PROMOTER VARIATION AND EXPRESSION LEVELS OF INFLAMMATORY GENES *IL1A, IL1B, IL6* AND *TNF* IN BLOOD OF SPINOCEREBELLAR ATAXIA TYPE 3 (SCA3) PATIENTS

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ABSTRACT

Age at onset in spinocerebellar ataxia type 3 (SCA3/MJD) is incompletely explained by the size of the CAG tract at the *ATXN3* gene, implying the existence of genetic modifiers. A role of inflammation in SCA3 has been postulated, involving altered cytokines levels; promoter variants leading to alterations in cytokines expression could influence onset. Using blood from 86 SCA3 patients and 106 controls this work aimed to analyse promoter variation of four cytokines (*IL1A, IL1B*, *IL6* and *TNF*) and to investigate the association between variants detected and their transcript levels, evaluated by quantitative PCR. Moreover, the effect of APOE isoforms, known to modulate cytokines, was investigated. Correlations between cytokine variants and onset were tested; the cumulative modifier effects of cytokines and APOE were analysed. Patients carrying the IL6*C allele had a significant earlier onset (4 years in average) than patients carrying the G allele, in agreement with lower mRNA levels produced by IL6*C carriers. The presence of APOE*ε2 allele seems to anticipate onset in average 10 years in patients carrying the IL6*C allele; a larger number of patients will be needed to confirm this result. These results highlight the pertinence of conducting further research on the role of cytokines as SCA3 modulators, pointing to the presence of shared mechanisms involving *IL6* and *APOE.*

KEYWORDS: MJD, polyglutamine disease, mRNA levels, cytokines

INTRODUCTION

Spinocerebellar ataxia type 3 (SCA3/MJD; MIM#109150; ORPHA98757) is the most common spinocerebellar ataxia worldwide. The number of coding CAG repeats at the causative locus, *ATXN3*, explains from 50% to 75% of the age at onset variance (revised in Bettencourt and Lima 2011) therefore implying the existence of additional familial factors, namely genetic. Several genetic modifiers have been proposed: the number of CAG repeats at several expansion loci (Jardim et al. 2003; Raposo et al. 2015; Tezenas du Montcel et al. 2014); allelic variants at the apolipoprotein E (*APOE)* (Bettencourt et al. 2011; Peng et al. 2014) and glucosidase, beta, acid (*GBA)* genes (Siebert et al. 2012); variation in the 3'UTR at the *ATXN3* gene (Long et al. 2015) as well as the size of the normal SCA3 allele (França et al. 2012). In the *ATXN3* gene a repeat expansion above 50 triplets encodes an abnormally long polyglutamine (polyQ) stretch in the ataxin-3 protein (Maciel et al. 2001); mutant ataxin-3 is prone to misfolding and aggregation, triggering a cascade of pathological events (Evers et al. 2014). The putative role of inflammation, namely the behaviour of interleukine 1 alpha (IL1A), interleukine 1 beta (IL1B), interleukine 6 (IL6) and tumor necrosis factor (TNF), has been investigated in polyQ diseases (Olejniczak et al. 2015). In SCA3 brain tissue, IL1β and IL6 staining was found to be enhanced, as compared to controls; activated microglia and reactive astrocytes have also been observed (Evert et al. 2001, 2003, 2006). Recently, eotaxin was found to be higher in serum of SCA3 asymptomatic carriers and in patients (da Silva Carvalho et al. 2015). *IL1A* c.-889C>T, *IL1B* c.-511C>T, *IL6* c.-174G>C and *TNF* c.-308G>A localized at the promoter of respective cytokine genes have been related *in vitro*, *ex* and *in vivo* studies with differences in mRNA and/or protein levels of these cytokines (Dominici et al. 2002; Fishman et al. 1998; Hall et al. 2004; Wilson et al.

1997). Moreover, a link between APOE and cytokines has been investigated, since APOE modulates inflammatory and immune responses in an isoform-dependent manner (Zhang et al. 2011). We have previously shown that the APOE*ε2 allele was significantly associated with an earlier age at onset in a cohort of SCA3 Azorean patients (Bettencourt et al. 2011).

Given a possible role of inflammation in SCA3, we hypothesised that promoter variants leading to alterations of expression levels of cytokines could influence disease manifestation, namely onset. Using peripheral blood from a homogenous Azorean cohort of SCA3 patients the present work aimed to analyse variants in the promoter regions of four main cytokines: *IL1A* c.-889C>T (rs1800587), *IL1B* c.-511C>T (rs16944), *IL6* c.-174G>C (rs1800795) and *TNF* c.-308G>A (rs1800629), and to investigate the association between these variants and the respective transcript levels. Genotype-phenotype correlations were performed to test the loci previously reported as potential modifiers of SCA3 onset. Moreover, the cumulative modifier effects of cytokines loci and APOE were also tested.

SUBJECTS AND METHODS

Subjects

Eighty six Azorean SCA3 patients, confirmed as carriers of the *ATXN3* mutation and 106 apparently healthy controls, were included in this study. Controls were selected taking into account the ancestry, age and gender distribution of cases. The size of the (CAG)n tract was determined as previously reported (Bettencourt et al. 2008). Age at onset, defined as the age of appearance of gait disturbance and/or diplopia reported by the patient and/or a close relative, was recorded during clinical assessments performed at the Department of Neurology (Hospital do Divino Espírito Santo - HDES, Ponta Delgada). *APOE* genotypes from SCA3 patients were considered for statistical analysis; genotyping was performed as in Bettencourt and colleagues (Bettencourt et al. 2011). All samples were collected after informed consent. This study is a part of a project approved by the Ethics Committee of HDES.

DNA isolation and multiplex PCR-RFLP

DNA was extracted from all samples using standard procedures. A multiplex PCR–Restriction Fragment Length Polymorphism (RFLP) was developed to analyse variants in the promoter of four cytokines: *IL1A* c.-889C>T (rs1800587), *IL1B* c.-511C>T (rs16944), *IL6* c.-174G>C (rs1800795) and *TNF* c.-308G>A (rs1800629). The set of primers (0.2µM of each one per reaction) for each cytokine variant as well as multiplex PCR-RFLP reactions mixture and conditions are described in supplementary Table 1.

RNA isolation and qPCR

A subset of 54 SCA3 patients were selected to measure cytokines mRNA levels. Signs of inflammatory or infective conditions were annotated by accessing the clinical records of patients; patients presented any of the abovementioned conditions were not included. mRNA cytokine levels were also determined in 33 controls. Four pre-validated TaqMan Gene Expression Assays (Hs00174092_m1, Hs01555410_m1, Hs00985639_m1 and Hs99999043_m1 from Applied Biosystems) were used to measure cytokines mRNA levels. RNA isolation and quantification, cDNA synthesis, quantitative PCR (qPCR) conditions, as well as calculation of relative expression values have been performed as described elsewhere (Raposo et al. 2015).

Statistical analysis

Allele and genotype frequencies were estimated for all analysed loci and Hardy-Weinberg equilibrium (HWE) was tested. Allelic/genotypic frequencies for controls (N=106) were compared with available data for other European and non-European populations. An ANCOVA, using age at sampling as covariate, was run to compare transcript levels between cytokine genotypes. The effects of the CAG length in expanded allele on age at onset, as well as the presence/absence of each cytokine allelic variant were assessed using a linear fitting model. Equality of variances between groups was verified by the Levene's test. An ANCOVA, using the CAG length in expanded allele as covariate, was conducted to compare estimated age at onset between: (1) cytokine genotypes; or (2) cytokine alleles; or (3) interaction of APOE*ε2 allele and cytokine genotypes; or (4) interaction of APOE*ε2 allele and allelic variants.

For two or more pairwise comparisons (comparisons between cytokine genotypes), p-values were adjusted using the Bonferroni procedure. Significant effects resulting from the ANCOVA comparisons, obtained only for IL6 allelic variants, were further tested. These correlations were confirmed: a) using a generalized estimating equation test, where kinship was used as repetitive measure within-subjects; and b) using the subset of 38 unrelated SCA3 patients (patients who shared grand-parents were considered related). All statistical analyses were performed in IBM SPSS Statistics 22 (IBM Corp. Released 2013). A statistically significant result lower than 0.05 was considered for all tests performed.

RESULTS

Gender and age at collection for the studied subjects are shown in Figure 1. Genotypes for the *ATXN3* locus (N=86) in patients, as well as relevant clinical data are displayed in Figure 1.

Loci were in conformity with Hardy-Weinberg equilibrium expectations, with the exception of IL1A and IL6 loci in SCA3 patients (supplementary Table 2). Pairwise differentiation exact test failed to detect significant differences in allelic or genotypic *IL1A, IL1B* and *TNF* frequencies between SCA3 patients and population-matched controls. At the IL6 locus, a statistically significant difference was obtained when comparing all patients with controls, which should reflect the excess of the C allele in the patients group; no differences, however, were detected when considering only unrelated patients (supplementary Table 2).

In SCA3 patients, no significant differences were obtained when comparing mRNA levels by genotypes (Figure 2). The effects of promoter allelic variants on mRNA levels were confirmed: the IL1A*T allele, the IL1B*T allele, the IL6*G allele and the TNF*A allele were associated with higher mRNA levels (Figure 2), in accordance with previous studies. The mRNA levels by cytokine genotype varied similarly in controls (data not shown).

A negative correlation between the size of *ATXN3* expanded allele of SCA3 patients and age at onset was observed (N=86, $r=-0.804$, $p<0.0005$). The explanation of the age at onset variance provided by the CAG length in expanded allele was 65% ($F=154.1$, $p<0.0005$). An improvement of the previously model was observed only when the presence/absence of the IL6*C variant was added, which significantly contributed to

the variance of the age at onset, by additionally explaining 1.9% (N=86, Part correlation coefficient=0.138, $p<0.05$).

Patients carrying the IL1A*T allele or the IL1B*T allele or the IL6*C allele all showed a tendency for an earlier age at onset (adjusted for mean CAG length) compared to patients homozygous for IL1A*C allele or the IL1B*C allele or the IL6*G allele (supplementary Table 3). Age at onset was anticipated by 4 years in average in patients carrying the IL6*C (N=66) (*F(1,83)*=4.7, p=0.03). The use of a generalized estimating equation test accounting for relatedness, and the earlier onset in patients carrying the IL6*C allele confirmed that this result was not due to patients relatedness (Wald X^2 = 3.8; p=0.05); the same tendency was observed when analysing only unrelated patients (N=38). In the present cohort (N=86), the presence of the APOE*ε2 allele explained 3.4% (p=0.003) of variance in age at onset. The presence of *APOE**ε2 allele significantly anticipated onset in average 10 years in patients carrying one or two copies of the IL6*C allele (p=0.005, Figure 3). Fitting a linear model, estimated age at onset (*F(3,81)*=63.001, $p<0.0005$) using the *APOE* and *IL6* loci alongside with the CAG size in expanded allele could be calculated applying the formula: age at onset = 230.117 – 2.686 x (CAG_n in expanded allele) – 3.272 x (presence/absence of IL6*C allele) – 6.911 x (presence/absence of APOE*ε2 allele).

DISCUSSION

A significant association between *IL6* c.-174G>C variation and age at onset was, nevertheless, identified; patients carrying the IL6*C allele presented, in average, an onset four years earlier than the one displayed by patients homozygous for the G allele. We further observed a tendency for lower mRNA levels in patients carrying the IL6*C allele compared to GG homozygous, a result which is in agreement with previous findings (Fishman et al. 1998). Fishman and colleagues had suggested that *IL6* c.-174G>C variation is near to a glucocorticoid receptor (GR) binding site as well as G>C position could potentiate the creation for a binding site for the transcription factor nuclear factor 1 (NF1), implying, in both cases, the repression of transcription (Fishman et al. 1998). In our cohort, the low mRNA levels of IL6 produced by the IL6*C carriers were associated with a premature SCA3 onset, suggesting that in SCA3 patients' cells, such low levels could negatively contribute to cellular dysfunction, leading to the premature appearance of the first symptoms. Nishimura and colleagues (Nishimura et al. 2001) previously reported an association between IL1B*C allele and SCA6 onset; however, this association was not confirmed in our cohort of patients. In this study, an anticipation of onset (average of 10 years) was observed in patients carrying the APOE*ε2 allele and one or two copies of the IL6*C allele. Although there is no published data for IL6, APOE is known to suppress the secretion of TNF and IL1B, the APOE*ε2 isoform being associated with the lowest levels of secretion (Zhang et al. 2011).

Even considering the homogeneity features of our patient's cohort, since sample size in this study is limited, the genotype-phenotype associations described should be replicated in a larger Azorean sample, when available, as well as in independent

cohorts. Globally, results highlight the pertinence of further research on the role of cytokines as modulators of SCA3 onset, pointing to the presence of shared mechanisms involving *IL6* and *APOE.*

COMPETING INTERESTS

The authors declare no competing interests.

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310

311 FIGURE LEGENDS

312 Figure 1. Demographic data and clinical features for studied individuals.

313 Figure 2. Cytokines mRNA levels (shown as $2^{-\Delta Ct}$) by genotypes in 54 SCA3 patients.
314 Expression values were adjusted for age at blood collection (50 years).). In the
315 comparisons performed by *IL1A* or *IL6* genotype, Bonferroni adjusted p-values were
316 obtained by an ANCOVA procedure. *IL1B* and *TNF* mRNA levels were not successfully
317 quantified for one patient.

318 Figure 3. Estimated age at onset was calculated taking in consideration the cumulative
319 effect of APOE*e2 allele and IL6 variation. ≠[1]The difference was in average of 4 years
320 (ANCOVA, p=0.03). ≠[2]The difference was in average of 10 years (t-test, p=0.005). T-test
321 was calculated using the OpenEpi, version 3.03 (Dean AG, Sullivan KM, Soe MM. OpenEpi:
322 Open Source Epidemiologic Statistics for Public Health. www.OpenEpi.com, updated in
323 2014/09/22). APOE genotype was not successfully obtained in one patient.

324

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Multiplex PCR primers, size of amplified fragments, restriction enzymes, size of restriction fragments as well as PCR-RFLP reactions mixture and conditions.

Supplementary Table 2. Genotypic, allelic frequencies and p-values for the Hardy-Weinberg equilibrium test for each cytokine loci studied in all and unrelated SCA3 patients and population-matched controls. Differentiation exact test p-values are also shown.

Supplementary Table 3. Genetic and clinical features of the 86 SCA3 patients divided by cytokines alleles, as well as presence/absence of APOE*ε2 allele.

Figure 1

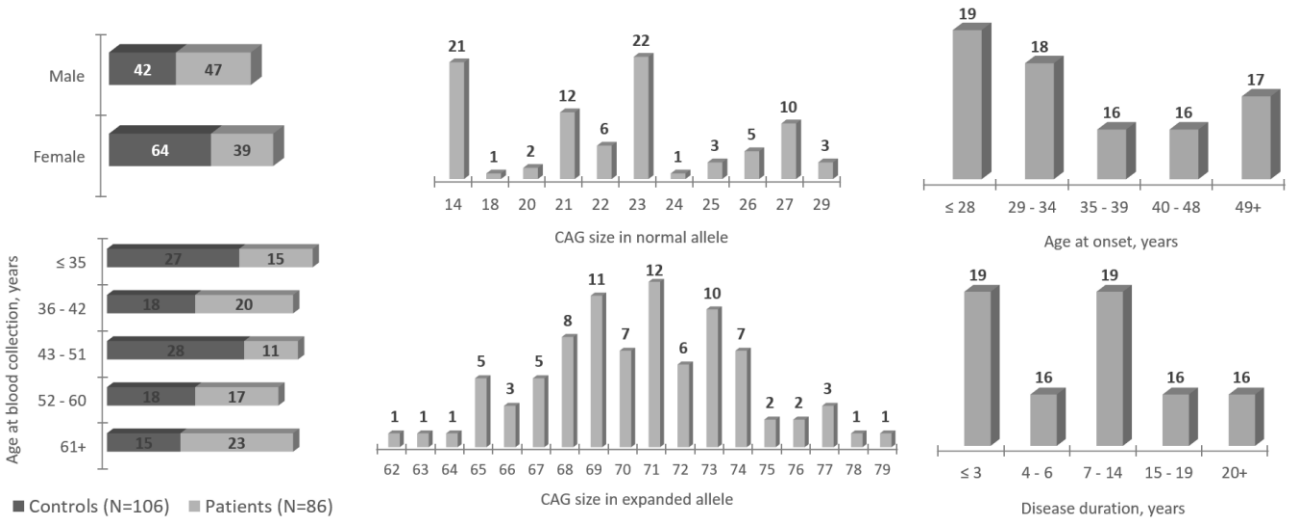


Figure 2

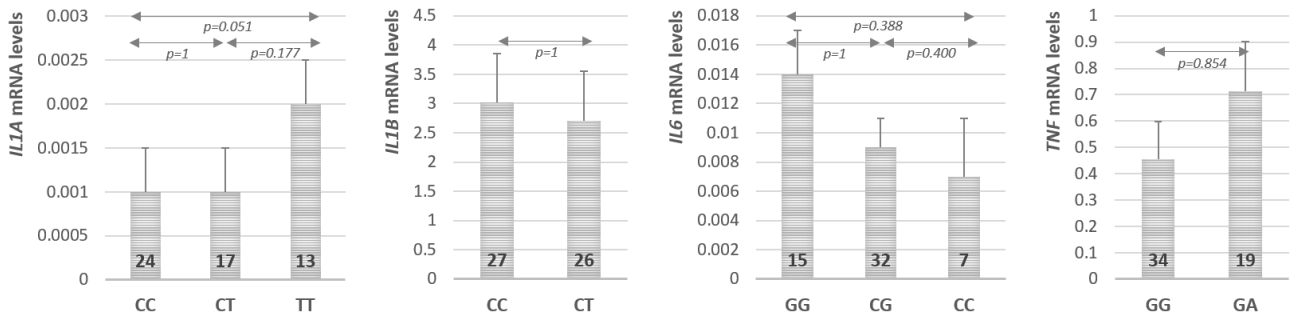
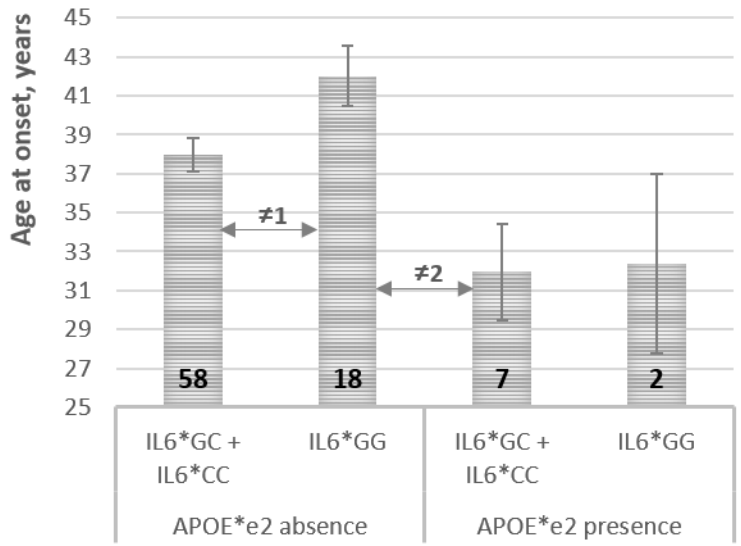


Figure 3



Supplementary Table 1. Multiplex PCR primers, size of amplified fragments, restriction enzymes, size of restriction fragments as well as PCR-RFLP reactions mixture and conditions.

	PCR		**RFLP**	
	Primers sequence 5' – 3'	**Product size (bp)**		
IL1α c.-889C>T				
IL1α-F[1]	TGTTCTACCACCTGAACTAGGC	99	*NcoI*	
IL1α-R[1]	TTACATATGAGCCTTCCATG		C → 79 + 20	T → 99
IL1β c.-511C>T				
IL1β-F[2]	TGGCATTGATCTGGTTCATCCA	244	*AvaI*	
IL1β-R	CCTGTCTGTATTGAGGGTG		C → 190 + 54	T → 244
IL6 c.-174G>C				
IL6-F	CAGAAGAACTCAGATGACTGGT	377	*NcoI*	
IL6-R	TGCAATGTGACGTCCTTTAC		G → 351 + 26	C → 377
TNFα c.-308G>A				
TNFα-F[3]	GAGGCAATAGGTTTTGAGGGCCAT	147	*NcoI*	
TNFα-R[3]	GGGACACACAAGCATCAAG		G →126 + 21	A → 147
Reaction mixture				
	0.4mM each dNTP 1x NH4 Buffer 3mM MgCl2 solution 1x HiSpec solution 2U BIOTAQ DNA polymerase (Bioline) 200ng genomic DNA		1X reaction buffer Tango (10X) 5U NcoI 5U AvaI (Thermo Fisher Scientific) 3µl PCR product	
	Total volume	*25µl*		*10µl*
Thermocycler/Thermoblock conditions				
	initial denaturation: 95°C, 5min *34 cycles* *denaturation: 95°C, 30s* *annealing: 58°C, 90s* *extension: 72°C, 45s* final extension: 72°C , 10min		Incubation: overnight, 37°C (the digested product run on a 14% PAGE gel and was revealed using a silver nitrate standard protocol)	

_ mismatch primers (point mutation)

[1]Primers pair: De Freitas NM, Imbronito A V., Neves AC, Nunes FD, Pustiglioni FE, Lotufo RFM. Analysis of IL-1A(-889) and TNFA(-308) gene polymorphism in Brazilian patients with generalized aggressive periodontitis. Eur Cytokine Netw. 2007;18(3):142–7; [2]Primer forward: Brett PM, Zygogianni P, Griffiths GS, Tomaz M, Parkar M, D'Aiuto F, et al. Functional Gene Polymorphisms in Aggressive and Chronic Periodontitis. J Dent Res. 2005 1;84(12):1149–53; [3]Primers pair: Moorchung N, Srivastava AN, Gupta NK, Ghoshal UC, Achyut BR, Mittal B. Cytokine gene polymorphisms and the pathology of chronic gastritis. Singapore Med J. 2007;48(5):447–54.

Supplementary Table 2. Genotypic, allelic frequencies and p-values for the Hardy-Weinberg equilibrium test for each cytokine loci studied in all and unrelated SCA3 patients and population-matched controls. Differentiation exact test p-values are also shown.

			SCA3 patients		Controls (C), N=106	*Differentiation test p-value#*	
			All, N=86	Unrelated (U), N=38		C. *versus* all	C. *versus* U.
IL1α c.-889C>T (rs1800587)							
Frequencies	Genotype	CC	0.424	0.474	0.491		
		CT	0.365	0.263	0.396	0.139	0.200
		TT	0.212	0.263	0.113		
	allele	C	0.606	0.605	0.689	0.099	0.153
		T	0.394	0.395	0.311		
			Hardy-Weinberg equilibrium				
			0.038	0.006	0.495		
IL1β c.-511C>T (rs16944)							
Frequencies	Genotype	CC	0.558	0.500	0.462		
		CT	0.419	0.474	0.481	0.136	0.523
		TT	0.023	0.026	0.061		
	allele	C	0.767	0.737	0.703	0.181	0.555
		T	0.233	0.263	0.297		
			Hardy-Weinberg equilibrium				
			0.141	0.403	0.163		
IL6 c.-174G>C (rs1800795)							
Frequencies	Genotype	GG	0.232	0.395	0.452		
		GC	0.628	0.447	0.462	0.002	0.253
		CC	0.140	0.158	0.086		
	allele	G	0.546	0.618	0.684	0.006	0.261
		C	0.454	0.382	0.316		
			Hardy-Weinberg equilibrium				
			0.019	0.739	0.316		
TNFα c.-308G>A (rs1800629)							
Frequencies	Genotype	GG	0.640	0.579	0.755		
		GA	0.337	0.421	0.226	0.121	0.122
		AA	0.023	0	0.019		
	allele	G	0.808	0.790	0.868	0.126	0.141
		A	0.192	0.210	0.132		
			Hardy-Weinberg equilibrium				
			0.726	0.171	1.000		

#p-value was calculated by exact G test in Genepop software (Raymond M. & Rousset F, 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J. Heredity, 86:248-249 Rousset, F., 2008. Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Mol. Ecol. Resources 8: 103-106).
Pairwise differentiation exact test (Excoffier L, Laval G, Schneider S. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol Bioinform Online. 2005;1:47–50) for genotypic frequencies between apparently healthy Azorean individuals versus individuals from:

(1) mainland Portugal – no differences significant for IL1β, IL6 and TNFα loci, no data available for IL1α locus (REFs);
(2) Europe – differences significant for IL6 and TNFα loci, not significant for IL1α and IL1β loci;
(3) Asia – differences significant for IL1α, IL1β and TNFα loci, no data available for IL6 locus;
(4) Africa – differences significant for IL1α and IL1β loci, not significant for TNFα locus, no data available for IL6 locus.
Genotypes from European, Asiatic and African samples were obtained in dbSNP, NCBI (http://www.ncbi.nlm.nih.gov/snp/).

Supplementary Table 3. Genetic and clinical features of the 86 SCA3 patients divided by cytokines alleles, as well as presence/absence of APOE*ε2 allele.

			CAG length			
	Alleles	N	Normal allele	Expanded allele	Age at onset*	Disease duration
IL1A c.-889C>T	C	37	22±4	71±3	39±1	12±8
	T	48	21±5	70±4	37±1	11±9
IL1B c.-511C>T	C	49	22±5	71±4	38±1	11±8
	T	37	22±5	70±4	38±1	11±9
IL6 c.-174G>C	G	20	20±5	70±4	41±2≠	11±7
	C	66	22±5	71±3	37±1≠	12±9
TNF c.-308G>A	G	55	22±4	71±3	37±1	12±9
	A	31	20±5	70±4	38±1	10±7
APOE ε2 allele	Absent	76	21±5	70±4	39±1≠	11±8
	Present	9	22±6	70±3	32±2≠	13±12

CAG length in normal and expanded allele, as well as disease duration is represented as mean ± standard deviation; *Age at onset was adjusted for mean CAG length and are represented as mean ± standard error; ≠ p<0.05 was considered statistically significant.