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# C-REACTIVE PROTEIN – PLAUSIBLE MARKER FOR PREECLAMPSIA

Gynecology

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## ABSTRACT

**Introduction:** Preeclampsia, a hypertensive disorder is known to complicate pregnancies accounting for maternal and fetal mortality. This condition is known to alter the serum level of C-reactive proteins (CRP) in preeclamptic patients. Hence the present study was conducted to assess if the alteration in the levels of CRP could be used as a marker for predicting preeclampsia and to check for their levels after the administration of L-Arginine.

**Material and methods:** This study recruited 186 Primigravidae women of age group 18 to 35 years. Doppler ultrasound was performed to categorize the participants into 4 groups. The recruited participants were subjected to blood tests to analyze the level of CRP. Further evaluation of their levels in the serum in subjects with abnormal doppler was performed after the administration of L-Arginine.

**Results:** The serum level of C-reactive protein was assessed in all the four categorized groups. Mean CRP values were higher in subjects with abnormal doppler i.e. Group 2 (2.6) and Group 3a (2.9) in comparison to subjects with normal doppler (Group 1) (2.1) which was statistically significant at $p<0.05$. The increased mean CRP levels reduced to (1.4) in Group 3b i.e. after supplementation with L-Arginine.

**Conclusion:** Altered level, i.e. increased levels of C-Reactive Protein could be used as a marker for preeclampsia.

## KEYWORDS

Preeclampsia; L-arginine; Doppler Ultrasound; C-reactive Protein; Nitric Oxide

## INTRODUCTION

Pre-eclampsia, a hypertensive disorder is known to complicate 3–8% of the pregnancies in Westernized countries. It accounts for about 10 – 15% of the overall maternal deaths[1]. It may be a lethal condition both for the mother and child increasing fetal and maternal mortality [2]. The maternal syndrome is characterized by hypertension, proteinuria and in its most severe form by thrombocytopenia, disseminated intravascular coagulation and hepatocellular damage. Many of these features are also found in systemic inflammatory response syndrome, suggesting an excessive maternal inflammatory response to pregnancy[3].

It is defined as "a new onset of elevated blood pressure of 140/90 mm Hg or more, recorded on two separate occasions at least 6 hours apart and in the presence of at least 0.3g of protein in a 24 hour urine, occurring after the 20th week of gestation in a previously normotensive patient, and resolving completely between 6-12 weeks after delivery" [4]. The pathologic process of preeclampsia remains incompletely elucidated, though factors implicated in the process have been identified which includes abnormal placentation, Oxidative stress, altered angiogenic factors, and Inflammation [5].

C-reactive protein (CRP) was identified way back in the 1930s as a plasma protein associated with one of the implicated process of preeclampsia i.e. acute inflammatory responses[6]. It is produced by the hepatocytes in the liver [7]. During a normal pregnancy, increased innate immune cell activation is seen in the periphery. In contrast, pregnancies affected by preeclampsia are associated with improper immune responses [8]. Inflammatory cytokines such as interleukin-6 and tumor necrosis factor-α are elevated in preeclampsia [9]. These cytokines stimulate the production of C - reactive protein[10], which is a highly objective and sensitive index of overall inflammatory activity in the body[11].

It is a well known concept that dysfunction of the L-Arginine–Nitric oxide system occurs in Preeclampsia [12]. The inflammatory marker C-reactive protein is associated with endothelial dysfunction, possibly due to a reduced bioavailability of Nitric oxide (NO)[13].

Hence the present study was conducted to analyze the levels of CRP in subjects with preeclampsia and to check for any change in the levels of CRP after the administration of L-Arginine. The mail aim of the present study was to analyze the level of CRP in subjects with normal and abnormal doppler and to analyze their levels after the administration with L-Arginine.

## METHODOLOGY

Doppler ultrasound of all enrolled participants was done to assess their proneness to preeclampsia following which the blood levels were analyzed to assess their serum CRP concentration. Doppler ultrasound has been used to assess the uteroplacental circulation and several studies have reported the high impedance in the uterine arteries, prior to the development of any clinical features of pre-eclampsia[3]. Over the last 5–10 years, interest in uterine artery Doppler assessment in predicting pre-eclampsia has shifted from the second trimester of pregnancy to the first trimester. There is now overwhelming evidence to show that abnormal uterine artery doppler in the first trimester is associated with abnormalities in trophoblastic invasions[14].

### Ethical Approval

Ethics committee approval was obtained from the Ethical Committee of Osmania Medical College as the study involved administering of a drug. Written informed consent was taken from the enrolled participants in their own language prior to the enrollment of cases.

### Study design

An interventional cohort study among pregnant women was conducted at the Institute of Obstetrics and Gynecology, Osmania Medical College, Government Maternity Hospital, Hyderabad and some referrals from the local practitioners in the old City of Hyderabad from August 2008 to December 2012. The participants included in the study were, Primigravidae women aged between 18-35 years. Participants were explained regarding the study protocol and written consent was obtained. All the participants were grouped into three categories based on the results of the doppler ultrasound i.e. Group 1 (Normal Doppler), Group 2 (Abnormal Doppler with no L-Arginine supplementation), Group 3 (Abnormal Doppler with supplementation of L - Arginine at around the 17th week of pregnancy). All the untreated group 2 patients placenta was sent for histopathological examination except 20 cases who were having hypertension.

All women in the Group 3 were given L-Arginine sachets of 3 gms/twice a day until delivery. All the cases received their regular supplementation of Iron, Calcium and Folic acid.

### Doppler Ultrasound Test

All the patients underwent the Doppler ultrasound of the uterine arteries. The equipment used was a GE Voluson 730 Expert Doppler unit having a pulsed wave, continuous wave and HPRF Doppler with

dual convex sector transducer. Doppler as a predictor for the development of preeclampsia was as high as 86.8%[15]. Doppler Ultrasound of the Uterine Arteries was analyzed in all women at the 1st Trimester of pregnancy (11-14 weeks) and between 17-22 weeks of pregnancy.

### C-Reactive Protein Estimation

A human highly Sensitive CRP ELISA Kit SK00080-02 was used for the estimation of CRP which contained recombinant CRP and antibodies raised against this protein.

### Sample Collection and Storage

**Serum:** A serum separator tube was used to collect the sample, which was allowed to clot for 30 minutes before centrifugation. The process of centrifugation was done for 15 minutes at 1000 x g and was stored at ≤ -20° C.

**Plasma:** Plasma samples were collected using EDTA as an anticoagulant and centrifuged for 15 minutes at 1000 x g within 30 minutes of collection.

### Sample Preparation

Collected serum and plasma samples needed a 10,000~20,000-fold dilution. For a 100-fold dilution 10 uL sample was added to 990 uL 1x Dilution Buffer. For a further 10,000-fold dilution, 10 µL of 100- fold diluted sample was added to 990 µL 1x Dilution Buffer. The final 20,000-fold dilution was obtained by adding 150 µL of 10,000-fold diluted sample to 150 µL 1x Dilution Buffer.

### Assay Procedure

All the reagents and working standards were prepared as per the manual instructions given by the ELISA kit[16]. 100 uL of prepared dilution buffer was added to each blank wells following which 100 uL of standard dilutions was added in reverse order of serial dilution from #8 to #1 per well and the plate was covered with a plate sealer and incubated at room temperature for 2 hrs on a microplate shaker. Each well was then aspirated and washed; the process was repeated thrice with 1x Wash Buffer (300 L). Whole plate was decanted after last wash to remove the traces of wash buffer. The plate was then inverted and blotted against clean paper towels following which 100 uL of detection antibody was added to each well and incubated for 2 hrs. The process of aspiration and wash was done as previously mentioned using the same guidelines after which streptavidin-HRP conjugate of 100 uL was added to each well and the process of incubation and aspiration was performed. After the process of aspiration and drying, 100 uL of substrate solution was added to each well and incubated. To determine any color change 100 uL of stop solution was added to each well. The optical density of each well was then determined using a microplate reader set at 450 nm.

### Calculation of Results

A standard curve was created by plotting the log of the known concentrations of the standard dilutions (X- axis) versus the log of its corresponding optical density (Y-axis) (Figure 1).

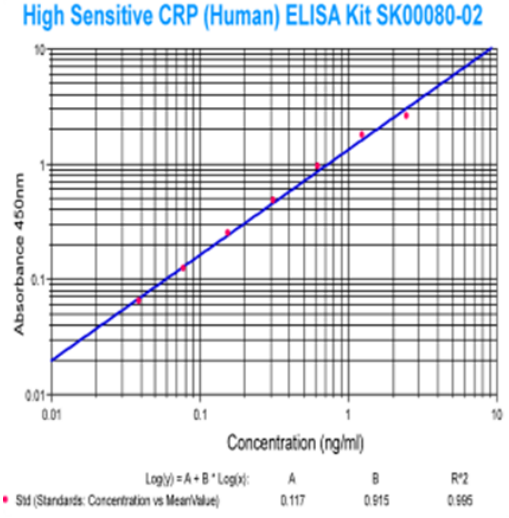



**FIGURE 1:** Standard graph for calculating CRP by ELISA kit.

### Linearity

To assess the linearity, the human serum and EDTA plasma samples were diluted with Dilution Buffer DB01 and the recovery of CRP in human serum samples were assayed by Human High Sensitive CRP ELISA Kit SK00080-02 (Figure 2). CRP estimation was fallowed as per the instructions and giudelines supplied along with the ELISA kit[16].

| SAMPLE | DILUTION FACTOR | ASSAYED (PG/ML) | FINAL (NG/ML) | RECOVERY (%) |
|---|---|---|---|---|
| HUMAN SERUM | 500 X | 920.312 | 4601 | 100 |
| HUMAN SERUM | 10000 X | 449.439 | 4494 | 97.7 |
| HUMAN SERUM | 20000 X | 214.250 | 4285 | 93.2 |
| HUMAN PLASMA | 5000 X | 268.125 | 1340 | 100 |
| HUMAN PLASMA | 10000 X | 130.251 | 1302 | 97.2 |
| HUMAN PLASMA | 20000 X | 66.194 | 1324 | 98.8 |

**FIGURE 2:** Linearity assessment of plasma and serum by ELISA kit.

The blood levels were repeated in Group 3 after the 32nd week of gestation to form a Group 3b in order to see whether there was any statistically significant improvement in the serum CRP levels.

In addition to the tests all cases had a routine obstetric profile which included complete blood picture, urine examination, blood sugar, thyroid profile, HIV and HBSAg. In cases with Preeclampsia, platelet count, renal and liver function tests with clotting profile were obtained. Women with severe preeclampsia were hospitalized and closely monitored.

### STATISTICALANALYSIS

Descriptive statistics were calculated for all variables. Mean values across the groups were compared using ANOVA followed by post hoc test. Pre and post values were compared by paired "t"-test. Further to study the associations with categorical variables, chi-square test was done. Level of significance was set as 0.05. SPSS version 19.0 was used for statistical analysis.

### RESULTS

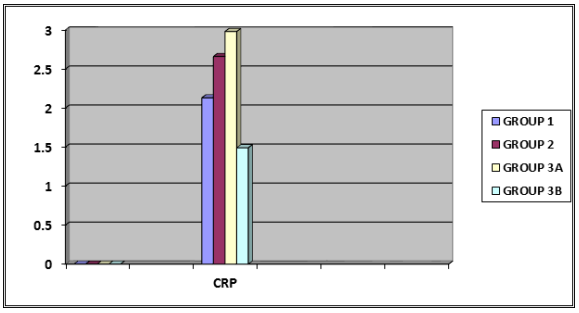
A total of 186 Primigravidae were recruited after scanning approximately 3800 women. All cases were monitored clinically throughout their pregnancy every 4 weeks up to 28th week and once in 2 weeks until the 36th week and weekly thereafter.

The C-Reactive Protein values in the serum were analyzed and there was a statistically significant abnormal level in those cases that developed Preeclampsia (i.e. those with abnormal doppler). The histopathological examination revealed that the presence of calcification the untreated group.

**TABLE 1:** Mean CRP values in various groups (Group 1, 2, 3a, 3b).

| | NUMBER | MEAN | Std deviation | Std error | P value |
|---|---|---|---|---|---|
| **CRP GROUP** | | | | | |
| **1** | 49 | 2.1 | 2.9 | 0.4 | < 0.05 |
| **2** | 50 | 2.6 | 0.7 | 0.1 | " |
| **3a** | 84 | 2.9 | 4.4 | 0.4 | " |
| **3b** | 60 | 1.4 | 0.7 | 0.09 | " |

Mean CRP values were higher in subjects with abnormal doppler i.e. Group 2 (2.6) and Group 3a (2.9) in comparison to subjects with normal doppler (Group 1) (2.1) which was statistically significant at p<0.05 as shown in table1. The increased mean CRP levels reduced to (1.4) in Group 3b i.e. after supplementation with L-Arginine.




**GRAPH 1:** Serum levels of CRP in the categorized groups before and after supplementation with L-Arginine.

The levels of serum CRP showed a mean of 2.13 ng/l in Group 1 (Graph 1) but elevated levels in Group 2 (2.66 ng/l) & Group 3a (2.98 ng/l) and a fall in Group 3b women (1.49) i.e. in subjects with L-Arginine supplementation which was statistically significant at p value <0.001.

**TABLE 2:** Levels of Serum CRP Before and After Supplementation

| | Group 1 Normal Doppler | Group 2 Abnormal Doppler | Group 3 (Abnormal Doppler before supplementation) | Group 4 (After supplementation with Arginine) |
|---|---|---|---|---|
| CRP (Normal level<3 ng/l ) | 91.8% (<3 ng/l) | 58% (<3 ng/l) | 64.3% (<3 ng/l) | 91.7% (<3 ng/l) |

Normal Levels of serum CRP were seen in 91.8% of the subjects with normal doppler (Table 2). In contrast, a lesser percentage of subjects i.e. 58% of them in the group 2 and 64.3% in group 3 showed the normal range of values. After administration with L-Arginine it is seen that 91.7% of subjects reverted back to their normal range of values which was statistically significant ($p<0.01$).

## DISCUSSION

The present study assessed the level of serum CRP in about 186 pregnant women with normal and abnormal doppler. Further evaluation of the serum level of these CRP was done after the administration of L-Arginine in patients with abnormal doppler.

KK Mandal et al[17] conducted a study to assess the serum uric acid and C-reactive protein levels in 52 preeclamptic and normal pregnant women in the third trimester of pregnancy. A higher mean of 8.14 was seen in preeclamptic women in comparison to subjects with normal pregnancy (6.28). This was in accordance with our study where a higher mean CRP were found in subjects with abnormal doppler i.e. group 2 (2.6) and group 3a (2.9) in comparison to subjects with normal doppler group 1 (2.1).

MA Samakoosh et al[18] conducted a case control study to compare C-reactive protein (CRP) level and fibrinogen concentration obtained from pregnant women with pre-eclampsia by comparing with normal pregnancies. Maternal serum CRP was higher in pregnant women with pre-eclampsia when compared with their respective normals. The mean value for maternal serum of women with preeclampsia was 4.4 ± 5 g/dl and of those with non preeclampsia was 2.2 ± 0.8 g/dl. Statistically significant differences between maternal serum CRP and pre-eclampsia have found ($p < 0.001$). This was in agreement with our study where a higher mean was found in groups prone to preeclampsia than the subjects with normal doppler.

Previous literature by H Ayatollahi et al[19] and AJ Onuegbu et al[20] also reported significant difference in the means serum CRP between normal pregnant women and preeclamptic women ($P<0.05$) which was in line with the present study.

In the present study serum CRP levels were higher in group 2 (2.66 ng/l) and group 3a (2.98 ng/l) i.e. in subjects with abnormal doppler than in subjects with normal doppler (group 1) (2.13 ng/l). A study by D. Mihu, et al [21] showed similar results where serum CRP concentration was significantly higher ($p< 0.001$) in preeclampsia patients when compared to their respective normal pregnant women.

Anothr study conducted by Kumuru S et al [22] also stated similar results of elevated serum CRP in preeclamptic women compared to normal pregnancy.

The significantly higher serum CRP in subjects with abnormal doppler indicate that it might be associated with preeclampsia. CRP being a sensitive marker of tissue damage and inflammation played a significant role in eliciting the inflammatory response characteristics of preeclampsia [23]. Zimmerman et al [24] and Mantovani and Dejana [25] demonstrated a relationship between endothelial dysfunction and inflammation, and it was further revealed that markers of endothelial activation or inflammation have an active role in pre-eclampsia[26].

This study went further analysis of the serum C - reactive protein estimation after the administration of L-Arginine a nitric oxide donor and revealed that normal levels of serum CRP (<3 ng/l) was seen in 91.8% of the subjects with normal doppler (Group 1). In contrast, a lesser percentage of subjects i.e. 58% of them in the group 2 and 64.3% in group 3 showed the normal range of values. After administration with L-Arginine it was seen that 91.7% of subjects reverted back to their normal range of values which was statistically significant ($p<0.01$). None of the previous cases compared their CRP data after administering the L-Arginine in subjects predisposing to preeclampsia (Abnormal doppler). Therefore, to the best of our knowledge this is the first study took part in advancement of serum CRP estimation after giving the drug. Also managed to control the disease condition of preeclamsia in pregnant women.

In preeclampsia there is an increased uteroplacental circulation resistance related with the alterations in the L-Arginine-NO-cyclic GMP pathway[27]. Endothelial dysfunction could be related with the presence of raised concentrations of CRP that alters the production and degradation of Nitric oxide[28].

This study proved that maternal inflammatory response, as measured using C-reactive protein is different for subjects with normal and abnormal doppler. These elevated levels of CRP occurring as a result of dysfunction of endothelial cells [29] could be used as risk marker for predicting preeclampsia.

## CONCLUSION

In conclusion it is observed that subjects with normal doppler had serum CRP levels in the statistically normal range. Participants with abnormal uterine artery doppler showed an elevated CRP. Hence this altered i.e. increased level of CRP could be used as a plausible marker to predict preeclampsia in pregnant women. Additionally, L-Arginine supplementation in those with doppler abnormality showed up a reversal of the values back to normal. This study gives a further scope to research where L-Arginine supplements could be used to improve endothelial function resulting from altered production of nitric oxide.

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