CHAPTER III

DETERMINATION OF AUTOANTIBODY PROFILE (ANTINUCLEAR AND ANTIPHOSPHOLIPID AUTOANTIBODY) IN STUDY COHORT AND ITS ASSOCIATION STUDY WITH SPONTANEOUS ABORTIONS.

3.1 Introduction

Spontaneous abortion, commonly known as miscarriage, is a significant event that occurs in around 10% of clinically recognized pregnancies during the early stages (before twenty weeks of gestation)(1-2).The immune system plays a critical role in maintaining a delicate balance between accepting the developing fetus as a foreign entity and safeguarding the maternal system(3-5). This balance is crucial for successful pregnancy outcomes(6-8). However, when there is an imbalance or disruption in the immune system, it can lead to improper placental development, especially in cases of early pregnancy failure(9-12).

Early pregnancy process involves development and differentiation of the maternal decidua,crucial for successful placenta formation (13-15).Following implantation, specialized cells called cytotrophoblasts infiltrate the uterine decidua and transform into extravillous trophoblasts(16-18). By the eighth week of pregnancy, these extravillous trophoblasts invade the decidua and establish direct communication with the maternal uterine spiral arteries while also supplying blood to the surrounding tissue (19-21). EVTs breakdown the spiral arteries' muscular walls and replaces the endothelial cells that line them, while also remodeling the small, adrenergic-sensitive, high-resistance vessels into wide, adrenergic-insensitive, low-resistance conduits and falicitating increase in blood flow to meet the demands of the developing fetus(22-25). Notably, during the initial invasion of the spiral arteries, the trophoblasts form loosely cohesive plugs within the vessel lumens(26-28). While these plugs temporarily impeding the passage of maternal red blood cells, creating an environment with reduced oxidative stress that promotes early embryonic development, but also while still enabling the movement of plasma to reach the placenta (29-31).

APS is an acquired autoimmune disorder with elevated levels of classical aPLs namely anti β-2 glycoprotein I (aβ2GP1), Lupus anticoagulant(LA) and antiCardiolipin (aCL) in the plasma (31-35) .It is found in 0–5% of the general population and in up to 40% of women suffering from stillbirth and/or recurrent miscarriage, termed as obstetrical antiphospholipid syndrome (OAPS) (36-38). APS can be classified as primary or secondary, depending on its association with other autoimmune conditions. Secondary APS is commonly found in patients with various autoimmune diseases, with systemic

lupus erythematosus (SLE) being the most frequently associated condition (39-40). In contrast, primary APS is diagnosed in patients who consistently test positive for antiphospholipid antibodies and display clinical manifestations of thrombotic events(41-42).

The major subpopulation of antibodies responsible for the thrombotic symptoms of obstetrical antiphospholipid syndrome (OAPS) is considered to be β2GPI-dependent APLA (43). This is because β2GPI, which is consistently present on the surface of placental cells, is targeted by these antibodies (42-43). Their presence has been found to hinder trophoblast invasion and differentiation, thereby affecting normal placental development (44-46). Normally, β2GPI is a plasma protein, and anti-β2GPI antibodies bind to it when it is complexed with phosphatidylserine on plasma membranes(47-48). Typically, phosphatidylserine remains within the inner leaflet of cell membranes, inaccessible to aPL or β2GPI in the bloodstream. However, when cells are damaged, activated, or undergo apoptosis, phosphatidylserine can become exposed, allowing β2GPI to bind to it (49-51). Binding of anti-phospholipid antibodies to monocytes' surface and endothelial cells' multiprotein complexes leads to upregulation of tissue factor expression through intracellular signaling pathways (52-54). The binding of aPL to β2GPI can activate various downstream signaling pathways and elicit effects such as cellular activation, complement cascade activation, and inflammation, depending on the cell type(55-57). Additionally, aPL can bind to β2GPI associated with membrane receptors such as Toll-like receptors (TLRs), apolipoprotein E receptor 2, and low-density lipoprotein receptors (LDLRs).This binding may result in inappropriate receptor activation and signaling or facilitate the internalization of aPL into the cell (58-62). β2 glycoproteins is also the cofactor in the binding of aCLto negatively charged phospholipids. Specifically, anticardiolipin antibodies bind to the complex formed by β2 glycoproteins and cardiolipin, which is a negatively charged phospholipid found in the mitochondrial membranes of eukaryotic cells (63-64). aCL present in patients with APS interfere with the normal mitochondrial function of neutrophils and monocytes, causing an increase in the production of reactive oxygen species and subsequent expression of tissue factor (65-68). In addition ,LA autoantibodies, prolong in vitro coagulation tests that depend on phospholipids, such as the activated partial thromboplastin test and kaolin clotting test (69-70) .This

interaction is important for the natural anticoagulant function of β2 glycoproteins, and any disruption in this system can lead to thrombosis (71-72). Notably, occurrence of both aCL and aβ2GPI antibodies have been associated with pregnancy complications. However, with conflicting findings (73-75).While some studies have not found a significant correlation between these antibodies and pregnancy loss, aCL has been identified as the most prevalent among the obstetric population with adverse pregnancies (76-80). Elevated levels of APLA have been observed history of multiple pregnancy failures as compared to single pregnancy loss (80-82).

Presence of ANA has correlated with increased plasma C3a levels and reductions in plasma C3 levels in earlier reports (83-84).In addition , it is reported to activate plasmacytoid dendritic cells through Toll-like receptor-9,leading to increased production of inflammatory cytokines such as interferon-alpha (85-88).The role of ANA in early pregnancy loss remains a subject of controversy (89-91). It is hypotheised that ANAs may contribute to inflammation in the uterus, hindering its ability to provide a conducive environment for embryo implantation (92-93). High incidence of ANAs has been reported in women with history of miscarriages, specifically, a high prevalence of low-titre ANAs has been observed in the blood samples of individuals with early pregnancy losses (94,95).

Considering that data on autoantibody profile in North-east India is sketchy, we triedto understand potential links between their presence and the occurrence of SAB. The study investigated occurrence of APLA specifically in conjunction with ANA in our study population, and their association with history of SAB.

3.2. Materials and methods

3.2.1. Study site, study design, and participants

A hospital-based case-control study was designed with 101 obstetric participants as the study cohort. The study was conducted at Tezpur Medical College and Hospital (TMCH) and Gogoi Nursing Home Complex (GNHC) in Tezpur, Assam. Obstetric participants with history of one or more fetal losses during 10-12 weeks of pregnancy (N=54, SAB cases) and age-matched women with history of least one live birth (N=47 ,control group) were recruited in the study during their routine visit to outpatient department (OPD). Ethical permission was obtained from the Institutional Ethics

Committee of Tezpur Medical College and Hospital (TMCH) with sanction numbers IEC/14. All participants were provided with a detailed patient information sheet and a patient consent form as per the guidelines of Indian Council of Medical Research (ICMR). The forms were provided in both English and the local language and participants were recruited for the study only after obtaining their written informed consent and that of their guardians.

Participants got screened for bad obstetric history (BOH) history of thrombosis and routine clinical examination during their visit to OPD. Transvaginal ultrasound was performed by the clinical staff to confirm spontaneous abortion.

Peripheral blood was collected by clinical staff for serological studies. However, if available, efforts were made to utilize the blood collected during routine examinations.

The following were the exclusion and inclusion criteria of the study-Inclusion Criteria

1. Reproductive age group of median age ,irrespective of pregnancy status
2. History of SAB /RSAB (case)
3. For Control group history with minimum one childbirth

Exclusion Criteria

1. Uterine anomalies
2. Hormonal imbalance .
3. History of neonatal death/ any debilitating diseases .

The information on obstetric history of the participants ,ethnicity, demographic and other characteristics were collected and recorded in the form of proformas by the research staff of both the hospitals.

Two participants who tested positive for VDRL and rubella were excluded from the study .

3.2.2 Blood sample collection and clinical investigations.

Preparation of plasma

2 ml of venous blood was withdrawn from study participants ,1ml collected in anticoagulant-treated tubes (10% 0.5m EDTA) filled vials. Plasma was separated by centrifugation for 10 minutes at 1,000-2,000 x g using a refrigerated centrifuge (cells were retained for DNA isolation). Centrifugation for 15 minutes at 2,000 x g depletes platelets in the plasma sample. The resulting supernatant was plasma which was transferred into a small aliquoates in clean microcentrifuge tubes using a pipette. The samples were maintained at 2-8°C while handling and after transferring, the tubes were stored at -20 degree for future use.

3.2.2.1 Antiphospholipid antibody (APLA) : β-2 glycoprotein I (aβ2GP1) IgG/ IgM and anti Cardiolipin (aCL) IgG/ IgM) profile in obstetric participants in relation to pregnancy.

Plasma samples, which had been previously aliquoted and stored at -20 degrees celsius, were thawed once. Subsequently, the samples were allowed to reach room temperature (30 minutes). The samples were diluted in a 1:101 ratio using buffer.

Commercial sandwich ELISA kits were utilized for the detection of autoantibodies, including aβ2GP1 IgG, aβ2GP1 IgM, aCL IgG, and aCL IgM respectively. Precoated ELISA plates were used following the manufacturer's protocol. In the ELISA assay, 100 microliters of diluted samples were added to the plates, which were precoated with autoantibodies. Calibrators, positive control, negative control, and dilution buffer (used as a blank) were also added to the plate. All samples were loaded in triplicates. After incubation with the primary antibodies, a ready-to-use secondary antibody, anti-human IgG horseradish peroxidase (HRP) conjugated antibody, was added to the ELISA plates. This secondary antibody specifically targeted human IgG antibodies, facilitating the detection and amplification of the antigen-antibody complex.

The levels of autoantibodies in the provided calibrators and the cut-off values for determining positivity are mentioned in the Table1 . The absorbance of the samples was measured at 450 nm, using an OD of 620 nm as the reference wavelength.

3.2.2.2 Antinuclear antibody (ANA) profile in obstetric participants in relation to pregnancy

Quantitative determination of Antinuclear Antibodies (Ig(GAM)) was performedusing the ANA Screen ELISA method. The sandwich ELISA procedure was conducted following the manufacturer's instructions, as mentioned previously. The kit provided cut-off values (Table 1) for interpreting the results. Additionally, a set of 5 calibrators with known levels of ANA antibodies was included to prepare a standard graph and determine the antibody levels in the test samples.

3.2.3 Statistical analysis

For statistical analysis of the data, XLSTAT software (2015 and 2018.7 versions) was used. Correlation analysis was performed between the expression aβ2GP1 IgG/, aβ2GP1 IgM ,anti aCL IgG, anti aCL IgM and ANA Ig(GAM)) in SAB. One sample t-test and Student's t-test were used for comparison between the mean values. A p-value < 0.05 was considered statistically significant.

Table 1: Threshold Values for Positive titre results and concentrations of autoantibodies in Calibrators.

	aCL IgG(u/ml)	aCL IgM (u/ml)	aβ2GP1 IgG (u/ml)	aβ2GP1 IgM(u/ml)	ANA Ig (GAM)) (u/ml)
Cal 1	31.25	31.25	6.25	6.25	31.25
Cal 2	62.5	62.5	12.5	12.5	62.5
Cal 3	125	125	25	25	125
Cal 4	250	250	50	50	250
Cut off valuesfor positive result	48	44	7	7	55

3.3. Results

3.3.1 Clinical profile of the obstetric participants

The study participants were reproductive age matched years with a median age of 26 years. The obstetric history and clinical profile of participants has been shown in (Table 2).

The participants were ruled out for VDRL (venereal disease research laboratory test), Hepatitis B surface antigen (HBs Ag), anti HCV (Hepatitis C virus), HIV 1 and 2 antigens. Thyroid-stimulating hormone (THS) was taken as normal in the range from 0.4 and 4.0 milli units per liter (mU/L) in the study cohort as per 2017 guidelines for American Thyroid Association.

Table 2: Obsteric history and pregnancy status of study cohort.

Characteristic	**Total participants (N=101)**	
Obsteric history	**Frequency**	**Gestational age of pregnancy in pregnant women (in weeks)**
Healthy	47 (42.34%)	-
SAB	46(41.44%)	-
RSAB	8(7.20%)	-
Pregnant (n=4)		
SAB	0	8-12 weeks
RSAB	2 (1.98%)	8-12 weeks
Healthy	2(1.98%)	8-12 weeks
***Non pregnant (n=97)**		
SAB	46(45.54%)	NA
RSAB	6(5.94%)	NA
Healthy	45(44.55%)	NA

*Non-pregnant women were the participants who had either given birth or experienced spontaneous abortion within the last 3-6 months from the study.

3.3.2. aβ-2 glycoprotein I and anti Cardiolipin antibody profile in study cohort and its association with pregnancy failure.

The study investigated plasma samples obtained from obstetric participants who had a history of spontaneous abortion, as well as those with a history of healthy pregnancies. The levels of aβ-2 glycoprotein I IgG/IgM antibodies and aCL IgG/IgM antibodies were analyzed in these samples. and association study was conducted to investigate any potential correlations between these antibody levels and the clinical characteristicsof the participants. The calibration run of the calibrators provided in kits for both aB2GP1 and acL gave a straight line, indicating a high degree of linearity. The calculated R2 value of 0.89-9 suggested a strong correlation between the expected and measured values, indicating the accuracy of the test (Appendix 1)

Among the total participants, aCL IgG antibodies were detected in a total of 15 participants, including those with a history of healthy pregnancies, spontaneousabortion (SAB), and recurrent spontaneous abortion (RSAB) (Table 3). When comparing the titres of positive participants, it was found that the range of aCL IgG levels was notably higher in two RSAB participants (ranging from 100 u/mL to 149 u/mL), followed by SAB participants (ranging from 63 u/mL to 70.9 u/mL), and healthy participants (ranging from 46.2 u/mL to 88.3 u/mL) (Figure 7a).

aCL IgM antibodies were detected in 7 obstetric participants, of which 6 had a historyof SAB. These SAB participants exhibited higher titre ranges (ranging from 45 u/mL to 210.3 u/mL) compared to the single positive healthy participant (68.1 u/mL) (Figure7b). When comparing the mean titre of autoantibodies between healthy and SAB participants for both IgG and IgM antibodies, no significant difference was observed. However, when comparing RSAB and healthy participants for aCL IgG levels, a significant difference was found, with significantly higher levels in RSAB participants (t-test, p=0.04) (Table 4).

In context of aβ2GP1, a negative result for aβ2GP1 IgG was noted in the study cohort. Interestingly, out of the 4 cases that tested positive for aβ2GP1 IgG, all had a history of SAB, with levels ranging from 6.8 u/mL to 51.8 u/mL (Figure 7c) .

We observed positive correlation (pearson's p=0.02) between aβ2GP1 IgM and aCL IgG in healthy pregnancy while a negative correlation (pearson's p=0.03) was

observed between aβ2GP1 IgM and aCL IgM in SAB history participants (Figure 8a and Figure 8b).

Table 3: Frequency of Positive Cases of Antiphospholipid Autoantibodies in the Study Cohort Based on Obstetric History

Obstetric history of participants	SAB history(n=46)	RSAB history(n=8)	Healthy participants (n=47)
aCl IgG positive	4(8%)	2(25%)	9(19%)
aCl IgM positivite	6(13.04%)	0	1(2.12%)
aβ2 GP1 IgG positivite	0	0	0
aβ2 GP1 IgM positivite	4(8.69%)	0	0

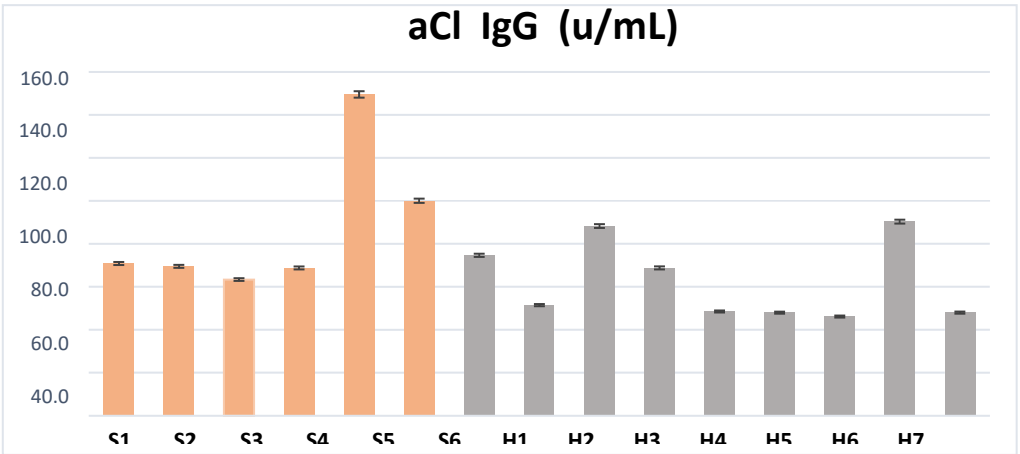

Figure 7a : Assessment of expression of aCl IgG autoantibody by ELISA. Autoantibody levels were expressed in u/ml as given in the manufacturer's protocol . Titre of aCl IgM positive cases in study cohort are shown above.

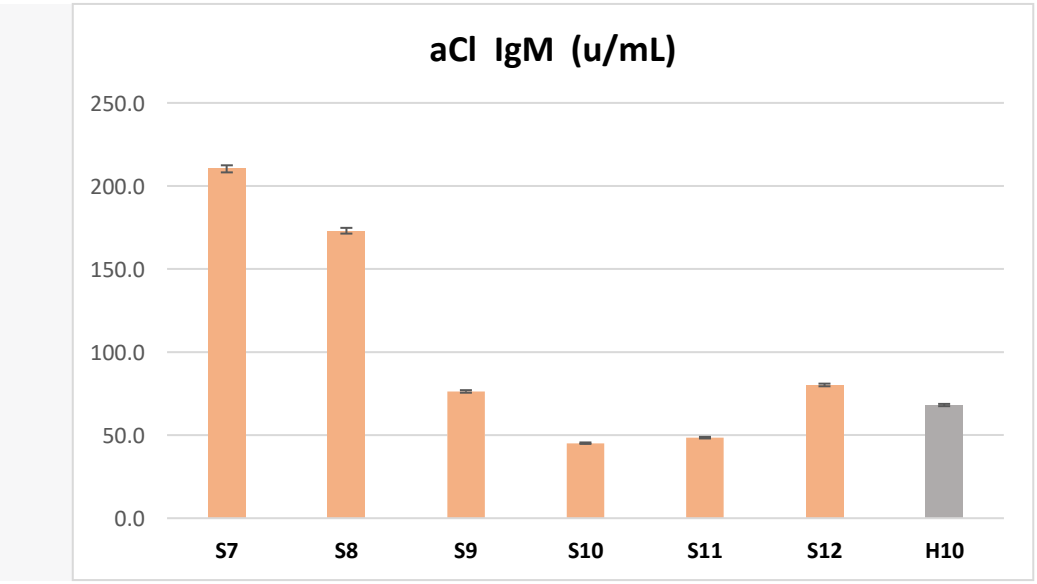

Figure 7b : Assessment of expression of aCl IgM autoantibody by ELISA. Autoantibody levels were expressed in u/ml as given in the manufacturer's protocol . Titre of aCl IgM positive cases in study cohort are shown above.

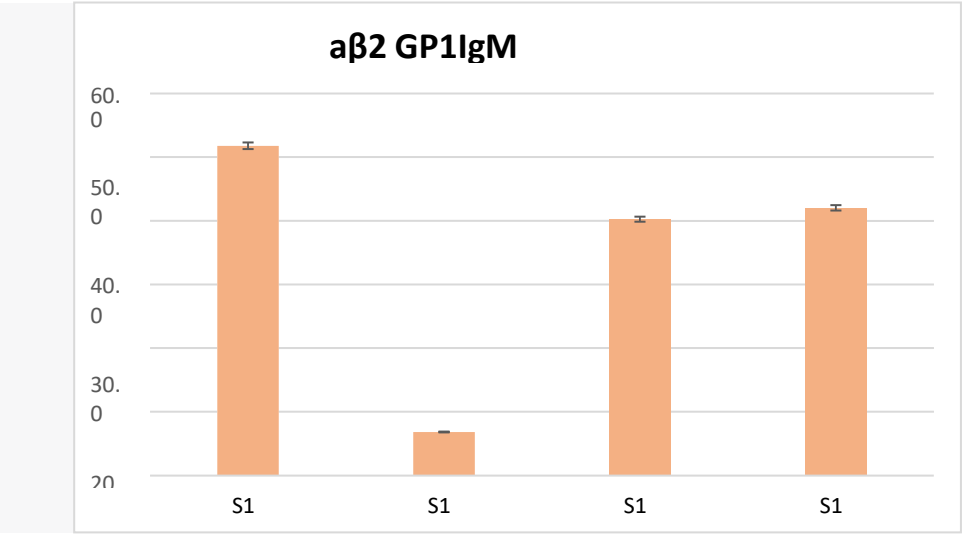

Figure 7c: Assessment of expression of aβ2 GP1 IgM autoantibody by ELISA.

Autoantibody levels were expressed in u/ml as given in the manufacturer's protocol . Titre of aCl IgM positive cases in study cohort are shown above.Titer of aβ2 GP1 IgM positive cases in study cohort are shown in the figure.

Table 4: Comparison of Mean Titers of APLAs in SAB and Healthy Participants (Significant P-values at α=0.05 Level)

	Mean titre of autoantibodies in SAB participants (u/mL± SE)		Mean titer of autoantibodies Healthy participants (u/mL± SE)	Comparision of means in SAB and healthy participants (Student t test , p value)
	SAB	RSAB		
aCl IgG	68.1±1.6	124.7±24	62.7±6	(Between RSAB and healthy participants 0.004
aCl IgM	105.5±23		68.1	
aβ2GP1IgM	35.2±9.7			

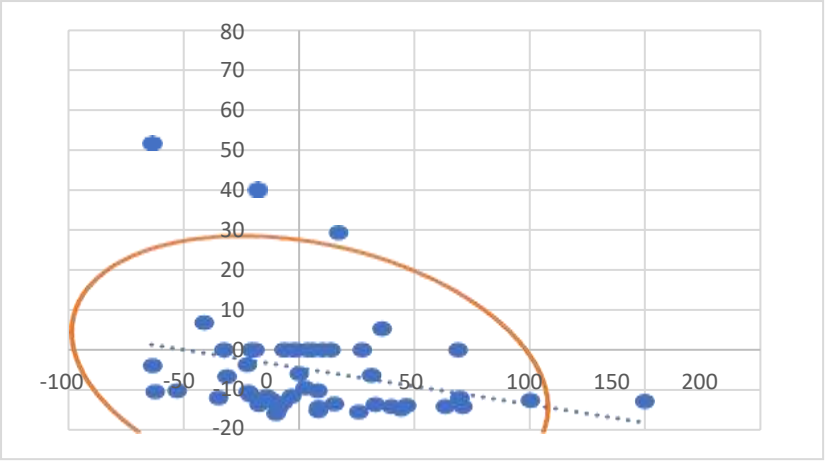

Figure 8a : Correlation analysis of expression of aβ2 GP1 IgM and aCl IgG in healthy participants .A significant negative correlation was observed with Pearson's r =-0.28, p=0.03.A p<0.05 was considered as statistically significant.

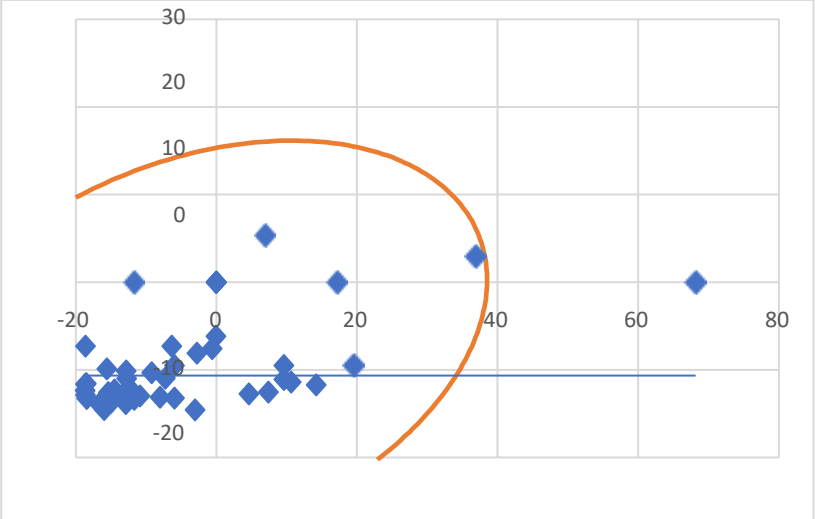

Figure 8b: Correlation analysis of expression of aβ2 GP1 IgM and aCl IgM in healthy participants .A significant positive correlation was observed with Pearson's r =0.3, p=0.02.A $p<0.05$ was considered as statistically significant.

3.3.2. ANA Ig (GAM) profile in study cohort and its association with pregnancy failure.

Among the obstetric participants, a positive result for ANA was observed in a total of 10 participants.Among these, the majority were participants with a history of healthy pregnancies (14.89%) while the remaining participants (6.52%) had a history SAB (Table 5) . When comparing the titres of ANA autoantibodies, the range of levels was found to be between 57.99 u/mL and 130.76 u/mL in the healthy group, and between 91.00 u/mL and 104 u/mL in the SAB group (Figure 9).

However, no significant correlation was observed between the levels of ANA autoantibodies and the obstetric history of the participants.

Table 5: Frequency of Positive Cases of ANA autoantibodies in the study cohort based on obstetric history.

Obstetric history of participants	**ANA Positivity**
SAB history participants (n=46)	3(6.52%)
RSAB history participants (n=8)	0
Healthy participants (n=47)	7(14.89%)

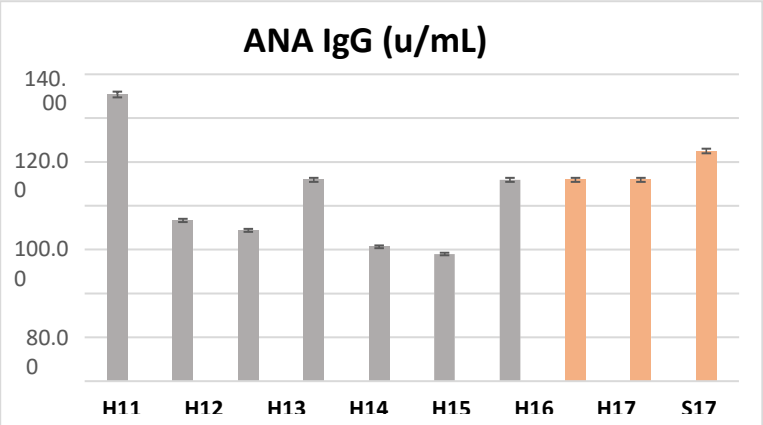

Figure 9: Assessment of expression of ANA IgG autoantibody by ELISA.

Autoantibody levels were expressed in u/ml as given in the manufacturer's protocol.

3.4 Discussion

Antiphospholipid syndrome (APS) , recognized as a significant risk factor for adverse pregnancy outcomes ,accounts for 8 to 42% of early pregnancy failures (96).These failures can be attributed to either thrombotic (coagulation) or non-thrombotic (inflammation) events (97-99). Elevated levels of aβ2GP1 and aCL have been consistently associated with the diagnosis of APS in the general population (100-101). In our study, we observed a higher incidence of aCL compared to other autoantibodies

in both the healthy history group and SAB history group. This finding emphasizes the predominant presence of aCL in the general population. Our results align with earlier studies by Su et al., which also reported the persistence of aCL in the general population (102-103).

We observed higher levels of aCL IgG were observed in RSAB cases compared to healthy individuals, but not in cases of SAB. This prompted towards the increased levels of aCL in obs particioants with history of multiple pregnancy failures rather than a single occurrence. This finding is consistent with earlier reports that have demonstrated an increased prevalence of elevated aPL antibodies in cases with multiple pregnancy failures (104,105). However, in contrast to other studies, we observed only a marginal decrease in APL levels during pregnancy, and these changes were not associated with the outcome of pregnancy.

Interestingly, aβ2GP1 IgM was predominantly observed in SAB history group, which is consistent with earlier reports identifying aβ2GP1 as a significant factor contributing to thrombosis and adversely affecting pregnancy outcomes (106). The negative correlation between aβ2GP1 and aCL autoantibodies in SAB, along with the increased presence of aβ2GP1, suggests its potential independent contribution to SAB . However, it is important to note that the number of aβ2GP1-positive samples in our study was limited, which restricts our ability to draw definitive conclusions. Although a predominance of ANA was observed in the healthy history participants, with a low frequency in SAB history group, the comparable range of ANA titers between the SAB and healthy groups suggests that ANA is equally distributed in our study cohort, regardless of obstetric history (107).

Our data collectively suggests a prevalence of primary APS in studied obstetric population .No observed correlation between APLA and ANA suggests that presence of one antibody does not necessarily indicate the presence or impact of the other, in relation to SAB. Furthermore,the presence of autoantibodies in women following fetal loss suggests that the persistence of these autoantibodies is not limited to the state of pregnancy.

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Salient findings of Chapter III

We observed a higher incidence of aCL compared to other autoantibodies in both the healthy history group and SAB history group. It emphasizes the predominant presence of aCL in the general population. In addition, higher levels of aCL IgG were observed in RSAB cases compared to healthy individuals, but not in cases of SAB. However, we did not find any significant association between aCL and SAB in our studied population. This prompted towards the increased levels of in obs particioants with history of multiple pregnancy failures rather than a single occurrence. Although B2GP1 IgM positivity was only observed in participants with a history of SAB, the frequency was too low to draw meaningful conclusions.

No significant association of ANA autoantibodies with pregnancy outcomes was noted in individuals with a history of SAB/RSAB. The lack of significant correlation between APLA and ANA suggests that the presence of one antibody does not indicate the presence or impact of the other, especially in the context of SAB. Nonetheless, the detection of autoantibodies in women after experiencing fetal loss indicates that these autoantibodies can persist beyond pregnancy.