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CARD15/NOD2 polymorphisms are associated with severe pulmonary sarcoidosis

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ABSTRACT: **Sarcoidosis and Crohn's disease are heterogeneous systemic diseases characterised by granulomatous inflammation. *Caspase recruitment domain* (*CARD*)*15* is a major susceptibility gene for Crohn's disease, and specifically for ileal and fibrostenotic subtypes. The *C-C chemokine receptor* (*CCR*)*5* gene has been associated with both parenchymal pulmonary sarcoidosis and perianal Crohn's disease.**

This study explored associations between *CARD15* polymorphisms, *CCR5* haplotype and distinct pulmonary sarcoidosis subtypes. 185 Caucasian sarcoidosis patients were genotyped for *CARD15* and *CCR5* polymorphisms. The genetic data were compared with 347 healthy controls and were examined for associations with serial pulmonary function tests and chest radiographs. *CARD15* genotypes did not differ between the unselected sarcoidosis cohort and controls. However, patients carrying the functional *2104T* (*702W*) polymorphism were more likely to have radiographic stage IV disease at 4-yr follow-up. All patients possessing both *CARD15 2104T* and *CCR5 HHC* haplotype had stage IV disease at presentation. Carriage of *2104T* was associated with worse forced expiratory volume in 1 s, whereas carriage of the *CARD15 1761G (587R)* polymorphism was associated with better lung function.

For the first time, an association between two *CARD15* polymorphisms and specific sarcoidosis phenotypes has been demonstrated, as well as an additive effect of possessing *CARD15 2104T* and *CCR5 HHC* haplotype.

KEYWORDS: ***Caspase recruitment domain 15*, *C-C chemokine receptor 5*, lung function tests, polymorphism, sarcoidosis**

Sarcoidosis and Crohn's disease are idiopathic systemic granulomatous disorders of the lung and intestine, respectively, with variable involvement of the skin, eyes and joints. There are associations between phenotypic variability and human leukocyte antigen class II alleles in both sarcoidosis (*DQB1*0201* with Lofgren's syndrome and erythema nodosum [1]; *DQB1*0602* with severe pulmonary disease [1, 2]) and Crohn's disease (*DRB1*07* with ileal involvement [3]; *DRB1*0103* with extraintestinal manifestations [4]). Furthermore, the *HHC* haplotype of the *C-C chemokine receptor* (*CCR*)*5* gene (which promotes T-cell recruitment and activation) is a marker for parenchymal involvement [5] but not for sarcoidosis overall, whereas the 32 bp deletion (Δ32) polymorphism of *CCR5* is associated with the subgroup of Crohn's patients with perianal disease [6].

The *caspase recruitment domain* (*CARD*)*15* gene (also known as nucleotide oligomerisation domain (*NOD*)2) is a major susceptibility gene for Crohn's disease [7, 8]. The *CARD15* gene encodes an intracellular protein (CARD15) of the NOD family, involved in innate immunity through recognition of bacterial pathogen-associated molecular patterns. CARD15 is composed of a nucleotide binding domain and 10 leucine-rich repeats [7, 9], which recognise muramyl dipeptide, a component of bacterial cell wall peptidoglycan. The three major Crohn's disease-associated variants, all located in this region, result in either amino acid substitutions (*C2104T* (*R702W*) and *G2722C* (*G908R*)) or premature truncation of the protein (*3020 insertion C* (*1007fs*)). In European and North American cohorts, the risk of disease increases up to threefold with carriage of a single variant allele and 20-fold with possession of two variant alleles [10]. Nine other *CARD15* single nucleotide polymorphisms (SNPs), including *T1761G* (*R587R*) and *C802T* (*P268S*), are also associated with

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Crohn's disease, but in these cohorts are in linkage disequilibrium with the three previously mentioned variants [11]. Notably, *CARD15* is associated with specific Crohn's subtypes: ileal and fibrostenotic disease.

CARD15 is the disease gene for Blau syndrome, a rare autosomal dominant granulomatous disorder characterised by arthritis, uveitis and a skin rash [12, 13]; early-onset sarcoidosis has also been associated with *CARD15* mutations [14]. However, in these diseases, the mutations are distinct from those of Crohn's disease.

The similarities between Crohn's disease and sarcoidosis, and the genotype–phenotype associations observed in the subtypes of the diseases which highlight their heterogeneity, justify the investigation of *CARD15* in sarcoidosis. Previous studies of *CARD15* in unselected sarcoidosis cohorts have provided conflicting results, with no association found in three studies [15–17], but an association with the *G2722C* (*G908R*) polymorphism in one report [18]. However, specific subgroups of sarcoidosis patients were not investigated in any of these cohorts.

Both *CARD15* and *CCR5* genes are of importance in innate immunity, and have been associated with specific subtypes of Crohn's disease and sarcoidosis. Therefore, carefully phenotyped patients with sarcoidosis were genotyped for 1) five Crohn's disease-associated *CARD15* polymorphisms, and 2) the *CCR5 HHC* haplotype, to test the hypothesis that polymorphisms in these genes would be associated with specific sarcoidosis phenotypes, and to investigate potential gene–gene interactions.

METHODS

Sarcoidosis patients

A total of 185 Caucasian patients were included in this study. All were recruited from the sarcoidosis clinic of the Royal Brompton Hospital (London, UK) a tertiary referral centre with patients mainly from the south east of England. In all patients, sarcoidosis was diagnosed histologically, and according to the criteria defined in the American Thoracic Society (ATS)/European Respiratory Society (ERS)/World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) consensus statement on sarcoidosis [19].

Written patient consent was obtained from all subjects. The Ethics Committees of the Royal Brompton Hospital gave authorisation for the study.

Controls

The control population comprised 347 white Caucasian subjects from south-east England, healthy as judged by checks, including medical history, physical examination and routine laboratory blood testing at regular intervals, during a 10-yr period before blood was taken for DNA extraction.

Genotyping for CARD15 *and* CCR5 *polymorphisms*

SNPs were determined using sequence-specific primers and PCR [20, 21]. Five *CARD15* SNPs were studied in all 185 patients (nomenclature according to [11]): *802 C>T* (*P268S, exon4, rs2066842*), *1761 T>G* (*R587R, exon 4, rs1861759*), *2104C>T* (*R702W, exon 4, rs2066844*), *2722G>C* (*G908R, exon8, rs2066845*) and *3020insC* (*1007fs, exon 11, rs2066847*). Genotyping for eight polymorphisms of the *CCR5* gene (*-5563(A/G), -3900(C/A), -3458(T/G), -2459(G/A), -2135(T/C), -2086(A/G), -1835(C/T)* and *Δ32*) was also undertaken as previously described and haplotypes assigned, to identify those possessing the *HHC* haplotype (*-2459G/-2135T/-2086G/-1835C*) [5]. 104 of the subjects had been included in the previous study [5] and 56 additional cases were genotyped.

Radiography

Chest radiographs for each patient were examined and compared in order to determine disease severity and course; these were evaluated independently by two experienced pulmonary radiologists [5] at presentation and 2- and 4-yr follow-up. Chest radiographic data were available for 177 of the 185 patients at presentation to the Royal Brompton Hospital, 158 at 2 yrs and 126 at 4 yrs. These differences were due to the inclusion of a minority of patients with more recent diagnoses who have not been followed up for long enough to be included in the 2- and 4-yr analysis. Chest radiograph staging was classified according to the joint ATS/ERS/WASOG consensus statement on sarcoidosis [19]. Table 1 shows the chest radiograph staging for patients genotyped for *CARD15* polymorphisms only, and for patients genotyped for both *CARD15* and *CCR5* polymorphisms, respectively.

Pulmonary function testing

Pulmonary function tests included forced expiratory volume in 1 s (FEV_1) and forced expiratory vital capacity (FVC) assessed by spirometry and transfer factor of the lung for carbon monoxide ($T_{L,CO}$) as measured by the single breath technique. Both were expressed as % predicted. These data were available for 174 of the 185 subjects at presentation, 149 at 2 yrs and 136 at 4 yrs.

TABLE 1 Chest radiograph staging of sarcoidosis patients genotyped for the *caspase recruitment domain (CARD)15*, and both *CARD15* and *C-C chemokine receptor* (*CCR*)*5* single nucleotide polymorphisms, at presentation, 2 yrs and 4 yrs

	Presentation	2 yrs	4 yrs
CARD15			
Subjects n	177	158	126
Stage 0	22 (12.4)	33 (20.9)	37 (29.4)
Stage I	33 (18.6)	24 (15.2)	12 (9.5)
Stage II	39 (22.0)	21 (13.3)	10 (7.9)
Stage III	16 (9.0)	11 (7.0)	6 (4.8)
Stage IV	67 (37.9)	69 (43.7)	61 (48.4)
CARD15* and *CCR5			
Subjects n	160	144	115
Stage 0	21 (13.1)	31 (21.5)	35 (30.4)
Stage I	29 (18.1)	22 (15.3)	9 (7.8)
Stage II	35 (21.9)	17 (11.8)	10 (8.7)
Stage III	14 (8.8)	11 (7.6)	6 (5.2)
Stage IV	61 (38.1)	63 (43.8)	55 (47.8)

Data are presented as n (%), unless otherwise stated.

Data analysis

Genotype and allele frequencies were determined by direct counting. Haplotypes were deduced by PHASE, version 2, a statistical haplotype reconstruction method [22].

Statistical analyses were performed using SPSS version 14 (SPSS, Chicago, IL, USA). Categorical data were analysed by Chi-squared contingency tables or Fisher's exact test as appropriate. Continuous data were analysed using the Mann–Whitney U-test or Kruskal–Wallis test as appropriate. A value of $p<0.05$ was considered significant.

RESULTS

CARD15 polymorphisms: unselected sarcoidosis cohort

In order to determine whether there were any associations between sarcoidosis in general and *CARD15* polymorphisms or haplotypes, allele and haplotype frequencies were compared for all sarcoidosis patients and the control population. These comparisons are shown in tables 2 and 3. Table 2 summarises the allele frequencies of the five *CARD15* polymorphisms investigated in the sarcoid and control populations. Both populations were in Hardy–Weinberg equilibrium for all genotype frequencies. No significant differences were found between the unselected sarcoidosis cohort and controls. Table 3 shows the *CARD15* haplotypes and their frequencies: there were no significant differences in *CARD15* haplotypes between the two groups.

Of the functional, Crohn's disease-associated polymorphisms, 2104T occurred only on haplotype 4, 3020insC on haplotype 5 and 2722C on haplotype 6. 1761G occurred only on haplotype 1, whereas 802T occurred on haplotypes 3–6 (table 3).

TABLE 2 *Caspase recruitment domain (CARD)15* allele frequencies in sarcoidosis and controls

Polymorphism	Amino acid	Allele frequency	
		Sarcoidosis[#]	Controls[¶]
802 (rs2066842)			
C	Pro	289 (78.1)	505 (72.8)
T	Ser	81 (21.9)	189 (27.2)
1761 (rs1861759)			
T	Arg	222 (60.0)	439 (63.3)
G	Arg	148 (40.0)	255 (36.7)
2104 (rs2066844)			
C	Arg	351 (94.9)	658 (94.8)
T	Trp	19 (5.1)	36 (5.2)
2722 (rs2066845)			
G	Gly	368 (99.5)	684 (98.6)
C	Arg	2 (0.5)	10 (1.4)
3020 (rs2066847)			
WT		367 (99.2)	683 (98.4)
ins C	Fs	3 (0.8)	11 (1.6)

Data are presented as n (%). WT: wild-type; ins C: insertion C; Fs: frameshift. [#]: n=185; [¶]: n=347.

CARD15, CCR5 and radiographic stage IV disease in sarcoidosis

Associations between *CARD15* polymorphisms, the *CCR5 HHC* haplotype and chest radiographic staging were investigated. Carriage of the *CARD15 2104T* polymorphism (haplotype 4) was associated with a higher frequency of radiographic stage IV disease than non-carriers at 4 yrs of follow up (76.9% *versus* 45.1%, OR 4.1, 95% CI 1.0–15.5; p=0.04). There were no significant differences between carriers and non-carriers of the other *CARD15* polymorphisms or haplotypes and either parenchymal disease (stage II–IV) or stage IV disease at presentation, 2 yrs or 4 yrs.

We have previously shown an association between carriage of the *CCR5 HHC* haplotype and parenchymal disease at presentation (radiographic stage $\geq$II *versus* stages 0 and I) [5]. In the current, larger, cohort (56 additional cases) the *HHC* haplotype was significantly increased in patients with stage IV compared with stage 0–III disease at presentation (n=160, 72.1% *versus* 46.5%, OR 3.0, 95% CI 1.5–5.9; p=0.003), 2 yrs (n=144, 68.3% *versus* 45.7%, OR 2.6, 95% CI 1.3–5.1; p=0.01) and 4 yrs (n=115, 69.1% *versus* 46.7%, p=0.02, OR=2.6, 95% CI=1.2–5.5).

Having established the influence of the *CCR5 HHC* haplotype on radiographic stage IV disease and having seen for the first time a significant association between one of the functional Crohn's disease-associated *CARD15* SNPs (*2104T*, haplotype 4) and stage IV disease, we investigated the influence of *2104T* in sarcoidosis patients subtyped according to their *CCR5 HHC* haplotype status. Patients were divided into four groups: 1) Group 1 (n=7): carriage of both *CCR5 HHC* and *CARD15 2104T*; 2) Group 2 (n=83): carriage of only *CCR5 HHC*; 3) Group 3 (n=8): carriage of only *CARD15 2104T*; and 4) Group 4 (n=62): carriage of neither.

All seven of the subjects carrying the *HHC* haplotype and *CARD15 2104T* (haplotype 4) had radiographic stage IV disease at presentation, 2 yrs and 4 yrs (fig. 1).

No differences were found when the subgroups were similarly analysed for change in chest radiograph status (at 4 yrs follow-up *versus* at presentation).

TABLE 3 *Caspase recruitment domain (CARD)15* haplotypes and their carriage frequencies in sarcoidosis and controls

	802	*1761*	*2104*	*2722*	*3020*	Carriage frequency	
						Sarcoidosis[#]	Controls[¶]
Haplotype 1	C	**G**	C	G	WT	123 (66.5)	212 (61.1)
Haplotype 2	C	T	C	G	WT	116 (62.7)	205 (59.1)
Haplotype 3	**T**	T	C	G	WT	52 (28.1)	122 (35.2)
Haplotype 4	**T**	T	**T**	G	WT	17 (9.2)	35 (10.1)
Haplotype 5	**T**	T	C	G	**ins C**	3 (1.6)	11 (3.2)
Haplotype 6	**T**	T	C	**C**	WT	2 (1.1)	10 (2.9)

Data are presented as n (%). Bold signifies a variant allele. WT: wild-type; ins C: insertion C. [#]: n=185; [¶]: n=347.

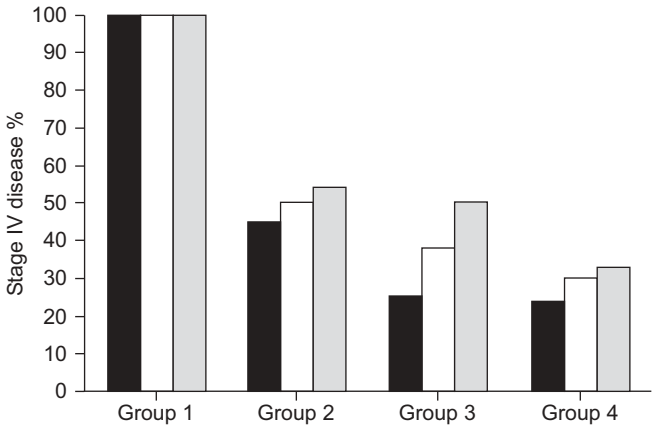

FIGURE 1. Frequencies of chest radiograph stage IV disease related to *C-C chemokine receptor* (*CCR*)*5 HHC* and *caspase recruitment domain* (*CARD15*) *2104T* (haplotype 4) status. Group 1 (n=7 at presentation and 2 yrs and n=6 at 4 yrs): carriage of both *CCR5 HHC* and *CARD15 2104T*; Group 2 (n=83 at presentation, n=73 at 2 yrs and n=60 at 4 yrs): carriage of only *CCR5 HHC*; Group 3 (n=8 at presentation and 2 yrs and n=6 at 4 yrs): carriage of only *CARD15 2104T*; Group 4 (n=62 at presentation, n=56 at 2 yrs and n=43 at 4 yrs): carriage of neither. All of the subjects carrying *CCR5 HHC* and *CARD15 2104T* had stage IV disease at presentation (p=0.0003, Kruskal–Wallis test), and at 2 yrs and 4 yrs. ■: at presentation; □: 2 yrs; ■: 4 yrs.

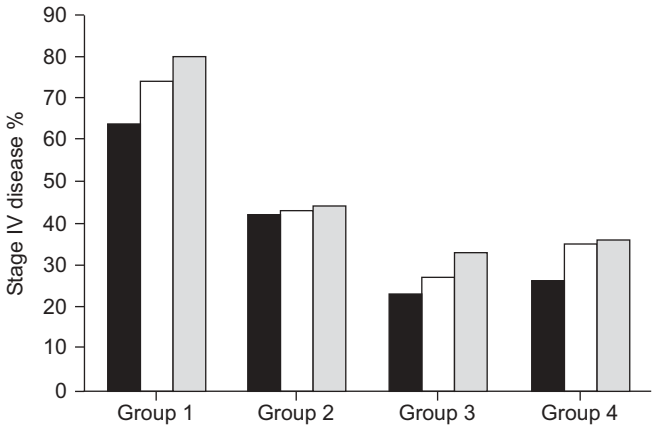

FIGURE 2. Frequencies of chest radiograph stage IV disease related to *C-C chemokine receptor* (*CCR*)*5 HHC* haplotype and *caspase recruitment domain* (*CARD*)*15 802T* (haplotypes 3–6) status. Group 1 (n=28 at presentation, n=27 at 2 yrs and n=25 at 4 yrs): carriage of both *CCR5 HHC* and *CARD15 802T*; Group 2 (n=62 at presentation, n=53 at 2 yrs and n=41 at 4 yrs): carriage of only *CCR5 HHC*; Group 3 (n=31 at presentation, n=30 at 2 yrs and n=24 at 4 yrs): carriage of only *CARD15 802T*; Group 4 (n=39 at presentation, n=34 at 2 yrs and n=25 at 4 yrs): carriage of neither. The *CARD15 802T* polymorphism in combination with the *CCR5 HHC* haplotype was also significantly associated with stage IV disease at presentation (p<0.005), 2 yrs (p<0.005), and 4 yrs (p<0.005), Kruskal–Wallis test. ■: at presentation; □: 2 yrs; ■: 4 yrs.

The *CARD15 802T* polymorphism in combination with the *HHC* haplotype was also significantly associated with radiographic stage IV disease (p=0.002 at presentation, 2 yrs and 4 yrs) (fig. 2; groups as described for fig. 1 but for *802T* carriage rather than *2104T*). Further analysis revealed, however, that this was a consequence of the known linkage disequilibrium [11] between *802T* and *2104T* (table 3).

No significant associations were found for *2722C*, *3020insC* or *1761G*, or their respective haplotypes.

CARD15 *polymorphisms and pulmonary function tests*

To clarify whether the carriage of *CARD15* polymorphisms and/or haplotypes acted as an index for disease severity, the possession of *CARD15* polymorphisms (and hence haplotypes, table 3) was investigated in relation to lung function tests.

Table 4 shows the carriage of the *802T*, *1761G* (haplotype 1) and *2104T* (haplotype 4) alleles and lung function tests at presentation, 2 yrs and 4 yrs. Carriage of the *CARD15 1761G* allele (haplotype 1) was associated with better lung function, as defined by $T_{L,CO}$, than non-carriage of the *G* allele at presentation (p=0.001), 2 yrs (p=0.006) and 4 yrs (p=0.002). The differences were significant at all time points, and interestingly a clear gene–dose effect can be seen at presentation: *GG* homozygotes have better $T_{L,CO}$ than *TG* heterozygotes, whereas *TT* homozygotes have the worst $T_{L,CO}$ (fig. 3).

Figure 4 shows the mean comparisons of $T_{L,CO}$ with *CARD15 1761* genotypes at presentation, 2 yrs and 4 yrs. Carriers of the *1761G* allele had a higher FEV_1 (p=0.02) at presentation compared with non-carriers. These patients also had a higher FVC at presentation (p=0.01); there was a trend towards significance for FVC at 2 yrs (p=0.07); and at 4 yrs the difference was again significant (p=0.02) (table 4). Of note, this cohort with better lung function did not possess haplotype 4, which includes the *2104T* allele.

Consistent with the worse radiographic stage associations, patients carrying the *2104T* allele (haplotype 4) had a worse median FEV_1 at presentation (79.3% *versus* 92.3%; p=0.04), 2 yrs (73.8% *versus* 93.3%; p=0.02) and 4 yrs (77.6% *versus* 92.4%; p=0.05), and a worse median FVC at 4 yrs follow-up (89.3% *versus* 100.3%; p=0.04) than patients not carrying the *2104*T allele.

No significant differences were seen between the carriage of *802T*, *3020insC* or *2722C*, and pulmonary function tests.

DISCUSSION

Sarcoidosis and Crohn's disease are both characterised by inflammation, granuloma formation and their systemic nature, with frequent extrapulmonary and extraintestinal manifestations respectively. They both display considerable phenotypic heterogeneity and degrees of disease severity, and important genetic associations have been identified for both conditions. The similarities between sarcoidosis and Crohn's disease have prompted several studies of the Crohns susceptibility gene *CARD15/NOD2* in unselected sarcoidosis cohorts [15–18]. Of these, one has reported an association with the *2722C* (*G908R*) variant [18] and one [17] found an increased transmission of two of the alleles but concluded that these mutations played no major role in their study population.

The *CARD15* polymorphisms studied are specifically associated with Crohn's disease subtypes, and it was hypothesised that they may be associated with subtypes of sarcoidosis. The *CCR5* gene has also been associated with both diseases [5, 6],

TABLE 4 Carriage of *caspase recruitment domain* (*CARD*)*15 802T*, *1761G* (haplotype 1) and *2104T* (haplotype 4) alleles in relation to lung function tests at presentation, 2 yrs and 4 yrs

	802T				*1761G* (haplotype 1)				*2104T* (haplotype 4)			
	n	FEV_1	FVC	$T_{L,CO}$	n	FEV_1	FVC	$T_{L,CO}$	n	FEV_1	FVC	$T_{L,CO}$
Presentation	174											
Carrier	64	93.05	98.35	76.45	118	93.2	100.1	81.8	17	79.3	86.8	68.8
Non-carrier	110	90.95	98	80.45	56	85.9	94.3	71.4	157	92.3	98.6	78.8
p-value		0.42	0.5	0.2		**0.02**	**0.01**	**0.001**		**0.04**	0.3	0.07
2 yrs	148											
Carrier	59	94.3	101.1	77.45	99	92.3	101.3	80.8	15	73.8	94.1	71.9
Non-carrier	89	91.2	98.7	78.6	50	89.7	97.9	71.9	133	93.3	99.9	79.5
p-value		0.6	0.9	0.5		0.2	**0.07**	**0.006**		**0.02**	0.2	0.1
4 yrs	136											
Carrier	53	91.3	96.8	73.7	89	92.2	101.5	81.3	13	77.6	89.3	71.8
Non-carrier	83	92.4	100.2	78.4	47	89.1	91.2	70.5	123	92.4	100.3	77.3
p-value		0.3	0.2	0.06		0.2	**0.02**	**0.002**		**0.05**	**0.04**	0.2

Data are presented as median % predicted values, unless otherwise stated. Bold signifies a significant value. FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; $T_{L,CO}$: transfer factor of the lung for carbon monoxide.

and this gene was further investigated to allow subclassification of the cohort on the basis of their *CCR5* haplotype.

Significant associations have been demonstrated between both a functional *CARD15* SNP (*2104T*, haplotype 4) and a synonymous *CARD15* SNP (*1761G*, haplotype 1), and severe pulmonary sarcoidosis phenotypes. An association between the *CCR5 HHC* haplotype and chest radiographic stage IV disease has been established. Of note, all patients possessing both the *CCR5 HHC* haplotype and *CARD15 2104T* allele had radiographic stage IV disease at presentation.

Patients possessing the functional *2104T* allele (haplotype 4) had worse FEV_1 values at presentation, 2 yrs and 4 yrs, as well as worse FVC at 4 yrs. The carriage of this polymorphism was also associated with an increased risk of radiographic stage IV disease at 4 yrs.

The *CARD15* gene product plays an important role in innate immunity by influencing recognition of specific bacterial patterns, and this is impaired in those possessing the Crohn's disease-associated functional variants. The precise mechanism through which these variants result in increased disease susceptibility remains uncertain. Transient transfection experiments have suggested that the impaired binding of muramyl dipeptide decreases nuclear factor (NF)-κB activation [23], with consequent impaired microbial clearance. In *CARD15* mutant mice, however, increased downstream NF-κB signalling has been demonstrated [24] with subsequent inflammation. The association of one of the functional mutations with a severe

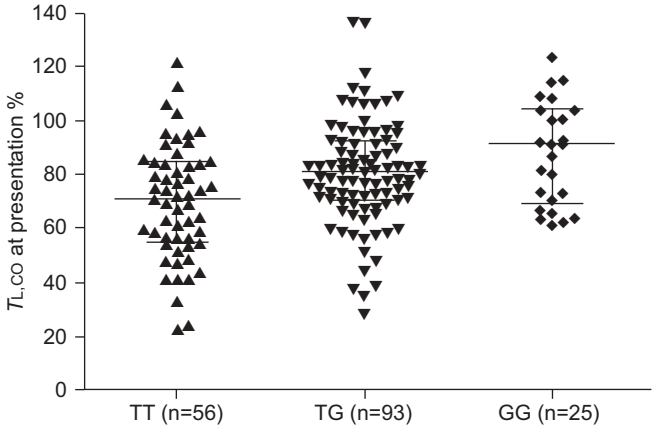

FIGURE 3. Influence of *caspase recruitment domain* (*CARD*)*15 1761* genotype on the transfer factor of the lung for carbon monoxide ($T_{L,CO}$). There was a significant gene–dose effect between *CARD15 1761* genotype (GG>TG>TT) and the level of $T_{L,CO}$ at presentation (p=0.001). Median and interquartile ranges are shown by horizontal lines.

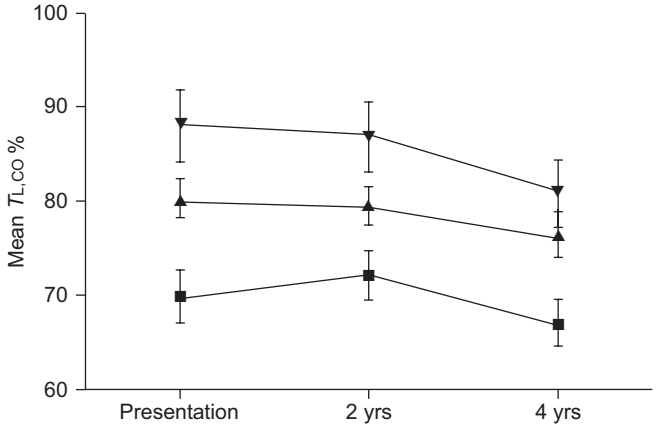

FIGURE 4. Mean ± SEM comparisons of transfer capacity of the lung for carbon monoxide ($T_{L,CO}$) with *caspase recruitment domain* (*CARD*)*15 1761* genotypes at presentation, 2 yrs and 4 yrs. Individuals with the GG genotype (>TG>TT) had significantly higher mean $T_{L,CO}$ levels at presentation (p=0.001), 2 yrs (p=0.009) and 4 yrs (p=0.003). ■: TT; ▲: TG; ▼: GG.

pulmonary sarcoidosis phenotype offers an intriguing insight into possible dysregulated responses to bacteria in this cohort.

We have previously found associations between the *CCR5 HHC* haplotype and parenchymal lung disease in a cohort of 104 British patients [5]. In this larger cohort, we have elucidated a significant association with pulmonary radiographic stage IV disease. These findings are consistent with our previous results, suggesting that dysfunction of this gene, of major importance in T-cell activation, could result in abnormal trafficking of T-cells to the lung and hence parenchymal abnormalities culminating in fibrosis [5].

All patients carrying the *CCR5 HHC* haplotype and the *CARD15 2104T* polymorphism had pulmonary stage IV disease at presentation. It is of interest that these two genes are both involved in innate immunity as discussed above. CARD15-deficient mice have been shown to lack the normal inhibition of the toll-like receptor (TLR) 2-mediated NF-κB response, with consequent T-helper (Th) cell type 1 overactivation [25]. CCR5 also enhances T-cell activation [26]. It is hypothesised that the possession of *CARD15 2104T* contributes to an impaired response to bacteria, whether commensal or pathogenic, with subsequent abnormal T-cell activation that is further enhanced by the possession of *CCR5 HHC* in this cohort of patients.

The *CARD15 1761G* allele, present in 40% of the sarcoidosis cohort, was associated with significantly better lung function parameters (not only $T_{L,CO}$ but also FVC) than the wild-type allele at all time-points. This suggests that carriage of this polymorphism is associated with less diffuse lung disease. A significant gene–dose effect was also observed (fig. 3). In Crohn's disease, this polymorphism is protective: there is a lower frequency of Crohn's disease in those possessing the *CARD15 1761G* allele [11]. This suggests that the polymorphism confers a favourable phenotype. It is, however, a synonymous SNP.

Possible explanations for the association of this synonymous SNP with a specific disease phenotype may include linkage disequilibrium with another, functional SNP, as yet unidentified. This hypothesis would suggest that it is acting as a marker. Alternatively, there has been considerable interest in ''silent'' polymorphisms, and a functional study has shown that the product of a synonymous polymorphism can differ significantly from the wild-type protein [27]. It was hypothesised that the synonymous polymorphism may affect the timing of cotranslational folding and so alter the structure of binding sites. Further functional studies of the *1761G* SNP will be required to investigate this.

In this study, chest radiographs were evaluated independently by two experienced pulmonary radiologists and chest radiographic appearances remain the accepted means of staging sarcoidosis [19]. However, pulmonary computed tomography (CT) scoring may be a more sensitive means of assessing lung disease pattern, including fibrosis and disease severity [28], and further studies investigating carriage of these genetic polymorphisms in relation to CT score may serve to confirm the association between lung fibrosis and these genes. Indeed, the relative insensitivity of chest radiographs may explain the lack of association found for *CARD15 1761G* and radiographic stage, when significant associations with lung function were found. Quantification of lung function change with continuous variables may be more precise than the categorical, descriptive chest radiographic staging system, and thus more likely to be able to identify associations [29].

A recent study using blood mononuclear cells has shown that a combination of NOD2 and TLR-2 ligand stimulation induced a higher secretion of the pro-inflammatory cytokines tumour necrosis factor (TNF)-α and interleukin (IL)-1β in sarcoidosis patients than healthy controls [30]. These cytokines are important in determining the inflammatory response in active sarcoidosis, and indeed granuloma formation. It will be informative to perform further such studies in individuals subclassified according to their *CARD15* genotype. Specifically, it would be of interest to investigate whether those possessing the *1761G* allele have a reduced predisposition to TNF-α and IL-1β secretion compared with those with the wild-type allele, and whether those with the *2104T* polymorphism have significantly greater secretion of these cytokines.

In conclusion, neither the *CARD15* nor the *CCR5* variants were more prevalent overall in the sarcoidosis cohort than the controls, confirming the hypothesis that these are not disease susceptibility genes but rather are associated with disease modification and/or progression after sarcoidosis is established. These results provide further evidence for genetic heterogeneity in determining the phenotype in sarcoidosis.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

None declared.

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