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BTNL2 gene SNPs as a contributing factor to sarcoidosis pathogenesis in a cohort of Greek patients

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ABSTRACT

Sarcoidosis is a multisystemic granulomatous disease of unknown etiology that primarily affects adults between the ages of 20 and 40 years old. It is characterized by the activation of Th1 lymphocytes resulting in the production of inflammatory cytokines and the formation of noncaseating epithelioid cell granulomas in affected tissues. The lungs and lymphatic system are the ones most frequently affected. The disease usually presents spontaneous remission in the first two years and, in a few patients, the disease progresses to pulmonary fibrosis or other fatal complications depending on the affected organ. The pathogenesis of sarcoidosis is still not clearly defined, and is considered an interaction between the environment and risk alleles in many genes.

The present case control study consisted of 146 Greek patients with sarcoidosis and 90 healthy volunteers from the same ethnic group. The coding and neighboring intronic regions of the *BTNL2* gene were sequenced and risk alleles were compared amongst the two groups. Thirty-seven different variants were detected from which 12 were synonymous substitutions and 25 non-synonymous. With the help of *in silico* tools (SIFT, PolyPhen, PROVEAN, PMut and EX_SKIP), 13 variants were classified as possible pathological risk variants including 4 novel ones. The most common risk variants contributing to phenotypic modulation of sarcoidosis were p.S360G and p.S334L, with the latter contributing to a more severe disease stage with

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extra-pulmonary manifestations such as skin granulomas and relapses being more common.

Introduction

Sarcoidosis is a multisystemic immune disorder of unknown aetiology that was first described as a separate medical condition in 1877. The disease is characterized by the formation of non-caseating epithelioid cell granulomas in involved organs. It has a worldwide incidence and it commonly affects young and middle-aged individuals between the ages of 20 and 40 years while later onset of the disease is not unusual. The incidence is estimated at around 16.5/100.000 in men and 19/100.000 in women; being higher in women and in people of African origin (Costabel and Hunninghake, 1999; Hunninghake et al., 1999; Iannuzzi et al., 2007; Nunes et al., 2007). The lung and the lymphatic system are predominately affected but virtually every organ may be involved (Iannuzzi, 2007; Nunes et al., 2007). Lung involvement alone is present in 86%–92% of cases or in association with extra-pulmonary localizations in about 50% of cases (Nunes et al., 2005). One-third or more of patients are asymptomatic, with incidental abnormalities on chest radiographs (Lynch et al., 2007). Most frequently affected extra-pulmonary sites include the skin, eyes, liver, lymph nodes, heart and central nervous system (CNS; Costabel, 2001; Nunes et al., 2007). The clinical course and expression of pulmonary sarcoidosis is variable. In at least half of cases, a benign course is followed with spontaneous resolution within less than 12–36 months, while in up to 30% of patients, the course is chronic (Lynch et al., 2007; Nunes et al., 2005). When sarcoidosis affects the skin, eyes, or liver, it causes significant morbidity (Rose et al., 2008). Mortality is estimated between 0.5% and 5% with severe disease leading to respiratory insufficiency and death from pulmonary or cardiac complications (Nunes et al., 2007). Clinical evidence of cardiac sarcoidosis has been reported in only 10% of patients, underscoring the importance of early detection (Mehta et al., 2008).

Diagnosis can be established when typical clinical-radiological findings are supported by histologic evidence of epithelioid granulomas in more than one organ system and by exclusion of other disorders known to cause granulomatous disease (Agostini et al., 2000; Costabel and Hunninghake, 1999; Hunninghake et al., 1999).

The etiology of sarcoidosis is unknown. Both environmental and hereditary factors have been proposed, supported by reports of familial clustering and associations between sarcoidosis and genetic polymorphisms (Rybicki et al., 2001; Smith et al., 2008). It seems that the development of sarcoidosis is probably the end result of exogenous agents (viruses and bacteria) acting on a genetically susceptible background triggering an immune response and leading to the formation of pathognomic granulomas (Costabel, 2001; Iannuzzi et al., 2007; Saidha et al., 2012).

The immune response in sarcoidosis is primarily mediated by the accumulation of activated CD4 + T-cells of the Th1 type and macrophages at sites of ongoing inflammation, notably in the lung. Cytokines and other mediators produced by these cells contribute to granuloma formation (Costabel, 2001; Smith et al., 2008). The proinflammatory macrophage cytokines IL-1, IL-6 and TNF-a are essential to induce and maintain granuloma formation, and all are increased in sarcoidosis, whereas the anti-inflammatory cytokine IL-10 is associated with resolution of the granuloma, suppressing the inflammatory response (Ziegenhagen and Muller-Quernheim, 2003).

In addition to cytokines and their receptors, other cell-signaling molecules are involved in regulating immune responses. Associations between class I HLA-B8 antigens and acute sarcoidosis (Grubic et al., 2011) and HLA class II antigens are reported (Grunewald et al., 2010a; Grunewald et al., 2010b). Valentonyte et al. (Valentonyte et al., 2005) reported an association of the butyrophilin-like 2 (*BTNL2*) gene on chromosome 6p21.32 (OMIM #612387) with sarcoidosis, and others have confirmed this association in African Americans and Whites (Rybicki et al., 2005a; Rybicki et al., 2005b). The *BTNL2* gene is a member of the immunoglobulin gene superfamily (B7 receptor family) resides in the class II major histocompatibility complex (MHC) region of chromosome 6p and is expressed on activated B and T cells.

BTNL2 appears to regulate T-cell activation, implicating the gene in inflammatory autoimmune diseases (Lopez Herraez et al., 2013; Mitsunaga et al., 2013; Nguyen et al., 2006; Suzuki et al., 2012).

Given previous publications of the association of a specific SNP (rs2076530; Li et al., 2006; Morais et al., 2012; Valentonyte et al., 2005; Wijnen et al., 2011) in the *BTNL2* gene and sarcoidosis, we sequenced all exons and adjoining intronic regions of the *BTNL2* gene (ENSG00000224242.3, gene sequence: NM_019602.1) on a clinically well-characterized cohort of Greek patients with sarcoidosis and a control population. Our aim was to replicate previously published results indicating *BTNL2* gene variants as risk alleles for sarcoidosis in the Greek population and also investigate their association with specific disease phenotypic features and prognosis.

Materials and methods

Patients

This was a case-control study. The patient group consisted of (a) 146 Greek sarcoidosis patients (56 males and 90 females with mean age 46 ± 12.7; range, 21–73 years), with mean age of disease onset 43.1 ± 13.3 and (b) a general population control group ($n = 90$; 37 males and 53 females with a mean age of 46 ± 18.4; range, 18–77 years). The average time for observation for all patients was 5 years. There were no familiar sarcoidosis cases. The control group consisted of healthy volunteers. None of the controls had a history of pulmonary or other inflammatory disease.

Table 1
Clinical details of patients with sarcoidosis.

	Total*		Male	Female
Patients	146	(100.0%)	56 (38.4%)	90 (61.6%)
Age (years, mean ± SD)	46	±12.7	40 ± 11.8	50 ± 11.9
Age of onset (years, mean ± SD)	45	±12.5	39 ± 12.1	48 ± 11.4
Disease stage				
0	10	(6.8%)	2 (1.4%)	8 (5.5%)
I	64	(43.8%)	22 (15.1%)	42 (28.8%)
II	52	(35.6%)	22 (15.1%)	30 (20.5%)
III	15	(10.3%)	5 (3.4%)	10 (6.8%)
IV	5	(3.4%)	5 (3.4%)	0 (0.0%)
Smoking				
Yes	102	(69.9%)	27 (18.5%)	75 (51.4%)
No	31	(21.2%)	21 (14.4%)	10 (6.8%)
Ex	13	(8.9%)	8 (5.5%)	5 (3.4%)
Diagnostic tests				
Biopsy (of non-caseating epitheloid cell granulomas in tissue/organ affected)	135	(92.5%)	45 (30.8%)	90 (61.6%)
Lymph nodes	32	(21.9%)	10 (6.8%)	22 (15.1%)
Skin	27	(18.5%)	5 (3.4%)	22 (15.1%)
Lung	13	(8.9%)	4 (2.7%)	9 (6.2%)
Endrobrochial	31	(21.2%)	12 (8.2%)	19 (13.0%)
Transbronchial	32	(21.9%)	14 (9.6%)	18 (12.3%)
Clinical-radiological	54	(37.0%)	27 (18.5%)	27 (18.5%)
Hypercalciuria	35	(24.0%)	19 (13.0%)	16 (11.0%)
Heart involvment	9	(6.2%)	3 (2.1%)	6 (4.1%)
Eye involvment	9	(6.2%)	2 (1.4%)	7 (4.8%)
Skin	32	(21.9%)	6 (4.1%)	26 (17.8%)
Löfgren	27	(18.5%)	11 (7.5%)	16 (11.0%)
Nervous system	2	(1.4%)	0 (0.0%)	2 (1.4%)
Patients with relapses	36	(24.7%)	14 (9.6%)	22 (15.1%)
Follow-up available >2 years (average 5 years)	121	(82.9%)	43 (29.5%)	78 (53.4%)
FVC%	99	±20	93 ± 23	102 ± 17
FEV1%	94	±21	90 ± 23	96 ± 20
DLCO%	83	±24	85 ± 23	82 ± 25

* The percentages are based on total number of samples.

Table 2
List of variants and genotype frequencies in sarcoidosis and control groups.

Residue	Variation ID (dbSNP)	Exon	Nucleotide (MAF)	SIFT	PolyPhen	PROVEAN	PMut	EX_SKIP	Genotype	Sarcoidosis (*n*:146), counts (frequency)	Controls (*n*:90), counts (frequency)	*p*-value*
H60H c.180C/T	rs28362683	2	C/T(0.133)	-	–	–	–	Yes	CC	127 (86.9%)	85 (94.44%)	0.44
									CT	19 (13.01%)	4 (4.44%)	0.045
									TT	0 (0%)	1 (1.11%)	0.38
W94R c.280A/T	rs28362682	2	A/T(0.1327)	Tolerated	Benign	Neutral	Pathological	No	TT	127 (86.9%)	89 (98.88%)	0.21
									TA	16 (10.96%)	1 (1.11%)	0.0088
									AA	3 (2.05%)	0 (0%)	0.29
D118N c.352G/A	rs143211074	2	C/T(0.003)	Deleterious	Possibly damaging	Deleterious	Neutral	Same chance	GG	144 (98.63%)	90 (100%)	0.92
									GA	2 (1.4%)	0 (0%)	0.52
									AA	0 (0%)	0 (0%)	1
A142P c.424G/C	NOVEL	2	C/G	Tolerated	Possibly damaging	Deleterious	Neutral	Same chance	GG	145 (99.3%)	90 (100%)	1
									GC	1 (0.68%)	0 (0%)	1
									CC	0 (0%)	0 (0%)	1
G143D c.428G/A	rs115653647	3	C/T(0.037)	Deleterious	Probably damaging	Deleterious	Neutral	No	GG	145 (99.3%)	90 (100%)	1
									GA	1 (0.68%)	0 (0%)	1
									AA	0 (0%)	0 (0%)	1
G145G c.435G/T	rs57116766	3	C/A(0.132)	–	–	–		No	GG	145 (99.3%)	89 (98.88%)	1
									GT	0 (0%)	1 (1.11%)	0.38
									TT	1 (0.68%)	0 (0%)	1
S149N c.446G/A	rs60263670	3	C/T(0.138)	Tolerated	Possibly damaging	Neutral	Neutral	No	GG	145 (99.3%)	89 (98.88%)	1
									GA	0 (0%)	1	0.38
									AA	1 (0.68%)	0 (0%)	1
S149T c.446G/C	NOVEL	3	G/C	Tolerated	Benign	Neutral	Neutral	No	GG	145 (99.3%)	90 (100%)	1
									GC	1 (0.68%)	0 (0%)	1
									CC	0 (0%)	0 (0%)	1
T165I c.494C/T	rs78587369	3	G/A(0.032)	Tolerated	Possibly damaging	Neutral	Pathological	Yes	CC	144 (98.6%)	89 (98.88%)	1
									CT	2 (1.37%)	1 (1.11%)	1
									TT	0 (0%)	0 (0%)	1
P171P c.513A/T	rs59129682	3	T/A(0.133)	–	–	–	–	No	AA	141 (96.57%)	89 (98.88%)	0.85
									AT	4 (2.74%)	0 (0%)	0.16
									TT	1 (0.68%)	1 (1.11%)	1
R181Q c.542G/A	rs28362681	3	C/T(0.133)	Tolerated	Benign	Neutral	Pathological	No	GG	141 (96.57%)	89 (98.88%)	0.85
									GA	4 (2.74%)	0 (0%)	0.16
									AA	1 (0.68%)	1 (1.11%)	1
K196E c.586A/G	rs2076523	3	T/C(0.370)	Tolerated	Benign	Neutral	Neutral	No	AA	108 (73.97%)	73 (81.11%)	0.49
									AG	38 (26.03%)	15 (16.66%)	0.13
									GG	0 (0%)	2 (2.22%)	0.14

A202V c.605C/T	rs28362680	3	C/T/G/A(0.170)	Tolerated	Benign	Neutral	Neutral	Yes	CC	139 (95.2%)	87 (96.665)	0.92
									CT	6 (4.1%)	1 (1.11%)	0.26
									TT	1 (0.68%)	2 (2.22%)	0.56
A202A c.606G/A	rs147483338	3	C/T					No	GG	145 (99.3%)	90 (100%)	1
									GA	1 (0.68%)	0 (0%)	1
									AA	0 (0%)	0 (0%)	1
R209R c.627G/A	rs60036207	3	C/T(0.133)	–	–	–	–	No	GG	140 (95.89%)	89 (98.88%)	0.77
									GA	4 (2.74%)	0 (0%)	0.16
									AA	1 (0.68%)	1 (1.11%)	1
N210N c.630C/T	rs78107756	3	G/A(0.038)	–	–	–	–	Yes	CC	144 (98.6%)	88 (97.77%)	1
									CT	2 (1.36%)	2 (2.22%)	0
									TT	0 (0%)	0 (0%)	1
E242G c.725A/G	Novel	4	T/C	Tolerated	Possibly damaging)	Deleterious	Neutral	Yes	AA	145 (99.31%)	90 (100%)	1
									AG	1 (0.68%)	0 (0%)	1
									GG	0 (0%)	0 (0%)	1
E279E c.837G/A	rs41333546	5	C/T (0.032)	–	–	–	–	No	GG	143 (97.9%)	87 (96.665)	0.92
									GA	3 (2.05%)	2 (2.22%)	1
									AA	0 (0%)	1 (1.11%)	0.38
R281K c.842G/A	rs41355746	5	C/T(0.032)	Tolerated	Probably damaging	Neutral	Neutral	No	GG	143 (97.9%)	87 (96.665)	0.92
									GA	3 (2.05%)	2 (2.22%)	1
									AA	0 (0%)	1 (1.11%)	0.38
D283V c.848A/T	rs34423804	5	T/A(0.038)	Tolerated	Benign	Neutral	Pathological	Yes	AA	143 (97.9%)	87 (96.665)	0.92
									AT	3 (2.05%)	1 (1.11%)	1
									TT	0 (0%)	2 (2.22%)	0.14
M295V c.883A/G	rs41417449	5	T/C(0.032)	Tolerated	Benign	Neutral	Neutral	Yes	AA	145 (99.31%)	88 (97.77%)	0.92
									AG	1 (0.68%)	2 (2.22%)	0.56
									GG	0 (0%)	0 (0%)	1
E307E c.921G/A	rs41392447	5	C/T(0.032)	–	–	–	–	No	GG	143 (97.9%)	87 (96.665)	0.92
									GA	3 (2.05%)	2 (2.22%)	1
									AA	0 (0%)	1 (1.11%)	0.38
V313V c.939A/G	rs2076529	5	T/C(0.365)	–	–	–	–	Yes	AA	93 (63.7%)	67 (74.44%)	0.27
									AG	50 (34.24%)	22 (24.44%)	0.18
									GG	3 (2.05%)	1 (1.11%)	0.38
S334L c.1001C/T	rs28362679	5	G/A(0.019)	Deleterious	Probably damaging	Deleterious	Neutral	Yes	CC	122 (83.56%)	79 (87.77%)	0.70
									CT	23 (15.75%)	11 (12.22%)	0.58
									TT	1 (0.68%)	0 (0%)	1
D336N c.1006G/A	rs41441651	5	C/T(0.032)	Tolerated	Possibly damaging	Neutral	Neutral	WT/MUT same chance	GG	143 (97.9%)	88 (97.77%)	1
									GA	3 (2.95%)	1 (1.11%)	0.38
									AA	0 (0%)	1 (1.11%)	0.38
Q350Q c.1050G/A	rs9268480	6	C/T(0.201)	–	–	–	–	No	GG	113 (77.39%)	68 (75.55%)	0.84
									GA	32 (21.92%)	18 (20%)	0.76
									AA	1 (0.68%)	4 (4.44%)	0.072

(continued on next page)

Table 2 (*continued*)

Residue	Variation ID (dbSNP)	Exon	Nucleotide (MAF)	SIFT	PolyPhen	PROVEAN	PMut	EX_SKIP	Genotype	Sarcoidosis (*n*:146), counts (frequency)	Controls (*n*:90), counts (frequency)	*p*-value*
S360G c.1078A/G	rs2076530	6	T/C(0.377)	Tolerated	Benign	Neutral	Neutral	Yes	AA	86 (58.9%)	51 (56.66%)	0.83
									AG	55 (37.67%)	27 (30%)	0.31
									GG	5 (3.42%)	12 (13.33)	0.0062
L366L c.1096C/T	rs76868526	6	G/A(0.032)	–	–	–	–	Yes	CC	143 (97.9%)	89 (98.88%)	0.92
									CT	3 (2.95%)	1 (1.11%)	0.38
									TT	0 (0%)	0 (0%)	1
P379L c.1136C/T	rs28362678	6	G/A(0.159)	Tolerated	Benign	Neutral	Pathological	No	CC	124 (84.93%)	74 (82.22%)	0.77
									CT	21 (14.38%)	15 (16.66%)	0.72
									TT	1 (0.68%)	1 (1.11%)	1
M380I c.1140G/A	rs28362677	6	C/T(0.158)	Tolerated	Benign	Neutral	Neutral	WT/MUT same chance	GG	124 (84.93%)	74 (82.22%)	0.77
									GA	22 (15.068%)	16 (17.77%)	0.60
									AA	0 (0%)	0 (0%)	1
P393Q c.1178-1179CA/AG	rs41521946	6	G/T(0.121)	Tolerated	Benign	Neutral	Neutral	Same	CACA	124 (84.93%)	74 (82.22%)	0.77
									CAAG	21 (14.38%)	16 (17.77%)	0.59
									AGAG	1 (0.68%)	0 (0%)	1
S404S c.1212A/C	rs41449245	6	T/G(0.158)	–	–	–	–	Yes	AA	123 (84.24%)	74 (82.22%)	0.84
									AC	23 (15.75%)	16 (17.77%)	0.73
									CC	0 (0%)	0 (0%)	1
S406A c.1215 T/G	NOVEL	6	T/G	Tolerated	Possibly Damaging	Neutral	Neutral	No	TT	144 (98.6%)	90 (100%)	0.92
									TG	2 (29.2%)	0 (0%)	0.52
									GG	0 (0%)	0 (0%)	1
G412C c. 1234G/T	NOVEL	6	G/T	Deleterious	Probably damaging	Neutral	Pathological	Yes	GG	144 (98.6%)	90 (100%)	0.92
									GT	2 (29.2%)	0 (0%)	0.52
									TT	0 (0%)	0 (0%)	1
H414H c.1242C/T	rs41535850	6	G/A(0.158)	–	–	–	–	Yes	CC	123 (84.24%)	77 (85.55%)	0.92
									CT	23 (14.75%)	12 (13.33%)	0.72
									TT	0 (0%)	1 (1.11%)	0.38
A430D c.1289C/A	NOVEL	6	C/A	Tolerated	Possibly damaging	Neutral	Pathological	Yes	CC	145 (99.31%)	90 (100%)	1
									CA	1 (0.68%)	0 (0%)	1
									AA	0 (0%)	0 (0%)	1
E454C c.1360G/T	rs28362675	6	C/G/A(0.03)	Deleterious	Probably damaging	Neutral	Pathological	No.	GG	142 (97.26%)	90 (100%)	0.77
									GT	4 (2.74%)	0 (0%)	0.16
									TT	0 (0%)	0 (0%)	1

BTNL2 gene sequence: NM_019602.1 (ENSG00000454136), numbering according to HGVS nomenclature-cDNA sequence with +1 corresponding to the A of the ATG translation initiation codon.
**p*-values calculated by double-sided Fisher's exact test.

Whole blood samples from the patients were obtained from the Outpatient Clinic of the Respiratory Department of the University of Athens and the 8th Department of Respiratory Medicine of the "Sotiria" Chest Disease Hospital of Athens. In all patients, the diagnosis of sarcoidosis was established by typical clinical and radiological findings and in many cases supported by histological evidence of non-caseating epithelioid cell granulomas in affected tissue/organ. Detailed clinical data of the patient populations are shown in Table 1.

All individuals included in the study were of Greek origin. The study was approved by the Ethics Committee of the University of Athens and all subjects participating signed an informed consent.

DNA extraction

Genomic DNA was extracted from 3 ml of peripheral blood, using the commercially available kit Nucleospin Blood L (MACHEREY-NAGE).

BTNL2 gene

Butyrophyillin-like-2 (MCH class II associated) *BTNL2* (MIM: 606000, Gene ID:56244) sequences were downloaded from Ensembl Genome Browser (www.ensemble.org/index.html; gene sequence ENST0000022424.3; NM_019602.1).

The presence of mutations in the 6 exons and neighboring intronic regions (50–150 bp on each side of each exon) of the *BTNL2* gene was assessed by Sanger sequencing. Sequencing analysis was performed with the DYEnamic™ dye terminator kit-Megabase (GE HealthCare LLC), run on the Megabase 1000/4000 series automated sequencer (GE HealthCare, LLC) and analyzed with the BioEdit software. The primers were designed using Primer 3 (primer sequences are shown in Supplementary Table 1).

Mutation evaluation

The sequence of the gene including known variations were downloaded from http://www.ensembl.org/Homo_sapiens/Transcript/Variation_Transcript/. With the use of bioinformatics tools such as SIFT (Ng and Henikoff, 2003), PolyPhen-2 (Adzhubei et al., 2010), PMut (Ferrer-Costa et al., 2004), PROVEAN (Choi et al., 2012) and EX_Skip (Raponi et al., 2011) in combination with the clinical phenotype, we have attempted to elucidate the effect of these variants, including the novel ones found in this study.

Statistical analysis

Statistical analysis was performed using SPSS 17.0, p values were calculated using double-sided Fisher's test (significant p values <0.05).

Results and discussion

The present study examined the entire coding sequence and neighboring intronic regions of the *BTNL2* gene in 146 patients with sarcoidosis and in 90 healthy controls of the same ethnic origin. Thirty-seven different variants were detected from which 12 were synonymous substitutions and 25 were non-synonymous. In addition, 16 intronic variants were detected (Supplementary Table 2), from which 7 were novel. Overall, the intronic variants showed similar frequencies between cases and controls. The majority of individuals from both groups carried more than one variant with 31.5% of cases and 44.4% of controls having the wild type sequence for all regions sequenced.

We used *in silico* tools (SIFT, PolyPhen, PROVEAN, PMut and EX_SKIP; Adzhubei et al., 2010; Choi et al., 2012; Ferrer-Costa et al., 2004; Ng and Henikoff, 2003; Raponi et al., 2011) to classify the variants as benign or pathological and EX_SKIP to determine if they affected exon skipping. If more than 2 of the SIFT, PolyPhen, PROVEAN, and PMut tools classified the variant as deleterious, pathological or possibly damaging, we considered them as being possible risk variants.

Amongst the non-synonymous substitutions, six were novel, p.A142P, p.S149T, p.E242G, p.S406A, p.G412C and p.A430D (not reported in the HGMD: http://www.hgmd.org/), and were not found in the

Table 3
Genotype and clinical characteristics of patients carrying probable risk variants.

Case	Main variant	Other risk variants*	Sex/age (years)	Disease stage	Other clinical characteristics	DLCO %†
38	p.S360G		F/52	Stage 0	Uveitis	81
103	p.S360G	p.T165I, p.D283V, p.S334L, p.G454C, p.M295V	M/25	Stage 0	Löfgren	94
7	p.S360G		F/56	Stage 0		81
77	p.S334L	p.G412C	F/55	Stage 0	Skin granulomas	91
96	p.S334L		F/49	Stage 0	Skin granulomas	100
21	p.A202V	p.A202V	F/59	Stage 0	Skin granulomas	85
2033	p.S360G		F/38	Stage I	Hyperalciuria, Löfgren	93
221	p.S360G		F/65	Stage I	Löfgren	66
119	p.S360G	p.A430D	M/34	Stage I	Uveitis, hypercalciuria	95
2019	p.S360G	p.D118N, p.A148P	F/28	Stage I	Parotid gland swelling	86
180	p.S360G		M/40	Stage I, Löfgren	Relapse	90
97	p.S360G		F/50	Stage I		69
102	p.S360G		F/49	Stage I		123
25	p.S360G		F/72	Stage I		89
2035	p.S360G		F/47	Stage I		109
95	p.S360G		F/51	Stage I	Skin granulomas	113
2026	p.S360G		M/64	Stage I	Skin granulomas	121
149	p.S360G		M/30	Stage I	Hyperalciuria	74
2028	p.S360G	p.A202V	F/55	Stage I	Skin granulomas, heart, pulmonary hypertension, nodulous erythema	91
2027	p.S360G		F/40	Stage I	Heart, hypercalciuria, hypocalcemia	77
33	p.S360G		F/25	Stage I	Skin granulomas	79
19	p.S360G	p.S334L	F/27	Stage I	Skin granulomas, heart, nodulous erythema, relapse	82
71	p.S360G		F/62	Stage I	Löfgren	83
178	p.S360G	p.S360G	F/39	Stage I	Löfgren, parotid gland swelling	111
166	p.S360G		M/27	Stage I	Hypercalciuria	100
165	p.S360G	p.S360G	F/55	Stage I		93
90	p.S360G	p.G143D, p.A202V	M/36	Stage I	Löfgren	102
2020	p.S360G		M/44	Stage I	Hypercalciuria	79
18	p.S360G		M/43	Stage I	Pleural fluid, hypercalciuria, hepatomegaly	128
274	p.S360G	p.G412C	F/27	Stage I	Hypercalciuria	100
37	p.S360G		M/21	Stage I		97
50	p.S360G		M/58	Stage I		107
167	p.S334L		M/36	Stage I	Löfgren, skin granulomas	86
54	p.S334L		M/36	Stage I	Löfgren, nodulous erythema, skin granulomas	118
42	p.S334L		F/54	Stage I	Hypercalciuria	96
101	p.S334L		F/47	Stage I	Löfgren	88
64	p.S334L		F/37	Stage I	Nodulous erythema, skin granulomas, CNS, heart, relapse	115
86	p.S334L		F/41	Stage I	Löfgren	102
181	p.A202V		F/60	Stage I	Löfgren, nodulous erythema, hypercalciuria, skin granuloma, relapse	98
227	p.S360G		F/54	Stage I	Hypercalciuria, uveitis	67
2034	p.S360G		M/35	Stage I	Hypercalciuria, uveitis, relapse	98
194	p.S360G		M/41	Stage I		80
63	p.S360G	p.D283V, p.G454C, p.A202V	M/41	Stage I	Relapse	59
72	p.S360G		F/46	Stage II		54
2069	p.S360G		M/32	Stage II		70
2068	p.S360G		M/43	Stage II		47
61	p.S360G		M/44	Stage II	Pulmonary hypertension	56
65	p.S360G		F/68	Stage II	Cervical lymph gland swelling	82
2025	p.S360G		M/28	Stage II		85
94	p.S360G		M/41	Stage II	Nodulous erythema	109
9	p.S360G		M/25	Stage II		76

Table 3 (*continued*)

Case	Main variant	Other risk variants*	Sex/age (years)	Disease stage	Other clinical characteristics	DLCO %†
2054	p.S360G		M/48	Stage II	Heart, hypercalciuria, Löfgren	112
15	p.S360G		F/59	Stage II	Hypercalciuria, nodulous erythema, uveitis, face nodules	74
39	p.S360G		M/32	Stage II		112
27	p.S360G		F/73	Stage II	Hyperalciuria, hypocalcemia	88
85	p.S360G		M/41	Stage II	Hypercalciuria, hypocalcemia	84
56	p.S360G	p.S360G	M/26	Stage II		110
213	p.S360G		M/34	Stage II	Hypercalciuria	104
52	p.S360G		F/49	Stage II	Pulmonary hypertension, relapse	57
2023	p.S360G	p.A202V	M/30	Stage II	Hypogammaglobulinemia, relapse	39
163	p.S360G	p.S360G, p.T165I, p.D283V, p.G454C, p.A202V	F/40	Stage II	Hypercalciuria, hypocalcemia, relapse, pulmonary hypertension	102
264	p.S334L		F/60	Stage II		71
59	p.S334L		F/71	Stage II	Relapse, nodulous erythrema	104
222	p.S334L		M/32	Stage II	Pulmonary hypertension, relapse	84
17	p.S334L		F/47	Stage II	Nodulous erythema, skin	68
2031	p.S334L		M/32	Stage II	Hypercalciuria, skin	97
173	p.S334L		F/49	Stage II	Relapse	65
57	p.S334L		F/29	Stage II		73
239	p.E242G		M/52	Stage II	Hypercalciuria, relapse	57
104	p.S360G	p.S334L	M/50	Stage III		91
168	p.S360G	p.S360G	M/49	Stage III	Relapse	71
89	p.S360G		F/50	Stage III	Hyperalciuria, relapse	60
60	p.S360G		F/48	Stage III	Uveitis	95
268	p.S360G		F/48	Stage III		95
114	p.S360G	p.D118N	M/58	Stage III	Skin granulomas	66
46	p.S334L		F/50	Stage III		91
196	p.S334L		F/58	Stage III		63
162	p.S334L		F/39	Stage III	Hypercalciuria, hypocalcemia, skin, relapse	69
20	p.S334L		M/39	Stage III	Hypercalciuria, skin granulomas, hypocalcemia, relapse	69
235	p.S334L	p.S334L	F/48	Stage III	Hypercalciuria, heart, relapse	87
78	p.S360G		M/37	Stage IV		40
2029	p.S334L			Stage IV		85
49	p.G454C		M/40	Stage IV	Hypercalciuria, hypocalcemia, pulmonary hypertension, relapse	102

*, Variants characterized as probable risk and/or causing exon skipping by the *in silico* method (see Methods); †DLCO: diffuse lung capacity for CO.

control group. One heterozygote for the minor allele was detected for the novel variants p.A142P, p.S149T, p.E242G and p.A430D while for p.S406A and p.G412C, two heterozygotes were found amongst the patient group.

The *in silico* evaluation characterized variants p.D118N, p.A142P, p.G143D, p.T165I, p.E242G, p.S334L, p.G412C, p.A430D and p.E454C as conferring possible risk causing changes in the structure and function of the produced protein, and probably contributing to the clinical phenotype along with other genetic or environmental causes. Additionally, 15 variants, amongst them 3 novel, 5 synonymous, and the known splicing allele p.S360G (rs2076530), were identified as causing exon skipping by the EX_SKIP software (Table 2). More specifically, heterozygosity for the known sarcoidosis risk splicing variant p.S360G (rs2076530; Li et al., 2006; Morais et al., 2012; Valentonyte et al., 2005; Wijnen et al., 2011) was frequent in both cases (37.67%) and controls (30%) showing a higher frequency of homozygotes for the ancestral allele (G) amongst the control group (13.3% vs. 3.42%; $p = 0.0062$) rather than the sarcoidosis patients. Significant statistical difference in frequency of heterozygotes for the minor allele between cases and controls was found for p.H60H ($p = 0.045$) and p.W94R ($p = 0.0088$). Variant p.H60H, although synonymous, was identified probably causing exon skipping by EX_SKIP (Table 2).

Amongst the 146 patients, 27 presented with Löfgren syndrome (a benign form of sarcoidosis), eleven of which carried possible risk variants. The clinical characteristics of patients carrying either novel or previously

reported variants deemed possible risk variants and/or affecting splicing are shown in Table 3. In general, the patients carrying the novel variants (cases 119, 2019, 274, 77 and 239; Table 3), either alone or in combination with other risk variants, presented with mild lung disease (stage 0 or I) and good response to treatment, except for the one carrying p.E242G, which developed stage II pulmonary sarcoidosis with relapses. All, however, presented with extra-pulmonary manifestations as well.

Previous studies have implicated rs2076530 (p.S360G) as a risk allele for autoimmune inflammatory diseases such as sarcoidosis and myositis, although the immunological basis of this association is not yet clear (Li et al., 2006; Morais et al., 2012; Valentonyte et al., 2005; Wijnen et al., 2011). The same variant was also frequently found in both the Greek cases and controls (Table 2) agreeing with previous studies (Morais et al., 2012; Rybicki et al., 2005a). The second most common "possible risk variant" as characterized by the *in silico* study for the Greek population was p.S334L (15.75%), with another 11 nonsynonymous "possible risk variants" [p.D118N (cases 2019 and 114), p.A142P (2019), p.G143D (90), p.T165I (103, 163), p.A202V (63, 2028, 2023, 90, 163, 181, 21), p.E242G (239), p.D283V (63, 103, 163), p.M295V (103), p.G412C (274, 77), p.A430D (119) and p.E454C (63, 103, 163, 49)], found in 1 to 4 cases; the majority in combination with other risk variants (Table 3).

Studying the coinheritance of other risk variants in the 60 patients carrying rs2076530, we found that five also carried p.A202V. The largest proportion of these (4/5, 80%) had chronic disease with relapses, pulmonary hypertension, calciuria and cardiac complications. However, a patient with Löfgren syndrome (case 90), a benign form of the disease, was compound heterozygote for p.G143D, p.A202V and p.S360G, perhaps moderating the expression of the disease. Detection of p.A202V in homozygosity (case 21) in the absence of any other risk variants was associated with mild lung disease but in combination with relapses and exo-pulmonary complications. While the combination of p.D118N, p.A142P and p.S360G (case 2019) presented with mild lung disease, normal DLCO and parotid gland swelling, while the coinheritance of p.A430D and p.S360G (case 119) resulted in mild pulmonary disease with hypercalciuria and uveitis. Five p.S360G homozygotes (168, 56, 178, 165) with one also coinheriting 4 other risk variants (163) were detected. Four of these p.S360G homozygotes presented with mild pulmonary disease while case 163 presented with stage II disease accompanied by extra-pulmonary complications and relapses. One might conclude that the coinheritance of more than one risk variant contributed to the more severe phenotype (Table 3).

From the total of 146 sarcoidosis cases, we had a follow-up duration for five years on average for 121. They were divided into two subgroups. Subgroup A consisting of 81 patients with non-persistent pulmonary disease stage 0 or I, including 11 patients with Löfgren syndrome and subgroup B composed of 40 patients with persistent pulmonary disease stage II, III or IV. The most common variant p.S360G (rs2076530) was detected in 37 patients (45.7%) in subgroup A and in 12 patients (30%) in subgroup B.

The second most common variant p.S334L was detected in heterozygosity with no other risk variants in 19 cases, the majority of which had stage II or III pulmonary disease and almost all presented extra-pulmonary manifestations and relapses. A homozygous p.S334L case (235) presented with stage III disease, relapses and extra-pulmonary complications with a large proportion having skin granulomas. Perhaps the inheritance of this variant in patients with sarcoidosis increases the likelihood of skin manifestations. Variant p.S334L coinherited with p.S360G (104, 19) was also associated with severe pulmonary disease with relapses, heart and skin symptoms, while case 103 with the variant combination: p.S334L, p.T165I, p.D283V, p.E454C, and p.M295V had Löfgren syndrome with spontaneous recovery without therapy. The additional coinheritance of the other risk variants could act as disease modifiers (Table 3).

Finally, variant p.E454C alone or together with others could be characterized as a risk allele related to chronic disease with relapses, pulmonary hypertension, hypercalciuria and hypercalcemia.

One of the limitations of our study is the small number of cases and controls; however, a major strength is that the cases and controls were of the same ethnic origin, avoiding population stratification. Additionally, the cases were well characterized clinically into different sarcoidosis disease stages. Finally, none of the previous studies had sequenced the whole coding region of the *BTNL2* gene.

Conclusions

The clinical phenotypes of complex diseases are most likely due to the interaction between multiple causative or contributory alleles, as well as non-genetic determinants (Cooper et al., 2013). Sarcoidosis being a multifactorial/complex disorder, the requirement for genetic risk alleles in many genes and/or environmental

factors has been proven to be necessary for the disease to manifest clinically (Grunewald, 2010). In this case control study, the combinations of multiple causative or contributory risk variants in the *BTNL2* gene, implicated in the causation of sarcoidosis, were found to affect the final clinical phenotype of the disease with p.S360G and p.S334L contributing to a more severe disease stage with extra-pulmonary manifestations such as skin granulomas and relapses.

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References

Adzhubei, I.A., Schmidt, S., Peshkin, L., Ramensky, V.E., Gerasimova, A., Bork, P., Kondrashov, A.S., Sunyaev, S.R., 2010. A method and server for predicting damaging missense mutations. Nat. Methods 7, 248–249.

Agostini, C., Adami, F., Semenzato, G., 2000. New pathogenetic insights into the sarcoid granuloma. Curr. Opin. Rheumatol. 12, 71–76.

Choi, Y., Sims, G.E., Murphy, S., Miller, J.R., Chan, A.P., 2012. Predicting the functional effect of amino acid substitutions and indels. PLoS One 7, e46688.

Cooper, D.N., Krawczak, M., Polychronakos, C., Tyler-Smith, C., Kehrer-Sawatzki, H., 2013. Where genotype is not predictive of phenotype: towards an understanding of the molecular basis of reduced penetrance in human inherited disease. Hum. Genet. 132, 1077–1130.

Costabel, U., 2001. Sarcoidosis: clinical update. Eur. Respir. J. Suppl. 56s–68s.

Costabel, U., Hunninghake, G.W., 1999. ATS/ERS/WASOG statement on sarcoidosis. Sarcoidosis Statement Committee. American Thoracic Society. European Respiratory Society. World Association for Sarcoidosis and Other Granulomatous Disorders. Eur. Respir. J. 14, 735–737.

Ferrer-Costa, C., Orozco, M., de la Cruz, X., 2004. Sequence-based prediction of pathological mutations. Proteins 57, 811–819.

Grubic, Z., Peros-Golubicic, T., Stingl, K., Zunec, R., 2011. The investigation of HLA microsatellites influence in predisposition to sarcoidosis among Croatians. Vasc. Lung Dis. 28, 18–26.

Grunewald, J., 2010. Review: role of genetics in susceptibility and outcome of sarcoidosis. Semin. Respir. Crit. Care Med. 31, 380–389.

Grunewald, J., Brynedal, B., Darlington, P., Nisell, M., Cederlund, K., Hillert, J., Eklund, A., 2010a. Different HLA-DRB1 allele distributions in distinct clinical subgroups of sarcoidosis patients. Respir. Res. 11, 25.

Grunewald, J., Idali, F., Kockum, I., Seddighzadeh, M., Nisell, M., Eklund, A., Padyukov, L., 2010b. Major histocompatibility complex class II transactivator gene polymorphism: associations with Löfgren's syndrome. Tissue Antigens 76, 96–101.

Hunninghake, G.W., Costabel, U., Ando, M., Baughman, R., Cordier, J.F., du Bois, R., Eklund, A., Kitaichi, M., Lynch, J., Rizzato, G., Rose, C., Selroos, O., Semenzato, G., Sharma, O.P., 1999. ATS/ERS/WASOG statement on sarcoidosis. American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and other Granulomatous Disorders. Vasc. Diffuse Lung Dis. 16, 149–173.

Iannuzzi, M.C., 2007. Advances in the genetics of sarcoidosis. Proc. Am. Thorac. Soc. 4, 457–460.

Iannuzzi, M.C., Rybicki, B.A., Teirstein, A.S., 2007. Sarcoidosis. N. Engl. J. Med. 357, 2153–2165.

Li, Y., Wollnik, B., Pabst, S., Lennarz, M., Rohmann, E., Gillissen, A., Vetter, H., Grohe, C., 2006. BTNL2 gene variant and sarcoidosis. Thorax 61, 273–274.

Lopez Herraez, D., Martinez-Bueno, M., Riba, L., Garcia de la Torre, I., Sacnun, M., Goni, M., Berbotto, G.A., Paira, S., Musuruana, J.L., Graf, C.E., Alvarellos, A.J., Messina, O.D., Babini, A.M., Strusberg, I., Marcos, J.C., Scherbarth, H., Spindler, A.J., Quinteros, A., Toloza, S.M., Moreno, J.L., Catoggio, L.J., Tate, G., Eimon, A., Citera, G., Catalan Pellet, A., Nasswetter, G.G., Cardiel, M.H., Miranda, P., Ballesteros, F., Esquivel-Valerio, J.A., Maradiaga-Cecena, M.A., Acevedo-Vasquez, E.M., Garcia Garcia, C., Pons-Estel, B.A., Alarcon-Riquelme, M.E., 2013. Rheumatoid arthritis in Latin Americans enriched for Amerindian ancestry is associated with loci in chromosomes 1, 12, and 13, and the HLA class II region. Arthritis Rheum. 65, 1457–1467.

Lynch 3rd, J.P., Fishbein, M.C., Saggar, R., Zisman, D.A., Belperio, J.A., 2007. Idiopathic pulmonary fibrosis. Expert. Rev. Respir. Med. 1, 377–389.

Mehta, D., Lubitz, S.A., Frankel, Z., Wisnivesky, J.P., Einstein, A.J., Goldman, M., Machac, J., Teirstein, A., 2008. Cardiac involvement in patients with sarcoidosis: diagnostic and prognostic value of outpatient testing. Chest 133, 1426–1435.

Mitsunaga, S., Hosomichi, K., Okudaira, Y., Nakaoka, H., Kunii, N., Suzuki, Y., Kuwana, M., Sato, S., Kaneko, Y., Homma, Y., Kashiwase, K., Azuma, F., Kulski, J.K., Inoue, I., Inoko, H., 2013. Exome sequencing identifies novel rheumatoid arthritis-susceptible variants in the BTNL2. J. Hum. Genet. 58, 210–215.

Morais, A., Lima, B., Peixoto, M.J., Alves, H., Marques, A., Delgado, L., 2012. BTNL2 gene polymorphism associations with susceptibility and phenotype expression in sarcoidosis. Respir. Med. 106, 1771–1777.

Ng, P.C., Henikoff, S., 2003. SIFT: predicting amino acid changes that affect protein function. Nucleic Acids Res. 31, 3812–3814.

Nguyen, T., Liu, X.K., Zhang, Y., Dong, C., 2006. BTNL2, a butyrophilin-like molecule that functions to inhibit T cell activation. J. Immunol. 176, 7354–7360.

Nunes, H., Soler, P., Valeyre, D., 2005. Pulmonary sarcoidosis. Allergy 60, 565–582.

Nunes, H., Bouvry, D., Soler, P., Valeyre, D., 2007. Sarcoidosis. J Rare. Dis. 2, 46.

Raponi, M., Kralovicova, J., Copson, E., Divina, P., Eccles, D., Johnson, P., Baralle, D., Vorechovsky, I., 2011. Prediction of single-nucleotide substitutions that result in exon skipping: identification of a splicing silencer in BRCA1 exon 6. Hum. Mutat. 32, 436–444.

Rose, A.S., Tielker, M.A., Knox, K.S., 2008. Hepatic, ocular, and cutaneous sarcoidosis. Clin. Chest Med. 29, 509–524 ix.

Rybicki, B.A., Iannuzzi, M.C., Frederick, M.M., Thompson, B.W., Rossman, M.D., Bresnitz, E.A., Terrin, M.L., Moller, D.R., Barnard, J., Baughman, R.P., DePalo, L., Hunninghake, G., Johns, C., Judson, M.A., Knatterud, G.L., McLennan, G., Newman, L.S., Rabin, D.L.,

Rose, C., Teirstein, A.S., Weinberger, S.E., Yeager, H., Cherniack, R., 2001. Familial aggregation of sarcoidosis. A case-control etiologic study of sarcoidosis (ACCESS). Am. J. Respir. Crit. Care Med. 164, 2085–2091.

Rybicki, B.A., Hirst, K., Iyengar, S.K., Barnard, J.G., Judson, M.A., Rose, C.S., Donohue, J.F., Kavuru, M.S., Rabin, D.L., Rossman, M.D., Baughman, R.P., Elston, R.C., Maliarik, M.J., Moller, D.R., Newman, L.S., Teirstein, A.S., Iannuzzi, M.C., 2005a. A sarcoidosis genetic linkage consortium: the Sarcoidosis Genetic Analysis (SAGA) study. Vasc. Diffuse. Lung Dis. 22, 115–122.

Rybicki, B.A., Walewski, J.L., Maliarik, M.J., Kian, H., Iannuzzi, M.C., 2005b. The BTNL2 gene and sarcoidosis susceptibility in African Americans and Whites. Am. J. Hum. Genet. 77, 491–499.

Saidha, S., Sotirchos, E.S., Eckstein, C., 2012. Etiology of sarcoidosis: does infection play a role? Yale J. Biol. Med. 85, 133–141.

Smith, G., Brownell, I., Sanchez, M., Prystowsky, S., 2008. Advances in the genetics of sarcoidosis. Clin. Genet. 73, 401–412.

Suzuki, H., Ota, M., Meguro, A., Katsuyama, Y., Kawagoe, T., Ishihara, M., Asukata, Y., Takeuchi, M., Ito, N., Shibuya, E., Nomura, E., Uemoto, R., Nishide, T., Namba, K., Kitaichi, N., Morimoto, S., Kaburaki, T., Ando, Y., Takenaka, S., Nakamura, J., Saeki, K., Ohno, S., Inoko, H., Mizuki, N., 2012. Genetic characterization and susceptibility for sarcoidosis in Japanese patients: risk factors of BTNL2 gene polymorphisms and HLA class II alleles. Invest. Ophthalmol. Vis. Sci. 53, 7109–7115.

Valentonyte, R., Hampe, J., Huse, K., Rosenstiel, P., Albrecht, M., Stenzel, A., Nagy, M., Gaede, K.I., Franke, A., Haesler, R., Koch, A., Lengauer, T., Seegert, D., Reiling, N., Ehlers, S., Schwinger, E., Platzer, M., Krawczak, M., Muller-Quernheim, J., Schurmann, M., Schreiber, S., 2005. Sarcoidosis is associated with a truncating splice site mutation in BTNL2. Nat. Genet. 37, 357–364.

Wijnen, P.A., Voorter, C.E., Nelemans, P.J., Verschakelen, J.A., Bekers, O., Drent, M., 2011. Butyrophilin-like 2 in pulmonary sarcoidosis: a factor for susceptibility and progression? Hum. Immunol. 72, 342–347.

Ziegenhagen, M.W., Muller-Quernheim, J., 2003. The cytokine network in sarcoidosis and its clinical relevance. J. Intern. Med. 253, 18–30.