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ORIGINAL ARTICLE

Flowcytometric Expression of CD11b and Detection of Netosis Markers (Calprotectin, Protease 57 and PADI4) in Women with RPL at Basrah Province

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ABSTRACT

Background: *pregnancy is defined as a special immunological condition when the mother's immune system protects the fetus against infection.* ***Objective:*** *the current study is designed to evaluate the role and expression of NETosis markers (Calprotectin, Protease 57 and PADI4) by ELISA and CD11b by Flowcytometry in women with Recurrent Pregnancy Loss (RPL) at Basrah Province.* ***Methodology:*** *this prospective cohort study was conducted from January 2022 to July 2023, included 75 participants (50 RPL patients and 25 controls). Blood samples were analyzed using ELISA to measure the expression of Protease 57, Calprotectin, and Peptidyl Arginine Deiminase Type IV (PADI4), also the surface expression of CD11b on activated neutrophils was assessed via flow cytometry.* ***Results:*** *calprotectin demonstrated a significant increase, with mean values of 5.43 in RPL patients versus 4.66 in controls (p=0.021). Protease 57 exhibited significantly elevated with mean levels in the RPL group (3.28) compared with controls (1.15, p=0.0001). CD11b expression, assessed via flow cytometry was revealed significant upregulation in RPL patients. The mean fluorescence intensity of CD11b was 17.55, substantially higher than the 11.43 observed in controls (p=0.0001).* ***Conclusion:*** *the findings of this study provide significant insights into the intricate relationship between recurrent miscarriages and the immune system, specifically through the lens of neutrophil extracellular traps (NETs) and the activation marker CD11b.And further elucidates the underlying immune mechanisms. Notably, the investigation into NETosis markers, alongside the assessment of PADI4, calprotectin, and Protease 57 levels, illuminates the complex interplay of inflammatory responses and neutrophil activation in the context of miscarriage.*

INTRODUCTION

The term miscarriage (Latin: abortus spontaneous) refers to the spontaneous ejection of the fertilized ovum, either whole or partially, into the uterus before the embryo is ready to survive on its own[1]. In fact, there are several ways by which the induction of local maternal tolerance of foreign fetal tissue is necessary for a successful pregnancy[2]. In general, miscarriages can be classified into two types as either mother-dependent or fertilized ovum-dependent. About 5–10% of all pregnancies end in miscarriage due to immune system-related pregnancy problems, thus medical professionals and academics are particularly interested in understanding the processes behind miscarriage[3]. In the general population, for instance, estimates of RPL's prevalence vary. The reason for this is frequently inconsistent choices about which populations belong in the denominator and numerator. Women who have had three or more miscarriages may be included in the numerator [4]. The highly inflammatory features of the multifunctional cytokine in reproductive biology may have an effect on the processes of fertilization and implantation[5]. There may be common underlying risk factors for other unfavorable pregnancy outcomes such as miscarriage. Numerous researches have investigated the relationship between a woman's past miscarriages and her potential risk of developing other pregnancy difficulties[6] .

Netosis is a kind of controlled cell death that depends on the development of neutrophil extracellular traps (NET), where polymorphonuclear (PMN) granulocytes create net-like structures of decondensed chromatin and proteases. These structures contain antibacterial compounds that restrict and limit infections, so halting the spread of infections[7]. NET formation the physiological response of neutrophils has been identified as a distinct mechanism of cellular death from necrosis and apoptosis[8] . NETosis, like necrosis, is a kind of cell death in which the integrity of the membrane is lost. Peptidyl arginine deiminase 4 (PAD4) is assumed to be the initiator of chromatin recondensation during NETosis[9]. The term "NETosis"

should only be applied in relation to cell death and not just in cases when NET creation is evident[10].

Additionally, neutrophil extracellular traps (NETs) are thought to have a significant role in both fertility and recurrent pregnancy loss at various stages of the reproductive cycle[11]. When neutrophils are infected with pathogens they become activated. Apart from the well-known processes of phagocytosis and degranulation, these cells may also produce neutrophil extracellular traps (NETs) in a multi-step process known as NETosis[12] .

The biological function of NETs in miscarriage has not been the subject of any research done to date. Studies involving a cohort of women with preeclampsia and recurrent miscarriages have shown that neutrophil activation and recruitment are triggered by bacterial infections caused by either *Brucella abortis* or *Listeria monocytogenes*. It has been established that women's listeriosis and the frequency of miscarriages are positively correlated[13]. According to other research, women with preeclampsia had significantly more NETs in the interstitial space of their placentas than women with normal gestations [11]. Placental inflammation has a significant correlation with premature birth, miscarriage, preeclampsia, and fetal development restrictions[14].

During pregnancy, the placenta has the ability to emit inflammatory cytokines and debris that activate neutrophils to create neural epithelial cells, or NETs. This can cause an obstruction of the intervillous space and encourage placental hypoxia, which can result in miscarriage[15]. There is mounting evidence that suggests NETS may play a role in thrombosis promotion in addition to their role as a host defense mechanism and cancer metastasis[16].

Numerous variables, including chromosomal abnormalities, infections, hormone imbalances, anatomical malformations, environmental causes, and immunological factors, contribute to the complex and multifaceted etiology of recurrent miscarriage. It is believed that 50% of the causes of miscarriage are due to the rejection of the fetus by the maternal immune system[17]. Approximately 50% of recurrent miscarriage cases are still unexplained and are experimentally treated with progesterone supplements, anticoagulants, or immunomodulatory techniques[18]. So the present study aims to evaluate the role of Netosis components (Protease 57, Calprotectin) in recurrent pregnancy loss in addition the role of PAD14, CD11b in activation of neutrophil for netosis.

METHODOLOGY

Study populations

This study had been conducted from January 2022 to July 2023. Blood samples were collected from women who were attending the Al- Basrah maternity and children hospital, with ages ranging from 18 to 41 years. The first age group (G1) was (18-29) years whereas the second age group (G2) ranged (30-41) years old. Prior to collection of samples, the participants were given a brief explanation about the experiment. Prior to their participation, all individuals provided written, informed consent. Historical medical records of the study population and some required data such as age.

Samples Collection

By using vein punctures, five µl (ml) of blood were collected from both normal pregnant and aborted women during the first trimester of pregnancy. Each sample was divided into 2 ml of blood and taken into an ethylene diamine tetra acetic acid (EDTA) tube to measure the CD11b with a kit Catalog No. E-AB-F1146C and CD45 with a kit Catalog No. E-AB-F1039J from Elabscience Biotechnology Inc.'s USA by using flow cytometry. 3 ml of blood were collected into a GEL tube to measure the levels of human protease 57 with a kit Catalog No. RDEEH4531, human PADI4 (PedtidyI Arginine Deiminase Type IV) with a kit Catalog No. RDEEH3496, and human calprotectin with a kit Catalog No. RDEEH4140 from My BioSource USA by using sandwich ELISA. The serum was separated and centrifuged for three minutes at 1500 rpm.

Statistical Analysis

The data were statistically analyzed using SPSS software and the significance of the observed differences, associations, or calculations were determined at p-value <0.05. Chi^2 and Fisher's Exact test was used to investigate the significance of associations. Mann-Whitney tests were used for differences between the groups of non-parametric data, and Spearman's test to examine nonparametric correlations. The Person test was used to examine the parametric correlations.

RESULTS

Table (1) and Fig. (1) showed that there was a highly significant statistical difference (p=0.0001) between the CD11b expression in patients (11.43) and its value (17.55) in control.

Table 1: Flow Flowcytometric expression of CD11b in patients and control

Parameter	Category	N	Mean	SD	*P*-value
CD11b	Patient	50	17.5528	6.28744	0.0001
	Control	25	11.4308	6.11407	

A statistically significant difference (*p*-value of 0.04) was found between patient and control groups regarding the CD11b levels in (18-29) age range group (Table 2). The patient group showed the highest level (16.089) as compared with the control group (10.655).

Table 2: Flow Flowcytometric expression of CD11b in patients and control, age range (18-29) group

Parameter	Group	N	Mean	SD	*P*-value
CD11b	Patient	23	16.089	6.44922	0.04
	Control	13	10.655	6.65107	

There was a statistically significant difference between the patients and control groups in CD11b levels in (30-41) age range group, as indicated in (Table 3), and highlighted by *p*-value 0.003. According to the findings, CD11b level was higher for patient group by recording 18.798 while it was lowest for control group (12.27).

Table 3: Flowcytometric expression of CD11b in patients and control of (30-41) age range group

Parameter	Group	N	Mean	SD	*P*-value
CD11b	Patient	27	18.798	5.98324	0.003
	Control	12	12.27	5.64082	

According to (Table 4), the levels of CD11b were significantly higher (20.508) (*p*-value = 0.012) in patients with (4-5) number of abortions in comparison with patients having (2-3) number of abortions (16.285).

Table 4: Expression levels of CD11b in patients with (2-3) and (4-5) abortions

Parameter	No. of abortion	N	Mean	SD	*P*-value
CDIIb	2-3	35	16.285	6.01420	0.012
	4-5	15	20.508	6.09420	

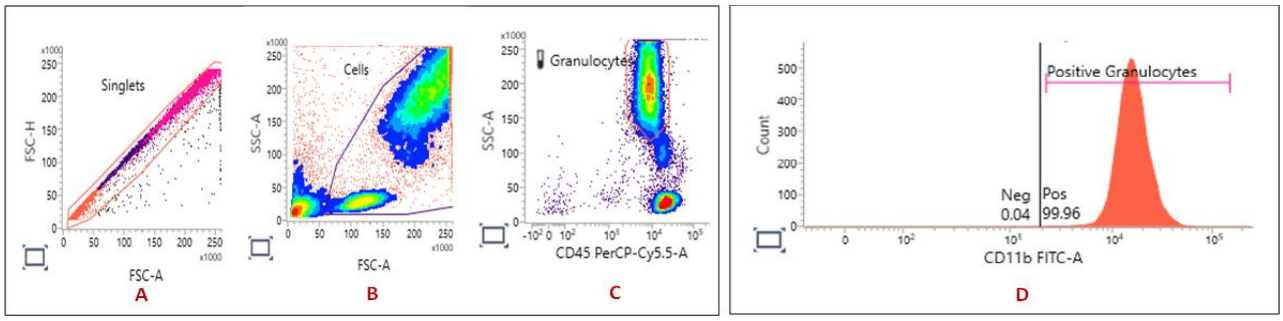

Fig. 1: Neutrophil cells gating by flow cytometry. A- granulocyte determination B- total neutrophil cells determination C- determination of CD45 expression levels. D- determination of CD11b expression levels

Table (5) shows that there were significant statistical differences in the NETosis markers, for PADI4, between patients and control groups. Calprotectin and Proteases 57 mean ranks were significantly higher (*p*=0.021, *p*=0.008, and *p*=0.0001 respectively) in patients compared to controls. Also, PAD14 didn't show significant differences between the two studied groups.

Table 5: Differences in expression levels of PADI4 and netosis markers levels among patients and control

Category		PADI 4	Calprotectin	Protes 57
Patients	N	50	50	50
	Mean	2.4638	5.4276	3.2782
	Median	1.7950	5.5550	2.0550
	SD	2.12250	1.36551	3.17664
	Minimum	0.30	2.75	.40
	Maximum	9.66	8.73	12.87
	Mean Rank	39.62	42.10	45.25
Control	N	25	25	25
	Mean	2.3064	4.6600	1.1532
	Median	1.5800	4.1900	.7200
	SD	2.27811	2.68484	1.29350
	Minimum	0.27	1.16	.31
	Maximum	8.28	11.98	5.30
	Mean Rank	34.76	29.80	23.50
***P*-value**		0.363	0.021	0.0001

The results of the current study at (18-29) age range group revealed that there is no statistically significant difference between the patients and control group in terms of the PADI4 level. While the results of the statistical analysis revealed that, there were statistically significant differences (*p*-value 0.494) in the levels of the NETosis indicators Calprotectin and Protes57 between the patients and control group, with the highest levels recorded in the patient group, (Table 6).

Table 6: Levels of PADI4, Calprotectin and Proteases 57 in patient and controls, (18-29) age range group

Group 18-29		PADI4	Calprotectin	Protes57
Patients	Mean	2.007	5.539	1.528
	N	23	23	23
	SD	1.466	1.316	1.463
	Minimum	0.295	2.748	0.397
	Maximum	6.512	8.044	7.366
	Median	1.7100	5.6700	1.0700
	Mean Rank	19.43	21.17	21.41
Control	Mean	2.395	4.426	1.035
	N	13	13	13
	SD	2.804	2.155	1.322
	Minimum	0.270	1.163	0.313
	Maximum	8.275	9.463	5.305
	Median	1.0000	4.2200	0.5900
	Mean Rank	16.85	13.77	13.35
***p*-value**		0.494	0.043	0.026

Table (7) shows the results of the statistical analysis of the indicators selected in this study. The results showed that there were no statistically significant differences between the patients and control group of (30-41) age range group in terms of PADI4 and Calprotectin levels, as shown in the Table. While recent data were observed between the patients and control group Calprotectin and Protes57 levels (*p*-value 0.049 and 0.001 respectively), with the highest levels recorded within the patients group.

Table 7: Levels of PADI4, Calprotectin and Proteases 57 in patients and control, (30-41) age range group

Group 30-41		PADI4	Calprotectin	Proteases 57
Patients	Mean	2.854	5.331	4.768
	N	27	27	27
	SD	2.516	1.424	3.490
	Minimum	0.521	3.184	0.578
	Maximum	9.658	8.726	12.870
	Median	2.2100	5.4400	3.6700
	Mean Rank	20.59	21.593	24.35
Control	Mean	2.208	4.913	1.281
	N	12	12	12
	SD	1.650	3.246	1.308
	Minimum	0.279	1.420	0.515
	Maximum	6.252	11.982	4.726
	Median	1.8400	4.0100	.7500
	Mean Rank	18.67	16.417	10.21
***p*-value**		0.642	0.199	0.001

The current data revealed that there were no statistical differences between the subgroups of the number of abortions (2-3 and 4-5) in their effect on the levels of the PADI4 and Calprotectin indicators (*p*-value 0.505 and 0.882 respectively) (Table 8).

The results indicated that there were significant differences (p=0.024 and p=0.010) in the levels of Proteases 57 in patients who have a history of (4-5) abortions.

Table 8: Levels of PADI4, Calprotectin and Proteases 57 in patients, according number abortion in in (2-3) and, (4-5) number of abortions group

Number of abortions		PADI4	Calprotectin	Proteases 57
2-3	Mean	2.401	5.396	2.897
	N	35	35	35
	SD	2.147	1.302	3.425
	Minimum	0.295	2.748	0.397
	Maximum	9.658	8.044	12.870
	Median	1.7800	5.6200	1.1700
	Mean Rank	24.60	25.70	22.03
4-5	Mean	2.611	5.499	4.167
	N	15	15	15
	SD	2.132	1.549	2.369
	Minimum	0.521	3.435	1.852
	Maximum	8.981	8.726	8.945
	Median	2.2800	5.4400	2.9400
	Mean Rank	27.60	25.03	33.60
	p-value	0.505	0.882	0.010

DISCUSSION

The relationship between recurrent abortions and the immune system has increasingly drawn more attention in clinical practice. The presence of neutrophil extracellular traps (NETs) within placental tissue, coupled with their elevated levels in the serum of women experiencing miscarriage, strongly suggests the involvement of NETs in the premature termination of pregnancy. This observation underscores the potential significance of NETs in adverse pregnancy outcomes, emphasizing the need for further research into the role of NETs in such scenarios[19,20]. The study focuses on women from specific age groups (18-29 and 30-41) in Basrah Hospital for maternity and pediatrics, providing a structured approach for examining the relationship between age and recurrent miscarriages within a defined population. Through subgrouping participants into categories based on age and the number of abortions, the study guarantees a focused analysis of these factors.

The results indicated that there were no statistically significant differences in age between the patients and

control groups (p = 0.624). However, analysis revealed a notable association between older patients and a history of multiple abortions, with 69.2% of older patients reporting multiple abortions compared to 34.3% of younger patients, a finding that was statistically significant (p= 0.0001). This suggests that advanced age may be a contributing factor to a higher incidence, despite that it does not affect the distribution of participants in the study. This observation aligns with a recent study by Zhang *et al*.[21]. Their study included 338 cases with spontaneous abortion and 1352 controls. It demonstrated a significant association, presented by a non-linear relationship, between the progression of age and the increased prevalence of spontaneous abortions, particularly in women older than 30 years[21]. Many previous studies demonstrated a positive relationship between advanced maternal age and recurrent abortion[22,23]. However, the determination of the perfect age for pregnancy has not been concluded yet, although some studies consider the age 30 a threshold for abortion[22,24], others considered the age 40[25] .

The observed significant difference in CD11b levels between patients and controls underscores potential immune dysregulation following abortion. CD11b, a key marker of immune cell activation and inflammation, exhibited higher expression levels in patients compared to controls. Sacks et al found that pregnant women exhibited higher expression levels of CD11b on the surface of leukocytes compared to non-pregnant woman[26].

This finding is consistent with Ye et al.'s[27] study that concluded that CD11b levels are higher in spontaneous abortion compared to non-pregnant females, which the authors explained by increased activation level of D-LDG during early pregnancy, irrespective of pregnancy outcome. The high CD11b expression may indicate an increased state of immune activation or inflammation within the decidual microenvironment, potentially influencing pregnancy outcomes. However, this contradicts the study by Faridi et al. That suggests that CD11b activation can have anti-inflammatory effects by inhibiting Toll-like receptor (TLR)-dependent inflammation and autoimmunity, thereby mitigating inflammatory damage[28]. Moreover, Sacks et al. found that pregnant women exhibited higher expression levels of CD11b on the surface of leukocytes compared to non-pregnant women[26].

In the present study, the analysis in the present study revealed a statistically significant difference (p=0.04) in CD11b levels between the two patients and controls for (18-29) age group, with the patient group exhibiting higher levels (16.089) compared to control (10.655). This discrepancy underscores the potential relevance of CD11b levels in differentiating between patient and control populations. Similarly, in the (30-41) age group, there was a significant difference in CD11b levels between patients and controls (18.798 vs. 12.27, p=0.003), with patients showing notably higher levels.

The correlation appears to be stronger in the older age group. This could potentially be attributed to the secretion of cytokines by CD11b+ cells present in the decidua and placenta. Zenclussen et al., in their study, utilized flow cytometry analysis and found increased numbers of CD11b+ cells in aborted placentas and deciduas compared to control pregnant mice. Their findings suggest that cytokines, produced not only by immunocompetent cells such as macrophages and mast cells but also by trophoblasts and decidua cells, plays a direct role in the pathology of abortion[29].

This supports the notion that alterations in CD11b levels may reflect underlying immune dysregulation, potentially driven by inflammatory processes, contributing to the observed differences between patients and controls. Moreover, studies have shown these inflammatory processes can induce fetal resorption by promoting ischemia through the activation of vascular endothelial cell procoagulants, leading to thrombosis and inflammation in mouse models.

Additionally, they depress levels of protein S, an anticoagulant protein, in experimental animals. This can further exacerbate cytokine release, endothelial cell damage, and the migration of white blood cells in response to endotoxin, potentially contributing to pregnancy complications[30,31].

However, the observation in the present study contrasts with finding of Ye et al. that stated CD11b expression in women with spontaneous abortion was significantly lower than in non-pregnant women. The reduced CD11b expression in spontaneous abortion women suggests potential implications for immune response in the pathogenesis of spontaneous abortion[27] .

The results also suggest that Cd11b might not only be a marker for the occurrence of recurrent miscarriages but could also reflect the recurrence rate of these events. Higher levels of Cd11b observed in the subgroup with 4-5 abortions in comparison with the subgroup with 2-3 abortions, with a p-value of 0.012. Elevated Cd11b levels, being a marker of NETosis, can point to the activation of neutrophils and their involvement in immunological responses which can be directly involved in the pathophysiology of recurrent pregnancy loss[32,33] . It is frequently correlated with the presence of maternal phospholipids which helps in activation of neutrophils[33]. Activated neutrophils discharge reactive oxygen species and proteolytic enzymes, resulting in damage to the decidual tissue and fetal loss. Tissue factor (TF) functions as a significant pro-inflammatory mediator in fetal injury induced by antiphospholipid antibodies (aPL)[34]. The correlation between elevated Cd11b levels and pregnancy loss can increase the potential to develop more targeting diagnostic and therapeutic approaches for women with a high risk of frequent miscarriages.

Peptidyl arginine deiminase 4 (PADI4) is a key enzyme involved in the citrullination process, a critical step in neutrophil extracellular trap (NET) formation known as NETosis. It also potentially holds a distinctive and fundamental function in the evolutionary process and early embryonic development among humans[35]. While no significant differences were found in PADI4 levels between patients and control groups in this study, its role in the context of the investigated condition may still be noteworthy.

PADI4-mediated citrullination of histones is crucial for chromatin recondensation during NETosis, contributing to the release of NETs and subsequent immune responses[36]. The lack of significant difference in PADI4 levels suggests its involvement may be more nuanced or regulated differently compared to other NETosis markers in abortion. Moreover, studies have shown that there is a slight decrease in PADI 4 levels in women who experienced miscarriages. This suggests the existence of a regulatory mechanism that suppresses the formation of NETs during pregnancy. According to existing literature, PADI 4 contributes to the generation of NETs, as evidenced by the positive correlation observed between PADI 4 and MPO levels in women who tested positive for NETs[20] .

The significantly higher levels of calprotectin in patients compared to controls suggest an increased neutrophil activation and potential dysregulation of inflammatory responses in abortion. Calprotectin is released by activated neutrophils during inflammatory processes and has been implicated in various inflammatory and autoimmune conditions[37]. Elevated levels of calprotectin may reflect ongoing inflammation and tissue damage. This aligns with current studies that show Increased levels of calprotectin are linked to elevated rates of abortion[38]. In addition, calprotectin possesses antimicrobial, cytostatic, antiproliferative, apoptosis-inducing, and chemotactic properties, playing a role in various physiological and pathological processes within the female reproductive tract[39].

CONCLUSIONS

The findings of this study provide significant insights into the intricate relationship between recurrent miscarriages and the immune system, specifically through the lens of neutrophil extracellular traps (NETs) and the activation marker CD11b. The observations underscore the nuanced roles these components play in the pathophysiology of recurrent pregnancy loss. While advanced maternal age and the number of previous abortions emerge as critical factors influencing the occurrence and frequency of miscarriages, the study further elucidates the underlying immune mechanisms, highlighting the differential expression of CD11b and NETosis markers in women experiencing recurrent miscarriages compared to controls. Notably, the investigation into NETosis markers, alongside the assessment of PADI4, calprotectin, and Proteases 57 levels, illuminates the complex interplay of inflammatory responses and neutrophil activation in the context of miscarriage.

Conflict of Interest

There is no conflict of interest.

Ethical approval

All parts of this study, which included pregnant women participants, were carried out in accordance with the ethical standards of the Ministry of Health, General Directorate of Health, Basra, Iraq, (Issue: 159 dated: 2-17-2022) as well as the verbal consent of the women before the study.

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