

Plasma progranulin levels predict progranulin mutation status in frontotemporal dementia patients and asymptomatic family members

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Mutations in the progranulin gene (*GRN*) are an important cause of frontotemporal lobar degeneration (FTLD) with ubiquitin and TAR DNA-binding protein 43 (TDP43)-positive pathology. The clinical presentation associated with *GRN* mutations is heterogeneous and may include clinical probable Alzheimer's disease. All *GRN* mutations identified thus far cause disease through a uniform disease mechanism, i.e. the loss of functional *GRN* or haploinsufficiency. To determine if expression of *GRN* in plasma could predict *GRN* mutation status and could be used as a biological marker, we optimized a *GRN* ELISA and studied plasma samples of a consecutive clinical FTLD series of 219 patients, 70 control individuals, 72 early-onset probable Alzheimer's disease patients and nine symptomatic and 18 asymptomatic relatives of *GRN* mutation families. All FTLD patients with *GRN* loss-of-function mutations showed significantly reduced levels of *GRN* in plasma to about one third of the levels observed in non-*GRN* carriers and control individuals ($P < 0.001$). No overlap in distributions of *GRN* levels was observed between the eight *GRN* loss-of-function mutation carriers (range: 53–94 ng/ml) and 191 non-*GRN* mutation carriers (range: 115–386 ng/ml). Similar low levels of *GRN* were identified in asymptomatic *GRN* mutation carriers. Importantly, ELISA analyses also identified one probable Alzheimer's disease patient (1.4%) carrying a loss-of-function mutation in *GRN*. Biochemical analyses further showed that the *GRN* ELISA only detects full-length *GRN*, no intermediate granulin fragments. This study demonstrates that using a *GRN* ELISA in plasma, pathogenic *GRN* mutations can be accurately detected in symptomatic and asymptomatic carriers. The ~75% reduction in full-length *GRN*, suggests an unbalanced *GRN* metabolism in loss-of-function mutation carriers

whereby more GRN is processed into granulins. We propose that plasma GRN levels could be used as a reliable and inexpensive tool to identify all GRN mutation carriers in early-onset dementia populations and asymptomatic at-risk individuals.

Keywords: Progranulin; ELISA; frontotemporal lobar degeneration; Alzheimer's disease

Abbreviations: bvFTD = behavioural variant frontotemporal dementia; FTLN = frontotemporal lobar degeneration; PNFA = progressive non-fluent aphasia; SD = semantic dementia; SPECT = single photon emission tomography

Introduction

Frontotemporal lobar degeneration (FTLD) is a genetically complex neurodegenerative disorder (Rademakers and Hutton, 2007). It is the second most common form of early-onset dementia after Alzheimer's disease, accounting for 5–10% of all dementia patients and 10–20% of patients with an onset of dementia before 65 years (Graff-Radford and Woodruff, 2007). Up to 50% of patients with FTLN report a family history of dementia, suggesting a strong genetic component to the disease. The clinical symptoms associated with FTLN are diverse including behaviour and personality changes, language disorders of expression and comprehension, cognitive impairment and sometimes motor neuron disease (McKhann *et al.*, 2001). Three clinical FTLN subtypes have been defined: behavioural variant frontotemporal dementia (bvFTD), progressive non-fluent aphasia (PNFA) and semantic dementia (SD). The most common neuropathology associated with this type of dementia is FTLN with ubiquitin- and TAR DNA-binding protein 43 (TDP-43) positive inclusions (FTLN-U) (Josephs *et al.*, 2004; Lipton *et al.*, 2004; Mackenzie *et al.*, 2006; Neumann *et al.*, 2006).

In 2006, we identified loss-of-function mutations in the progranulin gene (*GRN*) as a major cause of familial FTLN-U, explaining 25% of FTLN-U patients worldwide (Baker *et al.*, 2006; Cruts *et al.*, 2006; Gass *et al.*, 2006). To date, 63 different pathogenic *GRN* loss-of-function mutations have been reported in 169 genealogically unrelated FTLN families (Gijssels *et al.*, 2008a). *GRN* mutations are found scattered over the gene and include a variety of genetic alterations, such as nonsense and splice-site mutations as well as small insertions and deletions leading to a shift in the normal reading frame. Recently, the first complete genomic *GRN* deletion and a near complete deletion of *GRN* exons 1–11 were also reported (Gijssels *et al.*, 2008b; Rovelet-Lecrux *et al.*, 2008). Together these studies established the notion that all *GRN* mutations share the same pathogenic mechanism, i.e. the loss of 50% functional *GRN*, suggesting a haploinsufficiency disease mechanism.

The spectrum of clinical presentations associated with mutations in *GRN* is highly heterogeneous (van Swieten and Heutink, 2008). Behaviour changes are the most common symptoms, although prominent language impairment is a frequent occurrence during the course of the disease (Gass *et al.*, 2006; Le Ber *et al.*, 2007; Rademakers *et al.*, 2007; Beck *et al.*, 2008). Importantly, episodic memory deficits occur in the initial stage in 10–30% of *GRN* mutation carriers, occasionally leading to patients being diagnosed with Alzheimer's disease or an amnesic variant of mild cognitive impairment (Benussi *et al.*, 2008; Brouwers *et al.*, 2007; Josephs *et al.*, 2007; Kelley *et al.*, 2007; Rademakers *et al.*, 2007).

GRN encodes a secreted precursor protein composed of a signal peptide sequence and 7.5 tandem repeats of a rare 12 cysteine motif (Bateman and Bennett, 1998). While *GRN* has growth factor properties and a weak anti-inflammatory effect, it can be proteolytically cleaved by extracellular proteases, such as elastase, to form a family of peptides, ranging in size from 6 to 25 kDa, called granulins, which are strongly pro-inflammatory (Zhu *et al.*, 2002). Both *GRN* and the granulins are widely expressed and have been implicated in a range of biological processes including development, wound repair, inflammation and tumorigenesis (He and Bateman, 2003; He *et al.*, 2003). The exact function of *GRN* in the brain still remains to be determined; however, a recent *in vitro* study suggested that *GRN* may function as a neurotrophic factor (Van Damme *et al.*, 2008).

Based on the uniform haploinsufficiency disease mechanism associated with *GRN* mutations, we hypothesized that the analyses of *GRN* levels in plasma using an ELISA could be used to predict *GRN* mutation status in patients with FTLN and their asymptomatic family members. In addition, we sought to test the usefulness of a *GRN* ELISA in the identification of *GRN* mutation carriers in patient populations with other forms of early-onset dementia, such as probable Alzheimer's disease (McKhann *et al.*, 1984).

Methods

Participants

Our FTLN study cohort consisted of 219 patients from a consecutive clinical case series seen at Mayo Jacksonville by the Behavioural Neurology section. All patients agreed to be in the study and biological samples were obtained after informed consent. DNA and plasma samples were collected and stored using standard procedures. Plasma was available for 94.5% (207/219) of the patients. Each patient underwent a full neurological evaluation and all who were testable had a neuropsychological evaluation. Structural neuroimaging was performed in all patients, the majority having MRI but a few have CT. Functional imaging using either single photon emission tomography (SPECT) or PET was also performed in most patients. The majority of patients were followed over time clinically. The diagnoses of the bvFTD, SD and PNFA were made using the Neary criteria (Neary *et al.*, 1998). Patients who had Primary Progressive Aphasia and were fluent (FA) met Mesulam criteria (Mesulam, 2003). Retrospectively, many but not all of them would meet criteria for logopenic or mixed aphasia (Gorno-Tempini *et al.*, 2008). Those with the corticobasal syndrome (CBS) met the criteria outlined by Boeve (2007). Out of 219 patients with FTLN (50.7% women), 74 patients were diagnosed with bvFTD (including one with FTD-ALS), 33 with PNFA, 44 with SD, 42 with FA and 26 with CBS. The mean age at disease onset was 63.9 years (SD 8.3 years,

range: 44–85 years). In 39.7% (87/219) of the patients a positive family history, defined as having at least one first degree relative with a history of dementia or disease in the FTL spectrum including ALS, progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD), was observed. Twenty-one patients underwent autopsy: 16 patients showed pathological diagnoses within the FTL spectrum, while five patients showed Alzheimer's disease at autopsy.

In addition to the patients included in the consecutive FTL series, DNA and plasma samples of eight affected and 18 unaffected at-risk individuals from three previously published *GRN* mutation families ascertained at Mayo Jacksonville and Mayo Rochester were available (Baker *et al.*, 2006; Gass *et al.*, 2006; Kelley *et al.*, 2007; Rademakers *et al.*, 2007). DNA and plasma samples of 72 early-onset probable Alzheimer's disease patients (mean age at onset \pm SD: 57.9 ± 6.4 years; range: 37–65 years; 61% women) and 70 unrelated control individuals (mean age \pm SD: 66.3 ± 11.0 years; range: 46–85 years; 50% women) were also ascertained at Mayo Jacksonville. The clinical diagnoses of Alzheimer's disease were made using the NINCDS/ADRDA criteria (McKhann *et al.*, 1984). In collaboration with Dr Deramecourt we further obtained plasma samples from the French patient carrying a deletion of *GRN* exons 1–11 and two unaffected unrelated control individuals from France (Rovelet-Lecrux *et al.*, 2008).

Procedures

For sequencing analyses, all 12 coding exons and the non-coding exon 0 of *GRN* and exon 1 and exons 9–13 of *MAPT* were amplified by PCR using our previously published primers and protocols (Hutton *et al.*, 1998; Baker *et al.*, 2006). PCR products were purified using AMPure (Agencourt Biosciences, Beverly, MA) then sequenced in both directions using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reactions were purified using CleanSEQ (Agencourt Biosciences) and analysed on an ABI3730 Genetic Analyser (Applied Biosystems). *GRN* sequencing was previously performed in 64 patients from this cohort as part of the Mayo Clinic FTL series, while *MAPT* sequencing was not previously performed in this cohort (Gass *et al.*, 2006).

To investigate whether patients who carry the same *GRN* mutation are descendants from a common founder, we did a haplotype sharing study with eight short tandem repeat (STR) markers spanning a region of 6.9 Mb flanking *GRN* at chromosome 17q21. The STR markers D17S1299, D17S951, D17S1860, D17S934, D17S950, D17S806, TAUPROM [an STR marker located in intron 0 of *MAPT* (Gass *et al.*, 2006)] and the newly developed GRN_GT15 were PCR amplified with one fluorescently labeled primer and analysed on an automated ABI3730 DNA analyser. PCR primers used for GRN_GT15 were F: TCCCATTTCTCCCTTAGTTG and R: AAGTTGAGGCTG CAGGGTG. Alleles were scored using the GENOTYPER software (Applied Biosystems).

Genotyping of control individuals for *GRN* variant c.415T>C (p.C139R) was performed with a TaqMan chemistry-based allelic discrimination assay, with Assay by Design probes (Applied Biosystems) and an Applied Biosystems 7900 PCR system, followed by analysis with Sequence Detection System 2.2.1 software (Applied Biosystems).

To determine GRN expression levels in human plasma samples, we used the human Progranulin ELISA kit (Adipogen Inc., Seoul, Korea) using a 1:100 dilution of the plasma samples in $1 \times$ diluent following manufacturer's instructions. The wash solution was aspirated after each third wash to ensure that all residual wash solution was removed. To increase accuracy all samples were analysed twice in two independent experiments using six interplate control

samples for normalization. Based on our FTL population ($n=207$), the median coefficient of variation (CV) was 3.2%. Recombinant human GRN provided with the ELISA kit was used as a standard.

For Western blot analyses, albumin depleted plasma samples (SwellGel Blue Albumin Removal Kit, Pierce, Rockford, IL) and protein samples extracted from cerebellar brain homogenates of FTL patients with and without *GRN* mutations, were separated by SDS-PAGE using pre-cast 4–20% Tris-Glycine gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes. GRN levels were assessed using the GRN capture antibody and the GRN detection antibody used in the ELISA (Adipogen Inc.). GRN cleavage products were obtained by elastase treatment of recombinant GRN for 30 min at 25°C (Athens Research & Technology, Athens, GA).

Statistical analyses

GRN expression levels were compared between two groups using Mann-Whitney tests. The Kruskal-Wallis test was used to compare GRN expression across clinical FTL subtypes. Results were considered statistically significant for $P \leq 0.05$; no adjustments were made for multiple testing; however, results were interpreted cautiously in the light of the number of tests conducted in these exploratory analyses. Since there was no overlap in distributions of GRN expression levels in the *GRN* loss-of-function mutation carriers compared to the non-*GRN* carriers, we explored sensitivity and specificity for various cut-off points on a continuous scale by fitting normal distributions to the square root of the GRN levels. Ten thousand bootstrap samples were then obtained to construct 95% confidence intervals for sensitivity and specificity at a range of potential cut-off values.

Results

We performed systematic mutation analyses of *GRN* and *MAPT* through direct sequencing in all 219 patients diagnosed with FTL from a consecutive clinical case series ascertained at Mayo Jacksonville. In *MAPT*, we identified the known pathogenic mutation, IVS10+16C>T, in intron 10 in a single patient (NGR180), who presented with personality changes at the age of 55 and was diagnosed with bvFTD. His father, paternal uncle and aunt also had early-onset dementia. Imaging analyses in this patient showed bilateral temporal atrophy on MRI as well as bilateral temporal hypometabolism on PET.

In *GRN*, we identified five different pathogenic loss-of-function mutations in a total of eight patients with FTL and a previously published partial deletion of exon 11, IVS10-15_Ex11+178del, in a single patient with FTL (Table 1). We further identified four missense mutations, two silent mutations and one regulatory variant, 5'UTR-22C>T, in eight patients with FTL (Table 1). With the exception of the silent mutation, p.P578, these *GRN* variants were previously published, however, their pathogenic nature largely remains unknown. Samples were not available from other family members to test for segregation of these *GRN* variants. The presence of missense mutation p.C139R (c.415C>T), which had not been identified in control individuals in previous studies, was further excluded in 248 control individuals ascertained at Mayo Jacksonville. A summary of the clinical and pathological characteristics of the *GRN* mutation carriers is provided in Table 1.

Table 1 Clinical and pathological characteristics of GRN mutation carriers

| Patient (alias) | Predicted cDNA mutation Predicted protein mutation | Sex | Age at onset (death) | Family history | Clinical Dx | Initial clinical symptom | Structural neuroimaging | Functional neuroimaging | Pathology | Reference to patient |
|---|---|-----|----------------------|----------------|-------------|--------------------------|---|--|----------------------|---|
| <i>GRN</i> loss-of-function mutations | | | | | | | | | | |
| NGR019 (11696) | c.26C>A p.A9D | F | 56 | Y | PNFA | Aphasia | MRI: perisylvian (L>R) atrophy | Not done | | (Gass <i>et al.</i> , 2006; Kelley <i>et al.</i> , 2007) |
| NGR001 | c.388_391delCAGT p.Q130SfsX125 | M | 65 (75) | N | PNFA | Aphasia | MRI: temporal and perisylvian (L) atrophy | Not done | FTLD-U (NII) + HpScl | This study |
| NGR077 | c.388_391delCAGT p.Q130SfsX125 | M | 50 | Y | bvFTD | Personality | MRI: generalized atrophy | FDG-PET: frontal (L>R) hypometabolism | | This study |
| NGR228 | c.911_912insTG p.W304LfsX58 | M | 63 | N | bvFTD | Aphasia/ Personality | MRI: frontal (L>R) atrophy | FDG-PET: frontal (L>R) hypometabolism | | This study |
| NGR192 | c.911_912insTG p.W304LfsX58 | F | 49 | Y | FA | Aphasia | MRI: parietal, perisylvian and temporal (L) atrophy | FDG-PET: parietal and temporal (L) hypometabolism | | This study |
| NGR003 (PPA-1D) | c.998delG p.G333VfsX28 | M | 65 (73) | Y | PNFA | Aphasia | MRI: perisylvian (L) atrophy | Not done | FTLD-U (NII) + HpScl | (Gass <i>et al.</i> , 2006; Mesulam <i>et al.</i> , 2007) |
| NGR043 (2619) | c.1477C>T p.R493X | F | 64 (69) | Y | bvFTD | Personality | MRI: moderate frontal and temporal (L+R) atrophy | Not done | | (Rademakers <i>et al.</i> , 2007) |
| NGR068 (7756) | c.1477C>T p.R493X | M | 48 (50) | N | FA | Aphasia | MRI: mild ventricular enlargement in frontal horn | FDG-PET: temporal (L+R), frontal and parietal (L) hypometabolism | | (Rademakers <i>et al.</i> , 2007) |
| NGR247 | c.1414-15_1590del p.A472_Q548del | F | 79 (86) | N | SD | Anomia | CT: temporal atrophy (L) | Not done | | This study |
| <i>GRN</i> mutations with unknown pathogenic nature | | | | | | | | | | |
| NGR022 | c.55C>T p.R19W | F | 61 | N | bvFTD | Personality | MRI: frontal and temporal (L+R) atrophy | Not done | | (Gass <i>et al.</i> , 2006) |
| NGR139 ^a | c.415T>C p.C139R | M | 76 | Y | FA | Aphasia | MRI: mild generalized atrophy | FDG-PET: parietal and temporal (L) hypometabolism | | This study |
| NGR221 | c.970G>A p.A324T | M | 64 (70) | N | PNFA | Apraxia of speech | MRI: temporal and parietal (L>R) atrophy | Not done | CBD | This study |
| NGR039 | c.1297C>T p.R433W | M | 66 | N | PNFA | Aphasia | MR: sylvian and temporal (L) atrophy | Not done | | (Gass <i>et al.</i> , 2006) |
| NGR105 | c.1297C>T p.R433W | F | 61 | Y | SD | Anomia | MRI: temporal (L>R) atrophy | FDG-PET: left hemisphere hypometabolism | | This study |
| NGR167 | c.1297C>T p.R433W | M | 68 | Y | FA | Aphasia | MRI: slight enlargement of the sylvian fissure (L) | FDG-PET: temporal and perisylvian (L) hypometabolism | | This study |
| NGR084 | c.1341C>T p.H447 | F | 64 | Y | bvFTD | Personality | MRI: frontal (R>L) atrophy | FDG-PET: frontal (R>L) hypometabolism | | This study |
| NGR225 | c.1734G>A p.P578 | F | 55 | N | PNFA | Aphasia | MRI: temporal (L>R) atrophy | Not done | | This study |

a Patient NGR139 also carries the rare 5'UTR-22C>T promoter variant. PNFA=Progressive non-fluent aphasia. bvFTD=behavioural variant frontotemporal dementia. FA=Fluent aphasia. SD=Semantic dementia. CBD=Corticobasal degeneration. R=right. L=Left. MRI=Magnetic resonance imaging. CT=computerized tomography. FDG-PET=18F-fluorodeoxyglucose positron emission tomography. FTLD-U=frontotemporal lobar degeneration with ubiquitin inclusions. NII=intranuclear inclusions. HpScl=Hippocampal sclerosis.

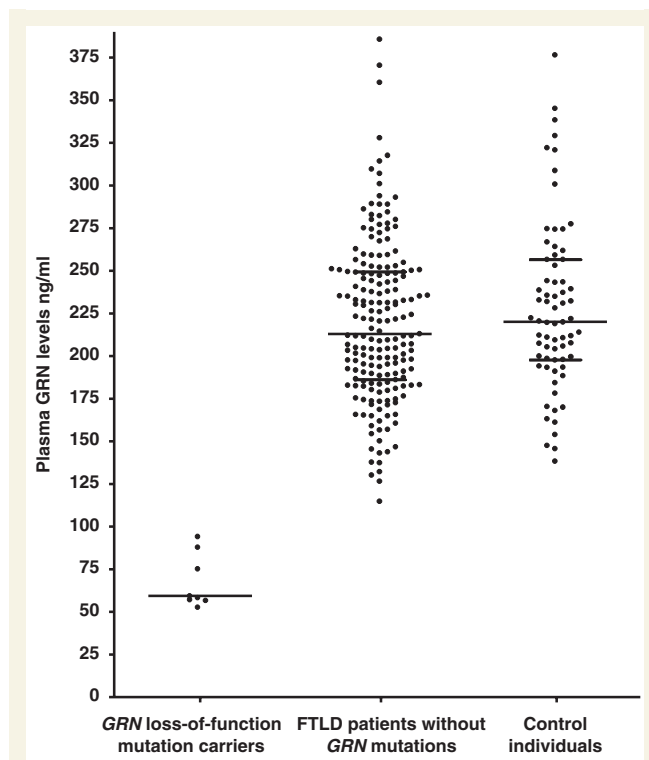


Figure 1 Plasma GRN levels in FTLN patients and control individuals. Plasma GRN protein levels (ng/ml) in patients with FTLN carrying loss-of-function *GRN* mutations ($n=8$), FTLN patients without *GRN* mutations ($n=191$) and healthy control individuals ($n=70$). Each data point represents an individual. Plasma levels are significantly decreased in loss-of-function *GRN* mutation carriers compared to non-*GRN* mutation carriers ($P<0.001$; Mann–Whitney test). For each group the median plasma level is indicated with a wide horizontal line. For larger groups (FTLN patients without *GRN* mutation carriers and healthy control individuals) 25% and 75% quantiles are shown with short horizontal lines.

Haplotype analyses using eight STR markers in a 6.9 Mb genomic region flanking *GRN* showed shared alleles for seven consecutive markers for patients NGR001 and NGR077 carrying p.Q130SfsX125 and shared alleles for all eight consecutive markers for patients NGR192 and NGR228 carrying p.W304LfsX58, suggesting a common genetic origin for each of these *GRN* mutations. A common founder for patients NGR043 and NGR068 carrying p.R493X had previously been reported (Gass *et al.*, 2006).

We then determined whether the levels of GRN in plasma could be used to distinguish *GRN* loss-of-function mutation carriers from non-*GRN* carriers in our FTLN series. Using a GRN ELISA we analysed a subset of 207 patients with FTLN from our consecutive cohort with plasma availability, including seven loss-of-function *GRN* mutation carriers and patient NGR247 carrying the partial *GRN* deletion, p.A472_Q548del. A significant reduction in GRN levels was observed in all patients carrying *GRN* loss-of-function mutations to about one third of the level observed in non-*GRN* carriers (Fig. 1; Table 2). GRN levels ranged from 53 to 94 ng/ml (mean value \pm SD: 68 ± 16 ng/ml) in mutation

Table 2 Summary of plasma GRN expression levels in patients with FTLN

| Variable | N | Plasma GRN levels (ng/ml) | | | P-value |
|---|-----|---------------------------|---------|---------|---------|
| | | Median | IQR | Range | |
| Loss-of-function <i>GRN</i> mutation ^a | | | | | |
| Yes | 8 | 59 | 57–81 | 53–94 | <0.001 |
| No | 191 | 213 | 186–249 | 115–386 | |
| Gender ^b | | | | | |
| Male | 95 | 203 | 183–233 | 130–370 | 0.0011 |
| Female | 96 | 233 | 196–258 | 115–386 | |
| Family history ^b | | | | | |
| Yes | 74 | 219 | 185–249 | 115–386 | 0.80 |
| No | 117 | 211 | 186–250 | 130–307 | |
| Clinical FTLN subtypes ^b | | | | | |
| bvFTLN | 62 | 218 | 189–258 | 143–370 | 0.61 |
| PNFA | 27 | 216 | 187–260 | 115–386 | |
| SD | 40 | 212 | 187–235 | 137–278 | |
| FA | 37 | 205 | 180–248 | 127–310 | |
| CBS | 25 | 230 | 188–249 | 130–360 | |

^a Patients carrying *GRN* mutations with unknown pathogenic nature were excluded.

^b Patients carrying *GRN* mutations were excluded.

carriers, while non-*GRN* carriers showed levels from 115 to 386 ng/ml (mean value \pm SD: 220 ± 47 ng/ml). Interestingly, GRN levels were variable among *GRN* mutation carriers with slightly higher expression in NGR019 (88 ng/ml) carrying the p.A9D mutation in the signal peptide sequence and NGR247 (94 ng/ml) carrying p.A472_Q548del, compared to the typical frameshift and nonsense mutation carriers (Fig. 2). Based on our data alone, any cut-off value between 94 and 115 ng/ml is associated with crude estimates of both sensitivity and specificity of 100%. By assuming normality of GRN levels after a square root transformation, we were able to explore continuous estimates of sensitivity and specificity with different cut-off values (Table 3). A cut-off value of 112 ng/ml maximized the average of the estimates of sensitivity and specificity. GRN levels in 70 control individuals ranged from 138 to 376 ng/ml (mean value \pm SD: 228 ± 50 ng/ml) comparable to patients with FTLN without *GRN* mutations (Fig. 1). As expected, normal GRN expression (259 ng/ml) was observed in our *MAPT* mutation carrier.

Within the group of patients carrying *GRN* mutations with unknown pathogenic nature, NGR139 (p.C139R) and NGR022 (p.R19W) showed relatively low expression of GRN, in the range of the lowest 3% of patients without *GRN* mutations (p.C139R: 124.2 ng/ml; p.R19W: 135.9 ng/ml) and below the GRN levels observed in control individuals. Although these GRN levels fall outside the range of the typical loss-of-function mutations, these mutations may induce a partial loss-of-function. In contrast, the GRN levels in the other missense mutation carriers and silent mutation carriers were well within the normal range (Fig. 2).

In the group of non-*GRN* mutation carriers, a significant gender difference in plasma GRN expression levels was observed with higher levels in females (mean value \pm SD: 230 ± 48 ng/ml) compared to males (mean value \pm SD: 210 ± 44 ng/ml) ($P<0.001$; Table 2). In exploratory analysis of subgroups by age and gender,

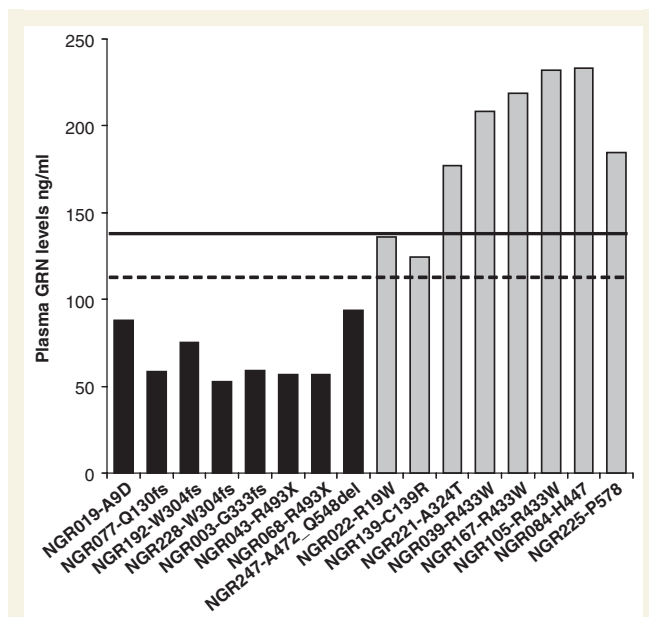


Figure 2 Specific plasma GRN levels in all types of *GRN* mutations. GRN expression is plotted for each patient with FTLD from our cohort carrying a *GRN* mutation. Black bars represent patients with FTLD carrying pathogenic *GRN* loss-of-function mutations; grey bars represent patients with FTLD carrying *GRN* mutations with unknown significance. The dashed line is the cut-off value for pathogenic *GRN* loss-of-function mutations based on the complete FTLD series and the black line indicates the minimum *GRN* expression identified in our control cohort (Fig. 1). Missense mutations p.R19W and p.C139R show *GRN* levels below the range detected in control individuals and may induce a partial loss of *GRN* function.

Table 3 Estimated sensitivity and specificity of the *GRN* ELISA assay using different cut-off values

| Plasma GRN level cut-point (ng/ml) | Sensitivity (95% CI) ^a | Specificity (95% CI) ^a |
|------------------------------------|-----------------------------------|-----------------------------------|
| 100 | 97.42 (86.90–100) | 99.88 (99.66–99.97) |
| 105 | 98.64 (90.67–100) | 99.80 (99.48–99.94) |
| 110 | 99.32 (93.37–100) | 99.67 (99.23–99.89) |
| 112 | 99.49 (94.62–100) | 99.61 (99.87–99.10) |
| 115 | 99.67 (95.35–100) | 99.49 (98.89–99.81) |
| 120 | 99.85 (96.75–100) | 99.23 (98.42–99.70) |
| 125 | 99.93 (97.79–100) | 98.86 (97.81–99.53) |
| 130 | 99.97 (98.56–100) | 98.36 (97.04–99.26) |
| 135 | 99.99 (99.11–100) | 97.71 (96.06–98.89) |

a. Estimate based on assumption of normally distributed GRN levels after square root transformation. Confidence intervals constructed by parametric bootstrap method.

it appeared that GRN levels may decrease with age in males, while remaining constant in females, however these findings did not reach significance (data not shown). Also, the gender difference was not confirmed in our control cohort. No significant differences in GRN plasma levels were observed between patients with a positive and negative family history of dementia

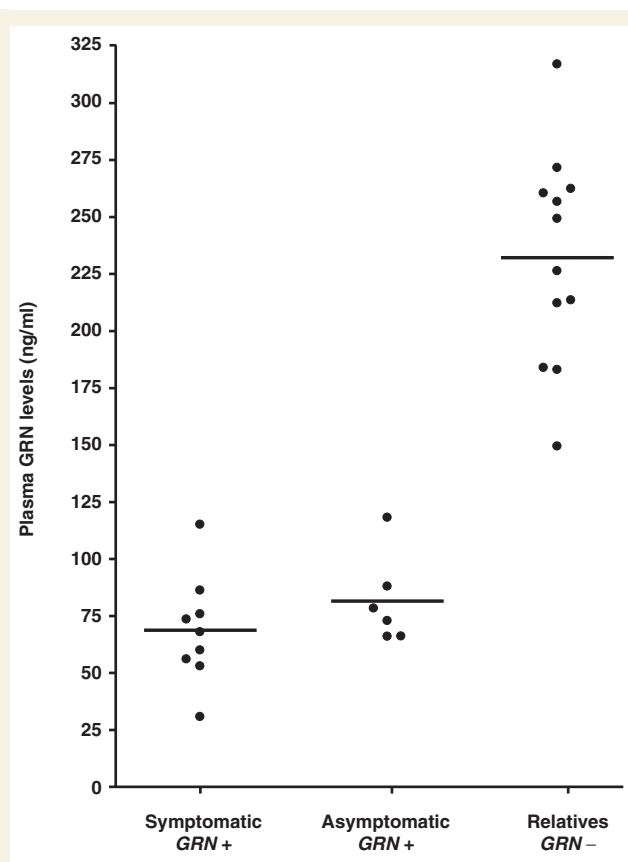


Figure 3 Plasma GRN levels in symptomatic and asymptomatic *GRN* mutation carriers in three *GRN* mutation families. Plasma GRN protein levels (ng/ml) in patients and relatives of three *GRN* mutation families ascertained in Mayo Jacksonville and Rochester: Symptomatic *GRN* mutation carriers ($n=9$), asymptomatic *GRN* mutation carriers ($n=6$) and relatives without *GRN* mutations ($n=12$). Plasma levels are significantly decreased in symptomatic and asymptomatic *GRN* mutation carriers compared to relatives without *GRN* mutations ($P<0.001$; Mann–Whitney test). Each data point represents an individual. For each group, the median plasma level is indicated with a wide horizontal line.

or between patients with each of the five clinical subtypes of FTLD (Table 2). Also, no correlation between GRN expression levels and the age of disease onset in *GRN* mutation carriers or non-*GRN* mutation carriers was observed.

To further characterize the effect of *GRN* mutations on the levels of GRN in plasma, we performed a GRN ELISA on nine affected (one proband included in the consecutive FTLD series and eight additional patients) and 18 unaffected at-risk individuals from three previously published *GRN* mutation families with typical loss-of-function mutations (p.T52HfsX2, p.T382SfsX30 and p.R493X) ascertained at Mayo Jacksonville and Mayo Rochester. Six of the 18 asymptomatic at-risk individuals were identified as *GRN* mutation carriers using DNA sequencing analyses. We observed significantly lower GRN levels in both symptomatic and asymptomatic *GRN* mutation carriers, than non-*GRN* mutation carriers ($P<0.001$; Fig. 3).

All patients in our FTLD cohort with GRN levels <112 ng/ml ($n=8$), were explained by pathogenic loss-of-function mutations identified by direct sequencing. Since the presence of a partial or complete *GRN* gene deletion/duplication is expected to have a similar effect on GRN expression, e.g. the loss of 50% of GRN, these data suggest the absence of *GRN* copy-number mutations in our series. To further confirm this finding, we performed a GRN ELISA on plasma obtained from the previously published French patient carrying a near complete deletion of *GRN* including exons 1–11 [patient II.4 from original publication (Rovelet-Lecrux *et al.*, 2008)] and two unrelated French control individuals. These analyses showed a strongly reduced expression of GRN (86.2 ng/ml) in the *GRN* deletion carrier, compared to control individuals (196.6 and 263.2 ng/ml).

Next, we performed a GRN ELISA in a population of 72 patients with a clinical diagnosis of early-onset Alzheimer's disease. In one patient, strongly reduced GRN expression levels (74 ng/ml) were observed and sequencing analyses showed a novel loss-of-function mutation c.592_593delAG in *GRN* exon 5, which is expected to result in a shift of the normal reading frame and a premature termination codon (p.R198GfsX19). This patient presented at the age of 63 years with an amnesic syndrome which was confirmed on neuropsychology. MRI showed mild atrophy including frontal atrophy. No family history of dementia was reported. He met NINCDS/ADRDA criteria for probable Alzheimer's disease (McKhann *et al.*, 1984). GRN levels in the other 71 patients with Alzheimer's disease without *GRN* mutations ranged from 143 to 368 ng/ml (mean value \pm SD: 238 ± 53 ng/ml), comparable to GRN levels in control individuals.

Finally, to gain more insight into the processing of GRN and to determine which GRN species are detected by the ELISA, we performed GRN Western blot analyses on plasma samples and brain homogenates of FTLD patients with and without *GRN* mutations. These studies indicated that the antibodies used in the ELISA could only detect full-length GRN, no intermediate GRN fragments or recombinant GRN cleavage products.

Discussion

The range of mutations identified in *GRN* over the past 2 years established GRN haploinsufficiency as the uniform disease mechanism underlying FTLD in *GRN* mutation carriers (Gijssels *et al.*, 2008a). Microarray data further showed significantly reduced GRN mRNA expression in peripheral blood in *GRN* loss-of-function mutation carriers compared to non-mutation carriers (Coppola *et al.*, 2008). These findings raised the important question as to whether expression of GRN in plasma could predict *GRN* mutation status and could be used as a biological marker to identify *GRN* mutation carriers.

To answer this question, we first studied a large consecutive series of clinical FTLD patients ascertained at Mayo Jacksonville, for which DNA and plasma samples were available. Direct sequencing analyses of *GRN* identified six different *GRN* loss-of-function mutations in nine FTLD patients explaining the disease in 4.1% (9/219) of the population and 5.7% (5/87) of FTLD patients with a positive family history. This *GRN* mutation frequency

is very similar to our previously reported mutation frequency of 4.8% in an unbiased subpopulation of US FTLD patients (Gass *et al.*, 2006). *GRN* mutations included typical nonsense and frameshift mutations as well as the previously reported p.A9D mutation in the signal peptide sequence of GRN, and a partial deletion of *GRN* exon 11, which extends from 15-bp upstream of exon 11 to 177-bp in exon 11 and is predicted to result in an in-frame deletion of exon 11 (p.A472_Q548del) (Gass *et al.*, 2006; Mukherjee *et al.*, 2006, 2008; Pickering-Brown *et al.*, 2006; Kelley *et al.*, 2007; Spina *et al.*, 2007). GRN expression studies in the complete FTLD cohort using a GRN ELISA demonstrated that plasma GRN levels were strongly reduced in all loss-of-function *GRN* mutation carriers compared to non-*GRN* mutation carriers ($P < 0.001$). Even though we observed a wide range in GRN expression in FTLD patients and control individuals, all mutation carriers showed significantly reduced GRN levels to about one third of the levels observed in non-*GRN* carriers and control individuals. In our series, a plasma GRN cut-off value of 112 ng/ml distinguished all *GRN* mutation carriers from non-*GRN* carriers and we therefore expect it to have close to 100% sensitivity and specificity in future samples. These results are in line with a recently published study by Ghidoni *et al.* (2008) who established that in an Italian FTLD population a GRN cut-off level of 110.9 ng/ml was 92.8% specific and 100% sensitive to identify *GRN* mutations. However, since there were only eight *GRN* mutation carriers in our FTLD population, further carriers will need to be assessed to more definitively decide on the optimal cut-off; in the meantime it may be prudent to use a higher threshold than 112 ng/ml to be more certain of a high sensitivity, without materially lowering specificity. For example, examining Table 3 we see that a cut-off value of 135 ng/ml has a lower confidence limit for sensitivity of >99%, and this is achieved with an estimated specificity of 97.7%.

Interestingly, we showed strongly reduced GRN expression in patients carrying all types of loss-of-function *GRN* mutations, including patient NGR019 carrying the p.A9D signal peptide mutation and patient NGR247 carrying the partial *GRN* deletion, p.A472_Q548del. We also confirmed similar low levels of GRN in a French patient with clinical Parkinson's disease carrying a near complete deletion in *GRN*.

Next, by analysing unaffected relatives carrying *GRN* mutations, we showed that the GRN reduction is independent of the disease status, suggesting that the GRN ELISA could also function as a diagnostic tool to identify asymptomatic *GRN* mutation carriers.

GRN sequencing analyses in our cohort of FTLD patients also identified four different *GRN* missense mutations with unknown pathogenic nature in six FTLD patients. The plasma GRN levels in these missense mutation carriers generally supported the previously predicted effect on *GRN* function based on *in silico* structural protein modeling analyses (Brouwers *et al.*, 2008; Gijssels *et al.*, 2008a). Mutation p.C139R was predicted to be likely pathogenic because of a destabilizing effect on the granulin fold by disrupting one of the cysteine disulfide bridges. This mutation had been previously reported in an Italian early-onset familial FTD patient and a Belgian late-onset Alzheimer's disease patient, while absent in >900 control individuals that were analysed by us and others (Bernardi *et al.*, 2008; Brouwers *et al.*, 2008).

We observed this mutation in a familial FTLD patient diagnosed with FA at the age of 76 years. His problem began with language difficulty including expression and comprehension. Sentence repetition was preserved. Picture description was normal except for some mild anomia. MRI showed mild non-asymmetrical atrophy. PET analyses showed hypometabolism in the left parietotemporal area and neuropsychometric testing indicated deficits in language, frontal function and preserved memory. In line with the predictions, low amounts of GRN were measured in the plasma sample of this patient (124 ng/ml), supporting the pathogenic potential of this mutation through a partial loss of GRN function. In contrast, all four patients carrying missense mutations p.A324T and p.R433W, which were predicted by *in silico* analyses to be tolerated, and both carriers of the silent *GRN* mutations (p.H447 and p.P578) showed GRN plasma levels within the normal range. Although we cannot exclude the possibility that these mutations abolish GRN function without reducing the overall expression of GRN, these findings suggested that plasma GRN levels may be a valuable tool in predicting the pathogenic potential of *GRN* mutations. We propose that individuals carrying intermediate levels of GRN (~110–140 ng/ml) undergo additional genetic testing to determine the presence of potential pathogenic *GRN* missense mutations.

Previous studies have reported clinical Alzheimer's disease diagnoses in patients carrying loss-of-function mutations in *GRN* (Boeve, 2007; Kelley *et al.*, 2007). In a clinicopathological study, we performed on the most common *GRN* mutation worldwide, p.R493X, 10% of the mutation carriers received a primary clinical diagnosis of Alzheimer's disease and memory impairment was the second most common initial clinical symptom, affecting 30% of the mutation carriers (Rademakers *et al.*, 2007). In this study, using our GRN ELISA as a screening tool, we analysed 72 patients with clinically diagnosed early-onset probable Alzheimer's disease for mutations in *GRN* and identified one patient (1.4% of the population) carrying a loss-of-function mutation. If a disease modifying treatment for either Alzheimer's disease or FTLD becomes available in the future, then, based on the finding above and knowing that that FTLD usually starts early, one could make the case that it would be reasonable to screen early-onset probable Alzheimer's disease cases with a plasma measure for GRN.

In our study, plasma GRN levels were ~75% reduced in mutation carriers with respect to non-*GRN* patients and controls, which is significantly more than expected based on the haploinsufficiency disease mechanism. A similar reduction in GRN expression was recently reported in an Italian series of *GRN* mutation carriers (Ghidoni *et al.*, 2008). In contrast, only 35–50% reductions in GRN mRNA levels have been reported in *GRN* mutation carriers (Baker *et al.*, 2006). These findings, together with the fact that we established that only full-length GRN and no granulins or intermediate GRN fragments can be detected by our ELISA, predict an unbalanced GRN metabolism in *GRN* mutation carriers, whereby the processing of GRN into granulins is increased. This would suggest that patients with *GRN* mutations may specifically lack full-length GRN, while maintaining normal levels of granulins. This is an interesting finding which may have implications for future therapeutic strategies, especially since GRN and the

proteolytically derived granulin fragments each have distinct biological properties, e.g. GRN functions as a growth factor or anti-inflammatory agent, while granulin peptides boost inflammation (Zhu *et al.*, 2002; He and Bateman, 2003; Ahmed *et al.*, 2007). In future experiments, detailed biochemical analysis of the secretion and proteolytic processing of GRN in mutation carriers and control individuals will be essential.

In conclusion, this large study of GRN expression in patients with FTLD, Alzheimer's disease and control individuals provides strong support for the use of a GRN ELISA as a reliable and inexpensive tool to identify *GRN* mutation patients and carriers. Moreover, compared to classical mutation screening of *GRN* by sequencing analyses, a GRN ELISA has the important advantage of identifying individuals carrying all types of mutations including *GRN* deletions.

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