MATERNAL CARDIOVASCULAR AND IMMUNE REGULATORY FACTORS BEFORE THE ONSET OF PRE-ECLAMPSIA

by

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I, Agata Ledwozyw confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Agata Ledwozyw

For Waldek And My Parents

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ABSTRACT

Pre-eclampsia is a pregnancy syndrome characterised by maternal hypertension and proteinuria and often associated with multi-organ dysfunction and fetal growth restriction. The aetiology of pre-eclampsia remains elusive, so a clearer understanding of the pathophysiology leading to the clinical syndrome would improve our ability to predict, prevent and treat this condition.

Pre-eclampsia is known to be associated with impaired utero-placental blood flow, maternal endothelial dysfunction and an exaggerated systemic inflammatory response. Women at risk of pre-eclampsia have classical cardiovascular risk factors, including obesity and chronic hypertension. Surprisingly, smoking during pregnancy has a protective effect against pre-eclampsia. The reason behind this paradox is unknown.

This thesis prospectively examines the sequence of changes in blood pressure, angiogenic and regulatory immune factors in a cohort of pregnant women recruited early in pregnancy and followed until childbirth. Pregnant women were assigned to three groups: low risk of pre-eclampsia, high risk of pre-eclampsia, or women who continued to smoke through pregnancy. Differences in prospectively determined immune-regulatory and angiogenic factors between groups were correlated with maternal blood pressure before the onset of pre-eclampsia, fetal growth restriction and pregnancy induced hypertension. Smokers were included to determine how they might be protected from developing pre-eclampsia, but vulnerable to fetal growth restriction.

Results provide further insight into the pathogenesis of pre-eclampsia and a novel algorithm to identify women at risk of pre-eclampsia. Attempts to identify a putative angiotensin II receptor stimulating autoantibody are also described.

Taken together, this thesis aims to enhance the understanding of the multiple pathological pathways leading to pre-eclampsia, in order to find novel pathways to improve the clinical outcome for mother and baby.

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LIST OF ABBREVIATIONS

Ang II	Angiotensin II
APCs	Antigen Presenting Cells
AT1-AA	Angiotensin II receptor 1 autoantibodies
AT1R	Angiotensin II receptor 1
ATP	Adenosine triphosphate
BCL	B-cell lymphoma
B_{Reg}	Regulatory B cells
CD	Cluster of differentiation
CHO cells	Chinese hamster ovary cells
CO	Carbon monoxide
conT	Conventional T cells
CTLA4	Cytotoxic T-lymphocyte protein 4 precursor
DAPI	4',6-Diamidino-2-Phenylindole
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ET1	Endothelin-1
FACS	Fluorescence-activated cell sorting
FGR	Fetal growth restriction
Flt-1	Fms-related tyrosine kinase 1
Foxp3	forkhead box P3
GARP	Glycoprotein A repetitions predominant
GATA-3	GATA Binding Protein 3
GITR	Glucocorticoid induced necrosis factor receptor related protein
H.PREG	Healthy pregnancy
HIF-1	Hypoxia-inducible factor 1-alpha

hiFBS	Heat inactivated fetal bovine serum
HLA	Human leukocyte antigen
HO-1	Heme oxygenase 1
HR	High risk
HSC	Hematopoietic stem cells
ICOS	Inducible T-cell COStimulator
IDO	Indoleamine 2,3-dioxygenase
IFNy	Interferon gamma
IgD	Immunoglobulin D
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IUGR	Intrauterine growth restriction
KDR	Kinase insert domain receptor
LAG3	Lymphocyte-activation gene 3
MHC	Major histocompatibility complex
MKI67	Marker Of Proliferation Ki-67
MMP2	Matrix metallopeptidase 2
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	norepinephrine
NO	Nitric oxide
Nrp-1	Neuropilin 1
PAPPA	Pregnancy-associated plasma protein A
PBMCs	Peripheral blood mononuclear cells
PCR	Protein: creatinine ratio
PET	Pre-eclampsia
PI	Propium iodide
PIGF	Placental growth factor
PIH	Pregnancy induced hypertension

RhoA	Rho-associated protein
RORc	Rorgamma
ROR-γt	RAR-related orphan receptor gamma
ROS	Reactive oxygen species
RTKs	Receptor tyrosine kinases
sFlt-1	Soluble fms-like tyrosine kinase-1
SM	Smokers
SMCs	Smooth Muscle Cells
TACTILE	T cell activation increased late expression
T-bet	T-box transcription factor
TCR	T cell receptor
TGF β	Transforming growth factor beta
T_{Reg}	Regulatory T cells
uDCs	Uterine Dendritic cells
uMCs	Uterine mast cells uNK
VEGF	Vascular endothelial growth factor

PUBLICATIONS

1. BenyahiaC, Ozen G, Orie N, **Ledwozyw A**, Louedec L, Li F, Senbel AM, Silverstein A, Danel C, Longrois D, Clapp LH, Norel X, Topal G. Ex vivo relaxations of pulmonary arteries induced by prostacyclin mimetics are highly dependent of the precontractile agents. *Prostaglandins Other Lipid Mediat.* **121,** 46–52 (2015).

2. Heimrath J, Paprocka M, Czekanski A, **Ledwozyw A**, Kantor A, Dus D. Pregnancy-induced hypertension is accompanied by decreased number of circulating endothelial cells and circulating endothelial progenitor cells. *Arch. Immunol. Ther. Exp. (Warsz).* **62,** 353–6 (2014).

3. Orie N. N., **Ledwozyw A**., Williams D. J., Whittle B. J. & Clapp L. H. Differential actions of the prostacyclin analogues treprostinil and iloprost and the selexipag metabolite, MRE-269 (ACT-333679) in rat small pulmonary arteries and veins. *Prostaglandins Other Lipid Mediat.* **106,** 1–7 (2013).

CHAPTER I : INTRODUCTION

1.1 ADAPTATION TO PREGNANCY

1.1.1 Maternal cardiovascular adaptation in healthy pregnancy

Healthy pregnancy requires the mother to make multiple physiological adaptations within the cardiovascular system. One of the main changes is vascular dilatation leading to a fall in systemic vascular resistance by almost 40%. An increase in heart rate and stroke volume leads to higher cardiac output and combined with a reduction in peripheral vascular resistance allows the blood pressure to remain stable (Robson et al. 1989). Post-implantation increases in cardiac output reach a maximum of 50% above the non-pregnant state by the 24th week of pregnancy and remains unchanged until childbirth (Rovinsky & Jaffin 1966). During normal pregnancy diastolic blood pressure decreases by 5-15mmHg, being the lowest at 22-24 weeks gestation and then towards the end of pregnancy comes back to preconception levels (Davey & MacGillivray 1988). Additionally, sodium and water retention leads to an increase in plasma volume to about 40% above non–pregnant levels (Halligan et al. 1993). All these changes contribute to an increase in uterine artery blood flow and are facilitated by remodelled and dilated spiral arteries.

During the non-pregnant state, the blood supply to the uterus is approximately 50 millilitres per minute whereas during pregnancy, at term uterine artery blood flow increases 10 fold to 500ml/min (De Swiet 1998). In order to enable an increase in placental perfusion a transformation in uterine arteries is made (Brosens et al. 1967). This is specifically achieved through changes in the spiral arteries of the uterus. Cytotrophoblast cells from the placenta invade maternal spiral arteries and replace endothelial and smooth muscles cells. This way the previously narrow arteries become converted to dilated, conduit vessels that are able to conduct much larger volumes of maternal blood to the placenta, at low pressure (**Figure 1.1**). The trophoblast invades the full depth of the decidua and into one-third of the myometrium (Pijnenborg 2010). Failure of placental invasion leads to compromised utero-placental blood flow and reduced development of the placenta. Poor placentaion is associated with pre-eclampsia and fetal growth restriction (Brosens et al. 2011).

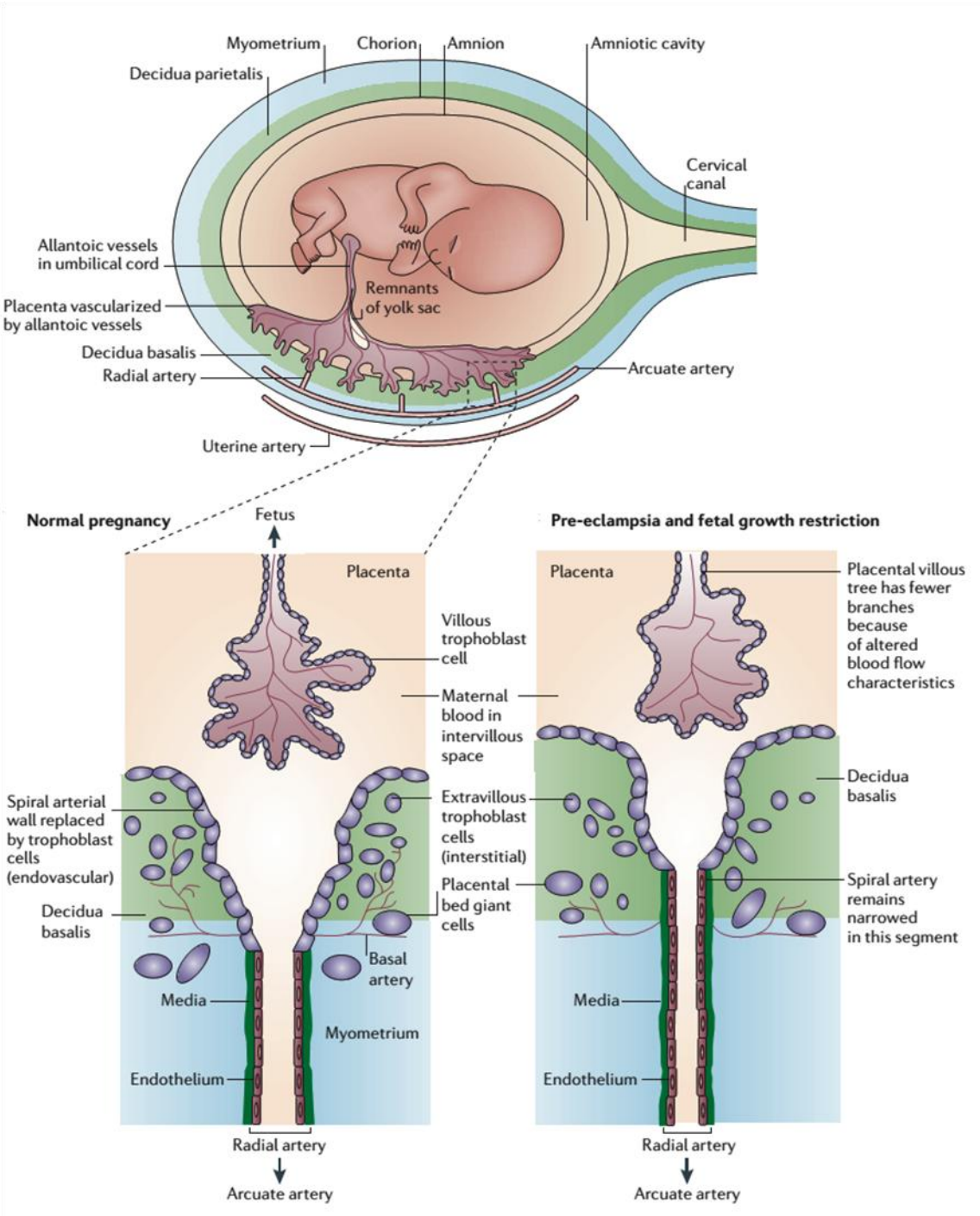

Figure 1.1 Uterine spiral arteries remodelling in human pregnancy.
In normal pregnancy (left) extravillous trophoblast cells (cytotrophoblast) from the placenta migrate into maternal spiral arteries and convert them to wide vessels that can conduct large volumes of blood into the intervillous space at low pressure. If the trophoblast invasion is shallow and spiral arteries are inadequately remodelled then the reduction in blood supply to placenta may lead to poor placental function, reduced fetal growth and pre-eclampsia (right). Copied from (Moffett & Loke 2006)

The very early stage of placentation in humans occurs in an environment with low oxygen tension (Burton et al. 2001). Decreased oxygen levels before the 10th week of pregnancy maintains trophoblasts in an immature but highly proliferative state. At this stage transforming growth factor beta 3 (TGFβ3) under the control of hypoxia-inducible factor 1 alpha (HIF-1α) inhibits trophoblast invasion (Caniggia et al. 2000). Once the spiral arteries open to the placental intervillous space and maternal blood reaches the placenta, the oxygen concentration increases. This leads to reduced expression of HIF-1α and TGFβ3 on trophoblast cells releasing trophoblast invasion into the spiral arteries. It has been suggested that if hypoxia persists beyond that point it may lead to failure in switching from proliferative to invasive trophoblast phenotype (Caniggia et al. 2000) which then could cause a shallow trophoblast invasion and inadequate spiral arteries remodelling.

1.1.2 Maternal immune adaptation in healthy pregnancy

In human pregnancy, placental trophoblast cells interact with the maternal immune system in two zones. One is at the interface between placental villous covered by syncytiotrophoblast and maternal blood. The second area of contact is within the uterine tissue, between the extravillous trophoblast (cytotrophoblast) and maternal immune cells in the decidua (**Figure 1.1**). The fetus and placenta share the same phenotype, which is half derived from the father and can be considered as a "semi-allograft". In order for the pregnancy to successfully progress the placenta has to be tolerated and protected from attack by the maternal immune system (Redman & Sargent 2010). This is partially possible because the syncytiotrophoblasts have no major histocompatibility complex (MHC) antigens on their surface making the placenta immunologically neutral (Moffett-King 2002). Additionally, cytotrophoblast cells (endovascular and interstitial) that invade maternal spiral arteries expresses a unique array of MHC class I molecules (Moffett & Loke 2006). They consist of HLA-C, HLA-E and HLA-G, but lack the typical stimulators of T-cell dependent graft rejection - HLA-A, HLA-B and HLA-D. They also do not express MHC class II molecules suggesting that trophoblast cells are not able to present their antigens directly to maternal $CD4^+$ T cells (Murphy et al. 2004; Moffett & Loke 2006)

HLA-C, HLA-E and HLA-G interact with uterine natural killer (uNK) cells (Moffett-King 2002; Carosella et al. 2008). HLA-G is known to directly act on uNK cell inhibitory receptors and through this interaction prevents the fetus from maternal immune attack. Cells of innate immune system that are important for implantation are present in the uterus even before the pregnancy. These cells usually differ phenotypically from the same cell types located in the periphery. Uterine NK cell comprise about 70% of total decidual leucocytes in comparison with 5-10% present of total leucocytes in peripheral blood (Moffett-King 2002). Uterine natural killer cells are not cytotoxic, they express high levels of CD56 marker and lack CD16, whereas peripheral NK subset is defined as $CD56^{dim}CD16^{+}$cells (Starkey et al. 1988; Kalkunte et al. 2008). Human uNK cells are known to express vascular endothelial growth factor A and C (VEGFA and VEGFC), placental growth factor (PLGF), angiopoietin 1 (Ang1) and 2 (Ang2) (Li et al. 2001), transforming growth factor beta 1 (TGFβ1) (Lash et al. 2006)as well as matrix metalloproteinase 2 (MMP2) (Naruse et al. 2009). Similarly to other tissues, the expression of VEGFA by uNK cells is induced by hypoxia (Cerdeira et al. 2013) and these cells have been shown to play a critical role in remodelling of spiral arteries and regulation of trophoblast cell invasion (Hanna et al. 2006; Rätsep et al. 2015).

Dendritic cells (uDCs) and mast cells (uMCs) are abundant in the uterus and were shown to be involved in implantation by promoting angiogenesis and trophoblast survival (Plaks et al. 2008; Woidacki et al. 2013). Uterine DCs in healthy pregnancy have an immature phenotype and produce mostly IL-10 (Kämmerer et al. 2003; Blois et al. 2004; Schumacher & Zenclussen 2014). It has also been revealed that uterine macrophages can communicate directly with trophoblast cells and by secreting pro-inflammatory cytokines promote growth and survival of trophoblast cells (Fest et al. 2007).

An enzyme, heme oxygenase-1 (HO-1) involved in degradation of heme, and its metabolite - carbon monoxide (CO) have been reported to promote implantation and placentation (Linzke et al. 2014; Zenclussen et al. 2011). HO-1 facilitates alloantigen tolerance by interrupting DCs maturation which leads to reduction in T cell responses and elevation in regulatory T cells numbers (Schumacher & Zenclussen 2014).

Therefore, heme oxygenase-1 may play an important role in influencing the maternal immune system to tolerating the fetus.

Another enzyme with immunosuppressive abilities called indoleamine-2,3-dioxygenase (IDO) is expressed in placenta and decidua (Ligam et al. 2005). IDO inhibits T cells proliferation by depriving them of essential for their development tryptophan (Terness et al. 2002; Munn et al. 1999; Lee et al. 2002) and has been shown to be essential in preventing rejection of allogeneic foetuses (Munn et al. 1998).

1.2 REGULATORY IMMUNE FACTORS

1.2.1 The immune system in humans

The principal role of the immune system is to protect the host against disease. This is achieved by the recognition and elimination of foreign antigens, formation of immunologic memory, and ability to discriminate between self- and non-self cells.

The human immune system can be divided to the innate and the adaptive immune system (Luckheeram et al. 2012). Both parts work in close collaboration. The innate immune system acts as a first line defence against pathogens. Cellular component of this system include natural killer cells, phagocytic cells such as neutrophils, dendritic cells and macrophages and pro-inflammatory cells such as eosinophils, basophils and mast cells (Delves & Roitt 2000). The adaptive immune system is divided into humoral immunity mediated by B lymphocytes and cellular immunity composed of T lymphocytes.

1.2.2 T lymphocytes

T cells recognise antigens through their highly specific T cell receptor (TCR) (Malissen et al. 2014; Klaus 2009). They are not able to detect soluble antigens so they interact with peptides associated with MHC class I or class II molecules and presented by APC cells (macrophages, dendrite cells, B cells). T cells are classified into two main populations according to the expression of their surface proteins so-called cluster of differentiation (CD) - CD4 and CD8.

$CD4^+$ T Cells recognise foreign peptides bound to MHC II, whereas $CD8^+$ T cells see those bound to MHC class I molecules (Matechak et al. 1996). Naïve $CD4^+$ T cells can differentiate into various subsets of "T helper cells" (T_H cells) depending on the cytokine milieu (**Figure 1.2**). The different subsets are T_H1, T_H2, T_H17, T follicular helper (T_{fH}) cells and regulatory T (T_{Reg}) cells (Ma et al. 2012).

$CD8^+$ T cells called cytotoxic T lymphocytes (T_C cells) can directly kill/lyse cancer cells, cells infected by pathogen, transplanted tissue or cells that are damaged (Klaus 2009).

After a primary exposure to an antigen a small proportion of $CD4^+$ T and $CD8^+$ T cells acquire memory phenotypes and are maintained for the purpose of a faster and more powerful secondary immune reaction (Sallusto et al. 1999).

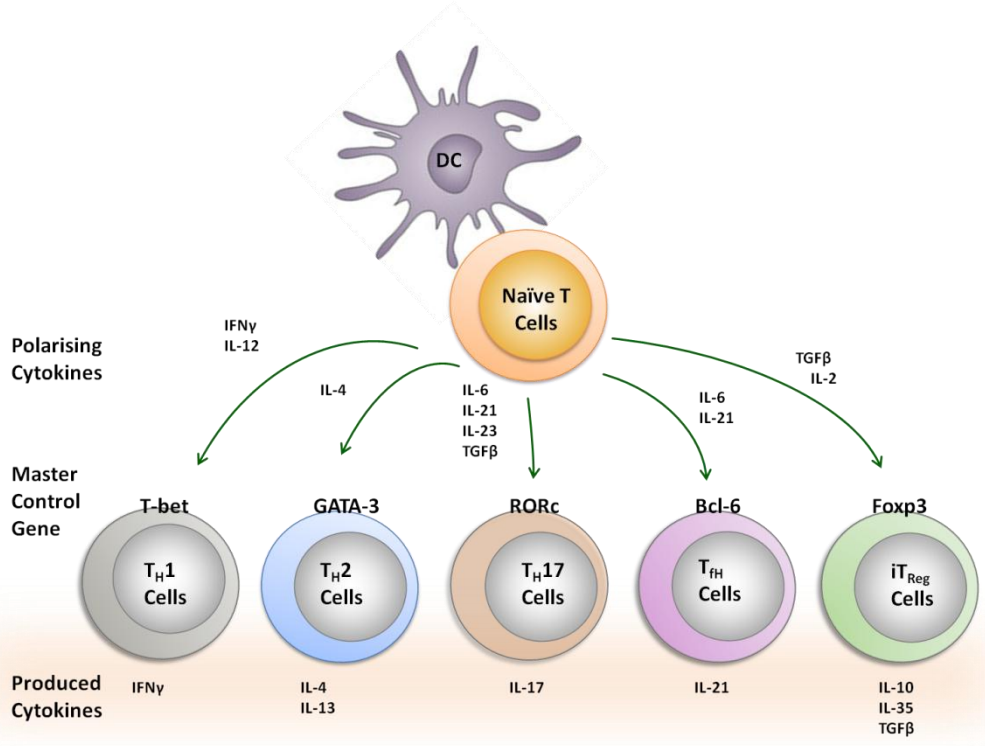

Figure 1.2 Depending on polarising conditions $CD4^+$ T cells can differentiate to different subsets.
The particular milieu triggers transcription of an individual master regulator and series of events lead to establishing new subsets cell type with specific function. Figure prepared on the basis of information provided in the review (Kaplan et al. 2015)

1.2.3 Regulatory T (TReg) cells

Memory T cells are required in order to maintain immunity whereas the role of T_{Reg} cells is to keep the immune system in check and prevent excessive inflammation and autoimmunity (Sakaguchi et al. 2007). T_{Reg} cells represent 5-10% of $CD4^+$ T cells in humans (Baecher-Allan et al. 2001) with key functions in transplantation tolerance (Waldmann et al. 2004), autoimmune diseases (Sakaguchi 2005) and aberrant immune tolerance induced by tumors (Lou 2013). Regulatory T cells are highly abundant in tumor tissues of various cancers including lung, breast, liver and pancreatic cancers (Zou 2006). Existing evidence indicates that T_{Reg} cell mediated immunosuppression may be one main mechanism allowing for escape of cancerous cells from attacks of the host's immune system (Zou 2006).

T_{Reg} cells are thought to be one of the major contributors to the tolerance of the feto-placental unit during pregnancy (Aluvihare et al. 2004; Teles et al. 2013). Recent reports indicate that T_{Reg} cells may influence placental development, by influencing the remodelling of spiral arteries and dampen the release of soluble sFlt-1 by reducing expression of sFlt-1 in placental tissue (Woidacki et al. 2015).

1.2.3.1 Regulatory T (Treg) Cells And Their Markers

Mouse T_{Reg} cells were first characterised in 1995 (Sakaguchi et al. 1995) and human T_{Reg} cells in 2001 (Levings et al. 2001; Ng et al. 2001). Both types of $CD4^+$ T cells express CD25 (interleukin 2 receptor α) marker. In 2003, Foxp3 was identified as a master regulator for mouse T_{Reg} cell development and function (Hori et al. 2003) and later confirmed that human T_{Reg} cells also express Foxp3 (Roncador et al. 2005). In addition to CD25 and Foxp3, other markers such as CTLA-4 (cytotoxic T lymphocyte antigen 4), HLA-DR, LAG3 (lymphocyte activation gene 3), GITR (glucocorticoid induced necrosis factor receptor related protein) and others have been associated with T_{Reg} cells (**Figure 1.3**) (Akbar et al. 2007; Rosenblum et al. 2015).

Unlike mice, human $CD4^+CD25^-$ effector T cells can upregulate expression of Foxp3 marker following activation (Walker et al. 2003; Yagi et al. 2004). Therefore, following immune stimulation Foxp3 might be detected in both - T_{Reg} cells and effector T cells. For this reason, Foxp3 may not be an ideal marker for identification of pure

populations of human T_{Reg} cells. It has been discovered that about 1-2% of the most strongly positive CD25 cells (Baecher-Allan et al. 2001) and Foxp3 (Gavin et al. 2006) exhibit the most immunosuppressive capacity. Since then T_{Reg} cells have been characterised as $CD4^{+}CD25^{hi}Foxp3^{hi}$ cells. Low expression levels of CD127 (the interleukin 7 receptor α chain) can be added for an even more precise T_{Reg} cell phenotype (Seddiki et al. 2006). Low expression of CD127 directly correlates with $Foxp3^{hi}$ (>90%) allowing for identification T_{Reg} cells without the need for intracellular staining for Foxp3 (Liu et al. 2006). However, even expression of these markers cannot be used with complete confidence. Upon activation, the conventional $CD4^{+}$ T cells downregulate CD127 and upregulate CD25 markers and those populations do not have regulatory properties (Mazzucchelli & Durum 2007). This is why few other molecules involved in activation, memory, trafficking and function of T_{Reg} cells have been used to characterise T_{Reg} cells with higher precision (**Figure 1.3**) (Sakaguchi et al. 2010; Thornton et al. 2010; Yadav et al. 2012).

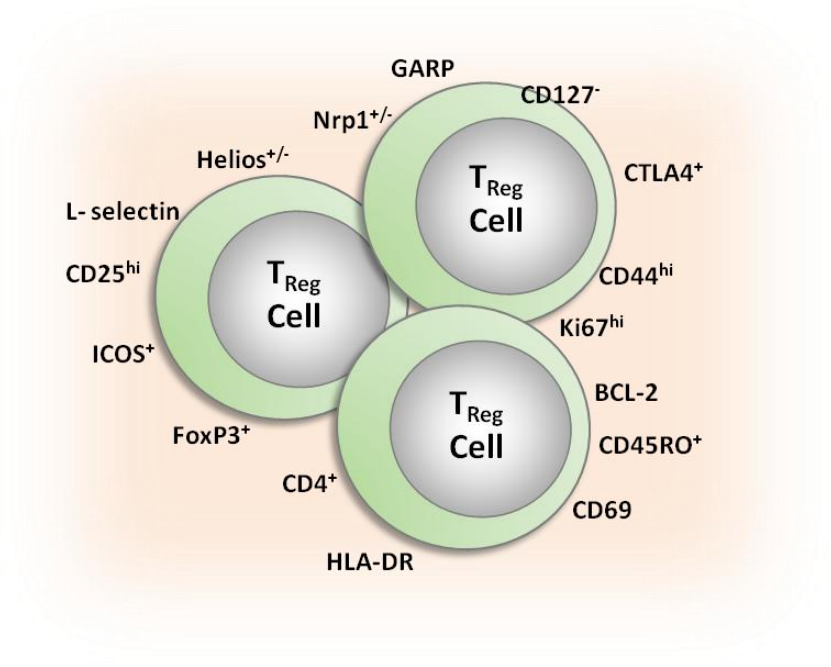

Figure 1.3 Selected membrane bound and intracellular markers used in identification of T_{Reg} cell populations.
HLA-DR (Human leukocyte antigen - antigen D related), Foxp3 (forkhead box P3), ICOS (inducible T cell co-stimulator), CD25 (interleukin 2 receptor α), L-selectin (CD62L, cell adhesion molecule), Helios (hematopoietic specific transcription factor), Nrp-1 (Neuropilin -1), GARP (Glycoprotein A repetitions predominant), CD127 (IL-7 receptor α chain), CTLA4 (cytotoxic T lymphocyte antigen 4), CD44 (cluster differentiation 44 involved in cell–cell interactions, cell adhesion and migration), Ki67 (MKI67, cellular marker for proliferation), BCL-2 (B-cell lymphoma 2), CD45RO (marker of memory T cells), CD69 (TACTILE; T cell activation increased late expression).

To date many T_{Reg} cell populations have been described. The best investigated include the $CD4^+CD25^+Foxp3^+$ T_{Reg} cells, interleukin 10 (IL-10) producing T_{Reg} cells – "T_R1"(Grazia Roncarolo et al. 2006), TGF_β producing T helper type 3 cells - "T_H3" (Faria & Weiner 2005), $CD4^-CD8^-$ (Zhang et al. 2000), $CD8^+CD28^-$ T cells (Najafian et al. 2003), $CD8^+$ $CD25^+FOXP3^+$ (Chaput et al. 2009).

Regulatory T cells can be grouped into main population of T_{Reg} cells that are capable of suppressing effector T cells and have similar phenotypes but differ in their stability and origin (Belkaid 2007). Regulatory $CD4^+$ T cells can be either natural T_{Reg} cells (nT_{Reg}) or induced T_{Reg} cells (iT_{Reg}). Natural T_{Reg} cells are thymus derived whereas iT_{Reg} (such as T_R1 and T_H3) are induced the periphery, having acquired their function following stimulation with a specific antigen, in a particular priming cytokine milieu.

Induced T_{Reg} cells mediate their inhibitory actions by secreting suppressive cytokines, such as TGF_{β} (Nakamura et al. 2001) and IL-10 (Kingsley et al. 2002). Whereas the regulatory activities of nT_{Reg} cells are independent from cytokines and involve direct interaction with antigen presenting cells (APCs) or $CD4^{+}CD25^{-}$ responder T cells (Shevach 2002). Natural and induced regulatory T cells differ in their origin and functional properties, they have a similar phenotype (Horwitz et al. 2008). Only few intra- or extracellular markers have the potential to differentiate between natural and induced regulatory T cells. These phenotypic markers expressed by thymus originating T_{Reg} cells include helios, a member of Ikaros family (Thornton et al. 2010) and neuropilin-1, a semaphorin III receptor (Weiss et al. 2012; Yadav et al. 2012).

Although the first cell populations with regulatory properties were identified within the $CD8^{+}$ T cell population (Gershon & Kondo 1970; Jandinski et al. 1976) these cells have been poorly characterised. Nonetheless, in recent years there have been more studies emerging that explore the immunosuppressive function of $CD8^{+}$ T_{Reg} cell. Regulatory $CD8^{+}$ T cells were reported to play an role in the recovery phase of experimental autoimmune encephalomyelitis (EAE) (Lee et al. 2008). Another $CD8^{+}$ T_{Reg} cells subset resembling the murine IL-10 secreting $CD4^{+}$ regulatory T cells have been shown to prevent the proliferation of naïve CD8 T cells to monocytes, immature and mature dendritic cells (Gilliet & Liu 2002).

1.2.3.2 Direct and indirect mechanisms of TReg cell mediated suppression

Direct Suppression

The known mechanisms by which T_{Reg} cells directly exert their suppressive activities include IL-2 deprivation, secretion of suppressive cytokines and cytolysis of target cells (Shevach 2009) (**Figure 1.4**).

T_{Reg} cells compete compete with effector T cells for IL-2. They express high affinity IL-2 receptor chain α (CD25) which binds and degrades IL-2 depriving effector T cells of this interleukin (Pandiyan et al. 2007). T_{Reg} cells can upregulate their expression of IL-2R much faster and to a greater extent than primed conventional T cells. However, once the conventional T (conT) cells such as $CD4^{+}CD25^{-}$ lymphocytes are

activated, they can shift the balance towards even higher IL-2 production and this way override inhibition carried out by T_{Reg} cells.

T_{Reg} cells express galectin-1 which is known to regulate cell signalling, activation, apoptosis, cytokine secretion (Rabinovich & Toscano 2009). They also secrete cytokines such as IL-10, IL-35 and TGF_{β} and this way are able to inhibit cell proliferation or even cause apoptosis of a target T cell (Grazia Roncarolo et al. 2006) (**Figure 1.4**).

Furthermore, T_{Reg} cells can mediate their suppressive actions by directly lysing their targets (Grossman et al. 2004). Upon stimulation of their receptor T_{Reg} cells release cytotoxic proteases called grazyme A (human) or grazyme B (mice) that initiate programmed $CD4^+$ and $CD8^+$ T cells death (Grossman et al. 2004).

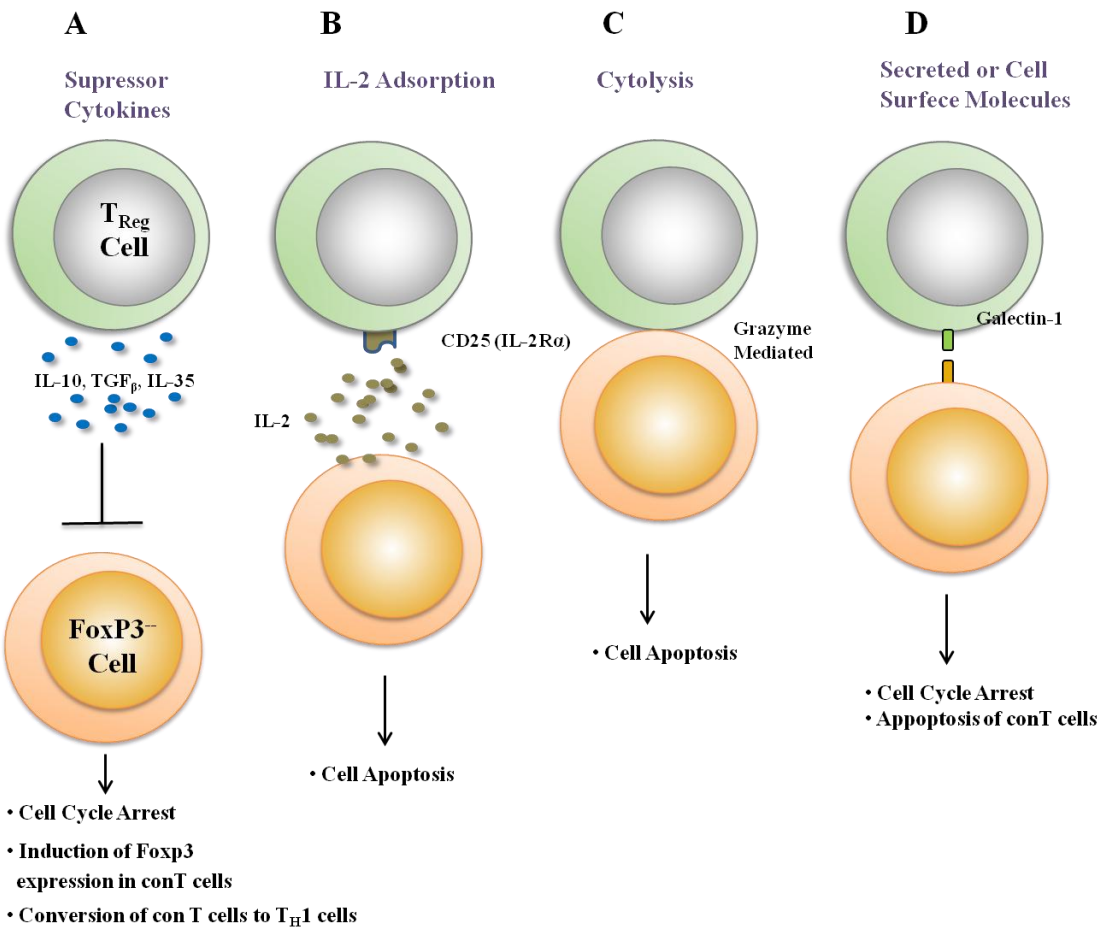

Figure 1.4 Mechanisms used by T_{Reg} cells to directly inhibit function of target T cells.
A) T_{Reg} cells inhibit function of responder T cells and myeloid cells directly by secreting pro-suppressive cytokines. B) Competition between effector cells and T_{Reg} cells for IL-2 leads to cell cycle arrest of the target cell and eventually its death. C) T_{Reg} cells have the ability to kill a target T cell by granzyme dependent mechanism. D) T_{Reg} cells are reported promote cell cycle arrest via expression of galectin-1. Figure prepared on the basis of information provided in the review by Dr Shevach (Shevach 2009).

Indirect suppression

Apart from directly mediated suppression, T_{Reg} cells can target cells indirectly by influencing the function of APCs and using them as mediators of their own actions (Tang et al. 2006).

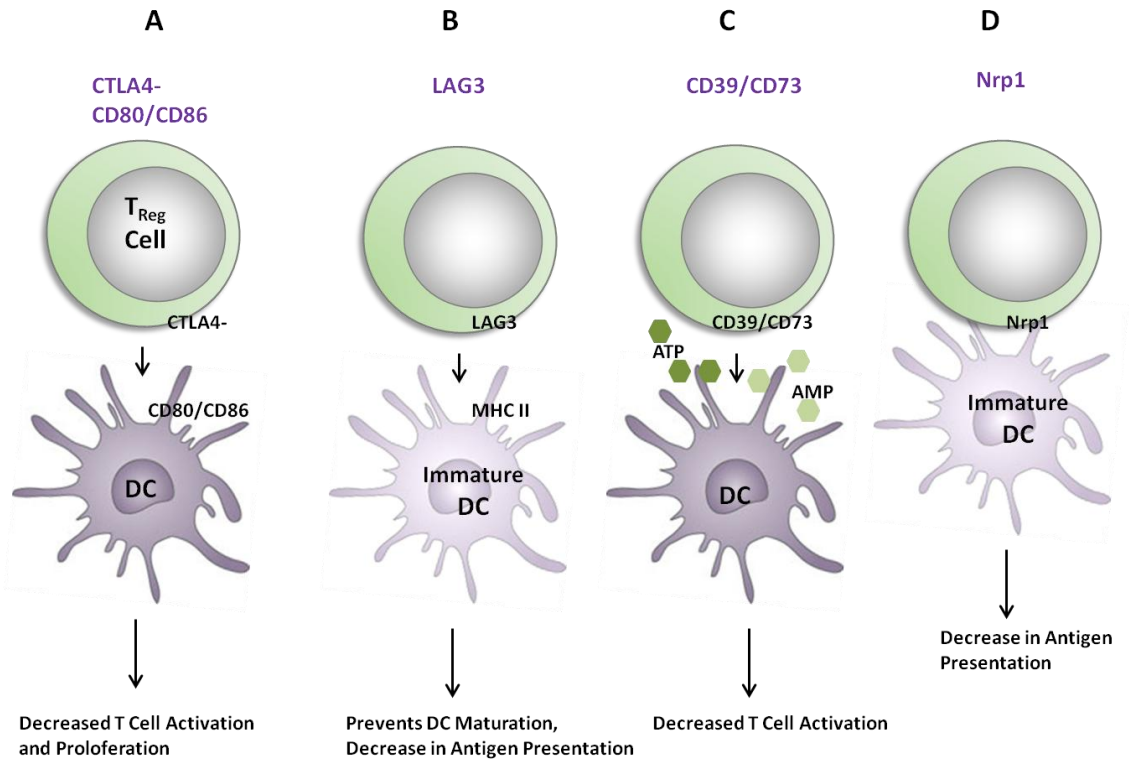

Figure 1.5 Mechanisms used by T_{Reg} cells to influence APCs and indirectly inhibit function of target T cells.
A) By interacting with APCs T_{Reg} cells down regulate their expression of CD80/CD86 costimulatory molecules and this way affect proliferation and activation of target cells. B) Binding to MHC class II on immature DC, T_{Reg} prevent them from maturation and limit their immunostimulatory capacity. C) Similarly, hydrolysis of pro-inflammatory ATP may be another mechanism used by T_{Reg} cells (Paust et al. 2004). D) Nrp1 is thought to facilitate long-lasting bonds between immature DC and T_{Reg} cells thus limits access of effector T cells to these APC. Figure prepared on the basis of information provided in the review by Dr Shevach (Shevach 2009).

T_{Reg} cells work by down-regulating expression of the CD80 and CD86 co-stimulatory molecules on dendritic cells which limits ability of DC to stimulate naïve $CD4^+$ T cells and leads to immunosuppression (**Figure 1.5**).

Additionally, lymphocyte activation gene 3 (LAG3), an immune checkpoint molecule present on human T_{Reg} cells is known to bind to MHC class II molecules on DCs and prevent them from maturation thus decreasing antigen presentation to target cells (Huang et al. 2004).

Adenosine triphosphate (ATP) present outside a cell indicates destruction of the tissue and induces pro-inflammatory effects of DCs. CD39 molecule expressed on regulatory T cells has been shown to inactivate ATP and this way inhibit APCs function (Deaglio et al. 2007).

Neuropilin 1(Nrp1) is a receptor for class III semaphorins and a co-receptor for vascular endothelial growth (VEGF) present on regulatory T cells (Campos-Mora et al. 2015; Pellet-Many et al. 2008). Nrp-1 has been shown to promote long‑lasting contacts between T_{Reg} cells and immature DCs this way limiting the ability of DCs to activate effector T cells (Sarris et al. 2008).

1.2.3.3 Regulatory B (BReg) Cells

B cells play many important roles within the immune system. They are known as antibody producing factories, antigen presenting cells and as agents involved in T cell stimulation. However, a subset of B cells with regulatory properties, B_{Reg} cells, have also been identified (Mizoguchi et al. 1997). The same as T_{Reg} cells, they are important in mediating immune homeostasis and self-tolerance (Mauri & Ehrenstein 2008). Their regulatory functions have been demonstrated in autoimmunity, experimental models of inflammation, cancer and transplantation. B_{Reg} cells can influence both the innate and adaptive parts of the immune system. Crucial to B_{Reg} cell function is IL-10, which supports T_{Reg} cell differentiation and inhibits production of various pro-inflammatory cytokines (Amu et al. 2010).

Regulatory B (Breg) Cells and Their Markers

Studies investigating B_{Reg} cells have identified various subsets of B cells with suppressive properties (Rosser & Mauri 2015)(described in **Table 1.1**).

B cells expressing high levels of CD1d molecule co-expressed together with CD5 and $CD21^{hi}$ (B10 cells) (Yanaba et al. 2008) or $CD21^{hi}$ and $CD23^{hi}$ (transitional two-marginal zone precursor cells) (Blair et al. 2009) are used to describe B_{Reg} cells in mice. They have been shown to conquer pathogen-driven and autoreactive immune dysfunctions (Kalampokis et al. 2013) while promoting the immune response to infections (Neves et al. 2010).

B_{Reg} Type	Human	Mouse	Key Features	Reference
B10 cells	$CD24^{hi}CD27^{+}$	$CD5^{+}CD1d^{hi}$	Present in blood (human), spleen (mice), produce IL-10, suppress monocytes, DCs and effector $CD4^{+}$ T cells	(Yanaba et al. 2008; Iwata et al. 2011; Matsushita et al. 2010; Horikawa et al. 2013)
Immature B cells	$CD19^{+}CD24^{hi}$ $CD38^{hi}$	-	Present in blood, at site of inflammation, produce high levels of IL-10, suppress T_H1 and T_H17 cells, are defective in patients with SLE and RA, induce T_{Reg} cells	(Flores-Borja et al. 2013; Mauri & Bosma 2012; Blair et al. 2010; Das et al. 2012)
Br1 cells	$CD19^{+}CD25^{hi}$ $CD71^{hi}$	-	Present in blood, produce IL-10 and IgG4	(Van De Veen et al. 2013)
Plasmablasts	$CD19^{+}CD24^{hi}$ $CD27^{int}$	$CD138^{+}CD44^{hi}$	Present in blood (human), dLNs (mice), produce IL-10, suppress DCs and effector $CD4^{+}$ T cells	(Matsumoto et al. 2014)
T2-MZP cells	-	$CD19^{+}CD21^{hi}$ $CD23^{hi}CD24^{hi}$	Present in spleen, produce IL-10, suppress effector $CD4^{+}$ and $CD8^{+}$ T cells, induce T_{Reg} cells	(Evans et al. 2007; Carter et al. 2011; Blair et al. 2009)
MZ cells	-	$CD19^{+}CD21^{hi}$ $CD23^{-}$	Present in spleen, produce IL-10, suppress effector $CD4^{+}$ and $CD8^{+}$ T cells, induce T_{Reg} cells	(Gray et al. 2007; Bankoti et al. 2012; Miles et al. 2012)
Plasma cells	-	$CD138^{+}MHC11^{lo}$ $B220^{+}$	Present spleen, produce IL-35 and IL-10, suppress effector $CD4^{+}$ T cells, neutrophils, NK cells	(Shen et al. 2014; Neves et al. 2010)
Tim-1^{+} B cells	-	$Tim\text{-}1^{+}CD19^{+}$	Present in spleen, produce IL-10, suppress effector $CD4^{+}$ T cells	(Xiao et al. 2012)

Table 1.1 Subsets of B cells with regulatory functions in human and mice.

The function and characteristics of B_{Reg} cells in humans have not yet been widely studied. Markers used in identifying human B_{Reg} cells and their function is described in **Table 1.2.**

Marker	Function	Refrence
CD1d	Presents lipid antigens to APCs	(Kawano et al. 1997)
CD5	Negatively regulates BCR signalling	(Kawano et al. 1997)
CD10	Marker for follicular lymphoma, Metalloprotease enzyme degrading small bioactive peptides	(Khin et al. 2003)
CD19	Involved in BCR activation	(Depoil et al. 2008)
CD24	Highly expressed on transitional and immature B cells Heat-stable antigen that provides co-stimulation to T cells	(Liu et al. 1992; Allman et al. 1992)
CD27	Marker of memory B cells, Induces B-cell activation and provides co-stimulation to T cells	(Iwata et al. 2011)
CD38	Induces B-cell cytokine production, Stimulates growth and prevents apoptosis of B cells, Indispensable for the regulation of levels intracellular calcium	(Blair et al. 2010)
CD40	Induces Fas expression and production of cytokines, Promotes B-cell proliferation and differentiation	(Blair et al. 2009)
IgD	Activated B cells	(Iwata et al. 2011)
IgM	Activates complement receptors	(Evans et al. 2007)

Table 1.2 Markers used for identification of human B_{Reg} cells.

Human B_{Reg} cells are often described as $CD19^{+}CD24^{hi}CD38^{hi}$ B cells, expressing variable levels of CD27 and high levels of CD1d molecules. They account for about 1% of peripheral blood mononuclear cells (PBMCs).

Although a primary agreement regarding the origin of regulatory $CD19^{+}CD24^{hi}CD38^{hi}$ B cells has been established, further research is required to clarify some of the existing discrepancies. Currently two models of regulatory B cell development have been proposed (Rosser & Mauri 2015). One suggests that B_{Reg} cells similarly to generated in thymus regulatory T, originate from a specific cell precursor. Whereas, the second model suggests that B_{Reg} cells take on their phenotype after exposure to a certain stimulus. The first proposed model is shown in **Figure 1.6**. To date, none of the studies investigating regulatory B cells managed to identify a unique marker for B_{Reg} cells such as Foxp3 existing for regulatory T cells.

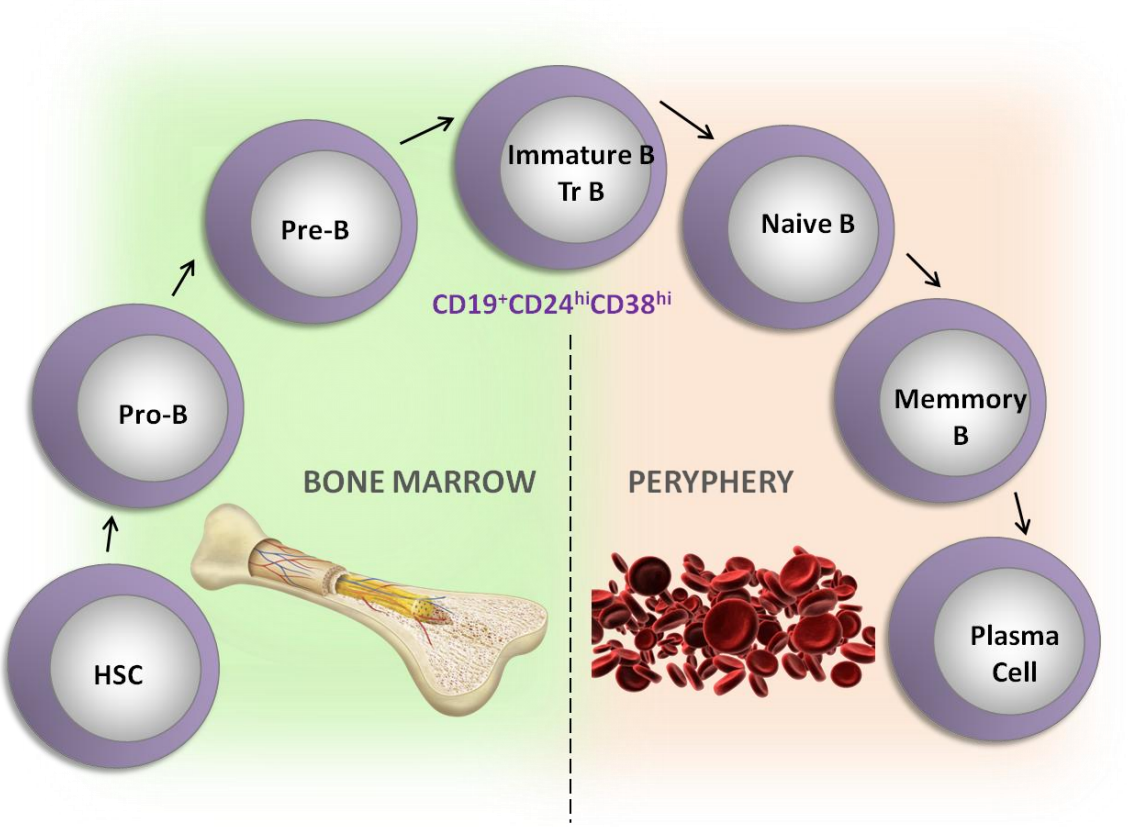

Figure 1.6 Schema representing proposed origin of regulatory B cells.
In the bone marrow, B cells development progresses from the hematopoietic stem cell (HSC), pro-B cell, pre-B cell and immature-B cell/transitional (Tr) stages. $CD19^{+}CD24^{hi}CD38^{hi}$ represent immature population of cells at the transition from bone marrow to periphery. Figure generated on the basis of information provided by (Stolp et al. 2014; Rosser & Mauri 2015; Lebien & Tedder 2008; Allman & Pillai 2008; Welner et al. 2008)

Mechanisms of Action of Regulatory B Cells

B_{Reg} cells exert their functions by various mechanisms and influence many aspects of the immune system (**Figure 1.7**). The main mode of action of B_{Reg} cells in mediated immune suppression in humans and mice involves secreting IL-10 cytokine (Mauri & Menon 2015; Iwata et al. 2011) which is further enhanced by signalling through CD80 and CD86 molecules (Blair et al. 2010). This way B_{Reg} cells are able to inhibit proliferation and dampen release of TNFα and IFNγ by $T_H1$cells (Yang et al. 2010). By reducing the levels of thymic transcription factor nuclear receptor RORγ, IL-10 secreting B_{Reg} cells suppress differentiation of T_H17 cells (Yang et al. 2012). What is important, IL-10 secreted by B cells has the ability to convert naïve as well as effector

$CD4^+$ T cells into Il-10 secreting T_{Reg} cells (T_R1) (Gray et al. 2007; Blair et al. 2009; Carter et al. 2012).

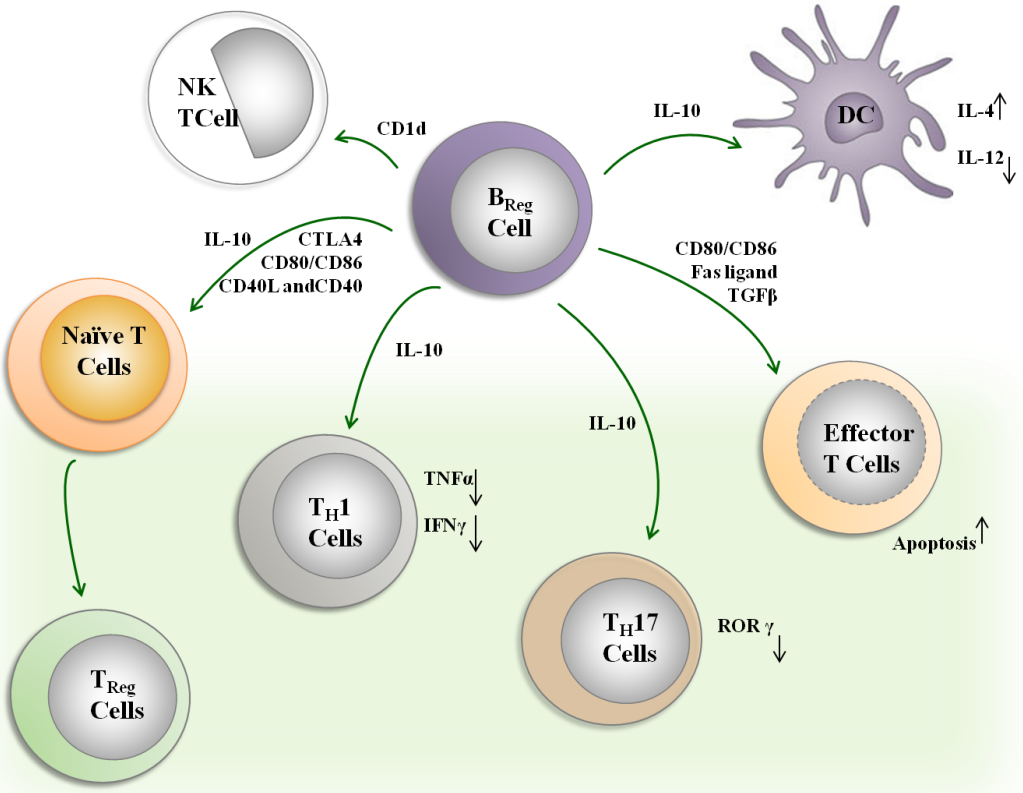

Figure 1.7 Mechanisms of B_{Reg} cells mediated suppression.
Expressed by B_{Reg} cells proteins and produced cytokine have multiple roles in regulation of immune response. IL-10 can induce T_{Reg} cells while down-regulating proliferation and differentiation of T_H1 and T_H17 cells. B_{Reg} cells can induce apoptosis of effector T cells and via CD1d pressed on their surface interact with NK T cells. Abbreviations: NK T cells, natural killer T cells; CTLA4, cytotoxic T lymphocyte protein 4; ROR-γt, transcription factor RORγt; TGFβ, transforming growth factor β; IFN-γ, interferon γ; TNF, tumour necrosis factor; T_H1, type 1 ; TH 17, type 17 T helper cells. Figure prepared on the basis of information provided in the review by Stolp et al (Stolp et al. 2014)

IL-10 produced by B cells can induce secretion and decrease production of IL-4 and IL-12 respectively by DC, hence shift the balance in T helper cells toward T_H2 cell type. (Moulin et al. 2000) Additionally, IL-10 secreting B_{Reg} cells can reduce the antigen presenting powers of DC (Matsushita et al. 2010) and dampen the production of pro-inflammatory cytokines (Saraiva & O'Garra 2010).

Interestingly, only a fraction of B_{Reg} cells actually secretes IL-10 (Nouel et al. 2014), suggesting that this small population of B cells supplies enough IL-10 to significantly influence the immune response. Although, IL-10 is their most powerful immunosuppressant, majority of B_{Reg} cells operate in an IL-10 independent manner or use a combination of various mechanisms. It has been suggested that activated B_{Reg} cells can produce TGFβ and express Fas ligand (FasL or CD95L) (Tian et al. 2001). Binding FasL with its receptor expressed by T_H1 cells leads to death of the target cell. Whereas secreted by B_{Reg} cells TGFβ reduces antigen presenting abilities of APCs. It has also been shown that TGFβ can induce Foxp3 expression on $CD4^+CD25^-$ T cells therefore make naïve T cell to acquire regulatory properties (Natarajan et al. 2012).

Regulatory B cells are currently under investigated, but their involvement in regulating the immune system led me to research their involvement in the progression of a healthy pregnancy.

1.3 CARDIOVASCULAR FACTORS IN PREGNANCY

Angiogenesis, the formation of new blood vessels from currently existing ones, is critical to the progression of a healthy pregnancy(Ferrara et al. 1996). Endogenous angiogenic factors need to be balanced to optimise placental development. An increase in pro- and decrease in anti- angiogenic components facilitates good placental development and function. Alterations in that balance are likely to lead to defective angiogenesis of the placenta, impaired uteroplacental vascularisation (Ahmed et al. 2000) and development of pre-eclampsia.

Some of the key molecules involved in the maintenance of a healthy pregnancy include vascular endothelial growth factor (VEGF), placental growth factor (PLGF) and their membrane bound receptors.

1.3.1 Vascular Endothelial Growth Factor

Vascular Endothelial Growth Factor (VEGF) induces proliferation of endothelial cells being one of the most important regulators of angiogenesis and vasculogenesis during development, as well as in the adult (Yancopoulos et al. 2000). It has the ability to upregulate the expression of endothelial cell nitric oxide synthase (eNOS) and nitric

oxide (NO) production this way contributing to vasodilation (Hood et al. 1998). When delivered to adult mice it inhibits development of dendritic cells (Gabrilovich et al. 1996), enhances production of B cells as well as generation of immature myeloid cells (Hattori et al. 2001). Importantly, VEGF also regulates hematopoietic stem cells (HSC) proliferation and survival during the hematopoietic repopulation by which VEGF influences blood-cells production (Gerber et al. 2002; Kirito & Kaushansky 2005).

The VEGF family consist of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E as well as placental growth factor (PLGF). VEGF is produced in close proximity to endothelial cells such as vascular smooth muscle cells and its expression is highly regulated by hypoxia, growth factors and oncogenes (Ferrara et al. 2003). Other cell types that express VEGF include activated macrophages (Fava et al. 1994) and trophoblast cells (VEGF-A and C) (Ferrara et al. 1992). VEGF-A acts through two related receptor tyrosine kinases (RTKs) VEGFR-1 (also known as Flt-1) and VEGFR-2 (also known as kinase domain region [KDR] or Flk-1). In contrast, PLGF and VEGF-B stimulate only VEGFR-1 receptor (Ferrara et al. 2003)(**Figure 1.8**).

Neuropilin-1 (Nrp-1) is a co-receptor for class III semaphorins (He & Tessier-Lavigne 1997) as well as for members of the VEGF family (Pellet-Many et al. 2008; Soker et al. 1998). In endothelial cells, Nrp-1 influences VEGF signalling by optimising presentation of ligands to the VEGF receptors and by stabilizing the created VEGF-VEGFR complexes.

Apart from the membrane-bound from, VEGF-R1 also exists as a soluble from (sVEGFR/sFlt-1) generated by alternative splicing of the fms-like tyrosine kinase (flt-1) gene (Kendall & Thomas 1993). Soluble Flt-1 is produced in high quantities by villous and extravillous trophoblast cells in the placenta (Clark 1998). Trophoblasts cells express much more sFlt-1 than the full length VEGFR1 (Flt-1) (Hornig et al. 2000). Circulating sFlt-1 binds free VEGF and PLGF therefore, decreases their availability to stimulate membrane bound receptors. Because of its anti-angiogenic properties, sFlt-1 is believed to pay an important role in pre-eclampsia (Helske et al. 2001).

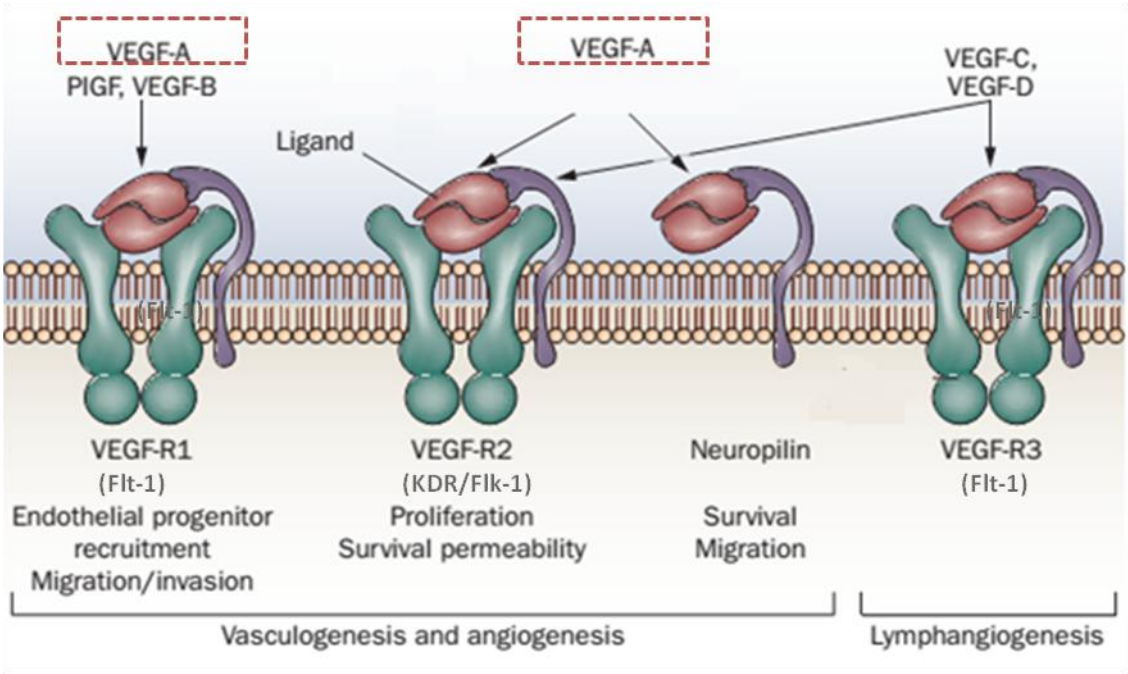

Figure 1.8 VEGF receptors family and their specific ligands mediate vasculogenesis, angiogenesis and lymphangiogenesis.
VEGF (known as VEGF-A) is the main stimulator of angiogenesis, activates Flt-1 and VEGFR-2 receptors. VEGF-C and VEGF-D stimulate lymphangiogenesisvia VEGFR-3. One subtype of VEGF, $VEGF_{165}$ binds to Neuropilin-1 (Nrp-1) and produces stronger angiogenic signals than other isoforms. Modified from (Grothey & Galanis 2009)

Measurements of VEGF concentration in blood of pre-eclamptic and healthy pregnant subjects have provided variable results that relate to difficulties in measuring free contrary to bound VEGF. The differences in free VEGF may be also unclear due to the fact that most of the measurements were performed in serum samples, not in plasma samples and the former may include VEGF released from activated platelets (Jelkmann 2001). Nevertheless, the general opinion is that VEGF levels are lower or unchanged in pregnancies affected by pre-eclampsia when compared to normotensive pregnancy (Levine et al. 2004; Tsatsaris et al. 2003)

1.3.2 Placental Growth Factor

Placental growth factor (PLGF) can modulate both endothelial cell function and trophoblast development (Khaliq et al. 1999; Athanassiades & Lala 1998; Torry et al. 2003). PLGF is a member of VEGF family and is closely related to VEGF-A (Ferrara & Davis-Smyth 1997). The PLGF name is derived from its first cloning from a human placental cDNA library (Maglione et al. 1991).

PLGF stimulates angiogenesis by binding and activating VEGFR-1 receptor. It plays an important role in many biological effects by influencing a wide range of different cell types. PLGF not only stimulates growth and maturation of blood vessels by acting on endothelial and smooth muscle cells (SMCs) but also recruits pro-angiogenic cell types such as macrophages to the site of an interest (Dewerchin & Carmeliet 2012). PLGF is known to induce vasodilatation, increase the proliferation of fibroblasts and SMCs, and stimulate the growth of collateral vessels (Bellik et al. 2005; Yonekura et al. 1999). PLGF has also been shown to inhibit the differentiation of dendritic cells as well as attract and activate macrophages that release lymphangiogenic and angiogenic factors (Selvaraj et al. 2003). PLGF is known to potentiate the bioactivity of VEGF *in vitro* and *in vivo* (Park et al. 1994) by synergising with VEGF and promoting angiogenesis (Carmeliet et al. 2001).

In the non-pregnant state PLGF can be detected in subjects affected by tumours (Han et al. 2014; Donnini et al. 1999). In this regard PLGF has a similar role in the development of invasive tumours as it does in the placenta (Carmeliet et al. 2001). Conversely to non-pregnant state, PLGF is measurable in high serum levels in healthy pregnancy (Chaiworapongsa et al. 2005). It is also highly expressed within the placenta (Khaliq et al. 1996; De Falco 2012). PLGF stimulates trophoblast growth and differentiation, suggesting a potential role in mediating trophoblast invasion of spiral arteries during placentation (Vuorela et al. 1997). PLFG promotes growth and development of the placenta thus low availability of that protein may contribute to poor placental development and impaired function (Arroyo et al. 2004).

1.3.3 Soluble Vascular Endothelial Growth Factor Receptor

As mentioned previously, soluble vascular endothelial growth factor (VEGF) receptor-1 (sFlt-1) freely circulates in the bloodstream as a truncated version of the membrane bound VEGF receptor. Although sFlt-1 can be produced by peripheral blood macrophages (Vuorela et al. 2009), mononuclear cells (Rajakumar et al. 2005), endothelial cells (Hornig et al. 2000) and vascular SMCs (Sela et al. 2008), the primary source for sFlt-1 in pregnancy is the placenta (Maynard et al. 2003).

Soluble Flt-1 binds both of VEGF and PLGF and thereby inhibits their biological functions through membrane bound receptors (Kendall & Thomas 1993). Recently, it has been suggested that apart from having an anti-angiogenic role, sFlt-1 can also act as a pro-inflammatory cytokine by priming endothelial cells and enhancing their sensitivity to other circuiting pro-inflammatory factors such as TNFα (Cindrova-Davies et al. 2011).

Soluble Flt-1 is found in high concentration in serum of women with pre-eclampsia (Levine et al. 2004). Due to its ability to block VEGF and PLGF mediated angiogenesis, and to promote inflammation, sFlt-1 is likely to mediate many of the features of pre-eclampsia. This includes reduced placental development and impaired maternal endothelium function.

1.4 PRE-ECLAMPSIA

Pre-eclampsia is a heterogeneous, multi-system syndrome of pregnancy defined by hypertension and proteinuria. Specifically, the gestational onset of hypertension exceeding 140/90 mmHg and proteinuria more than 0.3g/ 24h, arising de novo after the 20th week of gestation in a previously normotensive woman (Brown et al. 2001). If proteinuria is absent then the diagnosis is based on the presence of hypertension and confirmed systemic disease identified as elevated liver enzymes, renal insufficiency in the absence of other renal disease, and platelet count below 100,000/microliter, pulmonary odeama, cerebral or visual disturbances (Roberts 2013).

This unique disorder complicates approximately 5% of all pregnancies and is a major cause of maternal and perinatal mortality and morbidity worldwide with over 50,000 women and even more newborns dying from pre-eclampsia each year (Lozano et al. 2011; Ghulmiyyah & Sibai 2012). Pre-eclampsia accounts for around 12% of all maternal deaths during pregnancy and the puerperium (Bellamy et al. 2007).

Severe pre-eclampsia is associated with increased risk of maternal death and complications such as acute liver or renal failure, liver haemorrhage, convulsions and stroke (Ghulmiyyah & Sibai 2012). These complications are usually seen in early onset pre-eclampsia (before 32 weeks of pregnancy). Women with a history of pre-eclampsia

have an increased risk of incidence of cardiovascular disease (Bellamy et al. 2007). In later life they are almost four times more likely to develop hypertension, have an increased risk of ischaemic heart disease, venous thromboembolism and stroke (Bellamy et al. 2007). However, there seems to be no association between pre-eclampsia and future incidence of cancers.

Currently, the only existing cure for pre-eclampsia is the delivery of the placenta and therefore baby.

It is difficult to predict who may be affected by pre-eclampsia and therefore, to prevent this condition developing. Controlled cohort studies showed that increased risk of pre-eclampsia exist in women who previously had pre-eclampsia, have chronic chypertension (booking blood pressure > 140/90mmHg), pre-existing diabetes, multiple pregnancy, nulliparity and family history of pre-eclampsia, raised body mass index, or maternal age ≥ 40 (Duckitt & Harrington 2005).

1.4.1 Classification of pre-eclampsia

Pre-eclampsia can be classified as early onset, prior to 34 weeks, mid-onset between 34 and 37 weeks, and late onset, after 37 weeks (NICE 2010). Although, several other thresholds have been suggested to classify early onset of pre-eclampsia including < 32 weeks or < 36 weeks, less than 34 weeks is most commonly used (Soto et al. 2012; von Dadelszen et al. 2003).

Early onset pre-eclampsia is often associated with fetal growth restriction (FGR) and abnormal uterine artery Doppler (Murphy & Stirrat 2000; Ness & Sibai 2006). In contrast, women who develop late onset pre-eclampsia are less likely to have FGR, they have normal or slight increased uterine artery arterial resistivity index, and more favourable maternal and neonatal outcomes (Ness & Sibai 2006; Sibai et al.).

1.4.2 Pathogenesis of pre-eclampsia

The pathogenesis of pre-eclampsia is only partially understood. Before the onset of the clinical syndrome, reduction in placental perfusion due to poor remodelling of maternal spiral arteries results in placental hypoxia and ischemia. Under these circumstances it is thought that the placenta either releases factors into the systemic

circulation that results in maternal endothelial dysfunction or creates an environment that leads to endothelial dysfunction (Rodgers et al. 1988), oxidative stress and inflammation (Redman & Sargent 2009) as well as activation of the haemostatic system (Cadroy et al. 1993). Circulating factors include reactive oxygen spices (ROS) (Raijmakers et al. 2004), inflammatory cytokines such as tumour necrosis factor (TNF-α) (Szarka et al. 2010), placental microparticles (VanWijk 2002), soluble VEGF receptor (soluble fms-like tyrosine kinase-1, sFlt) (Maynard et al. 2005), soluble endoglin (sEng) (Venkatesha et al. 2006), endothelin 1 (ET1) (Taylor et al. 1990) and agonistic autoantibodies to the angiotensin II receptor (AT1-AA) (Wallukat et al. 1999). However there is no clear evidence that all these factors come from the placenta.

An increased expression of hypoxia-inducible factor 1-alpha (HIF-1α) and HIF-2α proteins have been identified in the placentas of women with pre-eclampsia (Rajakumar et al. 2004). Additionally, over-expression of HIF-1α in the placenta results in hypertension, proteinuria and fetal growth restriction in mice (Tal et al. 2010). These finding support the role of hypoxia in pre-eclampsia development. However, direct evidence is lacking as current technology makes it very difficult to investigate the environment within placenta of ongoing pregnancies in women who are destined to develop pre-eclampsia. HIF-1α expression is known to be upregulated not only by hypoxia but also by various inflammatory factors (Redman & Sargent 2009). These include cytokines, vasoactive peptides, tumour necrosis factor (TNF) and reactive oxygen spices (ROS).

Oxidative stress occurs when the production of antioxidants cannot overcome presence of reactive oxidant spices (ROS) (Burton & Jauniaux 2011). Oxidative stress is one of the key mechanisms indicated in the pathogenesis of pre-eclampsia (Cindrova-Davies et al. 2007). It has been shown to induce inflammation increasing the release of proinflammatory cytokines and chemokines. The source of oxidative stress in pre-eclampsia is thought to originate from hypoxic placental environment which occurred due to impaired spiral arteries remodelling (Burton & Jauniaux 2011). Different source of free radicals is also a free heme, the product of degradation of red blood cells. Protection from damaging effects of heme exists in the form of enzyme heme oxygenase (HO) that degrades heme to bilirubin, free iron and carbon monoxide (CO)

(George & Granger 2013). When bilirubin is known to have anti-oxidant properties, CO is a potent vasodilator, has anti-apoptotic properties and has been shown to promote angiogenesis (George & Granger 2013). Pregnant mice that are partially deficient in HO-1 form, develop hypertension, have small placentas and what is more have elevates plasma concentration of circulating sFlt-1 (Zhao et al. 2009). These all features are also key factors in pre-eclampsia. Recent reports suggest that pre-eclampsia, intrauterine growth restriction and miscarriage are all associated with diminished presence of HO-1 in the placenta (Schumacher & Zenclussen 2014) .

Interestingly, it has been revealed that prolonged exposure to paternal antigens present in seminal fluid prior to fluid prior to conception is associated with a reduced risk of pre-eclampsia (Saftlas et al. 2014). This finding suggests a clear relationship between impaired immune adaptation to pregnancy and pre-eclampsia.

Pre-eclampsia and fetal growth restriction (FGR, a fetus that has not achieved its genetically determined growth) have been shown to have similar placental pathology and the majority of women with pre-eclampsia have growth restricted fetuses. However, not all subjects with FGR develop pre-eclampsia and not all women with pre-eclampsia have small babies. To date it has not been explained why not all women who develop FGR also develop pre-eclampsia. Similarly, women who develop pregnancy induced hypertension have normally grown babies and lack typical pre-eclampsia-like symptoms such as proteinuria. It seems to be that hypertension is not the cause of preeclampsia. It is more of an adaptive response to the ischaemic placental environment.

1.4.3 Pre-eclampsia and increased sensitivity to Angiotensin II

It has been established that normotensive pregnant women are much more resistant to the effect of exogenously infused Angiotensin II (Ang II) than non-pregnant or pre-eclamptic subjects (Abdul-Karim & Assalin 1961; Grant et al. 1973). In vitro studies shown that rabbit arteries exposed to serum of pre-eclamptic women exhibit 2.9-fold increase in sensitivity to Ang II, and 1.6–fold increased sensitivity to norepinephrine (NE), than vessels exposed to serum from healthy pregnant patients (Tulenko et al. 1987). This cannot be associated with increased levels of Ang II as pre-

eclamptic patients have less circulating Ang II compare to those with healthy pregnancy(Massani et al. 1967)

To date the, this enhanced reactivity to Ang II in pre-eclampsia is not fully understood and can be considered as one of the most typical features related to pre-eclampsia. Recent studies show that this may have many grounds including Ang II mediated increase in endothelin-1 (ET-1) production (Wenzel et al. 2011), enhanced RhoA kinase pathway activation (Mishra et al. 2011), Angiotensin II receptor 1 (AT1) heterodimerization (AbdAlla et al. 2001), alteration in their desensitization (Zhang et al. 2013) or actions of Angiotensin II type 1-Receptor Autoantibody (AT1-AA)(Wenzel et al. 2011).

1.4.4 Angiotensin II receptor 1 autoantibodies and their role in pre-eclampsia

Women with pre-eclampsia appear to develop agonistic autoantibodies (AT1-AA) which activate the AT1 receptor and mimic the physiological actions of Ang II (Wallukat et al. 1999). The binding of the AT1-AA to the seven amino acid sequence of the second extracellular loop of the angiotensin II type 1 receptor stimulates a response from rat neonatal cardiomyocytes which can be blocked by administration of an AT1 receptor antagonist (Wallukat et al. 1999).

AT1-AA are detectable in serum of all women with reduced placental blood flow including those with pre-eclampsia, fetal growth restriction and patients who despite impaired uterine perfusion have a healthy pregnancy outcome (Walther et al. 2005). AT1-AA are not evident in healthy pregnant subjects with normal placentation. AT_1-AA levels closely correlate with severity of the pre-eclampsia (Siddiqui et al. 2010), but it is unclear at which point of gestation they appear for the first time.

Ang II has been suggested as a key regulator of sFlt-1 production and secretion during normal, healthy pregnancy (Zhou et al. 2007). Ang II through the AT1 receptor induces time and concentration dependent rise in sFlt-1 in human trophoblast cells. An additional activation of AT1 receptors by AT1-AA is suggested to act as the source of high levels of sFlt-1 in pre-eclampsia (Zhou et al. 2008). Several *in vitro* and *in vivo* studies suggest that in pregnancy AT1-AA can modulate molecules such as: intracellular Ca^{2+}(Thway et al. 2004), ET-1 (Wenzel et al. 2011), sFlt-1 (Lamarca et al.

2012), soluble Endoglin (sEng) (Taylor et al. 1990), inflammatory cytokines (TNFα, IL-6), ROS and NADPH oxidase (Parrish et al. 2011).

Angiotensin II type 1-Receptor Autoantibodies are considered to contribute to the development of pre-eclampsia by composing an important link between placental ischemia and endothelial dysfunction (Siddiqui et al. 2010).

1.4.5 Cigarette Smoking and Pre-eclampsia

An interesting, inverse association between cigarette smoking during pregnancy and incidence of pre-eclampsia have been reported (Conde-Agudelo et al. 1999; Llurba et al. 2013; England & Zhang 2007). This observation was expanded by ex vivo work in human placental explants culture exposed to cigarette smoke. Obtained results suggested that elevation in PIGF and decrease in sFlt-1 levels may be one of the mechanisms involved in protective effects of smoking (Mehendale et al. 2007). Latter findings confirmed that PLGF/sFlt-1 ratio is also higher in maternal blood of women who smoke during pregnancy (including those with abnormal uterine perfusion) compare with pregnant non-smokers (Llurba et al. 2013). Interestingly, the immunosuppressive regulatory Treg cells levels were also found to be higher in non-pregnant smokers than in non-smokers (Hampras et al. 2012).

Taking those findings together it can be suggested that smoking during pregnancy acts on some aspects of the immune or cardiovascular system and lowers risk of pre-eclampsia development.

At the same time, babies delivered by women who smoked thorough pregnancy have significantly lower birth weight when compared with offsprings whose mothers did not smoke during pregnancy and are more likely to suffer from fetal growth restriction than their non-smoking colleagues (Andersen et al. 2009; Vedmedovska et al. 2010; Jauniaux & Burton 2007)

1.5 THE AIMS OF THEIS

This thesis investigates the interplay between maternal phenotype and regulatory innune and cardiovascular factors.

The aims are:

1. To determine whether the profile of T_{Reg} and B_{reg} cells in women destined to develop pre-eclampsia is different from women destined to have a healthy pregnancy
2. To determine whether women who smoke are protected from pre-eclampsia by an alteration in the level of their T_{Reg} and B_{reg} cells or PIGF and sFlt-1.
3. To determine if B_{reg} cells are functionally different in women destined to develop pre-eclampsia.
4. To correlate immunoregulatory cells with maternal blood pressure.
5. To correlate T_{Reg} and B_{reg} cells with angiogenic factors.
6. To detect autoantibodies to anngiotensin II receptor 1 in women with pre-eclsmpsia.

CHAPTER II: MATERIALS AND METHODS

2.1 RECRUITMENT OF STUDY PARTICIPANTS

Approval for tissue collection was obtained from South East NHS Research Ethics Committee (REC reference: 13/LO/0287). I recruited 196 pregnant women who were inpatients or outpatients at the University College London Hospital (UCLH). Written consent was obtained from all the participants prior to entering the study. Thirty women were recruited once they developed the condition (pre-eclampsia [PET], fetal growth restriction [FGR], pregnancy induced hypertension [PIH]). They were identified on the Antenatal Care Unit as well as when attending antenatal clinics. In the prospective part of the study 166 subjects took part. All patients received written information about the study at their first midwife appointment. Patients of low risk of pre-eclampsia and the majority of patients who smoked during pregnancy were then recruited while attending their early pregnancy ultrasound scans at the Antenatal Unit. Women at high risk of pre-eclampsia were also recruited at their 1st trimester fetal scan and additionally at a 20-24 weeks fetal ultrasound scan appointment, as well as at any time before 30 weeks of pregnancy while attending high risk pregnancy management clinics lead by Dr David Williams and Dr Melissa Whitten. Those extra sources of recruitment were identified in order to “enrich” the cohort for patients at risk of pre-eclampsia.

High blood pressure at booking, maternal history of pre-eclampsia, pre-existing chronic hypertension and pre-eclampsia in previous pregnancy were inclusion criteria for the high risk group. The exclusion criteria for the entire study included multiple pregnancies, history of multiple miscarriages, pre-existing autoimmune conditions such as lupus or rheumatoid arthritis, thyroid problems, asthma, diabetes and STDs.

All patients had their individual study number assigned and collected blood samples were coded accordingly with that number.

At the each time when the blood sample was taken, the blood pressure measure was recorded and performed analysis protein content in urine (dipstick).

The recruited subjects did not receive any payment for participating in this study but were able to contact me directly if needed any guidance. Clinical characteristics of all participants were recorded and are described in table **Table 3.1, Chaper III.**

2.1.1 Definitions of Study Groups of Interest

1. Pre-eclampsia (PET): diagnosed as new onset of hypertension ≥ 140/90mmHg after 20th week of pregnancy accompanied by proteinuria greater than 0.3g/24 hours, a urine protein: creatinine ratio (PCR) > 30mmol/L and/or abnormal blood results indicating elevated liver enzymes, renal insufficiency or thrombocytopenia (NICE 2010).
2. Pregnancy Induced Hypertension (PIH): diagnosed as new hypertension presenting after 20 weeks of gestation without significant proteinuria (NICE 2010).
3. Fetal Growth Restriction (FGR): also known as Intrauterine Growth Restriction (IUGR) was confirmed if the fetal weight was below the 10th percentile for gestational age (Royal College of Obstetricians and Gynaecologist. 2013), in the absence of fetal or maternal disease, including infection, chromosomal abnormalities.
4. High Risk of Pre-eclampsia (HR): women with chronic hypertension, low PAPPA level (<0.3MOM), previous pre-eclampsia, nulliparity and family history of pre-eclampsia.
5. Smokers (SM): Women who smoked from before conception and continued to smoke at least 2 cigarettes per day throughout pregnancy. Smoking status was confirmed by measures of serum cotinine levels.
6. Healthy Pregnancy (H.PREG): normotensive pregnant women who had an uncomplicated pregnancy. Women who had an unanticipated complicated pregnancy outcome were excluded.

2.2 COLLECTION OF BLOOD SAMPLES

All peripheral blood samples were collected either by me or by qualified hospital staff. Gold top BD Vacutainer® SST™ tubes were used for samples destined to be processed for serum and green top BD Vacutainer tubes contain a sodium heparin anticoagulant for Peripheral Blood Mononuclear Cells (PBMCs). Up to 30ml of blood

was collected at a time. Samples in SST tubes were stored on ice and those with heparin in room temperature for up to 6 hours before processing.

2.3 PROCESSING BLOOD FOR SERUM

Blood collected in serum separating tubes (SST) were centrifuged at 1500g at 4^{o}C for 15 minutes, aliquoted into 1.8ml cryovials and frozen at -80^{o}C until needed.

2.3.1 The enzyme linked immunosorbent asssays (ELISA)

I used the enzyme-linked immunosorbent assays (ELISA) to assess the levels of circulating in serum cardiovascular factors. It is a plate based technique allowing for detection and quantification of proteins, peptides antibodies and hormones.

In order to assess prospective levels of serum soluble fms-like tyrosine kinase (sFlt-1) and Placental Growth Factor (PlGF) throughout pregnancy I used commercially available ELISA Quantikine® kits (R&D Systems, USA). Angiotensin 1 receptor antibody (AT1-AA) levels were measured using an ELISA kit from MyBioSource, USA. AT1-AA serum levels were also investigated by in-house made ELISA. All assays employed a quantitative sandwich enzyme immunoassay technique for measuring a factor of interest in the serum samples. In the principle, samples are incubated in a microplate pre-coated with monoclonal antibody directed against measured protein or chemical. Detection of the complex antibody-measured factor is possible after a substrate solution is added allowing for a colour to develop in proportion to the amount the measured factor bound in the initial step of the assay.

A solid phase competitive Cotinine ELISA kit (Abnova, Taiwan) was used to determine cotinine content in serum samples. In this assay, Cotinine enzyme and serum samples are added onto the 96 well plate pre-coated with anti-Cotinine antibody. Cotinine form serum and the HRP-conjugated enzyme compete for cotinine binding sites. Unbound factors are then removed by washing. Once the substrate is added, the intensity of the colour that develops is inversely proportional to Cotinine concentration in that sample.

All assays were preformed in accordance with protocols provided with the kits.

2.3.2 Human Soluble fms-like Tyrosine-1 (sFlt-1) ELISA

All components of the kit were brought to room temperature before use. Powdered sFlt-1 substrate was reconstituted with deionised water to obtain stock concentration of 20 ng/ml. Once the substrate was fully dissolved, half dilutions ranging from 2000 pg/ml to 31.2 pg/ml were prepared in Calibrator Diluent RD6-10. The same diluent was used as the zero standard (0pg/ml). 100 µl of Assay Diluent RD1-68 was then added into each of the well of a 96 well plate and followed by 100 µl of standard or serum sample. The plate was then covered with an adhesive strip and incubated at room temperature for 2 hours on a horizontal orbital shaker set to 500rpm. Before adding 200 µl of Human VEGFR1 Conjugate the plate was washed 4 times with Wash Buffer. Following 2 hours long incubation at the same condition as previously the plate was washed and 200 µl of Substrate Solution added. After 30 minutes incubation the reaction was terminated by adding 50 µl of Stop Solution. Optical density of each well was determined using a microplate reader set to 450 nm with correction wavelength at 540 nm. The minimal detectable dose of sFlt-1 was 3.5pg/ml.

2.3.3 Human Placental Growth factor (PLGF) ELISA

The substrate provided with the PLGF ELISA kit was resuspended in 1 ml of Calibrator Diluent RD6-11 and produced stock concentration of 1000 pg/ml. Further dilutions were then performed in half concentrations ranging from 500pg/ml to 15.6 pg/ml were achieved. Calibrator Diluent served as the zero standard (0pg/ml). 100 µl of Assay Diluent RD1-68 was then added into each of the well of a 96 well plate and followed by 100 µl of standard or serum sample. The plate was then covered with an adhesive strip and incubated at room temperature for 2 hours on a horizontal orbital shaker set to 500rpm. After that time 4 washes with Wash Buffer and 200 µl of human PLGF Conjugate were applied. After 2 hours of incubation at the same condition as previously the plate was washed and 200 µl of Substrate Solution added. Following 30 minutes incubation in dark at room temperature the reaction was terminated by adding 50 µl of Stop Solution. Optical density of each well was determined using a microplate reader set to 450 nm with correction wavelength at 540 nm. The minimal detectable dose of PLGF was 7pg/ml.

Serum concentrations of PLGF were calculated using a standard curve generated from the range of substrate readings.

2.3.4 Serum Cotinine ELISA

Ten microliters of serum samples and ready to use standards (concentrations range from 0 ng/ml to 100 n /lm) were pipetted into the 96-well plate provided. Hundred microliters of enzyme conjugate was then added to each of the wells and incubated in dark at room temperature for 1 hour. Wells were then washed 6 times with distilled water and 100 µl of Substrate reagent added. Following 30 minutes of incubation the reaction was stopped by adding 100 µl of Stop Solution to each ell. Absorbance measurements at 450 nm were performed using multiplate reader. The minimal detectable level of PLGF was 7pg/ml.

2.4 QUANTITATIVE DETERMINATION OF HUMAN AT1-AA CONCENTRATIONS IN SERUM.

2.4.1 Serum AT1-AA detection by commercially available ELISA kit.

All elements of the ELISA kit were brought to room temperature before using. Provided Standard was reconstituted in Sample Diluent to yield stock concentration of 20 ng/ml. Further dilutions were performed in half concentrations ranging from 10 ng/ml to 0.312 ng/ml were achieved. Sample Diluent served as zero standard (0pg/ml). Hundred microliters of standard or serum sample was added per well, covered with adhesive strip and incubated for 2 hours 37^{o}C. After decanting the liquid 100 µl of Biotin Conjugate was pipetted into each well and plate incubated for another hour at 37^{o}C. Plate was then washed with Wash Buffer followed by addition of 90 µl of HRP-avidin per well. After incubating at 37^{o}C for 15-30 minutes, 50 µl of Stop Solution was added into each of the wells. Microplate reader set to 450 nm with correction wavelength at 540 nm was used to determine the optical density of each sample.

The minimum detectable dose of AT-1AA for this assay was 0.078pg/ml.

2.4.2 Serum AT1-AA detection by in-house made ELISA.

Two peptides corresponding to second extracellular loop of Angiotensin II receptor 1 (AT1-R) (165-191, IHRNVFFIENTNITVCAFHYESQNSTL) were synthesised by Cambridge Peptides (Cambridge, UK) and GL Biochem Ltd (Shanghai, China). The protocol for this assay was designed on the basis of previously published methods for in-house prepared sandwich ELISA for the detection of autoantibodies to G-protein-coupled receptors (Fu et al. 1994; Zhang et al. 2013) with the help of Dr Charis Pericleous form Prof Anisur Rahman's labolatory, UCL.

Flat-bottom 96 well micro-titer plates with surface treatment providing high (MaxiSorp^{TM}) and medium (MediSorp™) affinity to molecules with mixed hydrophilic-hydrophobic domains were used in the assays (NUNC, Denmark). Plates were coated with 100 µl of AT1-R peptide in dilutions ranging from 1 µg/ml to 20 µg/ml prepared in 100 mmol/l of Na_2CO_3 (Sigma, UK), pH = 9 –11 and stored overnight at 4^{o}C.

The next day plates were washed with PBS-T buffer made of PBS (pH=7.4) and 0.1% (v/v) Tween 20 (Sigma Aldrich, UK). Wells were then saturated with 100 µl of 2% albumin bovine (wt/v) (Sigma Aldrich, UK) in PBS for 1 hour at 37^{o}C. After multiple washes with PBS-T, serum or isolated IgG dilutions in PBS were pippeted onto the microtiter plates and incubated for another 1 hour at 37^{o}C. As a commercially available AT-AA standard does not exist, uncojugated human IgG (Sigma, UK) was purchased in order to determine the detection sensitivity of the assay. Commercial IgG was added at the same stage as serum isolated IgG samples. After the incubation and washes, 100 µl of goat anti-human IgG antibody Alkaline Phosphatase conjugated (Sigma Aldrich, UK) (1:7000 dilution in PBS or PMT) were added into each well and incubated for 1 hour at 37^{o}C.

Following multiple washes with PBS-T buffer, 100 µl of Alkaline Phosphatase Substrate (pNPP Microwell Substrate System, KPL, USA) was then pipetted into each of the wells. Optical density at 405 nm was measured using and micro-plate reader following 15, 30 and 45 minutes incubation.

2.5 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)

Collected blood samples were diluted 1:1 with RPMI 1640 containing L-glutamine (Lonza, Belgium) and supplemented with 1% penicillin and streptomycin sulphate (Sigma/Aldrich, UK). Two volumes of this solution were then slowly pippeted into a Falcon Tube (BD Biosciences, UK) containing one volume of Ficoll-Paque PLUS (GE Healthcare, UK). PBMCs were then separated by density gradient centrifugation at 800g for 30 minutes at 21^{o}C with minimum acceleration and minimum brake. Samples before and after the centrifugation are shown on **Figure 2.1** The mononuclear cell layer was collected and washed twice with RPMI 1640 by centrifuging at 400g for 10 minutes at 4^{o}C.

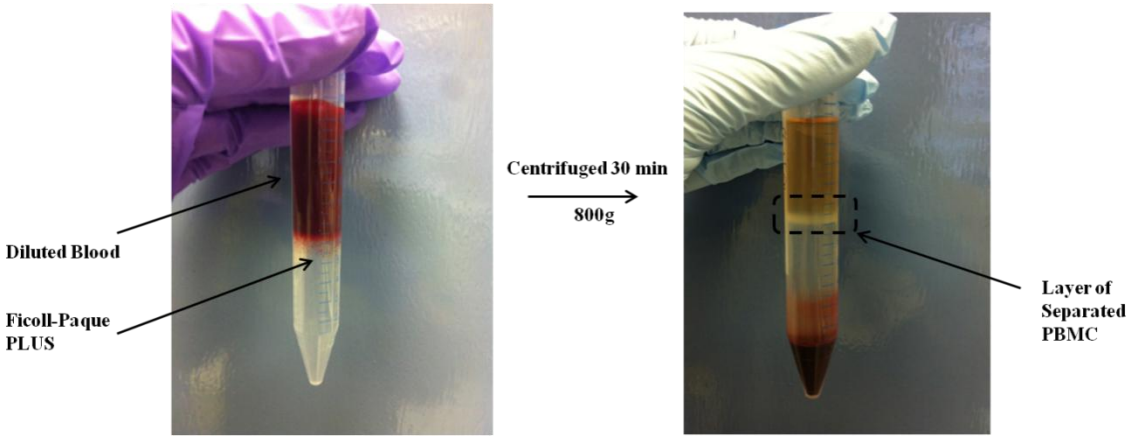

Figure 2.1 PBMCs isolation before (left) and after (right) the gradient-density centrifugation.

Isolated cells were then counted and their viability confirmed using ADAM-MC automated cell counter. After the cell count was determined they were re-suspended in a freezing medium and frozen. Both stages are described in detail in the next paragraph.

2.6 CELL COUNTING, FREEZING AND THAWING

2.6.1 Cell Counting with Automated Mammalian Cell Counter, ADAM-MC

The ADAM MC automated cell counter is design to perform cell counting and viability assessment using the propium iodide (PI) staining method of dead cells in

combination with advanced fluorescent microscopy image analysis. PI intercalates with cell DNA and stains the nucleous of that cell and fluorescent images are taken. The obtained images are analysed by integrated with the system software. All the steps are preformed automatically once the sample mixed with the dye is applied onto the disposable chip loaded into the counter.

For total cell count, 20µl of the cell suspension was mixed with 20µl of "AccuStain Solution T". Fifteen microliters of this cell lysate is then loaded into a specially designed chamber on the Accu-chip 4X. "Solution T" contains PI and a lysis buffer allowing for the dye to penetrate all the cells in a sample. This way the total number of cells will be counted. Similarly, for non-viable cell determination, 20µl of the cell suspension is mixed with an equal volume of "AccuStain Solution N" and mixed by vortexing. Again, 15µl of this cell lysate is also loaded onto the Accu-chip 4X. The "Solution N" contains only PI thus the dye will only stain cells with ruptured membranes thus determine only the non-viable cells.

The viability and cell number are automatically calculated and results provided as a cell number in 1ml of cell culture medium. Multiplying this value by the volume of prepared cell suspension allows for determining the total cell number in the investigated sample.

2.6.2 Cell Freezing and Storage

After the cell number was determined, the cells were re-suspended in freezing medium containing 90% of heat inactivated fetal bovine serum (Gibco/Thermo Fisher Scientific, UK) and 10% Dimethyl sulphoxide (DMSO) (Sigma, UK) and frozen at density $1x10^7$/1ml of the medium for PBMCs or $1x10^6$/1ml for CHO cells. Cryovials (Starlab, UK) containing the cell suspension were placed into "Mr Frosty" freezing container filled with propan-2-ol (Sigma, UK) and stored in -80^oC freezer. 24 hours later the cells were transferred to tank with liquid nitrogen for long term storage.

2.6.3 Cell Thawing

Peripheral blood mononuclear cells (PBMCs) cells were fast-thawed in 37^oC water bath. Cells were then resuspended in 20 ml of RPMI 1640 supplemented with

1% penicillin and streptomycin sulphate and additionally with 10% of heat inactivated FBS (hiFBS) for PBMCs. Cells were then washed by centrifuging at 400g for 10 minutes.

2.7 FLOW CYTOMETRY

Analysis of stained cells was performed by flow cytometry by using BD LSRFortessa™ (BD, UK) and on BD LSR II Flow Cytometer (BD, UK). For T_{Reg} or B_{Reg} cells identification, 100,000 PBMCs per sample were collected. Whereas when the analysis was performed for cultured cells the number of cells analysed was 20,000. Flow cytometry data was then analysed with FlowJo software (Tree Star Inc., USA).

2.7.1 Cell Intracellular and Extracellular Staining

Cells were stained using commercially available pre-conjugated antibodies. The optimal concentration for each of the antibodies was determined by titration prior to use in experiments (**Table 2.1**)**.** Antibody bound fluorochromes were chosen according to the level of cell expression of each particular marker. For the low expressed proteins like Foxp3 the brightest fluorochromes were chosen whereas for highly abundant markers such as CD4 less bright fluorochromes were used.

Antibody	Fluorochrome	Clone	Description	Company	Dilution	Cell Type
CD4	FITC	A161A1	T4/Leu-3	BioLegend	1:40	T_{Reg}
CD4	APC	RPA-T4	T4/Leu-3	eBioscience	1:40	T_{Reg}
CD25	BV421	M-A251	IL-2 receptor α chain	BioLegend	1:40	T_{Reg}
CD127	APC	A019D5	IL-7 receptor α chain	BioLegend	1:40	T_{Reg}
Helios	PerCP/Cy5.5	22F6	Transcription factors, Ikaros family	BioLegend	1:20	T_{Reg}
Foxp3	PE	PE236A/E7	Forkhead box protein P3	eBioscience	1:50	T_{Reg}
Nrp-1	Pe/Cy7	12C2	VEGF165R	BioLegend		T_{Reg}
TNFα	PE	MAB11	Macrophage cytotoxic factor (MCF)	eBioscience	1:50	T
IFNγ	PE	4S.B3	T cell interferon, Macrophage-activating factor (MAF)	eBioscience	1:50	T
CD19	PerCP/Cy5.5	HIB19	Known as B4	BioLegend	1:40	B_{Reg}
CD19	BV412	HIB19	Known as B4	BioLegend	1:50	B_{Reg}
CD24	APC	ML5	Ly-52, Heat Stable Antigen	BioLegend	1:40	B_{Reg}

CD38	Pe/Cy7	HIT2	T10,ADP-ribosyl cyclase	BioLegend	1:80	B_{Reg}
CD1d	PE	51.5	MHC-like, type I transmembrane protein	BioLegend	1:50	B_{Reg}
CD10	PE	H10a	Common acute lymphoblastic leukemia antigen (CALLA)	BioLegend	1:50	B_{Reg}
CD27	PE	M-T271	Known as S152	BioLegend	1:50	B_{Reg}
CD5	FITC	UCHT2	Leu-1, Ly-1	BioLegend	1:40	B_{Reg}
CD4 iso	FITC	RTK4530	-	BioLegend		T_{Reg}
CD4 iso	APC	P3.6.2.8.1	-	eBioscience		T_{Reg}
CD25 iso	BV	MOPC-21	-	BioLegend		T_{Reg}
Helios iso	PerCP/Cy5.5	HTK888	-	BioLegend		T_{Reg}
Foxp3 iso	PE	P3.6.2.8.1	-	eBioscience		T_{Reg}
Nrp-1 iso	Pe/Cy7	MOPC-173		eBioscience		T_{Reg}
TNFα iso	PE	P3.6.2.8.1	-	eBioscience		T
IFNγ iso	PE	P3.6.2.8.1	-	eBioscience		T
CD19 iso	PerCP/Cy5.5	MOPC-21	-	BioLegend		B_{Reg}
CD19 iso	BV412	HIB19	-	BioLegend		B_{Reg}
CD1d iso	PE	MOPC-11	-	BioLegend		B_{Reg}
CD10 iso	PE	MOPC-21	-	BioLegend		B_{Reg}
CD27 iso	PE	MOPC-21	-	BioLegend		B_{Reg}
CD5 iso	FITC	MOPC-21	-	BioLegend		B_{Reg}

Table 2.1 Antibodies used for the identification of various cell phenotypes such as T_{Reg}, B_{Reg} cells, $CD4^+$ TNFα or $CD4^+$IFNγ within PBMCs population

Regulatory T and B cell were quantified within $1x10^6$ previously frozen PBMC. Cells were thawed as described in paragraph 2.6.1 and counted. They were then re-suspended in FACS buffer containing PBS (Gibco/Thermo Fisher Scientific, UK) congaing 1% hiFBS, 2mM EDTA (Sigma UK) and 0.05% (wt/vol) sodium azide (Sigma UK). Concentration of cells was brought to $1x10^7$/ml and 100 µl of that suspension was transferred to 96-well U-bottom plates (Nunc, Denmark). In order to remove traces of any proteins, cells were washed twice in PBS by centrifuging for 1 minute at 800g at 4^oC. Hundred microliters of PBS congaing LIVE/DEAD fixable blue or violet fluorescent dye (Thermo Fisher Scientific, UK) at concentration 1 µl of dye per 1 ml of PBS was then added to each of the 96 wells containing cells. The plate was incubated in dark at 4^oC for 30 minutes. After that time cells were washed by centrifuging as previously. Fifty microliters of an antibody cocktail made up of PBS and antibodies for T_{Reg} or B_{Reg}

identification or their appropriate isotype controls were added to cells. Plate was then incubated for 25minutes at 4^{o}C in dark. After washing, cells were permeabilised and fixed by incubating them for 40 minutes in dark at room temperature with the Transcription Factor Fixation/Permeabilization buffer (eBioscence, UK). Following washing step they were stained for the intracellular markers using anti- Foxp3 and anti- Helios antibodies in Transcription Factor Permeabilization Buffer (eBioscence, UK) and incubated for 30 minutes in the same conditions as previously. Once the staining was completed the wells were washed and transferred to FACS tubes (BD Falcon, UK) for flow cytometry analysis as shown on **Figure 2.2**. Compensation controls were prepared as described in paragraph 2.7.2.

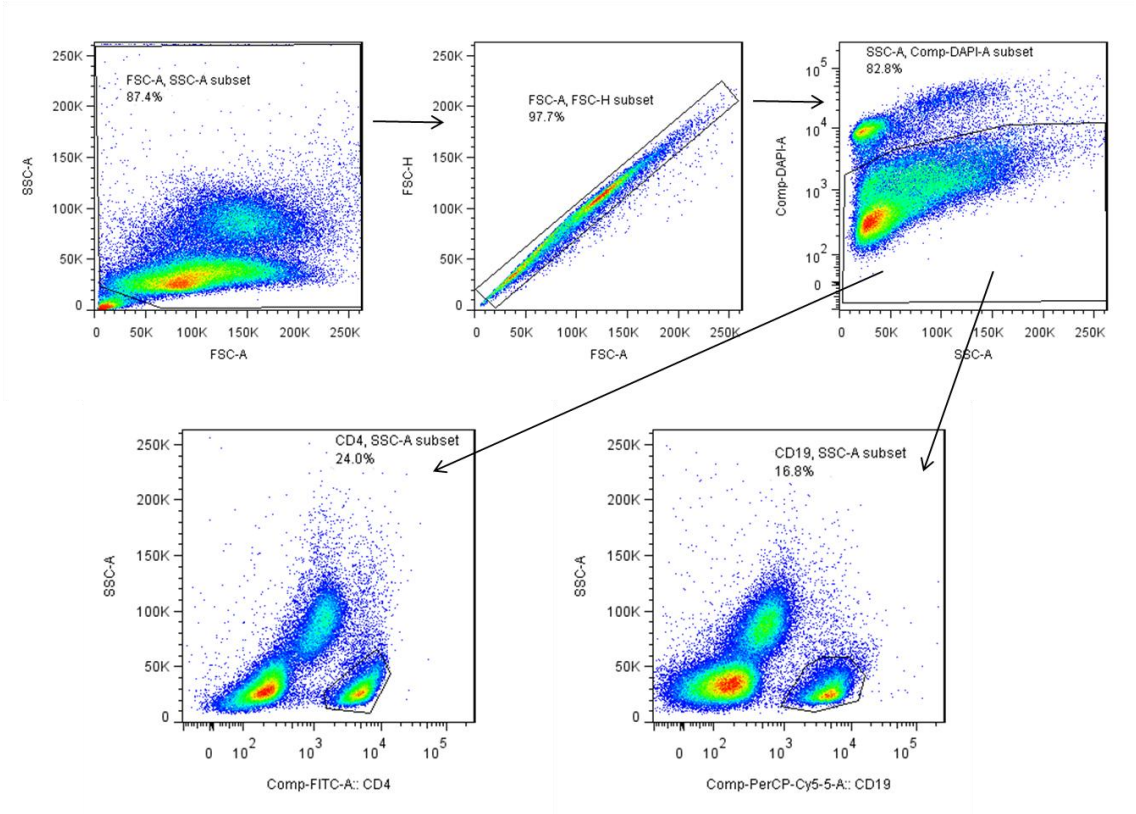

Figure 2.2 Gating strategy used in identification CD4^{+} Tell and CD19^{+} B cell subsets.
Cells were first gated to discriminate debris and then to identify singlets. Before gating for CD4 or CD19 positive subsets, all cells staining for DAPI (LIVE/DEAD fixable stain) were excluded.

2.7.2 Preparation of Compensation Controls for Flow Cytometry Experiments

In order to correct for spectral overlap during multicolour flow cytometry experiments, the colour compensation was performed. The antibody panels were design the way allowing for minimum compensation.

UltraComp eBeads (eBioscience, UK) were used to stain for single markers. One drop of the beads solution (about 50 µl) along with 1 µl of a particular florochrome – bound antibody was added to FACS tubes. One extra tube with unstained beads was also prepared. Tubes were then incubated at $4^{o}C$ in dark for 15 minutes. Beads were then washed in 2 ml of FACS buffer and centrifuged 5 minutes at 500g. The liquid was then decanted and beads re-suspended in 200 µl of FACS buffer. Prepared this way beads were used to calculate the level of compensation for all the fluorochromes during flow cytometry experiments.

2.8 REGULATORY T AND B CELLS FUNCTIONAL ASSAYS

2.8.1 TReg and BReg Cells Preparation and Fluorescence Activated Cell Sorting (FACS)

In order to assess the function of T_{Reg} and B_{Reg} cells, previously frozen PBMCs from healthy pregnant patients and women who developed pre-eclampsia were thawed as described earlier. Samples from both groups were gestational matched with average gestation for healthy patients being 30.67 week ± SEM 1.764 and from pre-eclampsia group 31 weeks ± SEM 1.472. Cells were then washed twice in PBS by centrifuging at 400g at $4^{o}C$ for 10 minutes, counted and stained with LIVE/DEAD cell marker (1µ of the dye diluted in 1ml of PBS). After 30minutes incubation at $4^{o}C$ in dark the cells were washed again and divided into two groups. Samples from each patient were divided into two – one stained for anti-CD4 APC and anti-CD25 BV421 antibodies for T_{Reg} cells identification and the other with anti-CD19 BV42, anti-CD24 APC and anti-CD38 Pe-Cy7 for B_{Reg} cells identification. Following the staining cells from each tube were again divided into two tubes each washed with PBS. A schematic description of the process is shown in **Figure 2.4**. Tube A with cells stained for T_{Reg} cell markers was divided into tube A and B. Both samples were then used to isolate the $CD4^{+}$ T cells with Tube B additionally having $CD4^{+}CD25^{hi}$ subset of cells depleted.

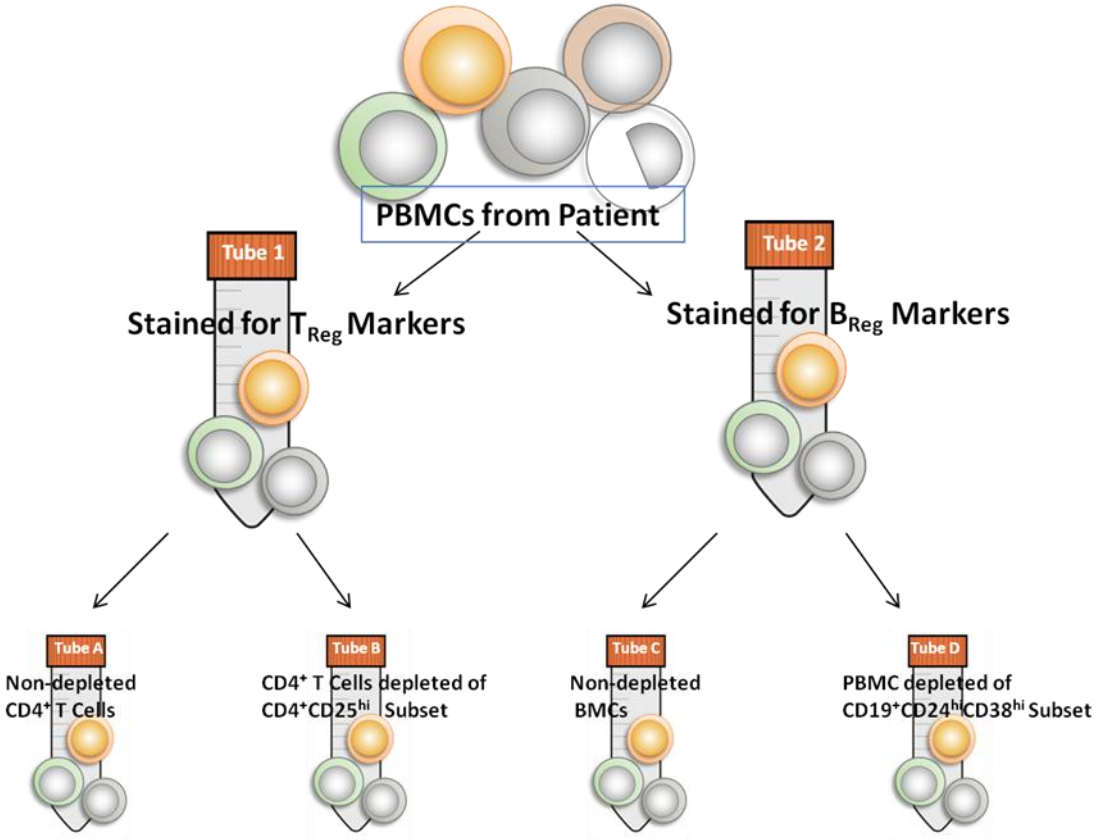

Figure 2.3 Cells preparation strategy for FACS

Similarly, tubes with cells stained for B_{Reg} cell markers were divided into two, C and D. Both samples were than analysed by cell sorter with sample C non-depleted, passed through the machine whereas sample D was depleted of $CD19^+CD24^{hi}CD38^{hi}$ subset**.** Gating strategy for cell sorting was the same as shown on **Figure 2.2.** Flow cytometry plots representing samples before and after T_{Reg} and B_{Reg} cells depletion are shown below on **Figure 2.4**.

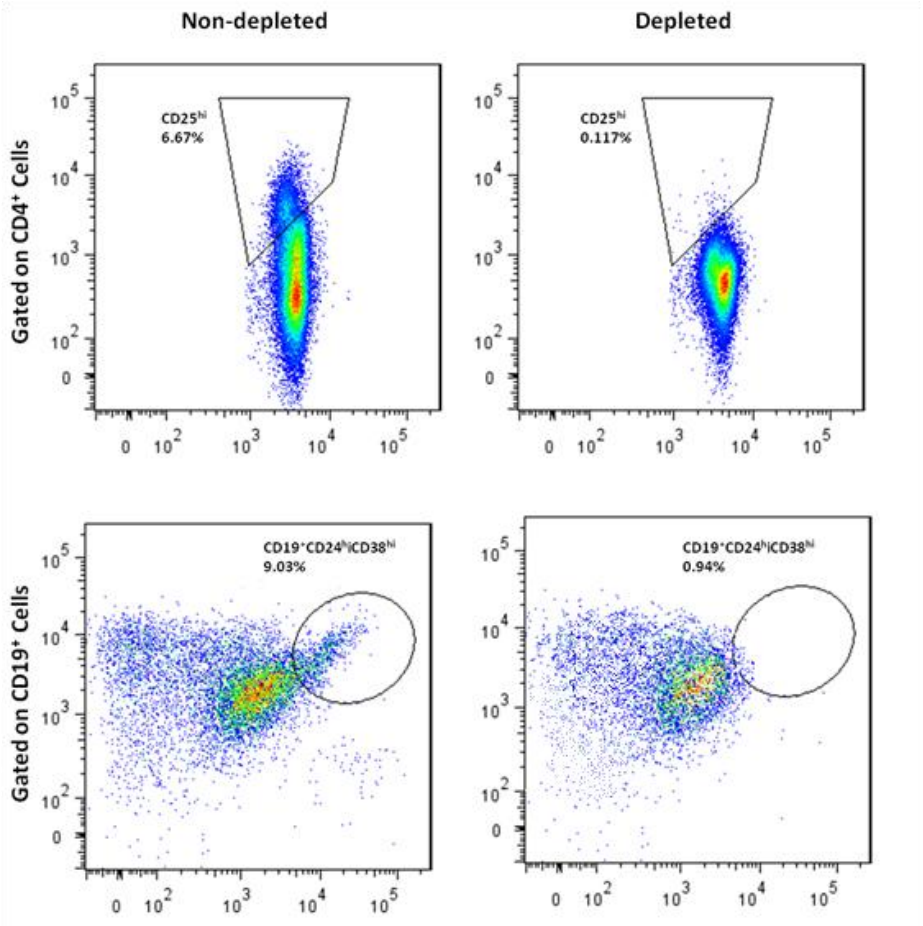

Figure 2.4 TReg and BReg Cells Preparation before and after FACS Sorting.
Left panel presents plots with cells before depletion (top) for T cells and (bottom) depleted for B cells. Panel on the right depicts cells distribution after $CD4^{+}CD25^{hi}$ (top) and $CD19^{+}CD24^{hi}CD38^{hi}$ (bottom) cells were depleted.

2.8.2 CD4+ T Cells Proliferation Assay

In order to determine cell proliferation, PBMCs were stained with carboxy-fluorescein diacetate succinimidyl ester (CFSE) (eBioscience, UK). CFSE easily crosses the cell membrane and covalently binds to intracellular protein. Once inside the cell, enzymes cleave the acetate groups and a fluorescent carboxy-fluorescein molecule is released. Cell divisions are then measured as halving of the CFSE fluorescence intensity. It is an easy and reliable method used to investigate cell proliferation that can be assessed by flow cytometry.

Cell culture conditions were optimised before performing a cell proliferation assay. For T cell receptor stimulation soluble or plate-bound functional grade purified anti-CD3 antibody (clone HIT3a, eBioscience, UK) and soluble anti-CD28 antibody (clone CD28.2, eBioscience UK) were present in the cell culture conditions. For plate bound-mediated stimulation plates were pre-coated for 12 hours with 0.5-2 µg/ml of anti-CD3 in PBS at 4^{O}C. As shown on **Figure 2.5** the combination of 2µg/ml of plate-bound anti-CD3 antibody and 2µg/ml of soluble anti-CD28 antibody provided the best conditions for cell proliferation.

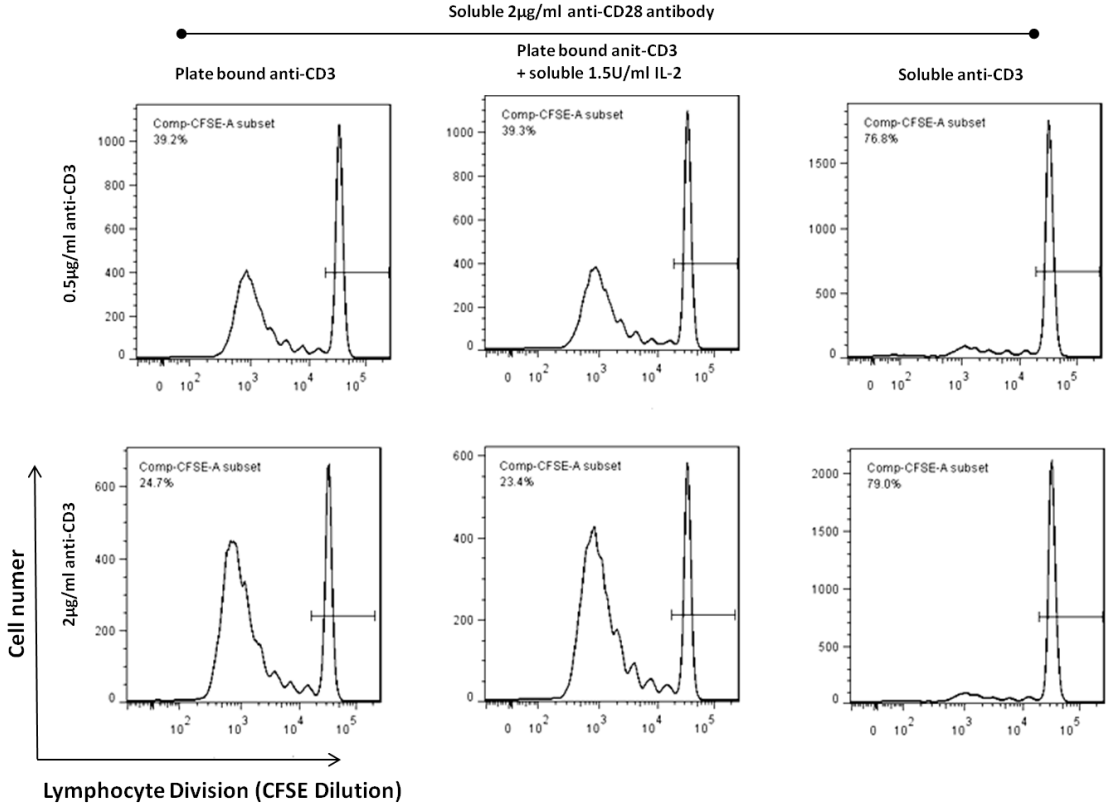

Figure 2.5 Investigation into the optimal conditions for CD4+ T cell proliferation.
Cells were cultured in presence of 2µg/ml of anti-CD28 antibody and plate-bond or soluble 0.5µg/ml or 2µg/ml anti-CD3 antibody. Additionally, 1U/ml IL-2 (Roche, Switzerland) was added to some of the conditions. Cells cultured with anti-CD28 antibody and with 2µg/ml plate-bound anti-CD3 antibody with or without IL-2 showed the greatest proliferation rate.

FACS sorted, non-depleted and depleted CD4^{+} cells were re-suspended in 3µM CFSE at a concentration of 5x10^{6}cell/ml. Following 10 minutes incubation on a roller in the dark the labelling was stopped by adding an equal volume of pure heat inactivated FBS (hiFBS) and incubated for another 5 minutes. In the next step, cells were washed 3

times with PBS by centrifuging at 400g for 10 minutes each time. Once the labelling was completed cells were re-suspended in complete RPMI 1640 media containing 10% hiFBS and 1% penicillin and streptomycin sulphate with the addition of 2µg/ml of anti-CD28 antibody. This way prepared cells were plated at a concentration of $2x10^5$cell/100µl in a flat bottom 96-well plate pre-coated with 2µg/ml of anti-CD3 antibody. Control samples were not stimulated with anti-CD3/CD28 antibodies. Cells were then cultured for 5 days in a cell culture incubator in a humidified atmosphere; temperature 37 °C and 5% CO_2.

On the 5^{th} day of culture, the cells were harvested with ice cold PBS supplemented with 1mM of EDTA. Following the protocol from section 2.8.1**,** cells were then labelled with LIVE/DEAD dye and surface stained with anti-CD4 APC and anti-CD25 BV421 antibodies. In the final stage, cells were fixed with Fixation Buffer (eBioscience, UK) and transferred to FACS tubes for flow cytometry analysis.

2.8.3 $CD19^+CD24^{hi}CD38^{hi}$ Depletion Assay

$CD19^+CD24^{hi}CD38^{hi}$ depletion assay conditions were optimised and a protocol published by the laboratory of Prof Claudia Mauri was followed (Blair et al. 2010). FACS sorted $CD19^+$ B cells depleted and non-depleted of $CD19^+CD24^{hi}CD38^{hi}$ were re-suspended in complete RPMI 1640 cell culture media supplemented with hiFBS and 1% penicillin and streptomycin sulphate. Five $x10^5$ cells were plated per well of flat-bottom 96 well plated pre-coated with 0.5µg/ml anti-CD3 antibody. Cells were then cultured for 72 hours in a cell culture incubator in humidified atmosphere; temperature 37 °C and 5% CO_2.

For the last 6 hours of culture, 50ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, UK), 250 ng/ml of Inomycin (Sigma Aldrich, UK) and 1 µl/ml of GolgiPlug (BD Bioscience, UK) were added. 72 hours after plating, the cells were harvested with cold PBS supplemented with 1mM of EDTA. Following protocol from section 2.6.1 cells were then ladled with LIVE/DEAD day and surface stained with anti-CD4 FITC and antiCD19 PerCP/Cy5.5 antibodies. In the next stage, prior intracellular staining cell were incubated with IC Fixation Buffer (eBioscience, UK) for 20 minutes in room temperature. Two subsequent washes with Permabilisation Buffer (eBioscience,

UK) were performed by centrifuging the cells suspension for 1 minute at 800g. Cells were then incubated for 25 minutes with anti-TNFα PE or anti- IFNγ PE antibodies in Permabilisation buffer. Once the staining was completed cells were washed and transferred to FACS tubes for flow cytometry analysis.

2.9 ASSAYS FOR THE DETECTION OF AT1-AA IN SERUM

Apart from the AT1-AA ELISAs described earlier, I used several other assays to detect ATI auto-antibodies.

2.9.1 Human IgG Gravity-Flow Purification Protocol

In order to isolate IgG from each patient of interest, I used maternal serum diluted 1:1 (1ml of patient serum and 1 ml of Binding Buffer) and applied to a NAB^{TM} Spin Column. Each column contained 1ml of immobilized protein G resin (Thermo Fisher Scientific, UK) and was equilibrated by adding 5ml of Binding Buffer (0.1M phosphate buffer, pH=7.2). Serum samples were diluted 1:1 (1ml of patient serum and 1 ml of Binding Buffer) and applied to the column. Flows through were collected and stored at -80°C for future experiments. Fifteen microliters of Binding Buffer was used to remove all unbounded proteins from the columns. Bound antibodies were eluted with 5 ml of Elution Buffer (0.1M glycine, pH=2.7). First 4ml of eluted samples were collected (previous experiments shown that last 1ml does not contain IgG) and neutralised by addition of 148µl of Neutralisation Buffer (1M Tris buffer pH=8.6).

During the next step, 4 ml samples of eluted antibodies were applied to the concentrators (50K, Amicon® Ultra-4 Centrifugal Filter Devises from Millipore) and centrifuged 20 minutes at 4500RPM. Wastes were removed. Phosphate buffered saline (PBS; 2mL) was applied to the samples and spun again at 4500RPM for 10 minutes. Flows through were removed and again 2ml of in phosphate buffered saline (PBS) applied. Samples were spun at 4500RPM for about 10 minutes, until the remaining volume reaches ~ 500 µl. Concentrated samples were transferred to Eppendorf tubes and PBS used to make the volume up to 1 ml. Samples were aliquoted and frozen at -80°C until use.

2.9.2 Luciferase bioactivity assay for the detection of AT1-AA

In order to investigate the presence and activity of the AT1-AA in isolated IgG fractions, an in-house luciferase bioassay was used as previously published by Prof Yang Xia laboratory (Siddiqui et al. 2010).

The principle of the luciferase bioassay is based on cloning the regulatory region of gene of interest (in this case, the Angiotensin II receptor 1) upstream of the luciferase gene in an expression vector. This construct is then introduced to DNA of cells such as CHO cells. Cells are then activated with factor (Ang II) which initiates downstream effects of the Angiotensin II receptor 1 and leads to transcription of the incorporated gene. Cells are then lysed in order to release all the intracellular proteins (including the luciferase). After adding luciferin (luciferase substrate) the enzymatic activity of luciferase is measured using a luminometer. Since AT1 receptor is connected to the luciferase reporter gene, the luciferase activity correlates directly with the AT1 receptor stimulation.

2.9.3 Production of CHO cells expressing constitutive AT1A receptor and bio-responsive NFAT-JDG reporter construct

Chinese hamster ovary (CHO) cells stably transfected with the rat ANG II receptor type 1A (CHO-AT1R) were prepared and kindly provided by Juliette Delhove form Dr Tristan McKay' laboratory at St George's, University of London.

Cloning of the Ang II receptor 1 (AngR1) into lentiviral expression cassette was preformed and pLNT-SFFV-AngR1-SV40-Neo generated where "SFFV" is a strong viral promoter and "SV40-Neo" is the Neomycin resistance selection cassette. High-titre preparations of pLNT-SFFV-AngR1-SV40-Neo and NFAT responsive reporter lentivirus pLNT-NFAT-FLuc-2A-eGFP where "NFAT" is 6 serial repeats of an NFAT binding consensus sequence and "FLuc" is firefly luciferase were prepared. CHO cells were then co-transduced with pLNT-SFFV-AngR1-SV40-Neo and pLNT-NFAT-FLuc-2A-eGFP. The double transgenic cell line was activated using the known NFAT agonists; PMA and ionomycin to verify function independent of Ang II receptor 1. This resulted in a significant up regulation of FLUc reporter activity. GFP expression in non-stimulated cells shows that there is basal NFAT activity under normal culture conditions. Double

transgenic CHO cells were then subjected to exogenous Ang II activation and again showed a significant up regulation of NFAT activity.

Once I have received ready to use CHO-AT1R cells I performed Western blot to confirm the level of AngR1 expression in cells pre- and post transfection.

2.9.4 Analysis of the expression of AngR1 in CHO Cells pre - and post –transfection: Western Blot

Western blot is commonly used to detect specific proteins present in an extract or homogenised tissue. One of the steps involves gel electrophoresis which is used to separate denatured proteins depending on the length of the polypeptide. Separated proteins are then transferred to a polyvinylidene difluoride (PVDF) membrane, and stained with antibodies specific to that target protein.

The protein content of CHO cell lysates was measured and 15 µg and 30 µg of protein extracted from pre - and post –transfection were investigated for AngR1 content. Samples were reduced by boiling at 100°C for 3 minutes with Sample Reducing Buffer (Life technologies, USA). This way prepared sample were then pipetted onto 10% Bis-Tris Gel and run in MOPS-SDS running buffer (Life technologies, USA) at 200V for about 45 to 60 minutes. A molecular weight marker (Fermantas, Cambridge UK) allowing for later identification of separated proteins was run alongside the samples. Using a Wet Transfer apparatus and Transfer buffer (Life technologies, UK) containing 10% methanol, the separated in the earlier step proteins were then transferred onto a PVDF. The transfer was conducted at 100V and took 1 hour. Following the transfer, PBS containing 5% skimmed milk (wt/v) and 0.1% Tween-20 (PBS-T) was used to block the membrane by incubating for one hour at room temperature.

After the blocking step, the membrane was incubated overnight at 4°C on a shaker (Eppendorf, UK) with goat-anti human ATR1 primary antibody (Abcam, UK) diluted 1:1000 in 5% skimmed milk in PBS-T. Next day, the membrane was washed with PBS-T and then incubated with goat anti-rabbit IgG-horseradish peroxidase secondary antibody (Santa Cruz, UK) diluted 1:1000 in 5% milk in PBST. The incubation was performed at room temperature and lasted 1 hour. Following the

incubation, the membrane was once more washed with PBS-T. The protein bands were visualized using Amersham ECL Plus kit (GE Healthcare Life Sciences, UK) and preserved on Amersham Hyperfilm ECL (GE Healthcare Life Sciences, UK). The developed hyperfilm was then scanned using the HP Desk-scan system.

2.9.5 Luciferase bio-assay

Chines Hamster Ovary -AT1R cells ($1x10^5$ cells) containing integrated copies of a gene encoding the rat AT1 receptor and 6x NFAT-driven luciferase constructs were plated on 24 well plates and cultured for 24 days in an incubator with humidified atmosphere; temperature 37 °C and 5% CO_2. Cells were maintained in Ham's F12 media (Lonza, Belgum) supplemented with 10% FBS (Gibco, Invitrogen UK), 1% penicillin and streptomycin sulphate (Sigma/Aldrich, UK), 2mM L-glutamine (Lonza, Belgum), and 8.75 g L-proline (Sigma Aldrich, UK). The next day the cell media was changed to serum-free. After 2 hours, cell arrest CHO-AT1R cells were treated with serum isolated IgG (1:500 - 1:10 dilutions), Ang II (0.1nM- 10 µM) in the presence or without Losatrtan (0.1µM-100µM). After 24 hours culture, CHO cells were lysed on a shaker in dark for 45 minutes using 100µl of Passive Lysis Buffer form the Dual-Luciferase® Reporter Assay System (Promega, UK). After mixing 20µl of that lysate with 100µl of the Luciferase Assay Reagent II (LAR II) (Promega, UK) the enzymatic activity was measured a Luminometer.

2.10 WIRE MYOGRAPHY

In order to compare the vascular reactivity of resistance vessels from women with pre-eclampsia and those who had a healthy normotensive pregnancy, I used wire myography. Wire myography allows an assessment of vaso-reactivity of small blood vessels *ex vivo*. The wire myograph equipment myograph (Model 620, Danish Myo Technology, Denmark) used in this project is shown in the figure below (**Figure 2.6**).

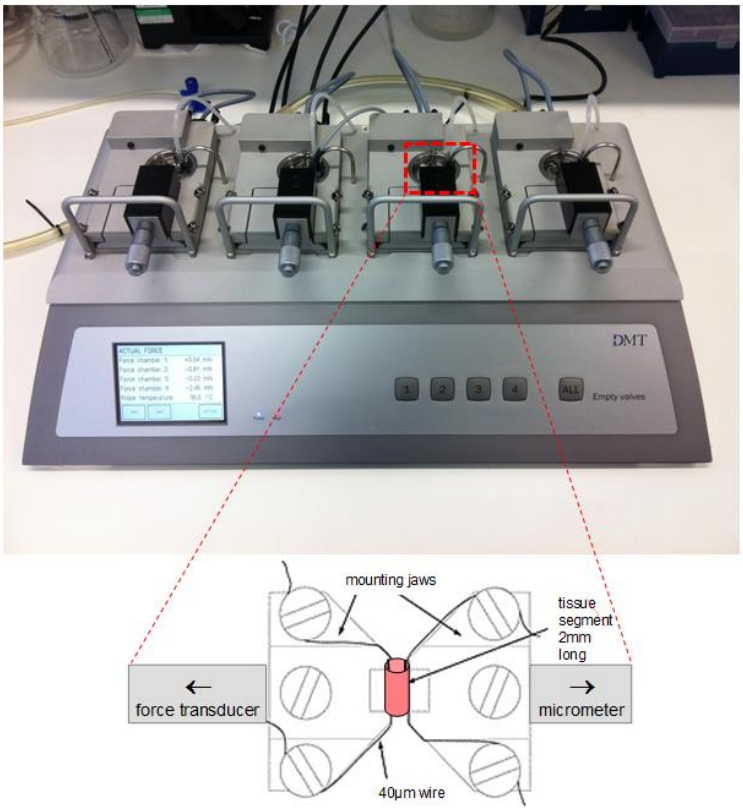

Figure 2.6 Multi chamber wire myograph.
Jaws are attached on one side to a micrometer which measures jaw separation, and on the other to a force transducer which measures the force exerted by the arterial contraction on the mounting wires.

2.10.1 Dissection and mounting of myometrial blood vessels

Biopsies of human myometrium were taken from the upper lip of the uterine incision at the time of lower segment caesarean section operations.

After collection, the biopsy specimens were placed in ice-cold physiological salt solution (PSS) of the following composition : NaCl 112mM, KCl 5mM, $CaCl_2$ 1.8mM, $MgCl_2$ 1mM, $NaHCO_3$ 25mM, KH_2PO_4 0.5mM, NaH_2PO_4 0.5mM, and glucose 10 mM, previously aerated with carbogen gas (95% O_2, 5% CO_2) and immediately transported to the laboratory. The fresh myometrial tissue was transferred to a Petri dish containing a layer of agar to hold fixing pins and kept moist with fresh cold PSS. The tissue was pinned and under a light microscope myometrial arteries identified and dissected within two hours from the moment of taking the biopsy.

Directly after dissection arterial segments of around 2mm in length were then mounted in a multi chamber wire for isometric tension recording (**Figure 2.7**). Each vessel segment was mounted on two 40µm stainless steel wires, one attached to a force transducer and one attached to a micrometer, via a pair of stainless steel jaws.

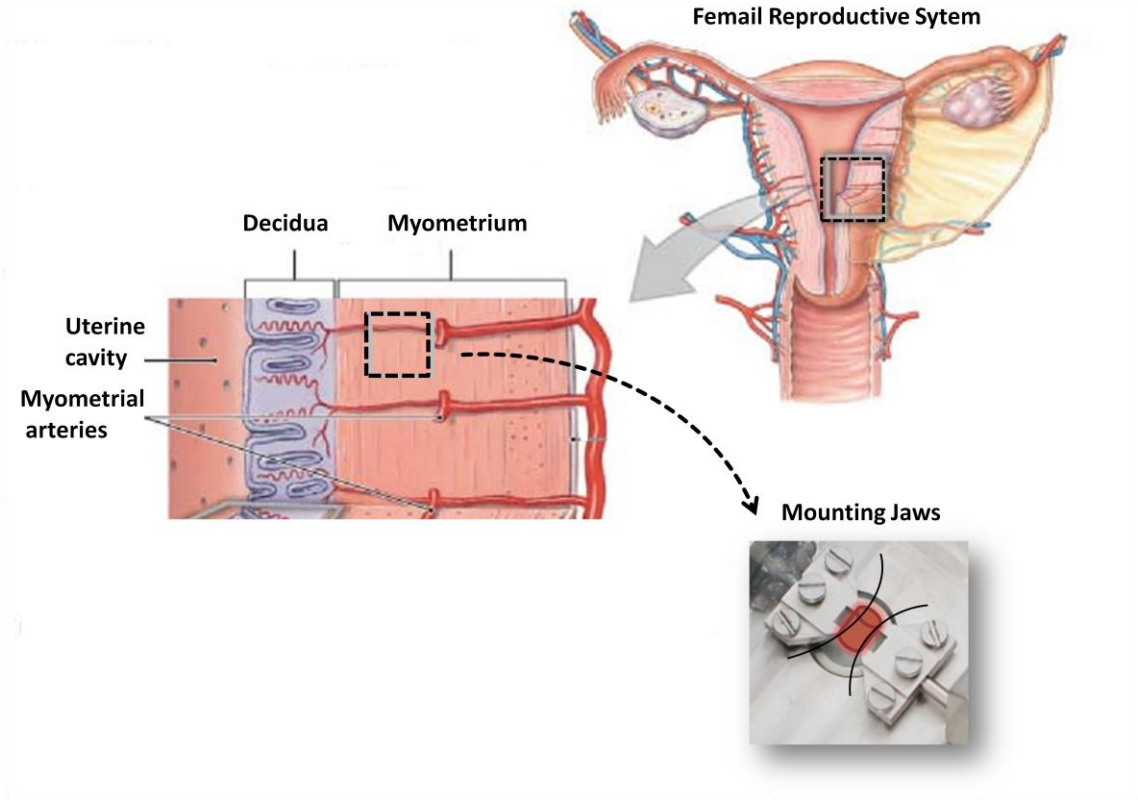

Figure 2.7 Graphical representation of vessels preparation for wire myography.
As shown in the picture utrain vessels form myometrium wre dissected and mounted in chambers of the wire myograph.

Each of the mounted vessels were bathed in 5ml of PSS, heated to 37°C and bubbled continuously with carbogen gas (95% O_2, 5% CO_2). Readings of force changes generated by mounted vessels were fed to a PC connected with the myograph and visualised using LabChart software.

2.10.2 Normalisation and vessels activation

Following equilibration for 30 minutes, a normalisation procedure was performed to determine the arterial lumen necessary for optimal force generation (Mulvany & Halpern 1977). It has been shown previously that for human small

myometrial arteries, maximal force generation is achieved when a segment is stretched to 90% of the diameter expected when exposed to a transmural pressure of 13.3kPa (100mmHg) (Andersen et al. 2011). This diameter is known as IC_{100} and 90% of this value as IC_1. In practical terms, this is achieved by step-wise distension of the vessel, with micrometer and force readings made at each step. The Laplace relationship is then applied to calculate IC_{100} from the intersection of the exponential tension-circumference plot and an isobar curve corresponding to 100mmHg.

In order to activate functional and the mechanical properties of the vessel segments and to confirm tissue viability, standard start procedures were performed 30 minutes after normalisation. Vessel contractions were stimulated by the addition 60mM KCl to the myograph chambers. After three minutes, when a plateau contraction was achieved, the chambers were washed four times with PSS over 20 minutes period. In order to test for endothelial function the second activation step involved application of 3µM Bradykinin at the peak of the 100nM U46619 (thromboxane A_2 mimetic) - induced contraction (**Figure 2.8**). Endothelial integrity was confirmed if a ≥50% relaxation was achieved; in practice an average relaxation of around 67% was observed.

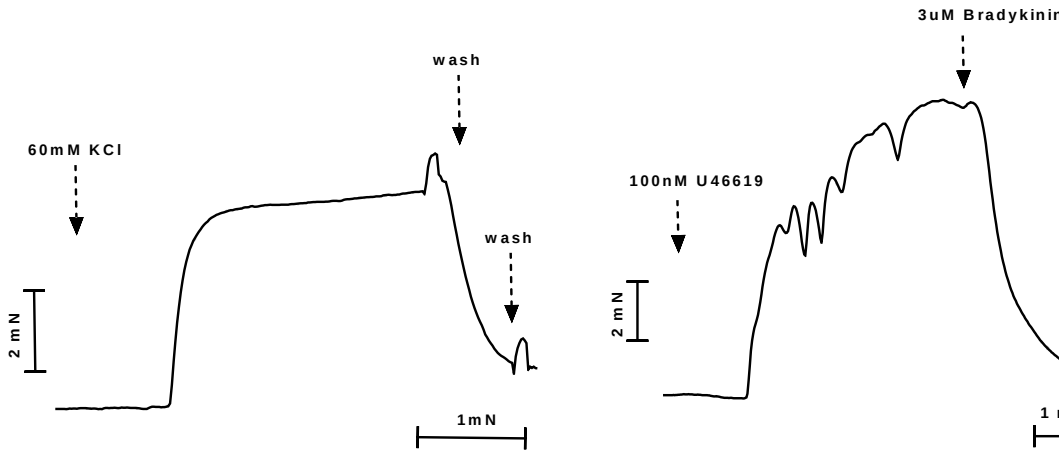

Figure 2.8 Raw traces depicting the standard activation protocol conducted on small myometrial artery from a healthy pregnant woman.

2.10.3 Experimental Procedure

2.10.3.1 Dose Response Curve to Angiotensin II

Cumulative concentrations (10^{-9} to 10^{-7}M) of angiotensin II were added to the vessels chamber. Changes in tone were expressed as a difference in force generated by vessels before the addition of the lowest concentration of the vasoconstricting agonist and subsequent doses.

2.10.3.2 Investigation of the effect exerted by pre-eclamptic IgG fractions on uterine arteries.

In order to test the vasoreactivity of maternal serum IgG on isolated blood vessels, I used the wire myograph. One vessel was dissected from a surgical biopsy specimen, taken at the time of caesarean section from women who either had pre-eclampsia or a healthy pregnancy. Each vessel was cut into two similar in length segments and mounted in myograph. After the so-called "wake-up" protocol described in paragraph 2.10.2 vessels were incubated for 15 minutes with 30μg/ml IgG fractions isolated either form healthy or pre-eclamptic serum (**Figure 2.9**)**.** Cumulative doses (10^{-9} to 10^{-7}M) of Ang II were then applied as described in paragraph 2.10.3.1**.** Vasoconstriction of vessels from women with preeclampsia and healthy pregnancy was compared.

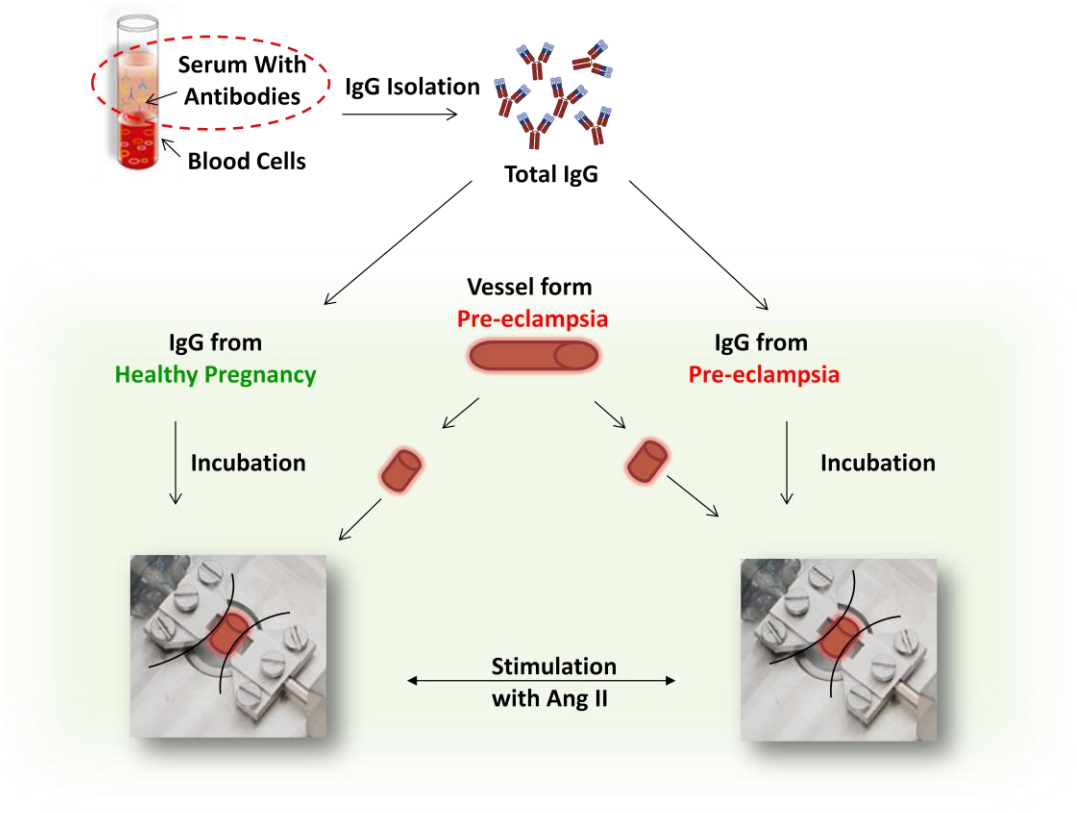

Figure 2.9 Schematic representation of the steps involves in the experiment investigating impact of IgG on pre-eclamptic blood vessels.

2.11 STATISTICAL ANALYSES

Several different statistical analyses were utilised across four results chapters. In each of the relevant chapters I provide details of used analysis. Here I present the summary.

2.11.1 Longitudinal Analyses

Data was analysed longitudinally with STATA *Version* 10. Linear mixed effects (growth curve) models, with both fixed and random effects were constructed. Mixed models were fitted using a *xtmixed* command. The fixed effect element included time-dependent variables (such as gestation) as well as clinical diagnosis variables. Whereas, the random effects component of the model consisted of random intercept and random slope terms. Random intercept allows for the overall level of the response (the factor being measured) to vary over women, and random slope shows that each woman had her own specific growth curve. Data was investigated for normality using

histogram (**Figure 2.10** and **Figure 2.11**)**,** Kolmogorov–Smirnov, D'Agostino & Pearson omnibus normality test and Shapiro-Wilk. Linear mixed models were then fitted on log-transformed values of each blood factor. Results are compared to the reference group such as normotensive pregnant women and expressed as coefficients with corresponding 95% confidence intervals, and p values, with <0.05 considered as a significant result.

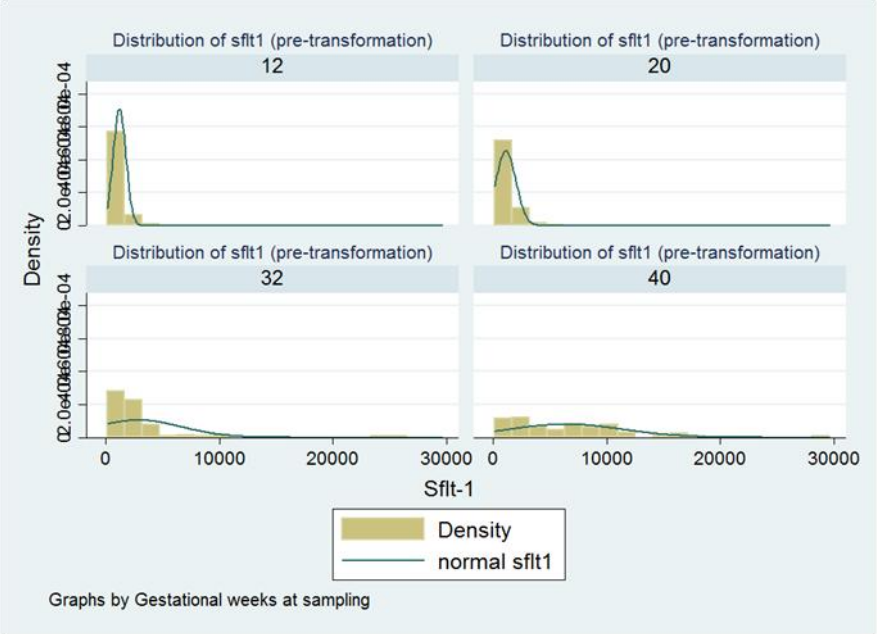

Figure 2.10 Figure showing examples of distribution sFlt-1 values before logarithmic transformation.
Mini graphs represent sFlt-1 measurements for 10-18 weeks (12), 19-27 weeks (20), 28-34 weeks (32) and 35-41 weeks (40) of pregnancy. The results were highly skewed.

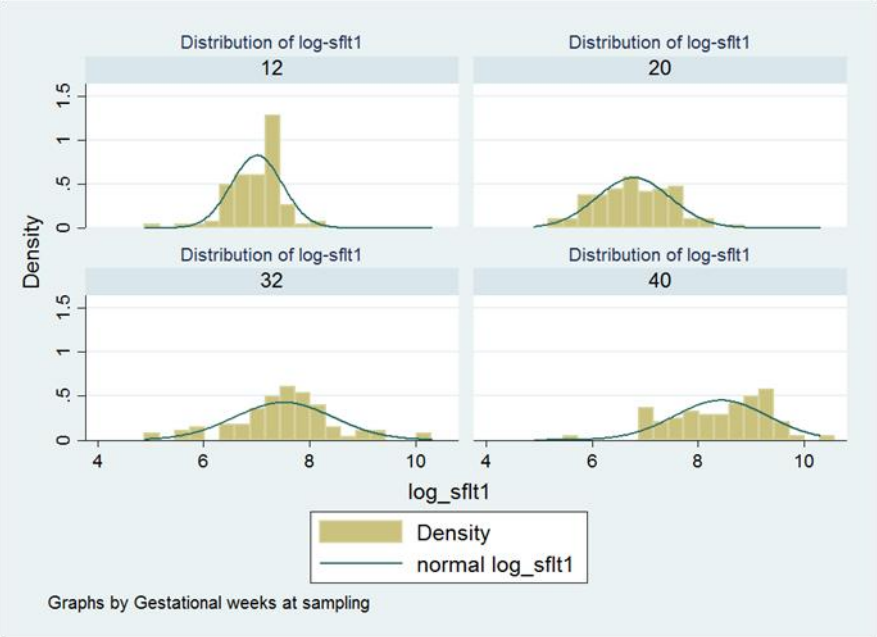

Figure 2.11 Figure showing examples of distribution sFlt-1 values after logarithmical transformation.
Mini graphs represent sFlt-1 measurements for 10-18 weeks (12), 19-27 weeks (20), 28-34 weeks (32) and 35-41 weeks (40) of pregnancy. Following logarithmic transformation, the data become normally distributed which is showed as a symmetrical distribution of the data about the mean.

2.11.2 Cross-sectional analyses

Graphpad Prism 5.0 software was used to perform most of the cross sectional statistical analyses. If data was normally distributed then an unpaired *t* test, a one-way ANOVA with a Bonferroni correction for multiple comparisons or Dunnet's test was used. When data was scattered but did not require transformation, tests such as Kurska Wallis and Mann–Whitney U-test were applied. Data are presented as means with standard errors of the mean, as well as medians with interquartile range (IQR), and p value of <0.05 interpreted as a significant.

2.11.3 Correlations

Correlations were calculated using Graphpad Prism 5.0. Correlations between between blood factors were performed using either Pearson's correlation coefficient test or Spearman's correlation coefficient test, depending on whether data were normally distributed. Data are expressed as correlation coefficients (r), with 95% confidence intervals and p values, and p <0.05 considered as significant.

CHAPTER III: SOLUBLE VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR 1 AND PLACENTAL DERIVED GROWTH FACTOR BEFORE AND DURING THE ONSET OF PRE-ECLAMPSIA

ABSTRACT

Cross-sectional and prospective studies have shown that there is an imbalance in circulating levels of pro-angiogenic (Placental Growth Factor; PIGF) and anti-angiogenic (Soluble fms-like tyrosine kinase-1; sFlt-1) factors before the clinical onset of pre-eclampsia. Paradoxically, women who smoke throughout pregnancy have a decreased risk of pre-eclampsia and a more pro-angiogenic profile in their serum. In order to determine the serological events that might protect against pre-eclampsia, or precede the onset of pre-eclampsia, I investigated the prospective changes in maternal serum sFlt-1 and PIGF levels from 10 weeks of gestation until childbirth in 3 groups of pregnant women; healthy women, women at high risk of pre-eclampsia, and women who continued to smoke during pregnancy.

The main findings are summarised below.

1. Women who developed pre-eclampsia had the lowest serum concentration of PIGF and the highest sFlt-1:PIGF ratio from all of the investigated groups.

2. Subjects who smoked during pregnancy had a higher serum PIGF concentration throughout pregnancy compared with all other groups. In particular, smokers had a higher serum PIGF level compared with women who had a healthy pregnancy, and much higher levels compared with those who developed pre-eclampsia, or fetal growth restriction.

3. Women who smoked during pregnancy had lower sFlt-1 levels compared with healthy women who had a healthy pregnancy and much lower than those who developed pre-eclampsia.

4. Women who smoked during pregnancy had a lower calculated sFlt-1:PIGF ratio compared with women who had a healthy pregnancy and a much lower level compared with women who went on to develop pre-eclampsia.

5. Women who smoked during pregnancy had a similar sFlt-1:PIGF ratio compared with women who were at risk of pre-eclampsia, but who had a healthy pregnancy outcome and also women who had a growth restricted offspring in the absence of hypertension.

6. My observations support the role of an elevated maternal serum sFlt-1:PIGF ratio for the diagnosis and prognosis of pre-eclampsia. Conversely, a low sFlt-1:PIGF ratio has negative predictive value for a diagnosis of pre-eclampsia.

7. Understanding the mechanism by which smoking elevates PIGF and suppresses sFlt-1 may improve our understanding into the aetiology of pre-eclampsia.

3.1 INTRODUCTION

Although the cause of pre-eclampsia is still not fully understood, an imbalance between pro-angiogenic factors such as PIGF and anti-angiogenic factors such as sFlt-1 appears to be one of the main pathogenic mechanisms involved in the development of this condition (Maynard et al. 2003). Women who develop pre-eclampsia have a reduced plasma concentration of PIGF and increased levels of sFlt-1, which is evident as early as 5 to 10 weeks before the clinical manifestation of the condition (Levine et al. 2004).

Pre-eclampsia is one of the main reasons for premature childbirth. It is associated with considerable neonatal morbidity and mortality, as well as high healthcare expenditure (Lozano et al. 2011; Ghulmiyyah & Sibai 2012). It is crucial to be able to identify women who are at risk of developing pre-eclampsia, especially early and severe onset disease, which is associated with a high risk for fetal death (Lisonkova & Joseph 2013).

Substantial efforts have been made to establish biomarkers that could predict pre-eclampsia. Research involving retrospective analysis (Richard J Levine et al. 2004), cross-sectional (Chaiworapongsa et al. 2004) and prospective studies (Romero et al. 2008; Zeisler et al. 2016; Noori et al. 2010) have shed some light into the mechanisms underlying this complex pregnancy related complication. However, it still remains unclear what role changes in blood levels of these factors have in the pathogenesis of pre-eclampsia.

Data from animal studies support the role for sFlt-1 as a causative factor in the development of pre-eclampsia. Infusion of adenovirus expressing sFlt-1 to pregnant rats induces a pre-eclampsia-like syndrome manifest as hypertension, proteinuria and glomerular endotheliosis (Maynard et al. 2003). Additional evidence comes from animal models in which induced uterine hypoxia results in increased production of sFlt-1, hypertension and proteinuria (Makris et al. 2007; Gilbert et al. 2007).

Women with classical risk factors for cardiovascular disease such as hypertension, obesity, hyperlipidaemia and diabetes are also at risk of pre-eclampsia

(Magnussen et al. 2007). The only exception to this association between classical risk factors for cardiovascular disease and pre-eclampsia is smoking, which paradoxically decreases the risk of pre-eclampsia by at least 32% (Conde-Agudelo et al. 1999). The reason behind this paradox is still unclear. Blood levels of sFlt-1 measured in men and non-pregnant women who smoke has been shown to be 50% lower than that of non-smokers (Belgore et al. 2000). Additionally, exposure of placental explants to cell culture medium infused with cigarette smoke resulted in decreased sFlt-1 secretion, with the lowest sFlt-1 levels present associated with the highest concentration of cigarette smoke (Mehendale et al. 2007). Also serum PIGF content in pregnant smokers have been reported to be significantly elevated above levels observed in non-smoking pregnant controls (Jeyabalan et al. 2008; Llurba et al. 2013). Aforementioned studies suggest a potential role of cigarette smoke in preventing pre-eclampsia by lowering the levels of sFlt-1 and increasing availability of free PIGF.

In this prospective study I collected blood samples from pregnant women at four time points during pregnancy. I measured levels of sFlt-1 and PIGF and calculated the sFlt-1:PIGF ratio. I analysed the results both longitudinally and at different time points during pregnancy to understand how these known indicators of pre-eclampsia might relate to the protective effect of smoking and yet still cause fetal growth restriction. In Chapter VI, I correlate angiogenic measures with changes in regulatory immune factors throughout healthy pregnancy and in pregnancies that develop into pre-eclampsia, pregnancy induced hypertension and fetal growth restriction.

3.2 METHODS

3.2.1 Study Design

This study was approved by the Research Ethics Committee (13/LO/0287; IRAS project ID: 108975) and all participants gave written informed consent. In one arm of the study women were recruited early in their pregnancy and followed until the delivery. The majority of them where recruited when attending a first trimester ultrasound scan. A number of patients were also recruited from high-risk pregnancy clinics to enrich the cohort for women at risk of pre-eclampsia.

Additionally, in order to increase the number of subjects for cross-sectional analysis I also recruited patients who had already developed pre-eclampsia.

At each appointment the participant's blood pressure was measured and a blood sample for serum and PBMCs extraction was collected.

Blood samples were collected at 10-18 weeks (N=152), 19-27 weeks (N=165), 28-34 weeks (N=157) and 35-41 weeks (N=154).

3.2.2 Subjects Characteristics

Demographic and clinical characteristics of the study participants are presented in **Table 3.1**. Birth percentile was calculated using GE ViewPoint5 software taking into account birth weight, gender, as well as maternal parameters such as height, weight and ethnicity.

3.2.3 Assessment of sFlt-1 and PIGF Levels

Serum levels of sFlt-1 and PIGF were measured using ELISA kits manufactured by R&D Systems, USA. Both factors were assessed in prospective samples obtained from patients who smoked during pregnancy and had a healthy pregnancy outcome (N=26), smokers who developed FGR (N=11), non-smokers who developed pre-eclampsia (N=12), FGR (N=9) or PIH (N=10), women at high risk of pre-eclampsia, but had healthy pregnancy outcome (N=24) and women who had a healthy pregnancy outcome (N=22), randomly chosen from 66 healthy subjects.

The number of serum samples for sFlt-1 analysis required earlier dilution (3-30 times) in order for the measured concentration to be within the range of a standard for that assay (31.2 pg/ml - 2000 pg/ml). Whereas the majority of samples used to measure PIGF levels did not require dilution prior to assay.

3.2.4 Statistical Analysis

Subjects in the prospective study were divided into those who developed pre-eclampsia (PET; N=12), pregnancy induced hypertension (PIH; N=10), non-smokers who developed fetal growth restriction (FGR; N=9), who smoked during pregnancy but had a healthy pregnancy outcome (SM; N=26), smokers with fetal growth restriction

(SM+FGR; N=11), those who were at risk of pre-eclampsia, but had healthy pregnancy outcome (HR; N=24) and finally those who were healthy and had healthy outcome of pregnancy (H.PREG; N=22). The purpose of this subdivision was to determine if there are any changes in measured factors that can be identified between the various groups.

Two out of 12 pre-eclamptic women had early-onset pre-eclampsia (<34 weeks), four had intermediate onset (≥34 and <37 weeks) and six patients were affected by late pre-eclampsia (≥37 weeks). Due to low numbers the decision was made to combine the data from those three subgroups into one pre-eclamptic group containing 12 subjects who developed pre-eclampsia at any gestation. Similarly, 3 patients who smoked developed early FGR (<34 weeks) and 9 who had late FGR (≥37 weeks) were pooled into one group. Eleven patients were excluded from the analysis as they developed pregnancy complications such as spontaneous premature delivery, intrauterine death and early neonatal death due to leukaemia, tested positive for HIV or had a twin pregnancy.

Cross-sectional analysis through pregnancy was performed using STATA Version 10, using a linear mixed effects model (growth curve) included both fixed and random effects. Due to the fact that the measured blood factors were not normally distributed the data was logarithmically transformed in order to achieve normality. The results are described as coefficients with 95% Confidence Intervals and p values. Detailed explanation of the analysis is described in Chapter II.

Cross-sectional analysis of the blood markers in serum of patients who already developed pre-eclampsia and their gestationally matched healthy controls and smoking subjects was analysed using GraphPad Prism 5.0 software. The data was analysed using a one-way Analysis of Variance (ANOVA) with Kruskal-Wallis test and Dunns post test for multiple comparisons. Data is presented as median with interquartile range.

3.3 RESULTS

Fifty-two of 166 recruited study participants had at least one of the following risk factors for pre-eclampsia: previous pre-eclampsia (N=17), maternal history of pre-eclampsia (N=6), chronic hypertension (N=25), elevated blood pressure at booking appointment (N=5), previous fetal growth restriction (N=3), high pulsality index at 20/24 weeks scan (N=17). Twelve women out of 52 (23%) from the high risk group developed pre-eclampsia, 8 developed pregnancy induced hypertension, 6 fetal growth restriction, 5 were excluded from the analysis and the remaining 24 had healthy outcome of pregnancy. Two patients out of 75 from the low risk group developed pregnancy induced hypertension, 3 fetal growth restriction, 3 had spontaneous pre-term delivery, 1 neonatal death and the remaining 66 were normotensive. The prospective study design is shown in **Figure 3.1**

Previous prospective studies have tended to combine data from patients who had a healthy pregnancy outcome irrespective of their first trimester risk of pre-eclampsia. This approach does not allow an investigation into why only some women at high-risk of pre-eclampsia actually go on to develop the disorder. For this reason, I grouped patients considered at-risk of developing pre-eclampsia, but who subsequently had a healthy pregnancy separately from low risk subjects who had a healthy pregnancy.

Eleven of the recruited subjects who smoked throughout the pregnancy had growth-restricted babies, but none of them developed pre-eclampsia or pregnancy induced hypertension.

3.3.1 Excluded cases

Three women from low-risk and 5 from high-risk group delivered spontaneously premature babies and were therefore excluded from the study. (The aetiology of pre-term labour is of interest, but beyond the scope of my project). One patient from the smoker group had a neonatal intrauterine death at 24th weeks and was also excluded from the analysis. Another case from the low-risk group resulted in a perinatal death due to neonatal leukaemia and was also excluded.

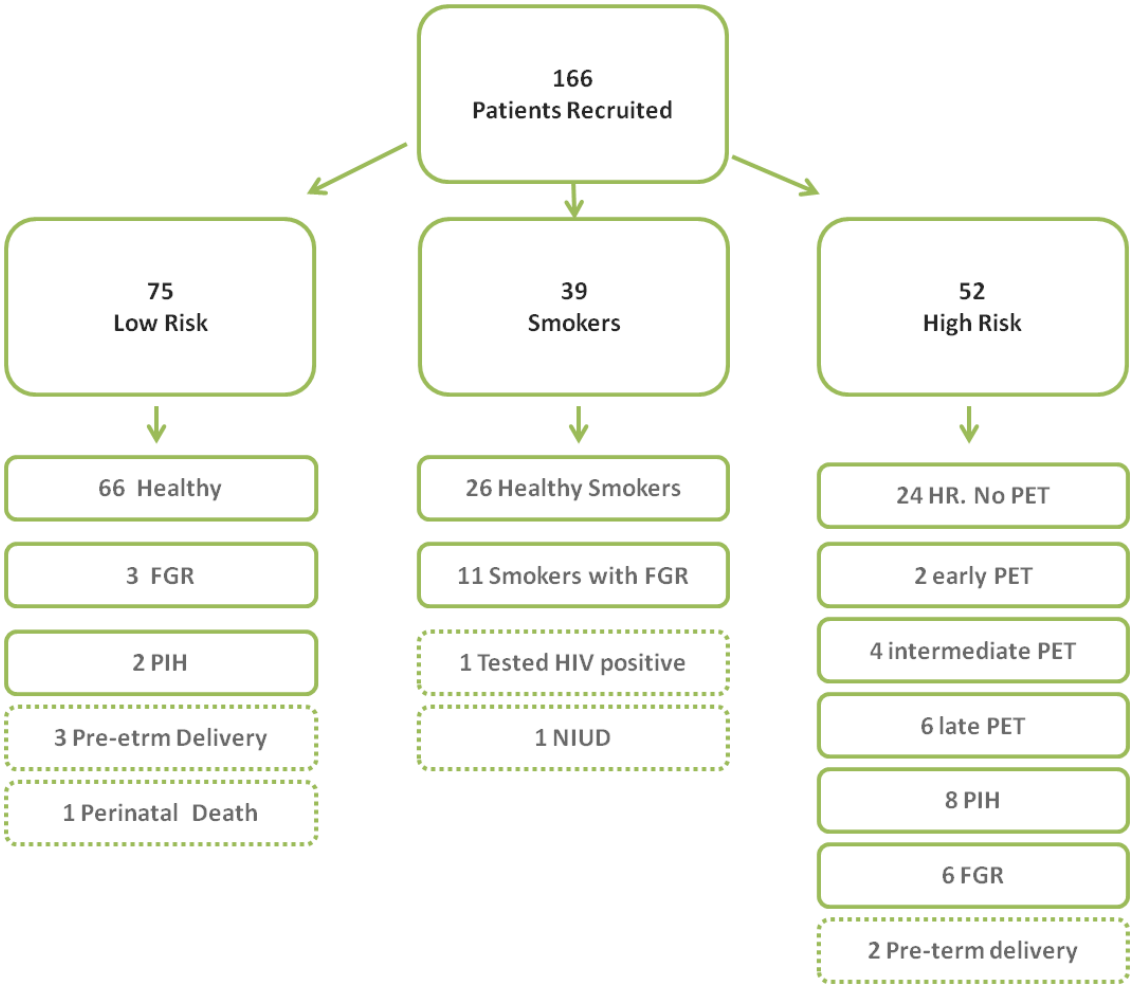

Figure 3.1 Prospective Study design.
166 subjects were recruited including 75 normotensive pregnant, low risk of pre-eclampsia, 52 women at high risk of pre-eclampsia and 39 smoking while pregnant. Patients in dashed line boxes were excluded from the final analysis.

3.3.2 Phenotype Data of Women Included in the Prospective Study

Key findings:

1. Women who developed pre-eclampsia had the highest body mass index (BMI) at 10-18 weeks in comparison with healthy controls ($p < 0.05$) (**Table 3.1**).
2. Smokers with and without fetal growth restriction were significantly younger than those who had healthy pregnancy ($p=0.01$)
3. Patients from all groups delivered babies with significantly lower birth weights compared with healthy controls ($p<0.0001$)

4. Babies born to mothers with pre-eclampsia and those affected by fetal growth restriction alone (smokers and non-smokers) had a significantly lower birth weight centile compared with healthy pregnant subjects ($p<0.001$)
5. Neonates from smokers, even born above the 10^{th} centile, were significantly lighter than babies born to healthy women (birth weight 358g ± 95.11g lighter (geometric mean and SEM, $p=0.01$, t-test).
6. No smokers developed pre-eclampsia.
7. Normotensive pregnant women had a significantly lower mean arterial pressure at 10-18 weeks compared with women who later develop pre-eclampsia (PET, $p=0.001$), pregnancy induced hypertension (PIH; $p=0.001$) and women who were at risk of pre-eclampsia, but had healthy pregnancy outcome ($p=0.001$) (**Figure 3.2**)

	H.PREG	PET	SM	SM+FGR	FGR	PIH	HR no PET	(p value)
Number (N)	66	12	26	11	9	10	24	-
Age (years)	34.4 (±2.8)	32.6 (±6.1)	30.6 (±5.9)	27.2 (±5.5)	35.8 (±5.3)	33.2 (±5.8)	34.9 (±3.8)	<0.0001
Average Serum Cotinine levels (Ng/ml)	0	0	188.4 (±99.1)	239.4 (±86.3)	0	0	0	0.1223
Nulliparous (%)	43 (65%)	6 (50%)	14 (53%)	8 (72%)	5 (55%)	5 (50%)	15 (62.5%)	
MAP at 10-18 Weeks (mmHg)	85.0 (±7.9)	101.2 (±11.2)	80.7 (±7.2)	93.2 (±10.3)	80.3 (±12.2)	101.2 (±7.7)	100.0 (±13.1)	<0.0001
BMI at 10-18 Weeks (kg/m^2)	23.2 (±3.7)	28.5 (±3.8)	25.7 (±5.5)	25.1 (±6.1)	21.4 (±3.8)	25.9 (±5.8)	27.1 (±6.4)	0.0015
Gestation at Delivery (weeks)	40.0 (±1.0)	36.2 (±2.2)	39.0 (±1.6)	35.9 (±2.9)	38.1 (±1.9)	38.7 (±1.1)	39.1 (±1.3)	<0.0001
Birth Weight (g)	3550 (±410.1)	2264 (±451.5)	3192 (±375.5)	1929 (±634.6)	2271 (±267.3)	3145 (±565.4)	3209 (±275.8)	<0.0001
Birth weight centile (SD)	69.1 (±23.3)	17.1 (±21.1)	55.2 (±27)	3.4 (±2.9)	3.0 (±2.7)	55.7 (±30.7)	52.3 (±29.7)	<0.0001
Ethnicity								
White Caucasian	53	8	19	9	5	9	17	
Black African/ Caribben	5	2	1	0	1	0	4	
Asian	4	1	3	2	3	0	1	
Hispanic	4	1	3	0	0	1	2	

Table 3.1 Characteristics of Prospective study participants and birth outcomes.
Abbreviations: pre-eclampsia (PET), pregnancy induced hypertension (PIH), women at high risk of PET, who but stayed healthy (HR no PET), fetal growth restriction (FGR), patients who smoked and normal outcome of pregnancy (SM), those who smoked and developed FGR (SM+FGR) and non-smokers with healthy normotensive pregnancy (H.PREG). One way ANOVA with Dunnet's test for normally distributed data such as birth weight was used and Kurska Wallis test for scattered data such as birth percentile and Mann–Whitney U-test for cotinine levels comparison. Data presented as mean with standard deviation.

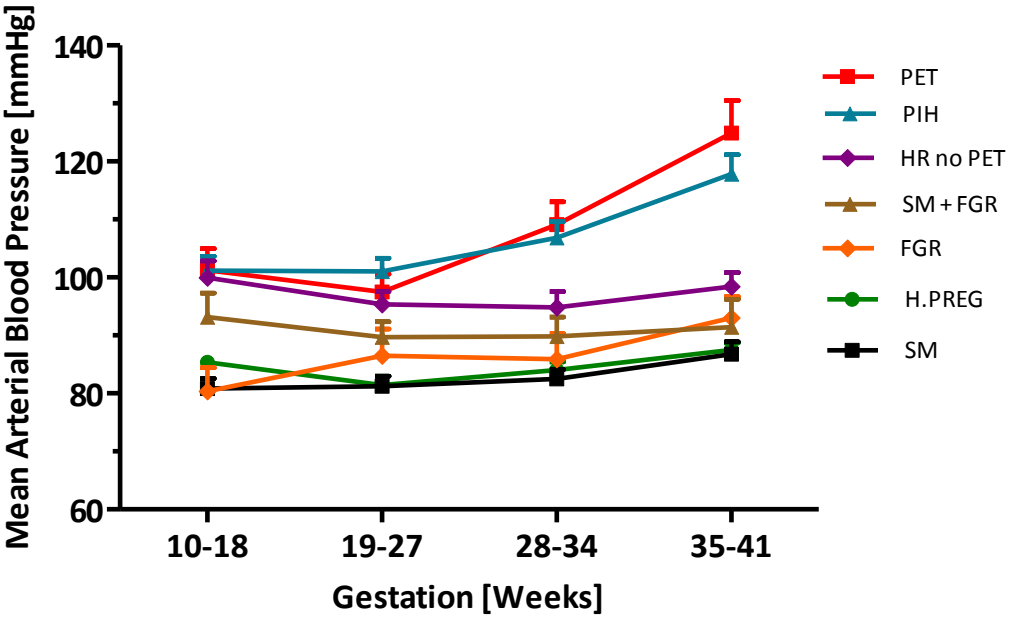

Figure 3.2 Maternal mean arterial blood pressure (MAP) between 10 and 41 weeks gestation.
Data presented for subjects who developed pre-eclampsia (PET; N=12), pregnancy induced hypertension (PIH; N=10), were at high risk of PET but stayed healthy (HR no PET; N=24), fetal growth restriction (FGR; N=9), patients who smoked and normal outcome of pregnancy (SM; N=26), those who smoked and developed FGR (SM+FGR; N=9) and non-smokers with healthy normotensive pregnancy (H.PREG; N=66). Geometric mean and estimated standard errors of the mean are reported on the figure.

Women who smoked and went on to develop FGR appeared to have higher cotinine levels compared with smokers who had babies born above the 10th centile however the difference was not statistically significant at any investigated time point.(**Figure 3.3**).

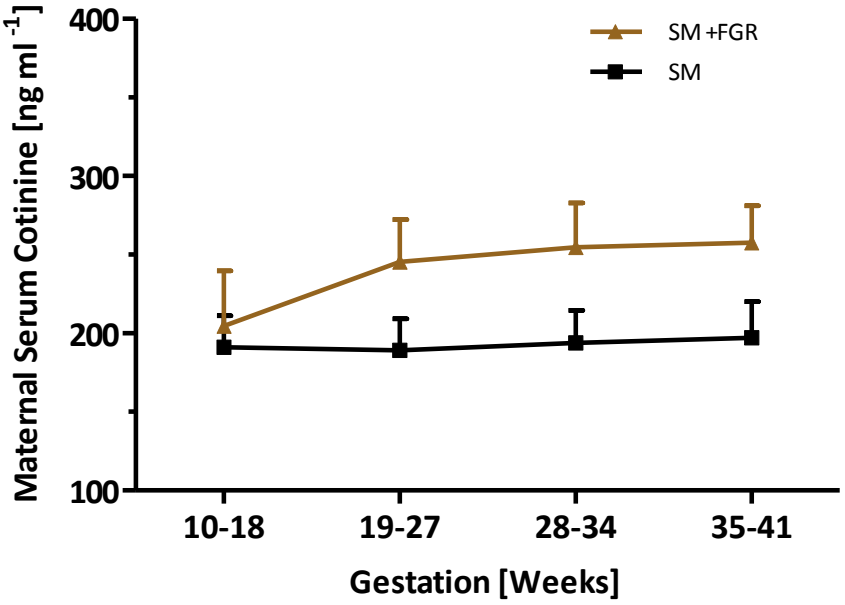

Figure 3.3 Maternal serum cotinine concentration between 10 and 41 weeks of gestation. Non-significant trend of cotinine levels being higher could be observed in group of patients who smoked and developed fetal growth restriction towards (SM+FGR; N=11) versus healthy smokers (SM; N=26). Cross-sectional analysis at each of the time points showed no significant difference between those two groups (non-parametric t-test, $p \leq 0.1223$). Geometric mean and estimated standard errors of the mean are reported on the figure.

3.3.3 Phenotypic Characteristics of Women recruited after the diagnosis of Pre-eclampsia

I recruited 30 women after the onset of pre-eclampsia and included 12 patients from prospective study who developed the condition.

Key points:

1. Women with early onset pre-eclampsia (< 34 weeks) had a significantly higher mean main arterial blood pressure (135.6 mmHg; SD 16.7 mmHg) at the point of diagnosis compared with subjects who developed late pre-eclampsia (≥37 weeks) (120.8 mmHg, SD 7.7mmHg, p=0.0315) (**Table 3.2**).
2. Women with late onset pre-eclampsia had babies born with a higher mean birth weight centile 31.0 (SD ±33.0,) compared with early onset pre-eclampsia 9.0 (SD ±11.6) (p=0.013)

	E PET	I PET	L PET	(p value)
Number (N)	22	6	14	-
Age (years)	32.9 (±5.4)	35.17 (±2.2)	30.43 (±6.4)	0.212
MAP at PET Diagnosis (mmHg)	135.6 (±16.7)	124.8 (±18.7)	120.8 (±7.7)	0.0355
PCR at PET Diagnosis (mg/mmol)	338.6 (±429.9)	195.3 (±115.8)	332.9 (±383.0)	0.8539
Gestation at Delivery (weeks)	30.6 (±2.5)	35.7 (±0.5)	37.6 (±1.1)	<0.0001
Birth Weight (g)	1287 (±383.1)	2097 (±245.0)	2608 (±393.4)	<0.0001
Birth weight centile	8.5 (±11.6)	11.6 (±10.4)	30.5 (±33.0)	0.013

Table 3.2 Clinical characteristics of patients who developed early pre-eclampsia (E PET; < 34 weeks), intermediate pre-eclampsia (I PET ≥ 34 - 37 weeks) and late pre-eclampsia (L PET, ≥ 37 weeks).
Thirty patients were recruited once they developed pre-eclampsia and 12 were part of prospective arm of the study and had pre-eclampsia as an outcome of pregnancy. One way ANOVA with Bonferroni correction multiple comparisons test was used for normally distributed data (age, MAP and birth weight), and Kruskal Wallis with Dunn's post hoc test for scattered data (PCR, gestation at delivery and birth percentile). Data presented as mean and standard deviation.

3.3.4 Comparison of Maternal Serum Angiogenic Factors at the Time of Pre-eclampsia Diagnosis

Data in this section was investigated for normality using histogram, Kolmogorov–Smirnov, D'Agostino & Pearson omnibus normality test and Shapiro-Wilk. When the data was not normally distributed the the Kruskal and Wallis one-way analysis of variance with pots hoc Dunn's multiple comparison test was used. Results are reported as median with interquartile range (IQR).

3.3.4.1 Soluble Flt-1 at the Time of Pre-eclampsia Diagnosis

Cross-sectional studies have shown that pre-eclampsia is associated with high serum concentration of sFlt-1 (Maynard et al. 2003). Published reports also suggest

that smoking may protect from pre-eclampsia by reducing levels of sFlt-1 (Kahn et al. 2011). My study confirmed that maternal serum levels of sFlt-1 are indeed significantly elevated in the peripheral blood of women affected by early (**Figure 3.4A**) and late (**Figure 3.4B**) pre-eclampsia.

Key findings:

1. Women who developed early pre-eclampsia (E PET; ≤ 34 weeks) had higher serum sFlt-1 levels 22706 pg/ml (IQR: 16275-25199 pg/ml) compared with gestation-matched normotensive, non-smoking women (H.PREG) 2118 pg/ml (IQR: 1355-3046 pg/ml) and smokers who had a healthy pregnancy outcome 1964 pg/ml (IQR: 1319-2615 pg/ml) (**Figure 3.4**A)
2. Women who developed late onset pre-eclampsia (L PET; ≥ 37 weeks) had a significantly higher serum sFlt-1 concentration (21317 pg/ml; IQR: 10467-26146 pg/ml) compared with healthy pregnant women (7041 pg/ml; IQR: 4615-9536 pg/ml) and smokers with a healthy pregnancy outcome (3826 pg/ml; IQR: 2448-6605 pg/ml).
3. At term, serum sFlt-1 increased in healthy normotensive women (7041 pg/ml), and remained apparently lower in smoking subjects (3826 pg/ml), but comparison did not reach statistical significance (**Figure 3.4B**).

3.3.4.2 PIGF at the Time of Pre-eclampsia Diagnosis

During healthy pregnancy serum PIGF levels are elevated, but in women destined to develop pre-eclampsia become reduced (Maynard et al. 2003). It has been suggested that smoking during pregnancy increases serum PIGF concentration, which may reduce the risk of pre-eclampsia (Llurba et al. 2013). The results of my study support what has been reported about PIGF in healthy pregnancy and pre-eclampsia.

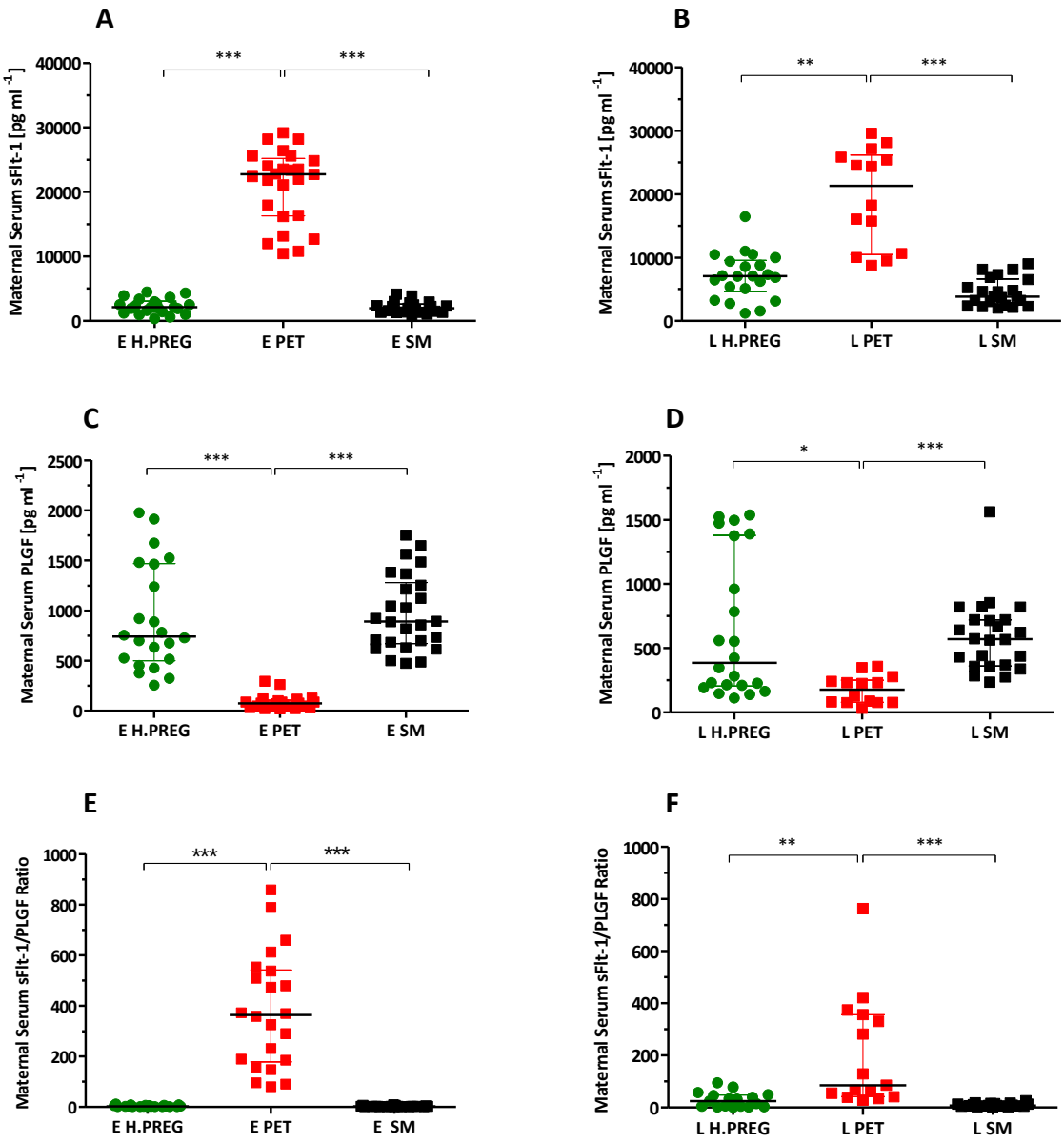

Figure 3.4 Circulating blood levels of sFlt-1, PIGF and the sFlt-1:PIGF ratio, during early (E, <34 weeks gestation) and late (L, >34 weeks) healthy pregnancy (H.PREG), pre-eclampsia (PET) and in women who smoke (SM).
Gestationally-matched healthy and smoking controls were included to rule out changes due to gestational age. Kruskal-Wallis one-way analysis of variance (ANOVA) with Dunn's post hoc was used in the analysis.

Key findings:

1. Maternal serum levels of PIGF in patients with early onset pre-eclampsia (E-PET) were significantly suppressed 73 pg/ml (IQR: 38.56-105.1 pg/ml) compared with gestational-matched healthy controls (740.2 pg/ml; IQR: 498.6-1467

pg/ml; p=0.001) and smokers (889.6 pg/ml; IQR: 670.6-1281 pg/ml; p=0.001) (**Figure 3.4C**).

2. Women with late onset pre-eclampsia (L PET), also had lower median serum concentrations of PIGF 175.4 pg/ml (IQR: 77.63-250.3 pg/ml) compared with 386.1 pg/ml (IQR: 206.4-1379 pg/ml; p<) in healthy pregnancy and 571.5 pg/ml (IQR: 361.4-721.5 pg/ml; p=0.001) in smokers (**Figure 3.4D**).

3.3.4.3 Soluble Sflt-1:PIGF ratio at the time of pre-eclampsia diagnosis

Previous studies have shown greater levels of the sFlt-1:PIGF ratio 5 weeks before and also during pre-eclampsia compared with healthy pregnancy (Zeisler et al. 2016; Levine et al. 2006). The level of sFlt-1:PIGF ratio positively correlates with disease severity and worse clinical outcomes (Pinheiro et al. 2014).

Key points from my own observations:

1. I confirmed that the ratio of sFlt-1:PIGF was much higher in women who had early onset pre-eclampsia (363.7 pg/ml; IQR: 177.8-541.2pg/ml) compared with gestational-matched healthy pregnant women (1.751 pg/ml; IQR: 1.180-5.659 pg/ml; p=0.001) and smokers who had a healthy pregnancy outcome (2.120 pg/ml; IQR: 1.27-2.88 pg/ml; p=0.001) (**Figure 3.4E**).
2. Subjects with late onset pre-eclampsia also had an elevated sFlt-1:PIGF ratio 85.19 pg/ml (IQR: 41.1-356.6 pg/ml) compared with normotensive pregnant women (24.90 pg/ml; IQR:3.72 - 47.90 pg/ml; p=0.01) and smokers (7.04 pg/ml; IQR: 4.689-14.12 pg/ml; p=0.001). (**Figure 3.4F**)

3.3.4.4 Soluble Flt-1 and PIGF in pre-eclampsia and pregnancy induced hypertension

Some published reports suggest that differences in sFlt-1 and PIGF are more pronounced in early compared with late onset preeclampsia (Wikström et al. 2007). I have confirmed that serum levels of sFlt-1 are higher and levels of PIGF lower in pre-eclampsia, regardless of early or late onset of the condition (Levine et al. 2006). Subjects who develop pregnancy induced hypertension (PIH), that is gestational onset hypertension without the multi-organ involvement that defines pre-eclampsia, have

serum concentrations of both factors that lie between healthy, normotensive mothers and those with pre-eclampsia.

Serum sFlt-1 levels

Key findings:

1. Women with pregnancy induced hypertension had serum levels of sFlt-1 (10260 pg/ml; IQR: 3846-12562 pg/ml) that were lower than found in women with early pre-eclampsia (22706 pg/ml; IQR: 16275-25199 pg/ml; p=0.001) and late pre-eclampsia (21317 pg/ml; IQR: 10467-26146 pg/ml; p<0.05), but above those measured in healthy pregnant women at term (H.PREG) (7041 pg/ml; IQR: 4615-9536 pg/ml) (**Figure 3.5A** and **Figure 3.5B**).
2. Surprisingly, I found that subjects who developed pre-eclampsia between 34 and 37 weeks of gestation (IPET; n=6) had suppressed serum sFlt-1 levels compared with women who had early onset of pre-eclampsia (11761 pg/ml; IQR: 8130-16143 pg/ml vs. 22706 pg/ml; IQR: 16275-25199 pg/ml; p<0.05). This may be explained by low numbers recruited in this gestational period.

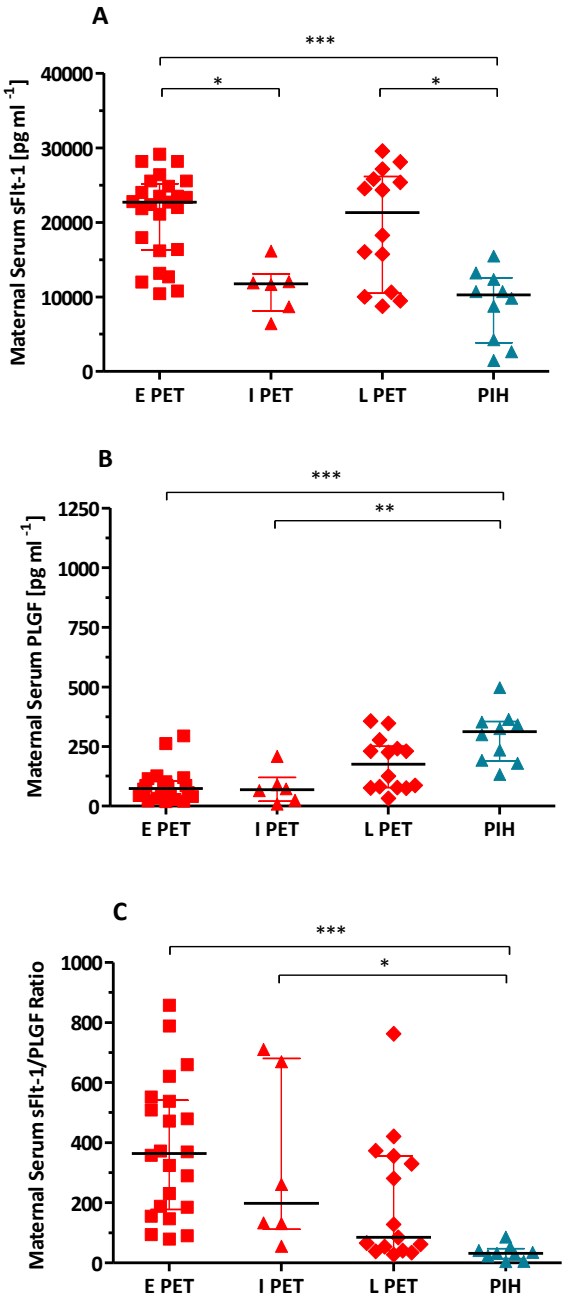

Figure 3.5 Circulating blood levels of sFlt-1, PIGF and the sFlt-1:PIGF ratio, during diagnosis of pre-eclampsia and pregnancy induced hypertension.
Early preeclampsia (E PET; ≤34 weeks gestation), intermediate gestation preeclampsia (I PET; 34-37 weeks) and late onset pre-eclampsia (L PET ≥37 weeks) compared with pregnancy induced hypertension (PIH). Kruskal-Wallis one-way analysis of variance (ANOVA) with Dunn's post hoc was used in the analysis.

Serum PIGF levels

Key findings:

1. I found that medium serum PIGF levels were higher in women with pregnancy induced hypertension 312.2 pg/ml (IQR: 189.5-355.2 pg/ml) compared with women who had early or intermediate onset pre-eclampsia (73 pg/ml; IQR: 38.56-105.1 pg/ml; p=0.001 and 68.63 pg/ml; IQR20.21-120.1 pg/ml; p=0.01 respectively) (**Figure 3.5B**).
2. Although, there was no statistically significant difference in serum PIGF levels between patients with different gestation of onsets of pre-eclampsia, there was a trend towards increasing PIGF levels as pregnancy progressed.
3. There was no significant difference in serum PIGF levels between women with late pre-eclampsia and pregnancy induced hypertension (175.4 pg/ml; IQR: 77.63-250.3 pg/ml vs.312.2 pg/ml; IQR: 189.5-355.2 pg/ml).

The sFlt-1:PIGF Ratio

Key findings:

1. Women who had early-onset pre-eclampsia and intermediate-onset pre-eclampsia had a higher sFlt-1:PIGF ratio (363.7 pg/ml; IQR: 177.8-541.2 pg/ml; p=0.001 and 197.5 pg/ml; IQR: 112.2-680.6 pg/ml; p<0.05, respectively) compared with women who had pregnancy induced hypertension (31.81 pg/ml; IQR: 14.71-46.93 pg/ml) (**Figure 3.5C**).
2. The estimated median values of the sFlt-1:PIGF ratio were not significantly different among all three pre-eclamptic groups, the sFlt-1:PIGF ratio was lowest for subjects with late-onset pre-eclampsia (85.19 pg/ml; IQR: 41.1-356.6 pg/ml).

3.3.5 Longitudinal and Cross-sectional Analyses of circulating angiogenic factors associated with different pregnancy outcomes

Data in this section was analysed using linear mixed effects model that is commonly employed in analysis of prospectively collected data from the same subjects. Values of sFlt-1, PIGF and sFlt-1:PIGF ratio did not pass the normality test

therefore were logarithmically transformed for further analysis. Results are expressed as an overall percentage change in levels of measured factors between 10 and 41 weeks of gestation according to pregnancy outcome (longitudinal analysis) or as percentage change in particular time points (cross-sectional analysis at 10-18, 19-27, 28-34, and 35-41 weeks). Analysed data is reported as coefficients with their corresponding 95% Confidence Intervals (95% CI) and p values.

Although statistical testing was performed on logarithmically transformed values, the geometric mean concentrations and estimated standard errors of the mean are reported on figures. The data points have been obtained by cross-sectional analysis at each of the gestational-age intervals of 10-18, 19-27, 28-34, and 35-41 weeks. The graphs represent changes seen in sFlt-1, PIGF and the sFlt-1:PIGF ratio during pregnancy according to pregnancy outcome.

3.3.5.1 Soluble Flt-1 levels before and during the onset of pre-eclampsia

Many earlier studies have shown that sFlt-1 concentrations are significantly increased above healthy pregnant levels 5-6 weeks before the clinical onset of pre-eclampsia (Levine et al. 2004). My own observations are in line with what has been previously reported about sFlt-1 in pregnancy. The concentration of serum sFlt-1 level in normotensive pregnant women remained constant until around 28 to 34 weeks then started to rise until child-birth. A similar pattern was observed for all other groups except for women who developed pre-eclampsia who showed an accelerated rise in sFlt-1 level and women who smoked and then developed fetal growth restriction (SM+FGR), who failed to show a rise in sFlt-1 level towards term (**Figure 3.6**).

Key findings:

1. Women who developed pre-eclampsia initially had similar sFlt-1 levels to healthy pregnant women at 10-18 weeks (-1.2% difference; 95%CI: -39.0 to 59.9%, p=0.960), which then rose to be +155.5% higher at 28-34 weeks (95% CI: 49.6 to 336.5%, 0.001) and then +229% higher at term (95%CI: 64.4 to 559.7%, p=0.001) (**Table 3.3 & Figure 3.6**).

2. Conversely, smokers who had a normal pregnancy outcome (SM) had -33.6% (95%IC: -53.8 to -4.5%, p=0.027) lower sFlt-1 levels in early pregnancy (10-18 weeks), compared with healthy pregnant women (H.PREG).
3. Women at high risk of pre-eclampsia, but who had a healthy pregnancy outcome (HR no PET) also had lower sFlt-1 levels, -35.2% (95%CI: -55.6 to -5.5%, p=0.024) compared with women who had a healthy pregnancy.
4. Throughout pregnancy smokers who had a healthy outcome (SM) had -45% lower levels of sFlt-1 (95%CI: -2.5 to -105.4%, p=0.036) compared with women who developed pre-eclampsia.
5. Women who smoked throughout pregnancy tended to have lower serum sFlt-1 levels by -21.8% (95%CI: -41.3 to 4.3%, p=0.094) compared with non-smoking subjects who had a healthy pregnancy **(Table 3.3**).
6. Non-smokers who developed fetal growth restriction without hypertension and smokers with normally grown babies had similar levels of sFlt-1 (difference of +1%; 95CI: -33 to 52.5%, p=0.96).
7. Women at risk of pre-eclampsia, but who remained normotensive (HR no PET) had lower serum sFlt-1 levels by -34% (95%CI: -50.9 to -11.3%, p=0.006) compared with healthy subjects and -41.9% lower (95%CI: -59.3 to -17.1%, p=0.003) than sFlt-1 levels observed in women who developed pre-eclampsia.

Difference in sFlt-1 Levels Throughout Pregnancy				
Group	Difference vs. Healthy	95% CI		p value
SM	-21.8	-41.3	4.3	0.094
PET	13.5	-20.7	62.6	0.485
PIH	-20.1	-46.0	18.1	0.257
FGR	-22.6	26.0	-49.2	0.231
SM+ FGR	6.0	-26.9	53.5	0.757
HR no PET	-34.0	-50.9	-11.3	0.006
Difference in sFlt-1 Levels at 10-18 weeks				
SM	-33.6	-53.8	-4.5	0.027
PET	-1.2	-39.0	59.9	0.961
PIH	-11.8	-47.9	49.2	0.639
FGR	-28.9	-62.8	35.8	0.301
SM+ FGR	-15.6	-48.9	39.3	0.507
HR no PET	-35.2	-55.6	-5.5	0.024
Difference in sFlt-1 Levels at 19-27 weeks				
SM	-12.0	-39.6	28.2	0.505
PET	-22.4	-50.8	22.6	0.278
PIH	-10.0	-46.2	50.5	0.688
FGR	-7.0	-44.7	56.2	0.783
SM+ FGR	23.9	-22.5	98.2	0.371
HR no PET	-18.7	-44.9	20.0	0.297
Difference in sFlt-1 Levels at 28-34 weeks				
SM	-1.3	-37.1	54.8	0.954
PET	155.5	49.6	336.5	0.001
PIH	-44.4	-68.5	-1.9	0.043
FGR	-28.3	-61.0	32.1	0.286
SM+ FGR	44.3	-16.8	150.3	0.191
HR no PET	-54.8	-71.9	-27.5	0.001
Difference in sFlt-1 Levels at 35-41 weeks				
SM	-26.1	-57.2	27.7	0.278
PET	229.3	64.4	559.7	0.001
PIH	14.8	-43.5	133.3	0.702
FGR	-37.4	-70.3	32.1	0.219
SM+ FGR	-43.9	-72.8	15.6	0.117
HR no PET	-39.5	-65.2	5.2	0.075

Table 3.3 Data presenting % change in serum sFlt-1 compared with women who had healthy pregnancy (H.PREG).
Values highlighted in green are significant. Red font indicates decrease whereas green indicates increase in sFlt-1 levels. Data is shown as coefficients with 95% CI and p values.

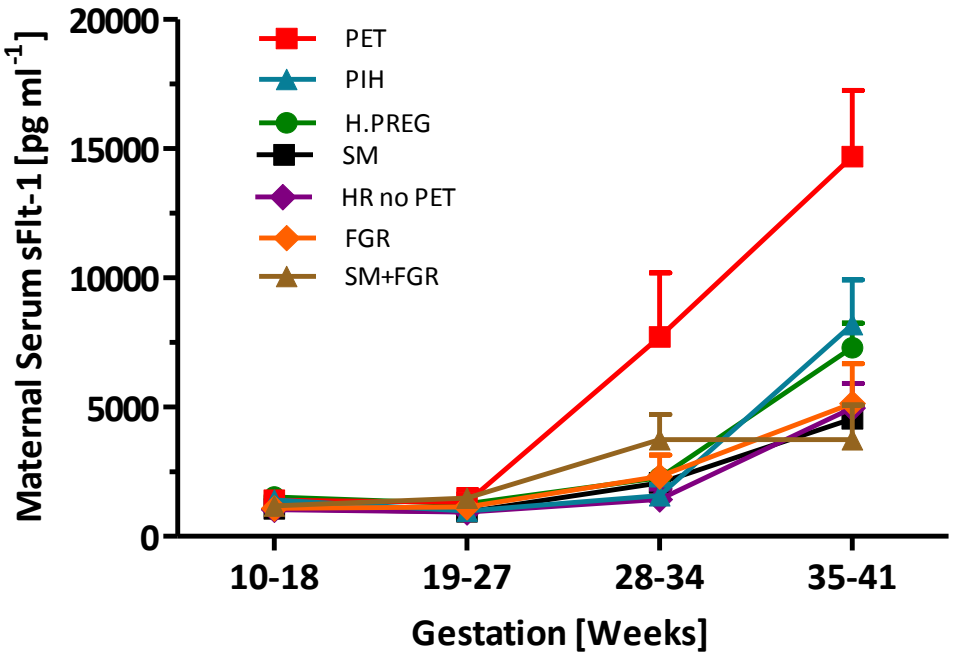

Figure 3.6 Circulating levels of sFlt-1, measured prospectively throughout pregnancy.
Levels in women who go on to develop pre-eclampsia (PET, N=12), pregnancy induced hypertension (PIH), fetal growth restriction (FGR, N=9), who smoked (SM, N=26), who smoked and were affected by fetal growth restriction (SM+FGR, N=11), were at high risk of pre-eclampsia but did not develop pre-eclampsia (HR no PET, N=24) in comparison with women who had a healthy pregnancy (H.PREG, N=22). Only women who were at high risk of pre-eclampsia (HR no PET), had significantly lower levels of sflt-1 throughout pregnancy. Data is displayed as geometric means and estimated standard error of the mean.

3.3.5.2 PIGF levels before and during onset of pre-eclampsia

Cross-sectional studies have shown that serum PIGF levels rise during normotensive healthy pregnancy, but from around 34 weeks gestation, fall gradually towards term, as sFlt-1 levels rise (Levine & Karumanchi 2005). I showed a similar pattern in PIGF levels (**Figure 3.7).** Women who developed pre-eclampsia showed an initial attenuated rise in PIGF concentration, which diminished from around 19-27th week of pregnancy, depending on the onset of clinical pre-eclampsia.

Key findings:

1. From 19-27 weeks, women who later developed pre-eclampsia had PIGF levels -43% lower (95%CI: -63.6 to -10.9%, p=0.014) than gestation-matched healthy women. At 28-34 weeks, serum PIGF levels decreased by -79.9% (95%CI: -87.6 to -67.4%, p<0.0001) in women who later developed pre-eclampsia, and by -77.3% (95%CI: -88.2 to -56.3%, p<0.0001) at term, compared with healthy normotensive pregnant women (**Figure 3.7** and **Table 3.4**)
2. At 10-18 weeks of gestation, women who developed pregnancy induced hypertension (PIH) had PIGF levels that were -44.8% lower (95%CI: -69.5 to 0.3%, p=0.049) in comparison with healthy pregnant subjects (H.PREG).
3. Although, in early pregnancy there was a non-significant increase in PIGF levels (+40.7%; 95%CI: -4.9 to 108.1%, p=0.088) in serum of smokers (SM), at 19-27 weeks sFlt-1 concentration was +48.6% (95% CI: 2.1 to 116.2%, p=0.039) above that measured in healthy controls.
4. From around 28-34 weeks of pregnancy, women who later developed fetal growth restriction (FGR) as well as smokers later affected by FGR (SM+FGR) had significantly decreased serum concentration of PIGF. For non-smoking women with FGR the levels were -53.1% (95%CI: -75.0 to -12.2%, p=0.018) lower at 28-34 weeks and even further decreased at term to -64.7% (95%CI: -83.2 to -26.1%, p=0.006) below the control levels (H.PREG). Whereas, serum PIGF concentration in smokers affected by FGR deteriorated from being -50.1% (95%CI: -72.8 to -8.5, p=0.025) lower than in healthy at 28-34 week to -84.7% (95%CI: -93 to -66.6%, p<0.0001) lower at term.

5. Throughout pregnancy, the overall PIGF levels in women who developed pre-eclampsia were -56.1% lower (95%CI: -68.7 to -38.2%, p<0.0001) than in healthy normotensive pregnant women (**Table3.3**).
6. Conversely, overall PIGF levels in smokers were over +196% (95%CI: 108.8 to 321.3%, p<0.0001) higher than those of women who later developed pre-eclampsia, +74.8% (95%CI: 13.9 to 168.3%, p=0.013) higher than women who had isolated fetal growth restriction (FGR) and +34% higher (95%CI: 0.8 to 78.2%, p=0.044) than normotensive healthy subjects.
7. Serum PIGF levels were suppressed by -34.5% (95%CI: -56.7 to -0.8%, p<0.001) in women with pregnancy induced hypertension and by -43.6% (95%CI: -46.4 to 23.7%, p=0.026) in smokers who later developed fetal growth restriction. An observation was made that overall PIGF serum content in healthy smokers is greater than in any of the other group (**Table 3.4**).
8. Women at high risk of pre-eclampsia, but who remained normotensive, had overall PIGF levels +80% higher (95%CI: 14.4 to 184.5%, p=0.012) than women who developed pre-eclampsia.
9. Throughout pregnancy, the overall PIGF levels from women who developed isolated fetal growth restriction (FGR) showed a tendency to be higher than women who developed pre-eclampsia (+56%; 95%CI: -8.4 to 165.5%, p=0.101).

Difference in PLGF Levels Throughout Pregnancy				
Group	Difference vs. Healthy	95% CI		p value
SM	34.0	0.8	78.2	0.044
PET	-56.1	-68.7	-38.2	<0.001
PIH	-34.5	-56.7	-0.8	0.046
FGR	-29.2	-56.7	15.8	0.166
SM+ FGR	-43.6	-65.9	-6.9	0.026
HR no PET	-18.6	-46.4	23.7	0.328
Difference in PLGF Levels at 10-18 weeks				
SM	40.7	-4.9	108.1	0.088
PET	-1.8	-41.0	63.5	0.943
PIH	-44.8	-69.5	-0.3	0.049
FGR	80.4	-15.6	285.7	0.128
SM+ FGR	-25.6	-61.6	43.9	0.379
HR no PET	42.5	-13.8	135.4	0.167
Difference in PLGF Levels at 19-27 weeks				
SM	48.6	2.1	116.2	0.039
PET	-43.0	-63.6	-10.9	0.014
PIH	-28.9	-58.7	22.4	0.219
FGR	-21.2	-56.3	41.9	0.427
SM+ FGR	-24.2	-57.8	36.1	0.354
HR no PET	-26.9	-55.1	19.0	0.208
Difference in PLGF Levels at 28-34 weeks				
SM	12.9	-25.2	70.5	0.563
PET	-79.9	-87.6	-67.4	<0.001
PIH	-42.3	-66.7	-0.3	0.049
FGR	-53.1	-75.0	-12.2	0.018
SM+ FGR	-50.1	-72.8	-8.5	0.025
HR no PET	-44.2	-66.7	-6.4	0.027
Difference in PLGF Levels at 35-41 weeks				
SM	30.8	-20.3	114.8	0.289
PET	-77.3	-88.2	-56.3	<0.001
PIH	-33.9	-66.2	29.2	0.226
FGR	-64.7	-83.2	-26.1	0.006
SM+ FGR	-84.7	-93.0	-66.6	<0.001
HR no PET	-36.8	-65.1	14.4	0.13

Table 3.4 Data presenting % change in serum PIGF compared with women who had healthy pregnancy (H.PREG).
Values highlighted in green are significant. Red font indicates decrease whereas green indicates increase in sFlt-1 levels. Data is shown as coefficients with 95% CI and p values.

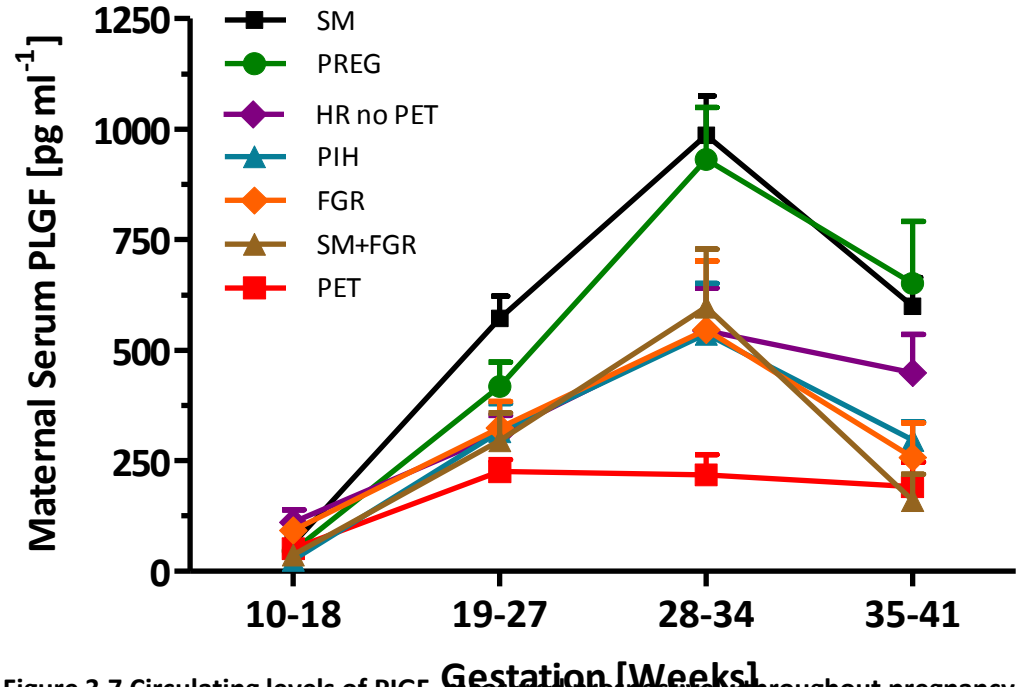

Figure 3.7 Circulating levels of PIGF, measured prospectively throughout pregnancy.
Levels in women who go on to develop pre-eclampsia (PET, N=12), pregnancy induced hypertension (PIH), fetal growth restriction (FGR, N=9), who smoked (SM, N=26), who smoked and were affected by fetal growth restriction (SM+FGR, N=11), were at high risk of pre-eclampsia but did not develop pre-eclampsia (HR no PET, N=24) in comparison with women who had a healthy pregnancy (H.PREG, N=22). Throughout pregnancy, smokers had PIGF levels that were more than 30% higher compared with healthy pregnant women. Conversely, women who went on to develop pre-eclampsia, pregnancy induced hypertension or who smoked with fetal growth restriction had lower levels of PIGF by 56%, 34% and 43% respectively.

3.3.5.3 Soluble Flt-1:PIGF ratio before and during onset of pre-eclampsia

The calculated ratio of serum sFlt-1:PIGF ratio is thought to be a more accurate measure of predicting and diagnosing pre-eclampsia than either of these biological factors alone (Wikström et al. 2007; Levine et al. 2006) (Zeisler et al. 2016).

Key findings:

1. At 10 to18 weeks of pregnancy, the values of sFlt-1:PIGF ratio were -52.5% (95%CI: -71.3 to -21.3%, p=0.004) lower in smokers, -63.3% (95%CI:-86.0 to -3.6%, p=0.042) decreased in FGR group and -54.9% less (95%CI: -75.2 to -17.9%, p=0.009) in high risk group than those of gestationally-matched healthy controls. (**Table 3.5** and **Figure 3.8**)
2. The ratio of sFlt-1:PIGF in healthy smokers (SM) group was similarly low at 19-27 weeks being 40.9% (95%CI:-62.8 to 6.0%, p=0.026) below the control levels (H.PREG).
3. Women who later developed pre-eclampsia had a sFlt-1:PIGF ratio that rose to 1170.4% (95%CI: 529.1 to 2465.4%, p< 0.0001) at 28-34 weeks above those of healthy controls and increased even further to 1444.4% (95%CI: 462.3 to 4141.6%, p<0.0001) higher at term.
4. Interestingly, values of the ratio for smokers who develop FGR were also significantly higher at 28-34 weeks (185.5%; 95%CI: 15.2 to 607.2%, p=0.023) as well as at term (349.3%; 95%CI: 31.6 to 369.7%, p=0.017) when compared to healthy controls. However the levels were still relatively low in comparison with those in pre-eclamptic group. (**Table 3.6**)
5. In overall, the ratio of sFlt-1:PIGF through the whole pregnancy was significantly higher in pre-eclampsia than in any other group being for example 145.1% (95%CI: -56.7 to -18.0%, p=0.002) over healthy pregnancy levels and over 320% (95%CI: 159.3 to 582.4%, p<0.0001) of those in healthy smokers.
6. Another interesting finding was that smokers who later developed fetal growth restriction had much higher sFlt-1:PIGF ratio than smokers with normally grown babies (205.3%; 95%CI: 57.3 to 492.3%, p=0.001).

Difference in sFlt-1/ PLGF Ratio Throughout Pregnancy				
Group	Difference vs. Healthy	95% CI		p value
SM	-40.4	-56.7	-18.0	0.002
PET	145.1	58.1	280.0	<0.0001
PIH	37.6	-22.3	143.4	0.269
FGR	-12.6	-55.5	71.6	0.692
SM+ FGR	63.0	-14.2	209.7	0.133
HR no PET	-23.4	-53.5	26.2	0.288
Difference in sFlt-1/ PLGF Ratio at 10-18 weeks				
SM	-52.5	-71.3	-21.5	0.004
PET	0.6	-48.6	96.8	0.987
PIH	61.8	-24.7	247.6	0.217
FGR	-63.3	-86.0	-3.6	0.042
SM+ FGR	16.8	-48.1	162.6	0.707
HR no PET	-54.9	-75.2	-17.9	0.009
Difference in sFlt-1/ PLGF Ratio at 19-27 weeks				
SM	-40.9	-62.8	-6.0	0.026
PET	36.2	-24.1	144.1	0.300
PIH	26.1	-39.0	160.8	0.531
FGR	20.3	-44.7	161.8	0.641
SM+ FGR	54.4	-27.4	227.9	0.259
HR no PET	9.3	-40.5	100.5	0.775
Difference in sFlt-1/ PLGF Ratio at 28-34 weeks				
SM	-13.1	-50.3	52.0	0.622
PET	1170.4	529.1	2465.4	<0.0001
PIH	-3.6	-58.4	123.3	0.931
FGR	6.2	-61.1	189.4	0.907
SM+ FGR	185.5	15.2	607.2	0.023
HR no PET	-21.9	-63.2	65.6	0.519
Difference in sFlt-1/ PLGF Ratio at 35-41 weeks				
SM	-47.7	-74.7	8.4	0.081
PET	1444.4	462.3	4141.6	<0.0001
PIH	66.5	-43.8	393.3	0.358
FGR	38.1	-59.4	369.7	0.605
SM+ FGR	349.3	31.6	369.7	0.017
HR no PET	8.3	-56.5	169.6	0.863

Table 3.5 Data presenting % change in serum sFlt-1:PIGF ratio compared with women who had healthy pregnancy (H.PREG).
Values highlighted in green are significant. Red font indicates decrease whereas green indicates increase in sFlt-1 levels. Data is shown as coefficients with 95% CI and p values.

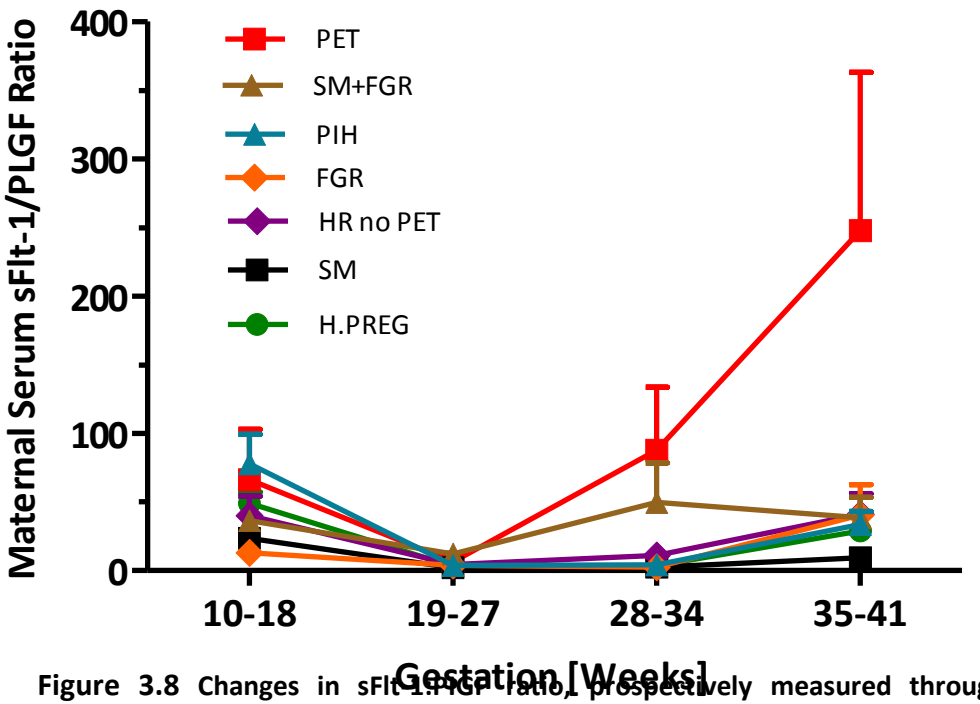

Figure 3.8 Changes in sFlt-1:PIGF ratio, prospectively measured throughout pregnancy.
Women who go on to develop pre-eclampsia (PET, N=12), pregnancy induced hypertension (PIH), fetal growth restriction (FGR, N=9), who smoked (SM, N=26), who smoked and were affected by fetal growth restriction (SM+FGR, N=11), were at high risk of pre-eclampsia but did not develop pre-eclampsia (HR no PET, N=24) in comparison with women who had a healthy pregnancy (H.PREG, N=22). Throughout pregnancy women who smoked had sFlt-1/PIGF ratio that was over 40% lower than women with a healthy pregnancy, and >80% lower than women who later developed pre-eclampsia.

sFlt-1	H.PREG				SM				PET				FGR			
	Difference %	95%CI		p value	Difference %	95%CI		p value	Difference %	95%CI		p value	Difference %	95%CI		p value
H.PERG	-	-	-	-	+27.8	-4.1	70.4	0.094	-11.9	-38.5	26.2	0.485	+29.1	-15.2	96.8	0.231
SM	-21.8	-41.3	4.3	0.094	-	-	-	-	-45.1	-2.5	-105.4	0.036	+1.1	-33	52.5	0.96
PET	+13.5	-20.7	62.6	0.485	+45.1	2.5	105.4	0.036	-	-	-	-	+46.6	-7.8	133.1	0.105
PIH	-20.1	-46	18.1	0.257	+2.1	-30.2	49.3	0.915	-29.7	-54.6	8.9	0.113	+3.1	-36.7	68.1	0.9
FGR	-22.6	26	-49.2	0.231	-1	-34.4	49.3	0.96	-31.8	-57.1	8.4	0.105	-	-	-	-
SM+FGR	+6.00	-26.9	53.5	0.757	+35.4	-5.5	94	0.098	-6.7	-38.6	41.9	0.745	+36.8	-14.7	119.5	0.191
HR no PET	-34.0	-50.9	-11.3	0.006	-15.7	-36.4	11.8	0.233	-41.9	-59.3	-17.1	0.003	-14.8	-43.9	29.3	0.449

PLGF	H.PREG				SM				PET				FGR			
	Difference %	95%CI		p value	Difference %	95%CI		p value	Difference %	95%CI		p value	Difference %	95%CI		p value
H.PERG	-	-	-	-	-34	-0.8	-78.2	0.034	124	124	124	<0.0001	+34.4	17.4	112	0.199
SM	+34.0	0.8	78.2	0.044	-	-	-	-	196.6	108.8	321.3	<0.0001	+74.8	13.9	168.3	0.013
PET	-56.1	-68.7	-38.2	<0.001	-63.9	-74.4	-49	<0.0001	-	-	-	-	-41.7	-63	-8.1	0.021
PIH	-34.5	-56.7	-0.8	0.046	-52.8	-69.4	-27.1	0.001	+45.8	-8.4	131.9	0.111	-14.5	-48.1	40.7	0.533
FGR	-29.2	-56.7	15.8	0.166	-43.9	-66.2	-6.8	0.026	+56	-8.4	165.5	0.101	-	-	-	-
SM+FGR	-43.6	-65.9	-6.9	0.026	-55.8	-73.2	-27.1	0.002	+24.4	-27.2	112.5	0.419	-25.4	-57.7	31.7	0.307
HR no PET	-18.6	-46.4	23.7	0.328	-35.5	-56.4	-4.6	0.029	80.4	14.4	184.5	0.012	7	-35.7	78.1	0.788

sFlt-1/PLGF	H.PREG				SM				PET				FGR			
	Difference %	95%CI		p value	Difference %	95%CI		p value	Difference %	95%CI		p value	Difference %	95%CI		p value
H.PERG	-	-	-	-	+72.1	16.7	153.7	0.007	-67.4	-67.4	-67.4	<0.0001	+48.2	24.7	76.2	<0.0001
SM	-40.4	-56.7	-18	0.002	-	-	-	-	-81.4	-88.8	-69.3	<0.0001	+44.8	19.7	75.1	<0.0001
PET	145.1	58.1	280	<0.0001	+320.7	159.3	582.4	<0.0001	-	-	-	-	-8.2	-25.9	13.7	0.430
PIH	+37.6	-22.3	143.4	0.269	+118	20.7	293.9	0.01	-56.8	-75.8	-23	0.005	-3.3	-22.7	21.0	0.769
FGR	-12.6	-55.5	71.6	0.692	46.9	-26.2	192.5	0.27	-73.1	-86.6	-46.1	<0.0001	-	-	-	-
SM+FGR	+63	-14.2	209.7	0.133	+205.3	57.3	492.3	0.001	-48.5	-75	6	0.071	+22.9	-1.2	52.8	0.064
HR no PET	-23.4	-53.5	26.2	0.288	27.7	-23.9	114.4	0.349	-75.7	-87.3	-53.7	<0.0001	+6.5	-12.5	29.5	0.529

Table 3.6 Data presenting % change in overall levels of sFlt-1, PIGF and sFlt-1:PIGF ratio.
Changes calculated for women who go on to develop pre-eclampsia (PET, N=12), pregnancy induced hypertension (PIH), fetal growth restriction (FGR, N=9), who smoked (SM, N=26), who smoked and were affected by fetal growth restriction (SM+FGR, N=11), were at high risk of pre-eclampsia but did not develop pre-eclampsia (HR no PET, N=24) in comparison with women who had a healthy pregnancy (H.PREG, N=22). Red font indicates decrease whereas green indicates increase in sFlt-1 levels. Data is shown as coefficients with 95% CI and p values.

3.3.5.4 Soluble Flt-1, PIGF and sFlt-1:PIGF ratio in actual numbers at different gestational-age intervals

In the previous paragraph the data was described as an overall percentage change in levels of measured factors but no actual levels were given. The data shown in **Table 3.7, Table 3.8** and **Table 3.9** of this paragraph is given as geometric means with estimated standard error of the mean.

Table 3.7 contains mean values of serum sFlt-1 concentrations and their standard errors of the mean, measured for all of the experimental groups at different gestational intervals. Numbers of samples measured per group at each of the time points are also displayed.

Table 3.8 contains mean values of serum PIGF concentrations whereas, **Table 3.9** describes mean values of sFlt-1:PIGF ratio measured over the time. Both tables have the same outline as **Table 3.7.**

Gestation	H.PREG			PET			SM			SM+FGR			FGR			PIH			HR no PET		
Weeks	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
10-18	1532	152.2	22	1392	137.5	9	1069	85.81	25	1178	108.8	10	1078	205.4	4	1411	339.1	7	1026	88.6	22
19-27	1256	198.3	22	1309	499.8	12	945.9	82.87	26	1499	391.1	11	1129	276.9	8	984.7	247.8	8	936.9	124.1	24
28-34	2265	245.9	22	7722	2472	12	2067	195.9	26	3730	974.3	11	2318	822.8	9	1578	410.2	10	1434	408	23
35-41	7295	955.3	22	14698	2547	10	4554	514.7	26	3744	1326	8	5138	1536	8	8185	1747	10	4961	954.4	24

Table 3.7 Maternal serum sFlt-1 concentration (pg/ml) in healthy pregnancy (H.PREG), pre-eclampsia (PET), smokers (SM), smokers with fetal growth restriction (SM+FGR), isolated fetal growth restriction (FGR), pregnancy induced hypertension (PIH), high risk group with healthy outcome of pregnancy (HR no PET) at different gestational-age intervals.
Values are presented as geometric means with estimated standard error of the mean.

Gestation	H.PREG			PET			SM			SM+FGR			FGR			PIH			HR no PET		
Weeks	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
10-18	46.19	6.459	22	51.16	12.07	9	62.38	7.243	25	36.93	5.153	10	92.46	19.56	4	24.81	6.571	7	110.6	28.61	22
19-27	418.2	54.41	22	255	27.36	12	572.4	49.82	26	295.9	62.46	11	324.7	59.39	8	316.2	63.11	8	310.8	41.33	24
28-34	932.3	117.5	22	217.9	45.55	12	986.5	45.55	26	598	130.2	11	546.4	155.1	9	536.9	114.8	10	543.7	97.07	23
35-41	650.9	140.6	22	190.9	56.09	10	599.8	65.4	26	160	59.17	8	257.5	77.68	8	296.9	40.49	10	448.9	86.72	24

Table 3.8 Maternal serum PIGF concentration (pg/ml) in healthy pregnancy (H.PREG), pre-eclampsia (PET), smokers (SM), smokers with fetal growth restriction (SM+FGR), isolated fetal growth restriction (FGR), pregnancy induced hypertension (PIH), high risk group with healthy outcome of pregnancy (HR no PET) at different gestational-age intervals.
Values are presented as geometric means with estimated standard error of the mean.

Gestation	H.PREG			PET			SM			SM+FGR			FGR			PIH			HR no PET		
Weeks	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
10-18	49.15	8.275	22	66.17	36.5	9	23.71	3.643	25	36.41	5.874	10	13.08	2.737	4	24.81	6.571	7	40.00	14.29	22
19-27	3.204	0.482	22	6.523	1.876	12	2.018	0.263	26	11.92	5.81	11	3.673	0.7622	8	3.755	0.965	8	4.562	1.435	24
28-34	3.519	0.6919	22	87.55	45.94	12	2.610	0.443	26	49.69	28.68	11	3.974	1.192	9	2.607	0.405	10	10.98	7.716	23
35-41	28.92	6.956	22	247.5	115.2	10	9.279	1.362	26	38.54	14.68	8	40.44	22.14	8	33.61	9.324	10	41.22	14.82	24

Table 3.9 Maternal serum sFlt-1:PIGF ratio in healthy pregnancy (H.PREG), pre-eclampsia (PET), smokers (SM), smokers with fetal growth restriction (SM+FGR), isolated fetal growth restriction (FGR), pregnancy induced hypertension (PIH), high risk group with healthy outcome of pregnancy (HR no PET) at different gestational-age intervals.

Values are presented as geometric means with estimated standard error of the mean.

3.4 DISCUSSION

Soluble Flt-1 is an antiangiogenic factor that acts by binding free PlGF and VEGF (Maynard et al. 2008). My work as well as already published research showed that during the last trimester of healthy normotensive pregnancy, the serum levels of sFlt-1 slowly increase and the levels of serum PIGF decrease (Noori et al. 2010). Observations from my study support the theory that pre-eclampsia is associated with an imbalanced relationship between pro-and anti-angiogenic factors.

The first trimester analysis showed similar circulating sFlt-1 levels in subjects who later went on to develop pre-eclampsia and those who had healthy normotensive pregnancy. Nevertheless, significantly raised serum sFlt-1 was detectable before the onset of pre-eclampsia occurred. This suggests that sFlt-1 alone may not be an important marker for prediction of this condition. Other studies also reported similar findings with changes in sFlt-1 concentration preceding the clinical manifestation of pre-eclampsia by about 5 weeks (Chaiworapongsa et al. 2005; Thadhani et al. 2004; R. J. Levine et al. 2004). Data from my study shows that once pre-eclampsia was established levels of sFlt-1 were similarly high in early and late onset pre-eclampsia which is in line with published reports (Noori et al. 2010; Levine et al. 2006).

Microalbuminuria is considered as a marker of endothelial dysfunction and it is evident in women at risk of preeclampsia (Bar et al. 1996). Healthy renal glomeruli have small pores in endothelial cells which are maintained by vascular endothelial growth factor (VEGF) (Kitamoto et al. 2001). Inhibition of VEGF by sFlt-1 results in disruption of this state and leads to proteinuria (Maynard et al. 2003). Therefore, in line with this information is finding from study that women with pre-eclampsia had a greater rise in sFlt-1 levels than women who had pregnancy induced hypertension or a normotensive healthy pregnancy. Gestational proteinuria is also associated with reduced serum levels of PIGF (Holston et al. 2009).

Development of a healthy placenta is partially driven by PIGF (Torry et al. 2004). My research showed even though smokers delivered on average 358g smaller babies than non-smoking healthy counterparts which suggests that some degree of placental under perfusion may be taking place (Wang et al. 1997) their levels of PIGF throughout pregnancy are maintained even higher than in healthy pregnancy. Published research

into why smoking may protect form pre-eclampsia has revealed, that maternal smoking during pregnancy increases the gene expression of PIGF in human placental specimens obtained as early as the 7th week of gestation (Kawashima et al. 2015). What is more this study also found that the ratio of PIGF gene and protein expression on invasive the trophoblast cells under oxygenation was significantly elevated when compared to non-smokers. Therefore it appears that in oxygenated conditions smoking stimulates the expression of PIGF in invasive trophoblast. As I described in Chapter I, this type of trophoblast is responsible for spiral arteries remodelling and therefore successful placentation (Caniggia et al. 2000). The abundant presence of PIGF in serum of smokers showed by me and others suggests that cigarette smoking may protect form pre-eclampsia by increasing the availability of free PLGF. Placenta growth factor could then not only enable the success of early pregnancy but also by increasing vasodilatation and inhibiting the differentiation of dendritc cells facilitate the progress of a healthy pregnancy.

In my thesis, I have shown that the increase in values of the sFlt1:PIGF ratio over healthy controls was not evident before the 27th week of gestation. This could be due to the fact that only 2 out of 12 subjects had early onset pre-eclampsia.

Published studies have shown the significant increase in sFlt-1:PIGF ratio as early as 17-20 weeks of pregnancy for those who develop early pre-eclampsia (Levine et al. 2006). Whereas for subjects who subsequently were affected by late onset of the condition the significant increase in the ratio was not evident until 25-28 weeks of pregnancy. Whatever the gestation of onset of pre-eclampsia, it appears the rise in sflt-1:plgf ratio precedes the clinical onset of signs and symptoms by 5-6 weeks. In that regard, measures of sFlt-1:PIGF ratio in early pregnancy could therefore be useful in identifying patients at low risk of future preterm pre-eclampsia.

My study showed that women who are affected by pregnancy induced hypertension have significantly lower sFlt-1:PIGF ratio than patients with pre-eclampsia. This observation supports the general believe that women who develop pregnancy induced hypertension and those with pre-eclampsia are thought to have different underlying pathologies. Women with pregnancy induced hypertension usually have normally grown babies in relation to a hyperdynamic maternal circulation,

as opposed to placental dysfunction seen in early pre-eclampsia (Bosio et al. 1999). Therefore, my results support the potential role for sFlt-1:PIGF ratio use in clinical setting in diagnosis of onset of pre-eclampsia. Later in pregnancy, in cases where gestational hypertension develops, but there is no clarity to make certain diagnosis, measures of sFlt-1:PIGF ratio may be able to distinguish between patients who will soon develop pre-eclampsia and those who will not. Currently all women with pre-eclampsia-like symptoms are hospitalised until pre-eclampsia is ruled out. Whereas some other cases that may need hospitalisation are over-looked. Although no prevention or treatment for pre-eclampsia exists, early detection and monitoring increase the chances of a better pregnancy outcome (Anon 2013).

As mentioned earlier, the late and early onset of pre-eclampsia were alike in the severity of the condition but surprisingly patients from the intermediate group (34-37 weeks) showed a trend toward a less severe onset. This is almost certainly due to the low numbers of women I recruited to the intermediate gestation onset of pre-eclampsia.

To my knowledge no study has addressed the similarities or differences in circulating cardiovascular factors of patients who were at risk but subsequently did not develop pre-eclampsia with those of healthy or pre-eclamptic subjects. One published report suggests that an early assessment of sFlt-1, PIGF or their ratio does not help to identify which women at high risk of pre-eclampsia (chronic hypertension or previous pre-eclampsia) may develop the condition (Sibai et al. 2008). This may simply reflect the fact that changes in angiogenic factor levels do not occur until 5 weeks before the clonical onset of pre-eclampsia.

Interestingly, data from my study revealed that in overall, patients at high risk of pre-eclampsia, but who had a healthy pregnancy not only had lower sFlt-1 levels, but higher PIGF levels and consequently a lower sFlt-1:PIGF ratio, which taken together is a pro-angiogenic profile that may have compensated for their other risk factors for pre-eclampsia. Women were recruited as high risk because of chronic hypertension and personal history of pre-eclampsia, but it appears that those who did not develop preeclampsia had good placental function as judged by elevated PIGF levels, even

superior to that of healthy pregnant women who had a normotensive pregnancy, which may have compensated for their underlying vulnerability to pre-eclampsia.

Others have shown that the gestational profile of sFlt-1 in healthy smokers is suppressed throughout the duration of pregnancy when compared to healthy pregnant women (Powers et al. 2005; Schmidt-Lucke et al. 2005; Levine et al. 2006). Although I found a similar trend in sFlt-1 in the current cohort of smokers, I observed a markedly elevated PIGF level, which decreased value of the calculated sFlt-1:PIGF ratio. In contrast to healthy pregnancy, a marked decrease in sFlt-1 was revealed when levels of that analyte in smokers were compared to those of latter pre-eclamptic subjects. In this study none of the smokers developed pre-eclampsia but others found that women who smoked and later were affected by pre-eclampsia had similar sFlt-1 levels before and at the onset of pre-eclampsia as healthy controls (Powers et al. 2005). This finding suggests that in patients who are bound to develop pre-eclampsia smoking may exert its protective function by restricting the elevation of sFlt-1 therefore limit the acceleration of condition.

It is tempting to speculate that the decrease in sFlt-1 and increase in PIGF mediated by smoking may contribute to the reduced risk of pre-eclampsia development in subjects who smoke during pregnancy. The potential mechanism involved in the protective role of smoking against pre-eclampsia could be mediated by the induction of placental heme oxygenase 1 (HO-1) by smoking. HO-1 has the ability to inhibit secretion of sFlt-1 from placental endothelial cells (Cudmore et al. 2007). Pre-eclamptic placenta show reduced HO-1 expression and increased sFlt-1 release (Maynard et al. 2003; Ahmed et al. 2000), whereas smokers show an increased expression of HO-1 in the placental basal plate and a dose-depended increase in HO-1 expression in a placental cell line incubated with cigarette smoke (Sidle et al. 2007). These observations may explain why smokers have reduced levels of circulating sFlt-1 during pregnancy and therefore a reduced prevalence of pre-eclampsia.

Although smoking during pregnancy protects from pre-eclampsia it increases the risk of fetal growth restriction (Meyer et al. 1976). Pre-eclampsia and fetal growth restriction have been shown to have similar placental pathology and the majority of

women with pre-eclampsia have growth restricted fetuses. However, not all subjects with FGR develop pre-eclampsia and not all women with pre-eclampsia have small babies. I found that women who had FGR without hypertension had a 75% lower sFlt-1:PIGF ratio compared with women who developed pre-eclampsia with FGR. Additionally, women who had an FGR pregnancy showed a reduction in serum PIGF level from around 28th week of pregnancy, in keeping with asymmetric growth restriction secondary to placental failure. As the source of PIGF is the placenta, this reduction in PIGF likely reflects reduced placental function.

There have been conflicting reports to whether circulating cardiovascular factors in patients who later develop fetal growth restriction are different to those of healthy or pre-eclamptic subjects. Some studies showed decreased PIGF and increased sFlt-1 presence when compared to healthy subjects (Wallner et al. 2007) whereas in other studies the levels are similar in both groups (Levine et al. 2006). The finding that non-smoking subjects who later develop fetal growth restriction have suppressed levels of PIGF could be potentially explained by the fact that the source of PIGF, the placenta is usually smaller in those women. The reason why patients who subsequently develop fetal growth restriction but not pre-eclampsia could be potentially explained by the observation that although their circulating PIGF is suppressed the secreted sFlt-1 is not high enough to cause the maternal onset of pre-eclampsia.

Although my study was underpowered due to low number of subjects in some of the groups, I showed that pregnant women who smoke during pregnancy have quite different cardiovascular profile to healthy pregnant non-smokers and women who ultimately develop pre-eclampsia. Despite the fact that 28% of smokers developed fetal growth restriction none of them was affected by pre-eclampsia or even pregnancy induced hypertension.

In summary, data from my work supports the paradox of smoking having protective effect against pre-eclampsia development. Further studies should investigate whether this effect persists in pregnant women who swap cigarette smoking for nicotine patches or vaping.

Any investigation into why pre-eclampsia develops or smoking protects should ensure that also women who developed pre-eclampsia despite smoking are included in the studies. They can potentially lead to discovery of the protective mechanism which could be implemented in preventing pre-eclampsia from happening. A major advantage of my study over those already published was that patients were carefully assigned into groups, taking into account all patient demographics and clinical data. Larger prospective studies would have a greater potential of unravelling the underlying pathology of pre-eclampsia, as opposed to PIH alone and FGR alone. Further, recognition that pre-eclampsia is a multi-system disorder with a multi-faceted pathophysiology involving an immune interaction between mother and the placenta, mother and her vasculature, alterations in angiogenic and vasoactive factors as well as metabolic changes. There will be several ways in which this cocktail of influences can ultimately lead to pre-eclampsia.

CHAPTER IV: REGULATORY T CELLS BEFORE AND DURING THE ONSET OF PRE-ECLAMPSIA

ABSTRACT

Regulatory T (T_{Reg}) cells are key players for a successful pregnancy. Reduced numbers and impaired function are implicated in pregnancy complications such as miscarriage (Jin et al. 2009; Sasaki et al. 2004) and pre-eclampsia (Sasaki et al. 2007). There have been a few cross-sectional studies, but no prospective analysis of how regulatory T cells in the peripheral blood change throughout pregnancy (Steinborn et al. 2010; Toldi et al. 2012; Prins et al. 2009; Aluvihare et al. 2004). Interestingly, outside of pregnancy, immunosuppressive $CD4^+CD25^+FOXP3^+$ T regulatory cells levels have been found to be higher in smokers compared with non-smokers (Hampras et al. 2012). As cigarette smoking during pregnancy is associated with a reduced incidence of pre-eclampsia I set out to investigate the changes in maternal peripheral blood of T_{Reg} cells from 10 weeks of gestation until childbirth in women who smoke, women who are at high risk of pre-eclampsia and healthy pregnant women. My main findings are summarised below.

1. Throughout pregnancy, smokers with uncomplicated pregnancy and non-smoking healthy controls had similarly high blood content of $CD4^+CD25^{hi}$ T_{Reg} cells.

2. Women who developed pre-eclampsia, and/or fetal growth restriction had a much lower percentage of $CD4^+CD25^{hi}$ T_{Reg} cells when compared with healthy pregnant women (-38.1%; 95%CI: -46.7 to -28.0%, $p < 0.0001$ for PET, and -32.5; 95%CI: -29.1 to -19.8; $p<0.0001$ for FGR).

3. The lower percentage of T_{Reg} cells in women who developed pre-eclampsia was evident from the first trimester of pregnancy, months before the onset of clinical pre-eclampsia.

4. Patients who went on to develop pregnancy induced hypertension (PIH) as well as women at risk of pre-eclampsia, but who had healthy pregnancy outcome (HR no PET) had decreased percentage of $CD4^+CD25^{hi}$ T_{Reg} cells, compared with healthy pregnant women (-34.7%; 95%CI: -44.6 to -23.1%, $p<0.0001$ for PIH and -28.2%;

95%CI: -36.5 to -18.7%, p<0.0001 for HR no PET) , however the levels were not as low as in pre-eclampsia.

5. Smokers who had a healthy pregnancy had the highest levels of natural T_{Regs} (nT_{Regs}), whereas women who develop pre-eclampsia had the lowest prevalence of natural T_{Reg} cells population.

6. CD4 positive lymphocytes from women with pre-eclampsia, which contained a low percentage of suppressive $CD4^{+}CD25^{hi}$ T_{Reg} cells, showed a greater proliferating rate than cells harvested from the peripheral blood of healthy pregnant women.

My observations support the theory that insufficiently expanded numbers of T regulatory cells contribute to the development of pre-eclampsia.

4.1 INTRODUCTION

During healthy pregnancy a mother must allow invasion and growth of the semi-allogenic placenta within her decidua. In order not to reject the half foreign conceptus, the maternal immune system must make profound changes. One of the proposed mechanisms is mediated by regulatory T (T_{Reg}) cells (Kahn & Baltimore 2010). Regulatory T cells are a subtype of $CD4^+$ T lymphocytes that regulates other members of the immune system including NK cells (Ghiringhelli et al. 2005), B cells (Lim et al. 2005) and T cells (Takahashi et al. 1998). In addition to function in suppressing autoimmune responses, in pregnancy T_{Reg} cells suppress aggressive allogeneic responses that may be directed against the fetus (Aluvihare et al. 2004). Two subsets of T_{Reg} cells have been described. Depending on where they develop, Treg cells are called natural, if they originate in the thymus and induced T_{Reg} cells if they are generated in the periphery (Bluestone & Abbas 2003).

Natural regulatory $CD4^+$ T (nT_{Reg}) cells are produced during early fetal and neonatal development (Darrasse-Jèze et al. 2005) and throughout life are involved in the surveillance of self-antigens and immune homeostasis (Sakaguchi 2004). Induced regulatory $CD4^+$ T (iT_{Reg}) are generated from naïve $CD4^+$ T cells after being activated by antigens (Apostolou et al. 2002). In healthy pregnancy the overall number of circulating T_{Reg} cells doubles compared with non-pregnant healthy controls (Somerset et al. 2004). In a mouse model, depletion of the total T_{Reg} cells leads to nearly complete fetal rejection, whereas depleting only iT_{Reg}, but not nT_{Reg} cells causes abnormal spiral artery remodelling and only partial fetal resorption (Samstein et al. 2012). The maternal decidua is the interface with the placenta where maternal immune cells directly encounter feto-placental antigens. Therefore, it is not surprising that the numbers of T_{Reg} cells in decidua are even higher than in peripheral blood (Heikkinen et al. 2004; Tilburgs et al. 2008). Their presence in the uterus before implantation of the blastocyst, facilitates the optimal environment for embryonic development, while T_{Reg} cell deficiency creates adverse conditions with infiltration of pro-inflamatory T_{H1} cells (Teles et al. 2013).

In addition to suppressing immune responses, T_{Reg} cells transfered to abortion prone mice was shown to boost numbers of the uterine mast cells positively influencing placentation and the remodelling of spiral arteries (Woidacki et al. 2015). There is a good amount of evidence suggesting that T_{Reg} cells are important for the implantation stage and maintenance of early pregnancy (Shima et al. 2010) but their role in the later stages of pregnancy is not well known. Current reports suggest that T_{Reg} cells may be less involved in the maintenance of an advanced pregnancy. According to experiments in mice, T_{Reg} cell depletion after implantation causes only a moderate increase in the abortion rate (Samstein et al. 2012).

There is good evidence that spontaneous abortions in human pregnancy is associated with a diminished number and reduced suppressive ability of regulatory T cells suggesting a major role for T_{Reg} cells in the regulation of maternal immune tolerance toward fetal-placental alloantigens (Jin et al. 2009; Arruvito et al. 2007). This early pregnancy data currently does not exist for pre-eclampsia. Many hypotheses generated to explain the pathology of pre-eclampsia suggest that the condition may be triggered by the inadequate development of maternal immune tolerance toward half-foreign placenta and fetus (Saito & Sakai 2003; Redman & Sargent 2003). The fact that pre-eclampsia is most common in the first pregnancy (Kenny et al. 2014), and that sperm exposure before pregnancy can induce tolerance to paternal human leukocyte antigens (HLA) and reduce incidence of pre-eclampsia (Saftlas et al. 2014) supports the theory of failed immune adaptation to pregnancy.

While most studies report decreases of T_{Reg} cells numbers in peripheral blood and decidua of women with pre-eclampsia (Hsu et al. 2014; Prins et al. 2009), there are some that were not able to support these findings (Steinborn et al. 2012; Paeschke et al. 2005; Sasaki et al. 2007).

Although a few studies have investigated the level of circulating regulatory $CD4^+$ T cells cross-sectionally at different stages of pregnancy (Steinborn et al. 2008; Steinborn et al. 2012; Somerset et al. 2004), to my knowledge there has been no published research that has followed the changes in circulating T_{Reg} cells throughout pregnancy in a prospective manner.

4.2 METHODOLOGY

4.2.1 Study Design and Subjects Characteristics

This study was approved by Research Ethics Committee (13/LO/0287; IRAS project ID: 108975), and all participants gave written informed consent. I studied the same cohort of 166 women as described in Chapter III. Blood for peripheral blood mononuclear cells (PMBCs) isolation was collected prospectively at the same time as the samples for sFlt-1 and PLGF measurements from all the subjects mentioned in the previous chapter. Similarly, patients who were recruited only once they developed pre-eclampsia had their blood taken for measures of cardiovascular and immune factors at the same time. However, not all of the patients with pre-eclampsia had blood drawn for the peripheral blood mononuclear cells (PMBCs) isolation due to the fact that the immune part of the project started later than the cardiovascular measures. Furthermore, in this chapter I describe 6 additional participants, healthy non-pregnant women, who also had their blood collected for assessment of T_{Reg} cell levels.

4.2.2 Assessment of Regulatory T Cells Numbers

Isolated PBMCs were stained with fluorescently labelled antibodies against CD4, CD25, CD127, Foxp3 and Helios as described in **Chapter II**. Helios was chosen as a marker to distinguish natural from induced regulatory T $CD4^+$ cells. Although there is a lot of discussion whether Helios is a specific marker for natural T_{Reg} cells, I found it to be suitable to discriminate between natural and inducible Tregs for the following reasons. Previously published report showed that the frequency of Helios positive cells within thymus-based $CD4^+CD8^-CD25^+Foxp3^+$ regulatory T cells was higher in mice than Neuropilin-1 (Nrp-1) positive cells (Singh et al. 2015). Nrp-1, a co-receptor for class III semaphorins and the VEGF family (He & Tessier-Lavigne 1997; Pellet-Many et al. 2008; Soker et al. 1998) is sugested as a marker for thymus derived regulatoy T cells (Papatriantafyllou 2012). Nrp-1 expression seems to appear in the later stages of T cell development than Helios and Foxp3. Majority of the studies investigating Nrp-1 as a marker of T_{Reg} cells were conducted in mice (Yadav et al. 2012; Weiss et al. 2012). When I attempted to measure Nrp-1 expressing $CD4^+Foxp3^+$ cells within collected PBMCs, the levels were almost impossible to detect (**Figure 4.1**).

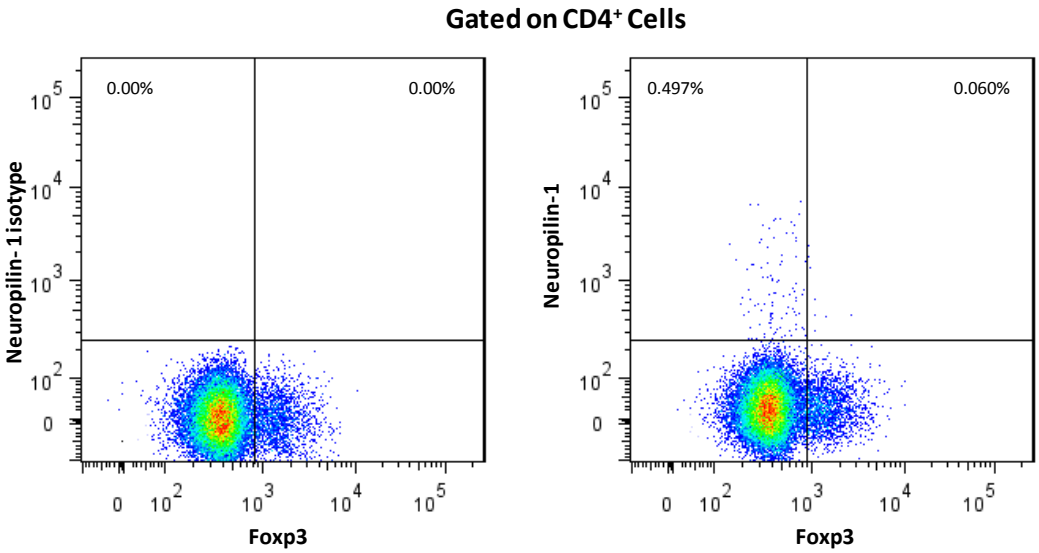

Figure 4.1 Gating strategy used in identification of Neuropilin-1 expressing $CD4^+Foxp3^+$ cells. The dot plots graph on the left shows the gating strategy used to identify Neropilin-1 whereas graph on the right depicts the percentage of detected $CD4^+Foxp3^+Nrp\text{-}1^+$ cells (0.06%).

Others have also found that the frequency of Nrp-1 positive T_{Reg} cells in peripheral human blood and secondary lymphoid organs is nearly undetectable (Milpied et al. 2009). Due to the difficulties with detecting $Nrp\text{-}1^+$ T cells, I have chosen Helios as a differentiatior between induced ans natural regulatory T cells.

I analysed stained cells using flow cytometry; BD LSRFortessa™ (BD, UK) and BD LSR II Flow Cytometer (BD, UK). Gating strategy for $CD4^+CD25^{hi}Foxp3$ cells was performed in a similar way to previously publish reports (Sasaki et al. 2007) and presented in **Figure 4.2**. I gated $CD4^+CD25^+CD127^-Foxp3$ as described in another study (Steinborn et al. 2012) and shown in figure **Figure 4.3.** I gated $CD4^+Foxp3^+Helios^{+/-}$cells as presented in **Figure 4.4**.

The frequency of regulatory $CD4^+$ T cells was measured using permutations of various surface markers. Cells were identified as $CD4^+Foxp3^+$, $CD4^+CD25^+Foxp3^+$, $CD4^+CD25^{hi}$ and $CD4^+CD25^+CD127^-$. My analysis revealed that in all of the cases the cell percentage within $CD4^+$ T population was very similar. Cells identified as $CD4^+CD25^+CD127^-$ are shown here only as an example of an alternative way of defining T_{Reg} cells.

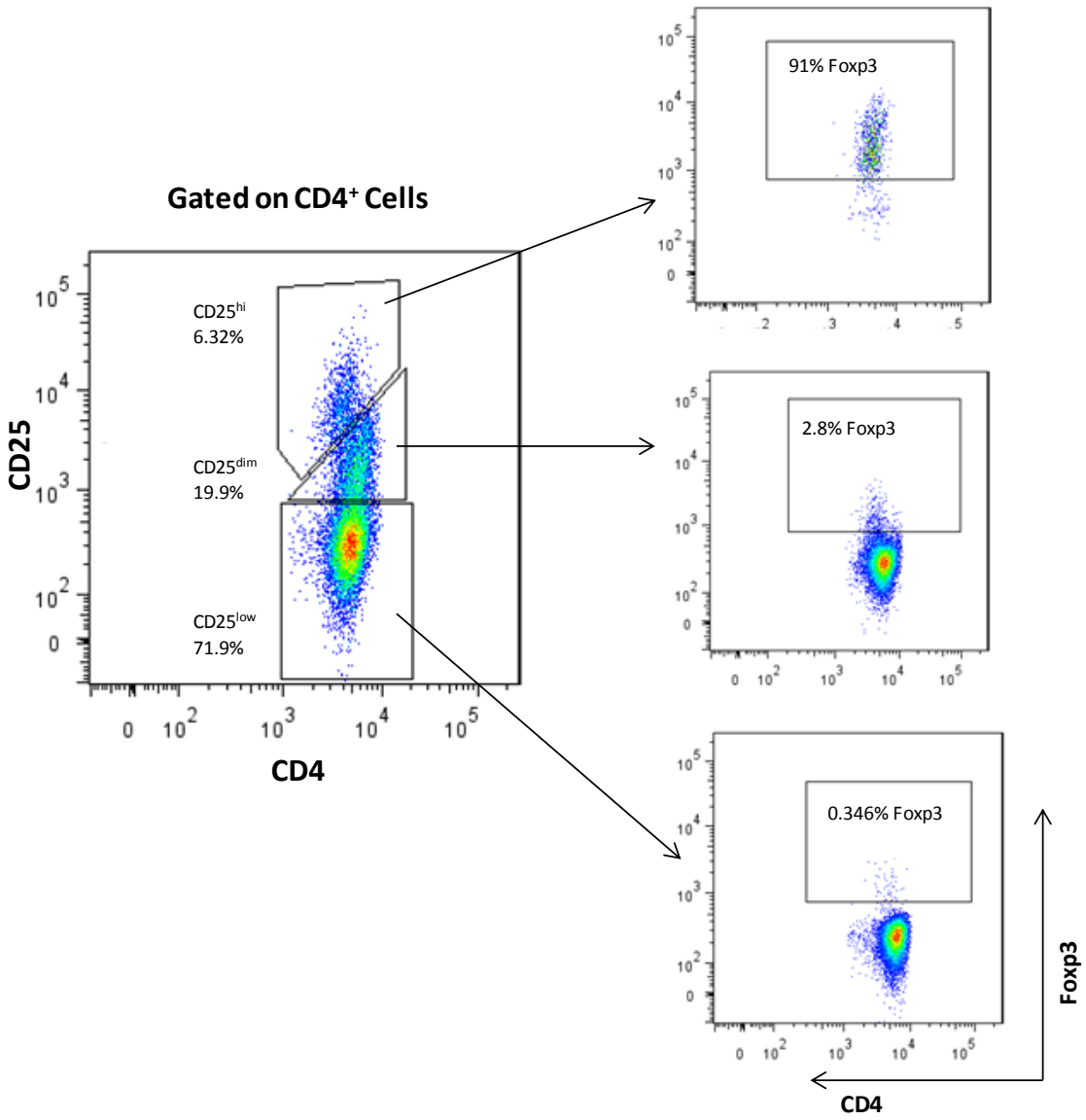

Figure 4.2 Gating strategy used for identification of $CD4^+CD25hiFoxp3$ cells.
$CD4^+$ T lymphocytes expressing high levels of CD25 marker (T_{Reg} cells) had the highest percentage of Foxp3 positive cells (91% in the case presented here), activated T cells ($CD25^{dim}$) had lower content of Foxp3 positive cells,2.8% (always below 10%), whereas Foxp3 expression within responder T cells ($CD25^-$) was usually blow 1% (0.346% here).

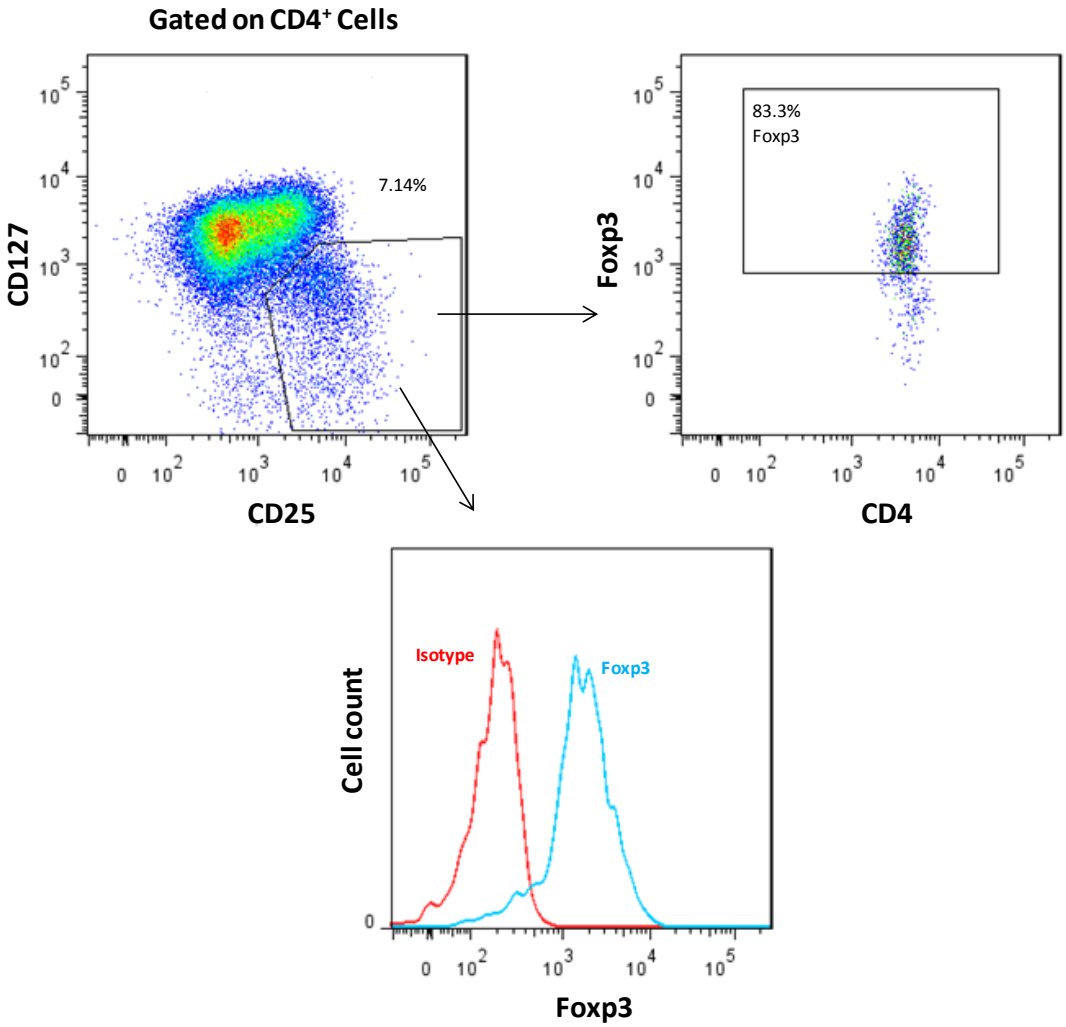

Figure 4.3 Gating strategy used in identification of $CD4^+CD25^+CD127^-$Foxp3 cells.
The percentage of Foxp3 positive cells within $CD4^+CD25^+CD127^-$ population was usually around 80% (top right figure). The histogram on the bottom shows content of Foxp3 cells within the maternal population in comparison with isotype control.

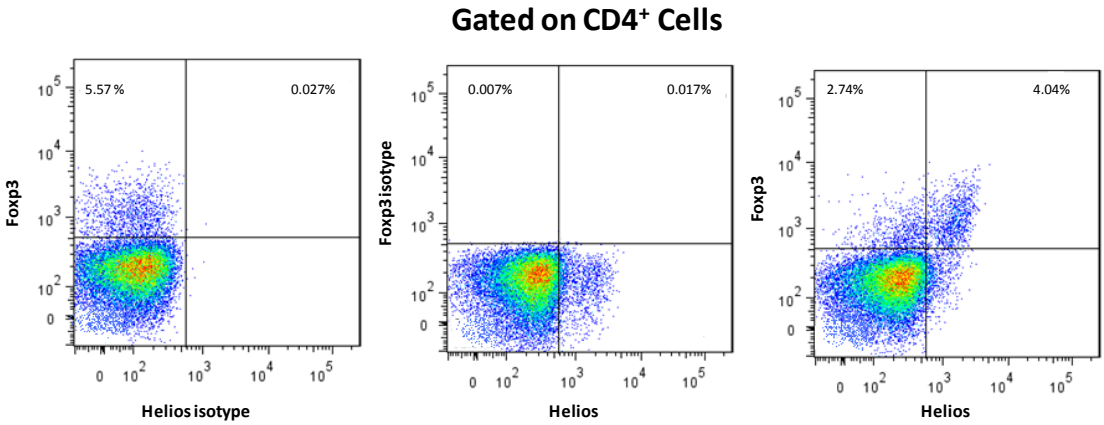

Figure 4.4 Gating strategy used for determining Foxp3^{+}Helios^{+} and Foxp3^{+}Helios^{-} cells within CD4 positive cells.

Cells known as natural T_{Reg}, expressing Helios marker are identified on the top right quadrant of the far right graph (4.04%) and Helios negative, induced T_{Reg} on the left (2.74%).

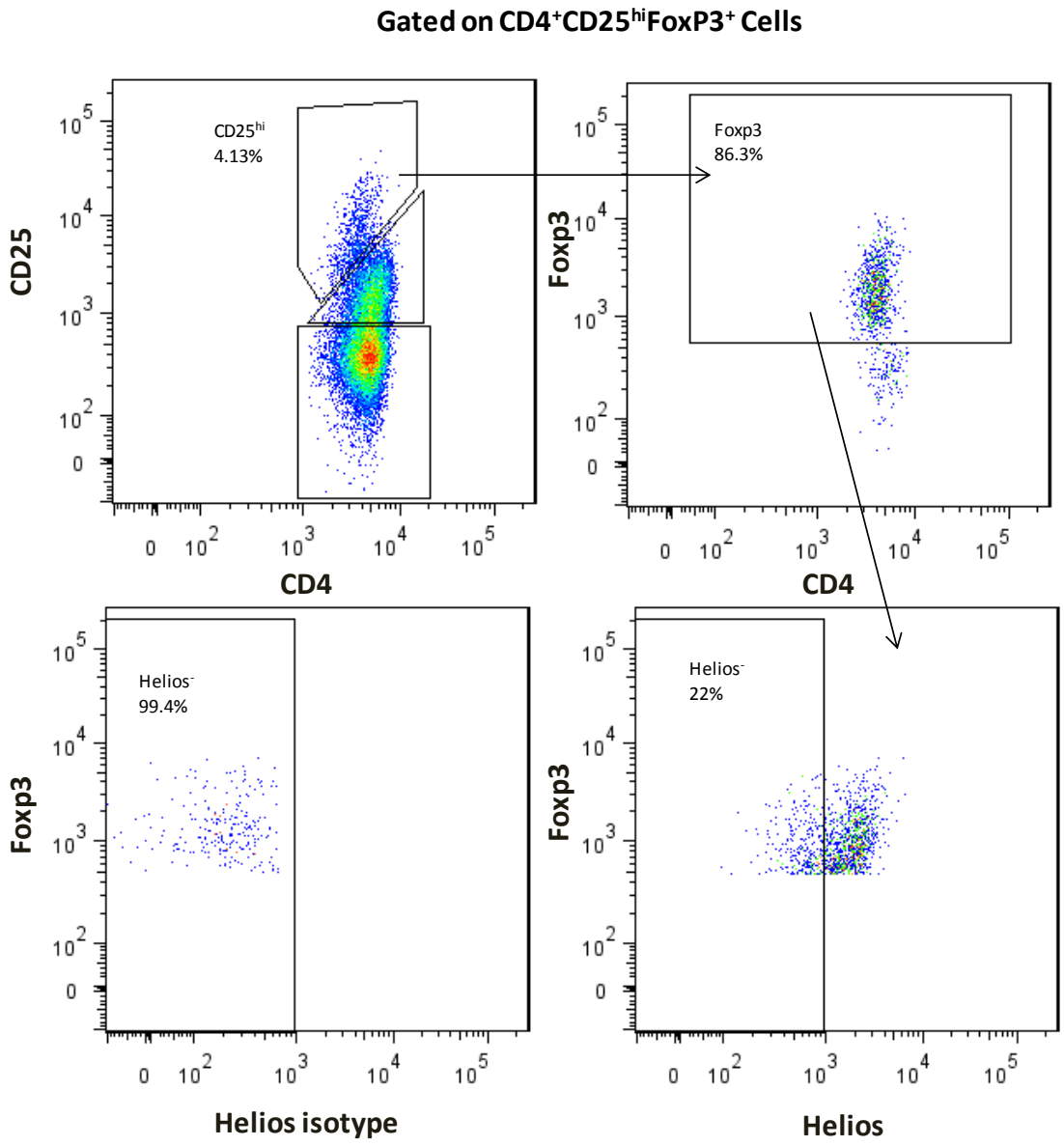

Figure 4.5 Gating strategy used for determining $CD25^{hi}Foxp3^{+}Helios^{+}$ and $CD25^{hi}Foxp3Helios^{-}$ cells within CD4 positive cells.
Cells were gated on regulatory T cells expressing high content of CD25 marker. Identified with this population Foxp3 cells were then analysed according to their expression of Helios based on standards fixed by isotype control.

Studies investigating T_{Reg} cells in pregnancy vary in terms of surface markers used in identifying those cells. Some investigators pin the analysis on $CD4^{+}CD25^{+}$ cells (Somerset et al. 2004; Toldi et al. 2012) or $CD4^{+}Foxp3^{+}$ (Hsu et al. 2012) and others measure $CD4^{+}CD25^{+}CD127^{-}$ (Steinborn et al. 2012) regulatory T cells.

When I compared the percentage of cells expressing CD25, but lacking CD127 marker with the population that have high levels of CD25, the numbers of identified

T_{Reg} cells were similar within investigated groups. However, the proportion of cells identified as $CD4^+CD25^+CD127^-$ was higher than T_{Reg} cells described as $CD4^+CD25^{hi}$ in healthy pregnant women and those who had pre-eclampsia **(Figure 4.6).** Healthy pregnancy (H.PREG) $CD127^-$ 7.11%; (IQR: 6.4 to 7.9%) compared with CD25hi 5.8% (IQR: 5.2 to 7.0%, p=0.0055) and PET $CD127^-$ 4.7% (IQR: 4.105 to 5.6%) vs. PET CD25hi 3.34%(IQR: 3.108 to 3.945%, p<0.0001).

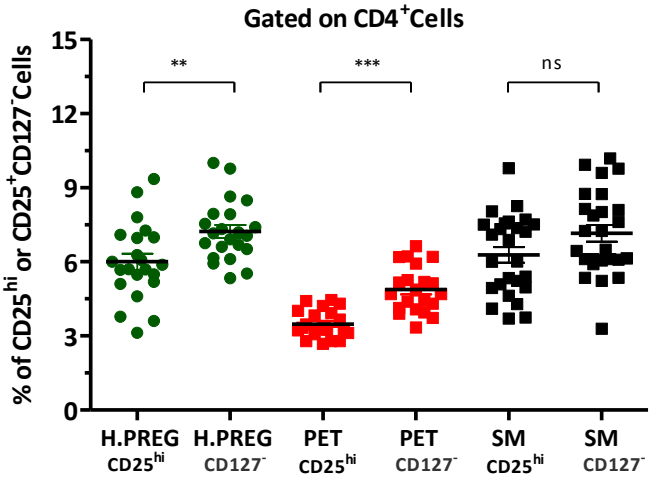

Figure 4.6 Comparison of subsets of T_{Reg} cells percentages defined as $CD4^+CD25^{hi}$ and $CD4^+CD25^+CD127^-$ at 27-34 weeks of gestation in healthy pregnant women (H.PREG, N=22), pre-eclampsia (PET) and smokers (SM).
The % of T_{Reg} cells described by lack of expression of CD127 marker were higher in healthy pregnant and in pre-eclampsia when compared to the cells identified using $CD25^{hi}$ marker. In either case the pattern in % levels of Tregs were similar between H.PREG, PET and SM regardless of the combination of markers used. Data was normally distributed therefore the pairs of $CD25^{hi}$ and $CD127^-$ populations were analysed using two tailed t-test (** = p <0.01, *** = p < 0.001, ns = non significant p = 0.0634). Data presented as medians with interquartile range (IQR).

Due to the fact that I conducted functional assays on regulatory T cells defined as $CD4^+CD25^{hi}$ cells, I also decided to describe this particular population throughout this whole chapter.

4.2.3 Statistical analysis

As in Chapter III, subjects from the cohort were divided depending on their pregnancy outcome into those who developed pre-eclampsia (PET; N=12), pregnancy induced hypertension (PIH; N=10), fetal growth restriction (FGR; N=9), who smoked

but had healthy pregnancy outcome (SM; N=26), smokers who were ultimately affected by fetal growth restriction (SM+FGR; N=11), women who were at risk of pre-eclampsia but did not develop the condition (HR no PET; N=24) and finally those who were healthy and had healthy outcome of pregnancy (H.PREG; N=66).

Statistical test used in the analysis of the data are described in detail in Chapter II. In brief, data was analysed longitudinally using a linear mixed-effects model and results are reported as coefficients with their corresponding 95% Confidence Intervals, and p values below 0.05 are considered as significant. In majority cases the data was scattered therefore prior to the analysis the values were logarithmically transformed in order to achieve normality. Due to the fact that the chances in frequency of T_{Reg} cells were shown to be independent of time/gestation the statistical model used in the analysis provided data on overall changes in levels of T_{Reg} cells through ought the pregnancy but not cross-sectional comparison at different time-points. Although statistical testing was performed on logarithmic transformed values, the geometric mean and estimated standard errors of the mean are reported on some of the figures for the visual clarity. The data points have been obtained by cross-sectional analysis at each of the gestational-age intervals of 10-18, 19-27, 28-34, and 35-41 weeks. The graphs represent changes seen in $CD4^{+}CD25^{hi}$ T_{Reg} cells percentage during pregnancy according to pregnancy outcome.

For cross-sectional investigation of T_{Reg} cells frequency I used the GraphPad Prism 5.0 software. Regulatory T cells levels at the 1^{st} trimester tested positive for normality distribution therefore the analysis was performed using one-way ANOVA corrected for multiple comparisons with Bonferroni's test and was expressed as mean with standard errors of the mean, with p-value of <0.05 as significant. Similarity, for the cross-sectional analysis of T_{Reg} cells in circulation of patients with established PET, PIH and their gestationally-matched healthy or smoking counterparts was performed by either one-way ANOVA corrected for multiple comparisons with Bonferroni's test if the data was normally distributed, or Kruskal-Wallis test with Dunn's post test for multiple comparisons if it was not. Data is presented as medians with interquartile range (IQR).

Analysis of data obtained in functional assay was performed using two-tailed t-test. Data is reported as mean with estimated standard error of the mean and p values below 0.05 are considered as significant.

4.3 RESULTS

4.3.1 Regulatory T cells at the Time of Pre-eclampsia Diagnosis

My research supports the view that levels of circulating regulatory T cells are reduced in pre-eclampsia. The proportion of $CD4^+$ T cells that express high levels of CD25 marker in the peripheral blood of women affected by early (**Figure 4.7 A**) and late (**Figure 4.7 A**) and intermediate pre-eclampsia (**Figure 4.8**) were indeed significantly reduced.

Key Results:

1. At 28-34 weeks of pregnancy, smokers and non-smoking healthy controls had elevated percentage of $CD4^+CD25^{hi}$ T_{Reg} cells compared to non-pregnant healthy women (5.795% [IQR: 5.170 to 7.018%3.775%] and 6.455% [IQR: 4.963 to 7.528%] respectively vs. 3.775% [IQR: 2.975 to 4.763%], $p<0.05$) (**Figure 4.7 A**)
2. Non-pregnant women and subjects who developed early pre-eclampsia had similar levels of T_{Reg} subset (3.775%; IQR: 2.975 to 4.763% vs. 3.34%; IQR: 3.180 to 3.945%).
3. Women who developed early pre-eclampsia (E PET; ≤ 34 weeks) had decreased percentage of $CD4^+CD25^{hi}$ T_{Reg} cells 3.34% (IQR: 3.180 to 3.945%) compared with gestation-matched normotensive, non-smoking women (H.PREG) 5.795% (IQR: 5.170 to 7.018%, p=0.001) and smokers (SM) 6.455% (IQR: 4.963 to 7.528%, p=0.001).
4. Patients with late onset of pre-eclampsia (L PET; ≥ 37 weeks) also had a significantly reduced proportion of $CD4^+CD25^{hi}$ T_{Reg} cells (3.245%; IQR: 3.083 to5.095%) compared with healthy pregnant women (5.67%; IQR: 4.743 to 7.093%, $p<0.05$) and smokers with a healthy outcome of pregnancy (6.225%; IQR: 4.613 to 7.550%, $p<0.01$) (**Figure 4.7 B**).

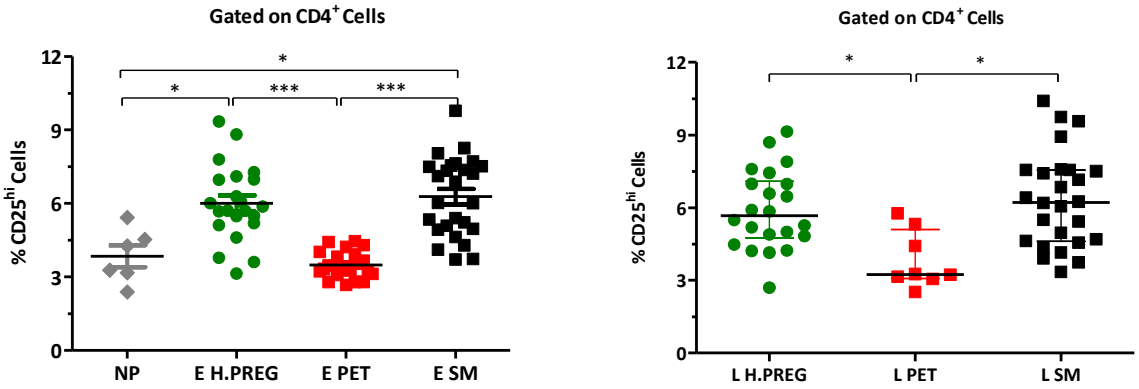

Figure 4.7 Peripheral blood levels of $CD4^{+}CD25^{hi}$ T_{Reg} cells, in non-pregnant women (NP), during early (E; <34 weeks gestation) and late (L; ≥ 37 weeks) in healthy pregnancy (H.PREG), pre-eclampsia (PET) and in women who smoked during pregnancy (SM).
Gestationally-matched healthy and smoking controls were included to rule out changes due to gestational age. Not all data was normally distributed therefore Kruskal-Wallis test with Dunn's post test for multiple comparisons was used (* p< 0.05, *** p<0.001). Data is presented as medians with interquartile range.

5. The percentage of $CD4^{+}CD25^{hi}$ T_{Reg} cells in early pre-eclampsia 3.34% (IQR: 3.180 to 3.945%), intermediate pre-eclampsia (3.885%; IQR: 3.088 to 4.540%) and late pre-eclampsia (3.245%; IQR: 3.083 to5.095%) were similar to pregnancy induced hypertension (3.975%; IQR: 3.475 to 5.195%).

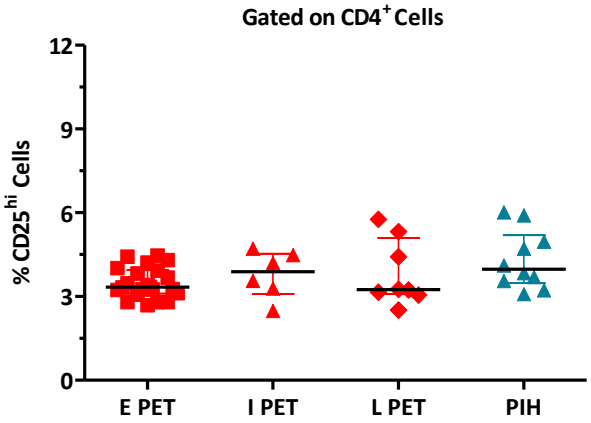

Figure 4.8 Peripheral blood levels of $CD4^{+}CD25^{hi}$ T_{Reg} cells, during diagnosis of early preeclampsia (E PET; ≤34 weeks gestation, N=22), intermediate gestation preeclampsia (I PET; 34-37 weeks, N=6) and late onset PET (L PET; ≥37 weeks, N=8) compared with pregnancy induced hypertension(PIH, N=10).
Kruskal-Wallis test with Dunn's post test for multiple comparisons was used (* p< 0.05, *** p<0.0001). Data is presented as medians with interquartile range (IQR).

4.3.2 Longitudinal and Cross-sectional analysis of Peripheral Blood T_{Reg} Cells Associated With Different Pregnancy Outcomes

4.3.2.1 Regulatory $CD4^{+}CD25^{hi}$ T Cells in Prospectively Collected Blood Samples

In my prospective study of pregnant women, the percentage of $CD4^{+}CD25^{hi}$ T_{Reg} cells in healthy pregnancy rose until around 19-27 weeks of gestation and then gradually decreased as pregnancy progressed (**Figure 4.9**). Interestingly, women who later developed pre-eclampsia (PET) had persistently low blood levels of $CD4^{+}CD25^{hi}$ T_{Reg} cells from the first trimester.

Key findings:

1. Throughout pregnancy smokers who had a normal pregnancy outcome (SM) had similar levels of $CD4^{+}CD25^{hi}$ T_{Reg} cells as healthy non-smokers (H.PREG) (-2.3%, 95%CI: -12.8 to 9.4%, p=0.684). Smokers had constant levels of Tregs throughout pregnancy (**Table 4.1**).
2. Women who developed pre-eclampsia, PIH, or fetal growth restriction had a significantly lower percentage of T_{Reg} cells compared with healthy pregnant women. Women who went on to develop pre-eclampsia had the lowest level of Tregs compared with healthy pregnant women (-38.1%; 95%CI: -46.7 to -28.0%, p<0.0001).
3. Non-smokers who later developed fetal growth restriction (FGR), women at high risk of pre-eclampsia, but who had a healthy pregnancy outcome (HR no PET) and subjects from pregnancy induced hypertension group (PIH), all had similar percentage of $CD4^{+}CD25^{hi}$ T_{Reg} cells to women who later developed pre-eclampsia. What is more, changes in T_{Reg} cells % seen throughout pregnancy were nearly identical in all four groups.
4. The prevalence of T_{Reg} cells in blood of women who smoked and developed FGR deteriorated as pregnancy progressed. These women showed a tendancy to lower $CD4^{+}CD25^{hi}$ Treg levels -15.1% (95%CI: -28.7 to 1.1%, p=0.065) compared with smokers who had a healthy pregnancy outcome.

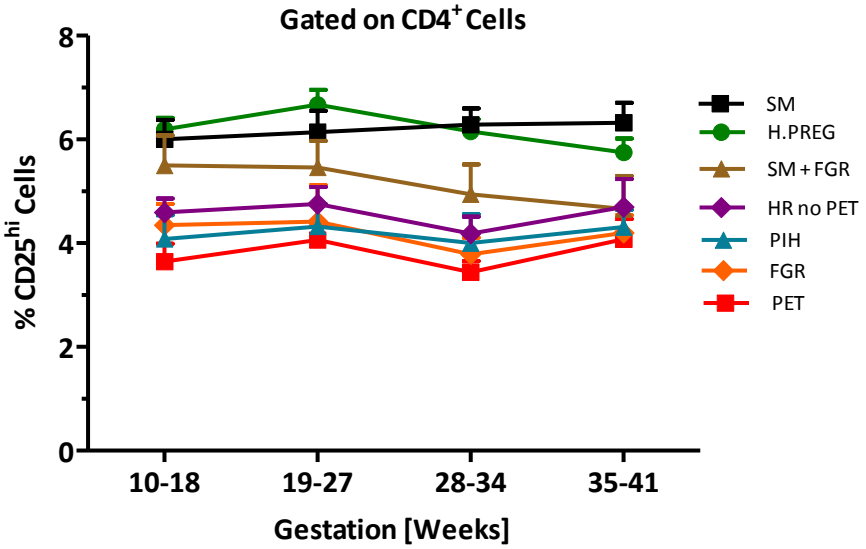

Figure 4.9 Changes in the % of $CD4^+CD25^{hi}$ T_{Reg} cells measured prospectively throughout pregnancy.
Data for women who go on to develop pre-eclampsia (PET; N=12), pregnancy induced hypertension (PIH; N=10), fetal growth restriction (FGR; N=9), who smoked (SM; N=26), who smoked and were affected by fetal growth restriction (SM+FGR; N=11), were at high risk but did not develop pre-eclampsia (HR no PET; N=24) in comparison with women who had a healthy pregnancy (H.PREG; N=66). Data presented as means with estimated standard error of the means.

$CD4^+CD25^{hi}$	H.PREG				SM				PET				FGR			
	Difference %	95%CI		p value	Difference %	95%CI		p value	Difference %	95%CI		p value	Difference %	95%CI		p value
H.PERG	-	-	-	-	+2.3	12.8	9.4	0.684	+61.5	39.0	87.6	<0.0001	+48.2	24.7	76.2	<0.001
SM	-2.3	-12.8	9.4	0.684	-	-	-	-	+57.7	33.2	86.8	<0.0001	+44.8	19.7	75.1	<0.001
PET	-38.1	-46.7	-28.0	<0.0001	-36.6	-46.5	-24.9	<0.0001	-	-	-	-	-8.2	-25.9	13.7	0.43
PIH	-34.7	-44.6	-23.1	<0.0001	-33.2	-44.3	-19.9	<0.0001	+5.4	-14.3	29.5	0.618	-3.3	-22.7	21.0	0.769
FGR	-32.5	-29.1	-19.8	<0.0001	-30.9	-42.9	-16.5	<0.0001	+8.9	-12.0	34.9	0.43	-	-	-	-
SM+FGR	-17.1	-29.1	-3.1	0.019	-15.1	-28.7	1.1	0.065	+33.8	9.6	63.5	0.005	+22.9	-1.2	52.8	0.064
HR no PET	-28.2	-36.5	-18.7	<0.0001	-26.5	-36.5	-14.9	<0.0001	16.0	-2.7	38.3	0.098	+6.5	-12.5	29.5	0.529

Table 4.1 Data presenting % change in overall $CD4^+CD25^{hi}$ T_{Reg} cells levels in pregnancy.
Healthy subjects (H.PREG), smokers (SM), women who go on to develop pre-eclampsia (PET), pregnancy induced hypertension (PIH), fetal growth restriction (FGR), who smoke and develop fetal growth restriction (SM+FGR), who were at risk but did not develop pre-eclampsia (HR no PET) compared with H.PREG, SM, PET and FGR levels. Values highlighted in green are significant. Red font indicates decrease (-) whereas green indicates increase (+) in T_{Reg} cells. Data is shown as coefficients with 95% CI and p values.

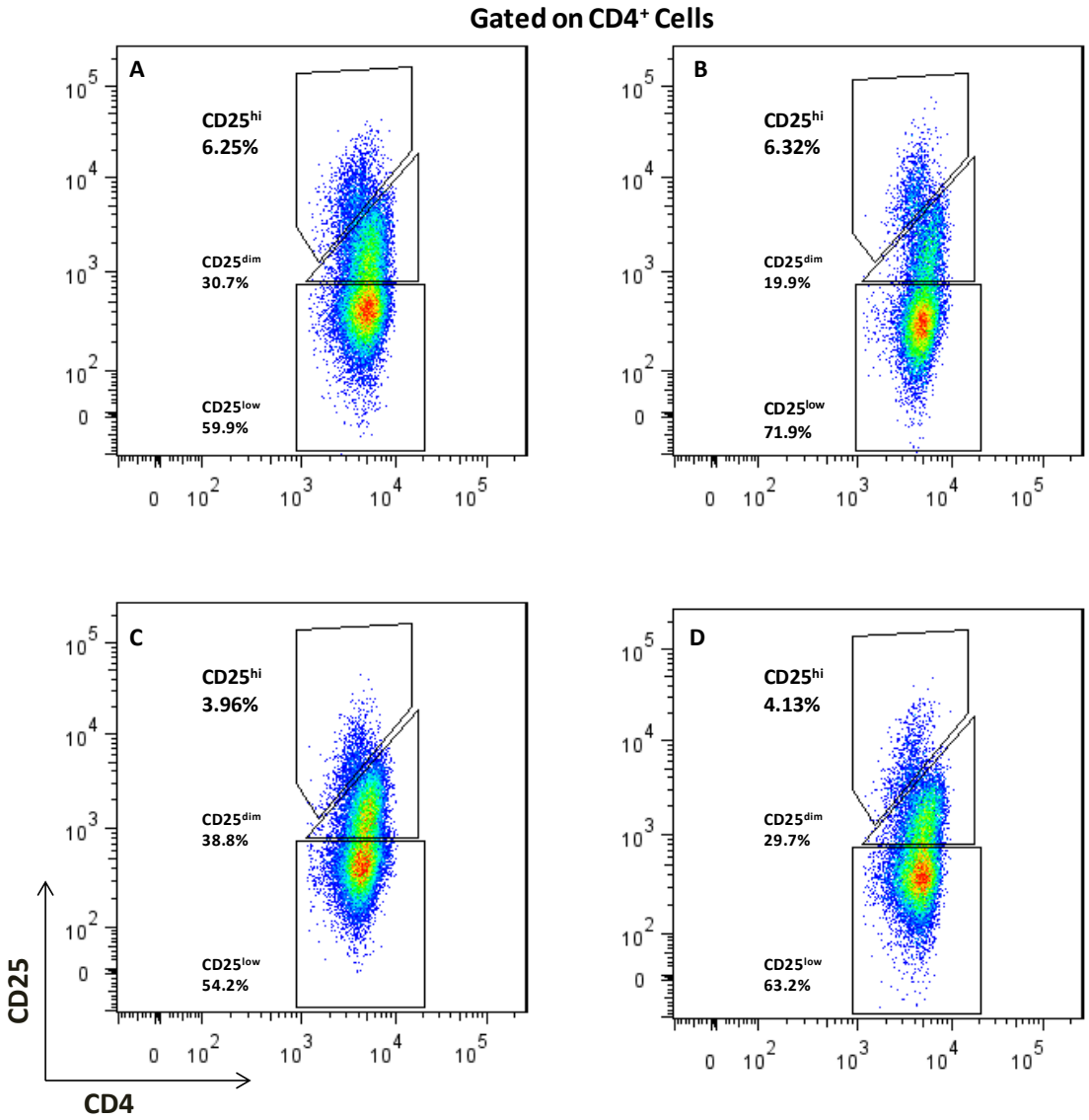

Figure 4.10 Representative flow cytometry plots of $CD4^{+}CD25^{hi}$ T_{Reg} cells in peripheral blood of pregnant women.

A) healthy pregnancy (H.PREG), B) smoked but had uncomplicated pregnancy (SM), C) developed pre-eclampsia (PET), D) non-smokers who developed fetal growth restriction (FGR). Dot plot graphs show examples of CD4 positive cells expressing high ($CD25^{hi}$, regulatory T cells) or average ($CD25^{dim}$, activated T cells) levels of CD25 surface marker, as well as those that lack CD25 ($CD25^{low}$, conventional T cells).

4.3.2.2 Circulating Regulatory $CD4^{+}CD25^{hi}$ Cells in Early Pregnancy (10-18 weeks)

My results support the possibility that regulatory T cells are similarly reduced in pre-eclampsia as in pregnancies affected by miscarriage, but to a lesser extent.

Key findings:

1. At 10-18 weeks of gestation, women who later developed pre-eclampsia had significantly reduced percentage of $CD4^{+}CD25^{hi}$ T_{Reg} cells (3.6%; IQR: 2.875 to 4.36%; p=0.001) compared to healthy controls (6.345%; IQR: 5.143 to 7.720%; p=0.01) and smokers (5.5%; IQR: 4.79 to 7.328%) (**Figure 4.11**).Women who later developed pregnancy-induced hypertension (PIH) and women at high risk of pre-eclampsia, but who remained normotensive (NR no PET) had a lower percentage of $CD4^{+}CD25^{hi}$ T_{Reg} cells (4.0%; IQR: 2.9 to 5.2%; p=0.001) and (4.6%; IQR: 3.443 to 5.130%; p=0.01) respectively, compared with levels in women who went on to have a healthy pregnancy.

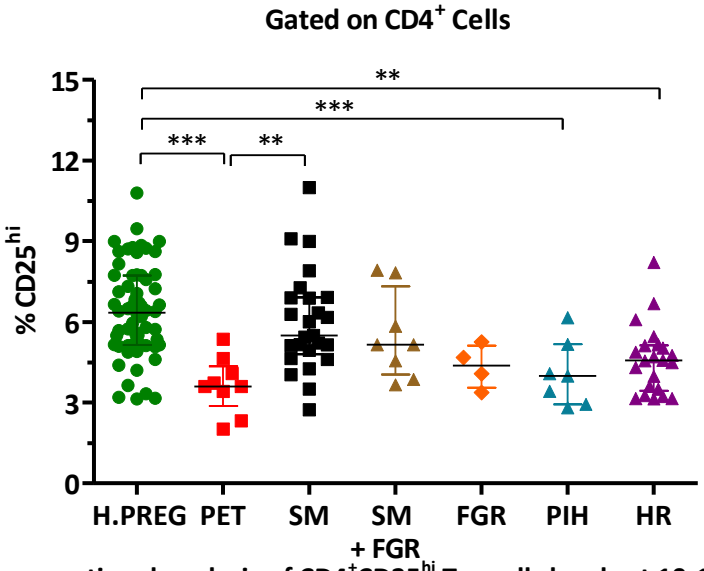

Figure 4.11 Cross-sectional analysis of $CD4^{+}CD25^{hi}$ T_{Reg} cells levels at 10-18 week of gestation. Data for women who had a healthy pregnancy (H.PREG; N=66), who go on to develop pre-eclampsia (PET; N=9), who smoked (SM; N=25), who smoked and were affected by fetal growth restriction (SM+FGR; N=8), developed isolated fetal growth restriction (FGR; N=4), pregnancy induced hypertension (PIH; N=7) or were at high risk but did not develop pre-eclampsia (HR no PET; N=22). The data was normally distributed therefore the analysis was performed using one-way ANOVA corrected for multiple comparisons with Bonferroni's test. Data is presented as median with interquartile range (** = $p < 0.01$, *** = $p < 0.001$)

2. At 10-18 weeks of gestation, non-smoking women who later developed isolated fetal growth restriction, had a lower percentage of $CD4^{+}CD25^{hi}$ T_{Reg} cells (4.380%; IQR: 3.555 to 5.115%) compared with women who went on to have a healthy pregnancy. (Due to low patient numbers in FGR group (n=4) the data was not significantly different.

3. At 10-18 weeks gestation, the estimated median values of the $CD4^{+}CD25^{hi}$ T_{Reg} cells were not statistically different between healthy pregnant women (H.PREG) level (6.345%; IQR: 5.143 to 7.720%), smokers who had a normal pregnancy outcome (SM) (5.5%; IQR: 4.79 to 6.905%) and smokers later affected by FGR (SM+FGR) (5.16%; IQR: 4.04 to 7.328%).

4.3.2.3 Natural $CD4^{+}Foxp3^{+}Helios^{+}$ and induced regulatory $CD4^{+}Foxp3^{+}Helios^{-}$ T cells before and during onset of pre-eclampsia.

Expansion of regulatory T cells is needed for a pregnancy to be successful. One report suggests that expansion in inducible T_{Reg} ($CD4^{+}Foxp3^{+}Helios^{-}$) rather than in natural T_{Reg} cells ($CD4^{+}Foxp3^{+}Helios^{+}$) accounts for this augmentation in healthy pregnancy (Hsu et al. 2012).

In my own observation both natural and inducible T_{Reg} cells were reduced in pregnancies that went on to be affected by pre-eclampsia (**Figure 4.13 & Figure 4.14**).

Key findings (natural T_{Reg} cells):

1. Throughout pregnancy, smokers who had a healthy outcome (SM) tended to have +7% (95%CI: -3.2 to 18.2%, p=0.185) higher proportion of $CD4^{+}Foxp3^{+}Helios^{+}$ natural T_{Reg} cells (nT_{Reg} cells) compared to healthy controls (H.PREG) **(Figure 4.12, Figure 4.13, and Table 4.2)**.
2. Women who developed pre-eclampsia (PET) and non-smokers with isolated fetal growth restriction (FGR) had a similar percentage of nT_{Reg} cells (+1%; 95%CI: -16.3 to 21.8%, p=0.918) which were around 26% (p<0.0001) lower than levels observed in healthy pregnancy (H.PREG).
3. The percentage of $CD4^{+}Foxp3^{+}Helios^{+}$ T cells was also diminished in blood of women at high risk of pre-eclampsia, but who had a healthy pregnancy outcome (HR no PET) (-15.4%; 95%CI: -23.7 to -6.3%; p=0.002) and pregnancy induced hypertension group (PIH) (-18.9%; 95%CI: -29.8 to -6.3%, p=0.005) however their levels were not as low as in PET or FGR cases.

4. Smokers who later developed fetal growth restriction (SM+FGR) showed an initial rise in nT_{Reg} cells similar to smokers with normally grown babies (SM) but from around 19-27th week of pregnancy the relative level of nTregs fell to levels similar to those who had FGR and did not smoke. There was no significant difference in overall percentage of nT_{Reg} cells between those two groups (+5.8%; 95%CI: -19.1 to 9.7%, p=0.443).

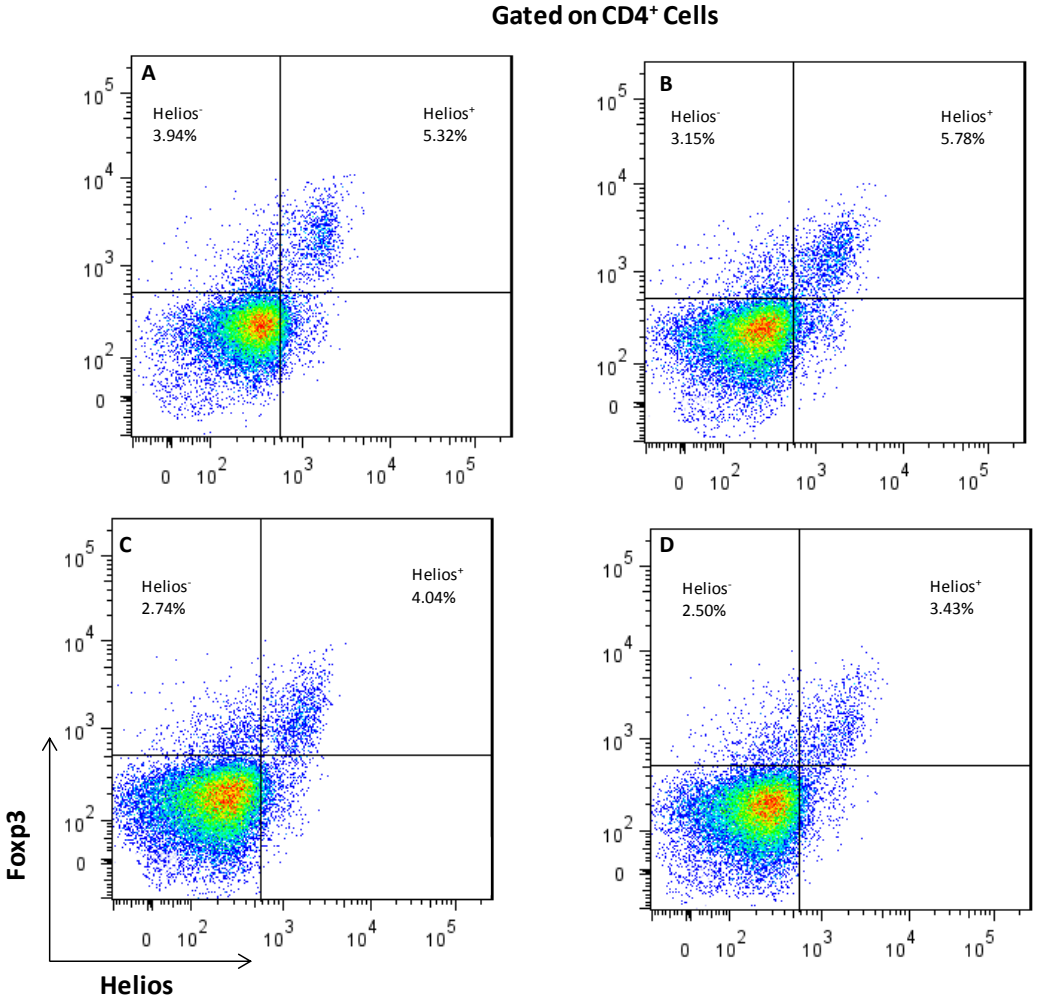

Figure 4.12 Representative flow cytometry plots of $CD4^+Foxp3^+Helios^-$ induced and $CD4^+Foxp3^+Helios^+$ natural T_{Reg} cells in peripheral blood of pregnant women.
A) had healthy pregnancy (H.PREG), B) smoked but had uncomplicated pregnancy (SM), C) developed pre-eclampsia (PET), D) non-smokers who developed fetal growth restriction (FGR).

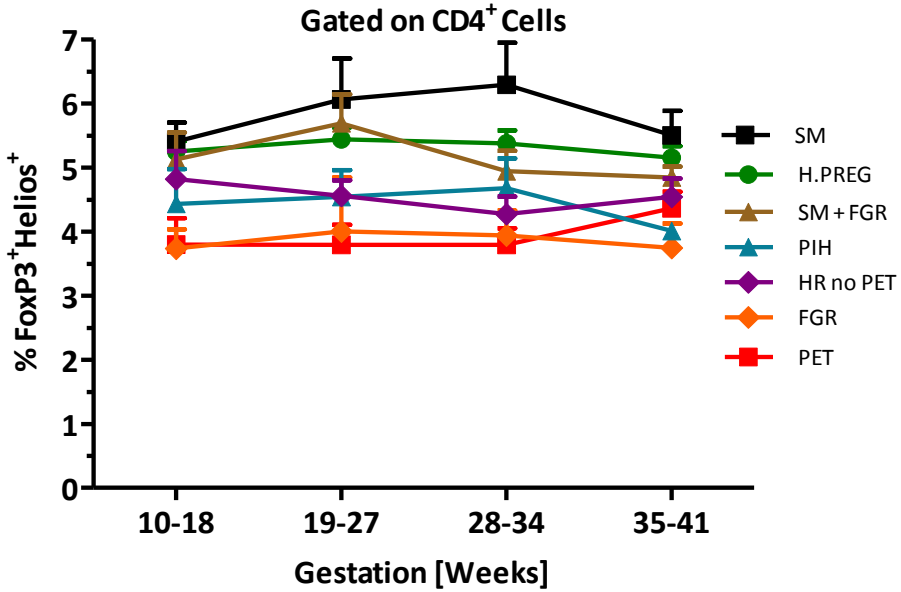

Figure 4.13 Changes in the % of $CD4^+Foxp3^+Helios^+$ natural T_{Reg} cells prospectively throughout pregnancy.
Data for women who go on to develop pre-eclampsia (PET; N=12), pregnancy induced hypertension (PIH; N=10), fetal growth restriction (FGR; N=9), who smoked (SM; N=26), who smoked and were affected by fetal growth restriction (SM+FGR; N=11), were at high risk but did not develop pre-eclampsia (HR no PET; N=24) in comparison with women who had a healthy pregnancy (H.PREG; N=66). Data presented as means with estimated standard error of the means.

nT_{Reg} Cells	H.PREG				SM				PET				FGR			
	Difference %	95%CI		p value	Difference %	95%CI		p value	Difference %	95%CI		p value	Difference %	95%CI		p value
H.PERG	-	-	-	-	-7.0	3.2	-18.2	0.185	+36.4	19.6	55.6	<0.0001	+35.1	16.0	57.3	<0.0001
SM	+7.0	-3.2	18.2	0.185	-	-	-	-	+45.9	25.9	69.1	<0.0001	+44.5	22.3	70.7	<0.0001
PET	-26.7	-35.7	-16.4	<0.0001	-31.5	-40.9	-20.6	<0.0001	-	-	-	-	-1.0	-17.9	19.4	0.918
PIH	-18.9	-29.8	-6.3	0.005	-24.2	-35.3	-11.1	0.001	+10.7	-7.6	32.5	0.269	+9.6	-10.0	33.3	0.358
FGR	-26.0	-36.4	-13.8	<0.0001	-30.8	-41.4	-18.2	<0.0001	+1.0	-16.3	21.8	0.918	-	-	-	-
SM+FGR	+0.8	-12.1	15.5	0.908	-5.8	-19.1	9.7	0.443	+37.5	15.5	63.7	<0.0001	+36.2	12.5	64.8	0.002
HR no PET	-15.4	-23.7	-6.3	0.002	-19.1	-25.3	-11.1	0.017	+8.4	-5.2	23.7	0.385	+12.2	-9.1	35.1	0.458

Table 4.2 Data presenting % change in overall $CD4^+Foxp3^+Helios^+$ natural T_{Reg} cells levels in the investigated groups of women.
Healthy subjects (H.PREG), smokers (SM), women who go on to develop pre-eclampsia (PET), pregnancy induced hypertension (PIH), fetal growth restriction (FGR), who smoke and develop fetal growth restriction (SM+FGR), who were at risk but did not develop pre-eclampsia (HR no PET) compared with H.PREG, SM, PET and FGR levels. Values highlighted in green are significant. Red font indicates decrease (-) whereas green indicates increase (+) in T_{Reg} cells %. Data is shown as coefficients with 95% CI and p values.

Key findings (induced T_{Reg} cells):

1. I found that peripheral blood $CD4^{+}Foxp3^{+}Helios^{-}$ induced T_{Reg} cells were highest in women who went on to have a healthy pregnancy (H.PREG) (**Figure 4.14 and Table 4.3**).
2. Subjects who later developed pre-eclampsia (PET) had the lowest percentage of iT_{Reg} cells being -38.2% (95%CI: -25.57 to -7.59%, p<0.0001) below levels observed in healthy controls (H.PREG).
3. At around 28-34 weeks smokers with uncomplicated pregnancies had similar levels of iT_{Reg} cells as non-smoking women with healthy pregnancy outcome (H.PREG), however throughout pregnancy they were reduced by 17.05% (95%CI: -25.57 to -7.59%, p<0.0001).
4. Women who developed pre-eclampsia (PET), isolated fetal growth restriction (FGR) or who were at risk of pre-eclampsia, but had healthy pregnancy outcome (HR no PET) all had similar iTreg cell levels.
5. Throughout pregnancy women who developed pregnancy induced hypertension (PIH) had $CD4^{+}Foxp3^{+}Helios^{-}$ iT_{Reg} cells -20.1% (95%: -31.6 to -6.5 %, p<0.0001) below healthy controls but +29.4% (95%CI: 6.4 to 57.4%, p=0.01) above pre-eclamptic levels.
6. Smokers and non-smokers who were latter affected by fetal growth restriction (SM+FGR and FGR) had similar percentage of iT_{Reg} cells to each other (-3.2%; 95%CI: -21.3 to 19%, p=0.754).

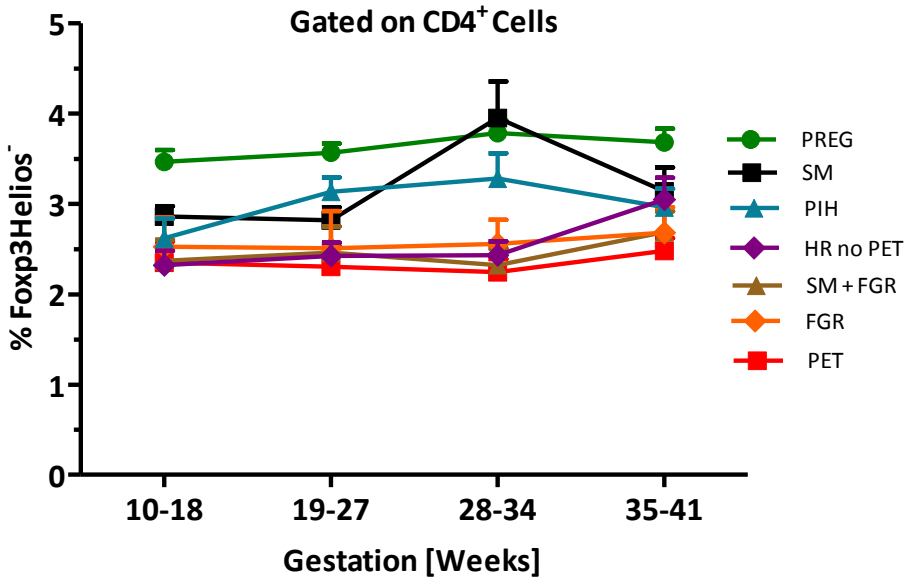

Figure 4.14 Changes in the % of $CD4^+Foxp3^+Helios^-$ induced T_{Reg} cells measured prospectively throughout pregnancy.
Women who go on to develop pre-eclampsia (PET; N=12), pregnancy induced hypertension (PIH; N=10), fetal growth restriction (FGR; N=9), who smoked (SM; N=26), who smoked and were affected by fetal growth restriction (SM+FGR; N=11), were at high risk but did not develop pre-eclampsia (HR no PET; N=24) in comparison with women who had a healthy pregnancy (H.PREG; N=66). Data presented as means with estimated standard error of the means.

iT_{Reg} Cells	H.PREG				SM				PET				FGR			
	Difference %	95%CI		p value	Difference %	95%CI		p value	Difference %	95%CI		p value	Difference %	95%CI		p value
H.PERG	-	-	-	-	+17.1	25.6	7.6	0.001	+61.9	40.4	86.7	<0.0001	+46.1	23.9	72.3	<0.0001
SM	-17.06	-25.57	-7.59	<0.0001	-	-	-	-	+34.3	14.4	57.6	<0.0001	+21.2	1.2	45.1	0.037
PET	-38.2	-46.4	-28.8	<0.0001	-25.5	-36.5	-12.6	<0.0001	-	-	-	-	-9.8	-26.3	10.5	0.318
PIH	-20.1	-31.6	-6.5	0.005	-3.6	-18.9	14.5	0.674	+29.4	6.4	57.4	0.01	+16.8	-5.6	44.5	0.151
FGR	-31.6	-42.0	-19.3	<0.0001	-17.5	-31.1	-1.1	0.037	+10.8	-9.5	35.7	0.318	-	-	-	-
SM+FGR	-33.8	-42.9	-23.2	<0.0001	-20.1	-32.3	-5.8	0.008	+7.3	-11.2	29.6	0.465	-3.2	-21.3	19.0	0.754
HR no PET	-31.8	-39.1	-23.5	<0.0001	-18.4	-30.3	-1.2	0.034	+11.8	-10.6	37.7	0.412	+17.1	-7.2	54.1	0.251

Table 4.3 Data presenting % change in overall $CD4^+Foxp3^+Helios^-$ induced T_{Reg} cells levels in the investigated groups of women.
Healthy subjects (H.PREG), smokers (SM), women who go on to develop pre-eclampsia (PET), pregnancy induced hypertension (PIH), fetal growth restriction (FGR), who smoke and develop fetal growth restriction (SM+FGR), who were at risk but did not develop pre-eclampsia (HR no PET) compared with H.PREG, SM, PET and FGR levels. Values highlighted in green are significant. Red font indicates decrease (-) whereas green indicates increase (+) in T_{Reg} cells %. Data is shown as coefficients with 95% CI and p values.

4.3.2.4 Regulatory $CD4^+CD25^{hi}FoxP3^+Helios^+$ natural and $CD4^+CD25^{hi}FoxP3^+Helios^-$ induced T cells

In the previous section, I demonstrated the levels of natural and induced regulatory T cells depending on the expression of the intracellular Helios marker within CD4 cells positive for Foxp3. I next wanted to discover the percentage of iT_{Reg} and nT_{Reg} cells within the population of regulatory T cells defined as $CD4^+CD25^{hi}$ T_{Reg} cells for each pregnancy group that I investigated. The gating strategy is shown in **Figure 4.5**, whereas **Figure 4.15** depicts examples of % iT_{Reg} cells in healthy pregnancy, smokers with uncomplicated pregnancy, pre-eclampsia and non-smokers with isolated fetal growth restriction.

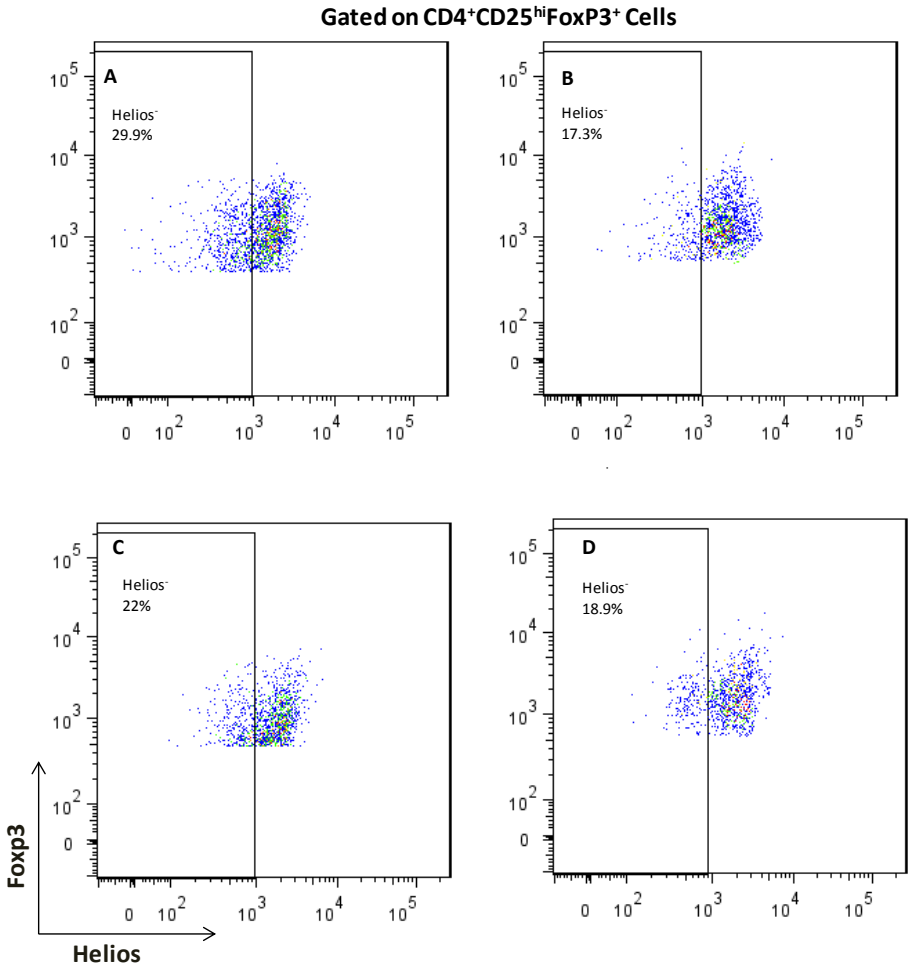

Figure 4.15 Representative flow cytometry plots of $CD4^+CD25^{hi}FoxP3^+Helios^-$ induced T_{Reg} and $CD4^+CD25^{hi}FoxP3^+Helios^+$ natural T_{Reg} cells in peripheral blood of pregnant women.
Women who A) had healthy pregnancy (H.PREG), B) smoked but had uncomplicated pregnancy (SM), C) developed pre-eclampsia (PET), D) non-smokers who developed fetal growth restriction (FGR). Numbers on each dot plot graph represent % of induced T_{Reg} cells. Dot plots graphs present raw data from 28-34 weeks interval.

Key findings:

1. Throughout pregnancy women who were normotensive and had a healthy pregnancy outcome (H.PREG) had the highest percentage of $CD25^{hi}FoxP3^+Helios^-$ induced T_{Reg} cells within the population of CD4 positive T lymphocytes (**Figure 4.16**).
2. During pregnancy, the levels of $CD4^+CD25^{hi}FoxP3^+Helios^-$ induced regulatory T cells gradually fell toward 28-34 weeks of gestation and then rose towards term in all studied groups. The only exception were women who went on to develop pre-eclampsia, who had low levels of iTregs that fell even further in the last trimester.

3. After a drop in iT_{Reg} $cell_s$ content at 28-34 weeks in patients who developed pregnancy induced hypertension (PIH), the percentage of iTregs rose to levels similar to those of healthy controls (H.PREG).
4. Smokers who had uncomplicated pregnancy (SM) and those who smoked and developed fetal growth restriction (SM+FGR) had the lowest content of iT_{Reg} cells (-44.1%; 95%CI: -50.3 to -37.2; $p<0.0001$ and -47.3%; 95%CI: -38.0 to -18.8; $p<0.0001$ respectively vs. healthy non-smokers), which means that they had the highest levels of natural (thymus originating) T_{Reg} cells.

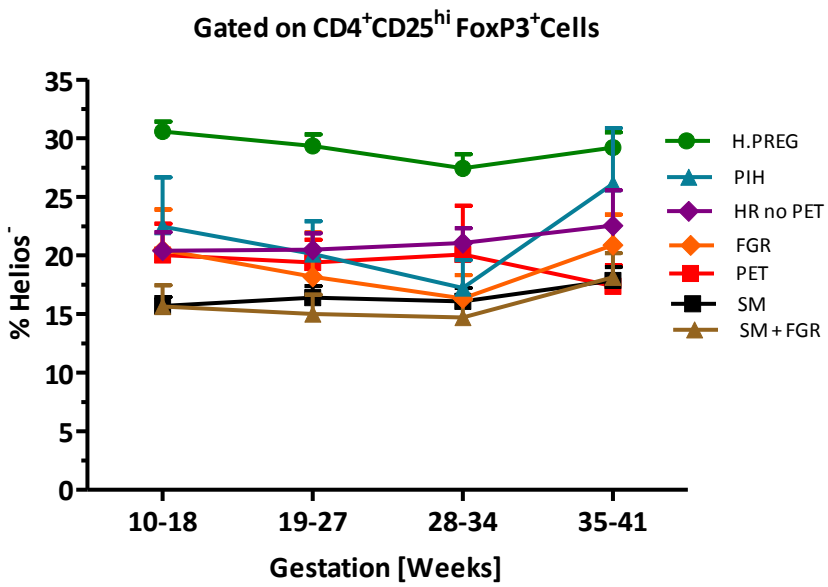

Figure 4.16 Changes in the % of $CD4^{+}CD25hiFoxP3^{+}Helios^{-}$ induced T_{Reg} cells measured prospectively throughout pregnancy.
Women who go on to develop pre-eclampsia (PET; N=12), pregnancy induced hypertension (PIH; N=10), fetal growth restriction (FGR; N=9), who smoked (SM; N=26), who smoked and were affected by fetal growth restriction (SM+FGR; N=11), were at high risk but did not develop pre-eclampsia (HR no PET; N=24) in comparison with women who had a healthy pregnancy (H.PREG; N=66). Data presented as means with estimated standard error of the means.

	iT_{Reg} Cells				nT_{Reg} Cells			
	Difference %	95%CI		p value	Difference %	95%CI		p value
SM	-44.1	-50.3	-37.2	<0.0001	+18.6	14.5	22.8	<0.0001
PET	-37.0	-46.3	-26.1	<0.0001	+14.5	9.1	20.0	<0.0001
PIH	-31.4	-42.9	-17.6	<0.0001	+12.0	6.0	18.3	<0.0001
FGR	-37.8	-48.9	-24.4	<0.0001	+14.9	8.4	21.8	<0.0001
SM+FGR	-47.3	-55.3	-37.8	<0.0001	+19.5	13.7	25.5	<0.0001
HR no PET	-29.0	-38.0	-18.8	<0.0001	+13.9	9.5	18.5	<0.0001

Table 4.4 Data presenting % change in overall $CD4^{+}CD25^{hi}FoxP3^{+}Helios^{-}$ induced T_{Reg} cells levels in blood of pregnant women.
Smokers (SM), women who go on to develop pre-eclampsia (PET), pregnancy induced hypertension (PIH), fetal growth restriction (FGR), who smoke and develop fetal growth restriction (SM+FGR), who were at risk but did not develop pre-eclampsia (HR no PET) compared to healthy subjects (H.PREG). Values highlighted in green are significant. Red font indicates decrease (-) whereas green indicates increase (+) in T_{Reg} cells %. Data is shown as coefficients with 95% CI and p values.

To summarise this chapter:

1. Overall, non-smoking healthy controls (H.PREG) and smokers who had a healthy pregnancy outcome, had a similar percentage of $CD4^{+}Foxp3^{+}$ T_{Reg} cells. However, within this population of T reg cells, smokers had relatively low levels of iT_{Reg} and high levels of nTreg cells (**Figure 4.13 and Figure 4.14**).
2. Both smokers and healthy pregnant women had a similar content of total $CD4^{+}CD25^{hi}$ cells, however healthy controls showed a relatively lower percentage of Foxp3 positive nT_{Reg} cells and higher level of iTregs. (**Figure 4.9 and Table 4.4)**.
3. Throughout pregnancy, women who later developed pre-eclampsia had decreased total $CD4^{+}Foxp3^{+}$ T_{Reg} population as well as the content of nT_{Reg} cells and iT_{Reg} cells. Interestingly, their nT_{Reg} cells in $CD4^{+}CD25^{hi}Foxp3^{+}$ group were elevated compared to healthy pregnancy (H.PREG), however the total percentage of $CD4^{+}CD25^{hi}$ was still lower than in healthy controls.

4.3.2.5 Proliferative Properties of $CD4^{+}$ T cells in healthy pregnancy and pre-eclampsia

To my knowledge there are only 5 studies investigating function of T_{Reg} cells in non-pregnant women, healthy pregnancy and pregnancy complicated by pre-eclampsia (Darmochwal-Kolarz et al. 2012; Steinborn et al. 2008; Steinborn et al. 2012; Santner-Nanan et al. 2009; Zeng et al. 2013). These studies used different T_{Reg} cells isolating techniques, responder cells, various surface markers and different stimulus for evaluation of suppressive abilities of T_{Reg} cells. Despite the differences in methodologies the results are consistent. They indicate that functional activity of T_{Reg} cells is significantly reduced in patients affected by pre-eclampsia when compared to those of healthy pregnancies.

Knowing that T_{Reg} cells from pre-eclampsia have reduced suppressive activities I aimed to investigate if the 38.1% decrease in percentage of $CD4^{+}CD25^{hi}$ T_{Reg} cells in the peripheral blood of women who developed pre-eclampsia that I observed (**Figure 4.9** &**Table 4.1**) makes a difference to the proliferative properties of $CD4^{+}$ T lymphocytes when compared to healthy pregnancy. I expected the $CD4^{+}$ T cells from women with pre-eclampsia to exhibit enhanced proliferation compared with those obtained from

healthy pregnancy. This would be explained by the fact that pre-eclamptic $CD4^+$ T contained less of the suppressive $CD4^+CD25^{hi}$ T_{Reg} cells than healthy controls.

I discovered that $CD4^+$ T lymphocytes isolated from peripheral blood of women with pre-eclampsia and stimulated with anti-CD3 and anti-CD28 antibodies did indeed show greater proliferative properties than $CD4^+$ T lymphocytes from healthy pregnant women (**Figure 4.17**). Only 87.16% (SEM: 2.833) of $CD4^+$ T cells obtained from healthy pregnant women proliferated compared with 96.40% (SEM: 1.164, p=0.0275) from women with pre-eclampsia (**Figure 4.18 A**). Conversely, the mean value of non-proliferated cells from healthy pregnant women was 12.75% (SEM: 2.89) and from women with pre-eclampsia 2.449% (SEM: 1.224) (**Figure 4.18 B**).

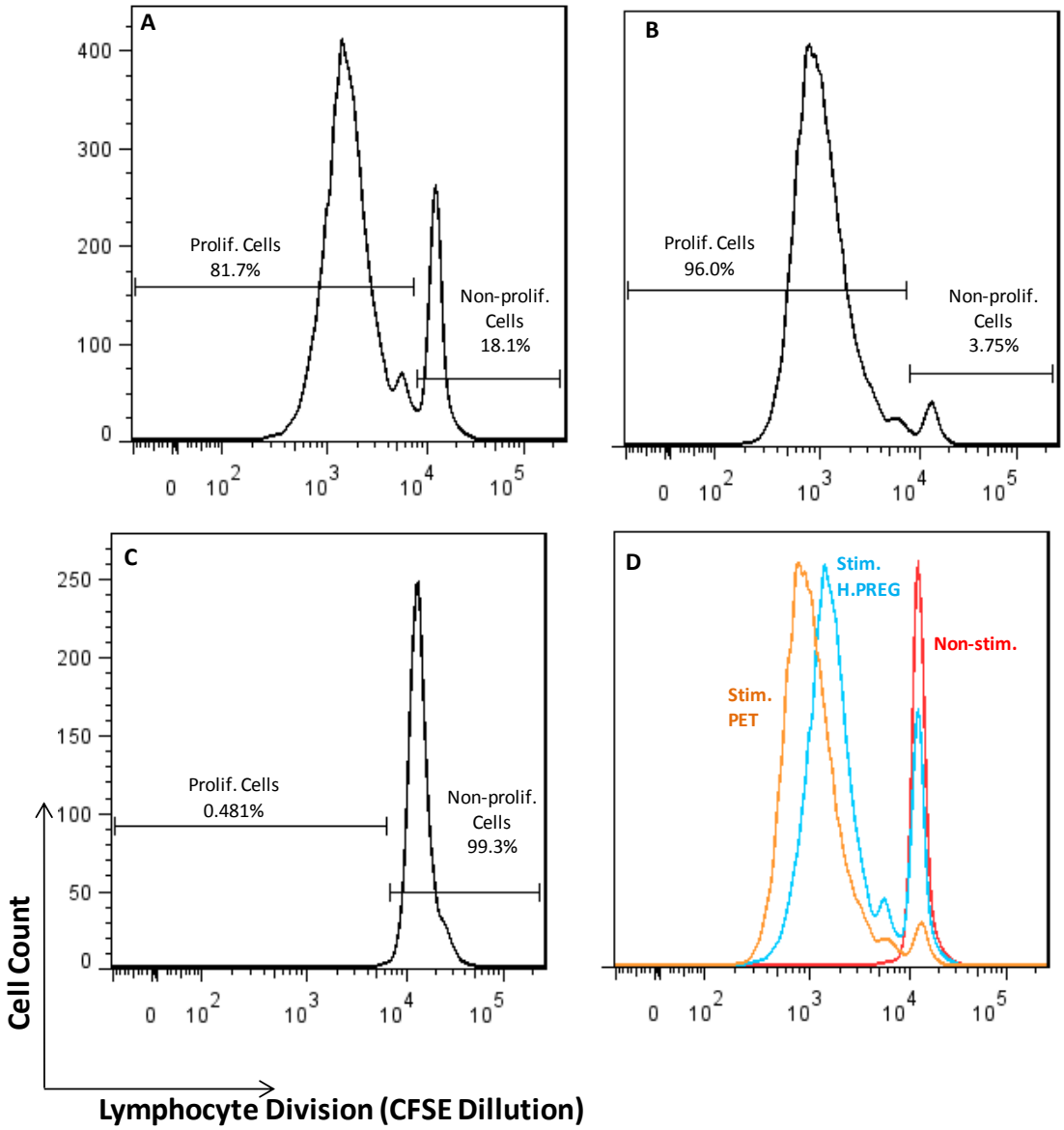

Figure 4.17 Representative histograms of proliferation CD4$^+$ T lymphocytes isolated from peripheral blood of A) a healthy pregnant woman (H.PREG) and B) a patient with pre-eclampsia (PET).

Cells were stimulated with anti-CD3/CD28 antibodies in culture for 5 days C) non-stimulated CD4$^+$ T lymphocytes cultured for the same length of time, D) data from A,B and C overplayed on one graph. Data representing 4 independent experiments, each conducted in triplicate. H.PREG; N=3, PET, N=4)

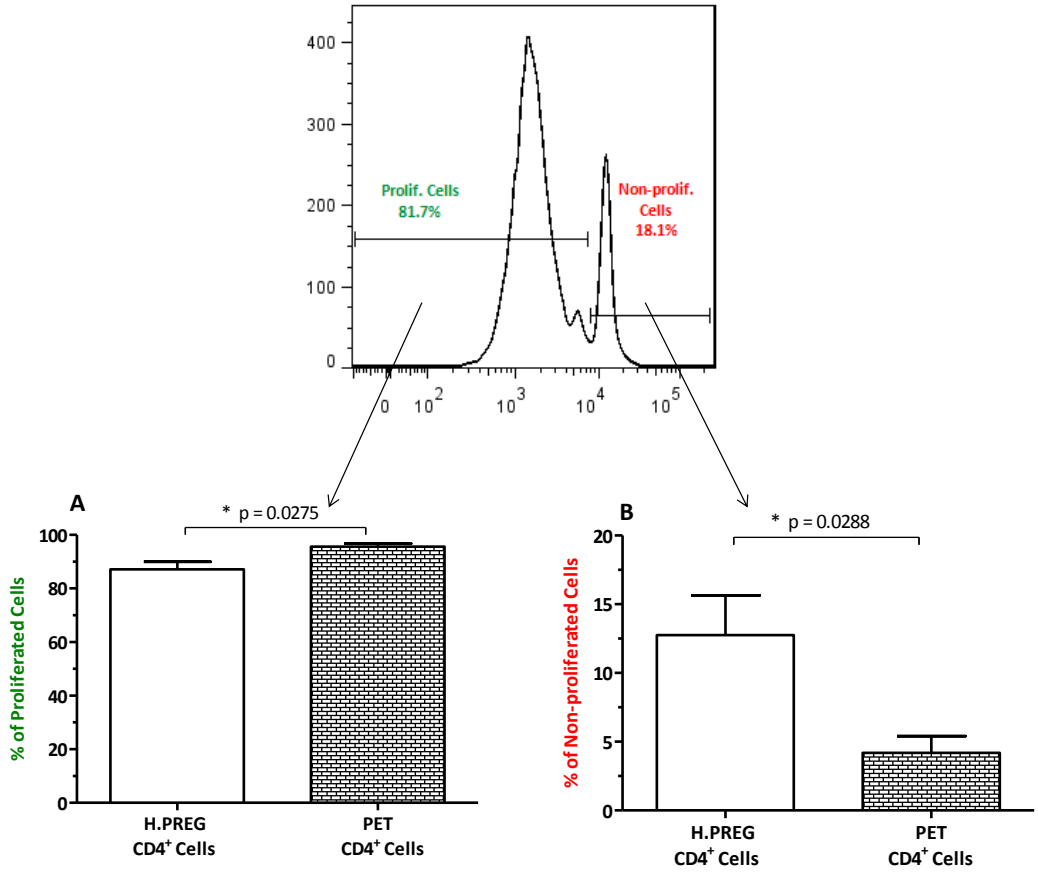

Figure 4.18 Comparison of proliferation of $CD4^+$ T cells isolated from 3 healthy pregnant and 4 pre-eclamptic women.
Cells were stimulated for 5 days with anti-CD3/CD28 antibodies After that time cells were arrested and the level of proliferation measured by flow cytometry. Graph A shows significantly greater level of proliferation of cells obtained from pre-eclampsia, whereas graph B depicts the difference in percentage of non-proliferated $CD4^+$ T cells. Data represents 4 independent experiments, each conducted in triplicate. Two-tailed t-test used to analyse the data. Data presented as mean with estimated standard error of the mean.

The impact of a decreased content of suppressive $CD4^+CD25^{hi}$ T_{Reg} cells within $CD4^+$ T lymphocytes population in pre-eclampsia is expressed as a greater ratio of proliferated to non-proliferated $CD4^+$ T cells compared a healthy pregnancy (28.19; SEM: 6.379 vs. 7.561; SEM: 1.608, p=0.0433) (**Figure 4.19**).

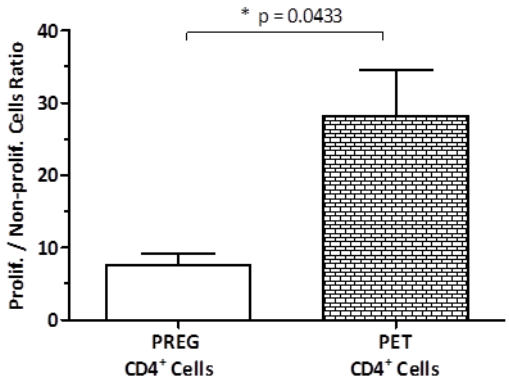

Figure 4.19 The ratio of proliferated to non-proliferated cells after 5 days of culture, obtained from healthy pregnancy and from pre-eclampsia.
Data represents 4 independent experiments, each conducted in triplicate. Two-tailed t-test used to analyse the data. Data presented as mean with estimated standard error of the mean.

Unfortunately, my attempts to culture $CD4^+$ T lymphocytes depleted of cells expressing high levels of CD25 were unsuccessful. Therefore, I cannot fully confirm that the increased level of proliferation seen in cells isolated from blood of women with pre-eclampsia was due to the diminished percentage of $CD4^+CD25^{hi}$ regulatory T cells. The data for this part was very inconsistent with the majority of cells dying during culture (**Figure 4.20**) with the rest unable to proliferate. Possible explanations for this failure are excessive time handling the cells before culture or a low number of cells plated.

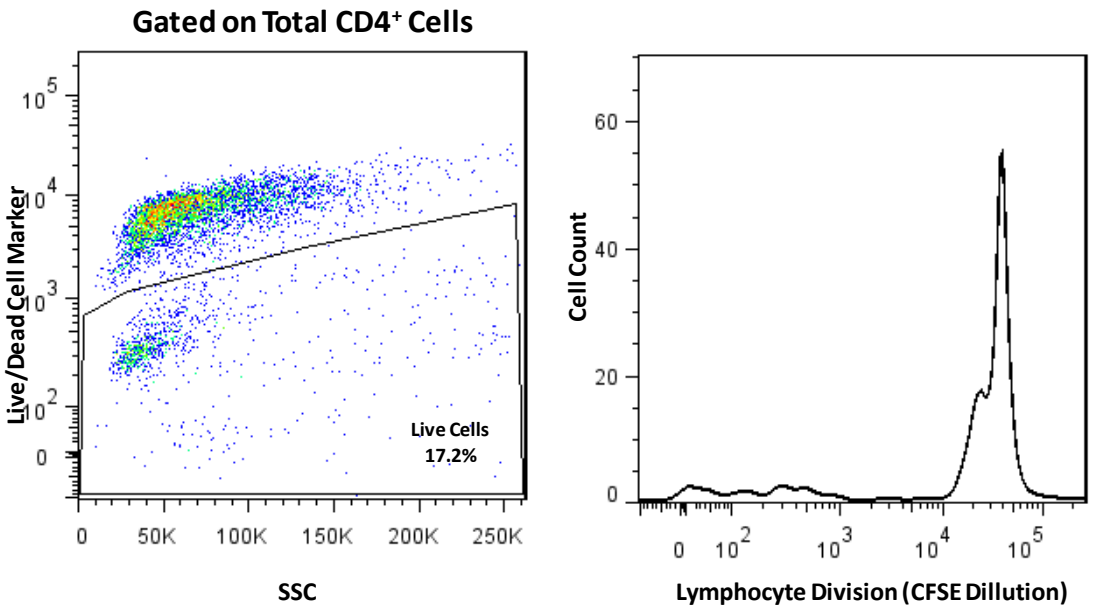

Figure 4.20 Graphs showing an example of raw data obtained after depleting $CD4^+$ T lymphocytes of $CD4^+CD25^{hi}$ cells.
Only a minority of cells survive, (in this illustrated example, just 17.2% were viable). The rate of their proliferation was very low (histogram on the right).

4.4 DISCUSSION

The advantage that my research has over already published studies is that it includes data obtained prospectively form the same patient. What is more, the subjects were carefully divided into appropriate groups according to the outcome of their pregnancy.

My research supports the general view that regulatory T lymphocytes play an important role in the development of a successful pregnancy (Guerin et al. 2009). My novel findings are that the levels of T_{Reg} cells had nearly doubled during the early weeks of healthy pregnancy and stayed elevated until childbirth. This observation strongly suggests that T_{Reg} cells are important at the time of placental implantation and in maintaining the progression of a healthy pregnancy. Conversely, from early pregnancy, women destined to develop pre-eclampsia, isolated fetal growth restriction or pregnancy induced hypertension are unable to expand their T_{Reg} cell population above levels found in non-pregnant women.

In a functional assay, I also showed that T_{Reg} cells from women with pre-eclampsia had a decreased ability to prevent proliferation of $CD4^+$ T lymphocytes when compared with cells obtained from healthy pregnant women. This result suggest that T-regulatory cells from women with pre-eclampsia were not only reduced in number, but were less able to suppress T cell proliferation to the same extend as the more abundant T_{Reg} cells from healthy pregnancy. Others have also shown that the suppressive properties of regulatory T cells from women with pre-eclampsia are down regulated compared with healthy pregnant women (Darmochwal-Kolarz et al. 2012; Steinborn et al. 2008; Steinborn et al. 2012; Santner-Nanan et al. 2009; Zeng et al. 2013). However, another study showed that despite the decreased numbers of T_{Reg} cells in pre-eclampsia, they were still able to suppress proliferation of autologous B cells (Zeng et al. 2013).

As I mentioned in the previous chapter, pre-eclampsia and fetal growth restriction have similar placental pathology, linked to impaired placentation (Benirschke et al 2012). It may not be surprising then that according to my data both groups have similarly low percentage of circulating regulatory T cells from the beginning of pregnancy.

None of the previous studies investigating T_{Reg} cells prevalence in pre-eclampsia have shown an association between severity, or the gestational onset of pre-eclampsia and the number of regulatory T cells (Toldi et al. 2012; Santner-Nanan et al. 2009; Hu et al. 2009). This is in line with my findings. When taking into account the underlying pathology, late onset pre-eclampsia is closer associated with pregnancy induced hypertension than with early pre-eclampsia, thus one could expect that the levels of circulating T_{Reg} cells in PIH and late PET should be higher at the beginning of the pregnancy and gradually fall closer to the clinical onset of the condition. However according to my data this does not seem to be the case. One possible explanation could be that women who develop late onset of PET and PIH have decreased levels of regulatory cells but the suppressive function of T_{Reg} cells is preserved and that is why onset of pre-eclampsia is delayed (late PET) or less severe (PIH) than in early PET and FGR. It could also be that their altered suppressive abilities decrease even further towards the end of pregnancy which enables the progression of PET or PIH. It has been

previously found that the suppressive activities of T_{Reg} cells defined as $CD4^{+}CD25^{+}CD127^{-}$ is highest at the beginning of a pregnancy but it declines in the later gestations (Steinborn et al. 2012). However, because of the lack of available literature on suppressive function of T_{Reg} cells form subjects with PIH, this theory would need to be tested empirically. I did not investigate the suppressive power of Tregs from women with late onset PET or PIH, or in early pregnancy.

Women with PIH have normally grown babies, which may suggest that the expansion/recruitment of T_{Reg} cells within the decidua is sufficient, but suppressed function or levels of cells circulating in the periphery fails to prevent the onset of hypertension. It has been suggested that in a pregnancy, the local/decidual expansion of T_{Reg} cells may be dependent on fetal alloantigens but the rise in regulatory T cells numbers in the periphery is more dependent on pregnancy hormones (Aluvihare et al. 2004).

I was surprised to find out that levels of regulatory T cells in women who were at risk of pre-eclampsia but had healthy pregnancy outcome were no different to levels observed in pre-eclampsia. Women at high risk of PET, but who had a healthy pregnancy outcome (HR no PET group) had suppressed T_{Reg} cells, but not as low as PET group. It could be speculated that although these two groups have low levels of regulatory T cells, they do not share other vulnerabilities to pre-eclampsia. Pre-eclampsia is a syndrome that includes maternal endothelial dysfunction (Powe et al. 2011). If women from 'HR no PET' group have a better lipid profile or healthier endothelium that is less sensitive to normal or elevated levels of circulating vasoconstrictor factors, then they may resist the onset of pre-eclampsia. It must always be remembered that pre-eclampsia is a multi faceted syndrome with several likely aetiologies (Pennington et al. 2011).

It is possible that T_{Reg} cells are involved in successful implantation but are dysregulated in periphery of subjects with hypertensive conditions. Studies in animals have shown that regulatory T cells play an important role in regulating hypertension. Their transfer into hypertensive mice reduces mean arterial pressure and cardiac hypertrophy (Kvakan et al. 2009). The discovery that in animal models T_{Reg} cells

improve vascular function and prevent blood pressure elevation caused by Angiotensin II (AngII) infusions (Barhoumi et al. 2011) strongly supports the importance of regulatory T cells in the prevention of pre-eclampsia. Indeed, as I will show later (Chapter VI)) one of the most important observations in the subjects who smoked during pregnancy was that they had a lower mean arterial pressure than healthy, non-smoking pregnsant women.

The fact that women with pre-eclampsia are hyper-sensitive to infusions of AngII has been known for decades (Gant et al. 1973). This sensitivity appears long before the onset of pre-eclampsia, even as early as 20 week of gestation. Therefore, it could be speculated that if Tregs are dysfunctional and/or low in number, they may be unable to ameliorate the vasoconstrictor actions of Ang II and subsequently prevent the onset of pre-eclampsia. What is more, women with pre-eclampsia appear to develop agonistic autoantibodies (AT1-AA) which activate the Ang II receptor 1 and mimic the physiological actions of Ang II (Wallukat et al. 1999). AT1-AA antibodies are detectable only in pregnant women with reduced placental blood flow including those with pre-eclampsia, fetal growth restriction (Walther et al. 2005). Therefore, although in my work women form HR no PET and PIH groups had low levels of circulating T_{Reg} cells, they had normally functioning placentas and possibly lacked AT1-AA in their serum.

Although outside of pregnancy levels of regulatory T cells were shown to be higher in smokers than non-smokers (Hampras et al. 2012), I found that subjects who continued to smoke and had a healthy pregnancy outcome, had a similar level of T_{Reg} cells in their blood as non-smoking healthy pregnant controls. Several other studies have shown that chronic exposure to cigarette smoke induces immunosuppressive changes such as lowering serum immunoglobulin titres, decreasing natural killer cell activity and T cell proliferative abilities (Johnson et al. 1990; Sopori 2002; Zeidel et al. 2002). Nevertheless, the effect of smoking in pregnancy on T_{Reg} cells and their function has not previously been investigated in pregnancy.

There is evidence that soluble components extracted from cigarette smoke are able to suppress key dendritic cell functions and favour the T_H2 immunity development (Vassallo et al. 2005). Cigarette smoking is associated with an increased susceptibility

to infections and cancers, and this is partially due to smoking induced changes in the immune system (Holt & Keast 1977; Ferson et al. 1979). Interestingly, chronic exposure to the vapour phase of cigarette smoke does not suppress the immune system, unlike the whole cigarette smoke (Sopori 2002). This indicates that some components of the particulate phase must be immunosuppressive. In cigarette smoke, most of the nicotine is within the particulate phase. Animals which are treated chronically with nicotine exhibit a significant loss of antibody responses and T cell proliferation, which resembles the effect of chronic exposure to cigarette smoke (Sopori 2002). Studies investigating nicotine-related impact on the immune system suggest, that after binding an antigen, T lymphocytes from nicotine-treated animals are not able to normally transmit the antigen-receptor mediated signals and this prevents them from proliferating (Geng et al. 1996).

Smoking is the only cardiovascular risk factor that protects against pre-eclampsia (Conde-Agudelo et al. 1999). In the current study, I showed that the levels of T_{Reg} cells in women who smoked during pregnancy were similar to non-smokers who had a healthy pregnancy outcome. It is possible therefore that the high pre-pregnancy levels of regulatory T cells in smokers are able to protect the mother form developing pre-eclampsia (Hampras et al. 2012). Regulatory T cells of smoking mothers might have been recruited to the decidua from periphery and facilitate good placentation. This could be possible as one report suggests that during pregnancy, T_{Reg} cells are not only induced locally in the feto-maternal interface but that they also migrate there from maternal peripheral blood (Tilburgs et al. 2008). On the other hand, pregnancies of smokers are often associated with miscarriages (Domínguez-Rojas et al. 1994), preterm birth (Chamberlain et al. 2013) and fetal growth restriction (Meyer et al. 1976). Therefore, in contradiction with aforementioned theory it is tempting to speculate that the increased prevalence of miscarriages, pre-term birth, or FGR in smokers, but reduced risk of pre-eclampsia could be related to well expand numbers of T_{Reg} cells in maternal periphery, but inadequate expansion or recruitment to the site of implantation. Unfortunately, the prevalence of regulatory T cells in decidua form smokers with uncomplicated pregnancy has not been yet reported.

I showed that in the early weeks of pregnancy, all smokers had a similar level of T_{Reg} cells to non-smoking healthy controls. However, as pregnancy progressed, smokers who developed a pregnancy affected by FGR showed a fall in their level of Tregs. This important finding suggests that the failing placenta may influence maternal immune adaptation to pregnancy. Based on my data it could be speculated that the protective effect of smoking against pre-eclampsia is transmitted through a higher level of regulatory T cells, which prevents an autoimmune or inflammatory pathway towards hypertension. There was a positive trend toward $CD4^{+}CD25^{hi}$ cells being elevated in women who smoked and developed FGR than in non-smokers with FGR. Eleven out of thirty-nine women (28%) who smoked, developed FGR. What is intriguing is that despite FGR, none of these pregnant smokers developed hypertension/pre-eclampsia.

Results from my study do not completely align with another published report investigating T_{Reg} cells. According to Hsu et al, a subset of decidual antigen presenting cells is responsible for the generation of T_{Reg} cells and that this process is defective in pre-eclampsia (Hsu et al. 2012). This group showed that healthy pregnant women and women affected by pre-eclampsia had a similar percentage of $CD4^{+}Foxp3^{+}Helios^{+}$ natural T_{Reg} cells, and it is the inadequate increase in $CD4^{+}Foxp3^{+}Helios^{-}$ induced T_{Reg} cells that is responsible for the limited expansion of overall T_{Reg} cell numbers in pre-eclampsia.

My results showed that the total $CD4^{+}Foxp3^{+}$ T_{Reg} cell levels are decreased in pre-eclampsia as well as both induced and natural T_{Reg} cells. However, when I investigated the levels of iT_{Reg} and nT_{Reg} cells within $CD4^{+}CD25^{hi}$ T cells, pre-eclamptic subjects indeed had suppressed levels of iTReg cells. Their nT_{Reg} cells were elevated when compared with non-smoking healthy controls but not elevated enough to exceed the level of T_{Reg} subset in controls. Interestingly, smokers with healthy pregnancy outcome also had suppressed iT_{Reg} cells levels, but significantly elevated nT_{Reg} cells. In non-smoking healthy controls iT_{Reg} cells were clearly those driving the total T_{Reg} cells expansion. One could expect that if regulatory T cells are behind mechanism decreasing their risk of pre-eclampsia, the expansion in T_{Reg} cells would be via smoking induced iT_{Reg} cells. It is possible that the balance in T_{Reg} cells subpopulations have

different profile in the deciduas of smokers, although previously published data for healthy pregnancies and pre-eclampsia showed that the nT_{Reg} and iT_{Reg} cells levels in deciduas correspond with those in periphery (Hsu et al. 2012). Nevertheless, in order to get the whole picture, the levels of regulatory T cells in the placental bed will need to be also measured.

In order to understand what kind of protective mechanism may be behind smoking, studies need to investigate the cross-talks between regulatory T cells and other members of the immune system such as dendritic cells, macrophages, B lymphocytes and NK cells. Reports outside of pregnancy on nicotine-induced alteration in function of dendritic cells (DCs) already exist (Guinet et al. 2004; Robbins et al. 2008; Yanagita et al. 2012; Nouri-Shirazi & Guinet 2012). All these studies agree, that nicotine can exert it immunosuppressive activities by impeding T_H1 mediated immunity. For example, one of the studies shows that exposure to nicotine induces differentiation of atypical human and mice DCs that lack the ability to develop T_H1 mediated immunity therefore it compromises hosts immune response to vaccination (Nouri-Shirazi & Guinet 2012). Antigen presenting cells (ACPs) such as dendritic cells as well as macrophages have been shown to have the ability to induce regulatory T cells (Sun et al. 2007; Belkaid & Oldenhove 2008; Denning et al. 2007). Published study on decidual APCs in human pregnancy suggests that in pre-eclampsia APCs were not able to induce iT_{Reg} cells to the same extend as APCs from healthy pregnancy did (Hsu et al. 2012). It is tempting to speculate that smoking could protect from pre-eclampsia by nicotine related alteration in dendritic cells function and regulatory T cell production.

My novel, prospective study found a clear reduction in regulatory T cell levels before the onset of clinical signs and symptoms of pre-eclampsia. I also revealed that as pregnancy progresses, T_{Reg} cell levels in smokers who go on to develop FGR decline but none of these women developed pre-eclampsia. My data revealed that T_{Reg} cells from women with pre-eclampsia do not have as strong anti-proliferative activity as T_{Reg} cells from healthy pregnancy. Women with pre-eclampsia not only have lower percentage of regulatory T cells but they seem to have altered function, which may make them more vulnerable to pre-eclampsia than women at high risk of pre-eclampsia but who have healthy pregnancy outcome.

In order to find out how broad is the involvement of regulatory T cell in pre eclampsia development, functional assays investigating the suppressive/anti-proliferative abilities of T_{Reg} cells obtained from periphery as well as decidua should be performed. Key groups for this type of investigation should include healthy pregnant controls, pregnant smokers, women form high-risk group and pregnancy induced hypertension, isolated FGR and pre-eclampsia.

CHAPTER V: REGULATORY B CELLS BEFORE AND DURING THE ONSET OF PRE-ECLAMPSIA

ABSTRACT

Regulatory B cells are immunosuppressive cells involved in modulation of immunological tolerance (Mauri & Menon 2015). The role of immune-suppressive B (B_{Reg}) cells in the progression of a healthy pregnancy or in the development of pre-eclampsia has not yet been assessed. Reduced numbers of Bregs and defective function of B_{Reg} cells have been associated with autoimmune diseases. However, research into their involvement in immune system regulation is still at an early stage (Blair et al. 2010; Flores-Borja et al. 2013; Bosma et al. 2012; Mauri & Menon 2015). In this study I set to investigate if there are any differences in frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells in pregnancy complications such as pre-eclampsia compared with healthy controls and if so, how early in the gestation they can be detected.

The main findings are summarised below.

1. Women who developed pre-eclampsia or fetal growth restriction had lower numbers of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells throughout pregnancy, compared with women who had a healthy pregnancy outcome. The frequency of Breg cells were reduced in women destined to develop pre-eclampsia form the first trimester of pregnancy.
2. Depletion of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells from PBMCs obtained from healthy pregnant women led to increase in frequencies of $CD4^{+}TNF\alpha^{+}$ and $CD4^{+}IFN\gamma^{+}$ T-cells supporting the involvement of B_{Reg} cells in immunosuppression of healthy pregnancy.
3. Depletion of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells from PBMCs of women with pre-eclampsia had no effect on the number of $CD4^{+}IFN\gamma^{+}$ and and $CD4^{+}IFN\gamma^{+}$ T cells. This observation suggests that the suppressive function of B_{Reg} cells from women with pre-eclampsia is defective.
4. Women who smoked during pregnancy had a similar $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells percentage compared with women who had a healthy pregnancy.

5.1 INTRODUCTION

Most knowledge about regulatory B cells comes from mice studies (Carter et al. 2011; Amu et al. 2010; Zhang et al. 2015). In 2010, immature B cells from healthy individuals defined as $CD19^{+}CD24^{hi}CD38^{hi}$ were shown to contain the highest proportion of IL-10 producing B cells (Blair et al. 2010). This population of B cells was able to suppress T_H1 and T_H17 cells differentiation and more importantly convert naïve $CD4^{+}$T lymphocytes to regulatory T cells (Flores-Borja et al. 2013).

The level and function of $CD19^{+}CD24^{hi}CD38^{hi}$ B cells has been investigated in several autoimmune diseases including rheumatoid arthritis (RA) and systemic lupus erythematous (SLE) (Blair et al. 2010; Flores-Borja et al. 2013; Bosma et al. 2012; Mauri & Menon 2015).

Regulatory $CD19^{+}CD24^{hi}CD38^{hi}$ B cells isolated from the peripheral blood of patients with SLE did not have the suppressive capacity seen in B_{Reg} cells originating from healthy individuals (Blair et al. 2010). Further support for a role of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells in immune system regulation has come from research performed in SLE patients treated with B-cell depletion therapy, Rituximab (Palanichamy et al. 2009; Anolik et al. 2007). Following CD20 B cell depletion, patients who had a higher ratio of immature to memory B cells had longer remission, suggesting immature B_{Reg} cells are associated with a better disease outcome. Elevated numbers of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells have also been correlated with reduced rates of kidney transplant rejection (Shabir et al. 2015). It has been proposed that high IL-10/TNFα ratio in $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells is associated with a better prognosis for renal transplant patients (Cherukuri et al. 2014). In this study, $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells were shown to suppress TNFα, IFNγ and IL-4 production by conventional $CD4^{+}CD25^{-}$ T cells to a similar extent as $CD4^{+}CD25^{hi}$ TReg cells.

Additionally, studies of graft-versus-host disease (GVHD) have revealed that $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells are involved in maintenance of transplant tolerance via suppressing responses of effector T cells (Nouel et al. 2014; Khoder et al. 2014).

These early studies support a central role for $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells in immune system regulation.

Information on levels and immunosuppressive function of B_{Reg} cells in pregnancy is limited mostly to murine studies (Jensen et al. 2013). It has been shown that when regulatory B cells are transferred to the abortion-prone mice, the dendritic cells become arrested in an immature state, numbers of regulatory T cells increase and fetus the becomes protected from rejection (Jensen et al. 2013). Levels of B_{Reg} cells were also higher in normally developing pregnancies than in abortion-prone animals. Additionally, the the fact that B_{Reg} cells have been shown to have protective properties towards tumour tissue (Inoue et al. 2006; Tadmor et al. 2011) suggests that they may also be involved in suppressing immune responses towards half foreign tissues such as placenta and fetus during human pregnancy.

One study assessed the frequency and function of $CD19^{+}CD24^{hi}CD27^{+}B_{Reg}$ cells in healthy human pregnancy, spontaneous abortion and non-pregnant women (Rolle et al. 2013). The authors found that levels as well as the immunosuppressive function of regulatory B cells were impaired in spontaneous abortion cases. What is more, they showed that numbers of $CD19^{+}CD24^{hi}CD27^{+}B_{Reg}$ cells during pregnancy rise above non-pregnant levels.

My study is the first to investigate regulatory $CD19^{+}CD24^{hi}CD38^{hi}$ B cells in human pregnancy and pregnancy complications such as pre-eclampsia.

5.2 METHODOLOGY

5.2.1 Study Design and Subjects Characteristics

This study was approved by Research Ethics Committee (13/LO/0287; IRAS project ID: 108975), and all participating subjects gave written informed consent. I measured $CD19^{+}CD24^{hi}CD38^{hi}$ B cells in blood samples from the same patients as described in Chapter IV, and at the same time points as measures for regulatory T cells and sFlt-1, PLGF.

Demographic and clinical characteristics of the study participants are presented in **Chapter III**, **Table 3.1** .

5.2.2 Assessment of $CD19^{+}CD24^{hi}CD38^{hi}$ B Cells Numbers

Isolated PBMCs were stained with fluorescently labelled antibodies against CD19, CD24, CD38, CD10, CD1d and CD27 as described in **Methods Chapter II**. Cells were analysed by flow cytometry by using BD LSRFortessa™ (BD, UK) and BD LSR II Flow Cytometer (BD, UK). Staining against CD10 and CD27 was used to determine a suitable gating strategy as previously described by Blair et al (Blair et al. 2010). Professor Mauri's group have shown that $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells express CD10 marker but lack CD27. Figure presenting gating strategy with the use of CD10 is shown below (**Figure 5.1**).

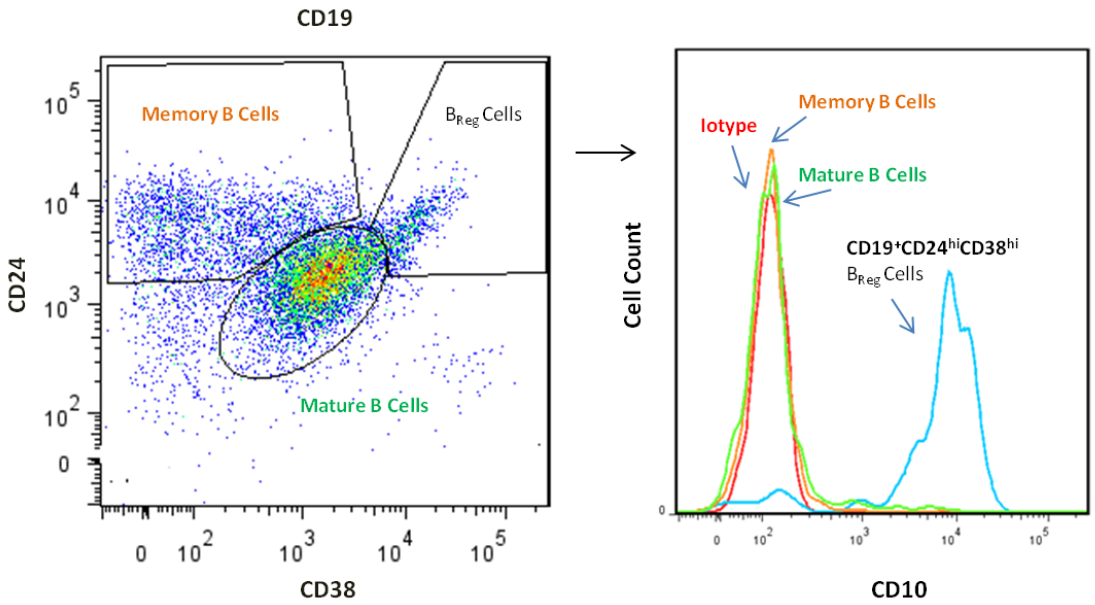

Figure 5.1 Gating strategy used in identifying $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} Cells.
Conversely to memory B cells ($CD19^{+}CD24^{hi}CD38^{-}$) and mature B cells ($CD19^{+}CD24^{int}CD38^{int}$), regulatory $CD19^{+}CD24^{hi}CD38^{hi}$ B cells are known to express CD10 marker (blue histogram). This feature was used to determine the optimal gate location for B_{Reg} cells identification.

5.2.3 Assessment of suppressive function of $CD19^{+}CD24^{hi}CD38^{hi}$ B Cells in healthy pregnancy and in pre-eclampsia.

Peripheral blood mononuclear cells (PBMCs) isolated from three healthy pregnant women and four women with pre-eclampsia were stimulated with anti-CD3

monoclonal antibody and cultured for 72 hours. In order to investigate the suppressive properties of $CD19^{+}CD24^{hi}CD38^{hi}$ B cells, they were depleted from PBMCs and cultured alongside untouched PBMCs from the same patient. If in pregnancy $CD19^{+}CD24^{hi}CD38^{hi}$ B cells have suppressive properties, once they are removed from the PBMCs population, the levels of $CD4^{+}TNF\alpha^{+}$ T cells and $CD4^{+}IFN\gamma^{+}$ T cells should increase. Gating strategy used in identifying CD4 positive cells is shown in **Figure 5.2** Figure 5.2 Gating strategy used in identifying $CD4^{+}CD19^{-}$ cells which were then gated according to presence of CD4 and TNFα or IFNγ.Cells were also stained for CD19 to ensure high purity of $CD4^{+}$ lymphocytes. Once $CD4^{+}CD19^{-}$ population has been identified, the cells were further investigated for TNFα and $IFN\gamma^{+}$ expressing cells (Figure 5.8).

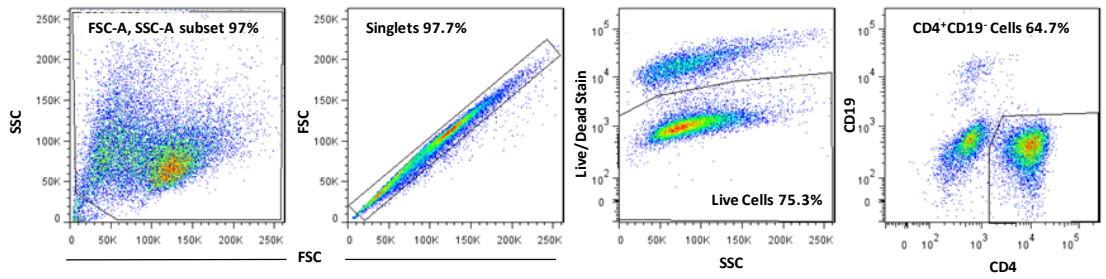

Figure 5.2 Gating strategy used in identifying $CD4^{+}CD19^{-}$ cells which were then gated according to presence of CD4 and TNFα or IFNγ.

5.2.4 Statistical analysis

As in previous chapters, subjects from the cohort were divided into subgroups depending on their pregnancy outcome: those who developed pre-eclampsia (PET; N=12); pregnancy induced hypertension (PIH; N=10); fetal growth restriction (FGR; N=9); smokers who had a healthy pregnancy outcome (SM; N=26); smokers who had a pregnancy affected by fetal growth restriction (SM+FGR; N=11); women at risk of pre-eclampsia but had a normal pregnancy outcome (HR no PET; N=24) and those who were healthy and had a healthy pregnancy outcome (H.PREG; N=66). As in **Chapter IV**, blood samples of an additional 18 patients who developed early, intermediate or late onset pre-eclampsia and 6 non-pregnant healthy women were investigated for $CD19^{+}CD24^{hi}CD38^{hi}$ B cells levels.

Symilary to the analysis performed in chapters III and IV, cross-sectional analysis at different time points during pregnancy (10-18, 19-27, 28-34 and 35-41 weeks) was performed using STATA Version 10, employing linear mixed effects model with fixed and random effects. If the data was not normally distributed therefore logarithmic transformation was performed in order to achieve normality. The results are described as coefficients with 95% Confidence Intervals and p values. The graphs represent percentage change in $CD19^{+}CD24^{hi}CD38^{hi}$ B cells during pregnancy according to pregnancy grouping. Statistical analysis was performed on logarithmic transformed values, but for visual clarity the geometric mean and estimated standard errors of the mean are reported in figures.

Data obtained in $CD19^{+}CD24^{hi}CD38^{hi}$ B cells functional assay was analysed with two-tailed pared t-test, p values below 0.05 were considered significant.

5.3 RESULTS

5.3.1 Regulatory $CD19^{+}CD24^{hi}CD38^{hi}$ B cells at the Time of Pre-eclampsia Diagnosis

Data from my research revealed that women with pre-eclampsia have lower levels of $CD19^{+}CD24^{hi}CD38^{hi}$ regulatory B cells in peripheral blood compared with gestational-matched healthy pregnant women and women who smoked whilst having a healthy pregnancy outcome (**Figure 5.3**).

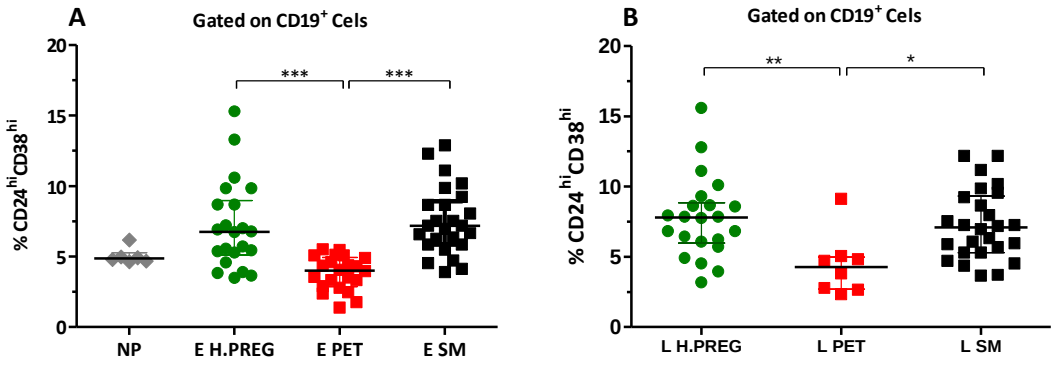

Figure 5.3 Peripheral blood levels of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells, during A: early pregnancy (E; <34 weeks gestation) and B: late pregnancy (L; ≥ 37 weeks).

Levels of peripheral Bregs in non-pregnant women (NP) compared with, healthy pregnant women (H.PREG), women with pre-eclampsia (PET) and women who smoked during pregnancy (SM). The levels of B_{Reg} cells were increased during healthy pregnancy and in smokers, whereas in women with pre-eclampsia are similar to those found in non-pregnant women. Not all data was normally distributed therefore Kruskal-Wallis test with Dunns post test for multiple comparisons was used (*p<0.05, **p<0.01, *** p<0.001). Data is presented as medians with interquartile range.

Key findings

1. Women who developed early pre-eclampsia (E PET) had a lower percentage of blood $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells (4.0%; IQR: 2.9 to 5.0%) compared with gestation-matched healthy mothers (H.PREG) 7.2%; (QR: 5.9 to 9.0%; p <0.001) as well as in smokers with uncomplicated pregnancy outcome (SM) (6.8%; IQR: 5.1 to 9.0; p <0.001).
2. Similarly, women with late onset pre-eclampsia (L PET) had a lower median percentage of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells 4.28% (IQR: 2.7 to 5.0%) compared with 7.82% (IQR: 6.0 to 8.8%, p <0.01) in healthy pregnancy and 7.9% (IQR: 5.31 to 9.3%, p<0.05) in smokers (**Figure 5.3B**).
3. The percentage of circulating $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells in the blood of healthy pregnant (H.PREG) and smoking women with healthy pregnancy outcome (SM) was elevated above non-pregnant (NP) levels (4.87%; IQR: 4.68 to 5.3%), however not-significantly so (**Figure 5.3A**). This may be explained by low numbers in non-pregnant group.
4. At 28-34 weeks, the blood content of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells was similar in healthy pregnant smokers and non-smoking controls (7.2%; [IQR: 5.9 to 8.95%] vs.6.755% [IQR: 5.1 to 8.9]).

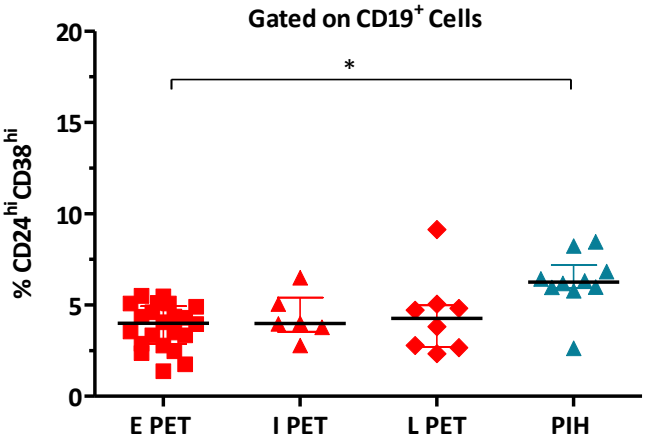

Figure 5.4 Peripheral blood levels of $CD19^{+}CD24^{hi}CD38^{hi}$ B cells, during a diagnosis of pre-eclampsia and pregnancy induced hypertension.
(E PET; ≤34 weeks gestation, N=22), intermediate gestation preeclampsia (I PET; 34-37 weeks, N=6) and late onset pre-eclampsia (L PET; ≥37 weeks, N=8) compared with pregnancy induced hypertension (PIH, N=10). Kruskal-Wallis test with Dunns post test for multiple comparisons was used (* p< 0.05, *** p<0.0001). Data is presented as medians with interquartile range (IQR).

5. Women with pregnancy induced hypertension (PIH) had blood levels of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells (6.6%; IQR: 5.9 to 7.2%) that were higher than found in women with early onset of pre-eclampsia (E PET) (4.0%; IQR: 2.868 to 4.948%, p <0.05) (**Figure 5.4**).
6. Levels of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells were similar regardless of gestational onset of pre-eclampsia. The median values for early pre-eclampsia were 4.0% (IQR: 2.9 to 4.%), for intermediate pre-eclampsia 3.4% (IQR: 3.5to 5.4%) and for late onset pre-eclampsia 4.28% (IQR: 2.7 to 5.0%).

5.3.1.1 Regulatory $CD19^{+}CD24^{hi}CD38^{hi}$ B cells in Prospectively Collected Blood Samples

Key findings:

1. Throughout pregnancy, women who later developed pre-eclampsia had levels of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells that were 31.2% (95%CI: -43.3 to -16.4, p<0.0001) lower compared with healthy pregnant subjects (H.PREG) and 37.7% (-50.0 to -22.5%, p<0.0001) lower compared with smokers who had an uncomplicated pregnancy outcome (SM) (**Figure 5.5** & **Table 5.1**).
2. Non-smokers who later developed fetal growth restriction (FGR) had the lowest percentage of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} lymphocytes from the beginning of the pregnancy. They were 46.7% (95%CI: -57.5 to -33.1%, p< 0.0001) below

normotensive healthy controls (H.PREG) levels at 10-18 weeks and further deteriorated toward term, being 62.1% (95%CI: -71.3 to -49.8%, p< 0.0001) lower. Overall, the percentage of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells in the blood of women with FGR was reduced by 46.7% (95%CI: -57.5 to -33.1%, p <0.0001) (**Table 5.2**).

3. Women who were at risk of pre-eclampsia, but who had a healthy pregnancy outcome had blood levels of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells that lie between healthy normotensive women and those with pre-eclampsia. They were 17.7% decreased (95%CI: -29.8 to -3.5%, p=0.017) compared with subjects who had a healthy pregnancy, but tended to be higher, +19.6% (95%CI: -4.7 to 50.1%, p=0.121) than levels from women who later developed pre-eclampsia.
4. There was no significant difference in the percentage of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells between healthy non-smokers (H.PREG), smokers with normally grown babies (SM) (+10.5%; 95%CI: -4.5 to 27.9%, p=0.177) and those who smoked and developed FGR (SM+FGR) (+4.5%; 95%CI: -14.5 to 27.8, p=0.644).

Representative examples of raw data for circulating $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells in blood of healthy pregnant women (H.PREG), smokers with uncomplicated pregnancy outcome, women with pre-eclampsia and non-smokers with isolated fetal growth restriction are shown in **Figure 5.6 .**

Difference in $CD19^{+}CD24^{hi}CD38^{hi}$ B Cell Levels Throughout Pregnancy				
Group	Difference vs. Healthy	95% CI		p value
SM	+10.5	-4.5	27.9	0.177
PET	-31.2	-43.3	-16.4	<0.0001
PIH	-6.9	-24.6	14.9	0.504
FGR	-46.7	-57.5	-33.1	<0.0001
SM+ FGR	+ 4.5	-14.5	27.8	0.664
HR no PET	-17.7	-29.8	-3.5	0.017
Difference in $CD19^{+}CD24^{hi}CD38^{hi}$ B Cell Levels at 10-18 weeks				
SM	+ 14.6	-3.0	35.4	0.109
PET	-30.5	-45.9	-10.6	0.005
PIH	-14.2	-33.8	11.1	0.245
FGR	-34.4	-52.1	-10.1	0.009
SM+ FGR	+ 8.5	-15.0	38.6	0.513
HR no PET	-19.0	-33.2	-1.8	0.032
Difference in $CD19^{+}CD24^{hi}CD38^{hi}$ B Cell Levels at 19-27 weeks				
SM	+ 14.7	-2.8	-2.8	0.105
PET	-31.1	-44.6	-14.3	0.001
PIH	-12.4	-31.7	12.3	0.295
FGR	-21.7	-40.6	3.1	0.082
SM+ FGR	+ 1.9	-19.2	28.3	0.876
HR no PET	-14.5	-28.7	2.5	0.090
Difference in $CD19^{+}CD24^{hi}CD38^{hi}$ B Cell Levels at 28-34 weeks				
SM	+ 5.6	-12.4	27.3	0.568
PET	-31.3	-45.5	-13.3	0.002
PIH	+ 10.9	-13.4	42.0	0.412
FGR	-56.9	-67.2	-43.3	<0.0001
SM+ FGR	+ 7.3	-15.4	36.0	0.563
HR no PET	-12.9	-28.4	5.9	0.165
Difference in $CD19^{+}CD24^{hi}CD38^{hi}$ CB Cell Levels at 35-41 weeks				
SM	-3.0	-20.9	19.0	0.772
PET	-32.2	-47.9	-11.6	0.004
PIH	-14.8	-35.2	12.1	0.252
FGR	-62.1	-71.3	-49.8	<0.0001
SM+ FGR	-1.0	-25.9	32.3	0.946
HR no PET	-31.5	-44.9	-14.9	0.001

Table 5.1 Data presenting % change in circulating $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells compared with women who had healthy pregnancy (H.PREG).
Values highlighted in green are significant. Red font indicates decrease (-) in cells content. Data is shown as coefficients with 95% CI and p values.

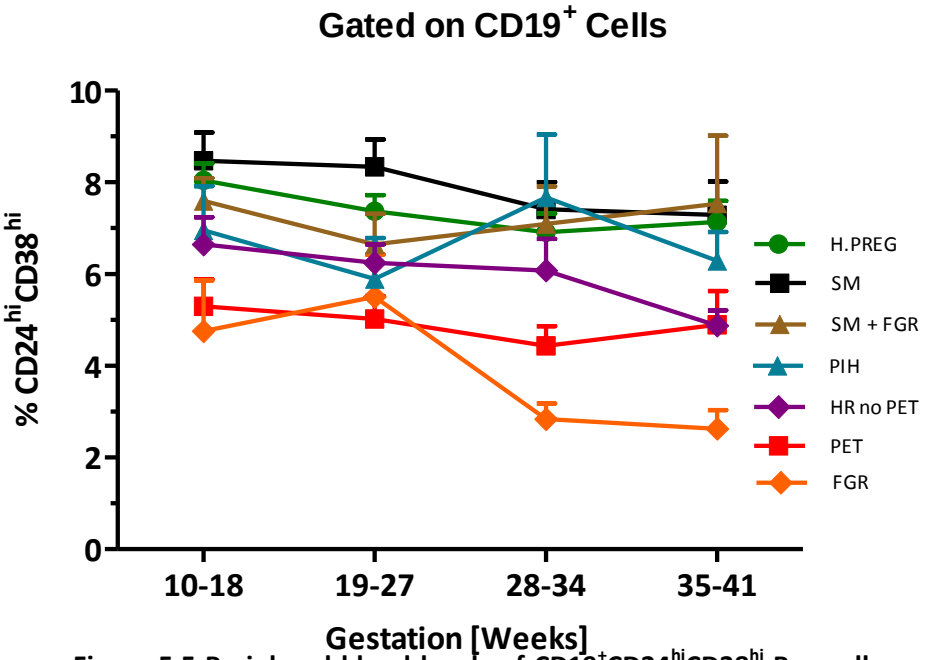

Figure 5.5 Peripheral blood levels of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells, measured prospectively throughout pregnancy.
Women who go on to develop pre-eclampsia (PET, N=12), pregnancy induced hypertension (PIH, N=10), fetal growth restriction (FGR, N=9), who smoked (SM, N=26), who smoked and were affected by fetal growth restriction (SM+FGR, N=11), were at high risk of pre-eclampsia but did not develop pre-eclampsia (HR no PET, N=24) in comparison with women who had a healthy pregnancy (H.PREG, N=66). Data presented as means with estimated standard error of the means.

$CD19^{+}CD24^{hi}CD38^{hi}$	H.PREG				SM				PET				FGR			
	Difference %	95%CI		p value	Difference %	95%CI		p value	Difference %	95%CI		p value	Difference %	95%CI		p value
H.PERG	-	-	-	-	-9.5	-21.8	4.7	0.177	+ 45.3	19.7	76.5	<0.0001	+ 87.5	49.5	135.2	<0.0001
SM	+ 10.5	-4.5	27.9	0.177	-	-	-	-	+ 60.6	29.1	99.8	<0.0001	+ 107.3	61.8	165.5	<0.0001
PET	-31.2	-43.3	-16.4	<0.0001	-37.7	-50.0	-22.5	<0.0001	-	-	-	-	+ 29.0	-2.3	70.5	0.072
PIH	-6.9	-24.6	14.9	0.504	-15.8	-33.3	6.4	0.148	+ 35.3	3.7	76.5	0.026	+ 74.6	30.6	133.4	<0.0001
FGR	-46.7	-57.5	-33.1	<0.0001	-51.8	-62.3	-38.2	<0.0001	-22.5	-41.3	2.4	0.072	-	-	-	-
SM+FGR	+ 4.5	-14.5	27.8	0.664	-5.4	-24.5	18.4	0.624	+ 51.9	17.3	96.7	0.002	+ 96.0	47.6	160.2	<0.0001
HR no PET	-17.7	-29.8	-3.5	0.017	-25.5	-38.3	-10.1	0.002	+ 19.6	-4.7	50.1	0.121	+ 54.4	19.6	99.3	0.001

Table 5.2 Data presenting % change in overall $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells levels in pregnancy.

Healthy subjects (H.PREG), smokers (SM), women who go on to develop pre-eclampsia (PET), pregnancy induced hypertension (PIH), fetal growth restriction (FGR), who smoke and develop fetal growth restriction (SM+FGR), who were at risk but did not develop pre-eclampsia (HR no PET) compared with H.PREG, SM, PET and FGR levels. Values highlighted in green are significant. Red font indicates decrease (-) whereas green indicates increase (+) in B_{Reg} cells %. Data is shown as coefficients with 95% CI and p values.

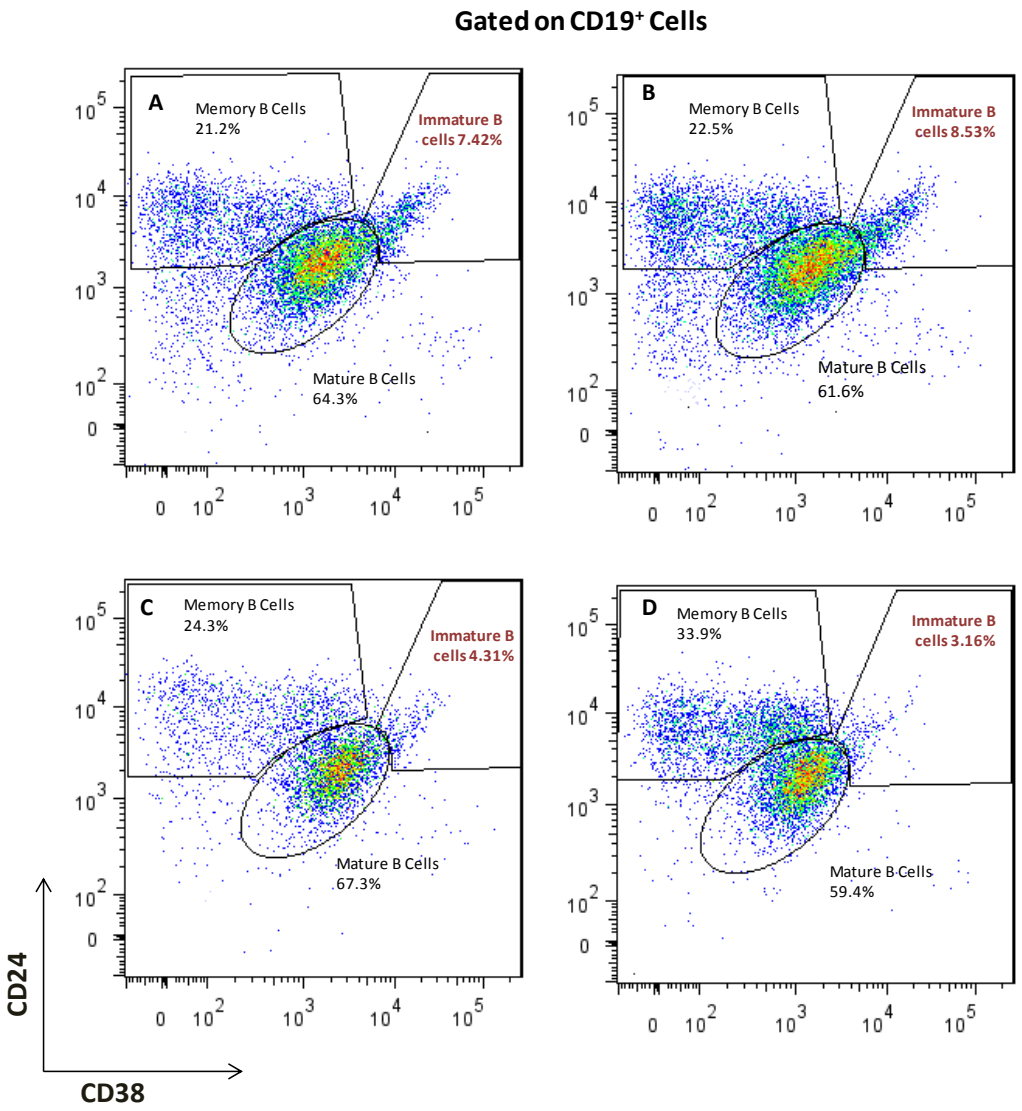

Figure 5.6 Scatter plots of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells levels in peripheral blood of pregnant women.

A) had healthy pregnancy (H.PREG), B) smoked but had uncomplicated pregnancy (SM), C) developed pre-eclampsia (PET), D) non-smokers who developed fetal growth restriction (FGR). Dot plot graphs show examples of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells (Immature B Cells), $CD19^{+}CD24^{int}CD38^{int}$ B cells (Mature B cells), $CD19^{+}CD24^{hi}CD38^{-}$ B cells (Memory B cells).

5.3.1.2 Immunosupressive Role of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells in healthy pregnancy and pre-eclampsia

There is emerging evidence to support a role for $CD19^{+}CD24^{hi}CD38^{hi}$ B cells in the regulation of the immune system in certain disease states(Blair et al. 2010; Shabir et al. 2015; Rosser & Mauri 2015). To date, only $CD19^{+}CD24^{hi}CD27^{+}$ B_{Reg} cells with phenotype more similar to memory ($CD27^{+}$) than immature $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells ($CD27^{-}$) have been investigated in healthy pregnancy or pre-eclampsia (Rolle et al. 2013).

In this chapter I have shown that the level of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells is decreased in the peripheral blood of women who go onto develop pre-eclampsia, long before the clinical onset of the condition. Following from that finding I wanted to determine whether the activity of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells was different between women with a healthy pregnancy and those with pre-eclampsia. Due to insufficient numbers of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cell in the peripheral blood of women with pre-eclampsia; I investigated if the depletion of this cell population from PBMCs obtained from healthy pregnancies or patients with pre-eclampsia may influence cytokine production by $CD4^{+}$ T lymphocytes.

Flow cytometry sorting was used to deplete $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells from PBMCs derived from healthy pregnant donors and women with pre-eclampsia. Depleted PBMCs as well as undepleted PBMCs were then stimulated with plate-bound anti-CD3 monoclonal antibody and cultured for 72 hours. The depletion of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells from healthy PBMCs significantly increased the frequency of $CD4^{+}TNF\alpha^{+}$ T cells (12.89%; ±1.36 SEM vs. 19.50%; ± 1.43 SEM; p=0.003) and $CD4^{+}IFN\gamma^{+}$ T cells (15.37%; ±1.08 SEM vs. 21.16%; ± 1.31 SEM; p=0.0029) compared to undepleted PBMCs from the same patient **Figure 5.7**.

In contrast to healthy controls, depletion of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells from PBMCs of pre-eclamptic women did not lead to increase in frequency of $CD4^{+}TNF\alpha^{+}$ T (18.85%; ± 4.586 SEM vs. 18.43%; ± 4.29 SEM; p=0.539) or $CD4^{+}IFN\gamma^{+}$ T cells (20.98%; ± 3.03 SEM vs. 20.44%; ± 2.94 SEM; p=0.503). This finding provides evidence for involvement of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells in modulating immune responses in

pregnancy as well as the impairment to their function in pre-eclampsia. Examples of raw data for $CD4^{+}TNF\alpha^{+}$ T and $CD4^{+}IFN\gamma^{+}$ T cells frequencies in PBMCs with or without $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells depletion are presented in **Figure 5.8**

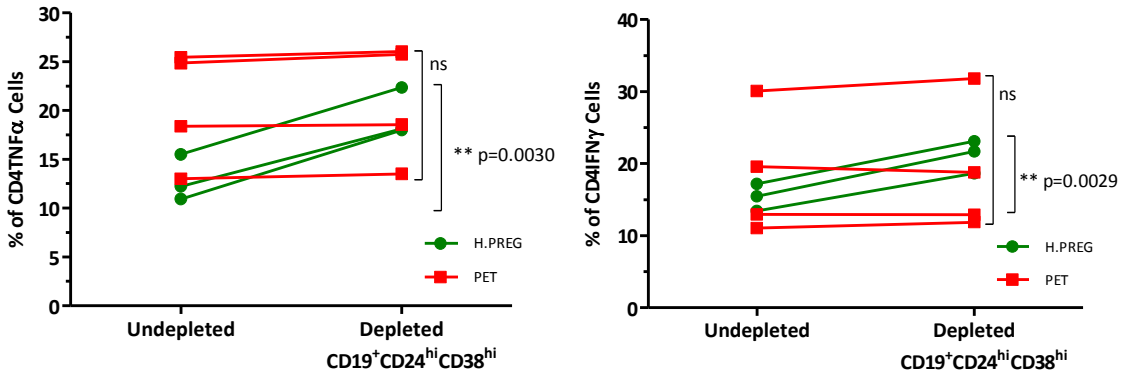

Figure 5.7 Graphs show the differences in frequency of CD4+TNFα+ T cells and CD4+IFNγ+ T cells measured within PBMCs depleted and non-depleted of CD19+CD24hiCD38hi BReg cells. Data represents four independent experiments, each conducted in triplicate. Two-tailed paired t-test used to analyse the data. Data presented as mean with estimated standard error of the mean.

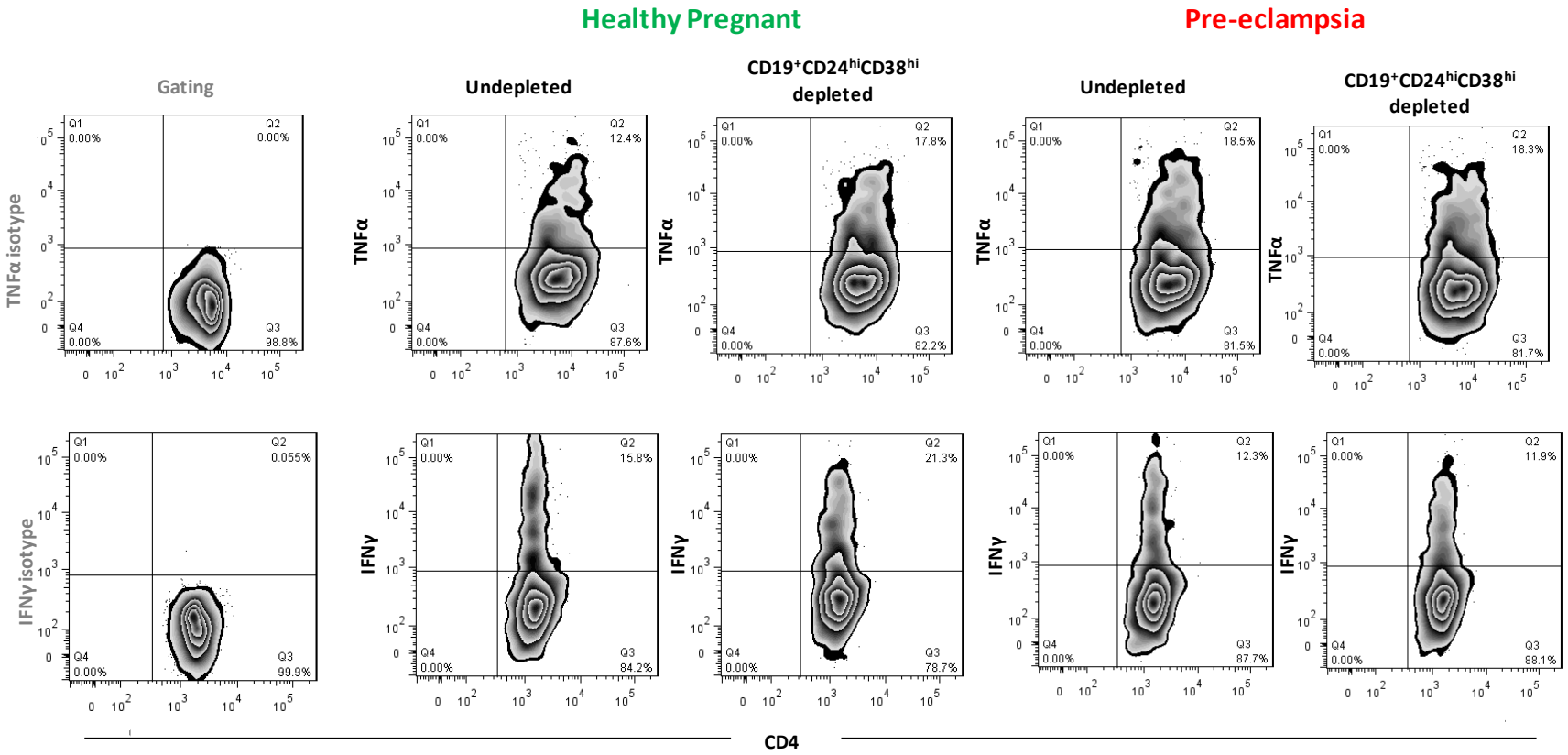

Figure 5.8 Representative flow cytometry plots for $CD4^+TNF\alpha^+$ T cells and $CD4^+IFN\gamma^+$ T cells from experiment performed on PBMCs isolated from blood of women who healthy pregnant women and subjects who developed pre-eclampsia.

Far left panel depicts gating strategy used to identify TNFα and IFNγ positive T cells within CD4+CD19- population, two middle columns shows corresponding CD4+TNFα+ T cells and CD4+IFNγ+ T cells levels before and after CD19+CD24hiCD38hi BReg cells depletion from healthy PBMCs whereas two columns on the right represent data for PBMCs obtained from pre-eclamptic subject.

5.4 DISCUSSION

Little is known about regulatory immune systems involved in maintenance and progression of a healthy human pregnancy. Studies investigating B cells in pregnancy have revealed that B cell lymphopoiesis is strongly suppressed (Muzzio et al. 2014; Medina et al. 1993). It has been suggested that this may be a mechanism aiming to reduce numbers of potentially autoreactive B cells which could recognise fetal antigens and lead to pregnancy failures. Regulatory B cells have in recent years been shown to have immunoregulatory properties and a defect in B_{Reg} cells function is associated with SLE or RA (Flores-Borja et al. 2013; Blair et al. 2010). Information about a potential link between B_{Reg} cells and pregnancy is very limited with only one available study investigating human regulatory B cells (Rolle et al. 2013).

In this chapter, I showed that levels of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells go up in the early weeks of pregnancy and although they slowly decrease towards partitution, they are still relatively high at term. This data is in line with the results of Rolle et al who showed that B_{Reg} cells rise in early pregnancy (Rolle et al. 2013). This scientific group showed that levels of $CD19^{+}CD24^{hi}CD27^{+}$ B_{Reg} cells with phenotype more similar to memory ($CD27^{+}$) than immature ($CD27^{-}$) B cells were significantly elevated in healthy pregnancy compared with non-pregnant women. Interestingly, women who were affected by spontaneous miscarriage had a similar low percentage of $CD19^{+}CD24^{hi}CD27^{+}$ B_{Reg} cells to non-pregnant subjects. Results obtained from both studies; Rolle et al. and mine, suggest that the lack of an appropriate expansion in B_{Reg} cells levels may be associated with pregnancy complications such as spontaneous miscarriage or pre-eclampsia. This theory is supported by my discovery that women who ultimately developed pre-eclampsia (PET) or isolated FGR had significantly lower levels of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells from the first trimester compared with women who had a healthy pregnancy outcome (H.PREG). Interestingly, women who ultimately developed fetal growth restriction showed the lowest frequency of regulatory B cells. Although at the beginning of the pregnancy women with FGR and PET women had similar levels of B_{Reg} cells, their drastically fall in the second half of the FGR pregnancy. Fetal growth restriction and pre-eclampsia have similar placental pathology with

shallow invasion of the placenta into the deciduas (Moffett & Loke 2006). Therefore one could expect that frequency of regulatory B cells would be the lowest in pre-eclampsia as this condition is usually associated with FGR and worsen by maternal onset of hypertension and proteinuria.

Autoimmune diseases such as SLE and rheumatoid arthritis also showed low frequency of peripheral blood $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells (Flores-Borja et al. 2013; Blair et al. 2010). This comparison suggests that pre-eclampsia and fetal growth restriction may also have an autoimmune element.

It has been reported that the ratio of T_H17/T_{Reg} cells is significantly elevated in patients with pre-eclampsia compared with healthy pregnant women (Santner-Nanan et al. 2009). My observations support the possibility that $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells could influence the right balance in T_H17 and T_{Reg} cells in healthy pregnancy. Indeed one study showed that in the absence of B_{Reg} cells, mice developed an exacerbation of inflammatory arthritis (Carter et al. 2011). This study identified that a lack of regulatory B cells led to a reduction in Foxp3 regulatory T cell levels, while supporting the rise of T_H17 and T_H1 cell mediated responses. This effect was ameliorated after the transfer of IL-10 producing B_{Reg} cells. They restricted the inflammation by inducing immunosuppressive rather than proinflammatory T cells.

Research in humans showed that although $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells obtained from RA were able to suppress the release of pro-inflammatory cytokines such as TNFα and IFNγ from conventional $CD4^{+}$T cells, but they failed to prevent the differentiation of T_H17 cells and were not able to induce fuctional T_{Reg} cells (Flores-Borja et al. 2013). Data from both studies suggest that B_{Reg} cells could be also directly or indirectly involved in the maintainance of the right balance of T_H17/T_H1 and T_{Reg} cells in human pregnancy and that disruption to B_{Reg} cells numbers and/or function could lead to pregnancy complications such as pre-eclampsia.

I showed that $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells from women with pre-eclampsia had no suppressive effect on fequencies of $CD4^{+}TNF\alpha^{+}$ and $CD4^{+}$T $IFN\gamma^{+}$ cells compared with the inhibitory effects of Bregs from healthy pregnant women. This suggest that $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells function as suppressors of immune responses in healthy

pregnancy but are defective in B_{Reg} cells from pre-eclampsia. Similar results were obtained by Rolle et al. who noticed that $CD19^{+}CD24^{hi}CD27^{+}$ B_{Reg} cells obtained from patients affected by spontaneous miscarriage had decreased ability to secrete IL-10 and suppress TNFα release (Rolle et al. 2013).

Regulatory B cells have been reported to exhibit their regulatory functions through multiple pathways, similar to regulatory T cells. It remains then to investigate which of the mechanism is faulty in pre-eclampsia and leads to development of this condition. It could be that disruptions to various mechanisms composing the suppressive properties of B_{Reg} cells could produce different pregnancy complications, one could predispose to pre-eclampsia, another to fetal growth restriction, or pregnancy induced hypertension.

The levels of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells seen in PIH were similar to levels in women with a healthy pregnancy outcome, but surprisingly women at risk of pre-eclampsia, but who had healthy pregnancy (HR no PET) had significantly reduced levels of B_{Reg} cells. This is a surprising finding as the majority of patients who developed PIH originated in the HR group. One would therefore expect that the frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells levels seen in HR no PET group would be similar to healthy controls, rather than similar to women who developed pre-eclampsia. Those patients stayed healthy whereas others who went on to develop PIH obviously had a pregnancy complication. It is possible therefore that the pathophysiological mechanism for PIH is non-immune and does not involve Bregs. However, to investigate this observation further, the regulatory properties of B_{Reg} cells from PIH and HR no PET groups would need to be measured.

Another possibility is that T_{Reg} cells as well as B_{Reg} cells are only a small fraction of all cells involved in the protection of healthy pregnancy and cannot be investigated or interpreted in isolation. Interleukine-10 and TGFβ are in the centre of immune system regulation (Saraiva & O'Garra 2010; Pickup et al. 2013). It has been shown that immune-suppressive properties of human $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells are independent from TGFβ (Blair et al. 2010), but the role of IL-10 in maintenance of a healthy pregnancy still needs to be determined. Although reports vary, there is

general agreement that TGFβ and IL-10 levels are reduced in pre-eclampsia (Sharma et al. 2007).

To date, regulatory B cells have been researched mostly in autoimmune conditions. My work provides a very early step into the role of regulatory B cells in healthy pregnancy and its complications. Much remains to be understood about regulatory B cells in humans, especially in pregnancy when gestational adjustments may influence changes not seen in non-pregnant healthy individuals. It is essential to understand the origin and role of B_{Reg} cells in healthy human pregnancy as they may possess an answer to how to prevent complications such as pre-eclampsia or fetal growth restriction.

CHAPTER VI : THE RELATIONSHIP BETWEEN MATERNAL IMMUNO-REGULATORY AND ANGIOGENIC FACTORS IN THE PREDICTION OF PRE-ECLAMPSIA, PREGNANCY-INDUCED HYPERTENSION AND FETAL GROWTH RESTRICTION

ABSTRACT

Pre-eclampsia is a multi-system disorder of pregnancy, which evolves out of the mother's inability to meet the physiological demands of healthy pregnancy. In this regard, there is reduced peripheral vasodilatation, impaired placental angiogenesis and attenuated immuno-regulatory changes. I investigated whether interactions between factors from different biological systems, in particular the immune and cardiovascular systems, could provide insight into the origins of pre-eclampsia.

In this chapter, I aimed to identify potential relationships between immuno-regulatory T and B cells, pro and anti-angiogenic factors sFlt-1, PIGF, sFlt-1:PIGF ratio and mean arterial blood pressure and their potential in predicting pre-eclampsia occurrence. My main findings are summarised below.

1. The level of regulatory T cells, (but not regulatory B cells), at 10 to 18 weeks gestation, was inversely correlated with mean arterial blood pressure (MAP) during the first trimester and at delivery.
2. The level of regulatory T cells, (but not regulatory B cells), measured at 10-18 weeks, as well as at term, showed an inverse relationship with sFlt-1 and sFlt-1:PIGF ratio evaluated at delivery.
3. At 10 to 18 weeks, receiver-operating characteristic (ROC) curves generated for a basic model based on demographic and clinical characteristics (smoking, maternal age, BMI, family history of pre-eclampsia, personal history of pre-eclampsia and levels of PAPP-A) seems to be a good predictive model for pre-eclampsia with area under to curve equal 0.862 (95%CI: 0.729 to 0.994). Extending this model for variables such as values of regulatory T and B cells, PIGF and mean arterial blood pressure increased the AUC up to 0.892 (95%CI: 0.765 to 1.000).

6.1 INTRODUCTION

Work in animals showed that transfer of $CD4^+$ T cells from pregnant rats with reduced uterine perfusion pressure (RUPP), the animal model of pre-eclampsia, to healthy pregnant rats leads to a significant increase in blood pressure, secretion of sFlt-1 and pro-inflammatory cytokines such as IL-6 and TNFα (Wallace et al. 2011).

Conversely, transfer of regulatory T cells to abortion prone mice results in enhanced remodelling of spiral arteries and decreased expression of sFlt-1 in placental tissue suggesting a clear association between regulatory immune and cardiovascular factors in pregnancy (Woidacki et al. 2015). My study has confirmed that women with pre-eclampsia have elevated serum levels of sFlt-1 and reduced levels of PIGF and regulatory T cells (Maynard et al. 2003; Levine et al. 2004; Santner-Nanan et al. 2009). However, to date no other study in humans has investigated the level of regulatory T cells before the onset of pre-eclampsia, nor their correlation with soluble cardiovascular factors such as sFlt-1, PIGF and mean arterial pressure, either once pre-eclampsia has developed or before its onset.

The early identification of women at risk of pre-eclampsia, allows the introduction of disease modifying prophylaxis, and intensive antenatal surveillance, to introduce treatment and allow timely intervention to minimize complications for mother and a child. Daily supplementation with low doses of aspirin from 12 weeks of pregnancy has been shown to reduce the incidence of preeclampsia and fetal growth restriction (Bujold et al. 2010). At 20 – 24 weeks, an association between high resistance uterine artery Doppler waveforms and pre-eclampsia has been established and this discovery helps to refine a woman's risk of pre-eclampsia.

However, there is a continuous search for markers that could be used early in pregnancy to identify women at risk of pre-eclampsia. Many markers have been investigated, they include PIGF, sFlt-1, soluble endoglin (sEng) (Noori et al. 2010), asymmetric dimethylarginine (ADMA) (Bian et al. 2015), pregnancy associated plasma protein-A (PAPP-A), human chorionic gonadotropin (hCG) (Spencer et al. 2008) and placental protein 13 (PP-13) (Nicolaides et al. 2006). Unfortunately none of them have proved to be effective enough for the first trimester prediction of future pre-eclampsia.

6.2 METHODOLOGY

6.2.1 Study Design and Subjects Characteristics.

Data described in **Chapters III – V** was here combined in order investigated relationship between soluble cardiovascular and immune factors such as regulatory T and B cells.

Demographic and clinical characteristics of the study participants are presented in **Chapter III, Figure 2.1**

6.2.2 Statistical analysis

Correlation analysis was performed on logarithmically transformed values if the data proved to be scattered. In this case Pearson correlation coefficient was used to investigate a potential association between factors. If the data was scattered, then Spearman test was implemented. In both cases correlation coefficients (r), 95% confidence intervals and p values were recorded ($p < 0.05$ was considered significant).

Mean Arterial Pressure values were normally distributed therefore analysed with two-tailed t-test sand shown as medians with 95% confidence intervals and calculated p value.

STATA Version 10 was used to construct receiver–operating characteristics (ROC) curves and to calculate the area under the curves (AUC) generated for various models. Data presented as AUC, 95% confidence intervals and standard error.

6.3 RESULTS

6.3.1 Correlations between regulatory T and B cells, mean arterial blood pressure and soluble angiogenic and anti-angiogenic factors.

6.3.1.1 Association Between T_{Reg} and B_{Reg} Cells and Mean Arterial Blood Pressure at 10 -18 weeks of gestation.

At 10 – 18 weeks of pregnancy, women who later developed pre-eclampsia (PET), pregnancy induced hypertension (PIH) or were at risk of pre-eclampsia, but stayed healthy (HR no PET) had higher blood pressures compared with women who

had a healthy pregnancy outcome (H.PREG), smokers with uncomplicated pregnancies (SM) and non-smokers who subsequently developed fetal growth restriction (presented in **Figure 3.2, Chapter III**). Furthermore, smokers with healthy pregnancy outcome had significantly reduced median blood pressure (81.83mmHg; 95%CI: 76.83 to 85.50, p=0.0033) compared with non-smoking healthy controls (85.33mmHg; 95%CI: 79.67 to 92.33) (**Figure 6.1**).

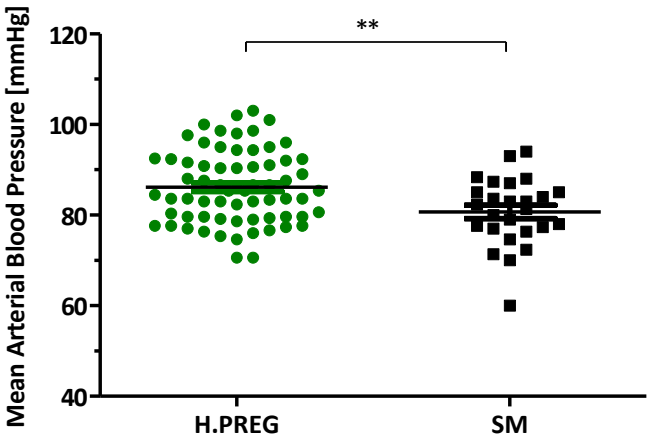

Figure 6.1 Maternal mean arterial blood pressure (MAP) between 10 and 18 weeks gestation for women who had a healthy normotensive pregnancy (H.PREG) and women who smoked and had a normal pregnancy outcome (SM).
Two-tailed t-test used for the analysis and data is presented as medians with 95% confidence intervals, p=0.0033.

Based on my previous observations, that showed first-trimester women who later developed pre-eclampsia had lower levels of regulatory T and $CD19^{+}CD24^{hi}CD38^{hi}$ B cells in their peripheral blood, I wanted to investigate if there was an association between the level of circulating regulatory cells and MAP.

At 10 to 18 weeks of pregnancy, there was an inverse correlation between $CD4^{+}CD25^{hi}$ T_{Reg} cells and mean arterial blood pressure (r= -0.4442, 95%CI: -0.5711 to -0.2749, p<0.0001) (**Figure 6.2A**). In other words, a high blood pressure correlated with a lower level of T_{Reg} cells. The association between T_{Reg} cells and MAP at 10-18 weeks was even stronger when analysing women according to whether or not they had a hypertensive pregnancy outcome. By including only healthy normotensive pregnant women, women who later developed pre-eclampsia and women who developed pregnancy-induced hypertension there was a stronger inverse correlation between T_{Reg}

cells and MAP (r=- 0.6329; 95%CI: -0.602 to -0.4589, p<0.0001) (**Figure 6.2B**). However, when each of the study groups was investigated separately, the inverse correlation between T_{Reg} cells and MAP was only evident for healthy controls (H.PREG) (r= -0.386; 95%CI: -0.6027 to -0.1201, p=0.0045) (**Figure 6.2C**).

$CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells did not show any correlation with blood pressure measurements at 10 to 18 weeks of pregnancy in any of the study groups (**Figure 6.3**)

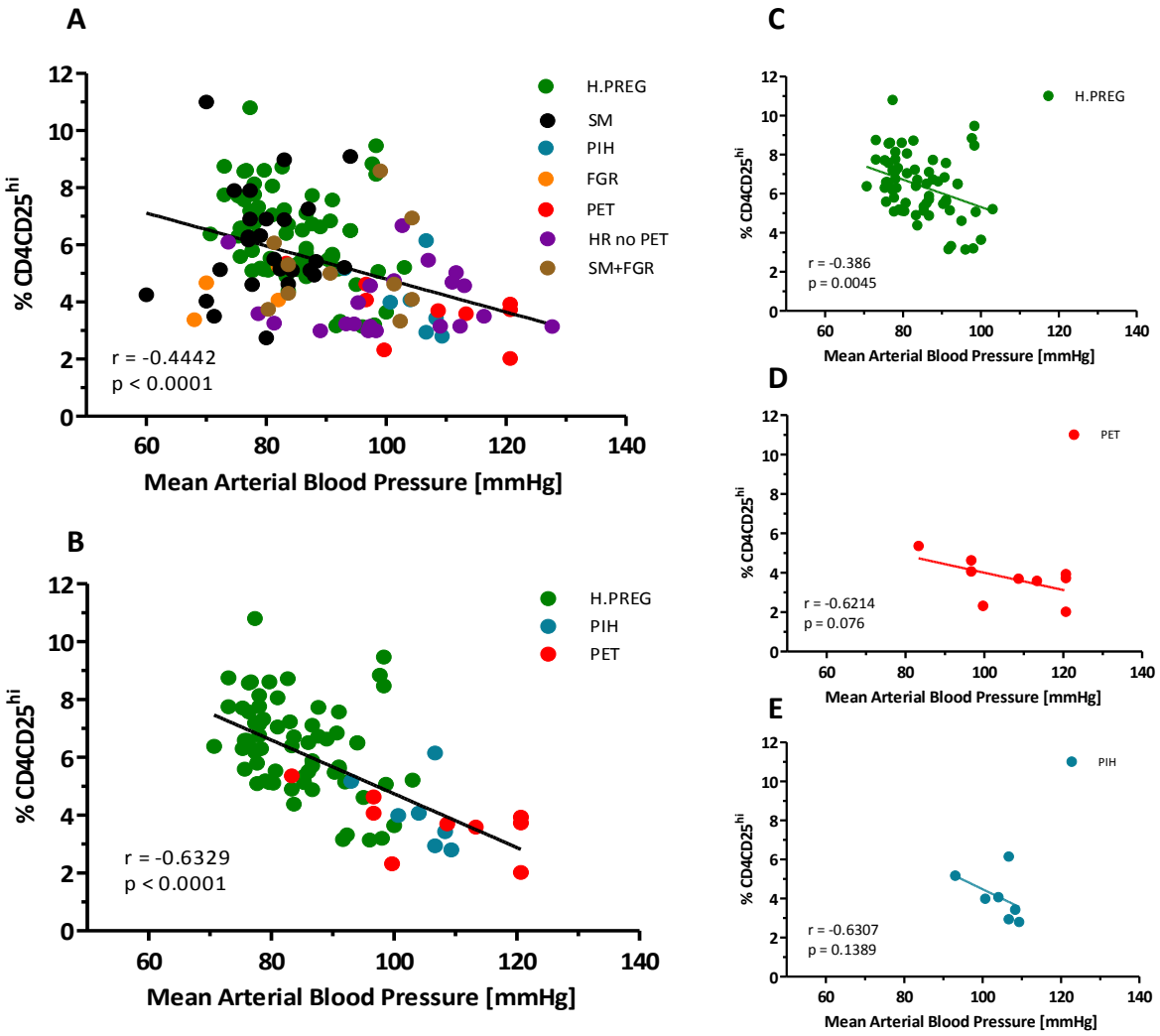

Figure 6.2 10–18 weeks' Gestation: Correlation between $CD4^{+}CD25^{hi}$ T_{Reg} cells and mean arterial blood pressure (MAP).
A) Correlation for women who go on to develop pre-eclampsia (PET, N=9), pregnancy induced hypertension (PIH=7), fetal growth restriction (FGR, N=4), smokers (SM, N=26), smokers who had fetal growth restriction offspring (SM+FGR, N=10), at high risk of pre-eclampsia but did not develop pre-eclampsia (HR no PET, N=22), and women who had a healthy pregnancy (H.PREG, N=66). B) Data for H.PREG, PET and PIH only. C) H.PREG, D) women who developed PET, E) PIH.
Data analysed with Spearman correlation coefficient, r and p values are provided in each of the graphs.

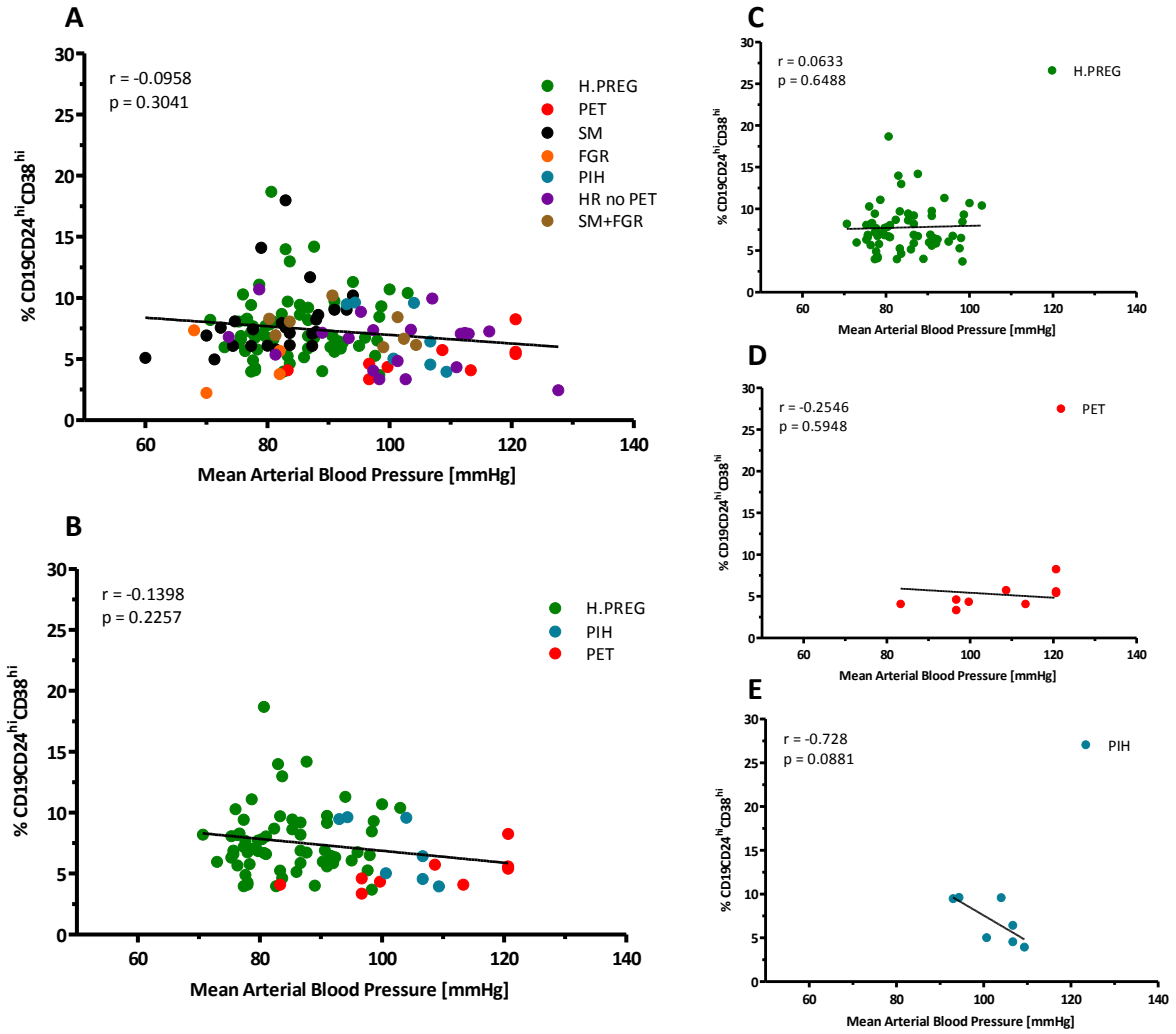

Figure 6.3 10 – 18 weeks: Correlation between $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells and mean arterial blood pressure (MAP).

A) Correlation for women who go on to develop pre-eclampsia (PET, N=9), pregnancy induced hypertension (PIH=7), fetal growth restriction (FGR, N=4), who smoked (SM, N=26), who smoked and were affected by fetal growth restriction (SM+FGR, N=10), were at high risk of pre-eclampsia but did not develop pre-eclampsia (HR no PET, N=22) and women who had a healthy pregnancy (H.PREG, N=66). B) Data for H.PREG, PET and PIH only. C) H.PREG, D) PET, E) PIH.

Data analysed with Spearman correlation coefficient, r and p values are provided in each of the graphs.

6.3.1.2 Association Between Mean Arterial Blood Pressure at 10 - 18 weeks of gestation and T_{Reg} Cells Levels at delivery.

Further analysis into associations between mean arterial blood pressure and $CD4^{+}CD25^{hi}$ T_{Reg} cells levels revealed that the higher MAP at 10 to 18 weeks the lower regulatory T cell percentage at delivery (r=-0.556; 95%CI: -0.7023 to -0.3647, p<0.0001) (**Figure 6.4A**).

Probably due to small sample sizes the only sub-group correlation for MAP and T_{Reg} cells in this setting was observed for healthy controls with an uncomplicated pregnancy outcome (H.PREG) (r= -0.3977; 95%CI: -0.597 to -0.1631, p=0.001)(**Figure 6.4B**).

There was no correlation between $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells and MAP, therefore I focused only the association between $CD4^{+}CD25^{hi}$ T_{Reg} cells and mean blood pressure.

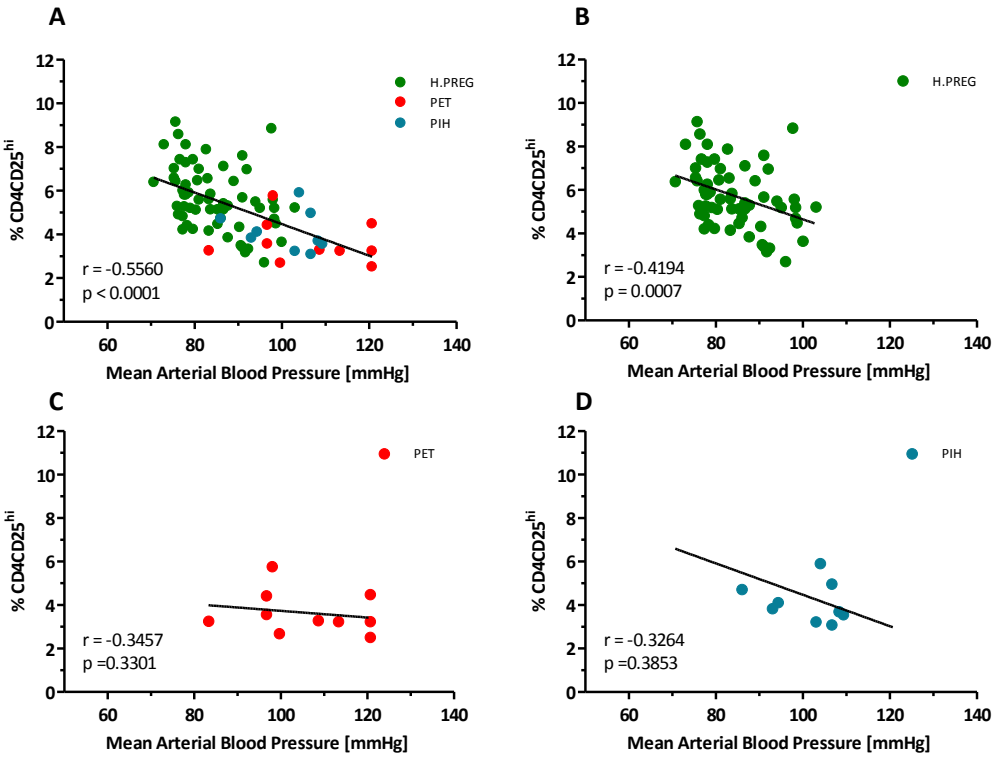

Figure 6.4 Correlations between mean arterial blood pressure (MAP) measured in the first trimester and $CD4^{+}CD25^{hi}$ T_{Reg} cells levels at delivery.
A) Correlations for women who go on to develop pre-eclampsia (PET, N=9), pregnancy induced hypertension (PIH=7) and women who had a healthy pregnancy (H.PREG, N=66). B) Correlations between T_{Reg} cells and MAP in H.PREG, C) PET, D) PIH. Data analysed with Spearman correlation coefficient, r and p values are provided in each of the graphs.

6.3.1.3 Association Between T_{Reg} Cells Levels at 10 - 18 weeks of gestation and Mean Arterial Blood Pressure at delivery.

There was an inverse association between frequencies of T_{Reg} cells measured early in pregnancy and blood pressure readings at parturition (r= -0.5795; 95%CI: -0.7231 to -0.3880, p<0.0001) (**Figure 6.5A**). As shown in **Figure 6.5A**, women with a healthy normotensive pregnancy outcome (green dots) were distinctly different from women who had a hypertensive pregnancy outcome (red and blue dots).

Although the inverse correlation between $CD4^{+}CD25^{hi}$ T_{Reg} cells and MAP was still evident when analysing healthy normotensive pregnant women (H.PREG) (r= -0.2817; 95%CI: -0.5233 to 0.0018, p=0.045) (**Figure 6.5B**), there was a very strong association in women who developed pregnancy induced hypertension (r=-0.8929; 95%CI: not provided by the test, p=0.0123) (**Figure 6.5D**). Interestingly, there was no correlation

between Treg cells in early pregnancy and MAP at delivery for women who developed pre-eclampsia (r=0.06667; 95%CI not provided by the test, p=0.8801) (**Figure 6.5C**).

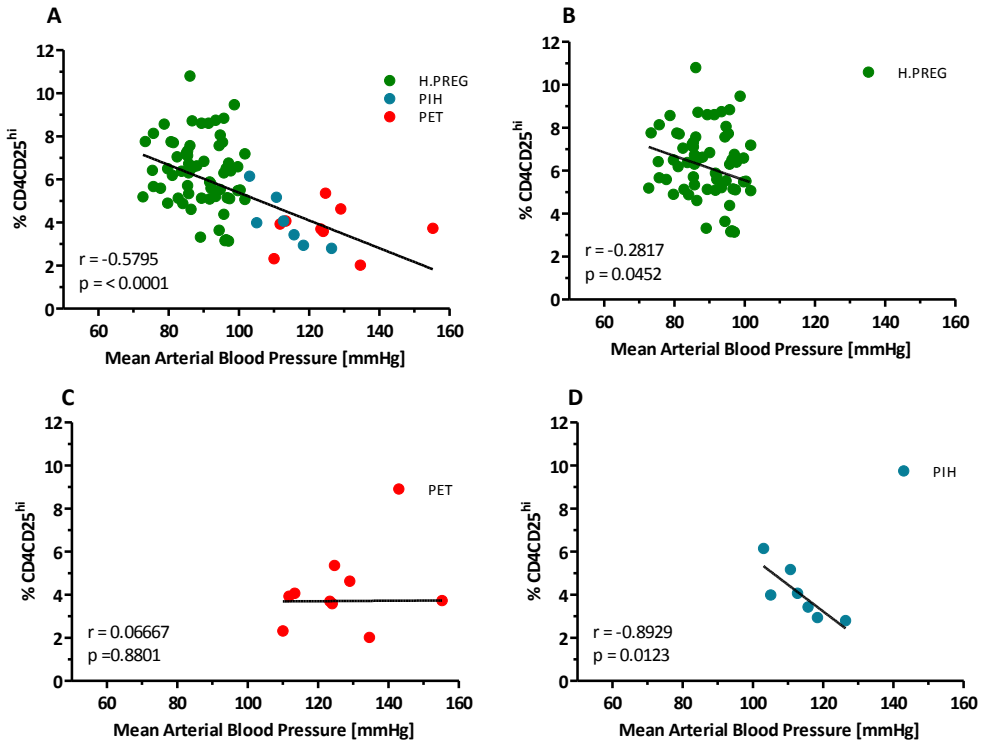

Figure 6.5 Correlations between CD4^{+}CD25^{hi} T_{Reg} cells levels evaluated in the first trimester and mean arterial blood pressure (MAP) measured at delivery.
A) Correlations for women who go on to develop pre-eclampsia (PET, N=9), pregnancy induced hypertension (PIH=7) and women who had a healthy pregnancy (H.PREG, N=66). B) - D) correlations between T_{Reg} cells and MAP in subgroups: B) – H.PREG, C) – PET, D) – PIH. Data analysed with Spearman correlation coefficient, r and p values are provided in each of the graphs.

6.3.1.4 Association Between Levels of T_{Reg} and B_{Reg} Cells at 10 - 18 Weeks of Gestation and at Delivery.

As described in **Chapters IV and V**, I showed that the levels of regulatory T and B cells are higher in the peripheral blood of pregnant smokers and non-smokers with a healthy pregnancy outcome compared with women who go on to have pre-eclampsia. In this section, I investigated whether there was a correlation between CD4^{+}CD25^{hi} T_{Reg} and CD19^{+}CD24^{hi}CD38^{hi} B_{Reg} cells that might predict an adverse pregnancy outcome.

At 10 to 18 weeks gestation, I discovered that $CD4^{+}CD25^{hi}$ T_{Reg} were positively correlated with $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells (r=0.3426; 95%CI: 0.1810 to 0.4861, p=0.0011) (**Figure 6.6A**).

Interestingly, a strong association between both T_{Reg} and B_{Reg} cells existed only for smokers with healthy pregnancy outcome (p=0.6135; 95%CI: 0.26 to 0.8229, p=0.0024) and was not present in any other study group (**Figure 6.6B**).

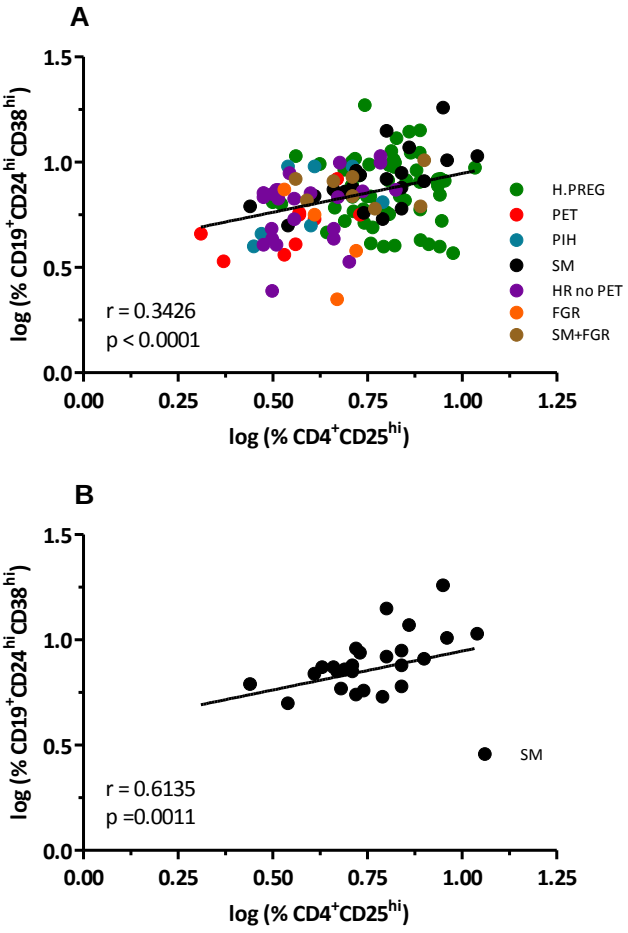

Figure 6.6 At 10–18 weeks Gestation: Correlation between $CD4^{+}CD25^{hi}$ T_{Reg} and $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells.

A) Correlation for women who go on to develop pre-eclampsia (PET, N=9), pregnancy induced hypertension (PIH=7), fetal growth restriction (FGR, N=4), who smoked (SM, N=26), who smoked and were affected by fetal growth restriction (SM+FGR, N=10), were at high risk of pre-eclampsia but did not develop pre-eclampsia (HR no PET, N=22) and women who had a healthy pregnancy (H.PREG, N=66). B) Correlation for smokers (SM; N=26). Prior to the analysis values were logarithmically transformed and data analysed using Pearson correlation coefficient, r and p values are provided in the graph.

When the same analysis was performed on maternal blood collected at delivery, the data showed no correlation between $CD4^{+}CD25^{hi}$ T_{Reg} and $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells neither in the whole cohort (r= 0.1709; 95%CI: -0.007928 to 0.3391, p=0.061) nor within individual groups (**Figure 6.7)**

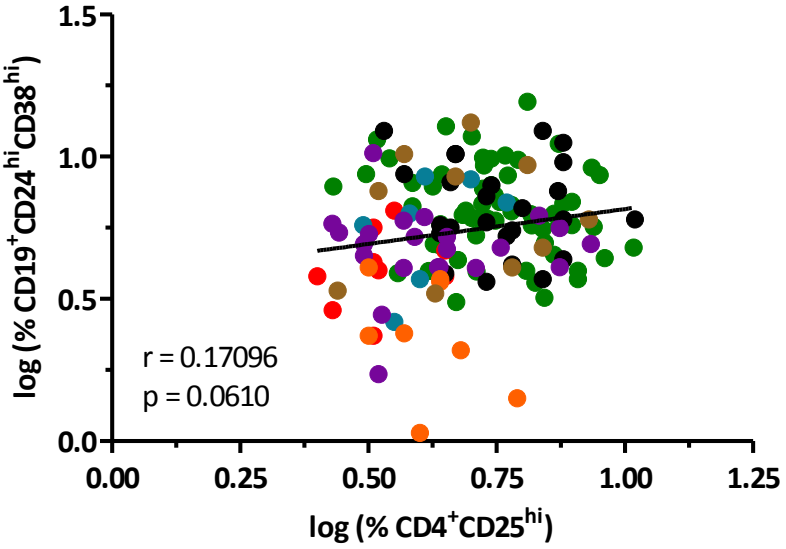

Figure 6.7 Correlations between $CD4^{+}CD25^{hi}$ T_{Reg} and $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cells at delivery. Correlation for women who go on to develop pre-eclampsia (PET, N=10), pregnancy induced hypertension (PIH=9), fetal growth restriction (FGR, N=8), who smoked (SM, N=26), who smoked and were affected by fetal growth restriction (SM+FGR, N=8), were at high risk of pre-eclampsia but did not develop pre-eclampsia (HR no PET, N=22) and women who had a healthy pregnancy (H.PREG, N=66). Prior to the analysis values were logarithmically transformed and data analysed using Pearson correlation coefficient, r and p values are provided in the graph.

6.3.1.5 Association Between T_{Reg} and B_{Reg} Cells Levels at 10 - 18 Weeks of Gestation and Circulating Angiogenic Factors at Delivery.

I showed that there was an inverse correlation between circulating $CD4^{+}CD25^{hi}$ T_{Reg} cells in early pregnancy and sFlt-1 levels and sFlt-1:PIGF ratio at delivery, but a positive correlation between first trimester Treg levels and PIGF levels at delivery (**Figure 6.8**). The correlation coefficient for T_{Reg} cells and sFlt-1 was -0.3419 (95%CI: -0.5644 to -0.07316, p=0.0141), for T_{Reg} cells and PIGF was 0.3954 (95%CI: 0.1373 to 0.6033, p=0.0037), for T_{Reg} cells and sFlt-1:PIGF ratio -0.4240 (95%CI: -0.6229 to -0.1735, p=0.0016).

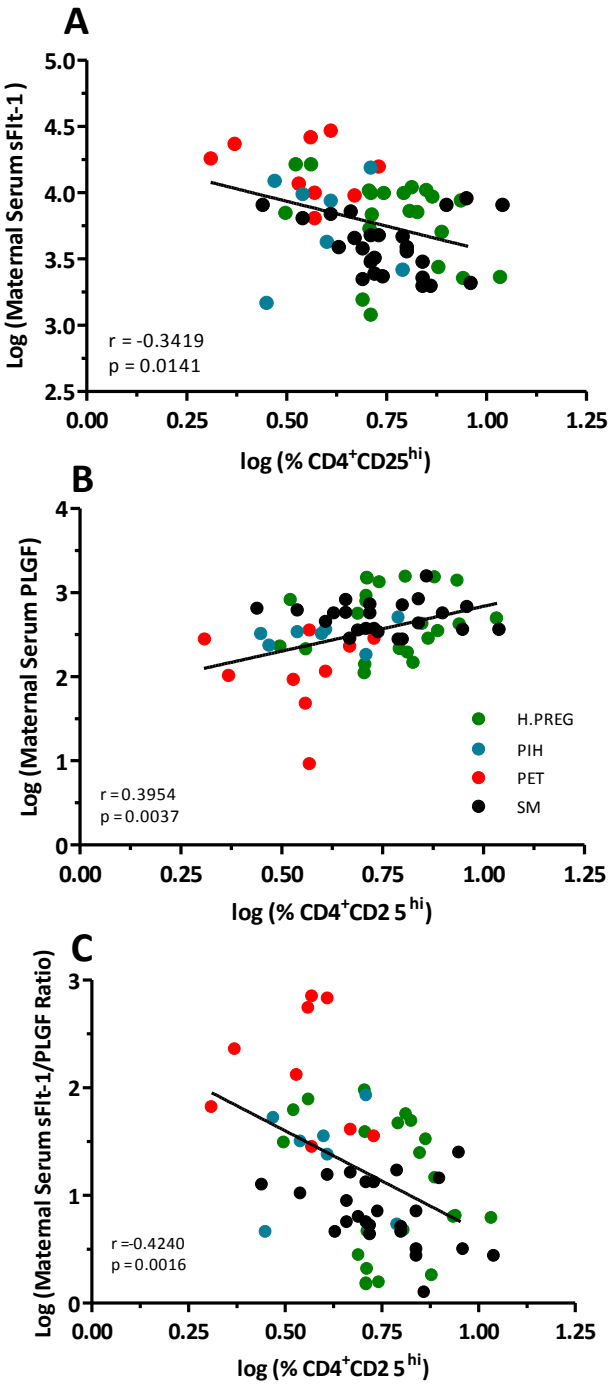

Figure 6.8 Correlations between $CD4^{+}CD25^{hi}$ T_{Reg} cell levels at 10 - 18 weeks and A) sFlt-1. B) PIGF C) sFlt-1:PIGF ratio at the delivery.

Correlation for women who go on to develop pre-eclampsia (PET, N=9), pregnancy induced hypertension (PIH=7), who smoked (SM, N=26), and women who had a healthy pregnancy (H.PREG, N=22). Prior to the analysis values were logarithmically transformed and data analysed using Pearson correlation coefficient, r and p values are provided in the graph.

Although the correlations between $CD4^{+}CD25^{hi}$ T_{Reg} cells levels and soluble angiogenic factors were seen in the cohort as a whole, the results were not replicated for individual patients groups. This could be explained by the small sample size of individual groups. Only smokers with a healthy pregnancy (n=26) outcome tended to have a inverse correlation between T_{Reg} cells levels in the first trimester and sFlt-1:PIGF ratio at term (-0.3470; 95%CI: -0.6527 to 0.05593, p=0.0893).

There was no association between $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cell levels at 10 - 18 weeks and sFlt-1, PIGF and sFlt-1:PIGF ratio at delivery (**Figure 6.9**).

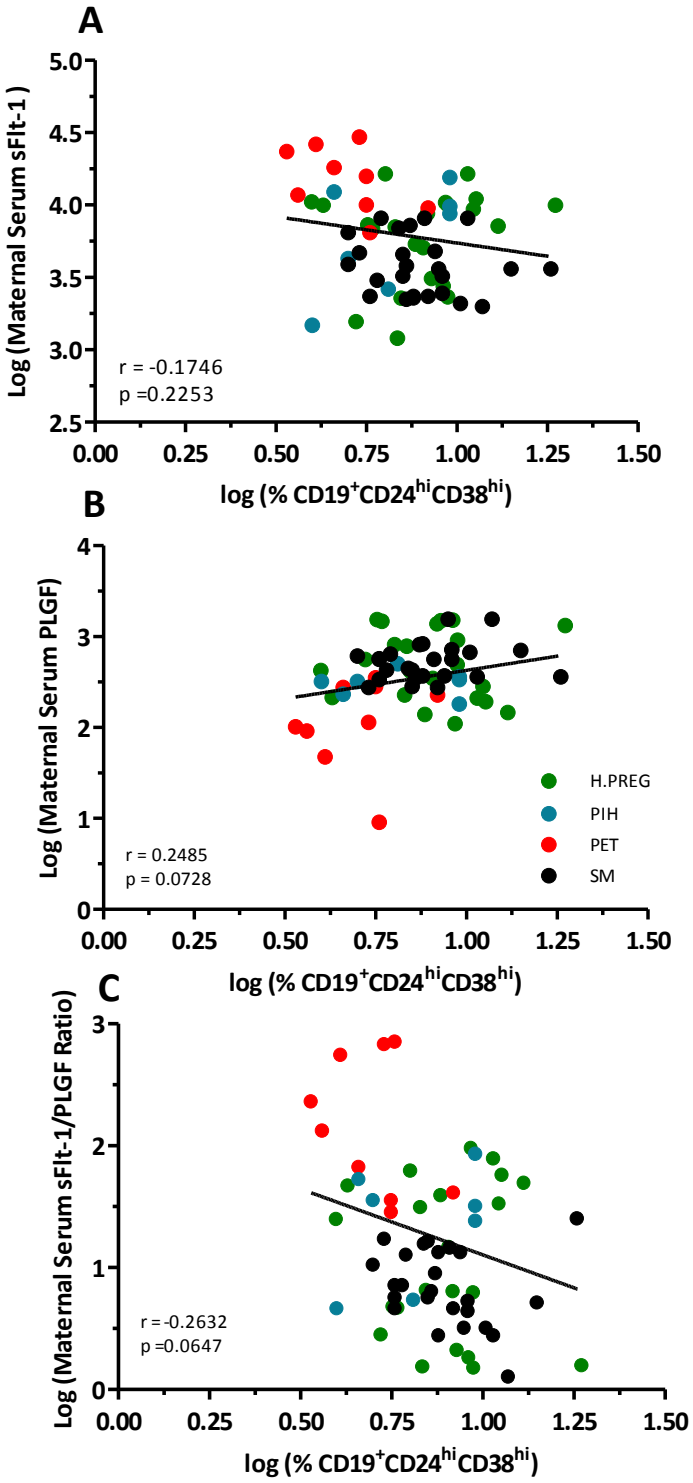

Figure 6.9 Correlations between $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cell levels at 10 - 18 weeks and A) sFlt-1. B) PIGF C) sFlt-1:PIGF ratio at the delivery.

Correlation for women who go on to develop pre-eclampsia (PET, N=9), pregnancy induced hypertension (PIH=7), who smoked (SM, N=26), and women who had a healthy pregnancy (H.PREG, N=22. Prior to the analysis values were logarithmically transformed and data analysed using Pearson correlation coefficient, r and p values are provided in the graph.

6.3.1.6 Association Between T_{Reg} and B_{Reg} Cells Levels and Circulating Angiogenic Factors at Delivery.

I have already shown that at clinical diagnosis of pre-eclampsia, women had significantly suppressed levels of PIGF, regulatory T and B cells and elevated sFlt-1, sFlt-1:PIGF ratio when compared to smokers and non-smokers with healthy pregnancy outcome. In this section I aim to investigate if there is an association between the earlier described immune and angiogneic factors at around delivery time.

At delivery, I found an inverse correlation between frequencies of $CD4^{+}CD25^{hi}$ T_{Reg} cells and serum levels of sFlt-1 (-0.3310; 95%CI: -0.5668 to -0.04490, p= 0.0247) as well as the levels of $CD4^{+}CD25^{hi}$ T_{Reg} cells and sFlt-1:PIGF ratio (-0.4395; 95%CI: -0.6473 to -0.1709, p= 0.0022) (**Figure 6.10**). Conversely, a positive correlation existed between $CD4^{+}CD25^{hi}$ T_{Reg} cells and PIGF levels (+0.4198; 95%CI: 0.1474 to 0.6330, p=0.0037).

I did not observe any association between B_{Reg} cell levels and values of sFlt-1, PIGF or sFlt-1:PIGF ratio in blood samples obtained around delivery time (**Figure 6.11**).

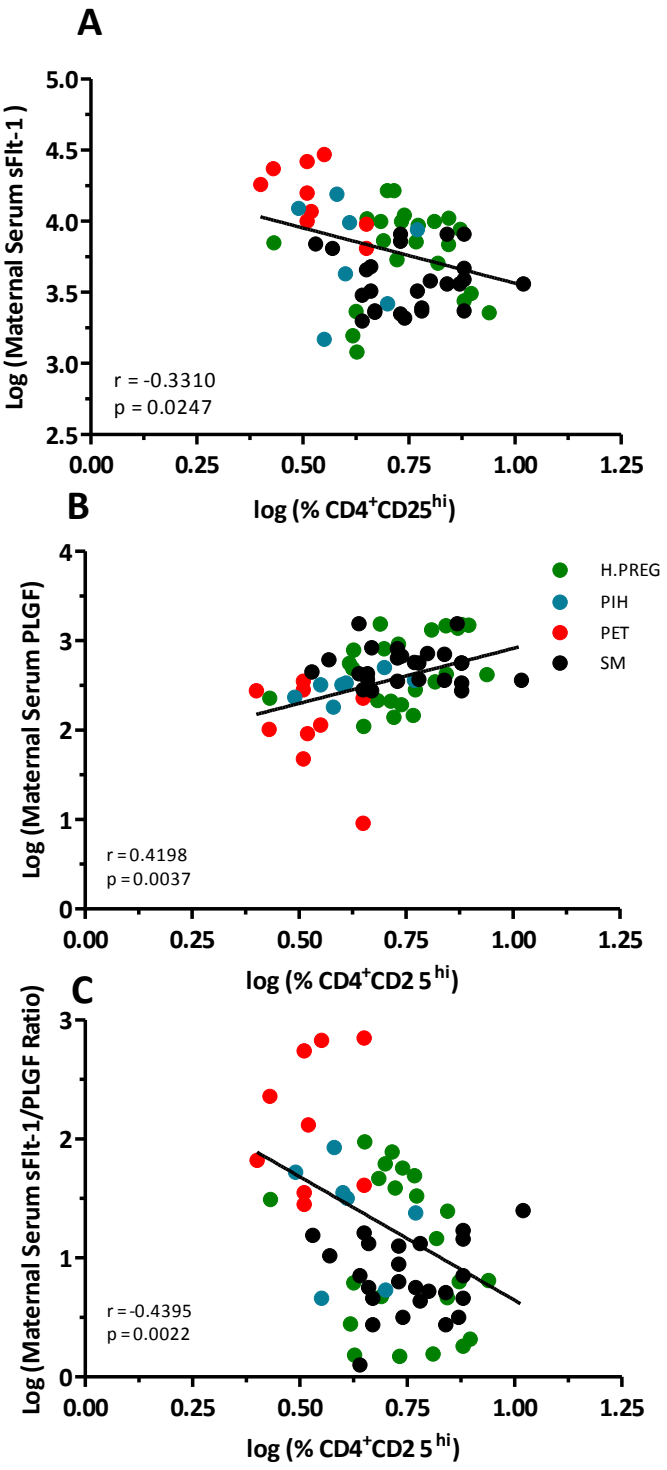

Figure 6.10 Correlations between $CD4^{+}CD25^{hi}$ T_{Reg} cell levels and A) sFlt-1. B) PIGF C) sFlt-1:PIGF ratio at delivery.

Correlation for women who go on to develop pre-eclampsia (PET, N=9), pregnancy induced hypertension (PIH=7), who smoked (SM, N=26), and women who had a healthy pregnancy (H.PREG, N=22). Prior to the analysis values were logarithmically transformed and data analysed using Pearson correlation coefficient, r and p values are provided in the graph

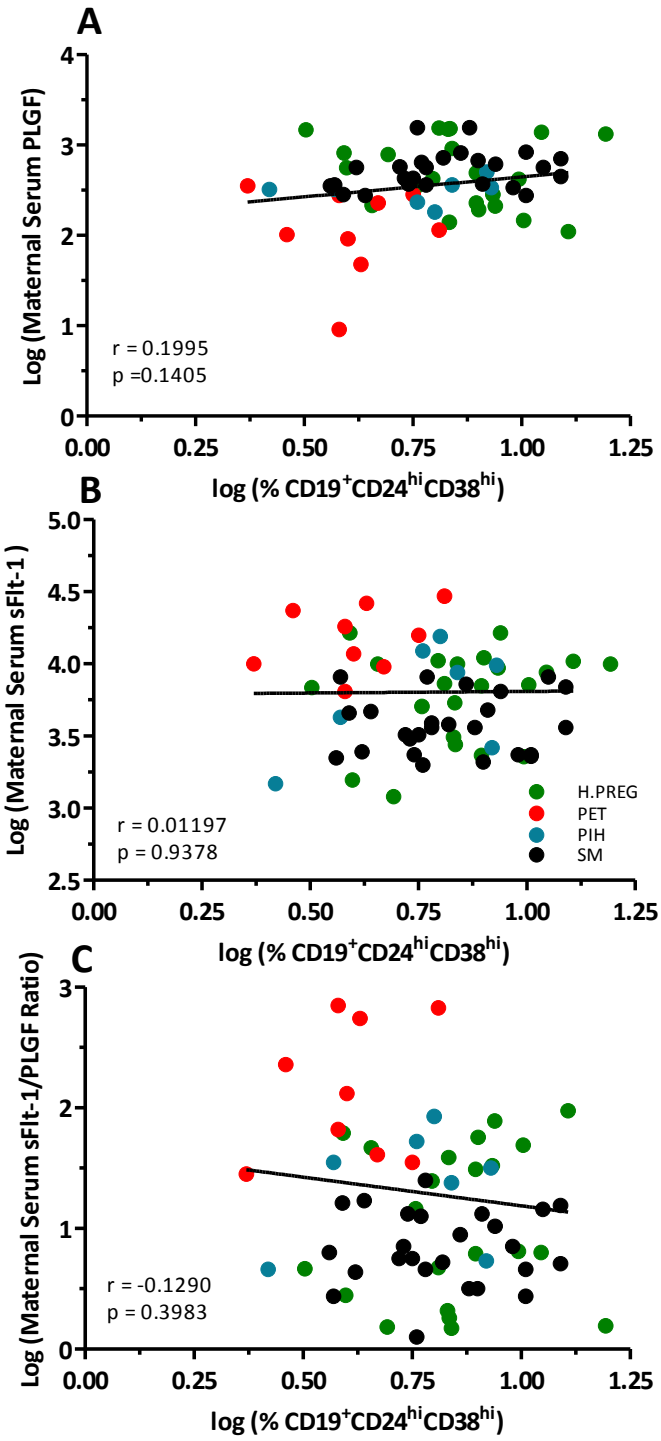

Figure 6.11 Correlations between $CD19^{+}CD24^{hi}CD38^{hi}$ B_{Reg} cell levels and A) sFlt-1. B) PIGF C) sFlt-1:PIGF ratio at the delivery.

Correlation for women who go on to develop pre-eclampsia (PET, N=9), pregnancy induced hypertension (PIH=7), who smoked (SM, N=26), and women who had a healthy pregnancy (H.PREG, N=22). Prior to the analysis values were logarithmically transformed and data analysed using Pearson correlation coefficient, r and p values are provided in the graph.

6.3.2 Regulatory Immune and Soluble Cardiovascular Factors in the Prediction of Pre-eclampsia.

Receiver operating characteristic curves (ROC) were generated for five different models based on a combination of variables in order to determine which selected factors have the best pre-eclampsia predictive power. Two sets of models were generated - one for data gathered at 10 to 18 weeks and the second for data from 19 to 27 weeks.

Basic model was based on: smoking, maternal age, BMI, family history of pre-eclampsia, personal history of pre-eclampsia and levels of PAPP-A measured at 10-14 weeks of pregnancy.

Basic + (T_{Reg} & B_{Reg}) model was based on the basic model with addition of level of regulatory T and B cells at 10-18 weeks and at 19-27 weeks. The combination of both T_{Reg} and B_{Reg} cell levels rather than either group alone, was chosen for their greater positive influence on area under the curve (AUC).

Basic + (PIGF) model was based on the basic model with addition of PIGF data.

Basic + (T_{Reg} & B_{Reg}) + (PIGF) model was based on the basic model with addition of regulatory T and B cells as well as PIGF data.

Basic + (T_{Reg} & B_{Reg}) + (PIGF) + (MAP) model was based on the basic model with addition of regulatory T and B cells, PIGF data and MAP. As I showed earlier in this chapter, elevated mean arterial blood pressure is a high risk factor for pregnancy complication, including pre-eclampsia. In order to investigate the role of the other variables in prediction of pre-eclampsia MAP values were added only in the final model.

Key findings:

1. The receiver-operating characteristic (ROC) curves identified 10 to 18 weeks (AUC: 0.862; 95%CI: 0.729 to 0.994) and 19 to 27 weeks (ACU: 0.864; 95%CI: 0.733 to 0.993) basic models alone to be good predictors of preeclampsia (**Table 6.1**).
2. Addition of measurements for regulatory T and B cells increased the area under the curve for both basic models; however the AUC at 10 to 18 week was most

impacted by this addition increasing to 0.929 (95%CI: 0.857 to 1.000) vs. 0.897 (95%CI: 0.768 o 1.000) at 19 to 27 weeks.

10 – 18 weeks	AUC	St.Error	95%CI	
Basic	0.862	0.068	0.729	0.994
Basic + (T_{Reg} & B_{Reg})	0.929	0.037	0.857	1.000
Basic + (PLGF)	0.872	0.059	0.756	0.988
Basic + (T_{Reg} & B_{Reg}) + (PLGF)	0.893	0.063	0.769	1.000
Basic + (T_{Reg} & B_{Reg}) + (PLGF) + (MAP)	0.892	0.065	0.765	1.000
19 – 27 weeks	**AUC**	**St.Error**	**95%CI**	
Basic	0.864	0.067	0.733	0.994
Basic + (T_{Reg} & B_{Reg})	0.897	0.066	0.768	1.000
Basic + (PLGF)	0.783	0.078	0.630	0.935
Basic + (T_{Reg} & B_{Reg}) + (PLGF)	0.849	0.079	0.695	1.000
Basic + (T_{Reg} & B_{Reg}) + (PLGF) + (MAP)	0.873	0.056	0.765	0.982

Table 6.1 Areas under the curve (ACU) for all the receiver-operating characteristic (ROC) models.
Table includes the basic model, basic model extended by inclusion of regulatory T and B cells, basic model extended for PIGF, basic model extended of both immune factors and PIGF, and with addition of mean arterial blood pressure (MAP) for prediction of preeclampsia at 10 to 18 weeks and 19 to 27 weeks. Values presented calculated ACU, standard error and 95% confidence intervals.

3. Addition of PIGF to the basic model slightly increased the AUC at 10 to 18 weeks (0.872, 95%CI: 0.756 to 0.988) but had a small negative effect on ROC curve at 19 to 27 weeks bringing the ACU to 0.783 (95%CI: 0.630 to 0.935).
4. Combining basic model with regulatory T and B cells and PIGF measured at the first trimester increased the AUC to 0.893 (95%CI: 0.769 to 1.000) which was similar when MAP was added on top of all of the variables 0.892 (95%CI: 0.765 to1.000) (**Figure 6.12**). At 19 to 20 week, a combination of the basic model with immune factors and PIGF had a negative effect on predictive power of the model as the AUC slightly decreased to 0.849 (95%CI: 0.695 to 1.000), addition of MAP brought the AUC to 0.873 (95%CI: 0.765 to 0.982).

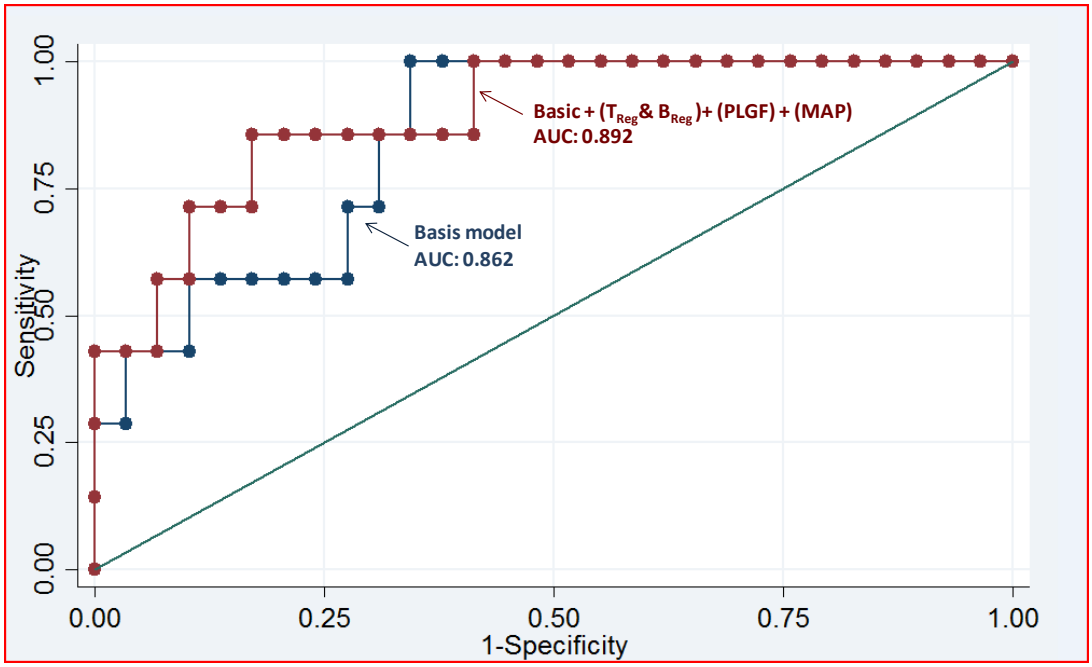

Figure 6.12 An example of receiver-operating characteristic (ROC) curve.
ROCs at 10 to 18 weeks generated for the basic model (blue) and basic model in combination with regulatory T and B cells, PIGF and mean arterial blood pressure. The greater the area under the curve (ACU) the better predictive power of the model.

5. When all the extended models at 10 to 18 weeks and 19 to 27 weeks were compared to the basic models no difference in predictive powers of these models over the basic was discovered (**Table 6.2**). The basic model was similarly good to any of the other models.
6. Although no statistically different form the basic model, the receiver operating characteristic analysis of the model generated for 10 to 18 weeks of pregnancy combining immune factors as well as clinical and demographic data provided the highest value of AUC form all of the investigational models (0.929; 95%CI: 0.857 to 1.000).

10 – 18 weeks	p value
Basic vs. [Basic + (T_{Reg} & B_{Reg})]	0.703
Basic vs. [(Basic + (PLGF)]	0.747
Basic vs. [Basic + (T_{Reg} & B_{Reg}) + (PLGF)]	0.524
Basic vs. [Basic + (T_{Reg} & B_{Reg}) + (PLGF) + (MAP)]	0.417
19 – 27 weeks	**p value**
Basic vs. [Basic + (T_{Reg} & B_{Reg})]	0.084
Basic vs. [(Basic + (PLGF)]	0.859
Basic vs. [Basic + (T_{Reg} & B_{Reg}) + (PLGF)]	0.174
Basic vs. [Basic + (T_{Reg} & B_{Reg}) + (PLGF) + (MAP)]	0.132

Table 6.2 Comparison of areas under the curve (ACU) for all of the generated models.
This includes the basic model and basic model extended for regulatory T and B cells, basic model extended for PIGF, basic model extended of both immune factors and PIGF, and with addition of mean arterial blood pressure (MAP) for prediction of preeclampsia at 10 to 18 weeks and 19 to 27 weeks. P Values presented for the comparisons between ACU of the basic model and the AUC of the other, extended models.

6.4 DISCUSSION

I have confirmed that low levels of circulating PIGF and high levels of sFlt-1 can be detected before the clinical onset of pre-eclampsia (Kusanovic et al. 2009; Moore Simas et al. 2014). In my work, concentration of PIGF in serum from women who were destined to develop pre-eclampsia were in average 56% below the levels seen in a healthy pregnancy. The difference was apparent as early as 19-27 weeks of gestation and further increased with pregnancy progression. Whereas, the levels of sFlt-1 in pre-eclampsia group were significantly elevated from around 28-34 weeks of pregnancy. These changes are known to correlate with the severity of pre-eclampsia and have been widely investigated for their prognostic value in identifying pregnant women at risk of developing pre-eclampsia (Zeisler et al. 2016; Chappell et al. 2013).

Pre-eclampsia (PET) is not a homogenous disease, but rather a syndrome with multiple clinical manifestations. Some suggest that early and late onset pre-eclampsia have a different aetiology (von Dadelszen et al. 2003; Huppertz 2008). Others consider fetal growth restriction (FGR), an outcome of poor placental perfusion, to be a defining characteristic of pre-eclampsia, as opposed to pregnancy-induced hypertension, which

is usually associated with an appropriately grown baby (Mifsud & Sebire 2014; Ness & Sibai 2006). Although pregnancies affected by FGR share the same placental pathology as pre-eclampsia, many women with fetal growth restriction do not develop hypertension or proteinuria. Given the multifaceted pathology associated with pre-eclampsia, it is highly unlikely that a single biomarker e.g. one angiogenic pathway, will successfully identify all women at risk of preeclampsia. Rather, an algorithm of multiple factors will be important.

In order to gain better understanding of the changes occurring in pre-eclampsia I investigated soluble factors that belong to two different systems – cardiovascular and immune. As women who smoke during pregnancy are protected against pre-eclampsia, I included a group of smokers to find out whether I can reveal any changes present in their blood, but absent in the blood of healthy pregnant non-smokers. The potential findings could provide insight into the protective role of smoking against pre-eclampsia.

My work showed that in the first trimester, the levels of circulating regulatory T cells were inversely associated with blood pressure measurements performed at the time of a blood sample collection. This finding was also true for intergroup correlations in normotensive controls who later had uncomplicated pregnancy. Current reports suggest that T_{Reg} cells may be less involved in the maintenance of an advanced pregnancy as T_{Reg} cell depletion after implantation causes only a moderate increase in the abortion rate (Samstein et al. 2012). However, the close association between T_{Reg} cells and MAP in early stage of healthy pregnancy suggests that regulatory T cells may be not only involved in the implantation but also in the maintenance of a healthy pregnancy.

Only nine patients who later developed pre-eclampsia were investigated at this early stage of pregnancy. Although, there was a trend showing that the higher mean arterial blood pressure (MAP) a patient had the lower was her level of regulatory T cell this association was not statistically significant. The fact that the association between T_{Reg} cells and MAP was absent in pre-eclampsia suggests that maybe development of this condition is not directly dependent on T_{Reg} cell numbers but more on their faulty

function. However, it could be also that this group was underpowered for this type of analysis.

No women from the low risk group, non smokers, developed pre-eclampsia. It is difficult to speculate if low levels of circulating regulatory T cells pre-dispose to pre-eclampsia or they are the result of changes in blood pressure. In all studied women the level of circulating T_{Reg} cells strongly correlated with MAP, but in women who developed pre-eclampsia there was no such correlation. Interestingly, the strongest negative correlation between T_{Reg} cells in the first trimester and MAP at delivery was in women who later developed pregnancy-induced hypertension (PIH). Women who develop PIH tend to have well grown babies and hypertension is generated from a maternal vulnerability to chronic hypertension (Zhang et al. 1997; Granger et al. 2001). This observation suggests low maternal Treg levels in early pregnancy may be a novel risk factor for PIH, rather than pre-eclampsia.

Interestingly, non-smokers who were later affected by fetal growth restriction, at 10 to 18 weeks of gestation, had similar levels of regulatory T cells to pre-eclamptic subject but their blood pressure readings were normal. As it is shown in **Figure 6.2A**, some smokers also had similar levels of regulatory T cells to women from PET or FGR group but they did not develop pre-eclampsia. Despite suppressed levels of T_{Reg} cells, these smokers had very low blood pressure with mean value even below levels recorded for healthy non-smokers. It was a surprising finding as outside of pregnancy smoking is associated with elevated blood pressure (Primatesta et al. 2001). This T_{Reg} cells – MAP association strongly suggest that in order to develop pre-eclampsia both, low T_{Reg} levels and high MAP are needed. If a woman has only low T_{Reg} levels then she may develop isolated FGR. Whereas, when low T_{Reg} levels are present in a smoker, then the woman is protected from either adverse outcome through a different mechanism.

When I investigated whether blood pressure readings in the first trimester were associated with regulatory T cells levels present at delivery, the data showed an inverse correlation between these two variables. The same observation was made for T_{Reg} cells levels assessed in first trimester and values of MAP at delivery. On the basis

of this information it is difficult to assess whether the level of T_{Reg} cells in early pregnancy influences future MAP in later pregnancy or the other way around. My data simply suggest that regulatory T cells and MAP are related. Interestingly, the frequency of T_{Reg} cells and MAP for women with pre-eclampsia did not exist. This observation suggests that MAP in the first trimester does not influence regulatory T cell levels around delivery. However, it is difficult to conclude that there is definitely no association between T_{Reg} cells at the beginning of the pregnancy and MAP at delivery as all patients with pre-eclampsia were on antihypertensive treatments that might have affected the blood pressure readings. Blood pressure could be then closely dependent on how well patient is responding to the treatment and when during the day the measurements were performed. I have chosen mean arterial blood pressure as an indicator of blood pressure levels rather than systolic or diastolic blood pressure reading as the MAP provides less variability.

As I showed in **Chapters IV and V**, non-smokers who were subsequently affected by pre-eclampsia or isolated fetal growth restriction had low levels of both regulatory T and B cells from early weeks of pregnancy until childbirth. Outside of pregnancy a cross-talk between regulatory B and regulatory T cells exists. B_{Reg} cells have been shown not only to induce new T_{Reg} cells (Olkhanud et al. 2011) but also to recruit them into the sight of inflammation and this way modulate their immune-suppressive capacity (Amu et al. 2010). However, when I investigated the associations between these two subsets I found that although a positive correlation was seen during the first trimester, this relationship was lost at delivery.

Additionally, I did not observe any association between the level of regulatory B cells and blood pressure measurements at any stage of the pregnancy. I can only speculate that although B-regs may influence T-regs during early pregnancy, they are not subsequently involved in the evolution of maternal MAP, which appears to be dictated by T-reg levels operating independently. However, in the first trimester, regulatory T cells were closely associated with maternal blood pressure levels even within the normotensive healthy pregnant group, but the same was not true for regulatory B cells. In my study, the frequency of Bregs in association with clinical parameters appeared to be completely random. Outside of pregnancy the same

population of human regulatory $CD19^{+}CD24^{hi}CD38^{hi}$ B cells have been confirmed to be associated with allograft tolerance in recipients of kidney transplant (Newell et al. 2010). Increased numbers B_{Reg} cells positively correlated with the state of tolerance.

A previously published study from our group found a strong inverse association between serum concentration of sFlt-1 at onset of pre-eclampsia and blood pressure reading at 10 to 17 weeks of pregnancy (Noori et al. 2010). This observation shows that women with a high MAP in early pregnancy require lower levels of sFlt-1 to develop PET. Separately, it is true a high MAP in first trimester is a risk factor for PET.

My data also revealed that the frequency of regulatory T but not B cells at 10 to 18 weeks was inversely associated with serum levels of sFlt-1 as well as the sFlt-1:PIGF ratio at delivery. Moreover, a positive correlation between regulatory T cells and PIGF also existed. The same observations were true for associations between T_{Reg} cells and the soluble angiogenic factors at delivery. Without functional studies showing direct crosstalk between regulatory T cells and sFlt-1, PIGF, it is difficult to draw strong conclusions but my data clearly suggests that all these factors may be closely related and implicated in pre-eclampsia development.

Others have shown that women with pre-eclampsia appear to develop agonistic autoantibodies (AT1-AA) which activate the AT1 receptor and mimic the physiological actions of a potent vasoconstricting factor- angiotensin II (Wallukat et al. 1999). An additional activation of AT1 receptors by AT1-AA is suggested to act as the source of high levels of sFlt-1 in pre-eclampsia (Zhou et al. 2008). Therefore it could be speculated that in pre-eclampsia, low levels of regulatory T cells with impaired function, may fail to suppress autoreactive T and B cells which then could lead to autoantibody production and subsequent elevation of serum sFlt-1 concentration. In this case regulatory T cells would be the direct link between increase in sFlt-1 levels in pre-eclampsia and the onset of the condition.

In line with previously published research, my data showed that sFlt-1 has a predictive value for pre-eclampsia in the later stage of pregnancy (Levine et al. 2004). This published study showed that sFlt-1 increases in serum of women who are destined to have pre-eclampsia around 5 to 10 weeks before the clinical manifestation

of the condition. The majority of patients from my study developed late onset pre-eclampsia with significant increase in sFlt-1 seen around the 28 weeks of pregnancy.

Similarly my data also confirmed that PIGF may be a useful as a screening test for pre-eclampsia when measured in the second trimester or later. Others found that in the second trimester, low concentration of PIGF is a reliable marker for prediction of early onset of pre-eclampsia, but not for late onset pre-eclampsia (Crispi et al. 2006; Crispi et al. 2008). Taking into account that only two out of twelve patients form my study had early pre-eclampsia, my data suggest that PIGF could be a good predictive biomarker for any onset of pre-eclampsia, but I concede numbers are small.

The ratio of sFlt-1 and PIGF is being broadly investigated by academic investigators as well as pharmaceutical companies for its diagnostic potential (Zeisler et al. 2016; Herraiz et al. 2015). According to results from my study, sFlt-1:PIGF could be useful in identifying women at risk of pre-eclampsia if measured from around 28th week of pregnancy.

My current data suggests that out of three cardiovascular system-related variables that I measured - sFlt-1, PIGF, sFlt-1:PIGF ratio, low levels of PIGF in early weeks of pregnancy may be the best predictive tool for pre-eclampsia in the early weeks of pregnancy. Multiple logistic regression analysis identified PIGF as a risk factor as early as the second trimester. In the final analysis of the data, multiple logistic regression models were able to identify combined blood markers in prediction of pre-eclampsia. Receiver-operating characteristics (ROC) curves generated for various models starting from a simple model based on demographic and clinical patient characteristics as well as models extended for variables such as PIGF and regulatoy T and B cells.

Published models investigating sFlt-1:PIGF ratio as a predictor of pre-eclampsia within 4 weeks of the clinical onset were able to achieve values that were above 0.8 AUC and this is considered as very high in this type of analysis (Zeisler et al. 2016). Similar, data was published for PIGF with Area Under The Curve (ACU) being close to 0.9 (Chappell et al. 2013). Therefore, I was surprised to find out that in my study a basic model taking into account only factors such as maternal age, history of pre-

eclampsia, BMI and smoking and PAPP-A measured at 10 -14 weeks provided a very high value for the area under the curve that was over 0.8. It is possible that my results are due to the inclusion of:

- highly phenotyped cohorts of patients with various pregnancy outcomes
- good record of clinical and demographical data for each of the patients
- only women from the high risk group developed pre-eclampsia
- none of the smokers developed pre-eclampsia

These features could have contributed to the basic model achieving such a high area under the ROC curve value. Unfortunately, due to the fact that my cohort consisted of only 166 patients, including just 12 women who developed pre-eclampsia, I was not able to use a validation cohort to assess the predictive performance of any of the developed models. Therefore it will be necessary for the models developed in my work to be tested in different cohorts of women in order to validate if they were not enhanced by the onset of pre-eclampsia only in patients who were already at risk of developing this condition.

Other models that included PIGF, regulatory T and B cell levels, in addition to MAP, gave similar results to the basic model alone. Interestingly, the receiver operating characteristic analysis of the model generated for 10 to 18 weeks of pregnancy combining immune factors as well as clinical and demographic data provided the highest value of AUC form all of the investigational models. This finding again suggests that using regulatory T (and possibly B cells) as biomarkers for pre-eclampsia alone or in combination with other soluble, serum based markers currently under investigation may enhance the predictive value of screening or diagnostic test for pre-eclampsia.

Results from my work introduce regulatory T cells as another possible risk factor closely related to pregnancy outcome. The fact the levels of T_{Reg} cells were associated with MAP, PIGF and sFlt-1 suggests that pursuing further research into their involvement in the maintenance of healthy pregnancy or pre-eclampsia development may unravel the mystery behind the development of pre-eclampsia .

CHAPTER VII:THE SEARCH FOR ANGIOTENSIN II TYPE 1 RECEPTOR AUTOANTIBODIES

7.1 INTRODUCTION

Women with pre-eclampsia appear to develop agonistic autoantibodies (AT1-AA) which activate the AT1 receptor and mimic the physiological actions of Ang II (Wallukat et al. 1999). AT1-AA are considered to contribute to the development of pre-eclampsia by composing an important link between placental ischemia and endothelial dysfunction (Siddiqui et al. 2010).

Pregnant mice injected with either total IgG or AT1-AA purified from pre-eclamptic patients develop placental ischaemia, fetal growth restriction, hypertension and proteinuria - typical symptoms of pre-eclampsia (Cissy C Zhou et al. 2008; Irani et al. 2009). Pregnant rats with a chronic reduction in uterine perfusion pressure (RUPP) or low dose TNFα infusion develop AT1-AA (Verlohren S, Herse F 2006). In both models, AT1-AA appearance was associated with a rise in blood pressure compared with healthy pregnant rats. This effect is not seen in non-pregnant animals. Interestingly, administration of the angiotensin II receptor 1 (AT_1) or endothelin 1 receptor A (ET_A) blockers significantly reduced blood pressure in RUPP model but has no effect in healthy pregnant rats (LaMarca et al. 2008). Those studies suggested that AT1-AA are the secondary event to abnormal uterine perfusion rather than the main cause of this fault and together with ET-1 through activation of the AT_1 and ET_A receptors may contribute to hypertension in pre-eclamptic subjects.

In a healthy pregnancy, Ang II through the AT1 receptor induces a time and concentration dependent rise in sFlt-1 in human trophoblast cells (Zhou et al. 2007). An additional activation of AT1 receptors by AT1-AA is suggested to act as the source of high levels of sFlt-1 in pre-eclampsia (Cissy Chenyi Zhou et al. 2008).

Taking into account the potential role of AT1-AA in the pathogenesis of pre-eclampsia and in view of my own findings of suppressed regulatory T and B cells in women destined to develop pre-eclampsia, I aimed to investigate their involvement in the development of this pregnancy-related condition. I wanted to test whether the levels of AT1-AA will correlate with the levels of regulatory T and B cells. I speculated

that regulatory T cells can suppress B-cell proliferation and immunoglobulin production and this way control AT1-AA production in a healthy pregnancy.

7.2 METHODS

Detailed methods are described in **Chapter II**. In summary, I used multiple techniques while attempting to detect autoantibodies to Angiotensin II receptor one.

I used:

1. A bioassay based on Chinese hamster ovary (CHO) cells stably transfected with Ang II receptor type 1 (CHO-AT1R).
2. Commercially available ELISA
3. Multiple home-made ELISAs
4. Wire myography

Unfortunately, I was not able to use rat neonatal cardiomyocytes, which other researchers have used to detect AT-AA (Wallukat et al. 1999). Other groups have successfully used CHO-AT1R cells (Siddiqui et al. 2010) as well as home-made and commercial ELISAs ((Zhang et al. 2013) Sahay et al. 2014) to detect AT-1AA.

7.3 RESULTS

In this chapter, I will show the techniques I used during my attempts to measure AT1-AA in serum of pregnant women. I will show examples of data to support my claims.

CHO-ATR1 cells responded well to Ang II which is known to stimulate the AT1 receptors, but no changes in reactive light units (RLU) over non-stimulated cells where seen for lysates of CHO-AT1R cells that were incubated with IgG form women with pre-eclampsia or healthy pregnant women (**Błąd! Nie można odnaleźć źródła odwołania.**).

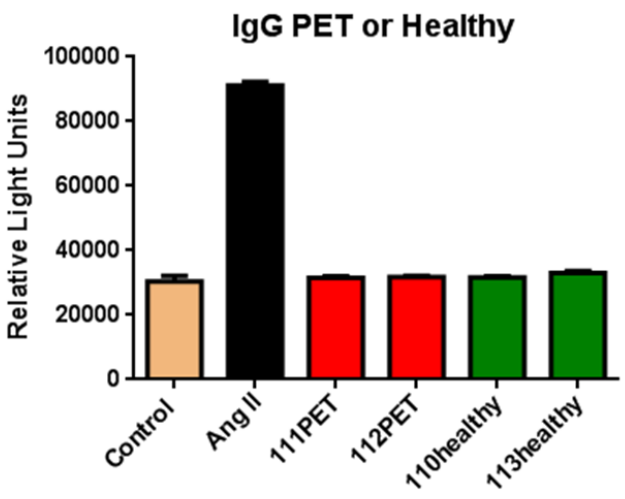

Figure 7.1 Representative data for CHO-AT1R bioassay.
Luciferase activity for CHO-AT1R cells without any stimulation (control), stimulated with 100nM Ang II, IgG from patients with pre-eclampsia (111PET & 112PET) or healthy pregnancy (110healthy & 113healthy).

When the CHO cells bioassay did not work I tried to use ELISA. Unfortunately, despite multiple attempts I was not able to detect any presence of AT1-AA in serum of patients with pre-eclampsia.

7.3.1 Wire myography.

First I confirmed that myometrial vessels obtained from women with pre-eclampsia are more sensitive to stimulations with Ang II than vessels from normotensive pregnancy. **Figure 7.2** shows representative recordings from Ang II dose-response experiments. In arteries from healthy pregnant controls, contractions elicited by Ang II were very weak (**Figure 7.2A**). After reaching maximal response, due to rapid desensitization of Ang II receptors type 1, vessels relaxed immediately and did not respond to further higher doses of Ang II. Two out of six vessels from normotensive pregnant women did not respond to Ang II (**Figure 7.2B**), even though their responses to high potassium, U46619 and Bradykinin were normal.

In contrast to the pattern observed with healthy vessels, arteries obtained from patients with pre-eclampsia exhibited significantly greater sensitivity to Ang II. Raw traces, showing examples of contractions induced by Ang II from pre-eclamptic myometrial vessels can be seen on **Figure 7.2**. Some of the responses were recorded as a single, but strong contraction followed by immediate receptor desensitization and vessel relaxation (**Figure 7.2C**). Other vessels showed weaker but, sustained contractions with slow receptor desensitization (**Figure 7.2D**).

Unfortunately, when I used IgG from serum of patients with pre-eclampsia there was no vasoconstrictor response to IgG alone neither enhanced vasoconstriction to Ang II. A graph with representative raw myography data is shown (**Figure 7.4**).

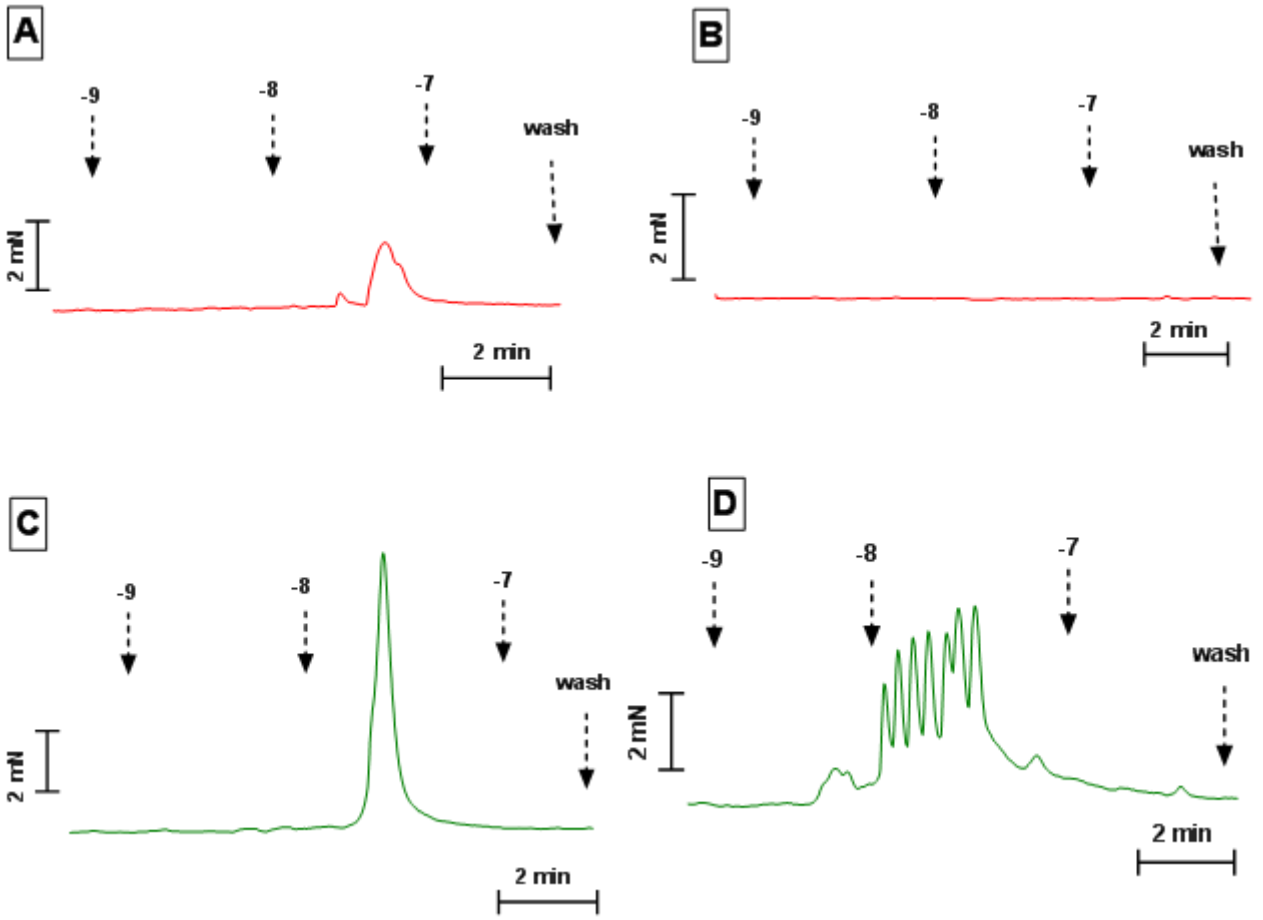

Figure 7.2 Raw traces showing representative force recording from two segments of myometrial arteries from healthy patients at 39th week (A&D) and two from patients with pre-eclampsia at 37th week (C&D).
Arrows indicate the addition of each Angiotenisn II dose (10^{-9} to 10^{-7}M).

Due to inconsistent responses to Ang II, results are presented as a median of areas under the curves generated in response to addition of cumulative concentration of Ang II (**Figure 7.3).** Ang II stimulated contraction in arterial segments obtained from patients with pre-eclampsia were clearly augmented with median area under the curve 307.5mN*s compared to 9.15mN*s in healthy subjects. This pattern of Ang II mediated hypersensitivity of pre-eclamptic compared to healthy vessels was observed due to higher force generation and also by prolonged response to the agonist without immediate receptor desensitization.

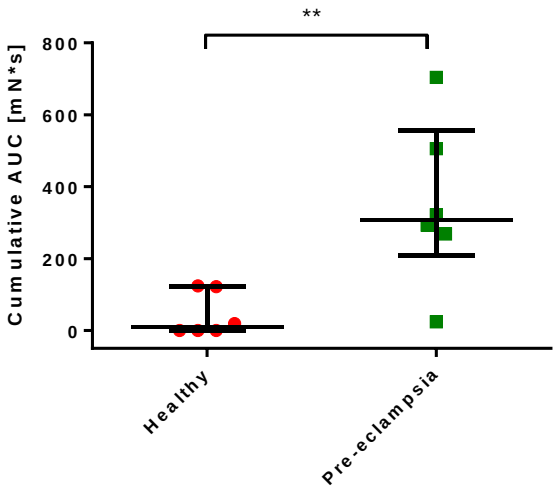

Figure 7.3 Scatter dot plot depicting the difference in surface area under the curves generated by cumulative concentration responses (10^{-9} to 10^{-7}M) induced by Angiotensin II in 6 myometrial arteries from four healthy pregnant women and two patients with pre-eclampsia.

Data is shown as median with interquartile range. ** = P<0.01; t – test with nonparametric Mann-Whitney.

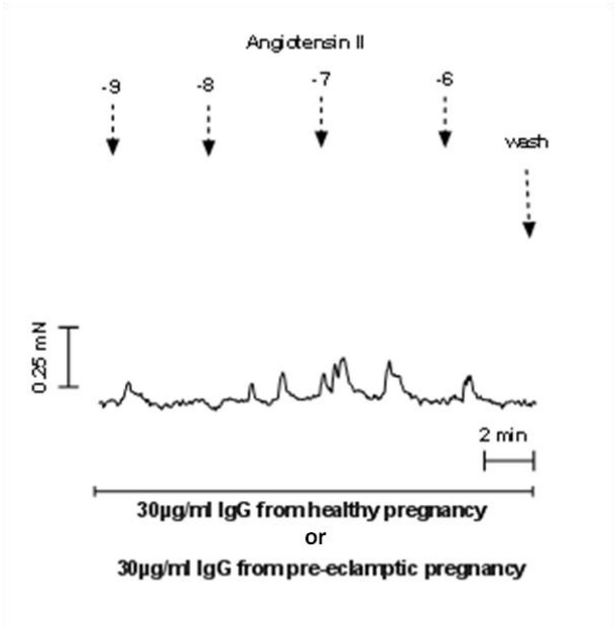

Figure 7.4 Raw trace showing an example of force recording from a segment of myometrial artery from a patient with pre-eclampsia treated with 30 µg/ml of total IgG isolated from healthy serum or (B) or pre-eclamptic.
Arrows indicate the addition of each Angiotenisn II dose (10^{-9} to 10^{-6}M).

7.4 DISCUSSION

I am convinced that AT1-AA are present in serum of women with pre-eclampsia. Many research laboratories have published data supporting the exostemce of angiotensin II autoantibodies. (Cissy Chenyi Zhou et al. 2008; Walther et al. 2005; LaMarca et al. 2008). Unfortunately, I was not able to detect them. Another group from Mexico were also unable to detect AT1-AA. They also used CHO cells based bioassay and concluded that preeclampsia in Mexican women is not associated with circulating AT1-AA.

Since I was not able to detect any autoantibodies to Ang II receptor 1, the next step was to set up a collaboration with a German group who use neonatal cardiomyocytes to investigate the presence of these autoantibodies in women with pre-eclampsia.

CHAPTER VIII: GENERAL DISCUSSION

Pre-eclampsia is a multisystem complication of pregnancy which according to the reports of World Health Organisation is responsible for over 50,000 maternal and 12% of all neonatal deaths each year. What is more, pre-eclampsia predisposes to future cardiovascular diseases. The fact that pre-pregnancy risk factors for pre-eclampsia are similar to those reported for cardiovascular diseases (Magnussen et al. 2007) could explain the existing association between these two disorders (Bellamy et al. 2007). However, most of the women with cardiovascular risk factors do not develop pre-eclampsia. In my work only 23% of women from high risk group were ultimately affected by pre-eclampsia (PET). What is observed in a hospital setting is that the majority of women suffering from pre-eclampsia did not have prior cardiovascular risk factors. This suggests that pre-eclampsia can be associated not only with faulty maternal peripheral adaptation to pregnancy but also with failure of immune system to fully accept the half-foreign fetus and placenta. The evidence exists that a maternal immune reaction against paternal factors may be a big part of the mechanism. Data for epidemiologic studies revealed that pre-eclampsia in most of the cases is associated with a first pregnancy (Hernández-Díaz et al. 2009). Prolonged exposure to paternal antigens present in seminal fluid prior to pregnancy reduces a risk of pre-eclampsia (Saftlas et al. 2014). However, if after the first pregnancy the partners is changed than the risk of pre-eclampsia increases (Basso et al. 2001). These findings suggest clear relationship between impaired immune adaptation to pregnancy and pre-eclampsia. Paradoxically, smoking during pregnancy decreases the risk of pre-eclampsia by at least 32% (Conde-Agudelo et al. 1999). The reason behind it is still unclear.

In the prospective study of pregnant women described in my thesis none of the 39 smokers have been affected by pre-eclampsia. Despite the fact that 11 of the women (28%) developed fetal growth restriction (FGR) they did not progress to pre-eclampsia. They also did not had a pregnancy induced hypertension (PIH). Although, subjects who smoked during pregnancy had similar levels of peripheral regulatory T and B cell when compared to healthy non-smokers, they had the highest serum PIGF concentration throughout pregnancy compared with all other groups. Smokers also had the lowest calculated sFlt-1:PIGF ratio compared. These results suggest a

potential role of cigarette smoke in preventing pre-eclampsia by lowering the levels of sFlt-1 and increasing availability of free PIGF. Smokers have been shown to have an increased expression of heme oxygenase 1 (HO-1) in the placental basal plate and a dose-depended increase in HO-1 expression in a placental cell line incubated with cigarette smoke (Sidle et al. 2007). It is possible inhaled cigarette smoke induces HO-1 which then inhibits secretion of sFlt-1 from placental endothelial cells and through this pathway reduces the chances of pre-eclampsia occurrence (Cudmore et al. 2007).

My study of pregnant women has identified several important immune and cardiovascular factors that precede, and appear to predict the onset of pre-eclampsia fetal growth restriction and pregnancy induced hypertension. Pregnant women who later developed PET, FGR or PIH had similarly low percentage of circulating regulatory T cells from the very early weeks of pregnancy. However, subjects who were affected by PIH or FGR had very different to PET profile of angiogenic factors. The values of sFlt:PIGF in PIH were over 50% lower than in pre-eclampsia whereas women with FGR the ratio was even 70% lower. This observation suggests that disruption to immune adaptation to pregnancy and imbalance between pro- and anti-angiogenic factors is required for a woman to develop pre-eclampsia. Interestingly, this changes were detectable as early as the first trimester for regulatory T cells and around 28 - 34 weeks for the sFlt:PIGF ratio.

My data showed that women who have low levels of T_{Reg} cells but normal profile of angiogenic factors do not develop pre-eclampsia, but isolated fetal growth restriction. The lack of endothelium disease seen by well balanced levels of sFlt-1 and PIGF suggests that despite the failure in immune adaptation to pregnancy seen as low T_{Reg} and B_{Reg} levels, these women did not develop pre-eclampsia. Additionally, women who were affected by PIH had similar to FGR profile of T_{Reg} cells and angiogenic factors. The only detected by me difference lied in elevated blood pressure readings from the beginning of the pregnancy in PIH group. According to my findings levels of regulatory T cells did not seem to influence the blood pressure readings at the end of PIH pregnancies as no association between these two variables was observed. However, there was an inverse correlation between mean arterial blood pressure in the first and

T_{Reg} cells in last suggesting that low T_{Reg} cells form early weeks of pregnancy may be a risk factor for PIH.

Findings from my work suggested that pregnant women should be monitored for their levels of circulating regulatory T cells and angiogenic factors from early week of pregnancy as they may help to identify patients at risk of developing pre-eclampsia or fetal growth restriction.

Only one study before mine assessed the frequency and function of B_{Reg} cells in human pregnancy women (Rolle et al. 2013). The authors found that levels as well as the immunosuppressive function of regulatory B cells are impaired in spontaneous abortion cases when compared to healthy pregnancy. What is more, they showed that numbers of $CD19^{+}CD24^{hi}CD27^{+}B_{Reg}$ cells during pregnancy rises above non-pregnant levels. I also saw decrease in levels or regulatory B cells in pregnancy complications such as re-eclampsia, fetal growth restriction or pregnancy induced hypertension. Interestingly, women who ultimately developed fetal growth restriction showed the lowest frequency of regulatory B cells. This was a surprising finding as pre-eclampsia is an extension to FGR therefore the lowest frequency of regulatory B cells should be seen in that group if B_{Reg} cells were involved in PET development. Further analysis into B_{Reg} cell showed that the frequency of B_{Reg} cells in association with a patient's clinical parameters appears to be completely random. Therefore, it could be suggested that although their function seems to be impaired in pre-eclampsia as shown by the lack of ability to suppress $CD4^{+}TNF\alpha^{+}$ and $CD4^{+}IFN\gamma^{+}T$ cells, their levels are not that reliable indicators of the disease progression.

Having completing my thesis, it is clear to me that pre-eclampsia is a multifaceted syndrome with multiple components that might add up through different pathways and mechanisms to the same syndrome in different women. Therefore, different women with pre-eclampsia or predisposition to pre-eclampsia may require different therapies to prevent or treat the condition.

According to my data assessment of regulatory T cells as well as sFlt-1 and PIGF may hold a promise of identifying at risk of future pre-eclampsia and help differentiate between women who are likely to develop pre-eclampsia or pregnancy induced

hypertension. When, sFlt-1 and PIGF may serve as good biomarkers for pre-eclampsia, regulatory T cells could additionally have a direct therapeutic application.

Preclinical studies outside of pregnancy have shown that freshly isolated or *ex vivo* expanded T_{Reg} cells can prevent both local and systemic organ and tissue destruction (Tang et al. 2004; Tarbell et al. 2007). Notably, *ex vivo* expansion of regulatory T cells from patients with SLE corrected their functional defect (Valencia et al. 2007) suggesting that *ex vivo* expanded, autologous T_{Reg} cells may have a beneficial effect in SLE patients.

No doubt more research into regulatory T cells involvement in healthy pregnancy and in pre-eclampsia is needed. Nonetheless, adoptive transfer T_{Reg} cells harvested from peripheral blood of women with low T_{Reg} cells level identified in early pregnancy, represents an exciting immunotherapeutic strategy for prevention of fetal growth restriction or hypertensive complications of pregnancy (June & Blazar 2006). Data from my and other studies suggests that regulatory T cells may play an important role not only during the implantation process but also in ensuring healthy pregnancy progress thought (Steinborn et al. 2008; Steinborn et al. 2012; Somerset et al. 2004). If that is really the case, then regulatory T cells could be used not only as a biomarker in pre-eclampsia prognosis but also as a treatment. Women with low levels of regulatory T cells at the beginning of their pregnancy could then have their T_{Reg} cells harvested, expanded *ex vivo* and transferred back as a treatment. This procedure would hopefuly prevent or ameliorate potential pregnancy complications.

The advantage that my research had over already published studies is that it includes data obtained prospectively form the same patient. What is more, the subjects were carefully divided into appropriate groups according to the outcome of their pregnancy.

Probably the biggest limitation to my study was a low number of subjects in many of the investigated groups. This is due to the same problem as seen every day in the pregnancy clinics - it is very difficult to identifying women at risk of pre-eclampsia in very early weeks of gestation.

Nevertheless, I hope that my observations have advanced our understanding of some of the pregnancy–related complications. I believe my work has shown that pre-eclampsia, pregnancy induced hypertension and fetal growth restriction are indeed very different conditions.

I showed that levels of regulatory T and B cells on smokers are no different to healthy pregnancy. This suggests that either the function of regulatory cells is greatly enhanced in smokers or the protective effect of smoking lies more on the side of angiogenic factors. I have not investigated the changes that occur at the site of maternal-fetal interface in smokers. Nevertheless, in order to get the whole picture, future studies should investigate the levels and function of decidual regulatory T cells. This may provide valuable information not only why pre-eclampsia develops but it can also shed light on a mechanism that protects smokers from developing pre-eclampsia.

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