Title
A C6orf10/LOC101929163 locus is associated with age of onset in C9orf72 carriers

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A C6orf10/LOC101929163 locus is associated with age of onset in C9orf72 carriers


*Appendix 1.

The G4C2-repeat expansion in C9orf72 is the most common known cause of amyotrophic lateral sclerosis and frontotemporal dementia. The high phenotypic heterogeneity of C9orf72 patients includes a wide range in age of onset, modifiers of which are largely unknown. Age of onset could be influenced by environmental and genetic factors both of which may trigger DNA methylation changes at CpG sites. We tested the hypothesis that age of onset in C9orf72 patients is associated with some common single nucleotide polymorphisms causing a gain or loss of CpG sites and thus resulting in DNA methylation alterations. Combined analyses of epigenetic and genetic data have the advantage of detecting functional variants with reduced likelihood of false negative results due to excessive correction for multiple testing in genome-wide association studies. First, we estimated the association between age of onset in C9orf72 patients (n=46) and the DNA methylation levels at all 7603 CpG sites available on the 450k BeadChip that are mapped to common single nucleotide polymorphisms. This was followed by a genetic association study of the discovery (n=144) and replication (n=187) C9orf72 cohorts. We found that age of onset was reproducibly associated with polymorphisms within a 124.7 kb linkage disequilibrium block tagged by top-significant variation, rs9357140, and containing two overlapping genes (LOC101929163 and C6orf10). A meta-analysis of all 331 C9orf72 carriers revealed that...
every A-allele of rs9357140 reduced hazard by 30% ($P = 0.0002$); and the median age of onset in AA-carriers was 6 years later than GG-carriers. In addition, we investigated a cohort of C9orf72 negative patients ($n = 2634$) affected by frontotemporal dementia and/or amyotrophic lateral sclerosis; and also found that the AA-genotype of rs9357140 was associated with a later age of onset (adjusted $P = 0.007$ for recessive model). Phenotype analyses detected significant association only in the largest subgroup of patients with frontotemporal dementia ($n = 2142$, adjusted $P = 0.01$ for recessive model). Gene expression studies of frontal cortex tissues from 25 autopsy cases affected by amyotrophic lateral sclerosis revealed that the G-allele of rs9357140 is associated with increased brain expression of LOC101929163 (a non-coding RNA) and HLA-DRB1 (involved in initiating immune responses), while the A-allele is associated with their reduced expression. Our findings suggest that carriers of the rs9357140 GG-genotype (linked to an earlier age of onset) might be more prone to be in a pro-inflammatory state (e.g. by microglia) than AA-carriers. Further, investigating the functional links within the C6orf10/LOC101929163/HLA-DRB1 pathway will be critical to better define age-dependent pathogenesis of frontotemporal dementia and amyotrophic lateral sclerosis.

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A novel age of onset modifier in C9orf72 carriers

The G\textsubscript{4}C\textsubscript{2}-repeat expansion in C9orf72 is the most common known cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Gijselinck et al., 2012) in Caucasians. It accounts for about 37% familial and 7% sporadic ALS patients; as well as 25% familial and 6% sporadic FTD patients (Rademakers, 2012) with age and sex dependent disease penetrance (Murphy et al., 2017). High phenotypic heterogeneity of C9orf72 patients also includes a wide range in disease age of onset (27–74 years) and duration (0.5–22 years) (Gijselinck et al., 2016). Yet, genetic modifiers of age of onset in C9orf72 patients are largely unknown [only the T-allele of rs1990622 in TMEM106B was associated with a later age of onset of FTD, but not ALS (Gallagher et al., 2014; van Blitterswijk et al., 2014)]. Detection of the age of onset modifier(s) might increase the accuracy of predicting age of onset in asymptomatic mutation carriers, which is important for clinical trials focused on early intervention.

Age of onset could be influenced by genetic and environmental modifiers, both of which may trigger epigenetic changes, such as DNA methylation at CpG sites (Zhang et al., 2016). Indeed, there is no a strict dichotomy between action of genetic and epigenetic factors; they often work in concert. Genome-wide DNA methylation profiles of identical twins are much more similar than between fraternal siblings (Zhang et al., 2016), demonstrating that many epigenetic changes are genetically controlled (e.g. the repeat expansion causes hypermethylation of the C9orf72 locus leading to downregulation of C9orf72 expression) (Xi et al., 2015b; Gijselinck et al., 2016). The DNA methylation levels of some CpGs are age-related allowing the estimation of DNA methylation age based on the cumulative assessment of 353 CpGs included on the genome-wide 450K BeadChip. Currently, DNA methylation age is the most accurate predictor of chronological age across multiple tissues (Horvath, 2013), but may in fact reflect biological age better than chronological age. Indeed, we recently reported that increased DNA methylation age acceleration (DNA methylation age minus chronological age) is
associated with earlier age of onset in C9orf72 patients analysed on the 450K BeadChip after exclusion of CpGs mapped to common single nucleotide polymorphisms (SNPs) (Zhang et al., 2017).

CpGs are the most mutable sites in the human genome because methyl-C can spontaneously deaminate to T (e.g. 35% of all coding mutations occur at CpG sites) (Lek et al., 2016). Hence, in the current study we tested the hypothesis that age of onset in C9orf72 patients is associated with some common SNPs causing a gain or loss of CpG sites (CpG-SNPs) and thus resulting in DNA methylation changes. Allele-specific DNA methylation is largely attributed to CpG-SNPs (Shoemaker et al., 2010), which have often been detected within promoter regions, transcription factor binding sites and DNase I hypersensitive sites (Gagliano et al., 2016), thus regulating the level of gene expression. CpG-SNPs belong to a group of methylation quantitative trait loci (Hannon et al., 2016), which are linked to some mental disorders (Gagliano et al., 2016).

We combined epigenetic and genetic approaches to map functional variants (CpG-SNPs) associated with age of onset in C9orf72 carriers. Such a study design reduces the likelihood of false negative results due to excessive correction for multiple testing in genome-wide association studies (GWASs). Our study also includes suggestions on how the significant SNPs exert their effects (e.g. by influencing gene expression).

### Materials and methods

#### Participants

Informed consent was obtained from all participants in accordance with the respective ethics review boards. Sample characteristics are presented in Table 1 and Supplementary Table 1 for C9orf72 carriers, and Supplementary Table 2 for C9orf72 negative patients. Briefly, our study included patients diagnosed with bulbar or limb onset ALS, behavioural FTD (bvFTD), semantic dementia, progressive non-fluent aphasia (PNFA), and FTD-ALS. All patients were of Caucasian origin and diagnosed at hospitals specializing in neurodegenerative disorders using established clinical criteria for ALS (Brooks et al., 2000) and FT (Neary et al., 1998), including the revised diagnostic criteria for bvFTD (Rascovsky et al., 2011) and language variants of FTD (Gorno-Tempini et al., 2011). Age of onset was defined as the age at which the first disease symptoms appeared, including initial bulbar or limb symptoms in ALS, and cognitive dysfunction in judgement, language, memory, or changes in behaviour or personality in FTD. Age of onset was either self-reported (for ALS) or obtained from unaffected family members (for FTD).

The discovery cohort was recruited from Canada, Italy, Spain, UK, USA or Argentina and consisted of 144 C9orf72 carriers, including 21 symptomatic and 22 asymptomatic carriers from 16 pedigrees. The independent replication cohort was obtained from centres (different from those that collected the discovery cohort) participating in the International FTD-Genomics Consortium (IFGC; https://ifgcsite.wordpress.com/) (Ferrari et al., 2014). It consisted of 187 unrelated FTD or FTD-ALS C9orf72 carriers from the USA, Canada, UK, France, Belgium, Italy, Germany, Spain, Sweden, the Netherlands and Australia. Information about family relatedness was obtained from the clinical notes of the neurologists who collected the samples. In addition, the presence of relatedness in the replication cohort was previously assessed as part of a GWAS that identified and excluded all first-degree relatives (through identity by descent for any pair with an estimate <0.125) (Ferrari et al., 2014).

For a follow-up study of unrelated C9orf72 negative patients, we investigated 2142 FTD and 164 FTD-ALS patients from the IFGC (Ferrari et al., 2014), as well as 328 sporadic ALS patients from the ALS clinic at Sunnybrook Health Sciences Centre, Toronto (Supplementary Table 2), which also provided frontal cortex from 25 unrelated ALS autopsy cases without an expansion in C9orf72 (<=30 repeats) for the gene expression studies (Supplementary Table 3).

#### Procedures

Blood genomic DNA was extracted using a QIAGEN kit. First, we analysed the genome-wide DNA methylation data from the 450K BeadChip (Illumina) that was previously generated using bisulfite converted DNA of 46 Canadian C9orf72 carriers (Zhang et al., 2017) to discover common CpG-SNPs with minor allele frequencies >5% that are associated with age of onset. The raw data were preprocessed and analysed using the minfi package in R-project (Aryee et al., 2014). The β-value was used to estimate the DNA methylation level of each CpG-site (β-value of 0: non-methylated; β-value of 1: completely methylated).

All participants of the discovery and replication cohorts (n = 331) were carriers of an expansion in C9orf72 (>30 repeats) based on previous analysis by repeat-primed PCR (Ferrari et al., 2014; Xi et al., 2015b). Genotypes for rs9357140, rs2143466 and rs1990622 were obtained by Sanger sequencing in the discovery cohort (Supplementary Table 4). For the

### Table 1 Sample characteristics of the discovery and replication C9orf72 datasets

<table>
<thead>
<tr>
<th></th>
<th>Discovery cohort</th>
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<th>Replication cohort</th>
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<tbody>
<tr>
<td></td>
<td>Unrelated carriers</td>
<td>Symptomatic carriers from 16 families</td>
<td>Asymptomatic carriers from 16 families</td>
<td>Unrelated carriers</td>
</tr>
<tr>
<td>Number of cases</td>
<td>101 (54.4)</td>
<td>21 (47.6)</td>
<td>22 (45.5)</td>
<td>187 (55.6)</td>
</tr>
<tr>
<td>Sex, male, n (%)</td>
<td>55 (54.4)</td>
<td>10 (47.6)</td>
<td>12 (51–63)</td>
<td>104 (55.6)</td>
</tr>
<tr>
<td>Age of onset, years, mean (range)</td>
<td>59.82 (37–78)</td>
<td>55 (48–60)</td>
<td>NA</td>
<td>57.2 (34–80)</td>
</tr>
</tbody>
</table>

NA = not applicable.
replication dataset, these SNPs together with eight SNPs in strong linkage disequilibrium (LD) with rs9357140 and rs2143466 (R² > 0.9) were either genotyped or imputed using the latest data from the Haplotype Reference Consortium (McCarthy et al., 2016) (Supplementary Table 5).

Genotypes for rs9357140 in a follow-up cohort of 2634 unrelated C9orf72 negative patients with ALS, FTD or FTD-ALS, were obtained by TaqMan™ assay (C_9782529_10, ThermoFisher Scientific) for 328 ALS patients, or imputed from IPGC-GWAS for 2306 FTD and FTD-ALS patients (Ferrari et al., 2014) using the latest data from the Haplotype Reference Consortium (McCarthy et al., 2016).

To measure the degree of LD, we extracted R² values (range from 0 to 1 with higher values indicating a higher LD) from the LDlink tool (https://analysistools.nci.nih.gov/LDlink) using the 1000 Genomes European population data. We searched for known variants within the boundaries of the LD block (R² > 0.8) tagged by the top significant SNP (rs9357140) using the ‘proxy search’ in LDlink. Functional predictions for the missense SNPs were based on the PolyPhen-2 and SIFT data available from the Exome Aggregation Consortium database (Lek et al., 2016). Using the UCSC genome browser, the LD-block was also analysed for transcriptional factor binding sites and DNase I hypersensitivity.

To detect genes whose expression is associated with rs9357140, we searched for expression quantitative trait loci (eQTL) using Genotype-Tissue Expression (GTEx v7) data from 48 types of human tissues (GTEx Consortium et al., 2017). The GTEx portal (https://www.gtexportal.org/) was used to analyse the association between rs9357140 genotypes and gene expression by a linear regression method. Normalized effect size (NES) was defined as the slope of the linear regression.

To quantify gene expression, total RNA was extracted from human frontal cortex of ALS cases without C9orf72 expansions using the QIAzol plus RNeasy® Mini Kit (QIAGEN) and reverse transcribed to cDNA using oligo dT primers and the AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies). Quantitative RT-PCR was conducted for 25 samples (Supplementary Table 3) with an RNA integrity number > 6.5 (based on an Agilent 2100 Bioanalyzer). To select endogenous control genes for the frontal cortex, we assessed four housekeeping genes including HPRT1 (MIM: 308000; Hs00999909_m1), UBC (MIM: 191340; Hs00824723_m1), B2M (MIM: 107900; Hs00999907_m1), and RPLP0 (MIM: 180510; Hs00420895_gH) (ThermoFisher Scientific) in nine samples (n = 3 per each rs9357140 genotype). We used Normfinder (Andersen et al., 2004) to identify the least variable housekeeping genes (B2M and RPLP0) in our samples (Supplementary Table 6). We measured expression of HLA-DRB1 transcript variant 1 (MIM:142857; Hs0414264_mH) and all C9orf72 transcripts (MIM:614260; Hs00376619_m1) (ThermoFisher Scientific) in triplicate for 25 samples with different rs9357140 genotypes: AA (n = 9), AG (n = 8) and GG (n = 8). Relative quantification was calculated with the ddCt method by geometric mean of housekeeping gene expression (B2M and RPLP0).

Statistical analyses
We used the linear regression model of the R minfi package to assess the genome-wide association between the DNA methylation status of CpG-SNPs and age of onset in C9orf72 patients, as well as to evaluate the false discovery rate to generate adjusted q-values (Zhang et al., 2017). We used a Manhattan plot to prioritize significant variants (P < 0.01 and q < 0.05) for further genetic study, and a Q-Q plot to highlight potential confounders using the R qman package (Turner, 2018).

To assess if genotypes affect age of onset, we used a Cox proportional hazard regression model (R survival and survminer packages) (Grambsch, 2000) adjusting for sex, rs1990622 genotypes, disease phenotypes, and censoring age of last follow-up for the 22 currently asymptomatic C9orf72 carriers. To adjust for relatedness in the Cox proportional hazard regression analysis of the discovery cohort, we created an indicator number for each family; then used the coxph function of the R coxme package with a frailty approach (Ripatti and Palmgren, 2000). The hazard ratio (HR) with 95% confidence interval (CI) is presented. To analyse the association between genotypes and age of onset in the C9orf72 disease subgroups, we used multivariate linear regression with an additive, dominant or recessive model adjusting for sex, rs1990622 genotypes, or DNA methylation age-acceleration. We also used multivariate linear regression to analyse the association between genotypes and age of onset in C9orf72 negative disease subgroups (adjusting for sex). We present the linear regression coefficient (B) with standard error (SE) and percentage of response variance explained by the linear regression model (r²). Results of additive model were presented, unless otherwise specified.

We used a meta-analysis (R metafor package) with a fixed-effect model to assess the pooled effect size of the Cox regression coefficient (logHR) from the discovery and replication stages (Trinh et al., 2016). We performed a trend analysis using the Cochran–Armitage test to analyse if rs9357140 genotypes are associated with C9orf72 disease subgroups. A non-parametric Mann–Whitney U-test or Kruskal–Wallis test was used to assess differences in age of onset or gene expression among two or more groups where appropriate. Sex and rs1990622 genotype adjusted P-values are shown, unless otherwise specified. The results with P < 0.05 were accepted as statistically significant.

Data availability
The data that support the findings of this study are available on request from the corresponding authors (E.R., M.Z.). The data are not publicly available because of information that could compromise the privacy of the research participants.

Results
Epigenetic analysis suggested CpG-SNPs associated with age of onset
The study design is presented in Fig. 1. First, we estimated the association between age of onset in a Canadian cohort of 46 unrelated C9orf72 patients and DNA methylation levels at 7603 common CpG-SNPs available on the 450K BeadChip. Age of onset was significantly associated with DNA methylation levels (q < 0.05) at three CpG-SNPs (rs12763379 on 10q24.2; rs9357140 and rs2143466 on 6p21.3):
$P = 9.6 \times 10^{-6}$, $P = 6.0 \times 10^{-6}$ and $P = 1.8 \times 10^{-5}$, respectively (Fig. 2A and Supplementary Table 7). However, rs12763379 in PYROXD2 was removed from follow-up study because of its overlap with insertion/deletion variations and single tandem repeats precluding reliable genotyping.

Genetic association study confirmed the association between rs9357140/rs2143466 and age of onset

Multivariate linear regression suggested that rs9357140 genotypes control the gain or loss of DNA methylation at CpG-site cg18698799 ($P < 1.0 \times 10^{-4}$), thereby underlying the association with age of onset: adjusted $P = 2.2 \times 10^{-5}$, $B = 7.01$ (SE: 1.47) (Fig. 2B). The association remained significant after adjusting for DNA methylation age-acceleration: $P = 2.7 \times 10^{-4}$, $B = 6.72$ (SE: 1.68). AA-carriers have significantly lower DNA methylation levels compared to AG-carriers ($P = 2.2 \times 10^{-5}$, Mann-Whitney U-test) or GG-carriers ($P = 4.7 \times 10^{-5}$, Mann-Whitney U-test); mean $\beta$-value: 0.04 (AA-carriers) versus 0.54 (AG-carriers) versus 0.88 (GG-carriers) (Fig. 2B). Similar results were observed for rs2143466 (Supplementary Fig. 1). The Q-Q plot suggested that there are no other confounders for the association (Supplementary Fig. 2). Both SNPs belong to a strong 124.7 kb LD-block ($R^2 > 0.8$) on chr6:32213638–32338386 containing two overlapping genes: a long non-coding RNA (LOC101929163) and C6orf10—an uncharacterized testes-specific gene with rs9357140 mapped to intron 9 and rs2143466 mapped to intron 14 (Fig. 2).

Next, we enlarged our discovery dataset to 144 carriers by genotyping rs9357140 and rs2143466 in 98 recently collected C9orf72 carriers, including 101 unrelated symptomatic carriers and 16 families with 21 symptomatic and 22 asymptomatic C9orf72 carriers (Fig. 1 and Table 1). To obtain the median age of onset for different SNP genotypes, we used the Kaplan-Meier estimate, censoring age of last follow-up for asymptomatic carriers. The median age of onset difference between rs9357140 AA- and GG-carriers was 12 years: 67 years for AA (95% CI: 60–71), 59 years for AG (95% CI: 56–64) and 55 years for GG genotype (95% CI: 54–60) (Fig. 3A). Cox proportional hazard regression analysis also revealed that age of onset in C9orf72 carriers is significantly associated with rs9357140 genotypes: adjusted $P = 1.1 \times 10^{-4}$, $HR = 0.43$ (95% CI: 0.28–0.66), suggesting that every A-allele could reduce hazard by 57% (Fig. 3B and Table 2). A similar association with age of onset was also observed for rs2143466: adjusted $P = 1.1 \times 10^{-4}$, $HR = 0.43$ (95% CI: 0.28–0.68) (Supplementary Fig. 3).

The replication study validated the association between rs9357140/rs2143466 and age of onset

In the replication stage (Fig. 1 and Table 1), we obtained genotypes from the IFGC-GWAS (Ferrari et al., 2014) for 10 SNPs tagged by rs9357140 ($R^2 > 0.9$) (Supplementary Table 5) for 187 C9orf72 patients with a median age of onset of 58 years and interquartile range (IQR) of 51–80 years. Cox proportional hazard regression analysis showed that age of onset was significantly associated with
Figure 2 Genome-wide DNA methylation analysis of the CpG-SNPs in C9orf72 patients. (A) Manhattan plot presenting the association between DNA methylation status of CpG-SNPs and age of onset, including a locus on chr6:32160000–32580000 with two age of onset-associated CpG-SNPs (rs9357140 and rs2143466 indicated by the box). Arrows indicate the transcriptional direction of each gene (5' to 3'). ‘Me’ in red represent methylation sites controlled by rs9357140 and rs2143466. The LD block tagged by rs9357140 (R² > 0.8) is highlighted in green. (B) Genotypes of rs9357140 are significantly associated with DNA methylation status: P < 1.0 × 10⁻⁶, B = −0.39 (SE: 0.01); and age of onset: P = 2.2 × 10⁻⁵ adjusted for sex and rs1990622 genotypes, B = 7.01 (SE: 1.47). The dashed line represents the linear regression trend.
rs9357140: adjusted $P = 0.03$, HR = 0.79 (95% CI: 0.64–0.98), suggesting that every A-allele reduced hazard by 21% (Table 2 and Fig. 3). As expected, similar associations with age of onset were observed for rs2143466: adjusted $P = 0.025$, HR = 0.79 (95% CI: 0.64–0.97) (Supplementary Fig. 3) and for the other eight SNPs within the LD block listed in Supplementary Table 5 (data not shown).

### Table 2 The Cox proportional hazard regression results for the association between the rs9357140 genotypes and age of onset in the discovery and replication cohorts

<table>
<thead>
<tr>
<th></th>
<th>Discovery (n = 144)</th>
<th>Replication (n = 187)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (95% CI)</td>
<td>0.59 (0.45–0.77)</td>
<td>0.79 (0.64–0.98)</td>
</tr>
<tr>
<td>$P$-value for AA versus AG versus GG</td>
<td>0.0011</td>
<td>0.029</td>
</tr>
<tr>
<td>Adjusted HR (95% CI)*</td>
<td>0.43 (0.28–0.68)</td>
<td>0.79 (0.64–0.98)</td>
</tr>
<tr>
<td>Adjusted $P$-value for AA versus AG versus GG*</td>
<td>0.00011</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*The hazard ratio (HR) and $P$-value was adjusted for sex, rs1990622 genotypes, disease phenotypes and family relationship in the discovery stage. HR was adjusted for sex, rs1990622 genotypes and disease phenotypes in the replication stage.

Meta-analysis revealed overall effect of rs9357140/rs2143466 on age of onset

We conducted a meta-analysis of logHR in all 331 C9orf72 carriers using a fixed-effects model and observed that every
A novel age of onset modifier in C9orf72 carriers

We also observed that age of onset differed significantly among the FTD subtypes ($P < 0.01$, Kruskal-Wallis test). Cox proportional hazard regression analysis revealed that the A-allele of rs9357140 reduced hazard by 30% (pooled HR = 0.70, $P = 0.0003$) (Fig. 3B). Again, a similar effect was observed for rs2143466 (pooled HR = 0.70, $P = 0.0002$) (Supplementary Fig. 3). The association between age of onset and rs9357140 was also significant in 304 unrelated C9orf72 patients: adjusted $P = 2.3 \times 10^{-5}$, $B = 3.2$ (SE: 0.67) (Supplementary Table 1). The median age of onset of rs9357140 AA-carriers was 6 years later than GG-carriers: 62 years (IQR: 57–68) versus 56 years (IQR: 50–62).

Subgroup analyses of the association between rs9357140 and age of onset

The association between age of onset and rs9357140 was evident in unrelated C9orf72 patients with either pure ALS ($n = 59$; adjusted $P = 0.002$, $B = 4.97$, SE: 1.53) or pure FTD ($n = 174$; adjusted $P = 0.0008$, $B = 2.82$, SE: 0.83), but not in patients with FTD-ALS ($n = 71$; adjusted $P = 0.125$, $B = 2.63$, SE: 1.69) (Supplementary Table 1 and Supplementary Fig. 4A–C). A similar result was observed for rs2143466 (Supplementary Fig. 4D–F).

Notably, we found no significant difference in age of onset among patients affected by pure ALS, pure FTD or FTD-ALS; or ALS/FTD subtypes (bulbar ALS, limb ALS, unspecified ALS, bvFTD, semantic dementia, PNFA, unspecified FTD): $P > 0.05$, Kruskal-Wallis test (Supplementary Fig. 5). Multivariate linear regression analysis in different disease subtypes revealed that age of onset was associated with rs9357140 genotypes in limb ALS ($n = 35$) and bulbar ALS ($n = 23$) under a dominant model (adjusted $P < 0.05$), and bvFTD ($n = 157$) under a recessive model (adjusted $P < 0.05$), but not in FTD-ALS patients ($n = 71$) (Supplementary Table 1).

To evaluate if rs9357140 genotypes modify disease phenotypes, we performed a trend analysis using the Cochran–Armitage test to analyse the association between rs9357140 genotypes and C9orf72 disease phenotypes (ALS versus FTD, ALS versus FTD-ALS, or FTD versus FTD-ALS) under an additive model (AA versus AG versus GG) (Supplementary Table 8); and found no statistically significant results ($P > 0.05$).

Age of onset in C9orf72 negative patients is associated with rs9357140

We analysed 2634 C9orf72-negative patients with ALS, FTD or FTD-ALS (Supplementary Table 2), and found a significant association between rs9357140 genotypes and age of onset (adjusted $P = 0.007$ for recessive model) (Supplementary Table 2). Subgroup analysis detected a significant association only in the largest subgroup of FTD patients ($n = 2142$, adjusted $P = 0.01$ for recessive model) with a small effect size ($B = 1.44$, SE: 0.55) (Supplementary Table 2). The association is evident in the bvFTD patients ($n = 1364$, adjusted $P = 0.035$ for recessive model), but not the other smaller FTD subtypes (Supplementary Table 2).

The expression of HLA-DRB1 and LOC101929163 is associated with rs9357140

Since rs9357140 and rs2143466 control the loss or gain of CpG-sites and therefore DNA methylation levels (Fig. 2), we hypothesized that CpG-SNPs at the C6orf10/LOC101929163 locus may modulate age of onset by regulating gene expression. We used the public eQTL dataset of 48 types of human tissue (GTEx portal) to analyse if genotypes of the top-significant tagging SNP (rs9357140) are associated with the expression of nearby genes (10 top-significant hits are shown in Supplementary Table 9). Among brain tissues, the A-allele of rs9357140 was associated with reduced expression of the LOC101929163 locus compared to AG-carriers ($P = 7.6 \times 10^{-6}$, NES = –0.66 in the nucleus accumbens, part of the basal ganglia; Supplementary Fig. 6A); and HLA-DRB1, encoding major histocompatibility complex, class II, DR beta 1 ($P = 4.1 \times 10^{-6}$, NES = –0.42 in the frontal cortex; Supplementary Fig. 6B).

To validate the link between rs9357140 genotypes and HLA-DRB1 expression, we conducted quantitative RT-PCR using frontal cortex from 23 unrelated ALS cases (Supplementary Fig. 6C). Mann-Whitney U-test confirmed that AA-carriers had significantly lower HLA-DRB1 expression compared to AG-carriers ($P = 0.001$) or GG-carriers ($P = 0.000003$). Of note, C9orf72 expression did not differ among the rs9357140 genotypes ($P > 0.05$, Mann-Whitney U-test, Supplementary Fig. 7) and was not correlated with HLA-DRB1 expression (adjusted $P = 0.23$, linear regression).

Bioinformatics analysis predicted multiple DNase I hypersensitivity sites within the LD-block associated with age of onset

The LD-block associated with age of onset contains 196 known variants tagged by rs9357140 ($R^2 > 0.8$), including five missense substitutions with minor allele frequencies of 0.36–0.38 and conflicting functional predictions by
rs9357140 were also moderately associated with age of onset in C9orf72-negative patients although the effect size was small (e.g., the median age of onset in AA-carriers affected by FTD was 1 year later than GG-carriers).

Recently, a key tool for connecting phenotypes to genetic variations has emerged from gene expression studies. Since the locus with significant SNPs may not be the actual disease-related target, cis-acting eQTLs can provide a mechanistic link between SNPs and the biological processes they affect (GTEx Consortium et al., 2017). In our study, the minor A-allele of rs9357140 (top-significant SNP within the C6orf10/LOC101929163 locus) is associated with reduced brain expression of LOC101929163 (in nucleus accumbens) and HLA-DRB1 (in frontal cortex), while the major G-allele is associated with their increased expression (Supplementary Fig. 6). Future functional studies have to investigate if the non-coding RNA LOC101929163 is a modulator of HLA-DRB1 expression (e.g., affecting transcriptional factors relevant to HLA-DRB1). The major histocompatibility complex class II protein HLA-DR is implicated in neurodegenerative diseases as a marker of activated microglia (Walker and Lue, 2015) and is important in initiating immune responses by presenting peptides derived not only from exogenous but also endogenous proteins, such as peptides resulting from autophagy of intracellular proteins by lysosomes (Dengjel et al., 2005).

Our results support the notion that microglial/autophagy pathways play key roles in modulating C9orf72 disease, the pathogenesis of which might involve both gain and loss of function mechanisms (Hardy and Rogaeva, 2014). Normal function of C9orf72 is essential for the lysosome/autophagosome pathway and immune responses in macrophages or microglia (O’Rourke et al., 2016; Shi et al., 2018). For instance, transcriptome and histologic analyses of C9orf72 carriers support the idea that decreased C9orf72 expression leads to altered microglial function and neuroinflammation (O’Rourke et al., 2016), while increased C9orf72 levels could be neuroprotective (McGoldrick et al., 2018; Shi et al., 2018). It is important to investigate if rs9357140 GG-carriers, which have an earlier age of onset and upregulated HLA-DRB1, are in a more pro-inflammatory state (e.g. by microglia) than AA-carriers.

Our survey of the literature and the GWAS catalogue database (https://www.ebi.ac.uk/gwas/) revealed that SNPs within or close to the C6orf10/LOC101929163 locus (Supplementary Fig. 8) are associated with autoimmune disorders (multiple sclerosis, rheumatoid arthritis, systemic sclerosis, Grave’s disease and asthma), as well as neurodegenerative diseases (FTD, Parkinson’s disease and Alzheimer’s disease) (Lambert et al., 2013; Ferrari et al., 2014; Lu et al., 2017), highlighting the role of the immune system in neurodegeneration (Supplementary Table 11). Notably, several dementia genes are linked to microglia/immune function (e.g. TREM2 and CD33) (Lambert et al., 2013). Our study of C9orf72-negative patients suggests that the C6orf10/LOC101929163 locus could be a modest age of onset modifier for the general
population of FTD patients. Intriguingly, another two SNPs (rs9268877 and rs9268856) near this locus have been reported in a case-control GWAS as modifiers of FTD risk (Ferrari et al., 2014). Of note, rs9357140 is not in LD with rs9268877 and rs9268856 representing an independent association signal (Supplementary Table 11), yet the mechanism behind the association with age of onset or disease risk could be similarly pointing to the functional significance of the HLA-DRA/HLA-DRB5 locus.

Notably, age of onset estimation is more objective for ALS (self-reported) than FTD (reported by family members) (Pottier et al., 2018). Hence, the less significant result in the replication C9orf72 cohort enriched in FTD patients (72.7% versus 27.8% in the discovery stage) may be explained by a less accurate age of onset estimation (Supplementary Table 12). In addition, the subgroup analysis could be further complicated by the less accurate estimation of age of onset for the complex FTD-ALS phenotype and reduced statistical power for the smaller subgroup.

One of the limitations of our study is the lack of unified deep phenotyping for each patient and healthy control data, however our findings set the basis for future research (e.g. aimed at investigating the link between CpG-SNPs and disease phenotype, risk, progression or severity). Another limitation is the absence of information on the expansions size in our study participants, because C9orf72 genotyping was done by repeat-primed PCR. This is of note, since repeat length examined by Southern blot was inversely correlated with age of onset (van Blitterswijk et al., 2013), and the clinical data support disease anticipation in C9orf72 families, which is evident by an earlier age of onset across successive generations (van Blitterswijk et al., 2013; Xi et al., 2015a; Van Mossevelde et al., 2017). However, C9orf72 repeat expansions are difficult to size accurately by Southern blot because of their large size (up to several thousand repeats) and somatic mosaicism masking the true length of the expansion (Xi et al., 2015a; McGoldrick et al., 2018). It would also be important to understand the genetic-epigenetic links across human tissues relevant to neurodegenerative disorders, since DNA methylation changes reflect the complex interactions between genes, environmental factors, and ageing (Zhang et al., 2016).

Our findings suggest that CpG-SNPs at the C6orf10/LOC101929163 locus might modify age of onset in C9orf72 carriers belonging to the entire ALS-FTD spectrum by controlling DNA methylation and gene expression (e.g. HLA-DRB1). CpG-SNPs at the C6orf10/LOC101929163 locus might also be age of onset modifiers for general FTD patients to a lesser extent. Understanding the functional mechanisms of the C6orf10/LOC101929163/HLA-DRB1 pathway (e.g. to investigate if the non-coding RNA LOC101929163 is a modulator of HLA-DRB1 expression) might prove critical for identifying biomarkers and/or designing drugs to modify age of onset in C9orf72 driven disease. Finally, the detected CpG-SNPs could be used to better predict age of onset in C9orf72 asymptomatic carriers in preventive clinical trials (e.g. based on the Genetic Frontotemporal dementia Initiative study) (Rohrer et al., 2015), for designing conditional and/or modifiers studies in the sporadic FTLD spectrum, such as based on IFGC related projects (https://ifgcsite.wordpress.com/) and for genetic counselling.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at Brain online.

Appendix I


References


GTEx Consortium; Laboratory, Data Analysis &Coordinating Center (LDACC)—Analysis Working Group; Statistical Methods groups—Analysis Working Group; Enhancing GTEx (eGTEx) groups; NIH Common Fund, et al. Genetic effects on gene expression across human tissues. Nature 2017; 550: 204–13.


Dengie J, Schoor O, Fischer R, Reich M, Kraus M, Muller M, et al. Autophagy promotes MHC class II presentation of peptides from...


Horvath S. DNA methylation age of human tissues and cell types. Genome Biol 2013; 14: R115.


