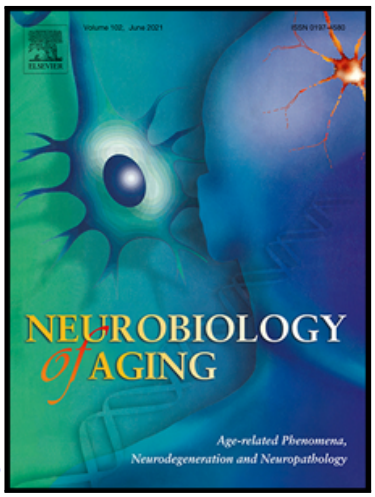

Protein interaction network analysis reveals genetic enrichment of immune system genes in frontotemporal dementia

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Highlights

- Protein interaction networks (PINs) can be used for disease gene prediction.
- We created an FTD-PIN to prioritize candidate genes for genetic analysis in FTD.
- We detected an enrichment of missense variants in the *TNFAIP3* gene in FTD patients.
- TNFAIP3-protein plays a role in immune signaling and neuroinflammation.
- Integration of PINs are useful to increase power in identifying disease-risk genes.

Protein interaction network analysis reveals genetic enrichment of immune system genes in frontotemporal dementia

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Abstract

To further unravel the complex genetic etiology of frontotemporal dementia (FTD), we hypothesized that interactors of the protein products of known FTD genes might be involved in the molecular pathways towards disease. We therefore applied protein interaction network (PIN) analysis to prioritize candidate genes for rare variant association. We created an FTD-PIN starting from known FTD genes downloading their physical interactors and performed functional enrichment analyses. We identified overrepresented processes in FTD and selected genes (n=440) belonging to the FTD processes for rare variant analysis in a Belgian cohort of 228 FTD patients and 345 controls. SKAT-O analysis suggested *TNFAIP3* as the top gene (P = 0.7 x10^{-3}) reaching near test-wide significance (P = 2.5x10^{-4}). We then analyzed the TNFAIP3-subnetwork within the FTD-PIN which indicated enrichment of several immune signaling networks, suggesting that disrupted immune signaling may be implicated in *TNFAIP3*-related FTD.

Our study demonstrates that integration of PINs with genetic data is a useful approach to increase the power for rare variant association analysis. Furthermore, we present a computational pipeline for identifying potential novel therapeutic targets and risk-modifying variants.

Keywords

protein-protein interaction network, rare variant association analysis, frontotemporal dementia, candidate gene prioritization

1. Introduction

Frontotemporal dementia (FTD) is a common form of early-onset dementia with a wide variety of clinical and pathological presentations. In clinical practice, a first distinction is made between the behavioral variant (bvFTD) and the language variant, primary progressive aphasia (PPA), which can

present as semantic variant (svPPA) or non-fluent PPA (nfvPPA) (Gorno-Tempini et al., 2011; Rascovsky et al., 2011). Notably, a combination of behavioral and language symptoms is not uncommon. Besides bvFTD and PPAs, FTD is frequently accompanied by Parkinsonian disorders such as corticobasal syndrome, progressive supranuclear palsy or motor neuron disease (MND), particularly amyotrophic lateral sclerosis (ALS, FTD-ALS). Neuropathologically, the neurodegeneration affects mainly the frontal and temporal lobes, referred to as frontotemporal lobar degeneration (FTLD). Up to 90% of patients develop either Tau (FTLD-Tau) or TDP-43 (FTLD-TDP) immunoreactive neuronal and glial inclusions (Mackenzie and Neumann, 2016). In the majority of the remaining patients, the cellular inclusions are positive for the FET (FUS-EWS-TAF15) proteins (FTLD-FET), and very rarely the inclusions contain the proteins of the ubiquitin-proteasome system (FTLD-UPS) but are negative for Tau, TDP-43 or FET proteins (Mackenzie and Neumann, 2016). Approximately 40% of patients have a positive family history of FTD or related neurodegenerative disease, with an autosomal dominant inheritance pattern in 10-27% of all FTD patients (Forrest et al., 2019; Goldman et al., 2005; Rohrer et al., 2009; Wood et al., 2013). Over two decades of genetic research have been pivotal in elucidating the autosomal dominant forms of FTD. To date, up to 25% of familial cases can be explained by the presence of high-penetrant, rare coding variants in *MAPT*, *GRN* or several other rarer Mendelian genes. Another 25% of the familial cases are explained by a hexanucleotide (G_4C_2) repeat expansion in the 5'UTR of the *C9orf72* gene (Ferrari et al., 2019). However, little is known regarding the etiopathogenesis of non-familial, sporadic FTD cases, with only 10% of the patients explained by a mutation in one of the Mendelian FTD genes. The molecular events triggering sporadic FTD are poorly understood, with evidence suggesting a causative mix of both genetic risk factors and environmental exposures (Ferrari et al., 2019). More studies and larger cohorts are needed to span this knowledge gap. Efforts to elucidate the genetics of sporadic or non-mendelian FTD include genome-wide association studies (GWAS) and rare variant association (RVA) studies, which showed limited success so far in both discovery power and replication rate across

studies. Importantly, marked disease heterogeneity of FTD complicates the cohort-based genetic studies.

This limited success underscores the need for strategies to prioritize rare and low-frequency variants from bulk genetic data. To this end, tools for integration of gene expression and protein-protein interaction (PPI) data, with either GWAS or sequencing data, have become popular to unravel the genetic etiology of genetically complex neurodegenerative disorders (Manzoni et al., 2020; Sonawane et al., 2019). These tools are based on the observation that proteins encoded by genes causing or associated to the same disease, interact with each other more frequently than expected by random chance (Barabási et al., 2011; Goh et al., 2007), forming modules contributing to the same cellular processes (Bauer-Mehren et al., 2011; Marbach et al., 2016; Oti and Brunner, 2006). Several approaches have been developed to accurately identify these disease modules, assuming that genes coding for other proteins in the module may be interesting candidate disease genes (Wang et al., 2011). One of these approaches, weighted PPI network analysis (WPPINA), has been developed to identify altered biological processes and candidate genes in FTD (Ferrari et al., 2017), and was later applied to prioritize candidate genes in a GWAS of Parkinson's disease (Ferrari et al., 2018).

In the present study, we updated and further automated one of these network strategies (WPPINA) and used it to construct a 2-layered protein interaction network (PIN) centered around known FTD genes (FTD-PIN). We applied this computational biology approach to identify highly interconnected and therefore likely essential proteins and enriched cellular processes, based on which we prioritized candidate genes for RVA on case-control sequencing data.

2. Materials and Methods

2.1. Construction of the protein interaction network

The PINs were built in a 2-step approach as described before (Ferrari et al., 2017). Briefly, the protein products of genes associated with the phenotype of interest were defined as 'seeds'. Direct interactors of the seeds (i.e. 1st layer interactions) were downloaded from peer-reviewed literature using PINOT (http://www.reading.ac.uk/bioinf/PINOT/PINOT_form.html) (Tomkins et al., 2020); stringent filtering was applied and PINOT downloaded PPIs from the following databases: BioGRID (Stark et al., 2006), bhf-uclb, IntAct (Kerrien et al., 2012), MINT (Licata et al., 2012), UniProt (Bateman et al., 2015), MBInfo (https://www.mechanobio.info/), InnateDB (Breuer et al., 2013). To ensure reproducibility of the PPIs, only interactions with a final score >2 (i.e., interaction is either detected by at least 2 methods (method score) or reported in at least 2 publications (publication score)) were retained in the PIN. Subsequently, the 1st degree interactors served as seeds to download their direct interactors (i.e., 2nd layer interactions), and filtered again for a final score >2. In both layers, all interactions with polyubiquitin (UBB, UBC and UBD) were discarded, as those are considered nonspecific consequences of protein ubiquitination prior to degradation. The 2-layered PIN therefore consisted of the combination of the seeds, 1st, and 2nd layer interactors. All downloads from the web-resource PINOT were performed in May 2020.

We calculated the inter-interactome degree (Ferrari et al., 2017) for each node in the 2-layered PIN. Briefly, for each single node this is defined as the number of seeds connected via that node. Nodes that able to bridge at least 60% of the seeds were considered as the inter-interactome hubs (IIHs). We subsequently extracted the core network composed of the IIHs and their direct interactors, hereafter referred to as core PIN.

The FTD core PIN was further filtered to retain only those proteins with evidence for expression in human brain frontal and temporal cortices. We downloaded tissue specific gene expression data from Braineac (http://www.braineac.org/) and retained the genes with a detected transcript (mean expression level > 1) in the frontal or temporal cortices. The interactions were retained if both interaction partners passed the expression filter.

2.2. Functional enrichment

We performed enrichment analysis of Gene Ontology Biological Processes (GO:BP) (Ashburner et al., 2000; Carbon et al., 2021) terms on the genes from the PIN using g:Profiler (g:GOSt, https://biit.cs.ut.ee/gprofiler/gost; analysis performed in September 2020) (Reimand et al., 2019). g:Profiler runs a Fisher's test and we used $P < 0.05$ as the significance threshold after multiple testing correction following the g:SCS method (g:Profiler). The enriched terms were then grouped into custom 'semantic classes' and larger 'functional blocks' by semantic similarity. Each enriched term received a weight according to its size in GO (term size) and the corrected "*P* value" of the enrichment analysis. Specifically, each term received a weight directly correlated with its -logP and inversely correlated with its size as: $term\ weight = -\log(Pvalue)/term\ size$. We then used the term weight to count the weighted number of supporting GO:BP terms per functional block and semantic classes to identify overrepresented processes in the PIN.

Enrichment of gene ontology terms on the genes from TNFAIP3-subnetwork was done as described above (analysis performed in June 2021) for GO:BP, cellular component (GO:CC) and molecular function (GO:MF) .

2.3. Study population and dataset

The patient cohort consisted of 228 index patients with FTD or FTD-ALS (mean age at onset 64.8±10.5 years, range 29-88 years). Specifically, the cohort consisted of 124 bvFTD, 49 PPA, 10 FTD-ALS and 45 mixed FTD phenotype. The research participants were recruited across Flanders-Belgium through a multicenter collaboration within the framework of the Belgian Neurology (BELNEU) Consortium. Diagnosis of FTD and subtypes was based on established clinical criteria (Agosta et al., 2015; Brooks et al., 2000; Gorno-Tempini et al., 2011; Rascovsky et al., 2011). All patients recruited via the BELNEU consortium were screened for mutations in the major genes associated with AD (*APP, PSEN1*, *PSEN2, APOE*, *TREM2, SORL1, ABCA7*), FTD and ALS (*C9orf72, GRN, MAPT, VCP, TARDBP, FUS, SOD1, TBK1*), prion disease (*PRNP*), and Parkinson's disease (PD: *LRRK2, PARK2*, and *SNCA*). Identified pathogenic mutation carriers were excluded from the analysis.

Ancestry matched and quality-controlled control sequencing data on 345 unaffected and unrelated Belgian individuals (n=345) was obtained through collaboration with the Cognitive Genetics group, Center of Medical Genetics, University of Antwerp.

All research participants or their legal representatives signed informed consent for participation in clinical and genetic research. The local medical ethics committees approved the clinical study protocols and informed consent forms at the collaborating sampling sites. The genetic study protocols and informed consent forms were approved by the ethics committee of the Antwerp University Hospital and the University of Antwerp, Belgium.

2.4. Whole exome sequencing and genetic analysis

Whole exome sequencing (WES) was performed on an Illumina NextSeq500 for the FTD patients and a HiSeq4000 platform for the control participants. Both datasets were subjected to post-sequencing quality control, alignment to the human reference genome (GRCh38) and variant calling with the in-house GenomeComb tool (Reumers et al., 2012; http://genomecomb.sourceforge.net/). We checked the ancestry for both cohorts by running a principal component analysis using the Hapmap3 populations and retained only the samples with European ancestry.

Both datasets were additionally subjected to a post-variant calling quality control procedure at both variant and sample level. At the sample level, we removed individuals with high missing call rate (>90%) and cryptic relatedness. For the variant quality control, we first set the genotypes with depth of coverage (DP) < 15X and allelic ratio > 3 as missing. Then, sites that were missing in at least 90% of the samples and/or significantly deviated ($P < 1x10^{-6}$) from the Hardy-Weinberg equilibrium were removed from both datasets to get rid of potential platform-specific artefacts. We extracted the variant sites mapping to the RefSeq coordinates of the genes prioritized from the FTD-PIN analysis and merged patient and control genotype files. Finally, we removed the sites that were differentially missing between case and control dataset ($P < 1x10^{-6}$), as well as sites with high missing call rate (>90%) in the complete dataset.

We validated the variants of interest identified in patients by Sanger sequencing using specific primers flanking the variants designed using Primer3 (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

2.5. Statistical analyses

We tested gene-based RVA with weighted SKAT-O from SKAT R package using phred scale predicted pathogenicity scores from combined annotation dependent depletion (CADD) as the variant weights. We included all protein altering variants based on the RefSeq transcripts, with minor allele frequency (MAF) < 1% in the gnomAD exomes Non-Finnish European cohort. We set the maximum cohort allele frequency to 0.01 for single variants and limited the analysis to the genes with ≥5 carriers with ≥1 qualifying variant. The analysis was corrected for gender.

2.6. Software

All PIN analyses were performed with in-house developed R scripts (https://www.r-project.org/). Networks were visualized using the Cytoscape 3.7.0 software (Shannon, 2003). WES data processing and analysis were conducted in a Linux environment. Mutation diagram was drawn in R.

3. Results

3.1. The FTD-PIN

We aimed at prioritizing candidate genes for rare variant analysis in FTD. Hereto, we selected 14 established/replicated FTD genes and risk factors (Table 1) as 'seeds' to create a FTD-PIN. Then, the FTD-PIN was built as detailed in materials and methods (Fig. 1).

The 2-layered PIN (seeds + 1st and 2nd layer interactors) consisted of 9,633 nodes and 38,538 edges (Supplementary Table 1). Within this network, we identified 319 inter-interactome hubs (IIHs) connecting >60% of the input FTD genes (Supplementary Figure 1) and, from this, extracted a core PIN composed of 731 nodes and 5,723 edges representing the most interconnected part of the 2-layered PIN (Supplementary Table 1). As a final step, we filtered the core PIN for tissue-specific gene expression; in particular, we allowed in the network only genes with a detectable transcript in the brain frontal and temporal cortices based on Braineac. We finally obtained 725 genes to be used as

input for the functional enrichment analysis (FTD-PIN, Supplementary Figure 2). The full list of interactions of the quality controlled 2- layered PIN, and inclusion status in the core PIN and FTD-PIN are reported in Supplementary Table 2.

3.2. Functional enrichment reveals central processes in FTD pathogenesis

The FTD-PIN represents the most connected part of the 2-layered PIN, containing all the proteins that are part of most (>60%) of the FTD seed interactomes and therefore likely to contain proteins involved in convergent disease pathways. We performed functional enrichment of the GO:BP terms on the FTD-PIN using g:Profiler and grouped all significantly enriched terms ($P_{corrected} < 0.05$) into functional blocks by semantic similarity (Supplementary Table 3). The functional blocks were further divided into semantic classes to specify different processes in a same block, e.g. the functional block *waste disposal* is divided into the classes *ubiquitin – proteasome*, *ubiquitin – proteasome – ER*, *autophagy*, *autophagy – mitophagy*, and *unfolded protein response (UPR)*. Considering the hierarchical structure of GO, we excluded terms with sizes > 1000 proteins and terms that could not be assigned to a specific semantic class, to reduce the statistical noise caused by general terms. This resulted in exclusion of 41.6% of the enriched terms, retaining 614 out of the 1051 GO:BPs for further prioritization (Supplementary Table 3).

To prioritize the functional blocks, we simply counted the number of enriched GO:BP terms supporting each block and semantic class, weighing the terms by the enrichment *P*-value and term size. The prioritized functional blocks and semantic classes were defined as groups with counts above the third quartile. The cut-off counts to prioritize classes were 3.81 and 0.40, for the functional blocks and semantic classes, respectively.

Following this procedure, 4 functional blocks were prioritized from the functional enrichment: *waste disposal*, *cell death*, *response to stimulus* and *immune system* (Fig. 2A). The leading functional block was *waste disposal*, with the *ubiquitin – proteasome* being the most dominant cellular process within *waste disposal* and over all overrepresented semantic classes. The 4 functional blocks were made up of 28 semantic classes including 16 with counts above the cut-off threshold of 0.44 (Fig. 2B,

Supplementary Table 4). The prioritized FTD cellular processes were made up of 146 GO:BP terms contributing to the 16 semantic classes (Supplementary Table 3). We then extracted the genes from the FTD-PIN contributing to the enrichment of the 146 GO:BP terms resulting in a list of 440 candidate genes for rare variant burden analysis (Supplementary Table 5).

3.3. WES data analysis and rare variant association testing on prioritized genes

We extracted the variants mapping to the 440 candidate genes from the WES data of 228 FTD patients and 345 unaffected controls. The merged quality-controlled dataset included 13,645 variants. We performed optimal sequence kernel association test (SKAT-O) on the prioritized genes with the SKATBinary function from the SKAT R package using CADD scores as variant weights. We calculated the genomic inflation based on the median test statistics and observed no inflation ($\lambda \sim 0.88$) (Fig. 3). The study-wide Bonferroni corrected significance threshold was set to 1.13×10^{-4} for the prioritized 440 genes. None of the genes passed stringent Bonferroni correction, but the tumor necrosis factor alpha induced protein 3 (*TNFAIP3*) gene reached the lowest *P*-value nearing study-wide significance ($P_{raw} = 6.91 \times 10^{-4}$, $P_{bonferroni} = 0.304$).

3.4. Enriched genetic variation in *TNFAIP3*

TNFAIP3 contained 8 missense variants present in 10 patients and 2 controls distributed across the protein (Table 2, Fig. 4). Furthermore, the TNFAIP3 protein was identified as one of the IIHs (connected to 9 seeds) in the 2-layer PIN, was part of all four overrepresented functional blocks (Supplementary Table 5). This topological and functional centrality suggest a potential role in FTD via common pathways of multiple causal genes.

The average age at onset of the *TNFAIP3* carriers was 64.1±7.1 years ranging between 56 and 75 (Table 2). Of note, all patients, except for the 3 carriers of the p.Thr647Pro variant, presented with an early-onset disease presentation (age at onset < 65 years) (Table 2). Majority of the patients presented with bvFTD phenotype (n=6, n=1 bvFTD-ALS). Therefore, we repeated SKAT-O for *TNFAIP3* by limiting the patient cohort to the bvFTD patients (n = 124), which confirmed the suggestive enrichment of *TNFAIP3* variants with bvFTD ($P_{raw} = 0.0021$).

3.5. TNFAIP3-related biological processes

As one of the IIHs, TNFAIP3 is strongly interconnected in the FTD-PIN; it interacts with 8 of the 14 FTD seeds (via 1 intermediate node) and directly with TBK1, an established causative FTD gene (Freischmidt et al., 2015; Gijselinck et al., 2015) and a known activator of NF-kB (Pomerantz, 1999) (Fig. 5, Supplementary Table 2). The *TNFAIP3* gene contributes to the enrichment of GO:BP terms classified in a number of different semantic classes according to our classification system, in particular TNFAIP3 is relevant for the enrichment of: 1) *waste disposal – ubiquitin proteasome*, 2) *immune system – innate*, *immune system – cytokine signaling*, *immune system – NF-kB signaling, immune system – interferon signaling* and *immune system – toll-like signaling*, 3) *cell death – signaling pathway* and 4) *response to stimulus – signaling*.

Due to this complex functional scenario, we decided to focus on the specific connectivity of TNFAIP3 protein within the core FTD-PIN and tried to prioritize some of these functions within the FTD context. We extracted the subnetwork of TNFAIP3 from the FTD-PIN, which consisted of TNFAIP3, the 9 FTD seeds it is connected to and the 27 intermediate nodes bridging TNFAIP3 to the seeds (Fig. 5). This subnetwork contained 37 nodes and 107 edges. Then, we tested the enrichment of GO:BP, GO:CC and GO:MF with g:Profiler to analyze the 27 intermediate nodes bridging TNFAIP3 to the seeds. As described before, we analyzed the GO:BP and the GO:CC and GO:MF terms excluding those with term sizes > 1000 to increase specificity. Semantic analysis (performed as described before) resulted in the selection of *immune system* and *cell death* (Fig. 6A, Supplementary Figure 3) functional blocks as prioritized mechanisms describing the functional profile of the TNFAIP3 FTD interactome. Looking into the semantic classes, the TNFAIP3-subnetwork showed a role in immune system signaling, such as cytokine, toll-like as NF-kB (Fig. 6A). Interestingly, *cell death - mitochondria* was the most significantly enriched semantic class in the overall TNFAIP3 subnetwork followed by *signaling* (Fig. 6A). Similarly, enrichment of the GO:CCs highlighted several immune signaling protein complexes, in particular the CD40 receptor complex (GO:0035631) was the most significantly enriched. Eleven proteins in the entire genome are annotated as part of the CD40 receptor complex

and 4 of them are part of the TNFAIP3 subnetwork (Fig. 6B). Looking at the GO:MFs, the most significant terms were related to *ubiquitination*, with *ubiquitin and ubiquitin-like protein ligase binding* (GO:0031625, GO:0044389) and *polyubiquitin modification-dependent protein binding* (GO:00319593) (Fig. 6C). The most specific GO:MF terms were *tumor necrosis factor binding* (GO:0043120) and *NF-κB-inducing kinase activity* (GO:0004704). Both terms had 5 associated proteins in the ontology, and 2 of them are part of the TNFAIP3 subnetwork.

3.6. Specificity of the gene prioritization pipeline

To evaluate the specificity of the FTD-PIN, we applied the same pipeline to a group of genes associated with a different trait. We retrieved 14 genes associated with aplastic anaemia (MIM: 609135) in Human Phenotype Ontology (HPO) from DisGeNET (https://www.disgenet.org/, November 2021) (Supplementary Table 6). The 2-layer anemia-PIN contained 4443 nodes of which 3663 were overlapping with the 2-layer FTD-PIN. Similarly, we identified 10 IIHs in the anemia-PIN as the nodes connecting at least 60 % of the 14 anemia seeds and extracted a core network. The final anemia-PIN contained 102 nodes of which 44 were shared with the FTD-PIN. Functional enrichment on the anemia-PIN resulted in prioritization of the GO:BP terms supporting *chromatin, response to stimulus, DNA metabolism*, and *protein metabolism* (Supplementary Figure 4). The only common functional block with the FTD-PIN, *response to stimulus*, showed different patterns of the semantic classes contributing to the enrichment. In the FTD-PIN, the enrichment was driven by *signaling mechanisms*, whereas in the anemia-PIN, this block was represented by *response to stress – radiation*.

4. Discussion

In this study, we applied weighted protein-protein interaction network analysis to prioritize genes for rare variant association analysis in FTD. To this purpose, we combined interactomes of different known FTD genes (seeds) to identify common disease related mechanisms. Our analyses identified *waste disposal*, *immune system*, *response to stimulus* and *cell death* as converging pathways across FTD genes. This, in turn, allowed us to identify and extract from the FTD-PIN potential key proteins

involved in these disease-associated cellular processes. This list of genes was then followed up in downstream genetic investigations, supporting a role for *TNFAIP3* as a new, potential gene whose polymorphism may contribute to FTD risk.

Despite the enormous progress in FTD genetics, the success rate of gene identification studies has been low when moving beyond the classical linkage studies in extended FTD pedigrees. Sequencing studies in large ALS and FTD cohorts have so far identified rare variants in *TBK1* (Freischmidt et al., 2015; Gijselinck et al., 2015), demonstrating the power of rare variant approaches in large cohorts. However, later studies could not replicate this success, emphasizing the genetic complexity of FTD and the need for additional methods to support investigation of rare variants and increase discovery power. In this regard, *in silico* system biology approaches have the potential to help in resolving this missing heritability issue (Manzoni et al., 2020). In the present study, we applied an *in silico* protein interaction network approach and hypothesized that proteins in close interaction with known disease players are more likely to have a role in FTD. We updated the previously described WPPINA pipeline (Ferrari et al., 2018, 2017) to identify disease processes and prioritize candidate genes relevant for FTD. This led to the selection of a small fraction of the protein coding genes in the genome for downstream genetic analyses in a balanced case-control cohort, increasing our statistical power in rare variant association tests.

We could not reach study-wide significance, indicating the difficulty of reaching the stringent Bonferroni significance in rare variant association analysis of complex traits, especially when dealing with non-large cohorts. However, we were able to identify *TNFAIP3* as a suggestive gene (P = 6.91×10^{-4}) reaching near study-wide significance (P = 1.13×10^{-4}).

Another major challenge in rare variant association studies of FTD, is the high clinical heterogeneity. Our cohort also consisted of a spectrum of clinical FTD sub-phenotypes. A typical approach to improve power in such cases is to focus on a smaller subset of patients with distinct clinical manifestations (Lee et al., 2014). However, the clinical subgroups in our cohort were relatively small to make meaningful observations. As the majority of the patients with *TNFAIP3* variants had the

bvFTD phenotype, we also tested this gene in the group of bvFTD patients only (n = 124), which corresponded to approximately half of the total patient cohort (n = 228). Even though gene burden of *TNFAIP3* was still nominally significant, it was much lower compared to the total FTD cohort, indicating that even in the largest clinical subtype the power was limited.

4.1. TNFAIP3 protein plays a role in cytokine-mediated immune response

The *TNFAIP3* gene encodes the tumor necrosis factor alpha (TNFα) induced protein 3 (TNFAIP3, P21580, also referred to as A20), which is a zinc-finger protein and an ubiquitin-editing enzyme (Hymowitz and Wertz, 2010). Its expression is induced by the TNFα as a response to inflammatory stimuli to regulate nuclear factor kappa B (NF-κB) activity (Hymowitz and Wertz, 2010). The NF-κB signaling pathway has already been linked with known FTD genes such as *TBK1, SQSTM1* and *CYLD* creating a direct link with neuroinflammation and FTD (Bright et al., 2019; Dobson-Stone et al., 2020). The TNFAIP3 protein is highly expressed in mature microglia where it acts as a mediator of microglia activation (Voet et al., 2018; Zhang et al., 2014). Its deficiency was shown to result in upregulation of inflammatory genes and many disease-associated microglia markers including apolipoprotein A, resembling the microglia in neurodegenerative conditions in mice (Voet et al., 2018). Interestingly, *Tnfaip3* knockout mice also exhibit axonal damage, a reported phenotype observed in neurons expressing a recently described FTD-ALS causing mutation in the *CYLD* gene (Dobson-Stone et al., 2020). These two proteins are shown to be recruited to the signaling complexes via the linear ubiquitin chain assembly complex (LUBAC; GO:0007192, Fig 6C) upon TNF stimulation to enhance (CYLD) or suppress (TNFAIP3) cell death (Draber et al., 2015). Together, these observations support a role for TNFAIP3 in FTD, through altered microglial function, axonal damage and cell death. Enrichment of GO:CCs supports this link via cytokine induced immune signaling protein complexes: *CD40 receptor complex* (GO:0035631) a part of the *TNF receptor superfamily complex* (GO:0002947) and the *LUBAC complex* (GO:0071797); and cell death protein complexes *ripoptosome* (GO:0097342) and *cell-death inducing signaling complex* (GO:0031264) (Fig. 6C). In the GO:BP enrichment of our core FTD-PIN, signaling processes of the *immune system* and *response to*

stimulus were among the prioritized FTD processes (Fig. 2, S4 Table), and were even more predominant in the TNFAIP3-subnetwork (Fig. 6A). This suggests that even in non-carriers of *TNFAIP3* risk-modulating variants, TNFAIP3 may still be implicated in the FTD disease process and serve as a potential target in disease modifying therapeutic approaches.

Variants in the *TNFAIP3* gene have previously been linked to B cell lymphoma and several autoimmune diseases, including multiple sclerosis, with a causal link for heterozygous premature termination codon mutations with early-onset Behçet-like autoimmune disease; yet no genetic association with neurodegenerative diseases has been described ((IMSGC), 2013; Ma and Malynn, 2012; Zhou et al., 2016). Genetic overlap between immune system diseases and FTD has previously been reported with enrichment of immune-related genetic variation in FTD GWASs (Broce et al., 2018) and increased prevalence of autoimmune diseases in patients with FTD (Miller et al., 2013). Here, we identified another autoimmune disease gene, *TNFAIP3*, as another possible link between autoimmunity and FTD. Previously, missense variants in the *TNFAIP3* gene were suggested to confer autoimmune disease risk by reducing TNFAIP3 expression (Ma and Malynn, 2012). Reduced TNFAIP3 expression has also been shown in blood of patients with Parkinson's disease, however the relationship of genetic variants to this reduced expression was not reported (Perga et al., 2017). Further characterization of the *TNFAIP3* missense variants identified in our study is needed to understand their role in disrupted immune signaling pathways and in conferring FTD risk.

4.2. Inferring disease development through protein interaction networks

In search for novel FTD associated genes, we analyzed a set of genes that are likely to play a role in disease-relevant cellular processes in a cohort of patients with clinical FTD without a previously known genetic cause. Our findings indicated that rare missense variants with intermediate-to-low effect size in the *TNFAIP3* gene could increase the risk of developing FTD. Identification of genes with such variants are highly challenging through typical genetic approaches (Manolio et al., 2009) thus require novel approaches to complement and to interpret genetic analyses.

In addition to prioritization of candidate genes for candidate gene identification studies as we have done in the present study, PINs can also contribute to understanding key elements in disease pathology and key proteins that drive subtype-specific disease development. This is particularly relevant for FTD, with its broad clinical and pathological spectrum, observed even in carriers of the same mutation. This suggests that there are different drivers of disease resulting in different pathological and clinical outcomes. Application of PINs on a stratified patient cohort by a defined pathological subtype and/or known genetic mutation therefor has the potential to identify such driver processes/proteins directing disease development toward a specific molecular subtype.

4.3. Limitations of the study

There are a number of limitations to our study. First, we acknowledge that our cohort size was limited and not powerful enough to observe an exome-wide significant association (Auer et al., 2016). This is a common challenge in genetic research of complex heterogenous diseases, particularly when aiming to identify rare variants with low-to-medium effect sizes and reduced penetrance. Identification and replication of such variants will require development of innovative bioinformatic and biostatistical approaches.

Another limitation of our study is the publication or ascertainment bias, in that some proteins/genes are more studied than others and therefore are more annotated in protein interactions databases and in Gene Ontology. Another similar problem comes from the fact that protein interaction and functional databases are incomplete by definition as they constantly grow in parallel with the number of research outputs that are produced. This is exemplified by the comparison between this current publication and the previously published FTD-PIN (Ferrari et al., 2017), obtained following the same pipeline; the 2-layered interactome in our study contained 2 times more nodes and about 4 times more interactions than reported before.

We took a different approach to prioritize functional blocks communal to multiple FTD seeds, however, in both studies similar waste disposal and signaling processes were among the enriched pathways. Furthermore, we obtained a different enrichment profile when the pipeline was applied

to another trait. This shows that, despite the biases towards well-studied interactions and pathways, with the current knowledge, we were able to obtain trait-specific network and pathways.

5. Conclusions

We used protein interaction analysis as a tool to identify overrepresented FTD pathways and, candidate genes for rare variant association in FTD. To our knowledge, this was the first time that protein network analysis was applied to inform rare variant association studies on next-generation sequencing data in FTD. The created FTD-PIN once again emphasized the enrichment of immune system processes, and the genetic findings supported the link between autoimmune disease and FTD. The genetic power of our study was limited to identify robust genetic association; however, we show that this pipeline can aid in candidate gene prioritization in underpowered genetic studies of complex diseases. Furthermore, our approach offers a computational framework to identify and study disease pathways and genes in complex diseases such as FTD.

Submission declaration and verification

We further state that all authors have reviewed the contents of the manuscript being submitted, approve of its contents and validate the accuracy of the data. We confirm that the data contained in the manuscript being submitted have not been previously published, are not been submitted elsewhere and will not be submitted elsewhere while under consideration at Neurobiology of Aging.

Ethical assurances

All research participants or their legal representatives signed informed consent for participation in clinical and genetic research. The local medical ethics committees approved the clinical study protocols and informed consent forms at the collaborating sampling sites. The genetic study protocols and informed consent forms were approved by the ethics committee of the Antwerp University Hospital and the University of Antwerp, Belgium.

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Disclosure statement

The authors report no competing interests.

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Figure 1. Construction of FTD-PIN for candidate gene prioritization. Direct interactors of the i) FTD seeds and ii) their interactors are downloaded from PINOT in two steps. Interactors that are not replicated (FS < 3) and ubiquitin proteins were removed from the PIN at both steps. Then the FTD-PIN was created as the nodes bridging at least 60% of the seeds and expressed in the Frontal and Temporal cortices. FS: final score, PIN: protein-protein interaction network, UBB: Ubiquitin B, UBC: Ubiquitin C, UBD: Ubiquitin D. NameB denotes interaction partner in PINOT output.

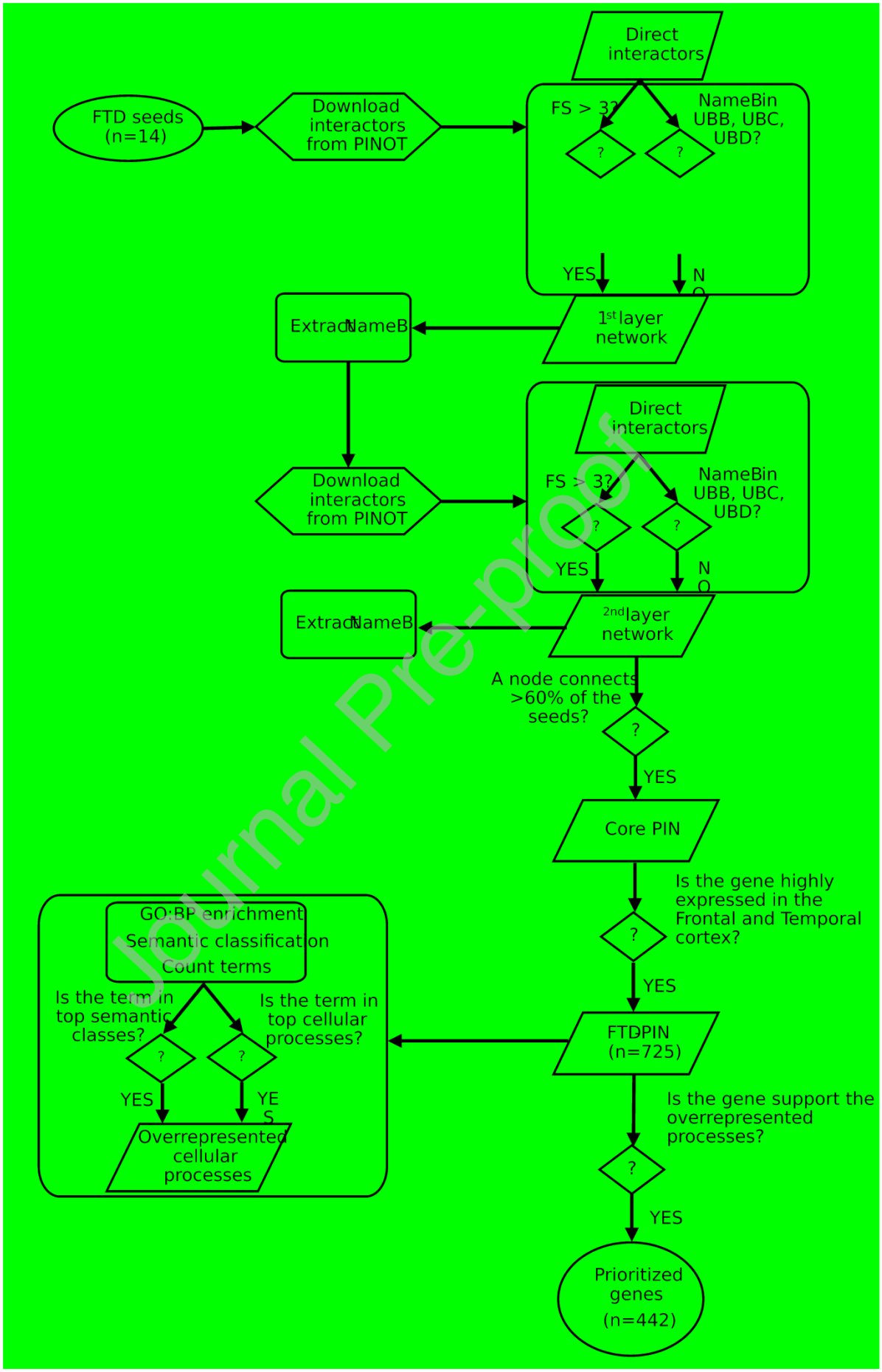

Figure 2. Distribution of weighted counts per functional blocks and semantic classes. (A) Weighted count of GO:BP terms per functional blocks, overrepresented processes (counts above the 3rd quantile (3.81)) are shown in dark blue. (B) Weighted count of GO:BP terms per semantic class of the prioritized functional groups shown in A. Four functional blocks are color-coded and the prioritized semantic classes with counts above the 3rd quantile (0.40) are shown in darker shade.

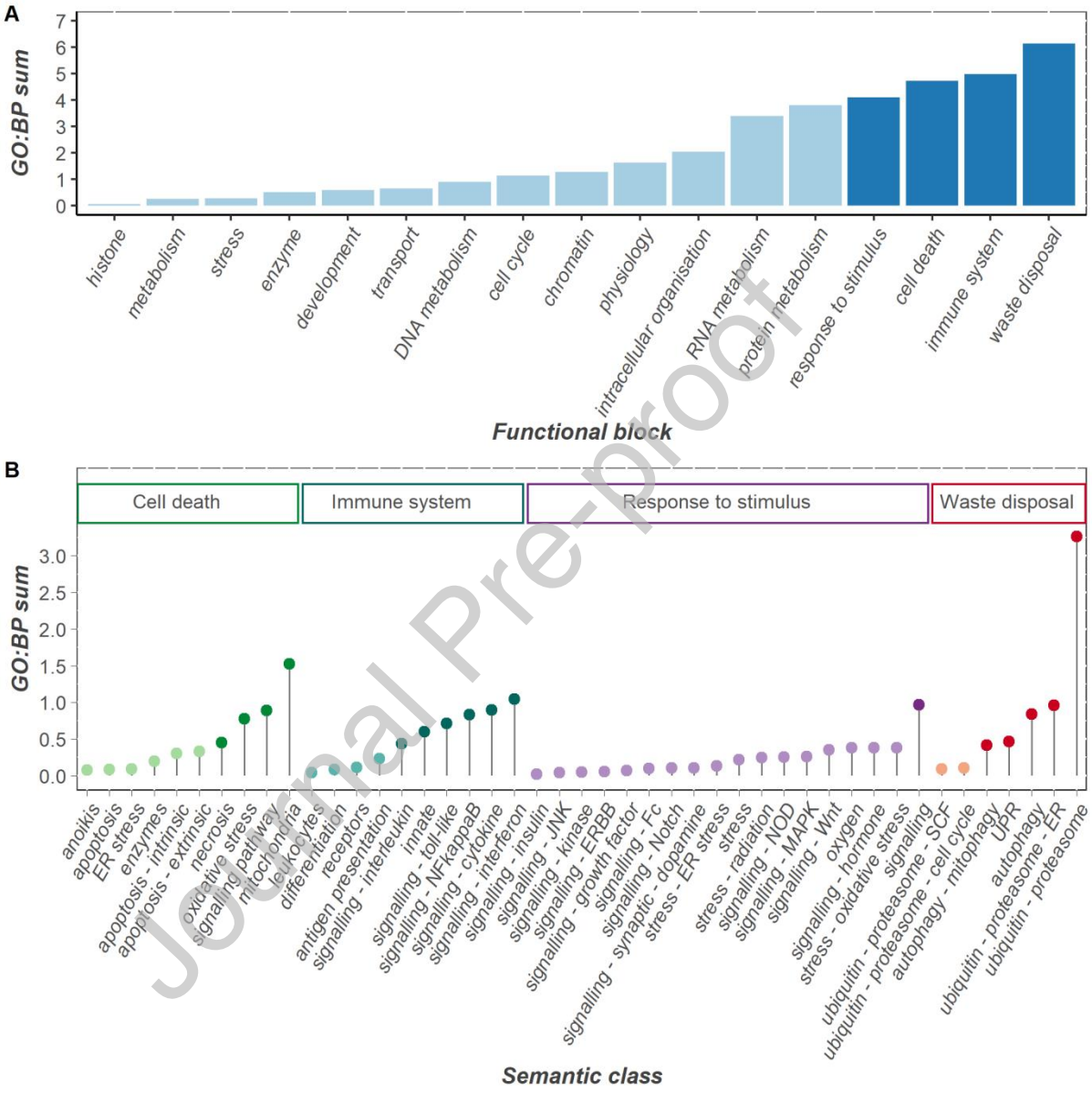

Figure 3. QQ-plot of SKAT-O test applied on prioritized genes.

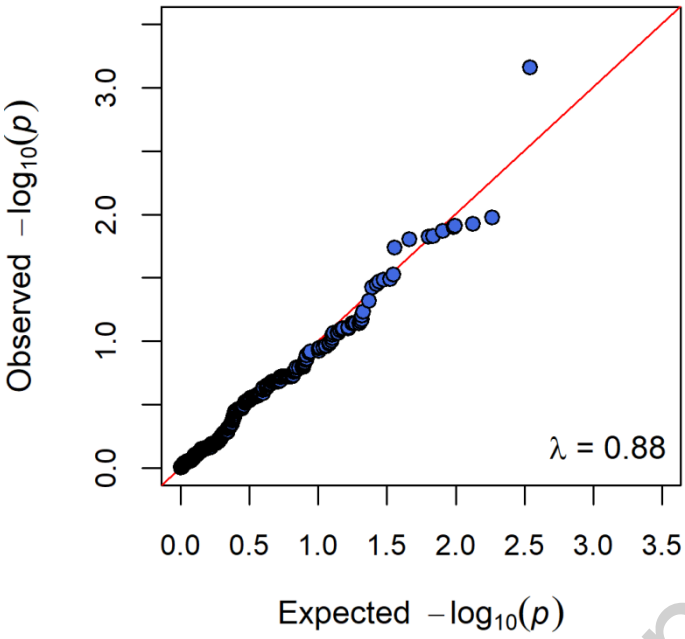

Figure 4. **Variants included in the statistical analysis in the *TNFAIP3* gene.** Patient variants are shown in red; control variants are shown in green. Left bar indicates the CADD score of the variants, the point size corresponds to the number of variants in the cohort. Protein domains are retrieved from Uniprot (ID: P21580). OTU: Ovarian tumor~~, ZF: Zinc finger~~.

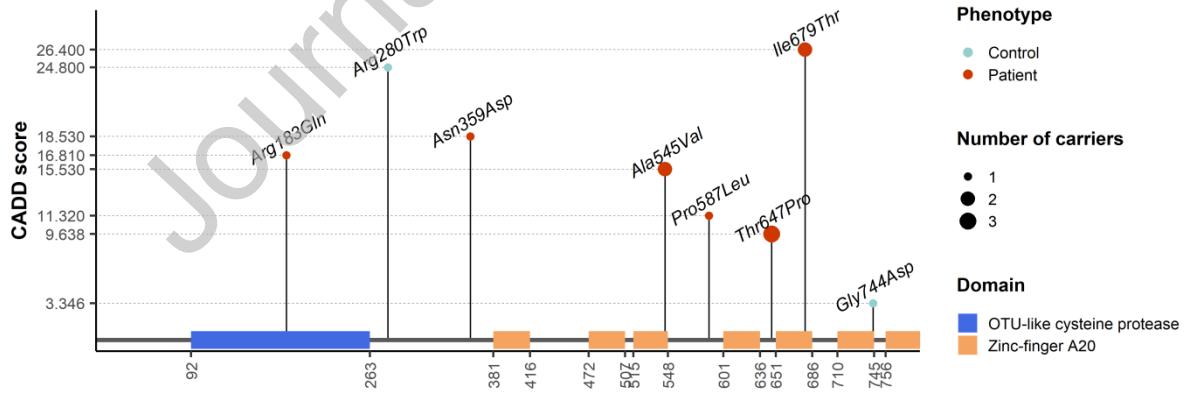

Figure 5. TNFAIP3 subnetwork in the FTD-PIN. Only the direct interactors connected to a seed are shown. TNFAIP3 in orange, seeds in light blue.

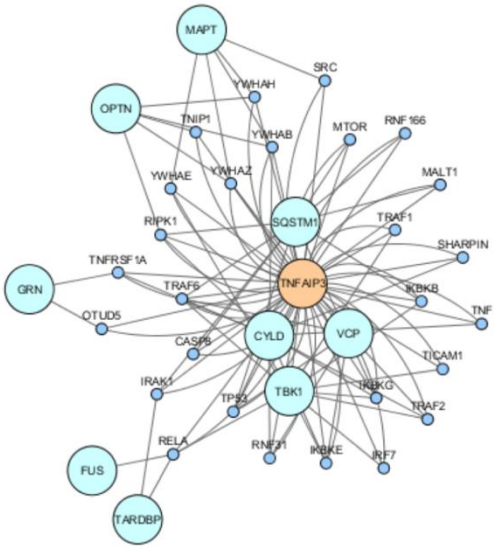

Figure 6. Gene ontology enrichment of the TNFAIP3 subnetwork.(A) Distribution of the weighted count of GO:BP terms supporting top two functional blocks into semantic classes. (B) Enrichment of gene ontology cellular component (GO:CC) terms. (C) Enrichment of gene ontology molecular function (GO:MF). The graphs are colored based on the input used for g:Profiler enrichment; green: only the interactors linking TNFAIP3 to the seeds (n = 27), orange: 27 TNFAIP3 interactors, 9 connected seeds and TNFAIP3. In B and C, the point size (recall) reflects the ratio of proteins contributing to the enrichment of a term with respect to its size (intersection size / term size, reported in g:Profiler output).

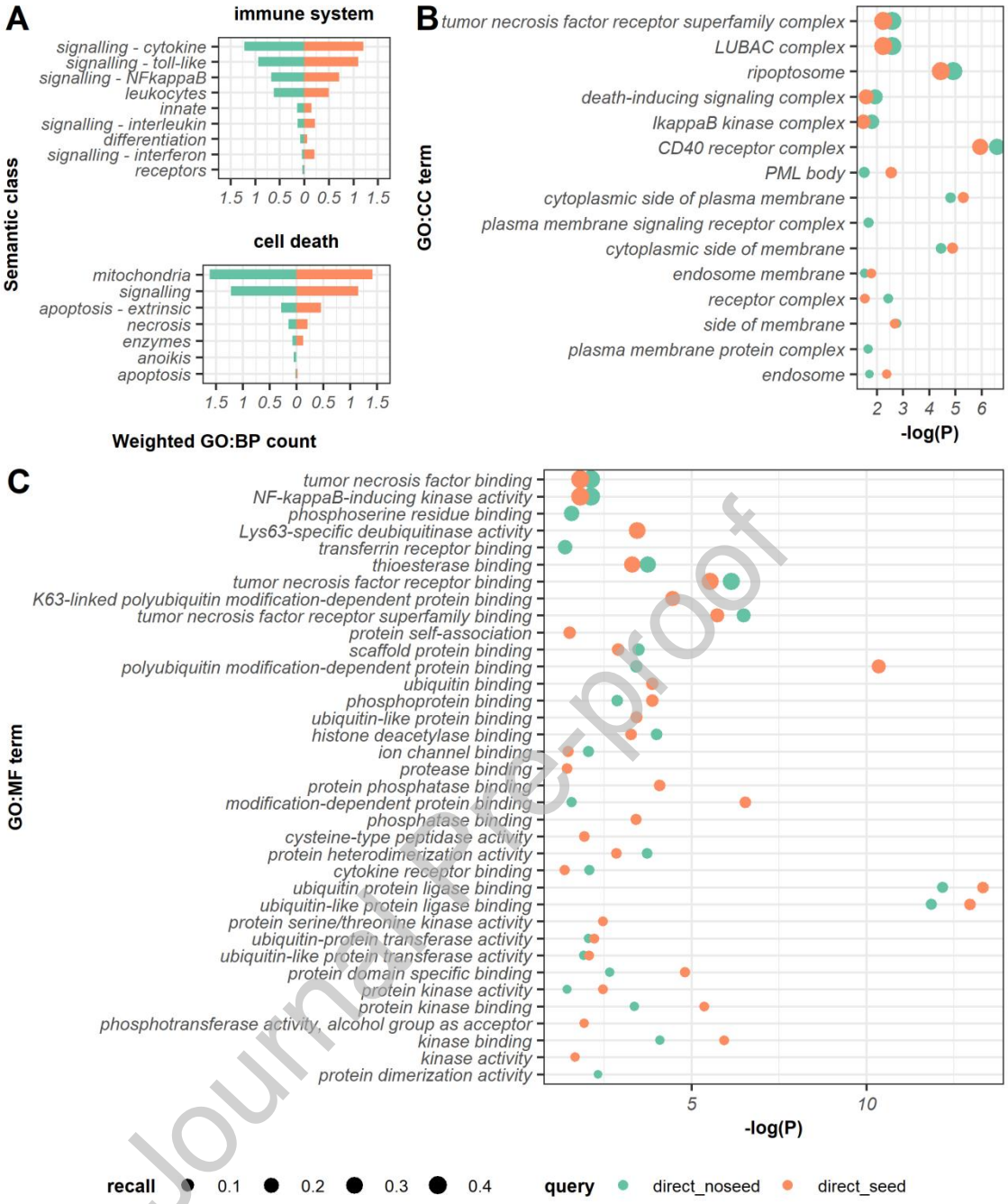

Table 1. FTD seed genes and associated phenotypes.

Gene name	Uniprot ID	Pathological subtype	Clinical phenotype	Part of the FTD-PIN?	Reference
MAPT	P10636	FTLD-Tau	FTD	Yes	(Hutton et al., 1998)
GRN	P28799	FTLD-TDP	FTD	Yes	(Baker et al., 2006; Cruts et al., 2006)
VCP	P55072	FTLD-TDP	IBMPFTD, ALS, FTD-ALS	Yes	(Johnson et al., 2010; Watts et al., 2004)
CHMP2B	Q9UQN3	FTLD-U	FTD	Yes	(Skibinski et al., 2005; van der Zee et al., 2008)
TARDBP	Q13148	FTLD-TDP	FTD, ALS, FTD-ALS	Yes	(Benajiba et al., 2009; Quadri et al., 2011)
FUS	P35637	FTLD-FUS	FTD-ALS, ALS	Yes	(Broustal et al., 2010; Van Langenhove et al., 2010)
TMEM106B	Q9NUM4	FTLD-TDP	FTD risk factor	No	(Van Deerlin et al., 2010)
C9orf72	Q96LT7	FTLD-TDP	FTD, ALS, FTD-ALS	No	(DeJesus-Hernandez et al., 2011; Renton et al., 2011)
UBQLN2	Q9UHD9	FTLD-TDP	ALS, FTD, FTD-ALS	Yes	(Deng et al., 2011; Synofzik et al., 2012)
SQSTM1	Q13501	FTLD-TDP	FTD, ALS, FTD-ALS, PDB	Yes	(Fecto et al., 2011; Le Ber et al., 2013; van der Zee et al., 2014)
CHCHD10	Q8WYQ3	NA	FTD-ALS	Yes	(Bannwarth et al., 2014)
TBK1	Q9UHD2	FTLD-TDP	FTD, ALS, FTD-ALS	Yes	(Cirulli et al., 2015; Freischmidt et al., 2015; Gijselinck et al., 2015)
OPTN	Q96CV9	FTLD-TDP	ALS, FTD-ALS	Yes	(Pottier et al., 2015)
CYLD	Q9NQC7	FTLD-Tau or FTLD-TDP	FTD-ALS	Yes	(Dobson-Stone et al., 2020)

IBMPFTD: Inclusion body myopathy with Paget disease of bone and frontotemporal dementia, PDB: Paget disease of bone

Table 2. Variants and patient demographics of *TNFAIP3* variant carriers in our cohort

Carrier	Exon	CDS change	AA change	dbSNPv151	CADD phred	GnomAD exomes NFE MAF[%]	Phenotype	AAO/AAR	Gender	FH
IID114	4	c.548G>A	p.Arg183Gln	rs375378882	16.81	0.008	FTD	62	m	F
IID463	6	c.838C>T	p.Arg280Trp	rs150198888	24.8	0.004	control	NA	m	NA
IID23	7	c.1075A>G	p.Asn359Asp	rs1004332178	18.53	0.003	bvFTD	60	m	S
IID103	7	c.1634C>T	p.Ala545Val	rs142752989	15.53	0.090	bvFTD	60	m	S
IID213	7	c.1634C>T	p.Ala545Val	rs142752989	15.53	0.090	bvFTD	61	m	F
IID56	7	c.1760C>T	p.Pro587Leu	rs150056192	11.32	0.025	bvFTD-ALS	59	m	S
IID25	8	c.1939A>C	p.Thr647Pro	rs142253225	9.638	0.238	bvFTD	75	f	S
IID105	8	c.1939A>C	p.Thr647Pro	rs142253225	9.638	0.238	bvFTD	75	m	S
IID219	8	c.1939A>C	p.Thr647Pro	rs142253225	9.638	0.238	PPA	75	m	U
IID87	8	c.2036T>C	p.Ile679Thr	rs140610274	26.4	0.010	PPA	56	m	F
IID147	8	c.2036T>C	p.Ile679Thr	rs140610274	26.4	0.010	bvFTD	58	f	U
IID361	9	c.2231G>A	p.Gly744Asp	rs150355046	3.346	0.072	control	NA	m	NA

IID: Individual ID number in the dataset; CDS: coding DNA sequence; AA: amino acid; dbSNPv151: the Single Nucleotide Polymorphism Database version 151; CADD phred: Combined Annotation Dependent Depletion phred score; GnomAD exomes NFE MAF: the minor allele frequency in the genome aggregation database Non-Finnish European whole exome sequencing dataset v2.1.1; AAO: Age at onset; AAR: Age at referral; FH: Family history status (F: familial, S: sporadic; U: unknown); m: male; f: female. Variants are reported by Refseq transcript NM_001270508. MAF reported in percentage.