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RESEARCH ARTICLE

Familial amyloid polyneuropathy in Portugal: New genes modulating age-at-onset

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Abstract

Objectives: Familial amyloid polyneuropathy (FAP ATTRV30M) shows a wide variation in age-at-onset (AO) between clusters, families, and among generations. We will now explore some candidate genes involved in altered disease pathways in order to assess their role as genetic modifiers of AO, using a family-centered approach. **Methods**: We analyzed 62 tagging SNPs from nine genes-*NGAL*, *MMP-9*, *BGN*, *MEK1*, *MEK2*, *ERK1*, *ERK2*, *HSP27*, and *YWHAZ* – in a sample of 318 V30M Portuguese patients (106 families), currently under follow-up. A generalized estimating equation analysis was used to take into account nonindependency of AO between relatives. Also, an *in silico* analysis was performed in order to assess the functional impact of significant variants associated with AO. **Results**: We found for the first time variants from six genes (*NGAL*, *BGN* (in the female group), *MEK1*, *MEK2*, *HSP27*, and *YWHAZ*) that were significantly associated with early- and/or late-onset. Then, we confirmed a strong synergistic interaction between *NGAL* and *MMP-9* genes. Additionally, by an *in silico* analysis, we found some variants for *MEK1* gene that may alter binding of the transcription factors and that influence the regulation of gene expression regarding microRNA binding sites and splicing regulatory factors. **Interpretation**: These findings showed that different genetic factors can modulate differently the onset of disease's symptoms and revealed new mechanisms with clinical implications in the genetic counseling and follow-up of mutation carriers and could contribute for development of potential therapeutical targets.

Introduction

Familial amyloid polyneuropathy (FAP) ATTRV30M is an autosomal dominantly (AD) systemic amyloidosis with variable clinical presentation, age-at-onset (AO), and phenotype severity.[1] It is characterized by extracellular amyloid deposits of fibrillary transthyretin (TTR) that results in degeneration of the peripheral nerves and it is caused by a point mutation in the *TTR* gene (chr18q12.1) (OMIM 176300). More than 100 different mutations have been identified,[2] but the Val30Met (V30M) missense mutation is the commonest worldwide.

Typically, a disease of adult-onset,[3] FAP ATTRV30M has not only shown a wide variation in AO between clusters but also within the same focus.[4–8] In Portugal, where it was first described,[3] it was characterized as having onset between 25 and 35 years. Nowadays, AO in Portuguese patients varies from 19–82 years.[9] However, given the large anticipation detected in Portuguese patients,[9] AO variability observed between generations is our target.

Earlier genetic studies focused on some candidate genes that can modify AO of FAP ATTRV30M, using a case–control approach,[10,11] but they did not take into account that early- and late-onset are not separate entities, since they may coexist within the same family.

In our recent study, we used for the first time a family-centered approach concluding that amyloid P component,

serum (*APCS*) and plasma retinol-binding protein 4 (*RBP4*) genes have an important role in AO variation and revealed for the first time the androgen receptor (*AR*) gene as an AO modifier both in males and females.[12]

Now, additional candidate genes related with other FAP ATTRV30M signaling pathways were selected. We used the same sample derived from the large Portuguese registry.[12]

A study using nerve and salivary glands biopsies found that biglycan (BGN), neutrophil gelatinase-associated lipocalin (NGAL), and matrix metalloproteinase-9 (MMP-9) proteins were upregulated in FAP ATTRV30M when compared to controls. BGN seems to be increased in the earliest stages of TTR deposition in the form of nonfibrillar aggregates, whereas NGAL and MMP-9 were only overexpressed at a later stage of disease progression when fibrillary deposits were formed.[13]

Monteiro et al., 2006 previously showed that extracellular signal-regulated kinases 1/2 (ERK1/2) showed increased activation in FAP ATTRV30M salivary gland and nerve biopsies. ERK1/2 kinases (MEK1/2) activation was also upregulated in peripheral nerves, with phosphorylation of ERK1/2. Therefore, this may represent an early signaling cascade leading to cytotoxic effects of TTR aggregates.[14]

Furthermore, heat shock proteins (HSPs) have been involved in several neurodegenerative diseases including FAP ATTRV30M and an increased expression of heat shock 27 kDa protein 1 (HSP27) related to the presence of extracellular TTR deposition in human FAP nerve, skin, and salivary gland biopsies was found, as compared to controls.[15]

Moreover, it has been described that tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (14-3-3zeta or YWHAZ) expression levels decreased with aging.[16] Also, Vieira et al., 2013 showed that TTR regulates YWHAZ protein levels and so the absence of TTR correlated with decreased levels of YWHAZ in the hippocampus in young/adult TTR null mice when compared to TTR wild-type animals, although no changes in gene expression were found.[17]

The aim of our study was to assess for the first time whether variants in these candidate genes have a modifier role in AO variation between generations in FAP ATTRV30M families and to look for a possible interaction between them.

Subjects and Methods

Subjects

From the largest FAP ATTRV30M registry worldwide (at UCA-CHP, Porto), we collected DNA samples and clinical data concerning 318 patients (106 families). Details are described at length at Santos et al., 2016.[12]

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

DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes, using the standard salting out method[18] or from saliva, using ORAGENE kits according to the manufacturer's instructions (DNA Genotek, Inc.).

Selection of SNPs and genotyping

A total of 62 tagging single-nucleotide polymorphisms (SNPs) were selected (Table 1) with Haploview v.4.1,[19] using an r^2 threshold of 0.80 (as measure of linkage disequilibrium) and with a minor allele frequency of 0.10%.

A multiplex polymerase chain reaction (PCR) amplification for 56 tagging SNPs was performed and genotyping was carried out by a SNaPshot reaction. To genotype rs350911, rs7698, rs983583, and rs1451637, PCR products were digested using *TfiI*, *HinP1I*, *PsiI*, and *BfaI* restriction enzymes, respectively, and loaded in QIAxcel multicapillary electrophoresis system (Qiagen). The rs12906411 and rs2289858 genotyping was performed by sequencing. Primers' design and genotyping techniques are described in more detail elsewhere.[12]

Design and statistical analysis

Our family-centered approach meant that several members of the same family were included in the analysis; therefore, each patient was "nested" in his/her family. We used generalized estimating equations (GEE),[20] since AO is nonindependent between members of the same family. The design and statistical analysis were described elsewhere.[12]

Results

We present a family-centered study of variants in nine candidate genes involved in FAP ATTRV30M signaling pathway.

In this study with 318 Portuguese FAP ATTRV30M patients (106 families) with a mean AO of $\sim$39 years, we unraveled for the first time some polymorphisms associated with AO variation in FAP ATTRV30M, as presented in Table 2. No significant results were found to be associated with AO variation regarding *MMP-9*, *ERK1*, and *ERK2* genes (data not shown).

Table 1. Tagging SNPs selected for each gene.

Candidate genes								
NGAL	*MMP-9*	*BGN*	*MEK1*	*MEK2*	*ERK1*	*ERK2*	*HSP27*	*YWHAZ*
				rs2289860				
				rs350916				
				rs1979013				
			rs1549854	rs350911		rs2276008		rs17365305
		rs2266862	rs745796	rs10250		rs13515		rs4734497
	rs1805088	rs7062216	rs9672789	rs2289858		rs1063311		rs1376672
	rs3918249	rs1126499	rs1432442	rs350903	rs7698	rs2298432		rs17365661
rs12006030	rs3918256	rs2073479	rs12906411	rs10412325	rs1143695	rs7286558	rs11769502	rs7835096
rs3780836	rs3787268	rs2269404	rs16949939	rs350897	rs11865086	rs3827303		rs3134354
	rs2250889	rs1042103	rs11630608	rs350895		rs8141815		rs35083016
	rs17577	rs743642	rs7181936	rs7258366		rs9610417		rs983583
			rs8039880	rs350894		rs5755694		rs1451637
				rs1823059				
				rs12609676				
				rs12459484				
				rs350887				

Table 2. Significant results of the analysis of *NGAL*, *BGN*, *MEK1*, *MEK2*, *HSP27*, and *YWHAZ* SNPs and AO variation taking into account intrafamilial nonindependency.

Gene	SNP	Genotypes	B	95% CI	*P*-value
NGAL	rs3780836	CC (reference)	–	–	–
		CT	−6.07	[−11.15; −0.98]	0.019
BGN **(**female group)	rs2269404	CC (reference)	–	–	–
		TT	10.48	[1.03; 19.93]	0.030
MEK1	rs8039880	AA (reference)	–	–	–
		GG	−7.02	[−14.03; 0.00]	0.050
	rs11630608	TT (reference)	–	–	–
		CC	−12.75	[−20.98; −4.53]	0.002
		CT	−9.38	[−14.21; −4.55]	$P < 0.001$
	rs16949939	CC (reference)	–	–	–
		CT	26.15	[14.34; 37.96]	$P < 0.001$
	rs745796	TT (reference)	–	–	–
		CC	−10.49	[−19.50; −1.47]	0.023
MEK2	rs1823059	CC (reference)	–	–	–
		TT	17.08	[4.13; 30.03]	0.010
HSP27	rs11769502	CC (reference)	–	–	–
		CT	−6.664	[−11.30; −2.02]	0.005
YWHAZ	rs17365305	GG (reference)	–	–	–
		GA	−6.759	[−12.97; −0.55]	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.

The role of *NGAL* and *BGN* genes

In *NGAL* gene, the CT genotype ($P = 0.019$) of rs3780836 was significantly associated with an earlier onset corresponding to a decrease of 6 years in mean AO (Table 2). For the other SNP assessed and for the haplotypic analyses, no significant results were found.

Since *BGN* gene is located in the X chromosome, the analyses were stratified by gender and the genotypic analyses were only performed in the female group.

Regarding the allelic analyses performed in the male patients group, no significant result was found associated with AO (data not shown).

Importantly, in the female group, the TT genotype ($P = 0.030$) of rs2269404 was significantly associated with a later AO, an increase of 10 years in disease onset (Table 2). In the haplotypic analyses performed for the female group, we found a significant result when the C-G-T-C-C-A-G haplotype is present ($P = 0.036$) associated with a later onset.

Regarding parental transmission for these genes, no significant differences were found.

MEK1 and *MEK2* genes and AO

For *MEK1* gene, we found four SNPs significantly associated with AO: the CC genotype ($P = 0.002$) and the CT genotype ($P < 0.001$) of rs11630608 and the CC genotype ($P = 0.023$) of rs745796 were associated with an earlier onset and this variation corresponds from 9 to 13 years in disease onset for these polymorphisms (Table 2). On the other hand, the CT genotype ($P < 0.001$) of rs16949939 was associated with a mean increase of 26 years in AO (Table 2).

Regarding the *MEK2* gene, we found that the TT genotype ($P = 0.010$) of rs1823059 was associated with a later AO, an increase of 17 years (Table 2).

In the haplotypic analyses, no significant results were found (data not shown).

Concerning parental transmission of the SNPs to the affected children, we found a differential transmission for allele C of rs11630608 and allele C of rs745796 in the *MEK1* gene. Nonaffected fathers transmitted more often than expected these alleles that are involved in an earlier onset. In addition, for the rs11630608, sons of non-affected fathers received more often than expected the C allele ($P = 0.012$), while for the rs745796, daughters of non-affected fathers received more often than expected the C allele ($P = 0.013$) (data not shown).

Regarding *MEK2* gene, for the rs1823059, we found that nonaffected fathers transmitted more often than expected the T allele that is associated with a later onset ($P = 0.015$). For the other SNPs, we did not find any significant differences in parental transmission (data not shown).

HSP27 and *YWHAZ* genes and AO variation

We found that the CT genotype ($P = 0.005$) of rs11769502 for *HSP27* gene and the GA genotype ($P = 0.033$) of rs17365305 for *YWHAZ* gene, were significantly associated with earlier onset and the difference corresponds to a decrease of 7 years in mean AO (Table 2). For these genes, we also performed haplotype-based analysis, but no differences were found (data not shown).

For these genes, no significant differences were found in parental transmission.

Functional impact and gene–gene interactions

To explore the functional impact of the SNPs associated with AO variation, we performed an in silico analysis using FuncPred and is-rSNP. Particular attention was paid to rs745796 of *MEK1* gene since some SNPs in LD (rs10851759, rs11071895, rs12914079, rs4776791, rs7403574, and rs8043062) may alter transcription factors' binding (TFB) sites. In addition, the is-rSNP algorithm highlighted that this SNP may also significantly affect the ability of one transcription factor to bind to DNA (LM120, $P = 0.001$) (Fig. 1). This analysis also predicts that rs537 (which is in LD with rs745796) may affect microRNA binding sites.

Additionally, we found that rs11071896 and rs17851970 (which are in LD with rs11630608 of *MEK1*), rs1030986 and rs16953566 (which are in LD with rs16949939 of *MEK1*) may alter the recognition sites for splicing regulatory factors.

A strong synergistic interaction was found with the MDR analysis, as shown in the dendogram (Fig. 2) for the best model, between the rs17577 of the *MMP-9* gene

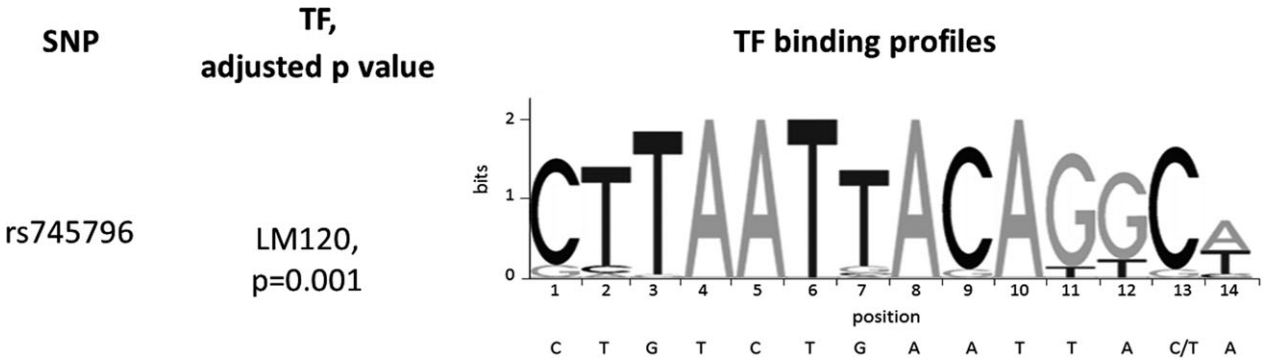

Figure 1. The sequence logo of the one transcription factor (TF) binding site potentially disrupted by *MEK1* rs745796.

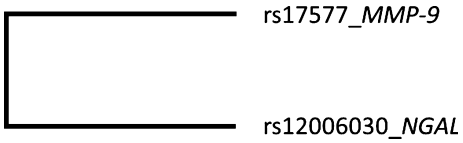

Figure 2. Dendogram showing a strong synergistic interaction among neutrophil gelatinase-associated lipocalin (*NGAL*)-*MMP-9* genes (darker lines suggest a synergistic relationship – shorter the lines, stronger the interaction).

and rs12006030 of the *NGAL* gene, with a testing balanced accuracy (TBA) of 0.59 and a cross-validation consistency (CVC) of 10/10. After permutation testing, this model was still significant ($P = 0.037$).

Discussion

Research on age-at-onset (AO) variation has been central in several dominant diseases including FAP ATTRV30M, since it might lead to a better understanding of the disease pathogenesis mechanisms. Thus, this study addresses the identification of variants of possible candidate genes as AO genetic modifiers in FAP ATTRV30M. To the best of our knowledge, this is the first study that examines the association of these six potential candidate genes (*NGAL*, *BGN*, *MEK1*, *MEK2*, *HSP27*, and *YWHAZ*) linked to several FAP ATTRV30M signaling pathways with AO, using a family-centered approach.

NGAL and *BGN* variants associated with AO variation

We examined variants in genes linked to remodeling of the extracellular matrix (ECM)-related components as *NGAL*, *MMP-9*, and *BGN* due to its overexpression in FAP ATTRV30M. In our sample, we found that the CT genotype of rs3780836 in the *NGAL* gene was associated with an earlier onset and we hypothesize that this variant could be a genetic risk factor for the FAP ATTRV30M patients. On the other hand, we found that in the female group, the rare genotype (TT) of rs2269404 of the *BGN* gene was associated with a later onset, leading us to suggest that this variant can have a possible protective effect in females. We also performed an MDR analysis for detection of gene–gene interaction, which is a powerful statistical tool of multilocus data reduction to improve the detection of genotypic combinations that predict disease risk.[21] We found a strong synergistic interaction between *NGAL* and *MMP-9* genes, confirmed by a 1000-fold permutation test. In addition, this study confirm the data already described in a previous study, using FAP ATTRV30M nerve and salivary glands biopsies, which showed that NGAL forms a complex with MMP-9 and where expression of these genes seems to overlap.[13] Therefore, this was the first study that explored the possible involvement of the variants of these genes associated with AO variation, using a family-centered approach. Furthermore, NGAL and MMP-9 were only overexpressed at a later stage when amyloid fibrils were already present, while BGN was upregulated in the earliest stages of TTR deposition, when nonfibrillar TTR aggregates were already present, but could coexist with TTR fibrils.[13] Similarly, Cardoso et al., 2008 corroborated the observations reported for human tissues,[13] but using an FAP ATTRV30M transgenic mice model.[22]

Role of *MEK1* and *MEK2* variants in AO variability

Although molecular signaling mechanisms in FAP ATTRV30M are not fully understood, a previous study provides evidence for the involvement of the MEK-ERK MAPK signaling pathway in disease pathogenesis.[14] Therefore, we selected *MEK1/2* and *ERK1/2* as candidate genes due to their role as mediators of the cytotoxic effects of TTR aggregates in different stages of disease progression.

In a study using human FAP ATTRV30M nerve biopsies, MEK1/2 activation was found upregulated in both asymptomatic carriers and patients when compared to controls. Furthermore, phosphorylation levels of MEK1/2 were decreased in later symptomatic stages.[14] MEK1/2 is activated after phosphorylation and may lead to ERK1/2 activation in response to a variety of hormones, growth factors, and oxidative stress, which can regulate transcription and translation.[23,24] When ERK signaling cascade is early activated, it can lead to increased cell proliferation and TTR aggregates expression levels. This will lead to cytotoxic effects by TTR aggregates and to an earlier AO.[14] Likewise, it was shown in peripheral nerves of a FAP transgenic mouse model an increased ERK1/2 activation when TTR deposits occurs when compared to control animals, where older animals (17 months) had twice the activation of younger ones (2 months).[14] Additionally, it was shown a sequential activation of MEK1/2 and ERK1/2 in brains with early stage of neurofibrillary degeneration.[23]

We found that four variants in *MEK1* were associated with an earlier AO and one variant associated with a later disease onset. Interestingly, we found a differential parental transmission regarding rs11630608 and rs745796 in the *MEK1* gene where the nonaffected fathers added a genetic risk effect to AO variation. Moreover, sons of nonaffected fathers in the case of rs11630608 and daughters of nonaffected fathers in the case of rs745796 have an increased susceptibility for earlier AO when they receive

the rare allele (C). Additionally, we found that the rs1823059 TT genotype of *MEK2* was associated with a later AO. Furthermore, for the rs1823059 in the *MEK2* nonaffected fathers added a protector genetic effect to AO variation leading to a later AO.

The adverse or protective effects associated with early- and late-onset of MEK1/2 variants point to an effect of these genes in our sample. We also found a possible modulatory effect on AO associated with the noncarrier chromosome (a transacting effect).

In silico analysis revealed some variants in LD with rs745796 of *MEK1* gene that may alter binding of the transcription factors LM120 promoting upregulation of this gene. Furthermore, LM120 was predicted to have more affinity when the C allele is present, reinforcing our genotype analysis where the CC genotype was found to be associated with individuals with an earlier AO. As in other studies, this could lead to an early activation of this pathway.[23] Additionally, we also found other alterations that could influence the regulation of gene expression regarding microRNA binding sites and splicing regulatory factors. Therefore, the inhibition or activation of the factors involved in the *MEK1/2* signaling cascade can be good targets for the development of novel therapeutic approaches.

HSP27 and *YWHAZ* variants and AO

Several studies have reported the essential role of the heat shock proteins (HSPs) in various neurodegenerative disorders associated with protein aggregation since these are considered important for cellular defense mechanisms. Thus, it was already demonstrated that in the presence of protein misfolding and aggregation, a neuroprotective stress response mediated by HSPs can be induced in Alzheimer's disease (AD),[25] Parkinson's disease[26], and Huntington's disease.[27] However, activation of heat shock transcription factor 1 (HSF) is required for upregulation of the HSP synthesis. In a previous study, it was shown that in FAP ATTRV30M human nerve, skin, and salivary gland biopsies with extracellular TTR deposits, there is induction of intracellular activation of HSF1 and consequently an increase in the expression of HSP27 and HSP70.[15]

In this study, we selected the *HSP27* gene in order to investigate if it influences AO variation, since HSP27 upregulation was only observed in tissues with extracellular TTR deposition.[15] We found that rs11769502 of *HSP27* was associated with an earlier onset, reinforcing the important role of *HSP27* in FAP ATTRV30M. Therefore, the effect of this variant could induce an early neuroprotective intracellular stress response by increasing *HSP27* expression, activating the cell defense mechanism to prevent neurodegeneration in FAP ATTRV30M.

As with HSPs, YWHAZ protein might act as a neuroprotection mechanism against toxicity in a variety of neurodegenerative diseases with common cellular and molecular mechanisms including protein aggregation since this may function as a sweeper of misfolded proteins.[28] In a previous study in AD, the authors found that YWHAZ stimulates tau phosphorylation[29] and is upregulated in the patients' brains.[30] In another study, it was shown the specificity of TTR to regulate YWHAZ levels and decreased YWHAZ protein expression in the hippocampus of young/adult TTR null mice when compared to TTR wild-type animals.[17] Also, it was shown that YWHAZ expression levels decrease with aging.[16]

We found that rs17365305 of *YWHAZ* gene was associated with an earlier onset, leading us to suggest that in the presence of this variant, the potential risk effect may be increased and the YWHAZ-related defense mechanisms blocked. Therefore, the modulation of this variant will be important in order to protect early-onset patients.

In conclusion, the results of our study provide evidence for an association of DNA noncoding variants of genes in FAP ATTRV30M pathways that may have phenotypic implications, particularly, in AO variation. However, our study does not preclude the possibility that other genes involved in these or other pathways may act as genetic modifiers of AO. Although an in silico analysis has been performed to predict functional impact of significant variants, functional studies will be important to deepen our knowledge. Moreover, in the future, it would also be interesting to replicate our study in other FAP ATTRV30M populations.

Therefore, with this study, we reveal for the first time, using a family-centered approach, that variants of *NGAL*, *BGN*, *MEK1*, *MEK2*, *HSP27*, and *YWHAZ* may act as potential genetic modulators of AO in FAP ATTRV30M, which could be useful for the development of novel therapeutic approaches, improve patient care, and aid in the genetic counseling of mutation carriers.

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Author Contributions

CL, AS: Conception and design of the study. TC, DS, MA-F, DM, CL, AS: Acquisition and analysis of data. DS, CL, AS: Drafting a significant portion of the manuscript or figures. TC, MA-F, DM, JS, IA, AS: Critical revision of the manuscript for important intellectual content. DM, CL, AS: Statistical expertize. TC, JS, IA, CL, AS: Obtaining funding. MA-F, IA, CL: Administrative, technical, or material support. JS, CL, AS: Study supervision.

Conflicts of Interest

D. Santos has received research support from a FCT fellowship (SFRH/BD/91160/2012) and received funding from Pfizer Inc for scientific meeting expenses (travel, accommodations, and registration). T. Coelho's institution has received support from FoldRx Pharmaceuticals, which was acquired by Pfizer Inc in October 2010; T. Coelho has served on the scientific advisory board of Pfizer Inc and 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. M. Alves-Ferreira has received research support from a FCT fellowship (SFRH/BD/101352/2014) and received funding from Pfizer Inc for scientific meeting expenses (travel, accommodations, and registration). J. Sequeiros, D. Mendonça, I. Alonso, C. Lemos, and A. Sousa report no disclosures.

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