Letter to the Editor

Age of onset in Huntington disease is influenced by CAG repeat variations in other polyglutamine disease-associated genes

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Sir,

We read with great interest the recent article by Tezenas du Montcel et al. who showed that the age of onset in several spinocerebellar ataxias (SCAs) is modulated by CAG repeat sizes in the normal range in other polyglutamine disease-associated genes (Tezenas du et al., 2014). Interestingly, the age of onset in patients with SCA3 was also influenced by the CAG repeat size in the HTT gene: Long normal HTT CAG repeat size was associated with a delayed age of onset in SCA3 patients (Tezenas du et al., 2014). Similarly, in a subsequent study in patients with SCA3 from mainland China, it was shown that the difference in CAG repeat size between the two HTT alleles interacted with the ATXN3 expansion and affected age of onset in these patients (Chen et al., 2016). A CAG repeat expansion in the HTT gene is the cause of Huntington disease (HD), the most common polyglutamine disease worldwide. Like other polyglutamine diseases, the age of onset in HD is inversely associated with the CAG repeat expansion size in the mutant allele which accounts for between 47 to 72% of the variance in age of onset in different HD populations (Cazeneuve and Durr, 2014). However, there is a wide distribution of age of onset in individuals carrying a mutation with an identical number of CAG repeats, suggesting the existence of other important (epi)genetic and/or environmental factors (Arning and Epplen, 2012; GeM-HD consortium, 2015). Given that the age of onset in SCA3 patients was recently found to be influenced by the HTT CAG repeat size, we wondered whether the age of onset in HD patients could also be influenced by the CAG repeat size variations in other polyglutamine disease-associated genes (PDAGs), particularly ATXN3. Therefore, we assessed the association between the number of CAG repeats in all known PDAGs and age of onset in a large cohort of HD patients.

We obtained clinical data and DNA samples from a subset (n=1000) of manifest HD patients participating in the European Huntington Disease Network REGISTRY study (http://www.euro-hd.net/html/registry). All these participants had entered the study before July 21, 2015. All participants in whom data on age of onset were available and in whom CAG repeat numbers in both alleles of each PDAG could be determined were included in the analyses. Using 10 ng of genomic DNA, two multiplex PCRs were performed in a TPprofessional thermocycler (Biometra, Westburg) with labelled primers flanking the CAG stretch for ATN1, ATXN1, ATXN7, CACNA1A and HTT in one mix and AR, ATXN2, ATXN3, and TBP in a second mix (Biolegio) (primers and PCR conditions are available upon request). Every PCR included a negative control without genomic DNA, a reference sample of CEPH 1347-02 genomic DNA and two positive control samples with predetermined 40 and 47 HTT CAG repeats (Applied Biosystems). Repeat size determination was performed by running the PCR products on an ABI 3730/3130 automatic DNA sequencer (Applied Biosystems) and analyzing the results with GeneMarker software (version 2.4.0).

To assess whether CAG repeat lengths in PDAGs were associated with age of onset in HD we applied multiple linear regression. Given the known exponential association between age of onset and mutant HTT CAG repeat size, the natural logarithmic transformation of age of onset was used as the dependent variable (Langbehn et al., 2004). We modelled the effect of each PDAG on age of onset separately by including its two alleles (with both linear and quadratic terms in order to account for potential non-linear effects) as well as their interaction as predictor variables while also adjusting for the effects of sex and CAG repeat sizes in both HTT alleles and their interaction (Aziz et al., 2009). For the AR gene only
CAG repeat size in the longer allele was used since men carry only one allele of this X-linked gene. Next, to assess whether the effect of mutant HTT CAG repeat size on age of onset was modified by CAG repeat lengths in other PDAG, the interaction between CAG repeat size in the mutant HTT allele and CAG repeat size in each of the two alleles of the other PDAG was additionally included. To reduce multicollinearity, particularly with respect to the interaction terms, all continuous predictors were centred around their respective means. To account for the effects of heteroscedasticity and influential points all statistical significance tests were based on robust estimators of standard errors. Moreover, to assure that the results were not unduly affected in case of deviations from model assumptions we also applied a non-parametric method by dividing the group based on median values of each PDAG and comparing differences in age of onset by the non-parametric Mann-Whitney U-test. Given the exploratory nature of this study no specific correction for multiple comparisons was applied. All tests were two-sided and significance level was set at p < 0.05. All analyses were performed in SPSS version 23.0 (IBM SPSS Statistics for Windows, IBM Corp).

The mean age of onset was 48.8 with a standard deviation of 12.2 years. The number of assessed samples per gene are summarized in Supplementary Table 1. The distribution of CAG repeat lengths followed a unique pattern for each gene and in some cases had a strong preference for a particular range of repeat lengths (Supplementary Table 1 and Supplementary Fig. 1). As expected age of onset was inversely associated with CAG repeat length in the expanded HTT allele (β = -0.060, p < 0.001) which accounted for 66.1% of the variation in age of onset in this cohort. Longer CAG repeat size in the larger ATXN3 allele was associated with a later age of onset in HD patients (β=0.003, p=0.048). Non-parametric comparison of age of onset between participants with CAG repeat sizes below the median versus those with CAG repeat sizes above the median in the larger ATXN3 allele confirmed this association (median age of onset: 47.6 vs. 50.0 years, p=0.025; Fig. 1). There was not a significant interaction between either of the ATXN3 alleles and the expanded HTT allele (p ≥ 0.20). However, there was a significant interaction between the CAG repeat size in the expanded HTT allele and the larger CACNA1A allele (β=-3.87 × 10⁻³ and p=0.011 for the interaction effect). Further scrutiny of this interaction revealed that for patients with a below median number of CAG repeats in the expanded HTT allele more repeats in the longer CACNA1A allele resulted in a later age of onset (median age of onset: 56.1 vs. 61.1 years, p=0.003), while for patients with an above median expansion the CACNA1A CAG repeat had little influence on the age-of-onset (Fig. 2). There was also a significant interaction between the CAG repeat size in the expanded HTT allele and the larger AR allele, with a model including a quadratic term for the AR CAG repeat size providing the best fit (β= -2.54 × 10⁻⁴ and p=0.035 for the interaction effect). Comparison of the medians in the total group showed that for patients with a below median number of CAG repeats in the expanded HTT allele more repeats on the longer AR allele tended to delay age of onset, while for patients with an above median expansion the longer AR CAG repeats tended to advance age of onset (Supplementary Fig.2). However, given that AR encodes for the androgen receptor, we also performed an additional analyses stratified by sex which demonstrated that the actual effect differed between males and females: in males a longer AR allele tended to delay age of onset in subjects with a relatively low expanded HTT CAG repeat size (median age of onset: 58.5 vs. 55.3 years, p=0.004), while in females a longer AR allele resulted in an earlier age of onset in subjects with a relatively larger expanded HTT CAG repeat size (median age of onset: 39.2 vs. 42.1 years, p=0.009) (Supplementary Fig. 3). Although
regression analysis suggested an effect of CAG repeat size in the smaller alleles of ATXN7 and TBP and a non-linear effect of the longer ATXN1 CAG repeat size, these effects were statistically non-significant when tested non-parametrically (data not shown).

In conclusion, we found that age of onset in patients with HD is modulated by CAG repeat sizes in the normal range of ATXN3, CACNA1A and AR. Our findings extend those of recent reports in SCAs (Tezenas du et al., 2014; Raposo et al., 2015; Chen et al., 2016), and provide further support for the notion that there may be a biological interaction between different PDAGs (Tezenas du et al., 2014; Tezenas du Montcel, 2015). However, given the exploratory nature of this study, larger studies are needed to confirm these preliminary findings in other cohorts of HD patients.

**Supplementary material**
- Supplementary table 1
- Supplementary figures 1, 2 and 3
- Acknowledgement list REGISTRY Investigators of the European Huntington Disease Network investigators

**Acknowledgements**
We would like to express our gratitude to all the patients who participated in the REGISTRY study. We also thank all the REGISTRY Investigators of the European Huntington Disease Network investigators for collecting the data (a list of all investigators is included in the supplementary material).

**Funding**
This study was supported by a seed fund (#570) from the European Huntington Disease Network. NAA is supported by a VENI-grant (#91615080) from the Netherlands Organization of Scientific Research and a Marie Sklodowska-Curie Individual Fellowship grant from the European Union (Horizon 2020, #701130). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Conflict of interest**
The authors declare no conflicts of interest.
References


Figure 1: Association between ATXN3 CAG repeat size and age of onset. Boxplots comparing the age-of-onset between participants with a below or above median number of CAG repeats in their larger ATXN3 allele (Mann-Whiney U-test p=0.025). Black horizontal lines represent medians, boxes display interquartile ranges and whiskers are 1.5 × interquartile range. Circles represent individual patient data with horizontally added jitter.
Figure 2: Interaction between the larger CACNA1A allele and mutant HTT CAG repeat size. Only in patients with mutant HTT CAG repeat size below median higher CACNA1A CAG repeat size was associated with a higher age-of-onset (Mann-Whiney U-test p=0.003). Black horizontal lines represent medians, boxes display interquartile ranges and whiskers are $1.5 \times$ interquartile range. Circles represent individual patient data with horizontally added jitter.