ANALYSIS OF TOTAL HLA-G LEVELS AND ITS ISOFORMS IN PLACENTAL MALARIA

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DECLARATION AND RECOMMENDATION

This thesis is my original work and has never been submitted to any institution known to me for any award.

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DEDICATION

My daughter **Lavender Kendi**

"Many of life's failures are people who did not realize how close they were to success when they gave up"

- Thomas Alva Edison.

ABSTRACT

The present study examined the total, membrane bound and soluble HLA-G in infected and uninfected placentas using quantitative real time PCR, ELISA and immunohistochemistry. The total HLA-G transcripts differed significantly (p = 0.009) between infected and uninfected primigravidae. The membrane bound HLA-G transcripts were significantly more abundant than the soluble HLA-G in infected placentas (p = 0.04) in all mothers. At the protein level infected placentas had higher mean levels of soluble HLA-G than uninfected and naïve sera. Soluble HLA-G concentration differed significantly between infected and uninfected primigravidae with a p of 0.001. Immunohistochemistry study demonstrated intense staining of HLA-G in infected placentas, with localization of membrane bound HLA-G protein in the syncytiotrophoblasts and near immune cells, when compared to uninfected placentas. Soluble HLA-G localized in the intervillous spaces and in the lumen of the stroma of forming fetal blood vessels. Soluble HLA-G concentrations in infected primigravidae and secundagravidae were negatively correlated to birth weight. Conversely, the concentration in infected multigravidae was positively correlated to birth weight and maternal age. Maternal age and the concentration of soluble HLA-G correlated negatively in all parities in a physiological pregnancy. However, in infected primigravidae and multigravidae the maternal age and soluble HLA-G correlated positively. The findings of this study indicate that there are high levels of HLA-G in infected placentas from all parities compared to the uninfected placentas. However, the levels of HLA-G in infected primigravidae are significantly higher than in uninfected with p value of 0.001. These results shows that HLA-G is involved in mediating anti-inflammatory response in *P. falciparum* infected placentas. The study findings suggest that interventions during pregnancy malaria should promote expression of HLA-G in the placenta to check down the effects of the inflammatory infiltrates in the *P. falciparum* infected placenta.

TABLE OF CONTENTS

CONTENTS **PAGE**

LIST OF TABLES

LIST OF FIGURES

LISTS OF ABBREVIATIONS

β-$_2$-m:	Beta-2 microglobulin is coded in chromosome 15 and associates with HLA–A and HLA–G for their proper functioning.
CTL:	Cytotoxic T lymphocytes
CSA:	Chondroitin sulfate A, a receptor for cytoadherence of infected erythrocytes in the placenta.
HELLP:	Hemolysis elevated liver enzymes low platelets count syndrome.
HBSS:	Hank's balanced salt solution
HCMV:	Human cytomegalovirus
HLA–G:	Human leukocyte antigen G, a non classical major histocompatibility antigen class 1b.
IL–10:	Interleukin 10, an anti-inflammatory (Th2) cytokine
ILT:	Immunoglobulin-like transcripts receptors for HLA-G (ILT2 and 4)
IUGR:	Intrauterine growth retardation
KIR2DL4:	Killer cell Immunoglobulin-like Receptor 2 long Cytoplasmic Domain 4
LIR:	Leukocyte immunoglobulin like receptors
MHC:	Major histocompatibility complex.
NK:	Natural killer cells
qRT-PCR:	Quantitative real time polymerase chain reaction for quantifications of mRNA expressed from a particular gene.
RT-PCR:	Convectional reverse transcriptase polymerase chain reaction
sVEGFR1:	Soluble vascular endothelial growth factor receptor 1.
Th1 and Th2:	Pro-inflammatory or Type 1 cytokines and anti-inflammatory or Type 2 cytokines.
TNF–α:	Tumor necrosis factor alpha, a pro-inflammatory (Th1) cytokine.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Malaria during pregnancy is a major cause of maternal morbidity and mortality in tropics. People living in areas where malaria is endemic; develop protective immunity against severe malaria and high-density parasitemia by adulthood. However, this protection is partially lost during pregnancy (McGregor, 1984; Tako *et al.,* 2005). In endemic areas women in their first and second pregnancies are most susceptible to *Plasmodium falciparum* infections (Fried and Duffy, 1996; Ordi *et al.,* 2001; Tako *et al.,* 2005). Women develop increasing resistance to pregnancy malaria over successive pregnancies, and this pattern of parity-specific resistance has been related to the acquisition of antibodies that inhibit adhesion of *Plasmodium falciparum*-infected erythrocytes to the placental receptor, chondroitin sulfate A (CSA) (Fried *et al.,* 1998; Ordi *et al.,* 2001; Tako *et al.,* 2005). Fried-Duffy model explains parity-specific resistance to placental malaria. However, it remains unknown how pregnancy-related immunomodulation may also contribute to malaria susceptibility and poor pregnancy outcomes (Fried and Duffy, 1996; Duffy and Fried, 2001).

The ability of *P. falciparum* to adhere to the CSA expressed by the trophoblast has recently been considered to play a major role in the marked susceptibility of pregnant women to infection (Fried and Duffy, 1996; Fried *et al*., 1998; Viebig *et al.,* 2005). Studies have shown that antibodies that inhibit *P. falciparum* adhesion to CSA are associated with increased birth weight, gestational age of newborns and reduced placental parasitemia (Fried *et al.,* 1998; Duffy and Fried, 2003). Alternatively, an immunosuppressive status, either general or local, has classically been postulated to be the major underlying determinant of malaria susceptibility during pregnancy (Rasheed *et al*., 1993; Fievet *et al.,* 1997; Ordi *et al.,* 2001). Although, several measures of maternal immunity are modified during pregnancy, and the placenta generates immunomodulatory factors including hormones, none of these have been conclusively related to malaria susceptibility or pregnancy outcomes (Duffy and Fried, 2001).

Placenta cells also called trophoblasts produce a variety of membrane-bound proteins and soluble products. These include members of the tumor necrosis factor (TNF) superfamily, complement regulatory proteins, class Ib human leukocyte antigen (HLA) molecules, progesterone, and prostaglandins, which could serve to thwart potentially harmful effects of immune cells and their products (Petroff *et al.,* 2003). These molecules are also involved in regulating immune responses of placental physiology (Hunt *et al*., 2005; LeMaoult *et al.,* 2005). Autocrine and paracrine factors such as steroid and polypeptide hormones, prostaglandins and anti-inflammatory cytokines that are present in the uterine environment during pregnancy change their secretory profiles during pregnancy (Hunt *et al.,* 2000).

Antigen-specific T and B lymphocytes which are responsible for acquired immunity are virtually absent from the maternal-fetal interface (Hunt *et al*., 2000). In contrast, non-antigen specific natural killer cells and macrophages which mediate innate immune responses are abundant and highly specialized in infected placenta (LeMaoult *et al.,* 2005).

Placenta malaria has been identified as a factor that can lead to hypertension in first time pregnant mothers (Muehlenbachs *et al.,* 2006). Muehlenbachs and colleagues found that the concentrations of soluble vascular endothelial growth receptor 1 (sVEGFR1) and its transcripts increased in first time placental malaria positive mothers. At the same time, infiltrating immune cells in intervillous spaces expressed vascular endothelial growth factor receptor (VEGF), the cognate ligand for sVEGFR1. This shows a conflict between the fetus and maternal system on who should deal with the parasites in the placenta. Low levels of HLA-G have been detected in pre-eclamptic term placenta (Hviid *et al*., 2004). Both pre-eclampsia and placental malaria infections are associated with low birth weight (Tako *et al.,* 2005).

HLA-G gene products dominate tissues within the placenta at a time when HLA-A and HLA-B molecules are largely absent (Hunt *et al*., 2000). This suggests that HLA-G plays a role in the materno-fetal interaction to maintain pregnancy until term (Hunt *et al*., 2000; Le Bouteiller and Solier, 2001).

Natural killer (NK) cells are key effectors of the human innate immune system and are essential in maintaining pregnancy (Rouas-freis and Carosella, 1997). Low NK cell cytotoxic activity is linked with increased sensitivity to human malaria infections (Moffett-King, 2002). In physiological pregnancy, the activity of NK cells is partly inhibited by HLA-G (Gonen-Gross *et al.,* 2003; Sartelet *et al*., 2005), among other factors. HLA-G5 isoforms down regulate maternal CD8+, CD4+ T cell reactivity, endothelial cell proliferation and migration (Fournel *et al*., 2000). This establishes a transient state of tolerance, which ensures implantation and placentation are successful (Hunt *et al.*, 2000).

Membrane bound HLA-G decreased in *P. falciparum* infected placenta, which may experience increased numbers of natural killer cells infiltrating into the intervillous spaces (Sartelet *et al.,* 2005). HLA-G isoforms have been shown to play antagonistic functions, with membrane bound HLA-G1 isoform acting as a restriction element and other isoforms as immunosuppressive factors. The antagonistic functions were found to depend on the concentrations of the products of HLA-G and the number of locally recruited CD8+ T cells. The varying balance could promote pro-inflammatory/pro-angiogenic cytokines in the placenta (Rajagopalan *et al*., 2006), the profile that develop during placental malaria (Fried *et al*., 1998). Several studies have shown that Th-1 cytokines are related to low birth weight infants born to placental malaria positive mothers (Fried *et al.*, 1998; Moormann *et al*., 1999; Ordi *et al*., 2001).

Soluble HLA-G proteins have been shown to have a long half-life in the body, because of their short cytoplasmic tail (Rajagopalan *et al.,* 2006), which has been shown to resist endocytosis and to take a long time to be moved to the endoplasmic reticulum (Rajagopalan *et al.,* 2006).

The current study examined the protein concentrations and mRNA transcript abundance of HLA-G isoforms in placental malaria positive mothers in an age and parity dependent manner compared to uninfected mothers from the same region. These levels were correlated with birth weight and maternal age.

1.2 Statement of the Problem.

In malaria endemic areas congenital parasitemia in infants born to placental malaria positive mothers is rare. However, placental malaria especially to first time mothers has been associated with low birth weight, intrauterine growth retardation (IUGR) and stillbirths. The actual mechanisms leading to these poor outcomes are not clear. It has been argued that maternal-fetal conflict could be an important factor in the pathogenesis of placental malaria. This conflict is thought to be controlled by many factors ranging from cytokines, placental peptides to nutritional requirements. The placenta lacks MHC class 1 proteins which are involved in intracellular antigen presentation to the cytotoxic T cells. HLA-G proteins are present in the placenta and may modulate the immune responses in placental malaria. Variations in concentrations of soluble HLA-G and membrane bound HLA-G, and the balance between them may play key roles in determining pregnancy outcome in infected women. A previous study demonstrated a decrease in membrane bound HLA-G with increased infiltration of immune cells in parasitised placentas (Sartelet *et al*., 2005). Soluble HLA-G which could play an anti-inflammatory role was not investigated. Also, immunohistochemistry which is a semi quantitative method for HLA-G analysis did not give conclusive information.

1.3 Objectives

1.3.1 General objective

The general objective of this project was to study the transcription levels and protein concentrations of human leukocyte antigen-G in placental malaria, and to relate these with maternal age and pregnancy outcome.

1.3.2 Specific objectives

- Determine levels of HLA-G isoforms in placental malaria positive and negative mothers by specific sandwich ELISA and immunohistochemistry.
- Determine HLA-G mRNA transcript abundance in placental malaria positive and negative women by quantitative real time polymerase chain reaction.
- Correlate HLA-G mRNA abundance and protein concentrations with birth weight.
- Analyse effects of parity and infection on HLA-G expression.

1.4 Hypotheses

- Levels of soluble HLA-G increase as compared to membrane bound isoforms in placental malaria positive mothers.
- The transcription levels and protein concentrations of HLA-G in placental malaria mothers are negatively correlated with birth weight.
- HLA-G mRNA transcript levels and protein concentrations are high in placental malaria.

1.5 Justification of the study

The pathogenesis of placenta malaria remains elusive. Severity and susceptibility have been related to adhesion and sequestration of infected erythrocytes to the placental bed. Several studies have focused on the feto-maternal exchange of various proteins as the cause of pregnancy complications and outcomes. However no study has investigated the role of non-classical MHC class 1b genes in pregnancy associated malaria and pregnancy outcome.

HLA-G has been found to play an important role in the maintenance of pregnancy by inhibiting alloreactive immune responses against a semi-allogeneic fetus. An immunohistochemistry study found that there is less expression of HLA-G with increased infiltration of inflammatory cells in *P. falciparum* parasitized term placenta (Sartelet *et al*., 2005). The study however did not look at the soluble HLA-G isoforms.

In this study, the variations in the expression of HLA-G isoforms during placental malaria were examined to determine whether HLA-G plays any role in placental malaria pathogenesis and pregnancy outcome. This is crucial in the search for proper interventions that would reduce poor pregnancy outcomes and levels of severity associated with placental malaria.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria Overview

Malaria is a mosquito-borne disease caused by *Plasmodium* parasite. People with malaria often experience fever, chills, and flu-like illness. Left untreated, they develop severe complications and die (Ferreira *et al.,* 2004).

Malaria causes about 350–500 million infections in humans and approximately 1-3 million deaths annually; representing, at least one death every 30 seconds. About 60% of the cases of malaria and more than 80% of the malaria deaths world-wide occur in Africa south of Sahara (WHO, 2005). This is due to a combination of factors including:

- Presence of a very efficient mosquito vector (*Anopheles gambiae*) assures high transmission of the parasite.
- The predominant parasite species is *Plasmodium falciparum*, which causes the most severe form of malaria
- Local weather conditions often allowing transmission to occur year round
- Scarce resources and socio-economic instability hindering efficient malaria control activities (WHO, 2005).

In areas of stable malaria transmission, susceptibility is greatest in children under the age of 5 years (Greenwood *et al*., 2005), and in pregnant women (Duffy and Fried, 2001; Ferreira *et al.,* 2004). The disease is caused by a protozoan parasite of the genus *Plasmodium*. The most serious forms of the disease are caused by *Plasmodium falciparum* and *Plasmodium vivax* (WHO, 2005). Other related species that infect man includes *Plasmodium ovale* and *Plasmodium malariae*.

Malaria occurs in over 100 countries and territories. Large areas of Central and South America, Hispaniola, Africa, the Indian subcontinent, Southeast Asia, the Middle East, and Oceania are considered malaria-risk areas. Yet malaria does not occur in all warm climates. For example, economic development and public health efforts have eliminated malaria from the southern

United States, southern Europe, Taiwan, Singapore, and all of the Caribbean islands except Hispaniola (WHO, 2005).

In Africa south of the Sahara, the principal malaria mosquito, *Anopheles gambiae*, transmits malaria efficiently. The type of malaria parasite most often found is *Plasmodium falciparum* which causes severe and often fatal disease (Duffy and Fried, 2001).

2.1.1 Malaria transmission

People get infected by malaria by being bitten by an infected female *Anopheles* mosquito. The *Anopheles* mosquitoes can transmit malaria and they must have been infected through a previous blood meal taken from an infected person.

When a mosquito bites, blood is taken in which contains the microscopic malaria parasites. The parasite grows and matures in the mosquito's gut for a week or more, then travels to the mosquito's salivary glands. When the mosquito next takes a blood meal, these parasites mix with the saliva and are injected into the bite sites. Once in the blood, the parasites travel to the liver and enter liver cells to grow and multiply. During this "incubation period", the infected person has no symptoms (Duffy and Fried, 2001).

After 8 days or several months, the parasites leave the liver cells and enter red blood cells, where they continue to grow and multiply (Ferreira *et al.,* 2004). After they mature, the infected red blood cells rupture, freeing the parasites to attack and enter other red blood cells. Toxins released when the red cells burst are what cause the observed typical fever, chills, and flu-like malaria symptoms (Duffy and Fried, 2001; Ferreira *et al.,* 2004).

2.1.2 Pregnancy malaria

Malaria infection during pregnancy is a major public health problem in tropical and subtropical regions throughout the world. In most endemic areas of Africa, pregnant women are the main adult risk group for malaria (WHO, 2005). The main burden of malaria infection during pregnancy results from infection with *P. falciparum* and *P. vivax*. The impact of the other two human malaria parasites namely *P. malariae* and *P. ovale* during pregnancy is less clear. Every

year at least 50 million women in malarious areas of Africa become pregnant with most of these women living in areas of relatively stable malaria transmission (WHO, 2005).

In pregnant women, malaria infection, often without fever may nevertheless cause anemia, hypoglycemia, pulmonary edema and placental dysfunction (Duffy and Fried, 2001). Pregnancy associated malaria (PAM) is mainly linked to a subpopulation of infected erythrocytes (IE) that adhere to chondroitin sulphate A (CSA) expressed by syncytiotrophoblasts in the placenta (Fried and Duffy, 1996; Viebig *et al.,* 2005). Placental isolates are functionally distinct because they do not bind to CD36, but instead bind to CSA (Fried and Duffy, 1996, Viebig *et al.,* 2005) and may bind other receptors like hyaluronic acid (Beeson *et al.,* 2000). Primigravidae are more susceptible to this sub population and with successive pregnancies, as they become resistant they develop broadly strain transcendent antibodies to the IE surface (Fried *et al.,* 1998). This suggests that a vaccine against placental associated malaria (PAM) is feasible. A large number of epidemiological studies on pregnant women in malaria endemic areas strongly support the concept that an anti-disease vaccine that blocks the adhesion of infected erythrocytes to CSA and thereby protects pregnant women is possible (Duffy and Fried, 2003). Fetal growth is impaired and babies born to primigravidae with placental malaria are on average 100 grams lighter than controls born to health primigravidae.

Sub-Saharan Africa is the region of the world hardest hit by malaria with infections estimated to cause 400,000 cases of severe maternal anemia and from 75,000-200,000 infant deaths annually. Maternal anemia contributes significantly to maternal mortality and causes an estimated 10,000 deaths per year due to malaria. Low birth weight (LBW) is the greatest risk factor for neonatal mortality and a major contributor to infant mortality (Wort *et al.,* 2006). Although many factors contribute to low birth weight, malaria is a major factor and one of the few, along with poor nutrition, anemia, and other infections, which are amenable to intervention once a woman becomes pregnant.

In small, observational studies, placental monocytes containing malaria pigment have been associated with decreased birth weight in first-born children and with severe maternal anemia. Dense intervillous monocytes infiltrations have been detected in placentas of 6.3% of Tanzanian

women, and were independently associated with LBW, primarily due to intrauterine growth retardation (IUGR) (Menendez *et al.,* 2000).

Parasites are unlikely to be directly responsible for the placental pathology, but leucocytes, through the production of non-chemotactic cytokines, might be associated with the thickening of the trophoblastic basement membrane causing a mechanical blockage of oxygen and nutrient transport across the placenta (Menendez *et al.*, 2000).

2.1.3 Pathologic changes in placental malaria

The weights of term placentas are significantly reduced during malaria infection when compared with those from normal women (Walter *et al.,* 1982). The morphological changes are a combination of the following features:

- Presence of parasites and leukocytes in the intervillous spaces.
- Macrophage concentration in the intervillous spaces.
- Malarial hemozoin and pigment deposits in the trophoblasts and hofbaurer cells (Placental macrophages).
- Excess perivillous fibrinoid deposits which persist for an unknown period of time after the parasites and monocytes clear.
- Syncytiotrophoblastic damage.
- Trophoblastic basal lamina thickening and proliferation of cytotrophoblasts.
- Scanty malaria pigment ingested by monocytes, associated with focal syncytial necrosis and proliferation of cytotrophoblastic cells (Walter *et al.,* 1982, Watkinson and Rushton., 1983; Kaushik *et al.,* 1992; Menendez *et al.,* 2000).

Excessive perivillous fibrinoid deposits are a constant histologic finding and are usually associated with syncytiotrophoblastic necrosis or ultrastructural damage such as partial microvilli loss, filamentous material accumulation in intracytoplasmic vacuoles, and "podocytelike" cytoplasmic projections on the basal surface (Walter *et al.,* 1982). Reduction in the exchange area of the syncytium exposed to the maternal blood is perhaps the cause of low birth weight and preterm deliveries (McGregor, 1984). Intervillous inflammation is the most frequent finding

associated with malaria and is especially severe in primigravidae (Yamada *et al.,* 1989; Ismail *et al*., 2000).

2.2 Placental Immune Responses

Pregnancy is a unique homeostasis between different systems of the body the end result of which favours the development of the fetus while preserving maternal health. Because of the potential immunological conflict between the mother and her antigenically different fetus, some sort of maternal immunomodulation is essential for survival of the conceptus (Duffy and Fried, 2001).

Several immunological parameters are measurably altered in the course of gestation including a requirement for Th2 responses to prevent fetal loss, although most microbes do not pose an increased threat for the pregnant women. Susceptibility is increased during pregnancy to some intracellular pathogens, including *P. falciparum* (Duffy and Fried, 2001).

Acquired humoral and cell mediated immune responses to a given antigen polarise by cross-inhibition of each other via Th1 and Th2 cells interactions. Th1 cells acts by releasing IFN-γ which inhibits Th2 cell replication, while Th2 cells release IL-4 and IL-10 which inhibits Th1 cell cytokines release (Duffy and Fried, 2001). Pregnancy-associated hormones such as progesterone inactivate mononuclear phagocytes functions such as cytokine production and inducible nitric oxide synthase (iNOS) expression. However, the monocytes activation markers such as CD11b, CD14, CD64 and basal levels of reactive oxygen intermediates are increased during pregnancy, although other markers such as HLA-DR and oxidative burst capacity are not elevated (Beeson *et al*., 2000).

In the developing human embryo, extravillous trophoblasts, which are in direct contact with maternal immune cells, express HLA-G (Kovats *et al*., 1990; Hunt *et al*., 2005). The main function of trophoblast HLA-G is to prevent NK cell attack on the fetus (Bainbridge *et al.,* 2001; Hviid *et al.,* 2004; Hunt *et al.,* 2005).

2.3 Non-classical MHC Class 1b genes: HLA-G

The HLA-G locus was first described by Daniel Geraghty and colleagues in 1987 (Geraghty *et al.,* 1987). The locus is sited at the telomeric part of the 6p21-3 chromosomal region in the vicinity of the HLA–A locus as shown in Figure 1.

HLA-G genes exhibit limited polymorphism, are expressed in lower amounts and have a more restricted tissue distribution, at least at the protein level (Geraghty *et al.,* 1987; Le Bouteiller and Valerie, 1997; Hunt *et al.,* 2005). Although their functions remain elusive, HLA-G molecules are capable of binding peptides. Their expression in trophoblast cells which are devoid of HLA class 1a molecules at their cell surface strongly suggests that HLA-G may have a functional importance in terms of maternal acceptance of the fetus and defense against infections.

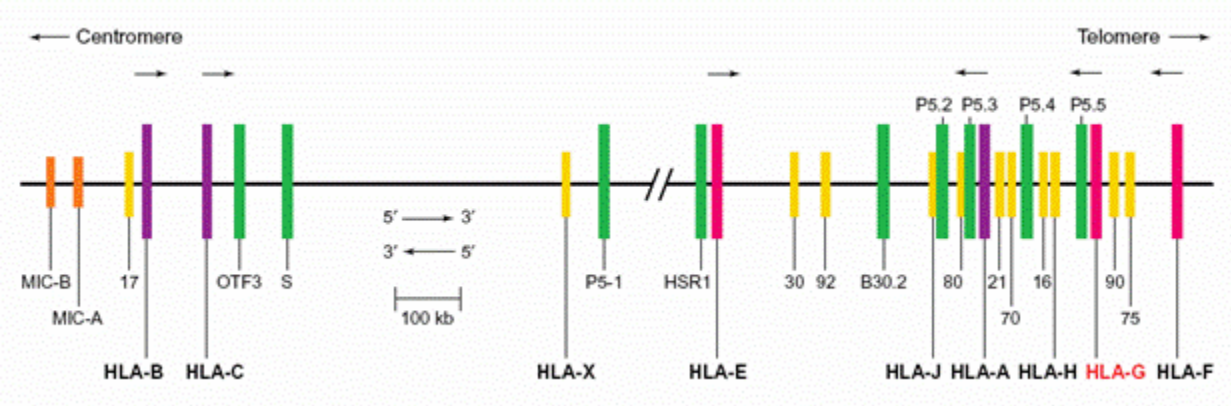


Figure 1: Schematic map of the HLA class 1 chromosomal region (6p21-3), showing the location of **classical (purple**), **non classical (red**) HLA class 1 genes, including **HLA-G**, **HLA class 1 pseudogenes** and **truncated genes** (**yellow**), **class 1 related-(orange**) genes and some (but not all) of the non-HLA class 1 genes (**green**) (Le Bouteiller and Valerie, 1997).

The HLA-G molecule inhibits the effector function of maternal NK cells via interaction with the killer cell immunoglobulin-like receptor 2 long cytoplasmic domain 4 (KIR2DL4) and the immunoglobulin-like transcripts 2 (ILT-2) which have inhibitory transmembrane motifs.

Immunoglobulin-like transcript-4 (ILT-4) also known as leukocyte immunoglobulin-like receptor 2, CD85d, and LILRB2, is a cell surface receptor expressed mainly on myelomonocytic

cells, whereas ILT-2 also known as leukocyte immunoglobulin-like receptor 1, CD85j, and LILRB1, is expressed on a wide range of immune cells including subsets of natural killer and T cells. Both ILTs contain immunoreceptor tyrosine-based inhibitory receptor motifs in their cytoplasmic tails that inhibit cellular responses by recruiting phosphatases such as Src homology 2 domain containing tyrosine phosphatase 1 (SHP-1). Human ILT-2 and ILT-4 compete with CD8 for MHC class 1 binding and bind preferentially to HLA-G than to HLA-A proteins (Sanders *et al.,* 1991; Shiroishi *et al.,* 2003).

HLA-G gene exhibit the typical structure of an MHC class 1 gene with similar exon and intron organization (Geraghty *et al.,* 1987) and association with beta 2-microglobulin (β_2m) and a conserved CD8-binding loop (Sanders *et al.,* 1991; Le Bouteiller and Blaschitz*,* 1999; Le Bouteiller and Solier, 2001). The first exon encodes a leader peptide, excised after entry into the endoplasmic reticulum (ER); the second, third and fourth exons encode the α-1, α-2 and α-3 external domains, respectively, of approximately 90 amino acids each. The α-3 domain of HLA-G can interact with the CD8 glycoprotein present on cytotoxic T lymphocytes (CTL) through the alanine site located at the position 245 (Sanders *et al.,* 1991). Like HLA class 1 a, they have a single N-linked glycosylation site (Asp86) located in the C-terminus of the α-1 domain, the intrachain disulfide bonds in the α-2 (Cys 101–Cys 164) and α-3 (Cys203-Cys 259) domains (Geraghty *et al.,* 1987; Le Bouteiller and Valerie*,* 1997; Le Bouteiller and Solier*,* 2001). The fifth exon encodes a hydrophobic transmembrane domain identical in length to that of class 1 a proteins.

HLA-G differs from the HLA class 1a genes in that a stop codon within exon 6 prevents translation of most of the cytoplasmic domain and thus translates six amino acids instead of 30 (Geraghty *et al.,* 1987; Le Bouteiller and Solier*,* 2001). Due to this lack of translation, the HLA-G cytoplasmic tail has lost a serine residue, a site of phosphorylation and a highly conserved tyrosine residue, the substrate of tyrosine kinase (Geraghty *et al.,* 1987). These losses may have functional implications in the signaling processes. The HLA-G mRNA is alternatively spliced into multiple isoforms that encode both membranes-bound and soluble proteins (Geraghty *et al.,* 1987; Le Bouteiller and Blaschitz*,* 1999; Le Bouteiller and Solier*,* 2001; Hunt *et al.,* 2005).

There are seven different HLA-G transcriptional isoforms (Hunt *et al*., 2005). Four of these encode membrane-bound products while the other three encode soluble proteins. The full length membrane-bound HLA-G1 isoform contains the three external domains, whereas the HLA-G2, -G3 and –G4 isoforms result in splicing out of exon 3, exons 3 and 4 or exon 4, respectively, and thus contain either one or two external domains (Le Bouteiller and Valerie*,* 1997; Le Boutteiller and Solier*,* 2001; Hunt *et al.,* 2005). Two actively secreted soluble HLA-G isoforms; HLA-G5 and HLA-G6 exhibit a unique feature which is the translation of intron 4, the presence of a stop codon within this intron preventing the translation of both transmembrane and cytoplasmic domains (Le Bouteiller and Solier*,* 2001). This intron-containing message thus yields soluble protein as shown in Figure 2. In contrast to alternatively splicing, exon 5 that gives rise to soluble truncated HLA-A and HLA-B arises from exon 5 instead of intron-containing mRNA like in HLA-G (Fujii *et al.,* 1994).

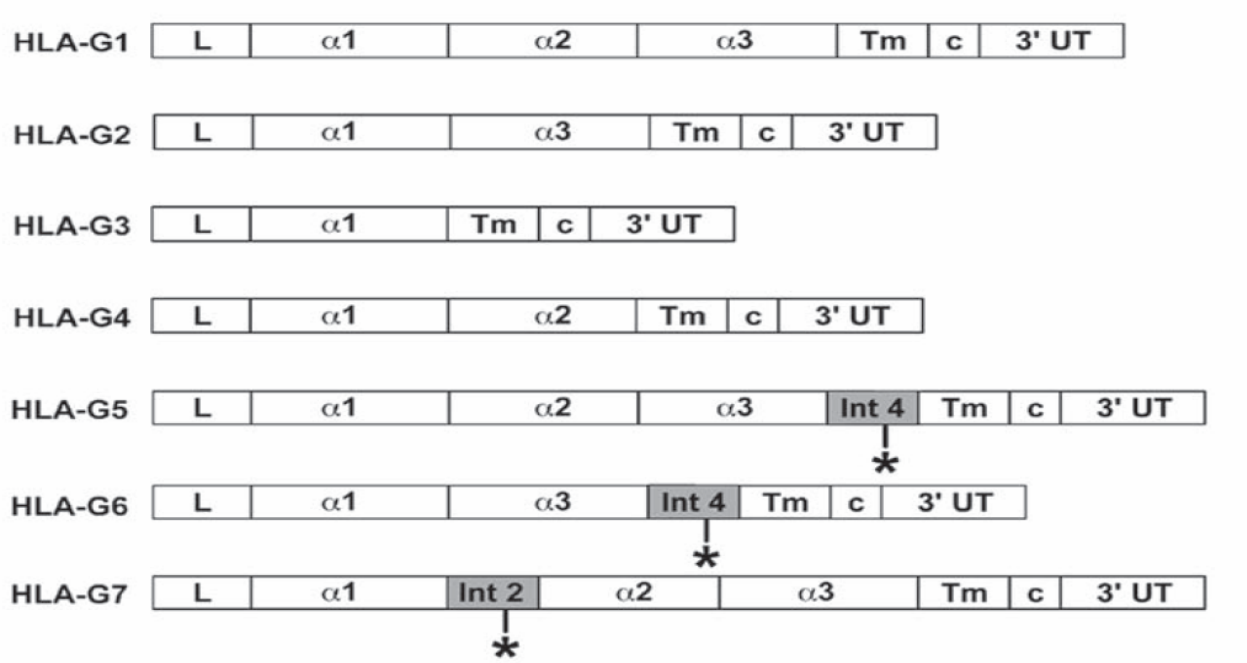


Figure 2: Schematic representation of messages derived by alternative splicing of the single HLA-G primary transcript. The three transcripts encoding soluble proteins are terminated in **intron 4** (HLA-G5, HLA-G6) and intron 2 (HLA-G7) by a stop codon (denoted by *), which precludes translation of the balance of the message, including the transmembrane and cytoplasmic domains. **L**, leader sequence; **Int**, intron; **Tm**, transmembrane; **C**, cytoplasmic; **UT**, untranslated (Pace *et al*., 2005).

Another potential soluble form, HLA-G7, with only the leader sequence and α-1 domain, has been described, but no secreted product has been detected to date (Pace *et al*., 2005).

HLA-G5 has been detected in maternal blood during pregnancy as well as in amniotic fluid (Fournel *et al.,* 2000). HLA-G5 protein is secreted by trophoblasts and has been detected in amniotic fluid in a wide concentration-range from 50 to 1500 ng/ml (Fournel *et al*., 2000) and maternal blood during pregnancy (Hunt *et al*., 2000; Hviid *et al.,* 2004).

Herpes simplex virus blocks intracellular transport of HLA-G by inducing synthesis of a transporter inhibitor (Schust *et al.,* 1996), and human cytomegalovirus (HCMV) gene products down regulates HLA-G expression in trophoblasts leading to low maternal immune tolerance of the fetus (Jun *et al.,* 2000).

2.4 Other Placental Associated Complications

Miscarriage and pre-eclampsia are the most common disorders of human pregnancy. Recent evidence demonstrates that chronic malaria increases the odds of hypertension in first time mothers by increasing the levels of soluble vascular endothelial growth factor receptor 1 (sVEGFR1) (Muehlenbachs *et al.,* 2006).

The oxidative stress or an imbalance in the oxidant/anti oxidant activity in utero-placental tissues plays an important role in the development of placenta-related diseases (Jauniaux *et al.,* 2006). Tumor necrosis factor alpha (TNF-α) and other pro-inflammatory cytokines may be important in the development of pre eclampsia, possibly in response to hypoxia (Conrad and Benyo, 1997). This has been implicated in the pathogenesis of intrauterine growth retardation by decreasing amino acid uptake by the fetus (Carbo *et al.,* 1995).

CHAPTER THREE
METHODOLOGY

3.1 Study Site

This study was conducted in Mother Offspring Malaria Study laboratories at Morogoro Regional Hospital, Tanzania and Seattle Biomedical Research Institute (SBRI), USA, between October 2006 and November 2007.

The study samples were collected from an on-going longitudinal cohort study in Morogoro Regional Hospital and Muheza Designated District Hospital Tanga in Tanzania. These are malaria endemic areas with year round transmission and seasonal fluctuations.

Ethical approval of the project was obtained from Ministry of Health Tanzania, as well as the Institutional Review Board (IRB) Seattle Biomedical Research Institute in accordance to guidelines on experimentation using human subjects. Informed consent was signed by all subjects prior to enrollment in the study.

3.2 Sample Size and Selection Criteria

Samples for this study included 30 placental tissues for immunohistochemistry and quantitative real time PCR and 144 sera for soluble HLA-G ELISA. Maternal age, parity and fetal birth weights were retrieved from the clinical records. Mothers with metabolic disorders such as diabetes, malnutrition and obesity were excluded from the study.

3.3 Determination of Parasitemia and Stage of the Placental Infection

Frozen placenta tissue sections of 5µm stained with Giemsa were examined microscopically at magnification of ×100 to determine placental infection with *Plasmodium falciparum*. The stage of infection was determined by examining the presence of asexual stages of the parasite, hemozoin pigment, fibrin deposits and inflammatory cells infiltrating intervillous spaces of the placental sections (Ordi *et al.,* 2001). The placental malaria was classified on the histological basis as acute, chronic and past infected (Ordi *et al.,* 2001).

3.4 Extraction of Total RNA and Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from frozen cryosections using RNeasy minikits (Qiagen, Hilden Germany). RNA quality was assessed by Nanodrop ND-1000 spectrophotometer, resulting in 260/280 ratios of 1.9 to 2.2. First strand cDNA was synthesized using superscript III enzyme (Invitrogen Carlsbad, California, United States) and anchored oligodT20 primers. Exon and intron spanning primers for HLA-G isoforms were designed with Primer 3.0 software™.

Primers for HLA-G1/2 representing membrane bound isoforms were;
5′-TCATGCTGAGATGGAAGCAG-3′ for forward; and
5′-TCTCCACAGCACAGCAGC-3′ for reverse (119 bp),

Primers for HLA-G5/6 for the soluble HLA-G isoforms were;
5′-GCTGAGATGGAGTAAGGAGGG-3′ for forward; and
5′-GGTGAAGGTGAGGGTCTCTG-3′ for reverse (105 bp) (Mitsdoerffer *et al.*, 2005).

Primers for total HLA-G were;
5′-AACCTCTTCCTGCTGCTCT-3′ for forward and
5'-GCGCTGAAATACCTCATGG-3' for reverse (81 bp product) (Validated by Yie *et al*., 2006).

Primers for endogenous control, trophoblasts expressed cytokeratin 7 were;
5′-GGCTGAGATCGACAACATCA-3′ for forward and
5′-CTTGGCACGAGCATCCTT-3′ for reverse (103bp product) (Muehlenbachs *et al.*, 2006). The regions flanked by these primers are shown in Figure 3.

Real time PCR was performed in triplicates using SYBR® green master mix and an ABI Prisms 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) with an annealing temperature of 60 ^{o}C. Threshold cycles (Ct) were normalised to endogenous control, cytokeratin-7 (KRT 7), and statistical tests performed on normalised Ct values. Data are represented as fold

difference from control gene, calculated using the formula; fold change = $2^{-\Delta\Delta Ct}$ (Yann *et al*., 2007)

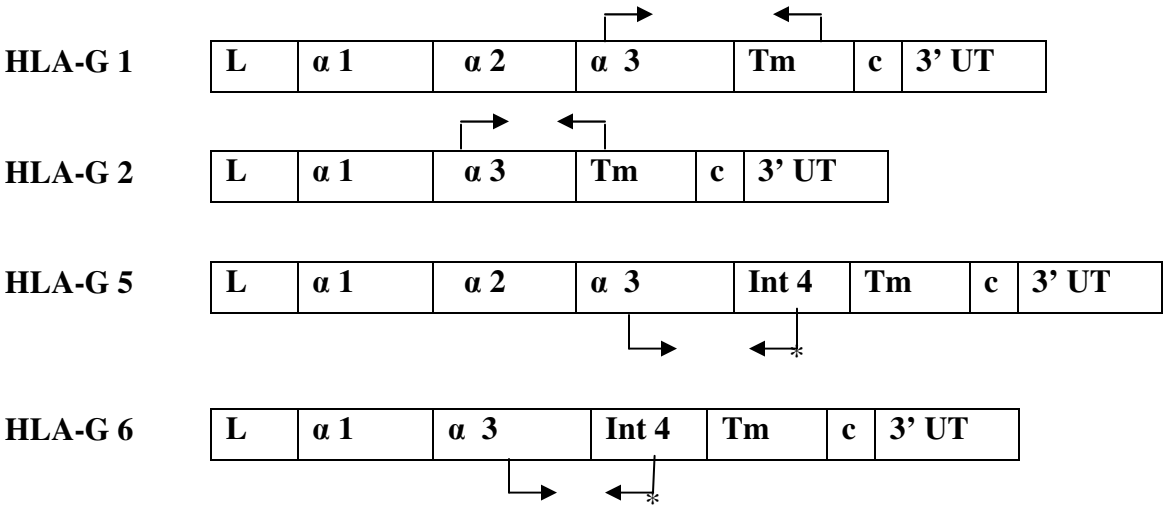


Figure 3: Illustrations of locations of the primer pairs used to amplify HLA-G1/2 and HLA-G5/6 isoforms respectively.

3.5 Specific Sandwich Enzyme Linked Immunosorbent Assay (ELISA)

Soluble HLA-G ELISA was carried on 144 serum samples obtained from malaria infected and uninfected placenta blood and naive males with a commercial kit (sHLA-G ELISA cat. No. RD194070100R Biovendor and Exbio Praha, a.s Czech republic). There were 134 serum samples from placental grinding and 10 naive male serum as a control. ELISA was done as per the manufacturers instructions. The data was stratified on *P. falciparum* infection stage for primigravidae and parity for all mothers studied.

3.6 Immunohistochemistry

Human term placenta was snap-frozen in liquid nitrogen and stored at –80^{o}C. Section of 5µm thickness were cryosectioned at -30^{o}C using cryostat (HM 505E, Microm Model). They were embedded on poly-L Lysine coated glass slides and fixed in 4% paraformaldehyde for 10 minutes. The sections were encircled with a pap pen to form a boundary on the tissue section to prevent the antibodies from spreading away from the section. The sections were blocked with 10% human serum and incubated for 30 minutes in a moisture chamber. They were then washed

three times with phosphate buffered saline (PBS) each time for eight minutes. They were then incubated for 30 minutes with the following primary antibodies on each section; mouse anti-human HLA-G1 (MEM-G/9 from Exbio, Praha, Czech Republic), HLA-G5 (5A6G7 Exbio, Praha, Czech Republic) at a dilution of 1:50 and mouse anti-human cytokeratin 7 dilution of 1:100 (clone OV-TL 12/30 Dakocytomation). Some sections were left out unstained with primary antibody to serve as negative controls. After washing three times with PBS, they were incubated with secondary antibody provided in a kit (Dako), in a moisture chamber for 30 minutes. After washing with PBS, three drops of chromogen were added to each section and developed till they turned golden brown. The reaction was stopped with water. Sections were counterstained with 10% Giemsa stain for 1 minute and cleared with xylene to remove pap pen. They were mounted with permount and coverslips and then observed under microscope at ×40 magnification. Photomicrographs were taken by use of Fuji® digital camera (Finepix A900, 9 megapixels) mounted on Olympus® light microscope. The intensity of staining from the sections was examined relative to the controls in *P. falciparum* infected and uninfected placentas.

3.7 Statistical Analysis

ELISA data for the concentration of soluble HLA-G, and the Ct values for quantitative real time PCR for infected and uninfected placentas were compared using two tailed Student's *t* test. $P \leq 0.05$ was considered to be significant. The immunohistochemistry sections were presented as photomicrographs and the intensity of staining was compared between infected and uninfected mothers' placental tissues. Linear regression was used to correlate the concentrations of soluble HLA-G with birth weight and maternal age.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Clinical Data

A total of 120 samples from malaria endemic area were studied. Of this, 54 were from infected mothers and 66 were from uninfected. Maternal age and fetal birth weight were recorded. This information is summarised in Table 4.1. The mothers were grouped in terms of number of births as primigravidae with one, secundagravidae for two and multigravidae for more than two times of birth.

Table 4.1: Summary of the clinical data for all mothers in the study

Parity	Mean maternal age (yrs)	Mean Birth weight (kg)	*P*
Primigravidae			
(Infected, n= 29)	20.65±3.23	2.79±0.41	
(Uninfected, n=31)	21.54±3.77	3.1±0.25	0.025
Secundagravidae			
(Infected, n=13)	23.77±3.86	2.90±0.25	
(Uninfected, n=21)	23.67±3.38	3.21±0.49	0.022
Multigravidae			
(Infected, n=12)	30.08±6.61	3.0±0.47	
(Uninfected, n=14)	31.29±3.89	3.24±0.41	0.17

The p values are for the comparison of the mean birth weight (kg) between *P. falciparum* infected and uninfected mothers by student t test. $P \leq 0.05$ was considered significant at 95 % confidence interval.

The maternal ages between infected and uninfected mothers in each parity were comparable. However, the birth weight differed significantly between infected and uninfected primigravidae with $p = 0.025$, and secundagravidae with $p = 0.022$, respectively. This information is shown in Table 4.1. The birth weight in multigravidae mothers did not differ significantly, although placental malaria uninfected had the highest birth weight, with $p = 0.17$. The maternal age and birth weight are shown as mean and standard deviation.

4.2 Microarray Data

Genome wide genes scan for seven chronic malaria infected and nine uninfected placentas from first time mothers was carried out with Affymetrix Human Chips array U133 plus 2.0 in our laboratory by Muehlenbachs *et al*., 2006 (unpublished). Four probes in the Affymetrix Human Chips were specific for HLA-G (Table 4.2). There was a significant increase in transcripts of HLA-G genes from chronically *P. falciparum* infected placentas compared to uninfected. There was a near two-fold change in transcripts abundance.

Table 4.2: Microarray data showing fold change in HLA-G transcripts from four probes in Affymetrix Human chips Array U133 plus 2.0.

Affymetrix I.D	Gene Title	*p*	Fold Change
211529_x_at	HLA-G	0.001	1.9
211528_x_at	HLA-G	0.002	1.8
210514_x_at	HLA-G	0.002	1.7
211530_x_at	HLA-G	0.03	1.5

Baseline survey result done at Mother Offspring Malaria laboratory by Muehlenbachs *et al*, 2006 baseline study, unpublished data).

4.3 Quantitative Real Time Polymerase Chain Reaction

4.3.1 Experimental validation

The quantitative real time PCR experiments were validated by observing that the polymerase chain reaction (PCR) efficiencies of the endogenous control and the target genes were approximately equal and there were no primer dimers which could give unspecific products. Plots of log of serial dilutions of the total RNA (ng) against the cycle threshold (Ct) values of each target gene and the endogenous control were constructed to ensure that the efficiencies were equal. The slope of the plots should be less than 0.1 for efficient PCR reactions. This is shown in plots 4a, b, c and d.

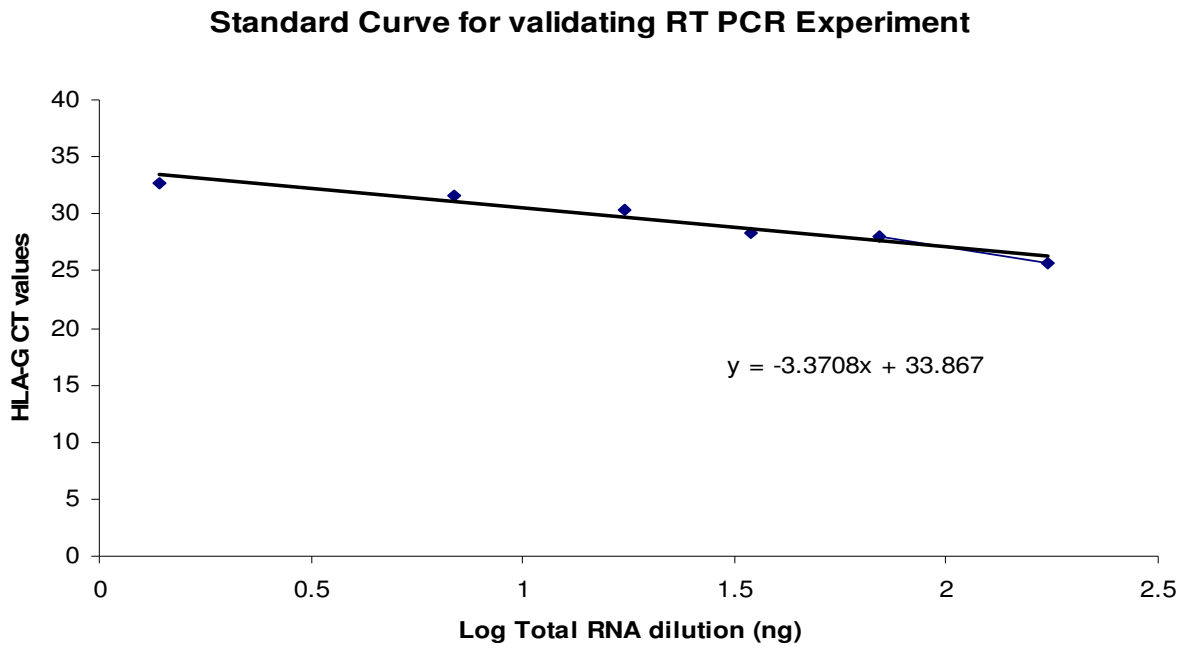


Figure 4 a: A plot of HLA-G Ct values against Log Total RNA dilutions (Slope =-3.37)

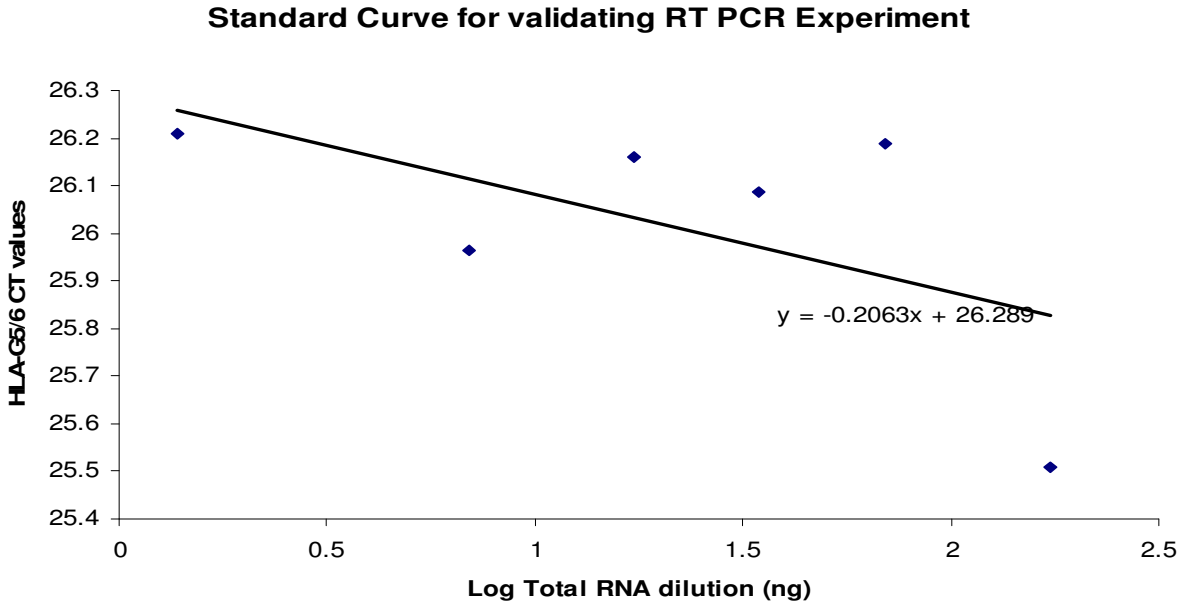


Figure 4 b: A plot of HLA-G5/6 Ct values against Log Total RNA dilutions (Slope=-0.20)

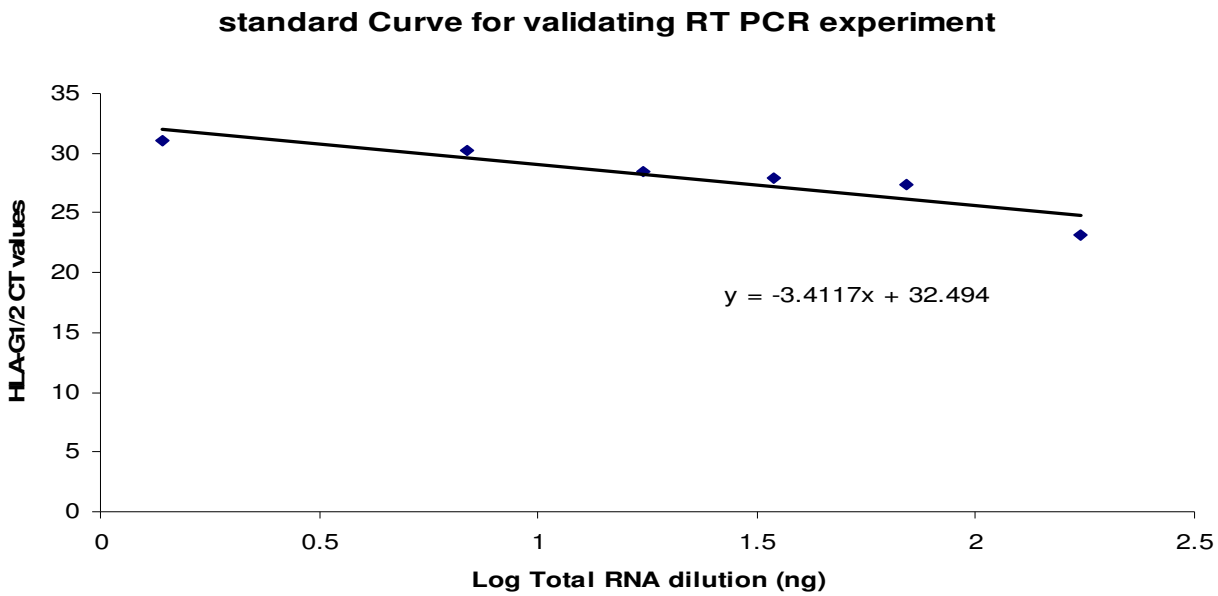


Figure 4 c: A plot of HLA-G1/2 Ct values against Log Total RNA dilutions (slope=-3.4)

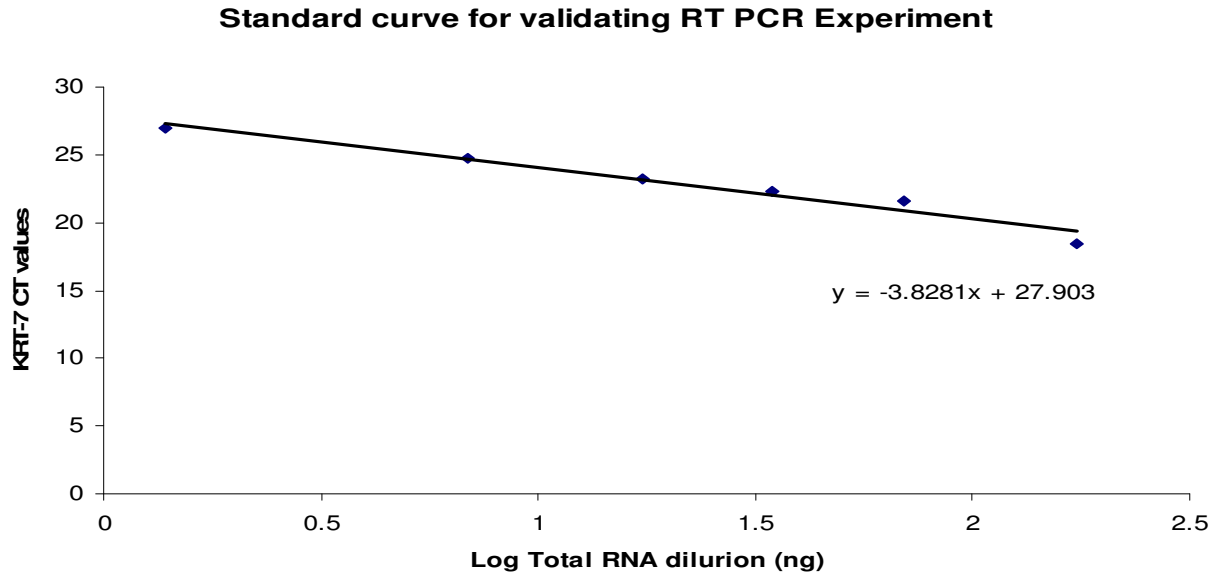


Figure 4 d: A plot of KRT-7 Ct values against Log Total RNA dilutions (Slope=-3.8)

The quantitative real time PCR specificity was confirmed by analysing the products in a gel electrophoresis. For a specific amplification of the target gene and absence of primer dimers a single amplicon is always expected from each primer pair. A single distinct band was detected in the validation step as illustrated on the plate 1.

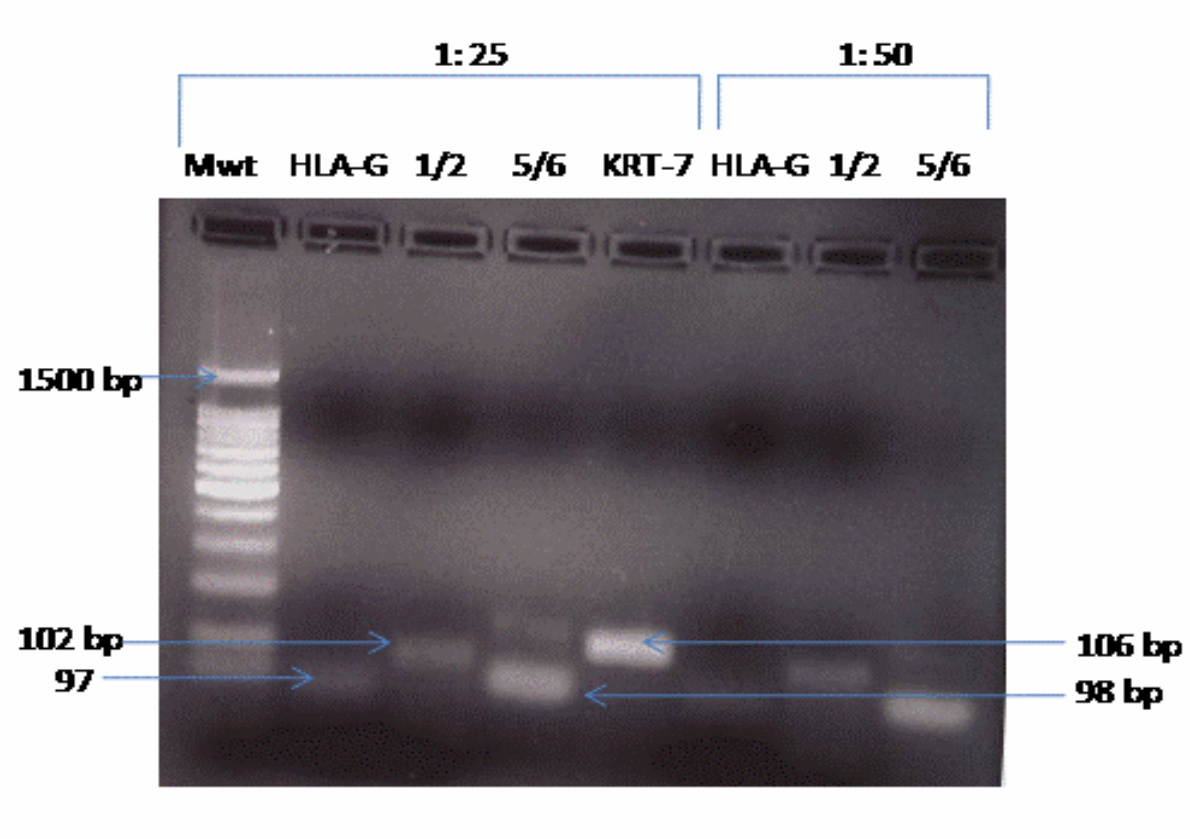
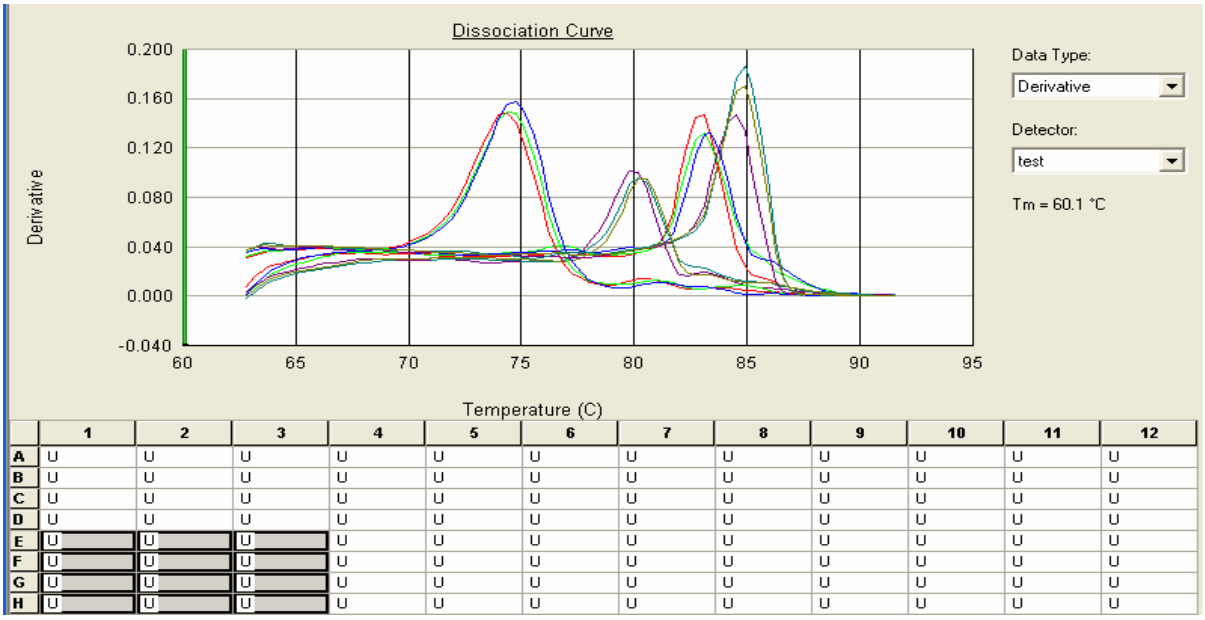


Plate 1: A 2% Agarose gel electrophoresis of the products of the quantitative real time PCR reaction. Mwt refers to the molecular weight marker in base pairs (bp). KRT-7 refers to cytiokeratin-7. This was done in two dilutions of the template; 1:25 and 1: 50.

From the agarose gel electrophoresis the following distinct bands were formed; 97 bp representing total HLA-G, 102 bp for HLA-G1/2, 98 bp for HLA-G5/6 and 106 bp for cytokeratin-7. This is illustrated in Plate 1.

To confirm that during the amplification process there were no primer dimers which could contribute to non-specific number of transcripts, the dissociation curves or melting curves were generated from all the wells. See Figure 5. There was a single peak from every well highlighted in the figure. Primer dimers, hairpins and/or non-specific amplification would be shown as irregular lines with several peaks.



	1	2	3	4	5	6	7	8	9	10	11	12
A	U	U	U	U	U	U	U	U	U	U	U	U
B	U	U	U	U	U	U	U	U	U	U	U	U
C	U	U	U	U	U	U	U	U	U	U	U	U
D	U	U	U	U	U	U	U	U	U	U	U	U
E	U	U	U	U	U	U	U	U	U	U	U	U
F	U	U	U	U	U	U	U	U	U	U	U	U
G	U	U	U	U	U	U	U	U	U	U	U	U
H	U	U	U	U	U	U	U	U	U	U	U	U

Figure 5: A dissociation curve showing single amplification peaks for the target. The triplicates in wells E, F, G and H represents; total HLA-G, membrane bound HLA-G1/2, soluble HLA-G5/6 and cytokeratin-7 respectively. E, F, G and H in the table show the transcription products for the total HLA-G, membrane bound HLA-G, soluble HLA-G and KRT-7, respectively. All these are in triplicates as loaded in the Nunc™ plates for qRT-PCR.

To ensure that the products had high copy number for quantitative analysis, the experiments were optimised so that the cycle threshold (Ct) values could be read below 29 in 40 cycles of PCR reactions as shown in Figure 6. The cytokeratin-7 which was used as an endogenous control had a high copy number compared to the target and was used to normalise the reactions to cater for the variations in the amount of template used in each of the 96 wells in a plate. The Ct values were used in comparative analysis to determine transcripts abundance.

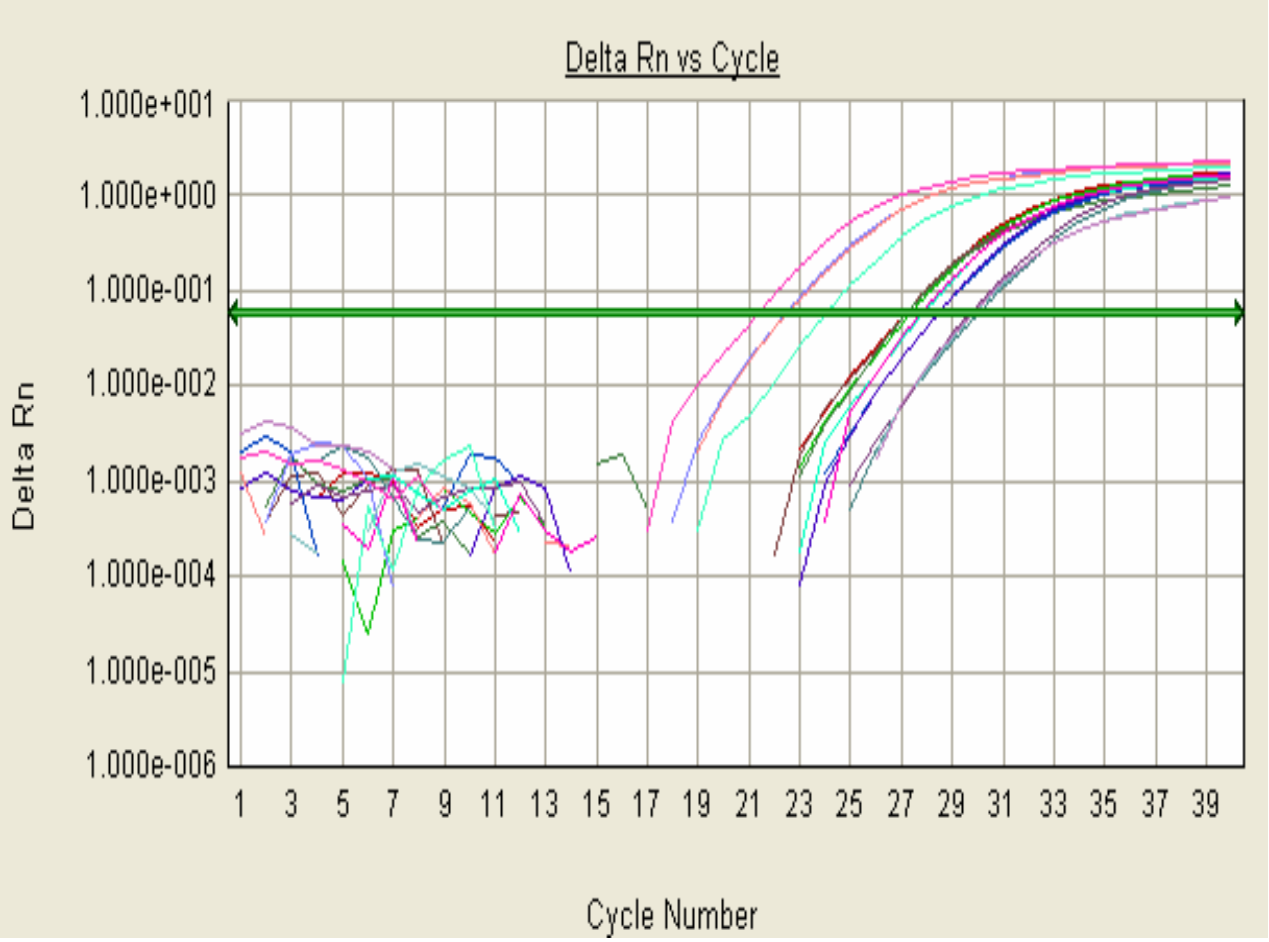


Figure 6: Illustration of the change in reaction against cycle numbers in 40 cycles. The curves show the formation of the products at real time during amplification. The curves crossed the threshold level at cycles less than 29. Values greater than 29 could not give high copy number and specific products.

To be sure that what was quantified were the products of alternative transcriptions taking place in the placenta, conventional reverse transcriptase PCR was carried out with primers specific for the full HLA-G isoforms (Pace *et al*., 2005). Four isoforms were clearly identified in 2 % agarose gel electrophoresis after reverse transcriptase polymerase chain reaction. A 775 bp band of HLA-G1, 585 bp band of HLA-G5, 369 bp band of HLA-G2 and 316 bp band of HLA-G6 were detected. In Plate 2, the numbers 5 and 6 had no product for they were negative controls without reverse transcriptase enzyme and the template mRNA. The primer for HLA-G1 was

meant to detect all the isoforms but there was no distinct band except one at 775bp, the rest was a smear indicating presence of other products.

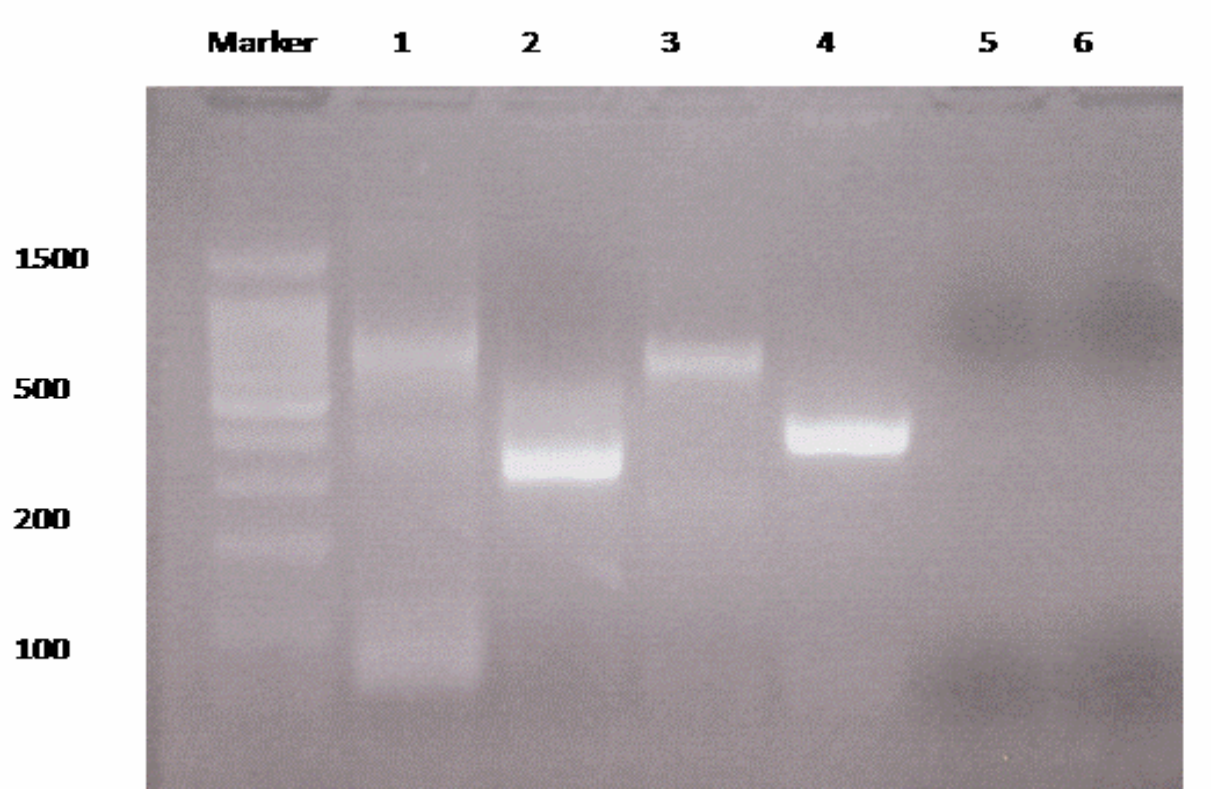


Plate 2: A 2% agarose gel electrophoresis of the HLA-G isoforms obtained from reverse transcriptase PCR of the placenta mRNA. 1, 2, 3, 4, 5 and 6 represents; HLA-G1, HLA-G2, HLA-G5, HLA-G6, control without reverse transcriptase and control without template mRNA respectively.

4.3.2 Results of the quantitative real time PCR

Transcripts abundance of the total, membrane bound and soluble HLA-G isoforms were analysed in 15 infected and 15 uninfected placentas using quantitative real time PCR. Ct values were normalized to cytokeratin-7 which is expressed at stable levels in the placenta. Statistical tests were performed on Ct values of infected and uninfected placentas by student t test at 95 % confidence interval. The $p \leq 0.05$ was considered significant. There was a significant difference between the Ct values of infected and uninfected HLA-G. This data is summarized in Table 4.3.

Table 4.3: Shows a summary of the real time PCR data showing mean normalised Ct values and the level of statistical significance. There are 15 placentas in each group.

Target genes	Mean Ct ±Stdev	*P*
Total HLA-G (isoforms 1, 2, 3, 4, 5, 6 and 7)		
Infected placentas	6.90±1.69	0.003
Uninfected placentas	7.63±0.88	
Membrane bound HLA-G (1/2)		
Infected placentas	7.41±2.43	
Uninfected placentas	6.46±1.83	0.04
Soluble HLA-G (5/6)		
Infected placentas	6.05±1.43	
Uninfected placentas	5.09±2.28	0.06

P values ≤ 0.05 at 95% confidence interval were considered significant by student t test. Ct stands for cycle threshold and Stdev is the standard deviation.

The data was represented as fold change in mRNA transcripts relative to uninfected. The fold changes were calculated by taking the average normalised Ct values from uninfected as a reference. To cater for the variations in the normalised Ct values for the uninfected placentas, the average Ct was subtracted from each sample and the fold change calculated using the formula; fold change = $2^{-\Delta\Delta Ct}$ (Yann *et al*., 2007). The average fold change for uninfected placentas which served as the reference point was 0±Stdev. Comparing the fold change between infected and the reference, there was a significant fold change in transcripts for the total and membrane bound HLA-G (Mb HLA-G). The transcripts of the soluble HLA-G in infected placentas were higher than those for uninfected though the difference was not significant. In this case the membrane bound HLA-G represents the isoforms HLA-G1 and HLA-G2, while the soluble HLA-G represent the intron-4 retain isoforms HLA-G5 and HLA-G6. The total HLA-G is the full molecule comprising all the seven isoforms. The primers for the total HLA-G spanned the entire leader sequence and the first exon which are present in all isoforms.

When comparing the isoforms transcripts abundance, the total HLA-G had higher transcripts than membrane bound and the soluble HLA-G. Soluble HLA-G had the lowest number of transcripts.

The quantitative real time PCR data was further stratified on the basis of parity. There were 13 infected and nine uninfected primigravidae. The student t test was used to test the statistical significance in the normalised Ct values to compare the difference. There was a significant difference ($p = 0.009$) in total HLA-G between infected and uninfected placentas.

Comparing membrane bound and soluble HLA-G, showed a statistically significant difference in mRNA transcripts abundance in infected and uninfected placenta. This is consistent with Yao *et al*., (2005) findings that the membrane bound HLA-G constitutes 80% and soluble HLA-G 20%. This study found that the membrane bound constituted 58% while soluble HLA-G constituted 42%. Primers which flank the 3' end are likely to give low transcripts abundance because the mRNA degrades faster from this end than from 5', and this might give inaccurate comparison results. However, it gives a general indication of the variation in the transcripts abundance for the two isoforms and thus can be confirmed by other techniques such as northern blot.

Table 4.4: A summary of the real time PCR data for 13 infected and 9 uninfected primigravidae placentas.

Target Genes	$_n\mu$Ct ±Stdev	Fold change	*P*
Total HLA-G (isoforms1, 2, 3, 4, 5, 6, and 7)			
Infected placentas	(8.31±2.33)	10.51±2.33	0.0009
Uninfected placentas	(9.79±1.30)		
Membrane bound HLA-G (1/2)			
Infected placentas	(7.60±2.73)	4.61±2.04	0.09
Uninfected placentas	(7.04±2.04)		
Soluble HLA-G (5/6)			
Infected placentas	(5.93±1.71)	2.05±1.75	0.08
Uninfected	(5.84±1.77)		

* $_n\mu$Ct stands for the normalised mean cycle threshold values. *P* values ≤ 0.05 at 95% confidence interval were considered to be significant by student t test.

The difference between total HLA-G in infected and uninfected placentas was significant with $p = 0.009$. The mean transcripts abundance for the membrane bound HLA-G1/2 and soluble HLA-

G5/6 was higher in infected than uninfected placentas although the difference was not significant. On average there was more than three fold change in transcripts of both membrane bound HLA-G1/2 and soluble HLA-G5/6 in infected primigravidae, while the total HLA-G fold change was about 10.

4.4 The Specific Sandwich Soluble HLA-G ELISA

The primigravidae were stratified by the stage of infection according to the histological placental malaria classifications. There were 20 acutely infected, classified on the basis of the presence of the parasites in intervillous spaces, inflammatory infiltrates and pigments; 13 past infections, without parasite but with pigments or fibrin deposits in intravillous spaces; 9 chronically infected, with parasite and no inflammatory infiltrates, and 18 uninfected placental sera. The concentration of soluble HLA-G was found to be significantly different between acute and past infected placenta (P = 0.007, and chi-square of 7.24). The acute and uninfected placenta had significant difference (p=0.04, chi square of 4.43). The past and chronic infected placentas did not differ significantly (P = 0.07, chi-square of 3.37). In total, the infected first time mothers had significantly higher soluble HLA-G than uninfected (P = 0.003, chi square of 8.71, for 29 infected and 31 uninfected primigravidae). This information is shown in the box plot in Figure 7.

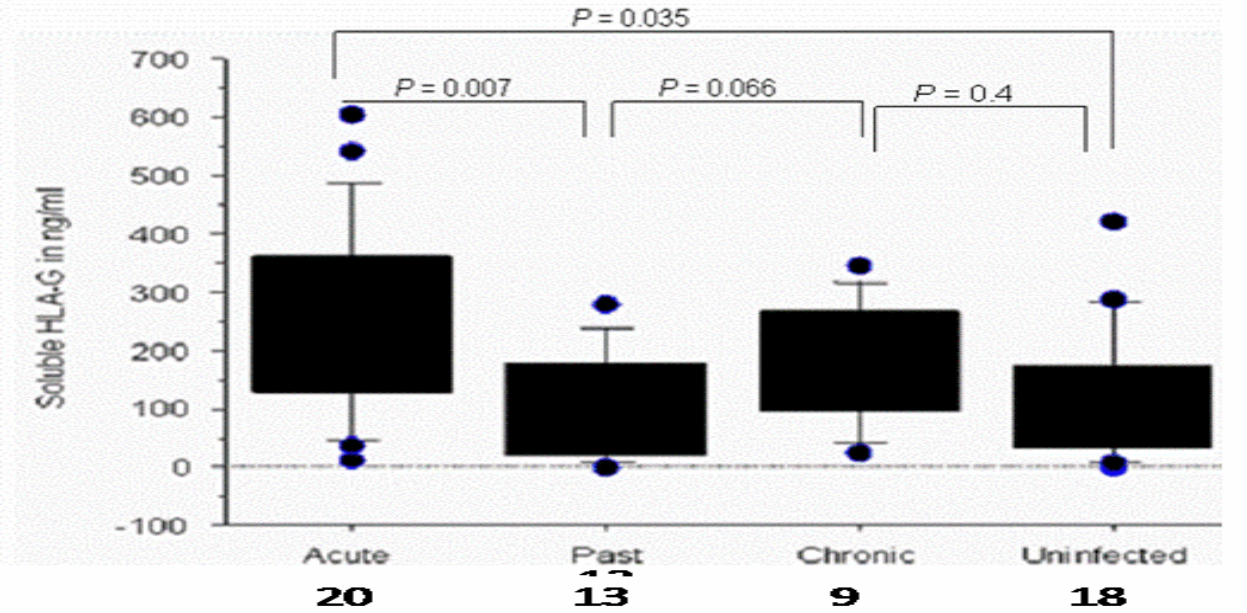


Figure 7: A box plot showing primigravidae soluble HLA-G concentrations (ng/ml) based on histological classification of placental malaria and in uninfected placentas (Bulmer *et al*., 1993 and Ordi *et al*., 2001).

The mean concentrations of soluble HLA-G for the massively inflamed or acute infected primigravidae was higher compared to that of the chronically and past infected placenta. The concentrations were higher in infected than uninfected primigravidae. However, the concentration for the past infected placentas was lower than that of the uninfected and the difference was not statistically significant (p = 0.4).

The soluble HLA-G concentrations were analysed in infected and uninfected sera for all parities. In total there were, 29 infected primigravidae, 31 uninfected, 13 infected secundagravidae and 21 uninfected and 12 infected multigravidae and 14 uninfected and 10 naïve male sera. There was a significant difference between infected and uninfected primigravidae with p=0.001 by student t test. For the rest of the groups there were no significant differences except on the infected primigravidae and secundagravidae.

Comparing the uninfected primigravidae with other parities either infected or uninfected, the uninfected primigravidae had the lowest soluble HLA-G concentrations. The difference between the uninfected primigravidae and other parities was significant. The p values are 0.02 and 0.007

with infected and uninfected multigravidae, 0.03 and 0.01 with infected and uninfected secundagravidae, respectively. However, the difference with naïve unexposed male sera was not significant with *p* value of 0.7. Also the difference between the infected primigravidae and other parities sera soluble HLA-G concentration was not significant.

Table 4.5: A summary of the mean soluble HLA-G concentrations for infected, uninfected mothers of all parities and naïve unexposed male sera.

Parities	Mean±stdev	*p*
Primigravidae (n=60)		
Infected	222.16±157.10, n=29	0.001
Uninfected	110.68±102.76, n=31	
Secundagravidae (n=34)		
Infected	199.27±114.09, n=13	0.5
Uninfected	231.67±190.06, n=21	
Multigravidae (n=26)		
Infected	254.23±184.35, n=12	0.9
Uninfected	261.76±172.80, n=14	
Naïve unexposed male	125.20±79.75, n=10	

The *p* values represent the comparisons of the infected and uninfected mean concentrations of soluble HLA-G by student t test. *P* values ≤ 0.05 at 95% confidence interval were considered significant.

The difference between the mothers sera irrespective of the infection and the naïve unexposed male were statistically significant except for the uninfected primigravidae who had mean concentrations lower than that of the naïve male with $p = 0.7$.

The concentration of soluble HLA-G was further analysed in all samples categorized as infected and uninfected irrespective of the parity. There were 54 infected, 66 uninfected and 10 naïve unexposed male sera. The infected had the highest mean soluble HLA-G concentration of 223.78±153.0 than uninfected who had 181.22±162.95. The difference was not significant with *p* of 0.14. The difference between the infected and the naïve unexposed male sera was significant with p of 0.006, while the difference between the uninfected mothers and naïve unexposed male was not significant with p of 0.096. This information is shown in the Figure 8.

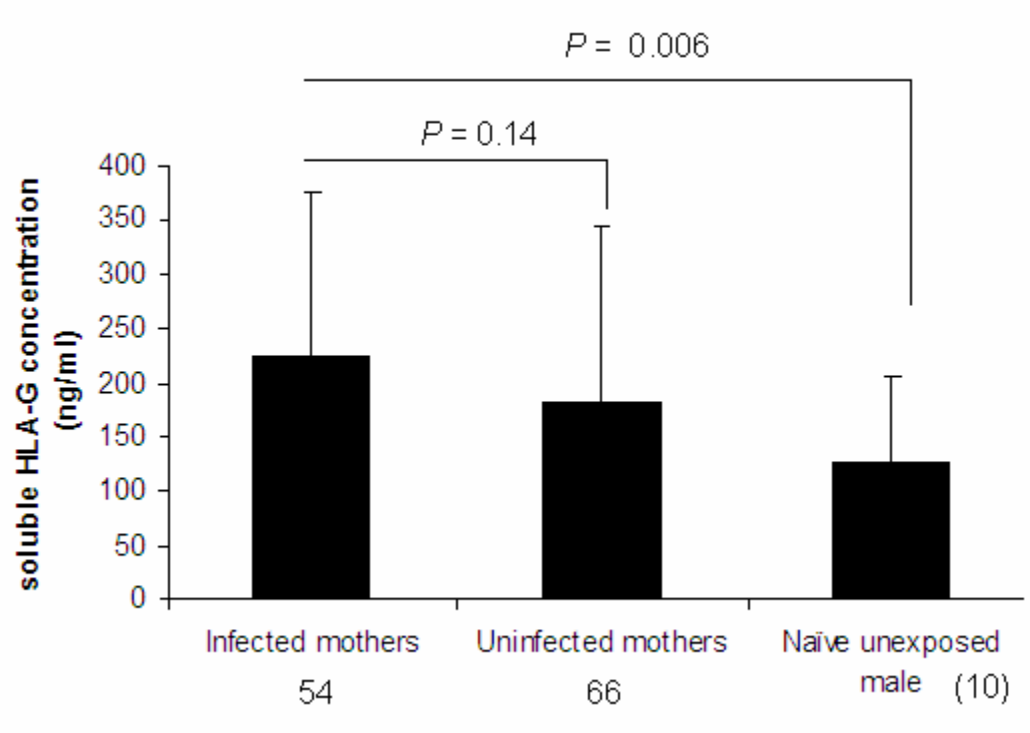


Figure 8: Plot of soluble HLA-G concentration (ng/ml) against the infected, uninfected mothers and naïve unexposed male sera.

The soluble HLA-G concentration of uninfected primigravidae was compared with other parities. It was found that there was a significant difference with other uninfected parities and the probability was 0.013 and 0.007 for the difference with uninfected secundagravidae and multigravidae, respectively. Compared with infected mothers of other parities, it was also found that uninfected primigravidae had significantly less soluble HLA-G than infected secundagravidae and multigravidae with p of 0.025 and 0.023, respectively.

4.5 Immunohistochemistry

Local expression of HLA-G and the intensity were investigated by immunohistochemistry in *P. falciparum* infected and uninfected placenta sections. The intensity of staining and the localization of membrane bound HLA-G and soluble HLA-G were determined. Mouse monoclonal antibody against membrane bound HLA-G1 (Clone MEM-G/9) and intron-4 specific mouse monoclonal anti-human HLA-G5 clone 5A6G7 was used (Exbio Praha, Czech Republic).

Trophoblasts were labeled with mouse anti-human cytokeratin-7. Dakocytomation kit was used in this study.

The membrane bound HLA-G1 was localized on the surface of the syncytiotrophoblasts, the endothelial lining of the forming fetal blood vessels and near immune cells in infected blood cells. The uninfected placental sections had low expression of HLA-G mainly on the syncytiotrophoblasts as compared to the infected. The HLA-G localized mostly on intervillous spaces along the syncytiotrophoblasts of the villi and the stroma while the intron-4 retaining soluble HLA-G1 was mainly found in the intervillous spaces and the fetal blood vessels. The intensity of staining was high in infected placentas for the soluble HLA-G.

4.5.1 Membrane bound HLA-G

The membrane bound HLA-G was evaluated in infected placentas with different stages of infections. These were acute, chronic, past and uninfected placentas. It was found that acutely infected placentas had high intensity of staining compared to others.

To ensure that there was no non-specific staining, infected placental section was stained with secondary antibody from Dako-Envision ® kit. This is shown by photomicrographs plates in the Figure 9. This was further compared by the similar sections stained with both primary and secondary antibodies against human membrane bound HLA-G. They were then visualized at ×40 magnification.

To ensure that the sections were proper cut, trophoblast cytoskeletons were stained with mouse anti-human cytokeratin-7 monoclonal antibody. Mouse anti-human cytokeratin-7 stains cytokeratin which is cytoskeleton for the placenta cells. This is illustrated by photomicrograph in Plate 3.

The membrane bound HLA-G was localised on the syncytiotrophoblasts, near infiltrating inflammatory cells and on the linings of the developing fetal blood vessels. This is shown in photomicrographs Plates in Figure 10.

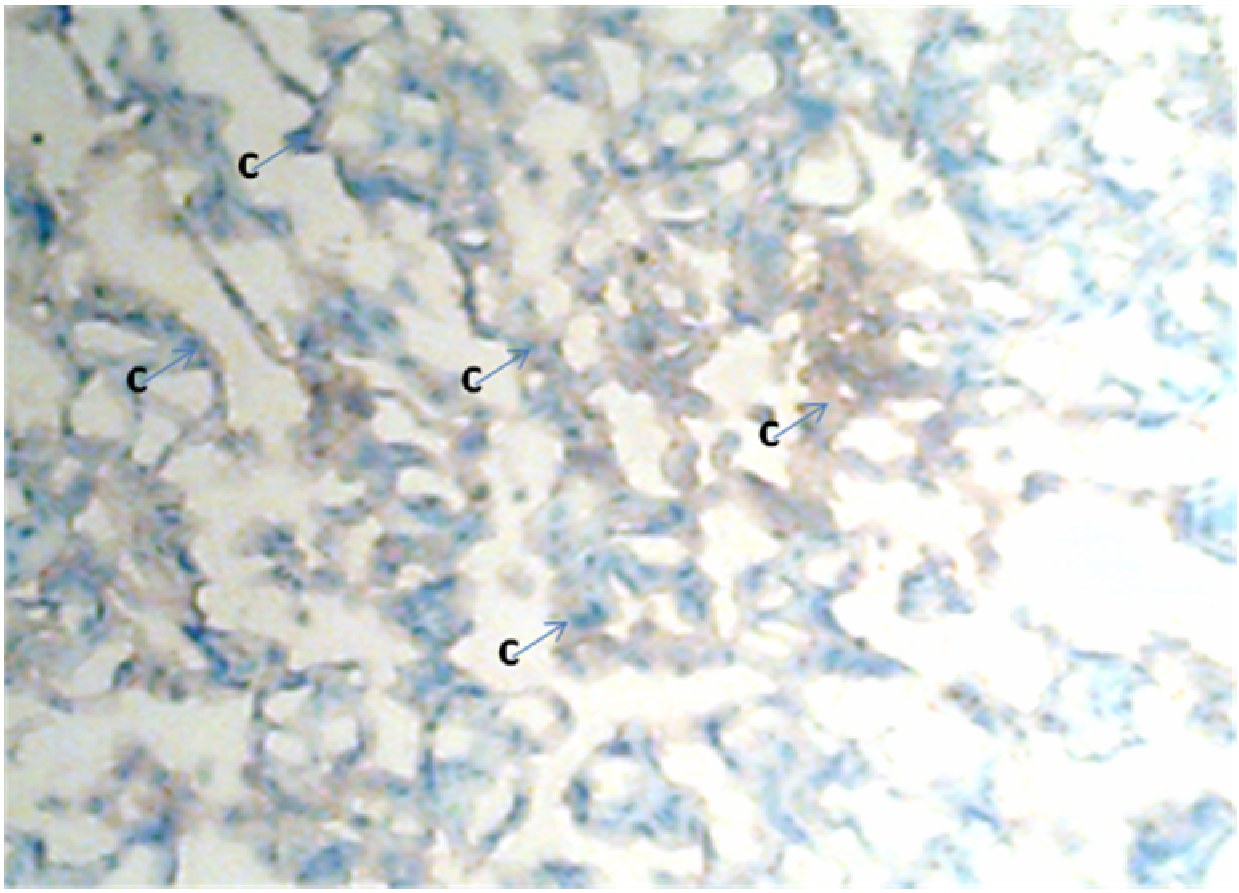


Plate 3: A primigravidae placental section showing cytoskeleton along the trophoblasts as stained with mouse anti-human cytokeratin-7 monoclonal antibody. **C** represents cytokeratin in the trophoblast. This stained dark brown, and mainly along the syncytiotrophoblasts. The photomicrographs were taken at a magnification of × 40, by use of Fuji® digital camera (Finepix A900, 9 megapixels) mounted on Olympus® light microscope.

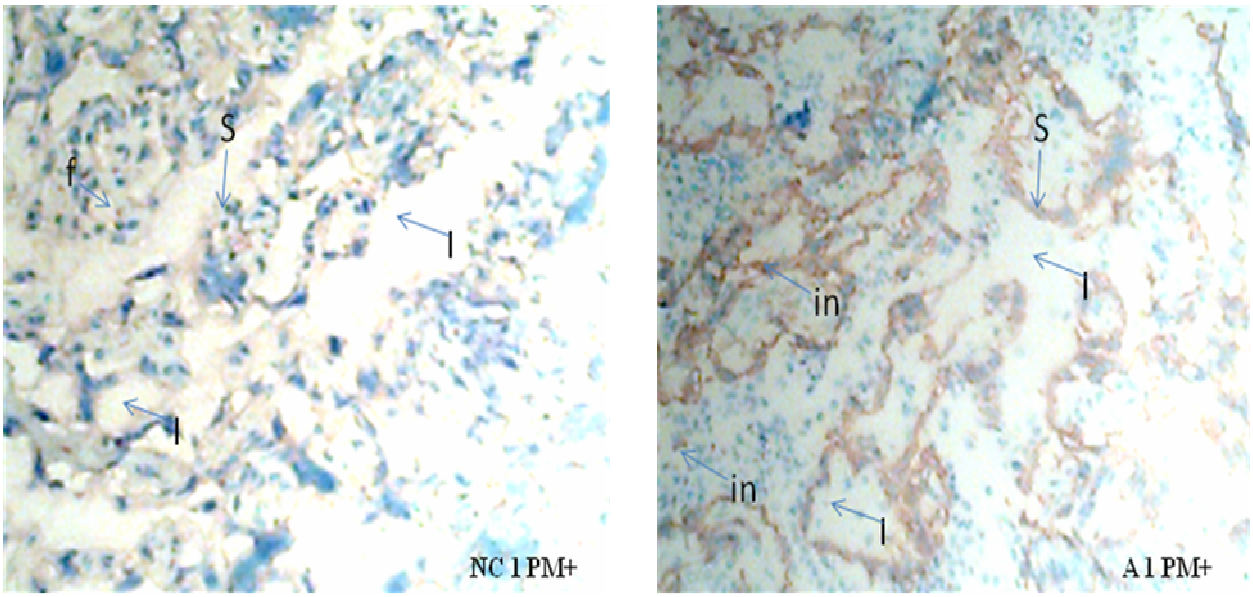


Figure 9: Shows photomicrograph plates of the negative controls. The Plate **NC 1 PM+** represents acutely infected placenta stained with secondary antibody from Dako-Envision® kit. The **A 1 PM+** represents acutely infected placenta stained with both primary and secondary antibodies for the membrane bound HLA-G. The photomicrographs were taken at the magnification of ×40. The photomicrographs were taken by use of Fuji® digital camera (Finepix A900, 9 megapixels) mounted on Olympus® light microscope

This was done to correct background due to non-specific staining of the HLA-G proteins in the placental section. Comparing the two sections, it was observed that the infected placenta had intense staining of membrane bound HLA-G proteins compared to non-specific stained section.

4.5.2 Soluble HLA-G

The sections were further stained with intron-4 retaining mouse anti-human HLA-G which labels the soluble HLA-G. It was observed that acutely infected placental sections had intense staining compared with others. The staining localised in the intervillous spaces and the lumen of the forming fetal blood vessel. This is shown in the Plates in Figure 10.

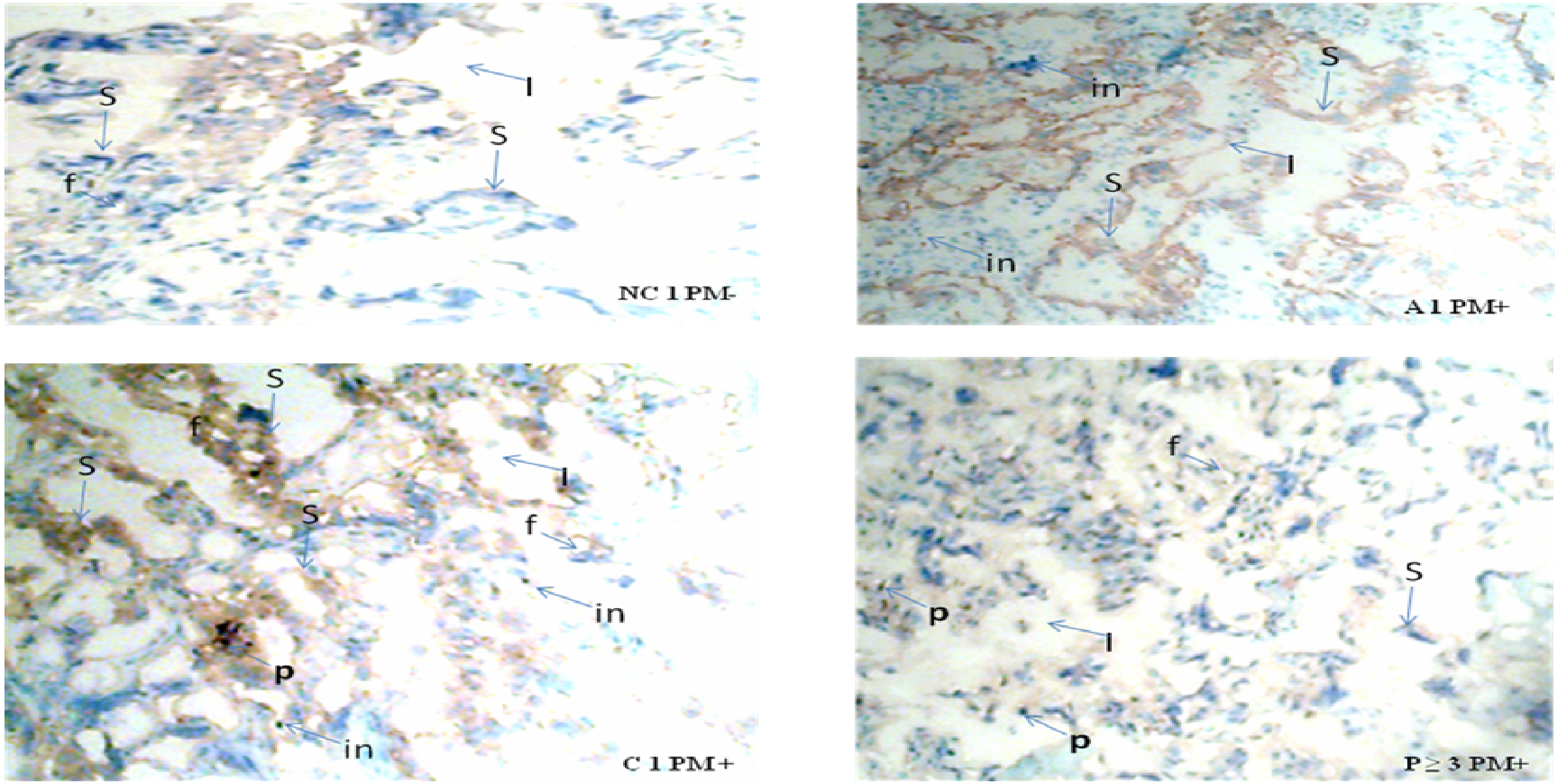


Figure 10: The photomicrographs Plates labeled **NC 1 PM**-, represents uninfected primigravidae placenta showing localization of membrane bound HLA-G along syncytiotrophoblasts. The **A1 PM+,** represents acutely infected primigravidae placental section. The **C1 PM+**, represents a chronically infected primigravidae placenta section and the **p≥3 PM+**, represents multigravidae placental section with past infections of *P. falciparum*. For all the plates the magnification was ×40. The parts labeled **I;** are intervillous spaces, **S;** are syncytiotrophoblast, **in;** are inflammatory infiltrates, **f;** are the fetal stroma and **p** are the pigments and fibrin deposits.

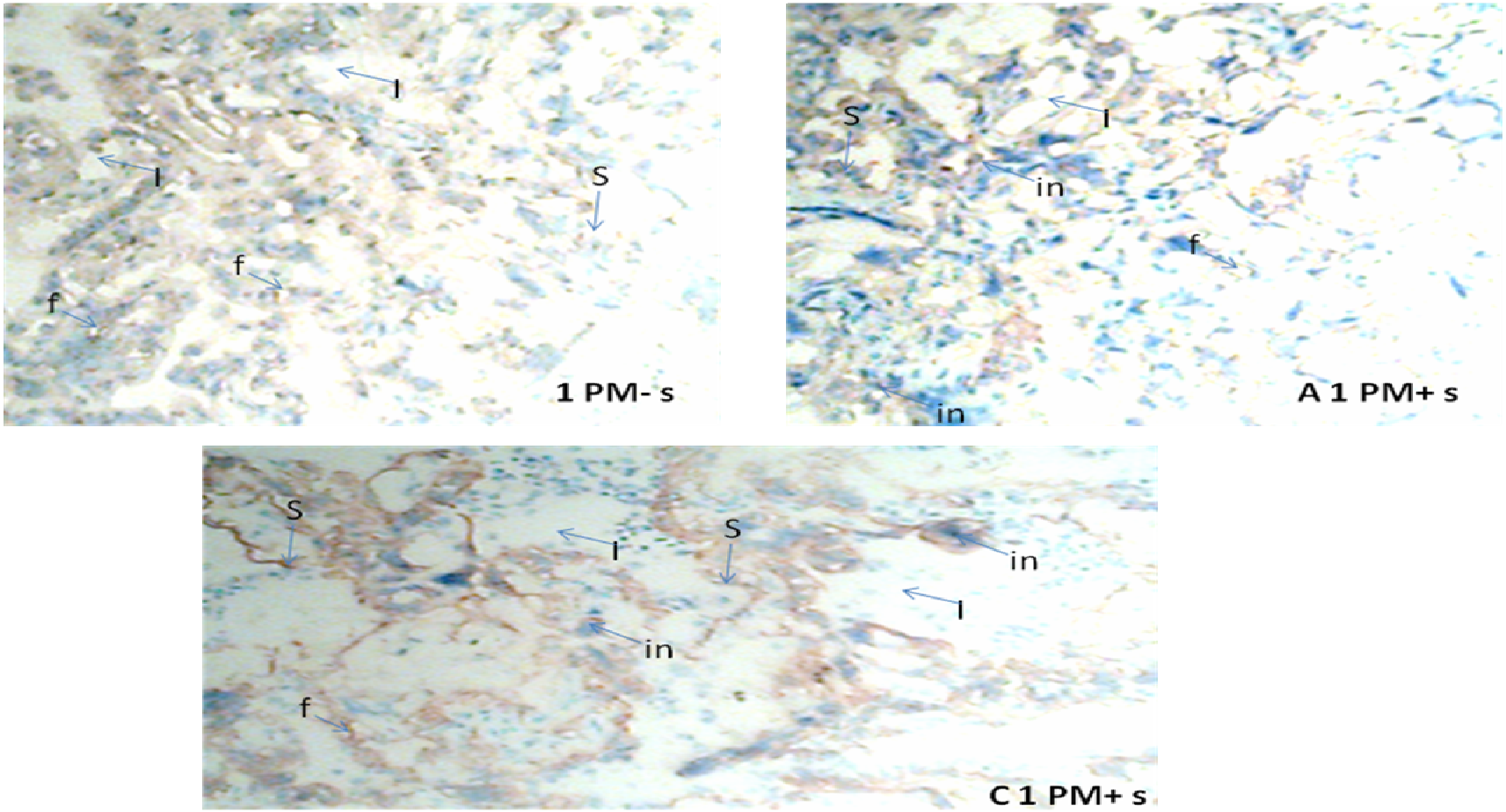


Figure 11: Photomicrograph plates shows localization of soluble HLA-G by the intron-4 retaining antibody (mouse anti human HLA-G5, Clone 5A6G7). The Plate labeled **1 PM-s** represents uninfected primigravidae placenta serving as a negative control. **A1 PM+s** represents acutely infceted placental section from primigravidae and C 1 PM+s is a chronically infected placental section from primigarvidae. The parts labeled **I;** are intervillous spaces, **S;** are syncytiotrophoblast, **in;** are inflammatory infiltrates and **f** are the fetal stroma. Photomicrographs were take at a magnification of × 40.

4.6 Correlations Analysis

The concentrations of soluble HLA-G were correlated with birth weight and maternal age. It was found that soluble HLA-G concentrations in infected primigravidae and secundagravidae are negatively correlated to birth weight with coefficients of correlation -0.16 and -0.18, respectively. Conversely, the concentration in infected multigravidae was positively correlated with a coefficient of correlation of 0.20.

For the uninfected mothers of all parities the concentrations of the soluble HLA-G was negatively correlated to the birth weight with correlations coefficients of -0.18, -0.19 and -0.08 for uninfected primigravidae, secundagravidae and multigravidae, respectively.

Maternal age and the concentration of soluble HLA-G correlate negatively in all parities in a physiological pregnancy. However, in infected primigravidae and multigravidae the maternal age and soluble HLA-G correlates positively with coefficients of correlations, 0.13 and 0.26, respectively.

4.7 Discussions

The microarray data of seven chronically *P. falciparum* infected and nine uninfected first time mothers showed approximately 2-fold increase in HLA-G transcripts in the infected primigravidae compared to uninfected controls (Muehlenbachs *et al.*, 2006 baseline survey unpublished data). The Affymetrix Human chip array U133 plus 2.0 used in this study had four probes for HLA-G. Though the transcripts of HLA-G were significantly high in infected placentas compared to uninfected, they could not show which isoforms were involved. However, this was crucial step in initial demonstration of the transcripts of HLA-G from the placental genome wide transcriptome profiling. However, this did not confirm whether these transcripts were translated into the protein.

The quantitative real time PCR demonstrated significantly high transcripts of total HLA-G in *P. falciparum* infected placentas compared to uninfected, $p = 0.003$. The transcripts of membrane bound HLA-G was found to be significantly higher in infected mothers than in uninfected with p

= 0.04. The high transcripts of HLA-G in infected placentas would be involved in coding for HLA-G proteins. These proteins would inhibit NK cells and other immune cells infiltrating the intervillous and intravillous spaces during placental malaria. Thus offering a mechanism of limiting deleterious effects of the immune infiltrates in the placenta and the fetus. However, the soluble HLA-G transcripts were not significantly different between infected and uninfected placentas with $p = 0.06$. In first time mothers, the transcripts of total HLA-G were significantly higher than in uninfected mothers with $p = 0.0009$. Although, the membrane bound and soluble HLA-G transcripts were high in infected placentas than uninfected, the differences were not significant.

The concentration of soluble HLA-G in infected primigravidae was significantly higher than in uninfected with $p = 0.001$. Although the transcripts of soluble HLA-G were not significantly higher in infected mothers, the high levels of soluble HLA-G concentrations was possibly from the products of the cleavage of membrane bound HLA-G (Park *et al*., 2004). In other parities, the difference was not significant although the infected mothers had high concentration than uninfected. The immunohistochemistry study demonstrated intense staining of HLA-G along syncytiotrophoblasts, lining of the forming fetal blood vessels and near immune cells and fibrin deposits in infected placentas.

The response of HLA-G seems to be determined by the type and/or intensity of infection. The inflammatory process is known to induce membrane bound and soluble HLA-G production (Wiendl *et al*., 2005). Transcripts of HLA-G1 and HLA-G5 have been demonstrated to be consistently found in lesional skin specimens of a psoriatic skin (Aractingi *et al*., 2001). Psoriasis is associated with hyperproliferation of epidermal keratinocytes a response mediated by T cells and cytokines (Aractingi *et al*., 2001). Increased expression of HLA-G was hypothesized as a mechanism for regulating infiltrating immune cells in psoriatic skin lesions. Consistent with this study, the high transcripts of membrane bound and soluble HLA-G in *P. falciparum* infected placentas would be involved in mediating an anti-inflammatory response in a similar mechanism.

The study findings contrast with Sartelet *et al*., (2005), findings where there was a low expression of HLA-G infected placentas with increased infiltration of the natural killer cells

especially in the basal plate of the placenta. Immunohistochemistry study of the concentration of the protein is a semi-quantitative analysis method and therefore could not give the exact levels of expression of HLA-G. Similarly, proteases cleavage of the membrane-anchored HLA-G makes immunohistochemistry unreliable to quantify the levels of HLA-G in the placenta. Another possible cause of the discrepancy could be the difference of the study population. Those study samples for Sartelet's work were drawn from hypoendemic area while those of the current study were from malaria hyperendemic area. This difference in malarial transmission intensity could be responsible for the differences in protein responsiveness to infections from the two populations.

HLA-G has been shown to be elevated in infectious diseases and cancer (Schust *et al*., 1996; Jun *et al*., 2000). Donaghy *et al*., (2006), demonstrated elevated circulating HLA-G levels in HIV infected patients with and without visceral leishmaniasis. Furthermore, HLA-G levels were increased in women with preterm birth due to intrauterine activation, and in women with hemolysis, elevated liver enzymes and low platelet count (HELLP) syndrome (Steinborn *et al*., 2003). In all these cases, the role of increased HLA-G was to inactivate immune cells at the site of inflammation either offering anti-inflammatory effects or promoting the pathogenesis of the disease.

Other studies have shown that HLA-G is up-regulated during infectious diseases (LeMaoult *et al*., 2003). It was found that HLA-G was expressed by neuronal cells after rabies virus infection (Lafon *et al*., 2005). Neuronal expression of HLA-G protein was observed upon challenge with interferon-β, a type 1 interferon critically involved in the immune response to rabies (Lafon *et al*., 2005). This was detected both at mRNA level and at the cell surface. Successful invasion of the nervous system by the rabies virus seems to be the result of a subversive strategy based on the survival of the infected neurons (Lafon, 2004). This strategy includes protection against virus-mediated apoptosis and destruction of T and natural killer cells that invade by redundant control of expression of the immunosubversive molecules FasL and HLA-G (Lafon, 2004). HLA-G would here represent a mechanism to show how the rabies virus escapes the immune system, therefore promoting destructive CNS damage under infectious conditions.

HLA-G would regulate central nervous inflammatory response by regulating the pro-inflammatory T cells via a negative feedback loop by inducible HLA-G expression, specifically on the resident microglia and the invading peripheral monocytes and macrophages (Lafon, 2004). Also HLA-G protein was found strongly expressed in brain specimens from patients with multiple sclerosis while it was rarely detectable in the non-pathological control specimens (Wiendl *et al*., 2005). HLA-G was also found in other disease entities such as meningitis and Alzheimer whereby expression was correlated to activation and MHC class II expression on microglial cells (Wiendl *et al*., 2005). The demonstration of HLA-G and its receptor ILT2 on CNS cells and in areas of microglia activation (Wiendl *et al*., 2005), implicate HLA-G as a contributor to the fundamental mechanisms regulating immune reactivity in the CNS. This pathway may act as an inhibitory feedback aimed to down-regulate the deleterious effects of T-cell infiltration in neuroinflammation.

HLA-G expression was also strongly down-regulated in human cytomegalovirus-infected monocytes after allogeneic stimulation (Jun *et al.,* 2000). Human cytomegalovirus unique sequence gene products 3 and 6 were found to down-regulate HLA-G, thus promoting pathogenesis in the placenta (Jun *et al*., 2000). This is an indication that induction and/or up-regulation of HLA-G expression might constitute a way to escape antiviral immunity for certain viruses. Thus, the immune cells may destroy the infected tissues because they are not inhibited by HLA-G and they are in massive numbers at the site of inflammation. Similarly, sera of HIV-infected patients were able to induce a strong HLA-G1 expression up-regulation by monocytes from healthy donors (LeMaoult *et al*., 2003).

HLA-G positive T-cells have been observed at sites of inflammation, such as inflamed skeletal muscle in myositic patients, suggesting a role they might be playing in inflammatory responses (Lafon *et al*., 2005).

Herpes simplex virus blocks intracellular transport of HLA-G by inducing synthesis of a transporter inhibitor (Schust *et al.,* 1996), and human cytomegalovirus (HCMV) gene products down regulates HLA-G expression in trophoblasts leading to low maternal immune tolerance of the fetus (Jun *et al.,* 2000).

In the present study, the levels of HLA-G were correlated with birth weight and maternal age. It was found that soluble HLA-G5 concentrations in infected primigravidae and secundagravidae are negatively correlated to birth weight with coefficients of correlation of -0.16 and -0.18, respectively. Conversely, the concentrations in infected multigravidae were positively correlated with a coefficient of correlation of 0.20. This would be because of the level of immunity to the placental malaria. Multigravidae are more immune than secundagravidae and primigravidae to pregnancy malaria. Similarly inflammatory responses are minimal in multigravidae compared to other parities. Therefore, the physiological level of HLA-G is found in this case as the effects of specific anti-adhesion antibodies counter the effect of *P. falciparum* (Fried and Duffy., 1996).

High transcripts of HLA-G in *P. falciparum* infected primigravidae are an indication that the placenta is mounting an anti-inflammatory response to counteract the effects of the massively infiltrating immune cells. In this case HLA-G might be thought to prevent destruction of the placenta by these immune cells and at the same time offering a state of tolerance to the placenta which could reduce the rate of their lysis and subsequently elimination.

Maternal age correlates negatively with the concentration of soluble HLA-G in all parities in a physiological pregnancy. However, in infected primigravidae and multigravidae the maternal age and soluble HLA-G correlates positively with coefficients of correlations of 0.13 and 0.26, respectively. Furthermore, in the infected primigravidae and secundagravidae, the concentrations of soluble HLA-G correlates negatively with birth weight. Similarly, the same negative correlation was observed in uninfected mothers of all parities. Interestingly, the infected multigravidae mothers' concentration of soluble HLA-G was positively correlated with birth weight. This indicates that soluble HLA-G might be playing a role in pathogenesis of placental malaria in multigravidae women.

The current study indicates that, HLA-G can only be used as an indicator of birth outcome in multigravidae women since there are not different relationships in other parities. However, this conclusion might be affected by the sample size which in this study was small.

HLA-G in primigravidae could possibly indicate the outcome in terms of fetal birth weight. Low birth weight is weight below 2.5 kilograms (Ordi *et al*., 2001). In this project it was found that infected primigravidae had significantly higher transcripts and concentrations of HLA-G compared with other groups. At the same time they have a low birth weight. Therefore, high levels of HLA-G in infected primigravidae would indicate low birth weight fetus. The mechanism leading to low birth weight remain obscure, but many molecules that are involved in placental malaria pathogenesis would indirectly show the relationship.

Taken together the data provide significant insights into the role of HLA-G in placental malaria. HLA-G represents an inducible molecule that may be important for maintaining an 'anti-inflammatory milieu' in the parasitised inflamed placenta. Since HLA-G down-regulates immune responses by interacting with virtually all cytotoxic cellular immune effectors including cytotoxic T cell and natural killer cells (Wiendl *et al*., 2003). At the same time the Wiendl *et al*, 2003, demonstrated that only low numbers of HLA-G cells are required to convey significant immune inhibitory effects. HLA-G represents an appealing self derived anti-inflammatory mechanism, possibly applicable as a strategy against the aggressive inflammatory response that occurs in the placenta of *P. falciparum* infected mothers. Importantly, the limited levels of HLA-G expression in placenta infected with *P. falciparum* are a major risk factor for various pregnancy complications such as miscarriages, abortion, pre-eclampsia and low birth weight infants.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The study found that the transcripts and protein of HLA-G was higher in infected placentas than in uninfected, with high levels in infected primigravidae. There was a significant difference in soluble HLA-G concentrations between infected and uninfected primigravidae. On the basis of immunohistochemistry the infected placentas stained more intensely than uninfected with HLA-G localizing on the syncytiotrophoblasts, fetal stroma, and near inflammatory infiltrates and pigment deposits in the infected placenta.

Also, HLA-G could be used to predict the birth out come in a parasitised mother based on the findings that in a physiological pregnancy there is a negative correlation between the concentration of HLA-G and the birth weight. However, this is not the case only with the infected multigravidae women who have a positively correlated.

The idea of local immunosuppression by HLA-G affecting disease activity and progression is appealing, both from an immunopathogenic and therapeutic view. Placenta HLA-G although being upregulated in *P. falciparum* infected mothers in response to inflammatory stimuli, might represent a regulatory principle balancing and controlling antigen specific activation as well as autologous T-cell activation *in-vivo*, which can lead to miscarriage. Upregulation of the immune-inhibitory HLA-G at sites of inflammation contributes to the limitation of organ damage and plays a role in the mechanism of tissue integrity.

5.2 Recommendations

It is recommended that in future studies:

1. Any future intervention strategy during pregnancy malaria should be aimed at raising the levels of HLA-G in the placenta. This will be important in counteracting the negative effects of inflammatory infiltrates during placental malaria infection. High levels of

HLA-G induced by a placental malaria vaccine or drug during intermittent preventive treatment would be important in mounting anti-inflammatory response.

2. All the isoforms of the HLA-G should be analysed and the transcripts sequenced. These can be used in production of recombinant proteins which could be used together with vaccines or intermittent preventive therapies to minimize the effects of placental malaria.
3. Western blot and northern blot methods should be used to make the quantification of HLA-G more accurate at the protein level. These would be used to raise expression of soluble HLA-G proteins which can be used to check down the infiltration of immune cells and fibrin deposits during placental malaria
4. In malaria hypoendemic areas it would be interesting to know how malaria pre-immunity would affect the expression of HLA-G.

REFERENCES

Aractingi S, Nicola B, Caroline L D, Manuelle V, Herve B, Laurence M, Louis D and Edgardo D C (2001). HLA-G and NK receptors are expressed in psoriatic skin: A possible pathway for regulating infiltrating T cells? *American Journal of Pathology* **159**: 71-77

Bainbridge D, Ellis S, Le Bouteiller P and Sarget I (2001). HLA-G remains a mystery. *Trends in Immunology* **22**: 548-552

Beeson J G, Rogerson S J, Cooke B M and Reeder J C (2000). Adhesion of *P. falciparum*-infected erythrocytes to hyaluronic acid in placenta malaria. *Nature Medicine* **6**: 86-90

Bulmer J N, Rasheed F N, Francis N, Morrison L and Greenwood B M (1993). Placental malaria. I. pathological classification. *Journal of Histopathology* **22**: 211-218

Carbo N, Lopez-Soriano F J and Argiles J M (1995). Administration of tumor necrosis factor-alpha results in a decreased placental transfer of amino acids in the rat. *Endocrinology* **136**: 3579-3584

Conrad K P and Benyo D F (1997). Placental cytokines and the pathogenesis of preeclampsia. *American Journal of Reproductive Immunology* **37:** 240-249

Donaghy L, Gross F, Amiot L, Mary C, Maillard A, Guiguen C and Gangneux J P (2006). Elevated circulating HLA-G levels in HIV infected patients with and without visceral leishmaniasis. Abstract number **0398**, presented in 16th European Congress of Clinical Microbiology and Infectious Diseases, held at Nice France on April 1-4, 2006

Duffy P E and Fried M (2003). Antibodies that inhibit *Plasmodium falciparum* adhesion to chondroitin sulfate A are associated with increased birth weight and gestational age of newborns. *The Journal of Infection and Immunity* **71**: 6620-6623

Duffy P E and Fried M (2001). Malaria in Pregnancy; deadly parasite susceptible host. 1st edition, Taylor and Francis, New York. Page 26-41

Ferreira U M, Nunes S M and Wunderlich G (2004). Antigenic diversity and immune evasion by malaria parasites. *Clinical and Diagnostic Laboratory Immunology* **11**: 987-995

Fievet N, Cot M, Ringwald P, Bickii J, Dubois B, Le Hersran J Y, Migot F and Deloron P (1997). Immune response to *Plasmodium falciparum* antigens in Cameroonian primigravidae: evolution after delivery and during second pregnancy. *Journal of Clinical Experimental Immunology* **107**: 462-467

Fournel, S. Aquerre-Girr M, Huc X, Lenfant F, Alam A, Toubert A, Bensussan A and Le Bouteiller P (2000). Soluble HLA-G1 triggers CD95/CD95-ligand-mediated CD8+ cells by interacting with CD8. *Journal of Immunology* **164**: 6100-6104

Fried M and Duffy P E (1996). Adherence of *Plasmodium falciparum* to Chondroitin sulfate A in the human placenta. *Science* **272**: 1502-1504

Fried M, Nosten F, Brockman A, Brabin B J and Duffy P E (1998**).** Maternal antibodies block malaria. *Nature* **395**: 851-852

Fujii T, Ishitani A and Geraghty D E (1994). A soluble form of the HLA-G antigen is encoded by a messenger ribonucleic acid containing intron 4. *Journal of Immunology* **153**: 5516-5524

Geraghty D E, Koller B H and Orr H T (1987). A human major histocompatibility complex class 1 gene that encodes a protein with a shortened cytoplasmic segment. *Proceedings of the National Academy of Science USA* **84**: 9145-9149

Gonen–Gross T, Gazit R, Achdout H, Hanna J, Mizrahi S, Markel G, Horejsi V and Mandelboim O (2003). Special organization of the HLA-G protein on the cell surface. *Journal of Human Immunology* **64**: 1011-1016

Greenwood B M, Bojang I L, Witty C J and Targett C A (2005). Malaria. *Lancet* **365**: 1487-1498

Hviid T V, Larsen L. G, Hoegh A M and Bzorek M (2004). HLA-G expression in placenta in relation to HLA-G genotype and polymorphisms. *American Journal of Immunology* **52**: 212-217

Hunt J S, Petroff G M, McIntire H M and Ober C (2005). HLA-G and immune tolerance in pregnancy. *FEBS Journal* **19**: 681-693

Hunt J S, Petroff M G and Burnett T G (2000). Uterine Leukocytes: Key players in pregnancy. *Seminars in Cell Development Biology* **11**: 127-137

Ismail M R, Ordi J, Menendez C, Ventura P J, Aponte J J , Kahigwa E, Hirt R, Cardesa A and Alonso P L (2000). Placental pathology in malaria: a histological, immunohistochemical, and quantitative study. *Journal of Human Pathology* **31**: 85-93

Jauniaux E, Poston L and Burton G J (2006). Placental-related diseases of pregnancy: involvement of oxidative stress and implications in human evolution. *Journal of Human Reproduction Update* **12**: 747-755

Jun Y, Kim E, Jin M, Sung H C, Han H and Geraghty D E (2000). Human cytomegalovirus gene products US3 and US6 down regulate trophoblasts class 1 MHC molecules. *Journal of Immunology* **164**: 805-811

Kaushik A, Sharma V K, Sadhana and Kumar R (1992). Malarial placental infection and low birth weight babies. *Journal of Communicable Diseases* **24**: 65-69

Kovats S, Main E K, Librach C, Stubblebine M, Fisher S J, and DeMars R (1990). A class I antigen, HLA-G, expressed in human trophoblasts. *Science* **248**: 220-223

Lafon M, Prehaud C, Megret F, Lafage M, Mouillot G, Roa M, Moreau P, Rouas-Freiss N and Carosella E D (2005). Modulation of HLA-G expression in human neural cells after neurotropic viral infections. *Journal of Virology* **79**: 15226-15227

Lafon M (2004). Subversive neuroinvasive strategy of rabies virus. *Archives of Virology Supplementary* **2004**: 149-159

Le Bouteiller P and Blaschitz A (1999). The functionality of HLA-G is immerging. *Journal of Immunological Reviews* **167**: 233-244

Le Bouteiller P and Valerie M (1997). HLA-G and Pregnancy. *Journal of Reviews of Reproduction* **2**: 7-13

Le Bouteiller P and Solier C (2001). Is antigen presentation the primary function of HLA-G? *Journal of Microbes and Infection* **3**: 323-332

LeMaoult J, Le Discorde M, Rouas-Freiss N, Moreau P, Menier C, McCluskey J and Carosella E D (2003). Biology and functions of human leukocyte antigen-G in health and sickness. *Journal of Tissue Antigen* **62**: 273-284

LeMaoult J, Rouas-Freiss N and Carosella D E (2005). HLA-G5 expression by trophoblast cells: the facts. *Journal of Molecular Human Reproduction* **11**: 719-722

McGregor IA. (1984). Epidemiology, malaria and pregnancy. *American Journal of Tropical Medicine and Hygiene* **33**: 517-525

Menendez C, Ordi J, Ismail M R, Ventura P. J, Aponte J J, Kahigwa E, Font F and Alonso P L (2000). The impact of placental malaria on gestational age and birth weight. *Journal of Infectious Diseases* **181:** 1740-1745

Mitsdoerffer M, Bettina S, Bernd C. K, Oliver N, Johannes D, Hans-Peter H, Michael W and Wiendl (2005). Monocyte-derived HLA-G acts as a strong inhibitor of autologous CD4 T cell

activation and is up regulated by interferon-β *in vitro* and *in vivo*: rationale for the therapy of multiple sclerosis. *Journal of Neuroimmunology* **159**: 155-164

Moffett-King A (2002). Natural killer cells and pregnancy. *Nature Reviews in Immunology* **2**: 656-663

Moormann M A, Sullivan D A, Rochford RA, Chensue W S, Bock J P, Nyirenda T and Meshnick R S (1999). Malaria and Pregnancy: placental cytokine expression and its relationship to intrauterine growth retardation. *The Journal of Infectious Diseases* **180**: 1987-1993

Muehlenbachs A, Mutabingwa T K, Edmonds S, Fried M and Duffy P E (2006). Hypertension and maternal-fetal conflict during placenta malaria. *Plos Medicine* **11**: 10.1371

Ordi J, Menendez C, Ismail RM, Ventura J P, Palacin A, Kahigwa, Ferrer B, Cardesa A and Alonso L P (2001). Placental malaria is associated with cell-mediated inflammatory responses with selective absence of natural killer cells. *The Journal of Infectious Diseases* **183**: 1100-1107

Pace J. L, Morales P. J, Phillips A and Hunt J S (2005). Analysis of the soluble isoforms of HLA-G, placenta and trophoblasts. In methods and protocols Volume 2. Humana Press Inc. Totowa, N. J. USA. Pages 181-189

Park G M, Lee S, Park B, Kim E, Shin J, Cho K and Ahn K (2004). HLA-G generated by proteolytic shedding inhibits NK-mediated cell lysis. *Journal of Biochemistry and Biophysical Research Communication* **313**: 606

Petroff M G, Lieping C, Teresa A. Phillips, Dagmar A, Peter S and Hunt J S (2003). B7 family molecules are favorably positioned at the human maternal-fetal interface. *Journal of Reproductive Immunology* **68**: 1496-1504

Rajagopalan S, Bryceson T Y, Kuppusamy P. S, Geraghty E D, Meer A V, Joosten I and Long O E (2006). Activation of NK cells by an endocytosed receptor for soluble HLA-G. *Plos Biology* **4**

Rasheed F N, Bulmer J N and Dunn D T (1993). Suppressed peripheral and placental blood lymphoproliferative responses in first pregnancies: relevance to malaria. *American Journal of Tropical Medicine and Hygiene* **48**: 154-160

Rouas-Freiss N and Carosella E D (1997). Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. *Proceedings of the National Academy of Science* **94**: 11520-11525

Sartelet H, Le-Hesran J Y, Graesslin O, Gaillard D, Fe M, Lechki C, Gaye A, LeBouteiller P and Birembaut P (2005). Less HLA-G expression in *Plasmodium falciparum* infected third trimester placentas is associated with more natural killer cells. *Journal of Placenta* **26**: 505-511

Sanders S K, Giblin P A and Kavathas P (1991). Cell-cell adhesion mediated by CD8 and human histocompatibility leukocyte antigen G, a nonclassical major histocompatibility complex class 1 molecule on cytotrophoblasts. *Journal of Experimental Medicine* **174**: 737-740

Schust D J, Hill A B and Ploegh H (1996) Herpes simplex virus blocks intracellular transport of HLA-G in placentally derived human cells. *Journal of Immunology* **157**: 3375-3380

Shiroishi M, Tsumoto K, Amano K, Shirakihara Y, Colonna M, Braud V M, Allan D S, Makadzange A, Rowland-Jones S, Willcox B, Jones E Y, Van der Merwe P A, Kumagai I and Maenaka K (2003). Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class 1 binding and bind preferentially to HLA-G. *Proceedings of the National Academy of Science* **100**: 8856-8861

Steinborn A, Rebmann V, Scharf A, Sohn C and Gross-Wilde H (2003). Placental abruption is associated with decreased maternal plasma levels of soluble HLA-G. *Journal of Clinical Immunology* **23**: 307-310

Tako A E, Zhou A, Lohoue J, Leke R, Taylor W D and Leke F G (2005). Risk factors for placental malaria and its effect on pregnancy outcome in Yaoundé, Cameroon. *American Journal of Tropical Medicine and Hygiene* **72**: 236-242

Viebig K N, Gamain B, Scheidig C, Le'polard, Przyborski J, Lanzer M, Gysin J and Scherf A (2005). A single member of the *Plasmodium falciparum* var multigene family determines cytoadhesion to the placental receptor chondroitin sulphate A. *European Molecular Biology Organization Report* **6**: 775-781

Walter P R, Garin Y and Blot P (1982). Placental pathologic changes in malaria. A histologic and ultrastructural study. *American Journal of Pathology* **109:** 330-342

Watkinson M and Rushton D I (1983). Plasmodial pigmentation of placentae and outcome of pregnancy in West African mothers. *Biomedical Journal* **287:** 251-254

Wiendl H, Mitsdoerffer M, Hofmeister V, Wischlusen J, Weiss E H, Dichgans (2003). The nonclassical MHC molecule HLA-G protects muscle cells from immune mediated lysis: implications for myoblast transplantation and gene therapy. *Brain* **126**: 176-185

Wiendl H, Ute F, Michel M, Carolyn J, Bettina S, Christine S, Jack A, Wolfgang B, Richard M, Amit B, Bernd C K and Michael W (2005). Expression of the immune-tolerogenic major histocompatibility molecule HLA-G in multiple sclerosis: Implications for CNS immunity. *Brain* **128**: 2689-2704

WHO (2005) World malaria report 2005: http://www.rbm.who.int/wmr2005/html accessed on 8th December 2006.

Wort U U, Hastings I, Mutabingwa T K and Brabin B L (2006). The impact of endemic and epidemic malaria on the risk of stillbirth in two areas of Tanzania with different malaria transmission patterns. *Malaria Journal* **5**: 1475-2875

Yamada M, Steketee R, Abramowsky C, Kida M, Wirima J, Heymann D, Rabbege J, Breman J and Aikawa M (1989). *Plasmodium falciparum* associated placental pathology: a light and electron microscopic and immunohistologic study. *American Journal of Tropical Medicine and Hygiene* **41**: 161-168

Yann K, Alen M, Sebastien P, Christian M and Nicolas M (2007). Statistical significance of quantitative PCR. *BMC Bioinformatics* **8**: 1-16

Yao Q Y, David H. Barlow and Ian L S (2005). Differential expression of alternatively spliced HLA-G in human preimplantation embryos and inner cell masses. *The Journal of Immunology* **175**: 8379-8385

Yie S, Li L, Li G, Xiao R and Librach L C (2006). Progesterone enhances HLA-G gene expression in JEG-3 Choriocarcinoma cells and human cytotrophoblasts *in vitro. Journal of Human Reproduction* **21**: 46-51