CHAPTER VI

CHARACTERIZATION OF EARLY ABORTUS AND TERM PLACENTA WITH RESPECT TO HISTOLOGY AND VASCULARIZATION IN SPONTANEOUS ABORTION AND IN HEALTHY PREGNANCY.

6.1 Introduction

The placenta undergoes a complex developmental process involving different cell types that are crucial for maintaining a healthy pregnancy(1-3). Among these cell types, cytotrophoblasts and syncytiotrophoblasts differentiate during early pregnancy(5-7). Syncytiotrophoblasts serve as a protective barrier between the mother and fetus, produce hormones and growth factors, and facilitate nutrient and waste exchange(8-12). Cytotrophoblasts, on the other hand, function as stem cells, aiding in the invasion of uterine arteries and contributing to placental remodeling. Following implantation, specialized cells called cytotrophoblasts infiltrate the uterine decidua and transform into extravillous trophoblasts (EVTs)(13-16). By the eighth week of pregnancy, these EVTs invade the decidua and establish direct communication with the maternal uterine spiral arteriesV via there interaction with decidual NK (dNK) cells while also supplying blood to the surrounding tissue (17-20). Additionally, mesenchymal cells, Hofbauer cells, fibroblasts, and pericytes mediate vascular development and nutrient transport, gas exchange, and hormonal regulation (21-23).

Remodeling of maternal spiral vessels takes place where uterine artery flow increases, resulting in the conversion of uteroplacental vessels that empty into the intervillous space (24-25). This transformation creates a high-capacitance, low-resistance system within the intervillous space, facilitating optimal nutrient exchange (26-28). This blood flow is vital for transporting oxygen, nutrients, and metabolic support to the developing fetus(29-30). At this time, the intervillous space undergoes significant changes, characterized by cellular proliferation and differentiation despite relatively low oxygen levels (31-33).Oxygen and nutrient exchange occur as maternal blood flows around the terminal villi in the intervillous space(33-35). Here the deoxygenated and nutrient-depleted fetal blood is carried from the fetus through the umbilical arteries, while oxygenated and nutrient-rich blood is transported back to the fetal systemic circulation via the umbilical vein (36-38). Disruptions in communication between EVTs and decidual immune cells can lead to uncontrolled immune responses and an imbalance in maternal-fetal immune tolerance (39-40).

$CD56^{+}$ $CD16^{-}$ dNK cells are a specialized type of trNK cells found at endometrial decidual tissue and display many unique phenotypic and functional characteristics compared with peripheral NK (pNK) cells and Tissue resident (trNK) cells. dNK exhibit distinct phenotypic and functional characteristics compared to peripheral NK cells (41). Most dNK cells display a $CD56^{+}CD16^{-}KIR^{+}$ phenotype, and apart from initiating remodelling of spiral arteries they also exhibit lower cytotoxicity, and higher cytokine secretion capabilities and mediating vasculogenesis via expression of growth factors such as growth factors: VEGFA and cytokines, favouring placentation (42,43) .

Vasculogenesis and angiogenesis, mediated by secretion of the growth factors and cytokines are the two major events contributing to development of placenta (44-47). Vasculogenesis involves the de novo formation of blood vessels from precursor cells derived from the mesoderm, while angiogenesis involves the creation of new vessels from pre-existing ones. The development of the placental vascular network starts early in pregnancy, with primary villi consisting of cytotrophoblasts and syncytiotrophoblasts (48-50). As development progresses, secondary villi form with a layer of connective tissue cells underneath. Villous capillaries develop from specific cells until around 10-12 weeks of gestation (50-52). Afterward, the capillaries undergo coiling, bulging, and sinusoid formation, resulting in the syncytiocapillary membrane (53-55). Although new vessel formation is not easily observed later in pregnancy, some capillary sprouts may be present. By about 32 days, the villous endothelial tubes connect with each other and with fetal allantoic vessels, establishing a primitive fetal-placental circulation (56-58). The process of forming new capillaries and expanding the villous vascular system continues until birth, favoring pregnancy(59-60).

Preeclampsia (PE), intrauterine growth restriction (IUGR) etc have been linked to impaired blood vessel formation and an imbalance in immune cells (61-63). Furthermore, inadequate physiological changes, such as fibrinoid necrosis and acute atherosis, negatively impact the remodeling of spiral arteries and can have detrimental effects on pregnancy(64-65). These abnormalities in the placenta, which result in reduced blood flow between the mother and fetus, are associated with restrictions in fetal growth (66-68).

Considering that placental vasculature varies throughout pregnancy it was pertinent to compare the histological features of early abortus and term placenta with a specific focus on the expression of CD56 and VEGFA in early abortus in relation to SAB.

6.2. Materials and methods

6.2.1. Study site, study design, and participants

The study site, study design and study participants are as described in section 5.2.1. Of the collected samples, 10 term placenta (5 healthy and 5 SAB history) and 20 early abortus samples (10 healthy, 10 SAB) were used for histological investigations in terms of NK characterization and vasculature.

The term placenta and conceptus product were collected by the clinical staff only after obtaining written informed consent of the participants and their guardians, as mentioned under section 4a.2.

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. Participants were recruited for the study only after obtaining their written informed consent and that of their guardians.

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 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 hospital and has been mentioned in

6.2.2. Staining and expression study.

6.2.2.1 Tissue fixation and tissue processing

Tissue samples were obtained and preserved in 10% neutral buffered formalin (NBF) for fixation. Fixed tissues were processed using paraffin embedding, a common method to prepare thin microscopic sections. Paraffin wax, with similar density to tissue, was used to embed the tissues. The embedded tissues were sectioned at a thickness of 4 μm using a rotary microtome, cutting from the middle of each specimen. The resulting tissue sections were mounted on clean gelatinized slides. The slides underwent histological analysis using H and E staining to study tissue morphology, and immunohistochemistry (IHC) was performed to assess the expression of the NK marker CD56 and the vascularization marker VEGFA.

6.2.2.2 H and E staining

In this H&E staining protocol, tissue slides are deparaffinized in xylene and then rehydrated using a series of graded alcohols. The slides are then stained with Hematoxylin for 5 minutes, followed by rinsing with water. To achieve contrast, the slides are differentiated in acid alcohol and rinsed again with water. Next, the slides are counterstained with Eosin for 2 minutes. After counterstaining, the slides are dehydrated with alcohol and cleared in xylene. Finally, the slides are mounted with a coverslip and examined under a light microscope for detailed histological analysis of the tissue samples. H & E sections were analyzed by light microscopy (magnification ×400). The observations were recorded by digital camera. Microscopic examination included examination of troploblast (proliferation, vascular invasion), stroma (inflammatory cells) and decidualization. The histopathologists examining the tissue sections were blinded to the clinical details of the cases.

6.2.2.3 Protein expression CD56 and VEGFA in abortus by Immunohistochemistry

Tissue sections were prepared from FFPE sections and processed for IHC as per the standard protocol. Goat anti-mouse HRP conjugated antibody was used as the secondary antibody (BD Biosciences, United States). Tissues showed 50% or more staining for all the three proteins were considered as positive. Staining intensity was calculated and samples were graded from 1+ to 4+ based on staining intensity.

6.2.3. Statistical analysis of data

Participants with incomplete data or incomplete investigations were excluded from the analysis. Statistical analysis of the data was performed using XLSTAT Biomed 2018.7 and 2015 versions. Correlation analysis was performed between the expressions of marker genes, marker proteins using the Pearson's Correlation test. Student's t-test was used for comparison between the mean values. A p-value < 0.05 was considered statistically significant.

6.3 Results

6.3.1 Microscopic Analysis of Histology in Early Abortus and Placenta

Vascular invasion by trophoblasts was observed in 3 SAB abortus but was absent in healthy abortus. In addition, SAB cases showed the presence of inflammatory cells in three samples, whereas healthy abortus samples did not exhibit such inflammation. The mean degree of decidua, representing the extent of specialized endometrial tissue, was comparable between healthy abortus samples (average of +2) and SAB abortus samples (average of +1.8), suggesting a similar level of decidua development in both groups.

In the placental analysis, necrotic areas were identified in two SAB placenta samples, indicating tissue damage and cell death. Conversely, necrosis was not observed in the healthy placenta samples. Table 28 presents the clinical findings of 30 tissue samples with respect to the histological variations observed between SAB and healthy cases.

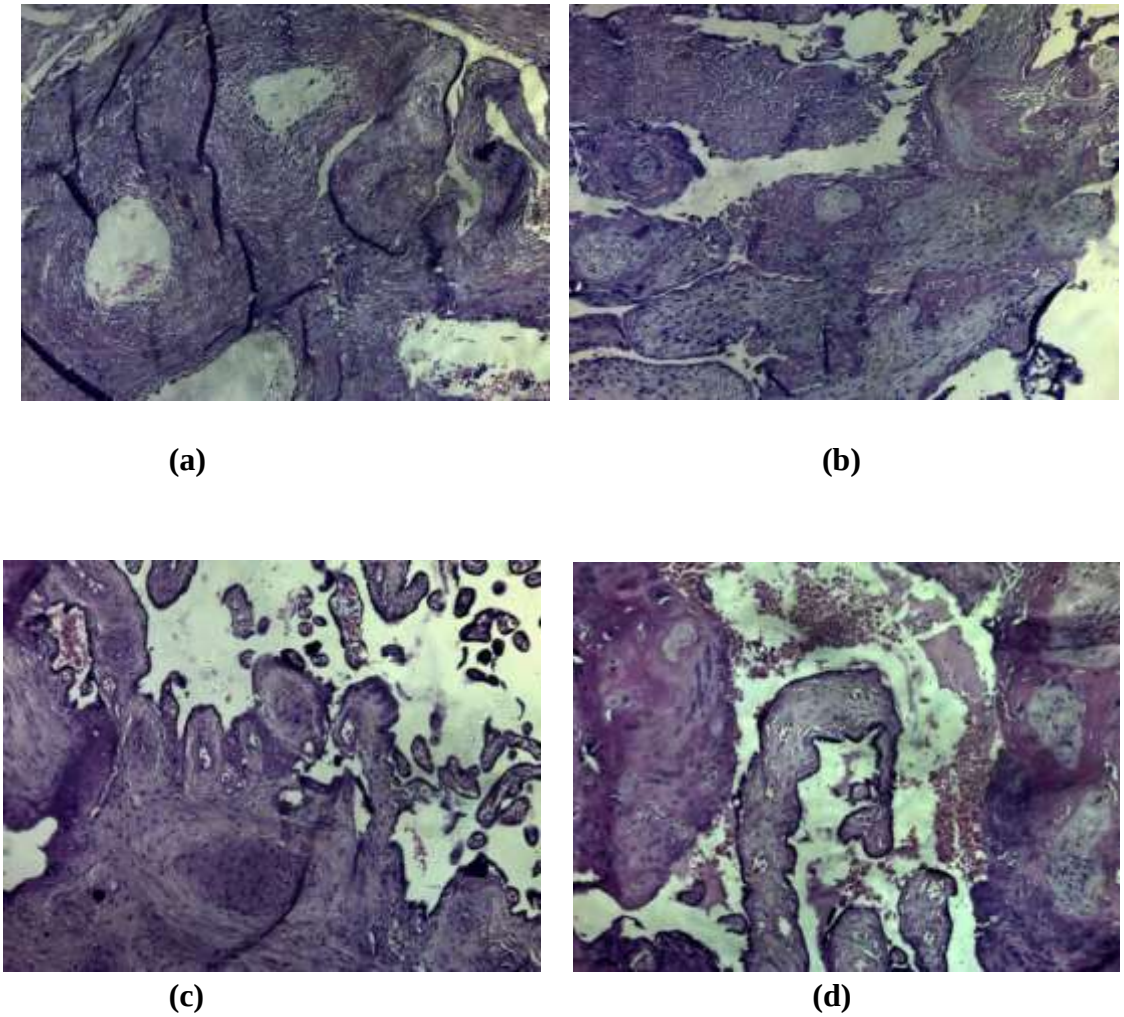


Figure 46: Graphical representation of histology of (A) abortus with spontaneous abortion (B) healthy abortus (C) Healthy term placenta and (D) placenta with history of SAB by H&E staining (100X). The image was captured in Axio Vert.A1 inverted microscope (Carl Zeiss, Oberkochen, Germany).

Table 28 a : Histological Findings of morphology, population, and vascularity of Villi in SAB abortus Cases, healthy abortus, healthy placenta, and term placenta with a history of SAB.

	Villi				
SL. No.	**Enlargement**	**Population**	**Hydrophic change**	**Degeneration**	**Vascular**
1 S ab	NONE	NONE	NONE		NONE
2 S ab	NONE	SMALL	NONE		NONE VASCULAR
3 S ab	NONE	SMALL	YES	MYXOID	NONE
4 S ab	NONE	NONE	NONE		NONE
5 S ab	NONE	NONE	NONE		NONE
6 S ab	NONE	NONE	NONE		NONE
7 S ab	NONE	NONE	NONE		NONE
8 S ab	NONE	NONE	NONE		NONE
9 S ab	NONE	NONE	NONE		NONE
10 S ab	NONE	NONE	NONE		NONE
11 H ab	NONE	NONE	NONE		NONE
12 H ab	NONE	ALL	SIZE	NONE	AVASCULAR
13 H ab	NONE	SMALL ROUND	NONE	MYXOID	AVASCULAR
14 H ab	NONE	SMALLROUND	NONE	RARE	AVASCULAR
15 H ab	NONE	SMALLROUND	NONE	MYXOID	AVASCULAR
16 H ab					
17 H ab	NONE	SMALL FEW	NONE	NONE	AVASCULAR
18 H ab	YES	SMALL,MEDIUM TO ROUND	YES	MYXOID	AVASCU
19 H ab					
20 H ab	NONE	SMALL TO ELONGATED	NONE	NONE	
21 S P	NONE	SMALL TO ELONGATED	NONE	NONE	2+
22 S P	NONE	SMALL TO ELONGATED	NONE	NONE	NONE TO 1+
23 S P	NONE	SMALL FEW	NONE	NONE	AVASCULAR
24 S P					
25 S P	NONE	SMALL TO MEDIUM	NONE	N0NE	3+
26 H P	NONE	SMALL TO MEDIUM	NONE	N0NE	3+
27 H P	NONE	SMALL TO MEDIUM	NONE	N0NE	3+
28 H P	NONE	SMALL TO MEDIUM	NONE	N0NE	3+
29 H P	NONE	SMALL TO MEDIUM	NONE	N0NE	3+
30 H P	NONE	SMALL TO MEDIUM	NONE	N0NE	3+

*__S ab__-spontaneous abortus, **H ab**-healthy abortus, **S p**- placenta with history of SAB, **H p**-healthy placenta

Table 28b: Histological findings of cell proliferation, vascular invasion of EVTs and necrosis, inflammation status in stromal cells in SAB abortus cases, healthy abortus, healthy placenta, and term placenta with a history of SAB.

	Trophoblast proliferation				**Stroma**			
SL. No.	**Polar/nonpolar**	**Proliferation**	**knot**	**Vascular invasion**	**Hemorrhage**	**Necrosis**	**Fibrin**	**Inflammatory**
1 S ab	RARE	NONE	NONE	NONE	NONE	1+	RARE	NONE
2 S ab	1+	NONE						POLYMORPHS ,LYMPHOCYTES
3 S ab	RARE	NONE	NONE		3+	2+	4+	NONE
4 S ab	SHEET	NONE	NONE		1+	NONE	NONE	NEUTROPHILS
5 S ab	SHEET	NONE	NONE	YES, AROUND CONGESTED BLOOD VESSELS	1+	NONE	NONE	LYMPHOCYTES
6 S ab	SHEET	NONE	NONE	YES, AROUND CONGESTED BLOOD VESSELS	1+	NONE	1+	LYMPHOCYTES
7 S ab	NONE	NONE		ONLY IMPLANTATION SITE VESSELS				
8 S ab	NONE	NONE	NONE					NONE
9 S ab	NONE	NONE	NONE	NONE	3+	1+		
10 S ab	NONE	NONE	NONE	NONE				
11 H ab	SHEET	NONE	NONE	NONE	1+	NONE	NONE	POLYMORPHS
12 H ab	NON POLAR	NONE			NONE	NONE	NONE	NONE
13 H ab	POLAR	NONE	NONE		NONE	RARE	2+	NONE
14 H ab	POLAR	NONE	NONE		NONE	NONE	NONE	NONE
15 H ab	POLAR	NONE	NONE		2+	2+	NONE	NONE
17 H ab	POLAR	SHEET	SHEET		2+			
18 H ab	POLAR				1+	1+	2+	
20 H ab	2+ POLAR	NONE	NONE	NONE	NONE	NONE	NONE	NONE
21 S P	POLAR	NONE	NONE		NONE	NECROSIS	2+	NONE
22 S P	POLAR	NONE	NONE		NONE	NECROSIS	2+	NONE
23 S P	POLAR	NONE	NONE		3+		1+	NONE
24 S P		RARE			3+		1+	
25 S P	POLAR	NONE	1+		NONE	NONE	1+	
26 H P	POLAR	NONE	1+		NONE	NONE	1+	
27 H P	POLAR	NONE	1+		NONE	NONE	NONE	
28 H P	POLAR	NONE	1+		NONE	NONE	NONE	
29 H P	POLAR	NONE	1+		NONE	NONE	NONE	
30 H P	POLAR	NONE	1+		NONE	NONE	NONE	

*__S ab__-spontaneous abortus, __H ab__-healthy abortus, __S p__- placenta with history of SAB, __H p__-healthy placenta

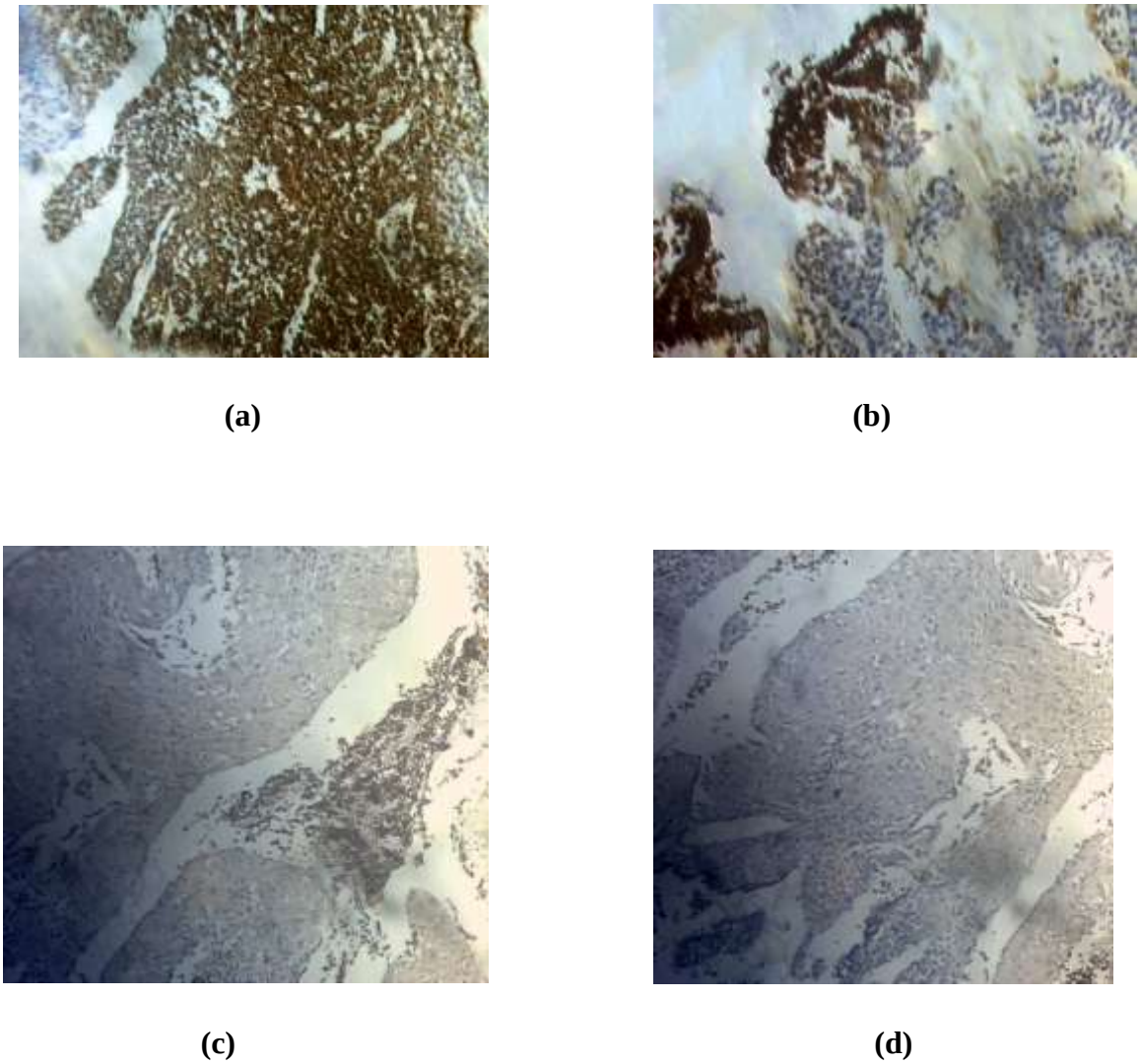
Table 28c : Histological findings of decidua ,endometrial gland along with other observations in SAB abortus cases, healthy abortus, healthy placenta, and term placenta with a history of SAB.

	Decidua	Endometrial gland	Others
SL. No.	**2+**	RARE	AMNIONIC SAC
1 S ab	3+	YES	PERIVASCULAR INFLAMMATION LYMPHO
2 S ab	3+	NONE	TROPHOBLAST SHEETS
3 S ab	3+	NONE	STROMAL VESSELS
4 S ab	3+	YES	
5 S ab	3+	YES	PERIVASCULAR INFLAMMATION_LYMPH
6 S ab		YES	
7 S ab	NO	SECRETORY GLAND ONLY	NO INFLAMMATION
8 S ab	NO		
9 S ab	3+	1+	
10 S ab	3+	NONE	
11 H ab	NONE	NONE	
12 H ab	3+	1+	CALCIFICATION, SOME SCALLOPED ELONGATED VILLI
13 H ab	3+		FIBROTIC VILLI
14 H ab	3+		
15 H ab			
16 H ab	3+		HIGHLY VASCULARISED EDEMATOUS STROMA
17 H ab			
18 H ab			
19 H ab	3+	1+	ONLY NECROSIS
20 H ab	NONE		FIBROTIC VILLI, CALICIFICATION
21 S P	NONE		CHORIO BASAL PLATE+, FIBROTIC VILLI?
22 S P			CHORIO BASAL PLATE+, FEW FIBROTIC VILLI?
23 S P	2+		
24 S P	NONE		
25 S P	NONE		CHORIO BASAL PLATE+
26 H P	NONE		CHORIO BASAL PLATE+
27 H P	NONE		CHORIO BASAL PLATE+
28 H P	NONE		CALIFICATION+ BASAL PLATE+
29 H P	NONE		CALIFICATION 3 +, BASALPLATE+
30 H P	NONE		CALIFICATION 3 +, BASALPLATE+

*__S ab__-spontaneous abortus, __H ab__-healthy abortus, __S p__- placenta with history of SAB, __H p__-healthy placenta

6.3.2 Expression of CD 56 and VEGFA by Immunohistochemistry

Tissues having greater than 50% staining for CD 56 and VEGFA were considered as positive for them. IHC results confirms the expression of both CD 56 and VEGFA was higher in healthy decidua as compared to SAB abortus (Figure 47).



(a) **(b)**

(c) **(d)**

Figure 47: Assessment of expression of CD56 in healthy abortus (A) and SAB abortus tissue (B) VEGF A in healthy abortus (C) and SAB abortus tissue (D) (100X) by Immunohistochemistry. The image was captured in the Axio Vert.A1 inverted microscope (Carl Zeiss, Oberkochen, Germany).

6.4 Discussion

Recent reports have highlighted the correlation between decidual NK cell expression and healthy placentation. Our findings, showing strong expression of CD56 protein in healthy samples, align with studies conducted by Wegman et al.(70) who reported that $CD56^{+}16^{-}$ NK cells secrete cytokines such as M-CSF and GM-CSF, which are believed to promote placental growth (71-72).And a compromise in CD56 protein ,compromises placentation via inadequate interaction of dNK with infiltrating EVTs, impairing the remodeling of maternal spiral arteries and leading to obstetric complications(73-74). However, our data is not in agreement with other findings that showed an increase in the mean number of $CD56^{+}$ cells in spontaneous pregnancy loss as compared to healthy pregnancy(75,76)

The presence of inflammatory cells in SAB abortus further confirms presence of proinflammatory environment, dampening placentation . This finding further supports the association between inflammation and adverse pregnancy outcomes(77,78). Interestingly, despite the similar extent of decidua in both groups, trophoblast invasion was only observed in the SAB group, whereas we expected to see more invasion in healthy abortus. The limitation of studying invasion is the varying sizes obtained via Dilation and curettage (D&C) performed by a clinician of healthy abortus, making it difficult to draw definitive conclusions regarding invasion patterns.

We observed moderate levels VEGFA expression, which along with high CD56, supports favorable vasculature and angiogenesis in healthy decidua (79,80). Conversely, low VEGFA expression in SAB cases indicates compromised vasculature (81,82). The low CD56 content in SAB may contribute to the inability to secrete growth factors and cytokines, which are essential for promoting placental development and maintaining a healthy pregnancy(83,84). Abnormal release of cytokines from the defective placenta can lead to placental insufficiency and systemic endothelial dysfunction, characterized by oxidative stress, increased inflammation, and various genetic and environmental factors (85,86).

Moreover, our findings indicate that the history of SAB may lead to physiological changes in the placenta rather than alterations in cell morphology(87). This is supported by the presence of necrosis in two cases with a history of SAB. Studies report the occurrence of lesions such as fibrinoid necrosis and intimal hyperplasia, involve perivascular lymphocyte infiltration, complement, and immunoglobulin deposits (88,89). It is reported to cause acute atherosis in the distal portions of spiral arteries, specifically at their tips within the decidua basalis, attributing to inadequate spiral artery remodeling(90,91) .In contrast, the abundance of syncytial knots in a healthy placenta confirms their role in placental circulation and nutrient exchange, contributing to a healthy pregnancy(92,93).

Taken together, our data suggest that SAB is associated with a proinflammatory environment characterized by low CD56 expression and compromised vascularization. In contrast, a healthy pregnancy is characterized by high CD56 expression, along with increased VEGF and cytokine production, supporting a favorable vascular environment.

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Salient findings of Chapter VI

Our findings on histology of the placenta suggests that a history of spontaneous abortion (SAB) may induce physiological changes in the placenta rather than alterations in cell morphology, as supported by the presence of necrosis in two cases with a history of SAB. Studies indicate the occurrence of lesions such as fibrinoid necrosis and intimal hyperplasia, involving perivascular lymphocyte infiltration, complement, and immunoglobulin deposits. These lesions are known to cause acute atherosis in the distal portions of spiral arteries, particularly at their tips within the decidua basalis, contributing to inadequate spiral artery remodeling. In contrast, the presence of syncytial knots in a healthy placenta confirms their role in placental circulation and nutrient exchange, which is crucial for a healthy pregnancy.

Collectively, the study confirms that SAB is linked to a proinflammatory environment characterized by low CD56 expression and compromised vascularization. Conversely, a healthy pregnancy is distinguished by high CD56 expression, increased VEGF, and cytokine production, promoting a favorable vascular environment.