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Relations between C9orf72 expansion size in blood, age at onset, age at collection and transmission across generations in patients and presymptomatic carriers

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Abstract

A (GGGGCC)n repeat expansion in C9orf72 gene is the major cause of frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS). The relations between the repeats size and the age at disease onset (AO) or the clinical phenotype (FTD vs. ALS) were investigated in 125 FTD, ALS, and presymptomatic carriers. Positive correlations were found between repeats number and the AO (p < 10^{-4}) but our results suggested that the association was mainly driven by age at collection (p < 10^{-4}). A weaker association was observed with clinical presentation (p = 0.02), which became nonsignificant after adjustment for the age at collection in each group. Importantly, repeats number variably expanded or contracted over time in carriers with multiple blood samples, as well as through generations in parent-offspring pairs, conversely to what occurs in several expansion diseases with anticipation at the molecular level. Finally, this study establishes that measure of repeats number in lymphocytes is not a reliable biomarker predictive of the AO or disease outcome in C9orf72 long expansion carriers.

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1. Introduction

Frontotemporal dementia (FTD) is the second cause of degenerative dementia in the presenium, after Alzheimer’s disease. A familial history of the disease is reported in half of the patients. Most of the familial cases reflect a monogenic origin with an autosomal...
dominant mode of inheritance. Symptoms of amyotrophic lateral sclerosis (ALS) are associated in 15% of patients with FTD. A GGGGCC (G4C2) repeat expansion in the first intron of chromosome 9 open reading frame 72 (C9orf72) is one of the major genetic cause of FTD and of ALS (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Most healthy individuals carry fewer than 24 repeats, whereas patients have up to thousands of hexanucleotide copies (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Three mechanisms might explain the pathogenicity of large repeat expansions: a C9orf72 loss-of-function, the formation of nuclear sense and anti-sense RNA foci, and dipeptide repeat proteins generated by repeat-associated non-ATG translation (Lagier-Tourenne et al., 2013).

C9orf72 disease is characterized by an important heterogeneity of clinical presentation (FTD, ALS) and of age of disease onset. Behavioral or motor symptoms can occur from the third decade of life, to a nearly incomplete penetrance in elderly mutation carriers (Le Ber et al., 2013). The size of the expansion also greatly varies among C9orf72 carriers and might explain a part of this clinical variability, particularly of the age at disease onset (AO) as reported in other repeat expansion diseases. Few studies have analyzed C9orf72 expansion sizes in blood, with controversial results concluding either to a positive (Beck et al., 2013), the absence (Dollicardo et al., 2014; Nordin et al., 2015; van Blitterswijk et al., 2013), or an inverse correlation with AO (Gjilencev et al., 2017; Van Morssevelde et al., 2017). Most of these studies were based on a limited number of C9orf72 carriers, thus precluding definite conclusions. Consequently, no clear information about the correlation of C9orf72 repeats number and clinical phenotypes is available to date, and further investigations are needed in large cohorts of patients.

Establishing whether the size of the expansion in blood can be used as a reliable peripheral marker predicting the age at onset and disease outcome in mutation carriers is critical when therapeutics based on antisense oligonucleotides or other molecules are being developed (Donnelly et al., 2013; Jiang et al., 2016). To address these questions, we quantified the expansion size of C9orf72 in peripheral lymphocytes from 125 carriers, which represents the largest cohort studied so far. The correlations between the expansion size and clinical phenotype, AO or age at blood collection, and the dynamic of the expansion in lymphocytes through time and over generations were analyzed.

2. Material and methods

2.1. Subjects and ethics

The estimate of G4C2 repeats number on the expanded allele was done in 125 C9orf72 carriers, including 73 symptomatic patients and 52 presymptomatic (PS) carriers. Genetic status has been established for all of them by repeat-primed PCR before, as previously described (Le Ber et al., 2013). Clinical data and blood samples were obtained after written informed consent for clinic-genetic studies was signed. Local ethic committee approvals were obtained for studies of symptomatic (IRB: CPP l’ile de France II, project #RBM 02-59) and presymptomatic carriers (RCB: 2015-A00856-43 CPP 68-15 Ile-de-France VI, for PrevDemAls and FTD-exome studies, respectively). A brief description of patients and PS cohorts is provided in Table 1. Among the patients (62 were French, 11 Italian), 42 presented isolated FTD, 12 had isolated ALS, and 19 had FTD and secondarily developed ALS. Eleven carriers (8 patients, 1 PS, 2 converters) from this cohort had multiple time points (2–4 times). Time intervals between first and last blood samples ranged from 7 months to 11 years (mean interval = 57.9 months; SD = 39.2 months).
2. Molecular analyses and sizing of C9orf72 repeat number by Southern blot

Genomic DNA was extracted from peripheral lymphocytes. Southern blots were then performed to size the repeat expansion (Beck et al., 2013). Briefly, 15 μg of genomic DNA was digested overnight with AluI (20 u) and Ddel (20 u) before electrophoresis. DNA was transferred to positively charged nylon membrane (Roche Applied Science) by capillary blotting and was baked at 80°C for 2 hours. The hybridization probe was an oligonucleotide from Eurofins MWG Operon (Germany) and comprised 5 hexanucleotide repeats (GGGGGGG)3 labeled 3' and 5' with digoxigenin (DIG). Filter hybridization was undertaken as recommended in the DIG Application Manual (Roche Applied Science) except for the supplementation of DIG Easy Hyb buffer with 100 μg/ml denatured fragmented salmon sperm DNA. After prehybridization at 48°C for 4 hours, hybridization was allowed to proceed at 48°C overnight. A total of 1 ng of labeled oligonucleotide probe were used per milliliter of hybridization solution. Membranes were washed initially in 2× standard sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS), while the oven was being ramped from 48°C to 65°C and then washed in fresh solution at 65°C for 15 minutes; further 15 minutes washes in 0.5× SSC, 0.1% SDS and 0.2× SSC, and 0.1% SDS at 65°C followed. Detection of the hybridized probe DNA was carried out with Digoxigenin antibody–HRP (Antibodies-online, 1/10,000 dilution) and revealed with ECL (“SuperSignal West Dura Extended DurationSubstrate” Fisher Scientific). Signals were visualized on Fluorescent Detection Film (Roche Applied Science) after 1–5 hours. All samples were electrophoresed against DIG-labeled DNA molecular-weight markers VII (Roche Applied Science). Hexanucleotide repeats number was estimated by visual interpolation with a plot (created in Microsoft Excel) of log10 base-pair number against migration distance and subtraction of the wild-type allele fragment size (199 bp). Mode and mean sizes were recorded for each individual with expanded repeats as described previously (Suh et al., 2011). The mode, defined as the point on the Southern blot with the highest intensity, was used for all the statistical analysis. For patients with multiple collections, modal number of repeats measured at the first sampling was used in correlation tests. Six technical duplicates were used to estimate the range of imprecision when sizing C9orf72 large expansions with this method. DNA extracted from the same blood collection of 6 patients was used in 2 different Southern blots. The absolute value of difference between 2 measures ranged from 16 to 172 repeats.

2.3. Comparisons and statistical analyses

Relations between repeats number and AO, age at blood collection, and clinical phenotype (FTD vs. ALS) were evaluated. We first investigated the association between repeats number and AO in patients with extreme AO: early (≤48 years; n = 23 patients) or late onset (>68 years; n = 25); AO, as well as the age at collection, was also considered as continuous variables in correlation analyses. Relations between the age at collection and repeats number were investigated in both symptomatic and PS carriers. We also compared repeats number in a subgroup of youngest patients with an age at onset <45 years (n = 16) and in presymptomatic carriers with an age at the time of blood collection ≤45 years (n = 32).

To accurately evaluate the impact of the repeats number on the phenotype, we compared 2 groups of patients: those with isolated ALS (n = 12) and those with isolated FTD (n = 42); cases presenting both phenotypes (FTD and ALS) were not included in any group in this analysis.

Finally, we assessed the dynamic of the expansion in lymphocytes in a given patient over time and over successive generations in familial cases. We evaluated whether the size varied over time in mutation carriers with multiple sample collections (n = 11) by comparing the expansion at each time point, regardless of the time interval during successive time points. The repeat size transmission across generations was evaluated by studying repeat size in 16 parent-offspring pairs belonging to 12 families from the whole cohort.

The F-test was used to compare variances of C9orf72 repeat number between PS and symptomatic patients, as the repeat number followed a normal distribution in both groups (Kolmogorov-Smirnov normality test p > 0.05). Conversely, the AO and age at collection did not pass normality tests in our cohort (Kolmogorov-Smirnov normality test p < 0.05). Consequently, Spearman’s rho (r) was used to estimate correlation levels with associated 2-tailed p value. A 2-tailed Mann-Whitney U test was performed for all statistical comparisons of means.

Since a previous study showed that gender could influence AO in patients with C9orf72 mutations (Le Ber et al., 2013), correlations between C9orf72 repeats number and AO were calculated using residual values of AO after adjusting for sex. In some analyses, repeats number was adjusted for the age at collection or AO. Residual values of repeats number were then used in correlation or in group comparisons analyses. GraphPad Prism Software v5.00 was used for data visualization.

3. Results

3.1. Clinical and molecular characteristics of patients and PS carriers

The mean AO in C9orf72 patients was 58 years (range: 37–77 years; median = 59; SD = 12.0). Although the mean AO was lower in ALS and FTDT-ALS patients than in patients with isolated FTD, none of these differences was significant between the 3 groups (p > 0.05 for all comparisons).

The mean age at first blood collection was 45.8 years in PS (range: 20–88 years; median = 43; SD = 16.7) and 61 years in symptomatic subjects (range: 39–80 years; median = 62; SD = 11.4). The mean age at collection was lower in ALS and FTDT-ALS compared to FTD but, here again, these differences were not significant (p > 0.05 for all mean comparisons).

The number of repeats ranged from 1459 to 3889 (mean = 2334; SD = 489.9) in PS carriers and from 1294 to 3320 (mean = 2426, SD = 480.7) in symptomatic patients. Means and variances of repeat number did not differ significantly between PS and symptomatic patients (p = 0.19 for Mann-Whitney U test and p = 0.87 for F-test). Details of repeat sizes distribution parameters in all carriers’ groups are shown in Table 1 and Supplementary Fig. 1.

3.2. Relations between repeat expansion size, age at onset, and age at blood collection

Mean repeats number was significantly lower in patients with early onset (AO ≤ 48, mean = 2186) than in patients with late onset (AO ≥ 68, mean = 2741) (p = 0.0001; Fig. 1A). When AO was considered as a continuous variable, repeats number was positively correlated with AO (r = 0.54; p < 0.0001) (Fig. 1B).

Relations between age at collection and repeats number were investigated as well (Fig. 2). We observed similar trends of correlations between repeats number and age at collection in patients (r = 0.51; p < 0.0001). Considering that the age at collection might influence the C9orf72 pathogenic expansion size in blood and could be a confounding factor in our analyses, repeats number was adjusted for age at collection and used in statistical analyses. The residual correlation of repeats number adjusted for age at collection with AO was no longer significant (r = −0.008; p = 0.95). In the same way, comparisons of adjusted repeats number for age at collection
between patients with extreme AO (EO vs. LO) became nonsignificant ($p = 0.84$). When repeats number was adjusted for AO, the residual correlation was slightly higher ($r = 0.12$) but nonsignificant. As attempted, AO and age at collection were highly correlated in our cohort ($r = 0.95$, $p < 0.0001$). Importantly, the same trend of correlation was found in PS individuals. The number of G4C2 repeats in the expanded allele was positively correlated with age at collection ($r = 0.65$; $p < 0.0001$). To go further, we compared the mean size of expansions in the subgroup of PS carriers with the youngest age at collection ($≤ 45$ years) and in patients who developed the disease before 45 years. Means of repeats number were lower in each subgroup compared to their respective whole cohort of patients and PS carriers. Difference of means between these 2 subgroups was not significant ($2037$ vs. $2147$, respectively; $p = 0.59$).

### 3.3. Relations between repeat number and clinical phenotypes (FTD vs. ALS)

Mean number of repeats was slightly lower in patients with ALS versus FTD ($2228$ vs. $2540$, respectively; $p = 0.024$, Fig. 1C). Comparisons of repeats number adjusted for the age at collection between these 2 groups became nonsignificant ($p = 0.33$).

### 3.4. Dynamic of the pathogenic expansion in lymphocytes over time

For a subset of the aforementioned samples ($n = 11$), multiple time points were available. These patients and PS individuals gave us the opportunity to measure expansion size changes over time individually and to evaluate relations with disease onset. We did not detect important repeats number differences or a clear trend toward expansion or contraction of the pathogenic expansion in the time intervals considered (Fig. 3). The absolute values of repeat number differences between collections ranged from 15 to 231. Among the carriers, 2 patients (patients 8 and 11, Fig. 3) developed symptoms between 2 blood collection times. These 2 converters did not present massive expansion or contraction of repeats number before and after disease onset. Little and opposite changes were observed for these 2 individuals (-173 repeats and +56 repeats for a time interval of 6.5 and 11 years, respectively).

### 3.5. Dynamic of the repeat expansion in parent-offspring transmissions

A total of 16 parent-offspring transmissions have been studied from the whole cohort, including 8 simple parent-offspring transmissions and 4 parents with 2 children each (Fig. 4). A reduction of repeats number from parents to children was observed in 11/16 pairs (mean $= -503$ repeats). Conversely, the repeats number increased over generations in 4/16 pairs (mean $= +399$ repeats). Nearly the same expansion size was detected in one parent-offspring pair. Expansion or contraction of repeats number over generations did not depend on the gender, the age at collection, nor the age at onset of the transmitting parent (Fig. 4).

### 4. Discussion

The intronic G4C2 pathogenic expansion in C9orf72 is the major genetic cause of FTD, FTD-ALS, and ALS (Cruts et al., 2013; Le Ber
et al., 2013; Majounie et al., 2012). We recently showed that genetic factors significantly influence the AO in C9orf72 families (Barbier et al., 2017). However, modifiers of AO in C9orf72 FTD remain not yet fully understood, and the search for genetic factors or markers predicting the disease outcome has become a priority to improve patients’ genetic counseling and set up clinical trials.

Because of the particular nature of the mutation, C9orf72 FTD and/or ALS belong to the family of repeat expansion diseases. As observed in other repeat expansion disorders, such as Huntington’s disease or Fragile-X syndrome, it has been proposed that the size of expansion could influence clinical features in C9orf72 disease. Estimate of repeats number from blood DNA has appeared as a potential noninvasive biomarker, despite the fact that blood estimates of repeats number may not exactly reflect what is occurring in the brain (van Blitterswijk et al., 2013). Consequently, some studies focusing on C9orf72 expansion size in peripheral lymphocytes were performed, leading to controversial conclusions (Table 2) (Beck et al., 2013; Cruts et al., 2013; Dols-Icardo et al., 2014; Gijselinck et al., 2016; Nordin et al., 2015; Russ et al., 2015; Suh et al., 2015; van Blitterswijk et al., 2013). Here, we found a positive correlation between repeats number from blood DNA has appeared as a potential noninvasive biomarker, despite the fact that blood estimates of repeats number may not exactly reflect what is occurring in the brain (van Blitterswijk et al., 2013). Consequently, some studies focusing on C9orf72 expansion size in peripheral lymphocytes were performed, leading to controversial conclusions (Table 2) (Beck et al., 2013; Cruts et al., 2013; Dols-Icardo et al., 2014; Gijselinck et al., 2016; Nordin et al., 2015; Russ et al., 2015; Suh et al., 2015; van Blitterswijk et al., 2013). Here, we found a positive correlation between repeats number from blood DNA and AO in a large cohort of 73 C9orf72 patients carrying long expansions, which is the largest cohort of carriers in whom blood expansion repeats number was measured to date.

A significant correlation between repeats number in blood and AO was found in 3 previous studies, based on smaller cohorts (van Blitterswijk et al., 2013; Nordin et al., 2015; Dols-Icardo et al., 2014). By contrast, an inverse correlation was evidenced in a Belgian cohort study that compared a group of 6 short expansion (<100 repeats) to 66 long expansion (>100 repeats) carriers (Gijselinck et al., 2016; Van Mossevelde et al., 2017). However, the methodology used in the Belgian cohort is distinct from other studies and ours, all studying AO as a quantitative trait in long expansion carriers. Small samples sizes, or potential distinct mechanisms in short and large expansion carriers, could explain divergent results. Also, these results suggest that C9orf72 pathogenesis could depend on crossing a repeat length, though it is unclear whether this interpretation holds in brain and other disease-relevant tissues.

Despite diverging conclusions on relations between repeats number and AO in the literature, converging evidences came out from association analyses of C9orf72 repeats number and DNA methylation state. Repeats expansion number has been showed to influence DNA methylation at the C9orf72 locus, longer expansions being associated with a higher methylation state in the 5′ regulatory region of the gene (Gijselinck et al., 2016; Russ et al., 2015; Xi et al., 2013). In this context, at least for longer expansions, down-regulation of expression of the expanded C9orf72 allele might counterbalance deleterious effects of the pathogenic expansion and slightly delay the disease onset.

However, and besides correlations with AO, we also detected a significant correlation between repeats number and age at collection in patients. Intriguingly, a very similar correlation was found in
our series of 52 PS carriers with an age at collection ranging from 20 years to 88 years, which is presently the cohort covering the most expanded range of age to look at relationship between expansion size and age at collection. This suggests that age at collection could be a major confounding variable, and that the observed correlations and associations are driven mostly by the age at collection. Indeed, when repeats number were adjusted for the age at collection, no significant result was observed from the analyses of correlation with AO, nor association with clinical phenotypes. Notably, these results are in line with those of Suh et al. who found a positive correlation between repeats number in blood and age at collection in C9orf72 patients, but not with age at onset after corrections for the age at collection and disease phenotypes (Suh et al., 2015). Finally, the collinearity between AO and age at collection prevents to determine statistically which variable, AO or age at collection, is driving the correlation with repeats number. This constitutes a limitation of this work as of other studies.

To go further, we focused on PS carriers with the youngest age at collection (<45 years) and patients who developed disease before the same age limit. Means of repeats number were not statistically significant between these young PS carriers and patients with a very early onset. Nevertheless, and assuming the hypothesis that low repeats number may be associated with earlier AO in our cohort of patients, all these young PS carriers (62% of the PS cohort) should be at high risk to develop disease in a very short time as they carry a number of repeats similar from the subset of patients who converted within the same range of age. However, the age-related penetrance before 45 years of age was previously estimated to be lower than 10% in 2 different cohorts of C9orf72 carriers (Le Ber et al., 2013; Majounie et al., 2012). All together, these results suggest that the lower number of repeats are more likely related to a younger age at collection rather than to the risk to develop disease, arguing in favor of a predominant influence of the age at collection on blood DNA repeats number estimates.

Given the correlation of the expansion size in blood with age at collection, and due to the dynamic nature of this mutation, it is reasonable to speculate that C9orf72 pathogenic expansions could evolve, expand or contract, with time in a given carrier. Data from C9orf72 individuals with multiple time points (with a mean time interval of 57.9 months) allowed to measure blood repeats number changes with time per patient, with a maximum time interval of 11 years. Importantly, we did not detect massive expansion or contraction of the expansion size with time, even in carriers with the longest follow-up interval (>10 years), or in the 2 carriers who converted between the first and last blood samples. The repeats number slightly varied over time in all cases. In addition, there was no clear and concordant trend toward expansion or contraction between first and last collections, as some carriers presented expansions, contractions, or even similar number of repeats over time. Smaller and more subtle changes might have happened in the interval between several samplings that cannot be precisely detected because of technical limitations of the Southern blot. Indeed, long expansions usually appear as a smear, preventing an accurate estimation of modal number of repeats but, up to now, this is the only method to size C9orf72 long expansions. The maximum difference due to the technical imprecision between 2 measures from the same individual’s DNA was up to 172 repeats. According to the slope of the regression lines between repeats number and age at collection, number of G4C2 repeats measured in blood DNA should increase by around 18 repeats per year. Theoretically, a longer period of follow-up (at least 20 years) should be needed therefore to reliably detect evolution of repeats number over time. So, in this study, the time intervals between multiple sample collections might have been too short to clearly observe the influence of the age on blood repeats number with this method. The weak
variations of observed repeats numbers more likely reflect the in-accurary of the measurement method rather than true variations. Young PS C9orf72 carriers, who will be followed for a longer period of time, should bring more valuable data to analyze the dynamics of the pathogenic expansion during lifespan and confirm, or not, whether blood repeats number increases over time.

Finally, the dynamic of long expansions through generations was investigated in 16 parent-offspring pairs. Importantly, measurable changes in repeats number have been observed, regardless of the gender or the age of transmitting parent. Unexpectedly, a contraction between successive generations was observed in the majority of transmissions (11/16). It is likely that a younger age at collection in offspring versus parents (mean difference of age at collection = 28 years) may partly explain this trend even though this hypothesis did not fit with all paired comparisons. Indeed, expansion or stable repeat number was observed as well, although less frequently. In line with these results, expansions as well as contractions of C9orf72 repeats have been previously reported in lymphocytes from parent-offspring pairs carrying short expansions (Gijselinck et al., 2016; Van Mossevelde et al., 2017). Importantly, these results demonstrate that blood C9orf72 long expansions do not systematically expand through generations and would argue against an anticipation phenomenon at the molecular level.

5. Conclusions

This study establishes that long repeat expansions are highly instable in lymphocytes and variable across time and generations. We observed that the C9orf72 expansion size in blood correlated with age of disease onset and moderately with clinical outcome. However, the age at sampling appeared as a major confounding factor, which could contribute to the observed trends. The correlation between repeats number and age at collection in PS individuals including young carriers who are likely to be more distant from the disease onset argues in favor of the last hypothesis. This study thus clearly supports that blood measure of long repeats cannot serve as a reliable noninvasive biomarker to predict disease onset or evolution toward FTD or ALS in C9orf72 disease, conversely to several other repeat expansion diseases. The identification of alternative biomarkers, easily measurable in accessible fluids, must therefore be a major research challenge as therapeutic era is coming.

Disclosure statement

The authors have no actual or potential conflict of interest.

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Appendix A. Supplementary data

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