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Characterization of a Subset of Patients With Rheumatoid Arthritis for Whom Current Management Strategies are Inadequate

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Objective. A subset of patients with seropositive rheumatoid arthritis (RA) do not mount a C-reactive protein (CRP) response during flares. We hypothesize that these patients are more likely to experience poor clinical care and less likely to respond to traditional therapy. This study questioned whether this presentation was associated with worse disease outcome and distinct immunological features.

Methods. Using Power Doppler ultrasound, 48 RA patients with active synovitis were recruited; 30 had normal (n)CRP (5 mg/L or less) and 18 had high (h)CRP (more than 5 mg/L) levels. All had equivalent disease burden assessed by other clinical and laboratory parameters.

Results. Time to diagnosis and time to first disease-modifying antirheumatic drug were significantly longer in nCRP compared with hCRP patients ($P < 0.05$). Significantly more nCRP patients needed escalation to biologics after 2-year follow-up ($P = 0.01$). The inflammatory milieu was also different between the two subgroups. Synergy between inflammatory cytokines observed in hCRP patients was lost in nCRP patients, and nCRP patients had significantly increased regulatory T-cell (Treg) frequencies that correlated positively with predictors of poor disease outcome. Conversely, hCRP but not nCRP patients demonstrated a significant upregulation of alternative complement pathway factors that correlated negatively with Treg frequency.

Conclusion. Patients with nCRP during flares of RA had an altered immunological profile compared with hCRP patients and experienced diagnostic delays and responded less favorably to conventional treatment.

INTRODUCTION

We now have multiple drug options for patients with rheumatoid arthritis (RA). Optimal disease management requires both good drug choice based on patient stratification and good assessment of response based on reliable measurement of disease activity (1) .

Common practice involves the use of the Disease Activity Score-28 (DAS-28), which includes examination of 28 joints for tenderness and swelling, a patient global visual analogue assessment (GVAS), and a measure of either C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR); the resulting numerical values divide disease activity into high, moderate, low, and remission (2). This is slightly complicated by the known discordance between cut-offs depending on the use of CRP versus ESR (3). Remission (primarily for early arthritis) or low disease activity (especially in long-standing disease) have been established as treatment targets (2). In general, normal serum CRP levels (below 5 mg/L) are considered to correlate well with good disease control (4,5). However, recent studies have highlighted the fact that disease activity scores based on acute phase response measurements do not reliably predict joint damage in all RA patients (6).

Musculoskeletal ultrasound (US) is a well-recognized adjunct to clinical assessment of disease activity in which

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increased synovial blood flow, demonstrated by Power Doppler signal, is a sensitive sign of active inflammatory disease (7–9). Furthermore, the presence of Power Doppler has prognostic significance, predicting the development of radiographic erosions and poor clinical outcome (10,11). Power Doppler is a sensitive and reliable method for assessing disease activity
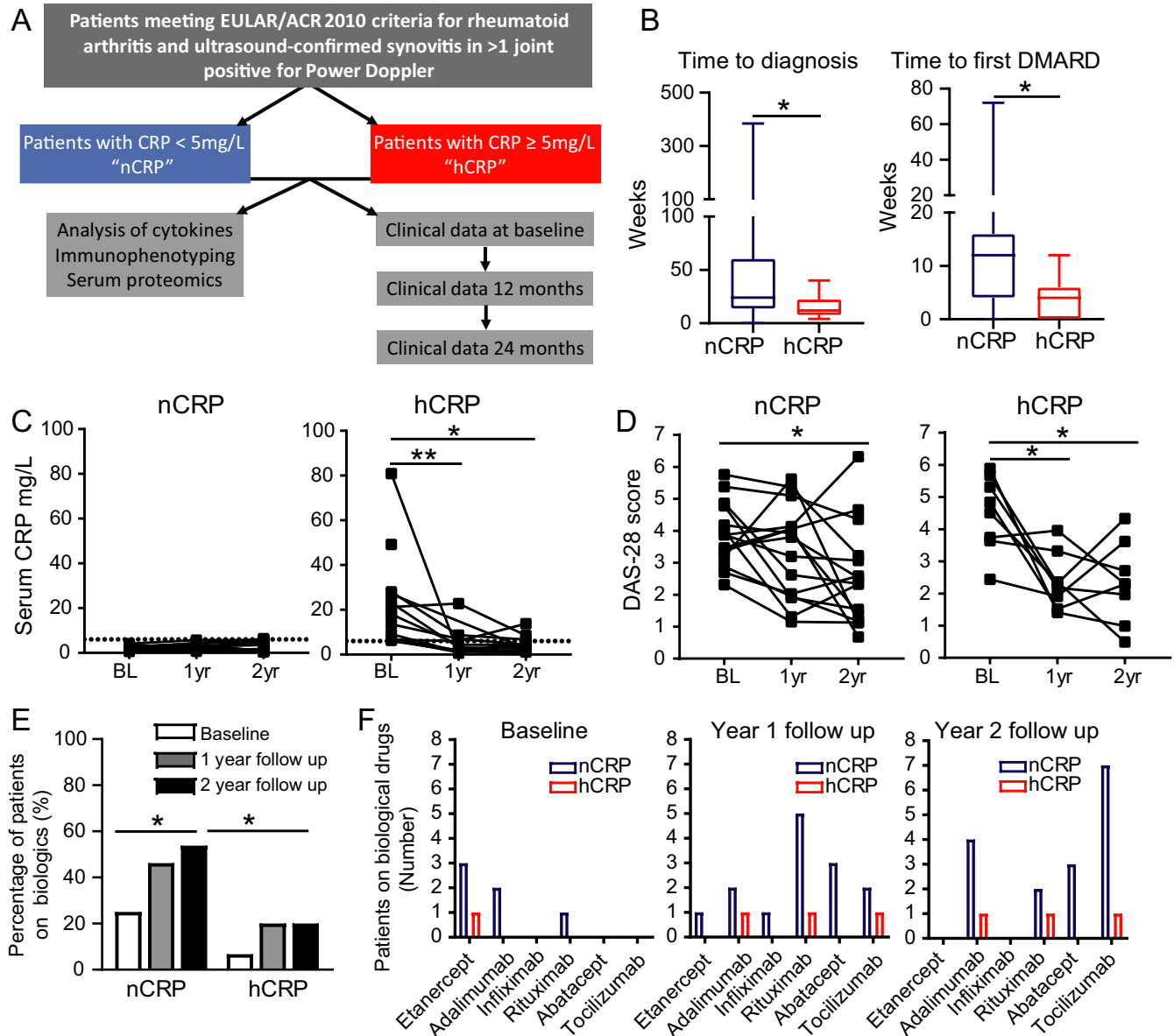
Figure 1. Patients with normal C-reactive protein (CRP) but active RA (nCRP) had a poor response to treatment. **A,** Schematic showing patient inclusion into the study at the University College London Hospital NHS Trust rheumatologist-led US clinic. US assessment of hands, wrists (22 joints), and feet (10 joints), if appropriate, was performed by two experienced consultant rheumatologists. Patients were recruited as they presented at clinic; consultant rheumatologists were blind to CRP levels at the time of scanning. Patients were treated as deemed necessary by the consulting physician according to National Institute for Health and Care Excellence (NICE) guidelines. Patients were followed-up at 1- and 2-years postrecruitment and assessed for treatment and disease activity. **B,** Time to diagnosis and time to first disease-modifying antirheumatic drug (DMARD) treatment were assessed where data was available (nCRP, n = 18; hCRP, n = 9). Box and whisker plot showing median and interquartile ranges, whiskers show minimum and maximum points. Mann-Whitney test; *P = 0.041 and *P = 0.013, respectively. **C,** Longitudinal CRP levels at baseline, 1- and 2-year follow-up. nCRP, n = 27; hCRP n = 15. Repeated measures analysis of variance (ANOVA) and Tukey post-test analysis; *P = 0.006; **P = 0.002. **D,** Longitudinal Disease Activity Score-28 (DAS-28) scores at baseline 1- and 2-year follow-up. nCRP, n = 15; hCRP n = 8. Repeated measures ANOVA and Tukey post-test analysis; *P = 0.01. Mean DAS-28 at baseline: nCRP = 3.9, hCRP = 4.5; 1-year follow-up: nCRP = 3.3, hCRP = 2.4; and 2-year follow-up: nCRP = 2.6, hCRP = 2.3. **E,** Total number of patients treated with biologics (%) at baseline, 1-, and 2-year follow-up. nCRP, n = 28; hCRP n = 15. Mean ± SE, 1-way ANOVA, and Tukey post-test analysis, *P = 0.05; unpaired t-test; *P = 0.02. **F,** Patients treated with biologic DMARDS during the study: nCRP (n = 16) and hCRP (n = 3). Patients followed longitudinally at baseline (nCRP, n = 6; hCRP n = 1), 1-year (nCRP, n = 13; hCRP n = 3), and 2-year (nCRP, n = 16; hCRP n = 3) follow-up.

and also predicts rapid radiographic progression in early arthritis (11–13).

Using US, we have identified a subgroup of patients with active, seropositive RA, whose CRP did not correlate with their disease activity; they had nCRP levels at a time when they had active, inflammatory joint disease that was confirmed on US. We hypothesized that these patients could be less well managed because the treating physician was falsely reassured by the nCRP and because this subgroup may be immunologically distinct from patients with classical RA who do mount an appropriate CRP response when flaring.

METHODS

Patients and healthy volunteers. Patients who met the European League Against Rheumatism/American College of Rheumatology 2010 criteria for RA and who were referred to the University College London Hospital NHS Trust rheumatologist-led US clinic and had US-confirmed synovitis (14) in at least one joint positive for Power Doppler signal were recruited. Exclusion criteria included presence of a comorbidity likely to result in a raised CRP (eg. cancer, infection), seronegativity for rheumatoid factor (RF) and/or anticyclic citrullinated peptide (CCP) antibodies, patients vaccinated in the last 3 months, or pregnant or breastfeeding women. Patients treated with anti-interleukin (IL)-6 therapy were also excluded because of known suppression of CRP (15). US assessment of the hands and wrists (22 joints), and feet if appropriate (10 joints), was performed using a GE Healthcare LOGIQ S8 Ultrasound machine using the OMERACT protocol by two experienced consultant rheumatologists (16). Synovial hypertrophy, Power Doppler, and erosion number were recorded for each joint. This was a "real-life" clinical study and consecutive patients were recruited from the rheumatologist-led US clinic. The scanning consultant rheumatologists were blind in terms of CRP levels at the time of scanning.

Patients were treated as deemed necessary by the consulting physician according to the National Institute for Health and Care Excellence (NICE) guidelines (nice.org.uk/guidance/ng100). Baseline demographic and clinical data were collected (Supplementary Table 1), and follow-up clinical and DAS data were collected in routine clinics at 12 ± 1 and/or 24 ± 1 months postrecruitment (Figure 1 and Table 1).

Peripheral blood (PB) was collected from adult healthy controls (HCs), with a mean age of 49.1 years (range 23-84). All participants gave informed written consent. Ethics approval for this study was given by the Hampstead Research Ethics Committee (14/LO/1506). Mononuclear cells were isolated from PB using Ficoll-Hypaque density gradient centrifugation.

Phenotypic analysis. PB mononuclear cells (PBMCs) were analyzed by flow cytometry after staining with LIVE/DEAD fixable cell stain for viability and surface and/or intracellular staining ex vivo with directly conjugated antibodies according to previously defined protocols (17,18) (see supplementary methods).

Serum cytokine levels. Serum IL-1β, tumor necrosis factor-α (TNFα), IL-6, IL-2, IL-4, IL-8, IL-10, IL-17A, IL-17F, IL-12/IL-23p40, and IL-21 were quantified simultaneously using human cytokine cytometric bead array (CBA) and CBA software (BD Biosciences).

Proteomics. Proteins expressed in plasma from eight patients with nCRP levels, eight patients with hCRP levels, and eight HCs were analyzed on SOMAscan (Slow Off-rate Modified Aptamer) Proteomic Assay, detecting over 1300 proteins and operated according to the manufacturer's instructions (https://somalogic.com/). Protein expression was reported as relative fluorescent units. Significantly differentially expressed proteins were analyzed using a functional enrichment analysis web-tool, WebGestalt (http://www.webgestalt.org/option.php). Overrepresentation analysis and Reactome enrichment categories were used. Venn diagram software (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used to identify similarities and differences between the groups.

Western blotting. Serum was diluted 1:400 and analyzed by Western blotting using a 4%-12% gradient agarose gel (Invitrogen). Gels were transferred using the Invitrogen Bolt system (Invitrogen) and polyvinylidene difluoride membranes blocked in 5% milk (MilliporeSigma) before overnight probing with anti-C3a/des-Arg antibody (Abcam) at 1:2500 followed by an horseradish peroxidase–conjugated anti-mouse antibody (1:5000). Membranes were exposed using ECL prime chemiluminescence reagent (Amersham) for 15 seconds using X-ray film. Bands were quantified using Analysis One densitometry software, and positivity was expressed as a fold change from HCs on the same blot.

Complement pathway test. The Compl300 assay (Euro Diagnostica/Wieslab) was carried out according to the manufacturer's instructions. Briefly: samples were diluted 1:101. Values were calculated by comparison to the positive and negative controls. C5a was measured using the C5a DuoSet (R&D Systems) as per the manufacturer's instructions. Serum samples were titrated and tested at 1:100 to fall within quantifiable range.

Statistical analysis. Significance testing was performed using Prism 8 software (GraphPad Software). Data were tested for normality using the D'Agostino and Pearson omnibus normality test, and differences between groups were assessed using nonparametric 2-tailed Mann-Whitney U test or multiple t tests with a false discovery rate (FDR) to account for multiple comparisons, as indicated in the figure legends. Categorical variables were compared with χ^2 or Fisher exact test. P values less than 0.05 were considered statistically sig-

Table 1. Demographic and clinical and disease features of patients in nCRP and hCRP subgroups at recruitment[a]

	Normal CRP	High CRP	P value
Number (n)	30	18	
Age, years: mean; SD (range)	49.1; 16.1 (23-84)	58; 17 (25-80)	0.093
Sex (F:M)	6.5:1	2:1	0.070[b]
Disease duration (years): median; IQR (range)	5; 15.25 (0-44)	11; 15 (0.5-40)	0.535
Clinical Assessments			
CRP, NR >5 mg/L: median; IQR (range)	1.95; 1.725 (<0.6-5)	11.3; 17.605 (5.6-80.9)	<0.0001[c]
Serum amyloid A, NR >10 mg/L Median; IQR (range)	4.6; 7.5 (2.8-46.5)	16.4; 43.8 (8.1-378)	<0.0001[c]
Erythrocyte sedimentation rate, NR 0-20 mm/hr: median; IQR (range)	9; 11 (2-112)	28; 30.5 (5-134)	0.010[c]
RF, IU/ml[d] Mean; SD (range)	183.4; 295.4 (9-1209.9)	150.6; 109.6 (24.6-352.7)	0.117
CCP antibodies units/ml: median; IQR (range)[d]	170; 285.5 (16-507)	160.5; 308.7 (1.5-459)	0.580
Total joints with Doppler signal, n Median; IQR (range)	3; 4.5 (1–21)	4; 4 (1-10)	0.647
Synovial hypertrophy, grades 1-3: Median; IQR (range)	11; 11 (3-25)	8.5; 8 (2-23)	0.715
Erosion number: Mean; SD (range)	7.1; 7.58 (0-24)	6.9; 6.45 (0-21)	0.908
Erosion number per year disease Mean; SD (range)	1.0; 1.1 (0-2.7)	0.5; 0.35 (0-1.4)	0.361
Tender joint count, n: Mean; SD (range)	9.7; 8.3 (1-28)	7.8; 6.9 (0-22)	0.549
Swollen joint count, n Mean; SD (range)	5.5; 5.3 (1-22)	5.1; 4.6 (0-15)	0.921
GVAS Median; IQR (range)	60; 40 (0-100)	70; 65 (10-100)	0.787
DAS			
DAS-CRP: Mean; SD (range)	4.2; 1.1 (2.5-6.5)	4.6; 1.5 (1.9-7.1)	0.354
DAS-ESR: Mean; SD (range)	4.5; 1.3 (2.6-7.2)	5.0; 1.9 (1.3-8.2)	0.294
Patients with high disease activity (DAS-28 >5.1) at recruitment; % (number)	25 (7/28[e])	52.9 (9/17[e])	0.057[b]
Patients with Moderate disease activity (DAS-28 >3.2-5.1) at recruitment: % (number)	53.6 (15/28[e])	23.5 (4/17[e])	0.047[b,c]
Patients with low disease activity (DAS-28 2.6-3.2) at recruitment; % (number)	17.9 (5/28[e])	5.9 (1/17[e])	0.251
Patients in remission (DAS-28 <2.6) at recruitment; % (number)	3.5 (1/28[e])	17.7 (3/17[e])	0.107[b]
Treatment			
DMARD treatment at recruitment, % (number)	53 (16/30)	50 (9/18)	0.822[b]
DMARDs + steroids, % (number)	16.6 (5/30)	11.1 (1/18)	0.597[b]
DMARDs + biologics, % (number)	16.6 (5/30)	0 (0/18)	0.141[b]
All biologics, % (number)	20 (6/30)	5.5 (1/18)	0.169[b]
Total number of DMARDs (current or failed) per patient	1.143	0.937	0.740[b]
Total DMARDs per year of disease: median; IQR (range)	0.17; 1 (0-4)	0.02; 0.15 (0-0.57)	0.020

(continued)

Table 1. *(Cont'd)*

	Normal CRP	High CRP	*P* value
Use of leflunomide/ methotrexate / hydroxychloroquine/ sulphasalazine/ azathioprine %	10/ 27/ 23/ 33/ 7	5/ 2/ 0/ 10/ 0	...
Total drugs per year of disease (including steroid, DMARD, and biologics): median; IQR (range)	0.15; 0.57 (0-1.08)	0.04; 0.15 (0-1.14)	0.239
Treatment naïve % (number)	26.6 (8/30)	27.7 (5/18)	>0.999[b]

Abbreviation: CCP, cyclic citrullinated peptide; CRP, C-reactive protein; DAS-28, Disease Activity Score-28; DMARD, disease-modifying antirheumatic drug; GVAS, global visual analogue assessment; hCRP, patients with high CRP (>5 mg/L) but active RA; IQR, interquartile range; nCRP, patients with normal CRP (≤5 mg/L) but active RA; NR, normal range; RA, rheumatoid arthritis; RF, rheumatoid factor.
[a] All results expressed as mean, SD, if normally distributed and as medians and IQRs if not normally distributed. Data analyzed using Mann-Whitney tests.
[b] Data analyzed using χ^2 test.
[c] Denotes significant value.
[d] All patients were positive for both RF and CCP antibodies.
[e] Data not available for all patients.

nificant. Matrix2png version 1.2.1 was used to create immune cell phenotype heat maps.

RESULTS

Patients with active RA but nCRP levels experienced diagnostic delays and were more likely to fail treatment with conventional disease-modifying antirheumatic drug. Using US to assess disease activity in patients with seropositive RA, an atypical subgroup that had active disease, as defined by the presence of Power Doppler, but normal serum CRP levels was identified. In order to investigate this observation, two groups of patients were recruited. They all had US-proven synovitis indicating active RA but were divided based on whether or not they had mounted a CRP response; either high-serum CRP (hCRP of 5 mg/L or greater) or normal-serum CRP (nCRP less than 5 mg/L) levels (Figure 1A for study design).

Strikingly, the time to diagnosis and time to first disease-modifying antirheumatic drug (DMARD) initiation were both

Table 2. nCRP patients had accelerated progression to biologics DMARDs compared with hCRP patients[a]

	Measured Variables and Outcomes	Normal CRP	High CRP	*P* value
1-Year follow-up	Patients with high disease activity at 1-year follow-up[e]: % (number)	30 (7/23)	0 (0/11)	0.040[b,c]
	DAS-28 (CRP) at 1-year follow-up: mean; SD, (range),	3.5; 1.6 (1.3-6.3)	2.7; 0.9 (1.4-3.9)	0.217
	Patients prescribed a new biologic at 1-year follow-up: % (number)	43.3 (13/30)	18.7 (3/16)	0.095[d]
2-Year follow-up	Patients with high disease activity at 2-year follow-up[e]: % (number)	5 (1/19)	0 (0/10)	0.460[c]
	DAS-28 (CRP) at 2-year follow-up: mean, SD (range),	2.6; 1.6 (0.5-6.3)	2.3; 1.2 (0.5-4.3)	0.597
	Patients with high[e] and moderate[f] disease activity at 2-year follow-up: % (number)	32 (6/19)	30 (3/10)	0.228[c]
	Patients being treated with biologics at 2-year follow-up: % (number)	61.5 (16/26)	20 (3/15)	0.010[b,d]

[a] Patients were followed up at 1 year and 2 years postrecruitment and assessed for treatment and disease activity. Follow-up for this study was from data collected in routine clinics, and some data were not available at the correct follow-up timepoints. All available data are reported. DAS follow-up data were included when the patient was seen at 12 ± 1 month, and/or 24 ± 1 month. Data are presented as means, SDs, or percentages (number) and were analyzed using the Mann-Whitney test.
[b] Denotes significance.
[c] Data analyzed using Chi-squared test.
[d] Data analyzed using Fisher's exact test.
[e] Disease control was assessed as high disease activity DAS-28 score greater than 5.1.
[f] Disease control was assessed as moderate disease activity; DAS-28 equal to or greater than 3.2-5.1 (see ref. 2).

significantly longer in the nCRP cohort (Figure 1B). However, all assessments of disease severity at recruitment were equivalent between the two groups (including DAS-28, swollen joint count, erosion number, total number of joints with Doppler signal, and CCP/RF titer; see Table 1). There was no correlation between disease activity (DAS-28) or CRP levels with any clinical characteristics, including age, disease duration, and RF (Supplementary Table 1 and data not shown). ESR and serum amyloid A (SAA) were also normal in nCRP patients (Table 1). Importantly, longitudinal analysis of nCRP patients identified that they were able to mount a CRP response under other circumstances, such as infection or surgery (Supplementary Figure 1A and B).

Throughout the study, all patients were treated according to NICE guidelines (https://www.nice.org.uk/guidance/ng100). Follow-up data from 1 and 2 years postrecruitment are shown in Figures 1C-F and Table 2. Importantly, patients in the nCRP cohort had nCRP levels throughout the study period, supporting the concept of this being a consistent phenotype. As predicted, hCRP patients had a significant reduction in CRP levels from baseline to 1- and 2-year follow-up (Figure 1C).

Despite persistently nCRP levels, 30% of patients in the nCRP cohort still had a DAS-28 score consistent with very active disease (DAS-28 score greater than 5.1) at 1 year postrecruitment compared with none in the hCRP cohort ($P = 0.040$). By the 2-year follow-up, 61% of nCRP patients had sufficiently active disease to necessitate escalation to biologic therapy (in the United Kingdom this requires a DAS-28 score of more than 5.1) compared with only 20% of the hCRP patients ($P = 0.010$) (Table 2 and Figure 1D). Moreover, nCRP patients had increased biologic drug usage (Figure 1E), but despite this, 32% of nCRP patients still had inadequate disease control at 2-year follow-up (Table 2). After 2 years, 25% (7 of 26) of nCRP patients were treated with tocilizumab, typically a third-line biologic, compared with 6% (1 of 15) in the hCRP group (Figure 1F).

The relationship between IL-6 and CRP was defective in nCRP compared with hCRP patients. Given the link between CRP and the inflammatory response, we questioned whether cytokine responses to inflammation were the same in both patient groups. Pro-inflammatory cytokines trigger CRP production during inflammation; however, despite having significantly different serum CRP levels, no significant differences in serum cytokine levels were detected between the two groups. In particular, IL-1β, IL-6, and TNFα, cytokines associated with both increased CRP and RA disease pathogenesis (19) were elevated significantly in both patient groups compared with HCs (Figure 2A and Supplementary Figure 2), which was true for patients treated with conventional DMARDS and biologics (Supplementary Table 2). Interestingly, IL-6 expression was significantly positively correlated with other proinflammatory cytokines (including IL-1β, TNFα,

IL-12/23, IL-21, and IL-17) in hCRP patients, but this synergy was lost in nCRP patients (Figure 2B). A similar pattern was observed when serum cytokines were directly correlated with CRP levels, whereas a positive correlation was observed between serum CRP and pro-inflammatory cytokines in hCRP patients, which was absent in the nCRP patients (Figure 2C).

Increased regulatory Treg frequency and IL-10 production in nCRP compared with hCRP patients. To assess potential changes in immune cell function in nCRP compared with hCRP patients, in-depth phenotyping of immune cell subsets was performed (Supplementary Figure 3A-H for gating strategies). Unique immune signatures were identified in both patient groups when compared with HCs and with each other, respectively (Figure 2D-F). Notably, Treg populations were significantly altered between nCRP and hCRP patients (Figure 2F).- Specifically, nCRP patients had increased naïve and CD161+ Treg frequencies and elevated Treg Foxp3 expression and reduced memory Treg frequencies compared with patients with hCRP (Supplementary Figure 4A and B). Tregs from nCRP patients produced significantly more IL-10 compared with those produced by hCRP patients and HCs (Supplementary Figure 4C) and had a significantly reduced interferon (IFN)-γ:IL-10 ratio, whereas hCRP patients had an increased IFN-γ:IL-17 ratio compared with HCs (Supplementary Figure 4D). Finally, Treg frequencies correlated significantly with markers of disease progression (such as number of erosions), disease activity (such as DAS-28), and chronic inflammation (synovial hypertrophy) in the nCRP but not in the hCRP patients (Figure 2G). Very few differences in B-cell phenotypes were detected between the two patient groups (Supplementary Figure 5).

Changes in complement activation may drive altered pathogenesis between nCRP and hCRP patients. To explore mechanisms that could explain the immunological changes between nCRP and hCRP patients, a serum proteomic analysis was performed. The proteomes of hCRP and nCRP patients were strikingly different when compared with HCs and with each other (Figure 3A and B). Proteins showing significant differences when compared with HCs were analyzed using Pathway Enrichment software (Figure 3C and D and Supplementary Table 3). Notably, proteins associated with complement and clot formation pathways were significantly enriched in hCRP patients compared with HCs and nCRP patients. This included significant upregulation of factors associated with alternative complement pathway activation, including Complement Factors B, H, and I; C5 and C9; and coagulation factor (F)IX, and downregulation of kallistatin in hCRP patients compared with nCRP patients (Figure 3E, Supplementary Figure 6, and Supplementary Tables 4 and 5).

Utilizing these data, we hypothesized a mechanism by which complement activation in hCRP patients was enhanced via the alternative pathway and other serine proteases (such as coagulation FXa, FIXa, FIIa, and kallikrein); however, complement
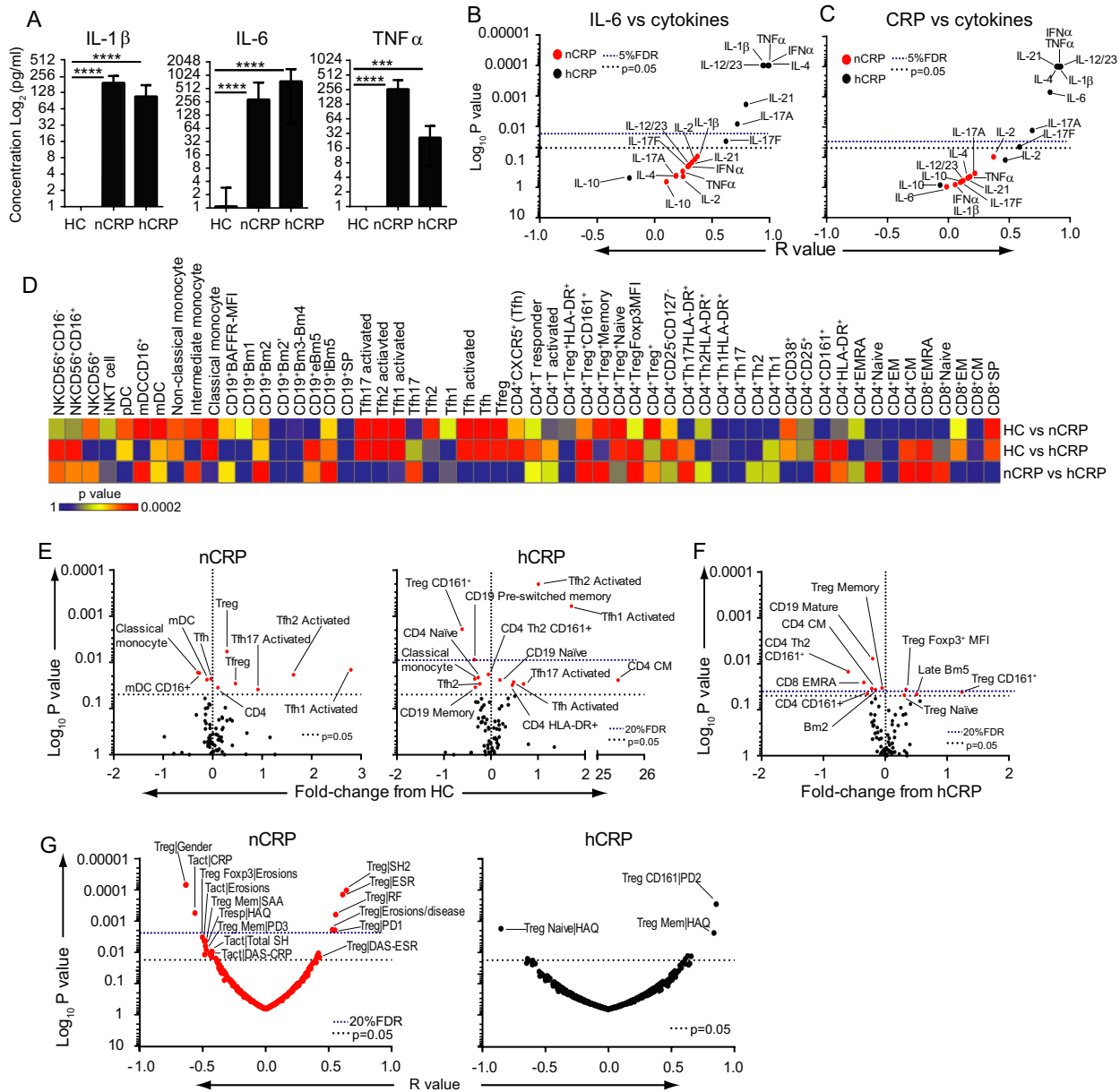
Figure 2. Altered cytokine and immune cell profiles in rheumatoid arthritis (RA) patients with normal (n) verses high (h) C-reactive protein (CRP) levels. Serum cytokine levels from nCRP (n = 21), hCRP (n = 13) and healthy controls (HCs) (n = 20). **A,** Serum interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α levels (mean ± SE). One-way analysis of variance, ***$P < 0.001$, ****$P < 0.0001$. Note: Patients on biologic therapies were excluded from this dataset (see Supplementary Figure 2 and Supplementary Table 2). Serum cytokine levels, IL-1β, TNFα, IL-2, IL-17A, IL-17F, interferon (IFN)γ, IL-8, IL-12/23, IL-10, IL21, IFNα, and IL-4 were correlated against IL-6 levels **(B)** and serum CRP levels **(C)** in nCRP (n = 21) and hCRP (n = 13) patients. Volcano plots comparing the significance of the correlation (Log$_{10}$ P value) versus the correlation coefficient (Pearson's correlation, R). Analyzed using unpaired t-tests and a false discovery rate (FDR) less than 0.05 (blue dotted line). Peripheral blood mononuclear cells (PBMCs) from nCRP (n = 26) and hCRP (n = 15) patients and HCs (n = 29) were assessed for CD4$^+$ and CD8$^+$ T-cell subsets, including regulatory (Treg), central memory (CM), effector memory (EM), effector memory CD45RA-re-expressing (EMRA), T follicular helper (Tfh) cells, and invariant natural killer T (iNKT) cells. CD19$^+$ B cell subsets (Bm1-Bm5), BAFF-receptor (R) expression, plasmacytoid and myeloid dendritic cells (p/mDC), NK cell, and monocyte subsets. Immune cell frequencies were compared between nCRP and HC, hCRP and HC, and nCRP and hCRP patients. Heatmap showing P values indicating significant differences (red) **(D)** and Volcano plots showing fold-change in immune cell subset frequencies **(E)** comparing HCs vs P value (Log$_{10}$ P value) in nCRP (left panel) and hCRP patients (right panel) and comparing nCRP and hCRP patients **(F)**. Analyzed using multiple unpaired t-tests and FDR 0.2% (blue dotted line). Populations $P = <0.05$ shown in red. **G,** Treg frequencies from nCRP (n = 26) and hCRP (n = 15) were correlated against clinical and disease features. Volcano plots comparing the significance of the correlation (Log10 P value) vs the correlation coefficient (Pearson's correlation, R). Analyzed using multiple unpaired t-tests and FDR 0.2% (blue dotted line).
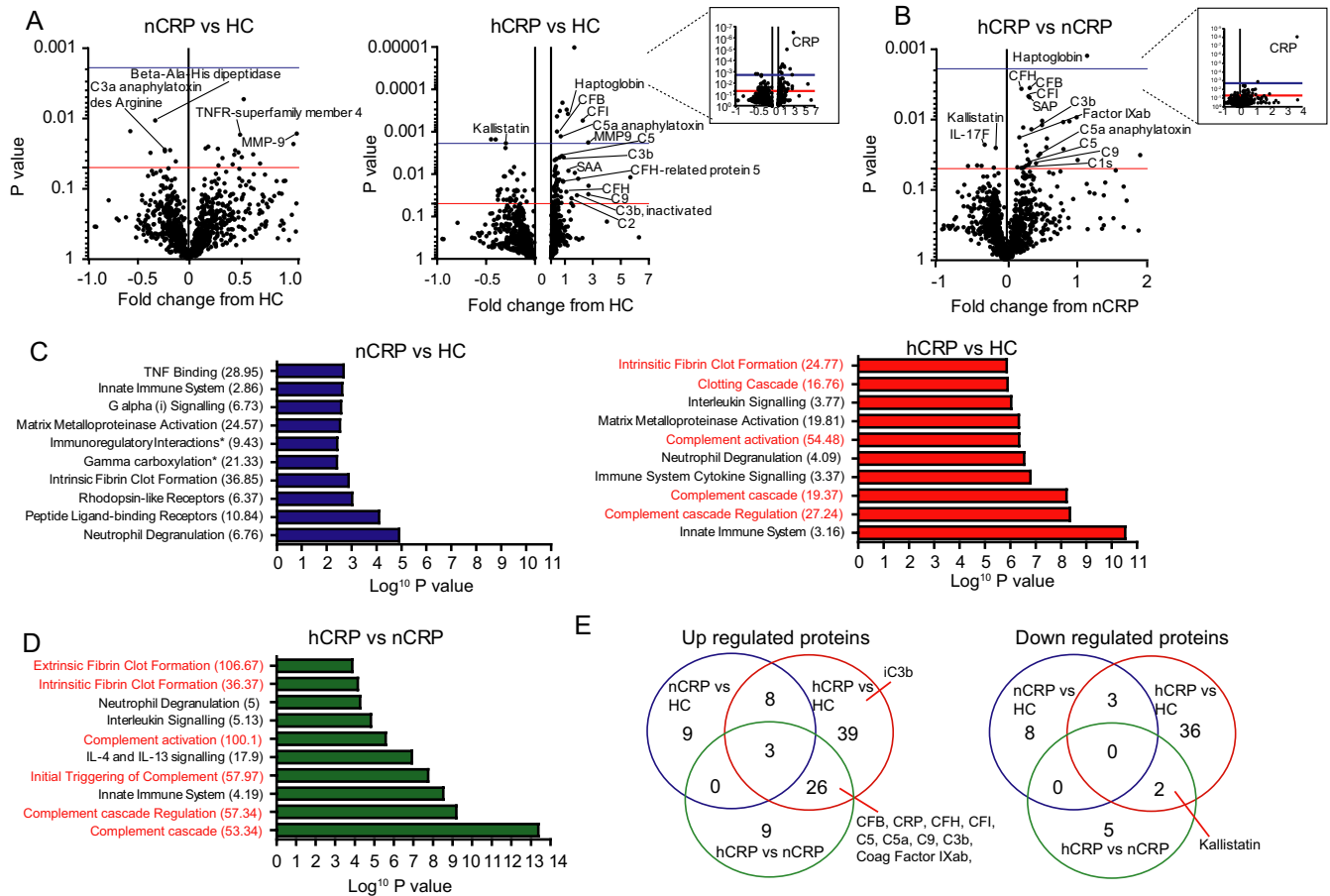
Figure 3. Complement activation pathways were elevated in rheumatoid arthritis (RA) patients with high (h) C-reactive protein (CRP) compared with normal (n)CRP. Proteomics (SOMAscan Proteomic Assay) analysis of serum from RA patients with nCRP or hCRP levels and healthy controls (HCs) (n = 8/group). Data analyzed using multiple *t*-tests and 10% false discovery rate (FDR) for multiple comparisons. Volcano plots showing ⁻log10 *P* value vs fold change from HC values for nCRP and hCRP proteomic analysis **(A)** and hCRP vs nCRP patients **(B)**. Red line shows *P* = 0.05, and blue line shows a 10% FDR value. CRP was significantly upregulated in hCRP patients as shown in subsections. Proteins were significantly different (*P* = 0.05) between nCRP and hCRP patients and HCs **(C)** or between hCRP and nCRP patients **(D)** and were analyzed using a functional enrichment analysis. Top 10 pathways are listed with the ratio of enrichment for each comparison. **E,** A Venn diagram showing the number of proteins whose expression was significantly different (*P* = 0.05) and were either up (positive fold change) or down (negative fold change) regulated. Lists of proteins are shown in Supplementary Tables 4 and 5.

activation in nCRP patients was predominantly achieved through the Classical and Mannose Binding Lectin (MBL) pathways, converging at C4b2b (Figure 4A and Supplementary Table 3). This mechanism was supported by the finding that hCRP patients had a reduced ability to activate the mannose lectin complement activation pathway (Figure 4B). Western blotting confirmed that C3, a central component of the complement system, was activated in both nCRP and hCRP patients (Figure 4C and D). However, although serum C3a correlated positively with CRP and ESR in hCRP patients, no correlation was seen in nCRP patients (Figure 4E), supporting the idea that complement activation was altered in hCRP patients compared with nCRP patients.

Finally, we investigated whether a relationship existed between altered expression of complement components and Treg frequency. Serum C5a levels were significantly and negatively correlated with Treg frequency in hCRP patients but not nCRP

patients (Figure 4F), suggesting a relationship between differential complement activation and immune cell phenotype in the patient subgroups.

DISCUSSION

This study describes a subset of seropositive RA patients who do not have an elevation in CRP despite active synovitis on US. When compared with patients who do have raised CRP during a flare, this nCRP subset was associated with a different immunological phenotype and with poor disease outcome measures.

Good outcome for patients with RA requires prompt diagnosis and rapid escalation of treatment when disease control is inadequate (20–22). In the United Kingdom, clinical decision making is guided by NICE (see Guideline 100), with initial treatment
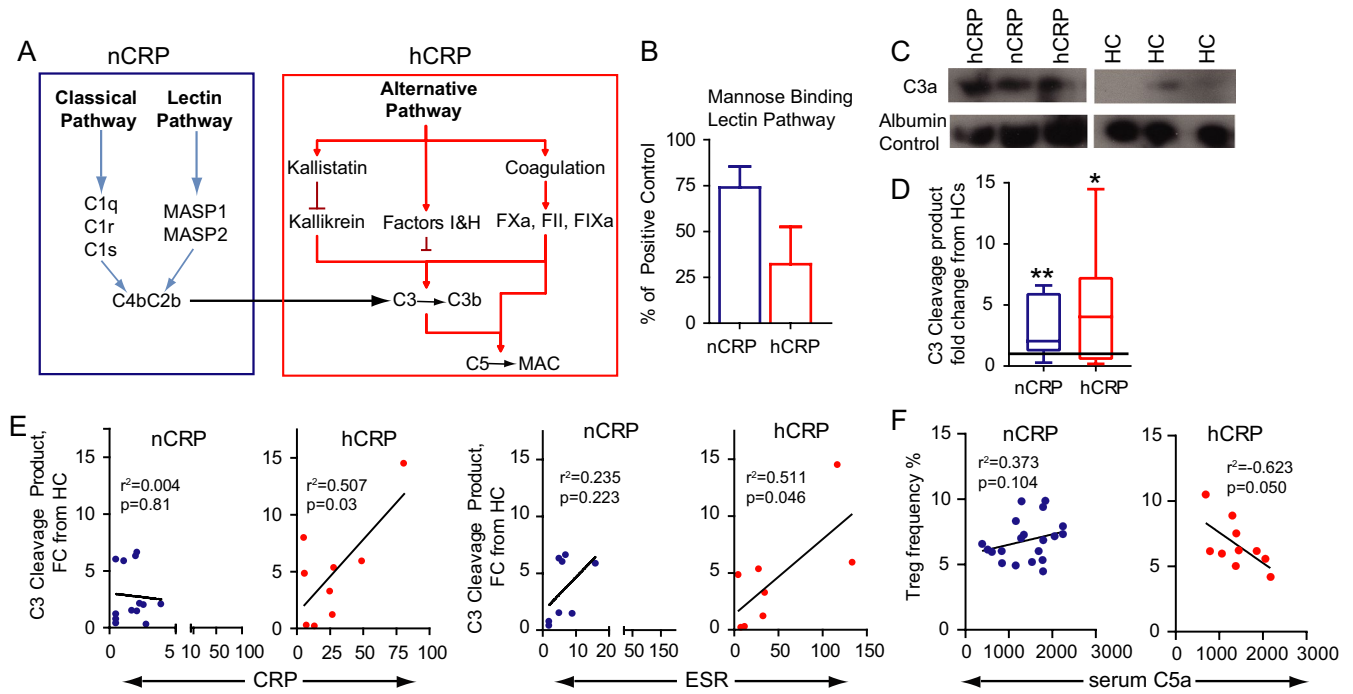
Figure 4. Proteins associated with the alternative complement pathway were significantly elevated in rheumatoid arthritis (RA) patients with high (h) C-reactive protein (CRP). **A,** Model outlining the proposed hypothesis that RA patients with synovitis and hCRP rely on increased activation of the alternative complement pathway compared with RA patients with normal CRP (nCRP), which rely on the Classical and Mannose Binding Lectin (MBL) pathway. This hypothesis was based on analysis of specific complement components identified as significantly altered between the patient groups and/or healthy controls (HCs). Abbreviation: F, factor; MAC, membrane attack complex; MASP, Mannan-binding lectin serine protease. **B,** Activation of the MBL complement pathway was assessed in serum from five nCRP and six hCRP patients. Mean ± SE. **C** and **D,** Serum from nCRP (n = 15) and hCRP (n = 8) patients was analyzed by Western blotting for C3a expression using albumin as loading control. Representative Western blots **(C)** and normalized densitometric values showing C3a expressed as fold change from HCs **(D)**. *T*-test; *P = <0.5, **P = <0.01. **E,** Serum C3a expression from panel **(D)** was correlated with CRP and erythrocyte sedimentation rate (ESR) levels; Pearson's correlation. **F,** C5a expression was measured by enzyme-linked immunosorbent assay in serum from nCRP (n = 20) and hCRP (n = 10) patients. Results were correlated against regulatory T cell frequency (%, refer to Figure 3); Pearson's correlation.

being conventional DMARDs, and escalation to biologics guided by NICE Technology Appraisal (TA375). The decision to start biologic DMARDs is permitted after the failure of two conventional DMARDs (generally including methotrexate), and ongoing disease with a DAS-28 score greater than 5.1. In this study, patients who had active disease were recruited. With the exception of CRP/ESR levels, baseline measures of disease burden were equivalent between the patients in the nCRP and hCRP subgroups; this included DAS-28, swollen joint count, erosion number, total number of joints with Doppler signal, and CCP/RF titer. The impact of this difference in CRP was stark; patients in the nCRP group experienced significant delays in both time to diagnosis and time to initiating DMARD therapy after diagnosis. This observation has implications for physicians: normal CRP and ESR neither exclude a diagnosis of RA nor are they markers of adequate disease control in this patient subset. Although these two statements may seem obvious, the data presented here suggest that clinicians are managing nCRP patients differently and less effectively.

Observational data from this study suggest that patient responses to conventional DMARDs might be less favorable in the nCRP subgroup, with an increased need for escalation to biologic drug treatment because of inadequate disease control. At 2-year follow-up, significantly more nCRP patients were escalated to biologic treatment, suggesting more aggressive disease and/or a lost window of opportunity to bring their disease under control with nonbiological DMARDs. This is despite the fact that it is harder for the patients in the nCRP cohort to make the DAS cut-off required for biologic treatment, seeing as inflammatory markers contribute to this score. Furthermore, in spite of an increased frequency of biologic treatment escalation in the nCRP cohort, 32% of patients in this group still had moderate/high disease activity at the 2-year follow-up.

In addition to delays in starting treatment, we suggest that part of the reason for the poor response to drug treatment is that nCRP patients are often consciously excluded from clinical trials—a raised CRP and/or ESR (variably defined, recently varying from CRP greater than or equal to 3 mg/L to greater than or equal to 6 mg/L (23–25), and ESR greater than 28 mm/hr) is listed as an inclusion criterion in many studies (26), which means that we do not have high-quality trial data to predict treatment response in the nCRP cohort. The reason for this may well be a desire to exclude patients who don't have active, inflammatory arthritis but score

highly on disease activity measures because of other factors, such as GVAS and tender joint count. The risk with this, however, is that truly active, nCRP-phenotype, seropositive RA patients get grouped inappropriately with fibromyalgia-dominant phenotype RA patients, thus limiting evidence on treatment response that can be extrapolated to the nCRP subgroup.

ESR and SAA were also normal in nCRP patients, suggesting that multiple pathways associated with activation of acute phase response were defective in this subgroup of patients. The risk of overreliance on raised acute phase reactants (APRs) was raised by data from The Consortium of Rheumatology Researchers of North America (CORRONA) database, a large United States registry of RA patients. The CORRONA study group investigated rates of raised APRs in their cohort of patients with RA who had active disease based on the Clinical Disease Activity Index (CDAI) (6,27). The CDAI is a composite score that is calculated using tender and swollen joint counts, and patient and physician global assessments. Of 9135 patients with active disease, defined as a CDAI of more than 2.8, 58% had normal APRs. APRs were considered to be elevated if CRP was greater than 8 mg/L and ESR was greater than 28 mmHg. The authors discuss the potential for underinvestigation and undertreatment of this group of patients. Interestingly, the use of biologics at recruitment was higher amongst patients with normal APRs, although it is not noted whether patients treated with tocilizumab were included in the study. However, this study again raises the possibility that nonbiologic DMARDs may be less effective in patients with normal inflammatory markers.

This research identified for the first time that RA patients with nCRP have a different immunological phenotype. Their pro-inflammatory cytokines did not exhibit the usual synergy one would expect, and the expected correlation with CRP levels was lost. The mechanisms underlying this observation remain unknown but are reminiscent of findings in patients with inflammatory bowel disease that also have raised IL-6 but low CRP (28). hCRP patients were characterized by an increased expression of alternative complement pathway proteins compared with nCRP patients.

Altered activation of complements could be connected to the observed changes in immune cell phenotype (29). T-cell stimulation can trigger alternative complement pathway activation via production of factors C3, C5, B, and D, generating the alternative convertase C3bBb (30). This stimulation can be maintained in an autocrine manner by C5a, which was significantly higher in hCRP patients. Moreover, this activation is mediated by the C3a and C5a receptors, which are associated with downregulation of Foxp3 in Tregs (31,32) as seen in the hCRP patients. CD161$^+$Foxp3$^+$ Tregs and Treg IL-17 production were also reduced in hCRP patients. This reduction could also be linked to higher circulating levels of C5a, as has been shown in other autoimmune disorders (33). Our results also point to a role for Tregs in disease progression because Treg frequency correlated with higher DAS-28 scores and erosion

scores in nCRP patients. Thus, differences in complement activation, immune cell subtypes, and inflammatory cytokines point to a difference in disease mechanism, which possibly could explain a difference in response to treatment (34).

In this study we ensured that only patients with RA who were seropositive for RF and anti-CCP antibodies were included. In our cohort, the rules for inclusion were consistent between the two groups and the average number of tender/swollen joints between the groups was the same. The strict inclusion criteria aimed to recruit homogeneous RA patient groups to minimize the inclusion of overlapping disease phenotypes, such as seronegative peripheral arthritis associated with seronegative spondyloarthropathy or psoriatic arthritis. However, because patients are referred for US when disease activity is not clear, fewer patients with raised CRP were recruited as these patients are less likely to need to be scanned.

From the data presented here, however, one might suspect further diagnostic and treatment delays for patients who are both seronegative for RF/anti-CCP and who do not mount a systemic inflammatory response.

A limitation of this study is the absence of longitudinal collection and analysis of blood samples. Therefore, a further study with larger patient numbers followed over time is required to answer questions raised by this work, including defining whether there may be two subgroups within the nCRP cohort—those who mount some sort of detectable CRP response between 0.6 and 5 mg/L and those whose CRP is continually less than 0.6 mg/L—as measured by the high-sensitivity CRP assays; further defining the role of complement activation in the nCRP and hCRP cohorts; and answering the questions raised surrounding treatment response.

In conclusion, the first thing that we hope will come from this study is the education of treating clinicians rather than equating a normal CRP with adequate disease control. These patients need other objective measures of their disease activity, such as US. The second need is for further mechanistic studies, perhaps exploiting the difference in complement activation, to inform clinical trial design for this neglected cohort.

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AUTHOR CONTRIBUTIONS

Drs. Bradford, McDonnell, Raj, Jury, and Manson wrote the manuscript, Drs. Isenberg and Ciurtin reviewed the manuscript, and all authors approved the final manuscript.
Study conception and design. Manson, Jury, Ciurtin
Acquisition of data. Bradford, McDonnell, Raj, Robinson, Cole, Ramakrishnan, González-Serrano, Mak, Eskiocak, Isenberg, Ciurtin, Manson,
Analysis and interpretation of data. Bradford, McDonnell, Raj, Cole, Ramakrishnan, González-Serrano, Eskiocak, Isenberg, Jury, Manson

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