

## EXTENDED REPORT

# CARD15 gene polymorphisms in patients with spondyloarthropathies identify a specific phenotype previously related to Crohn's disease

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**Background:** The association between spondyloarthropathy and Crohn's disease is well known. A risk for evolution to Crohn's disease has already been shown in the subgroup of patients with spondyloarthropathy associated with chronic gut inflammation.

**Objective:** To investigate whether the reported polymorphisms in the *CARD15* gene, a susceptibility gene for Crohn's disease, are associated with the presence of preclinical intestinal inflammation observed in spondyloarthropathies.

**Methods:** 104 patients with spondyloarthropathies were studied. All underwent ileocolonoscopy with biopsies between 1983 and 2004. The prevalence of three single nucleotide polymorphisms in the *CARD15* gene (R702W, G908R, and 1007fs) was assessed using restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR); the patients were compared with an ethnically matched Crohn's disease population and a control population.

**Results:** The carrier frequency of R702W, G908R, or 1007fs variants in the spondyloarthropathy populations (20%) was similar to the control population (17%), but increased to 38% in the spondyloarthropathy subgroup with chronic gut inflammation. This frequency was significantly higher than in the other spondyloarthropathy subgroups ( $p=0.001$ ) or the control group ( $p=0.006$ ), but not different from the Crohn's disease group (49%) (NS). This indicates that *CARD15* polymorphisms are associated with a higher risk for development of chronic gut inflammation.

**Conclusions:** *CARD15* gene polymorphisms clearly identify a subgroup of patients with spondyloarthropathies associated with chronic intestinal inflammation.

The spondyloarthropathies are a group of interrelated inflammatory diseases characterised by a pauciarthral, peripheral, asymmetrical arthritis with or without axial involvement, with ankylosing spondylitis as the prototype.<sup>1</sup> Reported prevalences of spondyloarthropathy vary between 0.2% and 1.9%.<sup>2-3</sup> Although an association with HLA-B27 is strong, recent genetic studies suggest a polygenic model of susceptibility.<sup>4-7</sup>

In up to 60% of spondyloarthropathy patients, articular involvement is associated with subclinical histological evidence of chronic or acute gut inflammation in the ileum or colon.<sup>8-10</sup> We described a long term evolution to overt Crohn's disease in 13% of patients with initial chronic gut inflammation.<sup>11-12</sup> The presence of chronic intestinal inflammation was not related to HLA-B27, but a weak association with HLA-B62 was found.<sup>11</sup>

The observed immunological similarities between spondyloarthropathy with gut inflammation and Crohn's disease support the concept that this subgroup of spondyloarthropathy patients can be considered a model for early immune alterations related to Crohn's disease. An enrichment of gut mucosal T cell lines with  $\alpha E\beta 7$  integrin and an increased expression of its ligand, E-cadherin, is found in the intestine in Crohn's disease as well as in spondyloarthropathy patients.<sup>13-15</sup> Recirculation of gut primed T cells to synovial tissue is one potential mechanism whereby gut and synovial inflammation could be linked. This hypothesis is supported by an altered expression of  $\beta 7$  integrins, which are highly expressed within the gut, on synovial T cells from patients

with spondyloarthropathies compared with rheumatoid arthritis.<sup>16</sup> Another potential mechanism includes trafficking of antigen presenting cells between gut and joints. Consistent with this was the augmented infiltration of gut mucosa and synovium with CD163 positive macrophages (producing interleukin 1 (IL1) and tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) in both Crohn's disease and spondyloarthropathy.<sup>17-18</sup> Finally, a comparable beneficial clinical effect of infliximab, a monoclonal antibody to TNF $\alpha$ , suggests a key role for this cytokine in both diseases.<sup>19-20</sup>

In 2001, a correlation was reported between polymorphisms in the *CARD15* gene and an increased susceptibility to Crohn's disease.<sup>21-23</sup> Three independent single nucleotide polymorphisms (SNPs) in *CARD15* are associated with Crohn's disease in around 30-46% of patients (one frame shift mutation (1007fs (SNP13)) and two missense mutations (R702W (SNP8) and G908R (SNP12)).<sup>22-24</sup> These variants increase the risk for Crohn's disease by a factor of 3 for heterozygotes and by a factor of 38 or 44 for, respectively, homozygous or compound heterozygous individuals.<sup>22</sup> Lower prevalences have been described in Crohn's disease patients in Scotland, Ireland, and northern Europe, whereas no association could be found in Japan.<sup>25-28</sup>

The *CARD15* gene encodes for an intracellular protein, which is expressed in monocytes, granulocytes, and dendritic, epithelial, and Paneth cells, and has binding affinity for

**Abbreviations:** CARD, caspase recruitment domain; ESSG, European Spondylarthropathy Study Group; SNP, single nucleotide polymorphism

bacterial cell wall components such as muramyl dipeptides.<sup>29</sup> The *CARD15* protein is involved in NFκB activation and in apoptosis by two N-terminal caspase recruitment domains (hence the term CARD), although its precise pathogenic role in Crohn's disease remains to be determined.<sup>29–31</sup>

*CARD15* gene polymorphisms have also been linked to another related disorder, Blau's syndrome, characterised by granulomatous inflammation of the uvea, skin, and joints.<sup>32</sup>

Several studies have been carried out to investigate the role of *CARD15* polymorphisms in spondyloarthropathies. These studies did not show an association with spondyloarthropathies or ankylosing spondylitis in particular.<sup>33–37</sup> An increased prevalence of *CARD15* polymorphisms was found in psoriatic arthritis though not in psoriatic skin disease.<sup>38–40</sup> However, a recent Italian study could not confirm this association.<sup>41</sup> Nevertheless, this finding could emphasise the importance of investigating the possible role of these genetic variants in specific clinical subpopulations of patients. In Crohn's disease, *CARD15* polymorphisms also seem to be related to certain clinical phenotypes.<sup>42–46</sup>

In view of the apparent correlation between gut inflammation in spondyloarthropathies and their clinical evolution to Crohn's disease, we investigated whether the presence of polymorphisms in this susceptibility gene for Crohn's disease is associated with gut inflammation in spondyloarthropathy.

## METHODS

### Study population

This study included 104 white patients with spondyloarthropathies (according to the ESSG criteria<sup>47</sup>), who underwent an ileocolonoscopy with concomitant ileal and colonic biopsies between 1983 and 2004. This population consisted of 74 male and 30 female patients with a mean age of 46 years (range 21 to 77). Spondyloarthropathy patients were systematically referred by the rheumatologist for an ileocolonoscopy with biopsies, independent of the presence of gastrointestinal symptoms.

Patients with a diagnosis of clinical Crohn's disease or psoriasis before the diagnosis of spondyloarthropathy were excluded from the study.

A subgroup of 54 patients with long term follow up since the time of their diagnosis of spondyloarthropathy (ranging from 17 to 49 years) was recently clinically reassessed. New follow up colonoscopies were not carried out.

The total spondyloarthropathy population consisted of 75 patients with ankylosing spondylitis according to the modified New York criteria<sup>48</sup> and 29 with an undifferentiated

form of spondyloarthropathy. Eighteen patients with ankylosing spondylitis had only axial involvement, while 57 also had peripheral disease (defined as the history or presence of peripheral arthritis, enthesitis, or both). Twenty five patients with undifferentiated spondyloarthropathy had peripheral disease and four only had axial involvement. These four patients had inflammatory low back pain and fulfilled the ESSG criteria but not the modified New York criteria for ankylosing spondylitis.

HLA-B27 status was known in 81 patients. In 53 patients both HLA-B27 and HLA-B62 status was known.

A population of 156 consecutive patients with Crohn's disease proven on clinical, endoscopic, and histological grounds was also included. This cohort included 57 male and 99 female patients with a mean age of 38 years (range 18 to 80). Prevalences were also compared with those in a control population of 140 individuals.

The study was approved by the local ethics committee. All patients signed their informed consent.

### Histological classification

A classification of histological lesions was used as reported in previous studies.<sup>8 11 12 49 50</sup> Three subgroups were distinguished: patients with normal gut histology, and those with acute and chronic inflammation.<sup>51</sup>

In acute inflammatory lesions normal architecture was well preserved. A mucosal and epithelial infiltrate of neutrophils and eosinophils was present, without a significant increase in lymphocytes. Small superficial ulcers covered with fibrin and neutrophils overlying hyperplastic lymphoid follicles were occasionally observed. The lamina propria was oedematous and haemorrhagic and contained mainly polymorphonuclear cells. The pattern of inflammation was similar to that seen in acute self limiting bacterial enterocolitis.

The principal features of chronic inflammatory lesions were mucosal architectural alterations with crypt distortion and atrophy in the colon, and villous blunting and fusion in ileal mucosa. In both ileum and colon there was an increased mixed cellularity and formation of basal lymphoid aggregates in the lamina propria. Whenever one of several biopsies featured chronic lesions, regardless of acute or active inflammation in other fragments, a diagnosis of chronic inflammation was made.

Although non-steroidal anti-inflammatory drugs may induce intestinal disorders, we and others excluded these drugs as factors in the aetiology of reported chronic inflammation.<sup>8 10 52</sup>

### *CARD15* genotyping (R702W, G908R, and 1007fs), and HLA-B27 and HLA-B62 typing

Genomic DNA was extracted from whole blood using the Qiagen blood and cell culture DNA kit (Westburg BV, Leusden, Netherlands). All patients were genotyped for R702W, G908R, and 1007fs using restriction fragment length polymorphism–polymerase chain reaction (RFLP-PCR), followed by separation of the DNA fragments on a 2.5% agarose gel. The missense mutation R702W (GenBank accession number G67950) abolishes the restriction site for *MspI*, resulting in an intact 130 base pair (bp) band for mutant alleles compared with two bands of 54 and 76 bp for wild type alleles (forward primer: 5'-CAGCCCTGATGACATTTCTCTT-3'; reverse primer: 5'-AGCCGCTCCTCTGCATCTCGTA-3'). The missense mutation G908R (GenBank accession number G67951) creates a restriction site for *HinPII*. The frameshift mutation 1007fs (GenBank accession number G67955) creates a restriction site for *NlaIV*. The presence of a mutant allele results in two bands of 219 and 41 bp, while the wild type allele produces a single 260 bp product (forward

**Table 1** Prevalence of *CARD15* variants in the populations according to subtypes defined at baseline

	n	Carriers of <i>CARD15</i> variant(s)
<b>Classification</b>		
Controls	140	24 (17%)
Crohn's disease	156	77 (49%)*
Spondyloarthropathy	104	21 (20%)†
Ankylosing spondylitis	75	16 (21%)
Undifferentiated spondyloarthropathies	29	5 (17%)
<b>Gut histology in the spondyloarthropathy population</b>		
Chronic inflammation	40 (38%)	15 (38%)‡
Acute inflammation	24 (23%)	0 (0%)
Normal histology	40 (38%)	6 (15%)

\* $\chi^2$ : p<0.001 (carrier frequency in Crohn's disease v control population).

† $\chi^2$ : p=0.5 (carrier frequency in general spondyloarthropathies v control population).

‡ $\chi^2$ : p=0.001 (chronic inflammation in patients with *CARD15* variant v chronic inflammation in patients without *CARD15* polymorphism).

**Table 2** Carrier frequency of *CARD15* variants in patients with spondyloarthropathies, Crohn's disease, and controls (%)

	Spondyloarthropathy (n = 104)				Crohn's disease (n = 156)				Controls (n = 140)			
	R702W	G908R	1007fs	Overall*	R702W	G908R	1007fs	Overall*	R702W	G908R	1007fs	Overall*
<i>CARD15</i> <sup>-/+</sup>	12 (12)	4 (4)	4 (4)	21 (20)	39 (25)	11 (7)	27 (17)	77 (49)	18 (13)	1 (1)	6 (4)	24 (17)
<i>CARD15</i> <sup>-/-</sup>	0 (0)	0 (0)	1 (1)		4 (3)	3 (2)	0 (0)		0 (0)	0 (0)	0 (0)	

Data are numbers of patients carrying R702W, G908R or 1007fs variants. *CARD15*<sup>-/+</sup>, heterozygous; *CARD15*<sup>-/-</sup>, homozygous. Overall, total number of patients in the group carrying at least one variant. \*The sum of all allelic *CARD15* variants is greater than the overall number of patients carrying at least one variant, as some patients carry two different single nucleotide polymorphisms, thus displaying compound heterozygous status.

primer: 5'-CTGAGCCTTTGTTGATGAGC-3'; reverse primer: 5'-TCTTCCAACCACATCCCCATT-3').

In the patients with known HLA-B27 and HLA-B62 status, typing of these markers was done using the microlymphocytotoxicity test, according to Terasaki and McClelland.<sup>53</sup>

**Statistical analysis**

Statistical significance was determined by the  $\chi^2$  test and odds ratios using SPSS (SPSS Inc, Chicago, Illinois, USA). Multivariate analysis (logistic regression) was carried out to investigate whether an association found in univariate analysis was independent of other genetic markers. Probability (p) values of less than 0.05 were considered significant.

**RESULTS**

We subdivided our cohort into three groups according to gut histology. Forty patients (38%) had normal histology, 24 (23%) had acute gut inflammation, and 40 (38%) had chronic gut inflammation (table 1).

**Univariate analysis**

**Prevalence of *CARD15* polymorphisms in the various populations**

The prevalences of *CARD15* polymorphisms in the total spondyloarthropathy (20%), specific ankylosing spondylitis (21%), and undifferentiated spondyloarthropathy (17%) populations did not differ significantly (table 1). All except one (homozygous for the 1007fs allelic variant) were heterozygous for at least one mutation. The prevalences of R702W, G908R, and 1007fs allelic variants in these spondyloarthropathy populations were 12%, 4%, and 5% respectively

(table 2). No compound heterozygosity was found. All carriers of *CARD15* polymorphisms in the spondyloarthropathy group had (a history of) peripheral disease (table 3). There were no significant differences in disease duration or the duration of the follow up period between the spondyloarthropathy patients carrying *CARD15* polymorphisms and the patients without these polymorphisms (data not shown).

In the Crohn's disease population, a carrier frequency of 49% (77 of 156 patients) was observed (table 1). Forty three Crohn's disease patients carried at least one R702W polymorphism, 14 carried at least one G908R polymorphism, and 27 carried at least one 1007fs polymorphism. Fourteen patients carried two polymorphisms, of which seven were homozygous and seven were compound heterozygous (table 2).

In the control group, 24 individuals (17%) carried *CARD15* polymorphisms (table 1). All except one (compound heterozygous for the R702W and 1007fs variant) were single heterozygotes (table 2).

The prevalence of polymorphisms in the spondyloarthropathy cohort (20%) was not different from that in the control group (17%) (p = 0.5; odds ratio (OR) = 1.22 (95% confidence interval (CI), 0.64 to 2.34)) and significantly lower than in our Crohn's disease population (49%) (p < 0.001; OR = 3.85 (95% CI, 2.17 to 6.83)).

**Association between *CARD15* polymorphisms and intestinal inflammation in spondyloarthropathy patients**

The carrier frequency in the subpopulation of spondyloarthropathy patients with chronic gut inflammation was 38% (15 of 40 patients) which was significantly higher than in the control population (p = 0.006; OR = 2.9 (95% CI, 1.33 to 6.30)) or the other spondyloarthropathy populations (p = 0.001; OR = 5.80 (2.02 to 16.68)) and not statistically different from that in our Crohn's disease population (49%, p = 0.2; OR = 1.62 (0.80 to 3.31)) (table 1).

Of all spondyloarthropathy patients carrying *CARD15* polymorphisms, 71% (15 of 21 patients) had chronic gut inflammation, none had acute inflammation, and 29% had normal histology (table 1). The only spondyloarthropathy patient carrying two *CARD15* variants also had chronic gut inflammation. In contrast, only 25 of 83 patients with a wild type genotype (30%) had chronic gut inflammation, 29% had acute inflammation, and 41% had normal histology. Consequently, the presence of *CARD15* polymorphisms was associated with a higher risk of the development of chronic gut inflammation.

There were no significant differences between the ankylosing spondylitis and the undifferentiated spondyloarthropathy groups for the prevalence of *CARD15* polymorphisms in patients with normal gut (3/29 v 3/11, respectively; p = 0.3; OR = 3.3 (95% CI, 0.5 to 19.4)), acute gut inflammation (0/13 v 0/11), or chronic gut inflammation (13/33 v 2/7; p = 0.7; OR = 1.6 (95% CI, 0.3 to 9.7)).

**Table 3** Prevalences of *CARD15* polymorphisms according to the presence of mainly axial or peripheral involvement in the total spondyloarthropathy group (n = 104), the ankylosing spondylitis group (n = 75), and the undifferentiated spondyloarthropathy group (n = 29)

	<i>CARD15</i>			
	Wild type	Variant	Total	
<b>Total spondyloarthropathy group</b>				
Axial	22	0	22	p = 0.006
Peripheral	61	21	82	
Total	83	21	104	
<b>Ankylosing spondylitis group</b>				
Axial	18	0	18	p = 0.006
Peripheral	41	16	57	
Total	59	16	75	
<b>Undifferentiated spondyloarthropathy group</b>				
Axial	4	0	4	p = 1.0
Peripheral	20	5	25	
Total	24	5	29	

In the subgroup of 54 patients who were clinically reassessed, four had evolved from histological chronic gut inflammation towards clinically overt Crohn's disease. Two of these four patients carried *CARD15* polymorphisms. The other 22 patients with chronic gut inflammation in this group did not develop clinical Crohn's disease.

#### Association between *CARD15* polymorphisms and HLA-B27 in spondyloarthritis patients

There was no significant association between the presence of these two genetic markers. Six of 34 HLA-B27 negative patients carried *CARD15* polymorphisms *v* 13 of 47 HLA-B27 positive patients ( $p = 0.3$ ; OR = 1.8 (95% CI, 0.6 to 5.3)).

#### Multivariate analysis

In the subgroup of 53 spondyloarthritis patients in whom both HLA-B27 and HLA-B62 status was known, logistic regression was undertaken (with the presence of chronic gut inflammation as the dependent variable). This showed that the association between chronic gut inflammation and *CARD15* polymorphisms ( $p = 0.01$ ; OR = 17.3 (95% CI, 2.0 to 152.3)) was independent of HLA-B27 ( $p = 0.42$ ; OR = 1.7 (0.5 to 6.0)) and HLA-B62 ( $p = 0.28$ ; OR = 2.5 (0.5 to 13.0)).

#### DISCUSSION

In this study we describe a novel and remarkably strong association between variants in a host defence gene located on chromosome 16 (*CARD15*) and a chronic form of gut inflammation in patients with spondyloarthropathies. The prevalence of *CARD15* polymorphisms in this subgroup of spondyloarthritis patients was not significantly different from that seen in patients with Crohn's disease.

Three single nucleotide polymorphisms have been associated with Crohn's disease.<sup>21–23</sup> One variant (1007fs) encodes a truncated protein which results in altered activation of NFκB in response to bacterial stimuli.<sup>29–31</sup> The two other single nucleotide polymorphisms (R702W and G908R) result in an amino acid substitution.

More recently, several groups assessed the linkage of *CARD15* variants in Crohn's disease to particular clinical phenotypes, but the results of these retrospective studies are disparate. The presence of two mutations has been linked to younger age at onset and preferential involvement of small bowel.<sup>43</sup> Preference for ileal involvement was also reported by Cuthbert *et al*<sup>42</sup> and by Ahmad *et al*.<sup>44</sup> Fibrostenosing disease was the dominant type in a study by Abreu *et al*.<sup>45</sup> In these studies, no association of *CARD15* variants with extraintestinal involvement could be shown.

Our study shows a new association between these three Crohn's disease associated variants in the leucine-rich region of the *CARD15* gene and a distinct subpopulation of patients with spondyloarthropathies. As in previous reports, the overall prevalence of mutations in spondyloarthritis patients was not statistically different from the prevalence in our control population.<sup>33–37</sup> However, unlike the previous studies, we identified a distinct clinical subgroup—characterised by the presence of chronic inflammatory gut lesions—with a high prevalence of *CARD15* polymorphisms similar to the Crohn's disease population and significantly higher than in the control population or the other spondyloarthritis patients. Previous studies from our group showed that these patients with chronic gut inflammation were at particular risk for progression to Crohn's disease.<sup>11, 12</sup>

Strikingly, in the present study none of the spondyloarthritis patients with only axial disease carried *CARD15* polymorphisms. Carriers of these polymorphisms all had (a history of) peripheral disease. This is in accord with previous studies of patients with ankylosing spondylitis where more

chronic gut inflammation could be found in those with peripheral disease than in those with strict axial disease.<sup>11</sup>

One previous study investigated *CARD15* polymorphisms in patients with ankylosing spondylitis, Crohn's disease, and ulcerative colitis.<sup>34</sup> It did not show a higher prevalence of *CARD15* variants in patients with ankylosing spondylitis plus Crohn's disease compared with idiopathic ankylosing spondylitis, ankylosing spondylitis plus ulcerative colitis, or healthy controls. However, the low prevalence of *CARD15* variants in the Crohn's disease population with ankylosing spondylitis was not compared with the prevalence in a general Crohn's disease population and it unexpectedly revealed a possible association between the G908R *CARD15* variant and ankylosing spondylitis plus ulcerative colitis.

Moreover, in a recent study we found an association between *CARD15* polymorphisms and the presence of radiological sacroiliitis in Crohn's disease patients, unrelated to the HLA-B27 status of these subjects. These data already suggested a role for the *CARD15* gene in the link between gut and joint inflammation.<sup>46</sup>

Our findings confirm the previous reported clinical, therapeutic, and immunological links between spondyloarthropathies and Crohn's disease and provide genetic proof for the association between these two disorders. As chronic gut inflammation in the majority of spondyloarthritis patients remains asymptomatic, this might suggest that *CARD15* polymorphisms could be linked to the development of (subclinical) chronic gut inflammation rather than to Crohn's disease as such.

The underlying pathogenic mechanisms that could explain the phenotypic expression of *CARD15* mutations in spondyloarthropathies need to be investigated. *CARD15* encodes a cytosolic protein that could play a role in spondyloarthropathies by interference with transport of antigens by macrophages from mucosal surfaces to the joints.<sup>35</sup> *CARD15* seems to function as an intracellular receptor for bacterial components, where the C-terminal leucine-rich repeat domain (LRR domain) is crucial for responsiveness. The cellular response to bacterial products has been shown to be altered in HEK293T cells transfected with expression plasmids containing any of the three SNPs.<sup>29, 31</sup> Moreover, expression of *CARD15* in myeloblastic and epithelial cells is enhanced by proinflammatory cytokines and bacterial components, through NFκB.<sup>30, 31, 56</sup> This response is likely to mediate cytokine production including TNFα, suggesting that upregulation of *CARD15* may be part of a positive regulatory loop and facilitate the response of the host to pathogens. A genetically determined disturbance of handling of bacterial products in the intestinal tract, leading to altered transport of antigens by macrophages to synovial tissue, is an interesting hypothesis that should be investigated in spondyloarthritis. A further identification and characterisation of inflammatory cells involved in gut and joint inflammation may also lead to new therapeutic targets.

#### Conclusions

A distinct phenotype associated with the three main Crohn's disease associated *CARD15* variants is reported in patients with spondyloarthropathies. Our data show that the presence of *CARD15* variants in spondyloarthritis patients strongly predisposes to chronic intestinal inflammation, defining a population at risk for evolution to Crohn's disease. However, the persistence of the subclinical character of the inflammation in a large proportion of patients may reflect the fact that Crohn's disease is a multigenic disease or alternatively that the heterozygous carriage of *CARD15* polymorphisms predisposes only to subclinical inflammation.

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