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# C9orf72 Protein Plasmatic Concentrations Are Similar between C9ORF72 Expansion Carriers and Noncarriers in Frontotemporal Dementia

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## Keywords

Frontotemporal dementia · C9ORF72 expansion · C9orf72 protein · Haploinsufficiency

## Abstract

**Background/Aims:** The aim of the study was to assess the theory of haploinsufficiency in C9ORF72 expansion carriers, the most frequent causative gene of frontotemporal dementia. **Methods:** Plasmatic concentrations of C9orf72 protein were measured in 33 patients suspected of familial frontotemporal dementia using an enzyme-linked immunosorbent assay. **Results:** No difference was observed between C9ORF72 expansion carriers (21.2% of patients) and noncarriers (78.8% of patients). C9orf72 protein determination is not a suitable biomarker for screening C9ORF72 expansion carriers. **Conclusion:** Our results provide new evidence against the hypothesis of haploinsufficiency leading to frontotemporal dementia in C9ORF72 expansion carriers.

## Introduction

Frontotemporal dementia (FTD) is the second cause of young-onset dementia, after Alzheimer's disease [1]. Up to 50% of FTD patients may present with a positive familial history and 10% of patients have a clear autosomal dominant history [2]. Three genes cause most of familial FTD: *MAPT*, *GRN*, and *C9ORF72*, cited by chronological order of discovery [3].

This last mutation consists of hexanucleotide GGGGCC repeat expansions between noncoding *C9ORF72* exons 1a and 1b [4] and is the most common genetic cause of FTD, accounting for 25% of all familial FTD [5].

Since its discovery in 2011, much research has been done to elucidate pathological mechanisms connecting *C9ORF72* expansion and the clinical and neuropathological disorders observed in FTD patients. Presently, 3 major hypotheses have emerged and are still debated [6]. The first one is a potential loss of function of C9orf72 protein because of the hexanucleotide long expansion: this theory is called haploinsufficiency [7]. Haploinsufficiency can be defined as an inactivation of one allele, leading to a reduction by half of protein levels [8]. This mechanism can already be observed in FTD patients harboring mutations in the progranulin gene (*GRN*), resulting in reduced secreted progranulin protein [9]. The second mechanism explaining disease with *C9ORF72* repeat expansion would be a gain of RNA toxicity [4]. Finally, the third hypothesis would be a toxicity caused by the formation of repeat-associated non-ATG proteins [10].

The aim of this study was to assess the *C9ORF72* expansion haploinsufficiency theory resulting in FTD, comparing plasmatic C9orf72 protein concentrations between *C9ORF72* expansion carriers and noncarriers.

## Material and Methods

### *Patients*

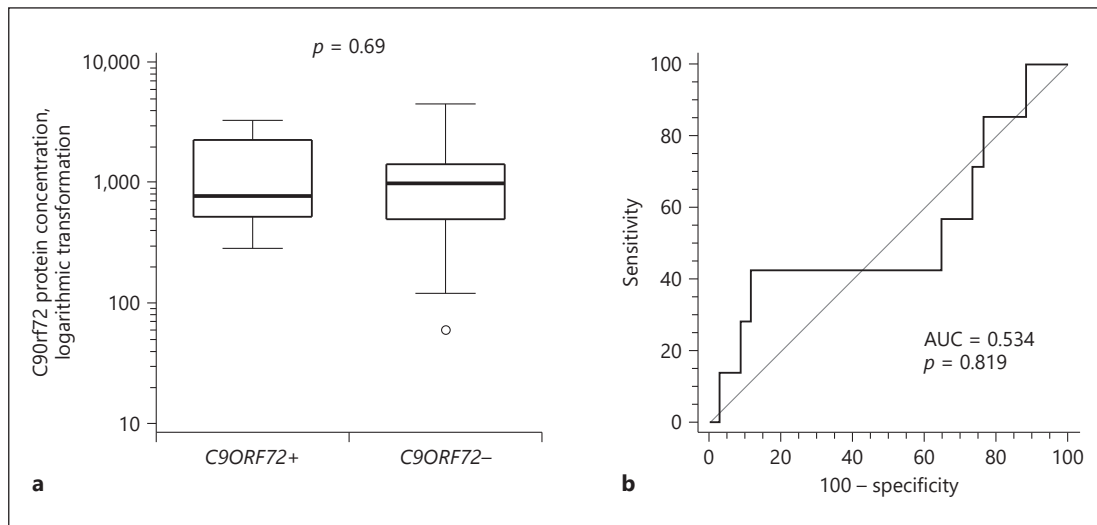
This study included a population of 33 patients aged from 39 to 87 years (16 men and 17 women) who were referred to Lyon and Grenoble Centers for Memory Resources and Research between 2014 and 2016 for a diagnostic evaluation of cognitive impairment. The patients met the diagnosis of probable behavioral variant of FTD (bvFTD) according to Rascovsky criteria [11]. The diagnosis of bvFTD was proposed in a multi-disciplinary consultation, taking into account clinical and neuropsychological observations, imaging analysis, and cerebrospinal fluid marker results (i.e., negative Alzheimer's disease profile [12]). Moreover, most patients were suspected of familial FTD [2] because of a family history of neurodegenerative diseases including FTD and/or ALS (amyotrophic lateral sclerosis) in particular. Following genetic counseling, all the patients included in this study gave informed written consent for FTD genetic testing and blood sampling was collected.

### *Genetic Screening for C9ORF72 Expansion*

After DNA extraction, the screening for GGGGCC repeat expansions in *C9ORF72* was performed using a repeat-primed PCR (RP-PCR), according to a protocol adapted from the international study from Akimoto et al. [13]. RP-PCR products were separated by capillary electrophoresis using an ABI 3130xL Genetic Analyzer (Life Technologies®, Carlsbad, CA, USA) with Genescan™ 500 ROX Size Standard (Life Technologies®). Data were analyzed using GeneMapper® Software 5 (Applied Biosystems®, Foster City, CA, USA). RP-PCR gave qualitative results for *C9ORF72* expansion screening, i.e., positive or negative. Positive cases harbored a typical saw tooth sloping pattern. A cutoff of 30 repeats was used to differentiate positive from negative cases. Otherwise, the genetic testing for *C9ORF72* was considered negative. All positive and negative results were confirmed using Southern blot analysis. Seven patients harbored *C9ORF72* repeat expansions; frequencies were 18.8% (3/16) for male patients and 23.5% (4/17) for female patients, respectively.

### *Plasmatic C9orf72 Protein Quantification*

EDTA whole blood samples were centrifuged at 2,000 *g* for 10 min. Two hundred microliters of plasma aliquots were prepared and stored at -80 °C until further analysis. C9orf72 protein quantification was performed using a Human C9orf72 ELISA kit (Cusabio®, College Park, MD, USA), according to the manufacturer's instructions. The quality of the assay was previously assessed by performing different plates with acceptable analytical criteria defined by good calibration models with  $r^2 > 0.99$ , a duplicate standard variability below 5%, a linearity of signals ( $r^2 = 0.996$ ) obtained for samples diluted from 1/5th to 1/30th and a within-run assay coefficient of variation at 5.1% ( $n = 4$  repetitions). Plasmatic C9orf72 protein concentrations (samples diluted 1/10th) were determined in duplicate in the same assay.



**Fig. 1.** **a** Distribution of C9orf72 protein concentration *C9ORF72* expansion carriers and noncarriers. **b** Diagnostic performances of C9orf72 protein to discriminate between *C9ORF72* expansion carriers and noncarriers (ROC curve).

**Table 1.** Demographic and biological characteristics of the cohort of patients

	<i>C9ORF72</i> carriers	<i>C9ORF72</i> noncarriers	All patients
<b>Demography</b>			
Patients, <i>n</i> (%)	7 (21.2)	26 (78.8)	33 (100)
Male/female, %	3/4	13/13	16/17
Age, years	70.9±9.3	67.7±10.7	68.4±10.3
<b>Plasmatic marker</b>			
C9orf72 protein, pg/mL	781 (531–2,256)	876 (447–1,328)	844 (485–1,378)

Age is expressed as mean ± standard deviation. C9orf72 protein concentrations are expressed as median (25th–75th percentile).

#### Statistics

All the statistics were performed on MedCalc® software, version 15.11.4. The distribution of C9orf72 protein concentrations was assessed using the d'Agostino-Pearson test. Mann-Whitney tests were performed to assess differences in C9orf72 protein concentration regarding the sex of patients and the presence/absence of *C9ORF72* expansion and Spearman's rho was assessed to measure a rank correlation between C9orf72 protein concentrations and age.

#### Results

Plasmatic C9orf72 protein concentrations ranged from 119 to 4,490 pg/mL in the whole cohort of 33 patients (Table 1). C9orf72 protein concentrations did not follow a normal distribution ( $p = 0.0001$ ). C9orf72 protein concentrations were not influenced by the sex ( $p = 0.10$ ) or the age of the patients ( $p = 0.71$ ).

Plasmatic C9orf72 protein concentrations were not different between *C9ORF72* expansion carriers and noncarriers ( $p = 0.69$ ). The area under the ROC curve was 0.549, with 95% confi-

dence interval ranging from 0.367 to 0.722. The Youden index was 0.313 and the associated criterion was >1,557 pg/mL, giving sensitivity and specificity of 42.9 and 88.5%, respectively (Fig. 1).

## Discussion

To our knowledge, our study is the first one reporting C9orf72 protein concentrations measured in the plasma of FTD patients. In this cohort of 33 patients suspected of familial FTD, C9orf72 protein concentrations varied widely from 119 to 4,490 pg/mL, with a median of 844 pg/mL, and were nondependent on the sex or age of the patients. More interestingly, no difference was observed between *C9ORF72* expansion carriers and noncarriers. Indeed, 7 patients presented a large GGGGCC *C9ORF72* expansion using the RP-PCR assay. A full concordance between this assay and Southern blot was obtained (data not shown). Despite the small size of the cohort, the percentage of positive cases was in good concordance with those presented by the different epidemiologic studies performed in Europe [5]. In the same way, no difference concerning sex ratio was observed between *C9ORF72* expansion carriers and noncarriers [14].

Our preliminary findings seem to suggest that haploinsufficiency may not explain clinical expression in bvFTD patients carrying *C9ORF72* expansion. Indeed, one could hypothesize that a potential haploinsufficiency would lead to a significant decrease of plasmatic C9orf72 protein concentration; this phenomenon was observed in *GRN* mutation carriers (symptomatic or not), leading to decreased plasmatic progranulin concentrations [15]. In *C9ORF72* expansion carriers, haploinsufficiency hypothesis was firstly suggested following the observation that all *C9ORF72* mRNA transcripts were decreased in the frontal cortex of FTD patients using quantitative real-time PCR [7, 16]. Unfortunately, immunoblotting assays in the brain were inconclusive, potentially due to nonspecific binding of antibodies [7]. Finally, recent findings showed that *C9ORF72* transcripts were also decreased in *MAPT* and *GRN* mutation carriers [17]. This lack of specificity means that a decrease of *C9ORF72* transcripts is not the only pathological process leading to FTD. In conclusion, plasmatic C9orf72 protein determination seemed not suitable to detect *C9ORF72* expansion carriers in our cohort of patients.

Another hypothesis is a *C9ORF72* gain-of-function mechanism, suggested by much in vitro and in vivo evidence [18]. RNA aggregates or foci were found in different brain regions of *C9ORF72* FTLD patients [19]. These foci might not cause a direct cellular dysfunction [20], but lead to a sequestration of RNA binding proteins and disrupt their normal activity [21, 22]. Finally, an unconventional translation of *C9ORF72* expansions into specific polypeptides was discovered in 2013 [23]. This process was also highlighted in other pathologies (myotonic dystrophy for example) whose molecular mechanism relies on abnormal genetic expansions [24]. Following a noncanonical translation [25], meaning the absence of an initiating codon and multiple reading frames, different proteins – poly(GP), poly(GA) and poly(GR) – can be synthesized in *C9ORF72* expansion carriers and are called C9RAN proteins [23]. The intrinsic toxicity of these C9RAN proteins remains unclear today. However, C9RAN proteins were discovered in the brain [23] and cerebrospinal fluid of symptomatic and asymptomatic *C9ORF72* expansion carriers [26, 27]. These proteins appear to be specific compared to controls or to other neurodegenerative diseases. Further analysis with larger cohorts including the whole spectrum of FTD-ALS should compare diagnostic performances of C9RAN and C9orf72 proteins in FTD.

## Statement of Ethics

Following genetic counseling, all the patients included in this study gave informed written consent for FTD genetic testing.

## Disclosure Statement

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