SOLUBLE HUMAN LEUKOCYTE ANTIGEN-G EXPRESSION IN PREGNANCY SUCCESS AND EARLY PREGNANCY LOSS IN KORLE-BU TEACHING HOSPITAL

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DECLARATION

ALL THE WORK RECORDED IN THIS THESIS IS ORIGINAL, UNLESS OTHERWISE ACKNOWLEDGED IN THE TEXT OR BY THE REFERENCES CITED. THIS WORK HAS ALSO NOT IN ITS PRESENT FORM OR OTHERWISE BEEN SUBMITTED TO THIS OR ANY OTHER UNIVERSITY FOR THE AWARD OF A HIGHER DEGREE.

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DEDICATION

THIS THESIS IS DEDICATED TO THE ALMIGHTY GOD,

MY FAMILY AND

TO THE MEMORY OF MY BELOVED

SANELA SETUTSI ADZOTOR HOSU-PORBLEY.

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My thanks, praises and worship to God most high, my tower of strength and sustenance, my God in whom is the depths of wisdom and knowledge.

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ABSTRACT

Background: Human Leukocyte Antigen (HLA) -G is a non-classical major histocompatibility complex (MHC) class I protein which has been described as being selectively expressed on the invasive trophoblast at the materno-foetal interface at the beginning of pregnancy. HLA-G has the potential role of protecting the trophoblast from cytotoxicity and enhancing maternal acceptance of the semi-allogeneic foetus by modulating the maternal immune system. HLA-G exerts several immunomodulatory effects, being beneficially implicated in embryo implantation and foetal survival. HLA-G inhibits the activation of the immune cells, and primes them into cytokine secretion profiles to control trophoblast invasion and maintain a local immunosuppressive environment for successful implantation and pregnancy survival. HLA-G has the ability to modulate the release of cytokines from human allogeneic peripheral blood mononuclear cells, and generate allogeneic cytotoxic T lymphocytes (CTLs) response in a concentration-dependent manner. Soluble isoforms of HLA-G has also been demonstrated to inhibit trophoblast invasion of the maternal decidua.

Setting/Location: The study was conducted at the Department of Obstetrics and Gynaecology in the Korle-Bu Teaching Hospital. Korle-Bu Teaching Hospital is a leading teaching hospital and a major referral centre in Ghana.

Aim: The aim of the study was to determine the role of soluble HLA-G in pregnancy success and early pregnancy loss in Korle-Bu Teaching Hospital.

Methods: This study involved eighty participants made up of twenty eight (28) normal pregnant women who had normal delivery, thirty two (32) women who had spontaneous or recurrent abortion, ten non-pregnant women and ten healthy men. A semi-structured questionnaire was administered to each consented participant to document their sociodemographic characteristics, and the history of pregnancy was

obtained from the clinic folders. 5ml of venous blood samples were collected from each consented participant and the plasma used to determine sHLA-G levels by Enzyme-linked immunosorbent assay (ELISA).

Results: The median sHLA-G levels were higher among women who had spontaneous abortions (66.5 U/ml) as compared to pregnant women who had normal delivery (49.53 U/ml), this was statistically significant. The first and second trimester sHLA-G levels of women who had spontaneous abortion are 66.53U/ml and 74.08U/ml respectively and was not statistically significant. The first and second trimester sHLA-G levels of pregnant women who had normal delivery are 39.73U/ml and 69.06U/ml respectively and were also not statistically significant. Healthy males had sHLA-G level (79.11 U/ml) as compared to healthy non-pregnant women (58.28 U/ml) but the difference was not statistically significant. Maternal sHLA-G levels was not statistically significant (P=0.26) in relation to maternal age and birth weight (P=0.38).

Conclusion: The results indicate that high levels of sHLA-G may adversely affect pregnancy outcome whilst reduced sHLA-G expressions may enhance pregnancy survival. There was not a significance difference between gestation and sHLA-G levels of women who had spontaneous abortion and normal pregnancy in pregnant women who had normal delivery. The sHLA-G levels were not affected by gender; healthy males had higher sHLA-G level as compared to healthy non-pregnant women, who were all in normal conditions of health but the difference was not statistically significant. This suggests that high sHLA-G levels in healthy individual may play a role in immunosurveilance. The history of contraceptive use had no effect on sHLA-G levels of women who had spontaneous abortion. Finally, there was no relationship between maternal age and corresponding sHLA-G levels, which had no effect on infant birth weight.

CHAPTER ONE

1.0 INTRODUCTION

Human Leukocyte Antigen (HLA) -G is a class Ib HLA molecule which was first characterized by its expression at the materno–foetal interface (Meer *et al*., 2004). It shares structural properties of its classic counterparts HLA-A, B and C (Alvaro et. al., 2005). However, unlike its counterparts, it is characterized by limited tissue distribution in healthy conditions and by the expression of seven different isoforms that can be either membrane bound (G1–G4) or secreted (G5–G7) (Meer *et al*., 2004). HLA-G1 is a full length isoform encoding the complete molecule with α1, α2 and α3 domains, transmembrane region and the intra cellular region of the class I heavy chain. The other HLA-G isoforms are alternatively spliced shorter transcripts, lacking regions complementary to one or more entire exons. Thus, HLA-G2 lacks exon 3, corresponding to the α2 domain; HLA-G3 lacks exon 3 and 4 and thus, only has the α1 domain; and HLA-G4 lacks exon 4 hence, the α3 domain. HLA-G5 and -G6 are equivalent to HLA-G1 and -G2, respectively, but they retain intron 4 which contain a stop codon. The anchoring transmembrane region could not be transcribed resulting in the expression of a soluble protein. Hence, they are also known as soluble HLA-G1 and HLA-G2. A further splice variant of HLA-G (HLA-G7) has also been reported. This isoform contains intron 2, which also has a stop codon, so that the resulting G7 protein would be a soluble HLA-G comprised of only the α1 region (Sargent *et al*., 2005). HLA-G has suppressive effects on natural killer (NK) cells, CD4 and CD8 T cells, B lymphocytes and antigen presenting cells such as macrophages and dendritic cells (Ober *et al*., 2006). Although HLA-G was found to inhibit the activation of the immune cells, its primary role is thought to be the modulation of cytokine secretion by

these cells to control trophoblast invasion and maintain a local immunosuppressive environment for successful implantation (Sargent *et al*., 2005).

In normal successful pregnancies, the developing foetal placental unit acts as an allograft which presents the allogeneic paternal HLA antigens to the mother. This presentation is done by the extravillous cytotrophoblast by expressing HLA-G (Le Bouteiller et. al., 2000) and lowered or reduced HLA-C prior to implantation (Onno *et al*., 1994, Redman *et al*., 2000). These antigens have the potential role of protecting the trophoblast from cytotoxicity. However, HLA-G helps in the maternal acceptance of the semi allogeneic foetus by modulating the maternal immune system (Hviid, 2005).

Expression of HLA class Ib genes in the trophoblast cells prevents cytolysis of trophoblast cells by resident NK cells. Resident natural killer (NK) (decidual) cells are a subpopulation of NK cells that reside in the decidua during pregnancy. Resident NK cells are poor killers of the usual NK cell targets and are programmed to recognise, attack and kill HLA null cells (Hunt, 2005). NK cells of unusual phenotype $CD16^{-}56$ bright are resident and abundant in the decidua in first and second trimester and reduce thereafter. HLA-G inhibits these NK cells through the NKAT3, CD96/NKG2A receptors preventing cytolysis and contributing to foetal survival (Munz *et al*., 1997; Perez-Villar *et al*., 1997; Soderstorm *et al*., 1997; Pende *et al*., 1997). NK cells also express immunoglobulin transcript (ILT2) and killer inhibitory receptor (KIR) 2DL4. ILT2 though expressed in low quantities on decidual NK cells (Trundley and Moffet, 2004; Navarro *et al*., 1999; Allan *et al*., 1999) binds HLA-G to prevent cell lysing. KIR2DL4 interaction with HLA-G may produce the cytokine interferon gamma (IFN-γ) at the materno-foetal interface (Kikuchi *et al*., 2003; Rajapalon *et al*., 2003). IFN-γ is a proinflammatory cytokine that can possibly drive immune cells into

immunosuppressive profiles, hence, serving the role of an anti-inflammatory cytokine and aiding pregnancy success. The trophoblastic cells of the placenta were further observed to resist NK cell– mediated lysis independent of HLA class I molecules (Sivori *et al*., 2000; Kikuchi-Maki *et al*., 2003). HLA-G effects are highly concentration dependent and its soluble isoforms reduce the ability of T cells to function effectively in the pregnant uterus. According to Hunt *et al*., (2004), about ninety-one percent of mothers, nullipara women and men lack antibodies to HLA-G in sera but women who had been pregnant even with multiple successful pregnancies showed anti-HLA-G antibodies in sera, suggesting interactions with B cells. However, this did not show any damage to the foetus resembling other anti HLA-G antibodies.

1.1 GENERAL OBJECTIVE OF THE STUDY

The aim of the study is to determine the role of sHLA-G expression in pregnancy success and early pregnancy loss in Korle–Bu Teaching Hospital (KBTH).

1.2 SPECIFIC OBJECTIVES

- To measure soluble HLA-G in normal pregnant women, women undergoing spontaneous or recurrent abortion in KBTH.

- To determine whether maternal sHLA-G plasma levels affect infant birth weight.

PROBLEM STATEMENT

Spontaneous abortion refers to a clinical condition that describes expulsion of products of conception before 20 weeks gestation (Ryan *et al*., 1999) without outside intervention. There are several deaths and disabilities' resulting from spontaneous abortion (Ahman *et al*., 2000) but it has been estimated to have the risk of one maternal death per 100,000 events (Meer *et al*., 2004). Worldwide, a little over 79 million spontaneous abortions occur per year (Pop. Council, 2000) and about 500,000 estimated deaths from pregnancy-related causes in a year as a result of induced abortions (Odlind *et al*., 1997). Spontaneous abortion is a major public health concern in Ghana. A study conducted at KBTH in Accra found that 18% of gynaecology admissions in the year 2000 were related to complications of abortion (Schwandt *et al*., 2010). Furthermore, of the total of 105 maternal deaths recorded at KBTH, 14% were due to complications of abortion (Schwandt *et al*., 2010). Ghana Maternal Health Survey in 2007 reports that more than one in 10 maternal deaths result from complications of abortion (Schwandt *et al*., 2010). Recurrent spontaneous abortion (RSA) of unknown etiology is a frustrating and emotionally charged clinical problem and it is one of the least understood pathological processes in spite of it being one of the most common pregnancy complications (Meka and Reddy, 2006). The emotional issues surrounding pregnancy loss become magnified exponentially when miscarriage occurs on a repetitive basis. A study conducted by Meka and Reddy (2006) showed 4-6% of all women attempting pregnancy experienced at least two miscarriages, of which 1-2% has three or more miscarriages and 12-14% of all pregnancies experienced clinical intrauterine pregnancy losses. The cause of repeated pregnancy loss is multifactorial and most women are not able to ascribe any reason for the

incident of spontaneous abortion to themselves (Laird, 2003). The etiology in approximately 50% of cases of spontaneous abortion is unknown, but it has been postulated that a proportion of these repeated pregnancy losses may be due to immune causes (Vercammen, 2008). One of the problems in understanding the underlying etiology of this immune failure is that the mechanisms by which the foetus is protected from the maternal immune system during normal pregnancy are not fully understood (Vercammen, 2008). Human leukocyte antigen (HLA)-G is thought to play a role in implantation. This non-classical HLA class Ib molecule has been demonstrated in human preimplantation embryos at the mRNA and protein level as well as in their culture supernatant, where the presence of soluble HLA-G (sHLA-G) has been reported a prerequisite for implantation (Shankarkurmar, 2004).

If soluble HLA-G has a role in pregnancy survival, then its expression may be altered in conditions with poor trophoblast invasion such as miscarriage and preeclampsia.

These observations point the way to the use of soluble HLA-G measurements as a possible diagnostic tool for these disorders.

JUSTIFICATION

HLA-G is a class Ib HLA which has gained much attention due to its multiple functions on the immune system. HLA-G exerts several immunomodulatory effects, being beneficially implicated in embryo implantation and foetal survival but, conversely, being potentially detrimental in tumours and viral infections (Vercammen, 2008). This two-edged sword behaviour suggests that HLA-G expression is under tight

regulation. However, to date, little is known about the regulation of this gene. All this features have made HLA-G an attractive target in different situations in which immune tolerance is involved, such as pregnancy and its complications, transplantation, cancer and viral infections, as well as in inflammatory and autoimmune diseases. sHLA-G has been shown to stimulate uterine natural killer (NK) cells to induce a unique profile of proinflammatory and proangiogenic mediators and cytokines favouring implantation and placentation (Vercammen, 2008). These observations point the way to the use of plasma soluble HLA-G measurements as a possible diagnostic tool or as a therapeutic tool and or target for predicting and achieving successful implantation and pregnancy outcome.

CHAPTER TWO

2.0 LITERATURE REVIEW

The term HLA refers to the Human Leucocyte Antigen Systems, which is located on and controlled by genes on the short arm of chromosome six (Messer *et al*., 1992). The HLA loci are part of the genetic region known as the Major Histocompatibility Complex (MHC) (Janeway *et al*., 2001). The MHC spans approximately 4Mb and encodes at least ~130 functional genes, of which more than 20% have functions in immunity, and it is the most gene-dense region of the human genome, as well as the region with the most disease associations (Sadki *et al*., 2011; Doherty and Zinkernagel, 1975).

The MHC genes are traditionally divided into three classes: the MHC class I and class II genes, which encode the antigen-presenting MHC molecules; and the class III genes, which encode complement, hormones and other proteins (Sadki *et al*., 2011). The peptide antigen-presenting MHC molecules are known as classical MHC molecules. There are also structurally related molecules of class I and class II that do not function in the presentation of peptide antigens to T cells and these are known as non-classical MHC molecules. The expression of HLA molecules is different between HLA class I and class II (Sadki *et al*., 2011).

HLA class I molecules are expressed on all nucleated cells, whereas class II molecules are expressed on antigen presenting cells (APCs): macrophages/monocytes, dendritic cells, Langerhans cells and B cells. Moreover, within the same class, different loci do not have the same level of tissue expression, HLA-C are naturally more weakly

expressed than HLA-A or HLA-B in class I and in class II, HLA- DR is strongly expressed than HLA- DP and HLA-DQ (Berrih *et al*., 1985).

2.1 Major Histocompatibility Complex (MHC) Class II

HLA-DP, -DQ and -DR loci are termed HLA Class II. Their function is to present antigens that originate from inside the cell (endogenous antigens) to cytotoxic T lymphocytes (CTLs). The tissue distribution of HLA Class II antigens is confined to antigen presenting cells, including B-lymphocytes, macrophages, dendritic cells, endothelial cells and activated T-lymphocytes (Sridhar). The expression of HLA Class II, on cells, which would not normally express them, is stimulated by cytokines like interferon γ (Janeway *et al*., 2001). HLA Class II molecules consist of two chains each encoded by genes in the HLA complex on Chromosome 6 (Janeway *et al*., 2001). MHC class II molecules comprise of non-identical and non-covalently associated polypeptide chains (α1, α2, β1 and β2) (Dahl and Hviid, 2011). These two chains have amino ends on the surface, a short transmembrane stretch and intra-cytoplasmic carboxyl ends, with the exception of the α1 domain, all domains are stabilized by disulphide bridges (Dahl and Hviid, 2011). The β chain is shorter than the α chain and contains the alloantigenic sites. A peptide binding groove is formed in between α1 and β1 domains with a beta pleated floor. The greatest polymorphic variability was found to occur in the amino acids sequence in the peptide binding region (Dahl and Hviid, 2011). This in turn determines the chemical structure of the groove and influences the specificity and affinity of peptide binding (Dahl and Hviid, 2011).

2.2 Major Histocompatibility Complex (MHC) Class I

Major Histocompatibility Complex (MHC) class I genes play an integral role in host defence against intracellular pathogens and tumours (Ribic, 2005). HLA Class I antigens are expressed on the surface of most nucleated cells (Ribic, 2005). Additionally, they are found in soluble form in plasma and are adsorbed onto the surface of platelets.

Erythrocytes were also found to adsorb HLA Class I antigens to varying degrees (Janeway *et al*., 2001). HLA-B7, A28 and B57 are recognizable on erythrocytes and are termed "Bg" antigens (Janeway *et al*., 2001). HLA class I genes are composed of eight exons and seven introns. Exon 1 encodes the signal sequence, exons 2, 3, 4 respectively encode for the extracellular domain (α 1, α 2, α3), exon 5 encodes the transmembrane portion and exons 6, 7, 8 encode the intra-cytoplasmic tail (Berrih *et al*.,1985).

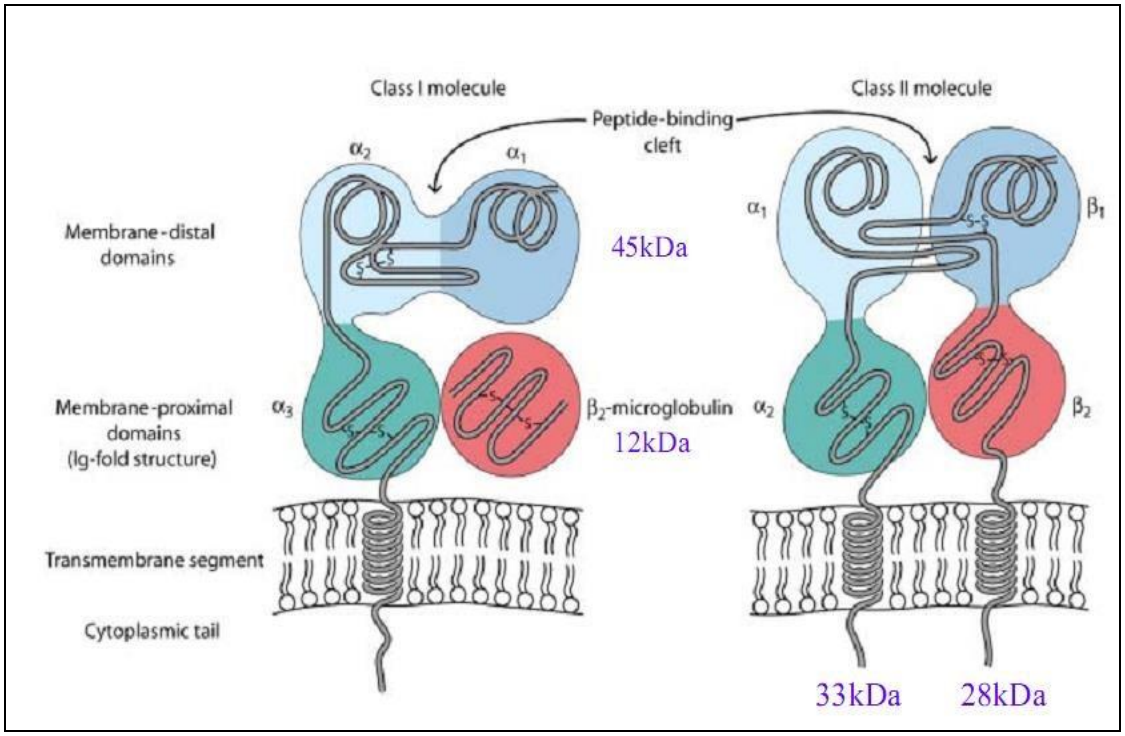

Figure 1: Structural differences in MHC class I and MHC class II molecules.

Credit: Kuby Immunology (www.ask4biology.com)

Class I MHC molecules contain two separate polypeptide chains, the heavier (44-47 KDa [kilodaltons]) alpha chain and the lighter (12 KDa) beta chain. The carboxyl end of α chain resides inside the cell while the amino end projects on the surface of cell with a short intervening hydrophobic segment traversing the membrane. The chain is coded by the MHC genes and has three globular domains α1, α2 and α3. β2-microglobulin is encoded by a gene on another chromosome. The α3 domain is non-covalently associated with the β2-microglobulin. Both α chain and β2-microglobulin are members of the immunoglobulin (Ig) superfamily. A peptide-binding groove is formed between α1 and α2 helices with beta-pleated sheet as its floor. A peptide of 8-10 amino acids long can be presented in this groove. The alloantigenic sites that carry determinants specific to each individual are found in the α1 and α2 domains (Dahl and Hviid, 2011). The greatest variability in amino acids (or polymorphism) occurs in the α1 and α2 sequences that line the wall and floor of the groove that binds the peptides.

The α1 and α2 domains also bind T cell receptor (TCR) of CD8 T lymphocytes. The parts of these domains that are in contact with TCR also show polymorphism. The immunoglobulin-like region of α3 domain is constant and non-covalently bound to β2-microglobulin. The CD8 molecules present on CD8 T lymphocytes binds to the conserved region of α3 (Dahl and Hviid, 2011).

There are two groups of MHC class I molecules; classical (HLA class Ia) and non-classical MHC class I molecule (HLA class Ib) (Adams & Parham 2001).

2.3 Classical MHC Class I Molecules (HLA CLASS Ia)

Classical MHC class I molecules are highly polymorphic, usually form trimmers on the cell surface and are mainly associated with antigen presentation. The cell surface glycopeptide antigens of the HLA-A, -B and -C series are called the classical HLA Class I antigens (Shankarkumar, 2004). HLA class Ia genes are among the most polymorphic genes described in the human genome. According to the IMGT/HLA data base, 1698 alleles of HLA-A, 2271 alleles of HLA-B and 1213 alleles of HLA-Cw have been identified. HLA-A, HLA-B and HLA-C also provide both stimulatory and inhibitory signals to natural killer (NK) cell immunoglobulin-like receptors (ILR). Although the three loci encode molecules with similar structure and function, the extent of their role in peptide presentation and ILR engagement varies across loci (Adams & Parham 2001).

2.4 Non-Classical MHC Class I Molecules (HLA Class Ib).

The non-classical class I genes, HLA- E, F, G, H, J, K and L are monomorphic and expressed on a more restricted set of cell types. They are not important as loci for peptide presenters (Shankarkumar, 2004). HLA-E, F and G were the first group of genes identified in the non-classical HLA family.

HLA-E is located between HLA-C and HLA-A, whereas HLA-F is located near HLAG and HLA-A (The MHC sequencing Consortium, 1999). They display a high level of similarity with class Ia genes and their protein products associate with β2-microglobulin (β2m). In contrast with classical genes they are almost non-polymorphic and show a very restricted expression pattern (Martinez *et al*., 2001). Differences do exist between the 3' cytoplasmic tail of class Ia and Ib genes (Heinrichs and Orr, 1990; Geraghty *et al*., 1987). HLA-G has a short cytoplasmic tail which is necessary for its much reduced spontaneous endocytosis (Davis *et al*., 1997). HLA-E is expressed on many different cells and tissues as the class 1a molecules (Lee *et al*., 1998). HLA-E binding groove has a unique amino acid substitution and a great affinity for signal peptides derived from HLA-G, an important role for the expression of HLA-E on trophoblast cells (Hviid, 2005). HLA-E was found to bind peptides derived from HLA-A, -B and -C leader sequences and in this manner forms ligands for the inhibitory CD94/NKG2A receptor of NK cells (Shankarkumar, 2004; Llano *et al*., 2003).

The function for HLA-F has not been well defined; however it has been shown to be bound by ILT2 and ILT4, so it may play a role similar to that of other non-classical in regulation of cytolytic cells (Janeway *et al*., 2001). HLA-F is has been detected on the invasive cytotrophoblast cells of the placenta (Hviid, 2005). The invasive

cytotrophoblast cells are the only cells of the placenta that express three class Ib molecules and may functionally substitute for one another (Ishitani *et al*., 2003).

2.5 Human Leukocyte Antigen (HLA) –G

Human leukocyte antigen G (HLA-G) is a non-classical major histocompatibility complex (MHC) class Ib antigen characterized by a limited polymorphism. HLA-G primary transcript generates 7 alternative mRNAs that encode membrane-bound (HLAG1, G2, G3, G4) and soluble (HLA-G5, G6, G7) protein isoforms (Abediankenari *et al*., 2007). HLA-G was first detected on extravillous cytotrophoblast cells. In nonpathological situations, HLA-G expression is restricted to the materno-foetal interface of the extravillous cytotrophoblasts, placental chorionic endothelium, thymic epithelial cells, and erythropoietic lineage cells from the bone marrow, as well as other immuneprivileged tissues such as the cornea, nail matrix, and autologous tissues such as the pancreas (Cai *et al*., 2012). HLA-G expression was detected in various types of human malignancies, and has been correlated with certain clinicopathological parameters in gastric carcinoma, lymphoma, ovarian and endometrial carcinoma (Cai *et al*., 2012). This suggested that HLA-G may promote tumour progression by suppressing immune regulation within tumour microenvironment, and thus helping tumour cells escape from anti-tumour immune surveillance. HLA-G expression was also detected in transplantation, multiple sclerosis, inflammatory diseases, and viral infections (Carosella *et al*., 2012).

HLA-G was found to bind to inhibitory receptors. Three HLA-G receptors have been described: ILT2/CD85j/LILRB1 (ILT2), ILT4/CD85d/LILRB2 (ILT4), and

KIR2DL4/CD158d (KIR2DL4) (Carosella *et al*., 2012). ILT2 is expressed by B cells, some T cells, some NK cells, and all monocytes and dendritic cells, but ILT4 is myeloid specific and only expressed by monocytes and dendritic cells (Carosella *et al*., 2012). KIR2DL4 expression is mainly restricted to the CD56bright subsets of NK cells, which constitute a minority of peripheral NK cells, but a majority of uterine NK cells (Carosella *et al*., 2012). Through these differentially expressed receptors, HLA-G can interact with B cells, T cells, NK cells, and antigen-presenting cells (APCs). Functionally, HLA-G1 was found to inhibit the cytolytic function of uterine and peripheral blood NK cells, the antigen specific cytolytic function of cytotoxic T lymphocytes, the alloproliferative response of CD4 T cells, the proliferation of T cells and peripheral blood NK cells and the maturation and function of dendritic cells (Carosella *et al*., 2012). Soluble HLA-G5 or soluble HLA-G1, which is generated by proteasomal cleavage from the cell membrane, has similar functions. The other HLA-G isoforms have been less well studied, and little is known about their function except that membrane-bound HLA-G2, HLA-G3, and HLA-G4 can inhibit NK-cell and cytotoxic T lymphocyte cytolysis in vitro (Carosella *et al*., 2012).

HLA-G1 is the full length isoform encoding the complete molecule which is the α1, α2 and α3 domains, the transmembrane region and the intracellular region of the class I heavy chain. The other HLA-G isoforms are alternatively spliced shorter transcripts lacking regions complementary to one or more entire exons. HLA-G2 lacks exon 3, corresponding to the α2 domain; HLA-G3 lacks exon 3 and 4 and thus, only has the α1 domain; and HLA-G4 lacks exon 4 and hence, the α3 domain. HLA-G5 and-G6 is equivalent to HLA-G1 and -G2, respectively, but are highly unusual in that, due to an incomplete splicing process, they retain intron 4 which contains a stop codon. This prevents the transcription of the anchoring transmembrane region, resulting in the

expression of soluble proteins. Hence, they are also known as soluble HLA-G1 and HLA-G2 (Sargent *et al*., 2005).

HLA-G is proposed the human functional homolog of murine Qa-2. Qa-2, the mouse Ped gene, is a MHC Class Ib protein with a defined function in regulation of preimplantation embryonic development (Comiskey *et al*., 2006). The Ped gene confers survival advantage to term and increases embryonic cleavage rates (Wu *et al*., 1999). Qa-2 positive mice exhibit enhanced birth and weaning weights (Warner *et al*., 2001). HLA-G and Qa-2 share structurally similar characteristics and interact with receptors of the innate and acquired immune systems (Comiskey *et al*., 2006). Similarities include expression of membrane-bound and soluble isoforms, increase in preimplantation growth rates and enhanced foetal survival, and have a shortened cytoplasmic tail (Comiskey *et al*., 2003).

2.6 Human Leukocyte Antigen (HLA) -G and Polymorphisms

The limited polymorphisms in HLA-G are located outside its binding groove (Hviid, 2005). Major groups of HLA-G alleles arise from single–nucleotide polymorphisms that change the amino acid sequence of HLA-G proteins, even still, further specific allelic variants are seen that arise from silent nucleotide variations (Hviid, 2005). However, WHO-acknowledged HLA-G alleles are not defined as polymorphisms in the noncoding region the HLA-G gene. Two amino acid substitutions in exon 2, 3 and 4 of HLA-G respectively define alleles HLA-G 0102 and HLA-G 0103, HLA-G 0104, and HLA-G 0106 respectively (Hviid *et al.,* 2001, 2002). Other existing alleles are: HLA– G 01010x group of alleles, as well as HLA-G0105N (a null alleles) with a third base deletion of codon 129 or the first base of codon 130 (Suarez *et al.,* 1997; Hviid *et*

al., 1997) in exon 3 leads to a conservative frame shift and is expected to be a non-functional protein and also responsible for decreased amounts of HLA-G1 isoforms (Pfeiffer *et al*., 2001). HLA-G0105N induces surface expression of HLA E which can interact with CD19/NKG2A in its implantation roles.

These HLA-G alleles may be classified into three: high secretors, the in-betweens and the low secretors. HLA-G0104 is a high secretor allele, -G01013 and –G 0105N are low secretor alleles, with –G01011 and –G 01012 falling between the two extremes (Hviid, 2005). Hence, the presence of an allele in an individual may determine the blood levels of HLA-G in that individual.

HLA-G alleles are distributed in different ethnic groups around the world. HLA–G 01010x group of alleles are predominant, with a frequency of about 80% in some Africans and 50-60% in Caucasians and Japanese. According to Ishitani *et al*., (1999), Ghanaians showed a high of 83% frequency of the allele HLA-G010101, 70% for African Americans; and a least of 10% for North Indians (Abbas *et al*., 2004). However, HLA-G010102 a member of the –G 01010x group of alleles which has a 14bp sequence in the 3'UTR of the gene is sparsely distributed in Africans (2.4%) but has a 30% frequency in other populations. Harrison *et al*., (1993) and Tamaki *et al*., (1993) stated that DNA sequence variations exist in the 5'URR (5'ustream regulatory region) in introns and also in the 3'UTR (3'untranslated region) of the HLA-G gene. In addition, Hviid, (2005) observed that sequences and polymorphisms in these same regions are important in HLA-G expression and regulation. Polymorphisms in position -725 are likely to be associated with the status of methylation and sHLA-G expression, and may have an association with spontaneous abortion (Ober *et al*., 2003).

The HLA-G gene and its transcript possess a 14bp deletion/insertion polymorphism in exon 8 of the 3'UTR (Harrison *et al*., 1993). HLA-G 010101 with 14bp deletions have

a significantly reduced expression compared to HLA-G010102 mRNA isoform having the 14bp sequence (Hviid, 2005). HLA-G010102 in transcription and alternate splicing showed additional mRNAs which lack the first 92bp of exon 8; these are variants of HLA-G1 and HLA-G5/G6. HLA-G 010103 alleles more closely resemble HLA-G 010101 alleles in their HLA-G mRNA expression. The HLA-G 010103 alleles showing this close resemblance to HLA-G 010101 have mRNA isoform with 14bp sequence and lack the first 92bp in exon 8, and are unique for HLA-G2 and possibly –G4 (Hviid, 2005). Rousseau *et al*., (2003) reports that HLA-G transcripts with the 92bp deletions are much more stable than the complete for the +14bp sequence in the 3'UTR (Hviid *et al*., 2002, 2004a) and women heterozygous for the 14bp polymorphism (Tripathi *et al*., 2004) risk having recurrent spontaneous abortion than their controls.

2.7 Membrane-bound and Soluble HLA-G

Pregnancy maintenance is said to be the prime role of HLA-G molecules and evidence from many studies suggest different isoforms of HLA-G as well as membrane-bound HLA-G, the shedded sHLA-G1, the soluble HLA-G5 may play different roles in pregnancy (Dahl *et al.*, 2013). HLA-G in peripheral blood is generated by alternative splicing of HLA-G transcript to produce soluble isoforms HLA-G5, G6, G7 (Hviid *et al*., 2000; Paul *et al*., 2000 ; Moreau *et al*., 1995; Fujii *et al*., 1994). HLA-G mRNAs retain intron 4 which has a stop codon are translated into sHLA-G isoforms (Hviid *et al*., 2000; Fujii *et al*., 1994). Membrane-bound isoforms may be shedded into peripheral blood to generate sHLA-G in a process requiring metalloproteinases (Park *et al*., 2004).

Soluble HLA-G in peripheral blood is also found in amniotic fluid (Rebman *et al*., 1998; Hunt *et al*., 2000). Rebman *et al*., (1998) and LeFriec *et al*., (2004) also reported peripheral blood monocytes are predominant in the secretion of HLA-G5, which is the central HLA-G molecule in male reproductive system; and detected in the tissues of the testis, epididymis, prostate gland (Langat *et al*., 2006) including sHLA-G in seminal fluid (Dahl *et al*., 2014) and in peripheral blood of men and non-pregnant women (Rizzo *et al*., 2007; Fuzzi *et al*., 2002; Hunt *et al*., 2000). Suggestion are that the predominating soluble HLA-G isoform in non-pregnant female and in male are HLA-G5, and during pregnancy the rise in sHLA-G in the maternal blood might primarily be a result of shedded HLA-G1 from trophoblast cell membranes in the placenta (Dahl *et al*., 2014). Many studies have detected sHLA-G in the culture media of preimplantation embryos in in vitro fertilization IVF procedures after 46-72hrs before transfer, and in single embryo cultures (Noci *et al*., 2005). Levels of sHLA-G in blastocyst media from IVF have shown high sHLA-G levels correlates with fertility success which is clinical pregnancy (Rebman *et al*., 2010; Vercammen *et al*., 2008; Hviid *et al*., 2006). Yie *et al*., 2005 also reported 386 embryo culture supernatants of which 69.9% were positive for sHLA-G after 72 hours, and observed live births rate in women who had an HLA-Gpositive embryos transferred and was significantly higher than those who had HLA-G– negative embryo transfers. sHLA-G negative preimplantation embryos culture used in IVF treatments failed with no signs of implantation (Sher *et al*., 2004; Fuzzi *et al.,* 2002). HLA-G mRNAs and protein expression was also detected in 2-16 –cell stage preimplantation embryos (Juriscova *et al*., 1996) and Hviid *et al*., (2006) confirmed HLA-G transcript have an association with increased cleavage rate when compared with those lacking HLA-G mRNAs. According to Hviid *et al*., (2006) expression of HLA-G is no guarantee for of

implantation. Regarding the many publications of the above, contradictions arise where human embryo culture supernatants from 8-cell morula embryos or beyond had no detectable sHLA-G (van Lierop *et al*., 2002) and large studies also recorded IVF treatment failures with sHLA-G positive preimplantation embryos (Hviid *et al*., 2006). Yie *et al*., (2005) again reported pregnancy and live births with sHLA-G negative IVF cycles.

One of the many receptors of HLA-G is KIR2DL4 and is mainly expressed on CD56bright NK cells which is absent in peripheral blood (Trowsdale and Moffet, 2008). Membrane-bound isoforms of HLA-G was found to induce inhibition of uterine NK cell mediated cytolysis through KIR2DL4 but not in peripheral NK cells which are devoid of this receptor (Ponte *et al*., 1999; Yu *et al*., 2006). Dahl *et al*., (2014) demonstrated peripheral NK cell inhibition by HLA-G1 in extravillous cytotrophoblast (EVT) cell lines and transfection in K562 cell lines showed HLA-G 5 is a more potent inhibitor of NK cell mediated cytolysis than HLA-G1. However, a study by Zhang *et al*., (2013) show that a combination of HLA-G5 and HLA-G1 has an additive effect on the inhibition of NK cytotoxicity. It has been shown that the expression of KIR2DL4 on the surface of uterine NK cells was higher in fertile women than among RSA women, indicating that the interaction between membrane-bound HLA-G and KIR2DL4 may favour induction of tolerance at the materno-foetal interface (Yan *et al*., 2007). HLAG1 may be most important in preeclampsia pathogenesis, however, low levels of its shedded sHLA-G have been found in the blood plasma of pregnant women with severe preeclampsia in late pregnancy (Dahl *et al*., 2014). Based on what is known today, it is possible that membrane-bound HLA-G interacts with inhibitory immune receptors to induce tolerance of the foetus, and at the same time sHLA-G serves as an activating molecule promoting proinflammatory cytokine secretion

allowing trophoblast migration and vascular remodelling (Dahl *et al*., 2014).In contrast, studies by McCormick *et al*., (2009) revealed sHLA-G actually inhibited trophoblast invasion, but this may be concentration dependent and tolerance at the materno-foetal interface may be contributed to by several immune cells interacting with HLA-G (Dahl *et al*., 2014).

2.8 Human Leukocyte Antigen (HLA) -G and Pregnancy

The human placenta is made up of several distinct subpopulations of trophoblast cells which originate from the progenitor cells of the trophectoderm layer of the blastocyst. HLA-G is selectively expressed by the trophoblast cells as early as the first trimester and throughout pregnancy. However, there are divergent patterns of expression of HLAG in the various subpopulations of trophoblastic cells (Hviid, 2005). These expressions are as follows; endovascular trophoblast cells express membrane-bound HLA-G (Proll *et al*.,1999), the syncytiotrophoblast cells express only sHLA-G like all other subpopulations of trophoblast cells (Ishitani *et al*.,2003;Kovats *et al*., 1990); moreover, the syncytiotrophblast (ST), which forms the placental interface with the maternal blood and was previously thought to be class I MHC negative, has now been shown to also express message for soluble HLA-G5 and-G6 (Solier *et al*., 2002), but the invasive extravillous cytotrophoblast (EVT) cells have the strongest expression of membranebound HLA-G. There is a good amount of evidence to support that the extravillous trophoblast cells express membrane-bound HLA-G1, soluble HLAG-5/-G6, and possibly other isoforms, whereas the HLA-G5 and HLA-G2/-G6 expression in villous trophoblast (VT) and syncytiotrophoblast cells have been proposed but are still a matter of controversy (Morales *et al*., 2003 and 2007; Ishitani *et al*., 2003). Hence

adequate expression of HLA-G at the foetal-maternal interface is important in the acceptance of the semiallogeneic foetus by the pregnant woman's immune system.

HLA-G was found to play an important role in immune tolerance during pregnancy (Tripathi, 2007). It was found to suppress the proliferation of alloreactive CD4+ T-cell, block the effector function of decidual monocyte/macrophage, inhibit cytolysis mediated by NK-cells and T cells, modulate the release of cytokines and shift decidual mononuclear cells toward the T helper (Th) 2 profile (Cai *et al*., 2012). Of the Th2 cytokines, interleukin (IL)-10 has been shown to induce HLA-G expression, which in return stimulates IL-10 expression (Cai *et al*., 2012). Cytokines have been shown to influence placental development, growth and invasion (Roth and Fisher, 1999). HLA-G also inhibits CD8+ and CD4+ T cell reactivity (Lila *et al*., 2001): soluble/membrane bound HLA-G critically regulate CD8+ T cells by eliminating alloreactive or antiparternal T cell by triggering surface expression and secretion of Fas ligands, resulting in apoptosis by the Fas/FasL pathway (Contini *et al*.,2003; Fournel *et al*., 2000; Solier *et al*., 2002) and another way of maternal tolerance is to prevent cytotoxic activity of CD8+ T cells against target cells by using ILT2 to transduce inhibitory signals as well as protecting target cells against T cell lysis (Contini *et al*.,2003; Shiroishi *et al*., 2003). T cells have the capacity to produce various cytokines and this is largely done by the expression of surface molecules. A study suggested that CD3+ peripheral blood T cells are at normal levels for non-pregnant recurrent aborters as compared with normal fertile women (Kwak-Kim *et al*., 2003). Measurement done by Kwak-Kim *et al*., (2003) revealed significantly low levels of CD3+ peripheral blood T cells in first trimester pregnancy than those who delivered live infants successfully. A decrease in the CD3+ T cell population in women who miscarried may be as a result of proportional increases in CD19+ B cells and or CD 56+ NK cells (Kwak-Kim *et al*.,

2003). Endometrial T cells (CD3+, CD8+CD4+TCR) are present in the menstrual cycle and early pregnancy, and significant in the implantation and maintenance of human pregnancies. However, very few T cells were detected in the endometrium. The effect of HLA-G on T cells depends on; numbers of T cells in decidua (Bulmer *et al*., 1988), and that trophoblastic cells in normal placentas are most unlikely to generate a cytotoxic T lymphocyte (CTL) cell response (Hunt *et al*., 2004). Two powerful, multifunctional leucocytes also known as antigen-presenting cells (APCs); macrophages and dendritic cells habit the endometrium of the decidua throughout pregnancy (Bulmer *et al*., 1988; Gardner and Moffet, 2003; Nehemiah *et al*., 1981). These are in close contact with the invasive trophoblast, uterine blood vessels and may play central roles in uterine and placental homeostasis (Bulmer *et al*., 1988; Gardner and Moffet, 2003; Nehemiah *et al*., 1981; Hunt *et al*., 1989). Decidual macrophages activated by expression of HLA class II and CD86 antigens appear to be programmed for immunosuppression. They function in producing inhibitory molecules that act on lymphocytes, reducing allogeneic and autologous T cell responses in comparison with monocytes and producing prostaglandins as well as spontaneously secreting antiinflammatory cytokines (also immunosuppressive) eg. IL-10 and TGF (Heikkinen *et al*., 2003; McIntire *et al*., 2004). Decidual macrophages also express surface markers and altogether cause immune invasion and activation of macrophages. IL-10 according to Moreau *et al*., (1999) activates HLA-G expression which further enhances IL10 secretion. Decidual dendritic cell (CD83+ type) are involved in immune cell inhibitory profiles by secreting IL-12 and induce T-helper 2 (Th2) cell in cocultures with naïve CD4+ T cells (Hunt *et al*., 2004). Placental HLA-G induces these APCs into those profiles by recognizing ILT2 and ILT4 receptors on the APCs (Petroff *et al*., 2002; Samaridis *et al*., 1997; Colona *et al*., 1997).

The nature of HLA-G and its tissue distribution strongly suggested that it might play a key role in preventing the trophoblast from being recognized as foreign and rejected by the mother's immune system (Sargent *et al*., 2005). Indeed, HLA-G can protect foetal trophoblastic cells from maternal NK cells through interaction with their inhibitory receptors. HLA-G expression by embryos seems to be a prerequisite to their implantation. Evidence for a placental contribution comes from the observation that soluble HLA-G levels were found to increase during the early stages of gestation in twin pregnancies (Sargent *et al*., 2005). The existence of soluble forms of HLA-G extends the potential for its systemic inhibitory action. Soluble HLA-G has been found in the serum of women in early pregnancy. However, the levels was not found to differ from those in preovulatory women (Sargent *et al*., 2005; Puppo *et al*.,1999; Rebman *et al*., 1999; Pfeiffer *et al*., 2000). Houcai *et al*., (2011) investigated soluble HLA-G in all trimesters in the serum of normal pregnant women and reported similarities. Soluble HLA-G expression was found to be altered in conditions with poor trophoblast invasion such as miscarriage and preeclampsia (Sargent *et al*., 2005). Soluble HLA-G in maternal blood may be largely produced by immune competent cells of the mother than the few by the trophoblast cells of the placenta (Steinborn *et al*., 2003). It has been shown that there is reduced HLA-G expression on the invasive cytotrophoblast in the decidua of women who miscarry (Sargent *et al*., 2005). Similarly, HLA-G expression by extravillous cytotrophoblast in the implantation sites of term placentas from preeclamptic women was found to be reduced compared to normal pregnancy (Sargent *et al*., 2005) and there is a corresponding decrease in soluble HLA-G in both the placentas and circulation of pre-eclamptic women (Sargent *et al*., 2005).

Soluble HLA-G levels lower than 9.95ng/ml possesses the relative risk of the development of placental abruption in the further course of pregnancy (Steinborn *et al*., 2003). Pfeiffer *et al*., 2000, in a study of 20 women experiencing spontaneous abortion, showed significantly reduced levels of soluble HLA-G in 9 weeks gestation with a sHLA-G measure of 25.9±3.9 SEM ng/ml as compared to 35.9±3.3 SEM ng/ml of 37 women with normal successful pregnancies. HLA-G 010103 and –G0105N alleles are reported by Pfeiffer *et al*., 2001 to be associated with recurrent spontaneous abortion, and – G0104 and –G0105 alleles with an increased risk of abortion (Aldrich *et al*., 2001). A great number of women with recurrent spontaneous abortion carried – G 0106 allele compared with women with normal successful pregnancy (Hviid, 2005). Blood levels of HLA-G and 14bp in the 3'UTR may be linked to recurrent spontaneous abortion. However, an interesting development showed that maternal sHLA-G plasma levels are not altered significantly during normal pregnancy and that very similar levels were found between non-pregnant and pregnant women even when different assays are used (Puppo *et al*., 1999; Rebman *et al*., 1999; Pfeiffer *et al*., 2000).

O'Brien *et al*., (2001) and Hylenius *et al*., (2004) both in independent case control studies reported that a reduced HLA-G mRNA expression in primipara women, as well as those who carry a foetus with +14/+14 HLA-G genotype have the greater risk of preeclampsia. Hylenius *et al*., (2004) further observed that -14bp polymorphisms HLA-G alleles are inherited more often from the father in heterozygous foetuses in pregnancies not complicated with preeclampsia and the opposite, +14bp HLA-G alleles are more inherited from the mother in foetuses in preeclamptic cases. He also did not find any evidence of HLA-G antigen incompatibility between mother and foetus in preeclampsia. Preeclampsia is a systemic disorder which evolves in the

second half of pregnancy in which a pregnant woman develops hypertension, proteinuria and often oedema due to abnormal vascular response and increased systemic vascular resistance, enhanced platelet aggregation, activation of coagulation cascades and endothelial cell dysfunction (Pregnancy, 2000; Hviid, 2006). Preeclampsia leads to intrauterine growth retardation and reduced placental blood flow to the foetus. Pathogenesis of preeclampsia is characterized excessively by maternal inflammatory response to pregnancy (Rebman *et al*., 1999) and is more of a Th1 response in contrast to normal pregnancy which is a Th2 response (Darmochwal-Kolarz *et al*., 1999). Preeclampsia affects 2-7% of pregnancies in varying degrees (Hviid *et al*., 2005).

Immune maladaptation is involved in preeclampsia however; it is not clear whether cytokine expressions are a direct aetiological factor. In an attempt to find a link between HLA-G alleles and preeclampsia: HLA-G0105N allele was found to have associations with preeclampsia in the Caucasian population (Hylenius *et al*., 2004) but not in the African American populations (Aldrich *et al*., 2000). Combined HLA-G genotype of mother and child predisposes to preeclampsia. Moreover significantly reduced levels of sHLAG in maternal serum had an association with preeclampsia; in a study of 20 preeclampsia women and 14 controls (Yie *et al*., 2004). Possibly +14/+14 bp HLA-G may predispose one to preeclampsia due to an aberrant and reduced expression in the placenta (O'Brien *et al*., 2001; Hviid, 2003). Abnormal HLA-G expression in preeclampsia may be associated with general pathology, supporting the pathogenic role of HLA-G in preeclampsia.

2.9 Human Leukocyte Antigen (HLA) -G and Diseases

HLA-G expression is evident in many different tumours, malignant haematopoietic diseases, inflammatory diseases and transplantations. However, HLA-G is particularly involved in oncology and transplantation (Tiago *et al*., 2010). HLA-G expression in disease was first described in tumour cells (Paul *et al*., 1998), since then, many studies have confirmed HLA-G expression in more than a thousand malignancies, where its gene transcription and protein expressions are switched on, and switched off in surrounding normal tissues (Rouas-Freiss *et al*., 2005).

HLA-G expression in tumours favours tumour progression, development and disease as HLA-G interactions with inhibitory receptors of NK cells prevents NK cytolysis of tumour cells (Tiago *et al*., 2010) hence impairing antitumuor immunity. Another means of escape of immune surveillance by tumours is the use of trogocytosis. Trogocytosis is a cell-to-cell contact-dependent uptake of membrane and associated molecules. It involves transfer of molecules at an area of the membrane as well as molecules not taking part in cell-to-cell crosstalk. Trogocytosis was studied in murine T cells where CD4+ and CD8+ T cells respectively acquired MHC class II and MHC class I molecules in antigen presenting cells (APCs) (Huang *et al*., 1999; Patel *et al*., 2001; Hudrisier *et al*., 1999). This was confirmed in humans as T cells acquired HLA-G1 from APCs and behaved in like manner as murine T cells (Tatari-Calderone *et al*., 2002; LeMaoult *et al*., 2007). These T cells acquiring HLA-G1 switch to immunosuppressive modes and those that acquire HLA-DR assume the roles of APCs (Tatari-Calderone *et al*., 2002). Hence, modulating the role and capabilities of the immune system toward tumours. NK cells may acquire MHC class1 (Sjostrom *et al*., 2001; Zimmer *et al*., 2003; Vanherberghen *et al*., 2004) and viral receptors from their

targets (Tabiasco *et al*., 2003). Activated NK cells may acquire HLA-G1 from tumour cells by trogocytosis, making them HLA-G-negative tumours which escape cytolysis (LeMaoult *et al*., 2007). Moreover, NK cells that acquire HLA-G1 ceases to proliferate, losses their cytotoxic abilities and become suppressor cells even inhibiting cytotoxic activity of other NK cells (Tiago *et al*., 2010).

Studies reveal HLA-G expression correlates with tumour progression in ovarian and breast carcinomas (Singer *et al*., 2003) and melanocytic lesions (Ibrahim *et al*., 2004). HLA-G levels were found to be high in patients with neuroblastoma (Morandi *et al*., 2007), and produced unfavourable outcomes in chronic lymphocytic leukaemia (Nuckel *et al*., 2005), gastric and colorectal cancers (Ye *et al*., 2007). Malignant haematopoietic diseases such as acute myeloid leukaemia, acute lymphoblastic leukaemia also expresses HLA-G molecules (Tiago *et al*., 2010).

Pregnancy-associated malaria (PAM), a peripheral or placental infection by *Plasmodium*, is a major public health concern due to significant adverse health effects on both mother and child (Moya-Alvarez *et al*., 2004). This problem is predominant in sub-Saharan Africa example Ghana (WHO, 2012). PAM is associated with increased malaria risk in infancy (Desai *et al*., 2007; Rachas *et al*., 2012) and has been associated with congenital malaria, increased malaria episodes, anaemia, and non-malaria episodes and fever episodes in infants (Tonga *et al*., 2013; Malhotra *et al*., 1997). The variation in HLA-G 3'UTR have been proposed to be associated with HLA-G gene expression levels and this regulatory region may play a role in the control of response to malaria (Garcia *et al*., 2013). The transmission of +3178 G allele and of the UTR-1 haplotype carries unique +3178 G transmission in children having lower intensity of the parasite during asymptomatic infection (Garcia *et al*., 2013). The base, Adenine

located at position +3178 has been associated with decrease mRNA stability in vitro and its due to an expansion of an AU-rich motif leading to a less stable mRNA (Yie *et al.*, 2008). The transmission of +3178 G is theoretically associated with increase in HLA-G expression in children. It has also been found that UTR-3 haplotype is associated with an increase in the level of intensity of infection together with mean levels of *Plasmodium falciparum* density (Garcia *et al.*, 2013). Studies in many populations have reported, the UTR-3 is associated with the coding allele group HLA-G01:04 (Castelli *et al.*, 2001) which is very frequent in the African populations (Donadi *et al.*, 2011). Rebmann *et al.*, (2001) have also reported the coding allele groups are associated with high sHLA-G production.

HLA-G is expressed in many inflammatory diseases and seems to shift T helper cells towards a Th2 profiles (Kapasi *et al.*, 2000; Carosella *et al.*, 2001) and may act as a tissue protective molecule in certain inflammatory diseases (Tiago *et al.*, 2010) such as psoriasis, multiple sclerosis and asthma. HLA-G is an attractive molecule for promoting the immune profile characteristic of asthma. This is because airway inflammation in asthma involves a T-helper cell type 2 skewing of molecules similar to pregnancy (Hunt *et al.*, 2005) with the expression of sHLA-G5 (Nicolae *et al.*, 2005). Individuals are genetically predisposed to over-expression of HLA-G in response to specific signals. Once secreted, HLA-G could promote cascade of events relating in worsening inflammation. A coincidence is also seen of HLA-G expression and Th2 phenomenon in allergic rhinitis were higher levels of sHLA-G expression was detected with a strong correlation between sHLA-G levels and clinical severity (Ciprandi *et al.*, 2009a and 2009b). Studies in rheumatoid arthritis reported lower levels of sHLA-G compared to healthy individuals, however, the levels correlates with

the presence of disease associated epitopes (Tiago *et al*., 2010). This may provide a link to genetic factors or a mere consortium with 2695 genotype markers across the MHC in 2321 T1D families which has put HLA-G at the main list of candidate genes susceptible for type -1 diabetics (Tiago *et al*., 2010).

The role of HLA-G in type 1 diabetic disease is not known, however, treatment of dendritic cells with IFN-beta induced monocytes to express HLA-G (Abediankenari *et al*., 2007). Comparisons of HLA-G expression in 20 normal patients and 20 diabetics showed low expression associated with dendritic cells of the diabetics. Dendritic cells expressing HLA-G mediate inhibition of autologous T cell activation, showing that HLA-G may prevent the immune pathway in diabetic pathogenesis (Abediankenari *et al*., 2007).

HIV infection is characterized by loss of HLA-A and HLA-B, but the expression of HLA-G remains unaffected or at least not decreased (Triphati and Agarwal, 2007). Along with inability of viral Nef to down regulate HLA-G, there could be some changes, particularly increased interleukin 10 indirectly influencing the expression of HLA-G (Navikas *et al*., 1995). This cytokine, interleukin 10 (IL10) up regulates expression of HLA-G (Triphati *et al*., 2003). Lozano *et al*., (2002) demonstrated that after HIV infection expression of HLA-G increased in all monocytes and some T lymphocytes. A contradictory report by Derrien *et al*., (2004) showed down regulation of HLA-G in HIV infection. Derrien *et al*., (2004) studied HLA-G expression in acute HIV infection, and their results are similar to other acute viral infections such as human cytomegalovirus and herpes simplex virus. These both decrease cell surface expression of HLA-G1, but the former particularly can increase HLA-G1 expression

upon reactivation (Fisher *et al*., 2000; Onno *et al*., 2000). Possibly the expression of HLA-G could be enhanced in the natural course of HIV infection so that the situation in chronic infection would be as shown by Lozano *et al*., (2002). Further, HLA-G polymorphism is also associated with the risk of HIV infection. Extensive study of HLA-G polymorphism in 456 HIV-seropositive and 406 HIV-seronegative African women showed significant association of G*0105N with protection from HIV-1 infection and G*010108 with susceptibility to infection (Matte *et al*., 2004). HLA-G*0105N is characterized by deletion of cytosine at position 130 of exon 3, which leads to frame shift and introduction of a stop codon in exon 4 (Ober *et al*., 1998). Hence HLA-G*0105N stops the production of a functional HLA-G molecule. This could be the likely reason for association of G*0105N with protection from HIV infection, would be that this impairs the function of HLA-G and so down regulation by HIV would be absent or decreased (Triphati and Agarwal, 2007). Lajoie *et al*., (2006) presented more extended and explicit data for HLA-G polymorphism in the same cohort and found that women carrying G*0105N had a 2.2fold decreased risk of HIV-1 infection compared with women without G*0105N. They also reported an HIV-seronegative woman who was homozygous for G*0105N.

2.10 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a common laboratory technique used to measure the concentration of analyte (antibodies or antigens) in solution. It involves separation of specific and non-specific interactions through serial binding to a solid surface, usually a polystyrene multi well plate to achieve quantitative results. ELISA is

quick and easy to carry out and its coloured end products correlate to the amount of analyte in the original sample. They are quick to use and designed to rapidly handle large number of samples and are popular choice for research and diagnostic targets. ELISAs can be quite complex, including various intervening steps and the ability to measure protein concentrations in heterogeneous samples such as blood. The most complex step in the process is detection, where multiple layers of antibodies can be used to amplify a signal. There are different ELISA formats; the most appropriate for this study is the sandwich ELISA. Sandwich ELISA has a high specificity, suitable for complex samples and is flexible and sensitive (www.abdserotec.com/an-introductionto-elisa.htm).

The human leukocyte antigen (HLA)-G is a HLA class Ib protein, which in contrast to the highly polymorphic classical HLA molecules shows limited polymorphism and restricted tissue distribution and has a unique alternative splice pattern (Ellis *et al*., 1986; Paul *et al*., 2000). HLA-G is expressed as several different splice variants including four membrane-bound (HLA-G1 to -G4) and three soluble isoforms (HLA-G5 to -G7). In addition, both membrane-bound β2-microglobulins (β2m)-linked and free dimers, membrane bound β2m-free heavy chains, and possibly soluble β2m-free dimers have been reported (Ishitani *et al*., 1992; Apps *et al*., 2007; Morales *et al*., 2007). According to Hunt, (2000) HLA-G5 which is spread throughout the placenta likely has mainly free heavy chains and lack β2m, however, detecting antibodies in reagents require light chain/heavy chain associate. Approximately 50 HLA-G alleles corresponding to 16 HLA-G proteins have been reported (The IMGT database; Nov. 2013). Investigations of HLA-G genetics in relation to risk of certain pregnancy complications have increased during recent years (Hviid *et al.,* 2001 and 2006; Dahl *et al*., 2012).

In most of the studies, sHLA-G has been determined with a commercially available sHLA-G enzyme-linked immunosorbent assay (ELISA) kit, based on the capture antibody MEMG/9, capturing sHLA-G1/-G5 in association with β2m and a detecting antibody against β2m. Interestingly, the study by Wu *et al*., (2009) who failed to report an association, used a different ELISA assay with a higher limit of detection. This could account for the differences in results, whereas there is no obvious explanation to the reported lack of association in the study by Zhang *et al*., (2003) examining children with atopic asthma and positive controls. In a study by Rizzo *et al*., (2009) sHLA-G1 and HLA-G5 were determined by performing two different ELISA assays: one capturing both sHLA-G1 and HLA-G5 and one capturing only HLA-G5 by the use of the monoclonal antibody (mAb) 5A6G7, which is specific for HLA-G5/-G6. It is interesting to note that low levels of sHLA-G in assays may be due to polymorphisms or undetectable HLA-G5/ sHLA-G1 (Hviid *et al*., 2004b). ELISA assays measuring sHLA-G in serum detects HLA-G molecules in a β2m-associated form, hence should detect both sHLA-G1 which is shed by membrane-bound HLA-G1 and soluble HLA-G5 isoforms however, shed HLA-G molecules may blur associations between sHLA-G protein expression and even its polymorphisms (Hviid, 2006). Observations by Fournel *et al*., (2000) showed that the varied differences in ELISA results depend on the specific antibodies used and whether they bind the β2m-associated HLA-G or intron 4-retaining sHLA-G isoforms.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Site

The study was conducted at the Department of Obstetrics and Gynaecology in the Korle-Bu Teaching Hospital (KBTH). Korle-Bu Teaching Hospital is situated in the nation's capital, Accra, Ghana. KBTH is the leading teaching hospital and the major referral centre in the country. It also serves as the teaching hospital of the University of Ghana Medical School, (UGMS), in Accra. The Department of Obstetrics and Gynaecology in KBTH is one of the biggest tertiary obstetrics and gynaecology care centre in the country. The Gynaecology Emergency Room (E/R) is a 24-hour OPD emergency clinic, fully equipped with all basic and necessary medical gadgets. There are doctors and nurses around the clock who attend to emergencies and referrals. The E/R receives many cases in a day; approximately thirty cases (not including the night cases) of which about 75% are complications of induced abortion and the rest are spontaneous abortions and other gynaecological emergencies. The E/R serves the needs of the local community who present with emergencies without the notice of referral letters. However, about 70% of the complicated pregnancy cases within the nation are referred to this department. Patients that are referred to this department originate from different social and ethnic groups as well as geographically distinct areas. Thus the demographics of the study participants enrolled in this study were not limited to a specific social group or ethnicity.

3.2 Inclusion Criteria

Pregnant women who had normal delivery, women diagnosed with spontaneous abortion(s), healthy non-pregnant women and normal healthy males were included in the study.

3.3 Exclusion Criteria

Participants with immuno-deficiencies, malignancies, co-morbid factors as well as those on immunosuppressive and immune stimulants therapies were ruled out of the study.

3.4 Study Participants

A total of eighty participants were involved in the study. Peripheral blood samples were obtained from thirty two women who attended the Gynaecology Emergency Room for treatment of spontaneous abortion or recurrent spontaneous abortion, twenty eight healthy normal pregnant women who had normal deliveries from the Gynaecology Unit of KBTH, Ghana as well as ten non-pregnant women and ten men drawn from the College of Health Sciences, UGMS.

Informed consent was obtained from or given by all participants. Participants were enrolled in the study if they met the following criteria: suffered/suffering from one or two and more miscarriages, had no immune deficiencies, malignancies, co-morbid factors as well as no current history of immunosuppressive and immune modulator

therapies. Screening was done for, intrauterine contour showing homogeneous echo pattern of the uterus, clotting profiles, and a semi-structured questionnaire (Appendix VII) was used for the socio-demographic history of both women experiencing spontaneous abortion and those with normal pregnancy and delivery. Non-pregnant women were enrolled based on negative urine pregnancy test (β subunit of human chorionic gonadotrophin) and last menstrual period, and healthy adult men as part of the study. Malaria screening was done for all the participants.

3.5 Collection of Blood Samples

Five millimetres of venous blood was obtained from each consented participant using butterfly needles (0.8×19mm×178mm, BD, USA) into heparinised vacuitainers (BD, USA) tubes.

Blood samples were transported from the Unit to the laboratory in ice chest within 1 hour of sample collection.

3.6 Separating Plasma from Blood Samples

Plasma was separated from heparinised blood samples by centrifugation from whole blood at 2000rpm for 10minutes. After centrifugation the plasma was collected and placed in cryo tubes and stored at -80°C until ready to use.

3.7 Preparation of Plasma for ELISA

On the day of running the ELISA the frozen plasma samples were allowed to thaw at room temperature (25°C). Plasma was diluted 10 times with dilution buffer. Plasma samples were aliquoted each with a new 100µl pipette tip into clean wells for dilutions.

The mixture was vortex to ensure proper missing of plasma and diluent.

3.8 Measuring sHLA-G levels by ELISA

The sHLA-G levels were measured by ELISA using the manufacturer's protocol. In brief, the buffers and solutions used were pre-prepared, and were diluted as outlined in Appendice I-II. Master Calibrators (standards) were reconstituted with distilled water and prepared in dilutions with dilution buffer as outlined in the Appendix III.

To the appropriate wells were added 100µl of standards, samples and dilution buffer and incubated at 2-8°C between 16-20 hours without shaking. After the incubation, the plate was washed manually for five times (5x) with 0.35ml of wash solution per well. The microtiter plate was then bloated on paper towels to remove excess wash solution and unbound analytes. This was followed by the addition of 100µl of Conjugate Solution. The microtiter plate was then incubated on an orbital plate shaker with shaking at 300rpm for 1 hour at room temperature (25°C). Wash procedure was repeated and remained the same. To each well, was added 100µl of Substrate Solution. Care was taken not to expose plate to direct sunlight, and plate was covered with

aluminium foil. The microtiter plate was then incubated for 25 minute at room temperature (25°C) without shaking. The Conjugate Solution which contains horse radish peroxidase (HRP) is allowed in incubation time to react with the Substrate Solution which is tetramethylbenzidine (TMB). This allows for colour development which is stopped by the addition of 100µl Stop Solution to each well of the microtiter plate (Appendix IV).

The absorbance of each well was determined within 5 minutes of addition of Stop Solution. A Micro plate reader with a reference wavelength of 630nm set to 450nm was used to read the microtiter plates. Spectrophotometric readings of the assays were done both at 650nm and 450nm, and the readings were subtracted to obtain the various absorbance. The sensitivity of the assay was 0.6Units/ml.

3.9 Statistical Analysis

Data was entered into a database and analyzed using Sigma-Stat Version 3.5. The data was analyzed for significant differences and/or associations between categorical data. Measures of centrality (median) was evaluated with Man-Whitney Rank Sum test, Kruskal-Wallis Rank Sum test and Bonferroni post-hoc test (pairwise). Descriptive statistics; box plot was used in the data presentation. P-values of <0.05 was considered statistically significant. Most analyses were conducted by comparing the sHLA-G levels in categorical data. Regression analysis was used to analyze and correct for possible confounding effects of age and infant birth weight.

CHAPTER FOUR

4.0 RESULTS

4.1 Description of Study Participant

A total of 80 participants were involved in the study. Thirty two (32) spontaneous abortion (SA) patients represented 40% of the population with mean age of 31.7, (17-41years). Twenty eight (28) normal pregnant women who had normal delivery (PND) had a mean age of 24.6; (16-35years) and represented 35% of the study population. Ten (10) non-pregnant women (NPW) had a mean age of 22.6; (18-37 years). Ten (10) normal healthy men (NM) used as negative controls had a mean age of 25.6; (22-33years) and represented 12.5% of the population. Table 1 list all study participants.

Table 1: Age Distribution of study participants;

Study Group	Total Number (%)	Age(M±SD)
Spontaneous abortion (SA)	32 (40)	31.7 ±3.2
Normal delivered (PND)	28 (35)	24.6 ±2.4
Non- pregnant women (NPW)	10 (12.5)	22.6 ±3.7
Health adult men(NM)	10 (12.5)	25.6 ±5.2

4.2 Levels of sHLA-G among study participants

Figure 2 shows sHLA-G levels in women who experienced spontaneous abortion (SA) and pregnant women who had normal delivery (NPD). sHLA-G levels in women who experienced spontaneous abortion (66.5 U/ml) was higher than those pregnant women who had normal delivery (49.35U/ml). This was statistically significant (P<0.05).

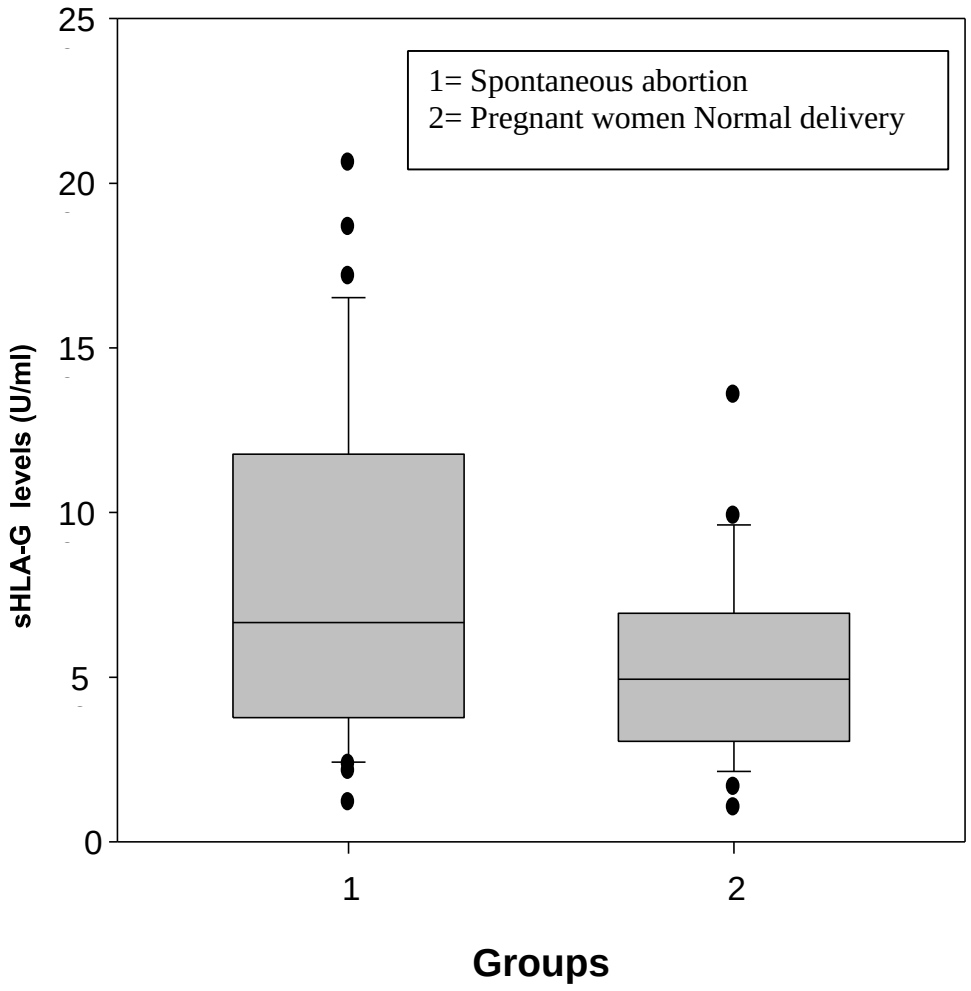

Figure 2: sHLA-G levels in spontaneous abortion patients (SA) and pregnant women who had normal delivery (NPD). Each box plot represents the median (dark band). P=0.03

The sHLA-G level in healthy non-pregnant women (58.28U/ml) was higher compared with sHLA-G levels in pregnant women who had normal delivery (49.36U/ml) and lower than women who had spontaneous abortion (66.53 U/ml). Figure 3 shows the differences in the median of the three.

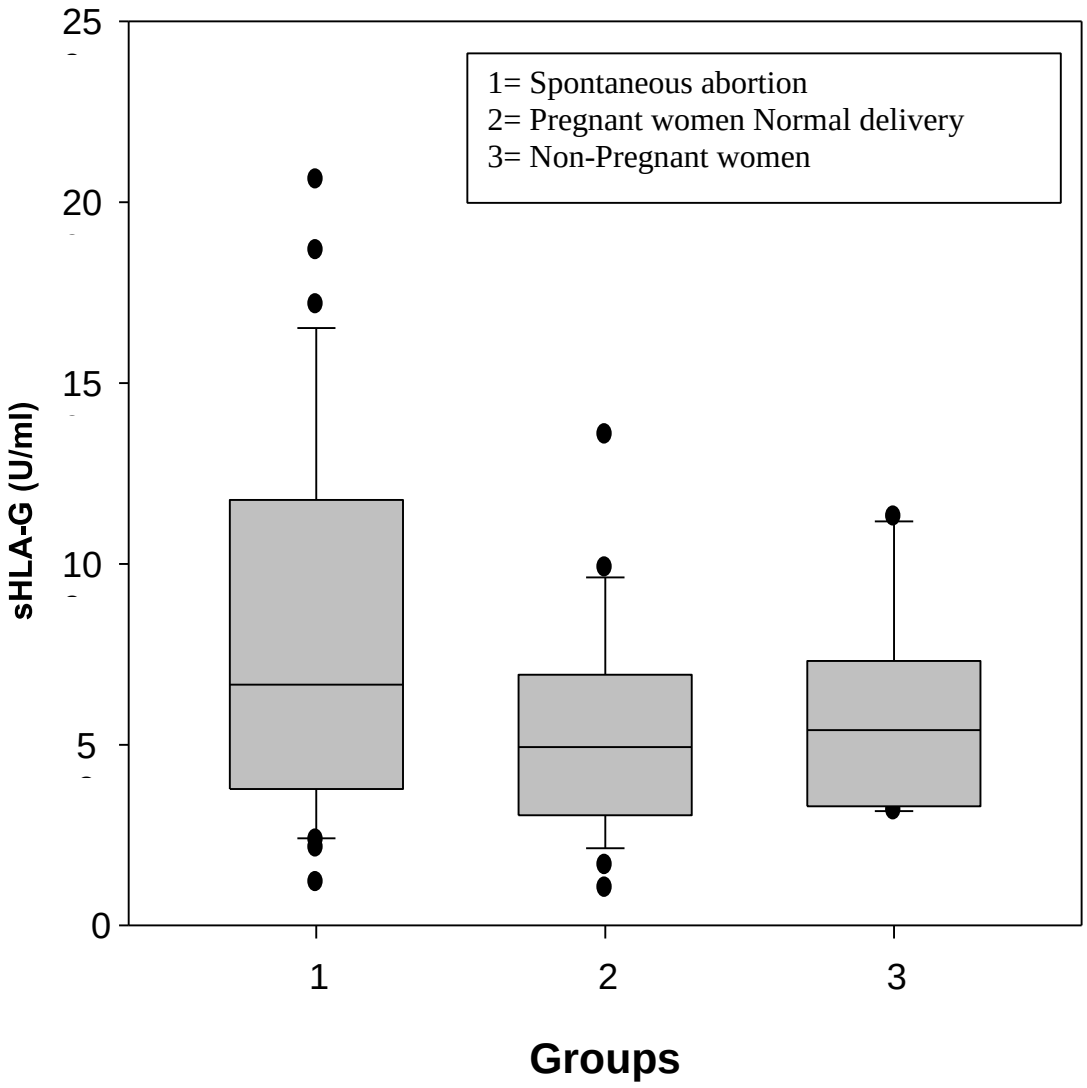

Figure 3: sHLA-G levels in spontaneous abortion patients (SA), pregnant women who had normal delivery (NPD), non-pregnant women (NPW).

Dark bands show the median sHLA-G levels in the groups.

4.3 Effect of Gender on sHLA-G Levels

The sHLA-G level of 79.11U/ml in normal healthy males was higher compared with the sHLA-G levels of 54.04 U/ml in healthy non-pregnant women. The difference was not statistically significant (P=0.18).

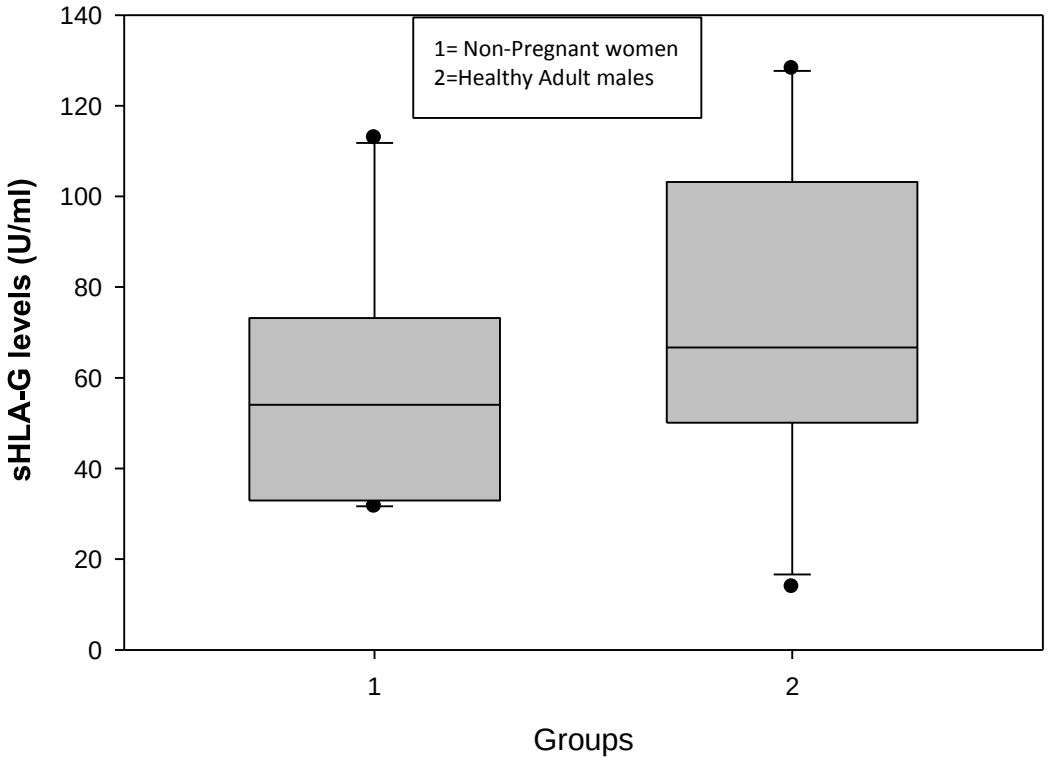

Figure 4: Comparison of sHLA-G levels in non-pregnant women (NPW) and normal healthy male (NM).

Each box plot represents the median (dark band). P=0.18

4.4 The impact of Gestation on sHLA-G levels

From the results, women in the first trimester who had spontaneous abortions (SA) showed a higher sHLA-G level of 66.53 U/ml as compared with pregnant women who had normal delivery (NPD) (sHLA-G of 41.94 U/ml). This was statistically significant (P= 0.04).

Women in the second trimester who had spontaneous abortions (SA) showed higher sHLA-G levels of 98.65 U/ml as compared with second trimester of pregnant women who had normal delivery sHLA-G level of 69.01 U/ml. However, this was not statistically significant (P=1.0).

There was no statistical significance difference (P=1.0) between first and second trimester sHLA-G levels in women who had spontaneous abortions (SA).

Pregnant women who had normal delivery showed a lower sHLA-G level of 39.73 U/ml in the first trimester compared with sHLA-G level of 69.06 U/ml in second trimester of pregnant women who had normal delivery. The difference was not statistical significance (P=0.08).

These data are shown in Figure 5.

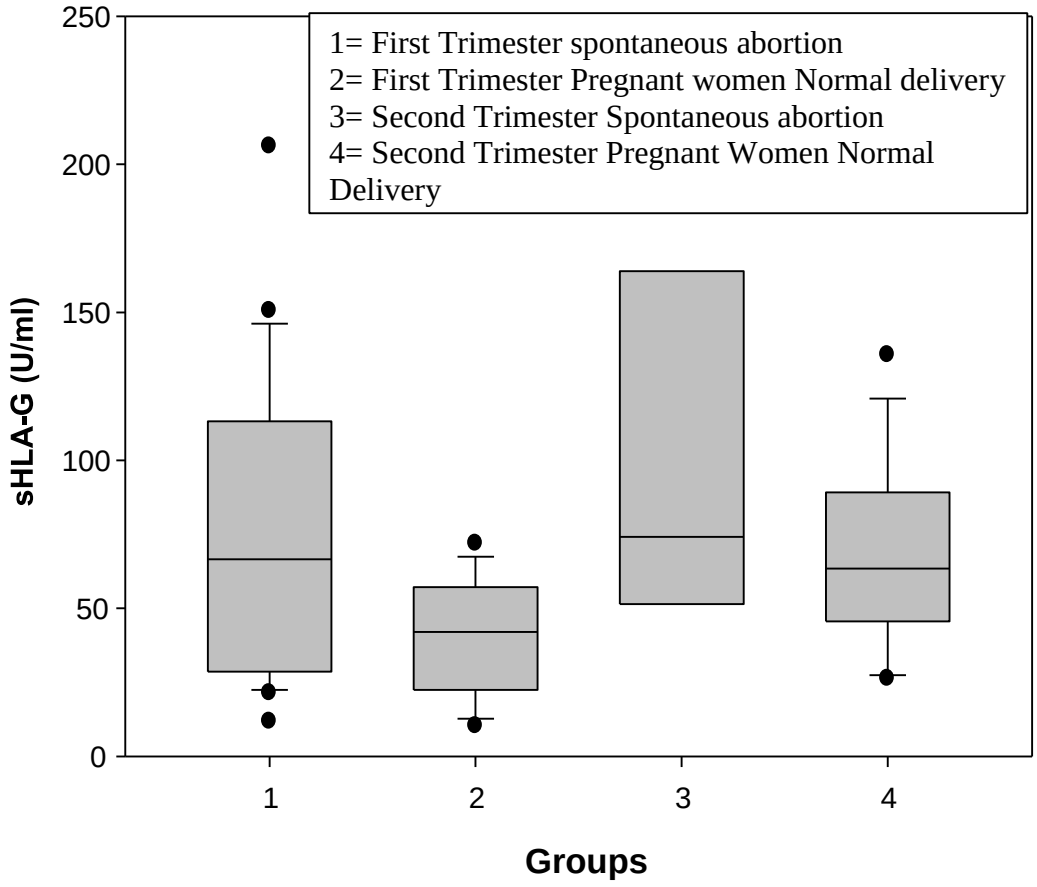

Figure 5: Differences in the levels of sHLA-G of first and second trimesters of normal pregnant women who had normal delivery (PND) and women who had spontaneous abortion. HLA-G in plasma was analyzed by ELISA. Data is presented as box plots with dark band as median.

4.5 Relationship between the levels of sHLA-G, maternal age, infant birth weight and contraception.

Results in Table 2 show the relationship between maternal age and sHLA-G levels as well as the sHLA-G levels of spontaneous abortion women and the history of contraception use.

There is no statistical significance difference (P=0.26) between maternal age and the corresponding sHLA-G levels.

Maternal sHLA-G levels have no statistical significant (P=0.38) on infant birth weight.

Women who had spontaneous abortion with the history of contraception use (84.68U/ml) was high compared to women who had spontaneous abortion with no history of contraception use (66.24U/ml). The difference was not statistically significant (P=0.62).

Table 2: Associations between sHLAG levels and Maternal Age, History of Contraception Use

	r	R^2	P value	Median sHLA-G level(U/ml)
MATERNAL AGE	0.083	0.007	0.26	----
INFANT BIRTH WEIGHT	-0.056	0.003	0.38	----
CONTRACEPTION			0.62	
YES (n=7)	----	-----		84.68
NO (n=25)	----	-----		66.24

r =correlation coefficient R^2 = coefficient of determination

CHAPTER FIVE

5.0 DISCUSSION

Foetal survival and pregnancy require an evolution of adaptive mechanisms that allows immunologic tolerance of the foetal allograft by the mother and at the same time maintaining immune competence against invading pathogens and HLA-G may represent one of the pathways of mechanisms for protecting the foetus (Ober *et al*., 2006). Recurrent spontaneous abortion (RSA) of unknown etiology is a frustrating and emotionally charged clinical problem. Spontaneous abortion is one of the least understood pathological processes in spite of being one of the most common pregnancy complications (Meka and Reddy, 2006). Identified causes are uterine malformations and genetic anomalies which account for 20-50% of the cases. Immunologic causes account for the rest of the causes (Vercammen, 2008). HLA-G altered expressions may predispose to RSA and clinical conditions in pregnancy. HLA-G is involved in reproduction; suppressing NK cell cytotoxic activity, regulating cytokines, inducing apoptosis in T cells, inhibit the activation of immune cells and controlling trophoblast invasion and maintenance of successful implantation (Sargent *et al*., 2005). HLAG expression is restrictive although it has been detected in much different tissues; however, its expression has been repeatedly described only on and by the trophoblast cells of the placenta (Kovats *et al*., 1990; Lila *et al*., 2001; Rebman *et al*., 2003).

One of the major findings from this study is that, women who had spontaneous abortion had higher sHLA-G levels of 66.5U/ml compared with pregnant women who

had normal delivery (49.35 U/ml) and the difference was statistically significant (P=0.03).

This suggests a possible role of sHLA-G in spontaneous abortion. This finding is in agreement with studies by Dahl and colleagues, (2014) who reported that HLA-G may inhibit trophoblast invasion in concentration-dependent manners, and that tolerance at the materno-foetal interface may be contributed to by the immune cells present in the decidua. Again, studies done by Hviid, (2006) have shown that sHLA-G positive preimplantation embryos failured to implant in IVF treatment. It has also been reported by McCormick *et al.*, (2009) that sHLA-G actually inhibited trophoblast invasion of the maternal decidua. Kanai *et al*., (2001), in in-vitro studies also demonstrated the ability of sHLA-G and/or mHLA-G to control the release of cytokines from human allogeneic peripheral blood mononuclear cells, which could generate allogeneic CTLs response in a concentration-dependent manner. Furthermore, soluble HLA-G levels could be altered in conditions with poor trophoblast invasion such as miscarriage and preeclampsia (Sargent *et al*., 2005).

Again, sHLA-G was detected in both healthy males and non-pregnant women, with the males showing higher sHLA-G level than the females but the difference was not statistically significant (P=0.18). These findings are consistent with Yie *et al*., 2004 which suggested that sHLA-G can be detected in all plasma from pregnant to non-pregnant women, and men (Rizzo *et al*., 2007; Fuzzi *et al*., 2002). HLA-G expression according to Lila *et al*., (2001); Rebman *et al*., (2003) and Hviid *et al*., (2004a) can be detected in serum, and plasma from men and women, but there are controversies in various studies as to whether sHLA-G can be found in the blood of all men and non-pregnant women under normal conditions of health. There again in literature, Ishitani

et al., (1999) in a comparative study, had indicated that there is a high of about 83% frequency of an HLA-G allele in Ghanaians. Based on these findings and this result, the current study suggests that high sHLA-G levels in healthy individuals may play a role in immunosurveilance in the Ghanaian populace. This finding is not in line with Vercammen (2008) who stated high HLA-G levels are present in disease conditions.

In this study, sHLA-G levels in pregnant women who had normal deliveries and healthy non-pregnant women were similar (sHLA-G levels of 53.13U/ml and 58.28U/ml respectively), showing no substantial differences. The result in this study is consistent with earlier studies which also found similar levels of sHLA-G between non-pregnant women and pregnant women even when different assays are used (Puppo *et al*., 1999; Rebman *et al*., 1999; Pfeiffer *et al*., 2000).

This study also looked at the effect of gestation on the levels of sHLA-G. sHLAG levels in first trimester gestation of spontaneous abortions were high compared to the first trimester of pregnant women who had normal delivery (P=0.04).

However, substantial differences were not seen in the second trimester between the two groups (P=1.0).

Other observations show that women who had spontaneous abortions had no substantial differences in the levels of sHLA-G between the first and second trimester gestations. Further findings in this study, reports that there are marked differences in the sHLA-G plasma levels of first trimester gestation (39.73 U/ml) which was not statistically higher (P=0.08) than the second trimester gestation (69.06 U/ml) of pregnant women who had normal delivery. However, findings by Hviid, (2006), which stated that maternal sHLAG plasma levels are not altered significantly during normal

pregnancy. Similar studies by Houcai *et al*., (2011) also reported similarities in soluble HLA-G levels in all trimesters in the serum of normal pregnant women.

Again, sHLA-G levels showed no relationship with age and infant birth weight. Infants born at 38 weeks and above to pregnant women who had normal delivery had birth weight ranging between 2.2kg to 4.7kg but their birth weights had no relationship with sHLA-G levels in maternal blood. However, studies by Hviid, et al., (2004b) and Hviid, (2006) report that HLA-G polymorphism (which influences HLA-G expression) was significantly associated with increased foetal and placental weight. Also in the mouse model, polymorphisms in the mouse Qa-2, a homolog of human HLA-G showed associations with increased foetal and placental weight (Comiskey et al., 2003). However, the current study was conducted in smaller populations.

Maternal age also had no effect on sHLA-G levels in women who had spontaneous abortion and pregnant women who had normal delivery. There may be no relationship between maternal age and maternal sHLA-G levels.

Finally, women who had spontaneous abortion with a history of contraceptive use had a higher sHLA-G level of 84.68 U/ml compared with women who had spontaneous abortions with no history of contraception but this was not statistically significant (P=0.62). Hence, the history of contraceptive use is not a determinant of maternal sHLA-G levels.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

- Soluble HLA-G levels were higher in the plasma of women who had spontaneous abortions than in normal pregnant women who had normal delivery. Many controversies surround HLA-G functions in pregnancy and its complications. However, the results of this current study are indicative that high levels of sHLA-G in circulation may play a role in spontaneous abortion but reduced sHLA-G levels in circulation may enhance pregnancy survival.

- High soluble HLA-G expression was detected in plasma of healthy non-pregnant women and healthy males in normal conditions of health. The current study suggests based on its findings and results that, high HLA-G levels are not present only in disease but high sHLA-G levels in healthy individuals may play a role in immunosurveilance.

- Soluble HLA-G levels did not alter substantially during the course of normal pregnancy in pregnant women who had normal delivery.

- Maternal age had no corresponding effect on maternal HLA-G levels, which in turn had no effect on infant birth weight.

6.2 RECOMMENDATION

- There is the need for more research on sHLA-G levels and cytokine profiles in larger population sample among Ghanaians. This may determine the combined effects of sHLA-G levels and cytokines on the outcomes of pregnancy in Ghana.

- The in-depth study of cytokines and sHLA-G and how they affect one another in pregnancy may serve as a therapeutic tool, as to how higher sHLA-G levels can be lowered to enhance pregnancy survival.

- Soluble HLA-G levels may be used as a diagnostic tool in women who may have spontaneous abortion and/or recurrent spontaneous abortion in Ghana.

- There is the need for public education on the effects of stigmatization and the risks of induced abortion, to allow people to freely seek proper medical care and to readily aid diagnosis of spontaneous abortions (miscarriages).

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APPENDICES

Appendix I:

Conjugate Solution 100x (Dilution)

Volume: 13ml

Components	Amount	Lot no.:	Amount used	Check steps done (☑)
Conjugate Solution	0.13ml	K21-033		
Conjugate Diluent	13ml	K00-600		

PROCEDURE

1. Pipette 0.13ml of Conjugate Solution into flask.
2. Add 13ml of Conjugate Diluent and mix.

Appendix II:

Wash Solution 10x (Dilution)

Volume: 500ml

Working solution: 1x

Components	Amount	Lot no.:	Amount used	Check steps done (☑)
Wash Solution	100ml	K00-579		
Distilled Water	50 ml	n/a		

PROCEDURE

1. Pipette 50ml of wash solution into a measuring cylinder.
2. Add 450mlof distilled water and mix.

Appendix III:

Master Calibrator stock solution

Volume: 500ml

Components	Amount	Lot no.:	Amount used	Check steps done (☑)
Master Calibrator	2 vials	K21-030S3		
Dilution Buffer	13ml	K00-595		
Distilled Water	0.8ml	N/A		

PROCEDURE

1. Pipette 0.8ml of distilled water into vial to obtain 625U/ml concentration of

   Reconstituted master calibrator (stock).

2 Aliquot 100µl of the stock into new a vial and add 400µl of the dilution buffer.

3. Aliquot 100µl into appropriate well.
4. Add 250µl of dilution buffer and pipette 100µl appropriate well
5. Repeat step 4 five times, to obtain specific dilutions for appropriate wells.

Appendix IV:

sHLA-G ELISA Plate

sHLA-G ELISA Plate												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Calibrator 125.00	Calibrator 125.00	SA01	SA09	SA17	SA26	AMC001	AMC009	AFC007	DP045	DP176	DP223
B	Calibrator 62.50	Calibrator 62.50	SA02	SA10	SA18	SA27	AMC002	AMC010	AFC008	DP088	DP194	DP228
C	Calibrator 31.25	Calibrator 31.25	SA03	SA11	SA19	SA28	AMC003	AFC001	AFC009	DP125	DP197	DP243
D	Calibrator 15.63	Calibrator 15.63	SA04	SA12	SA20	SA29	AMC004	AFC002	AFC010	DP137	DP198	DP277
E	Calibrator 7.81	Calibrator 7.81	SA05	SA13	SA21	SA30	AMC005	AFC003	DP007	DP142	DP202	DP283
F	Calibrator 3.91	Calibrator 3.91	SA06	SA14	SA22	SA31	AMC006	AFC004	DP010	DP155	DP204	DP285
G	Blank	Blank	SA07	SA15	SA23	SA32	AMC007	AFC005	DP042	DP158	DP208	DP263
H	Blank	Blank	SA08	SA16	SA24	SA33	AMC008	AFC006	DP043	DP175	DP222	DP291

Appendix V.

Median sHLA-G levels (U/ml) and P value for Categories Compared

Study Participants	Median sHLA-G levels (U/ml)	P value
Spontaneous Abortion	66.52	0.03
Pregnancy with Normal Delivery	49.35	
1st Trimester of Spontaneous Abortion	66.53	0.04
1st Trimester of Pregnancy with Normal Delivery	41.94	
2nd Trimester of Spontaneous Abortion	98.65	1.0
2nd Trimester of Pregnancy with Normal Delivery	69.05	
1st Trimester of Spontaneous Abortion	66.53	1.0
2nd Trimester of Spontaneous Abortion	74.08	
1st Trimester of Pregnancy with Normal Delivery	39.73	0.08
2nd Trimester of Pregnancy with Normal Delivery	69.06	
Non Pregnant Women	54.04	0.18
Healthy Adult Males	79.11	

Krukal-Wallis Rank Sum Test (Trimesters) P=0.015

Appendix VI

The characteristics of study participants

Sample ID	Age/Yrs.	Category	Contraceptive Usage	Gestation/ months	Infant Weight/ Kg	Units/ ml
SA01	41	Spontaneous Abortion	No	1	n/a	11.7
SA02	41	Spontaneous Abortion	No	5	n/a	186.5
SA03	46	Spontaneous Abortion	Yes	3	n/a	64.02
SA04	30	Spontaneous Abortion	Yes	4	n/a	140.7
SA05	39	Spontaneous Abortion	Yes	3	n/a	28.3
SA06	35	Spontaneous Abortion	No	1	n/a	141.8
SA07	30	Spontaneous Abortion	Yes	3	n/a	46.3
SA08	27	Spontaneous Abortion	No	1	n/a	112.3
SA09	23	Spontaneous Abortion	No	3	n/a	23.4
SA10	31	Spontaneous Abortion	No	4	n/a	63.5
SA11	28	Spontaneous Abortion	Yes	1	n/a	88.6
SA12	33	Spontaneous Abortion	No	3	n/a	206
SA13	29	Spontaneous Abortion	No	1	n/a	90.3
SA14	30	Spontaneous Abortion	No	2	n/a	27.2
SA15	22	Spontaneous Abortion	No	3	n/a	113.4
SA16	41	Spontaneous Abortion	No	2	n/a	66.2
SA17	25	Spontaneous Abortion	No	1	n/a	135.5
SA18	34	Spontaneous Abortion	Yes	4	n/a	84.7
SA19	17	Spontaneous Abortion	No	3	n/a	119.1
SA20	29	Spontaneous Abortion	No	2	n/a	68.5
SA21	41	Spontaneous Abortion	No	3	n/a	25.6
SA22	29	Spontaneous Abortion	No	4	n/a	49.1
SA23	40	Spontaneous Abortion	No	1	n/a	51.3

SA24	35	Spontaneous Abortion	No	1	n/a	29.4
SA26	33	Spontaneous Abortion	No	3	n/a	69
SA27	24	Spontaneous Abortion	No	2	n/a	62.9
SA28	37	Spontaneous Abortion	No	5	n/a	171.5
SA29	21	Spontaneous Abortion	No	4	n/a	58.5
SA30	25	Spontaneous Abortion	Yes	3	n/a	150.4
SA31	38	Spontaneous Abortion	No	3	n/a	66.8
SA32	25	Spontaneous Abortion	No	2	n/a	21.3
SA33	34	Spontaneous Abortion	No	4	n/a	34.8
AMC001	n/a	Normal Male	n/a	0	n/a	128.2
AMC002	n/a	Normal Male	n/a	0	n/a	41.4
AMC003	n/a	Normal Male	n/a	0	n/a	123
AMC004	n/a	Normal Male	n/a	0	n/a	96.5
AMC005	n/a	Normal Male	n/a	0	n/a	91.4
AMC006	n/a	Normal Male	n/a	0	n/a	66.8
AMC007	n/a	Normal Male	n/a	0	n/a	666.6
AMC008	n/a	Normal Male	n/a	0	n/a	52.9
AMC009	n/a	Normal Male	n/a	0	n/a	13.8
AMC010	n/a	Normal Male	n/a	0	n/a	54.6
AFC001	n/a	Non Pregnant Female	n/a	0	n/a	52.4
AFC002	n/a	Non Pregnant Female	n/a	0	n/a	32.1
AFC003	n/a	Non Pregnant Female	n/a	0	n/a	112.8
AFC004	n/a	Non Pregnant Female	n/a	0	n/a	55.7
AFC005	n/a	Non Pregnant Female	n/a	0	n/a	61.8
AFC006	n/a	Non Pregnant Female	n/a	0	n/a	31.6
AFC007	n/a	Non Pregnant Female	n/a	0	n/a	37.6
AFC008	n/a	Non Pregnant Female	n/a	0	n/a	63.5
AFC009	n/a	Non Pregnant Female	n/a	0	n/a	102.1
AFC010	n/a	Non Pregnant Female	n/a	0	n/a	33.2

DP007	28	Pregnancy with normal delivery	n/a	2	3.6	25.1
DP010	30	Pregnancy with normal delivery	n/a	2	3	10.1
DP042	30	Pregnancy with normal delivery	n/a	3	3	21.8
DP043	20	Pregnancy with normal delivery	n/a	5	3.5	82.4
DP045	30	Pregnancy with normal delivery	n/a	5	2.5	29.4
DP088	28	Pregnancy with normal delivery	n/a	3	3.6	22.9
DP125	28	Pregnancy with normal delivery	n/a	2	2.9	48.5
DP137	28	Pregnancy with normal delivery	n/a	3	3.4	33.7
DP142	25	Pregnancy with normal delivery	n/a	1	3.2	71.8
DP155	18	Pregnancy with normal delivery	n/a	2	2.7	56.3
DP158	20	Pregnancy with normal delivery	n/a	3	3.8	41.9
DP175	25	Pregnancy with normal delivery	n/a	2	2.2	16.5
DP176	26	Pregnancy with normal delivery	n/a	9	3.2	36.5
DP194	19	Pregnancy with normal delivery	n/a	6	3	98.7
DP197	21	Pregnancy with normal delivery	n/a	4	3.8	41.4
DP198	23	Pregnancy with normal delivery	n/a	4	3.3	60.7
DP202	25	Pregnancy with normal delivery	n/a	6	3.9	49.6
DP204	26	Pregnancy with normal delivery	n/a	3	3.8	60.7
DP208	31	Pregnancy with normal delivery	n/a	6	3	71.3
DP222	23	Pregnancy with normal delivery	n/a	4	3.9	61.8
DP223	19	Pregnancy with normal delivery	n/a	8	2.5	37
DP228	20	Pregnancy with normal delivery	n/a	6	2.6	135.5
DP243	17	Pregnancy with normal delivery	n/a	4	2.9	95.9
DP277	27	Pregnancy with normal delivery	n/a	3	3.1	57.9
DP283	16	Pregnancy with normal delivery	n/a	6	3	81.3
DP285	24	Pregnancy with normal delivery	n/a	2	2.3	49.1

DP263	35	Pregnancy with normal delivery	n/a	4	4.7	63.5
DP291	27	Pregnancy with normal delivery	n/a	4	3.9	26.1

Appendix VII

QUESTIONNAIRE

Identification number …… …… Date……..

Age……..Duration of Pregnancy:……..weeks

Marital Status: a. Single b. Married c. Widowed d. Separated e. Divorced

Do you work? …… If yes, what do you do? ……...

During the pregnancy, did you have any exposure to X-rays?

No Yes,
If yes how many times……

Herbal Drugs No Yes,

Alcohol No Yes,

Medications No Yes,

If yes please specify…….

Cigarettes No Yes,

During the pregnancy, did you have any viral infections/Rashes No Yes,

If yes please specify

……
……
……

Were there any complications during the pregnancy? No Yes, if yes please specify

……..

Do you have a history of infertility? No Yes,

Was the pregnancy the result of infertility treatment? No Yes,

How many pregnancies have you had, including any miscarriages, abortions, and this one: ……

Have you had recurrent pregnancy loss or a stillbirth? No Yes,

How many miscarriages have you had?.....

How many abortion have you had? ……

……
Were you on birth control pills before conception? No Yes, If yes please specify

……
……
……..

Are you

Diabetes? No Yes

Hypertensive? No Yes

Have you had a history of trauma or domestic violence? No Yes; if yes, please explain:

……..
……..

Have you ever had a blood transfusion? No Yes; if yes date…….

Do you have a history of a sexually transmitted disease? No Yes,

If yes which of the following: gonorrhoea, chlamydia, HPV syphilis