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Novel clinical associations with specific *C9ORF72* transcripts in patients with repeat expansions in *C9ORF72*

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Abstract

The loss of chromosome 9 open reading frame 72 (*C9ORF72*) expression, associated with *C9ORF72* repeat expansions, has not been examined systematically. Three *C9ORF72* transcript variants have been described thus far; the GGGGCC repeat is located between two non-coding exons (exon 1a and exon 1b) in the promoter region of transcript variant 2 (NM_018325.4) or in the first intron of variant 1 (NM_145005.6) and variant 3 (NM_001256054.2). We studied *C9ORF72* expression in expansion carriers ($n = 56$) for whom cerebellum and/or frontal cortex

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Compliance with ethical standards

was available. Using quantitative real-time PCR and digital molecular barcoding techniques, we assessed total *C9ORF72* transcripts, variant 1, variant 2, variant 3, and intron containing transcripts [upstream of the expansion (intron 1a) and downstream of the expansion (intron 1b)]; the latter were correlated with levels of poly(GP) and poly(GA) proteins aberrantly translated from the expansion as measured by immunoassay ($n = 50$). We detected a decrease in expansion carriers as compared to controls for total *C9ORF72* transcripts, variant 1, and variant 2: the strongest association was observed for variant 2 (quantitative real-time PCR cerebellum: median 43 %, $p = 1.26e\text{-}06$, and frontal cortex: median 58 %, $p = 1.11e\text{-}05$; digital molecular barcoding cerebellum: median 31 %, $p = 5.23e\text{-}10$, and frontal cortex: median 53 %, $p = 5.07e\text{-}10$). Importantly, we revealed that variant 1 levels greater than the 25th percentile conferred a survival advantage [digital molecular barcoding cerebellum: hazard ratio (HR) 0.31, $p = 0.003$, and frontal cortex: HR 0.23, $p = 0.0001$]. When focusing on intron containing transcripts, analysis of the frontal cortex revealed an increase of potentially truncated transcripts in expansion carriers as compared to controls [digital molecular barcoding frontal cortex (intron 1a): median 272 %, $p = 0.003$], with the highest levels in patients pathologically diagnosed with frontotemporal lobar degeneration. In the cerebellum, our analysis suggested that transcripts were less likely to be truncated and, excitingly, we discovered that intron containing transcripts were associated with poly(GP) levels [digital molecular barcoding cerebellum (intron 1a): $r = 0.33$, $p = 0.02$, and (intron 1b): $r = 0.49$, $p = 0.0004$] and poly(GA) levels [digital molecular barcoding cerebellum (intron 1a): $r = 0.34$, $p = 0.02$, and (intron 1b): $r = 0.38$, $p = 0.007$]. In summary, we report decreased expression of specific *C9ORF72* transcripts and provide support for the presence of truncated transcripts as well as pre-mRNAs that may serve as templates for RAN translation. We further show that higher *C9ORF72* levels may have beneficial effects, which warrants caution in the development of new therapeutic approaches.

Introduction

The most frequent genetic cause of frontotemporal lobar degeneration (FTLD) and motor neuron disease (MND) is a hexanucleotide repeat expansion in chromosome 9 open reading frame 72 (*C9ORF72*) [8, 22], which is pathogenic due to the formation of RNA foci, repeat-associated non-ATG (RAN) translation resulting in dipeptide-repeat proteins (c9RAN proteins), and/or a loss of *C9ORF72* expression [2, 8, 21]. It has been shown that C9ORF72 proteins localize to the nuclear membrane in healthy neurons and interact with components of the nuclear pore complex (i.e., Importin β1 and Ran-GTPase), implicating C9ORF72 proteins in nucleocytoplasmic import [29]. The presence of a repeat expansion in *C9ORF72* is also thought to compromise nucleocytoplasmic transport [11, 14, 30].

Although models have validated toxic gain-of-function mechanisms [5], they have been unable to confirm or exclude reduced *C9ORF72* expression as a pathogenic mechanism [6, 15, 16, 23]. At least three transcripts are generated from the *C9ORF72* gene: the GGGGCC

repeat is located between two non-coding exons (exon 1a and exon 1b) either in the promoter region of transcript variant 2 (NM_018325.4), or in the first intron of variant 1 (NM_145005.6) and variant 3 (NM_001256054.2, Fig. 1). Whereas variant 1 is predicted to result in a 222 amino acid long protein (exon 2–5, isoform b), variant 2 and variant 3 appear to result in 481 amino acid long proteins (exon 2–11, isoform a).

In *C9ORF72* expansion carriers, decreased levels of *C9ORF72* mRNA have been reported [8], which was confirmed by other studies. In the fontal cortex, for instance, reduced expression levels were detected for total *C9ORF72* transcripts [3, 6, 12, 28], variant 1 [12], and variant 2 [8, 10, 28]. A reduction was also described in the cerebellum for total *C9ORF72* transcripts [3] and variant 2 [28]. Interestingly, other experiments suggested that a repeat expansion in *C9ORF72* can lead to truncated expansion-containing abortive transcripts [13], and that the expansion may cause a shift from transcription of exon 1b (variant 2) towards transcription of exon 1a (variant 1 and variant 3) [23]. Nevertheless, studies focusing on *C9ORF72* expression have also revealed inconsistencies [3, 8, 12, 13, 17, 21, 22], potentially due to variability in sample size, nomenclature, methodology, tissue type, and RNA integrity.

Since large-scale studies have yet to be reported that evaluate *C9ORF72* expression systematically, we examined all known *C9ORF72* transcripts in a unique pathological cohort of *C9ORF72* expansion carriers. To further investigate intron 1 containing transcripts, we also assessed two intronic regions, one upstream of the repeat expansion (intron 1a), and one downstream of the repeat expansion (intron 1b). Our study uncovers decreased expression of specific *C9ORF72* transcripts, reveals novel associations with survival after onset, and provides evidence of truncated transcripts as well as pre-mRNAs that act as templates for RAN translation.

Materials and methods

Subjects

From the Mayo Clinic Florida Brain Bank, we selected all patients with *C9ORF72* repeat expansions, and a subset of patients with FTLD and/or MND without repeat expansions and control subjects without neurological diseases, for whom frozen cerebellum and/or frontal cortex were available, and for whom sufficient high quality RNA could be obtained for *C9ORF72* expression studies [RNA integrity number (RIN) above seven, Online Resource Materials and Methods]. To measure *C9ORF72* expression levels, we used two approaches: TaqMan gene expression assays (Life Technologies) and digital molecular barcoding (NanoString Technologies). For our *C9ORF72* expression studies using TaqMan assays, we were able to include a cohort of 56 *C9ORF72* expansion carriers [23 FTLD, 16 FTLD/MND, 14 MND, and 3 others (i.e., Alzheimer's disease and depressive pseudodementia)] [4], 31 patients without those expansions (9 FTLD, 13 FTLD/MND, and 9 MND), and 20 control subjects without any neurological disease (Table 1). The same cohort of expansion carriers was included in our expression studies using NanoString technologies (Online Resource Table 1). We evaluated associations with poly(GP) and poly(GA) levels for a total of 50 expansion carriers from this cohort (22 FTLD, 14 FTLD/MND, and 14 MND, Online Resource Table 2). Written informed consent was obtained before study entry from all

subjects or their legal next of kin if they were unable to give written consent, and biological samples were obtained with ethical committee approval.

Examination of *C9ORF72* transcripts

Total RNA was extracted from frozen cerebellum and frontal cortex using the RNeasy Plus mini kit (Qiagen), which includes a step to remove genomic DNA (gDNA) with gDNA Eliminator columns. RNA quality and quantity were determined with an Agilent 2100 Bioanalyzer using the RNA Nano Chip (Agilent Technologies). We performed a reverse transcription reaction with 300 ng of RNA as template, using an equal ratio of Random Hexamers and Oligo dT primers and the SuperScript III Kit (Invitrogen). Quantitative real-time PCR was then done on an ABI7900 PCR system (Applied Biosystems) using TaqMan gene expression assays. We assessed neuronal markers synaptophysin (*SYP*, Hs00300531_m1) and microtubule-associated protein 2 (*MAP2*, Hs00258900_m1), and endogenous control genes ribosomal protein, large, P0 (*RPLP0*, Hs00420895_gh) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Hs02758991_g1). Additionally, we examined total *C9ORF72* transcripts (Hs00376619_m1), *C9ORF72* variant 2 transcripts (custom) [8], and *C9ORF72* variant 3 transcripts (Hs00948764_m1). Values of *C9ORF72* variant 1 transcripts (Hs00331877_m1) were too low when using a TaqMan gene expression assay to allow inclusion of this variant, as reported previously [3]. Using R Statistical Software (R Foundation for Statistical Computing, version 3.2.0), we calculated the median of replicates and took the geometric mean of neuronal markers and endogenous control genes, and subsequently, determined expression levels relative to control subjects utilizing the Ct method.

Digital molecular barcoding was done in house by the manufacturer using 100 ng of RNA as template. We examined neuronal markers *SYP* and *MAP2*, and endogenous control genes *RPLP0* and *GAPDH*. The following targets were included in our analysis: total *C9ORF72* transcripts, *C9ORF72* variant 1 transcripts, *C9ORF72* variant 2 transcripts, *C9ORF72* intron 1a containing transcripts, and *C9ORF72* intron 1b containing transcripts (Fig. 1, Online Resource Table 3). *C9ORF72* variant 3 values were around background level when using digital molecular barcoding (consistent with another report [9]), and consequently, variant 3 was not included in our digital molecular barcoding analysis. The NanoStringNorm R package and nSolver Analysis Software (NanoString Technologies, version 2.5) were used to account for technical assay variation using the geometric mean method. Maximum background subtraction was then performed, and data were normalized to the geometric mean of neuronal markers and endogenous control genes.

Poly(GP) and poly(GA) immunoassays

Brain homogenates were prepared as previously described [1]. In brief, approximately 50 mg of cerebellum or frontal cortex was lysed in cold radio immunoprecipitation assay (RIPA) buffer and sonicated on ice. Lysates were cleared by centrifugation at $100,000 \times g$ for 30 min at 4 °C. The supernatant was collected and the protein concentration was determined by BCA assay. To prevent carry-over, the resulting pellet was resuspended in RIPA buffer, re-sonicated, and re-centrifuged. The RIPA-insoluble pellet was then extracted using 7 M urea buffer, sonicated and centrifuged at $100,000 \times g$ for 30 min at 22 °C. The protein

concentration of the urea-soluble supernatant was determined by Bradford assay. Homogenates were diluted in Trisbuffered saline (TBS) and equal amounts of protein for all samples from the same neuroanatomical region and fraction were tested in duplicate wells. Poly(GP) levels in each fraction were measured using a previously described sandwich immunoassay that utilizes an affinity purified rabbit polyclonal poly(GP) antibody (Rb9259) and Meso Scale Discovery (MSD) electrochemiluminescence detection technology [25]. We used serial dilutions of recombinant $(GP)_8$ to prepare the standard curve. Response values, corresponding to the intensity of emitted light upon electrochemical stimulation of the assay plate using the MSD QUICKPLEX SQ120, were acquired and background corrected prior to interpolation of poly(GP) levels using the standard curve. Poly(GA) levels in cerebellar lysates were similarly measured with an MSD-based poly(GA) sandwich immunoassay that utilizes a purified rabbit polyclonal poly(GA) antibody (Rb4333) provided by the Cleveland laboratory and a mouse monoclonal poly(GA) antibody (clone 5F2) provided by the Edbauer laboratory [18]. Serial dilutions of recombinant $(GA)_{50}$ were used for the standard curve. Total levels in a given tissue sample were calculated from levels in soluble and insoluble fractions and used for examining associations of intron 1 containing transcripts with poly(GP) or poly(GA) levels.

Statistical analysis

We summarized data with median and interquartile range (IQR). To compare expression levels of transcripts between groups (e.g. *C9ORF72* expansion carriers, patients without those expansions, and control subjects without neurological diseases), we used a Kruskal–Wallis rank sum test, and when this test was significant, we used a Wilcoxon rank sum test for each pairwise comparison ($p < 0.017$ considered significant after Bonferroni correction). Associations between transcripts measured by gene expression assays and digital molecular barcoding techniques (i.e., total *C9ORF72* transcripts and variant 2) were assessed using a Spearman's test of correlation; Spearman's correlation coefficient *r* and 95 % confidence intervals (CIs) were estimated ($p < 0.025$ considered significant after Bonferroni correction). Because intron 1a containing transcripts and intron 1b containing transcript were only detectable in a subset of samples, we also compared the number of subjects with and without those transcripts for each pairwise comparison with a Fisher's exact test, and we estimated odds ratios (ORs) and 95 % CIs ($p < 0.017$ considered significant after Bonferroni correction).

To assess the presence of associations within our overall cohort of expansion carriers, and within each disease subgroup (i.e., FTLD, FTLD/MND, and MND), associations of expression levels with variables were evaluated using a Spearman's test of correlation, a Wilcoxon rank sum test, or a Kruskal–Wallis rank sum test, as appropriate for the nature of the given variable. In addition, we investigated associations between expression levels and survival after onset in our overall cohort of patients with *C9ORF72* repeat expansions, and in each disease subgroup separately, using Cox proportional hazards regression models. Hazard ratios (HRs) and 95 % CIs were estimated, and deaths of any cause were utilized as our survival endpoint. All models in the overall cohort were adjusted for age at onset and disease subgroup. When investigating separate disease subgroups, models were adjusted for age at onset. In our Cox regression analysis, we considered the RNA expression level as a

dichotomous categorical variable using three different cutoff points (median, 25th percentile, and 75th percentile) in the given subject group. To adjust for multiple testing and to control the family-wise error rate at 5 %, we utilized a Bonferroni correction separately for each outcome and for each disease subgroup; p values <0.0063 were considered significant when evaluating associations of each outcome with variables studied, including disease subgroup, gender, age at onset, age at death, repeat length, and survival after onset (using three different cutoff points).

Given that intron 1a containing transcripts and/or intron 1b containing transcripts may serve as templates for RAN translation, we also determined whether those transcripts were associated with poly(GP) or poly(GA) levels using a Spearman's test of correlation. For each transcript, two tests were performed in the cerebellum [i.e., poly(GP) and poly(GA)] and one test in the frontal cortex [i.e., poly(GP)], and thus, p values below 0.025 and 0.050 were considered significant after Bonferroni correction, respectively. R Statistical Software was used for all statistical analyses.

Results

Reduction of specific *C9ORF72* transcripts

C9ORF72 expression levels differed significantly between *C9ORF72* expansion carriers, patients without expansions, and control subjects for total *C9ORF72* transcripts (p 5.65e-07) as well as for variant 1 (p 3.86e-05) and for variant 2 (p 1.86e-10, Tables 2, 3). For simplicity, we focused on comparisons with *C9ORF72* expansion carriers (Tables 2, 3); all results are shown in Online Resource Tables 4–5.

When using TaqMan assays, total *C9ORF72* transcript levels in expansion carriers were 67 % (IQR 53–82 %) in the cerebellum, which was significantly lower than those in patients without expansions (98 %, IQR 81–121 %, $p = 9.14e-06$) or those in control subjects (100 %, IQR 86–118 %, $p = 2.61e-05$, Table 2, Fig. 2). The cerebellar expression of variant 2 was 43 % (IQR 33–55 %) in expansion carriers as compared to 94 % (IQR 71–108 %, $p = 5.20e-09$) in patients without expansions, and 100 % (IQR 78–120 %, $p = 1.26e-06$, Table 2, Fig. 2) in control subjects. No significant difference was detected for variant 3 ($p = 0.12$). In the frontal cortex, total *C9ORF72* transcript levels in expansion carriers were 78 % (IQR 64–105 %), significantly lower than in patients without expansions (130 %, IQR 113–161 %, $p = 2.03e-07$) or in control subjects (100 %, IQR 85–122 %, $p = 0.02$, Table 2). Expression levels of variant 2 were 58 % (IQR 48–67 %) in expansion carriers as compared to 131 % (IQR 114–166 %, $p = 9.63e-12$) in patients without expansions, and 100 % (IQR 73–128 %, $p = 1.11e-05$, Table 2) in control subjects. After adjustment for multiple testing, no significant difference was observed for variant 3 ($p = 0.02$, Table 2).

NanoString technologies were utilized to examine expression levels of total *C9ORF72* transcripts, variant 1, and variant 2. In the cerebellum, total *C9ORF72* transcripts were significantly lower in expansion carriers (58 %, IQR 42–76 %) than in patients without expansions (80 %, IQR 67–93 %, $p = 0.003$) or in control subjects (100 %, IQR 88–119 %, $p = 1.59e-07$, Table 3). Cerebellar levels of variant 1 were also reduced in expansion carriers (47 %, IQR 31–63 %) as compared to patients without expansions (61 %, IQR 55–75 %, $p =$

0.01) and to control subjects (100 %, IQR 72–149 %, p = 9.04e-06, Table 3). In *C9ORF72* expansion carriers, the lowest levels were detected for variant 2: 31 % (IQR 19–47 %) in expansion carriers, 70 % (IQR 55–90 %, p = 7.27e-07) in patients without expansions, and 100 % (IQR 87–113 %, p = 5.23e-10, Table 3) in control subjects. Reduced levels of total *C9ORF72* transcripts were also observed in the frontal cortex of expansion carriers (72 %, IQR 62–83 %) versus patients without expansions (113 %, IQR 104–134 %, p = 1.42e-09) or versus control subjects (100 %, IQR 90–118 %, p = 2.35e-06, Table 3). For variant 1, expression levels were 69 % (IQR 49–90 %) in expansion carriers as compared to 110 % (IQR 88–138 %, p = 0.0001) in patients without expansions, and 100 % (IQR 78–139 %, p = 0.001, Table 3) in control subjects. Again, the most prominent decrease was observed for variant 2, which demonstrated expression levels of 53 % (IQR 34–63 %) in expansions carriers, 121 % (IQR 94–143 %, p = 1.84e-08) in patients without expansions, and 100 % (IQR 90–131 %, p = 5.07e-10, Table 3) in control subjects.

Subsequently, we assessed associations between expression levels obtained using TaqMan assays and NanoString technologies for transcripts measured by both methods (i.e., total *C9ORF72* transcripts and variant 2). We detected significant associations for total *C9ORF72* transcripts in the cerebellum (r = 0.84, 95 % CI 0.75–0.90, p < 2.20e-16) and frontal cortex (r = 0.72, 95 % CI 0.58–0.82, p = 1.58e-15, Fig. 2, Online Resource Table 6). Similar associations were detected for variant 2 in the cerebellum (r = 0.75, 95 % CI 0.61–0.84, p < 2.20e-16) and frontal cortex (r = 0.80, 95 % CI 0.71–0.86, p < 2.20e-16, Fig. 2, Online Resource Table 6).

Elevation of *C9ORF72* transcripts containing intron 1a

In the frontal cortex, we also discovered a significant difference in transcripts containing intron 1a (upstream of expansion, p = 0.0002, Table 3, Figs. 1, 2) when using NanoString technologies. For those transcripts, the relative expression was 272 % (IQR 98–481 %) in expansion carriers as compared to 0 % (IQR 0–108 %, p = 0.0005) in patients without expansions, and 100 % (IQR 0–234 %, p = 0.003) in control subjects. No such difference was detected in the cerebellum (p = 0.20). Intriguingly, for intron 1b containing transcripts (downstream of expansion) no significant differences were noted in the frontal cortex or in the cerebellum (p 0.11, Table 3). Since intron containing transcripts were undetectable in a subset of individuals, we also performed an alternative analysis using a Fisher's exact test, which aligned with aforementioned results: in the frontal cortex, intron 1a containing transcripts were undetectable in 12 % of expansion carriers as compared to 55 % of patients without expansions (OR 0.11, 95 % CI 0.03–0.42, p = 0.0003), and 40 % of control subjects (OR 0.20, 95 % CI 0.05–0.80, p = 0.02, Online Resource Table 7). We did not observe a significant difference in the cerebellum (p 0.06), nor did we note significant differences for intron 1b containing transcripts in the frontal cortex or in the cerebellum (p = 1, Online Resource Table 7).

Associations of specific *C9ORF72* transcripts with clinical features

Next, we explored *C9ORF72* expression within our group of expansion carriers. In both neuroanatomical regions, we noticed that expression levels of total *C9ORF72* transcripts, variant 1, variant 2, variant 3, and intron 1b containing transcripts did not differ significantly

between FTLD, FTLD/MND and MND patients (Online Resource Tables 8–9), nor did we detect associations with gender, age at onset, age at death or repeat length (Online Resource Tables 10–13). Importantly, we did detect a significant difference between disease subgroups for transcripts containing intron 1a in the frontal cortex ($p = 0.002$, Table 4, Fig. 2): for those transcripts, higher levels were observed in patients with FTLD (396 %, IQR 210–625 %) or FTLD/MND (373 %, IQR 158–441 %) as compared to patients with MND (52 %, IQR 0–186 %, $p = 0.001$ and $p = 0.006$, respectively). Interestingly, there was no significant difference between disease subgroups for transcripts containing intron 1a in the cerebellum. We did detect a significant association with survival after onset for variant 1 in both neuroanatomical regions: expression levels greater than the 25th percentile conferred a survival advantage (models adjusted for age at onset and disease subgroup, Table 5, Online Resource Tables 14–15), both in the cerebellum (HR 0.31, 95 % CI 0.14–0.67, $p = 0.003$) and in the frontal cortex (HR 0.23, 95 % CI 0.11–0.49, $p = 0.0001$). In our expansion carriers, median survival was reduced in patients with lower variant 1 levels (cerebellum: 3.0 years, IQR 1.9–4.4 years, frontal cortex: 2.7 years, IQR 1.7–4.0 years, Fig. 3) as compared to patients with higher variant 1 levels (cerebellum: 6.2 years, IQR 3.1–8.3 years, frontal cortex: 6.4 years, IQR 3.7–8.6 years, Fig. 3). We did not detect significant associations between other *C9ORF72* transcripts and survival after onset in our expansion carriers (Online Resource Tables 14–15). Of note, for simplicity, we focused on our overall cohort of expansion carriers; we have, however, also provided tables that contain analyses for disease subgroups (e.g. Online Resource Tables 12–13).

Associations of *C9ORF72* transcripts containing intron 1 with poly(GP) and poly (GA) levels

To further investigate our findings related to intron 1 containing transcripts, we evaluated associations between those transcripts and poly(GP) c9RAN proteins. Although we did not detect a significant association in the frontal cortex (p 0.20), we noticed a significant association in the cerebellum for intron 1a containing transcripts ($r = 0.33$, 95 % CI 0.04–0.59, $p = 0.02$) and, more prominently, for intron 1b containing transcripts ($r = 0.49$, 95 % CI 0.23–0.70, $p = 0.0004$, Table 6, Fig. 3). Based on these significant findings in the cerebellum, we subsequently assessed associations between intron 1 containing transcripts and cerebellar poly(GA) levels: we also detected significant associations for both intron 1a containing transcripts ($r = 0.34$, 95 % CI 0.03–0.60, $p = 0.02$) and intron 1b containing transcripts ($r = 0.38$, 95 % CI 0.08–0.62, $p = 0.007$, Table 6, Fig. 3).

Discussion

To evaluate the contribution of *C9ORF72* transcripts to disease pathogenesis, we set out to determine the expression of specific *C9ORF72* transcripts in patients with repeat expansions in *C9ORF72*. Our study revealed a decreased expression of total *C9ORF72* transcripts, variant 1, and variant 2, but not of variant 3, in expansion carriers as compared to non-expansion carriers and controls. Moreover, we discovered that higher levels of variant 1 were associated with prolonged survival after onset in expansion carriers. Interestingly, we also detected an increase in transcripts containing intron 1a, specifically in the frontal cortex

of expansion carriers with an FTLD phenotype, while in the cerebellum intron 1 containing transcripts were significantly associated with poly(GP) and poly(GA) levels.

Previous studies focusing on *C9ORF72* expression in expansion carriers revealed inconsistencies between laboratories and samples [3, 8, 12, 17, 21, 22], which could be attributed to relatively small sample sizes, investigated transcripts, differences in methods, variability between tissue or cell types, and the integrity of extracted RNA. For these reasons, we examined one of the world's largest cohorts of pathologically confirmed expansion carriers, evaluated all known *C9ORF72* transcripts, used TaqMan and NanoString techniques, assessed two brain regions, and only included samples with relatively high RIN values (Table 1).

We detected a significant decrease in expansion carriers as compared to patients without repeat expansions and to control subjects without neurological diseases for total *C9ORF72* transcripts, variant 1, and variant 2. The strongest association was observed for variant 2, whereas no significant difference was observed for variant 3. Excitingly, we also discovered an association between variant 1 and survival after onset. Only a few factors are currently known to be associated with survival after onset in patients with *C9ORF72* repeat expansions, including repeat length and genetic variants [e.g. rs5848 in granulin (*GRN*)] [26, 27]. The novel association we identified with variant 1 suggests that higher *C9ORF72* levels may have beneficial effects, which could have major implications for the development of new therapeutic approaches targeting *C9ORF72* (e.g. antisense oligonucleotides [ASOs]). Based on our findings, researchers should focus on ASOs that reduce GGGGCC-containing RNA foci and/or c9RAN proteins without altering *C9ORF72* expression [16]. Additionally, future studies could investigate variant 1 in patients for whom RNA has been extracted from blood to determine whether its transcript levels could also be used as a prognostic indicator.

We further detected elevated levels of intron 1 containing transcripts (i.e., intron 1a), in agreement with recent reports [9, 13, 17, 21, 31]. Importantly, we identified significant differences between disease subgroups, with the most prominent elevation in the frontal cortex of FTLD patients, which is the primary affected region in those patients. One could speculate that a similar increase in intronic transcripts might be present in the spinal cord of MND patients, but this requires additional study.

Given the fact that we noted an increased expression of transcripts containing sequences upstream of the repeat (intron 1a), but not of transcripts downstream of the repeat (intron 1b), our data suggest that at least a subset of intron containing transcripts in *C9ORF72* expansion carriers is truncated. It seems unlikely, however, that truncation occurs in all intron containing transcripts, especially since groups have been able to detect c9RAN proteins using antibodies flanking the repeat [31]. c9RAN protein pathology, in particular inclusions of poly(GP) and poly(GA), is abundant in neocortical regions, hippocampus, thalamus, and cerebellum [2, 18– 21, 24, 31]. While previous studies utilized immunohistochemical methods to investigate associations between c9RAN protein pathology and other features of disease [7, 18, 19, 24], we have now employed immunoassays to quantify poly(GP) and poly(GA) burden. Interestingly, our experiments showed a significant association of intron 1 containing transcripts (both intron 1a and intron

1b) with poly(GP) and poly(GA) levels in the cerebellum of *C9ORF72* expansion carriers. As mentioned above, one could hypothesize that the increase of intron 1a (but not intron 1b) containing transcripts in the frontal cortex is a reflection of truncated transcripts that contain only part of the expansion, and therefore, those transcripts may not serve as templates for RAN translation. This would explain the absence of significant associations between intron 1 containing transcripts and poly(GP) levels in the frontal cortex. In contrast, the cerebellum likely contains less truncated transcripts as no relative increase in intron 1a containing transcripts was seen in expansion carriers. Moreover, the significant associations of poly(GP) and poly(GA) levels with both intron 1a containing transcripts and intron 1b containing transcripts in this region (most profoundly for intron 1b) suggest that transcripts containing the entire first intron may serve as templates for RAN translation in the cerebellum.

In summary, our present study reveals associations with specific *C9ORF72* transcripts and provides support for the presence of truncated transcripts and pre-mRNAs that may serve as templates for RAN translation. Although these promising findings do require further investigation, they shed new light on disease pathogenesis and may point towards novel therapeutic approaches for *C9ORF72*-related diseases as well as the larger FTLD and MND disease continuum.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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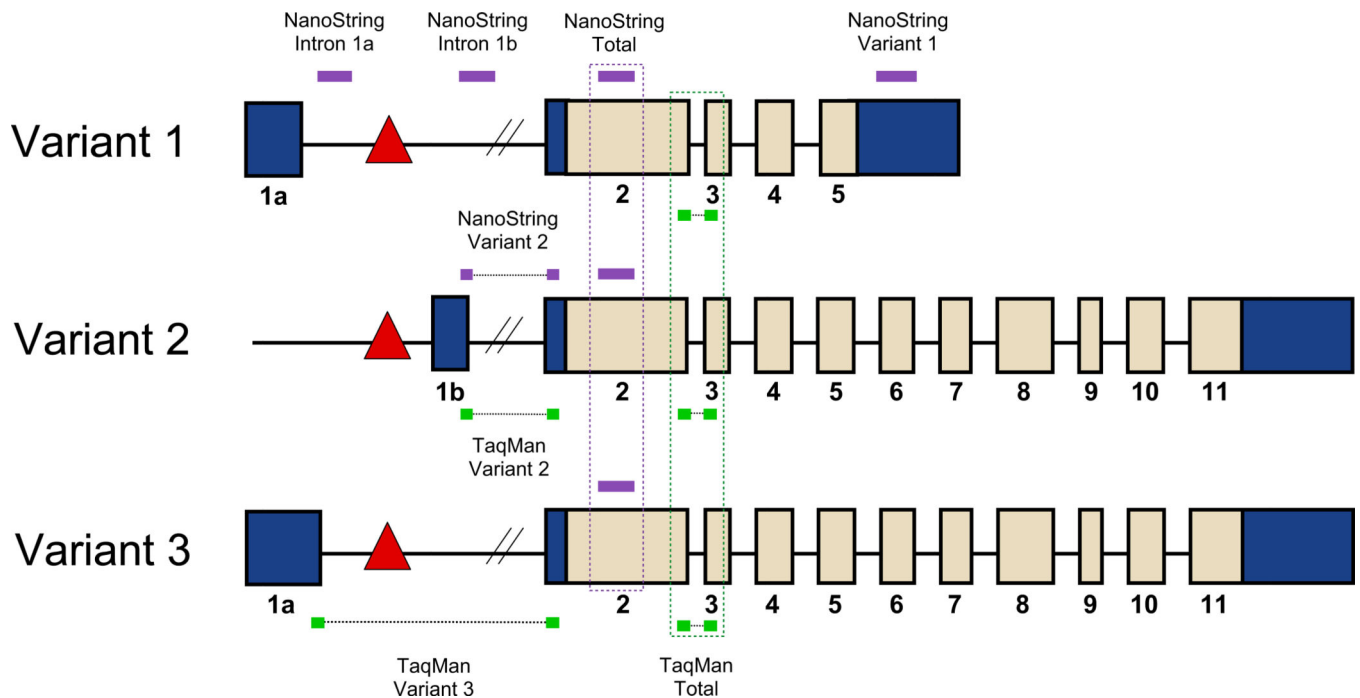
Fig. 1.

Localization of *C9ORF72* probes. The three known *C9ORF72* transcripts are visualized; the repeat expansion in *C9ORF72* is represented by a *red triangle* and located in the promoter region of variant 2 (NM_018325.4) or in the first intron of variant 1 (NM_145005.6) and variant 3 (NM_001256054.2). Whereas variant 1 is predicted to result in a 222 amino acid long protein (exon 2–5, isoform b), variant 2 and variant 3 appear to result in 481 amino acid long proteins (exon 2–11, isoform a). For our TaqMan assays, probes were used targeting total *C9ORF72* transcripts, variant 2, and variant 3 (*green*). Our digital molecular barcoding experiment contained probes for total *C9ORF72* transcripts, variant 1, and variant 2 as well as probes that target the region between exon 1a and the expansion (transcripts containing intron 1a) and between exon 1b and exon 2 (transcripts containing intron 1b, *purple*)
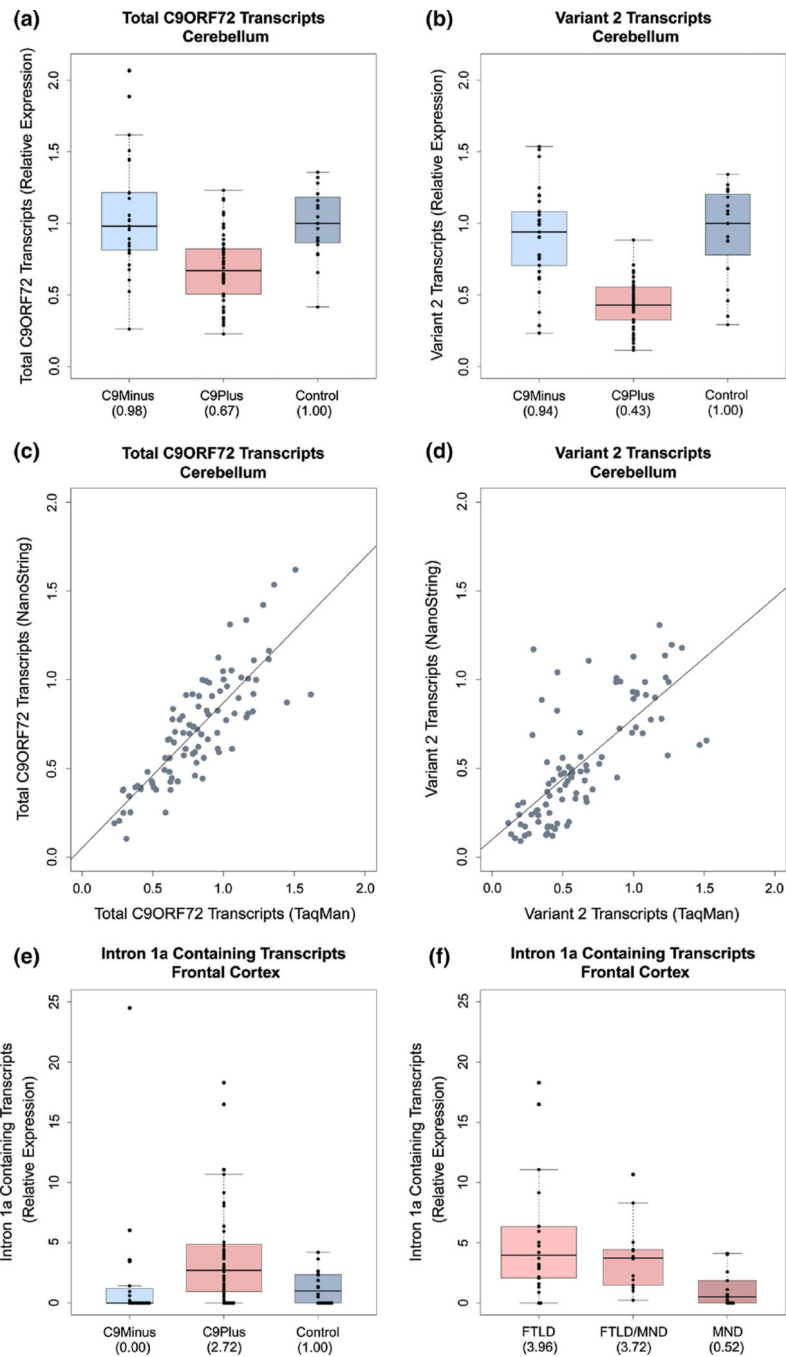
Fig. 2.
Expression of specific *C9ORF72* transcripts. *C9Plus* patients with *C9ORF72* repeat expansions, *C9Minus* patients without *C9ORF72* repeat expansions, and *Control* control subjects without neurological diseases. For each box plot, the median is represented by a *solid line* (values are specified between *brackets below panels*), and *each box* spans the IQR (25th percentile to 75th percentile). In the cerebellum, a reduction in total *C9ORF72* transcripts is observed in patients with *C9ORF72* repeat expansions as compared to patients without these expansions or to control subjects (**a** using TaqMan assays, Table 2).

Additionally, a decrease in variant 2 is detected in the cerebellum of *C9ORF72* expansion carriers as compared to patients without expansions or to control subjects (**b** using TaqMan assays, Table 2). In the cerebellum, there is an association between expression levels measured using TaqMan assays and NanoString technologies, both for total *C9ORF72* transcripts (**c** Online Resource Table 6) and for variant 2 (**d** Online Resource Table 6). In the frontal cortex, levels of intron 1a containing transcripts are higher in expansion carriers than in patients without expansions or in control subjects (**e** using NanoString technologies, Table 3), and within our cohort of expansion carriers, the highest levels appear to be present in patients with FTLD or FTLD/MND (**f** using NanoString technologies, Table 4)
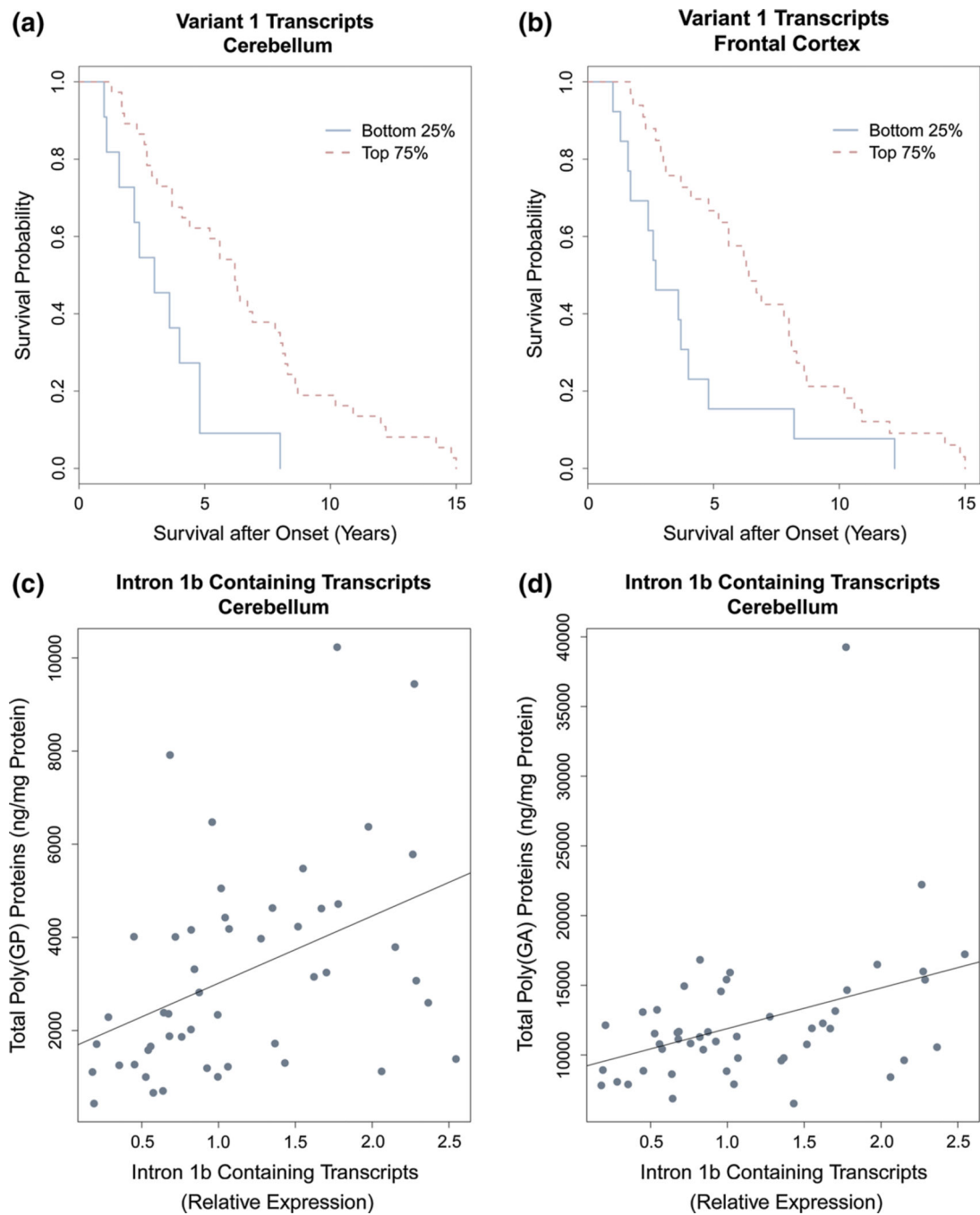
Fig. 3.
Associations with clinical and pathological characteristics. Associations with clinical and pathological variables are shown for our overall cohort of *C9ORF72* expansion carriers. Survival following disease onset, according to 25th percentile variant 1 levels, is displayed for the cerebellum (**a** using NanoString technologies, Table 5) and for the frontal cortex (**b** using NanoString technologies, Table 5). Additionally, associations between intron 1b

containing transcripts and poly(GP) levels (**c** using NanoString technologies, Table 6) or poly(GA) levels (**d** using NanoString technologies, Table 6) are shown for the cerebellum

Table 1

Subject characteristics in *C9ORF72* expression cohort (TaqMan; *n* = 107)

Variable	C9Plus cohort (*n* = 56)	C9Minus cohort (*n* = 31)	Control cohort (*n* = 20)
Subject characteristics			
Gender (male)	32 (57 %)	14 (45 %)	7 (35 %)
Age at onset (years)	62.0 (53.1–66.8)	–	–
Age at death (years)	66.5 (60.0–72.8)	72.0 (65.5–83.0)	87.5 (81.8–93.0)
Survival after onset (years)	5.0 (2.8–8.1)	–	–
RIN cerebellum (value)	9.4 (8.9–9.6)	9.4 (9.2–9.5)	9.3 (8.6–9.5)
RIN frontal cortex (value)	9.1 (8.6–9.7)	9.5 (9.1–9.7)	8.9 (8.6–9.2)
Repeat length cerebellum (kb)	12.2 (10.5–13.6)	–	–
Repeat length frontal cortex (kb)	25.6 (21.5–39.8)	–	–
Diagnosis			
FTLD	23 (41 %)	9 (29 %)	–
FTLD/MND	16 (29 %)	13 (42 %)	–
MND	14 (25 %)	9 (29 %)	–
Other	3 (5 %)	–	–
Available cerebellum	54 (96 %)	29 (94 %)	19 (95 %)
Available frontal cortex	52 (93 %)	29 (94 %)	20 (100 %)

Data are sample median (IQR) or number (%). Information was obtained for patients with (C9Plus) and without (C9Minus) expansions in *C9ORF72*, as well as for control subjects (Control); information was unavailable regarding age at onset (*n* = 5, C9Plus), age at death (*n* = 1, C9Plus), survival after onset (*n* = 5, C9Plus), and repeat length in frontal cortex (*n* = 2, C9Plus). We used "–" when information was not available for an entire group. This *C9ORF72* expression study was performed in both the cerebellum and frontal cortex. Cerebellum was acquired for 54 expansion carriers (23 FTLD, 15 FTLD/MND, 14 MND, and 2 other diseases), 29 patients without expansions (8 FTLD, 13 FTLD/MND, and 8 MND), and 19 control subjects. We obtained frontal cortex for 52 expansion carriers (22 FTLD, 14 FTLD/MND, 13 MND, and 3 other diseases), 29 patients without expansions (7 FTLD, 13 FTLD/MND, and 9 MND), and 20 control subjects

Table 2

Expression of *C9ORF72* transcripts using TaqMan assays

TaqMan		C9Plus versus C9Minus cohort			C9Plus versus Control cohort		
Group	*p* value[a]	C9Plus	C9Minus	*p* value	C9Plus	Control	*p* value
Cerebellum							
Total	**5.65e-07**	0.67 (0.53–0.82)	0.98 (0.81–1.21)	**9.14e-06**	0.67 (0.53–0.82)	1.00 (0.86–1.18)	**2.61e-05**
Variant 2	**1.86e-10**	0.43 (0.33–0.55)	0.94 (0.71–1.08)	**5.20e-09**	0.43 (0.33–0.55)	1.00 (0.78–1.20)	**1.26e-06**
Variant 3	0.12	0.92 (0.63–1.25)	1.10 (0.93–1.41)	–	0.92 (0.63–1.25)	1.00 (0.76–1.30)	–
Frontal cortex							
Total	**1.97e-07**	0.78 (0.64–1.05)	1.30 (1.13–1.61)	**2.03e-07**	0.78 (0.64–1.05)	1.00 (0.85–1.22)	**0.02**
Variant 2	**8.53e-13**	0.58 (0.48–0.67)	1.31 (1.14–1.66)	**9.63e-12**	0.58 (0.48–0.67)	1.00 (0.73–1.28)	**1.11e-05**
Variant 3	0.02	0.99 (0.74–1.41)	1.39 (0.97–2.01)	–	0.99 (0.74–1.41)	1.00 (0.80–1.54)	–

Data are sample median (IQR) or *p* value and obtained using TaqMan assays; normalized to the geometric mean of synaptophysin (*SYP*) and microtubule-associated protein 2 (*MAP2*). For simplicity, only comparisons with *C9ORF72* expansion carriers are shown, an extended version of this table is displayed in Online Resource Table 4. Information was obtained for patients with (C9Plus) and without (C9Minus) expansions in *C9ORF72*, as well as for control subjects (Control). For each brain region, three tests were performed (i.e., total *C9ORF72* transcripts, variant 2 transcripts, and variant 3 transcripts), and thus, *p* values below 0.017 (bold) were considered significant after Bonferroni correction

[a] A Kruskal–Wallis rank sum test was performed to determine whether expression levels differed between groups (*p* < 0.017 considered significant after Bonferroni correction); when significant differences were detected a Wilcoxon rank sum test was used for pairwise comparisons (*p* < 0.017 considered significant after Bonferroni correction); when no significant differences were detected a Wilcoxon rank sum test was not performed for pairwise comparisons (indicated with "–")

Table 3

Expression of *C9ORF72* transcripts using NanoString technologies

NanoString			C9Plus versus C9Minus cohort			C9Plus versus Control cohort		
Group	p value[a]		C9Plus	C9Minus	p value	C9Plus	Control	p value
Cerebellum								
Total	**1.18e-07**		0.58 (0.42–0.76)	0.80 (0.67–0.93)	**0.003**	0.58 (0.42–0.76)	1.00 (0.88–1.19)	**1.59e-07**
Variant 1	**9.45e-06**		0.47 (0.31–0.63)	0.61 (0.55–0.75)	**0.01**	0.47 (0.31–0.63)	1.00 (0.72–1.49)	**9.04e-06**
Variant 2	**2.19e-12**		0.31 (0.19–0.47)	0.70 (0.55–0.90)	**7.27e-07**	0.31 (0.19–0.47)	1.00 (0.87–1.13)	**5.23e-10**
Intron 1a	0.20		1.08 (0.50–1.86)	0.76 (0.26–1.40)	–	1.08 (0.50–1.86)	1.00 (0.44–1.88)	–
Intron 1b	0.91		1.00 (0.66–1.59)	0.98 (0.55–1.50)	–	1.00 (0.66–1.59)	1.00 (0.66–1.57)	–
Frontal cortex								
Total	**3.44e-11**		0.72 (0.62–0.83)	1.13 (1.04–1.34)	**1.42e-09**	0.72 (0.62–0.83)	1.00 (0.90–1.18)	**2.35e-06**
Variant 1	**3.86e-05**		0.69 (0.49–0.90)	1.10 (0.88–1.38)	**0.0001**	0.69 (0.49–0.90)	1.00 (0.78–1.39)	**0.001**
Variant 2	**1.04e-12**		0.53 (0.34–0.63)	1.21 (0.94–1.43)	**1.84e-08**	0.53 (0.34–0.63)	1.00 (0.90–1.31)	**5.07e-10**
Intron 1a	**0.0002**		2.72 (0.98–4.81)	0.00 (0.00–1.08)	**0.0005**	2.72 (0.98–4.81)	1.00 (0.00–2.34)	**0.003**
Intron 1b	0.11		1.57 (0.96–2.36)	1.24 (0.44–2.10)	–	1.57 (0.96–2.36)	1.00 (0.37–1.74)	–

Data are sample median (IQR) or *p* value and obtained using NanoString technologies; normalized to the geometric mean of synaptophysin (*SYP*) and microtubule-associated protein 2 (*MAP2*). For simplicity, only comparisons with *C9ORF72* expansion carriers are shown, an extended version of this table is displayed in Online Resource Table 5. Information was obtained for patients with (C9Plus) and without (C9Minus) expansions in *C9ORF72*, as well as for control subjects (Control). For each brain region, five tests were performed (i.e., total *C9ORF72* transcripts, variant 1 transcripts, variant 2 transcripts, intron 1a containing transcripts, and intron 1b containing transcripts), and thus, *p* values below 0.010 (bold) were considered significant after Bonferroni correction

[a] A Kruskal–Wallis rank sum test was performed to determine whether expression levels differed between groups (*p* < 0.010 considered significant after Bonferroni correction); when significant differences were detected a Wilcoxon rank sum test was used for pairwise comparisons (*p* < 0.017 considered significant after Bonferroni correction); when no significant differences were detected a Wilcoxon a rank sum test was not performed for pairwise comparisons (indicated with "–")

Table 4

Associations of *C9ORF72* transcripts with disease subgroups using NanoString technologies

NanoString			FTLD versus FTLD/MND cohort			FTLD versus MND cohort			FTLD/MND versus MND cohort		
Group	p value[a]		FTLD	FTLD/MND	p value	FTLD	MND	p value	FTLD/MND	MND	p value
Cerebellum											
Intron 1a	0.45		1.29 (0.66–2.09)	0.93 (0.53–1.35)	–	1.29 (0.66–2.09)	0.96 (0.25–1.53)	–	0.93 (0.53–1.35)	0.96 (0.25–1.53)	–
Intron 1b	0.33		1.06 (0.82–1.63)	0.87 (0.68–1.33)	–	1.06 (0.82–1.63)	0.82 (0.32–1.42)	–	0.87 (0.68–1.33)	0.82 (0.32–1.42)	–
Frontal cortex											
Intron 1a	**0.002**		3.96 (2.10–6.25)	3.73 (1.58–4.41)	0.49	3.96 (2.10–6.25)	0.52 (0.00–1.86)	**0.001**	3.73 (1.58–4.41)	0.52 (0.00–1.86)	**0.006**
Intron 1b	0.61		1.83 (0.97–2.51)	1.53 (1.04–2.24)	–	1.83 (0.97–2.51)	1.27 (0.55–2.30)	–	1.53 (1.04–2.24)	1.27 (0.55–2.30)	–

Data are sample median (IQR) or *p* value and obtained using NanoString technologies; normalized to the geometric mean of synaptophysin (*SYP*) and microtubule-associated protein 2 (*MAP2*). In total, within our cohort of *C9ORF72* expansion carriers, we examined eight different associations [i.e., disease subgroup, gender, age at onset, age at death, repeat length, and survival after onset (using three different cutoff points)] for each outcome, and thus, *p* values below 0.0063 (bold) were considered significant after Bonferroni correction; in this table, only one of those eight associations is displayed (disease subgroup)

[a] A Kruskal–Wallis rank sum test was performed to determine whether expression levels differed between groups (*p* < 0.0063 considered significant after Bonferroni correction); when significant differences were detected a Wilcoxon rank sum test was used for pairwise comparisons (*p* < 0.017 considered significant after Bonferroni correction); when no significant differences were detected a Wilcoxon a rank sum test was not performed for pairwise comparisons (indicated with "–")

Table 5

Associations of *C9ORF72* transcripts with survival after onset using NanoString technologies

NanoString			Overall cohort		FTLD cohort		FTLD/MND cohort		MND cohort	
Group	Survival after onset		Hazard ratio (95 % CI)	p value	Hazard ratio (95 % CI)	p value	Hazard ratio (95 % CI)	p value	Hazard ratio (95 % CI)	p value
Cerebellum										
Variant 1	>Median		0.49 (0.26 to 0.93)	0.03	0.43 (0.16 to 1.14)	0.09	0.93 (0.29 to 2.97)	0.90	0.20 (0.04 to 1.01)	0.05
	>25th Percentile		0.31 (0.14 to 0.67)	**0.003**	0.04 (0.004 to 0.40)	0.006	0.77 (0.18 to 3.28)	0.72	0.23 (0.06 to 0.88)	0.03
	>75th Percentile		0.65 (0.31 to 1.40)	0.28	1.14 (0.43 to 3.04)	0.79	2.09 (0.49 to 8.86)	0.32	–	–
Frontal cortex										
Variant 1	>Median		0.82 (0.44 to 1.51)	0.52	1.39 (0.52 to 3.71)	0.52	0.50 (0.16 to 1.64)	0.26	0.45 (0.12 to 1.76)	0.25
	>25th Percentile		0.23 (0.11 to 0.49)	**0.0001**	0.37 (0.12 to 1.14)	0.08	0.04 (0.004 to 0.42)	0.007	0.17 (0.04 to 0.79)	0.02
	>75th Percentile		0.90 (0.45 to 1.81)	0.77	1.07 (0.38 to 3.03)	0.89	0.97 (0.18 to 5.13)	0.97	0.27 (0.03 to 2.15)	0.21

Data are hazard ratio (HR) with 95 % confidence interval (CI) or *p* value and obtained using NanoString technologies; normalized to the geometric mean of synaptophysin (*SYP*) and microtubule-associated protein 2 (*MAP2*). In total, within our cohort of *C9ORF72* expansion carriers, we examined eight different associations [i.e., disease subgroup, gender, age at onset, age at death, repeat length, and survival after onset (using three different cutoff points)] for each outcome, and thus, *p* values below 0.0063 (bold) were considered significant after Bonferroni correction; in this table, only three of those eight associations are displayed (survival after onset with three different cutoff points). A Cox proportional hazards regression model was used, using a dichotomous categorical variable with three different cutoff points (median, 25th percentile, and 75th percentile) in the given subject group. We used "–" to indicate that the number of samples in a given group was too low to allow *p* value calculation

Table 6

Associations of *C9ORF72* transcripts with poly(GP) and poly(GA) levels using NanoString technologies

NanoString		Overall cohort		FTLD cohort		FTLD/MND cohort		MND cohort	
Group	Association	Spearman's *r* (95 % CI)	*p* value	Spearman's *r* (95 % CI)	*p* value	Spearman's *r* (95 % CI)	*p* value	Spearman's *r* (95 % CI)	*p* value
Cerebellum									
Intron 1a	Poly(GP)	0.33 (0.04 to 0.59)	**0.02**	0.24 (−0.21 to 0.59)	0.29	0.52 (−0.11 to 0.83)	0.07	0.25 (−0.35 to 0.64)	0.38
	Poly(GA)	0.34 (0.03 to 0.60)	**0.02**	0.57 (0.11 to 0.87)	**0.006**	0.36 (−0.27 to 0.79)	0.23	0.03 (−0.62 to 0.67)	0.92
Intron 1b	Poly(GP)	0.49 (0.23 to 0.70)	**0.0004**	0.48 (0.05 to 0.78)	0.03	0.80 (0.35 to 0.96)	**0.001**	0.35 (−0.23 to 0.73)	0.22
	Poly(GA)	0.38 (0.08 to 0.62)	**0.007**	0.61 (0.21 to 0.86)	**0.003**	0.49 (−0.18 to 0.90)	0.08	0.01 (−0.61 to 0.58)	0.96
Frontal cortex									
Intron 1a	Poly(GP)	0.19 (−0.12 to 0.47)	0.20	0.19 (−0.29 to 0.59)	0.40	0.59 (0.08 to 0.89)	**0.03**	0.49 (−0.10 to 0.84)	0.09
Intron 1b	Poly(GP)	0.11 (−0.19 to 0.40)	0.45	−0.21 (−0.59 to 0.22)	0.36	0.47 (−0.16 to 0.85)	0.09	0.20 (−0.48 to 0.74)	0.51

Data are Spearman's correlation coefficient *r* [95 % confidence interval (CI)] or *p* value and obtained using NanoString technologies; normalized to the geometric mean of synaptophysin (*SYP*) and microtubule-associated protein 2 (*MAP2*). For each transcript, two tests were performed in the cerebellum [i.e., poly(GP) and poly(GA)] and one test in the frontal cortex [i.e., poly(GP)], and thus, *p* values below 0.025 and 0.050 (bold) were considered significant after Bonferroni correction, respectively. A Spearman's test of correlation was used (*p* < 0.025 and *p* < 0.050 considered significant after Bonferroni correction for the cerebellum and frontal cortex, respectively)