CHAPTER IVa

INVESTIGATION INTO KIR-HLA COMBINED GENOTYPE, KIR COPY NUMBER VARIATIONS (CNV), HLA-G ISOFORMS AND KIR-HLA EXPRESSION IN RELATION TO SPONTANEOUS ABORTIONS .

4a.1 Introduction

Spontaneous abortion (SAB), the loss of fetus before twenty weeks of gestation is one of the early pregnancy complications observed in 10 % of clinically confirmed pregnancies(1-3). Its repetitive occurrence for two or more than two times, termed as Recurrent spontaneous abortion (RSAB) is observed in 2-3 % of early pregnancy loss (1-4). Chromosomal abnormalities hormonal abnormalities , uterine anomalies and autoantibodies attribute as major contributing factors of SAB (5-7). Although, anti-phospholipid antibodies - anti-cardiolipin and anti- β2 glycoprotein have been reported to reduce proliferation and invasion of decidua by extra-villous cytotrophoblasts cells (EVTs) thereby prohibiting a healthy placental vasculature in SAB, 40-50 % of the causes are yet to be elucidated (7-9).

The maternal decidua functions as maternal-fetal interface that facilitates maternal-fetal crosstalk leading to embryo implantation and placentation (10-12).The decidua comprises primarily of trophoblast cells of fetal origin, decidual stromal cells and influx of immune cells particularly natural killer (NK) cells (40-70%) along with macrophages and some T cells (13,14). The EVT expresses both HLA-C and non-classical HLA class I molecules, including HLA-E, HLA-F, and HLA-G (14-17). Although HLA-G expression is tissue restricted, it is abundantly expressed in trophoblast cells, mediating tolerance to the semi allogenic fetus and promoting angiogenesis and vascularization (17-19).

HLA-G levels in the maternal serum are reported to correlate positively with pregnancy outcome (20) HLA-G exhibits seven distinct isoforms due to alternate splicing of primary transcript, which are both membrane-bound (HLA-G1, G2, G3,G4) and soluble (sHLA-G: sHLA-G5, G6, G7) (21--23). HLA-G1 is the complete molecule with classical alpha chain structure, which is non covalently associated with the β-2-microglobulin chain, HLA-G5 isoforms closely resembles HLA-G1 in having the three extracellular domains of alpha chain but lacks the transmembrane domain (24-25). All the other isoforms are shorter and lack one or two domains of the heavy chain, either in extracellular (HLA-G1/G2/G3/G4) or transmembrane domain (HLA-G5/G6/G7) as shown in figure 4 of section 1.1.

Signalling by HLA-G is mediated via its interaction with receptors namely, ILTs and KIR2DL4 (26-27). ILT2 and ILT4, expressed on various immune cells (DCs, B cells, NK cells, and T cells) and myeloid cells respectively possess inhibitory signaling motifs (ITIMs) at their cytoplasmic domain (28-29). The interdomain region of ILT2 binds to β2M, while its D1 domain interacts with the α3 domain of HLA-G (27). ILT4 primarily interacts with the α3 domain of HLA-G and is capable of binding both β2M-associated and β2M-free isoforms (30). On the other hand , KIR2DL4, expressed on decidual NK cells, exhibits dual signaling functions through its cytoplasmic tail containing both activation (ITAM) and (ITIMs) motifs. It specifically binds to unique residues Met76 and Gln79in the α1 domain of HLA (31-34)

Notably, HLA-G has cysteine residues at position 42 in the α1 domain of heavy chain and tends to form homodimers via an intermolecular disulfide bond between the cysteine residue (32-33). Dimerization of HLA-G enhances its binding affinity to ILT receptors through geometric configurational changes, exposing more ILT binding sites (34-35). It also allows for simultaneous binding of two receptors to one HLA-G dimer, leading to enhanced transmission of the inhibitory signal, while the close proximity of intracellular domains augments intracellular signaling. KIR2DL4 cannot bind to HLA-G homodimers due to steric clashes caused by the juxtaposition of two protomers (36-37). Soluble HLA-G isoforms, which are mostly monomeric predominantly mediate signaling through KIR2DL4 (38-42). The soluble HLA-G isoforms are encocytised in the endosomes and upon interaction with KIR2DL4 in endosomes it mediates endosomal signaling for the secretion of numerous cytokines and chemokines and growth factors favourable for pregnancy(43-48) .

The KIR multigene family, located within the leukocyte receptor complex (LRC) on chromosome 19q13.4, consists of fourteen functional genes (KIR2DL1–5, KIR3DL1–3, KIR2DS1–5, KIR3DS1) and two pseudogenes (49-53). These genes are categorized into haplogroup-A and haplogroup-B based on their gene content, with haplogroup-B exhibiting variation in the combination of inhibitory and activating genes (54). ClassIb HLA-C molecules expressed on EVT serve as ligands for KIR2DL1/L2/L3/S1/S2(55). And a balance of activating and inhibitory signalling by KIRs is crucial for optimal NK activation, for maintenance of pregnancy (56-57).

HLA-C has two allotypes- HLA-C1 and HLA-C2, depending on the presence of asparagine (HLA-C1) or lysine (HLA- C2) at position 80 in the alpha 2 domain (58-

59). HLA-C2 acts as a cognate ligand for the inhibitory receptor KIR2DL1, delivering a robust inhibitory signal while also binding to the activating allele KIR2DS1(60-65). The inhibitory signaling mediated by KIR2DL1 and HLA-C2 is counterbalanced by activating signaling mediated by HLA-C2 and KIR2DS1(65-68). On the other hand, HLA-C1 serves as the cognate ligand for inhibitory receptors KIR2DL2 and KIR2DL3, although with less effective inhibitory signaling compared to KIR2DL1. Furthermore, KIR2DL2 and, to a lesser extent, KIR2DL3 exhibit some cross-reactivity with C2 ligands (69-70).

Population studies have identified different KIR-HLA genotypes associated with pregnancy outcomes, with the prevalence of specific genotypes varying among populations (71-73). For instance, the Japanese population shows a high prevalence of the AA genotype with HLA-C1 allotypes, while Asian populations tend to have a higher frequency of activating genes in the KIR B haplogroup and HLA-C2 allotypes, which are favorable for successful pregnancies (74-75). The impact of immune factors on pregnancy outcomes has been the focus of several studies (76-78). Maternal KIR2DS1 with paternal HLA-C2 has been reported as a protective factor (79-83) but conflicting results suggest that this combination poses a risk (84-86) . Lower levels of KIR2DL1 and KIR2DS1 in peripheral NK cells have also been associated with adverse pregnancy outcomes (87-90).This highlights the co-evolutionary relationship between KIRs and HLA class I molecules , under balancing selection for reproduction and survival (91-93).

While a balanced activation of NK cells via maternal KIR2DL1/S1 with fetal HLA-C is well documented for mother-neonate pairs (94-95), studies on first trimester abortus are limited (96-97). HLA-C expression study in abortus tissue would provide an insight into NK activation in SAB. In addition, given the limited availability of data on HLA-G isoforms,KIR2D-HLA-C genotype in early abortus, it was pertinent to investigate HLA-G isoforms, KIR-HLA-C combined genotypes, KIR CNVs and their expression in early pregnancy loss. This study is particularly novel considering the distinct genetic makeup of individuals from the northeastern part of India and to the best of our knowledge no previous data on HLA-G isoforms in the context of early pregnancy exists.

4a.2. Materials and methods

4a.2.1. Study site, study design, and participants

A hospital-based case-control study was designed with 214 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. The SAB history group (N=85) included age-matched obstetric participants with one or more fetal losses before 20 weeks of gestation, while the control group (N=129) included women with at least one live birth.

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.

Participants got screened to be enrolled in the study during their visit to clinic OPD for routine checkups. Effort was made by the clinical staff to collect sample left over from routine examination. Peripheral blood samples collected were used for genotyping, copy numbers variation (CNV) and serological study.

56 women were pregnant at the time of sample collection (N=24 SAB history participants , N= 32 healthy history participants) and went to full term delivery. Term placentas were collected for retrospective study. From among these women, cord blood could be collected from 46 neonates for mother-neonate pair combined study Cord blood from 10 neonates (of healthy group) were excluded from study due to incomplete information.

We included an additional group of 24 women who experienced spontaneous miscarriage between 12-15 weeks of pregnancy. Samples were collected from the products of conception, specifically from the spontaneous abortus, for these participants. Additionally, we recruited 17 women who underwent voluntary medically induced termination of pregnancy at the same gestational age. Samples from the conceptus obtained from these women served as control abortus samples.The conceptus product were collected by the clinical staff only after obtaining written informed consent of the participants and 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 hospitals.

4a.2.2 Extraction and Quality Assessment of DNA, RNA, and Protein from studied samples.

4a.2.2.1 DNA isolation from peripheral blood and umbilical cord blood

Study participants were enlisted during their visit to the clinic for routine checkup. Briefly 1ml of peripheral blood sample were collected by the clinical staff in tubes containing sodium EDTA anticoagulant. Blood samples were centrifugated at 1,500 x g for 10 minutes at at 4°C using . Supernatant plasma was separated and transferred to sterile 1.5 ml Eppendorf tubes in 100 µl aliquots . Aliquoted plasma samples were labelled and stored at −20 °C for further use . Meanwhile, the pellet containing RBCs were used for genomic DNA isolation by use of QIAamp, DNA Mini Kit (Qiagen,Hilden, Germany) following manufacturer's instruction . The quantity and quality of the isolated genomic DNA were assessed by Nano-VueTM plus spectrophotometer (GE Healthcare, Little Chalfont, United Kingdom.

one millilitre umbilical cord blood was collected by clinical staff in EDTA vials immediately after delivery. Samples were mixed by inversion 8–10 times after being drawn and then stored at -80°C until DNA isolation by the above-mentioned protocol.

4a.2.2.2 Isolation of RNA, cDNA and Protein from tissue samples

Placenta and abortus tissue samples were collected in 1.5 ml RNA later solution (Ambion, United States) and homogenized using a hand-held tissue grinder (G-Biosciences, United States). DNA, RNA, and protein were extracted using Allprep DNA/RNA/protein kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was converted to cDNA using the High capacity cDNA reverse transcription kit (Invitrogen, Applied Biosystems, Foster City, United States) according to the manufacturer's instructions. The quantity and quality of the isolated genomic DNA, cDNA and protein were assessed by Nano-VueTM plus spectrophotometer (GE Healthcare, Little Chalfont, United Kingdom).

4a.2.3. PCR based KIR genotyping and allotyping of HLA-C

4a.2.3.1 HLA-G isoform transcript study

PCR reaction was standardized for exon 2, exon 3 and exon 4 in a Veriti PCR (Applied Biosystems, United States) to amplify the exons of the HLA-G gene using cDNA as the template. Primer sequences were taken as described by Hviid et al. (Table 1). After optimization of PCR conditions, expression study of HLA-G isoforms was carried out in eighty HNSCC tissue samples in a total volume of 15 µL using 2 µL of template cDNA, 1X PCR buffer, 1.5mM MgCl2, 200µM of each deoxynucleotide triphosphate (dATP, dTTP, dCTP, dGTP), 0.5µM of each primer and 1.5U of Taq DNA polymerase. To determine whether the isoforms are membrane-bound or soluble, PCR was performed using exon 5 specific primer sequences as described by Van and Ober (98).The annealing temperature and PCR cycle conditions for amplification of exons are presented in **Table 6**. Isoforms of HLA-G were classified according to the presence the exon 2, exon 3, exon 4 and exon 5 as described by Amiot and Samson (99). The specificity of band amplification was determined by sequencing of 15% of the amplicons (Appendix 2).

Table 6 : Amplification Conditions for HLA-G Exon by PCR

Exons	PCR program
Exon 2	Initial denaturation of 94°C for 2 mins 30 cycles of 94°C for 30 secs, 64°C for 30 secs and 72°C for 1 min Final extension of 72°C for 10 mins Hold at 4°C
Exon 3	Initial denaturation of 94°C for 2 mins 35 cycles of 94°C for 60 secs, 61°C for 1 min 30 secs and 72°C for 2 mins Final extension of 72°C for 10 mins Hold at 4°C
Exon 4	Initial denaturation of 94°C for 2 mins 35 cycles of 94°C for 30 secs, 57°C for 30 secs and 72°C for 1 min Final extension of 72°C for 10 mins Hold at 4°C
Exon 5	Initial denaturation of 94°C for 5 mins 35 cycles of 94°C for 60 secs, 60°C for 60 secs and 72°C for 2 mins Final extension of 72°C for 10 mins Hold at 4°C

4a.2.3.2 KIR genotyping in cohort

Genotyping of 16 KIR genes was done in DNA of template by Polymerase chain reaction -sequence specific primming approach. PCR reaction was carried out in a total volume of 15 µL using 2 µL of template DNA, 1X PCR buffer, 1.5mM $MgCl_2$, 200µM of each deoxynucleotide triphosphate (dATP, dTTP, dCTP, dGTP), 0.5µM of each primer and 1.5U of Taq DNA polymerase as described previously. Sequences of primers used is provided in Table 7. Thermal cycling was performed in a Veriti PCR (Applied Biosystems, USA) with an initial hotstart step at 94°C for 4 minutes, followed by denaturation at 94°C for 30 seconds, annealing at 68°C for 30 seconds, and extension at 72°C for 30 seconds. These steps were repeated for 35 cycles, with a final extension at 72°C for 7 minutes, and a hold step at 15°C. KIR polymorphism and

haplotypes were determined by analyzing the presence or absence of PCR amplicons of specific band size as mentioned in Table 7.

Amplicons were run in electrophoretic chamber in 3% agarose gel with ethidium bromide .DNA ladder of adequate size was run along with the samples , to size the fragment of PCR amplicons. Each KIR genes were determined to be present by presence of amplicon band of the respective size. The validation of the PCR amplification was done using framework KIR genes as positive control for each PCR reaction (100).

Table 7 : Primer Sequences and band sizes of amplicon used for KIR polymorphism

Genes	Primer sequences	Amplicon size (bp)
	Group 1	
KIR3DL3	Forward GGAGCTTGTTTGACATTTACCATCT	207
	Reverse TGACAGAAACGGGCAGTGGGTC	
KIR2DS4del	Forward CTTGTCCTGCAGCTCCATCTATC	176
	Reverse GAGTTTGACCACTCGTAGGGAGC	
KIR2DL1	Forward GCARTGTTGGTCAGATGTCATGTTTGAAC	152
	Reverse AGGTCCCTGCCAGGTCTTGCG	
KIR2DS4	Forward TTCCTGGCCCTCCCAGGTCAC	87
	Reverse AAGGAAGTGCTCAAACATGACATCC	
	Group 2	
KIR2DL3	Forward TCTTCTTTCTCCTTCATCGCTGGTGCTG	533
	Reverse-a CCTGCAGGCTCTTGGTCCATTACAA	
	Reverse-b CTGCAGGCTCTTGGTCCATTACCG	
KIR2DL5	Forward TCCTGCAGCTCCAGGAGCTCATT	193
	Reverse CGGGTCTGACCACTCATAGGGT	
KIR2DL2	Forward AGGGAGGGGGAGGCCCATGAAT	171
	Reverse AACAAGCAGTGGGTCACTCGAGTT	
KIR2DL4	Forward GTATCGCCAGACACCTGCATGCTG	91
	Reverse CACCAGCGATGAAGGAGAAAGAAGGGA	
	Group 3	
KIR3DL2	Forward AGGCCCATGAACGTAGGCTCCG	150
	Reverse GGTCACTTGAGTTTGACCACACGC	
KIR2DS2	Forward GCACTTCCTTCTGCACAGAGAGGGGAAGTA	130
	Reverse TCTGTAGGTCCCTGCAAGGTCTTGCATC	
KIR2DS5	Forward CTGCACAGAGAGGGGACGTTTAACC	95
	Reverse GTCATGCGACCGATGGAGAAGTTGC	
KIR3DS1		80

	ForwardTACATGTTCACTCCAAGGCCAATTTCTCCATCGGTT	
	Reverse TCACTTCTAAATCTGTAGGTCCCTGCAAGGGCAC	
	Group 4	
KIR3DL1	Forward ATCCTCCTCCTCTTCTTTCTCCTTCATCT	565
	Reverse CTCGCTGTTGGCTGTTCTGTTC	
KIR2DS3	Forward GTGACCTTGTCCTGCAGCTCCT	
	Reverse GAGCCGAAGCATCTGTAGGTTCCTCCT	170
KIR2DS1	Forwarda GCARTGTTGGTCAGATGTCATGTTTGAAC	
	Reverse-a TAGGTCCCTGCCAGGTCTTGCC	153
	Reverse-b TAGGTCCCTGCCAGGTCTTGCT	

4a.2.3.3 Copy number variation study of KIR2DL1 and KIR2DS1

TaqMan Copy Number Assay (Applied biosystem,cat no-4403326) was used to test KIR2DL1 and KIR2DS1 genes in the 214 study cohort .The assay was performed according to the manufacturer's protocol. FAM dye-labeled MGB probe was used for CNV assays and VIC dye-labeled TAMRA probe was used for the RNaseP H1 RNA reference assay. For each reaction ,20 ng DNA template was used with TaqMan Genotyping Master-mix and run on the Applied Biosystems QuantStudio 3 system **.**The PCR protocol involved a 10-minute step at 95°C followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Real-time PCR results were analyzed using SDS v2.3 software with an auto baseline and manual Ct threshold of 0.2. Copy Caller software was used to perform sample copy number analysis by relative quantification (ΔΔ CT) using a comparative CT method.

4a.2.3.4 HLA-C allotyping

Multiplex real time PCR genotyping platform was used to determine the HLA class I ligands. Exon 2 and 3 of HLA-C loci were amplified using locus specific primers (101) . PCR reaction was carried out in a total volume of 15 µL using 2 µL of template DNA, 1X PCR buffer, 1.5mM $MgCl_2$, 200µM of each deoxynucleotide triphosphate (dATP, dTTP, dCTP, dGTP), 0.5µM of each primer and 1.5U of Taq DNA polymerase as described previously. Thermal cycling was performed in a Veriti PCR (Applied Biosystems, USA) with an initial hotstart step at 95°C for 7 minutes, followed by denaturation at 95°C for 30 seconds, annealing at 65°C for 1 minute, and extension at 72°C for 2 minutes. These steps were repeated for 45 cycles, with a final extension at 72°C for 7 minutes, and a hold step at 4°C.

PCR amplicons of the respective size were extracted by Qiaquick Gel Extraction kit (Qiagen, Hilden, Germany) and run in Real-time PCR platform ,Quant Studio 3 Time PCR System (Applied Biosystems, USA). Fluorescent probes and primers targeting ligand specific region for typing HLA variants. A non-polymorphic region of the amplicon was targeted as internal control. Samples were run in 96 well plate format . Constituents of master mix and the cycling conditions for Real-Time PCR and is presented in Table 8 and 9. The Ct values of each reaction determined presence or absence of targeted HLA. Molecular grade distilled water was used as non-template control (NTC) in the RT setup.

Table 8 : Master mix composition for HLA class I ligands RT PCR

Mastermix (10µL reaction volume)	
Composition	Concentration/reaction
2xTaqman universal PCR master mix	1x
Internal control primer	200nm
Internal control probe	150nm
HLA-C primer	200nm
HLA-C probe	150nm
DNA	5-15ng
H_20	Adjusting volume

Table 9: PCR programme for SSP -real time PCR for HLA class I ligands

PCR programe
95°c for 10 min
60 cycles for 95°c for 15 sec and 60°c for 1 min

4a.2.3.5. Transcript expression of *KIR 2DL1, KIR2DS1* and HLA-G in abortus and placenta.

Expression of KIR-2DL1, KIR-2DS1 and HLA-G was studied in term placenta and abortus samples. Gene expression study was performed using SYBR green-based assays (Applied Biosystems, USA) on QuantStudio 3 Time PCR System (Applied Biosystems, USA). *GAPDH* was used as the endogenous control for normalization of

expression levels. 10 term placenta from women with history of minimum two live births and without any medical anomalies were used as the calibrators for mRNA quantification by the comparative CT method. Primer sequences to amplify the genes KIR2DL1,KIR2DS1 have been mentioned above in Table 7**.** RT^2 Primer Assay were used for HLA-G (PPH23053B, Qiagen, Hilden, Germany) and GAPDH (PPH00150F, Qiagen, Hilden, Germany) respectively.

Real-time PCR was carried out in 10µL of reaction volume consisting of 1X Sybr green PCR master mix, 0.4 µM of each primer, 100ng of cDNA and water to adjust the volume. Cycling conditions for Real-Time PCR is presented in Table 1. The relative expression of the target genes was determined using the formula $2^{-\Delta\Delta ct}$ method which was derived as follows-

$\Delta\Delta C_t = \Delta C_t$ of patient – ΔC_t of the calibrator

where $\Delta C_t = C_t$ of target gene – C_t of endogenous control (GAPDH)

RQ (Relative quantification) = $2^{-\Delta\Delta Ct}$ which represents the fold change in expression of the target gene relative to the calibrator

Targetted genes were successfully amplified without any nonspecific amplification as presented in AppendixI.

4a.2.4. Expression of KIR2D , HLA-C, HLA-G proteins by ELISA

Levels of KIR-2D ,HLA-C and HLA-G were quantified by Indirect ELISA by use of primary antibodies raised in mouse -monoclonal KIR-2D (ThermoFisher Scientific) , HLA-C (Sigma-Aldrich, Merck,Germany) and HLA-G HLA (Invitrogen, Applied Biosystems, Foster City, CA, USA . Goat anti-mouse HRP conjugated antibody (BD Biosciences, United States) was used as the secondary antibody. Samples were run in triplicates and Optical Density (OD) was recorded in the Varioskan Lux multimode microplate reader (Thermo Fisher Scientific, United States).The mean optical density plus three standard deviations of the negative controls was used as the cut-off reading. The total protein levels were expressed as the ratio designated as arbitrary unit or AU. AU was calculated by dividing mean optical density of the sample by mean optical density plus three standard deviations of negative controls. Expression was further checked for β-actin protein using the ß-actin primary antibody (Invitrogen, Applied

Biosystems, Foster City, United States) as an internal control in the studied tissue samples.

4a.2.5. Validation of KIR2D and HLA -C expression by Immunohistochemistry.

Tissue sections were prepared from FFPE sections and processed for IHC as per standard protocol. HLA-G monoclonal antibody

Protein expression of KIR2D,HLA-C and HLA-G were validated by Immunohistochemistry (IHC) technique. Tissue sections were prepared from FFPE sections and processed for IHC as per the standard protocol. Primary antibodies each for KIR2D, HLA-C and HLA-G , as mentioned above) were used to along with Goat anti-mouse HRP conjugated secondary antibody for the experimentation Tissues showed 50% or more staining of all the proteins were considered as positive for all the three proteins.

4a.2.6. Statistical analysis

For statistical analysis of the data, XLSTAT software student version was used. Correlation analysis was performed between the expression of proteins. Student's t-test were used for comparison between the mean values. A p-value < 0.05 was considered statistically significant.

4a.3. Results

3.3.1 Clinical and demographic profile of the obstetric participants

The study cohort comprised individuals from three linguistic affinities namely Indo-European (IE), Austro-Asiatic (AA), and Tibeto-Burman (TB) populations in the reproductive age years with a median age of years. The demographic profile, obstetric history and clinical profile of participants has been shown in (Table 10).

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.

Among the participants included in the study cohort, 39% were individuals who experienced SAB, while 7% were individuals who experienced RSAB.

Table 10 : Demographic profile and Obstetric history of study cohort

Characteristic	**Total participants (N=214)**
Ethnicity	**Frequency**
IE	156(72.89%)
AA	32(14.95%)
TB	26(12.14%)
Obsteric history	
Healthy	129(60.28%)
SAB	85(39.71%)
Full term delivery	
Healthy history participants	40 (18.69%)
SAB history participants	16(7.27%)
Miscarriage	
MTP control	17(7.94%)
SAB miscarriage	24(11.21%)

4a.3.2 HLA-G and its association with Early Pregnancy Failure

The study utilized early abortus samples from cases of spontaneous abortion as well as term placenta samples with a history of spontaneous abortion, in comparison to their respective control groups, for HLA-G expression study.

4a.3.2.1 Characterization of HLA-G isoforms early abortus and placenta tissue.

Investigation of HLA-G isoforms revealed membrane bound isoforms (G1,G2,G3 and G4) were predominant as compared to the soluble form (chi-square, p=0.05) in both abortus and in placenta (chi-square, p=0.05). (Figure 10a,10b).

When the membrane bound isoforms were examined individually in early abortus , it was noted that proportion of HLA-G3 isoforms were significantly higher in SAB as compared to the healthy MTP abortus (z-test for two proportions /Two-tailed test, p=0.003). Presence of soluble isoforms: HLA-G5, HLA-G6, and HLA-G7 were noted in healthy abortus, while only HLA-G5 were detected in SAB abortus (Table 12)

In the analysis of placenta samples, it was observed that HLA-G1 and HLA-G4 isoforms were more prevalent in the healthy placenta as compared to the SAB history placenta. Conversely, the frequencies of HLA-G2 and HLA-G3 were found to be comparable between both the groups (Figure 2b). Furthermore, there was a significant difference in the proportion of HLA-G4 (z-test for two proportions / Two-tailed test, p=0.001) between the healthy and SAB history placenta samples .Minimal presence of HLA-G soluble isoforms was noted in placenta as showed in as shown in (Table 13)

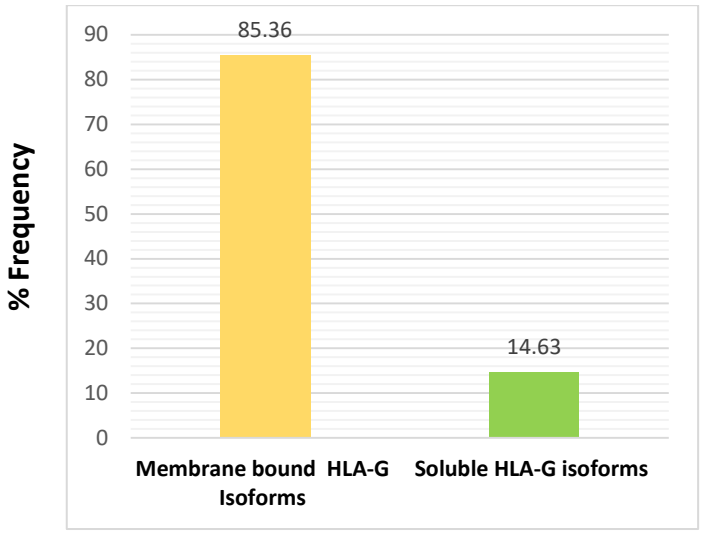


Figure 10 : Frequency of HLA-G isoforms in abortus tissue

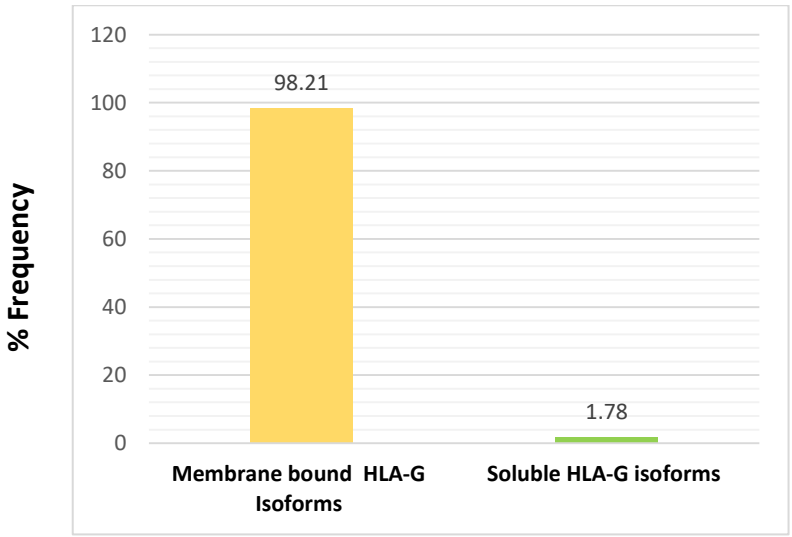


Figure 11 : Frequency of HLA-G isoforms in placenta .

Table 12: Proportions of HLA-G isoforms in placenta tissue

	HLA-G1	HLA-G2	HLA-G3	HLA-G4	sHLA-G5	sHLA-G6	sHLA-G7
Healthy Placenta (n=40)	25	15	12.5	45	2.5	0	0
SAB placenta (n=16)	12.5	25	37.5	25	0	0	0

Table13: Proportions of HLA-G isoforms in abortus tissue

	HLA-G1	HLA-G2	HLA-G3	HLA-G4	sHLA-G5	sHLA-G6	sHLA-G7
Healthy Abortus (n=17)	17.60	5.90	29.40	23.50	5.90	5.90	11.80
SAB Abortus (n=24)	3.30	0	50	8.30	4.20	0	4.20

4a.3.2.2. Docking study of HLA-G3, HLA-G4 isoforms

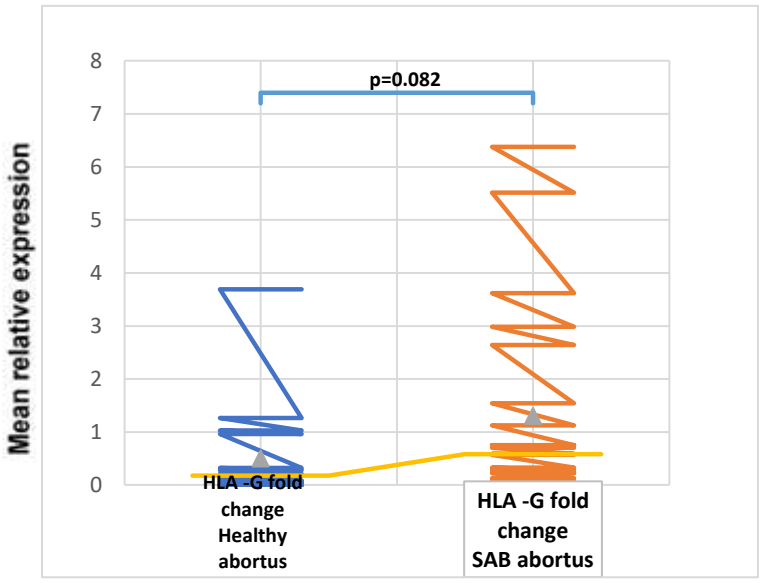
Based on the understanding that both isoforms of HLA-G, namely G3 and G4, possess an alpha 1 domain, it was anticipated that their binding and signaling would primarily occur through the KIR2DL4 receptor unlike ILT receptors which require an alpha 3 domain for binding.

Docking of HLA-G and KIR2DL4 receptor was performed to study their interaction , with a focus on different alpha units of HLA-G3 and HLA-G4. The binding energy in HLA-G4/KIR2DL4 complex was higher (16 kcal/mol) than HLA-G3/KIR2DL4 complex (14.3 kcal/mol),although it did not reach significance level **.**

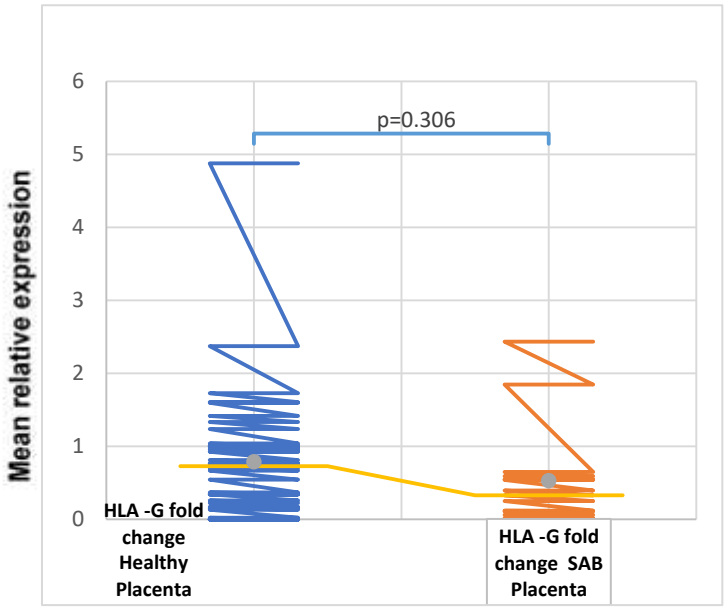
4a.3.2.3. Expression of HLA–G in tissue samples

SAB abortus had higher HLA-G transcript compared to healthy abortus. However, the protein levels were comparable between both SAB abortus and healthy abortus (Figure 12).In the placenta, the transcript level expression of HLA-G was found to be comparable between the SAB history placenta and healthy placenta.However, the

healthy placenta exhibited a higher expression of HLA-G protein as compared to the SAB placenta, although this difference did not reach statistical significance (Student's t-test, p=0.07) (Figure 13). Logistic regression analysis confirmed that HLA-G is a promising predictive marker for a healthy outcome of pregnancy with a p-value of 0.01 and AUC of 0.8

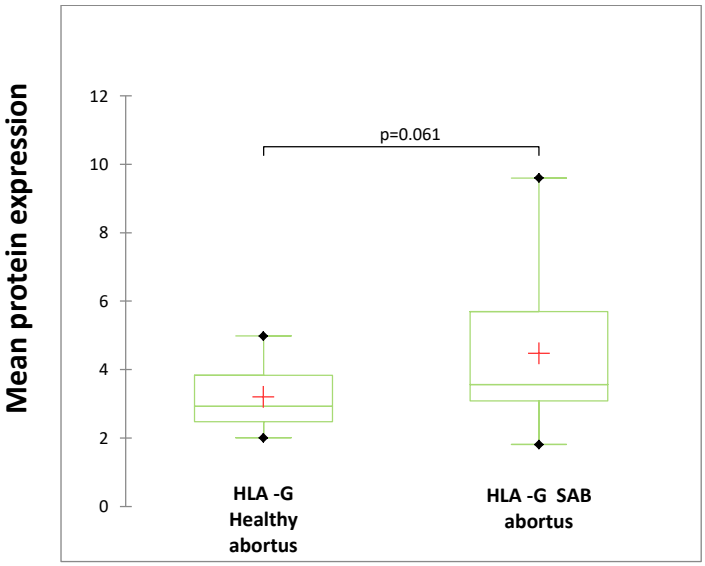


(a)

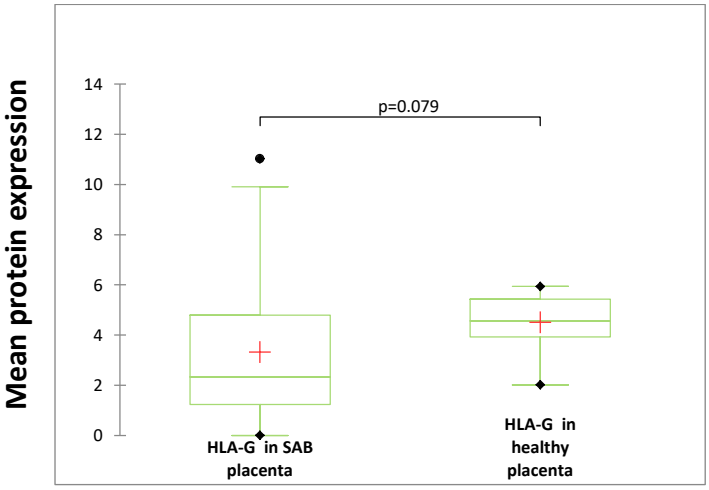


(b)

Figure 12 : Transcript expression of *HLA-G* in (a) abortus and in (b) placenta.Values indicate fold change. Mean relative expression was calculated as fold change.



(a)

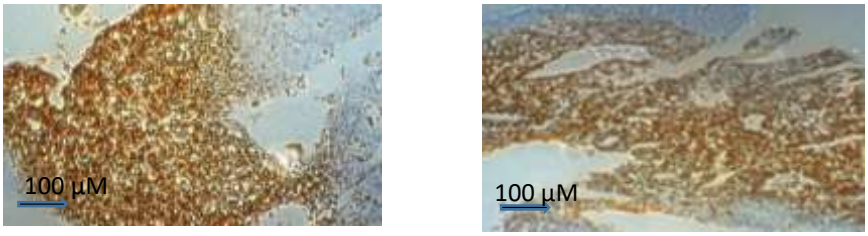


(b)

Figure 13 A and B: Assessment of HLA-G expression in abortus tissue and placenta tissue. Protein expression was calculated as the arbitrary unit (AU). A $p<0.05$ was considered as statistically significant.

4a.2.2.4. Validation of expression of HLA-G by Immunohistochemistry

IHC results confirmed the presence of HLA-G in tissue samples. Tissues having greater than 50% staining for HLA-G were considered as positive for HLA-G. Increased HLA-G protein was seen in healthy abortus (Figure 14)



(a) (b)

Figure 14 **:** Assessment of expression of HLA-G in healthy abortus (A) and SAB abortus tissue (B) (100X) by Immunohistochemistry. The image was captured in the Axio Vert.A1 inverted microscope (Carl Zeiss, Oberkochen, Germany).

4a.3.3 KIR-HLA-C study in relation to SAB.

4a.3.3.1. KIR-2DL1/2DS1– HLA-C combined genotype in mother neonate pairs

A total of 56 women had full-term delivery in our study, as mentioned earlier. Among them, 46 mother-neonate pairs were recruited for KIR-HLA-C combined analysis, comprising 22 pairs with a history of SAB and 24 pairs belonged to healthy group.

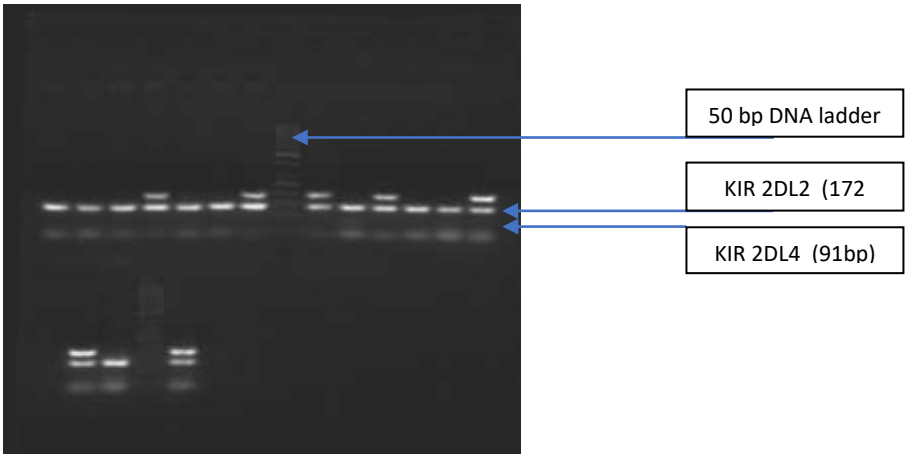
Maternal KIR genotype in conjunction with neonate HLA-C allotypes were compared between SAB history group and healthy group. All 16 KIR genes were amplified successfully by PCR –SSP approach (Figure 15).

All the SAB history mothers (n=22) had KIR2DL1+/S1+ genotype. Wherein , healthy history mothers had KIR2DL1+/S1+ (n=13) as well as KIR2SDL1+/S1- (n=10). This indicated towards higher prevalence of activating KIR2DS1 in healthy mothers(chi-square, p=0.003) as compared to SAB history mothers(Table 14)

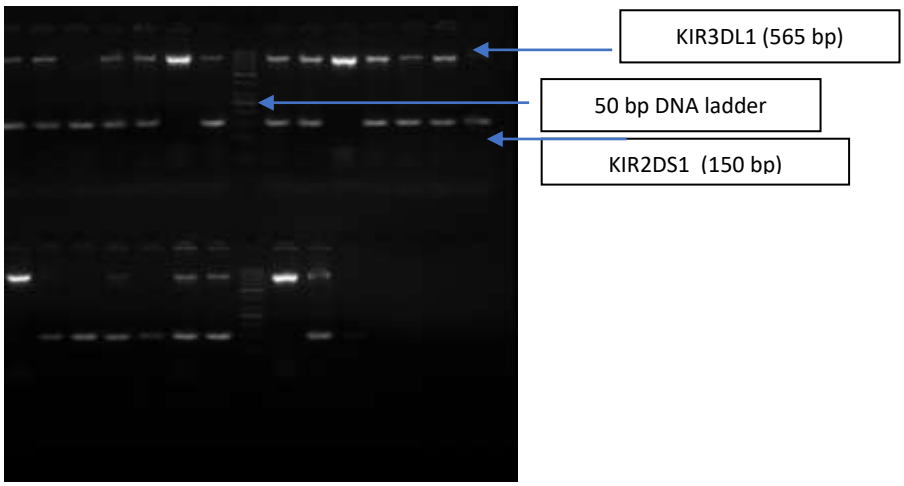
On examining the HLA-C allotype of neonates , we observed that HLA-C2 allele predominated in neonate belonging to healthy mothers than SAB mothers (chi-square , p=0.001) . In line with this ,C2C2 genotype was predominant in healthy neonates while C1C2 and C1C1 were predominant in neonates of SAB group mothers (Table 15).

Combined maternal KIR and neonate HLA-C data suggests that in case of SAB group, the maternal KIR2DL1/S1 get minimal cognate ligand HLA-C2 from neonates, attributing to suboptimal NK signalling .In case of healthy group higher maternal KIR2DS1 gets adequate cognate ligand HLA-C2 in neonates to activate NK cells and trigger downstream signalling (Table 16). However, whether the activation of NK in healthy participants via KIR2DS1 and HLA-C2, was overactivation, needs to be examined.

Given the dynamic nature of NK cell populations and their changing receptor expression during pregnancy we were tempted to examine the expression of KIRs and their genotypes in early abortus samples and term placenta.



(a)



(b)

Figure 15 : Agarose gel showing amplicons of KIR2DL4,KIR2DL2 (a) and KIR3DL1 and KIR2DS1 (b) in the study cohort

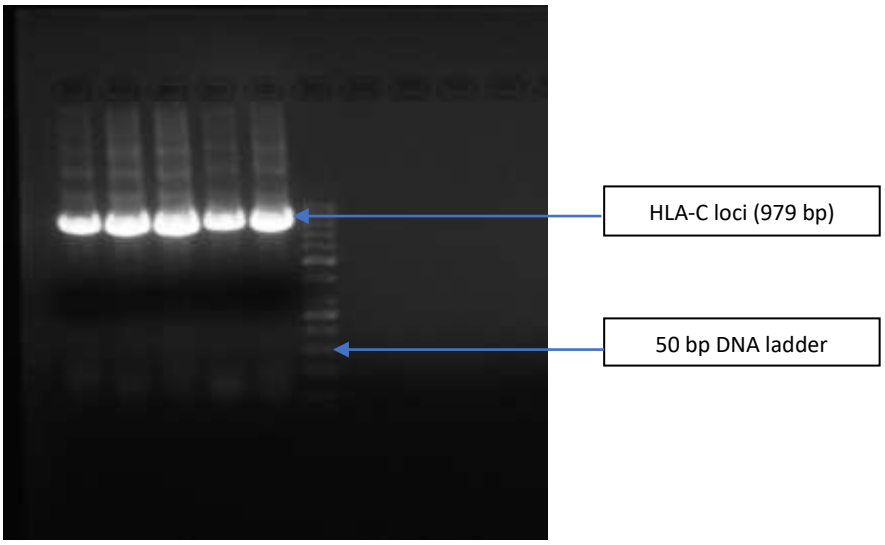


Figure 16a : Agarose gel showing the DNA bands of HLA-C loci (exon 2 & 3). 50 bp ladder was loaded in well no. 6.

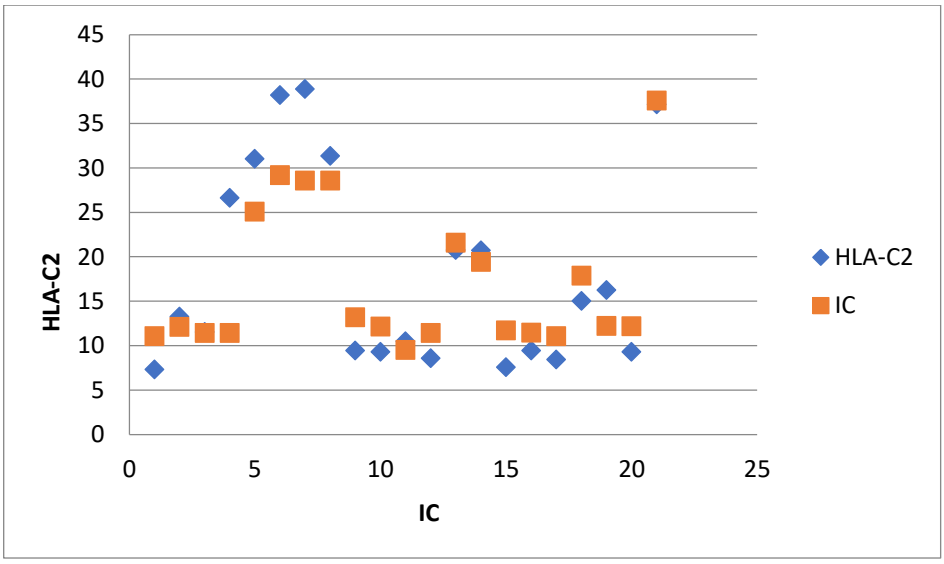


Figure 16b : The C_T values indicating presence or absence of the HLA-C2 in study cohort. Samples that carry the HLA-C2 ligand formed a distinct cluster based on their C T values. These samples showed similar levels of amplification for both the internal control (IC) and the specific reactions targeting the KIR ligands.Samples that lacked HLA-C2 ligand did not amplify ,however they showed amplification in the IC reaction.

Table 14 : Frequency of combined KIR2DL1/S1 in healthy and SAB mothers

Gene combinaton	**frequency (%)**	
	Healthy mothers (n=22)	**SAB history mothers (n=24)**
2DL1+/S1+	59.09	100
2DL1+/S1-	-	-
2DL1-/S1+	45.45	-

Table 15 : Frequency of HLA-C allotypes in neonates of healthy and SAB mothers

Gene combinaton	**frequency (%)**	
	Healthy mothers (n=22)	**SAB history mothers (n=24)**
C1C1	13.63	25
C1C2	18.18	37.5
C2C2	77.27	29.16

4a.3.3.2. KIR Genotype, its expression in conjunction of HLA-C in early abortus tissue

We further examined the KIR genotype, with a particular focus on KIR2DL1 and KIR2DS1, and their expression in relation to HLA-C in cases of early SAB abortus tissue.

The frequency of KIR2DL1 was found to be significantly higher (chi-square test, p=0.02) in SAB abortus compared to the healthy abortus. The distribution of the KIR2DL1+/S1+ genotype was similar between the SAB and healthy groups (Table 17). However, the KIR2DL1+/S- genotype was more prevalent in the SAB group. In

contrast, the presence of the KIR2DS1+/L1- genotype did not show a significant difference between the healthy and SAB abortus groups, with only a slight variation

observed in the frequency of KIR2DS1+ between the two groups (Table 17).

The transcript level of the KIR-2DL1 gene was found to be higher in SAB abortus compared to healthy abortus . Additionally, KIR2DS1 was also expressed in SAB abortus, however at a lower level than KIR2DL1. In contrast, KIR2DS1 expression was downregulated in the healthy group (Figure 17). The protein levels of KIR2D were found to be comparable between healthy abortus and SAB abortus cases. (Figure 18) However, the levels of HLA-C were significantly higher in healthy abortus compared to SAB abortus (Figure 19).

Higher iKIR2DL1 content in conjunction with low HLA-C levels suggested suboptimal interaction between KIR -HLA and poor activation of NK in SAB abortus.

Table 16 : Frequency of combined KIR-HLA-C genotype in mother neonate pairs .

Obstetric Population	**HLA-C allotype frequency (%) in neonates**		**Combined maternal KIR genotype - neonate HLA-C allotype frequency (%)**								
	C1	**C2**	**KIR2DL1+C2+**	**KIR2DL1+ KIR2DS1 +C2**	**KIR2DL1+ KIR2DS1+ C1+**	**KIR2DL2+C1+**	**KIR2DL3+C1+**	**KIR2DL1 +C1+**	**KIR2DL1+ KIR2DS1+ C1+**	**KIR2DL2+ C2+**	**KIR2DL3+ C2+**
SAB history mother (n=24)	87	56	72.73	45.45	22.72	50	4.55	27.27	22.72	4.54	45.45
Healthy mother (n=22)	22.72	86.36	54.17	66.66	63.63	4.17	63.63	63.63		50	4.16

Table 17 : Frequency of combined KIR2DL1/S1 in healthy and SAB abortus .

Gene combinaton	**frequency (%)**	
	Healthy abortus (n=17)	**SAB abortus (n=24)**
2DL1+/S1+	52.94	45.85
2DL1+/S1-	23.52	54.1
2DL1-/S1+	76.47	45.85

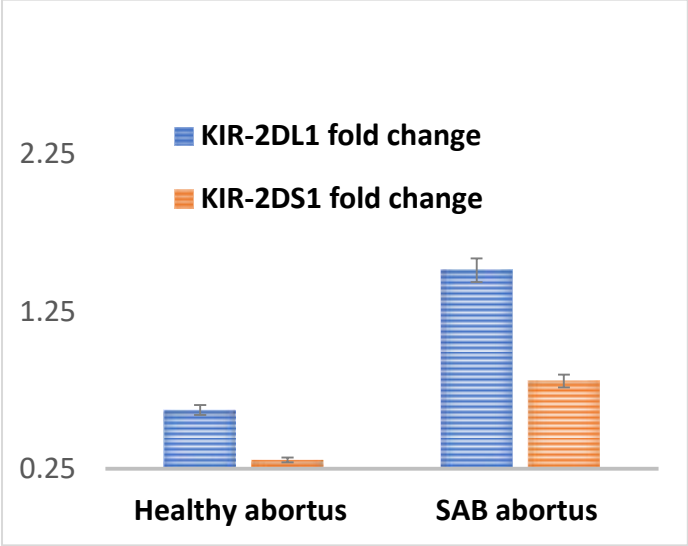
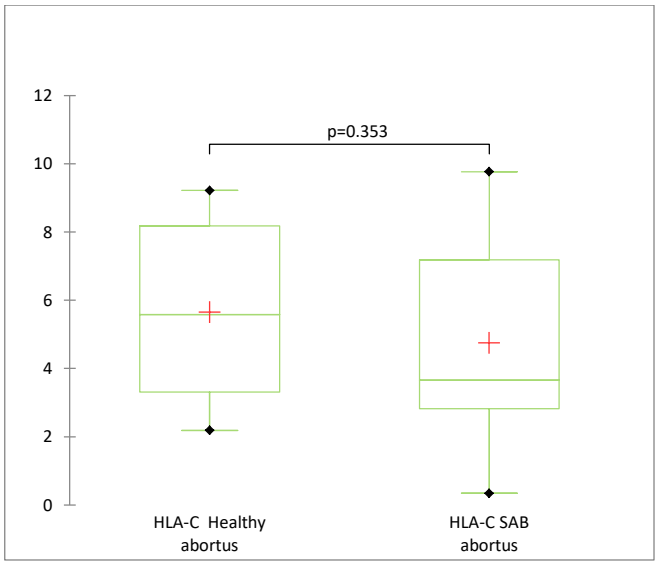
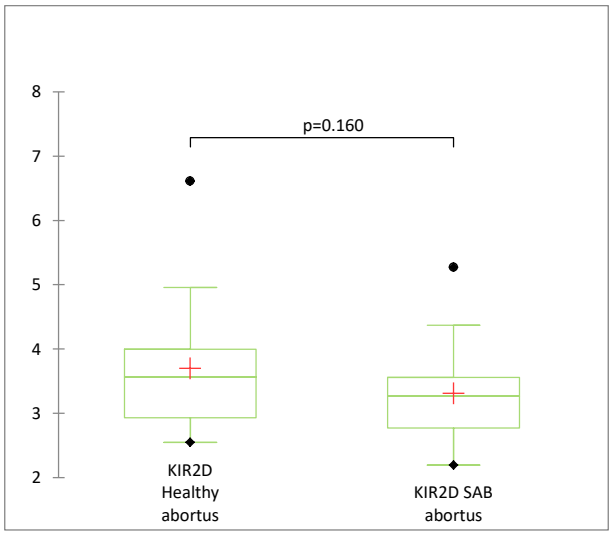


Figure 17 : Transcript expression of *KIR2DL1* and *KIR2DS1* in abortus .Values indicate fold change.



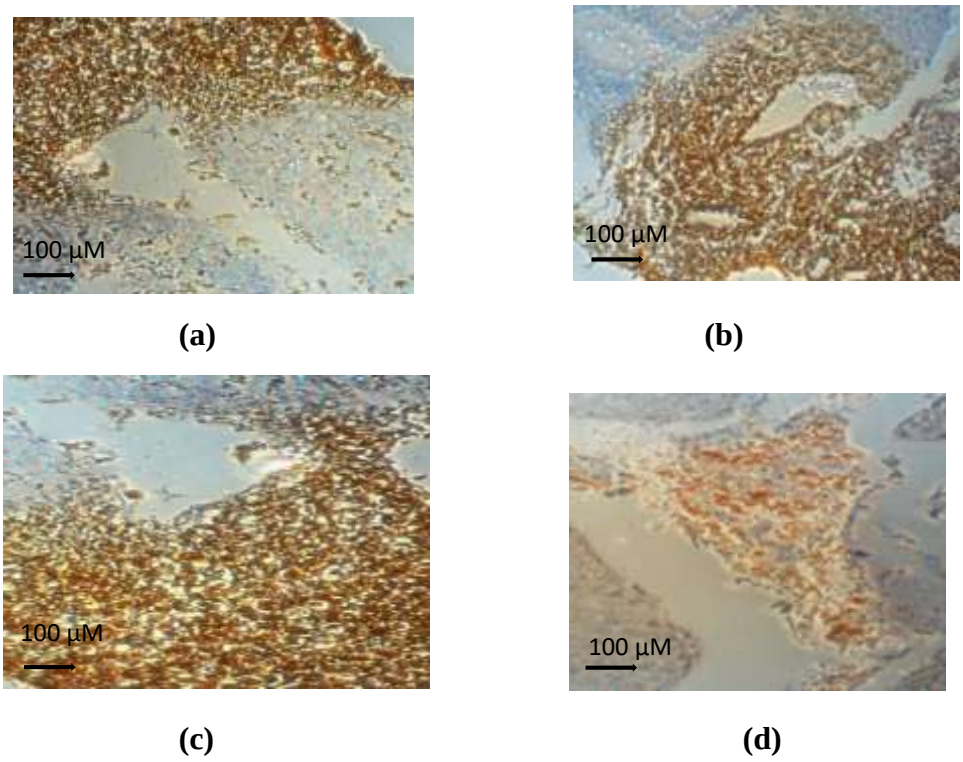
(a)



(b)

Figure 18: Assessment of KIR2D and HLA-C expression in (a) abortus tissue and in (b) placenta tissue.Protein expression was calculated as the arbitrary unit (AU) and error bars represent standard deviation from the mean.A $p<0.05$ was considered as statistically significant.

4a.3.3.2.1. Validation of KIR and HLA-C expression in early abortus tissue by IHC .



(a) **(b)**

(c) **(d)**

Figure 19: Assessment of expression of KIR 2D in healthy abortus (a) and SAB abortus tissue (b) (100X) , expression of HLA-C in (c) healthy and (d)SAB abortus by Immunohistochemistry. The image was captured in the Axio Vert.A1 inverted microscope (Carl Zeiss, Oberkochen, Germany).

4a.3.3.3. Analysis of KIR Genotype and Copy Number Variation in the Study Cohort, and Evaluation of KIR2D-HLA-C Expression in Term Placenta

4a.3.3.3.1 KIR genotype and Copy number variation in study cohort.

To rule out any impact of small sample size on the accuracy of KIR genotyping studies, the KIR genotype analysis was conducted on cohort of 214 participants.

Haplogroup BX dominated the study cohort as well as the mother-neonates (Table 18).The overall gene content observed in the mothers of paired samples were comparable to the larger study cohort (Figure 20, 21).

The frequency of the KIR2DL1 gene was found to be higher in women with a history of SAB compared to healthy women. In the SAB group, 62.35% participants KIR2DL1+/KIR2DS1+, while 37.36 % participants had KIR2DL1+/KIR2DS1- genotype Conversely, among the healthy participants, the frequency of KIR2DS1 was observed to be higher compared to the SAB participants. Furthermore, in the healthy group, KIR2DS1 was found to be present both with and without KIR2DL1 in the individuals (Table 19).

To understand the discrepancy in KIR distribution, we further explored the copy number variations (CNVs) of KIR2DL1 and KIR2DS1 genes in study cohort. The copy numbers of KIR-2DL1 and KIR-2DS1 genes were consistent with the observed gene frequency data. The mean copy number of maternal KIR2DL1 was found to be higher in the SAB group as compared to the healthy group (Figure 22). Conversely, the mean copy number of KIR-2DS1 was higher in the healthy group compared to the SAB group (Figure 22).. Furthermore, when comparing the mean copy numbers of KIR2DL1 and KIR2DS1 in the healthy group, it was determined that KIR2DS1 exhibited a significantly higher mean copy number compared to KIR2DL1(student t test, $p=0.001$).

Table 18: KIR locus profile of study cohort .

	2DL1	2DS4	2DS4 del	2DL2	2DL3	2DL5	2DS2	2DS5	3DS1	3DL1	2DS3	2DS1	HAPLO TYPE
S1	■	■		■		■	■						BX
S2	■	■		■		■	■						BX
S3	■	■		■		■	■		■			■	BX
S4	■	■		■		■	■					■	BX
S5	■	■		■		■	■		■			■	BX
S6	■	■		■		■	■			■		■	BX
S7	■	■		■		■	■						BX
S8	■	■		■		■	■						BX
S9	■	■	■	■		■	■		■				BX
S10	■	■		■		■	■	■					BX
S11	■	■		■		■	■			■	■	■	BX
S12	■	■		■		■	■			■	■	■	BX
S13	■	■		■		■	■			■	■	■	BX
S14	■	■		■	■	■	■	■			■	■	BX
S15	■	■		■		■	■				■	■	BX
S16	■	■		■		■	■	■	■		■	■	BX
S17	■	■	■	■		■	■				■	■	BX
S18	■	■		■		■	■			■			BX
S19	■	■		■	■	■	■						BX
S20	■	■		■		■	■	■	■				BX
S21	■	■		■	■	■	■						BX
S22	■	■	■	■		■	■		■	■			BX
S23	■	■		■		■	■						BX
S24	■	■		■		■	■						BX
S25	■	■		■		■	■				■		BX
S26	■	■		■		■	■		■				BX
S27	■	■		■		■	■	■		■		■	BX
S28	■	■		■		■	■			■		■	BX
S29	■	■		■		■	■			■		■	BX
S30	■	■		■		■	■	■		■	■	■	BX
S31	■	■		■		■	■		■	■		■	BX
S32	■	■		■		■	■			■		■	BX
S33	■	■		■		■	■			■		■	BX
S34			■				■	■	■			■	BX
S35			■				■	■	■		■	■	BX
S36			■				■	■	■		■	■	BX
S37			■				■	■	■		■	■	BX
S38			■				■				■	■	BX
S39	■	■		■			■				■	■	BX

	2DL1	2DS4	2DS4 del	2DL2	2DL3	2DL5	2DS2	2DS5	3DS1	3DL1	2DS3	2DS1	HAPLO TYPE
S40	■	■		■			■				■	■	BX
S41			■		■		■					■	BX
S42	■	■		■		■	■				■	■	BX
S43	■	■		■		■	■				■	■	BX
S44	■	■			■		■				■	■	BX
S45	■	■					■	■			■	■	BX
S46	■	■					■		■		■	■	BX
S47	■	■			■		■	■			■	■	BX
S48	■	■	■	■		■	■	■	■		■	■	BX
S49	■	■		■			■				■	■	BX
S50	■	■		■	■		■		■		■	■	BX
S51			■				■				■	■	BX
S52			■				■	■			■	■	BX
S53			■				■				■	■	BX
S54			■				■		■			■	BX
S55	■	■	■				■				■	■	BX
S56			■		■		■		■		■	■	BX
S57			■				■	■			■	■	BX
S58			■				■				■	■	BX
S59			■				■		■		■	■	BX
S60			■				■				■	■	BX
S61			■				■	■	■			■	BX
S62			■				■				■	■	BX
S63			■				■				■	■	BX
S64			■				■				■	■	BX
S65			■				■				■	■	BX
S66			■				■				■	■	BX
S67			■				■				■	■	BX
S68			■				■				■	■	BX
S69			■				■					■	BX
S70			■				■					■	BX
S71			■			■	■					■	BX
S72			■			■	■					■	BX
S73			■			■	■					■	BX
S74			■			■	■					■	BX
S75			■				■					■	BX
S76			■				■					■	BX
S77			■				■					■	BX
S78			■				■					■	BX

	2DL1	2DS4	2DS4 del	2DL2	2DL3	2DL5	2DS2	2DS5	3DS1	3DL1	2DS3	2DS1	HAPLO TYPE
S79			■				■		■			■	BX
S80			■				■		■			■	BX
S81			■				■		■			■	BX
S82			■				■					■	BX
S83			■				■					■	BX
S84			■				■					■	BX
S85			■									■	BX
S86	■	■			■			■				■	BX
S87	■	■			■			■				■	BX
S88	■	■			■			■				■	BX
S89	■	■			■			■				■	BX
S90	■	■			■			■				■	BX
S91	■	■			■			■				■	BX
S92	■	■			■			■				■	BX
S93	■	■			■			■				■	BX
S94	■	■			■			■				■	BX
S95	■	■			■	■		■				■	BX
S96	■	■			■	■		■				■	BX
S97	■	■			■	■		■				■	BX
S98	■	■			■	■		■				■	BX
S99	■	■			■			■				■	BX
S100	■	■		■		■	■						BX
S101	■	■		■		■	■						BX
S102	■	■		■		■	■						BX
S103	■	■		■		■	■		■				BX
S104	■	■		■		■	■						BX
S105	■	■			■			■				■	BX
S106	■	■			■			■				■	BX
S107	■	■			■			■				■	BX
S108	■	■			■			■				■	BX
S109	■	■			■		■	■				■	BX
S110	■	■			■		■	■				■	BX
S111	■	■			■			■				■	BX
S112	■	■			■			■				■	BX
S113	■	■			■			■				■	BX
S114	■	■			■	■		■				■	BX

	2DL1	2DS4	2DS4 del	2DL2	2DL3	2DL5	2DS2	2DS5	3DS1	3DL1	2DS3	2DS1	HAPLO TYPE
S115	■	■			■			■				■	BX
S116	■	■			■		■	■				■	BX
S117	■	■			■		■	■				■	BX
S118	■	■			■		■	■				■	BX
S119	■	■			■		■	■				■	BX
S120	■	■			■		■	■				■	BX
S121	■	■			■	■		■				■	BX
S122	■	■			■			■				■	BX
S123	■	■			■			■				■	BX
S124	■	■			■			■				■	BX
S125	■	■			■			■				■	BX
S126	■	■			■			■				■	BX
S127	■	■			■			■				■	BX
S128	■	■			■	■		■				■	BX
S129	■	■			■			■				■	BX
S130	■	■		■	■		■	■	■	■	■	■	BX
S131	■	■		■			■	■	■	■	■	■	BX
S132	■	■		■	■		■	■	■	■	■	■	BX
S133	■	■		■			■	■	■	■	■	■	BX
S134	■	■		■			■	■	■	■	■	■	BX
S135	■	■		■			■	■	■	■	■	■	BX
S136	■	■		■		■		■	■	■	■	■	BX
S137	■	■		■				■	■	■	■	■	BX
S138	■	■		■				■	■	■	■	■	BX
S139	■	■		■				■	■	■	■	■	BX
S140	■	■		■	■			■	■	■	■	■	BX
S141	■	■		■				■			■	■	BX
S142	■	■		■				■			■	■	BX
S143	■	■		■		■		■			■	■	BX
S144	■	■		■				■			■	■	BX
S145	■	■		■				■			■	■	BX
S146	■	■						■	■	■	■	■	BX
S147	■	■						■	■		■	■	BX
S148	■	■						■	■		■	■	BX
S149	■	■						■	■		■	■	BX
S150	■	■						■	■		■	■	BX
S151	■	■						■	■		■	■	BX
S152	■	■					■	■	■		■	■	BX
S153	■	■						■	■		■	■	BX
S154	■	■						■	■		■	■	BX
S155	■	■						■	■		■	■	BX
S156	■	■					■	■	■		■	■	BX

	2DL1	2DS4	2DS4 del	2DL2	2DL3	2DL5	2DS2	2DS5	3DS1	3DL1	2DS3	2DS1	HAPLO TYPE
S157	■	■						■			■	■	BX
S158	■	■			■						■	■	BX
S159	■	■			■	■					■	■	BX
S160	■	■			■	■	■				■	■	BX
S161	■	■			■	■					■	■	BX
S162	■	■			■	■		■					BX
S163	■	■			■	■					■	■	BX
S164	■	■				■		■			■	■	BX
S165	■	■				■		■			■	■	BX
S166	■	■				■					■	■	BX
S167	■	■				■	■				■		BX
S168	■	■				■					■		BX
S169	■	■				■					■		BX
S170	■	■				■	■					■	BX
S171	■	■			■	■						■	BX
S172	■	■			■						■	■	BX
S173	■	■			■						■		BX
S174	■	■			■						■		BX
S175	■	■			■						■		BX
S176	■	■			■						■		BX
S177	■	■			■						■		BX
S178	■	■			■						■	■	BX
S179	■	■			■						■	■	BX
S180	■		■		■						■		BX
S181	■	■						■			■	■	BX
S182	■		■					■			■	■	BX
S183	■		■								■		BX
S184	■		■								■	■	BX
S185	■		■			■		■				■	BX
S186	■		■			■						■	BX
S187	■		■			■		■					BX
S188	■		■			■	■						BX
S189	■		■		■	■		■					BX
S190	■		■		■	■		■			■		BX
S191	■		■		■	■						■	BX
S192	■	■				■						■	BX
S193	■		■			■						■	BX
S194	■		■			■	■					■	BX
S195	■		■			■					■		BX
S196	■		■		■	■					■	■	BX

	2DL1	2DS4	2DS4 del	2DL2	2DL3	2DL5	2DS2	2DS5	3DS1	3DL1	2DS3	2DS1	HAPLO TYPE
S197	■		■			■	■	■			■	■	BX
S198	■		■			■					■	■	BX
S199	■		■	■	■	■							BX
S200	■		■	■	■	■							BX
S201	■		■	■	■	■							BX
S202	■		■	■	■	■							BX
S203	■		■	■	■	■		■					BX
S204	■		■	■	■	■	■	■	■	■			BX
S205	■		■	■	■	■	■		■	■			BX
S206	■		■	■	■	■	■	■	■	■			BX
S207	■		■	■	■	■	■	■	■	■			BX
S208	■		■	■	■	■							BX
S209	■		■	■	■	■		■					BX
S210	■		■	■	■	■	■	■	■	■			BX
S211	■		■	■	■	■	■		■	■			BX
S212	■		■	■	■	■	■	■	■	■			BX
S213	■		■	■	■	■	■	■	■	■			BX
S214	■		■	■	■	■	■	■	■	■			BX

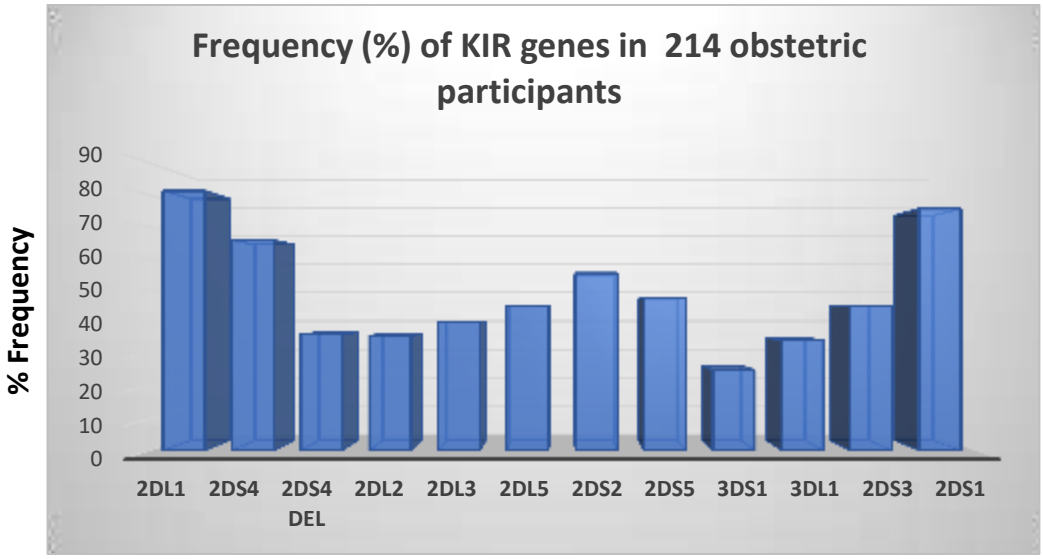


Figure 20: Frequency of KIR genes in 214 study particiopants

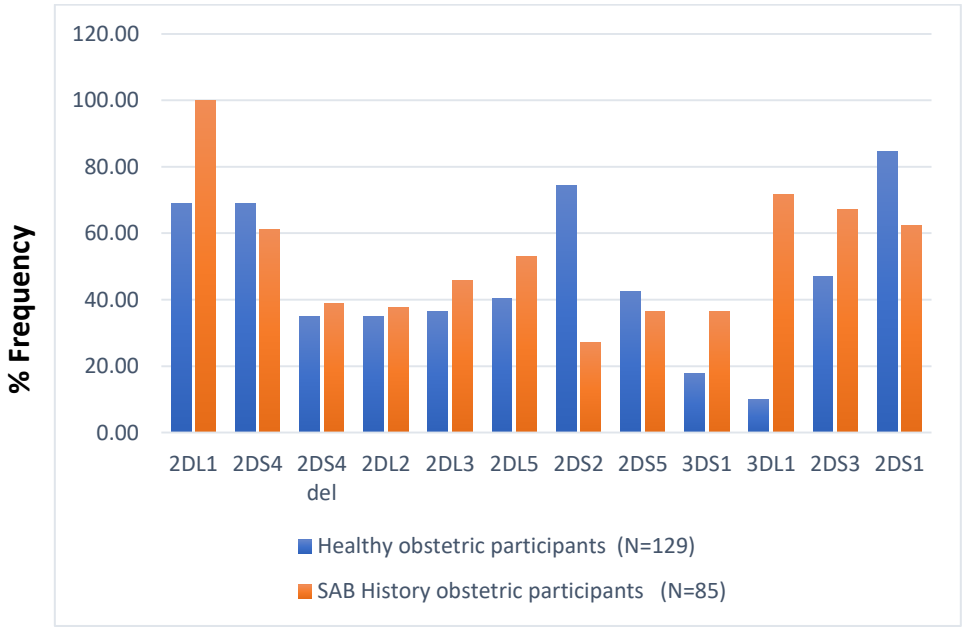
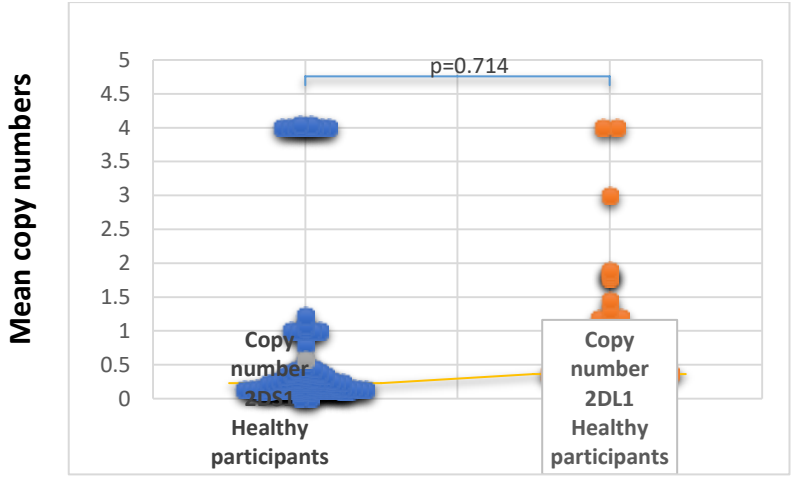


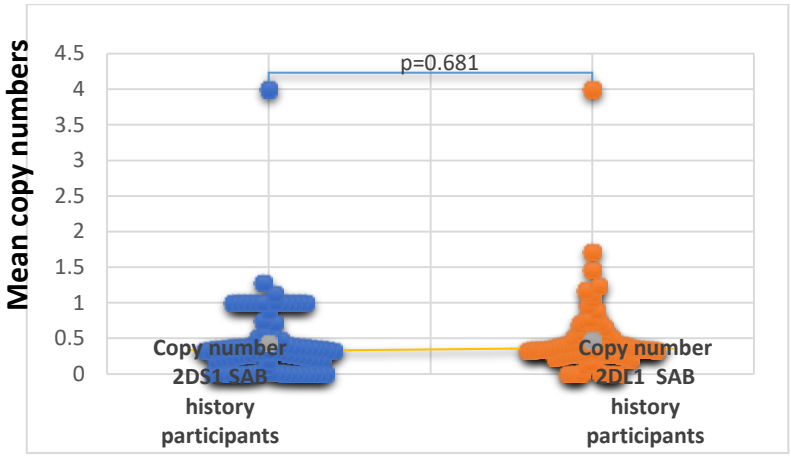
Figure 21: Frequency of KIR genes in 214 study participants based on their obstetric history .

Table 19 : Frequency of combined KIR2DL1/S1 in healthy and SAB history participants.

Gene combinaton	**frequency (%)**	
	Healthy obs participants (n=129)	**SAB history participants (n=85)**
2DL1+/S1+	77.52	91.20
2DL1+/S1-	-	100
2DL1-/S1+	31	-



(a)



(b)

Figure 22: Mean copy numbers of KIR 2DL1 and KIR2DS1 in (a) healthy participants and in (b) SAB history particpants.

4a.3.3.4.KIR2D-HLA-C expression in term placenta

The expression of KIR2DL1 and KIR 2DS1 was studied in term placenta of 56 women who delivered neonates including the above mentioned 46 mothers from mother - neonate pairs.

The transcript level expression of 2DL1 gene was higher in SAB placenta compared to their healthy placenta while levels of KIR2DS1 transcript was comparable between SAB and healthy placenta (Figure 23). KIR2D protein levels were higher in healthy

history placenta than SAB history placenta(p=0.0001) (Figure 23). HLA C was higher in SAB histpry placenta as compared to healthy placenta (p=<0.001) (Figure 24).

Given the involvement of KIRs in the development of various autoimmune diseases, we also investigated the autoantibody profile of the cohort, with a specific focus on their association with SAB.

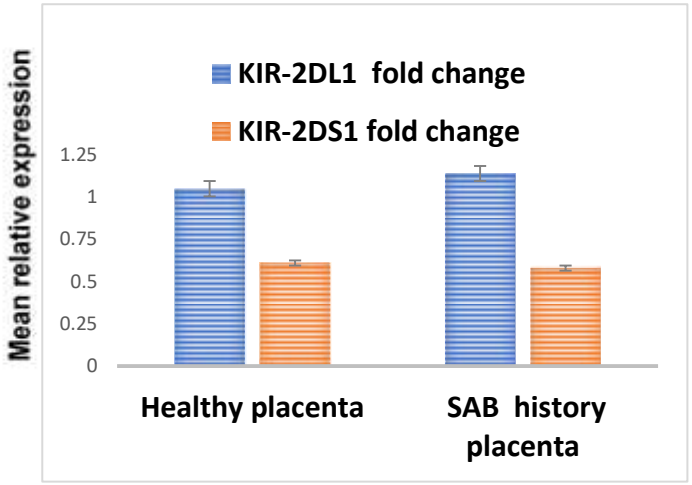
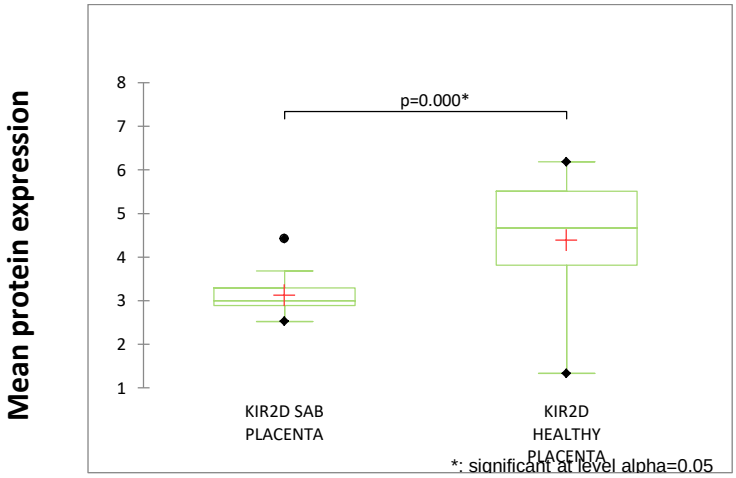
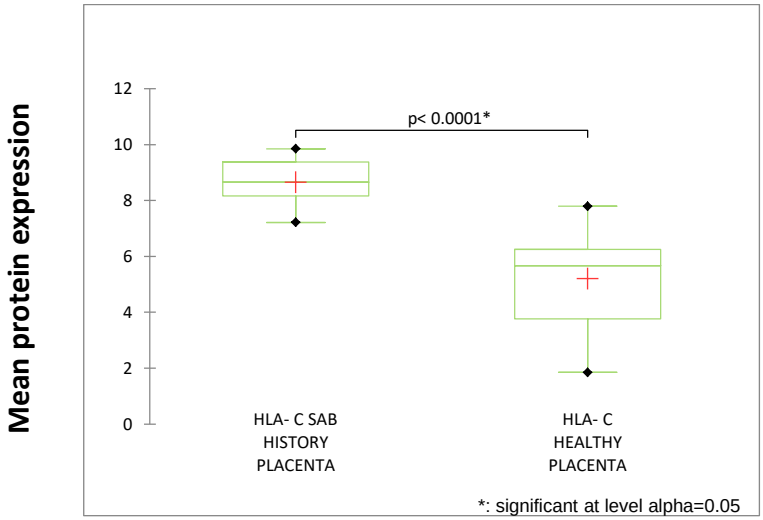


Figure 23: Transcript expression of *KIR2DL1* and *KIR2DS1* in placenta .Values indicate fold change.



(a)



(b)

Figure 24 : Assessment of (a) KIR2D and (b) HLA-C expression in placenta samples. Protein expression was calculated as the arbitrary unit (AU) . A $p<0.05$ was considered as statistically significant.

4a.4. Discussion

HLA-G mediated signalling is crucial to successful pregnancy in pregnancy outcome(102,103). HLA-G has seven isoforms that differ in extracellular domains, transmembrane domain structure (103-105). Recent investigations have reported that distribution of HLA-G isoform transcripts vary with developmental stages and region of the developing fetus (106-107), which tends to suggest temporal necessity of different isoforms at different stages of foetus development (108). We examined HLA-G isoforms in early abortus versus term placenta in relaion to early pregnancy loss. Membrane bound HLA-G3 and HLA-G4 isoforms were seen in the early abortus and in term placenta, with dominance of HLA-G4 in healthy placenta. This is in line with studies which showed predominance of membrane bound isoforms HLA-G1 and HLA-G2 in healthy placenta as compared to pre-eclampsiatic placenta (109-111). However, relation of isoform profile in early abortus with early pregnancy loss is yet to be elucidated (112). We tried to understand if a signalling difference by HLA-G3 and HLA- G-4 isoforms could explain the difference in pregnancy outcome.

HLA-G has a unique property of bearing a cysteine amino acid at position 42 in the α1 domain of its heavy chain(113). Hypothetically, both HLA-G3 and HLA-G4 possess this alpha 1 domain, enabling dimerization and facilitating favorable receptor

interaction and downstream signaling, and hence difference in their proportion may not necessarily affect pregnancy(114-115)]. However, Wang et al. reported the inability of G-3 to dimerize and lack of dimerization might affect the binding orientation of its receptors and hinder downstream signalling(116-119). A significant increase in proportion of HLA–G3 in early SAB abortus corroborated with earlier report where a positive correlation between HLA-G3 and reduced HLA–G level was noted in blood of women with preeclampsia (120-122).

Another unique property of HLA-G is the cleavage of membrane bound HLA-G isoforms by metalloproteases for endocytosis in the endosomal compartments to signal primarily from endosomes(123-125). And our data on increased soluble isoforms of HLA-G in abortus , particularly healthy abortus support a role of endosomal signalling for favorable decidualization in early pregnancy (126). Given that full length isoforms HLA-G1 and HLA-G5 were markedly higher in SAB abortus as compared to healthy abortus, its plausible that the HLA-G mediated signalling was optimal in SAB abortus . Interestingly ,we also observed all soluble isoforms (HLA-G5, HLA-G6, HLA-G7) in abortus samples but they were nearly absent in placenta samples, possibly suggesting the importance of these isoforms in the invading trophoblast for early decidualization (127-129)

HLA-C are also expressed on trophoblast ,and are cognate ligands for KIR receptors expressed on NK cells and studies have reported optimal KIR-HLA-C signalling a necessity for maintaining pregnancy (130-135).Correlation between activating KIR genes and the risk of pregnancy complications is reported to vary among different populations considering their different genetic makeup (135-138). And our population ,rich is B haplotypes are under stronger diversifying selection reproductive success, the risk of pre-eclampsia or extreme birth weight(139-142). Our data on mother-neonate pairs revealed that the SAB group had a dominance of maternal $KIR2DL1^{+}$ and $KIR2DL1^{+}$/ $KIR2DS1^{+}$ genotypes, with a lower presence of the HLA-C2 allele in neonates. Although, it could be argued that the presence of KIR2DS1 could counterbalance the inhibitory signaling of KIR2DL1, but its unlikely given the higher frequency and affinity of KIR2DL1 for HLA-C2 ligand leading to suboptimal signaling . Similar findings in our in larger cohort of 214 obsterric participants validate the findings in mother-neonate pair and are consistent with earlier findings.

Higher frequency of KIR2DS1-HLAC2 combined genotype in mother-neonate pairs ,favorable for pregnancy in our study cohort is supported by studies showing that KIR2DS1-positive females with fetal HLA-C2 have improved trophoblast invasion and spiral artery remodeling through the secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) as compared to pre-eclampsiatic women who lacked KIR2DS1(143-147). KIR2DS1's presence in uterine natural killer (uNK) cells mediates cytokines such as TGF-β, PlGF, and VEGF, which play a crucial role in immune reactions (148-152). However, contradicting these findings by Dambaeva et al. and others showed an association of KIR2DS1 with an increased HLA-C2 allelic frequency in early pregnancy failure (153-157) .

Higher copy number of KIR2DS1 in our study cohort with healthy pregnancy outcome is in synchority with study reports where females with the KIR B genotype the presence of activating KIR2DS1 bound to HLA-C2 were less prone to obstetrical complications (158-159). CNVs have a notable influence on disease susceptibility, particularly in the context of viral infections (160-162). The impact of CNVs, like 3DL1S1's effect on HIV control and the significant role of KIR2DL3 expression when combined with HLA-C in resolving hepatitis C virus infection, underscores the significance of CNVs in determining disease vulnerability (163-165). However, the understanding of CNVs' involvement, in context of KIRs in early pregnancy loss remains largely unexplored (166).Furthermore,an increased frequency of KIR2DL+S1-genotypes with high KIR2D and low HLA-C content in early SAB abortus indicates poor activation of NK cells as compared to healthy abortus. Our data supports that KIR2DL1 is a risk factor for pregnancy complications.

Additionally, our study found no association between autoantibodies, such as anticardiolipin and antinuclear autoantibodies, and SAB. Although B2GP1 IgM positivity was only observed in participants with a history of SAB, the frequency was too low to draw meaningful conclusions and it was supported by earlier reports where autoantibodies did not associate with pregnancy complications. This was in agreement with previous studies which showed no association of autoantibodies with pregnancy outcome (167-169). However, contradictory findings have been reported by others who showed an association of autoantibodies with early pregnancy failure (170-175).

Our findings collectively shows that the presence of membrane-bound HLA-G4, along with higher copy number of KIR2DS1 and KIR2DL1/KIR2DS1-HLAC2 combined

genotype in mother-neonate pairs, contributed to a balanced activation of natural killer (NK) cells and correlated with favorable pregnancy outcomes. While HLA-G3 isoforms in conjunction with higher KIR2DL1 or KIR2DL1+/S1+ - HLA C2 and compromised maternal tolerance, placentation in SAB, plausibly for inadequate NK activation. Higher HLA-C,KIR2D content in abortus than in term placenta showed its larger requirement in early pregnancy, in addition to soluble HLA-G isoforms for favoring placentation. Our data supports on differential expression of KIRs in early and term phase of pregnancy, emphasizing on necessity of NK expressed KIRs for EVT invasion and tissue remodeling in early decidua.

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Salient findings of Chapter IVa

The proportion of HLA-G3 isoforms was significantly higher in SAB compared to healthy abortus. Soluble isoforms (HLA-G5, HLA-G6, and HLA-G7) were detected in healthy abortus, while only HLA-G5 was found in SAB abortus. Significant difference in the proportion of HLA-G4 (z-test for two proportions / Two-tailed test, p=0.001) between the healthy and SAB history placenta samples. Minimal presence of HLA-G soluble isoforms was noted in placenta. TWe observed comparable levels of HLA-G protein between the elective abortus and SAB abortus but noted higher HLA-G levels in healthy placenta than SAB history placenta. Nonetheless, logistic regression analysis predicted HLA-G levels as predictive marker for a healthy outcome of pregnancy (AUC=0.8) with a p-value of 0.01.

Data on mother-neonate pairs revealed that the SAB group had a dominance of maternal KIR2DL1+ and KIR2DL1+/ KIR2DS1+ genotypes, with a lower presence of the HLA-C2 allele in neonates. Our findings collectively shows that the presence of membrane-bound HLA-G4, along with higher frequency KIR2DL1+/KIR2DS1+-HLAC2 and higher frequency and copy number of KIR2DS1 in mother-neonate pairs, contributed to a balanced activation of NK cells and correlated with favorable pregnancy outcomes. While HLA-G3 isoforms in conjunction with higher KIR2DL1 with lower KIR2DL1+/S1+ - HLA C2 combined genotype compromised maternal tolerance, placentation in SAB, plausibly for inadequate NK activation. Higher HLA-C, KIR2D content in abortus than in term placenta showed its larger requirement in early pregnancy, in addition to soluble HLA-G isoforms for favoring placentation. The novelty of the study lies in examining early conceptus and term placenta from case (spontaneous pregnancy loss) and control participants.

CHAPTER IV b

STUDY ON 3'UTR MEDIATED REGULATION OF HLA-G IN PREGNANCY.

4b.1 Introduction

HLA-G possesses a unique characteristic that sets it apart from classical HLA class I loci (1-6). While the coding region of HLA-G demonstrates minimal variation across global populations, there is relatively higher diversity observed in two non-coding regions, namely the 5' upstream regulatory region (5' URR) and the 3' untranslated region (3'UTR) (7-10). Unlike classical MHC class I molecules, which utilize enhancer A, the interferon-stimulated regulatory element (ISRE), and the SXY module in their proximal promoter for fine-tuning gene expression, HLA-G exhibits nucleotide sequence variations, mutations, and/or deletions in those regions (11-14) . As a result, HLA-G does not respond to NF-κB, interferon regulatory factor 1, and class II trans activator DNA-binding factors, indicating the involvement of alternative mechanisms in HLA-G transactivation (15-17). HLA-G demonstrates distinct alternative regulatory elements in its promoters, such as the locus control region (LCR), heat shock element (HSE), progesterone response element (PRE), hypoxia response element (HRE), three cAMP response elements, an additional functional ISRE, and three RAS response elements. These elements mediate the HLA-G expression in response to specific environmental signals, such as hypoxia and progesterone (18-20).

The HLA-G promoter region is associated with a limited number of disease-specific polymorphisms. Furthermore, epigenetic modifications, such as DNA demethylation and inhibition of histone acetylation have been found to impede the activation of the HLA-G gene (21-25). Notably, the HLA-G gene contains a 5' untranslated region (5' UTR) promoter sequence located between 1.1 and 1.4 kb from the transcription start site (ATG start 1) (22-24). This region contains binding sites for nuclear transcription factors that regulate the expression of HLA-G. Within this region, the 1000 Genomes project has identified variable sites, including position-762 situated between a cAMP response element (CRE) and interferon-stimulated regulatory element (ISRE), which are close to known regulatory elements (25-30). These variations exert an influence on the binding of transcription factors (Figure 25).

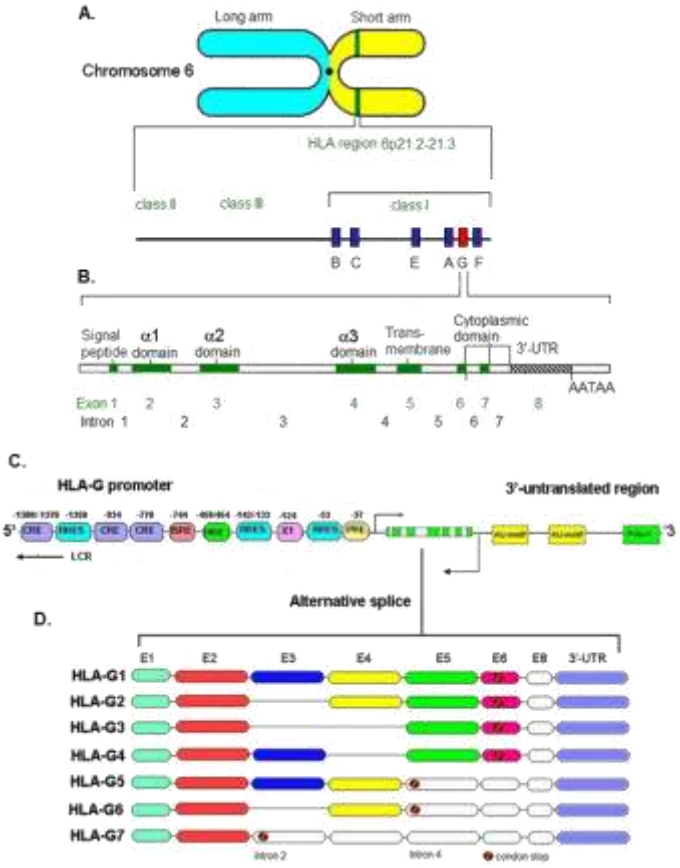

Figure 25: Schematic representation of the HLA-G gene, highlighting key polymorphisms in the5′upstream regulatory region and the 3′-untranslated region.

Exon 7 is absent in the mature mRNA of HLA-G, and exon 8 is not translated due to a stop codon in exon 6 which contains essential regulatory elements like polyadenylation signals and AU-rich elements (31-35). Both RNA-binding proteins (RBPs) and microRNAs (miRNAs), play a crucial role in binding to specific regulatory sites within the mRNA's 3' UTR, fine-tuning the gene's protein synthesis. The mature mRNA of HLA-G lacks exon 7 and exon 8 is not translated due to a stop codon in exon 6. The untranslated exon 8 forms the 3′UTR of the mature HLA-G RNA, which also includes important regulatory elements such as polyadenylation signals and AU-rich elements (36-42). RNA-binding proteins (RBPs) and microRNAs (miRNAs) binds to specific regulatory sites within the 3' UTR of the mRNA, thereby influence their translation, localization, and degradation (43-46) and

mutations in the 3' UTR binding sites can disrupt these regulatory mechanisms, impacting the gene's post-transcriptional expression (47-48).

MicroRNAs (miRNAs) are short non-coding RNA sequences that play a crucial role in regulating gene expression and cellular functions (48-52). They dynamically adapt to the placental environment, making them potential biomarkers for pregnancy complications such as preeclampsia, gestational diabetes, obesity, ectopic pregnancies, fetal growth restriction, recurrent pregnancy loss and preterm birth. The + 3142C > G SNP (rs1063320) at 3' UTR of HLA-G provides a binding site for placental microRNAs miR-148a, miR-148b, and miR-152 (53-60). These microRNAs bind to mutant ‘‘G‘‘ allele at 3'UTR with strong affinity which then restricts post-transcriptional processing of HLA-G mRNA and downregulates HLA-G protein expression (61,62). Elevated levels of miR-148a and miR-152 with altered HLA-G expression in the placenta has been associated with pregnancy related disorders such as preeclampsia (PE), fetal growth restriction and pre-term labor (61, 62). However, reports on whether microRNA 148a/ miR-152 mediated HLA-G expression is affected by presence of mutant “G” allele in its + 3142 3'UTR position remains debated. In addition, presence of 14 base IN/DEL polymorphism (rs371194629) at position +2960 of exon 8 is associated with stability of HLA-G mRNA and splicing of its isoforms. 14 bp IN removes 92 base pair sequences from mature mRNA, affects transcript stability, suppresses translation of HLA-G and is associated with low HLA-G levels in pregnancy complications such as pre-eclampsia (PE) and recurrent spontaneous abortion (RSAB) (63-68).

Dysregulation of HLA-G leads to deprivation from the necessary proangiogenic microenvironment for uterine spiral arteries remodeling during early placentation and causes early pregnancy failure (1-5, 69). Considering that we have observed difference in HLA-G isoform profile between healthy abortus and SAB abortus in earlier chapter, we further tried to understand the post transcriptional regulation. We investigated the impact of genetic variations on HLA-G regulation by examining the miRNA profile in the 3' untranslated region (3'UTR) of the *HLA-G* gene with status of heat shock factors (HSF) and DNA methyltransferase (DNMT1) in spontaneous abortions in 5'UTR.

4b.2. Materials and methods:

4b.2.1. Study site, study design, and participants:

A hospital-based case-control study was designed with 214 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. The SAB history group (N=85) included age-matched obstetric participants with one or more fetal losses before 20 weeks of gestation, while the control group (N=129) included women with at least one live birth.

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.

Participants got screened to be enrolled in the study during their visit to clinic OPD for routine checkups. Effort was made by the clinical staff to collect sample left over from routine examination. Peripheral blood samples collected were used for mutation studies.

56 women were pregnant at the time of sample collection (N=24 SAB history participants, N= 32 healthy history participants) and went to full term delivery. Term placentas, after successful delivery were collected for expression study.

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 hospitals.

4b.2.2 Extraction and Quality Assessment of DNA, RNA, and Protein from studied samples.

4b.2.2.1 DNA isolation from peripheral blood.

Study participants were enlisted during their visit to the clinic for routine checkup. Briefly 1ml of peripheral blood sample were collected by the clinical staff in tubes containing sodium EDTA anticoagulant. Blood samples were used for genomic DNA isolation by use of QIAamp, DNA Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instruction. The quantity and quality of the isolated genomic DNA were assessed by Nano-VueTM plus spectrophotometer (GE Healthcare, Little Chalfont, United Kingdom.

4b.2.2.2 RNA Isolation and cDNA Preparation

Placenta samples were collected in 1.5 ml RNA later solution (Ambion, United States) and homogenized using a hand-held tissue grinder (G-Biosciences, United States). DNA, RNA and protein were extracted using Allprep DNA/RNA/protein kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was converted to cDNA using the High capacity cDNA reverse transcription kit (Invitrogen, Applied Biosystems, Foster City, United States) according to the manufacturer's instructions. The quantity and quality of the isolated genomic DNA, cDNA and protein were assessed by Nano-VueTM plus spectrophotometer (GE Healthcare, Little Chalfont, United Kingdom).

4b.2.2.3 Isolation of microRNA and preparation of miR-specific cDNA from placenta.

MicroRNA (miR-148, miR-152) and a non-coding RNA control (RNU) were isolated from placental tissue using the mirVana™ miRNA Isolation Kit (Invitrogen, Applied Biosystems, USA). Subsequently CDNA was prepared as per Mastermix given in Table 20. The PCR conditions are the same as mentioned in section 4a.2.

Table 20: Composition of cDNA preparation master mix

100Mm dntps	.15 µl
Reverse transcriptase	.1µl
Buffer	1.5µl
RNase inhibitor	.19µl
Nuclease free water	4.16µl
RT primers (5x)	5 µl

4b.2.4. Association study of HLA-G +3142G/C SNP and 14 bp DEL/IN polymorphism with SAB.

Previous report from our lab showed that HLA-G haplotypes- UTR-5 and UTR-7 were the most frequent in our studied samples. SNP + 3142 C > G (rs1063320) and 14 bp DEL/IN polymorphism (rs66554220) at position + 2960 are present in both these haplotypes and hence were chosen for the study. We investigated these mutations due to its reported role in affecting HLA-G mRNA stability and diminishing expression.

PCR-based method was employed for genotyping of both + 3142C > G SNP and 14 base pair IN/DEL polymorphism in 214 obstetric participants (85 SAB and 129 control). Self-designed allele-specific primers (two forward and one reverse primer) used for confirming the presence of C > G polymorphism in samples for + 3142 C > GSNP association study. Predesigned primers from a previous study were used for the amplification of exon 8 for HLA-G 14 base pairIN/DEL polymorphism [17] (Table 21).

Table 21: Primer sequences for determination of association of SNP+3142C>G

+3142 C>G (rs1063320)	Primer Sequence (5'-3')	Amplicon size (base pair)
Forward primer 1	CTCTGTCTCAAATTTGTGGTG	337
Forward primer 2	CTCTGTCTCAAATTTGTGGTC	
Reverse primer	CTGGTGGGACAAGGTTCTACTG	
HLA G exon 8 (rs66554220)		
Forward primer	GTGATGGGCTGTTTAAAGTGTCACC-	210/224
Reverse primer	GGAAGGAATGCAGTTCAGCATGA	

4b.2.5. Expression of microRNA levels

The expression study of tissue-resident miR-148a and miR-152 was determined using TaqMan assays (Invitrogen, Applied Biosystems, USA). Approximately 200 ng of cDNA of each miRs was obtained from all tissue samples for transcript expression study and RNU48 was used as control microRNA for normalizing the miR-148a/152 expression levels.

The master mix composition and cycling conditions of Real time PCR are presented in Table 22 and Table 23. The relative expression of the target genes was determined using the formula $2^{-\Delta\Delta Ct}$ method as explained in section 4a.2.4.

Table 22: Composition of Real time PCR master mix

Composition	**Concentration/quantity**
Taqman Micro RNA assay	1X
Taqman master mix	1X
cDNA converted from miRNAs	100ng
Nuclease free water	Adjust the volume

Table 23: Cycling conditions of Real-time PCR

S. No	Gene	Primer
3	*HSF1*	PPH01055F-200,, RT^2 qPCR Primer Assays (Qiagen, Hilden, Germany)
4	*DNMT1*	FP – CATGAAGCATGAGAATGAGGCT RP - ACTGCACCAGTGAGATCAGGA

Table 24: Primers used in transcript expression of immunoregulatory genes.

PCR program
95°C for 10 mins 40 cycles of 95°C for 15 secs and 60°C for 1 min

4b.2.6. Expression of HSF1 and DNMT1 in placenta.

Transcript level expression of *DNMT1* and *HSF1* was done in the placental tissues using SYBR green-based assays (Applied Biosystems, USA) on QuantStudio 3 Real-time PCR Systems (Applied Biosystems, United States). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control for normalization of expression levels were used as the calibrators for mRNA quantification by the comparative CT method. RT^2 qPCR Primer Assays (Qiagen, Hilden, Germany) were used as shown in Table 24. Real-time PCR was carried out in 10 µL of reaction volume consisting of 2X. SYBR Green

PCR master mix, 0.4 μM of each primer, 100-200 ng of cDNA and water to adjust the volume. The relative expression of the target genes was determined using the formula $2^{-\Delta\Delta Ct}$method as explained in section. All the genes were successfully amplified in Real time PCR as presented in Appendix I.

4b.2.7. Statistical analysis

XLSTAT software, version 2018.7 was used for the statistical analysis. Frequencies observed for each polymorphism in both cancer and control samples were analyzed by Fischer's exact test. Correlation between the frequencies of two SNPs in cancer and SAB samples was analyzed using nonparametric tests. P-value < 0.05 was used as statistically significant.

4b.3 Results

4b.3.1. Association of HLA-G +3142G/C with SAB

In the study population, the predominance of heterozygotes GC over CC/GG was observed in the rs1063320 and predominance of homozygous insertion (II) was observed over heterozygous insertion-deletion (ID) /homozygous deletion (DD) for 14 bp IN/DEL polymorphism in + 2960 UTR region of HLA-G (Table 6). While SAB participants had a higher frequency of the ''IN" allele (Fisher's exact p =<0.0001), the participants with no history of spontaneous abortion had a higher frequency of the ''DEL" allele (Fisher's exact p =<0.0001) (Table 25).

Table 25: Frequency distribution of +3142 G/C SNP and 14 base pair polymorphism in Pregnancy with significant p-values

	SAB (n=59)	**Control (n=111)**	**p-value**
Genotype	11(18.96%)	18(16.21%)	
CC	29(50%)	40(36.03%)	
GC	21(36.20%)	52(46.84%)	
GG	3(5.17%)	84(75.67%)	<0.0001
DEL/DEL	1(1.72%)	5(4.50%)	<0.0001
IN/DEL			
IN/IN	55(94.82%)	30(27.02%)	<0.0001
Alleles			

C	51(43.96%)	76(34.23%)	
G	71(61.20%)	144(64.86%)	
IN	111(95.68%)	65(29.27%)	
DEL	7(6.03%)	173(77.92%)	<0.0001
Haplotype			
DEL/C	3(5.17%)	47(42.34%)	<0.0001
DEL/G	2(3.44%)	28(25.22%)	<0.0001
IN/C	37(63.79%)	11(9.90%)	<0.0001
IN/G	48(82.75%)	29(26.12%)	<0.0001

Given the predominance of GC genotype in the study participants and ‘‘G” allele provides microRNA binding site, we examined expression of tissue-resident miR-148a and miR-152

4b.3.2. Expression of miRs in placenta.

miR-148a and miR-152 upregulated in placenta samples. miR-148a levels were comparable between SAB and healthy group. However, miR-152 was downregulated in healthy as compared to SAB where it showed base level expression (Figure 26)

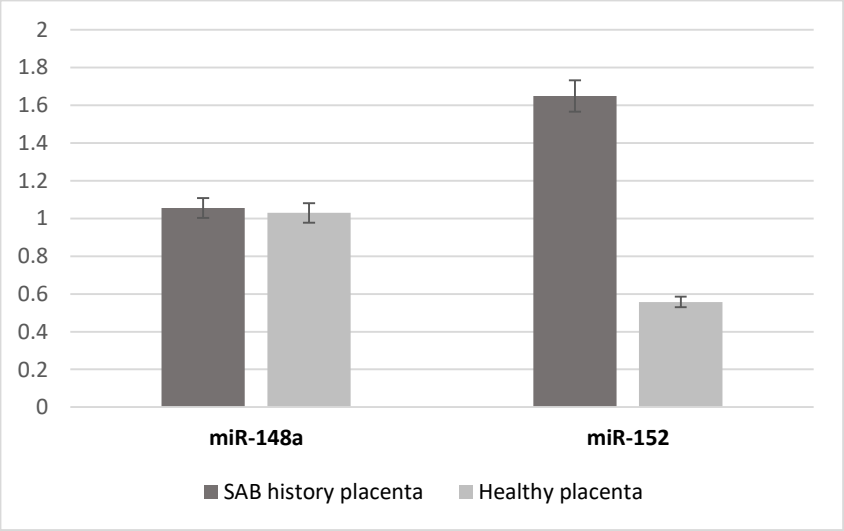

Figure 26: Expression of miRs in placenta. Data showed that tissue resident miRNAs were upregulated in both SAB (miR 148 A = 1.05 fold and miR152 = 1.64 fold) and healthy placentas (miR 148 A = 1.02 fold) However, miR 152 was downregulated (0.55 fold) in healthy placenta Error bars in the graph represented standard error of the mean.

In previous chapter section 4a.3.1section, we have shown low HLA-G expression in SAB placenta compared to healthy placenta. Positive correlation between both miR-148a and miR- 152 (Pearson's correlation =1, p =<0.001) were observed in SAB history placenta and between miR-148a and HLA-G protein (Pearson's correlation =.6, p =0.003) in SAB history placenta (Figure 27).

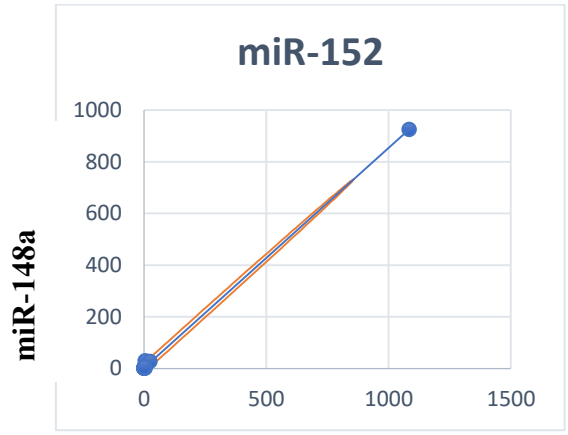

Figure a

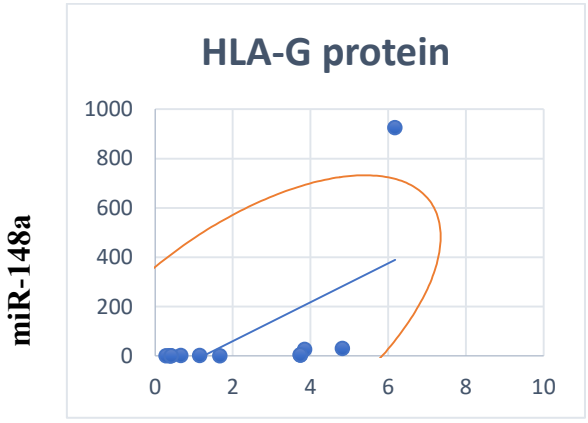

Figure b

Figure 27: Correlation analysis revealed a positive correlation of expression of miR-148a with levels of and miR-152 (3A) and between of miR-148a and HLA-G protein in SAB history placenta.

4b.3.3. Expression of DNMT1 and HSF1 in placenta.

DNMT1 transcript levels were higher in SAB placenta than healthy placenta. However, *HSF1* mRNA levels were higher in SAB compared to control (Figure 28). Both DNMT1 (p = < 0.0001) and HSF1 (p=<0.0001) showed positive correlation with HLA-G level in SAB control (Figure 29).

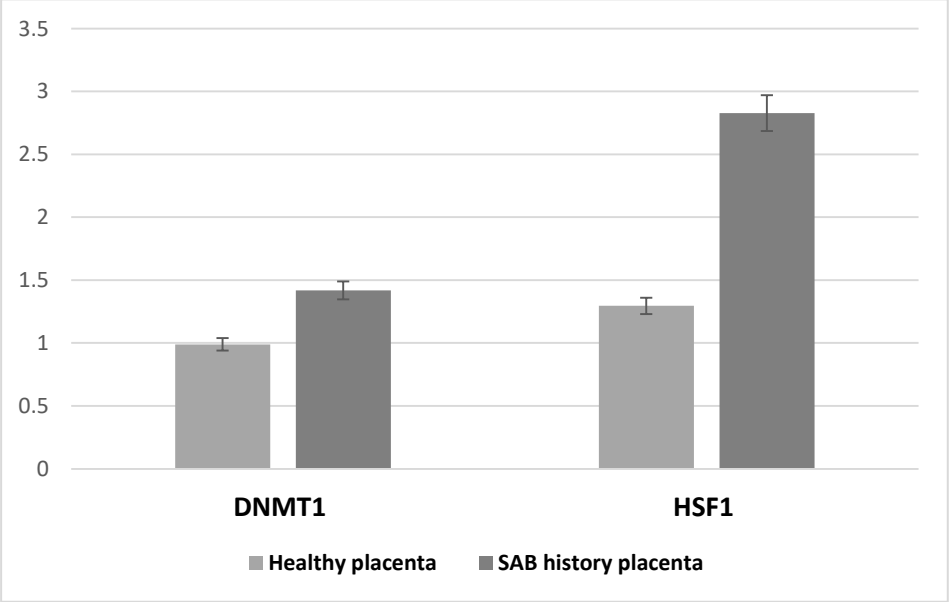

Figure 28: mRNA expression profile of genes regulating the HLA-G 5' upstream regulatory region (URR). DNMT1 (SAB = 1.418 fold and SAB control = 0.989 fold) and transcription factor HSF1 (SAB = 2.82 fold and SAB control = 1.29 fold) were upregulated in placenta samples irrespective of obstetric history. Error bars in the graph represented standard error of the mean.

Positive correlation between HLA-G and DNMT1 (Pearson's correlation=0.4, p =.02) were observed in SAB history placenta. However, in healthy placenta HSF1 and HLA-G showed positive correlation. (Pearson's correlation =.94, p =0.001) in SAB history placenta (Figure 29).

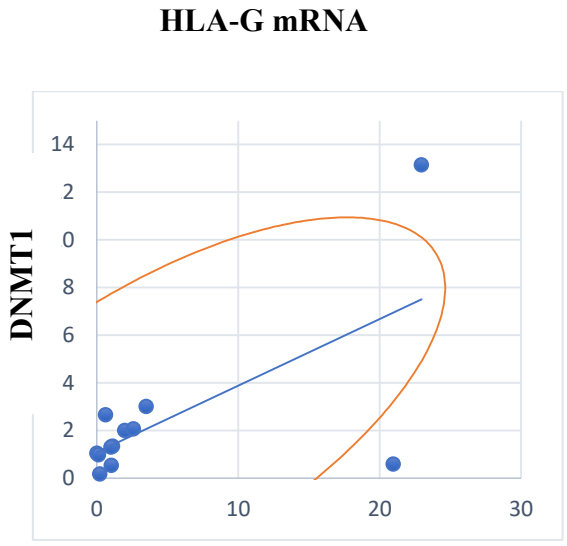

Figure 29a

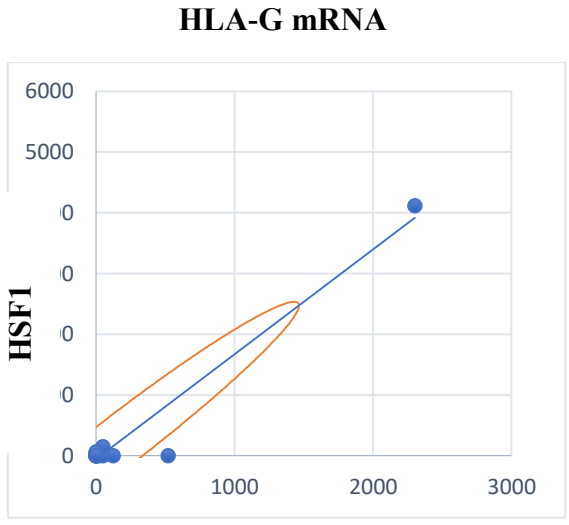

Figure 29b

Figure 29: Correlation analysis revealed a positive correlation of expression of DNMT1 and with levels of HLA-G mRNA in SAB (4A) and between HSF1 and HLA-G mRNA in healthy placenta.

4b.4 Discussion:

HLA–G regulation is of immense interest because although it exhibits a tissue-restricted expression (1–2). Increased HLA-G with ‘‘GC” heterozygote predominance in the study participants was suggestive of balancing selection in our population. Global data has reported the ‘‘G” allele to be the major allele with a frequency of 0.55 % in the global population (70). As per the report of thousand genome project, the 3'UTR of HLA-G exhibited 4.5 times higher nucleotide diversity (2.86%) as compared to the promoter (0.63%) and coding (0.64%) regions, both 3'UTR and 5'URR of HLA–G are under balancing selection in various population (70,71).

HLA-G is abundantly expressed in the placenta and its levels were seen to correlate with successful pregnancy outcome (72). Our study is concurrent with earlier findings where higher HLA G level was seen as a factor predictive of successful pregnancy in our study participants’ outcome by logistic regression analysis. We observed upregulation of HLA-G protein in SAB and SAB control placenta despite high levels of both the microRNAs. A comparison of HLA-G levels in healthy placenta vs SAB revealed increased expression of HLA-G in healthy placenta although it was not statistically significant. The expression of HLA-G in term placenta is very limited and Moniek et al (2019) had reported increased HLA-G level in term placenta of women with a history of recurrent spontaneous abortion (73). However, the factors which cause downregulation of HLA-G in early pregnancy loss remains debated (74).

To understand the observed difference in HLA-G levels in SAB placenta, we examined 3'UTR polymorphism and levels of miR-14a and miR-152. Our finding on higher levels of microRNAs in the SAB group are in agreement with previous investigations of Zhang et al who reported a higher level of miR-152 in preeclamptic animal models [8]. However, unlike the reports of earlier investigations, we noted no correlation between SAB and microRNAs levels (75). The mutant ‘‘G” allele in the + 3142 position is reported to provide higher binding affinity to placental resident microRNAs correlated positively with HLA-G transcript levels in the SAB group.

It is plausible that the upregulation of DNMT1 in the placenta of SAB history could be a compensatory response aimed at reducing the aberrant expression of HLA-G transcripts, as higher levels of DNMT1 were found to be positively associated with HLA-G transcript levels in SAB. Further investigation into the HLA-G promoter methylation is required because we did not examine the promoter-specific methylation state of HLA-G which is a crucial mechanism for HLA-G expression (16).

Our study findings indicate that the expression of HLA-G is influenced by the combination of 3' UTR polymorphism and the 5' untranslated region (5' UTR) of HLA-G genes, rather than being regulated by microRNAs (miRNAs) in SAB pregnancies.

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Salient findings of Chapter IVb

Our data on predominance of "GC" heterozygotes among the study participants suggests balancing selection in our population. Despite the upregulation of miR-148a and miR-152 in placenta samples, we found no correlation between these miRNAs in the placenta. Interestingly, we observed that HLA-G expression is influenced by the combination of the 14 bp DEL allele, which is in linkage disequilibrium with the SNP +3142 C > G, and the 5' URR HLA-G genes-HSF1. The upregulation of DNMT1 in the placenta of individuals with a history of spontaneous abortion (SAB) could be a compensatory response aimed at reducing the aberrant expression of HLA-G transcripts, as higher levels of DNMT1 were found to be positively associated with HLA-G transcript levels in SAB. Further investigation into the methylation state of the HLA-G promoter is required because we did not examine the promoter-specific methylation state of HLA-G, which is a crucial mechanism for HLA-G expression.