

## Research Article

# Expression and modulation of complement receptor 2 (CR2/CD21) in the pathophysiology of rheumatoid arthritis

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### ABSTRACT

**Background:** The involvement of B cells, complement activation and subsequent immune complex deposition has been implicated in the pathogenesis of rheumatoid arthritis (RA). Although the reduced expression of complement receptor 2 (CR2, CD21) on the B cells of RA patients is known for long, we aimed at determining the modulation and expression of CR2 on PBMC of healthy individuals and RA patients.

**Methods:** Sixty controls and 57 RA patients were enrolled. PBMC-CR2 transcript levels were correlated with the levels of C3, C3d and circulating immune complexes (CIC) in controls and patients and with DAS28 in patients only. CIC levels were determined by PEG precipitation, C3 levels by nephelometry and C3d levels were determined by enzyme linked immunosorbent assay (ELISA). Sixteen patients were recruited for 6 months follow up studies of transcript levels of PBMC correlated with DAS28 score. Appropriate statistical methods were used for the analyses of data.

**Results:** PBMC- CR2 transcript levels were declined in patients as compared to controls. PBMC-CR2 levels correlated negatively with DAS28 score. DAS28 correlated positively with levels of CIC, C3 and C3d. Levels of PBMC -CR2 increased in patients with decline in DAS28 scores in 6 months follow-up patients.

**Conclusions:** Low level of CR2 expression in patients may, thus, contribute significantly to the pathological manifestation of RA. Cause-effect relationships of the up regulation of CR2 on improvement of health condition with the pathophysiology of RA and their importance as putative disease markers is being confirmed.

**Keywords:** Rheumatoid arthritis, Complement receptor, Transcript, DAS-28, Disease marker

### INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by the presence of autoantibodies, joint inflammation and subsequent destruction of cartilage and bone.<sup>1</sup> The disease affects multiple organs, including the joints, skin, heart, lungs and eyes. However, the primary target of the abnormal autoimmune response is the synovial joints.<sup>2</sup> RA involves all elements of the immune response. It is initiated by immune complexes (ICs) and complement, perpetuated by cytokines and

affected by metalloproteinases.<sup>3</sup> The local production of IgG and rheumatoid factors (RF), which are auto antibodies directed against the Fc portion of IgG, along with the antibodies to anti-cyclic citrullinated peptide lead to complement activation, appear important in destructive events associated with the synovitis.<sup>4,5</sup>

The classical pathway can be initiated by several triggers present in the inflamed joint such as deposited autoantibodies, dying cells, and exposed cartilage proteins. B cells producing autoantibodies, which in turn

form immune complexes, contribute to RA pathogenesis partly via activation of complement.<sup>6,7</sup> The effects of complement are mediated through complement receptors. These are CR1 (CD35), CR2 (CD21), CR3 (CD11b/18), CR4 (CD11c/18), C3aR and C5aR.

The complement receptor type II (CD21, CR2) belongs to the super gene family of regulators of complement activation. CR2 is the functional receptor for C3d fragments on immune complexes and the Epstein-Barr virus (EBV) envelope protein gp350.<sup>8,9</sup> It is expressed on all mature B-lymphocytes, follicular dendritic cells and also on T-lymphocytes.<sup>10</sup>

CR2 on the surface of B cells appears in a trimolecular complex of CD81/CD19/CR2.<sup>11</sup> When it is coligated with the BCR, it mediates activation, proliferation and differentiation and lowers the threshold of antigen sensitivity of B cells.<sup>12</sup> The present study, the first of its kind would provide baseline data as the expression levels of CR2 on the PBMC in the healthy individuals and its relationship with the pathophysiology and clinical disease activity of RA. The study is expected to facilitate the evaluation CR2 as a disease activity marker for RA.

## METHODS

57 patients (51 females and 6 males) diagnosed with RA (18-52 years) were included in the study. All of the patients were DMARD (disease modifying anti rheumatic drug) treatment naïve at the time of their inclusion in this study. Diagnosis was done according to the criteria put forward by ACR for the classification of RA.<sup>13</sup>

The patients fulfilled at least four of the criteria either simultaneously or over a period of time. Laboratory investigations like serum and plasma levels of C3, anti-CCP, CRP, ESR, TJC (tender joint count), SJC (swollen joint count) and VAS were done for all the patients in the clinical investigation laboratory.

DAS28 score was used to calculate the disease activity. This study was approved by the institute's ethics committee and written informed consent was taken from all the participants at the time of enrolment. In addition, 16 patients were enrolled for 6 months longitudinal follow up studies. A further 60 matched healthy volunteers (49 females and 11 males) were enrolled in the study to serve as controls (age 18-41 years).

### Sample collection

Venous blood (5 - 7 ml) with or without anticoagulant (EDTA) was drawn from the controls and RA patients. The fluid fraction of blood (Plasma and serum) was separated from the cellular fraction by centrifugation at ~500xg at 4°C, aliquotted and stored at -70°C until further used for the estimation of C3, C3d and CIC levels. The packed cellular fraction was used for the isolation of PBMC to determine the levels of CR2.

### Quantification of CR2 transcripts

RNA was extracted from PBMC by using trizol reagent (Sigma-Aldrich, USA) according to the manufacturer's instruction. The RNA was quantified by spectrophotometer/ nano-drop and run on a formaldehyde gel to check the integrity, and 1mg RNA was converted to cDNA by reverse transcription using Expand RT-kit (Roche) in 20 ml reaction volume containing oligo-dT.

A negative control reverse transcription reaction was also performed without adding reverse transcriptase enzyme. A volume of 1 ml of cDNA obtained by reverse transcription was amplified with specific primers for CR2. PBMC transcript level was normalized by b-actin, which acted as an internal control. Primer pairs used for CR2 were: Forward: gcc gac acg act acc aac c; Reverse: agc aag taa cca gat tca cag c and for b-actin Forward: aga aaa tct ggc acc aca cc; Reverse: tag cac agc ctg gat ag ccaa.<sup>14</sup> PCR was done in a 25 ml reaction volume using Taq Polymerase (MBI Fermentas, Hanover, MD, USA).

The PCR products were run on 1.5% agarose gel with ethidium bromide. Semi-quantitative analysis was conducted using a computerized densitometry imager (Bio-Rad, Quantity one software) to obtain CR2/b-actin ratio.

### Estimation of C3

C3 level was estimated by using Minineph plus™ kit (The binding site, Grassobio, Italy) according to the manufacturer's instructions by nephelometry in the Department of Medicine, All India Institute of Medical Sciences, New Delhi.

### Estimation of C3d

The complement C3 fragment, C3d (35 kDa), a cleaved product of C3b, shares common epitopes with intact C3 and C3b. C3d levels were estimated using BMASSAY ELISA Kit (Biomedical Assay Co.Ltd. Beijing, China) according to the manufacturer's instructions.

### Circulating immune complexes (CIC) estimation

Circulating immune complexes (CIC) were estimated in plasma samples of RA patients and controls by the method described previously by Sai Baba et al.<sup>15</sup> CIC were precipitated from the plasma with 2.5% PEG-6000 (polyethylene glycol) at 4°C overnight, and the concentration of CIC was measured in a spectrophotometer (UV-160A UV-VIS Spectrophotometer, Shimadzu, Kyoto, Japan) using aggregated human gamma globulin as standard.

### Statistical analysis

The results are presented as means±standard deviation (SD) and in percentages. The differences in averages

between the study groups were examined by the Mann-Whitney test and independent sample t-test. The comparison between the two data in follow-up study was done by paired t-test.

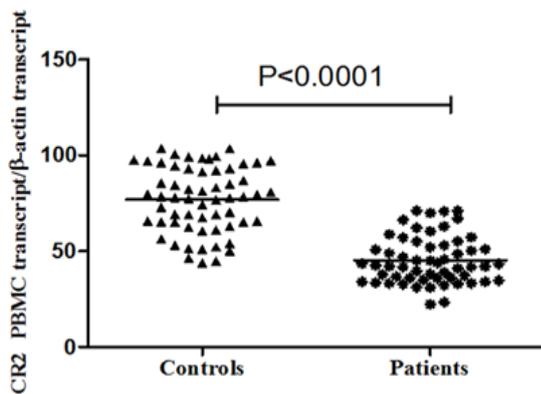
Correlation coefficient between the test parameters was assessed by applying Spearman's rank correlation test and the significance level was measured by two-tailed paired student's t-test. In all cases,  $P < 0.05$  was considered significant.

Data analysis was performed using SPSS software version 14 and Graph pad PRISM version 5.

## RESULTS

### CR2 expression at transcript level (RT-PCR)

There was a significant decline in the expression of CR2 in RA patients when compared to controls ( $p < 0.0001$ , Mann-Whitney test) in PBMC. The mean value for PBMC CR2 in controls ( $76.88 \pm 17.00$ ) was 40.9% higher than that of patients ( $45.36 \pm 12.44$ ) (Figure 1).



**Figure 1: CR2 expression at transcript level (RT-PCR).**

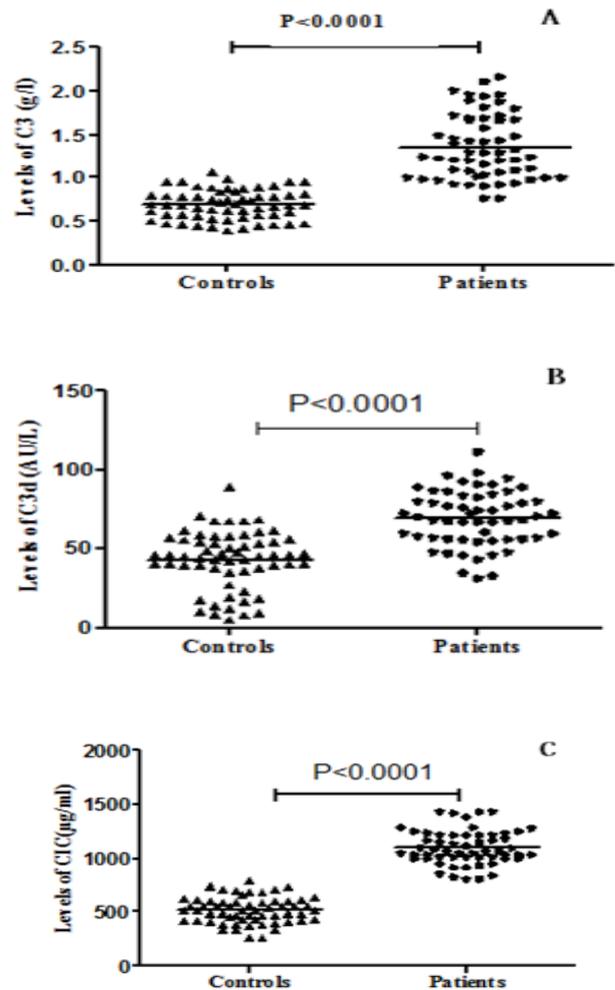
Level of CR2 in PBMC was determined by semi-quantitative RT-PCR. These plot shows the values of CR2 expression normalized by  $\beta$ -actin expression in controls ( $n=60$ ) and RA patients ( $n=57$ ). Each symbol represents one control (black triangles) or patient (black stars) and the bar within each group represents the mean value. The P value was determined by Mann-Whitney test.

### Levels of C3, C3d and CIC

In present study group there was a significant ( $p < 0.0001$ , Mann-Whitney Test) increase in the level of C3 in the patients. The mean levels of C3 were found to be  $0.68 \pm 0.17$ g/l in controls and  $1.36 \pm 0.37$ g/l in patients respectively (Figure 2 A). The mean levels of C3d were found to be  $42.92 \pm 18.29$  AU/L in controls and  $69.46 \pm 17.87$  AU/L in RA patients. Hence there was a significant ( $p < 0.0001$ , Mann-Whitney test) increase in the

level of C3d in RA patients (Figure 2 B).

The mean levels of CIC were found to be  $515.6 \pm 119.8$   $\mu$ g/ml in controls and  $1103 \pm 159.7$   $\mu$ g/ml in RA patients. Hence there was a significant ( $p < 0.0001$ , Mann-Whitney test) increase in the level of CIC in RA patients. In patients and controls, plasma CIC levels ranged from 50-1400  $\mu$ g/ml (Figure 2 C).



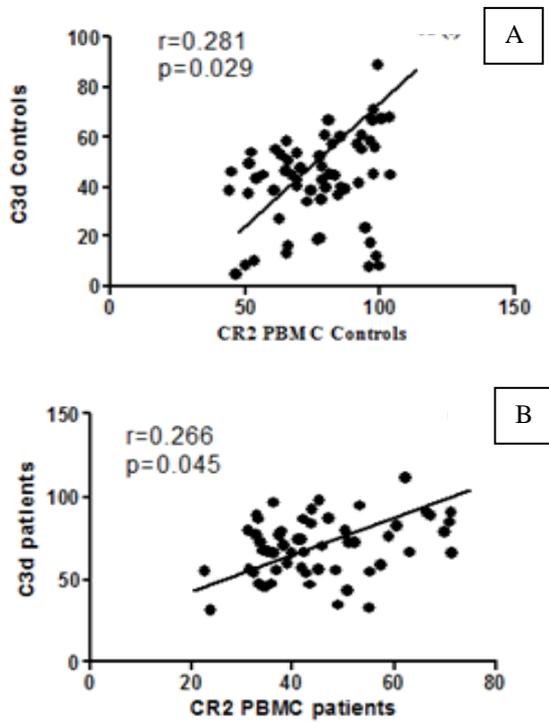
**Figure 2 (A, B and C): Levels of C3, C3d and CIC.**

These plot shows the absolute individual values of C3, C3d and CIC in both controls ( $n=60$ ) and RA patients ( $n=57$ ). Each symbol represents one control (black triangles) or RA patient (black stars), and the bar within each group represents the mean percentage value. The P values are derived by Mann-Whitney test.

### Correlation of CR2 PBMC transcript with clinical parameters

The relationship of PBMC CR2 transcript levels with C3, C3d and CIC were evaluated in both controls and patients. The relationship of CR2 transcript with DAS28 score was evaluated in patients. A significant positive correlation was observed between CR2 transcript in

PBMC in controls with C3d levels in controls ( $p=0.029$ ,  $r=0.281$ , spearman rho analysis) Figure 3 A and also with the patients ( $p=0.045$ ,  $r=0.266$ , spearman rho analysis) Figure 3 B.



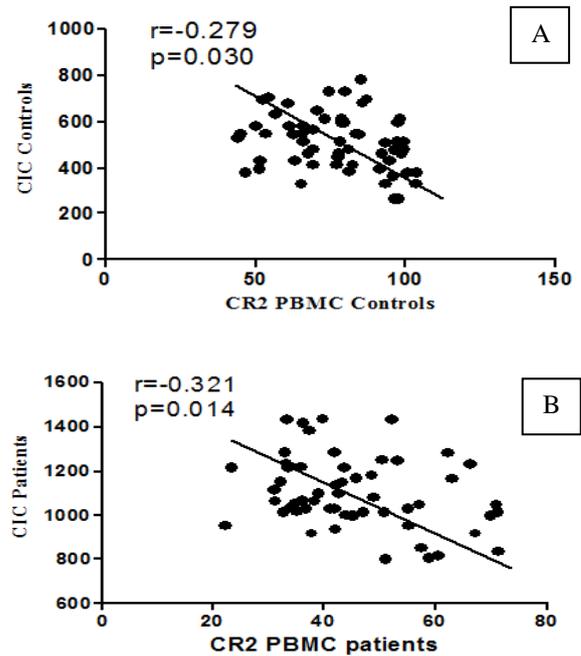
**Figure 3 (A and B): Correlation of CR2 PBMC transcript with C3d levels.**

These figures represent correlation between (A) CR2 PBMC transcript ( $n=60$ ) in controls with C3d levels ( $n=60$ ) in controls and (B) CR2 PBMC transcript ( $n=57$ ) in patients with C3d levels ( $n=57$ ) in patients. The  $p$  and  $r$  values indicated in the respective panels were calculated by two-tailed test and Spearman's rho analysis.

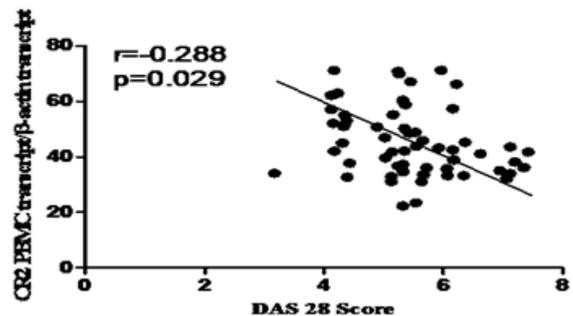
In RA patients, CR2 transcript in PBMC negatively correlated with CIC levels in controls ( $p=0.030$ ,  $r=-0.279$ , spearman rho analysis) Figure 4A and as well as in patients ( $p=0.014$ ,  $r=-0.321$ , spearman rho analysis) Figure 4B.

These figures represent correlation between (i) CR2 PBMC transcript ( $n=60$ ) in controls with CIC levels ( $n=60$ ) in controls and (ii) CR2 PBMC transcript ( $n=57$ ) in patients with CIC levels ( $n=57$ ) in patients. The  $p$  and  $r$  values indicated in the respective panels were calculated by two-tailed test and Spearman's rho analysis.

DAS28 score was calculated from the clinical history and laboratory investigations of the patients. A significant negative correlation was observed between PBMC CR2 transcript ( $p=0.029$ ,  $r=-0.288$ , spearman rho analysis) (Figure 5) with the disease activity score (DAS28) in RA patients ( $n=57$ ).



**Figure 4 (A and B): Correlation between CR2 PBMC transcript and CIC levels.**

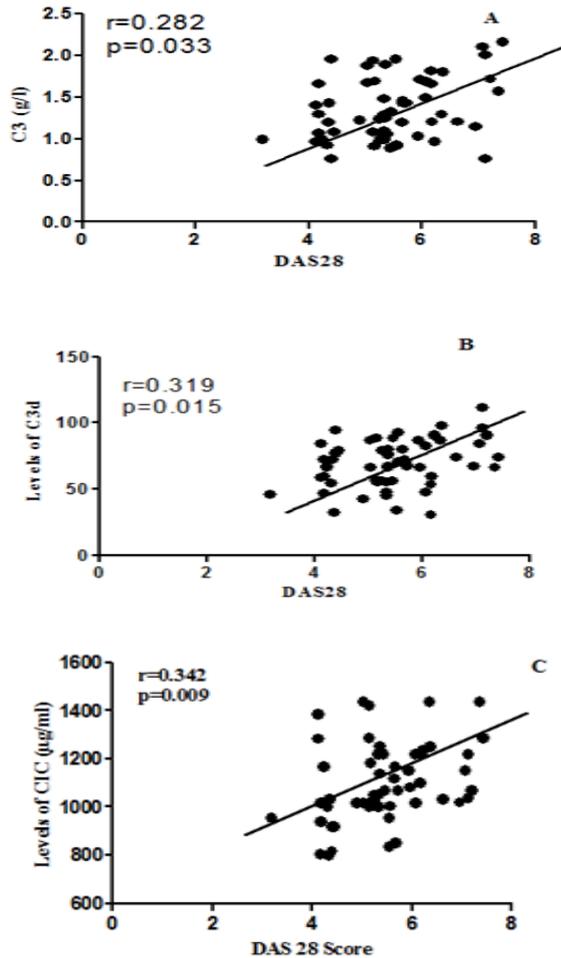


**Figure 5: Correlation between CR2 PBMC transcript in patients with DAS 28 score.**

This figure represents correlation between CR2 PBMC transcript ( $n=57$ ) in patients with DAS 28 score. The  $p$  and  $r$  value indicated in the panel was calculated by two-tailed test and Spearman's rho analysis.

**Correlation of DAS 28 score with clinical parameters (C3, CIC and C3d)**

A significant positive correlation was observed between the levels of C3 ( $p=0.033$ ,  $r=0.282$ , spearman rho analysis) (Figure 6 A), C3d ( $p=0.015$ ,  $r=0.319$ , spearman rho analysis) (Figure 6 B) and CIC ( $p=0.009$ ,  $r=0.342$ , spearman rho analysis) (Figure 6 C) with the DAS 28 score in naïve RA patients respectively.



**Figure 6 (A, B and C): Correlation of C3, C3d and CIC.**

These figures represent correlation between levels of C3, C3d and CIC (n=57) in patients with DAS 28 score. The p and r values indicated in the respective panels were calculated by two-tailed test and Spearman’s rho analysis.

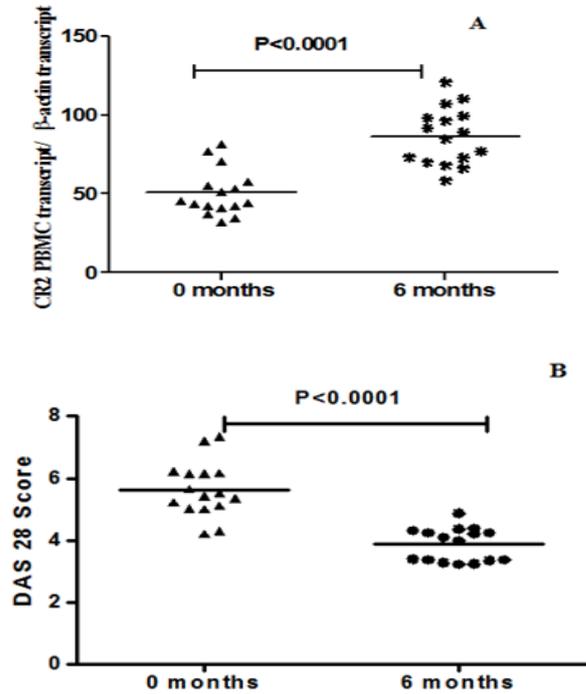
**Expression of CR2 in follow-up patients**

The follow up study brings the most conclusive evidence in understanding the factor as a determinant of the disease pathophysiology and modulation. A significant increase of CR2 expression at the transcript level in PBMC (p<0.0001, Mann-Whitney test) Figure 7 A was observed during 6 months of follow up when compared with the CR2 levels during the naïve RA.

Furthermore, there was a significant decrease in DAS 28 score in patients of 6 months follow than that of naïve RA patients (p<0.0001, Mann-Whitney test) Figure 7B.

Levels of CR2 PBMC transcript and DAS 28 score in 0 and 6 months follow ups. The plot shows the absolute individual values of CR2 PBMC transcript and DAS 28 in both 0 and 6 months follow ups (n=16). Each symbol represents one control (black triangles) or RA patient

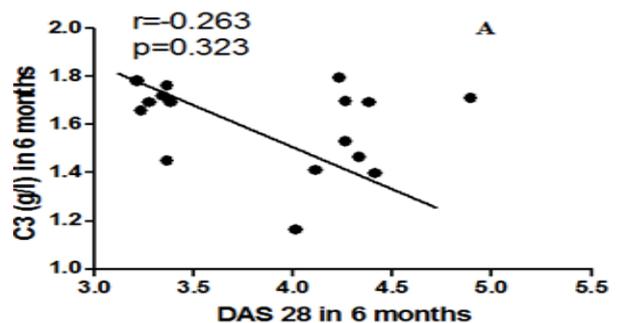
(black stars), and the bar within each group represents the mean percentage value. The P values are derived by Mann-Whitney test.

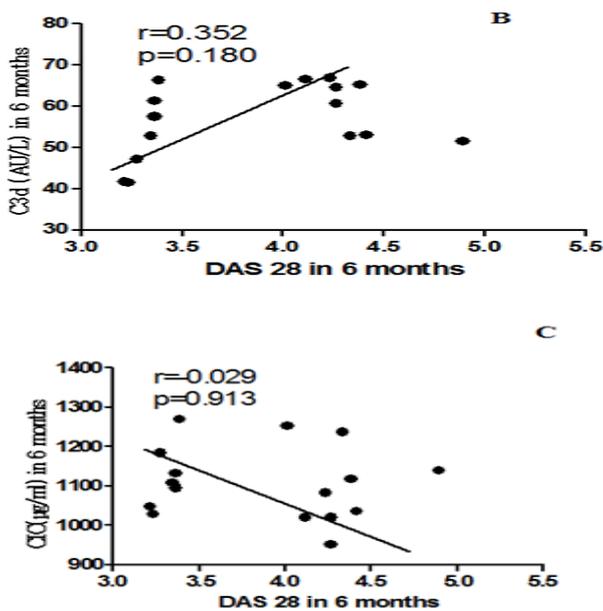


**Figure 7 (A and B): Levels of CR2 PBMC transcript and DAS 28 score.**

**Correlation of DAS 28 score with clinical parameters (C3, CIC & C3d) in follow up patients**

Correlations of C3, C3d and CIC with the DAS28 score were studied in 16 patients after 6 months of treatment. We found no significant correlation between C3d (p=0.180, r=0.352, spearman rho analysis) Figure 8 (A) with DAS28 score in 6 months of follow up. A moderately negative correlation was observed between C3 (p=0.323, r=-0.263, spearman rho analysis) Figure 8 (B) and CIC (p=0.913, r=-0.029, spearman rho analysis) Figure 8 (C) and DAS28 score in 6 months of follow up study respectively. Furthermore, there was a significant decrease in the levels of C3, C3d and CIC in patients of 6 months follow than that of naïve RA patients (p<0.05, Mann-Whitney test).





**Figure 8 (A, B and C): Levels of C3, C3d and CIC.**

These figures represent correlation between levels of C3, C3d and CIC (n=16; 6 months follow-ups) in patients with DAS 28 score in 6 months follow-ups.

The p and r values indicated in the panel were calculated by two-tailed test and Spearman's rho analysis, respectively.

## DISCUSSION

Out of the total 57 patients enrolled in the study, 51 were females and six were males, which put the sex ratio at 8.5:1. However, this sex ratio does not reflect the actual sex ratio of patients with RA visiting the hospital. The disease activity of the patients, assessed by DAS 28 score, showed a mean value of 5.63-3.87. This score is representative of a comparatively aggressive form of the disease. Data on the status of expression of CR2 in RA in humans are sparse. Available reports are not sufficient for the emergence of a clear picture regarding the role of this CR2 in the pathogenesis of RA. We found a significant decline in the mean level of PBMC- CR2 transcripts in patients with RA compared with controls.

To the best of our knowledge, much work is not carried out on this receptor in India. Studies show that decreased level of CR2 in peripheral blood B-cell upon activation and reduced expression of the CR2 by synovial fluid B and T lymphocytes in RA patients.<sup>15,16</sup>

Decreased CR2 expression is due to increased shedding of the receptor, however, RA patients shed the same amounts of CR2 as healthy individuals and they even display reduced levels of soluble CR2. Interestingly, synovial B cells express even less CR2 compared to peripheral blood B cells in RA patients.<sup>17</sup> This suggests disease-related modulation in the expression of CR2 in

patients. To gain an insight into the relation of CR2 with disease associated parameters, we determined the levels of C3, C3d and CIC in patients and controls, and DAS 28 score in patients, and evaluated their correlation with CR2 transcript. Levels of C3, C3d and CIC were increased in patients as compared to controls. The findings were indicative of immune complex overload, exaggerated complement activation and inflammatory conditions in the patients with RA.

Since C3 is the merging point in the activation of complement cascade by different pathways, C3 had been the molecule of focus in different investigations carried out on autoimmune disorder. The increased consumption of C3 in patients may be due to the lowering of the levels of CR2 in patients. C3 level was determined by a number of factors which include rate of synthesis determined partly by genetic factors rate of consumption and compensatory increase in the synthesis of C3 in inflammation which can mask increased consumption.<sup>17-19</sup>

Moreover, localized synthesis of C proteins and restricted activation of the complement cascade in inflamed tissues is not reflected by a decreased level of plasma C3 as exemplified by diseases like myasthenia gravis or membranous nephritis.<sup>20-22</sup> Hence it was not unexpected that C3 level is not associated with DAS28 score. However, there was no significant correlation between PBMC CR2 transcripts in with C3 levels in both controls and patients. We found a significant positive correlation between C3 and DAS28 score. C3 level is determined by a number of factors which include rate of synthesis, rate of consumption and compensatory increase in the synthesis of C3 in inflammations. Hence, a static measure of C3 protein does not always reflect the level of C3 consumption.

The level of C3d largely reflects the extent of C3 activation. The quantitation of C3d levels has allowed for an estimation of complement activation in patients with RA patients. Morrow et al shown that C3d levels were raised above normal in RA patients, presumably affecting the inflammatory processes involved in this disease.<sup>23</sup>

The interaction of C3d with CR2 plays an important role in B cell activation and maturation. C3d levels were associated with the severity of disease in RA and proved to be a much better indicator than C3 and plasma C3d levels correlated with clinical activity.<sup>24</sup> It has been reported that C3d level raised in synovial fluid from RA patients.<sup>25</sup> There was a significant positive correlation observed between CR2 transcript in PBMC in both control and in patients.

We found a significant positive correlation between C3d and DAS28. This cumulative findings observed in our patients is disease acquired may be due to exaggerated activation of complement and dysregulation of the complement cascade by CIC and CR2 respectively. The phenomenon is interlinked and overlapping.

CIC causes enhanced activation of the complement cascade in the plasma. Exaggerated complement consumption leading to the deficiency of complement components is one of the major consequences of immune complex overload.<sup>26,27</sup> Levels of CIC increased in RA patients suggesting immune complex overload which is the hallmark of autoimmune diseases. There was a significant positive correlation observed between CR2 transcript in PBMC in both control and in patients. We found a significant positive correlation between C3d and DAS28. We measured IC as a whole by PEG precipitation and therefore could not differentiate the size or the class of the constituent antibody.

Follow-up studies provide a clearer picture of the role of a factor in the pathology of a disease. Therefore, we carried out a follow-up study in a group of 16 patients. Many patients with poor prognosis do not attend the routine clinics and many patients who initially volunteered to participate in the investigation refused to continue further. These are some of the reasons why a systematic follow up of RA is difficult. We studied 16 volunteers systematically at 0 day who had not started with DMARD and 6 months follow up after treatment. The studies which were followed up beyond this period could not be systematized. The study revealed a statistically significant increase in the level of CR2 transcript in PBMC in the 6 months follow up patients. These findings suggest an intimate relationship between the level of CR2 transcript and disease activity in RA. This level was significantly lower in case of patients with naïve RA when compared with those with the follow ups. Thus, our follow up studies clearly showed a relationship between the up-regulation of the CR2 levels and their importance as putative disease marker.

To the best of our knowledge this is the first report on the status and modulation of CR2 transcript in PBMC in RA and its correlation with disease activity. These observations suggest an intimate relationship between CR2 and pathophysiology of RA and CR2 as a potential disease activity marker. A detailed investigation involving a larger number of study subjects and investigations at different time points during the course of the disease would bring further confirmation to our findings. It will be interesting to explore the modulators of CR2 expression in health and disease to gain further insight to the overall dynamics and role of CR2 in RA.

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