C-reactive protein polymorphisms influence serum CRP-levels independent of disease activity in ankylosing spondylitis

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Abstract

Objective

C-reactive protein (CRP) levels are frequently used to determine disease activity in patients with ankylosing spondylitis (AS), but these levels may not reflect disease activity. We therefore investigated the influence of common single-nucleotide polymorphisms (SNPs) in the CRP gene on CRP levels in AS patients. Additionally, the relation between CRP levels and BASDAI (Bath Ankylosing Spondylitis Disease Activity Index) was examined.

Methods

This exploratory cross-sectional study included 189 Dutch AS patients. CRP SNPs rs2794521, rs3091244, rs1800947 and rs876538 were genotyped and haplotypes constructed. Linear regression analysis was used for the association between SNPs and CRP levels, with correction for confounders non-steroidal anti-inflammatory drugs use, body mass index, smoking, age, gender and disease activity (BASDAI).

Results

Only 52% of AS patients with a high disease activity (BASDAI ≥4) showed a high CRP level (≥10mg/L), whereas the others did not. In AS patients, CRP levels changed with different genotypes, with genotype CA of tri-allelic (C>T>A) SNP rs3091244 showing higher CRP levels in comparison with genotype CC (CA: 18.6 mg/L vs. CC: 8.3 mg/L; p=0.02). Carriers of haplotype 5 (tagged by allele A of rs3091244) had a higher risk to express a CRP ≥10 mg/L (OR=2.9, 95%CI 1.0–8.3; p=0.05) when compared with non-carriers.

Conclusion

In AS, patients with high disease activity often do not show corresponding high CRP levels. We found that CRP levels vary with different CRP genotype in AS patients. Carrying distinct genetic variants might play a role in certain AS patients who show low CRP levels despite high disease activity (as well as high CRP levels with low disease activity). This observation may be important for the interpretation of disease activity scores that incorporate CRP levels, like the ASDAS.

Key words

ankylosing spondylitis, C-reactive protein, polymorphism, disease activity, disease activity score

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Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disease. It is characterised by sacroiliitis, a restricted motion of the spine, and chest expansion, due to inflammation and bone formation. In comparison to other rheumatic diseases (such as rheumatoid arthritis), it is difficult to assess clinical disease activity in AS, as a minority of patients exhibit peripheral arthritis (which makes a joint count less suitable) and in many cases, increased inflammatory parameters such as C-reactive protein (CRP) are absent.

Disease activity in AS has been measured with the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), a patient-oriented questionnaire, since 1994 (1). Active disease is specified as a BASDAI score ≥4 (on a 0–10 scale) and is used as an indicator to start treatment with TNF blocking agents (2).

Recently, the Assessment of SpondyloArthritis International Society (ASAS) has developed the Ankylosing Spondylitis Disease Activity Score (ASDAS), which incorporates plasma levels of CRP (3).

This new scoring system necessitates to obtain more information about the role of CRP in AS.

CRP is a plasma protein of the pentraxin family, known for its rapid increased expression as part of the acute phase reaction. CRP levels correlate with the BASDAI, but several studies found that CRP level does not necessary reflect disease activity, as many AS patients have normal or only minimally increased CRP levels (4-6). Previous studies showed that elevated CRP levels (≥10mg/L) were found in 50% of AS patients with active disease (6-8).

An explanation for the lack of increased CRP levels in some AS patients with high disease activity might be a genetic contribution to variation in CRP levels. In a healthy population, small variations in the low range of plasma CRP (<10 mg/L) are attributed to genetic factors as well as environmental factors such as body mass index (BMI), gender, age and smoking (9). Twin and family studies illustrate that genetic factors can contribute up to 40–50% of the phenotypic variation of baseline plasma CRP concentrations (10, 11). A meta-analysis of genome wide association studies (GWAS) in more than 80.000 individuals identified associations of CRP levels with single-nucleotide polymorphisms (SNPs) at loci within or close to the *CRP* gene and in other genes (12). To date, most disease association studies concerning the relationship between *CRP* gene polymorphisms and CRP levels were performed in relation to cardiovascular disease (13-18).

When compared with cardiovascular disease, higher ranges of serum CRP levels are used in the clinical evaluation of AS patients. As a whole different subject, high CRP levels are correlated with an increased risk to develop cardiovascular disease (19). Also, there is evidence of an increased mortality and morbidity due to cardiovascular disease in AS patients, thought to be regulated by the influence of inflammation on atherosclerosis (20).

Since CRP levels do not correlate with disease activity in every AS patient, we have investigated the contribution of *CRP* gene polymorphisms and haplotypes on CRP levels in AS patients. This influence is of clinical importance as it can interfere with diagnostic and treatment choices based on CRP levels. Additionally, we assessed the relation between CRP levels and BASDAI as a measure of disease activity.

Patients and methods

Patients

A cross-sectional study in AS patients was conducted in a study population of patients who were considered eligible for treatment with tumour necrosis factor (TNF) blocking agents and an unselected group of AS patients. All patients were anti-TNF naïve. AS patients fulfilled the 1984 modified New York criteria (21), were self-reported Dutch Caucasians and unrelated to any other patient participating in this study.

The study population consists of patients recruited at the VU University Medical Centre (VUmc) and the Jan van Breemen Research Institute|Reade (Reade), a large outpatient clinic in Amsterdam. Power calculations showed that our study had a >β=80% power to detect a change in mean CRP level of ≥5 mg/L,

which represents a clinically meaningful change for allele frequencies >5%.
The study was approved by the medical ethical committees of the VUmc and Reade and all participants gave written informed consent.

Outcome measures
Blood samples and all measurements, including CRP levels and questionnaires, were obtained at baseline, before anti TNF therapy was started, making sure that all measurements were of TNF naïve patients. When using the term "CRP levels" further on, we are referring to these TNF naïve CRP levels obtained at the start of each study (baseline).
Serum CRP levels were determined on Roche/Hitachi Modular P (at VUmc) and Cobas C (Reade) systems by a particle enhanced immunoturbidimetric assay. Intra- and inter-assay coefficients of variation for CRP were 1.9% and 3.4%, respectively. These measurements were conducted with a detection limit of 1 mg/L.
A CRP level ≥10 mg/L was considered a parameter of active disease in AS.
Several other clinical data were collected such as age, gender, HLA-B27 status, BMI, smoking status, disease duration and symptom onset duration, extra spinal manifestations of AS (presence of anterior uveitis, Inflammatory Bowel Diseases [IBD]) and psoriasis), medication use, and disease activity scores such as the BASDAI, the BASFI and the global disease activity score (VASpatient).

Genotyping the CRP *SNPs*
Genomic DNA was isolated from peripheral blood by standard procedures. Four common *CRP* gene tagging SNPs with published minor allele frequencies (MAF) of more than 5% that were associated with CRP levels or their proxies were selected on the basis of published work. Functional polymorphisms in the *CRP* gene region (dbSNP ID) rs2794521 T>C (17, 18) and triallelic SNP rs3091244 C>T>A located in the gene promoter (22), as well as rs1800947 G>C, a synonymous coding SNP in exon 2 (15) and SNP rs876538 G>A in the 3' flanking region (18, 23) were genotyped.
Genotyping was performed with appropriate quality control measures by the Taqman SNP allelic discrimination method with an ABI PRISM 7000 Sequence detection System (Applied Biosystems, Foster City, CA, USA) (details are available from the authors upon request).

Statistical analysis
Linear regression analysis was performed to identify the relation between the *CRP* polymorphisms and CRP levels. All analyses were also corrected for all confounders, including BASDAI, with all covariates entered simultaneously into the regression model.
On the basis of previous publications, gender, BMI, age, smoking status and NSAID use were selected as confounding variables (7, 23) and CRP levels were corrected for disease activity status in each patient using the BASDAI.
Using the linear regression analysis, the effect of the different genotypes and the effects of carriership of both the minor and the major alleles on CRP level were tested. For the triallelic SNP rs3091244, the effect of the different genotypes and the effect of carriership of both minor alleles were analysed. Because CRP levels were skewed to the right in all linear regression analyses, the natural logarithm of CRP was used as outcome variable. Results were presented as back-transformed geometric means for CRP levels.
Logistic regression analyses were performed to determine the effect of the genotype on the dichotomised CRP levels, with a cut-off point of 10 mg/L. The same confounding variables were also used as correction for the logistic analysis.
An independent samples *t*-test was used to determine the differences in CRP level between two groups with different disease activity (BASDAI score of ≥4 or <4) and H5 carriership.
The publicly available Haploview version 4.2 software (Cambridge, USA) was applied to estimate pairwise linkage disequilibrium (LD) between the four SNPs examined (24).
Haplotypes were constructed to determine the integrated effect of multiple SNPs on CRP levels. Genotype data of each of the four *CRP* SNPs were used to infer the frequency of the haplotypes present in the population using the program Phase v2.1. Only patients with >99% certainty in haplotype estimates were included in the analysis (25).
The same linear and logistic regression analyses were applied to determine the effect of the haplotype on CRP levels.
We present otherwise uncorrected two-tailed *p*-values. For all analyses, statistical significance was designated as a two-tailed *p*-value less than 0.05.
Statistical analysis was conducted using SPSS V20.0 (Armonk, NY, IBM Corp.).

Results

Patient characteristics
A total of 233 AS patients was selected for our study. Forty-two patients were excluded because of self-reported non-Caucasian ethnicity and 2 patients because they did not fulfil the predefined control criteria for polymorphism and haplotype determination, thus leaving 189 patients for statistical analysis.
The main characteristics of the 189 AS patients under study are listed in Table I. Our study population of AS patients encompasses a large variety in disease activity, with a high mean BASDAI of 4.9 (SD 2.2).
In total, 47.3% of the patients had a CRP level ≥10 mg/L. Mean BASFI was 4.5 (SD 2.7) and mean VASpatient 5.8 (SD 2.6). A high percentage of AS patients (50%) had peripheral arthritis. CRP levels did not significantly differ in patients with extra-spinal manifestations such as peripheral arthritis, anterior uveitis and IBD (data not shown).

Disease activity variables and CRP levels
CRP levels were significantly positively correlated with BASDAI (p=0.001, β 0.242), BASFI (p=0.001, β 0.275) and VAS (p=0.001, β 0.351). Figure 1 illustrates BASDAI values plotted against mean CRP levels, which shows that outliers in the lower and higher ranges of BASDAI strongly contribute to the association between BASDAI and CRP levels. In the intermediate ranges of BASDAI, no clear correlation is seen with CRP levels.

Table I. Characteristics of 189 patients with ankylosing spondylitis.

Characteristics	Mean±SD or n. (cumulative %)
Age (years)	47.8 ± 11.7
Male	145 (76.7)
Duration of disease (years)	15.0 ± 10.5
HLA-B27 positivity	185 (86.6)
Smoking status	73 (40.6)
Mean BMI (kg/m^2)	25.4 ± 4.3
Peripheral arthritis	94 (50.3)
Anterior uveitis	55 (29.3)
Psoriasis	21 (11.2)
IBD	14 (7.4)
Patients on NSAIDs	146 (78.1)
Patients receiving DMARDs	18 (9.6)
Patients on systemic steroids	14 (7.5)
Patients with CRP≥10 mg/l	87 (47.3)
BASDAI (0–10)	4.9 ± 2.2
Patients with BASDAI ≥4	125 (67.2)
BASFI (0–10)	4.5 ± 2.7
VASpatient (0–10)	5.8 ± 2.6

HLA-B27: human leucocyte antigen B27; BMI: body mass index; IBD: inflammatory bowel disease; NSAIDs: non-steroidal anti-inflammatory drugs; DMARDs: disease-modifying anti-rheumatic drugs; CRP: C-reactive protein; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; VAS: visual analogue scale.

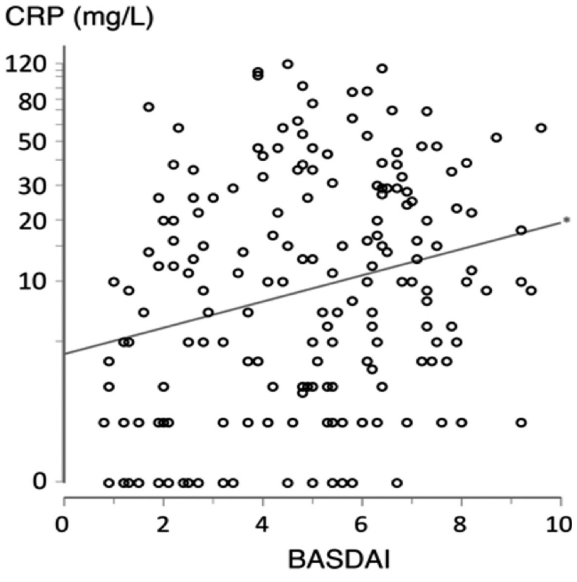

Fig. 1. CRP levels in mg/L (natural logarithm) for BASDAI (0–10).
Plotted CRP levels for different values of BASDAI show that outliers strongly contribute to the association between BASDAI and CRP levels in our study population.
*p=0.001, β 0.242
(linear regression)

CRP values ≥10 mg/L were found in 52% of the AS patients with a high disease activity (BASDAI≥4); the other patients with high disease activity did not express high CRP levels (≥10 mg/L).
BASDAI and BASFI were significantly positively associated (p<0.001, β 0.729), as well as BASDAI and VAS (p<0.001, β 0.768) and BASFI and VAS (p<0.001, β 0.657).

CRP *SNPs and CRP levels*
The genotype frequencies of all four SNPs were in Hardy-Weinberg equilibrium.
Allele frequencies of SNPs rs2794521 (T=0.68, C=0.32), rs3091244 (C=0.65, T=0.30, A=0.05), rs1800947 (G=0.96, C=0.04) and rs876538 (G=0.77, A=0.23) were in concordance with frequencies found in the Framingham Offspring cohort, a study of 1640 healthy unrelated participants of European descent (23).
As shown in Table II, all four SNPs genotyped showed differences in mean CRP level between genotypes, with only rs3091244 CA genotype showing statistical significantly higher CRP levels when compared with the reference genotype CC (p=0.02), also when corrected for BASDAI and other confounders (p=0.03). Patients with genotype CA show a strongly increased mean serum CRP level of 18.6 mg/L.
AS patients carrying the A-allele of rs3091244 (combined genotypes CA and TA) also had significantly higher CRP levels compared with patients homozygous for the C-allele (p<0.02).
Based on linear regression, the contribution of rs3091244 to serum CRP level variation was 3.2%.

CRP *haplotypes and CRP levels*
Six haplotypes defined by the four representative SNPs in the *CRP* region were constructed and coded H1-H6 in order of descending frequency (Fig. 2A). Linkage disequilibrium between the *CRP* SNPs is shown in Figures 2B and 2C.
Haplotype analysis showed that H5 was associated with CRP levels. A minority of 10.6% of the AS patients was a H5 carrier and these were all heterozygotes. H5 is tagged by the A allele of rs3091244 and AS patients who are carriers of H5 have higher mean serum CRP levels than non-carriers after correction for all confounders (p<0.03). Mean CRP level in H5 carriers is 15.4 mg/L compared with 8.6 mg/L in H5 non-carriers. This result reflects the same significant association as described above for the A-allele of rs3091244.
In clinical practice CRP values are often interpreted as being normal or elevated. For that reason we calculated the percentage of AS patients showing an elevated CRP level (≥10 mg/L), subdividing in groups by H5 carriership and disease activity (BASDAI), as shown in Table III. Table III also shows mean CRP level for each of these groups. Both disease activity and H5 carrier status influence mean CRP level as well as the percentage of patients showing a high CRP level.
Mean CRP level was elevated despite low disease activity in H5 carriers.
With small numbers of patients in each group, only disease activity (BASDAI) in the H5 non-carrier group was significantly correlated with mean CRP level. (p=0.003, independent samples t-test).
The results of the logistic regression analyses showed that AS patients carrying H5 had a 2.9-fold higher odds (95%CI 1.0–8.3; p=0.05) compared with non-carriers to have a CRP level ≥10 mg/L, after adjusting for the BASDAI and other potential confounders.
Correction for peripheral arthritis and HLA-B27 status did not change the results.

Discussion

In this exploratory study, we investigated the relationship between four common *CRP* gene polymorphisms and their haplotypes and plasma CRP levels in 189 anti-TNF naïve AS patients. Statistical significance was reached for the tri-allelic SNP rs3091244. AS pa-

Table II. Mean serum CRP values in mg/L for CRP SNP genotypes.

Genotypes	n (frequency)	Mean CRP (mg/L)	95%CI	*p*-value
rs2794521 T>				
TT	91 (48%)	10.6	ref	ref
CT	76 (40%)	7.9	6–11	0.17
CC	22 (12%)	8.0	4–14	0.40
rs3091244 C>T>A				
CC	75 (40%)	8.3	ref	ref
CT	77 (40%)	8.9	7–12	0.77
CA	17 (9%)	18.6	10–34	0.02*
TT	17 (9%)	8.3	4–16	0.98
TA	3 (2%)	5.4	1–24	0.58
AA	N.D.	-.-	-.-	-.-
rs1800947 G>C				
GG	175 (93%)	8.9	ref	ref
GC	14 (7%)	12.9	6–26	0.31
CC	N.D.	-.-	-.-	-.-
rs876538 G>A				
GG	108 (58%)	11.0	ref	ref
GA	69 (37%)	7.5	6–10	0.06
AA	9 (5%)	5.9	3–14	0.17

Mean serum CRP levels for different CRP SNP genotypes of rs2794521, rs3091244, rs1800947 and rs876538. CRP values were backtransformed after natural logarithmic correction. N.D.: not detected. *Significant result (linear regression).

tients with the CA genotype were associated with higher mean CRP levels (18.6mg/L) and patients homozygous for the major C-allele with lower mean CRP levels (8.3 mg/L).

H5 carriers (tagged by the A allele of rs3091244) also show higher mean CRP values in comparison with non-carriers and have a nearly threefold higher odds (OR=2.9) of expressing a CRP value ≥10 mg/L, independent of the disease activity (BASDAI).

Our observation that different genotypes of rs3091244 associate with different CRP levels in AS agrees with the findings of several large studies in general populations (18, 26), with Kathiresan *et al*. showing that (after stepwise selection among 13 *CRP* SNPs) rs3091244 explained the largest part of basal CRP level variance in the Framingham Offspring cohort study (23).

The effect of rs3091244 on CRP level variation was also established on the basis of haplotype analysis (15, 27). *In vitro* studies demonstrated rs3091244 to be functional, by affecting the transcriptional activity of the CRP gene (22).

Studies (23, 26) in a healthy population show a similar percentage of the CRP level variation explained by rs3091244 (1.8–3.4%), as in our cohort of AS patients (3.2%).

In a healthy population, different genotypes of rs3091244 entail a serum CRP level ranging between 1.6–2.4 and 1.8–3.4 mg/L (23, 26). In our cohort of AS patients, serum CRP levels ranged between 5.4 and 18.6 mg/L with different genotypes of rs3091244. In addition, a large study on a single harmful stimulus, acute coronary syndrome, found homozygote carriers of allele A of rs3091244 to show the highest CRP elevations, with serum CRP values ranging between 11–72 mg/L (28). One could hypothesise that in a situation with increased CRP production, the genetic influence on serum CRP level becomes more apparent and through this, causes a wide range in serum CRP level variation between different genotypes.

In our study population of patients with AS, the other SNPs studied (rs2794521, rs1800947 and rs876538) also showed different CRP levels with each genotype, but statistical significance was not reached, which may be due to their small effect size and our small study population.

Extensive research has been done worldwide to relate common SNPs to serum CRP levels in general populations. Top main effect SNPs were located within and near the CRP locus, in those of European, Asian as well as African American ancestry (among which rs3091244) (12, 29, 30).

Other strongly associated genes are APOC1 (apolipoprotein C1), HNF1A (hepatocyte nuclear factor 1 homeobox A), IL6R (interleukin-6 receptor), LEPR (leptin receptor), GCKR (glucokinase regulator), ASCL1 (achaete-scute complex homologue 1) and TREM2 (Triggering Receptor Expressed On Myeloid Cells 2) highlighting the immune response and metabolic regulatory pathways involved in the regulation of chronic inflammation (12).

These early protein quantitative trait loci (pQTL) studies revealed only IL6R was among 13 loci associated with AS in previous GWAS or 13 new AS risk loci identified in a dense SNP genotyping case-control association study on the Immunochip custom microarray with over 25.000 individuals (31). Whether these and other genes will influence CRP levels and contribute to susceptibility with AS remains to be determined in large scale studies in ethnically diverse human populations. Since a study on data mining of GWAS of several immune-mediated diseases in general populations was promising (which used constructed allelic scores that explain greater proportions of the variance in biological intermediates such as CRP), this approach might also be successfully applied in AS (32). However, to establish a causal relationship this study recommends follow-up with formal Mendelian Randomisation methodologies (33) when an association with AS is detected by an allelic score made up by known CRP level associated SNPs.

CRP and disease activity

This study shows that high disease activity (BASDAI) is correlated to high mean CRP levels in only half of the patients with AS. As a consequence of this, and as is known in clinical practice, many patients with AS do not express high CRP levels as a reflection of clinically significant inflammation.

H5 carriers have a 3-fold increased chance to express a higher mean serum CRP level than H5 non-carriers. On the other hand, AS patients who are H5 non-carriers could show low CRP values (<10 mg/L) despite high disease activity (BASDAI >4). This poses a possible explanation to why some AS

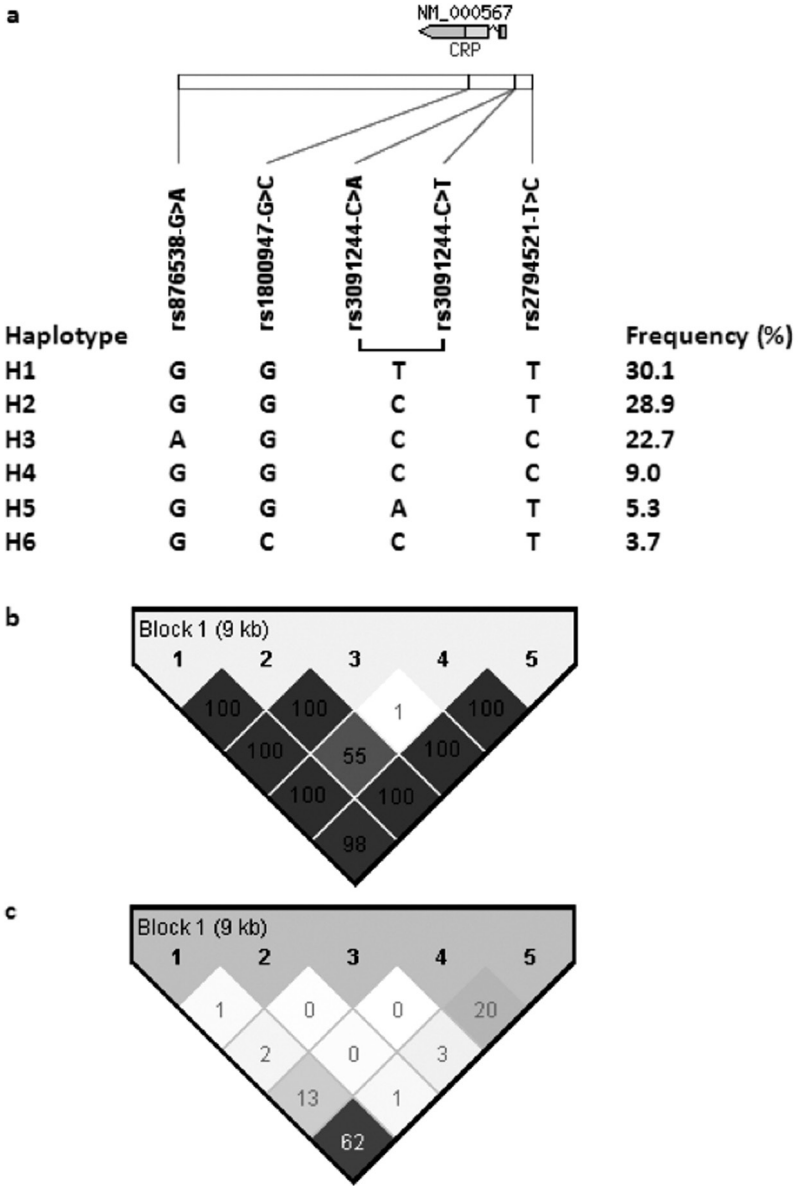

Fig. 2. SNPs in the region of the CRP gene at chromosome 1q21-q23 (157,942,341 - 157,951,720 bp; NCBI build 36) with their pairwise linkage disequilibrium (LD), haplotypes, and haplotype frequencies in AS patients.
a) Location, haplotypes and haplotype frequencies of the four SNPs (with dbSNP accession numbers) in the CRP region in 189 AS patients. In addition, one patient carried a recombinant haplotype (not shown).
b) LD plot showing pairwise LD between SNPs as D' x 100 (100=maximum possible value) created by the Haploview v4.2 programme. (24)
c) The r2 parameter, as determined with the Haploview programme, that quantitates intermarker LD is given (r2=0 indicates no LD, r2=100 indicates complete LD). CRP: C-reactive protein; H: haplotype; SNPs: single-nucleotide polymorphisms.

patients do not show an increase in CRP level despite high disease activity. The results are in concordance with findings in another rheumatic inflammatory disease by Rhodes *et al*. They found CRP haplotypes to influence acute-phase serum CRP levels in rheumatoid arthritis, implicating this effect could influence clinical decision making and lead to suboptimal treatment (34, 35). They show high serum CRP levels associated with the haplotype tagged by allele C of rs3093059, which is in very strong LD with allele A of rs3091244.

These findings are especially relevant as CRP levels are widely used in AS and have been included in the ASDAS, a new disease activity score. The ASDAS has been reported by van der Heijde *et al*. to be the best at discriminating between high and low disease activity at baseline and follow-up (3).

The present study showed that only 52% of AS patients with high disease activity show a corresponding rise in CRP level and shows that differences in genetic makeup influence CRP level.

Therefore, one should take into account the genetic influence on CRP levels when assessing disease activity with the ASDAS. This is even more important because clinical decisions, such as starting or stopping treatment with TNF blocking agents, are based on these disease activity parameters. This might lead to under- or overtreatment of AS patients depending on their specific genetic profile, also possibly influencing costs (36).

A limitation of this study might be the heterogeneous study population, including AS patients who are candidates for anti-TNF treatment and those who do not require this treatment. In order to minimise this difference, we have adjusted for all factors that might influence CRP level, especially the BASDAI score. The main limitation of this study is its small sample size. Nonetheless, we are able to show an influence of genetics on CRP-level with rs3091244 reaching statistical significance. Further studies with a larger population of AS patients are required to indicate more precisely the effect of SNPs in CRP and other genes on serum CRP levels.

CRP measurements were not done with a high sensitivity CRP (hsCRP) assay, which can detect CRP at a minimum level of 0.1 mg/L. However, this is of less importance in this study, as higher CRP values are more of interest in AS patients rather than the precise differences in low CRP values.

At the time of initiation of this study, the ASDAS of these patients was not yet available, therefore the BASDAI was used to adjust for disease activity status in the AS patients.

Conclusion

Many AS patients with high disease activity do not show a high CRP level. Our results show that genetic polymorphisms play a role in the variation of CRP levels in patients with AS. The importance of a genetic contribution on

Table III. Mean serum CRP level in mg/L as well as percentage (%) of patients showing a CRP level ≥10mg/L. Grouped by H5 carriers and H5 non-carriers and by high or low disease activity (BASDAI).

	Mean CRP	% CRP ≥10mg/L	Mean CRP	% CRP ≥10mg/L
	BASDAI ≥4 (n=125)		BASDAI <4 (n=61)	
H5 carrier (n=20)	18.5	67%	11.8	63%
H5 non-carrier (n=169)	10.5	50%	5.5*	35%

Mean serum CRP level for BASDAI <4 and >4 and for H5 carriers and non-carriers. Also, the percentage (%) of patients showing a CRP level >10mg/L with BASDAI <4 and >4 and for H5 carriers and non-carriers is depicted. BASDAI unknown; n=3 (* BASDAI <4 and >4 in H5 non-carriers, p=0.003, independent samples t-test).

CRP levels might be taken into consideration when clinical decisions in AS patients have to be made.

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