# Prevalence of Erythrocytopenia & Thrombocytopenia in Eastern Indian Population & its Correlation with Acute Antibody Mediated Renal Transplant Rejection & Cytomegaloviral Nephropathy

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## ABSTRACT

**Objective**: Our objective was to study the prevalence of thrombocytopenia & erythrocytopenia post renal transplantation in Eastern Indian population. Thrombocytopenia & erythrocytopenia are common phenomena prevailing post transplant rejection. It is found to occur in patients developing HCMV (human cytomegaloviral nephropathy), post transplantation. Whereas in the case of none rejected patients thrombocytopenia is unlikely to occur.

**Methods**: Several methodologies such as the serological cross match, HLA-cross match DSA luminex, renal biopsy including C4d staining, hematological RBC & platelet count monitoring were adopted in the study, along with these the serum creatinine levels of the rejected patients were tested by making use of several biochemical approaches. Statistical analysis of the data obtained from the laboratories, were also done by the software prism 4.1.

**Results**: Out of total population of 30 patients selected randomly, 24 were found to have successful transplants as for them a significant increase in both platelet & RBC count were noticed post transplantation where as for the other 6 patients a significant decrease in the RBC & platelet count was observed post transplantation along with a significant increase in the serum creatinine levels. There was also a significant decrease in the GFR (glomerular filtration rate) which was an indication of some sort of graft dysfunctioning. All the patients were checked for viral nephropathy & the above 6 patients were found to develop HCMV nephropathy. For the above mentioned 6 patients, the presence of C4d marker in their renal peritubular capillaries after performing the immunehistochemical C4d staining was a key indicator of acute antibody mediated rejection.

**Conclusion**: Our study clearly reveals that Thrombocytopenia & erythrocytopenia was quite common in the patients with acute antibody mediated renal transplant rejection. A low RBC & platelet count persisted in them even after transplantation owing to allograft rejection, & HCMV nephropathy. Whereas for the patients with successful transplants events such as thrombocytopenia & erythrocytopenia were nevertheless unlikely.

## INTRODUCTION

Thrombocytopenia & erythrocytopenia are well recognized complications prevailing post transplant

rejection. Recent finding suggests a significant correlation between HCMV nephropathy & thrombocytopenia, with HCMV having a direct CPE (cytopathic effect) on megakaryocytes primarily.[1,2,3] HCMV infection however has also been found to suppress haematopoeisis i.e. formation of RBC from the bone marrow.[4] HCMV infection has been found to provoke allograft rejection by triggering several immunological cascades, through different mechanisms one of which can be cited as "molecular mimicry'. The HCMV proteins are very much similar to the HLA DR β chain sharing a common epitope with it, which causes any Ab produced against

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HCMV to bind to the β chain of HLA DR. This mimicry often results in elevated production of cytokines such as IFN-γ along with the increased expression of other adhesion molecules namely intracellular adhesion molecule (ICAM) which intensifies the chances of allograft rejection.[5,6] From the biochemical point of view, there also exists a significant correlation between renal transplant rejection & serum creatinine levels. Elevated levels of serum creatinine are often observed in case of renal transplant rejection. Creatinine serves as an important indicator of renal health, being an essential byproduct chiefly excreted by the functional kidneys by means of glomerular filtration. If GFR (Glomerular filtration rate) or CrCl (creatinine clearance) is deficient, a significant rise in the serum creatinine is observed. The rise in serum creatinine post transplant rejection is primarily due to the marked damage caused to the nephrons. Whereas for the patients having, successful transplantation all the above mentioned haematological complications are unlikely.

## MATERIALS AND METHODS

### Patients
The data was collected randomly for 30 patients undergoing renal transplantation over the year 2012-2015 who were either admitted or attended by Medica Superspeciality Hospital in Kolkata, India.

### Methods Applied in the Case study
The blood samples collected from the patients were subjected to the following lab procedures mentioned below.

### DSA Cross Match
To determine specificity of HLA Ab, dicrete tests were performed using life codes LSA class I/II phycoerythrin conjugated goat Antihuman IgG Ab was used as secondary Ab.[7] The Sample analysis was done on a life match fluoroanalyzer using LUMINEX 1001S v.2.3 as software for data acquisition and quick type for Life match as analysis software. For determination of an individual bead recognition by an anti HLA Ab in the recipient's serum, the MFI or mean fluorescence intensity was considered. A serum sample was deemed positive in the light of the raw MFI value i.e. when the raw MFI value was >3000.

### Lymphocyte Cross Match or Serological Crossmatch (CDC)
Pre transplant lymphocytotoxicity test was performed using CDC (Complement Dependent cytotoxicity) cross match test with added anti human globulin (AHG).[8] Incubation was conducted using 1 µl of donor lymphocyte suspension & 1µl of recipient sera in a 72 well containing terasaki plate at RT for 30mins, followed by anti human globulin addition & incubation for 1-2 mins. 5µl of rabbit complement was added to each well & mixture was kept undisturbed at RT

for 60 mins. 2µl of eosin dye was added to the mixture & was examined under an inverted Phase contrast microscope. The results were considered positive when more than 10% of the donor lymphocytes were killed by the anti HLA Abs present in the recipient serum. DTT (Di thio threitol) was used for inactivation of IgM Ab in the recipient's sera.

### Kidney Biopsy
Patients underwent protocol biopsy post renal transplantation to determine allograft dysfunction & rejection.

### Diagnosis of Rejection
Acute Antibody mediated rejection was diagnosed using the criteria defined by Banff classification.

### Immunohistochemistry
C4D Staining was performed with Envision Flex Mini Kit (DAKO) Denmark & Novo link Min Polymer Detection System (Leica UK) with primary Ab supplied by Cell Mark, USA[9] (Figure 2).

### RBC & Platelet Count Monitoring
Auto hematology analyzer MINDRAY 6800 was used for measuring the Differential count (i.e. RBC & platelet count) of the patients pre & post transplantation.

### Serum Creatinine Level
The serum creatinine levels of the rejected patients were measured by the ortho clinical diagnostics VITROS 250 system making use of VITROS CREA slides.[10,11]

### RT PCR
Real time PCR was done for quantification of viral DNA by the use of Rotor -Gene Q 50512241 instrument & Artus CMV RG PCR kit 4503263. Pathogen Detection by PCR was based on the amplification of specific regions of the pathogen genome.

### Statistical Analysis
All statistical analysis was done using Prism 4.1 software.
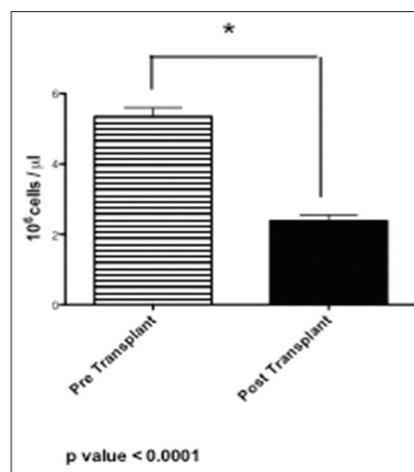
## RESULTS

We had selected a total population of 30 patients undergoing renal transplantation, out of which 24 had successful transplantation as for them there was a significant increase in both RBC & platelet count post transplantation. Whereas for the other 6 patients the story was somewhat different as they all developed thrombocytopenia & erythrocytopenia post renal transplantation. Several tests namely DSA Cross match luminex[7], lymphocyte cross match or serological cross match (CDC), RBC & platelet count monitoring, were performed for all 30 patients. Several additional biochemical tests were performed for the 6 patients developing thrombocytopenia which included

the measurement of serum creatinine levels which was significantly high post transplantation compared to that of pre transplantation indicating some sort of nephropathy, rejection or graft dysfunctioning. Percutaneous needle renal biopsy including C4d staining was performed for the above 6 mentioned patients who were presumed to have allograft rejection owing to drastic increase in serum creatinine levels & significant drop in both RBC & platelet count post transplantation.[9] The presence of C4d marker in the renal peritubular capillaries of the above mentioned 6 patients was a key indicator of acute antibody mediated rejection. For these rejected patients the mean MFI value for both Anti HLA class I &II IgG were found to be much greater than that of the non rejected patients. RT PCR was performed for all the 30 patients. No viral nephropathy such as CMV, BKV, HCV, HBV, was detected in the 24 patients having successful transplantation, where as for the 6 patients with acute antibody mediated rejection Real time PCR quantification of HCMV viral load had shown a mean viral peak of 5632 copies/ml. Table 1 depicts the total no of cases considered i.e. 30, the total no of patients developing thrombocytopenia & erythrocytopenia (A) Being 6, the total no of successful transplants (B) i.e. 24. The no of determinants i.e.(n). The mean value of the platelet & RBC count post transplantation for the 24 patients having successful transplantation was $3.64 \times 10^5/\mu l \pm 0.2$ & $4.79 \times 10^6/\mu l \pm 0.19$ respectively. The mean value of the platelet & RBC count post transplantation for the patients with allograft rejection & nephropathy developing thrombocytopenia & erythrocytopenia was $1.14 \times 10^5/\mu l \pm 0.21$ & $3.38 \times 10^6/\mu l \pm 0.29$ respectively, along with the p value which was found to be <0.0001. Figure 1 depicts the RBC count of the rejected patient's pre & post transplantation which was $5.34 \times 10^6/\mu l \pm 0.29$ & $3.38 \times 10^6/\mu l \pm 0.29$ respectively. Figure 2 depicts the platelet count of the 6 patients including patients having rejection & nephropathy (developing thrombocytopenia & erythrocytopenia), pre & post transplantation which was $2.75 \times 10^5/\mu l \pm 0.21$ & $1.14 \times 10^5/\mu l \pm 0.21$ respectively. Figure 3 shows the mean RBC count of the 24 patients (with successful transplantation), pre & post transplantation which was $3.49 \times 10^6/\mu l \pm 0.19$ & $4.79 \times 10^6/\mu l \pm 0.19$ respectively. Figure 4 depicts the platelet count of these 24 patients pre & post



**Figure 1:** Depicting the significant decrease in RBC count post transplantation in the patients having thrombocytopenia & erythrocytopenia. Bar 1 & 2 corresponds to the RBC count pre & post transplantation respectively
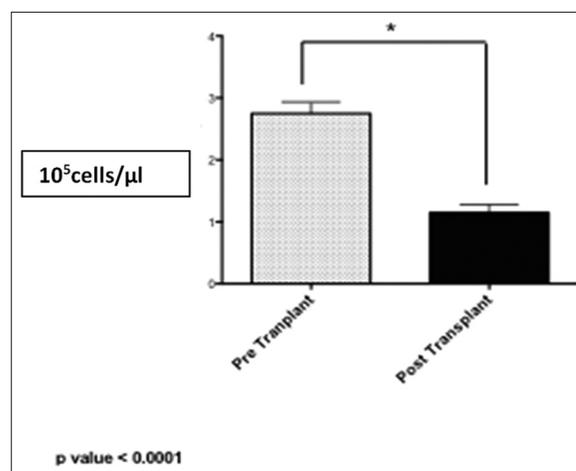


**Figure 2:** Depicting the significant reduction in platelet count in the patients developing thrombocytopenia post transplantation. Bar 1 & 2 corresponds to the pre & post transplantation platelet count respectively
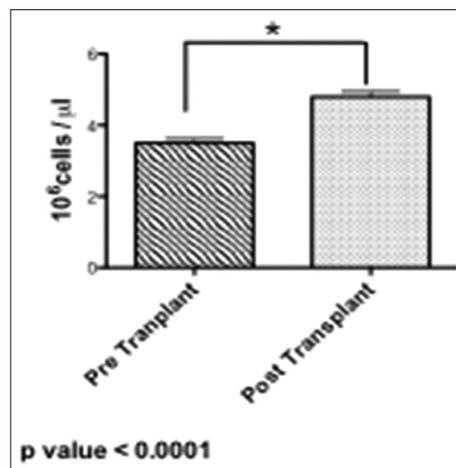


**Figure 3:** Depicting the significant increase in RBC count post transplantation incase of the patients having successful transplantation. Bar 1 & 2 corresponds to the pre & post transplantation RBC count respectively
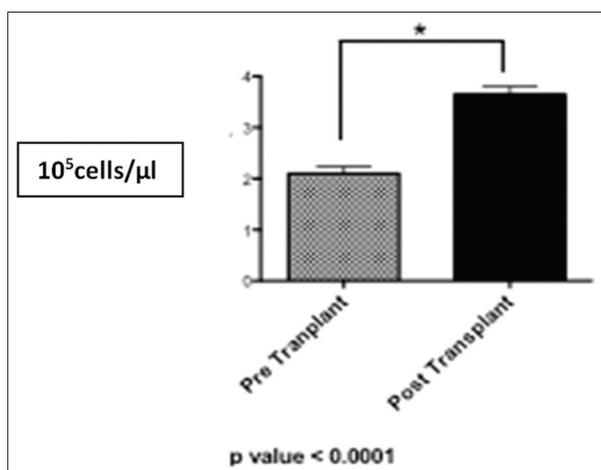
**Table 1: Depicting platelet and RBC count in both rejection and non rejection episodes**

| No of cases n=30 | Platelet count ($\times 10^5/\mu l$) post transplantation | RBC count ($\times 10^{\wedge 6}/\mu l$) post transplantation |
|---|---|---|
| A, n | 6 | 6 |
| B, n | 24 | 24 |
| Mean A | 1.14 | 3.38 |
| Mean B | 3.64 | 4.79 |
| t value | 7.5±0.2 | 6.77±0.24 |
| P value | <0.0001 | <0.0001 |

A: Rejection related data,  B: Non rejection related data, n: No of determinants

transplantation which was 2.14 ×10⁵/μl±0.2 &3.64× 10⁵/μl± 0.2. Figure 5 depicts the serum creatinine levels of the



**Figure 4:** Depicting the significant increase in platelet count post transplantation in the patients having successful transplantation. Bar 1 & 2 corresponds to the pre & post transplantation platelet count respectively
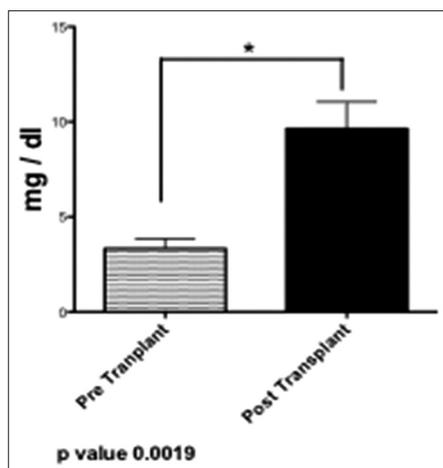


**Figure 5:** Depicting the significant increase in the serum creatinine level post transplantation in the 6 patients with thrombocytopenia. Bar 1 & 2 corresponds to the serum creatinine level pre & post transplantation respectively
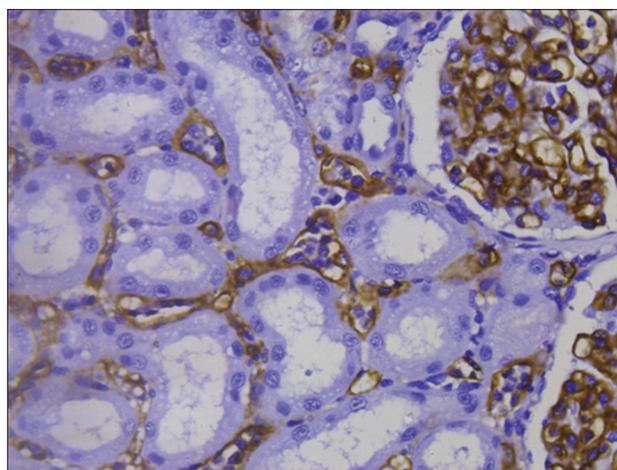


**Figure 6:** Photograph showing Post Transplant C4d deposition in CDC cross match negative DSA cross match positive cases with donor specificity

6 patients having rejection pre & post transplantation which was 5.33mg/dl±0.92 & 8.18mg/dl±0.92 with an increase of 2.85mg/dl. The mean MFI value of the patients with successful transplantation was found to be 245.1±35.42 & 292.3±32.9 for Anti HLA class I & II IgG respectively. The mean MFI value of the 6 patients having rejection was found to be 9806± 3107 & 7349± 2666 for Anti HLA class I & II IgG. The serological CDC crossmatch showed clear negative results for all the 30 patients. The photograph of C4d staining given below shows the deposition of C4d marker in the renal peritubular capillaries of the rejected patients depicting acute antibody mediated rejection.

## DISCUSSION

The above recorded data clearly suggests that acute antibody mediated rejection & immune-hematological events can commence simultaneously. Even mild immune-hematological events can herald full blown symptoms of renal rejection & this observation is worth this report. Among the 30 patients that we selected with mean age of 46 years ± 12 who had undergone renal transplantation, 6 patients were found to develop thrombocytopenia & erythrocytopenia post transplantation i.e. significant reduction in both platelet & RBC count post transplantation. Where as significant increase in both RBC & platelets were observed in the other 24 patients having successful transplantation. The Luminex based donor specific Ab cross match for all the 24 patients having successful transplantation were found to have clear negative results pre and post transplant with a mean MFI value <250 post transplantation, so the chances of donor specific Ab mediated immune activation was abrogated, where as for the other 6 patients Luminex based donor specific Ab cross match was found to be positive post transplant with a mean MFI value >9000.[7] Real time PCR was also done for all the 30 patients & only the 6 patients having rejection were found to develop HCMV nephropathy. In case of the non rejected patients or patients having successful transplantation their RBC & platelet count increased post transplantation which can be attributed to implantation of the new functional transplant (kidney) of the donor, which meant that normal erythropoietin formation had taken place which led to the increase in RBC Count.[12] From the clinical biochemistry point of view, the serum creatinine levels also increased post transplantation for the 6 patients by a mean value of 2.85mg/dl. Their Glomerular filtration Rate (ml/min/1.73m²) was also calculated by using the formula $175 \times (S_{Cr})^{-1.154} \times (Age)^{-0.2}$. The mean GFR Post transplantation was found to be 14.5ml/min/1.73m²[2] compared to 21.8ml/min/1.73m² in case of pre transplantation indicating graft dysfunctioning. HCMV can trigger several immunological cascades thereby provoking allograft rejection, HCMV infections can often lead to thrombocytopenia as recent findings suggests[1,2].

But the exact mechanism leading to such haematological complications or its interrelation with HCMV infection couldn't be ascertained which itself constitutes a limitation in our study. Whereas erythrocytopenia observed in case of the rejection episodes can be attributed to the interference with the normal process of erythropoeisis in general, owing to allograft rejection as kidney is known to play an important role in the production of normal erythropoietin.

## CONCLUSION

From the above clinical study conducted, we can conclude that there exists a significant correlation of haematological events such as erythrocytopenia & thrombocytopenia with acute antibody mediated renal transplant rejection & HCMV infection. Post transplant HCMV infection can trigger all graft rejection by exhibiting ''molecular mimicry''. Whereas for the patients having successful transplants occurrence of such events are never the less unlikely.

## ACKNOWLEDGEMENT

### Conflicts of Interest
The authors thereby declare they have no conflict of interest.

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