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Large Normal Alleles of ATXN2 Decrease Age at Onset in Transthyretin Familial Amyloid Polyneuropathy Val30Met Patients

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Objective: Transthyretin (TTR)-related familial amyloid polyneuropathy (FAP) is an autosomal dominant neurological disease, caused most frequently by a Val30Met (now classified as Val50Met) substitution in *TTR*. Age at onset (AO) ranges from 19 to 82 years, and variability exists mostly between generations. Unstable oligonucleotide repeats in various genes are the mechanism behind several neurological diseases, found also to act as modifiers for other disorders. Our aim was to investigate whether large normal repeat alleles of 10 genes had a possible modifier effect in AO in Portuguese TTR-FAP Val30Met families.

Methods: We analyzed 329 Portuguese patients from 123 families. Repeat length (at *ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *TBP*, *ATN1*, *HTT*, *JPH3*, *AR*, and *DMPK*) was assessed by single and multiplex polymerase chain reaction, using fluorescently labeled primers, followed by capillary electrophoresis. We used a family-centered approach, and generalized estimating equations were used to account for AO correlation between family members.

Results: For *ATXN2*, the presence of at least 1 allele longer than 22 CAGs was significantly associated with an earlier onset in TTR-FAP Val30Met, decreasing mean AO by 6 years (95% confidence interval = -8.81 to -2.19, $p = 0.001$). No association was found for the remaining repeat loci.

Interpretation: Length of normal repeats at *ATXN2* may modify AO in TTR-FAP Val30Met and may function as a risk factor. This can be due to the role of *ATXN2* in RNA metabolism and as a modulator of various cellular processes, including mitochondrial stress. This may have relevant implications for prognosis and the follow-up of presymptomatic carriers.

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Transthyretin (TTR)-related familial amyloid polyneuropathy (FAP) is an autosomal dominant systemic amyloidosis characterized by extracellular amyloid deposits, and it is caused by a point variant in the *TTR* gene (chr18q12.1; OMIM 176300). Although >100 variants have already been identified, Val50Met (classically known as Val30Met, NM_000371.3) is the most frequently described disease-causing variant in this gene, associated with large clusters.¹

TTR-FAP was initially described in Portugal, by Andrade, as a disease with age at onset (AO) mostly between 25 and 35 years.² Nowadays, in Portuguese families, TTR-FAP Val30Met shows a wide variation in AO (19–82 years).³ Furthermore, it was found that early onset (AO < 40 years) and late onset (AO ≥ 50 years) cases, and even asymptomatic carriers aged 95 years, may coexist in the same family,^{4,5} where offspring often show an earlier

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AO than their affected parent (anticipation). Recently, Lemos et al showed that anticipation was a real biological phenomenon still lacking explanation.³

Since 1991, a new molecular mechanism responsible for several neurogenetic disorders was found, legitimizing anticipation in several diseases such as myotonic dystrophy type 1 (DM1).⁶

More recently, the role of expanded repetitive tracts as genetic modifiers of other genetic diseases has been evidenced. Previous studies showed that an intermediate-length (CAG)_n expansion in the coding region of the ataxin-2 (*ATXN2*) gene, responsible for spinocerebellar ataxia type 2 (SCA2), was a major contributor to amyotrophic lateral sclerosis (ALS).⁷⁻⁹

Additionally, other authors found that the normal repeat length in other spinocerebellar ataxia (SCA) genes also modulated AO of different SCAs.¹⁰⁻¹² Repeat size in the *ATXN2*, atrophin 1 (*ATN1*), and huntingtin (*HTT*) genes, interacting with *ATXN3*, may modulate AO in Machado–Joseph disease (MJD/SCA3).¹⁰ These results, however, were not replicated in an Azorean cohort.¹³

HTT, TATA-box binding protein (*TBP*), and *ATXN7* are directly involved in transcription and transcriptional regulation,¹⁴⁻¹⁹ whereas *ATXN1* and *ATXN2* may interfere with RNA metabolism.^{20,21} In DM1, repeat expansions may affect splicing of the same target RNAs.²² Variants in androgen receptor (*AR*) are associated with variable AO in TTR-FAP Val30Met in both men and women.²³

Recently, some studies have focused on possible modifier genes of AO in TTR-FAP Val30Met, using case–control^{24,25} and family-centered approaches^{23,26},

however, the role of repeat loci has not yet been explored. To the best of our knowledge, only 1 study has looked for triplet repeat expansions as a possible explanation for anticipation in TTR-FAP Val30Met; however, only a small sample of 9 Portuguese affected parent–offspring pairs with large anticipation (>12 years) and 19 noncarrier individuals were compared, no major differences being found.²⁷ We now focused on AO variability, applying more recent technologies for accurate and reproducible determination of repeat length of normal repeats in 10 genes related to neurodegenerative disorders, using a family-centered approach. We tested the hypothesis that larger normal alleles in these genes are more prone to be associated with an earlier onset of TTR-FAP Val30Met than smaller normal alleles.

Subjects and Methods

Patients

At the Unidade Corino de Andrade - Centro Hospitalar do Porto (CHP; Porto, Portugal), the largest TTR-FAP Val30Met patient's registry worldwide has been collected and clinically characterized over the

past 75 years. We retrieved a total of 329 patients with known AO. These patients belonged to 123 different families with at least 2 generations affected, coming from various geographical areas of the country. For each patient, AO was established by the same team of neurologists specializing in TTR-FAP Val30Met; AO was defined as the appearance of first symptoms (usually sensorial loss or autonomic dysfunction), reported by the patient and coinciding with an abnormal neurological and/or neurophysiological examination. In the smaller number of cases where cardiac or kidney involvement is the sole symptom, its manifestation also defines onset of the disease.

Distribution of Normal Alleles in Noncarriers of Val30Met Variant

To assess, for each candidate gene, the distribution of normal repeat length, we analyzed 70 healthy persons (140 alleles), spouses and unaffected siblings from the same families, molecularly confirmed as noncarriers of the Val30Met variant. They had the same geographical origin and were matched by family to our patients, but did not bear the Val30Met mutation.

Sample Collection and Storage

DNA samples were collected at CHP and stored at the Predictive and Preventive Genetics Center, Institute for Molecular and Cell Biology, Instituto de Investigação e Inovação em Saúde, a bio-bank authorized by the National Commission for Data Protection to collect and store diagnostic and research samples and associated data. Written informed consent was obtained for all participants. The CHP's ethics committee approved the study.

DNA Analyses

Genomic DNA was extracted from peripheral blood leukocytes, using the standard salting-out method,²⁸ or from saliva, using ORAGENE kits, according to the manufacturer's instructions (DNA Genotek, Kanata, ON, Canada). DNA samples were quantified in a NanoDrop spectrophotometer.

Repeat Selection and Genotyping

A set of 10 candidate genes that show CAG/CTG trinucleotide repeat expansions associated with different neurological disorders were selected to explore the impact of repeat size at these loci on AO of TTR-FAP Val30Met (Table 1).

Repeat length was determined by a polymerase chain reaction (PCR) amplification assay, using fluorescently labeled forward primers. Sequences for PCR primers were designed with Primer3Plus software, and the presence of hairpins and secondary structures was ascertained with AutoDimer v1.0 (primer sequences available upon request).

To amplify the set of 10 short tandem repeats, single-plex PCR amplifications were performed in a final volume of 10.94 µl for *HTT* and 12.50 µl for *ATXN2*, *ATXN7*, *TBP*, and myotonic dystrophy protein kinase (*DMPK*), using 6.25 µl of HotStar Taq Master Mix Kit (Qiagen, Hilden, Germany). In each PCR reaction, primer concentration was 0.71 µM, 0.8 µM, 1 µM, 0.4 µM, and 0.24 µM, respectively. All reactions used 1.25 µl of dimethylsulfoxide (10%), except for *TBP* gene, and 20 ng of DNA. Cycling conditions are available upon request.

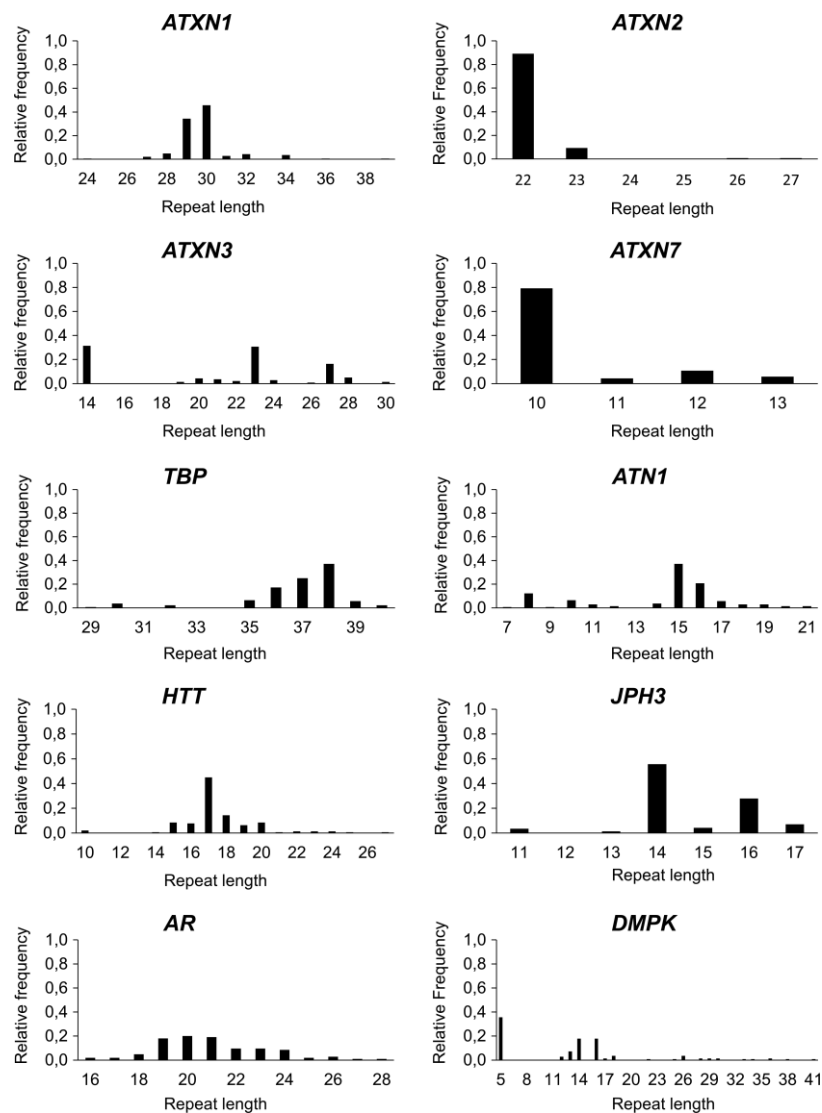


FIGURE 1: Repeat length distribution of the 10 genes studied in our sample of 70 noncarriers of Val30Met.

Then, 2 multiplex PCR reactions were carried out (one multiplex 1 for *ATXN1*, *ATN1* and junctophilin 3 [*JPH3*] and the other multiplex 2 for *ATXN3* and *AR*), in a final volume of 12.50 μ l, using 6.25 μ l of Multiplex PCR Master Mix (Qiagen). Primer concentration for multiplex 1 was 0.5 μ M, 0.25 μ M, and 0.6 μ M, respectively, and for multiplex 2 it was 0.62 μ M for all primers and 20 ng of DNA. PCR products were mixed with the Liz-500 size standard (Applied Biosystems, Foster City, CA). Size of fragments was determined by capillary electrophoresis using the ABI-PRISM 3130 XL Genetic Analyzer (Applied Biosystems) and analyzed with GeneMapper v4.0 software (Applied Biosystems). Automated DNA sequencing of representative alleles from each locus in the reverse direction using Big Dye Terminator Cycle Sequencing v1.1, Ready Reaction (Applied Biosystems), according to the manufacturer's instructions, was performed to determine the exact correspondence between fragment size and repetitive tract length.

Classification of Allele Size according to the Number of CAG/CTG Repeats

For *ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *HTT*, and *DMPK*, we classified normal alleles as short, medium, intermediate–short, and intermediate–large according to the classification of Tezenas du Montcel et al¹⁰ or the criteria of Gene Reviews²⁹ (Table 2).

For *TBP*, *JPH3*, and *AR* loci, there is no description of intermediate alleles; also, there were none found in our population for *ATN1*. Therefore, we considered a different approach for their analysis (see the Design and Statistical Analysis section); alleles were classified as short if their size was equal to or smaller than the median and large if they were larger than the median.

Design and Statistical Analysis

For *ATXN1*, *ATXN2*, *ATXN3*, *ATXN7*, *HTT*, and *DMPK*, genotypes were divided into 2 categories: (1) both alleles were of short or medium length and (2) there was at least 1 intermediate

TABLE 1. Characteristics of All Repeat Loci Selected and Associated Diseases

Gene	Chr Location	Repetitive Motif	Disease
<i>ATXN1</i> (ataxin-1)	6p22.3	(CAG) _n (CAT) _n (CAG) _n	Spinocerebellar ataxia type 1
<i>ATXN2</i> (ataxin-2)	12q24.1	([CAG] _n CAA [CAG] _n) _n	Spinocerebellar ataxia type 2
<i>ATXN3</i> (ataxin-3)	14q21	(CAG) ₂ CAA AAG CAG CAA (CAG) _n	Machado–Joseph disease
<i>ATXN7</i> (ataxin-7)	3p21.1-p12	(CAG) _n	Spinocerebellar ataxia type 7
<i>TBP</i> (TATA-box binding protein)	6q27	(CAG) _n (CAA) _n (CAG) _n	Spinocerebellar ataxia type 17
<i>ATN1</i> (atrophin 1)	12p13.31	(CAG) _n	Dentatorubral–pallidoluysian atrophy
<i>HTT</i> (huntingtin)	4p16.3	(CAG) _n	Huntington disease
<i>JPH3</i> (junctophilin 3)	16q24.3	(CTG) _n	Huntington disease–like type 2
<i>AR</i> (androgen receptor)	Xq12	(CAG) _n	Spinal and bulbar muscular atrophy
<i>DMPK</i> (myotonic dystrophy protein kinase)	19q13.3	(CTG) _n	Myotonic dystrophy type 1

Chr = chromosome.

allele. For *TBP*, *JPH3*, *ATN1*, and *AR*, we divided genotypes into 3 categories: (1) both alleles were equal to or smaller than the median, (2) at least 1 was larger than the median, and (3) both alleles were larger than the median.

For each locus, genotypes where both alleles were of short/medium length or equal to or smaller than the median

were considered as the reference and compared to all genotypes including at least 1 intermediate allele.

Our family-centered approach means that we included in the analysis several members of the same family, where each patient was “nested” in his/her family. To account for nonindependency of AO between members of the same family, we used generalized

TABLE 2. Classification of Short, Medium, Intermediate-Short, and Intermediate-Large Alleles for All Candidate Genes Studied

Genes	Normal Alleles		Intermediate Alleles	
	Short	Medium	Intermediate–Short	Intermediate–Large
<i>ATXN1</i>	<36	—	—	36–38, pure repeat
<i>ATXN2</i>	<22	22	23–26	27–29
<i>ATXN3</i>	<16	16–24	—	25–37
<i>ATXN7</i>	<10	10–11	—	12–15
<i>TBP</i>	—	—	—	—
<i>HTT</i>	≤26	—	—	27–35
<i>JPH3</i>	—	—	—	—
<i>ATN1</i>	<37	—	—	37–48
<i>AR</i>	—	—	—	—
<i>DMPK</i>	≤37	—	—	38–50

Based on Tezenas du Montcel et al¹⁰ and

estimating equations (GEEs), by performing a weighted analysis adjusted for gender.³⁰ Therefore, we assessed whether there was association of the length of the various repeats with AO (as the dependent variable). The unstandardized coefficient (*B*) corresponds to the mean AO variation observed in the individuals carrying a specific genotype when compared with the reference category. To correct for multiple testing, we applied a Bonferroni correction (α was set at 0.005 in the GEE analysis, as we tested 10 genes).

We also analyzed parent–offspring transmissions, assessing whether larger alleles were more likely to be transmitted by the affected father or by the affected mother, using a Fisher exact test. All statistical analyses were performed with SPSS Statistics v23 (IBM, Armonk, NY). A *p* value <0.05 was considered statistically significant.

Results

We assessed the length of repeats at both alleles of a set of 10 loci in a sample of 329 Portuguese patients and in 70 of their noncarrier relatives. To search for potential modifier effects of the repetitive motif length on AO variation, we analyzed a sample of 329 TTR-FAP Val30Met patients belonging to 123 different families with a mean AO of 39.83 \pm 13.21 years (range = 53 years). Mean AO in males (38.09 \pm 13.96 years, range = 51 years) was lower than in female patients (41.39 \pm 12.33 years, range = 53 years), as described in the literature,^{3,4,23} and this difference was statistically significant (95% confidence interval [CI] = -6.17 to -0.43, *p* = 0.024).

Distribution of Repeat Lengths in the Sample of Noncarriers of Val30Met

Table 3 describes the range of normal alleles for each gene, as established in the literature and the range found in our sample, the median, and interquartile range. The Figure 1 shows the distribution of those repeat lengths in our sample.

This sample is very much like those in other countries, if we consider error margins for normal CAGs,^{10,11} as well as in previous studies in the Portuguese population for Huntington disease^{13,31}; MJD/SCA3^{13,32,33}, SCA1, SCA2, SCA7, and dentatorubral pallidolusian atrophy^{13,33}, and SCA17,³³ in terms of both range and allele frequencies.

Analysis of Repeat Length and AO Variation

No significant association with AO in TTR-FAP Val30Met patients was seen for repeat length in *ATXN1*, *ATXN3*, *ATXN7*, *TBP*, *ATN1*, *HTT*, *JPH3*, *AR*, and *DMPK* (Supplementary Table 1).

ATXN2 (CAG) with >22 Alleles Is Associated with an Earlier Onset in TTR-FAP Val30Met Patients

Normal CAG repeats in *ATXN2* may range from 14 to 31 repeats (see Table 3). In our sample, the normal

TABLE 3. Distribution as Described in the Literature and in Our Sample of Number of Normal Alleles for the Genes Analyzed for Noncarriers of Val30Met

Genes	Normal Alleles	
	Literature, Range	Our Sample, Range; Median (IQR)
<i>ATXN1</i>	6–38; 39–44 CAT interrupted	24–39; 30 (1)
<i>ATXN2</i>	14–31	22–27; 22 (0)
<i>ATXN3</i>	12–44	14–30; 23 (10)
<i>ATXN7</i>	4–19	10–13; 10 (0)
<i>TBP</i>	25–42	29–40; 37 (2)
<i>HTT</i>	≤26–35	10–27; 17 (3)
<i>JPH3</i>	6–28	11–17; 14 (2)
<i>ATN1</i>	6–35	7–21; 15 (6)
<i>AR</i>	9–36	16–28; 21 (4)
<i>DMPK</i>	5–37	5–41; 14 (11)

IQR = interquartile range.

ATXN2 (CAG)_n alleles were less polymorphic, displaying only 6 different sizes, ranging from 22 to 27 repeats (see Table 3). The (CAG)₂₂ allele was the most frequent (88%), followed by (CAG)₂₃ (9%; see Fig 1), as seen in other populations.

The homozygous genotype for 22 CAG repeats (*n* = 261) was considered the reference category against which all other genotypes (*n* = 68 patients), where at least 1 allele with >22 CAGs was present, were compared. Mean AO of the 261 TTR-FAP Val30Met patients homozygous for (CAG)₂₂ was 40.92 \pm 13.55 years (range = 53 years), whereas mean AO of the 68 patients carrying at least 1 allele longer than 22 CAGs was 35.63 \pm 10.93 years (range = 50 years), and this difference was statistically significant (95% CI = 1.79 to 8.78, *p* = 0.003). For the reference category, mean AO was

38.92 \pm 14.12 years in males and 42.81 \pm 12.75 years in females. This difference was statistically significant (95% CI = -7.18 to -0.61, *p* = 0.020).

In the group carrying at least 1 allele longer than 22 CAGs, mean AO was 34.45 \pm 12.82 years in males and 36.51 \pm 9.35 years in females. However, this difference was not statistically significant (95% CI = -7.72 to 3.59, *p* = 0.466).

Using a family-centered approach, we found that patients carrying at least 1 *ATXN2* allele larger than 22 repeats had an earlier mean onset (almost 6 years; 95%

CI = -8.81 to -2.19, $p = 0.001$) after adjusting for sex (Supplementary Table 2). This effect was not sex-dependent and remained significant after adjustment for multiple comparisons, using the conservative Bonferroni correction ($p = 0.005$). When we analyzed the relationship between gender of transmitting (affected) parent and size of the alleles, we found that large normal alleles were equally transmitted from the mother or the father ($p = 0.698$). Analysis of transmissions was only performed for the *ATXN2* gene, as it was the only one where larger alleles showed a significant association with AO.

Discussion

Several genetic modifiers of AO in TTR-FAP Val30Met have already been identified,^{23–26} but the possible role of large normal alleles in genes containing a repeat motif had never been considered and deserved particular attention. With this in mind, our strategy was to assess whether the normal CAG/CTG repeat length in 10 genes could act as a genetic modifier influencing the AO of TTR-FAP Val30Met patients.

Large normal *ATXN2* alleles showed a strong association with AO, patients carrying at least 1 normal allele with >22 repeats in *ATXN2* being prone to earlier onset.

Previous studies have shown that length of normal CAG repeats in *ATXN2* is quite variable (14–31 CAGs), depending on the population, with 22 and 23 CAG repeats being the most common alleles,^{34,35} similarly to what was found in our sample, where its range was 22 to 28. The CAG repeat in *ATXN2* is responsible for SCA2 when its length is >32 to 34 units, correlating with AO and severity.^{36,37} Some SCA2 patients with 32 to 33 repeats show later onset.^{38,39} Moreover, previous studies have suggested that intermediate-length CAG repeats in *ATXN2* (27–33 units) are associated with increased risk for some diseases and modulate AO in ALS^{7,8,40} and Parkinson disease (PD),⁴¹ but seemingly not for hereditary spastic paraplegias.⁴² Intermediate repeat expansions in *ATXN2* associate with PD in Asian populations,^{43,44} indicating that different genetic background may play a role.

Ataxin-2 is one of the polyQ proteins, highly expressed in various neuronal and non-neuronal tissues, including the brain,⁴⁵ and has been involved in the regulation of several biological processes such as RNA-mediated metabolism, translation regulation, cytoskeleton reorganization, Ca^{2+} homeostasis, and mitochondrial stress.⁴⁶ The hallmark of TTR-FAP Val30Met is the presence of extracellular deposits of TTR aggregates and amyloid fibrils in several tissues; particularly, in peripheral nerves, diverse molecular pathways associated with degeneration have been confirmed using in vivo samples and cell culture

studies, including activation of nuclear factor κ B, proinflammatory cytokines, oxidative stress, and endoplasmic reticulum (ER) stress.⁴⁷ Oxidative stress may be further increased by the presence of (CAG)_{23–28} alleles at the *ATXN2* locus and contribute to the processing of misfolded proteins, which in turn cause ER stress with enhanced reactive oxygen species production, thereby creating a feedback loop.⁴⁸

Another interesting feature is that when *ATXN2* interacts with ALS pathogenic pathways, an altered localization of *ATXN2* has been observed.⁷ These altered mechanisms can lead to an early AO, because *ATXN2* intermediate-length repeats can alter protein stability or degradation affecting ataxin-2 levels, which may promote interaction with other proteins.⁷ In ALS, it was also found that *ATXN2* intermediate alleles modulate disease pathways via its RNA-dependent interaction with other ALS-related proteins, such as FUS and TDP-43.⁴⁹ Another study found that coexpression of *ATXN2* intermediate allele, combined with C9orf72 depletion, increases ataxin-2 aggregation, leading to neuronal toxicity.⁵⁰ Therefore, it would be interesting to assess ataxin-2 interaction with other TTR-FAP Val30Met-associated proteins.

To confirm these hypotheses, future studies focused on level of protein expression with (CAG)_{23–28} are needed. Our results lead to the hypothesis that (CAG)_{23–28} in *ATXN2* might influence TTR aggregation and amyloid fibril formation, contributing as a risk factor for neurodegeneration. Furthermore, they may also indicate that a population-specific genetic profile at *ATXN2* influences the ability to confirm/discard the potential modifier effect in AO variation; for this reason, replication studies should be performed in different populations.¹³

Importantly, Tezenas du Montcel et al also found in a large cohort that repeat-encoding genes with normal length can be modulators of AO for other SCAs, confirming that these interactions may occur in different populations and disorders.¹⁰

Our findings support the hypothesis that large normal alleles in *ATXN2* may modulate AO in TTR-FAP Val30Met. Although no significant association was found for the other genes, we cannot exclude a role for dynamic mutations other than the ones studied. We had a special concern with statistical power, taking into account (1) sample size (one of the largest sample collections of TTR-FAP Val30Met available worldwide); and (2) that the statistical analysis focused on a parametric test (GEE), which is a powerful statistical method, and multiple testing corrections to prevent type I errors. The results obtained also reinforce the importance of replication studies in other populations to confirm these results. Unraveling factors that contribute to modulating AO is important for

understanding the disease pathogenesis, for improving genetic counselling, and for the follow-up of presymptomatic carriers.

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Author Contributions

All authors contributed to the conception and design of the study and the acquisition and analysis of data. D.S., A.S., and C.L. drafted a significant portion of the manuscript or figure.

Potential Conflicts of Interest

Nothing to report.

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