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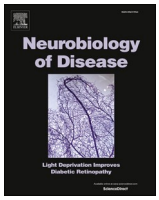

Plasma lysosphingolipids in *GRN*-related diseases: Monitoring lysosomal dysfunction to track disease progression

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Abbreviations: CDR + NACC FTLD, Clinical Dementia Rating plus scale plus National Alzheimer's Coordinating Center Frontotemporal Lobar Degeneration; CLN-11, neuronal ceroid lipofuscinosis-11; EYO, expected years to onset; FTD, frontotemporal dementia; FTD-*C9orf72*, FTD patients carrying a heterozygous *C9orf72* expansion; FTD-*GRN*, FTD patients carrying heterozygous *GRN* mutations; FTD-ng, FTD patients without any identifiable genetic cause; GCase, β-glucocerebrosidase; HC, healthy controls; LGB3, lysoglobotriaosylceramide; LGL1, glucosylsphingosine d18:1; LSD, lysosomal storage disease; LSM18:1, lysosphingomyelin d18:1; LSM509, lysosphingomyelin 509; lysoSPL, lysosphingolipids; NfL, neurofilament light chain; PGRN, progranulin; PSAP, prosaposin; PS-*GRN*, presymptomatic heterozygous *GRN* carriers; QC, quality control; SPL, sphingolipids; SPMase, sphingomyelinase; UPLC, ultraperformance liquid chromatography..

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ABSTRACT

GRN mutations are among the main genetic causes of frontotemporal dementia (FTD). Considering the progranulin involvement in lysosomal homeostasis, we aimed to evaluate if plasma lysosphingolipids (lysoSPL) are increased in *GRN* mutation carriers, and whether they might represent relevant fluid-based biomarkers in *GRN*-related diseases.

We analyzed four lysoSPL levels in plasmas of 131 *GRN* carriers and 142 non-carriers, including healthy controls and patients with frontotemporal dementias (FTD) carrying a *C9orf72* expansion or without any mutation. *GRN* carriers consisted of 102 heterozygous FTD patients (FTD-*GRN*), three homozygous patients with neuronal ceroid lipofuscinosis-11 (CLN-11) and 26 presymptomatic carriers (PS-*GRN*), the latter with longitudinal assessments. Glucosylsphingosin d18:1 (LGL1), lysosphingomyelins d18:1 and isoform 509 (LSM18:1, LSM509) and lysoglobotriaosylceramide (LGB3) were measured by electrospray ionization-tandem mass spectrometry coupled to ultraperformance liquid chromatography.

Levels of LGL1, LSM18:1 and LSM509 were increased in *GRN* carriers compared to non-carriers ($p < 0.0001$). No lysoSPL increases were detected in FTD patients without *GRN* mutations. LGL1 and LSM18:1 progressively increased with age at sampling, and LGL1 with disease duration, in FTD-*GRN*. Among PS-*GRN* carriers, LSM18:1 and LGL1 significantly increased over 3.4-year follow-up. LGL1 levels were associated with increasing neurofilaments in presymptomatic carriers.

This study evidences an age-dependent increase of β-glucocerebrosidase and acid sphingomyelinase substrates in *GRN* patients, with progressive changes as early as the presymptomatic phase. Among FTD patients, plasma lysoSPL appear to be uniquely elevated in *GRN* carriers, and thus might serve as suitable non-invasive disease-tracking biomarkers of progression, specific to the pathophysiological process. Finally, this study might add lysoSPL to the portfolio of fluid-based biomarkers, and pave the way to disease-modifying approaches based on lysosomal function rescue in *GRN* diseases.

1. Introduction

Frontotemporal dementias (FTD) are neurodegenerative diseases mainly affecting behavior, social cognition, executive functions and language, usually beginning between the age of 50 and 65 years (Moore et al., 2020), and are most frequently associated with abnormal TDP-43-positive neuronal inclusions. Heterozygous loss-of-function mutations in *GRN*, the gene coding for progranulin (PGRN) protein, are the most frequent genetic causes of the familial forms of FTD (Baker et al., 2006; Cruts et al., 2006), together with *C9orf72* repeat expansions (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Most pathogenic *GRN* mutations cause PGRN haploinsufficiency, heterozygous carriers having less than half of normal circulating PGRN levels in plasma (Ghidoni et al., 2008; Sellami et al., 2020). Besides, homozygous *GRN* mutations, associated with undetectable plasma PGRN, cause neuronal ceroid lipofuscinosis-11 (CLN-11), a rare childhood/juvenile lysosomal storage disease (LSD) (Smith et al., 2012; Huin et al., 2020).

Progranulin is involved in inflammation, tumorigenesis, neuronal survival and outgrowth (Bateman and Bennett, 2009; Paushter et al., 2018). In addition, PGRN plays a role in lysosomal homeostasis, and several findings support the contribution of lysosomal dysfunction to the pathomechanism of *GRN*-related diseases (Götzl et al., 2014; Kao et al., 2017; Valdez et al., 2017; Ward et al., 2017; Arrant et al., 2019; Zhou et al., 2019; Huang et al., 2020; Boland et al., 2022). Noteworthy, PGRN is processed in the lysosome, and it promotes the delivery of prosaposin (PSAP) to the lysosome, thereby indirectly regulating the biological activity of lysosomal enzymes (Zhou et al., 2015, 2017). Indeed, inside the lysosome, PSAP is cleaved into saposin peptides that serve as activators of lysosomal enzymes implicated in the degradation of sphingolipids (SPL). PGRN deficiency is associated with decreased saposin levels (Paushter et al., 2018), leading to impaired degradation of SPL and their accumulation in brains of heterozygous *GRN* patients and $Grn^{-/-}$ mice (Zhou et al., 2015, 2019; Arrant et al., 2019; Feng et al., 2020). In addition, lysosomal dysfunction caused by PGRN deficiency impairs effective ganglioside clearance, resulting in accumulation of different ganglioside species in both human brains and murine models (Boland et al., 2022).

Elucidating the pathophysiology of *GRN*-related diseases, and identifying appropriate disease-tracking biomarkers are major challenges, as therapeutic options targeting progranulin deficiency are upcoming (Boeve et al., 2022). In complex inherited LSD such as Gaucher, Fabry and Niemann-Pick type A/B diseases, specific SPL, and their deacylated derivatives lysosphingolipids (lysoSPL), accumulate in brain and other tissues due to severely decreased enzymatic activity. In the aforementioned sphingolipidoses, lysoSPL are detectable in plasma, and their levels have been validated as diagnostic biomarkers and to monitor treatment effectiveness (Dekker et al., 2011; Pettazzoni et al., 2017; Piraud et al., 2018; Hurvitz et al., 2019; Polo et al., 2019). We hypothesized that lysoSPL levels might be increased also in blood of *GRN* carriers, and might serve as potential biomarkers in *GRN* diseases too. To support this hypothesis, we investigated plasma levels of four lysoSPL in a large cohort of FTD patients carrying heterozygous *GRN* mutations

(FTD-*GRN*), in three patients with CLN-11 and, longitudinally, at the early stage of *GRN* disease in presymptomatic heterozygous carriers.

2. Materials and methods

2.1. Participants

The studied population consisted of 131 individuals carrying *GRN* mutations and 142 non-carriers. *GRN* carriers included 102 FTD-*GRN* patients, 3 patients with CLN-11 related to homozygous *GRN* mutations and 26 presymptomatic heterozygous *GRN* carriers (PS-*GRN*). *GRN* mutations are listed in Supplementary Table 1. Plasma progranulin dosage was performed in all carriers and 138 non-carriers using ELISA method with the progranulin-human-ELISA kit (Adipogen, Coger SAS, France), according to the manufacturer's instructions and as described previously (Sellami et al., 2020).

The 102 FTD-*GRN* patients have been investigated in the context of standard clinical care in expert centers within the French research network on FTD (Sellami et al., 2020). Their demographic and clinical characteristics are summarized in Table 1. All patients fulfilled diagnostic criteria for behavioral variant FTD (Rascovsky et al., 2011). For descriptive purposes, we also included three CLN-11 homozygous *GRN* patients reported elsewhere (Huin et al., 2020). Their mean age at onset and sampling were much lower than those of FTD-*GRN*, as expected for a childhood/juvenile disease (Table 1).

The 26 PS-*GRN* (asymptomatic relatives of FTD-*GRN* patients) were enrolled in a prospective study (Predict-PGRN, clinicalTrials NCT04014673) (Saracino et al., 2023). Among them, 17 had two blood samplings, with mean interval of 3.4 ± 1.5 years between the first and the second sample. Proximity to prodromal and clinical onset was estimated with the Clinical Dementia Rating plus NACC FTLD (CDR + NACC FTLD) (global score, sum of boxes) (Miyagawa et al., 2020). None developed clinical symptoms during this time interval.

The group of 142 non-*GRN* mutation carriers included 43 healthy controls (HC), 44 FTD patients carrying a heterozygous *C9orf72* expansion (FTD-*C9orf72*), and 55 patients without any identifiable genetic cause (FTD-ng), after extensive genetic evaluations including NGS.

All participants gave written informed consent, and the study was approved by the local ethics committees of Paris-Necker Hospital and of "Assistance Publique – Hôpitaux de Paris" Ile de France VI.

2.2. Plasma sampling

Blood samples were collected in EDTA tubes using the same protocol for all participants in fasting state. All samples were analyzed in the Pitié-Salpêtrière Hospital laboratory, involved in the diagnosis of both LSD and neurodegenerative diseases. Samples were centrifuged at 2000*g* for 20 min at +4 °C, aliquoted by fraction of 500 μL, and then frozen in polypropylene tubes at −80 °C until assay. All plasma measurements were performed using standardized procedures, blinded to the clinical and genetic status.

2.3. Plasma lysosphingolipid measurements

SPL are mainly divided into ceramides, phosphosphingolipids (mostly represented by sphingomyelins), glycosphingolipids, and their deacylated derivatives lysoSPL, including glucosylsphingosine, lysosphingomyelin and lysoglobotriaosylceramide. Glucosylsphingosine d18:1 (LGL1), lysoglobotriaosylceramide (LGB3), lysosphingomyelin d18:1 (LSM18:1) and its carboxylated analog lysosphingomyelin 509 (LSM509), are increased in the plasma of patients suffering from Gaucher (LGL1), Fabry (LGB3) and Niemann-Pick type A/B and C (lysosphingomyelins) diseases.

LSM18:1, LSM509, LGL1, LGB3 were measured simultaneously in plasma by electrospray ionization-tandem mass spectrometry (TQD, Waters), coupled to ultraperformance liquid chromatography (UPLC-Acquity, Waters). Briefly, in polypropylene tubes, 100 μL of plasma were mixed with 200 μL of a mixture of internal standards in methanol, consisting of LSM-d17:1, Glycine-lysoGb3 and ^{13}C6-lysoGL1. The mixture was vortexed during 1 min, standing 15 min at room temperature, and then centrifuged 10 min at 10000*g*. The supernatant was injected in the UPLC – Tandem Mass Spectrometry system. Reverse phase liquid chromatography was performed on an UPLC C18 Ethylene Bridged Hybrid (BEH) column (2.1 × 50 mm, 1.7 μm) at 45 °C (mobile phase A: ultrapure water, mobile phase B: 100% methanol, 0.02% acetic acid). To separate LGL1 from galactosylsphingosine, plasmas were also analyzed by using a Hydrophylic Interaction Chromatography – UPLC BEH column (2.1 × 50 mm, 1.7 μm), as reported (Sidhu et al., 2018). LysoSPL were detected in a positive mode with multiple reaction monitoring. For lysoSPL quantification, calibration curves were made by a serial dilution of a mixture of LSM18:1, LGL1 and LGB3.

The precision of the method was investigated by the intra-day and inter-assay imprecision, assessed by analyzing quality control (QC) samples at two different nominal concentrations (high QC and low QC). The results of QC samples demonstrated acceptable accuracy and precision (Supplementary Table 2). One patient with Niemann-Pick type A/B, one with Gaucher, and one with Fabry disease were used as "positive controls" for the method, and had elevated plasma levels of the corresponding lysoSPL compared to HC (Supplementary Table 3).

2.4. Plasma NfL measurements

Plasma neurofilament light chain (NfL) measurements were performed in 54 carriers (35 FTD-*GRN* patients, 19 PS-*GRN*) included in the study using Single Molecule Array (Quanterix, USA), as previously described (Saracino et al., 2021).

2.5. Statistical analysis

We first compared the three groups of non-*GRN* carriers (HC, FTD-*C9orf72* and FTD-ng) between them, to exclude any differences in demographic characteristics and plasma lysoSPL levels. We then compared *GRN* carriers – including PS-*GRN*, FTD-*GRN* and CLN-11 – and non-carriers. Additional comparisons were performed between the three groups of FTD patients (*GRN*, *C9orf72* and non-genetic). Non-parametric tests were used as data were not normally distributed.

Demographic and clinical characteristics were compared using Kruskal-Wallis test and Fisher's exact test when appropriate. Dunn's test and pairwise Fisher's exact test were then performed for pairwise comparisons using Benjamini-Hochberg correction.

Plasma lysoSPL were compared between the studied groups and the *GRN* mutation types using either Mann-Whitney-Wilcoxon test or Kruskal-Wallis test with Dunn's test for post-hoc analyses. The associations of lysoSPL levels with age at sampling, age at onset and disease duration (for FTD-*GRN*), or expected years to onset (EYO, for PS-*GRN*) were evaluated using Spearman's correlation test. Among *GRN* carriers, the correlations of plasma lysoSPL between each other and with plasma NfL levels, and CDR + NACC FTLD scores, were analyzed with Spearman's test as well.

For the individuals who underwent repeated blood samplings over time we used linear mixed effects models to test for significant differences between the two time-points, with age at first sampling as covariate.

All results were considered significant for p-values ≤ 0.05. Multiple testing of plasma lysoSPL was handled with Benjamini-Hochberg method. Statistical analyses were performed with the R 4.2.2 software (The R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Population of GRN carriers and non-carriers

There were no differences in demographic characteristics and lysoSPL levels between the three groups of non-*GRN* mutation carriers (Supplementary Table 4), allowing to merge them into a unique group for further comparisons with *GRN* carriers. Among non-carriers, plasma LSM18:1, LSM509, LGL1 and LGB3 levels did not differ according to the gender, and did not vary with the age at sampling (Supplementary Fig. 1).

Characteristics and comparisons between non-carriers, PS-*GRN*, FTD-*GRN* and CLN-11 are summarized in Table 1. As expected, the mean age at sampling was higher in FTD-*GRN* compared to CLN-11 and to PS-*GRN* ($p < 0.0001$), and plasma progranulin levels were lower in all *GRN* carriers compared to non-carriers ($p < 0.0001$, Table 1).

3.2. Plasma lysosphingomyelins and glucosylsphingosine levels are increased in GRN carriers

Among the four lysoSPL analyzed, plasma LSM18:1, LSM509 and LGL1 levels were significantly higher in the 131 *GRN* carriers compared to non-carriers (Table 1 and Fig. 1). LGB3 levels did not differ according to *GRN* mutation status, nor between the different groups of *GRN* carriers, and were not considered, therefore, in further analyses. As in non-carriers, none of the lysoSPL differed according to the gender in *GRN* carriers.

Lysosphingomyelins (LSM18:1 and LSM509) were elevated in each group of *GRN* carriers, regardless of their clinical status. LSM509 was even higher in CLN-11 patients, even if the reduced sample size precluded further comparisons. On the other hand, LGL1 levels were only increased in *GRN* patients, and not different between PS-*GRN* and non-carriers (Fig. 1).

There was no impact of the different mutation types (nonsense, frameshift, splice site) on the levels of any of the lysoSPL (Supplementary Fig. 2). Moreover, there was a slight trend in favor of higher lysoSPL levels in individuals with lower plasma progranulin dosage, though not reaching statistical significance (Supplementary Fig. 3).

3.3. Glucosylsphingosine and lysosphingomyelins in FTD-GRN patients

LSM18:1 and LGL1 plasma levels increased with age at onset ($\rho = 0.254$, $p = 0.01$; and $\rho = 0.203$, $p = 0.04$) and age at sampling ($\rho = 0.294$, $p = 0.003$; and $\rho = 0.268$, $p = 0.006$) in FTD-*GRN* patients (Fig. 2A), whereas no such effect was observed for age at sampling in non-carriers (Supplementary Fig. 1). Only LGL1 levels increased with disease duration in the FTD-*GRN* group ($\rho = 0.239$, $p = 0.016$) (Fig. 2B).

To determine if the increase in plasma lysoSPL levels is a *GRN* gene-specific pathological process, we directly compared their levels between FTD-*GRN*, FTD-*C9orf72* and FTD-ng (Table 2 and Fig. 3). Plasma levels of LGL1, LSM18:1 and LSM509 were all significantly higher in FTD-*GRN* than in FTD patients not carrying *GRN* mutations, whose levels were not different from those found in HC, as already shown.

3.4. LSM18:1 and LGL1 longitudinally increase in presymptomatic GRN carriers

In the group of 26 PS-*GRN*, LSM18:1 and LSM509 levels were in the same range as those observed in patients, whereas LGL1 levels were similar to those of non-carriers (Table 1). There was no association between any of the lysoSPL and the age at sampling or the EYO (data not shown).

In the 17 PS-*GRN* who underwent follow-up plasma sampling after a mean interval of 3.4 ± 1.5 years, LSL18:1 and LGL1 levels displayed a significant increase over time ($p < 0.0001$ and $p = 0.0002$, respectively) (Fig. 4 and Supplementary Table 5).

Plasma lysoSPL levels were not associated with changes in the CDR + NACC FTLD scores; however, all PS-*GRN* remained asymptomatic during their follow-up.

3.5. Correlations of plasma lysoSPL between each other

In the overall population of *GRN* carriers, LSM18:1 and LSM509 levels were significantly correlated to each other ($\rho = 0.438$; $p < 0.0001$), whereas there was no such association with LGL1 levels (Supplementary Fig. 4).

Table 1
Demographic characteristics, plasma lysoSPL and progranulin levels in the studied populations.

	Non-carriers (a)	PS-*GRN* (b)	FTD-*GRN* (c)	CLN-11 (d)	*p*-value	corrected *p*-value
Number of cases	142	26	102	3	–	–
Gender (F/M)	78/64	14/12	50/52	2/1	0.78	–
Age at sampling (years)	b,d	a,c	b,d	a,c		
Mean ± SD	61 ± 11.3	38.8 ± 11.0	62.2 (±7.2)	38.3 (±23.6)	***<0.0001****	-
Median (Q1; Q3)	63.5 (56; 69)	39 (32.3; 45)	63 (58; 67)	36 (26; 49.5)		
LSM18:1 (nM)	b,c,d	a	a	a		
Mean (±SD)	2.3 (±0.6)	3.7 (±1.6)	3.1 (±1.1)	4.8 (±2.2)		
Median (Q1; Q3)	2.2 (1.9; 2.7)	3.6 (2.5; 4.7)	3.0 (2.3; 3.4)	4.9 (3.8; 5.9)	***< 0.0001****	***< 0.0001****
LSM509 (nM)	b,c,d	a,d	a,d	a,b,c		
Mean (±SD)	0.3 (±0.2)	0.5 (±0.5)	0.4 (±0.3)	1.3 (±0.6)	***< 0.0001****	***< 0.0001****
Median (Q1; Q3)	0.2 (0.2; 0.3)	0.3 (0.2; 0.6)	0.3 (0.2; 0.7)	1.0 (1.0; 1.5)		
LGL1 (nM)	c,d	c,d	a,b	a,b		
Mean (±SD)	1.2 (±0.6)	1.3 (±0.7)	2.4 (±1.4)	3.5 (±1.2)	***< 0.0001****	***< 0.0001****
Median (Q1; Q3)	1.1 (0.8; 1.5)	1.3 (1.0; 1.6)	2.1 (1.4; 3.0)	3.3 (2.9; 4.1)		
LGB3 (nM)						
Mean (±SD)	0.4 (±0.2)	0.3 (±0.1)	0.4 (±0.3)	0.5 (±0.1)	0.43	0.43
Median (Q1; Q3)	0.4 (0.3; 0.5)	0.3 (0.2; 0.4)	0.4 (0.3; 0.5)	0.6 (0.5; 0.6)		
Progranulin (ng/mL)	b,c,d	a	a	a		
Mean (±SD)	119.4 (±29.6)	39 (±13.8)	36.0 (±11.4)	0	***< 0.0001****	-
Median (Q1; Q3)	113.0 (98.3; 131)	38.5 (27.8; 49.5)	35.0 (29.0; 43.0)	0		

Data are given as mean (± SD) and as median (Q1; Q3). Significant differences are indicated in bold, and the groups (a,b,c or d) compared to which the difference is significant are indicated in the top of the table cells. CLN-11: neuronal ceroid lipofuscinosis 11 patients with homozygous *GRN* mutations; FTD-*GRN*: frontotemporal dementia patients with heterozygous *GRN* mutations; F; females; M: males; PS-*GRN*: presymptomatic heterozygous *GRN* carriers; Q1: first quartile; Q3: third quartile: SD: standard deviation.

3.6. Correlations of plasma lysoSPL with markers of disease severity

To evaluate the association of plasma lysoSPL with the progression of the neurodegenerative process, we studied their correlation with plasma NfL levels, a well-known marker of neuroaxonal degeneration. Plasma NfL measured 7.2 ± 2.8 pg/mL in PS-*GRN* and 90.2 ± 44.6 pg/mL in FTD-*GRN*. In PS-*GRN*, NfL levels were higher in individuals who were closer to phenoconversion, based to their EYO ($\rho = 0.355$; $p = 0.039$). On the other hand, plasma NfL were not correlated with age at onset ($\rho = -0.294$; $p = 0.10$), age at sampling ($\rho = -0.232$; $p = 0.19$), or disease duration ($\rho = 0.207$; $p = 0.24$) in patients (Supplementary Fig. 5A). In PS-*GRN* there was a significant association between plasma LGL1 and NfL levels ($\rho = 0.347$; $p = 0.038$), whereas no significant associations emerged between any of the lysoSPL and NfL levels in patients (Supplementary Fig. 5B).

4. Discussion

Among the consequences of progranulin deficiency, lysosomal dysfunction may play a relevant role in the pathomechanism common to FTD and CLN-11 (Paushter et al., 2018; Arrant et al., 2019; Boland et al., 2022). To determine whether progranulin deficiency in *GRN* carriers is accompanied by changes in the levels of storage products in plasma, as in LSD, we measured four lysoSPL in plasma from 131 patients and presymptomatic individuals carrying *GRN* mutations. Notably, this study also included three patients with CLN-11 disease, an extremely rare condition linked to homozygous *GRN* mutations and complete loss of progranulin.

Plasma lysosphingomyelins (LSM18:1 and LSM509) were significantly higher in all *GRN* mutation carriers compared to non-carriers, whereas LGL1 was only increased in FTD-*GRN* and CLN-11 patients, but not in presymptomatic carriers. Of note, LSM509 levels were even higher in homozygous CLN-11 patients compared to heterozygous

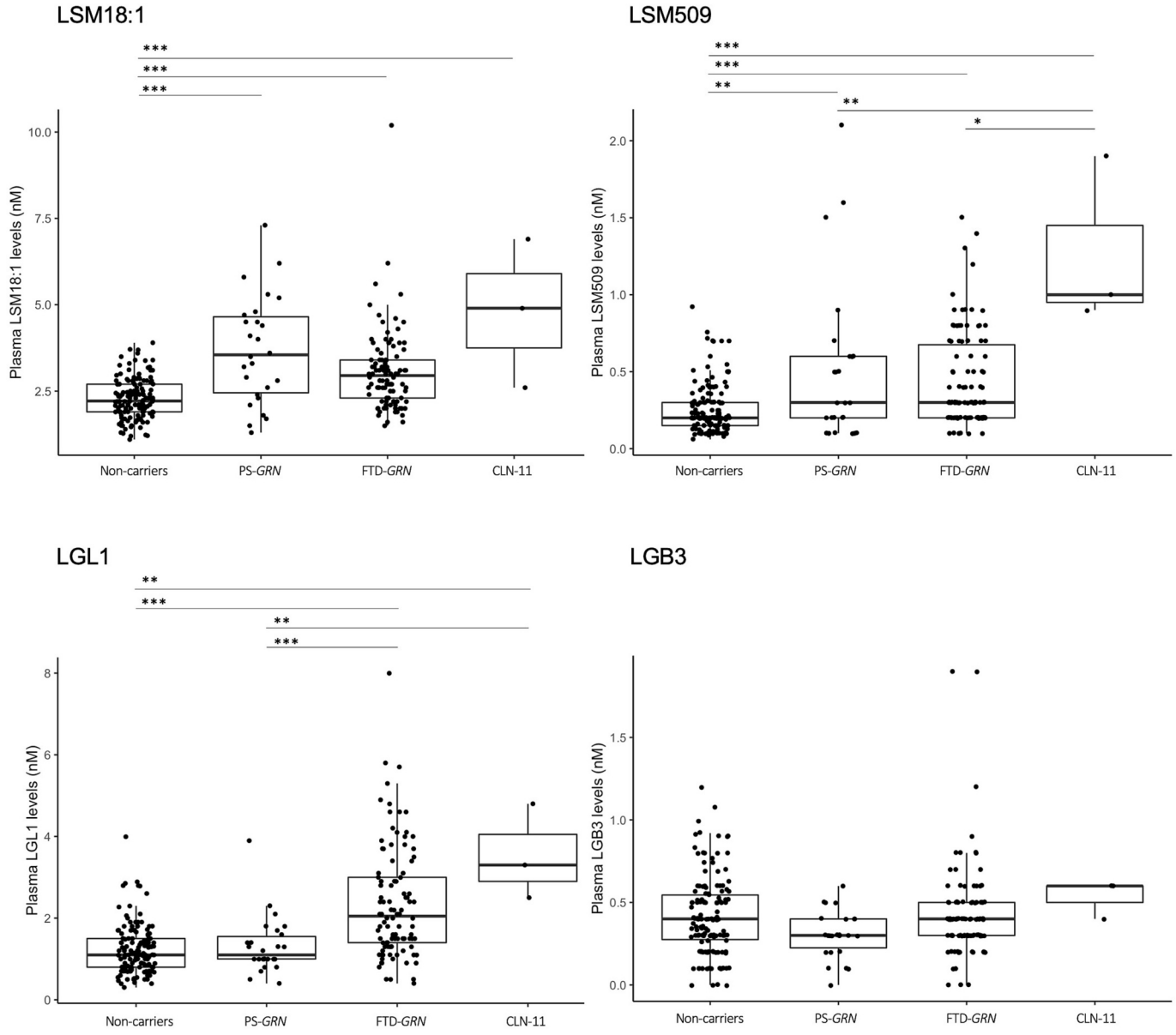

Fig. 1. Plasma lysoSPL levels in the studied population. Plasma LSM18:1, LSM509, LGL1 and LGB3 levels in 131 *GRN* carriers and 142 non-carriers. Asterisks indicate statistically significant differences between the groups after post-hoc Dunn's test (*** for $p < 0.001$, ** for $p < 0.01$, * for $p < 0.05$). CLN-11: neuronal ceroid lipofuscinosis 11 patients with homozygous *GRN* mutations; FTD-*GRN*: frontotemporal dementia patients with heterozygous *GRN* mutations; PS-*GRN*: presymptomatic heterozygous *GRN* carriers.

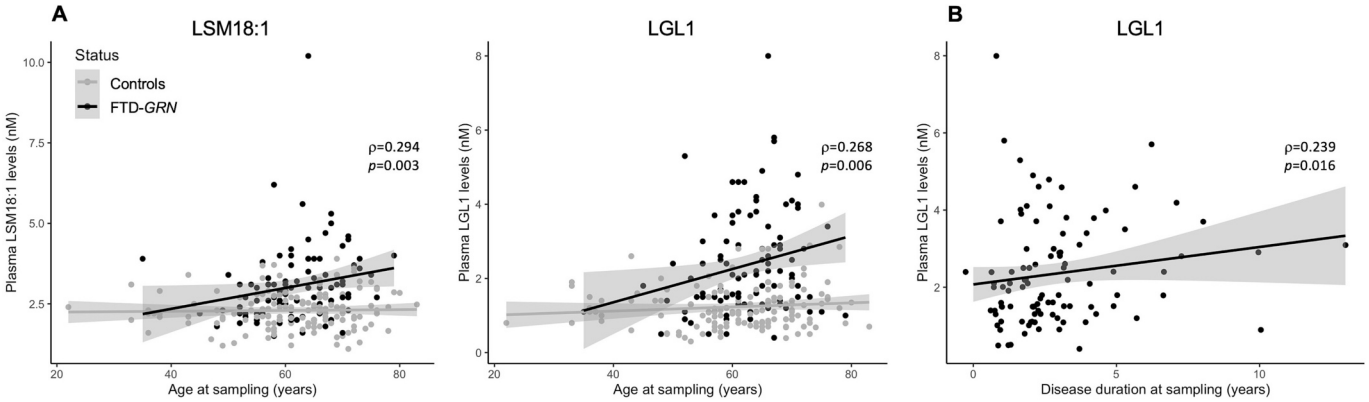

Fig. 2. Association of plasma lysoSPL levels with age at sampling and disease duration in FTD-*GRN*. A. A positive correlation with age was found for plasma LSM18:1 levels ($\rho = 0.294$; $p = 0.003$) and LGL1 levels ($\rho = 0.268$; $p = 0.006$) in FTD-*GRN* patients. Notably, no lysoSPL levels correlated with age at sampling in controls (see also Supplementary Fig. 1). B. Plasma LGL1 levels were positively correlated with disease duration in FTD-*GRN* patients ($\rho = 0.239$; $p = 0.016$). FTD-*GRN*: frontotemporal dementia patients with heterozygous *GRN* mutations.

Table 2
Comparison of plasma lysoSPL levels between the three groups of FTD patients, including *GRN* mutation carriers, *C9orf72* expansion carriers and patients with non-genetic FTD.

	FTD-*GRN*	FTD-*C9orf72*	FTD-ng	*p*-value	corrected *p*-value
Number of cases	102	44	55	–	–
Gender (F/M)	50/52	24/20	28/27	*0.83*	–
Age at sampling (years)					
Mean (±SD)	62.2 (±7.2)	62.0 (±7.9)	64.1 (±8.9)	*0.52*	-
Median (Q1; Q3)	63 (58; 67)	64 (57.8; 67.3)	64 (57.5; 71)		
LSM18:1 (nM)	b,c	a	a		
Mean (±SD)	3.1 (±1.1)	2.3 (±0.5)	2.3 (±0.6)	< ***0.0001****	< ***0.0001****
Median (Q1; Q3)	3.0 (2.3; 3.4)	2.3 (2.0; 2.6)	2.3 (1.9; 2.8)		
LSM509 (nM)	b,c	a	a		
Mean (±SD)	0.4 (±0.3)	0.3 (±0.1)	0.3 (±0.2)	< ***0.0001****	< ***0.0001****
Median (Q1; Q3)	0.3 (0.2; 0.7)	0.2 (0.2; 0.3)	0.2 (0.2; 0.4)		
LGL1 (nM)	b,c	a	a		
Mean (±SD)	2.4 (±1.4)	1.3 (±0.5)	1.2 (±0.7)	< ***0.0001****	< ***0.0001****
Median (Q1; Q3)	2.1 (1.4; 3.0)	1.1 (1.0; 1.7)	1.1 (0.7; 1.3)		

Data are given as mean (± SD) and as median (Q1; Q3). Significant differences are indicated in bold, and the groups (a,b or c) compared to which the difference is significant are indicated in the top of the table cells. F; females; FTD-*C9orf72*: patients carrying *C9orf72* repeat expansion; FTD-*GRN*: frontotemporal dementia patients with heterozygous *GRN* mutations; FTD-ng: patients with non-genetic FTD; M: males; Q1: first quartile; Q3: third quartile: SD: standard deviation.

carriers, whilst a similar trend, not reaching statistical significance, was present for the other lysoSPL species. A fourth analyte, LGB3, did not differ between groups. These results provide supportive evidence that sphingolipid degradation defects are associated to the pathophysiology of *GRN*-related diseases, possibly throughout previously demonstrated abnormal lysosomal enzymatic activities (Zhou et al., 2019; Valdez et al., 2020). Notably, LGL1, LSM18:1 and LSM509 levels were not different from control subjects in FTD patients carrying *C9orf72* expansions – the other major genetic cause of FTD with TDP-43 aggregation – or with non-genetic forms of FTD. Thus, increased levels of those analytes are not common to all forms of FTD, nor are they linked to abnormal neuronal TDP-43 aggregation, but rather lysoSPL elevation more likely represents a process related to *GRN* haploinsufficiency. Importantly, there was no association between lysoSPL and age at sampling in controls, as previous works already pointed out (Murugesan et al., 2016; Kubaski et al., 2022). Taken together, these observations suggest that the increase of lysosomal storage products is not related to normal ageing, but could be triggered by PGRN deficiency.

The mechanisms by which the activity of lysosomal enzymes is impaired by progranulin deficiency are not fully understood, and may involve the interactions with other pivotal proteins in the intralysosomal network, such as PSAP and pro-cathepsin D (Tayebi et al., 2020). PGRN is involved in PSAP internalization into the lysosome (Zhou et al., 2015, 2017). PSAP is then cleaved by cathepsin D into mature saposins (A to D) which are essential coactivators of several lysosomal enzymes including, but not limited to, β-glucocerebrosidase, acid sphingomyelinase, and galactocerebrosidase (Paushter et al., 2018; Zhou et al., 2019; Arrant et al., 2019; Tayebi et al., 2020; Valdez et al., 2020). Additionally, PGRN enhances the maturation of pro-cathepsin to its active form cathepsin D which, in turn, activates saposins (Valdez et al., 2017; Chen et al., 2018; Butler et al., 2019; Tayebi et al., 2020). Therefore, PGRN deficiency likely results in reduced mature saposins which could, in turn, impair the aforementioned enzymatic activities leading to the increase of SPL and lysoSPL levels (Fig. 5). This is supported by impaired lysosomal enzyme activities in brain, fibroblasts and iPSC-derived cortical neurons from heterozygous *GRN* patients (Götzl et al., 2014; Valdez et al., 2017, 2020; Ward et al., 2017; Arrant et al., 2019; Boland et al., 2022), and lysosomal vacuolization and lipofuscin accumulation in brain of *Grn*−/− mice, a model of *GRN*-related FTD (Ahmed et al., 2010; Tanaka et al., 2014). Since lysosomes are ubiquitously expressed in all cells except red blood cells, the impairment of lysosomal activities in the presence of PGRN deficit concerns all cells and tissues, including circulating leukocytes (Dekker et al., 2011; Polo et al., 2019), which makes plasma lysoSPL concentrations raise accordingly, though at a lesser degree.

Accumulation of LGL1 results from decreased activity of β-glucocerebrosidase (GCase) in lysosomes. GCase activity is reduced in a context of PGRN deficit, as shown in frontal cortex of FTD-*GRN* patients and in *Grn*−/− mice (Arrant et al., 2019; Zhou et al., 2019), possibly due to decreased saposins levels (Zhou et al., 2019; Valdez et al., 2020), lysosomal mislocalization, or incomplete glycosylation of GCase (Arrant et al., 2019). The partial recovery of GCase activity with the addition of PGRN-derived peptides in mouse and human models of *Gba1/GBA1* mutations further supports the crucial role of PGRN in GCase activity (Zhao et al., 2023). Our results are thus consistent with reduced GCase activity in *GRN* disease, suggesting the use of plasma LGL1 as a repeatable and non-invasive assay to monitor this lysosomal enzymatic dysfunction.

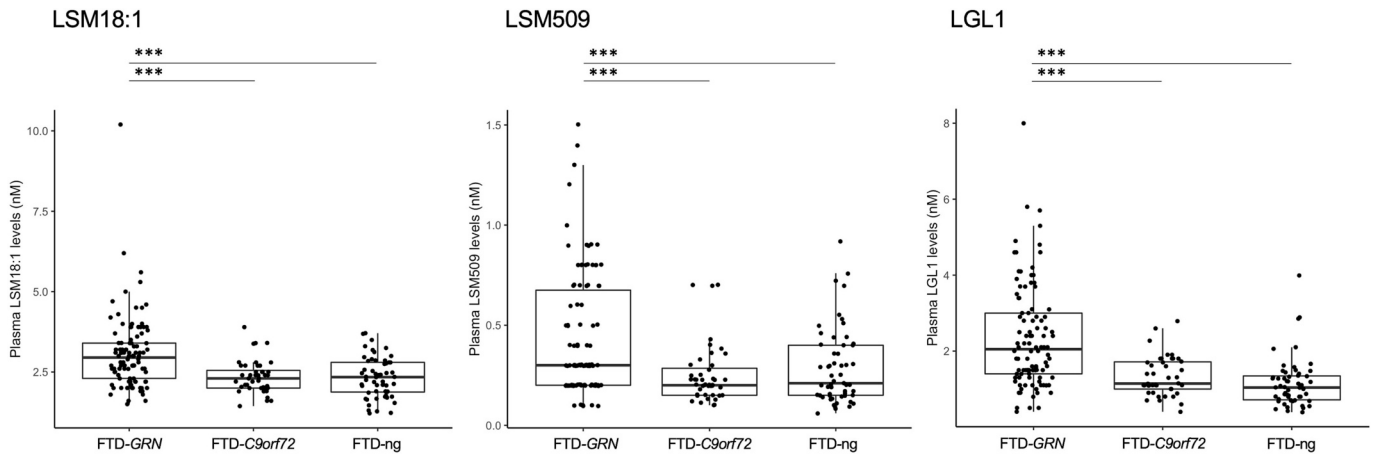

Fig. 3. Comparison of plasma lysoSPL levels between FTD patients carrying heterozygous *GRN* mutations, *C9orf72* expansions and with non-genetic forms. Asterisks indicate statistically significant differences between the groups after post-hoc Dunn's test (*** for $p < 0.001$). FTD-*C9orf72*: patients carrying *C9orf72* repeat expansion; FTD-*GRN*: frontotemporal dementia patients with heterozygous *GRN* mutations; FTD-ng: patients with non-genetic FTD.

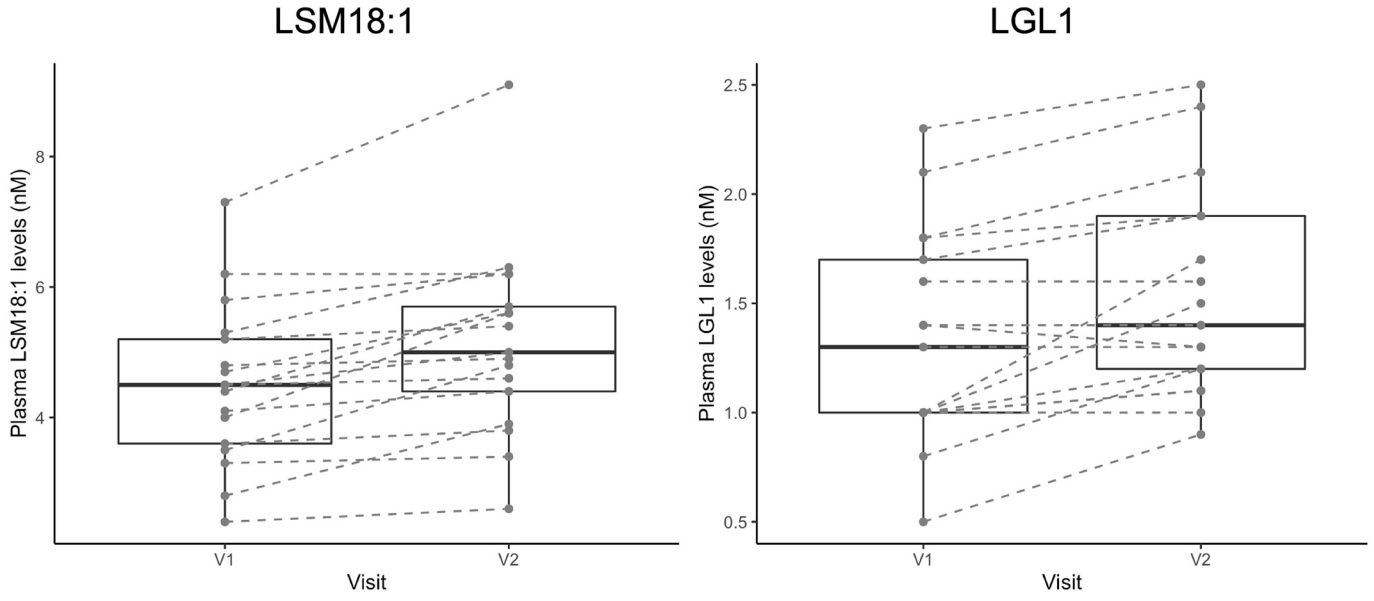

Fig. 4. Longitudinal increases in plasma LSM18:1 and LGL1 levels in PS-*GRN* undergoing follow-up sampling. Linear mixed effect models including baseline age as covariate disclosed statistically significant increases over time ($p < 0.0001$ for LSM18:1 and $p = 0.0002$ for LGL1, after Benjamini-Hochberg correction). PS-*GRN*: presymptomatic heterozygous *GRN* carriers; V1: visit 1; V2: visit 2.

Additionally, our study suggests that PGRN deficit not only affects GCase function, but also likely impairs lysosomal acid sphingomyelinase (SPMase) activity in a similar manner, possibly based on the reduced availability of functional saposins in the lysosome. Indeed, *GRN* mutation carriers displayed increased levels of plasma LSM18:1 and LSM509 compared to non-carriers. Lysosphingomyelins are increased, at higher level, in Niemann-Pick type A/B diseases caused by SPMase deficiency, another enzyme which needs saposin co-activation (Ni and Morales, 2006; Xiong et al., 2016). Moreover, elevated plasma lysosphingomyelins in all *GRN* carriers regardless of their clinical stage indicates greater vulnerability of SPMase activity to PGRN deficiency, even long before phenoconversion, and suggests that the dysregulation of different enzymatic activities may be a dynamic process throughout the overall disease course. The evidence of significant changes in plasma PSAP/saposin levels in association with lysoSPL increases would strengthen the hypothesized relationship between progranulin deficit, impaired prosaposin cleavage, and reduced enzymatic activities in *GRN* carriers.

However, the PGRN/PSAP pathomechanism is not unequivocal for all lysosomal enzymes. Indeed, there was no detectable accumulation of LGB3, a substrate of α-galactosidase, in *GRN* carriers, which is in line with normal α-galactosidase activity reported in brain tissues from FTD-*GRN* patients (Arrant et al., 2019). The latter study and the present findings suggest that this enzyme is not, or only mildly, affected by PGRN deficiency, and that impaired PSAP processing may not be the only factor contributing to disease pathogenesis. Our results collectively suggest that several intertwined mechanisms linking PGRN deficit to elevated lysoSPL are likely involved, thus resulting in different effects on the levels of lysosomal metabolic byproducts. Overall, a wider unbiased metabolomic approach may be necessary to evidence even more relevant differences in other lipid-based markers.

Another finding of this study is the age-dependent increase of LSM18:1 and LGL1 levels in FTD-*GRN* patients, whereas no such association emerged in non-*GRN* carriers. This suggests that lysosomal dysfunction gradually worsens with the progression of the pathological process at the clinical stage of the disease, which is further supported by the higher LGL1 levels in patients with the more advanced disease at

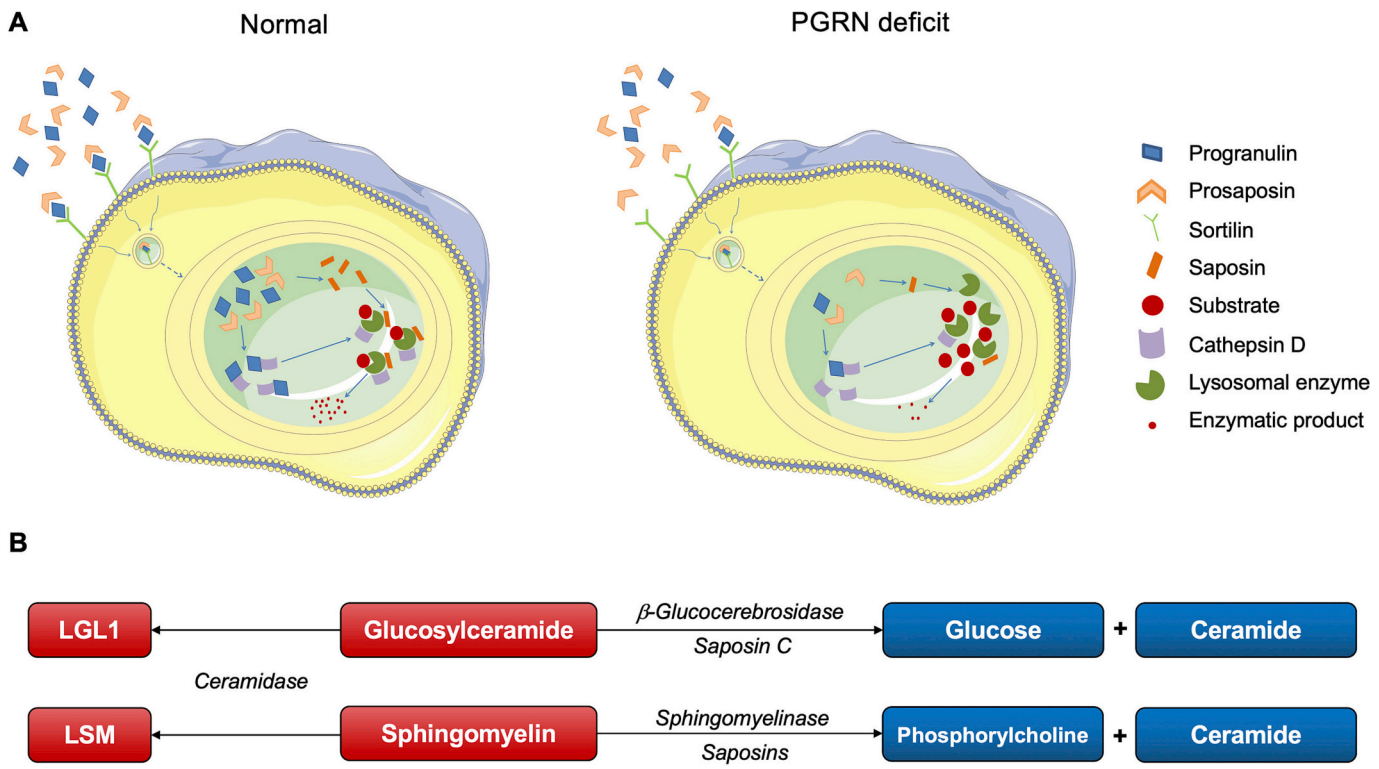

Fig. 5. Schematic model of the role of progranulin in the lysosome and possible mechanism leading to lysoSPL accumulation in presence of progranulin deficit. A. In a normal cell, internalization of PSAP, via endosome, is mediated by the binding of PGRN-PSAP complex to sortilin. Once in the lysosome, PGRN enhances the maturation of pro-cathepsin to its active form cathepsin D. PSAP is then cleaved by cathepsin D into saposins that are key activators for lysosomal enzymes activity and SPL degradation. In *GRN*-mutated cells, the PGRN haploinsufficiency leads to lower PSAP internalization resulting in the impairment of the enzymatic activities and substrates (SPL, lysoSPL) accumulation. B. Scheme of the enzymatic pathways involved in SPL degradation and lysoSPL production. LysoSPL: lysosphingolipids; PGRN: progranulin; PSAP: prosaposin; SPL: sphingolipids.

sampling. Although needing replication in independent cohorts, these results hold promise for a role of plasma LGL1 and LSM18:1 as potential biomarkers for tracking disease trajectory at the full-blown stage. Due to their direct link with the pathophysiological process, plasma lysoSPL appear to be uniquely increased in *GRN* mutation carriers, thus serving as innovative easily-accessible, non-invasive biomarkers of progression in *GRN*-related diseases, in addition to or in combination with other, less specific biological markers such NfL, neuronal pentraxins, and glial fibrillary acidic protein (van der Ende et al., 2019, 2020; Heller et al., 2020).

To further test the hypothesis of an ongoing alteration of sphingolipid metabolism throughout the overall disease process, we longitudinally analyzed the same lysoSPL levels in a group of *GRN* carriers at the presymptomatic phase. Two analytes, LSM18:1 and LGL1, displayed longitudinal increases in PS-*GRN*, over a mean follow-up time of 3.4 years. Of note, this change was already detectable long before clinical conversion, PS-*GRN* carriers being at a relatively young age at baseline on average (<40 years) and without any noticeable clinical progression during their follow-up. LSM18:1 was higher compared to non-carriers at baseline, and displayed the more sustained increases. On the other hand, LGL1 was less impacted in the presymptomatic phase, its levels being lower at baseline and displaying a smoother increase over time. This is in line with the observation of normal GCase activity in the brain tissue at presymptomatic stage in *GRN* carrier (Arrant et al., 2019). Of note, LGL1 levels were higher in presymptomatic carriers with elevated NfL levels, whose increase heralds phenoconversion (van der Ende et al., 2019; Rojas et al., 2021; Staffaroni et al., 2022). Not surprisingly, LGL1 levels were not associated with NfL levels in patients, as NfL massively increase around the phenoconversion, then their levels do not reflect disease progression in symptomatic individuals (van der Ende et al., 2019). Although preliminary, these results point out a different dynamic of impaired lysoSPL degradation in the presence of PGRN deficiency, and suggest a dysregulation of plasma lysoSPL in a time-dependent manner along the entire course of *GRN* disease. In particular, lysosphingomyelin levels might serve as early tracers of preclinical stage, whereas LGL1 increases may occur smoothly and at a later stage, with sustained increases during the clinical phase.

The current study has some limitations. Firstly, the group sizes were limited but nevertheless they do represent large cohorts for rare genetic diseases. In particular, the inclusion of three CLN-11 was rather unique, as CLN-11 cases are exceptional, with very few families described worldwide (Huin et al., 2020). Our results suggest a dose effect of PGRN deficiency on the impairment of lysoSPL degradation, but this cannot be ascertained owing to the rarity of CLN-11 cases. Secondly, longitudinal samples were available for a limited number of presymptomatic carriers. Overall, though promising, these results need to be validated in larger independent cohorts to confirm the magnitude of lysoSPL change over time, in particular in carriers undergoing phenoconversion. Additionally, the concentrations of lysoSPL in *GRN* carriers were of moderate amplitude, not comparable to those in LSD patients (Polo et al., 2019). This was however expected, as the mechanism of lysosomal enzyme dysfunction is indirect and mediated by PGRN haploinsufficiency, rather than directly related to homozygous mutations in genes coding for the corresponding enzymes. Nonetheless, differences in our population were significant and, notably, in the same range as those found in patients with Parkinson's disease carrying heterozygous *GBA1* mutations (Pchelina et al., 2018).

In conclusion, our study provides important findings demonstrating increased levels of LGL1, LSM18:1 and LSM509 in the plasma of *GRN* carriers, thereby reinforcing the hypothesis that lysosomal dysfunction contributes to some extent to the mechanism of *GRN*-pathogenesis. Overall, our work suggests that plasma lysoSPL may become useful,

easily accessible progression biomarkers in FTD caused by *GRN* mutations. Identifying novel candidates in the fast-moving context of innovative biomarkers is a major challenge for monitoring forthcoming therapeutic trials and measuring drug response. This study sheds light on lysoSPL assay in research settings for *GRN*-related diseases, and paves the way to new disease-modifying or preventive approaches based on lysosomal dysfunction rescue or substrate reduction.

Disclosure

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2023.106108.

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