.

1.4.3.1.2 miRNAs in FTD

A recent study [45] examined the plasma miRNA signature in a cohort of FTD patients: two miRNAs, miR-34a-5p and miR-345-5p, showed increased expression, and two were downregulated, miR-200c-3p and miR-10a-3p. Notably, miR-34a-5p was upregulated in presymptomatic carriers with the *C9ORF72* mutation, suggesting the mutation influences miRNA levels. Additionally, levels of miR-345-5p progressively increased from healthy controls to presymptomatic *C9ORF72* to symptomatic patients. Other studies evaluated the regulation of GRN expression by miRNAs. Rademakers and colleagues in 2008 [379] described an allelic variant in GRN 3'UTR region associated with the disease and corresponding to a predicted binding site for miR-659. Other miRNAs associated with regulation of progranulin levels involved miR-107 and miR-29b [380,381]. Interestingly, it was reported that the TMEM106B gene is repressed by miR-132/212 cluster that is a post-transcriptional mechanism that increases intracellular levels of progranulin [382]. Several miRNAs identified to be differentially expressed in post-mortem tissue, blood and CSF could represent a specific signature for the disease as was recently found for miR-335-5p and let-7e in the CSF of FTD patients compared with controls [383,384].

1.4.3.2 LncRNA

LncRNAs are located in the nucleus and a few in the cytoplasm. They are mostly polyadenylated and transcribed by RNA polymerase II, and derive from all genomic regions, including intergenic areas and near or inside of protein-coding genes [385]. lncRNAs are involved in several cellular processes, but their major role is in the regulation of gene expression patterns. They exert this function through interactions with chromatin modifiers, DNA, RNA, and RNA-binding proteins (RBPs) [385,386]. At the transcriptional level, lncRNAs affect chromatin organization, the formation of nuclear speckles and RNA polymerase II activity [386,387]. At the post-transcriptional level, lncRNAs, interacting with various RNAs and proteins, regulate splicing, mRNA turnover and protein translation [385,386,388,389]. LncRNAs can also be a trap for microRNAs and RBPs, influencing their availability to other molecules, particularly mRNAs. At the post-translational level, lncRNAs form scaffolds to assemble functional ribonucleoprotein complexes, affecting protein stability [385,386]Moreover, lncRNAs are involved in neural differentiation and synaptic plasticity. By altering these processes, they can contribute to neurodegeneration.

1.4.3.2.1 lncRNA in AD

Expression of lncRNA BACE1-antisense is selectively increased in AD brains and competes with miR-545-5p binding to stabilize BACE1 mRNA. This will finally result in increased expression of BACE1 that contribute to the formation of the toxic Aβ peptides [390].

LncRNA 51A is an antisense transcript of Sortilin-related receptor 1 (SORL1), involved in APP transport. The expression of lncRNA 51 causes alternatively spliced protein form [391]. This change causes the processing

of APP to shift in the direction of increased Aβ formation. The expression of 51A is increased in the cerebral cortex of Alzheimer's patients [392].

lncRNA 17A is highly expressed in cerebral tissue of AD patients. It drives alternative splicing of GABA B2 receptor. This event abolishes GABAB R2 intracellular signalling. 17A transcription increases amyloid β peptide (Aβ) secretion and $A\beta_{42}/A\beta_{40}$ peptide *ratio*. 17A synthesis can be induced by inflammatory stimuli [393].

The expression of BC200 RNA in the brains of AD patients is significantly upregulated [394]. It is involved in the regulation of protein synthesis in synapses, and thus regulating synaptic plasticity [395,396].

BDNF-AS inhibits BDNF mRNA transcription by altering chromatin at its locus, and BDNF is an important trophic factor involved in nerve growth and development, underlying its potential for the treating AD [395,396].

1.4.3.2.2 lncRNA in FTD

The expression of lncRNAs NEAT1_2 and MALAT1 are significantly increased in FTD [397]. In FTLD-TDP cases, it can be observed increase in the combination of TDP-43 and lncRNA NEAT1 in the cortex. In addition, the overexpression of lncRNA NEAT1 ameliorates the toxicity of TDP-43 in Drosophila and in TDP-43 protein disease yeast models [398].

2. AIM

AD and FTD are complex heterogeneous disorders with a strong genetic background, but the identification of a genetic cause is difficult given the multifactorial aetiology of the disorders and the overlap between them and other neurodegenerative diseases. Generally, early-onset cases have the stronger heritability, while late-onset dementia is less heritable but highly polygenic. The pleiotropic nature of dementia emerges, as a spectrum of phenotypes can result from the same or different mutations in the same gene. Many AD and FTD cases with an unclear family history of neurodegenerative disease remain unexplained as the genetic basis have been identified in a small percentage of apparently sporadic cases. This suggests that other genetic risk factors have not been revealed so far, which in combination with additional non-genetic factors could be responsible for the remaining familial and apparently sporadic patients.

In light of these evidences, the aim of this project was to explore the two most common forms of dementia using genetic and epigenetic approaches.

1. **Genetic study**: 188 subjects were investigated at the genomic level, using a Next Generation Sequencing (NGS) approach.

In particular, different custom panels were designed to screen the most common genes involved in AD and FTD, applied on a well characterized cohorts of patients. Specifically:

1.1 A small dimension NGS design was applied to 136 dementia patients homogeneous from a molecular point of view, with low CSF levels of Aβ or positivity to PET with Aβ tracer.

1.2 A larger NGS panel customised with 43-genes, able to screen causative genes and risk factors for AD and FTD, along with gene related to other neurodegenerative diseases causing dementia, was applied to a clinically heterogeneous cohort of patients.

1.3 SureSelect Custom Constitutional Panel 17Mb (CCP17) was applied to patients negative for the genetic screening of common causative genes but with strong family history for dementia and/or young onset of symptoms. It is a targeted NGS for inherited disease comprising more than 5000 genes, curated by field experts in human genetics research. It is, therefore, also called Clinical Exome.

2. **Epigenetic study**: a genetic FTD cohort consisting of 40 patients and 20 controls was investigated for the expression of miRNAs.

Advances in the study of human genome revealed that only 2% of DNA is translated into proteins, while the remaining part is mostly transcribed into non-coding RNA (ncRNA), with regulatory functions. Among them, microRNA (miRNA) recently raised great interest. MiRNA are small noncoding molecules that regulate gene

expression by suppressing translation of mRNA or by degrading it [399]. Therefore, they are pivotal actors in cellular processes such as proliferation, differentiation and apoptosis [357].

In genetic cases, the underneath pathology is known, therefore, this facilitates the enrollment in specific clinical trials. In non-genetic cases, it is impossible to predict the pathology in vivo. Therefore, it is important to meet the need for biomarkers for this form of the disease. In this scenario, miRNA have great potential as biomarkers, being present in several biological fluids. To this purpose, genetic FTD cases were enrolled in the project, since they represent the gold standard, being known the underlying pathology.

In particular:

2.1. A first discovery phase was performed to test the expression levels of 754 miRNAs in 30 patients carrying mutations in *C9ORF72*, *GRN* and *MAPT* genes, and 10 control subjects. This part of the project focused on exploring the expression profile of 754 miRNA.

2.2 Results were validated in additional 30 patients and 10 controls.

Statistical analyses were performed comparing controls with each genetic group, in order to find differences specific for each group and therefore for the mutated gene. Moreover, since *C9ORF72* and *GRN* mutated genes underlie the same pathology, expression profile of miRNA in these two groups were jointly compared with controls.

3. Materials and Methods

3.1 Genetic screening in dementia

3.1.1 Population

One hundred and eighty-eight patients with neurodegenerative diseases afferent to the Alzheimer unit of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico were enrolled for the study. All patients were referred to our Centre in suspicion of dementia. They underwent the standard clinical workup, comprising detailed medical history, physical and neurological examination, screening laboratory tests, Mini-Mental State Examination (MMSE); qualitative brain magnetic resonance imaging (MRI) or computed tomography (CT). The presence of significant vascular brain damage was excluded (Hachinski Ischemic Score < 4). All patients underwent lumbar puncture for the analysis of CSF biomarkers Aβ, total tau (tau), and tau phosphorylated at position 181 (Ptau). In case of borderline Aβ CSF levels (about ±10% of reference value), patients underwent Amyloid-PET. In table 1 shows demographic information of the patients screened.

Table 1. Demographics

	AD (n=133)	**bvFTD (n=46)**	**OTHER (n=9)**
Gender (male: female)	62:70	24:22	5:4
Mean age (SD) yrs	71.8 (7.6)	74 (7)	69
Mean age of onset (SD), yrs	68.9 (7.6)	69,5 (6,9)	67
Mean CSF $A\beta_{42}$ (SD), pg/ml	465.3 (94.3)	840,8 (279,4)	643,2 (253)
Mean CSF tau (SD), pg/ml	615.6 (420.1)	522 (420,7)	514,3 (232)
Mean CSF ptau (SD), pg/ml	78.5 (37.6)	73,9 (45)	80,5 (22)
APOE status			
ε4+	66	11	3
ε4-	67	35	6
Positive family history of neurodegenerative or psychiatric diseases (%)	100%	54%	100%
Relatives with neurodegenerative Disease (range)	3-8	1-4	2-5
Family history of psychiatric disease (%)	7,3%	7,7%	11%

SD= standard deviation; Yrs= years; AD= Alzheimer Disease; CBS= Corticobasal Syndrome; PSP= Progressive supranuclear palsy, bvFTD= behavioural variant Frontotemporal dementia. *LBD: Lewy body dementia; CCA: cerebral amyloid angiopathy; bvFTD: Logopenic variant FTD; MCI: mild cognitive impairment

The panels were designed to screen exons of targeted gens and 50 bps of flanking regions.

The small size panel comprised *APP, PSEN1, PSEN2* (causing familial AD[6]), *GRN* and *MAPT*[257] (associated with familial FTD) along with *NPC1* and *NPC2* genes (causing NPC[400]). This study originates from the observation that NPC and AD share clinical and neuropathological similarities: in the adult forms, NPC mimic AD clinic; moreover, neurofibrillary tangles [183] and amyloidogenic processing [184] have been observed in NPC patients.

The Dementia panel (Table 2) comprised: the common genes involved in AD and FTD, genetic risk factors and genes associated to neurodegenerative diseases, known to cause dementia.

Table 2. Dementia Panel

Gene	*Coding Protein*	*Associated disease*	*Mode of transmission*
APOE	Apolipoprotein E	Alzheimer's Disease	Risk factor
APP	Amyloid precursor protein	Alzheimer's Disease	Autosomal dominant
ATP13A2	Lysosomal 5 P-type ATPase	Kufor-rakeb	Autosomal recessive
C19ORF12	Protein unknown	Mitochondrial membrane protein associated neurodegeneration (MPAN)	Autosomal recessive
CHCHD10	Coiled-coil-helix-coiled-coil-helix domain containing 10	FTLD/ALS	Autosomal dominant
CHMP2B	Charged multivesicular body protein 2B	FTLD	Autosomal dominant
CP	Ceruloplasmin	Aceruloplasminemia	Autosomal recessive
CSF1R	Colony stimulating factor 1 receptor	HDLS	Autosomal dominant
DCTN1	Dynactin subunit 1	Perry syndrome/ALS	Autosomal dominant
EIF2B 1-5	Eukaryotic translation initiation factor 2B subunits	Leukoencephalopathy with vanishing white matter (VWM)	Autosomal dominant
FTL	Ferritin light chain	neuroferritinopathy	Autosomal dominant
FUS	FUS RNA binding protein	FTLD/ALS	Autosomal dominant
GBA	Glucosylceramidase beta	Gaucher Disease	Autosomal recessive
GFAP	Glial fibrillary acidic protein	Alexander disease	Autosomal dominant
GRN	progranulin	FTLD	Autosomal dominant

LRRK2	Leucine rich repeat kinase 2	PD type 8	Autosomal dominant
MAPT	tau	FTLD	Autosomal dominant
MATR3	Matrix protein	ALS/FTLD	Autosomal dominant
NOTCH3	Notch	CADASIL	Autosomal dominant
NPC1 e 2	NPC intracellular cholesterol transporter 1	Niemann-Pick type C	Autosomal recessive
PANK2	Pantothenate kinase 2	Pantothenase kinase-associated neurodegeneration	Autosomal recessive
PFN1	Profiling 1	ALS/FTLD	Autosomal dominant
PLA2G6	Phospholipase A2 group VI	Phospholipase A2-associated neurodegeneration (PLAN)	Autosomal recessive
PRNP	Prion Protein	Creutzfeldt-Jacob disease, fatal familial insomnia, Kuru Gerstmann-Straussler disease, Huntington disease-like,	Autosomal dominant
PARKAR1B	Protein kinase cAMP-dependent type 1 regulatory subunit beta	FTD/PD/AD like dementia	Autosomal dominant
PSEN1 and 2	Presenilin 1 and 2	AD	Autosomal dominant
SNCA	Alpha synuclein	PD	Autosomal dominant
SORL	Sortilin related receptor 1	AD	Risk factor
SQSTM1	Sequestosome 1	Paget disease/FTD	Autosomal dominant
TARDBP	TAR DNA binding protein	FTD	Autosomal dominant
TBK1	TANK binding kinase 1	FTD/ALS	Autosomal dominant
TMEM230	Transmembrane protein 230	PD	Autosomal dominant
TREM2	Triggering receptor expressed on myeloid cells	Nasu-Hakola	Autosomal dominant
UBE3A	Ubiquitin protein ligase E3A	Angelman Syndrome	Autosomal dominant
VCP	Valosin containing protein	Paget disease/IBMFTD	Autosomal dominant

3.1.2 Libraries preparation

To create DNA libraries, HaloPlex target enrichment system was used.

- 50ng of gDNA was fragmented using 16 restriction enzymes (RE) in the format of eight double digestion.
  - Each DNA is diluted to 1.8 ng/µL in 10mM Tris buffer (pH 8.5).
  - A reaction mix was prepared combining 24,5 µL of RE buffer and 0.64 µL of Bovine serum albumin (BSA) per sample and dispensed in 8-well strip tube (2,8 µL per tube) .
  - 0,35 µL of each enzyme of the first set of enzymes was added to of the 8-well strip tube.
  - After, 0,35 µL of each enzyme of the second set was added.
  - 3,5 µL of the RE mix was dispensed in 8 well per sample.
  - 3,5 µL of diluted gDNA was added to RE mix.
  - The thermal cycle program for restriction digestion was as follow:

STEP	TEMPERATURE	TIME
STEP 1	37°C	30 min
STEP 2	4°C	Hold

- Fragments hybridized to probes targeting exons and 25 bp of exon padding to cover flanking regions: HaloPlex HS probes hybridized to both ends of target DNA, directing circularization. During hybridization, molecular barcodes and Illumina sequencing motifs, including index sequencing were incorporated into the targeted fragment.
  - The hybridization master mix was prepared combing 34 µL of Hybridization Solution and 5 µL of Probes.
  - 39 µL of mix were distributed to a new tube
  - 5 µL of index primer was added.
  - Digested DNA samples from each of the eight RE reaction were transferred and added to the hybridization mix.
  - Thermal cycler program for hybridization was as follow:

STEP	TEMPERATURE	TIME
STEP 1	95°C	5 min
STEP 2	58°C	2 h

- The hybridization buffer was then removed in preparation of the next step.
  - A mix of 20 µL of Stop solution and 80 µL of AMPure XP beads was added to each sample.
  - Samples were incubated 5 minutes at room temperature with continuous shaking at 1300 rpm.
  - On a magnetic plate, the cleared solution was discarded and 200 µL of 70% ethanol.
  - Again, on a magnetic plate, the cleared solution was discarded and samples were air-dried.

- Following, the circularized hybrids were closed in a ligation step.
  - DNA ligation master mix comprised for each sample 10 µL of Ligation solution, 0,6 µL of 1 mM rATP and 39,4 µL of nuclease-free water.
  - 50 µL of ligation master mix were added to each sample and beads were resuspended.
  - Samples were incubated at room temperature for 2 minutes.
  - Beads were collected through a magnetic plate and 47,5 µL of supernatant were transferred to a fresh tube
  - 2,5 µL of DNA Ligase were added to each sample
  - Samples were incubated in a thermal cycler at 55°C for 10 minutes

- The DNA-Probe hybrids containing biotin were captured on streptavidin beads.
  - 40 µL of Dynabeads suspension for each sample was cleared on a magnetic rack and 40 µL of Capture solution were added to the beads.
  - 40 µL of Capture solution + streptavidin bead mixture were added to each ligation reaction
  - Samples were incubated 15 minutes at room temperature with continuous shacking.
  - After that, the solution was cleared on a magnetic plate and the supernatant removed.
  - The beads were washed with 100 µL of Wash 1, cleared from the supernatant.
  - The washing step was repeated with 150 µL of Wash 2.

- Captured target libraries were amplified by PCR. PCR master mix was prepared as follow. For each samples:
  - 53,2 µL of Nuclease-free water,
  - 30 µL of Herculase II reaction buffer,
  - 0.8 µL of dNTPs
  - 4 µL of Primer 1
  - 8 µL of primer 2
  - 4 µL of Herculase II fusion DNA Polymerases.
  - 100 µL of PCR master mix were added to each sample. Thermal cycle program was:

STEP	N OF CYCLES	TEMPERATURE	TIME
1	1	98°C	2 min
2	24	98°C	30 sec
		60°C	30 sec
		72°C	1 min
3	1	72°C	10 min
4	1	8°C	Hold

- The next step was the purification of PCR products.
  - 40 µL of nuclease-free water and 100 µL of AMPure XP bead suspension were mixed and added to each sample.
  - Samples were incubated for 5 minutes at room temperature with continuous shaking.
  - The solution was cleared with the magnetic plate and 200 µL of 70% ethanol was added per sample.
  - The solution was cleared and additional 200 µL of 70% ethanol was added.
  - The solution was cleared and the beads were air-dried.
  - 45 µL of Elution Buffer were added to each sample, which was incubated at room temperature for 2 minutes
  - approximately 40 µL of cleared supernatant were removed to a fresh tube .

3.1.3 Qualitative and quantitative analysis of DNA

Libraries were validated and quantified using High sensitivity DNA kit (Agilent Technologies) containing chip and reagents designed for sizing and analysing DNA fragment. Each DNA chip contains an interconnected set of microchannels that is used for electrophoretic separation of nucleic acids acid fragments based on their size. The protocol used was as follow:

1. Prepare the gel-dye mix allowing the DNA dye concentrate and DNA gel matrix to equilibrate to room temperature for 30 minutes.
2. Pipette 15 µL of the blue capped dye concentrate into a red-capped DNA gel matrix vial.
3. Vortex for 10 seconds. Visually inspect proper mixing of gel and dye.
4. Transfer the gel-dye mix to the top receptacle of a spin filter.
5. Centrifuge for 15 minutes at room temperature at 2240 g ± 20 % .

Reagent and samples are loaded on a chip by mean of a priming station holding the chip and a syringe which create the pressure for filling.

6. Put a DNA chip on the chip priming station.

7. Pipette 9.0 µL of the gel-dye mix at the bottom of the well marked
8. Press the plunger of the syringe down until it is held by the clip.
9. Wait for exactly 60 seconds and then release the plunger with the clip release mechanism.
10. Wait for 5 seconds, then slowly pull back the plunger to the 1 ml position.
11. Open the chip priming station and pipette 9.0 µL of the gel-dye mix in each of the wells marked .
12. Pipette 5 µL of green-capped DNA marker into the well marked with the ladder symbol and into each of the 12 sample wells.
13. Pipette 1 µL of the yellow-capped DNA ladder in the well marked with the ladder symbol.
14. In each of the 12 sample wells pipette 1 µL of sample (used wells) or 1 µL of deionized water (unused wells)
15. Place the chip horizontally in the adapter of the IKA vortex mixer and vortex for 60 seconds at 2400 rpm.
16. Run the chip in the Agilent 2100 instrument within 5 minutes.

3.1.4 Clinical exome sequencing

Patients negative for the genetic screening but with family history for dementia and/or young onset were further investigated with SureSelect Custom Constitutional Panel 17Mb (CCP17). It is a targeted NGS for inherited disease comprising more than 5000 genes, curated by field experts in human genetics research. It is, therefore, also called Clinical Exome.

The protocol used was the following:

- 50 ng input gDNA were used, for a total volume of 7 µL
- DNA Fragmentation
  - For each sample, 2 µL of 5X SureSelect Fragmentation Buffer and 1 µL of SureSelect Fragmentation Enzyme were added.
  - Fragmentation conditions were:

STEP	TEMPERATURE	TIME
STEP 1	37°C	15 min
STEP 2	65°C	5 min
STEP 3	4°C	Hold

- **Repair and dA-Tail the DNA ends**
  - For each sample, 16 µL of End Repair-A Tailing Buffer and 4 µL of End Repair-A Tailing Enzyme were mixed and added
  - Thermal cycling was as follow:

STEP	TEMPERATURE	TIME
STEP 1	20°C	15 min
STEP 2	72°C	15 min
STEP 3	4°C	Hold

- **Ligation of the molecular-barcoded adaptor**
  - The ligation master mix was prepared mixing 23 µL of Ligation Buffer and 2 µL of T4 DNA Ligase for each sample.
  - 25 µL of ligation master mix and 5 µL of Adaptor Oligo mix were added to each end-repaired/dA-tailed DNA sample
  - The ligation thermal conditions were:

STEP	TEMPERATURE	TIME
STEP 1	20°C	30 min
STEP 2	4°C	Hold

- Sample purification
  - 80 µL of AMPure XP beads were added to each DNA sample
  - Samples were incubated for 5 minutes at room temperature
  - On a magnetic plate the supernatant was discarded
  - 200 µL of 70% ethanol in each sample was added
  - Ethanol was discarded and samples were dried in the thermal cycler, set to hold samples at 37°C for 1-2 minutes.
  - 35 µL of nuclease-free water were added and the sample was incubated for 2 minutes at room temperature
  - The supernatant was cleared and transferred to a fresh tube

- Amplification of library
  - Pre-Capture PCR mix was prepared adding per sample:

    10 µL of 5× Herculase II Reaction Buffer

    0.5 µL of 100 mM dNTPs mix

    2 µL of Forward Primer

    1 µL of Herculase II Fusion DNA Polymerase
  - 13,5 µL of PCR mix were added to each sample
  - 2 µL of the appropriate SureSelect XT HS Index Primer were mixed to each reaction.
  - PCR conditions were as follow:

STEP	N OF CYCLES	TEMPERATURE	TIME
1	1	98°C	2 min
2	9	98°C	30 sec
		60°C	30 sec
		72°C	1 min
3	1	72°C	5 min
4	1	4°C	hold

- Library purification
  - 50 µL of AMPure XP beads were added to each PCR amplification reaction and samples were incubated at room temperature for 5 minutes.
  - Beads were washed twice with 200 µL of 70% ethanol
  - Ethanol was discarded and samples were dried in the thermal cycler, set to hold samples at 37°C for 1-2 minutes
  - 15 µL of nuclease-free water were added to each sample that were incubated for 2 minutes at room temperature
  - The supernatant was cleared and transferred to a fresh tube

- **Quality and quantity assess**
  - 2100 Bioanalyzer and DNA 1000 Assay was used

- Hybridization of libraries
  - 500–1000 ng of each library sample were brought to the final volume of 12 µL using nuclease-free water.
  - 5 µL of SureSelect XT HS and XT Low Input Blocker Mix were added

- The Thermal cycle was as follow:

STEP	N OF CYCLES	TEMPERATURE	TIME
1	1	95°C	5 min
2	1	65°C	10 min
3	1	65°C	1 min
4	60	65°C	1 min
		37°C	3 sec
5	1	35°C	hold

- During segment 3 of the program, 13 µL of Probe Hybridization Mix were added to each samples, comprising

  2 µL of 25% RNase Block solution

  2 µL of probes

  6 µL of SureSelect Fast Hybridization Buffer

  3 µL of nuclease-free water

- Capture the hybridized DNA
  - 50 µL of Dynabeads MyOne Streptavidin T1 magnetic beads for each sample were washed three times with 200 µL of SureSelect Binding Buffer.
  - The beads were resuspended in 200 µL of SureSelect Binding Buffer
  - The entire volume of each hybridization mix was transferred into the washed beads and incubated for 30 minutes at room temperature
  - The supernatant was discarded and 200 µL of SureSelect Wash Buffer 1 were added.
  - The supernatant was discarded and 200 µL of Wash Buffer 2 were added
  - The samples were incubated for 5 minutes at 70°C in the thermal cycler
  - The wash buffer was removed and 25 µL of nuclease-free water was added.

- Amplification of captured libraries
  - Post-Capture PCR was prepared mixing:

    12,5 µL of nuclease-free water

    10 µL of 5× Herculase II Reaction Buffer

    1 µL of Herculase II Fusion DNA Polymerase

    0,5 µL of 100 mM dNTP Mix

    1 µL of SureSelect Post-Capture Primer Mix

- 25 µL of PCR mix were added to samples
- The PCR Thermal conditions were as follow:

STEP	N OF CYCLES	TEMPERATURE	TIME
1	1	98°C	2 min
2	9	98°C	30 sec
		60°C	30 sec
		72°C	1 min
3	1	72°C	5 min
5	1	4°C	hold

- On a magnetic plate, supernatant was transferred into a new tube.

- Library purification
  - 50 µL of AMPure XP beads were added to each PCR amplification reaction and samples were incubated at room temperature for 5 minutes.
  - Beads were washed twice with 200 µL of 70% ethanol
  - Ethanol was discarded and samples were dried in the thermal cycler, set to hold samples at 37°C for 1-2 minutes
  - 25 µL of nuclease-free water were added to each sample that were incubated for 2 minutes at room temperature
  - The supernatant was cleared and transferred to a fresh tube
  - after assessing quality and quantity of library, samples were pooled

3.1.5 Sequencing

For the custom panels, the sequencing pool was loaded on Illumina Cartridge V2 300 cycles and run on Illumina MiSeq platform (Illumina).

Sequencing of clinical exome was performed on NextSeq 550 platform (Illumina)

3.1.6 Bioinformatic analysis

The fastq NGS data were analysed using Alissa Align&Call software (Agilent Genomics) which aligned the reads to human genome reference (UCSC hg19, GRCh37, February 2009). Align&Call analysis pipeline

automatically performed quality control, filtering variants for robust coverage metrics and high phred score. Variant calling format (VCF) files from Align&Call were processed with the Expert Variant Interpreter (eVai) platform (enGenome, https://evai.engenome.com, version 2.7) to perform annotation and categorization of the variants. Variants were classified using a combined approach based on American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) criteria [401] automatically provided by the platform. ACGM/AMP criteria consist in twenty-eight items which describe each variant with respect to different characteristics, such as population allele frequency, segregation and functional data. The criteria are hierarchically organized in five groups of different levels of evidence to support pathogenic or benign classification: “Pathogenic”, “Likely pathogenic”, “Benign”, “Likely benign”, “Variant of Uncertain Significance” (VUS). Moreover, a score of pathogenicity is automatically assigned to variants by the software [402]. eVai platform also provides a list of condition and phenotypes associated to a gene, and the direct link to ClinVar database, a public archive of reports of the association variants-phenotype, supported by evidence (https://www.ncbi.nlm.nih.gov/clinvar/)

Moreover, the impact of the variants was assessed by *in silico* predictions using PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/) which evaluates the effect of missense substitutions on protein sequence and structure, and Mutation Taster (http://www.mutationtaster.org/), which accesses the effect of missense and nonsense substitutions, and intronic alterations, based on the effect on protein sequence.

3.1.7 Allelic discrimination

To validate variants found, allelic discrimination assay was performed. To this purpose, specific TaqMan probes (ThermoFisher Scientific) were used, able to detect Single Nucleotide Polymorphisms (SNPs) in genomic DNA samples. The probes are oligonucleotides complementary to the region of interest, containing in the 5’ end a fluorescent molecule, usually VIC® for wild-type nucleotide and FAM©® for the mutated nucleotide.

The genotyping reaction comprised:

12.5 µ*L of Genotyping Master Mix*

1 µL of probes

1 µL of genomic DNA

10.5 µL nuclease free water.

The RT-PCR was run of QuantStudio 12K flex system, with the following thermal profile:

STEP	N OF CYCLES	TEMPERATURE	TIME
1 - activation	1	95°C	10 min
2 - denaturation	40	95°C	15 sec
3 - annealing/elongation	1	60°C	1 min

3.2 MiRNA expression profile in genetic FTD

3.2.1 Population

For miRNA expression profile, I took advantage of samples from Genetic FTD Initiative, an international consortium of research centers across Europe and Canada, whose aim is to further knowledge in genetic FTD. Preliminary results have been obtain on a cohort comprising 10 C9ORF72 hexanucleotide expansion carriers, 9 GRN and 10 MAPT mutation carriers, and 9 healthy subjects. Results have been validated on a cohort comprising additional 10 subjects per group (Table 3).

Table 3. FTD patients' demographics

	C9ORF72 (N=20)	**GRN (N=19)**	**MAPT (N=20)**	**CONTROLS (N=19)**
Gender (male:female)	14:6	10:9	12:8	11:8
Mean age (SD) yrs	59,55 (12,8)	60,1 (12,5)	59,9 (12,5)	59,9 (12,6)
Mean age of onset (SD), yrs	58.4 (9.1)	60,2 (8.4)	59,1 (8,6)	n.a.

SD= standard deviation; Yrs= years, n.a.= not applicable

3.2.2 Total RNA extraction

Total RNA was extracted from whole blood collected in PAXgene tubes, containing reagents that lyse blood cells and stabilize RNA for downstream analysis. After blood collection, the PAXgene tubes have been incubated for at least 2 hours at room temperature to ensure complete lysis of blood cells. Tubes were stored firstly at 20°C and then at 80°C until used.

Before starting the procedure, tubes were incubated at room temperature for 2 hours.

RNA was purified using PAXgene® blood miRNA kit (PreAnalytix), following the protocol below:

1. Centrifuge the PAXgene tube at 4000 g for 15 min to pellet the samples.
2. Remove the supernatant and add 4 mL of RNAse-free water.
3. Vortex to dissolve the pellet and centrifuge for 15 minutes at 4000 g.
4. Remove the entire supernatant and 350 µL of Buffer BM1.
5. Vortex until the pellet is visibly dissolved, and centrifuge for 15 min at 4000 x g
6. Remove the entire supernatant by decanting or pipetting, and discard.
7. Add 350 µL of Buffer BM1, and vortex until the pellet is visibly dissolved.
8. Pipet the sample into a 1.5 ml microcentrifuge tube. Add 300 µL of Buffer BM2 and 40 µL proteinase K. Mix by vortexing for 5 s and incubate for 10 min at 55°C in
9. Pipet the sample into a PAXgene Shredder spin column (lilac) placed in a 2 ml processing tube, and centrifuge for 3 min at full speed (do not exceed 20,000 x g).

10. Carefully transfer the entire supernatant of the flow-through from the PAXgene Shredder spin column to a new 1.5 ml microcentrifuge tube without disturbing the pellet in the processing tube.
11. Add 700 µL of isopropanol (100%, purity grade p.a.), and mix by vortexing.
12. Pipet 700 µL of sample into the PAXgene RNA spin column (red) placed in a 2 ml processing tube. Close the lid gently, and centrifuge for 1 min at 8000–20,000 x g.
13. Place the spin column in a new 2 ml processing tube and discard the old processing tube containing flow-through.
14. Pipet the remaining sample into the PAXgene RNA spin column (red). Close the lid gently, and centrifuge for 1 min at 8000–20,000 x g. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.
15. Add 350 µL Buffer BM3 to the PAXgene RNA spin column. Close the lid gently, and centrifuge for 15 s at 8000–20,000 x g. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.
16. Add 10 µL DNase I stock solution to 70 µL Buffer RDD in a 1.5 ml microcentrifuge tube.
17. Mix by gently flicking the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.
18. Pipet the DNase I incubation mix (80 µl) directly onto the PAXgene RNA spin column membrane, and incubate on the benchtop (20–30°C) for 15 min.
19. Add 350 µL Buffer BM3 to the PAXgene RNA spin column. Close the lid gently, and centrifuge for 15 s at 8000–20,000 x g. Place the spin column in a new 2 ml processing tube and discard the old processing tube containing flow-through.
20. Add 500 µL Buffer BM4 to the PAXgene RNA spin column. Close the lid gently, and centrifuge for 15 s at 8000–20,000 x g. Discard the flow-through. Place the spin column in a new 2 ml processing tube and discard the old processing tube containing flowthrough.
21. Add another 500 µL Buffer BM4 to the PAXgene RNA spin column. Close the lid gently, and centrifuge for 2 min at 8000–20,000 x g.
22. Discard the processing tube containing flow-through and place the PAXgene RNA spin column in a new 2 ml processing tube (supplied). Centrifuge at 8000–20,000 x g for 1 min.
23. Discard the processing tube containing flow-through. Place the PAXgene RNA spin column in a new 1.5 ml microcentrifuge tube, and pipet 40 µL Buffer BR5 directly onto the spin column membrane. Close the lid gently, and centrifuge for 1 min at 8000–20,000 x g to elute the RNA.
24. Repeat the elution step (step 18) as described, using 40 µL Buffer BR5 and the same microcentrifuge tube.
25. Incubate the eluate for 5 min at 65°C in the shaker–incubator without shaking. After incubation, chill immediately on ice.
26. Store the RNA at –80°C.

3.2.3 Qualitative and quantitative analysis of DNA

RNA was analysed with 2100 Bioanalyzer and RNA 6000 Nano kit (Agilent Genomics) for qualitative and quantitative characterization (Agilent Genomics). RNA quality is indicated by RNA integrity number (RIN). It is determined by no longer the ratio of the ribosomal bands alone, but by the entire electrophoretic trace of the RNA sample, including the presence or absence of degradation products. The protocol above has been followed:

1. Heat denature the ladder for 2 min at 70 °C.
2. Cool down the vial on ice and prepare aliquots in RNase- free vials with the required amount for a typical daily use. Store aliquots at - 70°C.
3. Before use, thaw ladder aliquots and keep them on ice
4. Place 550 µL of Agilent RNA 6000 Nano gel matrix into the top receptacle of a spin filter and centrifuge for 10 minutes at 1500 g ± 20 %.
5. Aliquot 65 µL filtered gel into 0.5 ml RNase- free microfuge tubes.
6. Add 1 µL of RNA 6000 Nano dye concentrate to a 65 µL aliquot of filtered gel
7. Cap the tube, vortex thoroughly and visually inspect proper mixing of gel and dye. Store the dye concentrate at 4 °C in the dark again.
8. Spin tube for 10 minutes at room temperature at 13000 g.
9. Place the chip on the chip priming station.
10. Pipette 9.0 µL of the gel-dye mix at the bottom of the well marked  and dispense the gel-dye mix.
11. Close the chip priming station and wait for exactly 30 seconds and then release the plunger with the clip release mechanism.
12. Wait for 5 seconds, and then slowly pull back the plunger to the 1 ml position.
13. Open the chip priming station and pipette 9.0 µL of the gel- dye mix in each of the wells marked  .
14. Pipette 5 µL of the RNA 6000 Nano marker into the well marked with the ladder symbol and each of the 12 sample wells
15. Pipette 1 µL of the RNA ladder into the well marked 
16. Pipette 1 µL of each sample into each of the 12 sample wells.
17. Place the chip horizontally in the adapter of the IKA vortex mixer and vortex for 60 seconds at 2400 rpm.
18. Start the run is started within 5 minutes.

3.2.4 MiRNA retrotranscription and pre-amplification

100 ng of total RNA in 3 µLwere retrotranscribed to cDNA using Megaplex™ RT Primers (ThermoFisher). Two reverse transcription (RT) reactions were run per sample, each containing a pool of primers (A and B).

4.5 µL of RT mix were added to total RNA, containing:

RT reaction mix components	**Volume per reaction**
Megaplex™ RT Primers (10X), Pool A or Pool B	0.75 µL
dNTPs with Dttp (100mM)	0.15 µL
Multiscribe™ Reverse Transcriptase (50 U/µL)	1.50 µL
10X RT Buffer	0.75 µL
$MgCl_2$ (25mM)	0.90 µL
RNase Inhibitor (20 U/µL)	0.09 µL
Nuclease-free water	0.35 µL

RT was run following the thermal cycling conditions:

STAGE	**TEMP**	**TIME**
CYCLE	16°C	2 min
(40 CYCLES)	42°C	1 min
	50°C	1 sec
HOLD	85°C	5 min
HOLD	4°C	∞

2.5 µL of RT product were preamplified, adding 22.5 µL of PreAmp Reaction Mix containing:

PreAmp Reaction Mix components	**Volume per reaction**
TaqMan® PreAmp Master mix (2x)	12.5 µL
Megaplex™ PreAmp Primers (10X), Pool A or Pool B	2.5 µL
Nuclease-free water	7.5 µL

The Preamplification was run with the following parameters:

STAGE	TEMP	TIME
HOLD	95°	10 min
HOLD	55°	2 min
HOLD	72°	2 min
CYCLE	95°C	2 min
(12 CYCLES)	60°C	15 sec
HOLD	99.9°C	10 min
HOLD	4°C	∞

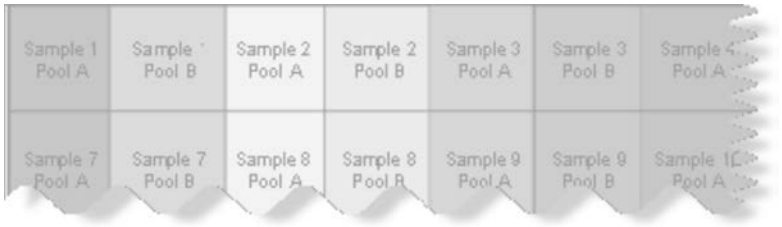
4 µL of each preamplification reaction have been diluted adding 156 µL of 1X TE pH8.0.

Per sample, 22.5 µL of TaqMan® OpenArray® Real-Time PCR Master Mix for Pool A and Pool B were dispensed in new tubes.

22.5 µL of diluted Pool A/Pool B preamplification product were added.

5 µL of each PCR reaction Mix were dispensed into each of 8 well on an OpenArray® 384-Well Sample Plate, as showed in the figure below:

Figure 11



The automated Accufill™ system was used to load the samples and PCR mix from 384-well plate to OpenArray ™ plate, a microfluidic system comprising about 3000 of 33nm of volume. Three samples can be loaded per OpenArray™ plate.

PCR was run on QuantStudio 12k Flex system.

3.2.5 Taqman MicroRNA Assay

miRNAs were validated with TaqMan® microRNA Assays.

They were specifically retrotranscribed using custom RT primer pools. Each 5X RT primer was diluted in 1X TE for a final concentration of 0,05X.

3 µL of total RNA was added to 12 µL of RT mix containing:

RT reaction mix component	**Volume**
RT primer pool	6 µL
dNTPs with dTTP (100mM)	0.30 µL
MultiScribe™ Reverse transcriptase (50U/ µL)	3 µL
10X RT buffer	1.50 µL
RNase Inhibitor (20U/ µL)	0.19 µL
Nuclease-free water	1.01 µL

After 5 minutes of incubation on ice, samples underwent the following thermal cycle:

STEP	**TEMP**	**TIME**
1	16°C	30 min
2	42°C	30 min
3	85°C	5 min
HOLD	4°C	∞

The PCR reaction mix was prepared as follow:

Components	**Volume**
TaqMan® microRNA Assays	0.5 µL
RT product	0.3 µL
PCR Master Mix	5 µL
Nuclease-free water	4.2 µL

PCR was run on QuantStudio 12k Flex system, following the thermal cycling condition below:

STEP	TEMP	TIME	CYCLES
ENZYME ACTIVATION	95°C	10 min	1
DENATURE	95°C	15 sec	45
ANNEAL/EXTEND	60°C	60 sec	

3.2.6 Bioinformatic analyses

Normalized CT values of miRNAs were used to analyze differences between healthy subjects and patients. Statistical significance of miRNAs modulation was assessed by Wilcoxon rank sum test. Significance was defined at the 5% level.

Principal Component Analysis and unsupervised hierarchical clustering based on the Euclidean distance metric were performed to individuate specific pattern of expression.

Average expression values of selected miRNAs were used to fit a binomial model in predicting of healthy subjects and patients.

Prediction scores from the classifier were used to evaluate the true positive rate (sensitivity) and the false positive rate (1-specificity) in a ROC curve. Performance of the curves was assessed by calculating the Area Under Curve (AUC) with 1000 bootstrap replicas for computation of the confidence bounds.

The analyses were completely conducted with Matlab R2022a.

3.2.7 Target prediction and pathway enrichment analysis

MiRNet (https://www.mirnet.ca/miRNet/home.xhtml) web tool was used to determinate miRNA-target interaction and pathways enrichment analysis. Targets were also associated to specific diseases by using DisGeNET (https://www.disgenet.org/home/).

4. Results

4.1 Genetic study

A total of 188 patients was screened using an NGS approach. 136 dementia patients were analysed with NPC panel, 52 patients were screened with Dementia panel. From the whole cohort, 35 patients with an interesting family history and/or early onset of the disease were further studied with the Clinical Exome. NGS was successfully completed for all the samples. An average of 97% of bases at >20x coverage was observed, with 98% high quality reads. The number of variants per sample was variable, depending on the gene panel applied.

4.1.1 NPC study

Patients screened with the NPC panel showed an average of 30 variants passing the quality control.

Five *NPC* variants in heterozygosis were found in seven patients (Table 4).

Two of these were previously found in NPC patients (in homozygosity or compound heterozygosity): the near-splicing variant c.441+1 G>A[403], located in the consensus-donor splice site of intron 4 of the *NPC2* (one with CBS and two with AD) and the missense *NPC2* p.V30M [404](in one AD patient). The former was predicted to be disease causing by Mutation Taster. The latter was predicted to be possibly damaging by PolyPhen software, while Mutation taster categorized it as tolerated.

Two were rare known variants: the missense p.K71R in *NPC2* and the nonsense p.Q241X in *NPC1*, each found in patients diagnosed with AD (Table 4). The former, according to Mutation Taster Software, is damaging since it causes a premature stop signal and therefore leads to an absent or disrupted protein product. This variant was identified in a patient The latter was predicted to have a disease-causing impact by Poly-Phen2 and Mutation Taster.

Moreover, a novel variant of uncertain significance was found in *NPC1* (NM_000271: exon11, c.T1708C, p.Y570H) in one patient with AD. It was predicted to be disease causing by *in silico* analysis performed with Mutation Taster software, while PolyPhen2 categorized it as polymorphism.

Table 4. Mutations identified in the present study

Mutation	dbSNP	Previously reported in	In silico predictions		Carrier's diagnosis
			PolyPhen2	Mutation taster	
***NPC2* c.441+1 G>A**	rs140130028	[403,405–407]	n.a.	D	CBS
					AD frontal variant
					AD frontal variant
***NPC2* p.V30M**	rs151220873	[404,405]	D	T	AD frontal variant
***NPC2* p.K71R**	rs142075589	[406]	D	D	AD
***NPC1* p.Q241X**	rs1064795718	/	T	D	AD
***NPC1* p.Y570H**	/	/	T	D	AD

CBS: Corticobasal syndrome; AD: Alzheimer's disease; n.a.: not applicable; D: disease causing; T: tolerated

To test whether the novel variant p.Y570H is a common polymorphism or may have a pathogenic role, the allelic frequency was investigated in a cohort of 200 healthy geriatric controls. The variant was absent in controls. The remaining variants were tested as well: three out of 200 controls (1.5%) presented two variants in heterozygosity. In particular, two controls displayed the c.441+1 G>A (MAF=0.005) and one healthy subject presented the variant p.K71R (MAF=0.0025), whereas no one showed the p.V30M and p.Q241X variants. There were no significant differences in MAF distribution for the detected variants in patients compared with controls. 5.2% of the cohort carried a variant as compared with 1.5% in healthy geriatric controls. Regarding the clinical phenotype, beside cognitive impairment, which was present in all patients studied, five out of seven carriers developed psychosis, mainly delirium, and two had extrapyramidal symptoms.

The screening also led to the identification of two patients who displayed causal mutations: one in APP (p.V717I) in a male patient diagnosed with AD at 55 years, and one in *GRN* (p.C157fs), predicted to lead to haploinsufficiency and previously associated with phenotypes of FTD spectrum [408], in a woman diagnosed with prodromal AD at 76 years of age.

4.1.2 Dementia panel and clinical exome

The results from the Dementia panel showed an average of 255 variants. The bioinformatic analysis led to the identification of 14 known and two novel rare variants in 13 genes, carried by 15 patients. All had MAF<1% and were pathogenic for at least one in silico prediction tool. ACMG/AMP guidelines were used to classify the variants[401].

Among all patients screened, 35 subjects, negative for the preliminary genetic screening, but with significant family history for dementia and/or young onset were further investigated. Genetic screening with the clinical exome approach provided a huge amount of data per patients (14000 variants on average) to be analysed. Since results were not-necessarily related to neurodegenerative diseases, a virtual panel of genes associated to dementia was applied. eVai platform also provides conditions and phenotypes associated to the genes where variants were called. Therefore, variants filtered by the bioinformatic tool as Pathogenic, Likely pathogenic or VUS were searched for references to dementia or neurodegeneration. This approach led to the identification of 10 known and 6 novel variants in 16 patients. All the results are shown in table 5.

Table 5. Results of Dementia panel and Clinical Exome screening

Gene	**Variant**	**dbSNP**	**ACMG/AMP criteria**	**Suggested Classification**	**Carriers' diagnosis**
AARS2	p.Arg199Cys	rs200105202	PM2, PP3, PP5	Pathogenic*	bvFTD
AARS2	p.Phe74Tyr	rs757169781	PM2, PP3	VUS	bvFTD
AARS2	p.Pro368fs	novel	PVS1, PM2	Likely Pathogenic	AD
CACNA1G	p.Trp1103Cys	novel	PP2, PM2, PP3	VUS	AD frontal variant
COL18A1	p.Glu301del	rs765971874	PM2, PM4	VUS*	AD frontal variant
CSF1R	c.1511-61C>T	novel	PM2, BP7	VUS	AD
CYP27A1	c.1184+1G>A	rs587778777	PVS1, PM2, PP5	Pathogenic*	-
DCTN1	c.3345+9G>A	rs762136929	PM2	VUS	AD
DCTN1	p.Cys191Thr	rs768268418	PM2	VUS	bvFTD
EIF2B1	c.253-27C>T	rs770625902	PM2, BP7	VUS	AD
GBA	p.Asn409Ser	rs76763715	PP5, PM2, PM1, PP3, PM5, PP2	Pathogenic*	bvFTD
GFAP	p.Glu223Gln	rs56679084	PM2, PP2, PP3	VUS	bvFTD
GRN	c.1179+3A>G	-	PM2, PP3	VUS	bvFTD
ITPR1	p.His2686fs	novel	PM2, PM4	VUS	AD
MAPT	p.Asp740Gly	-	PM1, PM2, PP3	VUS	LBD

NPC1	p.Asn222Ser	rs55680026	PP2, BS1	VUS*	MCI
PLA2G6	p.Ile131Phe	novel	PP2, PM2	VUS	Cerebral amyloid angiopathy
PSEN2	p.Thr18Met	rs143061887	PM2	VUS*	AD
PSEN2	c.141+15C>T	rs756117413	PM2, BP7	VUS	AD
SORT1	p.Pro15Ser	rs150163924	PM2,BP4	VUS	bvFTD
SORT1	p.Met428Thr	rs529921012	PM2	VUS	AD frontal variant
SNCA	p.Asp2Glu	novel	PM2	VUS	AD frontal variant
SQSTM1	p.Pro118Ser	rs200152247	PM2,BP4	VUS	bvFTD
SQSTM1	p.Pro392Leu	rs104893941	PM2, PP3, PP5	Likely Pathogenic*	bvFTD
SQSTM1	p.Tyr383*	novel	PVS1, PM2	Likely Pathogenic	AD frontal variant
TBK1	p.Ile73Val	rs751253214	PM2, BP4	VUS	bvFTD
TMEM106B	p.Lys129Arg	rs150868186	PM2	VUS	bvFTD
TREM2	p.Arg161K	novel	PM2, PP3	VUS	bvFTD
TREM2	p.Asp134Gly	rs28939079	PM2	VUS*	bvFTD
TYMP	p.Val208Met	rs121913039	PM1, PM2, PM5, PP3, PP5	Pathogenic*	AD

*= reported in ClinVar database. VUS: Variants of unknown significance; BP: supporting benign; PM: Pathogenic moderate; PP: Supporting pathogenic; PVS= pathogenic strong

4.1.2.1 Variants consistent with carrier's phenotype

Several variants were found in genes causative or known risk factors for the carrier's phenotype.

A VUS in *GRN* gene (*c.1179+3A>G*) was found in a patient diagnosed with FTD. Interestingly, the carrier also harboured a mutation in GBA gene (D409S) reported to be pathogenic in ClinVar database.

Two known VUS in *PSEN2* gene were carried by two patients with AD. The variant p.Thr18Met was reported in ClinVar with uncertain significance, c.141+15C>T is an intronic variant for whom no evidence was reported, but predicted by the bioinformatic tool to be VUS.

In a bvFTD patient, a rare variant of uncertain significance (I73V) was found in *TBK1* (TANK-Binding Kinase 1).

Three variants were found in *Sequestosome 1 (SQSTM1)* gene. Two were harboured by two patients diagnosed with bvFTD: p.Pro118Ser was classified a VUS, while Pro392Leu is reported to be pathogenic and is the most frequent variant among Paget disease patients [409]. The third is a novel variant predicted to be pathogenic by in silico prediction tools, and was instead carried by a patient diagnosed with the frontal variant of AD.

Two known variants of uncertain significance were found in *SORT1* gene: Pro15Ser variant was found in a patient with bvFTD, Met428Thr variant in a patient diagnosed with the frontal variant of AD.
A known VUS (Cys191Thr) was found in *DCTN1* gene, in a bvFTD patient. , c.3345+9G>A in a patient with AD.
Another interesting finding are two variants in *TREM2* (Triggering Receptor Expressed On Myeloid Cells 2) gene. Both variants were found in patients suffering from bvFTD. p.R161K is a novel variant, D134G is reported as uncertain in ClinVar, although it was found in Nasu-Hakola disease patients [410–412].

4.1.2.2 Variants non-classically associated to carriers' phenotype

Other variants belong to genes that were classically associated to different diseases.
Three variants were found in *AARS2* gene: two known variants were carried by bvFTD patients: p.Arg199Cys was reported to be pathogenic in ClinVar, p.Phe74Tyr was classified as VUS. The variant p.Pro368fs was novel and predicted to be likely pathogenic by bioinformatic tools. It was carried by a patient with AD.
A rare known variant of uncertain significance (p.Glu223Gln) in *GFAP* gene, classically associated to Alexander's disease [413]was found in a patient diagnosed with bvFTD.
A patient suffering from AD was carrier of a VUS in *EIF2B1* (c.253-27C>T), a gene causing Leukoencephalopathy with vanishing with matter.
In *CSF1R* (Colony stimulating factor 1 receptor) gene, causing hereditary diffuse leukoencephalopathy with spheroids (HDLS), a novel variant predicted as VUS was found in a patient diagnosed with AD.
A known VUS (c.3345+9G>A) was found in *DCTN1,* gene responsible for Perry Syndrome and ALS, in a patient with AD.
A patient diagnosed with Lewy Body Dementia (LBD) carried the variant p.Asp740Gly in *MAPT* gene.
A patient with Mild Cognitive Impairment (MCI) harboured a mutation in NPC1 (p.Asn222Ser), associated to Niemann-Pick type C disease.
The variant p.Ile131Phe in *PLA2G6* gene was found in another patient with AD.

4.1.2.3 Variant unexpected in the clinical setting

Following clinical exome sequencing, five mutations gained attention since were filtered by the bioinformatics tool being pathogenic or VUS and reported in ClinVar. Two were pathogenic: the first was p.Val208Met in *TYMP* (Thymidine Phosphorylase) gene, carried by an AD patient, the second was c.1184+1G>A in *CYP27A1 (*Cytochrome P450 Family 27 Subfamily A Member 1).
Three mutation were reported as VUS in ClinVar and were of interest as they occur in genes associated to processes possibly involved in AD: p.Glu301del in COL18A1 (encoding for collagen type XVIII), p.Trp1103Cys in *CACNA1G* gene (encoding for CaV3.1 T-type calcium channel) and p.His2686fs in *ITPR1* gene (encoding for an intracellular receptor for inositol 1,4,5-trisphosphate).

4.2 MiRNA expression profile in genetic FTD

To study miRNA expression profile of subjects, I took advantage of OpenArray technology, which allow the detection of 754 target simultaneously. I was able to find an average of 125 miRNA expressed per group.

4.2.1 *C9ORF72* expansion carriers vs Controls

4.2.1.1 MiRNA expression profile

Two miRNA were found to be significantly altered in patients compared to control group (Figure 12). Has-miR-20b and has-miR-223# were upregulated in patients (p-value 0,04347 and 0,03499 respectively).

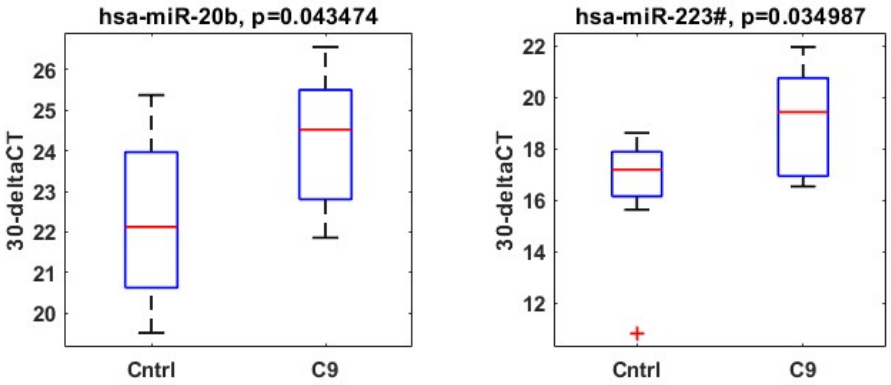


Figure 12. Box plot showing median of 30-ΔCt and min and max value for healthy controls (Ctrl) and *C9ORF72* expansion carriers (C9)

These two miRNAs underwent to PCA and hierarchical clustering (Figure 13 A and B respectively) and the plots show a good clustering, with *C9ORF72* group positioning on the right.

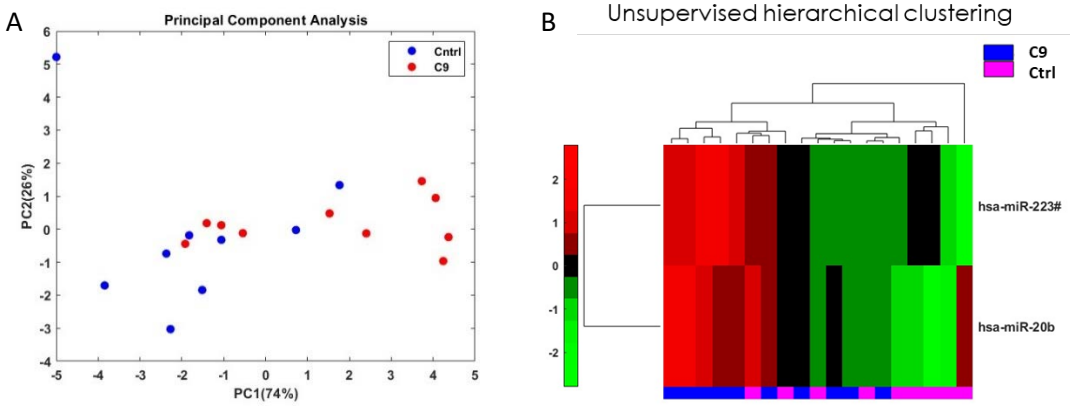


Figure 13. Principal component analysis (A) and Unsupervised Hierarchical clustering (B) showing how patients and healthy subject cluster due to miRNAs signature-expression pattern.

This 2 miRNAs signature predicted patients and controls with 80% sensitivity and 78% specificity [AUC= 0,87 (0,60-0,98)] (Figure 14).

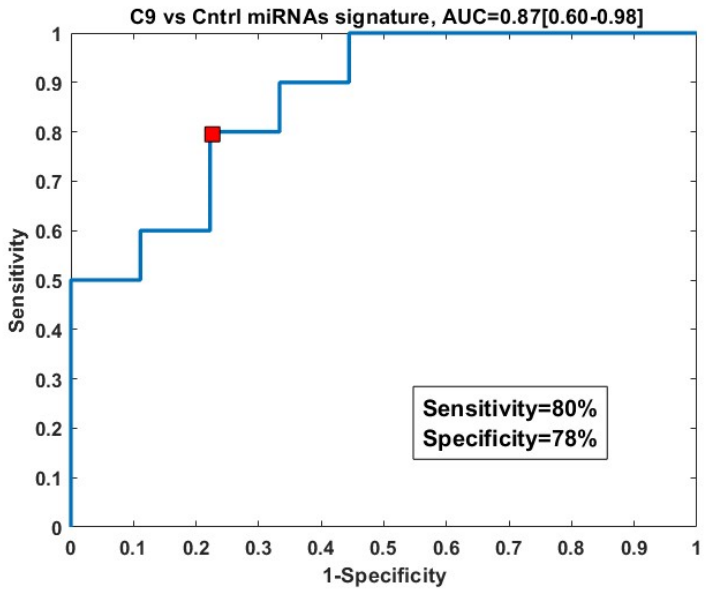


Figure 14. Receiver Operating Characteristic (ROC) curve showing sensitivity and specificity in predicting patients and controls. AUC: area under the curve.

4.2.1.2 Target prediction and pathway enrichment analysis

The figure 15 shows pathways associated to the miRNAs signature. Significance of the association is set at 0.05, and the pathways with higher p-values are at the top of the graph.

For *C9ORF72* signature, beside cell cycle regulation and p53 signalling pathways, in common to the other group, endocytosis appears to associate with high significance.

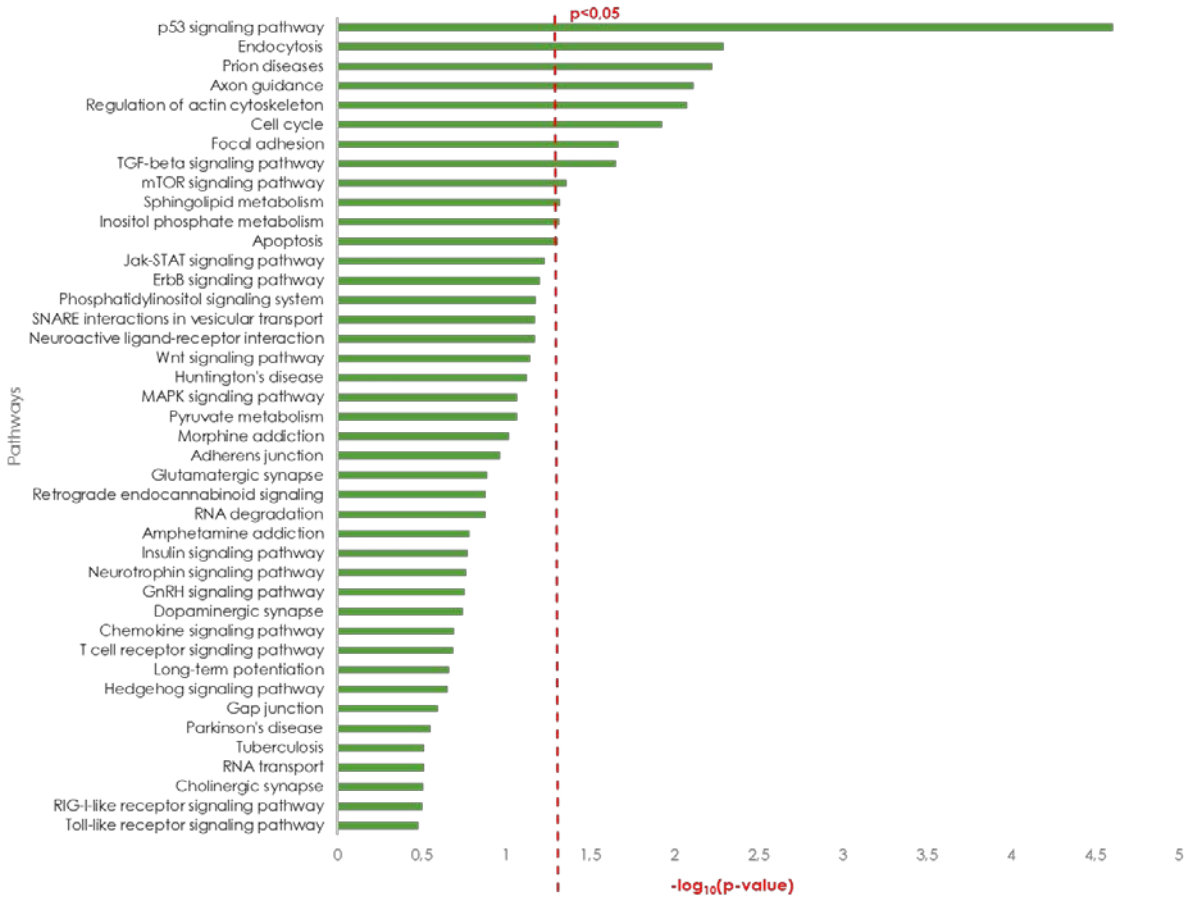


Figure 15. Biological pathway (y-axis) associated to the miRNAs signature. $-\log_{10}$(p-value), expressing the significance of this association, is reported on x-axis. Higher p-value are at the top of the graph.

4.2.2. *GRN* mutation carriers vs Controls

4.2.2.1 MiRNA expression profile

Six miRNA appeared to be significantly altered in patients compared to control group (Figure 16).

Five of them were upregulated in patients, namely hsa-miR-28-3p (p=0,03998), hsa-miR-342-3p (p=0,03147), hsa-miR-365 (p=0,3998), hsa-miR-576-5p (p=0,03998), hsa-miR-642 (0,03998). Conversely, hsa-miR-590-5p was downregulated compared to controls (p=0,02443).

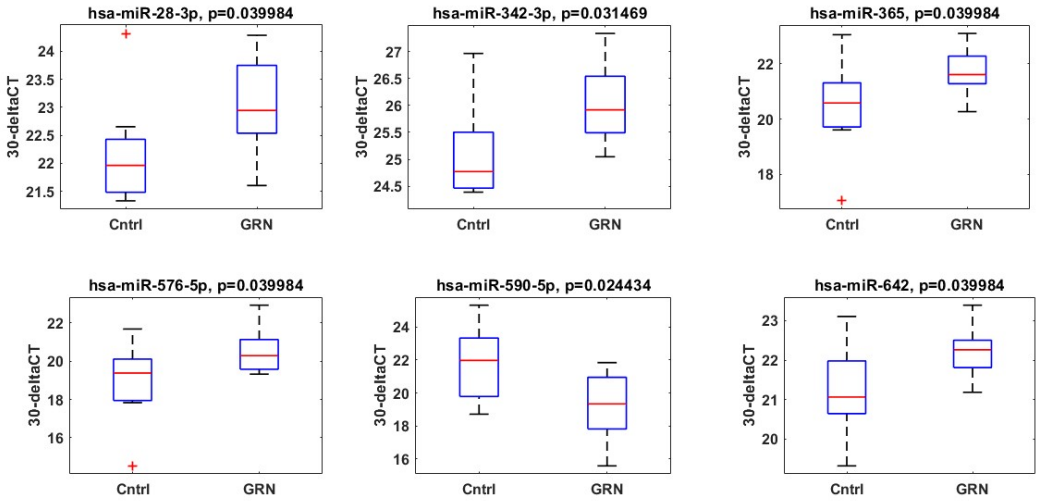


Figure 16. Box plot showing median of 30-ΔCt and min and max value for healthy controls (Ctrl) and *GRN* mutation carriers.

There is a good clustering of the patients and control group, with variability within GRN group, explained by PC2 (Figure 17).

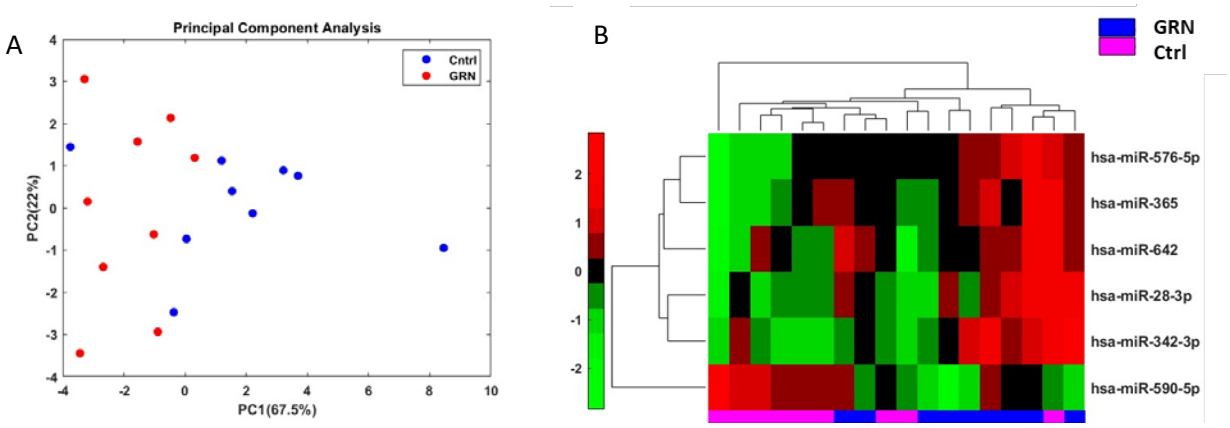


Figure 17. Principal component analysis (A) and Unsupervised Hierarchical clustering (B) showing how patients and healthy subject cluster due to miRNAs signature-expression pattern

Again, this signature predicts patients and controls with high sensitivity and specificity (figure 18)

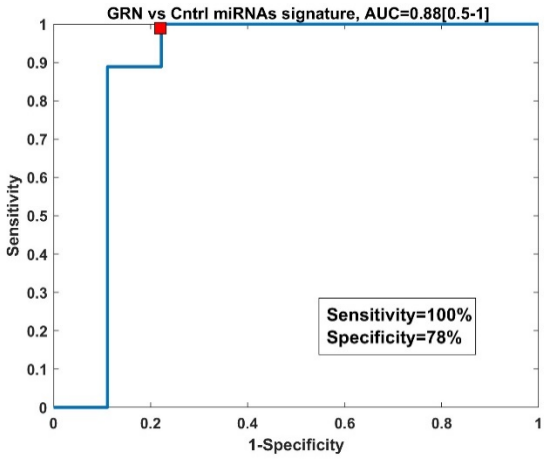


Figure 18. Receiver Operating Characteristic (ROC) curve showing sensitivity and specificity in predicting patients and controls. AUC: area under the curve.

4.2.2.2 Target prediction and pathway enrichment analysis

For *GRN* miRNA signature, target prediction (Figure 19) highlighted significant association with TGF-b, ErbB and neurotrophin signalling. This is in line with the trophic and neuroprotective function of progranulin.

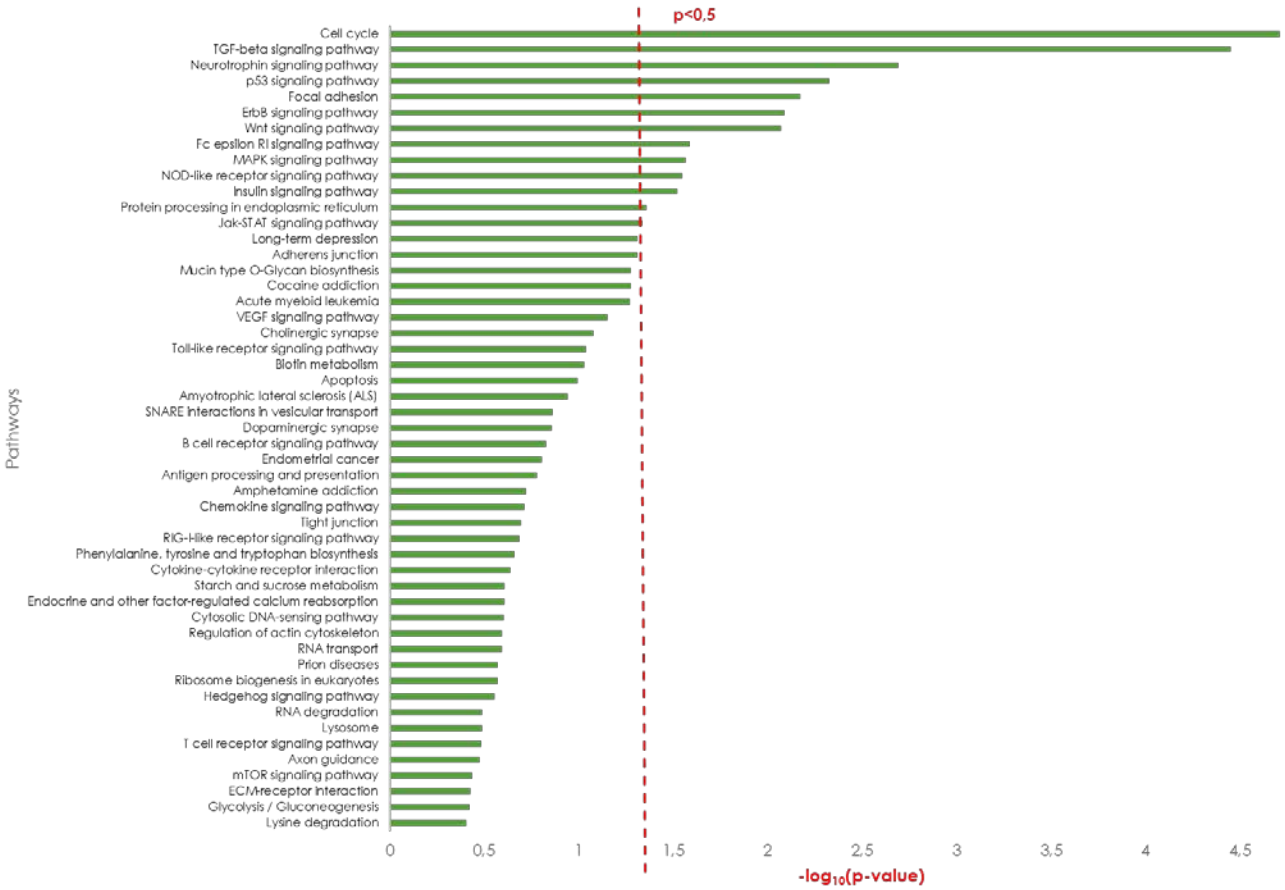


Figure 20. Biological pathway (y-axis) associated to the miRNAs signature. $-\log_{10}$(p-value), expressing the significance of this association, is reported on x-axis. Higher p-value are at the top of the graph

4.2.3 *MAPT* mutation carriers vs Controls

4.2.3.1 MiRNA expression profile

In the comparison between *MAPT* mutation carriers and control subjects, 9 miRNA were found to be differently expressed (Figure 21). Hsa-miR-146a, hsa-miR-192, hsa-miR-25, hsa-miR-28, hsa-miR-28-3p, hsa-miR-30c, and hsa-miR-576-3p were upregulated compared to control subjects (p= 0,01327, p=0,0006, p=0,04347, p=,02201, p=0,01721, p=0,03499, p=0,0222 respectively). Hsa-miR-339-5p and hsa-miR-532-3p were instead downregulated in patients (p=0,022 and p=0,04347, respectively).

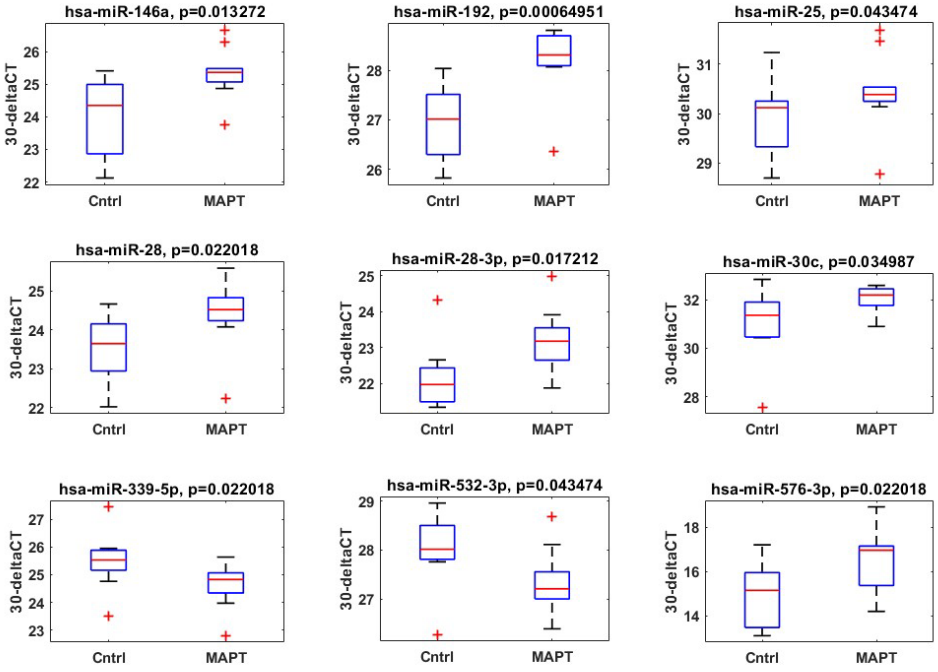


Figure 21. Box plot showing median of 30-ΔCt and min and max value for healthy controls (Cntrl) and *MAPT* mutation carriers

Due to this 9 miRNA signatures, there is an almost complete clustering of the two groups, as shown in figure 22.

A B

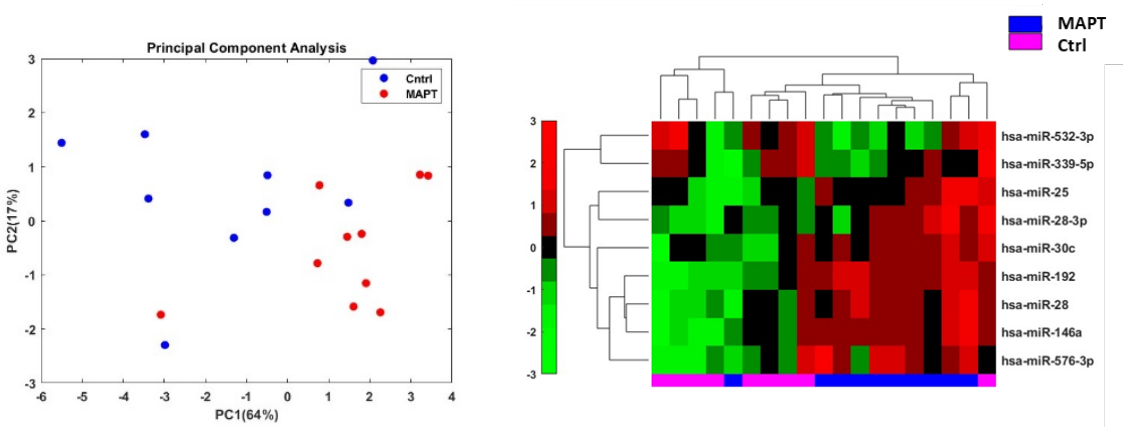


Figure 22. Principal component analysis (A) and Unsupervised Hierarchical clustering (B) showing how patients and healthy subject cluster due to miRNAs signature-expression pattern

It predicts patients and controls with 90% sensitivity and 100% sensitivity [AUC=0,94 (0,68-1)] (figure 23).

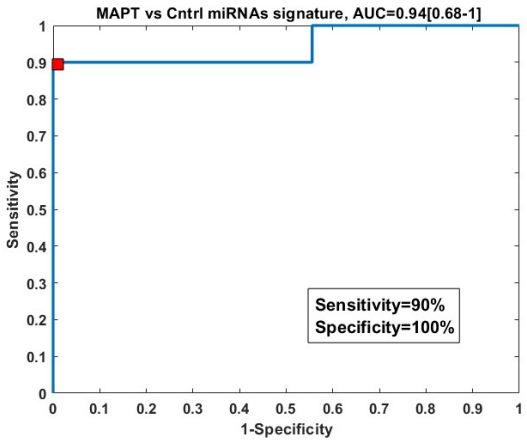


Figure 23. Receiver Operating Characteristic (ROC) curve showing sensitivity and specificity in predicting patients and controls. AUC: area under the curve.

4.2.3.2 Target prediction and pathway enrichment analysis

For MAPT miRNA signature, we can observe several pathways associated with high significance (Figure 24). ErbB signalling pathway and Apoptosis appear to be significant. Of note, compared to the other two group, there is a greater involvement of pathways associated to inflammation, namely TLR-, mTOR-, MAPK signalling pathways.

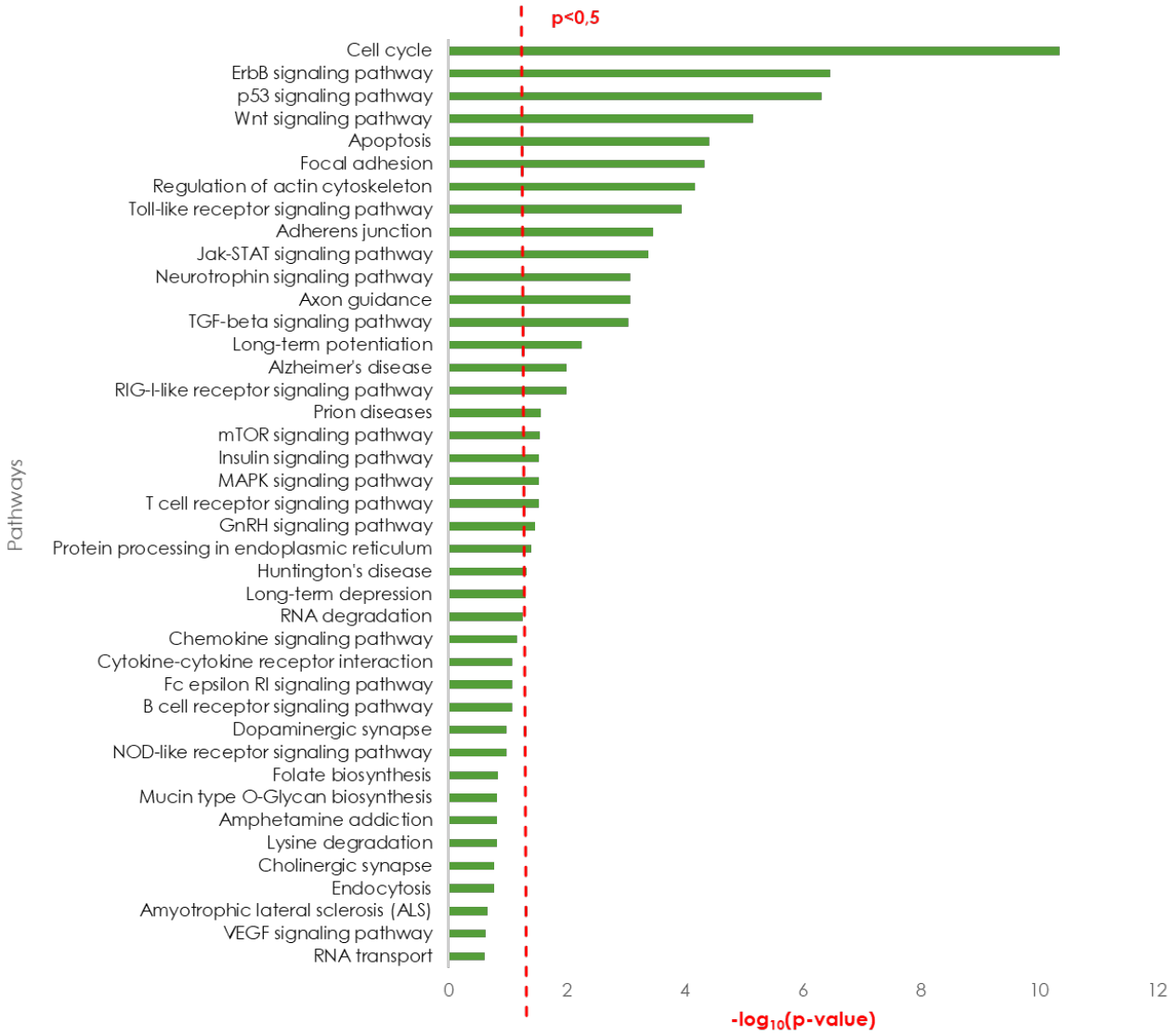


Figure 24. Biological pathway (y-axis) associated to the miRNAs signature. $-\log_{10}$(p-value), expressing the significance of this association, is reported on x-axis. Higher p-value are at the top of the graph.

4.2.4 Evaluation of underlying pathology

From a neuropathological point of view, FTD patients can be grouped in FTD-Tau, comprising carriers of *MAPT* mutations, and FTD-TDP43, comprising C9ORF72 and GRN mutation carriers. In order to find a signature related to the neuropathology underneath, the expression profile of miRNAs in MAPT group and the one of C9ORF72 and GRN carriers jointly were compared with control subjects. Four miRNAs appeared to be statistically significant (Figure 25).

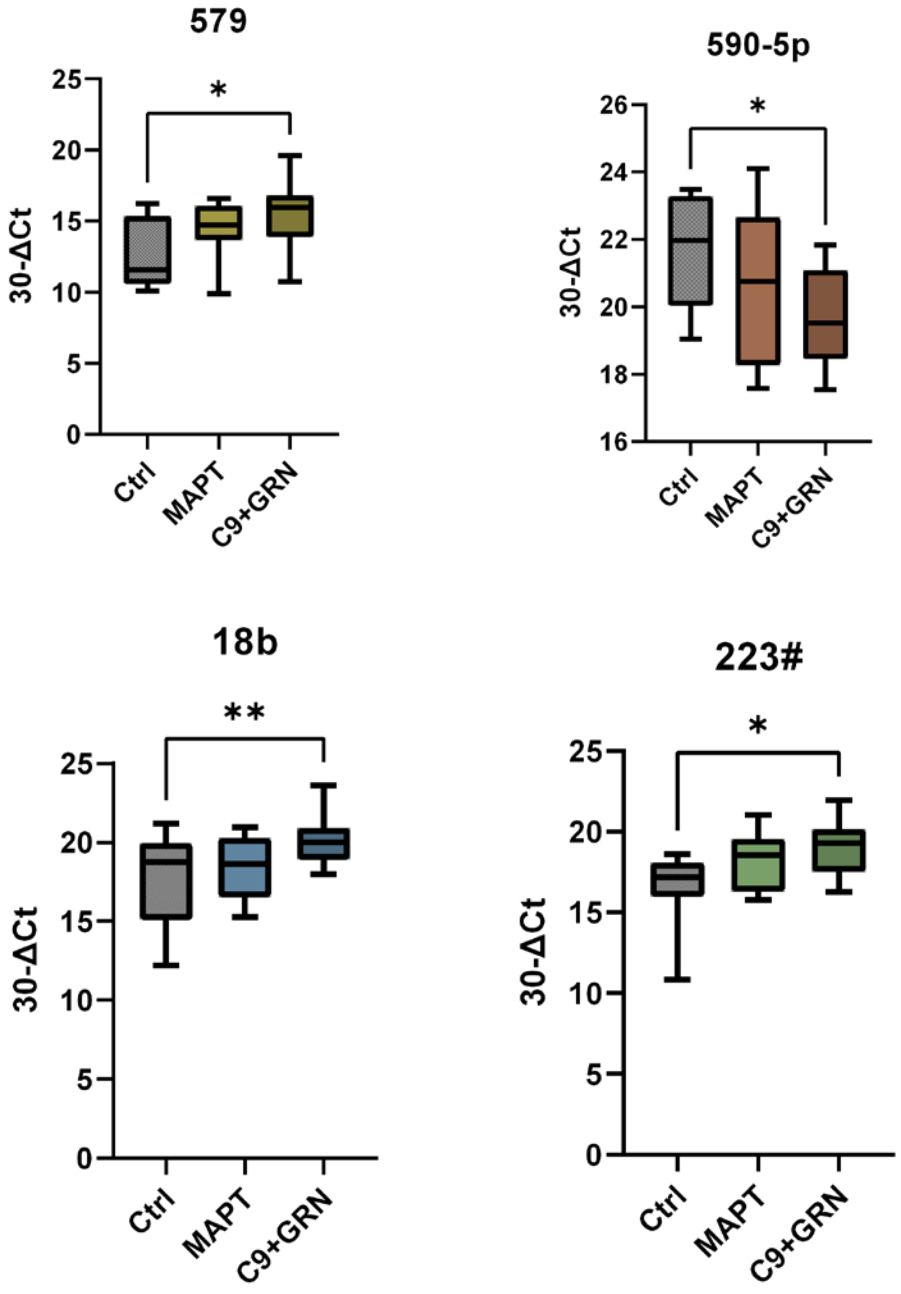


Figure 25. Box plot showing median of 30-ΔCt and min and max value for *GRN+C9ORF72 and MAPT* mutation carriers compared to healthy controls (Cntrl).

4.2.5 Validation of results

Results were validated with single TaqMan probes. Mean relative quantification (RQ) calculated with the $2^{-\Delta\Delta Ct}$ method.

For *C9ORF72* group (figure 26) both miRNAs identified in the discovery phase were validated (hsa-miR-20b p: 0,0373; hsa-miR-2223# p: 0,0307).

A B

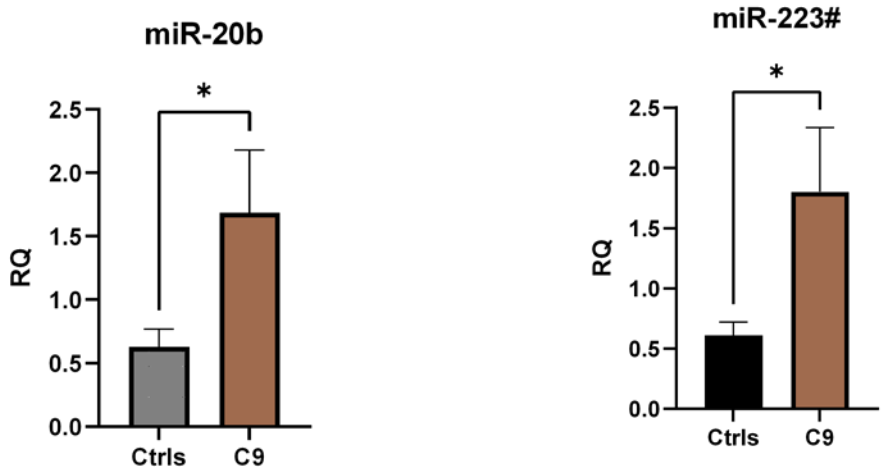


Figure 26. Comparison of mean RQ ± SEM between *C9ORF72* and Control group for hsa-miR-20b (A) and hsa-miR-2223# (B)

Concerning *GRN* signature, five miRNAs were validated (hsa-miR-28-3p, p: 0,0267 ; hsa-miR-365, p: 0,0313; hsa-miR-576-5p, p: 0,0409 ; hsa-miR-590-5p, p:0,0365. Fiure 27A, B,C,D, E). Significance was lost for has-miR-642 (p:0,01936. Figure 27F).

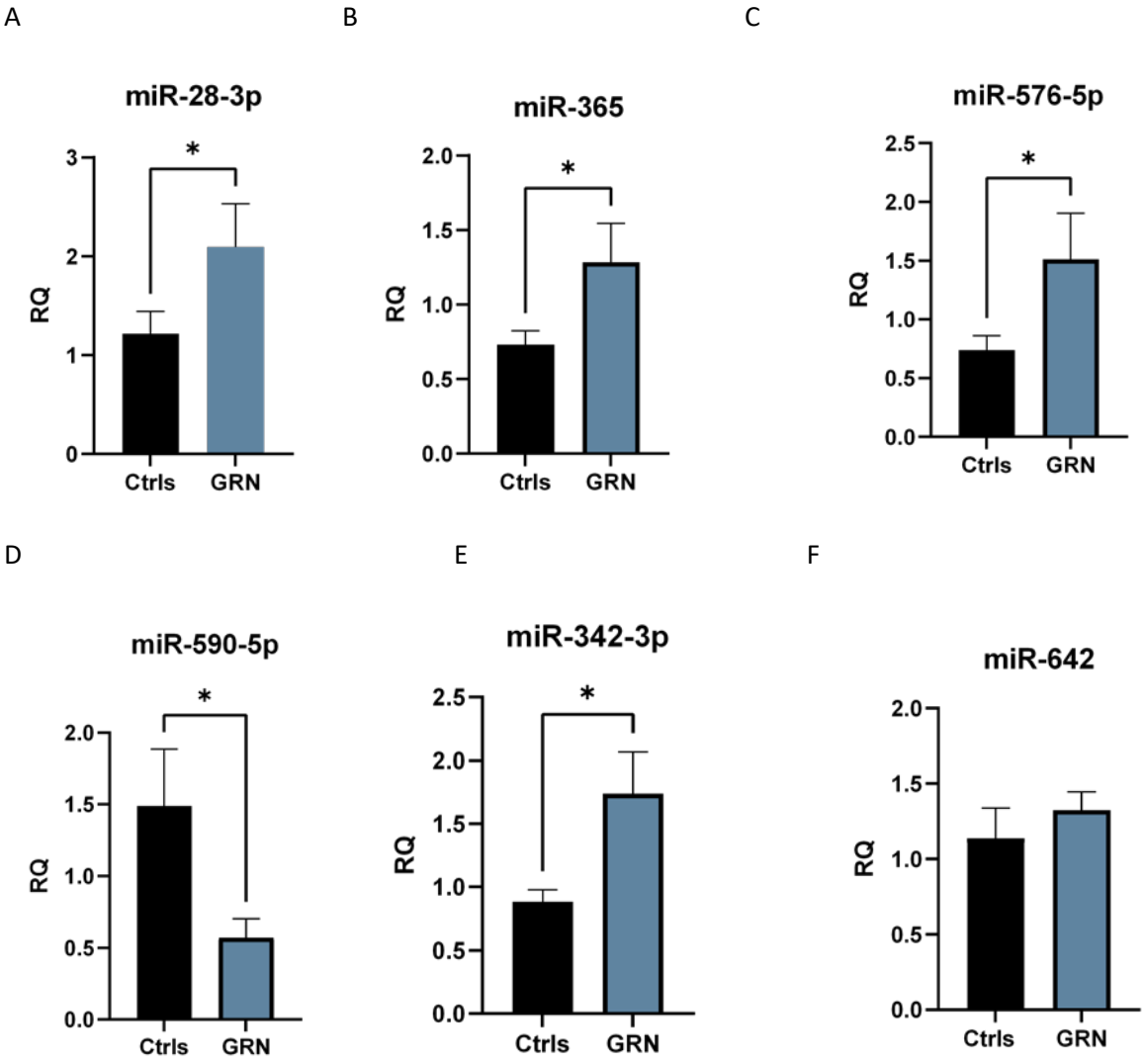


Figure 27. Comparison of mean RQ ± SEM between *GRN* and Control group for hsa-miR-28-3p (A), hsa-miR-365 (B), hsa-miR-576-5p (C), hsa-miR-590-5p (D), hsa-miR-342-3p (E), hsa-miR-642 (F)

For *MAPT* mutation carriers, significance was validated for hsa-miR-146a (p:0,0277), hsa-miR-192 (p:0,0155), hsa-miR-28 (p: 0,0380), hsa-miR-28-3p (p:0,0373), hsa-miR-339-5p (p:0,0391), hsa-miR-532-3p (p:0,0188) and hsa-miR-576-3p (p:0,0028), as shown in figure 33 A-G). the trend from the discovery phase was confirmed for hsa-miR-25 and hsa-miR-30C (p:0,0563 and p: 0,0551, respectively, figure 28 H and I)

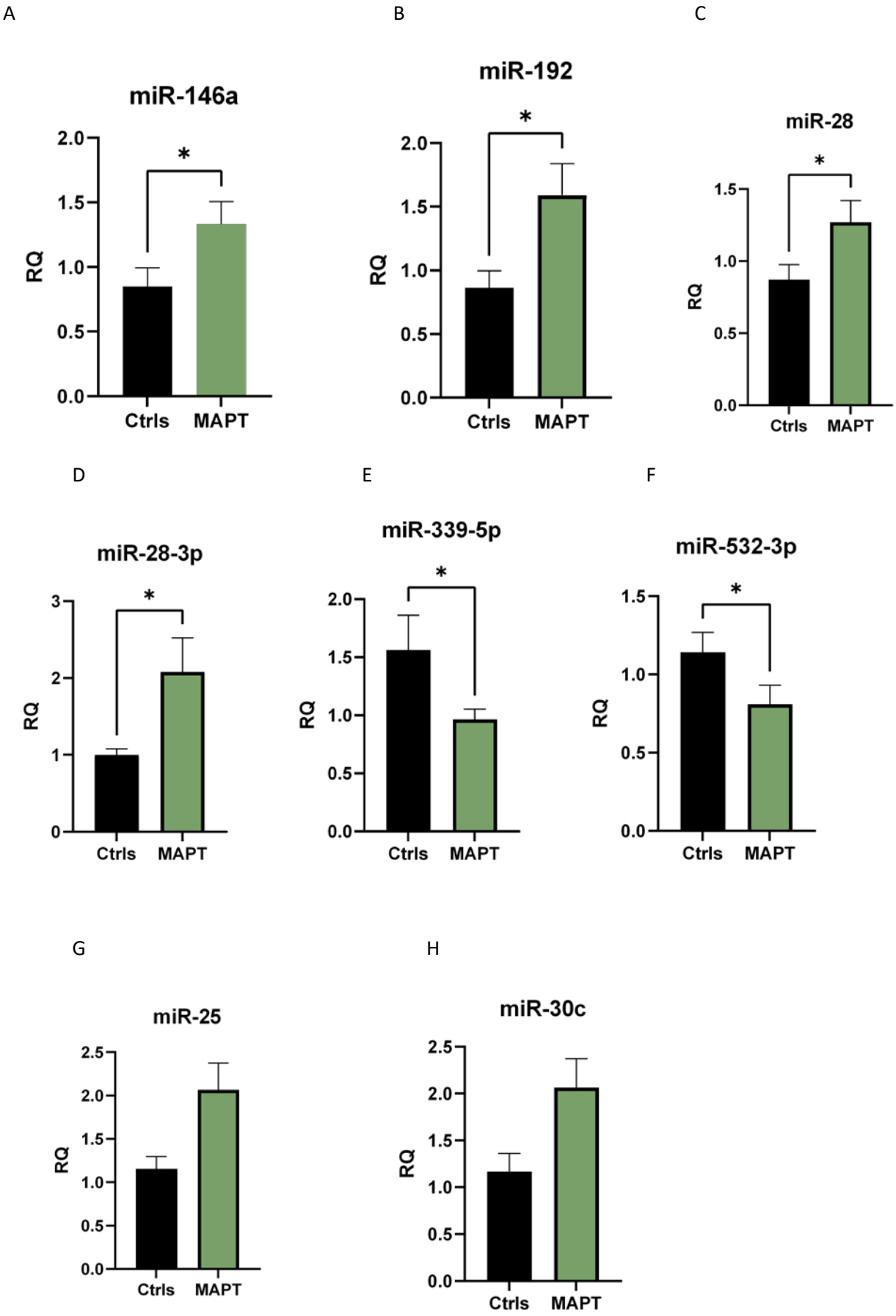


Figure 28. Comparison of mean RQ ± SEM between *GRN* and Control group for hsa-miR-146a (A), hsa-miR-192 (B), hsa-miR-28 (C), hsa-miR-28-3p (D), hsa-miR-339-5p (E), hsa-miR-532 (F), hsa-miR-25 (G), hsa-miR-30c (H)

5. Discussion

Alzheimer's disease and Frontotemporal dementia are two of the most common forms of neurodegenerative dementia. They are complex multifactorial disorders with a strong genetic background. For both the diseases, a familial and a sporadic form is recognised. Most of the cases are sporadic with no or less apparent familial aggregation and late-onset of the symptoms, while familial cases present Mendelian inheritance and predominantly earlier onset age. The dichotomy Familial-Sporadic form is an oversimplification that do not account for the complexity of the disease. There are early-onset cases without evidence for Mendelian transmission while, conversely, late-onset forms are frequently observed with a strong familial clustering, sometimes resembling a Mendelian pattern. Moreover, a considerable proportion of these cases is genetically determined [414,415]. Genetic, epigenetic and environmental factors interplay to influence this complex aetiology. In light of these evidences, this study aimed to explore AD and FTD from a genetic and epigenetic point of view.

For the genetic analysis, I took advantage of Next Generation Sequencing (NGS) technologies, rapid and cost-effective methods enabling the screening of several genes simultaneously. 188 patients were screened with customised genes panels, designed *ad hoc* according to the clinical phenotype.
136 patients with low CSF levels of Aβ or positivity to PET with Aβ tracer and a clear anamnestic family history for neurodegenerative diseases or psychiatric disorder were screened with the NPC panel. Seven of these patients carried mutation in *NPC1* and *NPC2* genes. Since the cohort was well characterized for the ongoing amyloid deposition in the brain, it can be speculated a correlation between the presence of *NPC1* and *NCP2* variants and the pathogenic amyloid cascade, leading to neurodegeneration. Regarding the clinical phenotype, beside cognitive impairment, which was one of the main inclusion criteria, five of seven carriers developed psychosis (70%) as compared with non-carriers (43%), mainly delusions, and two had extrapyramidal symptoms (28%, compared with 10% in non-carriers). Three out of four known variants found in the present study were previously found in the heterozygous status in three genetic screening conducted independently in cohorts of patients from different European countries affected by neurodegenerative diseases. In particular, the p.V30M mutation was found in one patient with PD [406] and one with CBS [405]. The c.441 + 1 G>A variant was present in patients diagnosed with PD, FTD [406], and CBS [405] and in a patient with psychosis [407]. This could suggest that *NPC* heterozygosity might promote neurodegeneration in combination with other environmental or genetic factors. Carriers account for 5.2% of the whole cohort. Although not statistically significant, the number of carriers is higher than in healthy subject cohort (1,3%). This result is in accordance with evidence previously reported in the Exome Variants Project (EVP, 2%) and the recent paper by Bremova and colleagues [416] (*NPC* heterozygosity of 1:200 in the general population)

and in line with previous studies in cohorts of patients with neurodegenerative or psychiatric disorders [405–407].

Following Dementia panel and clinical exome sequencing, both predictable and unconventional variants were found.

Two patients suffering from AD carried two variants in PSEN2. One of this, Thr18Met variant was first discovered in a PD patient interestingly presenting a CSF profile comparable with AD risk.

A patient with FTD displayed a variant in *GRN*, previously reported by Bartoletti-Stella and colleagues in a patient with reduced levels of plasma progranulin [417]. Interestingly the patient also carried a mutation in *Glucocerebrosidase (GBA).* The gene encodes for a lysosomal membrane protein that cleaves the beta-glucosidic linkage of glycosylceramide, an intermediate in glycolipid metabolism. GBA mutations cause the autosomal-recessive lysosomal storage disorder Gaucher's disease, but in the heterozygous status represent the most common risk factors for Parkinson's disease. D409S variant is a relatively common mutation in PD patients [418].

In a bvFTD patient, a VUS was found in *TBK1* gene, associated to FTLD-ALS spectrum. The gene encodes a multifunctional kinase regulating a number of cellular processes, including the innate immune system and inflammation, autophagy, and cell proliferation [290].

Both AD and FTD patients displayed variants in *SQSTM1*, *SORT1 and DCTN1.*

SQSTM1 encodes for a multifunctional protein that binds ubiquitin and regulates activation of the nuclear factor kappa-B (NF-kB) signalling pathway. Mutations in this gene result in sporadic and familial Paget disease of bone but are also proven to influence susceptibility to FTD [291].

SORT1 is a known FTD risk factor and has been proposed to be involved in AD pathogenesis as well [419]: the encoded protein is a neuronal receptor involved in intracellular protein transport and cellular signal transduction and in particular of progranulin [286].

DCTN1 encodes a subunit of dynactin, a microtubule-binding protein involved in the transport of molecules. Mutations in this gene are associated to Perry Syndrome, a brain disease characterized by parkinsonism, psychiatric changes, weight loss and hypoventilation[420]. Variants in this gene have been reported FTD/ALS patients [421,422]. Hori and colleagues reported variants in this gene also in elderly Japanese dementia patients [421,422].

Two bvFTD patients carried variants in *TREM2*. This gene encodes a membrane protein that forms a receptor signalling complex with the TYRO protein tyrosine kinase binding protein. The encoded protein functions in immune response and may be involved in chronic inflammation by triggering the production of constitutive inflammatory cytokines. Mutations in this gene are responsible for Nasu-Hakola disease. Interestingly, both homozygous and heterozygous mutations in *TREM2* have been found in patients with FTD-like phenotypes but without any bone-associated symptoms [423].

Interestingly, a patient diagnosed with LBD carried a VUS in MAPT. This variant was previously reported in an Italian patient with lower motor neuron disease [424]. MAPT variants are not frequently related to LBD: some studies report an association with *MAPT* H1 haplotype [425], and only the paper form Orme and colleagues reported a LBD patient harbouring a variant in *MAPT* gene [426]

VUS were found in genes classically associated to other phenotypes (*AARS2, GFAP, PLA2G6, EIF2B1, NPC1 a*nd CSF1R) but included in the screening as they can cause dementia. Although the clinical characteristics of the carriers do not reflect the phenotypes of such diseases, the presence of variants in these genes could indicate common pathways possibly involved in dementia aetiology.

AARS2 encodes for alanyl-tRNA synthetase 2, a mitochondrial enzyme that specifically aminoacylates alanyl-tRNA. Defects in this gene have been associated to two very different phenotypes: early-onset cardiomyopathy and late-onset leukoencephalopathy, the latter associated with a variety of clinical phenotypes dominated by dementia, psychiatric changes, movement disorders, and upper motor neuron signs [427].

CSF1R gene is responsible for hereditary diffuse leukoencephalopathy with neuroaxonal spheroids, and have been also found in patients with sporadic FTD [428].

The novel variant found in PLA2G6 falls in a domain mutated in late-onset presentation of phospholipaseA2-associated neurodegeneration, a disease presenting with ataxia and cognitive decline [429].

The variant in NPC1 found in a patient with MCI was also reported by Cupidi and colleagues in CBS patients [405].

Following clinical exome sequencing, five mutations gained attention since were filtered by the bioinformatic tool being pathogenic or VUS and reported in ClinVar. They were found in patients with AD.

CAGNA1G and *ITPR1* call into play Calcium homeostasis. The first encodes for a T-type calcium channel, the latter for a receptor for inositol 1,4,5-trisphosphate, implicated in calcium signalling. Ca2+ is a tightly regulated second messenger that is crucial for normal neuronal function. By binding to proteins, Ca2+ modulates different neuronal processes, such as energy production, survival and death, and plays an important role in learning and memory. A study by Rice and colleagues showed that the expression of CAGNA1G and of genes encoding inositol triphosphate receptors (IP3R) is altered in several brain regions with aging [430]. *Cacna1g* expression appears to be significantly decreased in the AD brain compared with aged, non-demented controls. Moreover, they proved that in an animal model, blockade of this calcium channel increases the Aβ production. Moreover, in animal studies, PSEN2 mutations modulate kinetics of inositol 1,4,5-trisphosphate-mediated calcium signals [431].

CYP27A1 encodes a member of the cytochrome P450 superfamily of enzymes, which is important for overall cholesterol homeostasis. Mutations in this gene cause cerebrotendinous xanthomatosis, a rare autosomal recessive lipid storage disease presenting in the childhood and causing progressive cognitive decline. The

gene synthesize 27-hydroxycholesterol (27-OHC), an oxidative derivative of cholesterol, which was proven by Wang and colleagues to affect learning and memory in mice, through tau phosphorylation [432]. Interestingly, 27-OHC shows high concentration in plasma of AD patient [433].

COL18A1 encodes for a type of heparan sulfate proteoglycans, molecules involved in several aspects of the pathogenesis of AD, since they have the ability to enhance Aβ fibrillization. In particular, collagen XVIII appears to be associated with vascular amyloid depositions and senile plaques in Alzheimer's disease brains [434].

TYMP gene encodes an angiogenic factor, which promotes angiogenesis in vivo and stimulates the in vitro growth of a variety of endothelial cells. It has a highly restricted target cell specificity, acting only on endothelial cells. Mutations in this gene have been associated with mitochondrial neurogastrointestinal encephalomyopathy. The mutation found here was reported by Martì and colleagues in a patient presenting impaired shot-term memory and inappropriate behaviour [435].

The second part of the project focused on the study of expression profile of miRNAs in a cohort of genetic FTD patients. To this purpose, I employed the OpenArray technology, which allowed the simultaneous detection of 754 miRNAs. Analysis was performed comparing three groups of mutation carriers, namely *C9ORF72*, *GRN* and *MAPT* groups, to control subjects.

For each group, the expression levels of a set of miRNAs were found to be significantly altered compared to control group. In particular, two miRNAs (has-miR-20b and has-miR-223#9) were found upregulated in *C9ORF72* group; in *GRN* mutation carriers five miRNAs (hsa-miR-28-3p, hsa-miR-342-3p, hsa-miR-365, hsa-miR-576-5p and hsa-miR-642) were overexpressed, one (hsa-miR-590-5p) was downregulated. In MAPT mutation carriers, seven miRNAs were overexpressed (hsa-miR-146a, hsa-miR-192, hsa-miR-25, hsa-miR-28, hsa-miR-28-3p, hsa-miR-30c, hsa-miR-576-3p), and two (hsa-miR-532-3p and hsa-miR-339-5p) were downregulated. These specific miRNA signatures predicted patients with high sensitivity and specificity. Pathways analysis was performed for each signature, and several biological processes have been highlighted with high significance association. Some were in common among the three groups, while others were more specifically associated to a mutated gene.

p53 signalling and cell cycle pathways appeared to be in common among the three group, associated with high significance. p53 is a transcription factor, critical for many important cellular functions involved in genome integrity, including cell cycle control, DNA damage response, and apoptosis [436]. The level and transcriptional activity of p53 massively increase in cells undergoing different types of stress including oxidative stress, DNA damage, telomere erosion or ribosomal stress [437]. p53 has previously been implicated in neurodegeneration. A significant increase in p53 levels and activity were detected in post-mortem CNS tissues of patients with ALS as well as in other neurodegenerative diseases, including Alzheimer

disease, Parkinson disease and Huntington disease [438]. Recently, Nof and colleagues associated p53 to C9ORF72 mutations, since they proved the ability of p53 reduction to rescue C9ORF72 mouse and fly models as well as iPSC motor neurons derived from C9ORF72 ALS patients, suggesting that p53 is one of the drivers of neurodegeneration caused by C9ORF72 mutations [439].

Concerning C9ORF72 group, endocytosis appeared to be a specific pathway. Endocytosis is pivotal process involved in cellular trafficking of molecules, influencing cell signalling and nutrient uptake. In neurodegeneration, dysfunction in endocytic membrane trafficking is a recurrent theme, often accounting for abnormal protein deposition. As reviewed by Tang, C9orf72 interacts with multiple members of the Rab small GTPases family, consequently exerting important influences on cellular membrane traffic and the process of autophagy. Its loss can impair endocytosis in neuronal cell lines, and attenuated autophagosome formation [440].

For GRN miRNA signature, target prediction highlighted significant association with TGF-β, ErbB and neurotrophin signalling. This is in line with the trophic and neuroprotective functions of progranulin.

TGF-β family is a group of pleiotropic cytokines with important neuroprotective functions. It is known to synergize with neurotrophins to protect neurons against insults and maintain neuronal health [441]. ErbB is a family of receptor tyrosine kinases through which neurotrophins exert their function [442].

For MAPT miRNA signature, several pathways can be observed, associated with high significance. ErbB signalling pathway and Apoptosis appear to be significant. Of note, compared to the other two group, there is a greater involvement of pathways associated to inflammation, namely TLR-, mTOR-, MAPK signalling pathways. Recently, inflammation is emerging as an important process in the pathology of FTD, although the timing and exact contribution to disease pathogenesis remains unclear. As reviewed by Bright and colleagues, several genes involved in FTD pathogenesis have also been implicated in neuroinflammation, indicating that it might contribute directly to the disease process in FTD rather than being secondary to neurodegeneration. [443]. Recent works tried to assess association with *MAPT*, finding a correlation between mutation/overexpression in the gene and neuroinflammation in animal models [444,445]

A further analysis was performed comparing MAPT and C9ORF72+GRN groups to control subjects, in order to identify miRNAs specific for the underlying pathology. Four miRNAs reached statistical significance.

Three (has-miR-579, has-miR-223# and has-miR-18b) were significantly overexpressed and one (has-miR-590-5p) was downregulated in C9ORF72+GRN group compared to controls. No statistical differences were found between *MAPT* and control groups. Therefore, it can be speculated that the dysregulation of these miRNAs could be involved in the neuropathological differences between the two groups.

6. Conclusions

Alzheimer's disease and Frontotemporal dementia are two of the most common forms of dementia, causing a progressive and irreversible cognitive decline. As the population ages, these pathologies are becoming a major global health issue and a burden for society. Therefore, is critical to deepen our knowledge of these types of pathologies, meeting the need for early diagnosis and effective treatments.
This project focused on the study of AD and FTD using genetic and epigenetic approaches.

For the first part of the project, a NGS methodology was used to explore the genomic background of dementia patients. Of 188 patients screened, 36 carried pathogenic mutations or variants of uncertain significance. Some of these variants occurred in causative genes or in genetic risk factors associated to AD and FTD *(GRN, PSEN2, SQSTM1, TREM2,* ect.). Other genes were classically associated to other phenotypes (*PLA2G6, AARS2, CSF1R*). Moreover, variants in genes unexpected in the clinical setting provide links to biological process that need to be further explored, as the case of the calcium signalling (*CAGNA1G* and *ITPR1).* The presence of an important family history for dementia in the population screened prompted me to further investigate the genetic background of this patients, searching for rare sequence variant that could further contribute to understanding the aetiology of dementias. The heterogeneous nature of the variants found in this study, both in AD and FTD patients, confirms the complex genetic background of these pathologies. It can be speculated that many genetic variations of small effect interact to increase risk of dementia, interplaying with epigenetic and environmental factors.

The second part of the project was based on the analysis of the expression profile of miRNAs in genetic FTD. Genetic cases have been chosen as gold standard for this study since the underlying pathology is known due to the occurrence of a mutation. A specific signature of miRNAs has been found, which can distinguish patients from healthy subjects with high sensitivity and specificity. This signature is different for each genetic group and therefore is able also to predict the underlying pathology. These findings have potential to be translated to non-genetic cases for whom is impossible to predict the pathology underneath in vivo and therefore is predicted the early enrollment of patients in clinical trials based on intervention on the protein deposition.

The advances in genomic and epigenetic technologies gave great impulse to the understanding of the molecular mechanisms underlying neurodegenerative dementia. Due to NGS techniques, new associated loci and a large number of variants of uncertain significance are being identified and in this scenario is crucial the translation of this genetic knowledge into the understanding of the affected molecular mechanisms. To this purpose, studying the expression profile of miRNAs and the interaction with targeted genes would give new

insights into the pathogenic processes that took place in these diseases. In this scenario, miRNAs would have great potential also as therapeutic target, hopefully in the next future.

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