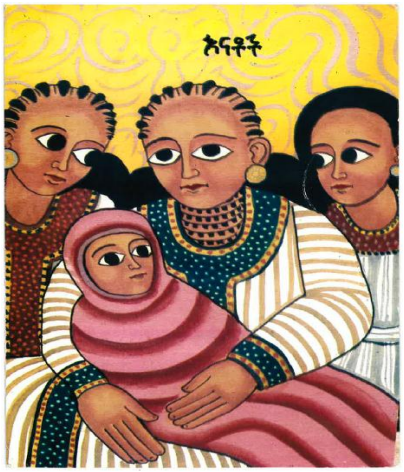
ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

GENETIC PREDISPOSITION FOR PREELAMPSIA IN A POPULATION OF PREGNANT WOMEN IN ADAMA CITY AND THE SUROUNDING AREAS, ETHIOPIA

Tsehayneh Kelemu Mihretie

Dissertation Presented in the Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Medical Biochemistry



December 2020

PhD supervisors

Main supervisors:

Daniel Seifu Melka, PhD
Associate Professor in Medical Biochemistry
Addis Ababa University, School of Medicine, College of health Sciences, Ethiopia

Stefan Hansson, MD, PhD
Professor, Senior consultant
Head Department of Obstetrics and Gynecology
Institute of Clinical Sciences Lund

Markos Abebe, PhD
Postdoctoral Scientist, Armauer Hanson Research Institute, Ethiopia

Co-supervisor:

Sisay Teklu Waji (MD)
Associate Professor in Obstetrics & Gynecology
Addis Ababa University, School of Medicine, College of Health Sciences, Ethiopia

Title of the project: Genetic predisposition for preeclampsia in a population of pregnant women in Adama and the surrounding areas, Ethiopia

Abstract

Introduction: Preeclampsia (PE) is a global health problem, which is the major cause of maternal and fetal deaths. The majority of PE related deaths occur in low- and middle-income countries with limited-resource. Currently there is no cure for the syndrome, delivery remains the only option. The etiology of PE is not elucidated yet. However, several studies suggest a hereditary predisposition for acquiring the disease. Accordingly, studies have showed the role of killer cell immunoglobulin-like receptor's (KIRs) expressed by uterine natural killer (uNK) cells and human leukocyte antigen-C (HLA-C) in familial predisposition of PE. In addition, recent evidences indicated the probable role of the CD99 expression in the pathogenesis of PE in a fetal sex dependent fashion. Such evidences are lacking in the Ethiopian context. Therefore, the general aim of the study was to evaluate the genetic predisposition for PE in a cohort of Ethiopian pregnant women.

Methods: A case-control study design was used. The study was conducted at Adama Regional Referral Hospital. We involved 288 (131 PE, 157 controls) study participants for the first part of the study and 241 (105 PE, 136 controls) study participants for the second part of the study. The demographic and clinical data was collected using questionnaire. The maternal venous and cord blood was collected, and DNA extracted using QIAGEN reagent. The DNA was amplified using traditional polymerase chain reaction (PCR) and real time quantitative PCR (RT qPCR) using TaqMan^{TM} Genotyping master mix (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, USA) and published primers. The data analysis was done using SPSS version 26.0. The Chi-Square test was used to evaluate the association between variables. The p-value <0.05 was used as cut-off point for the association of variables.
Results: the mean age, systolic and diastolic blood pressures were higher while gestation age was lower in PE group compared to controls. A statistically significant association was observed in frequency of KIRAA, KIR2DS1 and in a subset of non-self HLA-C between the PE and control groups. In addition, a statistically significant association was showed in maternal regulatory region low CD99 expression genotype (CC) between PE mothers of males compared to controls.

Conclusion: The KIRAA plays role in predisposition of PE while KIR2DS1 may play role in protection of Ethiopian pregnant women from PE. In addition, maternal regulatory region CC genotype may predispose mothers for PE in fetal sex-dependent fashion.

Key words: Preeclampsia, KIRAA, HLA-C2, male fetus, CD99

Popular summary

Preeclampsia (PE) is a health disorder that occurs during pregnancy starting from 20th week of gestation age. It is manifested by high blood pressure and protein in urine or signs and symptoms of maternal organ dysfunction or the defect in development of the fetus. Preeclampsia is the cause of death to both the mother and the fetus. Preeclampsia is the third leading cause of maternal death. More than 99% of maternal deaths occur developing countries out of which more than 69% of deaths are concentrated in sub-Saharan countries. The etiology of PE is not known though placenta is proposed to be the root cause of the disorder. So far, there is no curative treatment and currently termination of pregnancy remains the only option. Evidences show that maternal and fetal genetic makeup may increase the risk of acquiring PE. In addition, study results showed the increased risk for PE in pregnancies carrying male fetus. Accordingly, we aimed to investigate whether there was association between maternal and fetal genes with PE in study I and with pregnancies carrying male fetus in study II.

We conducted the study at Adama Regional and Referral Hospital, Ethiopia, which provides about 4000-5000 delivery services annually. A case-control study design was employed involving 288 and 241 participants in study I and study II respectively during December 2016 to August 2017 period. We investigated five maternal genes and, two fetal and maternal genes that were believed to have role during early pregnancy.

Only one of the five maternal genes showed association with PE. On the other hand, the gene that protects mothers from PE was more frequent in Ethiopian women compared to women from other African countries. In addition, PE was associated with subgroup of pregnancies carrying male fetus that possessed paternal gene that was not present in the mother.

In conclusion, Ethiopian women share similar genetic risk with women from other African countries while the Ethiopian women seem less predisposed to PE due to their genetic makeup compared to women from other African countries.

Acknowledgments

I would like to thank Department of Biochemistry, School of Medicine, College of Health Sciences, Addis Ababa University for sponsoring my study and facilitating the necessary conditions for my study. I would like to thank my colleagues at department of Biochemistry.

My gratitude extends to Armauer Hansson Research Institute (AHRI), Ethiopia for giving me the opportunity to join Biomedical Science Partnership Program (BSPP), and sponsoring my study. I would like to thank the staffs at AHRI for their help during my study.

I am grateful to Swedish International Development Agency (SIDA) for sponsoring my study. I would like to extend especial thanks to Dr Taye Tolera, the former Director for AHRI for facilitating my study.

I would like to thank Adama Referral Hospital and Adama Regional Laboratory for their help during data collection. My special thanks goes to Mr Feleke Belachew, Mr Legesse and Mr Tamene.

Most importantly, I would like to extend my thanks to all mothers who participated in this study without whom this study would not have been realized.

I would like to thank medical residents, obstetricians and midwifes at Adama Referral Hospital for their help during data collection. My special thanks goes to Dr Alemayehu, the former medical director for facilitating data collection.

My gratitude also goes to my supervisor Dr Daniel Seifu, who was helpful to me in various aspects of my study.

I would like to thank my supervisor Professor Stefan R. Hansson, Lund University, Sweden, who was helpful from the beginning to the end.

My thanks also go to Professor Stefan Hansson's team specifically Dr Lena Erlandsson and Eva Hansson at Lund University for their help and for training me in molecular techniques during my time at Lund University.

I would like to thank Dr Markos Abebe and Dr Sisay Teklu for being helpful during my study.

I would like to extend our thanks to CoLaboratory group for developing database for our study and providing us access to it.

Signature page

Examining Board

Name Signature Date

Members of Examining Board

Examiner: Birggita Essen (MD, PhD, Professor) _____ _____

Examiner: Shiferaw (MD) _____ _____

Examiner: N.Gnanassekaran (PhD) _____ _____

Supervisors:

Daniel Seifu (PhD) _____ _____

Stefan Hansson, MD, PhD, Professor _____ _____

_____

Maria Degef, PhD, Chair of the Department of Biochemistry

Statement of declaration

I hereby declare that the dissertation contains original research works.

_____

Tsehayneh Kelemu

Dedicated to:

This work is dedicated to my wife Bosena Mihret for being by my side all the time, our sons Engineer Muluken Tsehayneh and Dr Fikru Tsehayneh for being supportive from beginning to the end and our grandkids Hiruy Muluken, Amen Muluken, Liyuwork Fikru, Eldana Fikru and Hamelet Muluken for being the source of joy to the whole family.

Table of contents

List of publications

- Kelemu, T., et al. Polymorphism in killer cell immunoglobulin-like receptors and human leukocyte antigen-c and predisposition to preeclampsia in Ethiopian pregnant women population. *J Reprod Immunol*, 2020. **141**: p. 103169.

- Kelemu, T. et al. Association of Maternal Regulatory Single Nucleotide Polymorphic CD99 Genotype with Preeclampsia in Pregnancies Carrying Male Fetuses in Ethiopian Women. *Int. J. Mol. Sci.* **2020**, *21(16)*, 5837.

List of figures **Page**

List of tables **Page**

Abbreviations

cAMP: cyclic adenosine monophosphate

CSF: colony stimulating factor

CD: cluster of differentiation

DC: dendritic cell

dNTP: deoxy nucleoside triphosphate

DNA: deoxyribonucleic acid

EVT: extra villous trophoblast

EDTA: ethylene diamine tetra acetic acid

FGR: fetal growth restriction

G-CSF: granulocyte colony stimulating factor

GM-CSF: granulocyte macrophage colony stimulating factor

HLA: human leukocyte antigen

HELLP: hemolysis elevated liver enzyme low platelet count

IL: interleukin

ISSHP: international society for the study of hypertension in pregnancy

IUGR: intra uterine growth retardation

KIR: killer cell immunoglobulin-like receptor

PCR-polymerase chain reaction

PE- preeclampsia

qPCR- quantitative polymerase chain reaction

SNP- single nucleotide polymorphism

SPSS- statistical package for social science

Treg- T regulatory

Th- T helper

uNK- uterine natural killer

VEGF-vascular endothelial growth factor

CHAPTER ONE: INTRODUCTION

1.1. Preeclampsia

Maternal mortality is a global health burden. The United Nations set a target of reducing maternal death by 75% between 1990 and 2015 (MDG 5). The maternal mortality decreased from 376,034 to 292,982 between 1990 and 2013. In 2013, more than 99% of deaths occurred in developing countries and pregnancy-induced hypertension was the third leading cause of maternal death that accounted for 29,275 deaths worldwide. In Ethiopia, the maternal mortality ratio decreased by about 70% between1990 and 2013 (Kassebaum et al., 2014). Pregnancy related complications remain the major cause of maternal death where 99% occur in low middle-income countries (Duley, 2009). According to Hogan et al., the sub-Saharan African countries including Ethiopia are the most affected by maternal mortality. Ethiopia is one the six countries in the world contributed for more than 50% maternal deaths (Hogan et al., 2010). Globally, up to 15% of deaths are attributed to preeclampsia (PE) that affects 2 to 8% of all pregnancies (Duley, 2009). In Ethiopia, PE affects between 2.2-8.4% of pregnancies (Vata et al., 2015, Tessema et al., 2015). Preeclampsia is a pregnancy related syndrome that usually appears in the second half of pregnancy and is manifested by gestational hypertension accompanied with proteinuria or evidence of other maternal organ and/or utero-placental dysfunction (Brown et al., 2018). Globally, PE is the cause of death for more than 70, 000 mothers and 500, 000 fetuses and neonates (Brown et al., 2018) and majority of deaths occur in low and middle income countries (Firoz et al., 2011).

Phenotypically, PE can be classified based on period of onset and severity of the disorder. Based on period of onset, it can be classified as early onset if diagnosed/delivery before 34 weeks of gestation and late onset if diagnosed/delivery at 34 weeks of gestation and above

(Aneman et al., 2020). Early onset PE is severe, more recurrent (Redman and Sargent, 2005) and it is associated with adverse perinatal outcomes such as growth restriction (Aneman et al., 2020) compared to late onset PE (van Esch et al., 2017) which is associated with maternal endothelial dysfunction (Aneman et al., 2020). Preeclampsia has consequences for both the mother and the fetus. For instance, PE complicates maternal organs such as cardiovascular system, nervous system, hematological system, liver, and kidney which may progress to eclampsia and death while it causes intra uterine growth restriction (IUGR) and still birth and 15% of premature birth (iatrogenic) due to lack of treatment. Currently, there is no treatment to prevent development of PE. However, acetylsalicylic acid (ASA) administration from the first trimester, is recommended as a prophylactic drug preventing thrombosis in the placenta and thereby reduced risk of developing PE. The reduction in the incidence of preterm PE was showed by ASPRE multicenter study of ASA administration in pregnant women with risk of PE (Rolnik et al., 2017). Moreover, fetal monitoring for possible growth restriction using fetal biometry and frequent ultrasound measurement is recommended (Brown et al., 2018).

1.2. Normal pregnancy

1.2.1. Implantation

The proper nourishment and development of embryo requires implantation of developing embryo to the maternal endometrium (Ashary et al., 2018). The process of implantation requires a cross talk between the developing embryo and the maternal uterus (Ashary et al., 2018). The successful implantation occurs only during the period of window of implantation which requires hormone dependent morphological changes of the endometrium (van Mourik et al., 2009). The uterine epithelium does not allow implantation unless it undergoes embryo receptive changes (Nikas and Aghajanova, 2002). Endometrium regularly cycles between

proliferative and luteal phases (Okada et al., 2018). During luteal phase of menstrual cycle, the uterine endometrium undergoes progesterone induced transformation (Moffett-King, 2002) into a implantation receptive tissue (Okada et al., 2018), a decidua that is infiltrated by the uterine natural killer (uNK) cells, which compose about 70% of the leukocyte population (Loke et al., 1995).

The receptive uterine endometrium is characterized by increased vascularity, increased secretary capacity and pinopodes development (Norwitz et al., 2001). The receptive stage of the uterus occurs in mid-luteal phase 7-10 days after ovulation (Cha et al., 2012). Decidualized cells secrete chemokines that recruit immune cells (Vinketova et al., 2016). The level of progesterone is high during luteal phase through pregnancy period and endometrial response to this hormone is facilitated by binding with nuclear progesterone receptor on stromal cells (Conneely et al., 2002). Progesterone acts via protein kinse A signaling pathway activated by a second messenger cyclic adenosine monophosphate (cAMP) (Vinketova et al., 2016). The decidualization process begins in the upper part of endometrium and transforms elongated fibroblast-like endometrial stromal cells surrounding spiral arteries into rounded morphology in the presence of increased levels of progesterone. The decidualized cells fulfill different functions before development of placenta including, nutrient supply to the embryo, protection of the embryo from maternal immune attack, stimulation of trophoblast growth and invasion (Okada et al., 2018). The decidua cells secrete pro-invasive and anti-invasive factors that bind receptors on invasive embryonic cells (Sharma et al., 2016). These factors employ various signaling pathways such as mitogen-activated protein kinase (MAPK), janus kinase (JAK), extracellular-signal-regulated kinase (ERK), cAMP and protein kinase B (AKT) pathways that ultimately activate signal transducer and activator of transcription (STAT) in controlling the invasion process (Sharma et al., 2016) (figure 1.3.1.1).

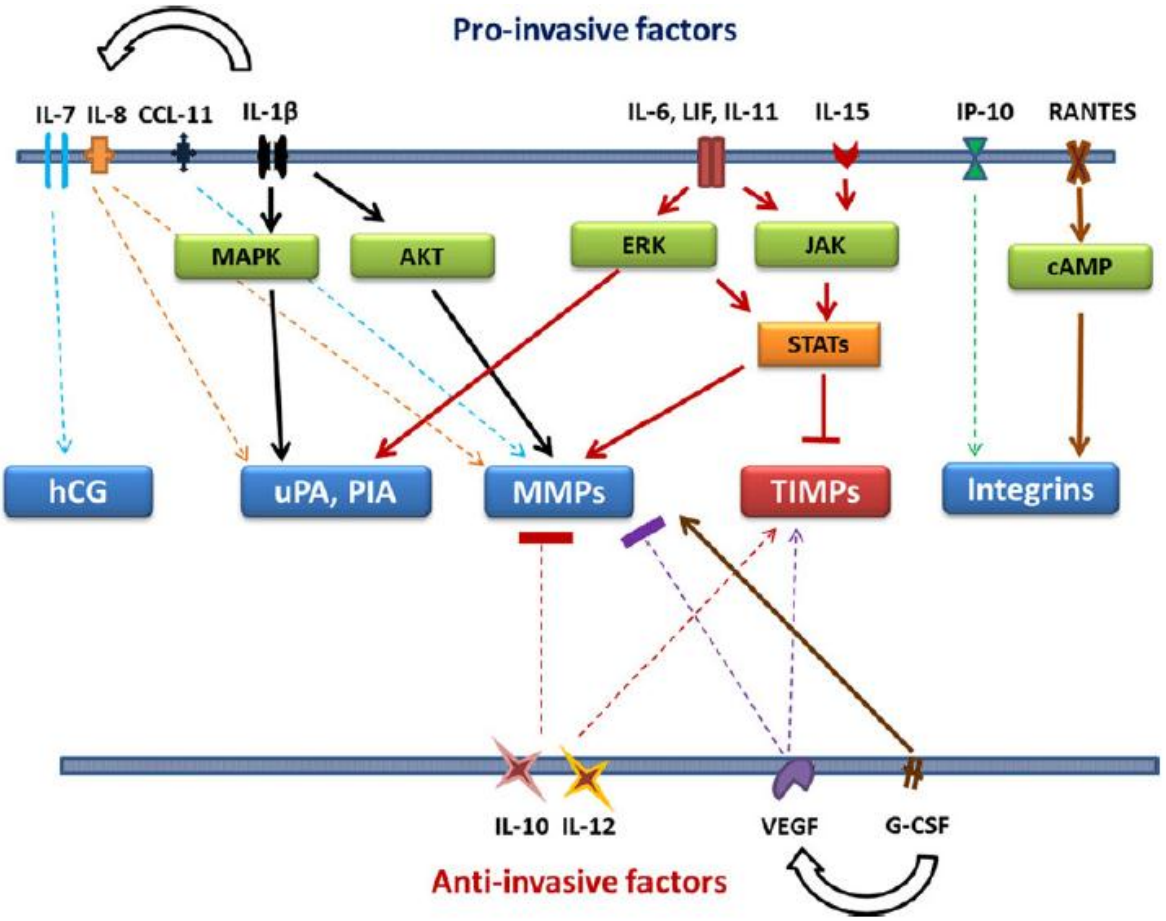


Figure 1.3.1.1. Decidual signaling molecules involved in invasive process of embryonic cells (Adapted from Sharma et al., 2016, American Journal of Reproductive Immunology). Matrix metalloproteinases (MMPs), Tissue inhibitors of metalloproteinases (TIMPs), urikase plasminogen activator (uPA), plasminogen inhibitor activator (PIA), human chorionic gonadotropin (hCG), mitogen-activated protein kinase (MAPK), janus kinase (JAK), extracellular-signal-regulated kinase (ERK), cAMP and protein kinase B (AKT), signal tranducer and activator of transcription (STAT), vascular endothelial growth factor (VEGF), granulocyte-colony stimulating factor (G-CSF).

Endometrial transformation for embryo receptivity involves the role of cytokines, hormones and growth factors (Singh et al., 2011). Within 24 to 48 hours of ovulation, fertilization takes place and the fertilized ovum undergoes rounds of cleavages until it forms morula, which passes through the fallopian tube and then enters uterine cavity where it is transformed into blastocyst with protective outer layer, zona pellucida (Red-Horse et al., 2004) (figure 1.3.1.2).

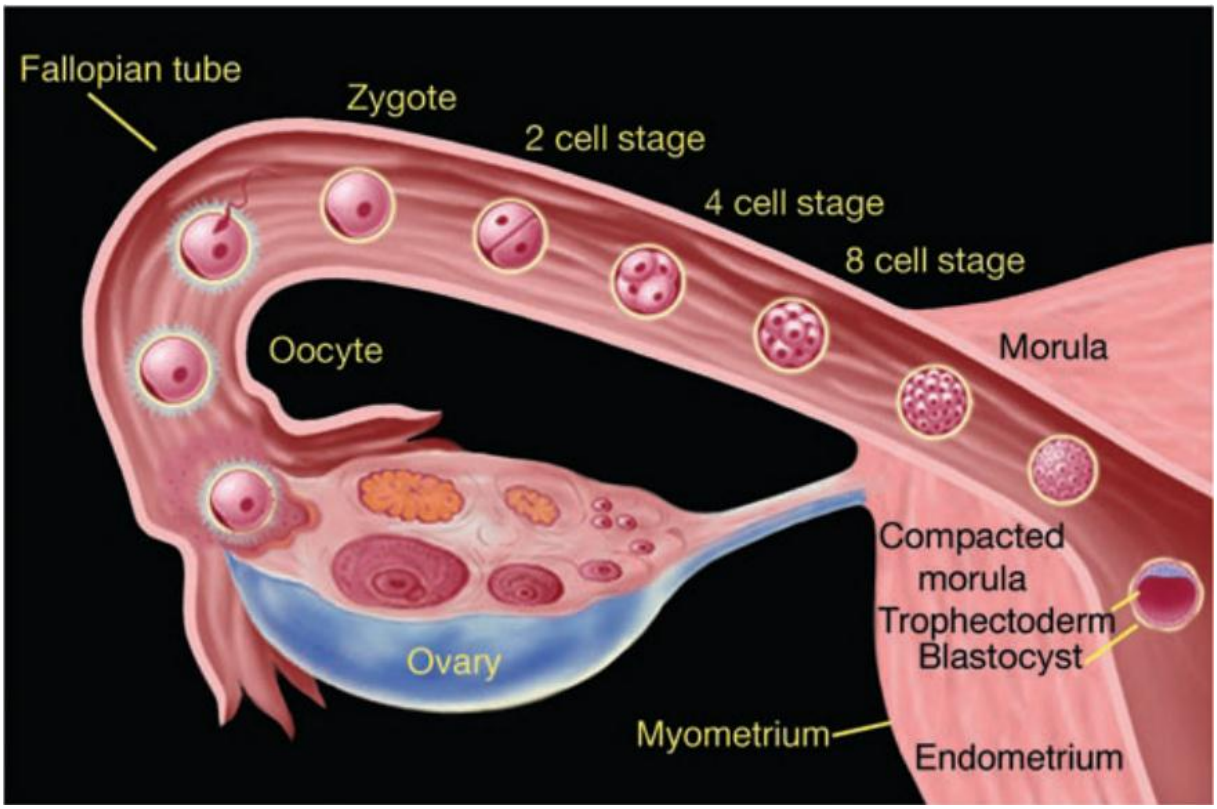


Figure 1.3.1.2. Blastocyst formation in humans (Adapted from Red-Horse et al., 2004, The Journal of Clinical Investigation)

Until establishment of blood supply, the early embryo is nourished by the nutrient contents of secretions from the uterine tube and uterine glands that include mixtures of amino acids, carbohydrates, lipids and proteins (Salamonsen et al., 2016). To gain access to the maternal nutrient supply the blastocyst should implant to the maternal decidua (van Mourik et al., 2009). The blastocyst emerges out of zona pellucida and implants to the uterine decidua enabling the embryo to sequester within the uterine wall (Red-Horse et al., 2004). Adhesion of blastocyst to endothelial lumen is preceded by shedding of zona pellucida and elongation to filamentous form (Spencer et al., 2004). The blastocyst expresses adhesion molecules and receptors that facilitate subsequent implantation following apposition, adhesion and invasion stages (van Mourik et al., 2009). The success of implantation depends on proper apposition of the embryo to the plantation site on endometrium (Massimiani et al., 2019). The blastocyst first adheres to microprotrusions (pinopodes) on the uterine epithelium then to the stromal

extracellular matrix then the blastocyst invades the endometrium penetrating through luminal epithelium into the stroma (Singh et al., 2011). The apposition of blastocyst to the implantation site is facilitated by the interaction of chemokines on the lumen of endothelium with chemokine receptors on the blastocyst (figure 1.3.1.3).

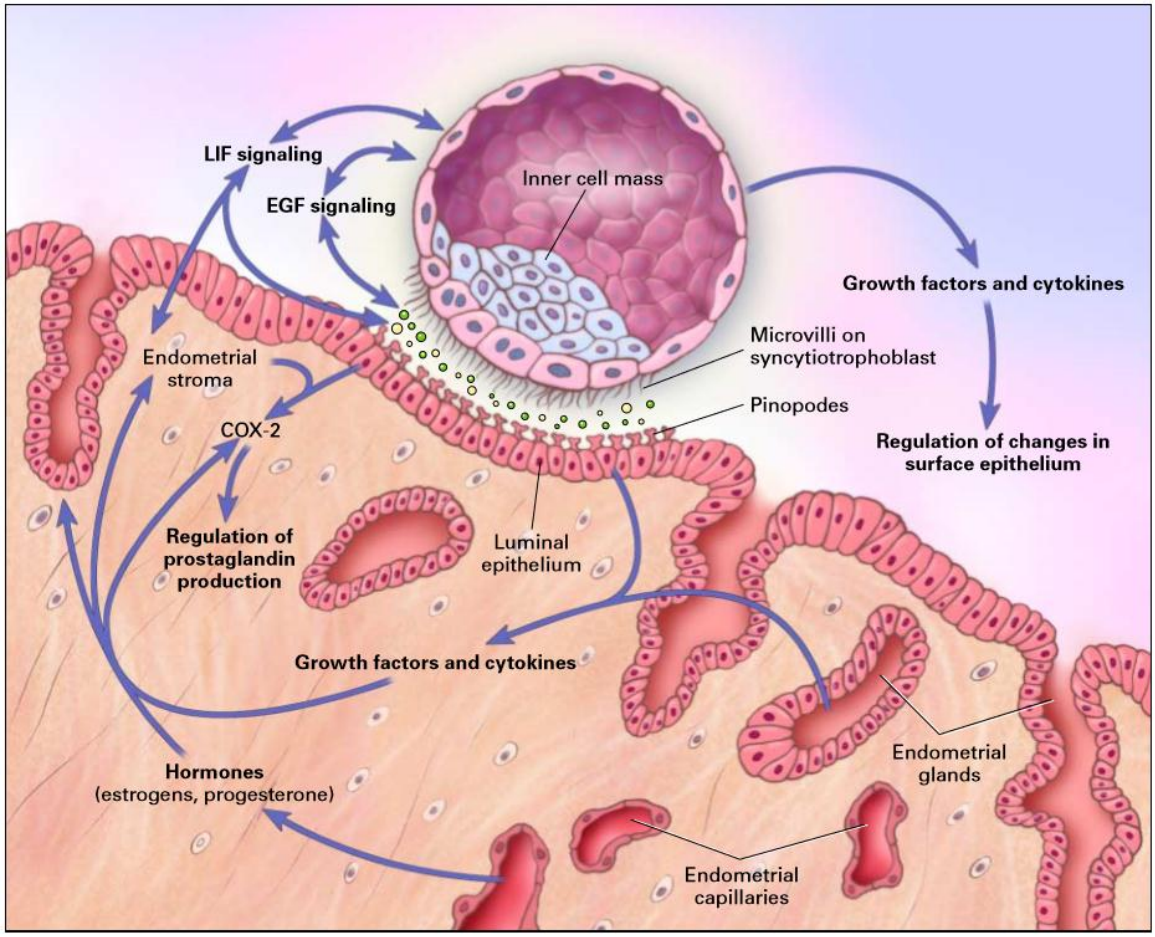


Figure 1.3.1.3. Blastocyst apposition and adhesion (Adapted from Norwitz et al., 2001, The New England Journal of Medicine)

During embryo apposition, endometrium secretes various chemokines including regulated upon activation normal T-cell expressed and secreted (RANTES), interleukin-8 (IL-8) and monocyte chemotactic protein (MCP)-1 on endometrium (Caballero-Campo et al., 2002) that bind to receptors on blastocyst including C-C chemokine receptor-5 (CCR5), CXCR1, and C-C chemokine receptor (CCR)-2B (Dominguez et al., 2003) respectively. The adhesion molecule L-selectin expressed on blastocyst plays important role in apposition of blastocyst to

the implantation site. It binds to oligosaccharide ligands on luminal epithelium (Dominguez et al., 2005). These chemokines play role as pro-inflammatory and chemotactic agents and facilitate leukocyte infiltration to implantation site on endometrium (Mikolajczyk et al., 2016, Baggiolini and Clark-Lewis, 1992, Deshmane et al., 2009). The leukocytes undergo transendothelial migration to the endothelial stroma involving adhesion molecules on leukocytes such as L-Selectin (Wedepohl et al., 2012) (figure 1.3.1.4).

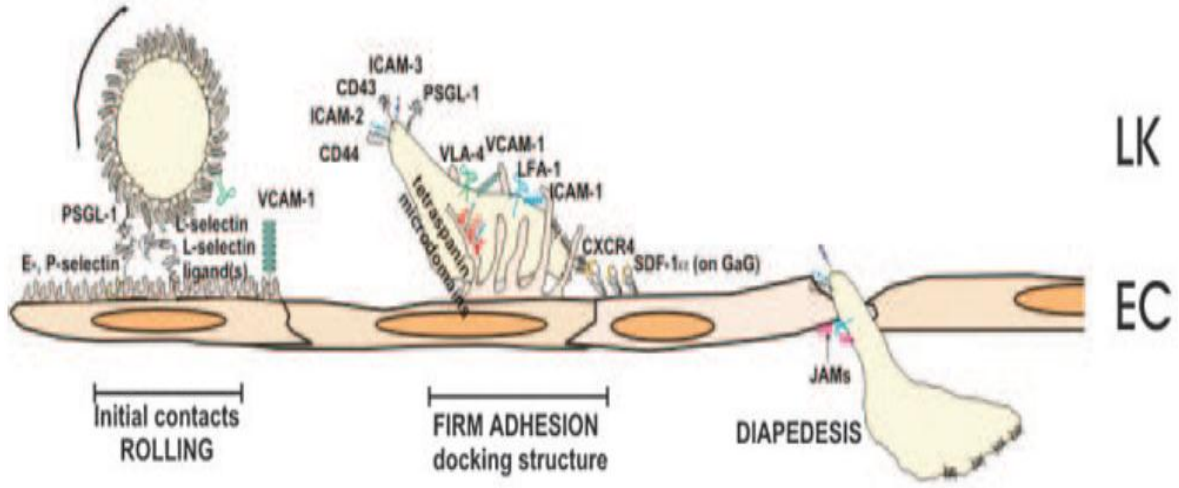


Figure 1.3.1.4. Leukocyte transmigration across uterine endothelium (Adapted from Dominguez et al., 2005, The FASEB Journal. Leukocyte (LK), Endothelial cell (EC).

During uterine receptive phase of the menstrual cycle (Nikas and Makrigiannakis, 2003), the luminal endometrium undergoes progesterone regulated morphological changes to structures known as pinopodes (Stavreus-Evers et al., 2001). The pinopodes are formed by temporary fusion of endometrial epithelial microvilli (Nikas and Aghajanova, 2002). The pinopode expresses adhesion molecules that facilitate interaction of blastocyst with endometrium (Massimiani et al., 2019). Then, blastocyst penetrates the endometrial epithelium and invades into the stroma assisted by metalloproteinases (Dominguez et al., 2005) (figure 1.3.1.5).

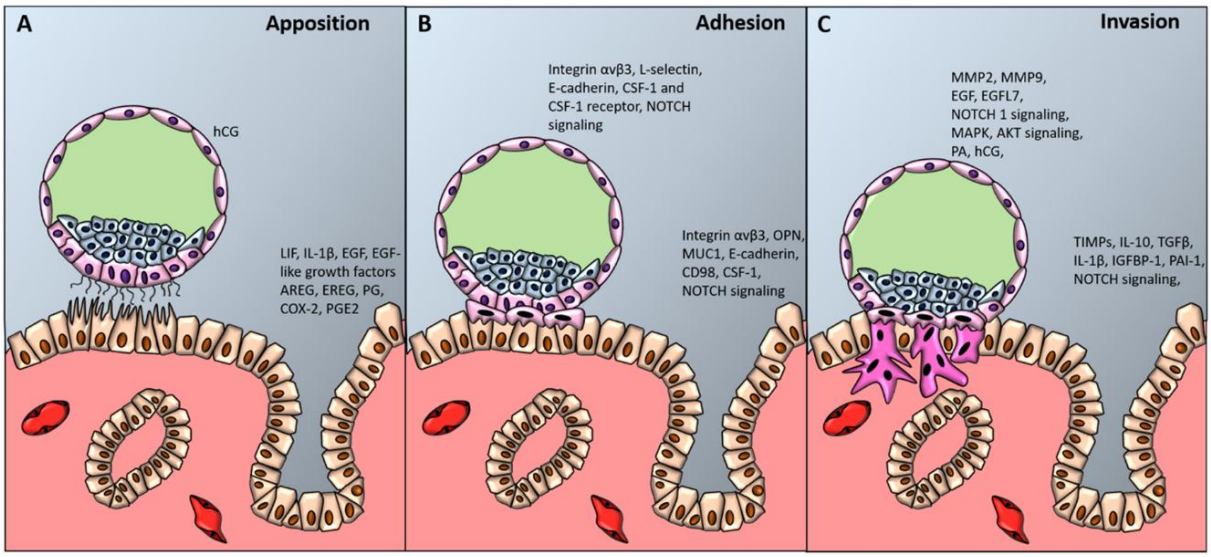


Figure 1.3.1.5. Implantation of embryo to the uterine endothelium (Adapted from Massimiami 2019, International Journal of Molecular Sciences)

The inner part of blastocyst constitutes embryonic cell mass while the outer part constitutes trophectoderm that differentiates into trophoblast cells that compose the placenta (Soares et al., 2018).

1.2.2. Placentation

The proper development of placenta is crucial for promoting the health of fetus and the mother (Knofler et al., 2019). Defect in placental development contributes to pregnancy complications such as PE (Fisher, 2015), miscarriage (Khong et al., 1987), IUGR and stillbirth (Freedman et al., 2019).

Placenta is a temporary organ that facilitates exchange of nutrient and oxygen from the mother to the developing fetus and clearance of fetal wastes (Knofler et al., 2019). Moreover, placenta serves as endocrine organ which produces various hormones needed for metabolic and physiological adaptations to pregnancy (Napso et al., 2018), growth of the fetus (Velegrakis et al., 2017) and protection of the fetus from maternal immune rejection (Mastorakos and Ilias, 2003). The proper fetal growth requires the adaptation of the maternal

systems including cardiac, pulmonary, immune and metabolic to pregnancy (Napso et al., 2018). Placental development starts following implantation of trophectoderm to uterine epithelium in 6-7 days (Knofler et al., 2019) and completely embedded in uterine wall 10 days after conception (van Mourik et al., 2009) (figure 1.3.2.1). The early placenta development and angiogenesis takes place under hypoxic condition that upregulates expression of vascular endothelial growth factor (VEGF) by uterine epithelial cells and decidual macrophages (Wheeler et al., 1995). Macrophages constitute about 20% of the decidual leukocyte population (Loke et al., 1995).

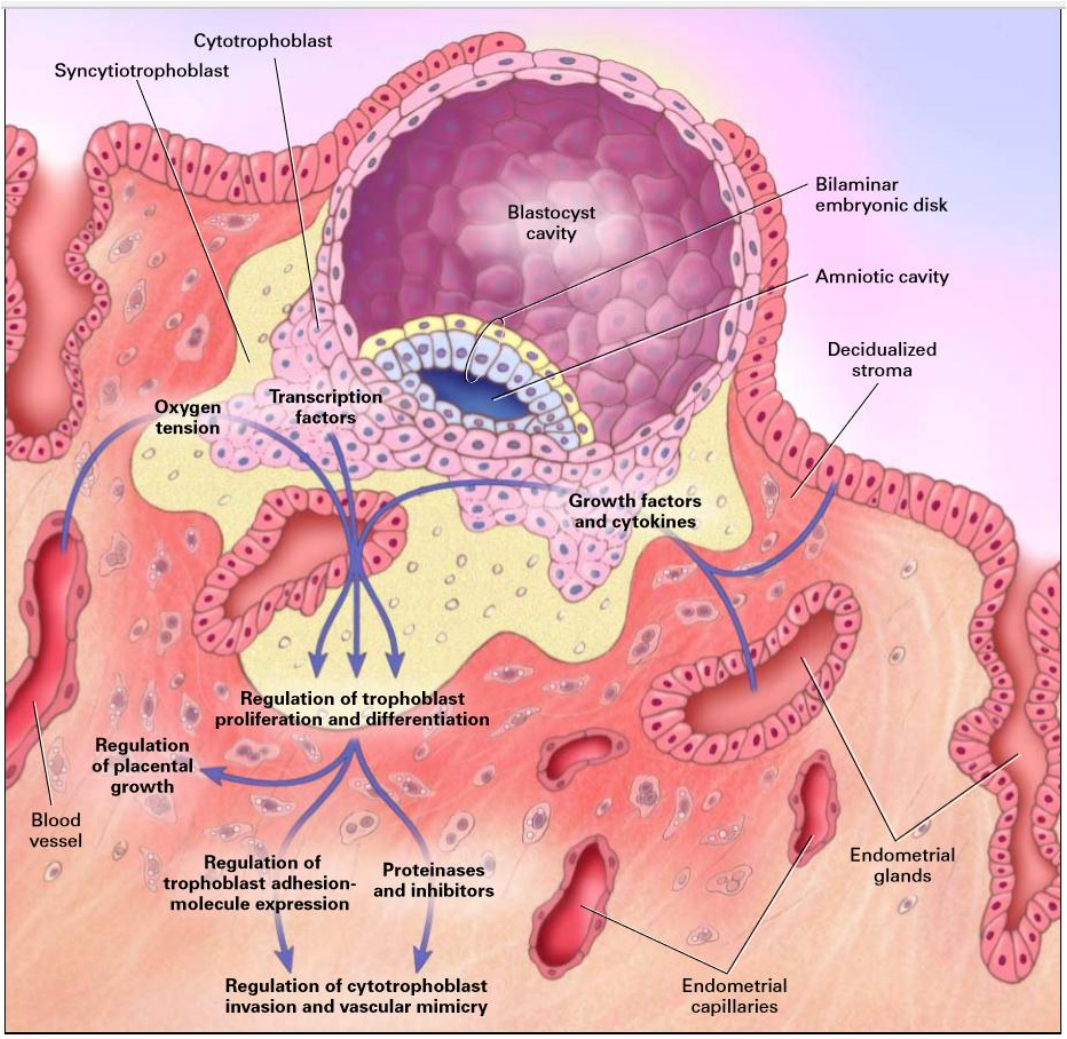


Figure 1.3.2.1. Implantation of blastocyst (Adapted from Norwitz et al., 2001, The New England Journal of Medicine)

The trophectoderm differentiates into villous trophoblast that cover villous tree and extra-villous trophoblast (EVT) cells that migrate into uterine decidua (Apps et al., 2009). The invasion of EVTs is facilitated by production of matrix metalloproteinases that degradation extracellular matrix whose further action is counter balanced by metalloproteinase inhibitors produced by decidua cells (Lala and Graham, 1990). The inner layer of placental villi is composed of cytotrophoblast, covered by syncytiotrophoblasts outer part while the core of villi contains fetal blood vessels (Moffett-King, 2002). The placenta attaches to the uterine wall with the help of EVTs followed by vascularization of the placental villi that establishes feto-maternal circulation (Chang et al., 2018). The uterine spiral artery delivers maternal blood to the intervillous space, which bathes the placental villi (Moffett-King, 2002).

1.2.3. Pregnancy immunology

From immunology point of view, pregnancy is considered as paradox phenomenon since genetically distinct mother and the fetus coexist peacefully (Poole and Claman, 2004). The maternal and fetal factors play role in creating immunotolerant environment that is protective of the semi-allogenic fetus form attack by the maternal immune system (Poole and Claman, 2004). The implantation of semi-allogenic fetus requires protection from maternal immune system by creating immunotolerant uterine environment. This is effected by increase in cells that secrete immunosuppressive cytokines and reduce secretion of inflammatory cytokines including T regulatory (Treg) cells and uterine natural killer cells (Massimiani et al., 2019).

During early pregnancy, the immune cells including uNK cells, Treg cells and dendritic cells (DCs) infiltrate the uterine decidua (Mor et al., 2011) and modulate immune responses (Aneman et al., 2020). The macrophages are tissue derivatives of peripheral monocytes that constitute second most abundant leukocytes in endometrial stroma (Ning et al., 2016). They secrete molecules that play role in immunomodulation during normal pregnancy and pro-inflammatory role in pathologic pregnancy depending on their phenotype (Ning et al., 2016).

The macrophage phenotype proportion shifts in different stages of pregnancy. The pro-inflammatory M1 macrophages become predominant in uterine decidua during the embryo implantation and placentation stages and the proportion shifts to mixture of pro-inflammatory M1 and immunomodulatory M2 macrophages during trophoblast invasion stage. Once the feto-placental is established, the M2 phenotype becomes predominant for the remaining period of pregnancy (Ning et al., 2016) (figure 1.3.3.1).

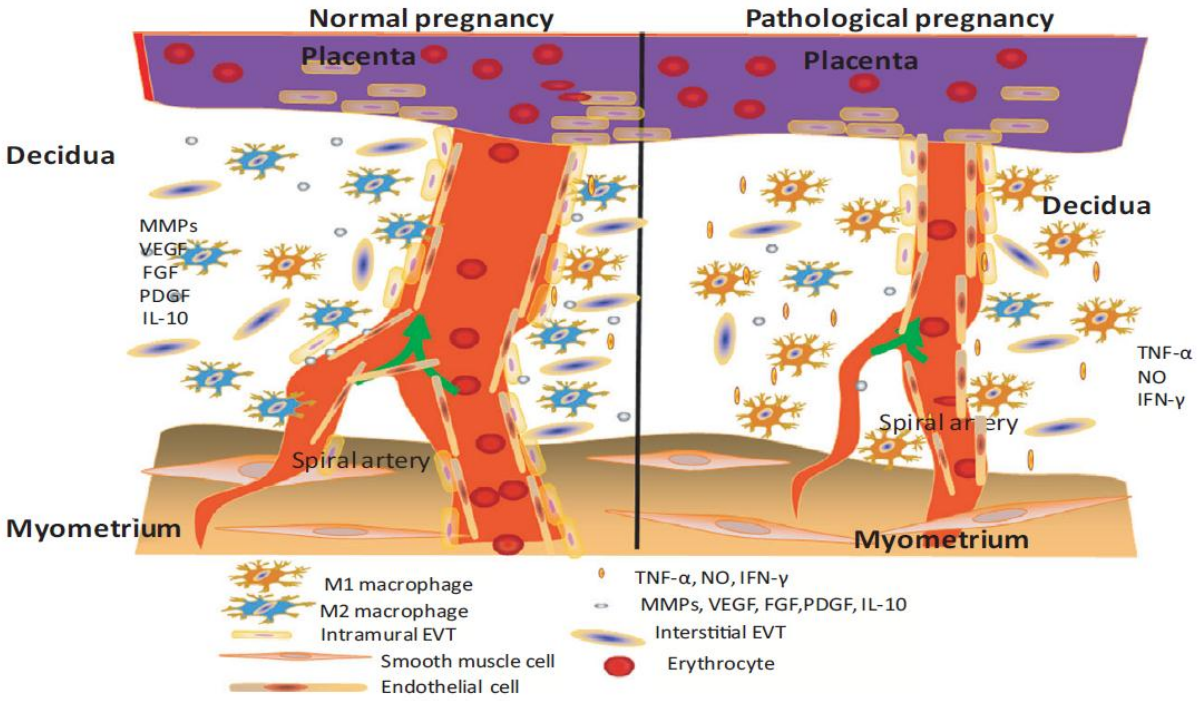


Figure 1.3.3.1. Macrophages in normal and pathologic pregnancy (Adapted from Ning 2016, Am J Reprod Immunol)

The DCs coordinate immunoregulation (Miyazaki et al., 2003) by orchestrating the differentiation of progenitor cell into T helper 1 (Th1), T helper 2 (Th2) and Treg cells (Saito et al., 2010). The decidual DCs are important players in success of embryo implantation on the receptive uterus and their number increases during the implantation (Saito et al., 2010). In normal pregnancy, the implantation stage constitute inflammatory phases with increased pro-inflammatory Th1 cytokine response (Mor et al., 2011). Normal pregnancy is considered as Th2 state (Mor et al., 2011). The Th2 immunosuppressive role is necessary for maintenance

of pregnancy (Granot et al., 2012). The decidual DCs promote Th2 response by promoting a transition from Th1 inflammatory response to Th2 anti-inflammatory response by facilitating differentiation of naïve CD4+ T cells into Th2 cells (Miyazaki et al., 2003). The predominance of Th2 response contributes to the tolerance against rejection of the allogenic fetus by the maternal immune system in normal pregnancy while Th1 response dominates in pathologic pregnancy such as PE (Saito et al., 2010).

The uNK cells express a range of receptors that bind with ligands expressed by trophoblasts and secrete cytokines that regulate trophoblast invasion (Moffett-King, 2002). The uNK cells secrete cytokines such as granulocyte macrophage colony stimulating factor (GM-CSF) and colony stimulating factor (CSF)-1 that promote trophoblast proliferation and differentiation (Jokhi et al., 1994, Jokhi et al., 1995) as well as leukemia inhibitory factor (LIF) that promote trophoblast invasion (Sharkey et al., 1999). Moreover, decidual Treg cells play role in maintenance immune tolerance during early pregnancy by inhibition of uNK cytotoxicity through secretion of ant-inflammatory cytokines such as interleukin-10 (IL-10) and hence protection of the trophoblast from maternal immune attack. In addition, Treg cells inhibit Th1 and Th17 cells and hence facilitate trophoblast invasion (Robertson et al., 2018). The association of Tregs deficiency with poor trophoblast invasion was showed in pregnancy complications such as PE and preterm delivery (Steinborn et al., 2012).

1.2.4. Uterine NK cells

Phenotypically, the uNK cells are $CD56^{bright}$ and $CD16^{-}$ cells district from $CD56^{dim}$ $CD16^{bright}$ peripheral circulating NK cells (Hiby et al., 1997). The uNK cells express combinations of killer cell immunoglobulin-like receptors (KIR) with extracellular and intracellular domains encoded by genes arranged in linear array on the long arm of chromosome 19 and are grouped into A and B haplotypes. The A haplotype which contains six inhibitory genes and B haplotype with variable gene content contains both activating and inhibitory genes and

maternal KIR genotype can be either AA, ABB or BB (figure 1.3.4.1). The genes encode activating and inhibitory receptors that interact with ligands expressed on fetal trophoblast cells and activate or inhibit cytokine secretion by uNK cells respectively (Moffett et al., 2015).

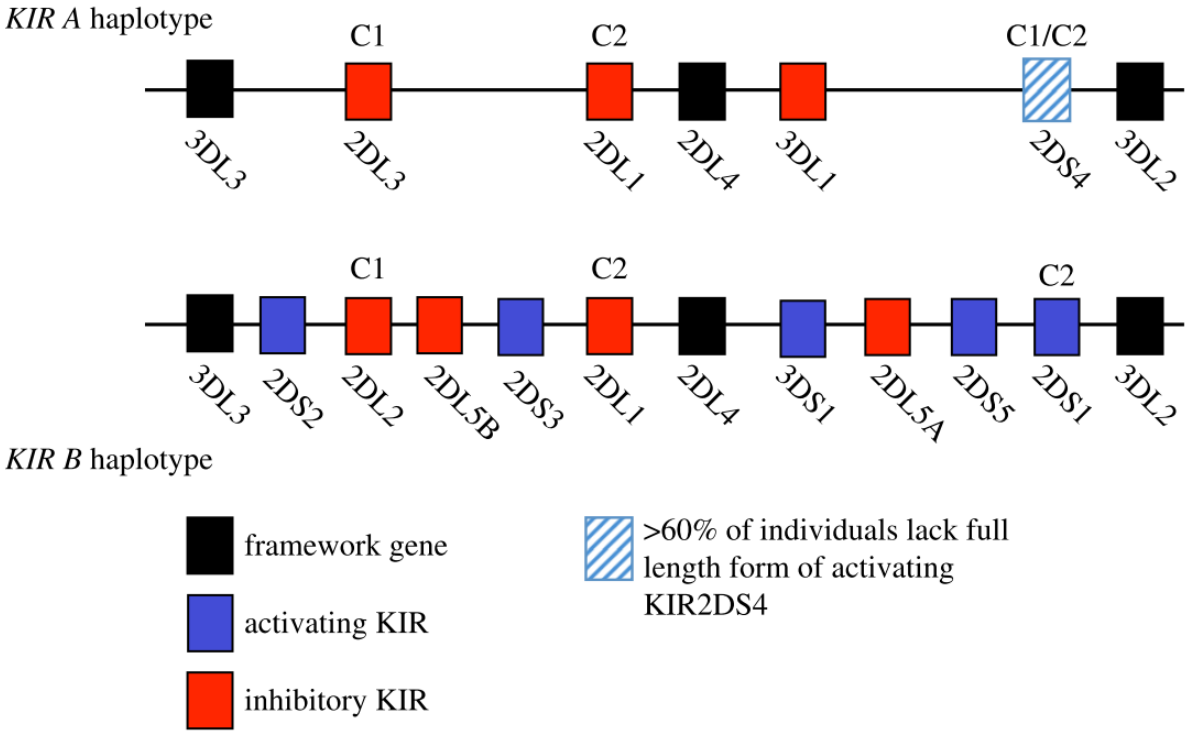


Figure 1.3.4.1. The distribution of KIR genes in A and B haplotypes with HLA-C binding specificities (Adapted from Moffett et al., 2015, Philos Trans R Soc Lond B Biol Sci)

The EVT cells express highly polymorphic human leukocyte antigen (HLA)-C and monomorphic HLA-G but they do not express HLA-A or HLA-B (Apps et al., 2009). Two groups of fetal HLA-C ligands, HLA-C1 and HLA-C2 expressed by trophoblast cells (Parham, 2005) plays key role in normal pregnancy and development of PE by interacting with maternal activating or inhibitory KIR receptors expressed on uNK cell (Redman and Sargent, 2010). Due to highly polymorphic nature of KIR and HLA-C genetic system, the receptor-ligand combination in each pregnancy would likely be different (Hiby et al., 2014). The C1 interacts with inhibitory receptors while C2 interacts with both activating and inhibitory KIR receptors (Moffett et al., 2015) (figure 1.3.4.2). The combination of maternal

KIRAA with fetal HLA-C2 of paternal origin results in pregnancy complications such as PE due to strong inhibition of uNK cells (Hiby et al., 2014) whereas combination with activating KIR is protective against pregnancy disorders (Hiby et al., 2010).

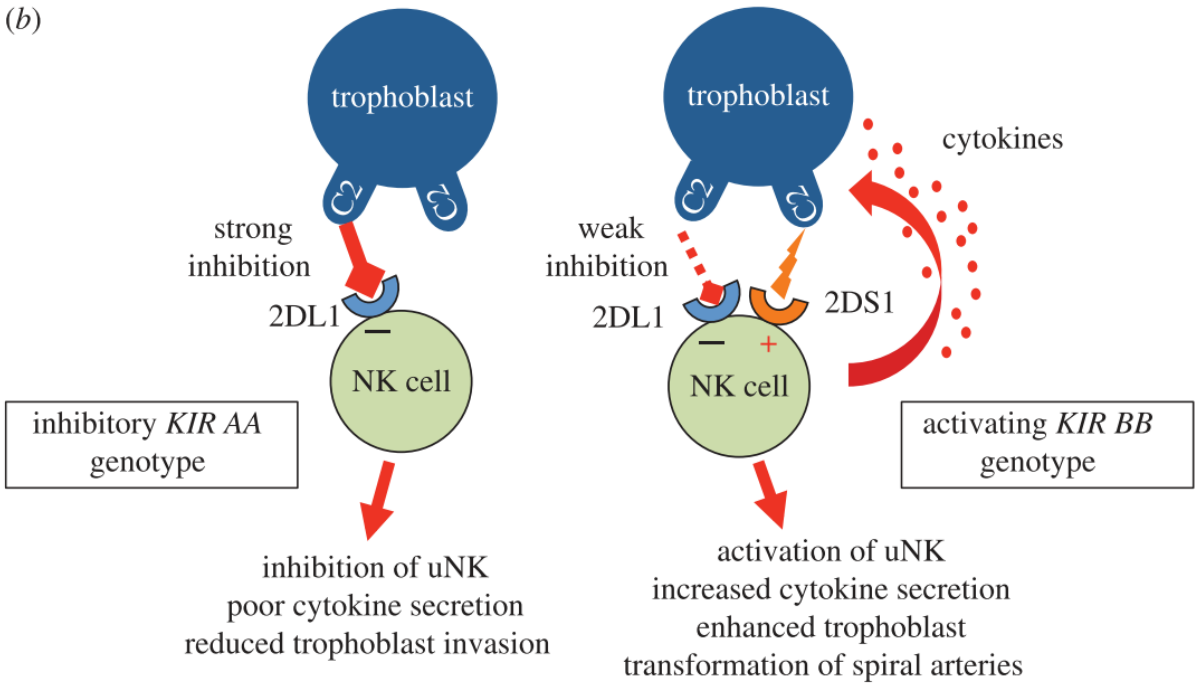


Figure 1.3.4.2. KIR-HLA-C interaction (Adapted from Moffett et al., 2015, Philos Trans R Soc Lond B Biol Sci)

The HLA-G plays a role in immune-regulation, it is highly expressed in the cytoplasm and in the membrane of EVTs cells protecting the fetus from maternal immune attack (Djurisic and Hviid, 2014). The HLA-G binds to inhibitory KIR2DL4 receptor on uNK cells (Rajagopalan and Long, 2012). The HLA-G expressed on trophoblast cells inhibits cytotoxicity of uNK cells and increases secretion of cytokines that promote angiogenesis (Gong et al., 2017, van der Meer et al., 2004).

The EVTs cells invade into uterine decidua (Soares et al., 2018) and the upper part of myometrium where they interact with uNK cells (Moffett et al., 2015). The invasion of trophoblasts is promoted by secretion of cytokines from activated uNK cells (Moffett et al., 2015). The KIRs expressed on uNK cells interaction with HLA-C ligands expressed on

trophoblasts followed by secretion of cytokines that stimulate proliferation and differentiation of trophoblasts cells (Veenstra van Nieuwenhoven et al., 2003). The trophoblast cells express receptors for cytokines secreted by uNK cells (Loke et al., 1995) and play role in remodeling of the spiral arteries (Moffett et al., 2015) (refer figure 9). In normal human pregnancy, proper perfusion of the feto-placental unit is ensured by remodeling of the uterine spiral arteries into wider diameter vessels with increased blood flow (Moffett et al., 2015) (figure 10). In pregnancies complicated by conditions such as preeclampsia, trophoblast interaction with uNK cells has inhibitory effect resulting in the shallow invasion of trophoblasts and defective uterine spiral arteries remodeling with decreased blood flow to the feto-placental unit (Moffett-King, 2002). Inadequate uterine artery transformation in preeclampsia causes IUGR and stillbirth of the fetus (Moffett-King, 2002). Poor trophoblast invasion and defective spiral artery remodeling also causes recurrent miscarriage (Hiby et al., 2010) (figure 1.3.4.3).

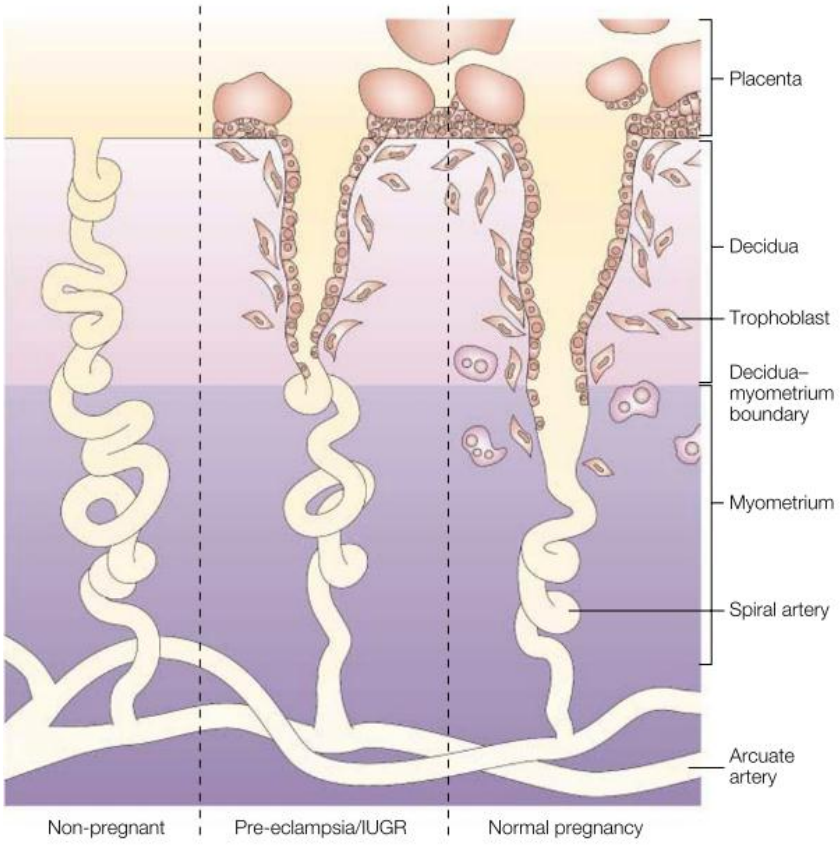


Figure 1.3.4.3. Spiral Artery remodeling in normal and complicated pregnancies (Adapted from Moffett-King, 2002, Nature Publishing Group)

1.2.5. Placental insufficiency

The placenta, an organ that develops from extraembryonic cells serves various functions that promotes the growth and development of the fetus. It supplies nutrients and oxygen to the developing fetus (Gude et al., 2004). The defective placental development results in fetal morbidity and mortality (Lo et al., 2018). The placental insufficiency results from chronic placental hypoxia (Thompson et al., 2016). The placental insufficiency due to impaired feto-maternal circulation results in oxygen deficient uterine environment with a risk of IUGR (Lo et al., 2018). Placental insufficiency causes pregnancy complications such as IUGR (Burton and Jauniaux, 2018) and preterm births (Redline, 2008). The placental insufficiency is affected by maternal ethnicity where Afro-Caribbean women showed the highest placental insufficiency (Audette et al., 2018).

1.3. Etiology of PE

Preeclampsia is a disease of unknown etiology (Dekker and Sibai, 1998). The placental ischemia was proposed to play role in the etiology PE (Page, 1948) that leads widespread endothelial dysfunction associated with maternal syndrome (Redman, 1991). In the updated model, PE develops in two stages. In stage 1 inflammatory factors are released into the maternal circulation from oxidatively stressed placental syncytiotrophoblast resulting from defective spiral artery remodeling and/or larger placenta (Redman, 1991, Staff, 2019). This may lead to early onset PE with IUGR or progress to stage 2 characterized by generalized vascular inflammation resulting in late onset with maternal signs such as hypertension and proteinuria (Staff, 2019).

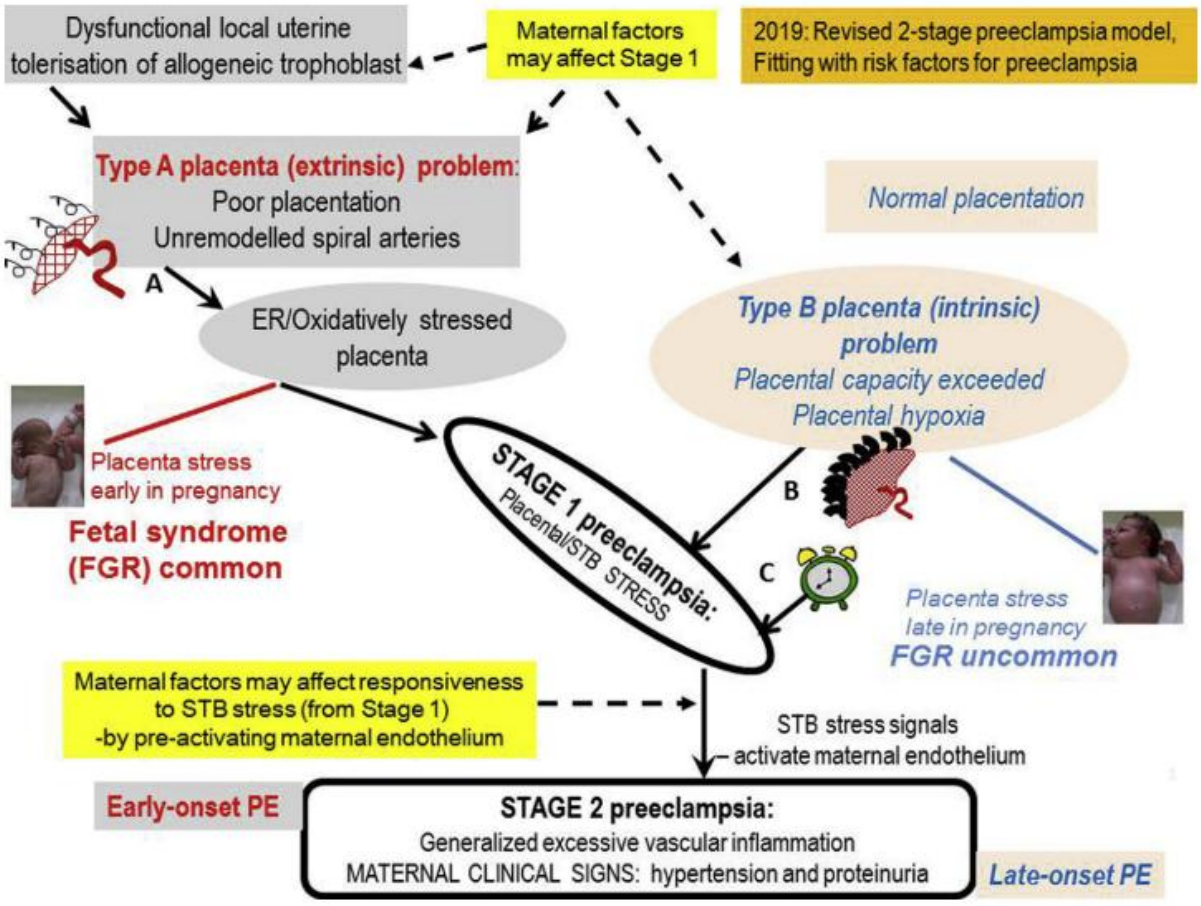


Figure 1.4.1. Modified two-stage model in development of preeclampsia (Adapted from Staff 2019, Journal of Reproductive Immunology)

1.4. Fetal sex and PE

Prenatal outcomes might be determined by difference in maternal immune responses in fetal sex dependent fashion (Al-Qaraghouli and Fang, 2017). The intrauterine environment puts differential selection pressure against male fetuses compared to female fetuses. The intrauterine morbidity and motility is higher in males than females (Myatt et al., 2014). The male fetuses are faced by placental environment adverse for their survival and growth (Clifton, 2010). The pro-inflammatory cytokine production is higher in male placenta than female placenta (Muralimanoharan et al., 2013). The neonatal morbidities such as respiratory distress syndrome are also higher in males than females. The small-for-gestation male fetuses are at increased risk of perinatal mortality compared to female fetuses (Voskamp et al., 2020).

The state of pregnancy is a co-existence of the allogeneic fetus with the mother, which necessitates tolerance in terms of immunological response. The lack of tolerance may end up with PE, which may have genetic origin. The lack of tolerance may be exacerbated by pregnancies, which carry male fetus with Y-chromosome genes of paternal origin. The fetal trophoblast cell expresses immunomodulatory molecule HLA-G that inhibits uNK cell cytolytic activity by interacting with surface receptor (Wedenoja et al., 2020). The expression of HLA-G was reduced in PE (Hara et al., 1996). The reduced expression of HLA-G was associated with underrepresentation of male fetuses in PE (Wedenoja et al., 2020).

The correlation between level of CD99 expression in male fetuses and PE was showed in previous study (Masoumi et al., 2017). The CD99 is widely expressed in various types of cells with roles in normal and disease conditions (Pasello et al., 2018). The level of expression of CD99 is regulated by polymorphic regulatory region SNP rs311103 GATA transcription factor binding site. The GATA1, GATA2 and GATA3 bind SNP rs311103G but not

rs311103C and plays role in differentiation of hematopoietic cells. The SNP rs311103C allele abolishes binding of GATA factor resulting in decreased CD99 expression while SNP rs311103G promote GATA binding resulting in increased CD99 expression (Moller et al., 2018, Yeh et al., 2018).

1.5. Clinical manifestations and management of PE

Preeclampsia adversely affects multiple organs of the mother and the developmental process of the fetus since endothelial dysfunction related to hypoperfusion of placenta plays central role in the pathophysiology the syndrome (Baumwell and Karumanchi, 2007). Preeclampsia is associated with clinical and biochemical manifestations such as high blood pressure, kidney dysfunction, liver damage, disorder of central nervous system, hemolysis, elevated liver enzymes, low platelet counts (HELLP), pulmonary edema, stroke, IUGR, placental abruption, preterm birth, neonatal respiratory distress and deaths of the mother and the fetus (Ghulmiyyah and Sibai, 2012, Armaly et al., 2018).

The management of PE endorsed by international society for the study of hypertension in pregnancy (ISSHP) takes following points into consideration: i) severity of hypertension ii) clinical status of the mother iii) developmental status of the fetus. iv) the resource setting of the service and v) gestation age. Accordingly, severe hypertension of $\geq$ 160/110mmHg is managed by urgent oral administration of nifedipine or intravenous administration of labetalol or hydralazine. The ISSHP recommends administration of magnesium sulfate until delivery for all preeclamptic women in low resource setting. In a situation where resource is limiting, administration of magnesium sulfate is recommended for PE women with severe hypertension and proteinuria or predictive signs of convulsion. The ISSHP recommends delivery at 37 weeks gestation for all PE women. In addition, delivery is recommended in PE women with severe hypertension unresponsive to three classes of antihypertensive agents or signs and

symptoms of worsening maternal organ dysfunction or non-reassuring fetal status (Brown et al., 2018).

1.6. Risk factors-ethnicity, hereditary factors for PE

The occurrence of PE shows ethnic variation and women of black race have higher chance of developing PE compared to Hispanic and Asian women (Ghosh et al., 2014). A study showed racial disparities in adverse pregnancy outcomes. The African-American women were at higher risk of having PE and intrauterine death compared to Hispanic women (Shahul et al., 2015). The risk of acquiring PE increases with: maternal age of 40 years and above, nulliparity, history of previous preeclampsia, family history of PE, multiple pregnancy, pre-existing medical conditions (Duckitt and Harrington, 2005). The parental genetic components play a role in susceptibility of PE (Roberts and Cooper, 2001). Preeclampsia may be triggered by maternal genes or fetal genes of maternal or paternal origin although maternal influence is stronger than the fetal (Skjaerven et al., 2005).

1.7. Environmental factors and PE

The maternal exposure to environmental pollutants such as fine environmental particles mainly particulate matter less than 2.5 micrometer size during the early pregnancy is associated with adverse pregnancy outcomes such as PE. The environmental pollutants affect placental function via epigenetic changes such as DNA methylation resulting in FGR. The increased methylation of placental DNA was showed on CpG sites of the promoter area of the investigated genes in pregnant women exposed to environmental particle during first and second trimesters (Cai et al., 2017). The study by Erlandsson et al., showed the increased secretion of inflammatory cytokines IL-6 and decreased secretion of hCG on first trimester trophoblast cells exposed to wood smoke air pollution particles containing polycyclic aromatic hydrocarbons. The study suggested the possible role of particles emitted during

wood burning in the pathogenesis of pregnancy complications such as PE (Erlandsson et al., 2020). In Ethiopian context, about 85% of the population make their living in agriculture and they do not have access for electric city supply. About 85% of the Ethiopian population make their living in agriculture and they do not have access for electric city supply. They depend on wood fire for cooking and are exposed for wood burning smoke.

1.8. Statement of the problem

Preeclampsia is a major health problem that causes mortality and morbidity to the mother and the fetus. The burden of the disease high in low-and-middle income countries. Women of black race are disproportionately affected by the disorder. The risk of developing the condition shows familial tendency. Parental genetic factors were suggested to contribute to the pathogenesis of the disease. Maternal and fetal genetic factors were suggested to paly role in the pathogenesis of the syndrome, the maternal contribution was showed higher. The exaggerated maternal immune response during early pregnancy was showed to play role in the pathogenesis of PE. The maternal uNK cells and fetal trophoblast cells were showed to play role in normal and complicated pregnancies. The uNK cells express set of KIR receptors while fetal trophoblast cells express HLA-C ligands. The KIR genes may encode activating or inhibitory receptors. The women with inhibitory genes are likely affected by PE when combined with fetal HLA-C. The KIR and HLA-C genes show polymorphism from individual to individual and hence receptor and ligand combination varies from pregnancy to pregnancy. Similarly, the HLA-C genes encode two groups of ligands and hence there is individual variation. Some combinations are protective from PE while other combinations are predisposing women for development of PE. In addition, pregnant women carrying male fetus are at increased risk of developing PE compared those carrying female fetus. Accordingly, this study was conducted to investigate whether the maternal and fetal genetic factors are associated with PE.

1.9. Significance of the study

The prenatal outcomes depend on processes that take place during early stages of pregnancy. In some cases, pregnancy may be complicated by pregnancy related disorders one of which is PE. Despite long years of efforts, the etiology of PE is not yet known. There is no curative treatment. However, the consequences can be minimized or avoided based on evidence-based care. Towards this effect, research is being conducted in different corners of the globe to generate data that can help in the stratification of pregnant women at genetic risk of developing PE.

So far, in Ethiopia, there is no molecular level research data regarding PE. Accordingly, the results from this study might in the future be implemented into clinical screening programs in to allow for early risk stratification of pregnant women in order to identify high-risk pregnancies that would benefit from prophylactic treatment with ASA.

1.10. Hypothesis of the study

We hypothesize that Ethiopian pregnant women may be predisposed to PE because of their genetic make-up.

CHAPTER TWO: OBJECTIVES

2.1. General objectives

The general objective of this study was to investigate whether genetic factors paly role in the pathogenesis of PE in a population of Ethiopian pregnant women.

2.2. Specific objectives

- To describe the demographic and clinical characteristics of the Ethiopian pregnant women population.
- To investigate the association of maternal KIR polymorphism with PE in Ethiopian pregnant women.
- To compare polymorphism in KIR genes in Ethiopians with other populations.
- To assess the association of maternal and fetal HLA-C polymorphism with PE in Ethiopian pregnant women.
- To describe the association of maternal SNP rs311103 polymorphism with PE in Ethiopian pregnant women.
- To assess the association of fetal SNP rs311103 polymorphism with PE in Ethiopian pregnant women.

CHAPTER THREE: MATERIALS AND METHODS

3.1. Setting

The different phases of the study were conducted at different institutions. The data and sample collection was done We conducted the study at Adama Regional Referral Hospital located in Oromia Regional State to the South East direction form the capital city of the country of Ethiopia at altitude of 1,712 meters above sea level. The hospital provides health services to the resident of Adama city and the surrounding areas. The hospital provides antenatal clinic follow up and delivery services to the pregnant women. The hospital is center for residency training of medical residents in different disciplines including Obstetrics and Gynecology. Each year, about 5000 delivery services are provided at the hospital. The blood and tissue samples collected at the hospital were processed and stored at Adama Regional Laboratory and the samples that need further processing were transported to Armauer Hanson Research Institute (AHRI). The DNA extraction and paraffin embedding of the placenta were conducted at AHRI. Then, the extracted DNA and paraffin embedded tissue were transported to Lund University. The genotyping experiment was conducted at Lund University, Department of Obstetrics and Gynecology, Faculty of Medicine.

3.2. Study design

A case-control study design was employed for this study. The study participants were residents of Adama city and the surrounding areas.

3.3. Study population

The study population consisted of pregnant women who have appeared to the delivery ward at Adama Regional Hospital during the study period. The recruitment of the study participants was conducted during from December 2016-August 2017. The enrollment of participants was

conducted based on the order of their appearance to the delivery ward during the above specified period. The selection of cases and controls was done by obstetricians and obstetrics and gynecology residents based on the national protocol (Management Protocol on Selected Obstetrics Topics, Federal Democratic Republic of Ethiopia, Ministry of Health, 2010).

3.4. Study participants

The case group pregnant women included in the study were in the age range of 18 to 45 with singleton pregnancy. The gestation age (GA) of the pregnant women was between 23 to 42 weeks. The included control group pregnant women were in the age range of 18 to 39 years with singleton pregnancy. The GA was between 26 to 44 weeks. A total of 288 study participant were included in Study I while we included 241 participants in the study II.

3.4.1. Inclusion criteria

The inclusion of the PE cases and controls was based on the demographic and clinical characteristics. The included PE cases were those pregnant women $\geq$ 18 years of age with singleton pregnancy, GA 20 weeks and above, sBP $\geq$140 mm Hg or dBP $\geq$90 mm Hg, with proteinuria or involvement of utero-placental dysfunction or sighs of maternal organ dysfunction. In the control group, we included pregnant women age 18 years and above women with normal blood pressure and no signs of maternal organ involvement.

3.4.2. Exclusion criteria

Originally, we recruited 345 pregnant women study participants. The pregnant women below the age of 18 years, lack of matching maternal venous blood and cord blood samples, participants without clinical data, samples without identifier for the case or control group, samples with duplicate codes, and multiple pregnancies excluded from the study.

3.5. Sample collection

The ethical issues were considered before data and sample collections. The letter of ethically approval was obtained from department of Medical Biochemistry, College of Health Sciences, Armauer Hanson Research Institute and National Ethics committees.

The sample and data collections were conducted after securing informed consent form the study participants. The information sheet and consent forms were prepared in English and Amharic languages. For those participants who can read and write, the Amharic version of the information sheet was given, and voluntary written informed consent secured. For those participants who cannot read and write, the information was read, and fingerprint was taken as a proof for informed consent.

We used a questionnaire designed for collection of demographic and clinical data. The standard operation procedures were prepared for sample collection and processing. Two rounds of training were given to data collectors about data collection and sample processing. During training, coding system was given special attention in order to match the same participant code with the code for maternal blood, cord blood and placenta samples. The questionnaire was filled by midwifes at Adama Referral Hospital delivery ward using face-to-face interview. The venous blood simple was collected in a test tube containing EDTA. Immediately after delivery cord blood was collected in a tube containing EDTA. The placenta tissue sample was collected in a bucket containing ice. The blood and tissue sample collections were done by trained midwife nurses. The maternal venous blood and cord blood were transported to Adama Regional Laboratory by porters working at delivery ward and then stored at -20^0C by medical laboratory technologist at Adama regional laboratory. The portions of placenta samples were preserved by medical laboratory technologist at Adama Regional Laboratory within about 30 minutes using liquid nitrogen for a brief moment and then

transferred to -80^{0}C freezer. The remaining portions of placenta samples were processed using formalin by trained medical laboratory technologist at Adama Regional Laboratory with in about 30 minutes after collection. The formalin processed placenta samples were transported to AHRI laboratory for further processing on the same day by principal investigator daily shuttling between Addis Ababa and Adama. This continued for about nine months. The stored blood and tissue samples were transported to AHRI laboratory using dry ice.

The blood samples were thawed to room temperature and the deoxyribonucleic acid (DNA) was extracted using QIAGEN DNA Mini Blood kit. The extraction was performed following the steps of cell lysis, DNA binding to the column, wash using wash buffers and DNA elution. The quality of eluted DNA was determined using Nano spectrophotometer and stored at -20^{0}C refrigerator. The genotyping experiment was done abroad in laboratory at Lund University, Sweden. The samples were exported in carbon dioxide ice (dry ice) after securing material transfer agreement from Ministry of Science and Technology, Federal Democratic Republic Ethiopia.

3.6. DNA extraction

The blood samples were thawed to room temperature and the deoxyribonucleic acid (DNA) was extracted using QIAGEN DNA Mini Blood kit. The DNA extraction was done at AHRI using QIAGEN Mini Blood Kit. The extraction was performed following the steps of blood cell lysis, binding of DNA to the column, wash using buffer and elution of the DNA. The maternal venous whole blood and fetal cord blood samples and reagents were thawed to room temperature. Twenty micro liters of Qiagen protease was transferred into 2 mL micro centrifuge tube and mixed with 200 μL of blood sample. Then, 200 μL of AL buffer was added to the mixture, vortexed and incubated at 56^{0}C for 10 minutes and briefly centrifuged. Two hundred micro liters of 96% ethanol was added and vortexed for 15 seconds and

transferred to Qiagen mini spin column. Then, 500 μL of AW1 buffer was added and centrifuged at 6000xg for 1 minute. The column was transferred to a 2 mL collection tube and 500 mL AW2 buffer added and centrifuged at 20,000xg. The column was placed onto a 2 mL collection tube and centrifuged for 1 minute. Finally, the column was placed onto 1.5 mL collection tube and then 200 mL AE buffer was added, incubated for 15 minutes at room temperature and then the extracted DNA harvested by centrifugation at 6000xg for 1 minute. The extracted DNA quality was measured using nano spectrophotometry and stored at -20^{0}C refrigerator at AHRI. The genotyping experiment was done abroad in laboratory at Lund University, Sweden. The samples were exported in dry ice after securing material transfer agreement from Ministry of Science and Technology, Federal Democratic Republic Ethiopia.

3.7. Analysis of KIR and HLA-C polymorphism using polymerase chain reaction (PCR)

The genotyping experiment was conducted at department of Obstetrics and Gynecology, Institute of Health Sciences, Lund University. The DNA extracted from maternal venous blood and cord blood were used for the KIR and HLA-C polymorphism analysis. The traditional polymerase chain reaction (PCR) method was used for genotyping of maternal and fetal genes. The published methods and primers by Martin et al, 2002 were used for genotyping of three inhibitory and two activating maternal KIR genes (Martin et al., 2002). The purchased reagents including four deoxynucleoside triphosphates (dNTPs), magnesium chloride, specific primers, PCR buffer, Q solution and Taq polymerase and DNA extract were used in the preparation of reaction mixture as prescribed by the manufacturer (QIAGEN, Germany). The mixture was subjected to preprogrammed denaturation, primer annealing and synthesis PCR conditions in a thermocycler machine (BIO RAD T100^{TM} Thermal Cycler).

Figure 3.7.1. Thermocycler machine used for PCR experiment

The KIR genotyping was done by agarose gel electrophoresis and autoradiography. The published methods and primers by Hiby et al 2004 were used for genotyping of two groups of maternal and fetal HLA-C (Hiby et al., 2004). The dNTPs, PCR buffers, primers, magnesium chloride, Q solution, Taq polymerase and DNA samples were used for preparation of reaction mixture as described by the manufacture (QIAGEN, Germany). The mixture was allowed to be amplified in preprogrammed thermocycler. The HLA-C genotyping was based data on the gel electrophoresis and gel documentation using autoradiograph. The control samples were run together with analysis samples and the results of analysis interpreted as positive and negative accordingly.

Materials used for PCR: we used extracted genomic DNA, gel electrophoresis apparatus with combs and tape, power supply, standard agarose (SAVEEN WERNER AB), 20x TBE buffer, GelRed nucleic acid stain and 6x loading buffer.

PCR procedure: we amplified fifty nanogram genomic DNA in 20 μL reaction mixture by adding 200 μM dNTP, 200 nM to 1.5mM of specific primers, 2mM MgCl2, 2μL PCR buffer, 4 μL Q solution and 0.5 U Taq polymerase (QIAGEN, Germany). Cycling was carried out in a BIO-RAD T100™ Thermal Cycler according to a previously described protocol (Hiby et al., 2004) programmed to cycle at 96^0C 1 minute for 1 cycle; 96^0C 25 seconds for 4 cycles, 65^0C 45 seconds for 4 cycles and 72^0C 30 seconds for 4 cycles; 96^0C 25 seconds for 26 cycles, 60^0C 45 seconds for 26 cycles and 72^0C 30 seconds for 26 cycles; 96^0C 25 seconds for 7 cycles, 55^0C 1minute for 7 cycles and 72^0C 2 minutes for 7 cycles and 72^0C 10 minutes for 7 cycles. The positive and negative control samples were used together with test samples. PCR products were electrophoresed on 2% agarose gels containing Gel Red Nucleic Acid Stain (BIOTIUM) and visualised under ultraviolet light. The size bands were determined using DNA ladder (GeneRuler 100bp Plus DNA Ladder, Thermo Scientific).

3.8. Analysis of SNP using real time quantitative real time PCR (RTqPCR)

The maternal and fetal DNA samples were used for the SNP analysis. The real time quantitative PCR (RTqPCR) method was used for analysis of SNP of maternal and fetal genotypes. The TagMan genotyping master mix reagents and published primers (Moller et al., 2018) were used for genotyping of three maternal and fetal genotypes including GG, GC and CC. The reaction mixture was prepared in duplicates as described by the reagent manufacturer (Applied Biosystems by Thermo Fisher Scientific, USA). The controls for each of the genotypes were used.

RTqPCR procedure: The DNA was used for SNP rs311103 genotyping based on the primers and methods previously described (Moller et al., 2018) using TaqMan^{TM} Genotyping master mix (Applied Biosystems by Thermo Fisher Scientific, Waltham, MA, USA). Briefly, the maternal and fetal genomic DNA were amplified in a 10 µL real time quantitative polymerase chain reaction containing 1 µL of 10 ng genomic DNA, 5 µL of 2_TaqMan Genotyping master mix, 0.25 µL of 40_ primer assay and 3.75 µL of distilled water. Cycling was carried out in a QuantStudio 3 thermocycler (Applied Biosystems by Thermo Fisher Scientific). Positive controls for the three genotypes, i.e., GG, GC and CC, and a negative control (DNase-free water) were included in each assay, together with test samples. All samples were run in duplicate.

3.9. Data management and statistical analysis

The database developed by CoLaboratory group was used for management of the collected data. The database was used for the management of demographic and clinical data collected using questionnaire (http://pregnancycolab.tghn.org/collect/). The database data and the laboratory analysis data were entered onto the SPSS version 26.0. Data was cleaned before analysis. The continuous and categorical variables were analyzed using the SPSS version 26.0. The tables and graphs were used for reporting the results. The Chi-square value was calculated and $p<0.05$, at 95% confidence interval was used as cut off value to interpret the association of variables. The risk for PE was assessed using odds ratio and confidence interval.

Participant code and data safety: Every participant was identified by a specific code. The PE cases and controls were given different identifier code. The maternal and fetal matching samples were given the same code number with a letter suffix that identifies the maternal from the fetal. The sequential coding system was used. The same code was used throughout the

study. The data was managed by the principal investigator using personal laptop with password which has no access for other individuals.

Quality control considerations: the standard operation procedure was prepared and applied during data collection, sample collection, sample processing, sample transport and storage. The quality of data and sample collections, timely transport and processing were secured by daily supervision by the principal investigator at the study site. The head midwife at delivery ward was assigned to supervise the daily data and sample collection process. The principal investigator made verbal communications with head nurse and data collectors when ever inconsistency was observed. The same done at Adama Regional laboratory. The quality of extracted DNA was assessed using nanospectrophotometer (Nano Drop ND 1000 Spectrophotometer) at 260/280 nm wavelength.

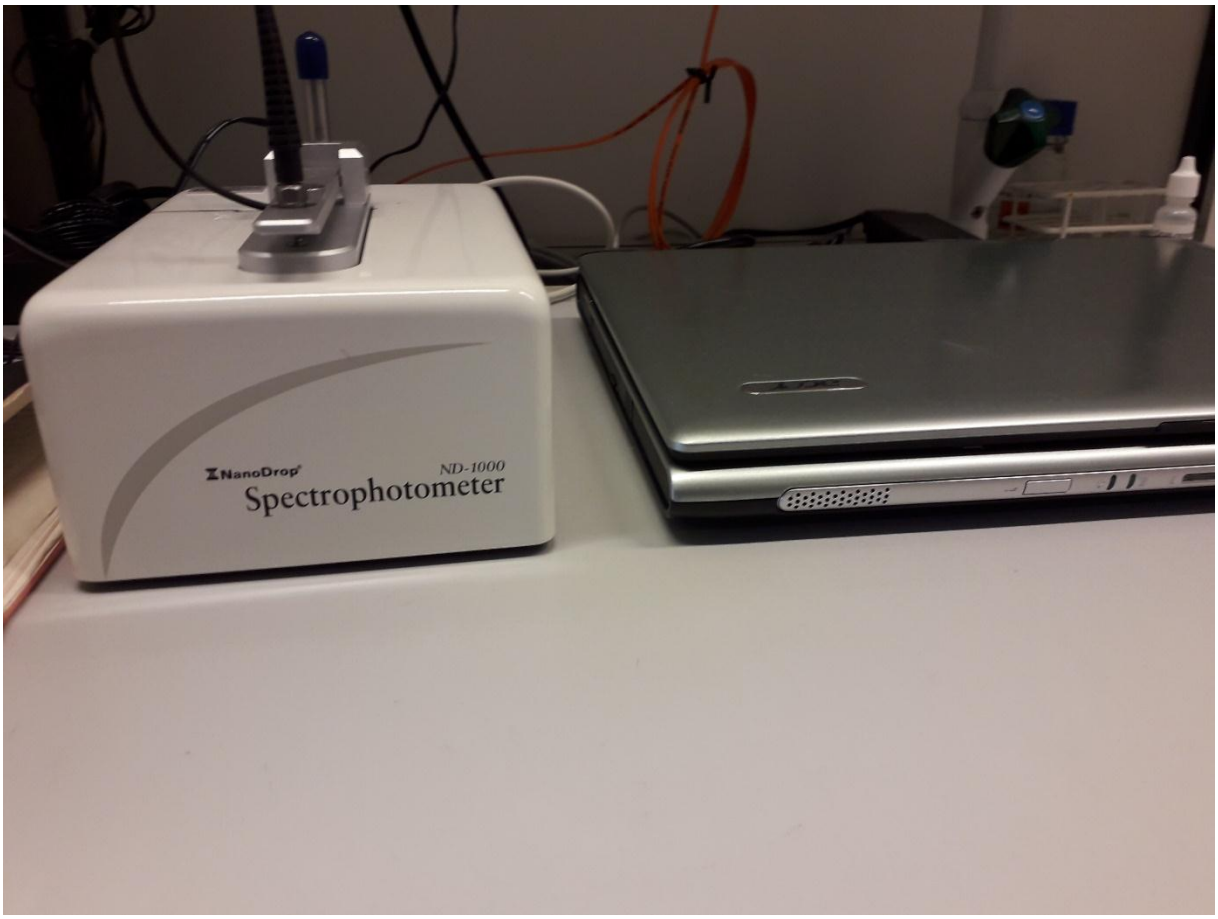


Figure 3.9.1. Nanospectrophotometer used for quality control of the DNA

The quality of DNA was further assessed by gel electrophoresis of genomic DNA using 1% agrose gel. Data were cleaned after entry into SPSS soft ware. In addition, control samples were used during the laboratory analysis.

CHAPTER FOUR: RESULTS

4.1. Polymorphism in killer cell immunoglobulin-like receptors and human leukocyte antigen-c and predisposition to preeclampsia in Ethiopian pregnant women population.

In the beginning, we recruited 345 study participants and then 32 were excluded due to missing of either or maternal or fetal blood samples or lack of clinical data and 313 DNA samples were genotyped and the data were entered into SPSS files. After further data editing we excluded seven samples due to lack of matching either maternal or fetal DNA samples or lack of a sample code that identifies the group of the participant as either control or PE. Further exclusion of participants with multiple pregnancies was made (control=9, PE = 9). The remaining 288 participants were included in the study. The missing values were recorded for blood pressure, GA and age in both groups. In control group, 14 values for systolic blood pressure (sBP), diastolic blood pressure (dBP) and gestation age (GA) were missing; nine were missing for age. In control group, 21 values for sBP and dBP, 14 values for GA and age were missing.

The study consisted of 288 participants grouped into case and the control groups. First, we described the demographic and clinical characteristics of our study cohort (Table 4.1.1).

The mean age, sBP and dBP was higher in PE group comped to controls while the mean GA in control group was higher compared to controls. Moreover, more than a quarter of the PE group showed early onset PE.

Table 4.1.1. Demographic and clinical characteristics of participants

Characteristics	Controls (n=157)	PE cases (n=131)
Age, mean	25.3 ± 5.0 SD	26.8 ± 5.1 SD
18-19	10.5% (15)	4.9% (6)
20-25	50.3% (72)	37.7% (46)
26-30	26.6% (38)	36.9% (45)
31-35	7.0% (10)	16.4% (20)
>35	5.6% (8)	4.1% (5)
sBP, mean	113.8 ± 8.7 SD	151.1 ± 16.6 SD
dBP, mean	72.4 ± 8.0 SD	98.6 ± 9.8 SD
GA, mean	271 days	253 days
Early onset PE	n.a	26.5% (31)
Late onset PE	n.a	73.5% (86)

*Data is presented as Mean ± SD. GA-gestational age; PE-preeclampsia; sBP-systolic blood pressure; dBP–diastolic blood pressu*re; *n.a-not applicable*

Then, we investigated whether there was association in polymorphism of KIR genotypes between the PE cases and controls (Figure 4.1.1). There was statistically significant difference in the frequency of KIR haplotypes between PE and controls, P=0.007, odds ratio at 95% confidence interval, 2.00 (1.20-3.33). The frequency distribution of KIRAA genotype was higher in PE cases compared to controls while the frequency of KIRAB/KIRBB genotypes was lower in PE cases compared to controls.

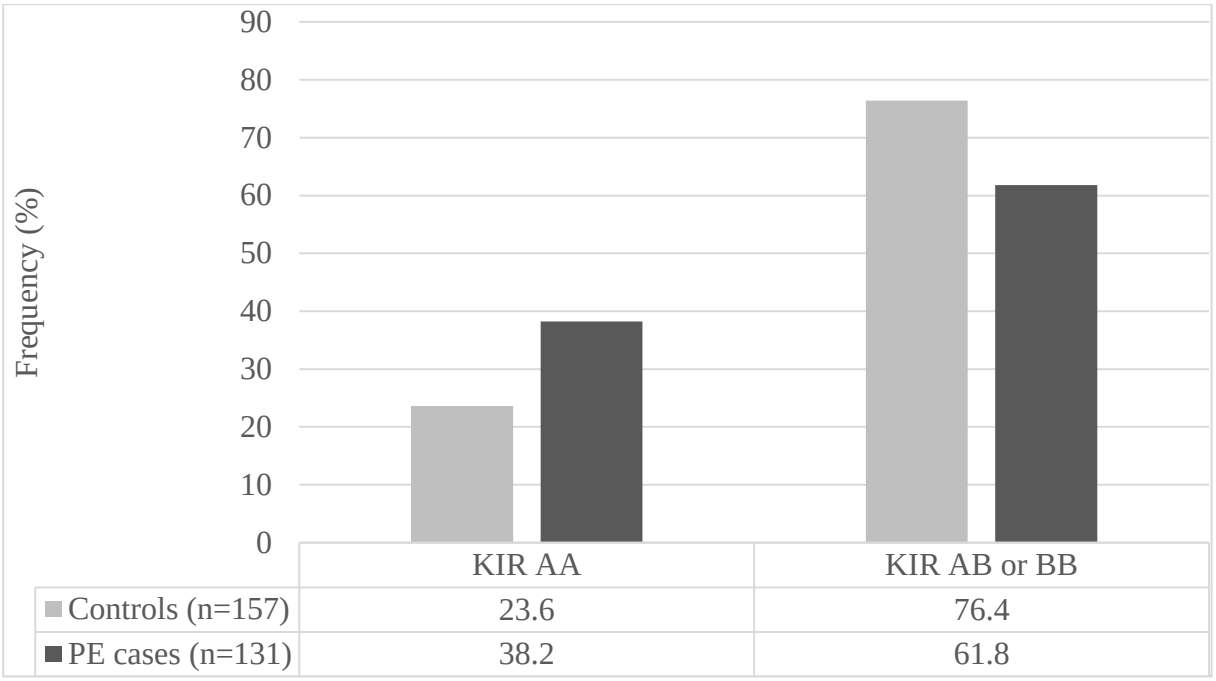


Figure 4.1.1. Frequency distribution of KIR haplotypes between PE and control pregnant Ethiopian women.

Next, the frequency distribution of inhibitory and activating genes were compared between PE case and controls of our cohort (Figure 4.1.2). There was no difference in frequency of inhibitory KIR2DL1 between the PE cases and controls (P>0.05). The frequency of inhibitory KIR2DL2 and KIR2DL3 was higher in controls than PE cases. However, the difference was not statistically significant. The statistically significant difference in frequency distribution of activating KIR2DS1 was observed between the PE cases and controls (P=0.013, odds ratio at 95% confidence interval 0.55 (0.35-0.88). The frequency was higher in controls compared to PE cases. The frequency of activating KIR2DS5 was higher in PE cases compared controls. However, the difference was not statistically significant (P>0.05).

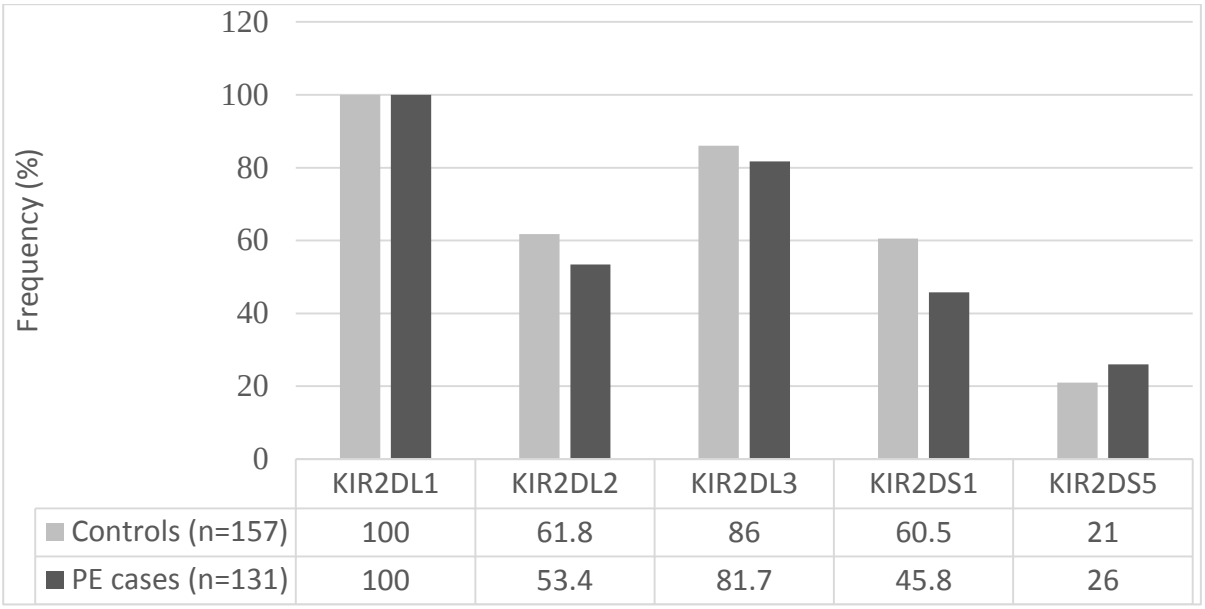


Figure 4.1.2. Frequency distribution of KIR genes between PE and control pregnant Ethiopian women

The frequency of inhibitory and activating KIR genes in Ethiopians with population from other countries were compared (Table 4.1.2). The notable difference was observed in frequency of activating KIR genes, KIR2DS1 and KIR2DS5. The frequency of KIR2DS1 was higher in a population of our study cohort compared to populations from other countries. However, the frequency of KIR2DS5 was lower in our study cohort compared to populations from other countries.

Table 4.1.2. Frequency of KIR genes in different populations.

	KIR Gene frequency (%)				
Population (n)	***KIR2DL1***	***KIR2DL2***	***KIR2DL3***	***KIR2DS1***	***KIR2DS5***
Ethiopia (n=288), this study	100	58.0	84.0	53.8	23.3
Israel Ethiopian Jews (n=31)	90.3	80.6	77.4	25.8	n.d
South Africa Black (n=167)	99.4	68.3	82.0	12.6	43.1
South Africa Caucasian (n=97)	99.0	57.7	88.7	39.2	39.2
Tunisia (n=114)	99.1	59.6	91.2	22.8	23.7
Nigeria Benin Yoruba (n=75)	100	38.4	92.0	14.7	n.d
Iran Fars Persian (n=248)	98.0	56.8	91.0	35.0	25.4
Yemen Jews (n=43)	97.7	46.5	95.2	31.0	n.d
India Western (n=161)	94.4	60.2	79.5	52.2	47.5
India Northeast Adivasi (n=101)	95.0	72.3	61.4	24.7	63.4
Sweden (n=102)	98.0	42.2	96.1	43.1	31.4

PE- Preeclampsia

We assessed whether there was association in polymorphism of maternal and fetal HLA-C groups with PE. The comparison was done in three subsets of mother-fetus pairs with different combinations of HLA-C groups (Table 4.1.3). There statistically significant difference was observed in a subset of mothers homozygous for HLA-C1 paired with heterozygous HLA-C fetus ($p<0.05$, $\chi 2= 4.8$ at 3.84 percentage point and 1 degree of freedom, odds ratio at 95% confidence interval, 2.8875 (0.8857-9.4135). The frequency was higher in pairs of PE group compared to control pairs. There was no statistically significant difference in frequency of the rest of mother-fetus HLA-C pairs between the PE cases and controls ($P>0.05$). The frequency of KIRAA genotype pregnant women carrying heterozygous fetus tended higher in PE group while the frequency of KIRAA genotype women carrying

homozygous HLA-C2 tended higher in controls compared to controls. However, there was no statistically significant difference in frequency between PE and controls (P>0.05).

Table 4.1. 3. Frequency distribution of HLA-C subsets between mother and fetus pairs in control and PE cases.

HLA-C Subgroup			
Mother	Fetus	Controls (n=157) % (n)	PE cases (n=131) % (n)
Non-self			
1+1	1+2	6.4 (10)	8.4 (11)
2+2	1+2	13.4 (21)	6.1 (8)
Missing self			
1+2	1+1	9.5 (15)	6.9 (9)
1+2	2+2	6.4 (10)	7.6 (10)
Self			
1+1	1+1	9.5 (15)	9.2 (12)
1+2	1+2	51.0 (80)	57.2 (75)
2+2	2+2	3.8 (6)	4.6 (6)

PE- Preeclampsia

Finally, we compared the frequency of KIRAA genotype pregnant women carrying heterozygous and homozygous HLA-C2 fetus (Figure 4.1.3). There was no statistically significant difference in frequencies of the genotypes between the PE and control groups.

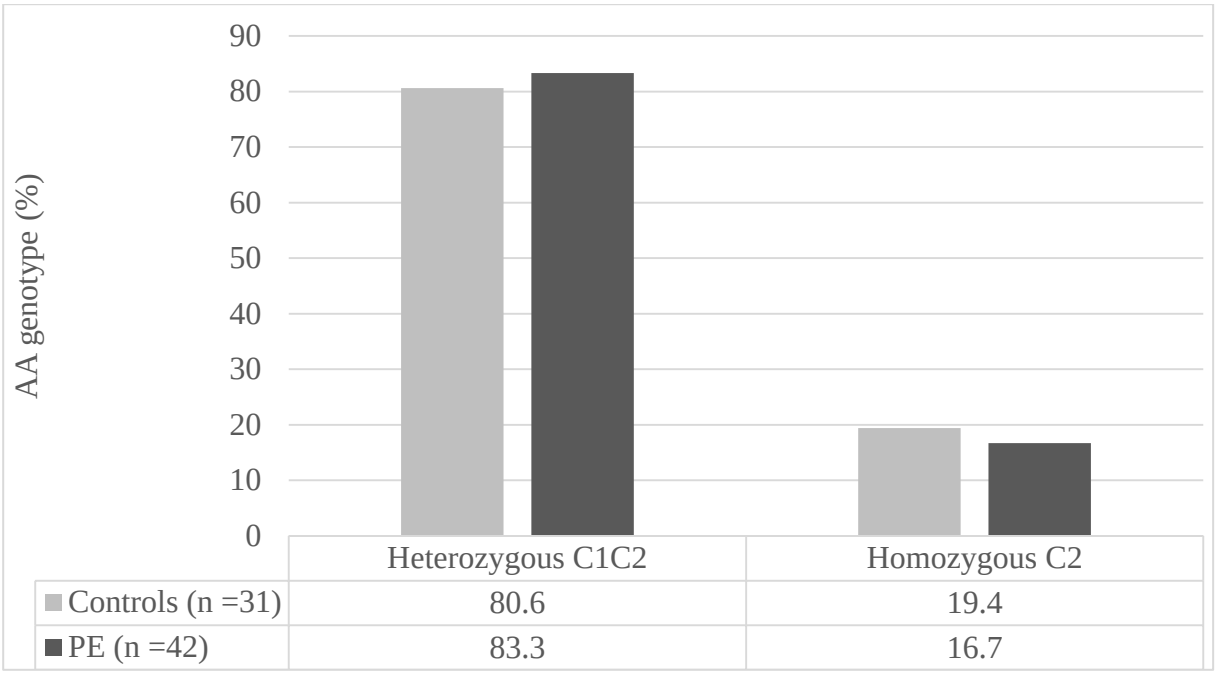


Figure 4.1.3. Frequency distribution of maternal KIR AA carrying heterozygous and homozygous HLA-2 fetus between PE and control pregnant Ethiopian women

4.2. Association of Maternal Regulatory Single Nucleotide Polymorphic CD99 Genotype with Preeclampsia in Pregnancies Carrying Male Fetuses in Ethiopian Women

The cohort consisted of 241 pregnant women participants grouped into two. The PE case group consisted of 105 participants while the control group consisted of 136 participants. We described the demographic and clinical parameters of our study cohort in PE cases and controls (Table 4.2.1). The mean age, sBP and dBP of PE cases tended higher compared to controls while the mean GA in PE cases tended lower compared to controls. About one fourth of the PE cases showed early onset PE.

Table 4.2.1. Demographic and clinical characteristics of study participants.

Characteristics	Controls (n= 136)	PE cases (n= 105)
Age	25.3 ±5	26.6 ± 5
sBP	114.0 ± 9	149.9 ± 17.2
dBP	72.4 ± 8.1	97.6 ± 10.1
GA	38+5 weeks	35+6 weeks
Early-onset PE	n.a	23.8% (25)
Late-onset PE	n.a	76.2% (80)

*Data is presented as Mean ± SD. GA-gestational age; PE-preeclampsia; sBP-systolic blood pressure; dBP–diastolic blood pressu*re; *n.a-not applicable*

We analyzed the frequency distribution of SNP rs311103 genotypes in subgroups of mothers and fetuses. We clustered high CD99 expression genotypes in one group G+ (GG and GC) and compared with low CD99 expression genotype (CC). First, we assessed the frequency distribution of high and low expression genotypes between PE and control mothers of male fetuses. We also assessed the frequency distribution of high and low expression genotypes between PE and control mothers of female fetuses (Table 4.2.2). There was a statistically significant association in frequency of low CD99 expression genotype between the PE and control mothers of males, $p < 0.05$ at $X^2 = 3.94$, percentage point 3.84, 1 degree of freedom, odds ratio at 95% confidence interval 3.08 (0.9663-9.8296).

Table 4.2.2. Frequency distribution of High and Low CD99 expression genotypes among subgroups of mothers.

Maternal genotype	PE mothers of male fetuses % (n=51)	Control mothers of male fetuses % (n=77)	PE mothers of female fetuses % (n= 54)	Control mothers of female fetuses % (n= 59)
G+	92.2 (47)	79.2 (61)	77.8 (42)	81.4 (48)
CC	7.8 (4)	20.8 (16)	22.2 (12)	18.6 (11)

PE-preeclampsia

Then, we compared the frequency distribution of high and low CD99 expression genotypes between subgroup of male fetuses of PE and controls. We also compared the frequency distribution of high and low CD99 genotypes between subgroup of female fetuses the PE and controls (Table 4.2.3). There was no statistically significant difference in frequency of the genotypes in male fetuses of PE and control subgroups ($P>0.05$). Similarly, no statistically significant difference was observed in frequency distribution of genotypes in female fetuses in PE and control groups ($P>0.05$).

Table 4.2.3. Frequency distribution of High and Low CD99 expression genotypes among subgroups of fetuses.

Fetal genotype	Male fetuses of PE mothers % (n=51)	Male fetuses of control mothers % (n=77)	Female fetuses of PE mothers % (n= 54)	Female fetuses of control mothers % (n= 59)
G+	80.4 (41)	76.6 (59)	75.9 (41)	79.7 (47)
CC	18.6 (10)	23.4 (18)	24.1 (13)	20.3 (12)

PE-preeclampsia

Finally, we assessed the frequency distribution of SNP rs311103 genotypes in maternal and fetal subgroups. First, we assessed the frequency distribution SNP rs311103 genotypes in

subgroup of mothers of male and female fetuses (Table 4 and 5). There was no association between frequency of genotypes between PE and control subgroup of mothers of male fetuses and subgroups of mothers of female fetuses (P>0.05).

Table 4.2.4. Frequency distribution of SNP rs311103 genotypes among maternal subgroups.

	Maternal groups			
Maternal Genotype	**PE mothers of male fetuses % (n=51)**	**Control mothers of male fetuses % (n=77)**	**PE mothers of female fetuses % (n=54)**	**Control mothers of female fetuses % (n=59)**
GG	41.2 (21)	36.4 (28)	40.8 (22)	40.7 (24)
GC	51.0 (26)	42.8 (33)	37.0 (20)	40.7 (24)
CC	7.8 (4)	20.8 (16)	22.2 (12)	18.6 (11)

PE-preeclampsia

No statistically significant difference was showed in frequency of genotypes between the subgroups of fetuses of the PE and control mothers (P>0.05).

Table 4.2.5. Frequency distribution of SNP sr311103 genotypes among fetal subgroups.

Fetal genotype	**Male fetuses of PE mothers % (n=51)**	**Male fetuses of control mothers % (n=77)**	**Female fetuses of PE mothers % (n=54)**	**Female fetuses of control mothers % (n=59)**
GG	31.4 (16)	29.9 (23)	40.8 (22)	30.5 (18)
GC	49.0 (25)	46.7 (36)	37.0 (20)	49.2 (29)
CC	19.6 (10)	23.4 (18)	22.2 (12)	20.3 (12)

PE-preeclampsia

CHAPTER FIVE: DISCUSSION

Human pregnancy involves deeper trophoblast invasion and longer GA. The invasion process involves communication between maternal and fetal cells via receptors and ligands. The receptor and ligand interaction convey signals that end up with cellular responses. Variable combinations of receptors and ligands result in diverse cellar responses that vary under normal and pathologic conditions (Parham and Moffett, 2013). The receptors and ligands are products of gene expression. The polymorphism of genes among individuals results in diverse combinations of receptors with ligands and variation in the response (Parham, 2005).

Human pregnancy is considered as paradoxical immunological phenomenon since the genetically distinct mother and the fetus co-exist (Poole and Claman, 2004). The maternal immune responses largely determine the outcome of pregnancy. Properly modulated maternal immune response results in normal pregnancy outcomes while exaggerated immune responses are associated with complications such as PE affecting the heath and survival of the fetus and the mother (Saito et al., 2010). The maternal and fetal genetic make-up and environmental factors play role in the pathogenesis of the PE (Moffett-King, 2002). The defective implantation and placentation were associated with certain feto-maternal genetic combinations that have role in trophoblast invasion during early pregnancy (Hiby et al., 2004). These combinations were showed to be more frequent in women of African ancestry (Nakimuli et al., 2015). Moreover, the occurrence of PE varies between pregnancies carrying male and female fetuses. Women carrying male fetuses are more predisposed to PE compared to the pregnancies carrying female fetuses (Broere-Brown et al., 2020). Women of black races are more affected by PE than other races (Nakimuli et al., 2014). Accordingly, in present study, we aimed to investigate the role of maternal and fetal genetic polymorphism in predisposition to PE in a population of Ethiopian pregnant women.

Maternal and fetal genetic factors contribute to more than 50% of genetic predisposition to PE, with maternal contribution being greater than that of the fetus (Cnattingius et al., 2004). The daughters of PE mothers are more than twice at greater risk of developing PE than those daughters of normal mothers and about 1.5 times more risk is transmitted from paternal line (Skjaerven et al., 2005). The genetic pathway may be either the daughter may inherit maternal genes that increase susceptibility to PE or the fetus may inherit from the daughter the genes that trigger PE (Skjaerven et al., 2005). A study showed the association of PE with 284 genes of which 103 of were also associated with preterm births emphasizing the role of PE in preterm delivery (Barbitoff et al., 2020). The study by Salonen et al., suggested that the genetic factors might play role in the development of PE (Salonen Ros et al., 2000). The study by Dekker et al., showed that PE is a genetic disease with familial tendency and involvement of a single gene or multiple genes inheritance pattern in PE susceptibility (Dekker and Sibai, 1998). Accordingly, we conducted a genetic predisposition study for PE on a population of Ethiopian pregnant women. To the best our knowledge, this study was conducted for the first time in Ethiopian population and hence would give additional dimension of approach to the existing reproductive health problem.

In the first part of the study, the demographic and clinical characteristics were described. The mean age showed higher tendency for the PE group. The frequency of pregnant women between ages 18 to 25 years tended higher for controls compared to PE cases while the frequency of the pregnant women in age range of 26 to 35 tended higher for PE cases compared to controls. The frequency of pregnant women above 35 years was higher in controls compared to PE cases. The majority of the study participants in both control and PE groups were in the age range of 26 to 35 years of age.

In our study cohort, the frequency of control group pregnant mothers between age 18 and 19 years old and age >35 was higher compared to the pregnant mothers in PE case group and the

control group were below 40 years of age while in PE group there were two mothers age 40 and 45 years old. The higher frequency of women age 18 to 19 and 35 year and above in control group compared to PE cases in our study cohort needs to be further investigated using larger sample size. A study by Khalil et al 2013, on singleton first trimester pregnancies with adjusted confounding factors showed that the maternal age is risk factors for the development of adverse pregnancy outcomes such as PE. The risk for the adverse pregnancies increases with advance in maternal age. The increased risk for miscarriage and PE was showed in those mothers age 40 years and above (Khalil et al., 2013). A study by Marozio et al., conducted on medical records of women aged 40 years and above delivered during fifteen years period showed that early onset PE was associated advanced maternal age (Marozio et al., 2019).

In our study cohort, the mean blood pressure was higher in PE cases compared to controls. It was also the primary diagnosis criteria for identification of PE cases. According to Roberts et al., 2005, increase in blood pressure results from endothelial dysfunction as one of the clinical manifestations of PE in the second stage PE development. Decreased perfusion of placental in early pregnancy results in release factors that initiate exaggerated inflammatory processes that results in endothelial activation and hypertension observed PE women. The pathological changes in kidney glomeruli contribute to the hypertension in PE (Roberts and Gammill, 2005). The study by Mishra et al., on normal and PE women undergoing caesarian section showed the role of neutrophil infiltration and release of reactive species in the pathogenesis of hypertension (Mishra et al., 2011).

More than one fourth of the PE cases in our cohort showed early onset PE diagnosed before 34 weeks of GA. Our finding showed higher prevalence of early onset PE compared to the global report (Aneman et al., 2020). Aneman et al., 2020 showed that the factors released from placental and immune cells play role in the pathogenesis of early and late onset PE (Aneman et al., 2020) (figure 5.1).

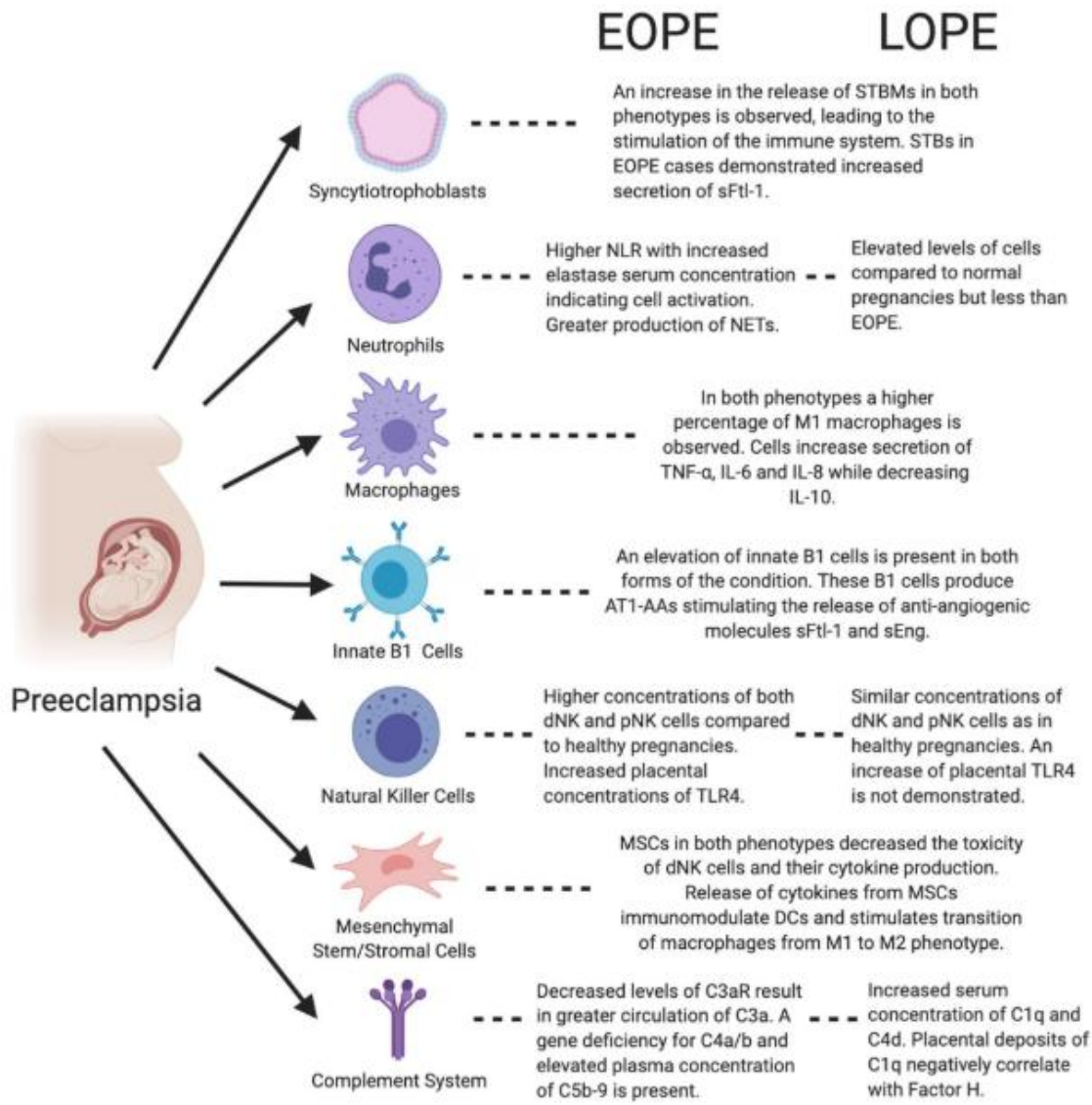


Figure 5.1. The role of placental and immune cells in early and late onset PE (Adapted from Aneman et al., 2020). AT1-AA, Angiotensin II type 1 receptor agonistic autoantibody; DC, Dendritic cell; dNK, Decidual natural killer; IL-6, Interleukin-6; IL-8, Interleukin-8; IL-10, Interleukin-10; MSC, Mesenchymal stem/stromal cell; NET, Neutrophil extracellular trap; pNK, Peripheral natural killer; NLR, Neutrophil to lymphocyte ratio; sEng, Soluble Endoglin; sFtl-1, Soluble fms-like tyrosine kinase-1; STB, Syncytiotrophoblast; STBM, syncytiotrophoblast micro-particles; TLR4, Toll-like receptor 4; TNF-α, Tumor necrosis factor-α.

The review by Armaly et al., indicated that early onset PE is associated with defective spiral artery remodeling resulting in placental malperfusion which causes increased secretion of soluble anti-angiogenic factor sFLT-1 and reduced secretion of a pro-angiogenic factor PlGF by oxidatively stressed placenta. The placental angiogenesis requires the role of PlGF, the main angiogenic factor. The high level of sFLT-1 promotes endothelial dysfunction by

reducing the level of PlGF (figure 5.2). The high level of sFLT-1 decreases the level of VEGF associated with injury of kidney podocyte and glomerular basement membrane accompanied by proteinuria (Armaly et al., 2018) (figure 5.3).

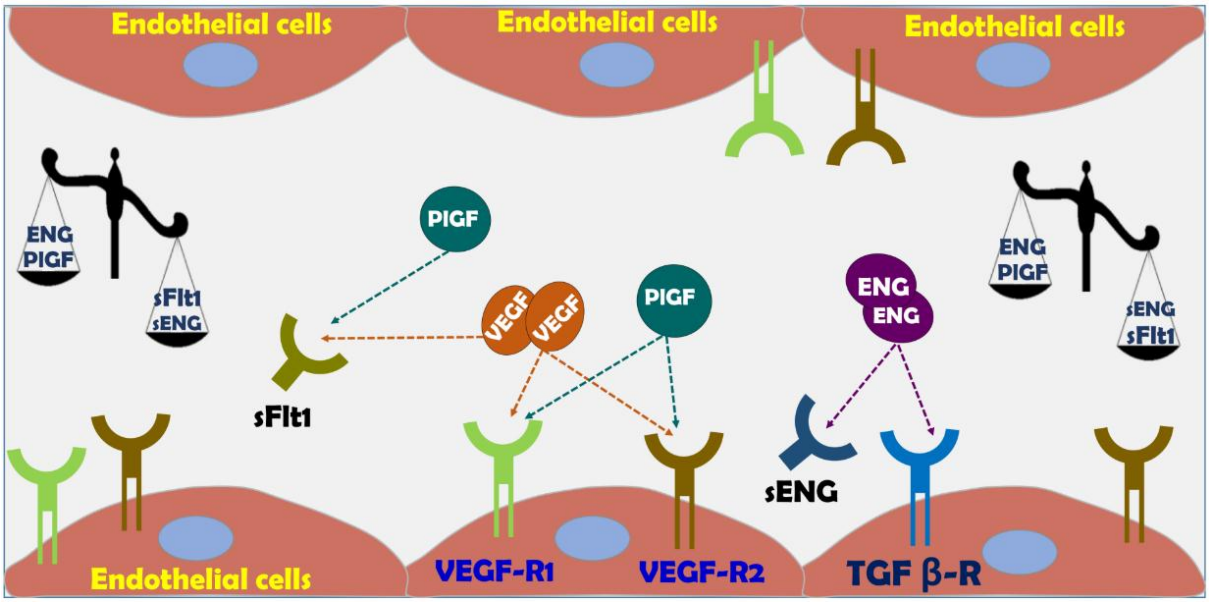


Figure 5.2. The role of angiogenic imbalance and vascular dysfunction in PE (Adapted from Armayl et al., 2018).

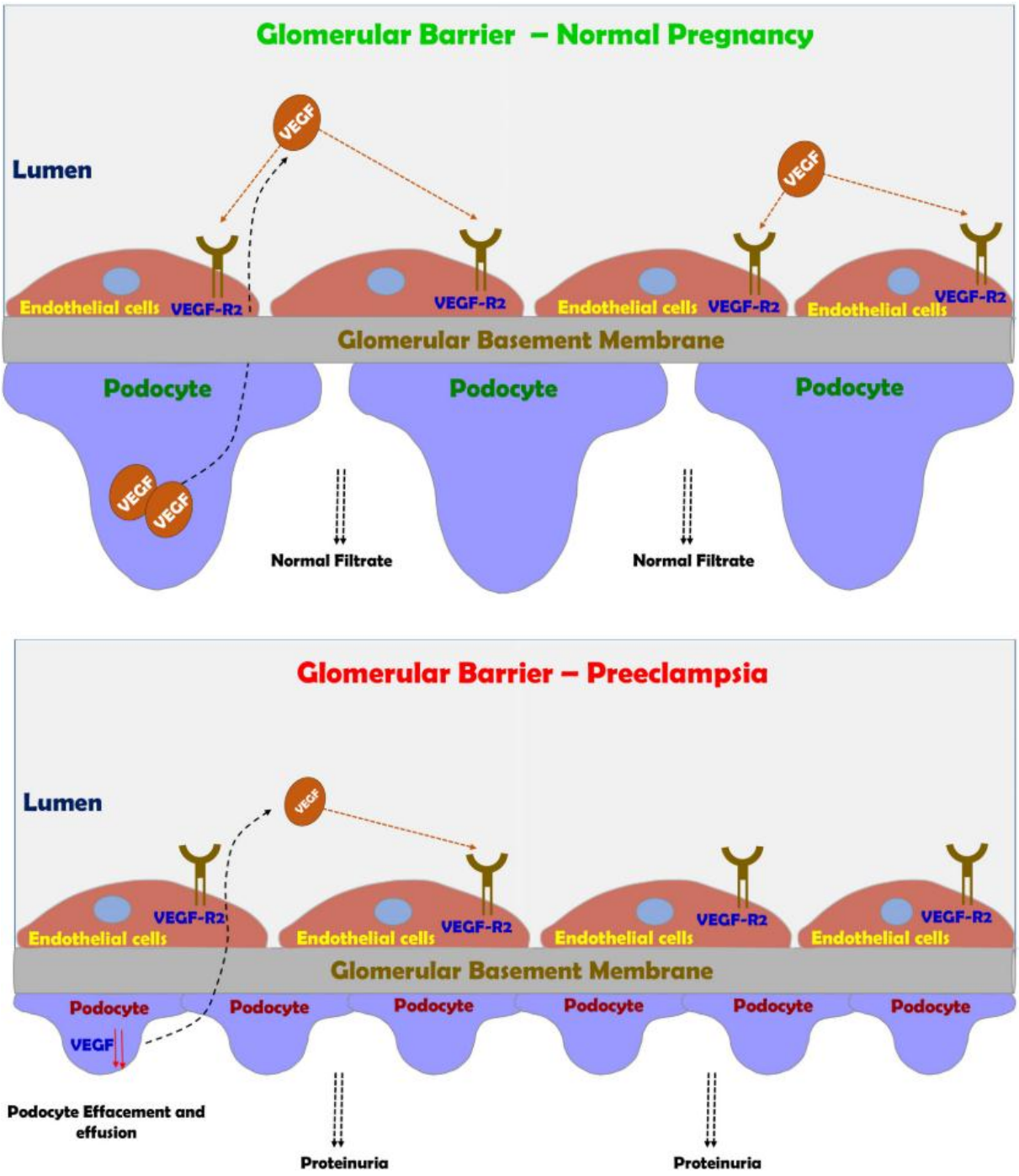


Figure 5.3. Renal function in normal and PE pregnancies (Adapted from Armayl et al., 2018).

The study by Seligman et al., showed that the formation of nitric oxide by damaged endothelial cells is decreased in PE contributing to the pathogenesis (Seligman et al., 1994). Förstermann, indicated the association of endothelial dysfunction with decreased bioavailability of nitric oxide due to its degradation by reactive oxygen species in oxidative

stress (Förstermann, 2010). Therefore, the high level of sFLT-1 in our cohort might have played a role in the pathogenesis of the early onset PE.

The maternal immune response in human pregnancy has important contribution in the pathogenesis of PE (Redman, 1992). The combinations of maternal KIR and fetal HLA-C play pivotal role in the pathogenesis of adverse pregnancy outcomes such as PE (Hiby et al., 2004). The KIR and HLA-C molecules are highly polymorphic and hence every pregnancy is likely to generate different combinations resulting in either normal or complicated pregnancy (Parham and Moffett, 2013). Accordingly, we investigated whether there was correlation between the polymorphism of maternal KIR and, maternal and fetal HLA-C genotypes with PE. We investigated five maternal KIR genes distributed in A and B haplotypes and then grouped into KIRAA and KIRAB/KIRBB genotypes. The A haplotype designation was based on the presence of inhibitory gene KIR2DL3 and the absence of KIR2DL2, KIR2DS1 and KIR2DS5 genes. The B haplotype designation was based on the absence of KIR2DL3 and the presence of KIR2DL2, KIR2DS1 and KIR2DS5 genes. There was a statistically significant association in the frequency of the KIR genotypes between PE cases and controls ($p<0.05$). The frequency of KIRAA genotype was higher in PE cases compared to controls. On the other hand, the frequency of KIRAB/KIRBB genotypes was higher in controls than in PE women. Moreover, our data showed the presence of the major inhibitory gene KIR2DL1 in all study participants. The study by Moffett el al., reviewed that both KIR2DL1 and KIR2DS1 bind HLA-C2 of the trophoblast cell resulting in opposite effects on the invasion of trophoblast cells and placentation (Moffett et al., 2015). From our finding, it seems that the absence or the presence of KIR2DS1 played determinant role in the pathogenesis of PE in our cohort. The inhibitory effect of KIR2DL1 on uNK cells function upon binding fetal HLA-C2 and subsequent placentation defect might may be dominant in the absence of maternal KIR2DS1. Hence, KIRAA genotype pregnant women in our cohort are likely more prone to develop PE

compared to those pregnant women with either KIRAB or KIRBB genotypes. Moreover, the pregnant women naïve for HLA-C2 (HLA-C1C1) carrying HLA-C1C2 fetus (non-self pair) were higher in frequency in PE group compared to similar pairs in control group. The result showed a statistically significant association in maternal and fetal pairs between PE and control groups. On the other hand, the comparison for the missing self HLA-C combination pairs specifically HLA-C1C2 pregnant women carrying HLA-C2C2 fetus showed higher frequency in control group compared to PE cases. The result showed no statistically significant association between the PE and control groups. Taken together, the extra fetal HLA-C2 may sensitize HLA-C2 naïve mothers for development of PE through inhibition of uNK cells by interacting with KIR2DL1 receptor in KIRAA genotype pregnant women. The consequent poor placental perfusion leads to in oxidative stress and release of placental factors into the maternal circulation promoting endothelial dysfunction observed in the pathological process during PE development (Redman and Sargent, 2005). Therefore, the women with HLA-C1C1 and KIRAA genotype combinations may be at risk to develop PE if their male partner is HLA-C2C2.

On the other hand, the higher frequency of KIRAB/KIRBB genotypes was higher in control pregnant women group compared to pregnant women in PE group. The frequency distribution showed a statistically significant. In addition, the frequency of the activating gene KIR2DS1 was higher in control group of pregnant women compared to PE and the result showed a statistically significant association. The increased frequency of the genotypes that contain the activating gene KIR2DS1 may indicate the likely role in the protection of pregnant women with KIRAB/KIRBB genotype from developing PE. The finding indicates that in uNK cells that express both activating KIR2DS1 and inhibitory KIR2DL1 receptors, the effect of activating receptors may off set the inhibitory receptor effect (Hiby et al., 2004). Hence, uNK cells may become activated to secrete cytokines that promote the trophoblast

invasion and proper placentation during early pregnancy. Hence, the binding of KIR2DS1 receptor and HLA-C2 on trophoblast might have played role in the normal placentation process. Hiby et al, conducted KIR and HLA-C genotyping study on a population of mothers in United Kingdom. They used matched DNA extracted from maternal blood and cord blood/or mouth swabs of babies. They analyzed 10 KIR genes and demonstrated higher frequency of KIRAA and KIR2DS1 in PE compared to controls with statistically significant association, which was in agreement with our study result. Unlike our study result, they found higher frequency of mothers with homozygous HLA-C1 confronted with fetal HLA-C1C2 in control mothers compared to PE group (Hiby et al., 2004). This may indicate difference in fetal HLA-C2 sensitization to PE between Ethiopian and European women. The frequency of KIR2DS5 also showed disparity between Ethiopian and European women. The study by Hiby et al., showed higher frequency of KIR2DS5 in control mothers compared to PE group (Hiby et al., 2004). Our study showed lower frequency of KIR2DS5 in PE mothers compared to controls. This may imply less contribution of KIR2DS5 in Ethiopian women for protection from PE compared to Europeans. However, the significance of our finding needs further investigation.

Nakimuli et al., investigated the distribution of 12 KIR genes and two HLA-C groups on a population of PE and control Ugandan group using DNA extracted from maternal and cord bloods. The study showed higher frequency of KIRAA genotype and KIR2DS1 in PE women compared to controls and higher frequency of KIR2DS5 in controls compared to PE women. The result for KIR2DS5 showed statistically significant association but not for KIR2DS1. A statistically significant association was showed for KIRAA between the two groups (Nakimuli et al., 2015). Their study also showed higher frequency of KIRAB&KIRBB in controls compared to PE. Our finding was in agreement with Ugandan study in that they share common genetic risk factor for the development of PE. Especially of note is the lower

frequency of KIR2DS5 in Ethiopian and Ugandan populations indicating some kind common role that it plays in relation to PE in African population which could be different from European population. Moreover, the frequency of KIRAA in Ethiopian and Ugandan PE women was comparable and higher than that showed for United Kingdom population. This indicates that the frequency PE predisposing genotype is more common in sub-Saharan African countries compared to Europeans. Even though the frequency pattern of KIR2DS1 is similar in a population of Ethiopian and Ugandan women, the frequency observed in Ethiopians was higher compared to Ugandans and populations from other countries (table 2). This should be noted whether it is the result of Ethiopia as origin of humankind or effect of genetic admixture due to population migration.

In the second part of the study, we investigated whether maternal and fetal polymorphism in regulatory region SNP rs311103 is associated with PE in fetal sex dependent fashion. Our study showed a statistically significant association in the frequency of maternal low CD99 expression genotype CC carrying male fetus between the PE and control group of pregnant women. There was underrepresentation of pregnant women with a low CD99 expression CC genotype carrying male fetuses in PE groups compared to controls. The study by Enninga et al., showed increased expression of pro-inflammatory in pregnancies carrying male fetus compared to pregnancies carrying female fetuses. In pregnancies carrying male fetuses, the proteins involved in signaling pathways of inflammation were found increased while in pregnancies carrying female fetuses, proteins involved in Th2 response signaling were found increased (Enninga et al., 2015). Accordingly, in Ethiopian CC women carrying male fetus, the immune response might be exaggerated causing adverse pregnancy outcomes such as PE and miscarriage while CC women carrying female fetus, the immune response might have resulted in normal pregnancy outcome (figure 5.4).

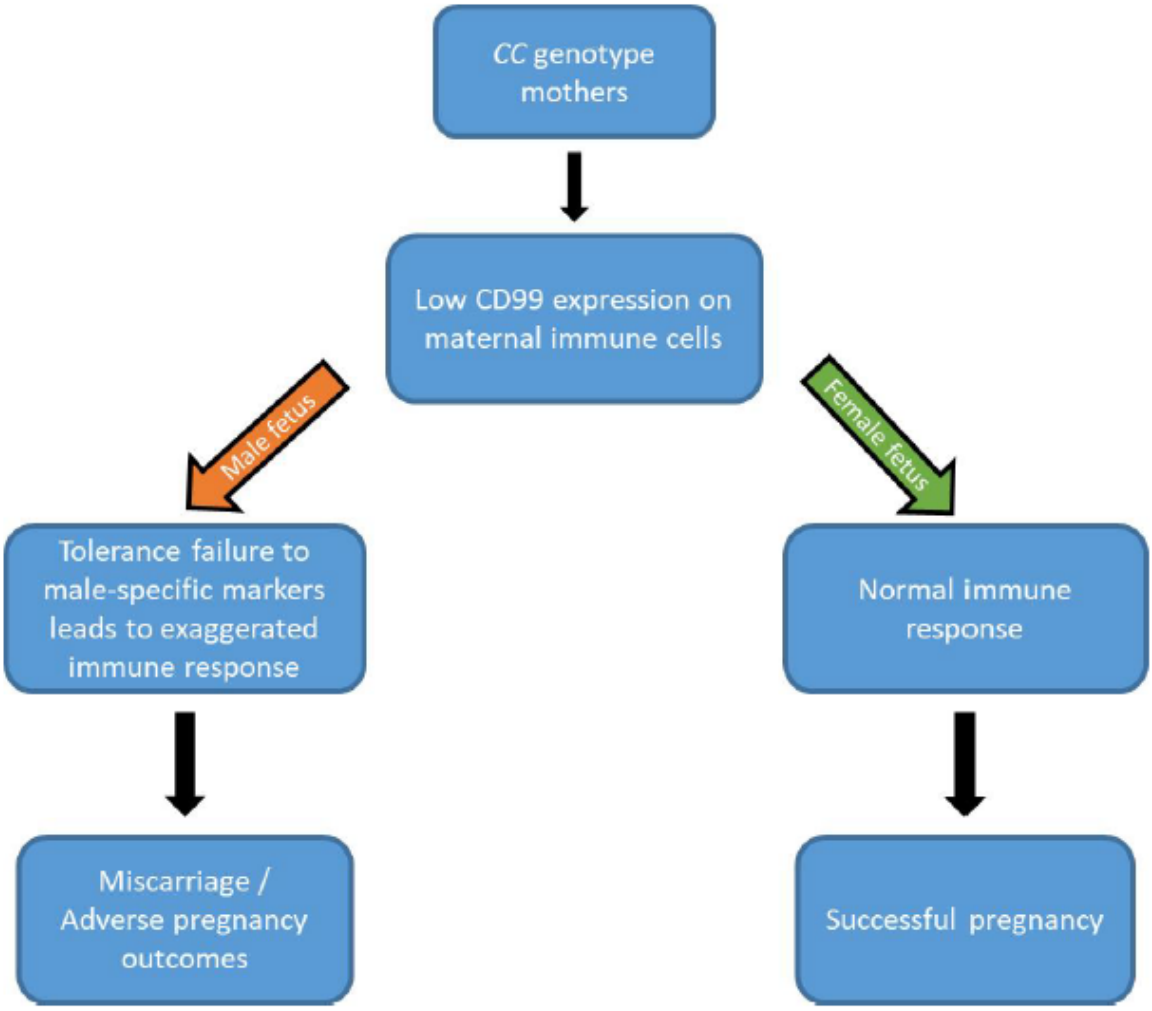


Figure 5.4. Adverse pregnancy outcomes in pregnancies carrying male fetus.

In addition, the frequency of male fetuses with low CD99 expression genotype (CC) of PE mothers tended to lower as compared with the male fetuses ontrol mothers. The GATA1 and GATA2 play role differentiation of erythroid precursors while GATA3 is important for differentiation of Th2 cells and NK cells. The expression of GATA3 is high in Th2 cells and very low in Th1 cells. The GATA3 transcription factor plays key role in differentiation and function of Th2 cells (Tindemans et al., 2014, Saito et al., 2010). The SNP rs311103C disrupts GATA3 binding resulting in low CD99 production (Yeh et al., 2018, Moller et al., 2018). Accordingly, Th2 differentiation and function might be inhibited shifting the balance towards Th1 inflammatory response resulting in adverse pregnancy outcomes (Saito et al.,

2010). The level of CD99 expression in heterozygous rs311103GC genotype males is higher compared to females (Moller et al., 2018). Therefore, we speculate that pregnant women with low CD99 expression genotype (SNP rs311103C) carrying male fetus might be predisposed to early pregnancy adverse pregnancy outcomes such as early onset PE, miscarriage and abortion due to Th1 mediated exaggerated inflammatory cytokine production. Moreover, we suggest that low CD99 and HLA-G expressions may show synergistic effect in the pathogenesis of PE in pregnancies carrying male fetus.

Historically, PE was first described as a disorder towards the end of 17^{th} century by Francois Mauriceau (Bell, 2010, Robillard et al., 2017) and despite tremendous progress, still it is a disease of theories (Phipps et al., 2019). The curative treatment is not yet available. There is disparity in the distribution of burden of PE in high and low-income countries as well as in white and black population. The majority of PE related deaths occur in African countries where resources are limited and access to health care is low (Duley, 2009).

The possibility of PE development due to factors other than genetic such as epigenetic modification related to environmental pollution needs to be investigated since Ethiopians primarily use wood fire for cooking. According to Lim et al., the exposure to environmental pollutants emitted from sources such as transport, wind dust and burning of biomass, is measured commonly by using the level of particulate matter (PM) < 2.5 μm level as an indicator. The carbonmonoxide (CO) level or fine particulate matter PM< 2.5 μm level is used as measure of personal exposure to household air pollution. Globally, the house hold air pollution is the major risk factor for the burden disease (Lim et al., 2012). Studies in Ethiopia showed high indoor air pollution with PM<2.5 μm size containing CO and nitrogen dixide (NO_2) (Tefera et al., 2016). The level of exposure to PM<2.5 μm and CO among Ethiopian adult females was higher compared to males (Lim et al., 2012).

Despite long years of effort, the etiology of PE is still unknown and there is no definitive curative treatment yet. Early detection of women at greater risk and administration of appropriate preventive measures would minimize the undesirable consequences of PE to the mother and the developing fetus (Rolnik et al., 2017). Hence, the results of our study may be important in the efforts of early stratification of women at greater risk to PE for possible administration of ASA as prophylactic treatment.

Summary study I

- KIRAA frequency was high in PE.
- KIR2DS1 frequency was high in control group.
- High frequency of non-self subgroup mothers homozygous for HLA-C1 carrying heterozygous fetus in PE group.
- Higher frequency of KIR2DS1 in Ethiopian population.

Summary study II

- There was a statically significant association in frequency of low CD99 expresser genotype with PE in mothers carrying male fetus.
- There was no statistically significant association in the frequencies of the remaining maternal or fetal genotypes with PE.
- Mothers with CC genotype carrying male fetus were underrepresented in PE group.

CHAPTER SIX: CONCLUSION

- Our study showed higher frequency of KIRAA in PE group and more than one fourth of the cases were early onset PE. KIRAA contains genes that encode inhibitory receptors. Hence, the cytokine secretion by KIRAA uNK cells might have been inhibited by interacting with fetal trophoblast HLA-C2 resulting in defective trophoblast invasion, spiral artery remodeling and poor feto-placental perfusion observed in PE.
- The frequency of KIR2DS1 gene was higher in control group women. This gene encodes activating receptor that interacts with fetal trophoblast HLA-C2 resulting in activation of uNK cells and secretion of cytokines that promote spiral artery remodeling and proper feto-placental perfusion observed in normal pregnancy. Moreover, higher frequency of KIR2DS1 in our study population compared to others might indicate either the gene originated in Ethiopian or effect of genetic admixture.
- The frequency of mothers naïve for HLA-C2 carrying fetus heterozygous HLA-C2 were higher in PE group. This group of mothers might have been sensitized by HLA-C2 of paternal origin for the development of PE.
- In PE group, mothers with low CD99 expression genotype (CC) carrying male fetus were underrepresented. This may indicate the role CD99 in success of pregnancy. Accordingly, those mothers with minor genotype might have experienced miscarriage or abortion during early pregnancy. However further study is recommended.

Strengths of the study

- The study was a case-control design and there was at least one control for every case.
- We used control samples to ensure the quality of the experiment.
- KIR study was the first study done on Ethiopian population.

- CD99 study was a novel study done in relation to PE.

Weaknesses of the study

- As a weakness, the collection of data and samples was not complete for some of the study participants, which decreased the number of participants.

Future perspective

The association of regulatory single nucleotide polymorphic CD99 with PE in pregnant women was a novel finding. The mechanism of SNP effect on PE needs to be further elucidated using gene expression and signaling studies. Moreover, the mechanism of possible synergistic effect of low HLA-G and CD99 in the pathogenesis of PE in pregnancies carrying male fetus needs to be further studied. Our study was based on single study site which may not represent different linguistic groups of Ethiopia. Therefore, we recommend further multicenter study with a larger sample size. Finally, we recommend further study if epigenetic mechanisms play synergistic role with genetic mechanisms in the pathogenesis of PE.

REFERENCES

<PE in LMIC.pdf>.

AL-QARAGHOULI, M. & FANG, Y. M. V. 2017. Effect of Fetal Sex on Maternal and Obstetric Outcomes. *Front Pediatr,* 5**,** 144.

ANEMAN, I., PIENAAR, D., SUVAKOV, S., SIMIC, T. P., GAROVIC, V. D. & MCCLEMENTS, L. 2020. Mechanisms of Key Innate Immune Cells in Early- and Late-Onset Preeclampsia. *Front Immunol,* 11**,** 1864.

APPS, R., MURPHY, S. P., FERNANDO, R., GARDNER, L., AHAD, T. & MOFFETT, A. 2009. Human leucocyte antigen (HLA) expression of primary trophoblast cells and placental cell lines, determined using single antigen beads to characterize allotype specificities of anti-HLA antibodies. *Immunology,* 127**,** 26-39.

ARMALY, Z., JADAON, J. E., JABBOUR, A. & ABASSI, Z. A. 2018. Preeclampsia: Novel Mechanisms and Potential Therapeutic Approaches. *Front Physiol,* 9**,** 973.

ASHARY, N., TIWARI, A. & MODI, D. 2018. Embryo Implantation: War in Times of Love. *Endocrinology,* 159**,** 1188-1198.

AUDETTE, M. C., LEVYTSKA, K., LYE, S. J., MELAMED, N. & KINGDOM, J. C. 2018. Parental ethnicity and placental maternal vascular malperfusion pathology in healthy nulliparous women. *Placenta,* 66**,** 40-46.

BAGGIOLINI, M. & CLARK-LEWIS, I. 1992. Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Lett,* 307**,** 97-101.

BARBITOFF, Y. A., TSAREV, A. A., VASHUKOVA, E. S., MAKSIUTENKO, E. M., KOVALENKO, L. V., BELOTSERKOVTSEVA, L. D. & GLOTOV, A. S. 2020. A Data-Driven Review of the Genetic Factors of Pregnancy Complications. *Int J Mol Sci,* 21.

BAUMWELL, S. & KARUMANCHI, S. A. 2007. Pre-eclampsia: clinical manifestations and molecular mechanisms. *Nephron Clin Pract,* 106**,** c72-81.

BELL, M. J. 2010. A historical overview of preeclampsia-eclampsia. *J Obstet Gynecol Neonatal Nurs,* 39**,** 510-8.

BROERE-BROWN, Z. A., ADANK, M. C., BENSCHOP, L., TIELEMANS, M., MUKA, T., GONCALVES, R., BRAMER, W. M., SCHOUFOUR, J. D., VOORTMAN, T., STEEGERS, E. A. P., FRANCO, O. H. & SCHALEKAMP-TIMMERMANS, S. 2020. Fetal sex and maternal pregnancy outcomes: a systematic review and meta-analysis. *Biol Sex Differ,* 11**,** 26.

BROWN, M. A., MAGEE, L. A., KENNY, L. C., KARUMANCHI, S. A., MCCARTHY, F. P., SAITO, S., HALL, D. R., WARREN, C. E., ADOYI, G. & ISHAKU, S. 2018. The hypertensive disorders of pregnancy: ISSHP classification, diagnosis & management recommendations for international practice. *Pregnancy Hypertens,* 13**,** 291-310.

BURTON, G. J. & JAUNIAUX, E. 2018. Pathophysiology of placental-derived fetal growth restriction. *Am J Obstet Gynecol,* 218**,** S745-S761.

CABALLERO-CAMPO, P., DOMÍNGUEZ, F., COLOMA, J., MESEGUER, M., REMOHÍ, J., PELLICER, A. & SIMÓN, C. 2002. Hormonal and embryonic regulation of chemokines IL-8, MCP-1 and RANTES in the human endometrium during the window of implantation. *Mol Hum Reprod,* 8**,** 375-84.

CAI, J., ZHAO, Y., LIU, P., XIA, B., ZHU, Q., WANG, X., SONG, Q., KAN, H. & ZHANG, Y. 2017. Exposure to particulate air pollution during early pregnancy is associated with placental DNA methylation. *Sci Total Environ,* 607-608**,** 1103-1108.

CHA, J., SUN, X. & DEY, S. K. 2012. Mechanisms of implantation: strategies for successful pregnancy. *Nat Med,* 18**,** 1754-67.

CHANG, C.-W., WAKELAND, A. K. & PARAST, M. M. 2018. Trophoblast lineage specification, differentiation and their regulation by oxygen tension. *Journal of Endocrinology,* 236**,** R43-R56.

CLIFTON, V. L. 2010. Review: Sex and the human placenta: mediating differential strategies of fetal growth and survival. *Placenta,* 31 Suppl**,** S33-9.

CNATTINGIUS, S., REILLY, M., PAWITAN, Y. & LICHTENSTEIN, P. 2004. Maternal and fetal genetic factors account for most of familial aggregation of preeclampsia: a population-based Swedish cohort study. *Am J Med Genet A,* 130a**,** 365-71.

CONNEELY, O. M., MULAC-JERICEVIC, B., DEMAYO, F., LYDON, J. P. & O'MALLEY, B. W. 2002. Reproductive functions of progesterone receptors. *Recent Prog Horm Res,* 57**,** 339-55.

DEKKER, G. A. & SIBAI, B. M. 1998. Etiology and pathogenesis of preeclampsia: current concepts. *Am J Obstet Gynecol,* 179**,** 1359-75.

DESHMANE, S. L., KREMLEV, S., AMINI, S. & SAWAYA, B. E. 2009. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res,* 29**,** 313-26.

DJURISIC, S. & HVIID, T. V. 2014. HLA Class Ib Molecules and Immune Cells in Pregnancy and Preeclampsia. *Front Immunol,* 5**,** 652.

DOMINGUEZ, F., GALAN, A., MARTIN, J. J., REMOHI, J., PELLICER, A. & SIMON, C. 2003. Hormonal and embryonic regulation of chemokine receptors CXCR1, CXCR4, CCR5 and CCR2B in the human endometrium and the human blastocyst. *Mol Hum Reprod,* 9**,** 189-98.

DOMINGUEZ, F., YÁÑEZ-MÓ, M., SANCHEZ-MADRID, F. & SIMÓN, C. 2005. Embryonic implantation and leukocyte transendothelial migration: different processes with similar players? *Faseb j,* 19**,** 1056-60.

DUCKITT, K. & HARRINGTON, D. 2005. Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies. *Bmj,* 330.

DULEY, L. 2009. The global impact of pre-eclampsia and eclampsia. *Semin Perinatol,* 33**,** 130-7.

ENNINGA, E. A., NEVALA, W. K., CREEDON, D. J., MARKOVIC, S. N. & HOLTAN, S. G. 2015. Fetal sex-based differences in maternal hormones, angiogenic factors, and immune mediators during pregnancy and the postpartum period. *Am J Reprod Immunol,* 73**,** 251-62.

ERLANDSSON, L., LINDGREN, R., NAAV, A., KRAIS, A. M., STRANDBERG, B., LUNDH, T., BOMAN, C., ISAXON, C., HANSSON, S. R. & MALMQVIST, E. 2020. Exposure to wood smoke particles leads to inflammation, disrupted proliferation and damage to cellular structures in a human first trimester trophoblast cell line. *Environ Pollut,* 264**,** 114790.

FIROZ, T., SANGHVI, H., MERIALDI, M. & VON DADELSZEN, P. 2011. Pre-eclampsia in low and middle income countries. *Best Pract Res Clin Obstet Gynaecol,* 25**,** 537-48.

FISHER, S. J. 2015. Why is placentation abnormal in preeclampsia? *Am J Obstet Gynecol,* 213**,** S115-22.

FÖRSTERMANN, U. 2010. Nitric oxide and oxidative stress in vascular disease. *Pflugers Arch,* 459**,** 923-39.

FREEDMAN, A. A., SILVER, R. M., GIBBINS, K. J., HOGUE, C. J., GOLDENBERG, R. L., DUDLEY, D. J., PINAR, H. & DREWS-BOTSCH, C. 2019. The association of stillbirth with placental abnormalities in growth-restricted and normally grown fetuses. *Paediatr Perinat Epidemiol,* 33**,** 274-383.

GHOSH, G., GREWAL, J., MÄNNISTÖ, T., MENDOLA, P., CHEN, Z., XIE, Y. & LAUGHON, S. K. 2014. Racial/ethnic differences in pregnancy-related hypertensive disease in nulliparous women. *Ethn Dis,* 24**,** 283-9.

GHULMIYYAH, L. & SIBAI, B. 2012. Maternal mortality from preeclampsia/eclampsia. *Semin Perinatol,* 36**,** 56-9.

GONG, H., CHEN, Y., XU, J., XIE, X., YU, D., YANG, B. & KUANG, H. 2017. The regulation of ovary and conceptus on the uterine natural killer cells during early pregnancy. *Reprod Biol Endocrinol,* 15**,** 73.

GRANOT, I., GNAINSKY, Y. & DEKEL, N. 2012. Endometrial inflammation and effect on implantation improvement and pregnancy outcome. *Reproduction,* 144**,** 661-8.

GUDE, N. M., ROBERTS, C. T., KALIONIS, B. & KING, R. G. 2004. Growth and function of the normal human placenta. *Thromb Res,* 114**,** 397-407.

HARA, N., FUJII, T., YAMASHITA, T., KOZUMA, S., OKAI, T. & TAKETANI, Y. 1996. Altered expression of human leukocyte antigen G (HLA-G) on extravillous trophoblasts in preeclampsia: immunohistological demonstration with anti-HLA-G specific antibody "87G" and anti-cytokeratin antibody "CAM5.2". *Am J Reprod Immunol,* 36**,** 349-58.

HIBY, S. E., APPS, R., CHAZARA, O., FARRELL, L. E., MAGNUS, P., TROGSTAD, L., GJESSING, H. K., CARRINGTON, M. & MOFFETT, A. 2014. Maternal KIR in combination with paternal HLA-C2 regulate human birth weight. *J Immunol,* 192**,** 5069-73.

HIBY, S. E., APPS, R., SHARKEY, A. M., FARRELL, L. E., GARDNER, L., MULDER, A., CLAAS, F. H., WALKER, J. J., REDMAN, C. W., MORGAN, L., TOWER, C., REGAN, L., MOORE, G. E., CARRINGTON, M. & MOFFETT, A. 2010. Maternal activating KIRs protect against human reproductive failure mediated by fetal HLA-C2. *J Clin Invest,* 120**,** 4102-10.

HIBY, S. E., KING, A., SHARKEY, A. M. & LOKE, Y. W. 1997. Human uterine NK cells have a similar repertoire of killer inhibitory and activatory receptors to those found in blood, as demonstrated by RT-PCR and sequencing. *Mol Immunol,* 34**,** 419-30.

HIBY, S. E., WALKER, J. J., O'SHAUGHNESSY K, M., REDMAN, C. W., CARRINGTON, M., TROWSDALE, J. & MOFFETT, A. 2004. Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success. *J Exp Med,* 200**,** 957-65.

HOGAN, M. C., FOREMAN, K. J., NAGHAVI, M., AHN, S. Y., WANG, M., MAKELA, S. M., LOPEZ, A. D., LOZANO, R. & MURRAY, C. J. 2010. Maternal mortality for 181 countries, 1980-2008: a systematic analysis of progress towards Millennium Development Goal 5. *Lancet,* 375**,** 1609-23.

JOKHI, P. P., KING, A., BOOCOCK, C. & LOKE, Y. W. 1995. Secretion of colony stimulating factor-1 by human first trimester placental and decidual cell populations and the effect of this cytokine on trophoblast thymidine uptake in vitro. *Hum Reprod,* 10**,** 2800-7.

JOKHI, P. P., KING, A. & LOKE, Y. W. 1994. Production of granulocyte-macrophage colony-stimulating factor by human trophoblast cells and by decidual large granular lymphocytes. *Hum Reprod,* 9**,** 1660-9.

KASSEBAUM, N. J., BERTOZZI-VILLA, A., COGGESHALL, M. S., SHACKELFORD, K. A., STEINER, C., HEUTON, K. R., GONZALEZ-MEDINA, D., BARBER, R., HUYNH, C., DICKER, D., TEMPLIN, T., WOLOCK, T. M., OZGOREN, A. A., ABD-ALLAH, F., ABERA, S. F., ABUBAKAR, I., ACHOKI, T., ADELEKAN, A., ADEMI, Z., ADOU, A. K., ADSUAR, J. C., AGARDH, E. E., AKENA, D., ALASFOOR, D., ALEMU, Z. A., ALFONSO-CRISTANCHO, R., ALHABIB, S., ALI, R., AL KAHBOURI, M. J., ALLA, F., ALLEN, P. J., ALMAZROA, M. A., ALSHARIF, U., ALVAREZ, E., ALVIS-GUZMÁN, N., AMANKWAA, A. A., AMARE, A. T., AMINI, H., AMMAR, W., ANTONIO, C. A. T., ANWARI, P., ÄRNLÖV, J., ARSENIJEVIC, V. S. A., ARTAMAN, A., ASAD, M. M., ASGHAR, R. J., ASSADI, R., ATKINS, L. S., BADAWI, A., BALAKRISHNAN, K., BASU, A., BASU, S., BEARDSLEY, J., BEDI, N., BEKELE, T., BELL, M. L., BERNABE, E., BEYENE, T. J., BHUTTA, Z., BIN ABDULHAK, A., BLORE, J. D., BASARA, B. B., BOSE, D., BREITBORDE, N., CÁRDENAS, R., CASTAÑEDA-ORJUELA, C. A., CASTRO, R. E., CATALÁ-LÓPEZ, F., CAVLIN, A., CHANG, J.-C., CHE, X., CHRISTOPHI, C. A., CHUGH, S. S., CIRILLO, M., COLQUHOUN, S. M., COOPER, L. T., COOPER, C., DA COSTA LEITE, I., DANDONA, L., DANDONA, R., DAVIS, A., DAYAMA, A., DEGENHARDT, L., DE LEO, D., DEL POZO-CRUZ, B., DERIBE, K., DESSALEGN, M., DEVEBER, G. A., DHARMARATNE, S. D., DILMEN, U., DING, E. L., DORRINGTON, R. E., DRISCOLL, T. R., ERMAKOV, S. P., ESTEGHAMATI, A., FARAON, E. J. A., FARZADFAR, F., FELICIO, M. M., FERESHTEHNEJAD, S.-M., DE LIMA, G. M. F., et al. 2014. Global, regional, and national levels and causes of maternal mortality during 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. *The Lancet,* 384**,** 980-1004.

KHALIL, A., SYNGELAKI, A., MAIZ, N., ZINEVICH, Y. & NICOLAIDES, K. H. 2013. Maternal age and adverse pregnancy outcome: a cohort study. *Ultrasound Obstet Gynecol,* 42**,** 634-43.

KHONG, T. Y., LIDDELL, H. S. & ROBERTSON, W. B. 1987. Defective haemochorial placentation as a cause of miscarriage: a preliminary study. *Br J Obstet Gynaecol,* 94**,** 649-55.

KNOFLER, M., HAIDER, S., SALEH, L., POLLHEIMER, J., GAMAGE, T. & JAMES, J. 2019. Human placenta and trophoblast development: key molecular mechanisms and model systems. *Cell Mol Life Sci,* 76**,** 3479-3496.

LALA, P. K. & GRAHAM, C. H. 1990. Mechanisms of trophoblast invasiveness and their control: the role of proteases and protease inhibitors. *Cancer Metastasis Rev,* 9**,** 369-79.

LIM, S. S., VOS, T., FLAXMAN, A. D., DANAEI, G., SHIBUYA, K., ADAIR-ROHANI, H., AMANN, M., ANDERSON, H. R., ANDREWS, K. G., ARYEE, M., ATKINSON, C., BACCHUS, L. J., BAHALIM, A. N., BALAKRISHNAN, K., BALMES, J., BARKER-COLLO, S., BAXTER, A., BELL, M. L., BLORE, J. D., BLYTH, F., BONNER, C., BORGES, G., BOURNE, R., BOUSSINESQ, M., BRAUER, M., BROOKS, P., BRUCE, N. G., BRUNEKREEF, B., BRYAN-HANCOCK, C., BUCELLO, C., BUCHBINDER, R., BULL, F., BURNETT, R. T., BYERS, T. E., CALABRIA, B., CARAPETIS, J., CARNAHAN, E., CHAFE, Z., CHARLSON, F., CHEN, H., CHEN, J. S., CHENG, A. T., CHILD, J. C., COHEN, A., COLSON, K. E., COWIE, B. C., DARBY, S., DARLING, S., DAVIS, A., DEGENHARDT, L., DENTENER, F., DES JARLAIS, D. C., DEVRIES, K., DHERANI, M., DING, E. L., DORSEY, E. R., DRISCOLL, T., EDMOND, K., ALI, S. E., ENGELL, R. E., ERWIN, P. J., FAHIMI, S., FALDER, G., FARZADFAR, F., FERRARI, A., FINUCANE, M. M., FLAXMAN, S., FOWKES, F. G., FREEDMAN, G., FREEMAN, M. K., GAKIDOU, E., GHOSH, S., GIOVANNUCCI, E., GMEL, G., GRAHAM, K., GRAINGER, R., GRANT, B., GUNNELL, D., GUTIERREZ, H. R., HALL, W., HOEK, H. W., HOGAN, A., HOSGOOD, H. D., 3RD, HOY, D., HU, H., HUBBELL, B. J., HUTCHINGS, S. J., IBEANUSI, S. E., JACKLYN, G. L., JASRASARIA, R., JONAS, J. B., KAN, H., KANIS, J. A., KASSEBAUM, N., KAWAKAMI, N., KHANG, Y. H., KHATIBZADEH, S., KHOO, J. P., KOK, C., LADEN, F., et al. 2012. A comparative risk

assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet,* 380**,** 2224-60.

LO, J. O., ROBERTS, V. H. J., SCHABEL, M. C., WANG, X., MORGAN, T. K., LIU, Z., STUDHOLME, C., KROENKE, C. D. & FRIAS, A. E. 2018. Novel Detection of Placental Insufficiency by Magnetic Resonance Imaging in the Nonhuman Primate. *Reprod Sci,* 25**,** 64-73.

LOKE, Y. W., KING, A. & BURROWS, T. D. 1995. Decidua in human implantation. *Hum Reprod,* 10 Suppl 2**,** 14-21.

MAROZIO, L., PICARDO, E., FILIPPINI, C., MAINOLFI, E., BERCHIALLA, P., CAVALLO, F., TANCREDI, A. & BENEDETTO, C. 2019. Maternal age over 40 years and pregnancy outcome: a hospital-based survey. *J Matern Fetal Neonatal Med,* 32**,** 1602-1608.

MARTIN, M. P., NELSON, G., LEE, J. H., PELLETT, F., GAO, X., WADE, J., WILSON, M. J., TROWSDALE, J., GLADMAN, D. & CARRINGTON, M. 2002. Cutting edge: susceptibility to psoriatic arthritis: influence of activating killer Ig-like receptor genes in the absence of specific HLA-C alleles. *J Immunol,* 169**,** 2818-22.

MASOUMI, Z., FAMILARI, M., KALLEN, K., RANSTAM, J., OLOFSSON, P. & HANSSON, S. R. 2017. Fetal hemoglobin in umbilical cord blood in preeclamptic and normotensive pregnancies: A cross-sectional comparative study. *PLoS One,* 12**,** e0176697.

MASSIMIANI, M., LACCONI, V., LA CIVITA, F., TICCONI, C., RAGO, R. & CAMPAGNOLO, L. 2019. Molecular Signaling Regulating Endometrium-Blastocyst Crosstalk. *Int J Mol Sci,* 21.

MASTORAKOS, G. & ILIAS, I. 2003. Maternal and fetal hypothalamic-pituitary-adrenal axes during pregnancy and postpartum. *Ann N Y Acad Sci,* 997**,** 136-49.

MIKOLAJCZYK, T. P., NOSALSKI, R., SZCZEPANIAK, P., BUDZYN, K., OSMENDA, G., SKIBA, D., SAGAN, A., WU, J., VINH, A., MARVAR, P. J., GUZIK, B., PODOLEC, J., DRUMMOND, G., LOB, H. E., HARRISON, D. G. & GUZIK, T. J. 2016. Role of chemokine RANTES in the regulation of perivascular inflammation, T-cell accumulation, and vascular dysfunction in hypertension. *Faseb j,* 30**,** 1987-99.

MISHRA, N., NUGENT, W. H., MAHAVADI, S. & WALSH, S. W. 2011. Mechanisms of enhanced vascular reactivity in preeclampsia. *Hypertension,* 58**,** 867-73.

MIYAZAKI, S., TSUDA, H., SAKAI, M., HORI, S., SASAKI, Y., FUTATANI, T., MIYAWAKI, T. & SAITO, S. 2003. Predominance of Th2-promoting dendritic cells in early human pregnancy decidua. *J Leukoc Biol,* 74**,** 514-22.

MOFFETT-KING, A. 2002. Natural killer cells and pregnancy. *Nat Rev Immunol,* 2**,** 656-63.

MOFFETT, A., HIBY, S. E. & SHARKEY, A. M. 2015. The role of the maternal immune system in the regulation of human birthweight. *Philos Trans R Soc Lond B Biol Sci,* 370**,** 20140071.

MOLLER, M., LEE, Y. Q., VIDOVIC, K., KJELLSTROM, S., BJORKMAN, L., STORRY, J. R. & OLSSON, M. L. 2018. Disruption of a GATA1-binding motif upstream of XG/PBDX abolishes Xg(a) expression and resolves the Xg blood group system. *Blood,* 132**,** 334-338.

MOR, G., CARDENAS, I., ABRAHAMS, V. & GULLER, S. 2011. Inflammation and pregnancy: the role of the immune system at the implantation site. *Annals of the New York Academy of Sciences,* 1221**,** 80-87.

MURALIMANOHARAN, S., MALOYAN, A. & MYATT, L. 2013. Evidence of sexual dimorphism in the placental function with severe preeclampsia. *Placenta,* 34**,** 1183-9.

MYATT, L., MURALIMANOHARAN, S. & MALOYAN, A. 2014. Effect of preeclampsia on placental function: influence of sexual dimorphism, microRNA's and mitochondria. *Adv Exp Med Biol,* 814**,** 133-46.

NAKIMULI, A., CHAZARA, O., BYAMUGISHA, J., ELLIOTT, A. M., KALEEBU, P., MIREMBE, F. & MOFFETT, A. 2014. Pregnancy, parturition and preeclampsia in women of African ancestry. *Am J Obstet Gynecol,* 210**,** 510-520.e1.

NAKIMULI, A., CHAZARA, O., HIBY, S. E., FARRELL, L., TUKWASIBWE, S., JAYARAMAN, J., TRAHERNE, J. A., TROWSDALE, J., COLUCCI, F., LOUGEE, E., VAUGHAN, R. W., ELLIOTT, A. M., BYAMUGISHA, J., KALEEBU, P., MIREMBE, F., NEMAT-GORGANI, N., PARHAM, P., NORMAN, P. J. & MOFFETT,

A. 2015. A KIR B centromeric region present in Africans but not Europeans protects pregnant women from pre-eclampsia. *Proc Natl Acad Sci U S A,* 112**,** 845-50.
NAPSO, T., YONG, H. E. J., LOPEZ-TELLO, J. & SFERRUZZI-PERRI, A. N. 2018. The Role of Placental Hormones in Mediating Maternal Adaptations to Support Pregnancy and Lactation. *Front Physiol,* 9**,** 1091.
NIKAS, G. & AGHAJANOVA, L. 2002. Endometrial pinopodes: some more understanding on human implantation? *Reprod Biomed Online,* 4 Suppl 3**,** 18-23.
NIKAS, G. & MAKRIGIANNAKIS, A. 2003. Endometrial pinopodes and uterine receptivity. *Ann N Y Acad Sci,* 997**,** 120-3.
NING, F., LIU, H. & LASH, G. E. 2016. The Role of Decidual Macrophages During Normal and Pathological Pregnancy. *Am J Reprod Immunol,* 75**,** 298-309.
NORWITZ, E. R., SCHUST, D. J. & FISHER, S. J. 2001. Implantation and the survival of early pregnancy. *N Engl J Med,* 345**,** 1400-8.
OKADA, H., TSUZUKI, T. & MURATA, H. 2018. Decidualization of the human endometrium. *Reprod Med Biol,* 17**,** 220-227.
PAGE, E. W. 1948. Placental dysfunction in eclamptogenic toxemias. *Obstet Gynecol Surv,* 3**,** 615-28.
PARHAM, P. 2005. MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol,* 5**,** 201-14.
PARHAM, P. & MOFFETT, A. 2013. Variable NK cell receptors and their MHC class I ligands in immunity, reproduction and human evolution. *Nat Rev Immunol,* 13**,** 133-44.
PASELLO, M., MANARA, M. C. & SCOTLANDI, K. 2018. CD99 at the crossroads of physiology and pathology. *J Cell Commun Signal,* 12**,** 55-68.
PHIPPS, E. A., THADHANI, R., BENZING, T. & KARUMANCHI, S. A. 2019. Pre-eclampsia: pathogenesis, novel diagnostics and therapies. *Nat Rev Nephrol,* 15**,** 275-289.
POOLE, J. A. & CLAMAN, H. N. 2004. Immunology of pregnancy. Implications for the mother. *Clin Rev Allergy Immunol,* 26**,** 161-70.
RAJAGOPALAN, S. & LONG, E. O. 2012. KIR2DL4 (CD158d): An activation receptor for HLA-G. *Front Immunol,* 3**,** 258.
RED-HORSE, K., ZHOU, Y., GENBACEV, O., PRAKOBPHOL, A., FOULK, R., MCMASTER, M. & FISHER, S. J. 2004. Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface. *J Clin Invest,* 114**,** 744-54.
REDLINE, R. W. 2008. Placental pathology: a systematic approach with clinical correlations. *Placenta,* 29 Suppl A**,** S86-91.
REDMAN, C. W. 1991. Current topic: pre-eclampsia and the placenta. *Placenta,* 12**,** 301-8.
REDMAN, C. W. 1992. Immunological aspects of pre-eclampsia. *Baillieres Clin Obstet Gynaecol,* 6**,** 601-15.
REDMAN, C. W. & SARGENT, I. L. 2005. Latest advances in understanding preeclampsia. *Science,* 308**,** 1592-4.
REDMAN, C. W. & SARGENT, I. L. 2010. Immunology of pre-eclampsia. *Am J Reprod Immunol,* 63**,** 534-43.
ROBERTS, J. M. & COOPER, D. W. 2001. Pathogenesis and genetics of pre-eclampsia. *Lancet,* 357**,** 53-6.
ROBERTS, J. M. & GAMMILL, H. S. 2005. Preeclampsia: recent insights. *Hypertension,* 46**,** 1243-9.
ROBERTSON, S. A., CARE, A. S. & MOLDENHAUER, L. M. 2018. Regulatory T cells in embryo implantation and the immune response to pregnancy. *J Clin Invest,* 128**,** 4224-4235.
ROBILLARD, P. Y., DEKKER, G., CHAOUAT, G., SCIOSCIA, M., IACOBELLI, S. & HULSEY, T. C. 2017. Historical evolution of ideas on eclampsia/preeclampsia: A proposed optimistic view of preeclampsia. *J Reprod Immunol,* 123**,** 72-77.
ROLNIK, D. L., WRIGHT, D., POON, L. C. Y., SYNGELAKI, A., O'GORMAN, N., DE PACO MATALLANA, C., AKOLEKAR, R., CICERO, S., JANGA, D., SINGH, M., MOLINA, F. S., PERSICO, N., JANI, J. C., PLASENCIA, W., PAPAIOANNOU, G., TENENBAUM-GAVISH, K. & NICOLAIDES, K. H. 2017.

ASPRE trial: performance of screening for preterm pre-eclampsia. *Ultrasound Obstet Gynecol,* 50**,** 492-495.
SAITO, S., NAKASHIMA, A., SHIMA, T. & ITO, M. 2010. Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. *Am J Reprod Immunol,* 63**,** 601-10.
SALAMONSEN, L. A., EVANS, J., NGUYEN, H. P. & EDGELL, T. A. 2016. The Microenvironment of Human Implantation: Determinant of Reproductive Success. *Am J Reprod Immunol,* 75**,** 218-25.
SALONEN ROS, H., LICHTENSTEIN, P., LIPWORTH, L. & CNATTINGIUS, S. 2000. Genetic effects on the liability of developing pre-eclampsia and gestational hypertension. *Am J Med Genet,* 91**,** 256-60.
SELIGMAN, S. P., BUYON, J. P., CLANCY, R. M., YOUNG, B. K. & ABRAMSON, S. B. 1994. The role of nitric oxide in the pathogenesis of preeclampsia. *Am J Obstet Gynecol,* 171**,** 944-8.
SHAHUL, S., TUNG, A., MINHAJ, M., NIZAMUDDIN, J., WENGER, J., MAHMOOD, E., MUELLER, A., SHAEFI, S., SCAVONE, B., KOCIOL, R. D., TALMOR, D. & RANA, S. 2015. Racial Disparities in Comorbidities, Complications, and Maternal and Fetal Outcomes in Women With Preeclampsia/eclampsia. *Hypertens Pregnancy,* 34**,** 506-515.
SHARKEY, A. M., KING, A., CLARK, D. E., BURROWS, T. D., JOKHI, P. P., CHARNOCK-JONES, D. S., LOKE, Y. W. & SMITH, S. K. 1999. Localization of leukemia inhibitory factor and its receptor in human placenta throughout pregnancy. *Biol Reprod,* 60**,** 355-64.
SHARMA, S., GODBOLE, G. & MODI, D. 2016. Decidual Control of Trophoblast Invasion. *Am J Reprod Immunol,* 75**,** 341-50.
SINGH, M., CHAUDHRY, P. & ASSELIN, E. 2011. Bridging endometrial receptivity and implantation: network of hormones, cytokines, and growth factors. *J Endocrinol,* 210**,** 5-14.
SKJAERVEN, R., VATTEN, L. J., WILCOX, A. J., RONNING, T., IRGENS, L. M. & LIE, R. T. 2005. Recurrence of pre-eclampsia across generations: exploring fetal and maternal genetic components in a population based cohort. *Bmj,* 331**,** 877.
SOARES, M. J., VARBERG, K. M. & IQBAL, K. 2018. Hemochorial placentation: development, function, and adaptations†. *Biology of Reproduction,* 99**,** 196-211.
SPENCER, T. E., JOHNSON, G. A., BAZER, F. W. & BURGHARDT, R. C. 2004. Implantation mechanisms: insights from the sheep. *Reproduction,* 128**,** 657-68.
STAFF, A. C. 2019. The two-stage placental model of preeclampsia: An update. *J Reprod Immunol,* 134-135**,** 1-10.
STAVREUS-EVERS, A., NIKAS, G., SAHLIN, L., ERIKSSON, H. & LANDGREN, B. M. 2001. Formation of pinopodes in human endometrium is associated with the concentrations of progesterone and progesterone receptors. *Fertil Steril,* 76**,** 782-91.
STEINBORN, A., SCHMITT, E., KISIELEWICZ, A., RECHENBERG, S., SEISSLER, N., MAHNKE, K., SCHAIER, M., ZEIER, M. & SOHN, C. 2012. Pregnancy-associated diseases are characterized by the composition of the systemic regulatory T cell (Treg) pool with distinct subsets of Tregs. *Clin Exp Immunol,* 167**,** 84-98.
TEFERA, W., ASFAW, A., GILLILAND, F., WORKU, A., WONDIMAGEGN, M., KUMIE, A., SAMET, J. & BERHANE, K. 2016. Indoor and Outdoor Air Pollution- related Health Problem in Ethiopia: Review of Related Literature. *Ethiop J Health Dev,* 30**,** 5-16.
TESSEMA, G. A., TEKESTE, A. & AYELE, T. A. 2015. Preeclampsia and associated factors among pregnant women attending antenatal care in Dessie referral hospital, Northeast Ethiopia: a hospital-based study. *BMC Pregnancy Childbirth,* 15**,** 73.
THOMPSON, L. P., PENCE, L., PINKAS, G., SONG, H. & TELUGU, B. P. 2016. Placental Hypoxia During Early Pregnancy Causes Maternal Hypertension and Placental Insufficiency in the Hypoxic Guinea Pig Model. *Biol Reprod,* 95**,** 128.
TINDEMANS, I., SERAFINI, N., DI SANTO, J. P. & HENDRIKS, R. W. 2014. GATA-3 function in innate and adaptive immunity. *Immunity,* 41**,** 191-206.

VAN DER MEER, A., LUKASSEN, H. G., VAN LIEROP, M. J., WIJNANDS, F., MOSSELMAN, S., BRAAT, D. D. & JOOSTEN, I. 2004. Membrane-bound HLA-G activates proliferation and interferon-gamma production by uterine natural killer cells. *Mol Hum Reprod,* 10**,** 189-95.

VAN ESCH, J. J. A., VAN HEIJST, A. F., DE HAAN, A. F. J. & VAN DER HEIJDEN, O. W. H. 2017. Early-onset preeclampsia is associated with perinatal mortality and severe neonatal morbidity. *J Matern Fetal Neonatal Med,* 30**,** 2789-2794.

VAN MOURIK, M. S., MACKLON, N. S. & HEIJNEN, C. J. 2009. Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. *J Leukoc Biol,* 85**,** 4-19.

VATA, P. K., CHAUHAN, N. M., NALLATHAMBI, A. & HUSSEIN, F. 2015. Assessment of prevalence of preeclampsia from Dilla region of Ethiopia. *BMC Res Notes,* 8**,** 816.

VEENSTRA VAN NIEUWENHOVEN, A. L., HEINEMAN, M. J. & FAAS, M. M. 2003. The immunology of successful pregnancy. *Hum Reprod Update,* 9**,** 347-57.

VELEGRAKIS, A., SFAKIOTAKI, M. & SIFAKIS, S. 2017. Human placental growth hormone in normal and abnormal fetal growth. *Biomed Rep,* 7**,** 115-122.

VINKETOVA, K., MOURDJEVA, M. & ORESHKOVA, T. 2016. Human Decidual Stromal Cells as a Component of the Implantation Niche and a Modulator of Maternal Immunity. *J Pregnancy,* 2016**,** 8689436.

VOSKAMP, B. J., PEELEN, M., RAVELLI, A. C. J., VAN DER LEE, R., MOL, B. W. J., PAJKRT, E., GANZEVOORT, W. & KAZEMIER, B. M. 2020. Association between fetal sex, birthweight percentile and adverse pregnancy outcome. *Acta Obstet Gynecol Scand,* 99**,** 48-58.

WEDENOJA, S., YOSHIHARA, M., TEDER, H., SARIOLA, H., GISSLER, M., KATAYAMA, S., WEDENOJA, J., HAKKINEN, I. M., EZER, S., LINDER, N., LUNDIN, J., SKOOG, T., SAHLIN, E., IWARSSON, E., PETTERSSON, K., KAJANTIE, E., MOKKONEN, M., HEINONEN, S., LAIVUORI, H., KRJUTSKOV, K. & KERE, J. 2020. Fetal HLA-G mediated immune tolerance and interferon response in preeclampsia. *EBioMedicine***,** 102872.

WEDEPOHL, S., BECEREN-BRAUN, F., RIESE, S., BUSCHER, K., ENDERS, S., BERNHARD, G., KILIAN, K., BLANCHARD, V., DERNEDDE, J. & TAUBER, R. 2012. L-selectin--a dynamic regulator of leukocyte migration. *Eur J Cell Biol,* 91**,** 257-64.

WHEELER, T., ELCOCK, C. L. & ANTHONY, F. W. 1995. Angiogenesis and the placental environment. *Placenta,* 16**,** 289-96.

YEH, C. C., CHANG, C. J., TWU, Y. C., CHU, C. C., LIU, B. S., HUANG, J. T., HUNG, S. T., CHAN, Y. S., TSAI, Y. J., LIN, S. W., LIN, M. & YU, L. C. 2018. The molecular genetic background leading to the formation of the human erythroid-specific Xg(a)/CD99 blood groups. *Blood Adv,* 2**,** 1854-1864.