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Next Generation Sequencing Analysis in Early Onset Dementia Patients

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Abstract.
Background: Early onset dementias (EOD) are rare neurodegenerative dementias that present before 65 years. Genetic factors have a substantially higher pathogenetic contribution in EOD patients than in late onset dementia.
Objective: To identify known and/or novel rare variants in major candidate genes associated to EOD by high-throughput sequencing. Common-risk variants of apolipoprotein E (*APOE*) and prion protein (*PRNP*) genes were also assessed.

[1]These authors contributed equally to this work.

[2]Data used in preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (http://adni.loni.usc.edu). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of ADNI investigators can be found at: http://adni.loni.usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf

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Methods: We studied 22 EOD patients recruited in Memory Clinics, in the context of studies investigating genetic forms of dementia. Two methodological approaches were applied for the target-Next Generation Sequencing (NGS) analysis of these patients. In addition, we performed progranulin plasma dosage, *C9Orf72* hexanucleotide repeat expansion analysis, and *APOE* genotyping.

Results: We detected three rare known pathogenic mutations in the *GRN* and *PSEN2* genes and eleven unknown-impact mutations in the *GRN*, *VCP*, *MAPT*, *FUS, TREM2,* and *NOTCH3* genes. Six patients were carriers of only common risk variants (*APOE* and *PRNP*), and one did not show any risk mutation/variant. Overall, 69% ($n = 9$) of our early onset Alzheimer's disease (EAOD) patients, compared with 34% ($n = 13$) of sporadic late onset Alzheimer's disease (LOAD) patients and 27% ($n = 73$) of non-affected controls (ADNI, whole genome data), were carriers of at least two rare/common risk variants in the analyzed candidate genes panel, excluding the full penetrant mutations.

Conclusion: This study suggests that EOD patients without full penetrant mutations are characterized by higher probability to carry polygenic risk alleles that patients with LOAD forms. This finding is in line with recently reported evidence, thus suggesting that the genetic risk factors identified in LOAD might modulate the risk also in EOAD.

Keywords: Alzheimer's disease, common variants, early onset dementia, frontotemporal dementia, Lewy body dementia, next generation sequencing, rare mutations

INTRODUCTION

The term early onset dementias (EOD) refers to a group of progressive neurodegenerative diseases, e.g., Alzheimer's disease (AD), frontotemporal dementia (FTD), or dementia with Lewy bodies (LBD), affecting individuals aged between 45 and 65 years, and it represents roughly 5% of dementia cases [1]. The symptoms of EOD are similar to those of late onset AD (LOAD) and FTD. However, EOD is thought to be more severe and typically causes a rapid decline in health [2, 3].

Both AD and FTD are pathologically heterogeneous disorders, characterized by a complex genetic architecture that is not yet completely understood. The heritability rates of the different dementia subtypes range from 40 to 80% with EOD showing a higher genetic component than late-onset dementia (for review [4]).

AD is clinically characterized by memory impairment and pathologically by the presence of amyloid-β (Aβ) peptide (the precursor of which is encoded by the *APP* gene) plaques and intraneuronal tangles of hyperphosphorylated forms of tau (a microtubule-associated protein encoded by the *MAPT* gene). The risk AD spectrum is composed of Mendelian genetic traits, genetic population risk factors (susceptibility genes), and nongenetic risk factors such as low cognitive reserve and head trauma [5, 6]. The apolipoprotein E gene (*APOE*) ε4 allele is a known population risk factor [7] that has been found to increase the risk of early onset AD (EOAD) [8]. Since its discovery, over 550 susceptibility genes have been suggested to increase the risk of AD [9], though the impact of most of these genes seems to be much lower than that of *APOE* [10, 11]. In particular, the common variants with small individual effects jointly modify the risk and age at onset of AD and dementia, showing a stronger effect in carriers homozygous for APOE ε4 [12].

Three genes have been identified to carry causative mutations for familial EOAD: amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) (for review [11]). The estimated mutation frequencies of these three genes are 1% for *APP*, 6% for *PSEN1*, and 1% for *PSEN2*. Together, they explain a genetic background of only 5–10% of EOAD patients, leaving a large group of autosomal dominant pedigrees genetically unexplained (for review [13]). This finding suggests that additional causal genes remain to be identified.

FTD is characterized by personality changes, language impairment, and deficits of executive functions associated with frontal and temporal lobe degeneration. At least nine autosomal dominant genetic traits have been associated with this pathology: mutations in *MAPT*, in the progranulin gene (*GRN*), and in the hexanucleotide repeat expansion *C9orf72* genes are the most common, with the highest prevalence of *GRN* mutations found in populations of northern Italy [14–17]. *GRN* null mutations cause protein haploinsufficiency, leading to a significant decrease in the circulating progranulin levels in plasma, serum, and cerebrospinal fluid (CSF) of mutation carriers [18–20]. Mutations in valosin-containing protein (*VCP*), TAR DNA-binding protein 43 (*TARDBP*), charged multivesicular body protein 2B (*CHMP2B*), fused in sarcoma (*FUS*), dynactin (*DCTN1*), and triggering receptor expressed on myeloid cell (*TREM2*) are rarer causes of this pathology [4, 21]. Mutations in

VCP [22], *TARDBP* [23], and *TREM2* [24] have been observed in Italian families with a history of FTD.

Interestingly, mutations in some of these genes, such as *MAPT*, *GRN*, and *C9orf72* have also been detected at low frequencies in AD patients, supporting the notion that a genetic heterogeneity exists for these diseases and that both diseases could form an AD-FTD disease continuum (for review [13]). An AD-like phenotype has also been described with the presence of a nonsense mutation in the prion protein gene (PRNP p.Q160*), which is responsible for inherited neurodegenerative spongiform encephalopathies [25]. In addition, the common coding polymorphism, methionine (M) to valine (V) at position 129 (M129V) in *PRNP* has been associated with EOAD, where the risk is higher for the VV genotype and is increased in patients with a positive family history [26].

The recent development of extremely powerful, massively, parallel DNA sequencing technologies allows for the systematic screening of individual genomes for DNA sequence variations at base-pair resolution, enabling researchers to address the missing hereditability question and, thus, to uncover novel and/or potentially pathogenic rare variants in candidate genes. As previously documented [27–29], targeted re-sequencing of a clinically significant gene panel may represent a powerful and cost-time-effective technique compared to the previously used sequential Sanger sequencing.

Recently, Cruchaga et al. [30], confirmed that the genetic factors identified in LOAD modulate the risk also in EOAD cohorts, where the burden of these risk variants is associated with familial clustering and earlier onset of AD. In the present study, we estimated the genetic load in EOAD and LOAD, by identifying known and novel, both rare and common risk variants in candidate genes. We applied next generation sequencing (NGS) analysis in a selected retrospective cohort of Italian EOD patients and compared the frequencies of variants found with those estimated in samples from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database.

MATERIALS AND METHODS

Participants

A retrospective sample of patients was recruited in the context of studies investigating genetic forms of dementia at IRCCS Istituto Centro San Giovanni di Dio Fatebenefratelli, Brescia Italy, Fondazione Casa Serena, Pontoglio, Brescia, Italy, and Fondazione Europea Ricerca Biomedica, Centro di Eccellenza Alzheimer, Ospedale Briolini Gazzaniga, Bergamo, Italy. Specifically, twenty-two patients fulfilled the following inclusion criteria for the present study: 1) phenotype of AD, FTD, or LBD and 2) early disease onset (<65 years old), or 3) family history suggestive of an autosomal dominant genetic form of dementia (i.e., high or medium risk of identifying a mutation according to Loy and Woods criteria [31, 32], as described below). Family history was collected through interviews with a first-degree relative or the spouse of the proband. The clinical and medical history of each family member was collected, and all of the available documentation for affected members was acquired. The probability of identifying a genetic mutation for AD or FTD was estimated considering the family medical history, the number of first and second-degree affected family members, and the age of symptom onset, according to the criteria developed by Loy and colleagues [31]. According to Loy et al.'s criteria for AD, we defined a probability of identifying a genetic mutation of ≥86% as a high risk, a probability of 68–85% as a medium risk, a probability of 15–67% as a low risk, and a probability <15% as apparently sporadic/unknown significance. Considering the same criteria for FTD patients, we considered an ≥88% probability of identifying a genetic mutation as a high risk, a 31–41% probability as a medium risk, and a probability <13% as a low risk. FTD pedigrees were also scored according to Wood's pedigrees classifications criteria [32, 33].

All participants were of Italian ancestry. Demographic features and clinical data (age at onset, MMSE) are reported in Table 1. As the sample was retrospectively pooled for the analysis, a standard protocol for biomarker characterization was not applied. Sixteen patients underwent one of the following examinations as part of their diagnostic exam: magnetic resonance imaging (MRI), positron emission tomography (FDG-PET) or single photon emission computed tomography (SPECT), and/or lumbar puncture. MRI and PET/SPECT scans were visually evaluated to determine medial-temporal atrophy and hypometabolism, respectively. CSF samples were processed with local procedures to determine the level of Aβ, tau, and p-tau. Positive diagnosis was determined based on established cut-offs.

Blood samples were collected from all patients. DNA and plasma were obtained according to standard procedures. Patients provided written informed

Table 1
Demographic, clinical features, and presence of rare/common risk variants in candidate genes in early onset dementia

Code	gender	age at onset	Clinical diagnosis	Genetic risk	MMSE	Ab	tau	p-tau	Atrophy	H-M	PRGN (ng/ml)	Gene	cDNA Change	Protein Change	Function	dbSNP	Pathogenetic	SIFT	PP2	LRT	MT	FATHMM	CADD	GERP++	phyloP	APOE	PRNP (M129 V)
4_1	F	60	AD	Apparently sporadic	n.a.	n.a.	n.a	n.a.	n.a.	n.a.	130	TREM2	G185A	R62H	Missense	rs143332484	unknown	T	B	N	N	T	8.315	–0.214	–0.173	ε4/ε4	Met/Met
5_1	M	57	AD	Unknown significance	10/30	+	+	+	+	+	104	GRN	G229A	V77I	Missense	rs148531161	unknown	T	B	N	N	T	10.3	–3.85	–0.864	ε3/ε4	Met/Met
15_1	M	45	AD	Unknown significance	n.a.	n.a	n.a	n.a	n.a.	n.a.	n.a.															ε3/ε4	Met/Val
16_1	M	51	AD	Unknown significance	4/30	n.a.	+	n.a.	–	n.a.	97															ε3/ε4	Met/Met
19_1	F	41	AD	Apparently sporadic	n.a.	+	+	n.a	+	+	111															ε2/ε3	Met/Met
20_1	F	57	AD	Unknown significance	25/30	+	+	n.a	–	+	115															ε3/ε4	Met/Met
21_1	F	48	AD	Unknown significance.	23/30	–	–	+	+	+	97															ε3/ε3	Met/Val
25_1	M	52	AD	Medium to high	26/30	+	–	–	+	+	104	FUS	*41G>A		3'UTR	rs80301724	n.a.									ε4/ε4	Met/Met
26_1	F	50	AD	Medium	14/30	n.a	n.a	n.a	+	+	86	PSEN2	A715G	M239V	Missense	rs28936379	yes	D	P	D	A	D	19.5	4.92	9.213	ε3/ε3	Val/Val
30_1	F	54	AD	Medium	12/30	+	n.a	n.a	–	n.a.	85	PSEN2	G717A	M239I	Missense	rs63749884	yes	D	D	D	A	D	27.8	4.92	9.734	ε3/ε3	Met/Val
												MAPT	*55delA		3'UTR	n.a.	n.a.										
32_1	F	60	AD	Medium	22/30	n.a	n.a	n.a	+	+	102	TREM2	G185A	R62H	Missense	rs143332484	unknown	T	B	N	N	T	8.315	–0.214	–0.173	ε4/ε4	Val/Val
												VCP	–216/–215 insGCTGCC		5'UTR		n.a.										
11_1	F	51	FTD	High	23/30	+/–	–	+	+	n.a.	108	GRN	–3895G>T		5'UTR	rs76783532	n.a.									ε3/ε3	Met/Val
29_1	M	56	FTD	High	n.a.	n.a	n.a	n.a	n.a.	n.a.	39	GRN	811_814del	L271fs	Deletion	n.a.	yes									ε3/ε3	Met/Val
14_1	M	57	LBD	Unknown significance	5/30	n.a	n.a	n.a	n.a.	n.a.	n.a.	TREM2	G259A	D87N		rs142232675	unknown	T	D	D	N	T	12.5	5.51	2.597	ε3/ε3	Val/Val
34_1	F	49	AD	Medium to high	15/30	+	–	–	–	+	149	GRN	C55T	R19W	Missense	rs63750723	unknown	T	B	N	N	T	8.7	0.13	0.17	ε3/ε4	Met/Val
35_12	F	54	AD	Medium	n.a.	n.a.	n.a.	n.a.	+	n.a	n.a.															ε3/ε4	Met/Val
36_1	M	n.a.	FTD-IBMPFD	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	145	VCP	C277T	R93C	Missense		unknown		P	D	M	D	3.8	19.39	2.70	ε3/ε4	Met/Val
37_2	F	62	AD	Low	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	138	NOTCH3	G3547A	V1183M	Missense	rs10408676		D	D		M	D	3.9	3.09	2.32	ε3/ε4	Met/Met
1413pcl	M	62	Familial Dementia	Unknown significance	5/30	n.a	n.a.	n.a.	n.a.	+	98															ε3/ε4	Met/Met
L028	F	63	AD	Low	28/30	+	+	n.a.	–	+	161	NOTCH3	3838-1G>T		Splicing											ε4/ε4	Met/Val
L029	F	62	FTD	High	18/30	n.a	n.a.	n.a.	+	+	n.a.	GRN	811_814del	L271fs	Deletion	n.a.	yes									ε3/ε3	Met/Met
L031	F	64	AD	Medium	4/ 27	n.a	n.a.	n.a.	n.a.	+	n.a.	PSEN2	G717A	M239I	Missense	rs63749884	yes	D	D	D	A	D	27.8	4.92	9.734	ε3/ε3	Met/Val

APOE, Apolipoprotein E (E4 risk allele); PRNP, Prion protein (G/val risk allele); PGRN/GRN, Progranulin; PSEN2, Presenilin 2; TREM2, Triggering receptor expressed on myeloid cell; FUS, Fused in sarcoma; MAPT, Microtubule-associated protein tau; VCP, Valosin-containing protein; A, known damaging; B, benign; D, damaging; N, neutral; P, possibly damaging; T, tolerant; n.a., not available; CADD, CADD phred-scaled (a score of 20 means 1% percentile highest scores of whole genome); dbSNP, single nucleotide polymorphism database (rs number); GERP, genomic evolutionary rate profiling score; GERP++, evolution score; MT, mutation taster; Phylop, phyloP100way vertebrate; PP2, PolyPhen2; SIFT, Sorting Intolerant from Tolerant; FATHMM, Functional Analysis through Hidden Markov Models (v2.3); LRT, Likelihood Ratio Test; AD, Alzheimer's disease; FTD, frontotemporal dementia; LBD, Lewy body dementia; FTD-IBMPFD, Inclusion Body Myopathy-Paget's disease of bone; "Genetic risk" denotes the estimated probability of identifying a genetic mutation base on Loy and Wood criteria for AD and FTD; "Unknown Significance" denotes lack of information about diagnosis or clinical details; "Apparently sporadic" indicates no other affected case in the family; H-M, Hypo metabolism.

consent. This study was approved by the local ethics committee (CEIOC, 62/2013).

NGS panel analysis screening

Genomic DNA was extracted from whole-blood samples with a commercially available kit according to standard procedures (GENTRA Minneapolis, MN, USA).

Due to logistics issues, some samples were analyzed through the use of the Ion Torrent PGM (Thermo Fisher Scientific, Waltham, MA USA) sequencer as NGS platform, by using a candidates genes panel, already described in Beck et al. [27]. Briefly, for library construction, 5 ng of genomic DNA were amplified using the Ion Ampliseq Dementia Research gene panel (Ampliseq™, Thermo Fisher Scientific, Waltham, MA USA), and the Ion Ampliseq™ Library kit 2.0, according to manufacturer's instructions. The generated amplicon library includes *PRNP, PSEN1, PSEN2, APP* (Amyloid Beta A4 Precursor Protein), *GRN, MAPT, TREM2, CHMP2B, CSF1R* (Colony Stimulating Factor 1 Receptor), *FUS, ITM2B* (Integral Membrane Protein 2B), *NOTCH3* (Notch 3), *SERPINI1* (Serpin Peptidase Inhibitor, Clade I (Neuroserpin), Member 1)*, TARDBP, TYROBP* (TYRO Protein Tyrosine Kinase Binding Protein), *VCP, SQSTM1* (Sequestosome 1). Amplicons were ligated to Ion Torrent Barcodes/adapters P1 using DNA ligase. A first step of Agencourt AMPure XP bead (Beckman Coulter Inc., Brea CA, USA) purification was followed by nick-translation of adapter-ligated products and PCR-amplification. A second purification step using AMPure beads was performed and the concentration and size of the libraries were determined using an Agilent BioAnalyzer DNA High-sensitivity LabChip (Agilent Technologies, Santa Clara, CA USA). After dilution to 100 pM, libraries were clonally amplified on Ion sphere™ particles (ISP) by emulsion PCR with the Ion PGM™ template OT2 200 kit on the Ion One Touch 2 instrument according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA USA). ISP were enriched using the Ion One Touch ES module, loaded on an Ion 314 chip kit V2 and sequenced with an Ion Torrent PGM System (Thermo Fisher Scientific, Waltham, MA USA).

The remaining samples were analysed with the Illumina MiSeq platform and the TruSight One Sequencing Panel (Illumina, Inc., San Diego, CA, USA). This panel includes 125,395 probes targeting a 12-Mb region spanning 4,813 genes, among which the genes investigated by Ion Torrent PGM (*PRNP, PSEN1, PSEN2, APP, GRN, MAPT, TREM2, CHMP2B, CSF1R, FUS, ITM2B, NOTCH3, SERPINI1, TARDBP, TYROBP, VCP, SQSTM1*). The data regarding the other all genes were not used. The obtained sequence reads were aligned to the hg19 human reference sequence using the Burrow–Wheeler Aligner (BWA version 0.7.12). Duplicated reads were removed with Picard tools. Local realignment, recalibration, and variant calling were conducted with the Genome Analysis Tool Kit (GATK version 3.30). In order to have comparable results between the two sequencing approaches, we extracted from TruSight One Variant Call Format file (i.e., VCF file), the variants located in the regions sequenced by Ion Torrent PGM panel using BEDTools [34].

APOE genotyping

Genetic variation at the *APOE* locus was determined by using the SNaPshot technique [35]. Briefly, assays for the *APOE* polymorphisms were performed using PCR reactions, which were subsequently combined to perform a single SNaPshot reaction. The amplification assay was designed with the following forward and reverse primers: APOE F: 5' CCAAGG AGCTGCAGGCGGCGCA 3' and APOE R: 5' GCC CCGGCCTGGTACACTGCCA 3'. A product of PCR-amplification was used as a template in the SNaPshot Multiplex assay. The following specific primers were used: SNAP APOE112:5' ACTGCACCAGG CGGCCGC 3' and SNAP APOE158:5'ATGCCGA TGACCTGCAGAAG 3'. Finally, the samples were analyzed, and allele peaks were determined using the ABI 3130xl genetic analyzer and the GeneMapper 4.0 program (Applied Biosystems, Foster City, CA, USA).

C9Orf72 hexanucleotide repeat expansion

PCR sizing of the GGGGCC hexanucleotide repeat was performed using previously published primers [36] on the ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA). The PCR reaction was carried out in a mixture containing 5% dimethylsulfoxide and 7-deaza-2-deoxy GTP in substitution for dGTP. Allele identification and scoring were performed using GeneMapper v4.0 software (Applied Biosystems).

GRN plasma level measurement

Plasma progranulin levels were measured in duplicate using an ELISA kit (Human Progranulin ELISA Kit, AdipoGen Inc., Seoul, Korea).

Statistical and bioinformatics analyses

To classify a variant as rare, its frequency should be lower than 1% in at least one of the three reference databases (1000 Genomes Project http://www.internationalgenome.org/, Exome Sequencing Project http://evs.gs.washington.edu/EVS/ and Exome Aggregation Consortium http://exac.broadinstitute.org) [37].

In order to predict the functional consequences of non-synonymous variations, we exploited eight different bioinformatics tools, namely: SIFT, PolyPhen-2, FATHMM, phyloP, MutationTaster, LRT, and CADD and GERP++ [38–45]. A variant is classified as damaging if for at least three tools the mutation is predicted to be deleterious.

Finally, to evaluate the mutation rate of the candidate genes selected in the NGS panel, we considered the gene damage index (GDI, a genome-wide, gene-level metric of the mutational damage that has accumulated in the general population), according to Itan et al. [46].

ADNI whole genome data

As a genetic replication cohort, we considered whole genome data from the ADNI database (http://adni.loni.usc.edu). From the whole genome data, we extracted the variants within the regions included in our sequencing panel and we applied the same variants annotation and classification performed for our sample.

The ADNI was launched in 2003 as a public-private partnership, led by Principal Investigator Michael W. Weiner, MD. The aim of ADNI project is to collect, validate and utilize heterogeneous clinical and biological data (including MRI and PET images, genetics, cognitive tests, CSF and blood biomarkers) to study the progression of AD. For up-to-date information, see http://www.adni-info.org.

RESULTS

Target screening: Plasma progranulin and C9ORF72 analyses

As a first step, progranulin plasma levels were assayed to screen for *GRN* null mutations (Table 1). One FTD patient was found to have progranulin plasma levels lower than the optimized cut-off value for null mutations detection of 61.55 ng/ml [19, 47]. For 5 samples, it was no possible to detect the progranulin levels, due to the lack of plasma samples from these patients.

None of the EOD patients carried the pathogenic hexanucleotide repeat expansion of *C9ORF72*. All patients were found to have less than 12 repeats [48].

NGS screening: Identification of known and unknown rare variants

Through the target re-sequencing of the 17 candidate genes panel, we identified fourteen rare variants in 68% of the selected EOD cases (15 patients) (Table 1). These variants were defined as "pathogenic" when previously described in the literature, and they were classified as "damaging" by bioinformatic tools or as "unknown impact" when no data were available in literature and no deleterious effect was predicted by bioinformatics tools.

Pathogenic variants

Among the 14 identified rare variants, three were classified as pathogenic and damaging and have been described in Italian pedigrees unrelated to the patients analyzed in the present study. Two variants were located in the *PSEN2* gene (p.M239V = rs28936379 code case: 26_1, [49]; p.M239I = rs63749884 code cases: 30_1, L031, [50]); and one variant was a p.L271fs null mutation in the *GRN* gene (code cases: 29_1; L029) (Table 1).

Finally, a heterozygous R93C mutation of the *VCP* gene was detected in a patient (Table 1, code: 36_1) affected by Paget's disease of bone diagnosed at 44 of age and no signs of dementia at the last examination (47 years). Family history showed multiple individuals with FTD and Paget's disease with autosomal dominant inheritance.

Variants of unknown impact

Two variants in the *TREM2* gene (R62H = rs143332484, D87N = rs142232675, [51]) (Table 1) were observed in three of the 22 patients (14%), including a patient with LBD.

Moreover, in *GRN* gene, we found variants in the 5'UTR (rs76783532) and two rare missense variants (V77I = rs148531161; R19W = rs63750723; Table 1), which have been reported as "pathogenic nature

unclear" and "not pathogenic", respectively, in online database (www.molgen.en.ua.ac.be/FTDmutations). Accordingly, these missense mutations did not influence the progranulin level in plasma (Table 1).

Other variants reported in online databases were those at 3'UTR of the *FUS* (rs80301724) and a missense mutation (V1183M = rs10408676) in *NOTCH3* gene resulting "damaging" according to bioinformatics tools.

Moreover, we detected rare mutations which have not been reported in the literature and databases. In particular, we found a mutation localized in the 5′UTR region and a missense mutation R93C (NM_007126: exon3: c.C277T:p.R93C) in the *VCP* gene, a deletion (NM_001203251:c.*55delA) in *MAPT* gene at 3'UTR region, a splicing variant (NM_000435: exon24: c.3838-1G>T) in *NOTCH3* gene.

Screening of known common variants

All EOD patients were genotyped for the rs429358 and rs7412 polymorphisms in the *APOE* gene and the rs1799990 polymorphism in the *PRNP* gene. *APOE* ε4 and *PRNP* 129Val are known to be risk alleles. The results indicated that the frequencies of the *APOE* ε4 carriers and the *PRNP* 129Val carriers were 59% for both. Twenty (91%) of the 22 subjects carried at least one of the two risk variants; of these subjects, 27% (six out of 22) carried both the *APOE* ε4 and the *PRNP* 129Val risk alleles. Six patients were carriers of at least one risk allele (*APOE* ε4 or *PRNP* 129Val) (Table 1).

One patient did not show any rare or common risk variants (Table 1).

Comparison to whole genome data

We compared and calculated the rare and common risk variants frequencies of the 17 candidate genes in our panel, with the data from the ADNI whole genome sequencing database. In the ADNI database, sporadic late-onset (38 patients), early onset (7 patients) AD cases, and 272 controls subjects were available. In our sample, the results indicated that, excluding the full penetrant mutations present in five patients, 9 patients (69%) showed ≥2 rare/common risk variants, compared with 13 (34%) and 73 (27%) observed in ADNI late-onset AD cases (LOAD) and controls, respectively (Chi-squared test: $\chi^2 = 15.8$, df = 4, $p = 0.003$; Table 2). If we add to our sample, the seven EOAD patients coming from ADNI database (n tot = 20), the percentage of genetic load did not change (65%, $n = 13$, Chi-squared test: $\chi^2 = 17.9$, df = 4, $p = 0.001$; Table 2).

DISCUSSION

This study suggests that EOD patients without full penetrant mutations are characterized by higher probability to carry polygenic risk alleles that patients with LOAD forms. This finding is in line with recently reported evidence [30], thus suggesting that the genetic risk factors identified in LOAD might modulate the risk also in EOAD.

Moreover, we confirmed the role of *GRN* and *PSEN2* genes in EOD, with the involvement of specific rare mutations already known. We also detected additional rare variants of unknown impact, located in the 5'/3' UTRs regulatory gene region of the *GRN*, *VCP*, *MAPT*, and *FUS* genes, missense mutations in *TREM2*, *GRN*, *NOTCH3*, and *VCP*, genes and a splicing variant in *NOTCH3* gene. According to the gene damage index (GDI) [46], mutations in these genes showed a value of "medium", suggesting that these genes are not frequently mutated in healthy populations. This finding further implies that mutations in these genes could be disease-causing.

Rare mutations of known significance

Twenty three percent of the cases carried one pathogenic mutation for dementia. In line with previous studies [14], mutations in the *PSEN2* gene, a rare cause of dementia worldwide, were frequent in our cohort (14%). Looking at the geographical distribution of *PSEN2* mutations described to date, it is noteworthy that 80% of these mutations were uncovered in two southern European countries, Italy and Spain. Thus, we can speculate that the non-homogeneous distribution of pathogenic mutations might be a result of genetic drift.

The *GRN* p.L271fs mutation is one of the most common GRN mutations worldwide. An analysis of this mutation in northern Italy showed that almost all families can be traced to a single founder. The origin of the mutation was dated to the Middle Ages at the turn of the first millennium, which explains the high frequency of this mutation in this geographic area [52].

Rare mutations of unknown significance

The role for novel variants of unknown significance in both common and rare dementia-associated genes

Table 2
Comparison of frequencies of rare (excluding the full penetrant) and common risk variants in our sample (This study) versus controls, late onset and early onset AD patients (LOAD and EOAD, respectively) obtained from ADNI database

		Variants										
		$n=0$	$n=1$				$n\geq 2$					
		total	Only rare	Only common	total	Only rare	Only common	1 rare + 1 common	1 rare + 2 common	2 rare + 1 common	2 rare + 2 common	3 rare + 1 common	Total
Groups	Sample (N)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
	Controls (272)	67 (25)	9 (7)	123 (93)	132 (49)	2 (3)	28 (38)	25 (34)	8 (11)	8 (11)	0 (0)	2 (3)	73 (27)$^{\$,£}$
ADNI	LOAD (38)	3 (8)	0 (0)	22 (100)	22 (58)	0 (0)	10 (77)	2 (15)	0 (0)	1 (8)	0 (0)	0 (0)	13 (34)$^{\$,£}$
	EOAD (7)	1 (14)	0 (0)	2 (0)	2 (29)	0 (0)	3 (75)	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)	4 (57)
This study	AD (13)	1 (8)	0 (0)	3 (100)	3 (23)	0 (0)	2 (22)	4 (44)	2 (22)	0 (0)	1 (11)	0 (0)	9 (69)$^{\$}$
ADNI + This study	EOAD + AD (20)	2 (10)	0 (0)	5 (100)	5 (25)	0 (0)	5 (38)	5 (38)	2 (15)	0 (0)	1 (8)	0 (0)	13 (65)$^{£}$

$^{\$}$Chi-squared test: Controls versus LOAD-ADNI versus AD-our study, $\chi^2 = 15.8$, df $= 4$, $p = 0.003$. $^{£}$Chi-squared test: Controls versus LOAD-ADNI versus EOAD-ADNI+AD-our study, $\chi^2 = 17.9$, df $= 4$, $p = 0.001$.

was not exhaustively elucidated. Recently, novel, likely pathogenic variants were described in Italian patients with dementia [53].

We found two AD patients carrying the R62H mutation in the *TREM2* gene, which has an unknown impact. A recent review on the correlation between *TREM2* and AD [54], showed a meta-analytic association of this mutation with the late onset form of the disease. Our results also showed its involvement in the early onset form of AD. Since the two patients were also homozygous for the *APOE* ε4 allele, this finding suggests interactions between *TREM2* and *APOE*, as already demonstrated *in vitro* [55, 56]. TREM2 is a lipid sensor that interacts with several AD risk factors involved in lipid metabolism, including *APOE*, which could decrease the threshold of disease occurrence [57].

In the specific case of two AD patients carrying the *GRN* p.V77I and R19W mutations, there is evidence of AD pathology in imaging and from biofluid biomarkers (Table 1). Since these missense mutations do not affect the progranulin levels, a pathogenic role of these mutations seems unlikely. However, we cannot exclude that they might have a pathogenic role other than "loss of function", as no functional studies have been performed. Their presence in AD patients might rather indicate that this gene could be implicated also in the pathophysiological mechanisms leading to AD dementia.

Our analyses showed the presence of additional rare variants located in the 5'/3' UTR regulatory gene region of the *GRN, VCP, MAPT*, and *FUS* genes. A recent study reported that 3'UTR SNPs, such as rs80301724 in the *FUS* gene, are present in microRNA binding sites and could impact the post-transcriptional regulation, resulting in overexpression of the protein [58]. Also missense mutations in *TREM2*, *GRN*, *NOTCH3*, and *VCP* genes and a splicing variant in *NOTCH3* gene were detected but, except for some information from bioinformatic tools, their specific functional impact was not assessed. The involvement of *NOTCH3* gene in dementia patients is interesting, both with a missense and with a splicing variant. This gene encodes a single-pass trans-membrane protein of 2321 amino acids, predominantly expressed in vascular smooth muscle cells in adults. It is well documented that *NOTCH3* mutations play a critical role in the pathogenetic mechanism of vascular smooth muscle cell degeneration linked to CADASIL, one of the most common hereditary forms of stroke [59]. A recent hypothesis of AD [60] suggested that in

CADASIL triggering events in the pathogenic cascade are not amyloid deposits but damaged blood vessels caused by inflammatory reactions that lead to ischemia, amyloid accumulation, axonal degeneration, synaptic loss, and eventually irreversible neuronal cell death. Inflammation and blood vessel damage are well recognized complications of AD, but what causes them and why the cerebral microvasculature is affected is still under debate [60]. Mutations in *NOTCH3* gene are known to provoke inflammatory reactions and damage the brain in a wide variety of diseases [59], thus it is possible that one or more mutations in this gene may damage the microvasculature of the brain eventually leading that leads to dementia. The V1183M mutation was classified as a polymorphism in an Italian population [61], though the A allele frequency observed was 0.006.

Inclusion body myopathy with Paget's disease of bone and/or FTD (IBMPFD) is a recently identified autosomal dominant disorder due to mutations in the *VCP* gene affecting muscle, bone, and brain. Interestingly, in our cohort we found the R93C (47832C>T) mutation in the *VCP* gene already described in patients with IBMPFD [62–65].

Common variants

In this study, we investigated the most established common risk variant for AD, the *APOE* ε4 haplotype. The functional role of this polymorphism in AD pathogenesis is unclear. However, there is now strong evidence that *APOE* ε4 could affect amyloid deposition [66]. Consistent with this evidence, in our cohort all APOE4 carriers with available CSF were amyloid positive (Table 1), except for the case code 19_1 of which we discuss separately (see below). The frequency of the ε4/ε4 genotype ($n = 4$, 17%, exact confidence interval 3–32%) was higher than that commonly observed in the Caucasian population (1000 genomes), which is reported to be 2%.

Moreover, we investigated the non-synonymous polymorphism p.Met129Val in the *PRNP* gene. Although there are no data on the functional effect of this polymorphism, we observed that the frequency of the risk variant allele G/Val was higher (59%) than the frequency reported in the general European population (frequency G/Val = 33% reported by the Exome Aggregation Consortium). A recent meta-analysis showed that the p.Met129Val allele was associated with decreased disease risk in late-onset AD, but not in EOAD [67].

Six patients from the present cohort carry only common risk variants. They could be sporadic cases with onset at the extreme end of expected age range. However, the hypothesis that EOD is caused by mutations in genes not included in the NGS panel cannot be ruled out. In this regard, whole genome sequencing could foster the investigation of additional genetic factors underlying apparently sporadic EOD. Nonetheless, this task was beyond the scope of the present work.

Additional observations

Interestingly, fifteen (68%) of the 22 patients carried at least one rare variant (*TREM2, GRN, PSEN2, MAPT, VCP, NOTCH3,* or *FUS*). Among these, fourteen subjects carried also a common variant (*APOE* and/or *PRNP*). This result supports the hypothesis that EOD results from the interconnected mechanisms leading to neurodegeneration, where multiple genes can be implicated in one or more systems. Indeed, recent biochemical approaches [55, 56] have shown interactions among these genes, such as between *TREM2* and *APOE in vitro*. These results strongly implicate a potential additive/synergic effect in EOD forms linked to the variable inter- and intrafamiliar expressivity. To indirectly assess this effect, we found through the ADNI database that, excluding the full penetrant mutations, 69% of our sample showed ≥ 2 rare/common risk variants, as compared to 34% and 27% in sporadic late-onset AD patients and controls, respectively. This indicates that the EOD is more often associated with rare variants or risk alleles, and this could be useful in the genotype-phenotype correlations. Moreover, <10% of subjects, in our cohort, and in late onset AD patients, compared to 25% in a control group, were not carriers of any of the examined variants, which strengthens the idea of using an NGS whole/exome genome approach in a larger sample.

One AD patient with a very early age of onset (41 years) showed neither rare nor common-risk variants. Even an exome clinic investigation detected no rare or common risk variants. The family history was negative, as no other first-degree relatives were affected (neither the parents nor three siblings, two of whom were older than the patient, presented with the disease). Misdiagnosis is unlikely since this patient was positive for all AD biomarkers (abnormal CSF amyloid and tau levels, hypometabolism on FDG-PET, and medial temporal atrophy on MRI). Although we considered the possibility that the

patient may show an extreme early-onset presentation of sporadic AD, this finding suggests that additional genes could be implicated in EOD, which strengthens the evidence that the panel of candidate genes needs to be expanded in the future.

For the first time, the D87N mutation in the *TREM2* gene was detected in a LBD patient with early onset. LBD is the second most common form of dementia after AD, with a prevalence rate of 4% in the general population [68]. The core symptoms of LBD include sleep disturbances, hallucinations, and cognitive deficits, accompanied within the first year by Parkinsonian motor symptoms. A recent twin study did not show a strong support for a genetic contribution to LBD. However, other studies have demonstrated that LBD aggregates in families and may have an autosomal inheritance pattern (for review [4]). To date, a few genetic markers have been identified. For instance, duplication and SNPs within α, β, γ−synuclein genes have been associated with increased risk of LBD [69, 70]. Moreover, mutations in the glucocerebrosidase (*GBA*) gene are more common in LBD, in addition to mutations in the *MAPT* or leucine rich repeat kinase 2 (*LRRK2*) genes (for review [4]). Only one genome-wide linkage study has been performed among patients with familial LBD. A locus on chromosome 2q35-q36 was identified, though none of the genes in this region could explain the relation with LBD [71]. Although further confirmation is needed, the presence of a *TREM2* mutation in an LBD patient adds a new actor to its genetic architecture. Mutations in *TREM2*, a microglial receptor, can lead to aberrant innate immune cell signaling, contributing to the initiation and propagation of several neurodegenerative phenotypes [72–83], including LBD. Moreover, this LBD patient was a carrier of the GG (Val/Val) *PRNP* risk genotype. This finding is in agreement with a previous study [84] that described a patient carrying the M232R mutation in the *PRNP* gene who developed dementia and died six years after onset. An autopsy revealed the patient had dementia with Lewy bodies, not Creutzfeldt-Jakob disease.

Conclusions

This study confirms the role of *GRN* and *PSEN2* mutations in EOD, in the Italian population and provides evidence for roles of novel rare mutations located in the 5'/3' UTRs regulatory gene region of the *GRN, VCP, MAPT*, and *FUS* genes, missense mutations in *TREM2*, *GRN*, *NOTCH3*, and *VCP*, genes and a splicing variant in *NOTCH3* gene, with a "medium" GDI value. As previously observed, mutations in the *PSEN2* gene, a rare cause of dementia worldwide, are frequent in Italian patients. We also confirmed that mutations in *GRN* gene were present in both FTD and AD phenotypes. Moreover, six patients were carriers of only common risk variants (*APOE* and *PRNP*), and one patient did not show any mutation/variant. Overall, 69% ($n = 9$) of our EAOD patients, compared with 34% ($n = 13$) of sporadic LOAD patients and 27% ($n = 73$) of non-affected controls, were carriers of at least two rare/common risk variants in the analyzed candidates' genes panel.

Though our findings are consistent with results obtained from large cohorts [12], independent replications in larger samples are warranted. To further validate the role of polygenic risk variants in EOD, a systematic screening of rare and common variants in dementia-associated genes should be implemented in prospective cohorts with full clinical and biomarker characterization.

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