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ARTICLE

Variants in *RBP4* and *AR* genes modulate age at onset in familial amyloid polyneuropathy (FAP ATTRV30M)

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Familial amyloid polyneuropathy (FAP) ATTRV30M is a neurodegenerative disorder due to point mutations in the transthyretin gene, with V30M being the commonest. FAP ATTRV30M shows a wide variation in age at onset (AO) between clusters, families and generations. Portuguese patients also show remarkable AO differences between genders. Genes found to be associated with FAP ATTRV30M pathways may act as AO modifiers. Our aim was to further explore the role of *APCS* and *RBP4* genes and to study for the first time the involvement of sex-linked genetic modifiers – *AR* and *HSD17B1* genes – in AO variation in Portuguese families. We collected DNA from a sample of 318 patients, currently under follow-up. A total of 18 tagging SNPs from *APCS*, *RBP4*, *AR* and *HSD17B1* and 5 additional SNPs from *APCS* and *RBP4* previously studied were genotyped. To account for nonindependency of AO between members of the same family, we used generalized estimating equations (GEEs). We found that *APCS* and *RBP4* were associated with late AO. In addition, rs11187545 of the *RBP4* was associated with an early AO. For the *AR*, in the male group three SNPs were associated with an early AO, whereas in the female group four were associated with both an early and later AO. These results strengthened the role of *APCS* and *RBP4* genes and revealed for the first time the contribution of *AR* genes as an AO modifier in both males and females. These findings may have important implications in genetic counseling and for new therapeutic strategies.
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INTRODUCTION

Familial amyloid polyneuropathy (FAP) ATTRV30M is an autosomal dominant systemic amyloidosis characterized by amyloid deposition of mutated fibrillar transthyretin (TTR) protein. The main clinical expression of this disease is a progressive peripheral sensorimotor and autonomic neuropathy due to a point mutation in the *TTR* gene (chr18q12.1) (OMIM 176300). Although more than 100 mutations have been found in the *TTR* gene,[1] V30M, NM_000371.3:c.148G>A (p.(Val50Met)) is the commonest.

Andrade[2] first described FAP in Northern Portugal as a disease occurring between 25 and 35 years. Variation in age at onset (AO) between clusters and within the same focus has been described.[3–6] Among Portuguese families, a remarkable wide variation in AO (19–82 years) has been observed, and an increasing number of late-onset cases (≥50 years) are being ascertained, including asymptomatic carriers aged 95 years.[4]

Variation in AO between generations has also been observed: late-onset parents often have early-onset offspring (<40 years) – an evidence for anticipation – whereas the reverse has never been observed. Recently, our group has shown that anticipation is a true biological phenomenon in FAP ATTRV30M.[6]

In order to clarify why there is such a striking AO variation in FAP ATTRV30M, an attractive strategy is to focus on modifier genes that can affect transcription through immediate gene transcript expression or translate into phenotypical alterations at multiple organizational levels.[7] Some modifier genes such as *amyloid P component, serum* (*APCS*), complement *C1QA* and *C1QC* and *plasma retinol-binding protein 4* (*RBP4*) have been unraveled so far but they only explain a small part of the AO variability in FAP ATTRV30M.[8,9] In a previous study, Soares *et al*[9] compared Portuguese patients in a classic case–control approach; these authors found that the variants studied in the *APCS* gene had a combined modifier effect when analyzing early-onset group *versus* controls, whereas the combination of one variant from *APCS* (rs6689429) and two variants from *RBP4* (rs7091052 and rs28383574) seemed to be involved with late-onset group.[9] No comparisons were made between early- and late-onset cases.

The *APCS* gene encodes a highly conserved plasma glycoprotein that is associated with amyloid deposition independently of protein origin. Apart from the plasma, APCS can also exist in amyloid deposits[10] and has the ability to bind to several ligands such as amyloid fibrils through a specific calcium-dependent mechanism.[10] In humans, the biological role of this protein has yet to be clarified. However, recently, it was found that the *APCS* has become an attractive therapeutic target in amyloid diseases.[11]

RBP4 gene encodes the specific carrier of retinol in the human plasma. *RBP4* is synthesized in the liver and when binding to all-*trans*-retinol circulates as a complex with TTR, forming a ternary RBP4–retinol–TTR complex. In the blood, the formation of the RBP4–retinol–TTR complex causes the apparent increase of molecular mass and thus prevents its loss through glomerular filtration by the kidneys, stabilizing the quaternary structure of TTR.[12]

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In several studies with Portuguese FAP ATTRV30M patients, women were found to have later onset than men.[4,13] Our group recently reiterated this finding.[6] Moreover, mother–son pairs showed larger anticipation whereas the father–daughter pairs showed only residual anticipation.[6] Therefore, to clarify gender-related differences associated with AO variation we will focus on sex steroid hormones as *androgen receptor* (*AR*) and *hydroxysteroid (17-β) dehydrogenase 1* (*HSD17B1*). Both affect TTR expression levels, but AR seems to have a stronger inducer effect in the TTR expression than HSD17B1.[14] The *AR* gene is located in the X chromosome and its nuclear transcription factor is activated to mediate binding of the androgenic hormones testosterone and 5α-dihydrotestosterone, whereas the *HSD17B1* gene may play an important role in regulating the local cellular levels of estradiol.[15]

Therefore, the aim of this study was to investigate whether variants of these candidate genes have a modifier effect in AO within FAP ATTRV30M families, what was not taken into account in previous studies.

We reassessed the role of *APCS* and *RBP4* genes and for the first time analyzed sex-linked genes (*AR* and *HSD17B1*) as possible modifiers for AO.

MATERIALS AND METHODS

Subjects

Unidade Corino de Andrade (UCA-CHP, Porto, Portugal) has the largest FAP ATTRV30M registry worldwide: over 2000 patients, belonging to more than 500 families, collected over 75 years and clinically well characterized. We concentrated on families with at least two generations affected and currently under follow-up at CHP. We achieved a sample of 318 patients from 106 families, coming from different geographical areas of the country.

For each individual, AO had been established by the same team of neurologists specialized in FAP ATTRV30M when the first sensorimotor symptoms were observed. The DNA samples of these patients were collected and stored at the Centro de Genética Preditiva e Preventiva (CGPP, Porto, Portugal) biobank, authorized by CNPD (National Commission for Data Protection).

All patients gave written informed consent and the study was approved by the Ethics Committee of CHP.

DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes using the standard salting-out method[16] or from saliva using ORAGENE kits according to the manufacturer's instructions (DNA Genotek, Inc., Kanata, ON, Canada).

Selection of SNPs and genotyping

We selected a total of 18 tagging single-nucleotide polymorphisms (SNPs) (Supplementary Table 1) through the degree of linkage disequilibrium (LD) existing between them, using Haploview v.4.1,[17] at an r^2 threshold of 0.80 (with a minor allele frequency of 0.10%). We also included five SNPs previously studied in order to replicate the results found by Soares *et al*.[9]

The SNP frequencies in the European population were obtained by resorting to the HapMap Project and dbSNP. All variants were submitted to the Leiden Open Variation Database shared installation (URL: http://databases.lovd.nl/shared/screenings?search_owned_by_=="Carolina%20Lemos"), with the following submission IDs: APCS: http://www.lovd.nl/APCS; AR: http://www.lovd.nl/AR; HSD17B1: http://www.lovd.nl/HSD17B1; RBP4: http://www.lovd.nl/RBP4 (patient IDs 38827–39346).

Primers were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Afterwards, the AutoDimer software (http://www.cstl.nist.gov/strbase/AutoDimerHomepage/AutoDimerProgramHomepage.htm) was used to assess the formation of hairpins and primer–dimer secondary structures. Single base extension (SBE) primers were designed as described in the literature.[18]

A multiplex reaction for 18 tagging SNPs was carried out using the QIAGEN Multiplex PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amplified products were purified with ExoSAP-IT (Amersham Biosciences, Uppsala, Sweden). Genotyping was performed by a SNaPshot reaction using the SNaPshot Multiplex kit (Applied Biosystems, Foster City, CA, USA). Final products were purified with SAP (Amersham Biosciences).

To genotype rs6689429, rs3758539, rs28383574 and rs7091052, PCR products were digested using *Bsa*JI, *Mnl*I, *Bsr*I and *Hin*P1I restriction enzymes and loaded in QIAxcel multicapillary electrophoresis system (Qiagen). For rs28383573, genotyping was performed by sequencing. In addition, uncertain genotypes were sequenced. Automatic sequencing was performed using the Big Dye Terminator Cycle Sequencing v1.1, Ready Reaction (Applied Biosystems) according to the manufacturer's instructions. Samples resulting from the SNaPshot reaction and sequencing were loaded in an ABI-PRISM 3130 XL Genetic Analyzer (Applied Biosystems). SNaPshot results were analyzed with the GeneMapper v4.0 software (Foster City, CA, USA).

Design and statistical analysis

Our family-centered approach means that we included in the analysis several members of the same family, and therefore each patient was 'nested' in his/her family. To account for nonindependency of AO between members of the same family, we used generalized estimating equations (GEEs).[19]

Therefore, we assessed any simultaneous association of the different variants with AO in FAP ATTRV30M (as the dependent variable) using the most common genotype as the reference category.

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. All statistical analyses were performed using IBM SPSS Statistics software (v.20; Armonk, NY, USA).

Haplotypes estimation was performed using SNPator software.[20] To estimate haplotype frequencies, the Haploview v.4.1 software was also used. Parental transmission was assessed using Fisher's exact test.

To detect gene–gene interactions, we used Multifactor Dimensionality Reduction (MDR) software (v.2.0).[21] Significant results were corrected for multiple testing, based on a 1000-fold permutation test, using the MDR Permutation Testing Module (v.1.0).

SNP Function Prediction (FuncPred), a bioinformatic tool, was used to predict putative SNP functional effects.[22]

RESULTS

Our patient sample shows a mean AO of ~39 years; however, mean AO in males (37.28) is lower than in females (40.52), as already described in the literature[4,6] (Table 1).

Using a candidate-gene approach with 318 Portuguese FAP ATTRV30M patients, we unraveled some variants for the first time associated with AO variation in FAP ATTRV30M, as presented in Table 2.

The role of *APCS* gene

Regarding the genotype GT for the rs28383573, we did not find a significant result. However, it is worth noting that one individual with the TT genotype presented a later onset and the difference corresponds to an increase of 26 years in mean AO (Table 2). For the other SNPs assessed, no significant results were found.

Table 1 Mean AO of male and female patients

Gender	*N*	*Range*	*Minimum*	*Maximum*	*Mean AO*	*SD*
AO						
Male	152	51	21	72	37.28	13.96
Female	166	53	21	74	40.52	12.30
Total	318	53	21	74	38.97	13.19

RBP4 gene and AO

We found that the CC genotype ($P=0.012$) and the CT genotype ($P=0.011$) of rs7094671 were associated with a later onset (increasing AO in 19 and 10 years, respectively). The CT genotype ($P=0.035$) of rs11187545 was associated with an earlier onset when compared with the TT genotype, with a decrease of 9 years in AO (although this is the only result that does not stand after multiple testing correction). Noteworthy, the individual with the CC genotype also presented an earlier onset.

Using MDR, we analyzed a possible interaction between *APCS* and *RBP4* genes but we did not find any strong interaction (data not shown).

The involvement of sex hormone genes

Taking into account that *AR* gene is located in the X chromosome the analyses were stratified by gender and the genotypic analyses were only performed in the female group. We found a total of five SNPs significantly associated with AO variation in the *AR* gene, three in the male group and four in the female group, including two in common for both genders.

Regarding the allelic analyses performed in the male patients group, significant results were found associated with a decrease in AO for the A allele ($P<0.001$) of rs17217069 and the G allele ($P=0.002$) of rs2361634, and this variation correspond to 10 and 9 years, respectively (Table 2).

In addition, in the female group, the CT genotype ($P<0.001$) of rs5919392, the GA genotype ($P=0.001$) of rs2361634 and the AT genotype ($P=0.033$) of rs5965433 were significantly associated with an earlier AO, and this variation correspond to 9 and 7 years for these variants. On the other hand, the CT genotype ($P<0.001$) of rs5919393 was significantly associated with a later AO (Table 2).

For rs5919393, in the male group the C allele ($P=0.015$) was associated with a decrease of 11 years, whereas in the female group this SNP was associated with an increase in AO and the differences in mean AO correspond to 7 years for the CT genotype (Table 2).

Concerning the *HSD17B1* gene, none of the SNPs were found to be significantly associated with AO variation. In addition, we did not find any significant interaction between *AR* and *HSD17B1* genes.

No haplotypic effect was found for any of the genes studied (data not shown).

Regarding parental transmission of the SNPs to the affected children, we found a differential transmission for allele T of rs28383573 in the *APCS* gene. We found that nonaffected fathers transmitted more often than expected this allele that is involved in a later onset.

Regarding *AR* gene, for rs5919392, we found that the affected mothers transmitted more often than expected the T allele that is associated with an early onset. For the other SNPs we did not find any significant differences in parental transmission.

A bioinformatic's analysis using FuncPred was also performed to assess the functional impact of the SNPs associated with AO variation of FAP ATTRV30M. This analysis predicted that one SNP in LD with rs5919393 (rs2473881) may alter transcription factor binding (TFB) sites in the *AR* gene, with a higher number of TFB sites when the A allele was present.

DISCUSSION

Mechanisms responsible for AO variation in FAP ATTRV30M are still elusive. Similar to several other neurodegenerative disorders, the *TTR* gene point mutation alone does not fully determine the AO variation or the course of the disease. Therefore, we have applied, for the first time, a family-centered approach also used in studies of other

Table 2 Significant results of the analysis of *APCS*, *RBP4* and *AR* SNPs and AO variation taking into account intrafamilial nonindependency

Gene	*SNP*	*Genotypes*	*B*	*95% CI*	*P-value*
APCS		GG (reference)	–	–	–
	rs28383573	TT[a]	26.17	(23.08; 29.25)	$P<0.001$
RBP4		CC (reference)	–	–	–
	rs7094671	TT	18.95	(4.10; 33.80)	0.012
		CT	10.42	(2.38; 18.46)	0.011
	rs11187545	TT (reference)	–	–	–
		CC[a]	−28.49	(−44.56; −12.42)	0.001
		CT	−8.98	(−17.06; 16.67)	0.035
AR (male group)		T (reference)	–	–	–
	rs5919393	C	−10.59	(−19.14; −2.04)	0.015
		G (reference)	–	–	–
	rs17217069	A	−10.38	(−14.22; −6.54)	$P<0.001$
		A (reference)	–	–	–
	rs2361634	G	−8.88	(−14.45; −3.31)	0.002
AR (female group)		CC (reference)	–	–	–
	rs5919392	CT	−8.53	(−12.76; −4.30)	$P<0.001$
		TT (reference)	–	–	–
	rs5919393	CT	6.63	(3.38; 9.87)	$P<0.001$
		AA (reference)	–	–	–
	rs2361634	GA	−6.96	(−11.00; −2.92)	0.001
		AA (reference)	–	–	–
	rs5965433	AT	−6.76	(−12.97; −0.56)	0.033

B, unstandardized coefficient (estimated quantitative effect of each genotype on AO variation compared with the reference genotype); CI, confidence interval; *P*-value, significance level was set to 0.05.
[a]Based in only one individual with this genotype.

diseases[19,23] and focused on modifier genes related with (1) *TTR* functional pathways involved in pathophysiological processes related to FAP ATTRV30M pathogenesis and also (2) sex-linked genes because of observed differences between genders and parent-of-origin effects associated with AO variation.

In a previous study, Soares *et al*[9] analyzed five SNPs (also studied by us) in a Portuguese sample of 92 patients and 85 controls using a classic case–control approach. Thus, for *APCS* gene the authors obtained significant results for the combination of rs6689429 and the rs2808661 genotypes associated with early onset when the early-onset group *versus* controls was compared; in addition, a joint effect of one SNP of the *APCS* gene (rs6689429) and two of the *RBP4* gene (rs7091052 and rs28383574) was associated with a later onset when the late-onset group *versus* controls was compared. In addition, in the study performed by Dardiotis *et al*[8] that analyzed only one SNP (rs2808661) of the *APCS* gene, also studied by us, the results were quite different as no significant modifier effect was found. We did not find any significant results for these SNPs, showing that different approaches can lead to different results. Different genetic risk factors can also be involved in different populations as in the case of the Cypriot sample. Furthermore, the sample size was quite small when compared with ours, showing that a larger sample, increasing the statistical power, is needed to draw some conclusion.

The role of rs28383573 in the *APCS* gene

Unlike other studies, we found in our sample a putative evidence of an association with a later onset for the *APCS* gene. However, it should be noted that the TT genotype (rare homozygous) of the rs28383573 only appears once in our sample. This is in accordance with the observed genotype frequencies in the European population (TT = 0.013), and therefore we think that this result is worth mentioning. Although one may question whether the effect found in this one individual is sufficient to associate this gene with AO, we look to this result as a hypothesis to be further explored because of the differential parent transmission effect found and the role of rare variants as genetic modifiers.

APCS has been associated with several amyloid deposits and it has been suggested that it stabilizes amyloid fibrils, protecting them from proteolytic degradation.[11] A study performed *in vitro* using *Drosophila* model showed that APCS binds to early TTR aggregates that are toxic to neuronal cells, therefore acting as a protective factor in inhibition of TTR-induced toxicity.[24] Therefore, this protective role of APCS could be increased by the presence of this variant, increasing the inhibition of TTR toxicity and preventing an earlier AO.

RBP4 gene variants associated with different AO patterns

One SNP (rs7094671) of the *RBP4* gene was associated with a late AO. In addition, rs11187545 was associated with an early AO.

As with rs28383573 of APCS gene, the CC genotype of the *RBP4* rs11187545 also appeared only once in our families but the CC genotype frequency of this SNP is also low (CC = 0.003) in the European population. As we found a significant result for the CT genotype, we consider that we cannot exclude the result for the CC genotype because it strengthens the involvement of the C allele of the rs11187545 in AO variability. *RBP4* and *TTR* variants cause either RNA or protein instability and result in abnormally low retinol levels.[25] Therefore, the lack of RBP4 or TTR alters the retinol levels and increases urinary excretion of RBP4–retinol complex.[26,27] The presence of these variants could therefore alter RBP4–TTR binding, preventing or increasing their binding to retinol, allowing us to hypothesize that the protective role of *RBP4* could be decreased because of damaged TTR stability increasing aggregates formation, leading to an earlier AO.

AR gene and AO variability

The anticipation effect in FAP ATTRV30M as a mechanism associated with patients' gender has already been previously described[6] and has reinforced the hypothesis that sex steroid hormone genes may also have a modifier role in the disease onset with the differences in AO between males and females.

Importantly, we found that rs5919393 was associated with an earlier onset in males and a later onset in females, leading us to suggest that this variant can have a possible protector effect in females. AR acts as a DNA-binding transcription factor that regulates positively or negatively gene expression.[28] From the *in silico* analysis we found that the A allele of the rs2473881, which is in LD with rs5919393, may alter TFB sites, promoting upregulation of this gene in the female late-onset group. Furthermore, the rs2361634 is associated with an earlier onset in both males and females, and we hypothesize that this variant could be a genetic risk factor to both genders. Interestingly, we found a differential parental transmission regarding rs5919392, showing that affected mothers add a risk genetic effect to AO variation. These differences in AO variation could also be partially explained by different androgen levels in circulation in males and females as males have higher androgen levels than females,[29] and this can induce TTR expression. Thus, as men have higher testosterone levels, increased levels of TTR will be produced possibly leading to early TTR amyloid deposits. Importantly, and confirming these observations, lower levels of TTR were found in women.[30] In a study using a mouse model with Alzheimer's disease it was shown that a decrease or absence of TTR influences the levels of sex hormones with a gender effect.[31] A positive association between sex hormones and TTR levels were found in other studies.[32,33] In addition, testosterone showed to have a neuroprotective role in animal studies,[34,35] and some studies have shown protective actions of sex hormones in several neurodegenerative diseases.[36]

Regarding the *HSD17B1* gene, although we did not found an effect in AO variation, this does not exclude other variants in other estradiol-linked genes as possible modifier candidate genes in FAP ATTRV30M.

All the results found seem to be due to the main effects of each gene, as no interaction between genes was observed.

Our study has several strengths: a large sample size for a rare disease and a family-centered approach that prevents population stratification, unlike a case–control study.[28] GEE analysis is also appropriate, as it corrects for familial correlations of AO and it tends to have a greater power to detect a statistically significant effect than other similar methods.[37] We also paid a special attention to statistical analysis by including multiple testing corrections.

However, further investigation around AO variability in FAP ATTRV30M is necessary to deepen our results and provide more insight into the underlying mechanisms involved. In addition, functional studies will also be required in the near future to confirm these results. The present study included only Portuguese patients, and thus replication of this approach in other populations would be very interesting.

Our findings confirmed the involvement of *APCS* and *RBP4* genes in the AO variation in FAP and this is the first study that unravels a new modifier gene – *AR* gene – as a potential modulator of AO variation in FAP ATTRV30M, particularly, sex linked. In conclusion, these findings may have important implications in genetic counseling of offspring and in the follow-up of mutation carriers.

CONFLICT OF INTEREST

D Santos has received research support from a FCT fellowship (SFRH/BD/91160/2012). T Coelho's institution has received support from FoldRx Pharmaceuticals that was acquired by Pfizer Inc. in October 2010; T Coelho has served on the scientific advisory board of Pfizer Inc. and has received funding from Pfizer Inc. for scientific meeting expenses (travel, accommodations and registration). She currently serves on the THAOS (natural history disease registry) scientific advisory board. Miguel Alves-Ferreira, Jorge Sequeiros, Denisa Mendonça, Isabel Alonso, Carolina Lemos and Alda Sousa declare no conflict of interest.

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