

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

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23 ABSTRACT

24 Age at onset in spinocerebellar ataxia type 3 (SCA3/MJD) is incompletely explained by
25 the size of the CAG tract at the *ATXN3* gene, implying the existence of genetic
26 modifiers. A role of inflammation in SCA3 has been postulated, involving altered
27 cytokines levels; promoter variants leading to alterations in cytokines expression could
28 influence onset. Using blood from 86 SCA3 patients and 106 controls this work aimed
29 to analyse promoter variation of four cytokines (*IL1A*, *IL1B*, *IL6* and *TNF*) and to
30 investigate the association between variants detected and their transcript levels,
31 evaluated by quantitative PCR. Moreover, the effect of APOE isoforms, known to
32 modulate cytokines, was investigated. Correlations between cytokine variants and
33 onset were tested; the cumulative modifier effects of cytokines and APOE were
34 analysed. Patients carrying the *IL6**C allele had a significant earlier onset (4 years in
35 average) than patients carrying the G allele, in agreement with lower mRNA levels
36 produced by *IL6**C carriers. The presence of APOE* ϵ 2 allele seems to anticipate onset
37 in average 10 years in patients carrying the *IL6**C allele; a larger number of patients
38 will be needed to confirm this result. These results highlight the pertinence of
39 conducting further research on the role of cytokines as SCA3 modulators, pointing to
40 the presence of shared mechanisms involving *IL6* and *APOE*.

41

42 KEYWORDS: MJD, polyglutamine disease, mRNA levels, cytokines

43

44 INTRODUCTION

45 Spinocerebellar ataxia type 3 (SCA3/MJD; MIM#109150; ORPHA98757) is the most
46 common spinocerebellar ataxia worldwide. The number of coding CAG repeats at the
47 causative locus, *ATXN3*, explains from 50% to 75% of the age at onset variance (revised
48 in Bettencourt and Lima 2011) therefore implying the existence of additional familial
49 factors, namely genetic. Several genetic modifiers have been proposed: the number of
50 CAG repeats at several expansion loci (Jardim et al. 2003; Raposo et al. 2015; Tezenas
51 du Montcel et al. 2014); allelic variants at the apolipoprotein E (*APOE*) (Bettencourt et
52 al. 2011; Peng et al. 2014) and glucosidase, beta, acid (*GBA*) genes (Siebert et al. 2012);
53 variation in the 3'UTR at the *ATXN3* gene (Long et al. 2015) as well as the size of the
54 normal SCA3 allele (França et al. 2012). In the *ATXN3* gene a repeat expansion above
55 50 triplets encodes an abnormally long polyglutamine (polyQ) stretch in the ataxin-3
56 protein (Maciel et al. 2001); mutant ataxin-3 is prone to misfolding and aggregation,
57 triggering a cascade of pathological events (Evers et al. 2014). The putative role of
58 inflammation, namely the behaviour of interleukine 1 alpha (IL1A), interleukine 1 beta
59 (IL1B), interleukine 6 (IL6) and tumor necrosis factor (TNF), has been investigated in
60 polyQ diseases (Olejniczak et al. 2015). In SCA3 brain tissue, IL1 β and IL6 staining was
61 found to be enhanced, as compared to controls; activated microglia and reactive
62 astrocytes have also been observed (Evert et al. 2001, 2003, 2006). Recently, eotaxin
63 was found to be higher in serum of SCA3 asymptomatic carriers and in patients (da
64 Silva Carvalho et al. 2015). *IL1A* c.-889C>T, *IL1B* c.-511C>T, *IL6* c.-174G>C and *TNF* c.-
65 308G>A localized at the promoter of respective cytokine genes have been related *in*
66 *vitro*, *ex* and *in vivo* studies with differences in mRNA and/or protein levels of these
67 cytokines (Dominici et al. 2002; Fishman et al. 1998; Hall et al. 2004; Wilson et al.

68 1997). Moreover, a link between APOE and cytokines has been investigated, since
69 APOE modulates inflammatory and immune responses in an isoform-dependent
70 manner (Zhang et al. 2011). We have previously shown that the APOE* ϵ 2 allele was
71 significantly associated with an earlier age at onset in a cohort of SCA3 Azorean
72 patients (Bettencourt et al. 2011).

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

83

84 SUBJECTS AND METHODS

85 *Subjects*

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

97

98 *DNA isolation and multiplex PCR-RFLP*

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

105

106 *RNA isolation and qPCR*

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

116

117 *Statistical analysis*

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

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

138 RESULTS

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

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

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

156 A negative correlation between the size of *ATXN3* expanded allele of SCA3 patients
157 and age at onset was observed (N=86, $r=-0.804$, $p<0.0005$). The explanation of the age
158 at onset variance provided by the CAG length in expanded allele was 65% ($F=154.1$,
159 $p<0.0005$). An improvement of the previously model was observed only when the
160 presence/absence of the *IL6**C variant was added, which significantly contributed to

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

163 Patients carrying the IL1A*T allele or the IL1B*T allele or the IL6*C allele all showed a
164 tendency for an earlier age at onset (adjusted for mean CAG length) compared to
165 patients homozygous for IL1A*C allele or the IL1B*C allele or the IL6*G allele
166 (supplementary Table 3). Age at onset was anticipated by 4 years in average in patients
167 carrying the IL6*C (N=66) ($F(1,83)=4.7$, $p=0.03$). The use of a generalized estimating
168 equation test accounting for relatedness, and the earlier onset in patients carrying the
169 IL6*C allele confirmed that this result was not due to patients relatedness (Wald $X^2 =$
170 3.8; $p=0.05$); the same tendency was observed when analysing only unrelated patients
171 (N=38). In the present cohort (N=86), the presence of the APOE* ϵ 2 allele explained
172 3.4% ($p=0.003$) of variance in age at onset. The presence of APOE* ϵ 2 allele significantly
173 anticipated onset in average 10 years in patients carrying one or two copies of the
174 IL6*C allele ($p=0.005$, Figure 3). Fitting a linear model, estimated age at onset
175 ($F(3,81)=63.001$, $p<0.0005$) using the APOE and IL6 loci alongside with the CAG size in
176 expanded allele could be calculated applying the formula: age at onset = $230.117 -$
177 $2.686 \times (\text{CAG}_n \text{ in expanded allele}) - 3.272 \times (\text{presence/absence of IL6*C allele}) - 6.911 \times$
178 $(\text{presence/absence of APOE*}\epsilon 2 \text{ allele})$.

179

180 DISCUSSION

181 A significant association between *IL6* c.-174G>C variation and age at onset was,
182 nevertheless, identified; patients carrying the IL6*C allele presented, in average, an
183 onset four years earlier than the one displayed by patients homozygous for the G
184 allele. We further observed a tendency for lower mRNA levels in patients carrying the
185 IL6*C allele compared to GG homozygous, a result which is in agreement with previous
186 findings (Fishman et al. 1998). Fishman and colleagues had suggested that *IL6* c.-
187 174G>C variation is near to a glucocorticoid receptor (GR) binding site as well as G>C
188 position could potentiate the creation for a binding site for the transcription factor
189 nuclear factor 1 (NF1), implying, in both cases, the repression of transcription (Fishman
190 et al. 1998). In our cohort, the low mRNA levels of IL6 produced by the IL6*C carriers
191 were associated with a premature SCA3 onset, suggesting that in SCA3 patients' cells,
192 such low levels could negatively contribute to cellular dysfunction, leading to the
193 premature appearance of the first symptoms. Nishimura and colleagues (Nishimura et
194 al. 2001) previously reported an association between IL1B*C allele and SCA6 onset;
195 however, this association was not confirmed in our cohort of patients. In this study, an
196 anticipation of onset (average of 10 years) was observed in patients carrying the
197 APOE*ε2 allele and one or two copies of the IL6*C allele. Although there is no
198 published data for IL6, APOE is known to suppress the secretion of TNF and IL1B, the
199 APOE*ε2 isoform being associated with the lowest levels of secretion (Zhang et al.
200 2011).

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

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

207 COMPETING INTERESTS

208 The authors declare no competing interests.

209

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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

325 SUPPLEMENTARY MATERIAL

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

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

333 Supplementary Table 3. Genetic and clinical features of the 86 SCA3 patients divided
334 by cytokines alleles, as well as presence/absence of APOE* ϵ 2 allele.

Figure 1

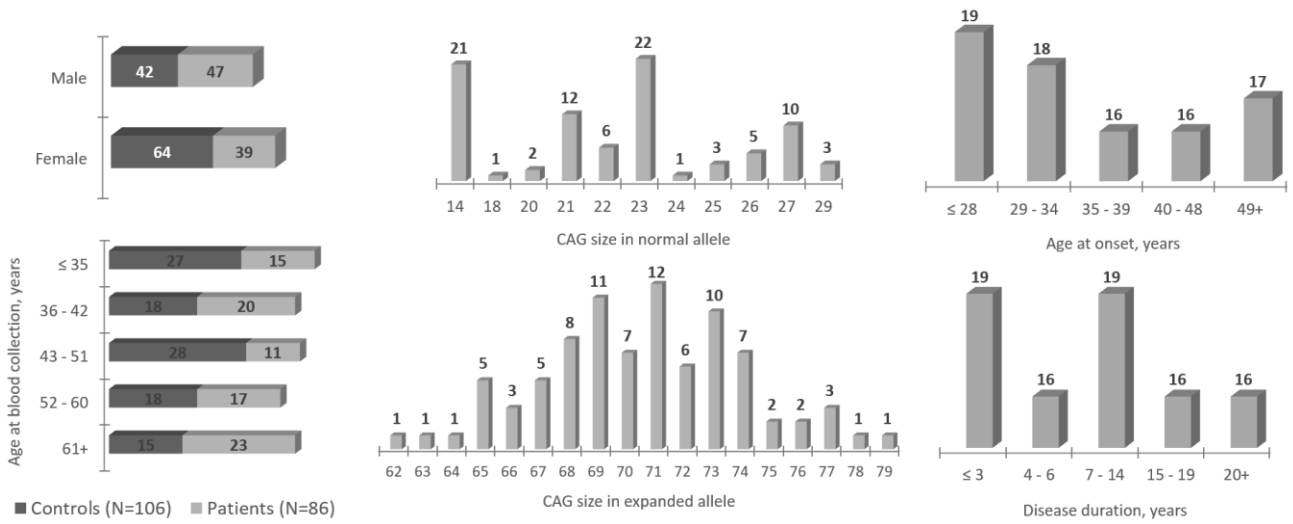


Figure 2

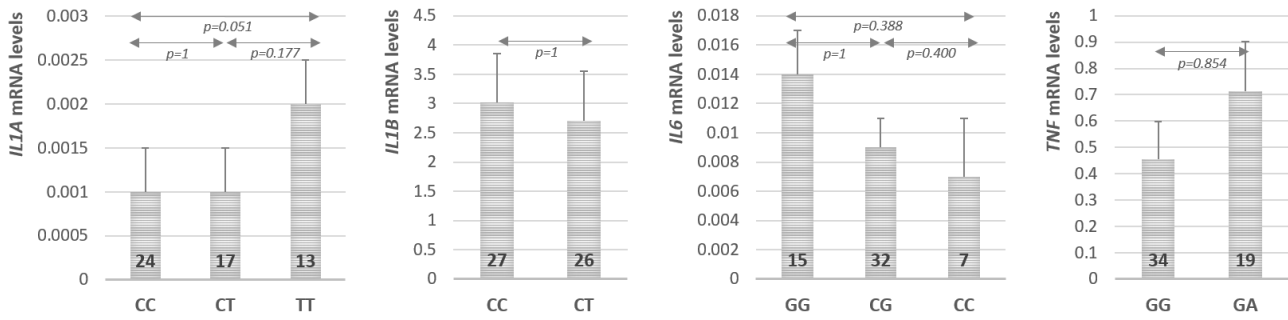
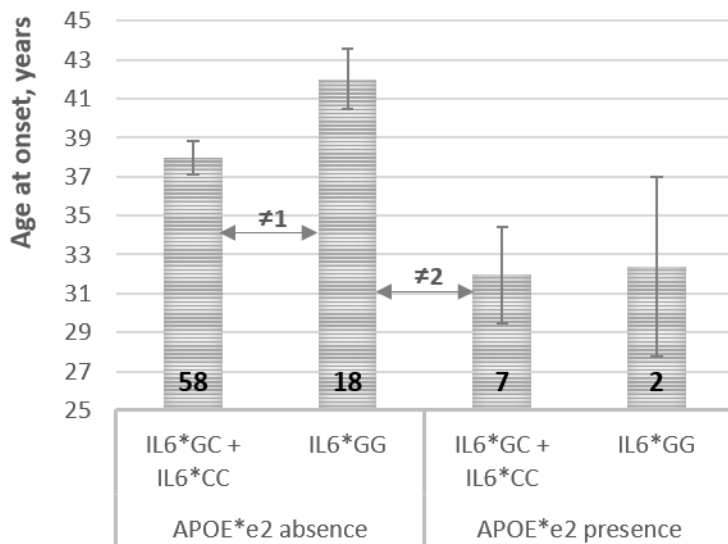


Figure 3



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) | |
| <i>IL1α c.-889C>T</i> | | | |
| IL1α-F ¹ | TGTTCTACCACCTGAACTAGGC | 99 | <i>NcoI</i> |
| IL1α-R ¹ | TTACATATGAGCCTTC ² CATG | | C → 79 + 20 T → 99 |
| <i>IL1β c.-511C>T</i> | | | |
| IL1β-F ² | TGGCATTGATCTGGTTCATCCA | 244 | <i>AvaI</i> |
| IL1β-R | CCTGTCTGTATTGAGGGTG | | C → 190 + 54 T → 244 |
| <i>IL6 c.-174G>C</i> | | | |
| IL6-F | CAGAAGAACTCAGATGACTGGT | 377 | <i>NcoI</i> |
| IL6-R | TGCAATGTGACGTCCTTTA ³ C | | G → 351 + 26 C → 377 |
| <i>TNFα c.-308G>A</i> | | | |
| TNFα-F ³ | GAGGCAATAGGTTTTGAGGG ³ CAT | 147 | <i>NcoI</i> |
| TNFα-R ³ | GGGACACACAAGCATCAAG | | G → 126 + 21 A → 147 |
| Reaction mixture | | | |
| | 0.4mM each dNTP | | |
| | 1x NH4 Buffer | | 1X reaction buffer Tango (10X) |
| | 3mM MgCl ₂ solution | | 5U <i>NcoI</i> |
| | 1x HiSpec solution | | 5U <i>AvaI</i> (Thermo Fisher Scientific) |
| | 2U BIOTAQ DNA polymerase (Bioline) | | 3μl PCR product |
| | 200ng genomic DNA | | |
| | <i>Total volume</i> | <i>25μl</i> | <i>10μl</i> |
| Thermocycler/Thermoblock conditions | | | |
| | initial denaturation: 95°C, 5min | | Incubation: overnight, 37°C |
| | 34 cycles | | (the digested product run on a |
| | denaturation: 95°C, 30s | | 14% PAGE gel and was |
| | annealing: 58°C, 90s | | revealed using a silver nitrate |
| | extension: 72°C, 45s | | standard protocol) |
| | final extension: 72°C, 10min | | |
| _ 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, Zygiogianni 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 | <i>Differentiation test p-value</i> [#] | | |
|--|----------|-----------------------------------|------------------------|------------------------|--|--------------|-------|
| | | 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 | 0.139 | 0.200 |
| | | CT | 0.365 | 0.263 | 0.396 | | |
| | | 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 | | |
| | | <i>Hardy-Weinberg equilibrium</i> | | | | | |
| | | 0.038 | 0.006 | 0.495 | | | |
| IL1β c.-511C>T (rs16944) | | | | | | | |
| Frequencies | Genotype | CC | 0.558 | 0.500 | 0.462 | 0.136 | 0.523 |
| | | CT | 0.419 | 0.474 | 0.481 | | |
| | | 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 | | |
| | | <i>Hardy-Weinberg equilibrium</i> | | | | | |
| | | 0.141 | 0.403 | 0.163 | | | |
| IL6 c.-174G>C (rs1800795) | | | | | | | |
| Frequencies | Genotype | GG | 0.232 | 0.395 | 0.452 | 0.002 | 0.253 |
| | | GC | 0.628 | 0.447 | 0.462 | | |
| | | 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 | | |
| | | <i>Hardy-Weinberg equilibrium</i> | | | | | |
| | | 0.019 | 0.739 | 0.316 | | | |
| TNFα c.-308G>A (rs1800629) | | | | | | | |
| Frequencies | Genotype | GG | 0.640 | 0.579 | 0.755 | 0.121 | 0.122 |
| | | GA | 0.337 | 0.421 | 0.226 | | |
| | | 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 | | |
| | | <i>Hardy-Weinberg equilibrium</i> | | | | | |
| | | 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 IL1 β , IL6 and TNF α loci, no data available for IL1 α locus (REFs);

(2) Europe – differences significant for IL6 and TNF α loci, not significant for IL1 α and IL1 β loci;

(3) Asia – differences significant for IL1 α , IL1 β and TNF α loci, no data available for IL6 locus;

(4) Africa – differences significant for IL1 α and IL1 β loci, not significant for TNF α 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 |
| <i>IL1A</i> c.-889C>T | C | 37 | 22±4 | 71±3 | 39±1 | 12±8 |
| | T | 48 | 21±5 | 70±4 | 37±1 | 11±9 |
| <i>IL1B</i> c.-511C>T | C | 49 | 22±5 | 71±4 | 38±1 | 11±8 |
| | T | 37 | 22±5 | 70±4 | 38±1 | 11±9 |
| <i>IL6</i> c.-174G>C | G | 20 | 20±5 | 70±4 | 41±2 [#] | 11±7 |
| | C | 66 | 22±5 | 71±3 | 37±1 [#] | 12±9 |
| <i>TNF</i> 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.