INFECTION AND IMMUNITY IN PREGNANCY AND PREECLAMPSIA

by

FANG XIE
MD, QingDao University Medical College, 1997

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ABSTRACT

Preeclampsia is a hypertensive disorder of pregnancy, which shares similar risk factors with atherosclerosis. There is increasing evidence to suggest that *Chlamydia pneumoniae* (*C. pneumoniae*) and cytomegalovirus (CMV) are linked with atherosclerosis and may trigger inflammation during pregnancy. However, the roles of these two microbes and the underlying immune mechanism remain unclear. Case control studies were preformed to examine the evidence of *C. pneumoniae* and CMV infection, immune response and Toll like receptor (TLR) gene variations in women with preeclampsia compared with normotensive intrauterine growth restriction (nIUGR), and normal pregnancy and non-pregnancy controls. At each stage, a systematic review and data synthesis methodology are applied to place our findings within the context of published studies in the area of *C. pneumoniae* and CMV infection and TLR gene variations in preeclampsia to clarify apparent discrepancies in these studies.

In the first paper, an increase of *C. pneumoniae* genomic DNA loads was detected in preeclampsia when compared with normal pregnancy and non-pregnancy controls. Data synthesis indicated *C. pneumoniae* IgG seropositivity was more prevalent in preeclampsia patients. The second paper detected that preeclampsia had higher CMV IgG antibody level than normal pregnancies. Further, anti-CMV IgG seropositivity was more prevalent in women with preeclampsia than nIUGR and normal pregnancy controls. The third paper discussed up-regulated neutrophil TLR2 and TLR4 protein as well as mRNA expression in preeclampsia where the difference was more markedly in an early-onset preeclampsia condition. Early-onset preeclampsia was associated with elevated mRNA expression of cryopyrin, NF-κBp50, NF-κBp65 and IL-1β, as well as increased pro- versus anti-inflammatory cytokine ratios (TNF-α/IL-10 and IL-6/IL-10). Finally, in the fourth paper, data synthesis has suggested that gene variations in TLR2 or TLR4 were associated with early-onset preeclampsia.

In summary, our findings indicated that infection with *C. pneumoniae* and CMV may trigger the maternal inflammation crossing the threshold development of preeclampsia. A better understanding of immunology and genetic basis of preeclampsia may reveal therapeutic opportunities for treatment of this disorder and lead to improved health for both mother and fetus.

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ANOVA	Analysis of variance
ARDS	Acute respiratory distress syndrome
ATN	Acute tubular necrosis
AT1	Angiotensin II receptor type 1
AAV-2	Adeno-associated virus-2
BP	Blood pressure
CAD	Cardiovascular disease
CARD	Caspase-activating and recruitment domain
CI	Confidence intervals
CMV	Cytomegalovirus
Cp	*Chlamydia pneumoniae*
CRP	C- reactive protein
DIC	Disseminated intravascular coagulation
EB	Epstein-Barr
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EOPET	Early onset preeclampsia
FBS	Fetal bovine serum
G	Gram
GA	Gestational age
H	Hours
HELLP	Hemolysis, elevated liver enzymes and low platelets
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen system (HLA)
HSV	Herpes simplex virus
IKK	IκB kinase
IL	Interleukin
INF-γ	Interferon γ
IQR	Interquartile range
IUGR	Intrauterine growth restriction
KW	Kruskal-Wallis ANOVA
LBD	Ligand-binding domain
LOPET	Late onset preeclampsia
LPS	Lipopolysaccharide
LRP	Leukocyte-rich plasma
LRRs	Leucine-rich repeats
MAP	Mean arterial pressure
MAPKs	Mitogen-activated protein kinases
MESF	Molecular equivalents of soluble fluorochrome
M-H	Mantel-Haenszell
MIF	Microimmunofluorescence
MyD88	Myeloid differentiation primary response gene 88
nIUGR	Normotensive intrauterine growth restriction
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NLRs	NOD-like receptors

NK cells	Nature killer cells
Non-Prg	Non-pregnancy controls
NPC	Normal pregnancy controls
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PBLs	Peripheral blood leukocytes
PCR	Polymerase chain reaction
PET	Preeclampsia
PGs	Eicosanoids
PI	Pulsatility index
PRR	Pattern recognition receptors
PSA	Prostate-specific antigen
RANTES	Regulated on activation, normal T cell expressed and secreted
RICK	Receptor-interacting serine/threonine protein kinase 2
RIP	Receptor-interacting protein
ROS	Reactive oxygen species
RR	Risk ratio
RT-PCR	Reverse transcription polymerase chain reaction
S/D	Systolis/Diastolis
SGA	Small for gestational age
SNPs	Single nucleotide polymorphisms
TAK1	Transforming growth factor beta activated kinase-1
TGF-β	Transforming growth factor beta
TIR	Toll/Interleukin-1 Receptor
TLR	Toll like receptor
TNF- α	Tumor necrosis factor-alpha
TRAF	TNF receptor-associated factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRAM	TRIF-related adaptor molecule

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CO-AUTHORSHIP STATEMENT

The study was primarily designed, conducted and analyzed by Dr. Peter von Dadelszen and Fang Xie. I, Fang Xie, preformed the experiments as part of my PhD studies, under the direct supervision of Peter von Dadelszen. Fang Xie and Dr. Peter von Dadelszen were responsible for data analyses for Chapters 2~6. Yuxiang Hu supported experiment design for Chapters 2~5. Drs Laura Magee, Eva Thomas, and Deborah Money were involved with study design in Chapters 2, 3 and 4. Dr Stuart Turvey helped with the study design in Chapters 4, 5 and 6. Drs Peter Leung and Keith Choi provided lab facilities and space for carrying on this project. Clinical coordinators, Pamela Lutley, Celine Basque and Monica Pearson assisted recruiting study subjects. Dr. Peter von Dadelszen and I are primarily responsible for writing the manuscripts.

CHAPTER 1: Introduction

1.1 Preeclampsia

Preeclampsia remains a leading cause of maternal and fetal morbidity and mortality. It is characterized by heterogeneous clinical and laboratory findings. The clinical findings of preeclampsia can manifest as either a maternal syndrome (hypertension, proteinuria, or various symptoms), or a fetal syndrome (growth restriction), or both (1). Despite increasing progress towards understanding the cause of preeclampsia and its phenotypes, the etiology of this serious disorder remains elusive (1). Current theories include abnormal placentation, cardiovascular maladaptation to pregnancy, genetic and immune mechanisms, an enhanced systemic inflammatory response, and angiogenic factors (2) (3). It seems probable that multiple factors are involved. The diagnostic criteria for subtypes of preeclampsia have not been consistently used among the published studies. Therefore, there is an urgent need to improve in methods of prediction, markers for confirming the diagnosis in various subtypes, prevention, or management of this disorder.

1.1.1 Epidemiology of preeclampsia

Preeclampsia has a bimodal frequency, being more common in young primiparous and older multiparous women and the overall incidence of preeclampsia complicates about 3% of all pregnancies. It remains a major cause of maternal and perinatal mortality and morbidity, and is particularly devastating in developing countries (4) (5). Nevertheless, there is disparity in reported rates of preeclampsia across studies. It results at least partly from the inconsistency in diagnostic criteria of the disorder, and variation in reported rates also may be due to the geographic, social, economic and racial differences.

There are several risk factors identified for the development of preeclampsia, including socio-demographical factors (extremes of reproductive age, socio-economic status, ethnic group), pregnancy factors (primigravida and previous preeclampsia), genetic factors or personal medical history (obesity, chronic renal disease, chronic hypertension, diabetes mellitus, and thrombophilia).

1.1.2 Pathogenesis of preeclampsia

The origins of preeclampsia lie in incomplete modification of the uterine spiral arteries (uteroplacental arteries) by invading extravillous cytotrophoblast in the first and second trimesters of pregnancy. Linking the placental intervillous space and the maternal endothelium is a soup of plausible endothelial-activating cells, subcellular fragments, lipid peroxides, eicosanoids and cytokines ('intervillous soup', Figure 1.1). At present, it is unclear which component(s) of this soup is/are primarily responsible for the endothelial cell activation, and therefore both the characteristic maternal phenotype of preeclampsia and the classical endothelial lesions of acute atherosis and renal glomerular endotheliosis. The maternal phenotype is one of exaggerated maternal systemic inflammation.

1.1.3 Diagnosis of preeclampsia

Diagnostic criteria for preeclampsia include the new onset of elevated blood pressure and proteinuria after 20 weeks of gestation. Features such as edema and blood pressure elevation above the patient's baseline are no longer diagnostic criteria. Severe preeclampsia is indicated by more substantial blood pressure elevations and a greater degree of proteinuria. Other features of severe preeclampsia include oliguria, cerebral or visual disturbances, and pulmonary edema or cyanosis (Table 1) (6, 7).

Diagnosis becomes less difficult if physicians understand where preeclampsia "fits" into the hypertensive disorders of pregnancy, including chronic hypertension, preeclampsia -eclampsia, preeclampsia superimposed on chronic hypertension and gestational hypertension (Figure1.2) (7).

1.1.4 Sub-classification of preeclampsia

Preeclampsia has been further subdivided into early- and late- onset preeclampsia, which is a more contemporary concept (8). It has been suggested that early- (before 34^{+0} weeks) and late- (after 34^{+0} weeks) onset preeclampsia has different etiologies and, therefore, a different clinical expression, but this is still a subject of considerable research. There are, however, some basic differences between the two groups (9):

A. The late-onset type of preeclampsia comprises more than 80% of all preeclampsia cases worldwide. Most of these late-onset cases are associated with (9):

- A normally grown baby with no signs of any growth restriction;
- A normal or only slightly altered behavior of the uterine spiral arteries (no changes in the Doppler waveforms or slight increase of the pulsatility index [PI]);
- No changes in the blood flow velocity characteristics of the umbilical arteries;
- An increased risk for pregnant women displaying an enlarged placental mass or surface (diabetes, multiple pregnancies, anemia, high altitude).

B. The early-onset type of preeclampsia comprises a small subset of all preeclampsia cases (5% to 20%, depending on the study), but comprises the most severe cases of respective clinical relevance. The typical features of this type of preeclampsia can be summarized as follows (9):

- An inadequate and incomplete trophoblast invasion of maternal spiral arteries;
- Changes of the blood flow within the placental bed spiral arteries and thus in the uterine arteries (notches and other changes [increased PI] of the Doppler waveforms);
- An increased peripheral resistance of the placental vessels may be one cause of abnormal blood flow characteristics of the umbilical arteries (increased systolis/diastolis (S/D) ratio in still preserved flow or absent and even reversed end diastolic blood flow velocity in these arteries);
- Clear signs of a fetal growth restriction.

Gestational age at diagnosis is an important clinical variable predicting both maternal and perinatal outcomes. Early-onset preeclampsia represents considerable additional maternal risk, as maternal mortality is some 20-fold higher at less than 32 weeks' gestation than when preeclampsia occurs at term (10). In addition, data indicate that early-onset preeclampsia may be a qualitatively different disease. This is supported by observations that the pathophysiology of early-onset preeclampsia differs from late-onset disease, in terms of neutrophil function (11) and cytokine levels (11-13). Also, there is compelling epidemiologic evidence that early onset disease (especially onset earlier than 28 weeks) is associated with greater risk for recurrence in later pregnancies (14), and increased risk for later cardiovascular disease and death (15). Being delivered at less than

37 weeks' gestation in a preeclampsia pregnancy increases the hazard for death from cardiovascular disease by 7 to 8 fold (16) (17). Furthermore, the concurrence of intrauterine growth restriction (birthweight in the lowest quintile), preeclampsia, and preterm birth (< 37 weeks' gestation) confers an adjusted hazard ratio for cardiovascular death of 16.1 (95% confidence interval (CI) 3.6 - 72.6) (16) compared with normotensive pregnancies delivered of appropriately grown fetuses at term. Previous research findings also indicated that perinatal morbidity and mortality are gestational age-dependent that is a given among diploid fetuses (18, 19). A greater than 50% chance of intact survival for a fetus delivered of a woman with preeclampsia arises only when the gestational age at delivery is at or older than 27^{+0} weeks' and/or the birthweight equal to or higher than 600 g (11). Moreover, early-onset preeclampsia, rather than that arising at term (20), is an important predictor of intrauterine growth restriction (21). In fact, recent data suggest that intrauterine growth restriction is a function of preeclampsia arising before 37 weeks' gestation (21), and that there is an increase in large babies among women with preeclampsia delivering after 37 weeks' gestation (20).

Preeclampsia is a heterogeneous disorder, and as with other diseases (e.g., type I and type II diabetes), progress in the understanding of this disorder would be assisted greatly if subtypes could be characterized (8). Women with early-onset preeclampsia may provide the most homogeneous data for differentiating the changes of preeclampsia from those of normal pregnancy as investigators attempt to advance knowledge.

1.1.5 Intrauterine growth restriction and preeclampsia

Intrauterine growth restriction (IUGR) is defined as the "failure to reach ones genetic growth potential". Since it is not possible to accurately quantify a fetus' "genetic growth potential", some clinical measures can be quantified added such as abdominal circumference (AC) below the 10^{th} percentile adjusted birth weight centiles and intrauterine growth curves (21). At term, the cutoff birth weight for IUGR is 2,500 g (21). IUGR can occur in isolation ('normotensive IUGR'), which many believe is the fetal syndrome of preeclampsia in isolation (23).

As with early-onset preeclampsia, incomplete placental implantation is a feature of normotensive IUGR (nIUGR) (22). What is it that causes one woman to develop the

maternal syndrome of preeclampsia, while another has a pregnancy complicated solely by IUGR? This is not fully understood. In part, this may be determined by a differential neutrophil-mediated maternal inflammatory response which characterizes both normal pregnancy and pregnancy complicated by nIUGR, but is accentuated in preeclampsia (23). Neutrophil activation by infection may be involved in the development of acute atherosis, as it is in atherogenesis (24).

IUGR also shares with preeclampsia an association with adverse long term cardiovascular outcomes. Epidemiology findings have showed that low birth weight is associated with endothelial dysfunction in young adults and may be relevant to the pathogenesis of atherosclerosis in later life. Recently an investigation about "long-term mortality after preeclampsia" (25) suggested that preeclampsia is related to widespread activation of the maternal vascular endothelium and increased later cardiovascular risks.

1.1.6 Excessive systemic inflammation and risk of preeclampsia

A systemic inflammatory response differs from local inflammatory responses, which are confined to specific tissues. Rather, a systemic response is diffuse, involving all cells and protein systems within the blood and, by secondary extension, affecting inflammatory networks outside the circulation in target organs (26).

Normal pregnancy is characterized by a mild systemic inflammatory response (23, 27). In preeclampsia, a similar response occurs, but is of greater intensity (27). Many indicators of a systemic inflammatory response are changed in normal pregnancy, ranging from leukocytic inflammatory markers and activity, to inflammatory changes in cytokine levels or clotting function, and are even more marked in preeclampsia (23). These changes not only affect leukocytes, but additional components to varying degrees (Table 1.2) (26).

Inflammatory response was equated with systemic sepsis, which causes hypotension and shock (27). Pro-inflammatory stimuli can impair endothelium-dependent relaxation (28). The hypotension of septic shock represents the end-stage decompensation of vascular homeostasis, a concept supported by animal experiments. A preeclampsia rat model, consisting of a single administration of a very low dose of endotoxin to pregnant rats, causes hypertension and proteinuria that persist until the end of pregnancy. High dose endotoxin causes shock in this model. Non-pregnant animals are unaffected by a similar dose (29). Infusion of TNF-α also causes hypertension in pregnant, but not in non-pregnant

rats (30). In these experiments, systemic inflammation leads to hypertension and some of the features of preeclampsia. Despite the above considerations, there is only a very limited place for anti-inflammatory interventions in the management or prevention of preeclampsia. The effectiveness of high dose corticosteroids to attenuate the HELLP (haemolysis, elevated liver enzymes and low platelets) syndrome is currently under discussion (31).

1.2 Chronic infection in preeclampsia and atherosclerosis

1.2.1 Preeclampsia and atherosclerosis

Acute atherosis changes in spiral artery of preeclampsia, like atherosclerosis, is characterized by focal endothelial disruption, fibrinoid necrosis of the arterial wall, the infiltration of perivascular spaces by mononuclear cells, an accumulation of lipid-laden macrophages, and lipoprotein deposition. As in atherosclerosis, evidence of inflammation can be within the fibrinoid debris of acute atherosis. The affected arteries may become partially or completely obliterated. This further contributes to the relative uteroplacental insufficiency, production of additional placental ‘soup’ for export into the maternal circulation and ischemia-reperfusion injury, a potent source of further activated neutrophils, lipid peroxides, and cytokines (Figure 1.1).

Both acute atherosis and atherosclerosis occur in the presence of hyperlipidemia (especially the presence of oxidized lipoproteins and increased plasma cholesterol) and involve a role for monocytes and macrophages. Previous research has illustrated that much about what is known about the pathogenesis of atherosclerosis is consistent with acute atherosis in preeclampsia (26).

Similar to atherosclerosis, a personal history of preeclampsia is associated with increased risk for the subsequent development of chronic hypertension, and with excess cardiovascular morbidity. Conversely, normotensive pregnancies, especially in women over 25 years old, predict long-term normotension, with up to two-fold reduction in the later development of hypertension.

1.2.2 Infection and inflammation in atherogenesis

1.2.2.1 Atherosclerosis is an inflammatory disease

At present, atherosclerosis is commonly accepted as an inflammatory disease (32)**.** While the contribution of the inflammatory components is significant in plaque development, there is emerging evidence that various human infectious pathogens can also be involved in the processes of endothelial injury, leading to smooth muscle cell migration and proliferative responses. These events are also major mechanisms in the formation of pathogenic lesions. Two of these pathogens, *Chlamydia pneumoniae* (*C. pneumoniae*) and cytomegalovirus (CMV), have generated major clinical interest. When in combination with other cardiovascular risk factors such as lipidemia and hypertension, these infectious agents can stimulate coronary heart disease and ischemic stroke (32).

1.2.2.2 *Chlamydia pneumonia* and cytomegalovirus in atherosclerosis

C. pneumoniae, an intracellular bacterial pathogen, is known as a leading cause of human respiratory tract infections worldwide. Over the last decade, several reports in the literature have suggested that infection with *C. pneumoniae* may contribute to the pathogenesis of atherosclerosis. In order to play a causative role in chronic disease, *C. pneumoniae* would need to persist within infected tissue for extended periods of time, thereby stimulating a chronic inflammatory response. *C. pneumoniae* has been shown to disseminate systemically from the lungs through infected peripheral blood mononuclear cells and to localize in arteries where it may infect endothelial cells, vascular smooth muscle cells, monocytes/macrophages and promote inflammatory atherogenous process. The involvement of *C. pneumoniae* in atherosclerosis was investigated by seroepidemiological and pathological studies, in vivo and in vitro studies, and in clinical antibiotic treatment trials (33).

CMV is a member of the herpesvirus group. Its replication is slow, and the virus induces characteristic giant cells with intranuclear inclusions. CMV is dormant within an infected person's body for life and most CMV infections are considered asymptomatic. Epidemiological, anatomo-pathological and physiopathological evidence are in favor of a relationship between CMV infection and three forms of arterial disease including coronary disease of the transplanted heart, post-angioplasty restenosis and atherosclerosis (34). Studies suggested that CMV might enhance lesion formation in various ways, such as

augmentation of the oxLDL uptake, altering monocyte adhesion or increasing the production of pro-inflammatory cytokines.

Potential mechanisms in atherogenesis include direct local effects of infectious *C. pneumoniae* and CMV (Figure 1.3) or amplification of the systemic inflammatory response (Figure 1.4).

1.2.3 Evidence of infection in preeclampsia

Epidemiologic studies have shown that maternal infection and preeclampsia are connected. A recently published systemic review and meta-analysis has reported that urinary tract infection and periodontal disease were significantly associated with an increased risk of preeclampsia (35). A number of infectious agents have been evaluated. These have included *H. pylori* (36), adeno-associated virus-2 (AAV-2) (37, 38), HIV (39), malaria (40), *Mycoplasma hominis* (41), Epstein-Barr (42), and herpes simplex virus 2 (42). However, there are conflicting results and current research findings indicate that preeclampsia is related more closely to total "infectious burden", than to specific pathogens (35).

Three clinical trials evaluated the effect of antibiotic treatment for bacteriuria during pregnancy on the risk of preeclampsia. One randomized controlled trial noted that treatment with sulphamethoxydiazine or sulphadimidine, as compared to placebo, did not affect the risk of preeclampsia (43). Two nonrandomized clinical studies from Germany (44) and Croatia (45) reported that, compared to women with non-treated bacteriuria, antibiotic treatment for bacteriuria was associated with a statistically significant reduction in the incidence of preeclampsia. The current conflicting clinical trial data suggest that antibiotic treatment may not affect well developed atherosclerosis, but might work for the early stage of atherosclerosis.

Of potential importance in activation of the inflammatory response is lipopolysaccharide (LPS). As stated, low doses of LPS infused into pregnant rats produce pathologic changes similar to those observed in preeclampsia (46, 47). In addition, following the ligation of bacterial LPS, trophoblast secretion of chemokines is significantly increased; this in turn results in increased monocyte and neutrophil chemotaxis, which might contribute to the pathogenesis of preeclampsia (48). LPS leads to release of pro-inflammatory cytokines through activation of two major pattern recognition receptors

(PRRs) present on innate immune cells (macrophages, NK cells and dendritic cells), i.e. the extracellular Toll-like receptor 4 (TLR4), and the intracellular nucleotide oligomerization domain 2 (NOD2) protein. Upon activation, these receptors signal cells of the adaptive immune system (mainly T cells) via production of nuclear factor kappa B and release of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) to constitute an inflammatory response necessary for effective clearance of the harmful pathogen. These observations suggest that systemic pro-inflammatory mediators such as LPS may be pathogenically linked to the development and progression of preeclampsia.

Several mechanisms have been proposed to explain how maternal infection might be involved in the etiology of preeclampsia or its manifestations. These include direct effects of infectious agents on the arterial wall, including endothelial injury or dysfunction, acute atherosis, and local inflammation that might cause relative uteroplacental ischemia (23). Of interest in the latter respect is a recent report noting increased circulating maternal soluble fms-like tyrosine kinase-1 levels (an antiangiogenic factor associated with preeclampsia) in nulliparas with placental malaria and hypertension (49). Recently, Lamarca *et al* (50), utilizing a first trimester extravillous cytotrophoblast cell line, reported that human cytomegalovirus impairs placentation through inhibition of cytotrophoblast invasion. Most hypotheses, however, focus on indirect effects mediated by enhancing the maternal systemic inflammatory response (51) (23), similarities between acute atherosis in preeclampsia and atherosclerosis (52), the increased risk of remote cardiovascular disease after an episode of preeclampsia (53), and the modest association observed between coronary heart disease and chronic infections by bacteria and viruses (32).

1.2.4 *Chlamydia pneumoniae* and CMV in preeclampsia

Inspired by the increasing evidence that infectious agents with *C. pneumoniae* and CMV significantly contribute to clinical cardiovascular disease, researchers have started to examine serologic evidence linking *C. pneumoniae* and CMV antibodies to preeclampsia (54). Several studies detected increased *C. pneumoniae* antibodies (55-58) in preeclampsia; evidence came from enzyme linked immunosorbent assays (ELISA) and microimmunofluorescence studies (59, 60). Furthermore, a genetic study reported that the presence of cytomegalovirus increased the risk of developing preeclampsia in women carrying specific HLA-DRB1 alleles (61). Two studies reported that women with

preeclampsia had higher seroprevalence of IgG antibodies to *C. pneumoniae* than controls (59) (58). One study found that parous women with previous preeclampsia had higher levels of IgG, IgM, and IgA antibodies to *C. pneumoniae* than parous women with no previous preeclampsia (57). These results need further confirmation in larger population.

1.3 Infection and innate immune response

Although in past decades the placenta has been proven to play a central role in the development of preeclampsia, recent investigations have increasingly suggested that inflammation and immune mechanisms, activated by infectious pathogens may be important in the pathogenesis of preeclampsia. Now, exciting discoveries of innate immune receptors have rekindled intense interests in the relationship between mechanisms of immune defense and infection seen in preeclampsia.

1.3.1 Cellular receptor and pathogen identification

Innate immunity is the first line to identify pathogen invasion. Significant advances in our understanding of the innate immune recognition have been made in the last decade following the identification of pattern recognition receptors which include Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (62). TLRs are pattern recognition receptors that detect motifs or signatures from bacteria, viruses, protozoa and fungi. NLRs detect mainly intracellular bacterial and viral genome. These two families of pattern recognition receptors contribute to the role of defence that the host possesses against microbial pathogens (62).

1.3.1.1 Toll like receptor signaling

TLRs recognize conserved pathogen-associated molecular patterns (PAMPs) on microorganisms. Different TLRs recognize different classes of PAMPs (63). TLR1, 2, 4 and 6 recognize lipopolysaccaide. The major ligands for TLR2 include the following: specific cell-wall components of Gram-positive and Gram-negative bacteria; mycobacteria; fungi; parasites; and viruses. TLR4 can recognize specific components of Gram-negative bacteria lipopolysaccharide (LPS). In contrast to these extracellular-localized TLRs, the so-called antiviral TLRs (TLR3, 7, 8, 9) have their recognition domains in an endosome

within the cell (e.g. double-stranded viral RNA in the case of TLR3) (64). Another class of TLRs binds protein ligands; flagellin, for example, is detected by TLR5 (65).

Activation of TLRs causes an immediate defensive response, including the production of an array of pro-inflammatory cytokines and antimicrobial peptides. Accumulating evidence has shown that individual TLRs can activate overlapping as well as distinct signaling pathways, ultimately giving rise to distinct biological effects (66) (Figure 1.5).

1.3.1.2 Cryopyrin inflammasome

Although TLRs are an important system for microbial sensing, they are not the only PRRs with this function. Components of viruses or bacteria that penetrate into the cytoplasm are recognized by cytosolic receptors, through which they cause cytokine production and cell motivation (65, 67). These cytosolic receptors appear to be divided into two subgroups: the NLR family, which includes at least 23 members that are either NALPs (NACHT-, LRR- and pyrin-domain-containing proteins) or NOD receptors; and a family of receptors that have an RNA-helicase domain connected to two caspase-recruitment domains (CARDs). NALP3 (cryopyrin) have been shown to form the assembly of the inflammasome, a crucial part of the innate immune response and a cytosolic complex of proteins that activates caspase-1 to process the proinflammatory cytokines IL-1β and IL-18, with NALP1 and NALP2.

Cryopyrin, a member of the NLR family, serves as an activator in response to specific toxins, endogenous danger signals, or microbial pathogens (68). Independent of TLR signaling, cryopyrin has been shown to form the assembly of the inflammasome, a crucial part of the innate immune response and a cytosolic complex of proteins that activates caspase-1 to process the pro-inflammatory cytokine IL-1β (69).

1.3.2 TLR and cryopyrin activation of NF-κB pathway

Signaling pathways via TLRs and NODs culminate in the activation of NF-κB (nuclear factor of kappa light polypeptide gene enhancer in B-cells) and/or mitogen-activated protein kinases (MAPKs), which regulate the expression of numerous immune and inflammatory genes (65, 70). The NF-κB family consists of five members that can

exist as dimers and a heterodimer composed of RelA and p50 is considered to be the most frequently activated during PRR signaling. The transcriptional activation of NF-κB is controlled by multiple nuclear proteins. A member of evolutionally conserved NOD1 and NOD2 signaling also leads to NF-κB and MAPK activation. PAMP stimulation induces self-oligomerization of NOD1 and NOD2, allowing the recruitment of the caspase-activating and recruitment domain (CARD) containing serine/threoine kinase receptor-interacting protein (RIP)-like interacting caspase-like apoptosis regulatory protein kinase (RICK). RICK subsequently activates transforming growth factor beta activated kinase-1 (TAK1), which finally activates NF-κB and MAPK (71, 72).

1.4 Innate immunity and preeclampsia

The immune system is classified into innate immune system and adaptive immune system (Fig. 1.7). When some microbes invade the body, the innate immune system recognizes these pathogens via TLRs on neutrophils/monocytes/macrophages and NK cells, and then the innate immune system is activated (73, 74). In this process chemokines are produced, which are a chemoattractant for immune cells, resulting in the accumulation of neutrophils and monocytes. At the same time, inflammatory cytokines such as IL-1, IL-6 and TNF-α produced enhance the activity of neutrophils and monocytes (74).

1.4.1 TLR expression in preeclampsia

The theory that TLRs are linked to preeclampsia is supported by correlations seen in clinical studies. Mazouni *et al* reported that TLRs mediated the imbalance between an inflammatory and anti-inflammatory pattern of monocytes in patients with preeclampsia (73). Another study found that TLR4 protein expression is increased in interstitial trophoblasts of patients with preeclampsia (74). In addition, TLR-4 positive trophoblasts from preeclampsia patients were frequently immunoreactive to activated nuclear factor-kappaB, tumor necrosis factor-alpha, and M30 (a specific apoptosis antigen for trophoblast) (74). Kim *et al* have suggested that, in preeclampsia, "danger signals" at the feto-maternal interface, recognized by trophoblasts through TLR4, may play a key role in the creation of a local abnormal cytokine milieu (74). This indicates a novel mechanism that links preeclampsia to activation of the innate immune system through TLR-4 (74).

Preeclampsia is associated with circulating neutrophil activation (75, 76). Our recent data found that, as compared to normal pregnancy controls, preeclampsia was associated with elevated neutrophil TLR2 and 4 mRNA and protein expressions (77).

A wealth of evidence suggests that activated TLR signaling can affect preeclampsia in multiple ways. TLRs play a central role in determining the Th1/Th2 balance of immune responses. TLR activation promotes the generation of a Th1-dominated immune response, and inhibits Th2 cytokine production (78). Th1 response up-regulation occurs at the feto-maternal interface in preeclampsia, and it is closely related to poor placentation and endothelial dysfunction (74, 79).

Recently, a paper has shown that TLRs also play a role in the regulation of immune cell migration by first trimester trophoblast cells (48). Following the ligation of TLR3 by the viral ligand, poly(I:C), or TLR4 by bacterial LPS, trophoblast secretion of chemokines is significantly increased; this in turn results in elevated monocyte and neutrophil chemotaxis (48). In addition, TLR3 stimulation induces trophoblast cells to secrete RANTES (Regulated on Activation, Normal T Expressed and Secreted) (48). These results suggest a novel mechanism by which first trimester trophoblast cells may differentially modulate the maternal immune system both during normal pregnancy, and in the presence of an intrauterine infection (48). Some studies have indicated that, in first trimester trophoblast cells, the apoptotic pathway may be activated through a heterodimer of either TLR2/TLR6 (80) or TLR2/TLR1 (48, 81).

1.4.2 TLR polymorphisms and susceptibility to preeclampsia

The rapid recognition of pathogens by the innate immune system is mediated through TLRs and binding of a microbe to a TLR results in activation of several inflammatory pathways (82, 83). Up-regulation of, and signaling by TLRs may be one of the mechanisms by which intrauterine inflammation triggers both parturition and preeclampsia (83).

Recent studies have shown maternal TLR4 polymorphisms may increase susceptibility to early-onset preeclampsia and HELLP syndrome (84). Additionally, we have found the presence of TLR2 Arg753Gln and two TLR4 SNPs (Asp299Gly and Thr399Ile) was associated with early-onset preeclampsia as compared with normal pregnancy controls (85).

A concern in all these studies is the wide range of allele frequencies in control groups. Problems could have been raised by population and ethnic differences; these may have been the cause of many conflicting results. Therefore, more extended studies are needed to establish whether there is an association between TLR gene polymorphisms and the risk of preeclampsia, and conclusions need be guarded at this time (85).

1.5 Summary and research objectives

The origin of preeclampsia lies in a mismatch between fetoplacental demands and uteroplacental supply, a situation that also arises in normotensive intrauterine growth restriction (nIUGR) (23). Why is there a differential response to the same underlying pathology? This is still poorly understood. To our knowledge, the development of preeclampsia predicts later heart attack and stroke through atherosclerosis. Much evidence shows an epidemiological link between chronic infection with *C. pneumoniae* and CMV and atherosclerosis. In our previous study, we have found that antibodies against *C. pneumoniae* and CMV are increased in early-onset preeclampsia compared to normotensive IUGR (55). Could the subclinical infection trigger the differential maternal response between preeclampsia and normotensive IUGR (23)? It is novel and may also provide an etiological link between a woman's experience of preeclampsia during her reproductive years and the subsequent increased risk of atherosclerosis.

1.5.1 Hypotheses

Preeclampsia shares similar risk factors with atherosclorosis. Accumulating evidence suggests that infection with *C. pneumoniae* and CMV are linked with cardiovascular disease and trigger inflammation in pregnancy. Thus in this study, we hypothesized that:

1. Maternal infection with *C. pneumoniae* and CMV may be associated with increased risks for preeclampsia.

As we know, innate immune response is the first line of defence against infectious dieases. Studies have reported that recognition of *C. pneumoniae* depends on exogenous ligands through TLR2 and TLR4 (86, 87); human CMV activates inflammatory cytokines via TLR2 (88). Cryopyrin, a NOD leucine-rich repeat protein, can identify pathogen PGN-derived molecules and is implicated in the regulation of the innate immune defence and

cell death pathways (85). TLRs and cryopyrin appear to play important roles in innate immunity as sensors of bacterial and viral components. In this regard, we hypothesized that:

2. TLRs and the cryopyrin inflammasome might be a potential link between the innate immune response to chronic infection and the development of preeclampsia.

Additionally, genetic variation in the population results in some individuals having a "subtle" but specific deficiency in the innate immune response (89). Common polymorphisms in TLR2 and TLR4 impair TLR signaling and are associated with cardiovascular disease (90) and the serious variant of preeclampsia, HELLP syndrome (100). Therefore, we hypothesized that:

3. Gene polymorphisms in TLR2 and TLR4 may increase the susceptibility to the development of preeclampsia.

1.5.2 Sample size calculation

For the sample size calculation, we used the level of anti-*C. pneumoniae* and anti-CMV as the primary outcome in this study. The hypothesis arose from our preliminary data from our nested case-control serology study, in which we found that titres of IgG against *C. pneumoniae* were higher in EOPET. The mean difference in IgG between PET and normal pregnancy was 0.19U/ml, with a mean standard deviation of 0.18 for the normal pregnancy group. The sample size for this project was based on these differences. Thus, to show a difference in anti- *C. pneumoniae* IgG levels of 0.19U/ml, assuming an α of 0.01 for multiple comparisons and α of 0.05 for two group comparison, and β of 0.2, we would require 25 women per group of each case, using non-parametric tests. Over 6500 women delivered annually at Children and Women Hospital of BC, of whom at least 600 have preeclampsia (60 early-onset preeclampsia). Therefore, we anticipated being able to recruit the required sample size within a year. In our experience, at least 50% of eligible women give their consent for participation. We anticipated that this sample size will also permit a similar investigation of anti-CMV IgG, TLR, cryopyrin, NF-κB subunits and cytokines (although different analytical appraches were takenfor individual experiments [see relevant chapters]).

1.5.3 Systematic review and data synthesis

To address the apparent discrepancies between our findings and those of previous studies and/or existing meta-analyses or systematic reviews, we planned to include our primary data within the context of the published literature through the objective process of systematic review and data synthesis.

A systematic review is a literature review focused on a single question that tries to identify, appraise, select and synthesize all high quality research evidence relevant to that question. It is crucial to evidence-based medicine. High quality systematic reviews seek to identify relevant evidence, select studies or reports for inclusion, assess the quality of each study, synthesize the findings from individual studies or reports in an unbiased way, interpret the findings and present a balanced and impartial summary of the findings with due consideration of any flaws in the evidence (91).

For the data syntheses described in this thesis (which were necessarily abbreviated due to constraints of presenting both primary and synthesized data within the relevant manuscripts) we took a standard approach, described below.

We included only English language studies that had completed the process of peer-review (meeting abstracts were excluded). Relevant citations were extracted from PubMed and Embase, as well as searching for relevant prior systematic reviews (35), the bibliographies of previous unstructured reviews, as well as the references of all studies identified by the strategies above. Keywords and medical subject headings were combined to generate lists of studies. Common to all searches, the key words were "pregn*," "pregnancy complication," "preeclam*," pre-eclam*," "preeclampsia," "pre-eclampsia," "hypertension," "gestational hypertension," "hypertensive disorder of pregnancy," "HDP," "PIH," "growth restriction," "small for gestational," and "IUGR.". For studies relevant to *C. pneumoniae,* we searched for "Chlamydia", "Chlamydiae", and "Chlamyophila." In the case of CMV, we searched for "cytomegalo* and" "herpesvir*." For TLR polymorphisms, we searched for "toll," "TLR," "polymorph*," "single nucleotide," and "SNP." Searches were preformed by me and Dr. von Dadelszen.

For relevant systematic review and data synthesis presented in the thesis, we have added a table of included studies as an addendum to Chapters 2, 3, and 5, as these were not included in the accepted papers, all of which are published.

1.5.4 Objectives

Objective 1: In Chapter 2, to test the prevalence differences of maternal infection with *C. pneumoniae* between preeclampsia and normotensive controls;

1) *C. pneumoniae* serology and genomic DNA loads were examined by ELISA and real-time PCR in women with preeclampsia and normal pregnancy, and in non-pregnancy controls, and
2) Through a systematic review and data synthesis of our findings and the published data, we examined anti-*C.pneumoniae* IgG seropositivity in preeclampsia and normal pregnancies.

Objective 2: In Chapter 3, to evaluate a possible link between the incidence of preeclampsia and maternal infection with CMV;

1) CMV serology of anti-CMV IgG, IgM and IgA was detected by using ELISA among women with preeclampsia, normotensive IUGR and normal pregnancy controls, and
2) A systematic review and data synthesis were preformed to analyze CMV infection in women with preeclampsia compared with normal pregnancy controls by combining our research findings with current literature results.

Objective 3: In Chapter 4, to elucidate whether TLR2, TLR4 and cryopyrin are potential links between innate immunity and preeclampsia;

1) Protein and mRNA expressions of neutrophil TLR2 and TLR4 were tested by flow cytometry and real-time PCR in normal and preeclampsia-complicated pregnancies,
2) mRNA expression of neutrophil NF-κB subunits (NF-κBp50 and NF-κBp65), cryopyrin and IL-1beta were examined by real-time PCR, and
3) Serum inflammatory cytokine profiles were detected by using a multiplex immunoassay.

Objective 4: In Chapter 5, to investigate the TLRs gene polymorphisms in normal and preeclampsia-complicated pregnancies;

1) TLR2 (Arg753Gln) and TLR4 (Asp299Gly and Thr399Ile) genetic frequency was detected by using an allelic discrimination assay, and
2) A systematic review and data synthesis were preformed to evaluate TLR2 and TLR4 gene polymorphism frequencies between preeclampsia and normal pregnancy controls.

Objective 5: In Chapter 6, to review TLR signaling in the pathogenesis of preeclampsia;

1) The relationship between TLR signaling and innate immune response was summarized, and
2) Current research findings on TLR expression and gene polymorphisms in preeclampsia were reviewed.

1.5.5 Thesis overview

This is a manuscript-based thesis including five published papers. During the review process of our submitted manuscripts, reviewers suggested merging data between early- and late-onset preeclampsia, for example, to increase statistical power, and this led to deviations from the planned study design in presentation of *C. pneumoniae* and CMV infection in early-onset preeclampsia, late-onset preeclampsia, normotensive IUGR, matched normal pregnancy and non-pregnancy controls. A summary table of the data by the original study design is presented in Chapter 7. The remainder of this thesis describes in detail the preformed experiments to address these specific aims.

To examine the postulated relationship between *C. pneumoniae* and preeclampsia (23, 55), in **Chapter 2**, we examined evidence of *C. pneumoniae* infection in preeclampsia using ELISA and real-time polymerase chain reaction (PCR) assays. Increased in *C. pneumoniae* whole blood genomic DNA was found in women with preeclampsia compared with normal pregnancy ($p<0.05$) and non-pregnancy controls ($p<0.05$). Data synthesis revealed that anti- *C.pneumoniae* IgG seroprevalence was associated with preeclampsia risk (RR 1.20, 95%CI 1.05 -1.36). Therefore, we proceeded to examine the postulated CMV infection and preeclampsia relationship.

In **Chapter 3**, we investigated CMV infection in normal and preeclampsia complicated pregnancies. Our data has shown that preeclampsia was associated with elevated anti-CMV IgG seropositivity and antibody index compared with normal

pregnancies (53% versus 40%, p<0.01; 1.06 [0.09, 4.52] versus 0.27 [0.06, 2.88], p<0.0001). No difference was observed in terms of CMV seroprevalence and antibody index level between normotensive IUGR and normal pregnancy controls. Data synthesis indicated the CMV infection was more prevalent in women with preeclampsia than normal pregnancy controls (RR 1.5, 95%CI 1.2-1.9). A greater understanding of the role of CMV infection in preeclampsia will provide useful information for the development of vaccination targets for disease prevention.

Therefore, in response to the data derived in Chapter 2 and 3, we next examined relevant cellular surface and intracellular receptors to infectious agents including *C. pneumoniae* and CMV, as well as detected the downstream effectors of these receptors. In **Chapter 4,** we examined the role of innate immune response through immune receptors TLR2, TLR4 and cryopyrin, in the development of preeclampsia. We observed that early-onset preeclampsia had increased TLR2 and TLR4 mRNA and protein expression (mRNA expression: TLR2, p<0.0001, TLR4, p<0.0001; protein expression: TLR2, p<0.01; TLR4, p<0.05) as well as elevated cryopyrin mRNA expression (p<0.001) compared with normal pregnancies. In addition, compared with normotensive IUGR, early-onset preeclampsia was correlated with elevated TLR4 mRNA expression (p< 0.05). Moreover, early-onset preeclampsia was associated with higher pro- versus anti-inflammatory cytokine ratio of TNF-α/IL-10 (p< 0.01) and IL-6/IL-10 (p< 0.001) than normal pregnancies. Our data indicated that TLRs and cryopyrin might be a potential link between innate immunity and preeclampsia, and may trigger for the differential inflammatory response existing between early-onset preeclampsia and normotensive IUGR.

As a consequence of finding relationships between TLR2 and TLR4 mRNA and protein expression and preeclampsia (particularly that of early-onset), in **Chapter 5** we explored common "TLR2 and TLR4 gene polymorphisms and their relationship with the risk to the development of preeclampsia". Through synthesis of our results and the published data, TLR2 (Arg753Gln) and TLR4 (Asp299Gly and Thr399Ile) gene polymorphisms appeared to be associated with increased preeclampsia risk (TLR2: RR 1.56, 95% CI 1.09- 2.23; TLR4: RR 1.31, 95% CI 1.04-1.67). Our data further suggested the association stronger for early-onset preeclampsia in particular (TLR2: RR 2.43, 95% CI 1.24- 4.76; TLR4: RR 2.06 95% CI 1.16- 3.67). A definitive and fully powered cohort study is required in the future to confirm this association.

To pull together our own findings and to interpret there is light of the published literature, in **Chapter 6** we have reviewed the rapidly progress in literatures correlating TLR signalling and preeclampsia. It has improved our understanding of the interplay between pathogen invasion, innate immune mechanisms and preeclampsia.

Finally, **Chapter 7** summarizes our research findings described in this thesis and proposes direction for future hypothesis driven investigation.

Table 1.1 Diagnostic criteria for preeclampsia (6, 7)

Preeclampsia
Blood pressure: 140 mm Hg or higher systolic or 90 mm Hg or higher diastolic after 20 weeks of gestation in a woman with previously normal blood pressure. Proteinuria: 0.3 g or more of protein in a 24-hour urine collection (usually corresponds with 1+ or greater on a urine dipstick test)
Severe preeclampsia
Blood pressure: 160 mm Hg or higher systolic or 110 mm Hg or higher diastolic on two occasions at least six hours apart in a woman on bed rest. Proteinuria: 5 g or more of protein in a 24-hour urine collection or 3+ or greater on urine dipstick testing of two random urine samples collected at least four hours apart
Other features: oliguria (less than 500 mL of urine in 24 hours), cerebral or visual disturbances, pulmonary edema or cyanosis, epigastric or right upper quadrant pain, impaired liver function, thrombocytopenia, intrauterine growth restriction

Table 1.2 The systemic inflammatory network is stimulated in preeclampsia relative to normal pregnancy (26)

Inflammatory markers	References
General markers of inflammation [a]	
Leukocytosis	Teronne *et al.* (2000) (92)
Increased leukocyte activation	Sacks *et al.* (1998) (93)
Neutrophil activation	Von Dadelszen *et al* (1999) (94)
Complement activation [b]	Haeger *et al.* (1989) (95)
Activation of the clotting system	Perry and Martin (1992) (96)
Activation of platelets	Konijnborg *et al.* (1997) (97)
Markers of endothelial activation	Taylor *et al.* (1991) (98)
Markers of oxidative stress	Gratacos *et al.* (1998) (99)
Hypertriglyceridaemia	Hubel *et al.* (1998) (100)
Pro-inflammatory cytokines [a]	
Tumour necrosis factor-α	Vince et al. (1995) (13)
Interleukin- 6	Greer et al. (1994) (101)
Interleukin- 8 [b]	Stallmach et al. (1995) (102)

[a] Significant changes occur relative to normal pregnancy.

[b] Not all authors agree.

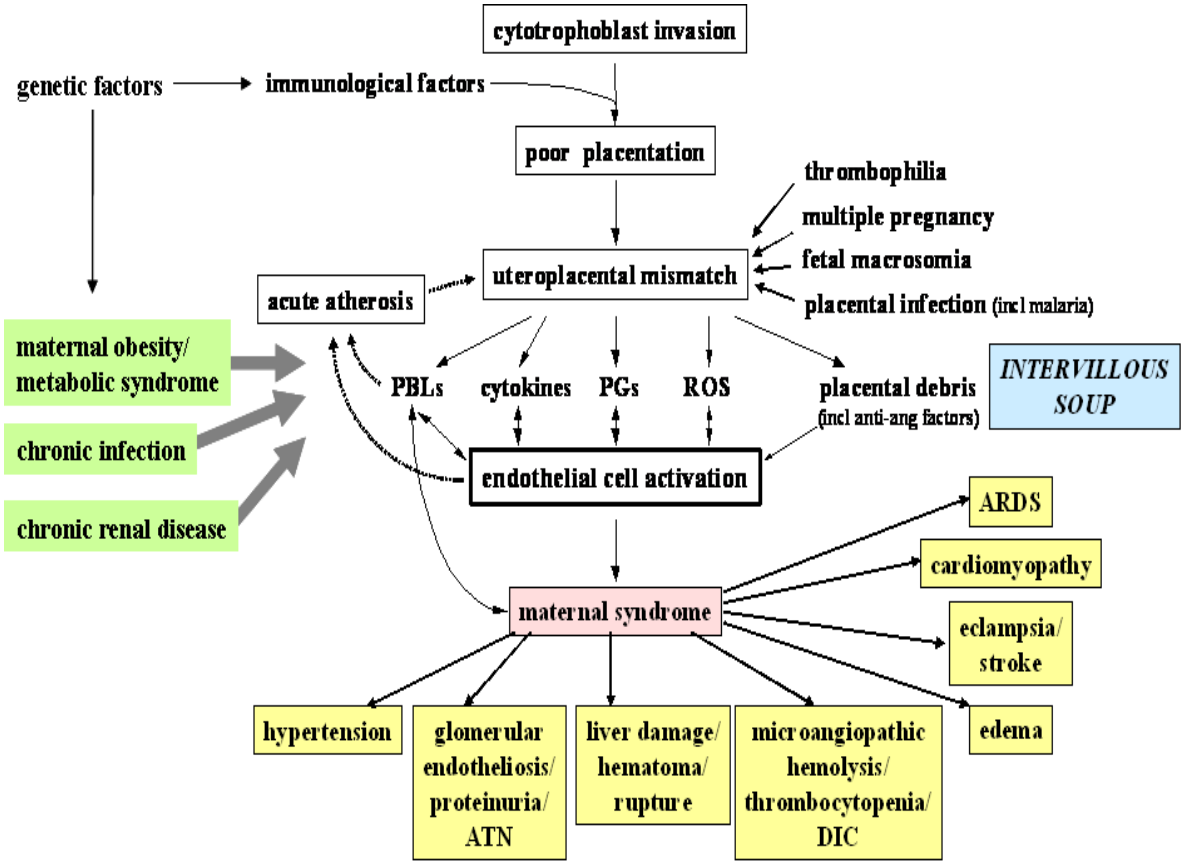

Figure 1.1 Pathogenesis of preeclampsia

In this model of preeclampsia, the maternal syndrome develops from a number of alternative pathways leading to uteroplacental mismatch, whereby the fetoplacental demands outstrip the maternal circulatory supply. In response to the mismatch, and probably due in part to recurrent ischaemia-reperfusion injury within the intervillous (maternal blood) space of the placenta and accelerated placental apoptosis, a soup of endothelium-damaging substrates is released with resulting endothelial cell activation and consequent development of the maternal syndrome of preeclampsia. Some elements of the soup, namely activated peripheral blood leukocytes, can cause direct end-organ damage. There is cross-talk between elements of the soup (modified from von Dadelszen et al 2002). **ARDS**: acute respiratory distress syndrome; **ATN**: acute tubular necrosis; **DIC**: disseminated intravascular coagulation; **PBLs**: peripheral blood leukocytes; **PGs**: eicosanoids; **ROS**: reactive oxygen species.

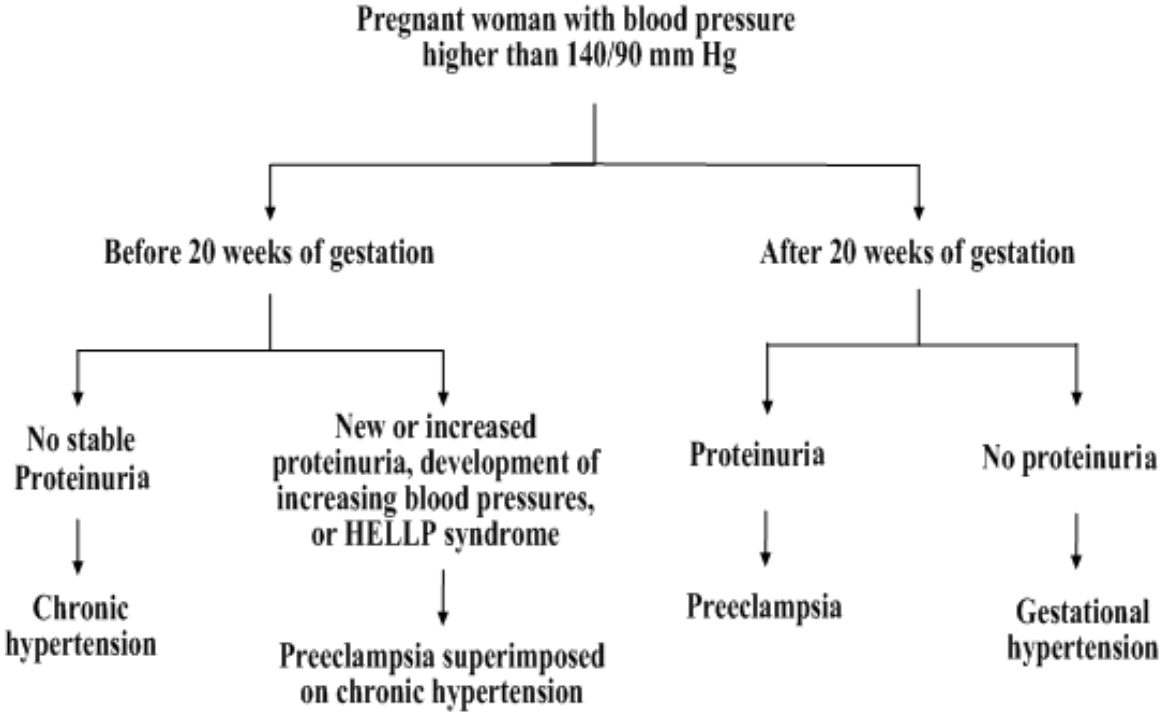

Figure 1.2 Preeclampsia as a hypertensive disorder of pregnancy (103)

Chronic hypertension is defined by elevated blood pressure that predated the pregnancy, is documented before 20 weeks of gestation, or is present 12 weeks after delivery (7). In contrast, preeclampsia-eclampsia is defined by elevated blood pressure and proteinuria that occur after 20 weeks of gestation. Eclampsia, a severe complication of preeclampsia, is the new onset of seizures in a woman with preeclampsia. Eclamptic seizures are relatively rare and occur in less than 1 percent of women with preeclampsia (104).

Preeclampsia superimposed on chronic hypertension is characterized by new-onset proteinuria (or by a sudden increase in the protein level if proteinuria already is present), an acute increase in the level of hypertension (assuming proteinuria already exists), or development of the HELLP (hemolysis, elevated liver enzymes, low platelet count) syndrome (6).

Gestational hypertension is diagnosed when elevated blood pressure without proteinuria develops after 20 weeks of gestation and blood pressure returns to normal within 12 weeks after delivery (4). One fourth of women with gestational hypertension develop proteinuria and thus progress to preeclampsia (105, 106).

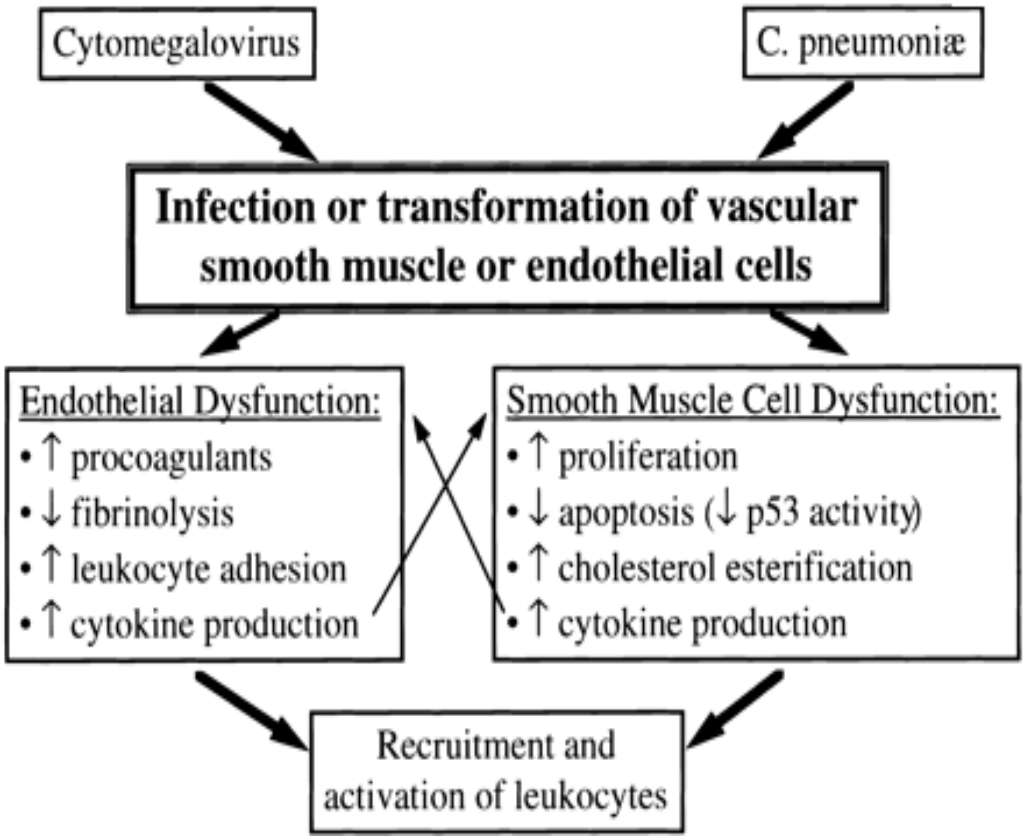

Figure 1.3 Direct effects of infectious agents with *C. pneumoniae* and CMV on intrinsic vascular wall cells

Microbial agents can infect and, in the case of viruses, transform vascular wall cells. Alternatively, the infected cells may survive but display deleterious functions such as those listed that could promote arterial lesion formation. Note potential cross talk between intrinsic vascular wall cells (endothelium and smooth muscle) and among vascular cells and leukocytes (107).

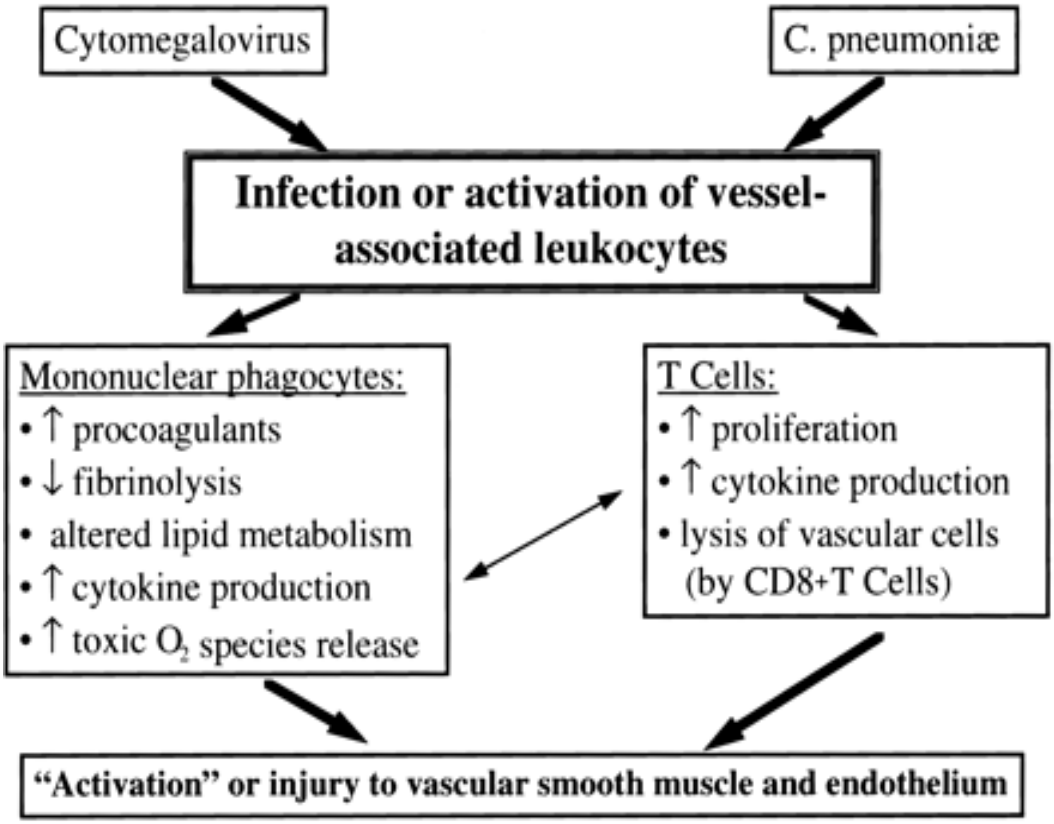

Figure 1.4 Indirect effects of infectious agents with *C. pneumoniae* and CMV on intrinsic vascular wall cells

Microbial agents may infect leukocytes, including macrophages and lymphocytes, already present in evolving atheroma. Infected leukocytes may be activated to express maladaptive functions such as those depicted, which may promote lesion evolution. Note potential cross talk between leukocytes and intrinsic endothelium and smooth muscle cells (107).

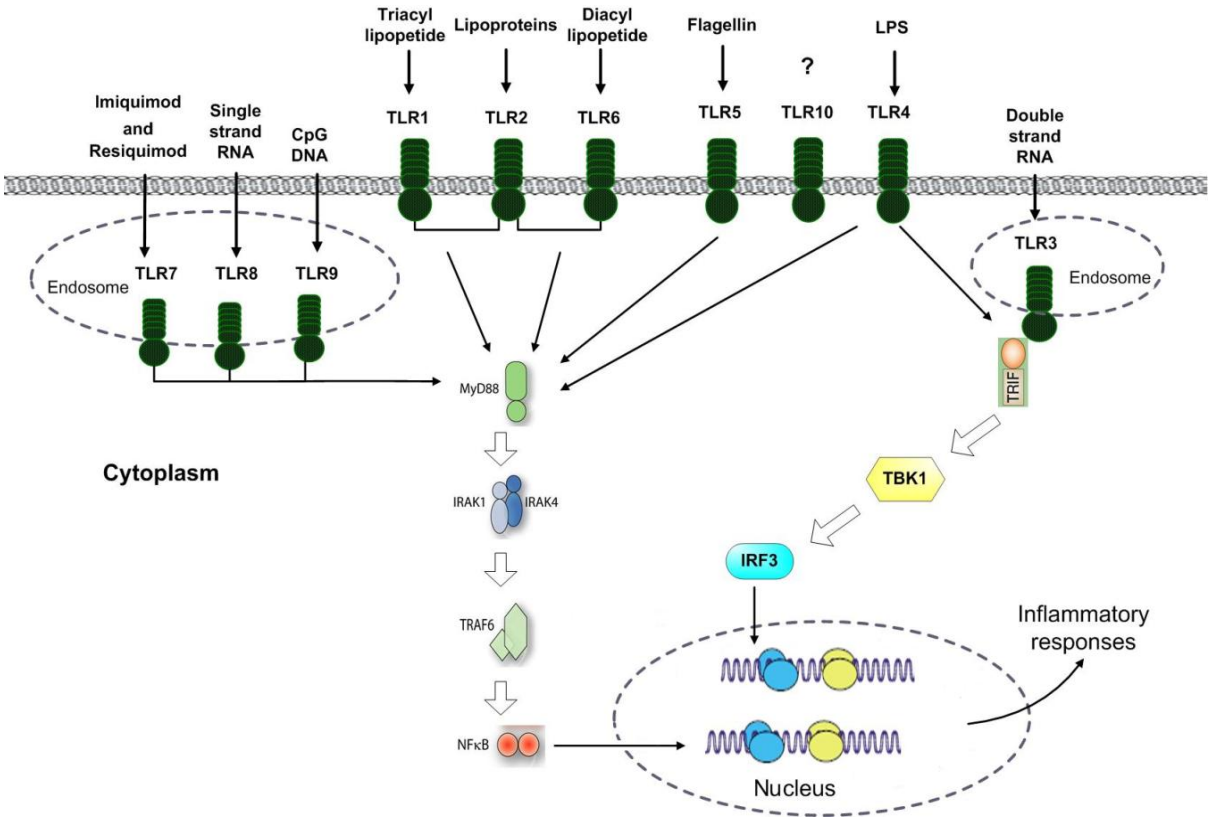

Figure 1.5 TLR signalling pathway

Each TLR activates a number of signaling pathways, some of which are common to all TLRs and some of which are specific to particular TLR types. This differential induction pattern is dependent largely on which cytoplasmic adaptor molecules are present to associate with the intracytoplasmic domain of the TLRs (108). These adaptors, which all contain Toll/Interleukin-1 Receptor (TIR) domains, include MyD88 (myeloid differentiation primary response protein -dependent and –independent pathways -such as TRIF (TIR-domain-containing adaptor inducing interferon β).

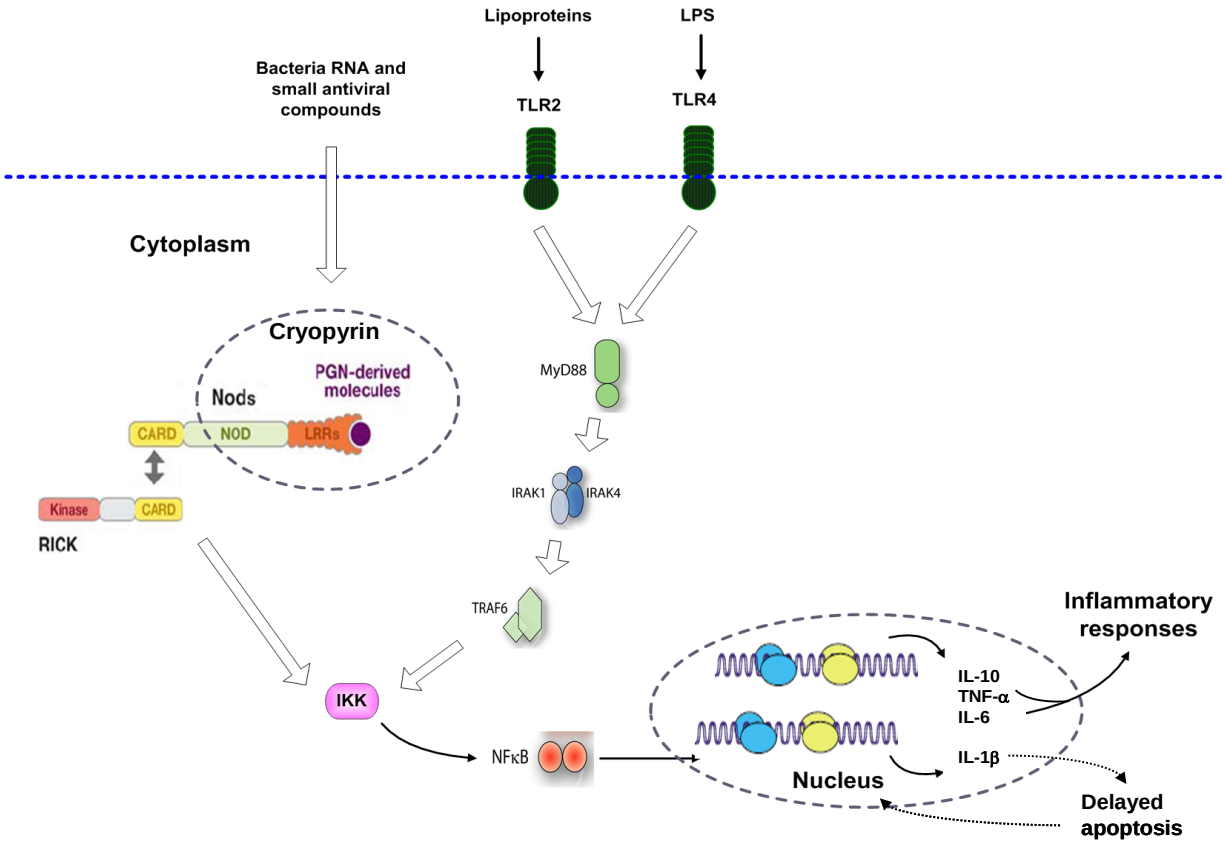

Figure 1.6 Activation of the NF-κB through TLR2, TLR4 and the cryopyrin inflammasome

The activation of either TLRs or the cryopyrin inflammasome by pathogens and/or other immune stimulants will result in downstream activation of NF-κB through IKK, with subsequent transcription of pro-inflammatory cytokines (such as TNF-α and IL-6), downregulation of anti-inflammatory cytokines (e.g., IL-10), and upregulation of the anti-apoptotic IL-1β. These changes are consistent with what is known of cytokine profiles and neutrophil apoptosis in women with preeclampsia. CARD: caspase-recruitment domain; IRAK: interleukin-1 receptor-associated kinase; IKK: IκB kinase; LPS: lipopolysaccharide; LRRs: leucine-rich repeats; MyD: myeloid differentiation primary response gene; NOD: nucleotide-binding oligomerisation domain; PGN: peptidoglycan; RICK: a CARD-containing protein kinase; TRAF: TNF receptor-associated factor

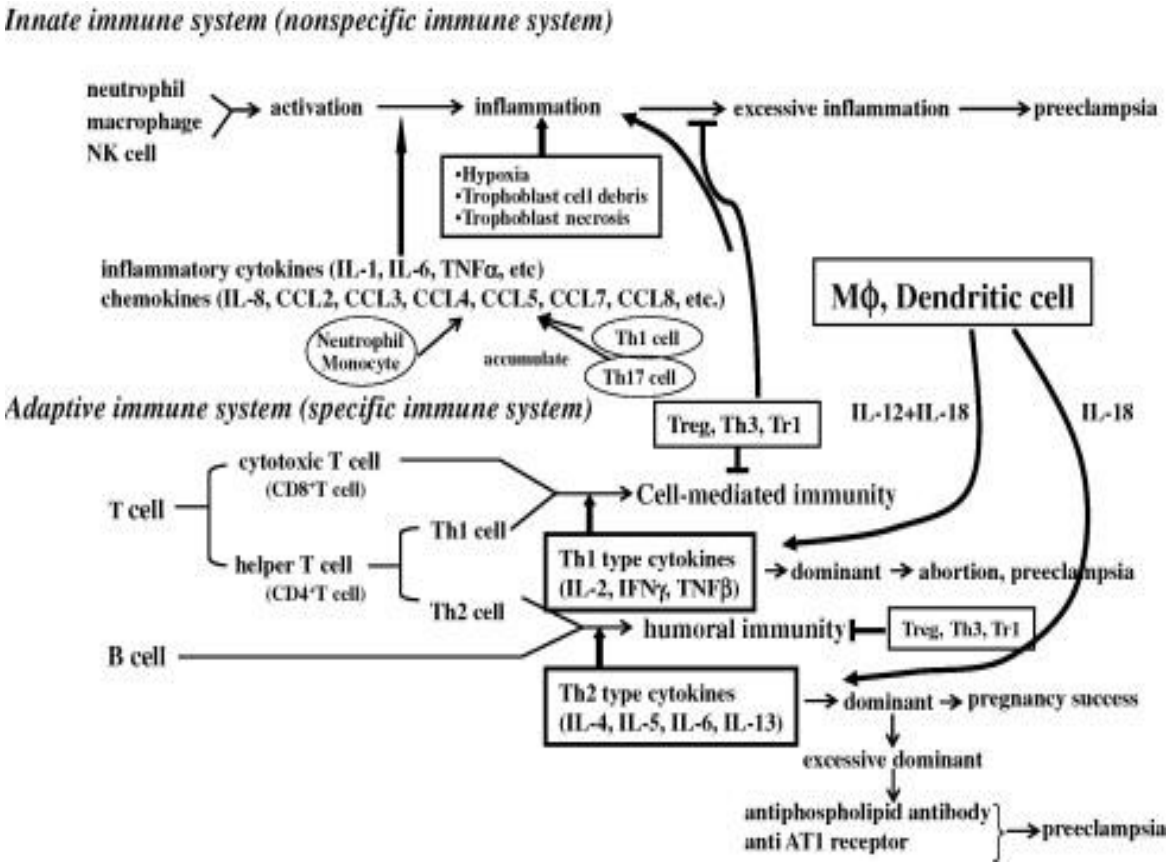

Figure 1.7 Innate immune system and adaptive immune system during pregnancy

Excessive activation of the innate immune system induces severe inflammation and also Th1 type immunity. These immune conditions may cause preeclampsia. Excessive Th2 type immunity may produce antiphospholipid antibodies or anti AT1 receptors resulting in preeclampsia. Dendritic cells take up these pathogens and present these antigens to T cells and B cells. Th2 cells and type 2 cytokines such as IL-4, IL-5, IL-6 and IL-13 play central roles in humoral immunity which is involved in antibody production. Furthermore, chemokines accumulate not only innate immune competent cells, but also adaptive immune competent cells.

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CHAPTER 2: *Chlamydia pneumoniae* Infection in preeclampsia[1]

2.1 Introduction

Preeclampsia is not only associated with increased maternal morbidity and mortality but also places the mother at increased risk for developing cardiovascular disease later in life (1). It is a multifactorial disorder and shares many risk factors and pathophysiologic features with atherosclerosis (2).

Increasing evidence has indicated that atherosclerosis may be associated with infection upon an intracellular organism, *Chlamydia pneumoniae* (*C. pneumonia*) (3, 4). Epidemiological studies have shown that increased *C. pneumoniae* antibodies in atherosclerosis (5, 6). Animal studies have demonstrated that *C. pneumoniae* exacerbates hypercholesterolemia-induced atherosclerosis with deficiency of LDL-receptor and apolipoprotein E (7, 8). The underlying mechanisms include direct effects of *C. pneumoniae* on the arterial wall as well as the indirect effects mediated by the augmentation of systemic inflammation (6). As there is a link between *C. pneumoniae* infection and atherosclerosis, could this association also exist in preeclampsia?

Previously, we observed an association between serum *C. pneumoniae* IgG levels in blood taken at the time of rubella serology (generally prior to 20 weeks of gestation) and the later development of early-onset preeclampsia (9). In this study, we tested the hypothesis that infection with *C. pneumoniae* may be a risk factor involved in the pathogenesis of the women after the clinical presentation of preeclampsia. To our knowledge, this is the initial study to examine both *C. pneumoniae* serology as well as whole blood genomic DNA (gDNA) copy numbers in women with preeclampsia.

2. 2 Methods

Ethics approval was granted from the University of British Columbia and the BC Women Hospital ethics and research boards. Informed consent was obtained to collect whole blood and clinical characteristics during specimen processing. Laboratory staff was blinded to the clinical characteristics during specimen processing. The presenting cases

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[1] "A version of this chapter has been published. **Xie F**, Hu Y, Magee LA, Money DM, Patrick DM, Brunham RM, Thomas E, von Dadelszen P. *Chlamydia pneumoniae* infection in preeclampsia. *Hypertension in Pregnancy* 2010;29(4):468-77."

were fifty women with preeclampsia. Women were diagnosed with preeclampsia using the criteria: blood pressure (BP) ≥ 140/90 mmHg on twice a day four hours apart and proteinuria > 300mg/24h, or ≥30mg protein/mmol creatinine, or > 1+ on catheterized urine specimen after twenty weeks of gestation. Two groups of controls consisted of 1) fifty-seven normal pregnant women matched for maternal age (± 5 years), gestational age at sampling (± 2 weeks), and parity (0, 1, ≥2) who went on to deliver at term (>37 weeks) following uncomplicated pregnancies. 2) 25 non-pregnant women aged between 20 to 40 years old, not using systemic hormonal contraception.

2.2.1 Clinical specimens

First, 16ml of venous blood was taken antenatally. Then, 200µl whole blood aliquots were saved for *C. pneumoniae* gDNA analysis. Serum was prepared by centrifugation. All specimens were frozen at -80℃ until analysis. Case-control pairs or trios were batch assayed to minimize any effect of inter-assay variability.

2.2.2 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was preformed according to the manufacturer's protocol to detect anti- *C. pneumoniae* IgG, IgM (Calbiotech, Sprint Valley, CA, USA) and anti- *C. pneumoniae* IgA (Calbiotech, Inc, Sprint Valley, CA). The results were expressed as Antibody Index as described by the kit's instruction.

2.2.3 Genomic DNA isolation

Genomic DNA extraction was from 200µl whole blood according to the manufacturer's instructions (DNA mini kit: Qiagen Inc., Canada). DNA was eluted in 60µl of AE buffer (elution buffer) and stored as small aliquots at -80℃ prior to analyses by PCR assays.

2.2.4 Quantitative TaqMan based real-time PCR

C. pneumoniae specific sequences were selected from VD4. VD4 is a highly conserved region of *ompA* gene encoding the major outer membrane protein (MOMP) of *C. pneumoniae* as previously described (10).

C. pneumoniae gDNA were amplified using highly specific quantitative TaqMan real-time PCR on a Sequence Detection System (ABI Prism 7300, Applied Biosystems). Amplification of 18s was preformed to examine gDNA degradation and PCR inhibition. The primer and probe sequences were as following (Table 2.1)

For *C. pneumoniae* gDNA detection, each PCR included serially diluted and purified organism-specific gDNA standards (Advanced Biotechnologies Inc.) and the tested samples. Tenfold serial dilutions ranging from 10 to 10,000 copies of purified *C. pneumoniae* gDNA were tested in triplicate and the C_T values were plotted against the copy numbers. Assay sensitivity was as low as 10 copies per PCR reaction, corresponding to 600 copies per ml. Tested gDNA loads were averaged from three replicates or counted as positive if one of the three wells was above the detection level. The DNA loads were expressed in Table 2 as the number of copies/ml of whole blood. An aliquot of 5μl of the extracted gDNA was added to 20μl of the reaction mixture containing 150nM each of *C. pneumoniae* forward and reverse primers, 150nM *C. pneumoniae* probe (final concentration), and 2 × TaqMan Universal PCR Master Mix (Applied Biosystems). The *C. pneumoniae* gDNA copy numbers were finally converted to copies per ml for data analysis and expression. The PCR conditions consisted of 1 cycle of 2 minutes at 50℃ and 1 cycle of 10 minutes at 95℃ followed by 45 cycles of 15s at 95℃ and 1 minute at 65℃.

2.2.5 Statistics

Data were analyzed using Prism 5.0 (GraphPad, San Diego, CA) software, with Fisher's exact and Chi-square tests for categorical variables, Mann-Whitney U tests for continuous variables, and Spearman's correlation, as appropriate. $P < 0.05$ was considered statistically significant.

The relative contribution of anti-*C. pneumoniae* IgG seroprevalence to the development of preeclampsia was estimated using Review Manager 5.0 (RevMan, Nordic Cochrane Centre, Copenhagen, The Cochrane Collaboration, 2008) and is presented as risk ratio (RR) with 95% confidence intervals (CI). The data synthesis was preformed by combining our data with current published reports (9, 11-15), respectively, using RevMan. The exposure (independent) variable was the prevalence of anti-*C. pneumoniae* IgG in included studies. The outcome (dependent) variable was the presence or absence of preeclampsia.

2.3 Results

2.3.1 Clinical characteristics

The clinical characteristics are shown in Table 2.2. Women with preeclampsia had higher mean artery blood pressure and delivered preterm and small-for-gestational age babies more frequently than the normal pregnancy controls. Women with preeclampsia had increased AST level (31.5 [20, 237]) and uric acid level (347 [249, 521]) compared with general population. It has been reported that AST normal range is 10~30 μM (http://www.nlm.nih.gov/medlineplus/ency/article/003472.htm) and serum uric acid normal range is 292 ± 58 μmol/L(http://en.wikipedia.org/wiki/Uric_acid).

2.3.2 Seropositivity, antibody indices, and whole blood gDNA for *C. pneumoniae*

Seroprevalence, antibody indices and gDNA loads for *C. pneumoniae* are shown in Table 2.3. The endogenous gene 18s was successfully amplified from all DNA samples, which indicated that nucleic acids extracted from whole EDTA blood were free of amplification inhibitors and no gDNA degradation. Women with preeclampsia had increased *C. pneumoniae* gDNA copy numbers compared with normal pregnancy (Mann-Whitney U test: $p<0.05$) and nonpregnancy controls (Mann-Whitney U test: $p<0.05$). No significant difference was observed in either seroprevalence or antibody indices of *C. pneumoniae* IgG, IgM, or IgA between study groups.

2.3.3 Correlation between genomic DNA loads and anti-*C. pneumoniae* IgG antibody indices

In women with measurable *C. pneumoniae* gDNA copy numbers, there was a correlation between copy numbers and anti-*C. pneumoniae* IgG antibody indices in individual women ($r^2 = 0.49$, Spearman's $p<0.0001$). Values for *C. pneumoniae* gDNA below the detectable threshold (600copies/ml) were randomly assigned values of 0 or 300 copy numbers/ml for calculation.

2.3.4 Data synthesis

Due to the imbalance in demographics between cases and controls in the study of Raynor *et al* (16), we excluded that study from the systemic review of anti-*C. pneuominae* IgG seroprevalence. The characteristics of studies included in this systemic review are

summarized in Table 2.4. For presentation of the data (Figure 2.1), we have divided the studies into those that presented data related to previous pregnancy history (11), IgG seroprevalence prior to 20 weeks' gestation (related to the later development of either early- or late-onset preeclampsia) (9, 12, 13), and IgG seroprevalence at the time of diagnosis of preeclampsia (11, 14, 15), including this study. The data synthesis reveals an association between anti-*C. pneumoniae* IgG seroprevalence and the risk for developing preeclampsia. This is most consistent for women with a prior history of preeclampsia, women destined to develop early-onset (but not so obviously late-onset) preeclampsia, and women with preeclampsia. There was no heterogeneity between studies.

2.4 Discussion

To our knowledge, this is the first study to report an association between *C. pneumoniae* gDNA copy numbers, anti- *C. pneumoniae* IgG seroprevalence and concentration, and the maternal syndrome of preeclampsia. It has been shown that detection of *C. pneumoniae* gDNA loads may be used as a helpful approach to diagnosis of *C. pneumoniae* infection in cardiovascular disease (17). Our data indicated that *C. pneumoniae* infection might be a risk factor involved in the pathogenesis of preeclampsia.

We recognize that *C. pneumoniae* gDNA copies were not detectable in some of women who developed preeclampsia. However, when gDNA copies were measurable, they were present at higher levels in women with preeclampsia and those levels correlated with anti-*C. pneumoniae* IgG concentrations. This is consistent with our premise that the maternal syndrome of preeclampsia is a 'multi-hit' phenomenon, whereby any individual woman will require sufficient stimuli (eg. either infectious agents and/or angiogenic factors) to trigger the maternal response characterized by endothelial cell activation and systemic innate inflammation. Therefore, the presence of a response to *C. pneumonaie* and/or specific IgG may be a stimulus that lowers the threshold for developing preeclampsia in some, but certainly not all, women.

It is possible that chronic infection may alter the intensity of the inflammatory response that has been associated with pregnancy and preeclampsia (18). *C. pneumoniae* modulates a cascade of events leading to endothelial activation (19, 20), inflammation (21) and platelet activation (22), which may be linked with preeclampsia. Studies found that *C. pneumoniae* results in procoagulant activation through altering the coagulation balance of

endothelial cells (20, 23). In addition, human monocytes infected with *C. pneumoniae* can produce cytokines as part of the cell-mediated immune response (21, 24). These cytokines include TNF-α, IL-6 and IL-1β, which have been reported raised in preeclampsia patients (24).

Three classes of serum antibodies, anti- *C. pneumoniae* IgG, IgM and IgA, were measured in the present study. Unlike our previous findings, we were unable to detect an association between *C. pneumoniae* antibodies and preeclampsia. In our earlier study, we found increased anti-*C. pneumoniae* IgG in women who were later to develop early-onset preeclampsia (9); the blood was drawn at the time of rubella testing in the late first and early-to-mid second trimester, prior to the onset of clinical disease. In the present study, we have examined *C. pneumoniae* IgG in women after the clinical presentation of preeclampsia. Similarly, Goulis *et al* found no difference in the antibody levels in women with normal, gestational hypertension and preeclampsia groups (11). Interestingly, their subgroup analysis of parous women has shown raised *C. pneumoniae* antibodies in the women with previous preeclampsia (11). These results may reflect that *C. pneumoniae* IgG is a first- or early mid-trimester marker, but for later risk lacks specificity. In addition, there is conflicting evidence on the association between *C. pneumoniae* seroprevalence and preeclampsia (9, 14, 16). In the data synthesis, we observed that the relationship between anti-*C. pneumoniae* IgG seroprevalence less than 20 weeks and later preeclampsia was more closely associated with early-, rather than late-onset disease, although the apparent heterogeneity between groups was not statistically significant. It is possible that the apparently stronger relationship between midtrimester anti-*C. pneumonaie* IgG and early-onset preeclampsia relates to a closer temporal relationship between the blood sampling and the onset of the clinical disease. An alternative explanation is that the origins of early- and late-onset preeclampsia vary, as we have postulated previously (25).

With respect to the data synthesis, we have observed a consistent relationship between anti-*C. pneumoniae* IgG seroprevalence and preeclampsia risk. However this relationship is not strong (risk ratio: 1.20), and systematic review is by its very nature retrospective and must be considered hypothesis generating. Some of the differences noted for *C. pneumoniae* serology in published studies may relate to the different ethnic structures in the study groups and confounding of the postulated relationship between *C. pneumoniae* infection and preeclampsia. Therefore, future studies are required that will

collect data on potential confounders such as ethnicity and country of birth. From the currently available data included in the systematic review, it is most probable that chronic infection with *C. pneumoniae* is a co-factor in the development of preeclampsia in a subset of women.

We recognize that others have observed an inconsistent relationship between single pathogen infection and preeclampsia (26). Recently, some studies have independently identified several candidate pathogens that may causally relate to preeclampsia (11, 14, 27). Instead of *C. pneumoniae* being the sole pathogen that may precipitate the response, multiple pathogens, and other constituents of the intervillous soup (28), may be involved and the risk of preeclampsia is to the aggregate pathogen load - the so-called 'pathogen burden'. Of note, increasing pathogen burden has been associated with increased C-reactive protein (CRP) levels (12). CRP is believed to reflect inflammation (29, 30); at least part of the association between pathogen burden and preeclampsia may be due to infection-induced inflammation. This role of infection-burden in preeclampsia needs to be studied further. Nevertheless, *C. pneumoniae*, or perhaps other intracellular pathogen activation, is a plausible candidate agent that may alter the risk of preeclampsia.

There are some limitations in this study. First, in our population, the sample size is relatively small. Future studies will be needed to address these issues in a larger cohort of patients. Secondly, we were not able to determine the temporal relationship between the infection and the onset of preeclampsia in the present study. We have generally found an interaction between gestational age and other biomarkers of preeclampsia (9, 31, 32), therefore we cannot exclude the gestational age effect. Finally, we did not perform serial examinations in women from the time of rubella serology until disease-onset and/or delivery, which may limit the interpretation of these findings.

In summary, our preliminary data has shown an association between preeclampsia, anti-*C. pneumoniae* IgG, and increased *C. pneumoniae* gDNA copy numbers in a subset of women. We suggested replicating these findings in a larger defined population, to determine whether intervention studies might be warranted.

Table 2.1 The sequences of *C. pneumoniae* VD4 primer pairs and probe used in real time-PCR

C. pneumonia	Sequence
Forward primer	5'-TCCGCATTGCTCAGCC -3'
Reverse primer	5'-AAACAATTTGCATGAAGTCTGAGAA-3'
Probe	VIC-TAAACTTAACTGCATGGAACCCTTCTTTACTAGG- TAMRA

Table 2.2 Maternal and perinatal clinical characteristics in women with normal and preeclampsia complicated pregnancies. Values are given as n(%) or median [range].

Characteristic	Preeclampsia (n=50)	Normal pregnancy (n=57)	P value
Maternal age (year)	34 [19, 43]	33 [24, 40]	0.38
Nulliparous	37 (74)	39 (68)	0.67
Gestational age at sampling (weeks)	34.4 [22.7, 40]	34.3 [21, 39.6]	0.97
Gestational age at delivery (weeks)	35.4 [26.3, 40.1]	39.6 [36.4, 41]	<0.0001
Mean artery blood pressure (mmHg)	123 [84, 139]	83 [80, 103]	<0.0001
Uric acid (μM)	347 [249, 521]	-	NA
Platelets ($x10^9$/L)	190.5 [77, 277]	204 [137, 269]	0.35
AST (μM)	31.5 [20, 237]	-	NA
Proteinuria (≥ '++')	26 (52)	0 (0)	<0.0001
Birth weight (g)	1840 [395, 4400]	3380 [2885, 4615]	<0.0001
SGA (<5^{th} centile)	19 (38)	0 (0)	<0.0001

SGA: Small for gestational age; the pregnancy was deemed to have achieved the outcome if any one fetus was born less than 5th percentile for gestational age and gender using multiethnic Canadian birth-weight charts.

Table 2.3 Anti- *C. pneumoniae* seroprevalence, antibody index and genomic DNA copy numbers in women with preeclampsia, normal pregnancy and non-pregnancy controls.

Variables	Preeclampsia (n=50)	Normal pregnancy (n=57)	Non-Pregnancy (n=25)
***Cp* seroprevalence (n (%))**			
anti- *Cp* IgG	32 (64)	32 (56)	19 (76)
anti- *Cp* IgM	1 (2)	0 (0)	0 (0)
anti- *Cp* IgA	38 (76)	36 (63)	15 (60)
***Cp* antibody index**			
anti- *Cp* IgG	0.67 [0.14, 2.17]	0.53 [0.13, 1.98]	0.60 [0.12, 1.58]
anti- *Cp* IgM	0.13 [0.01, 0.64]	0.13 [0.02, 0.38]	0.17 [0.01, 0.44]
anti- *Cp* IgA	1.85 [0.34, 4.51]	1.62 [0.43, 4.39]	1.57 [0.39, 3.54]
***Cp* gDNA measurable rate (n (%))**	13 (26)	13 (23)	5 (20)
***Cp* gDNA copy numbers/ml**	4500 [800, 36000]	2100 * [600, 6000]	800 * [600, 5400]

Cp- Chlamydia pneumonia Values are given as n (%) or median [range]. * $p<0.05$ (*Mann-Whitney U* test: versus preeclampsia).

Table 2.4: Characteristics of studies included in the systemic review

First Author, Year	Region, Country	Design	Participants	Adjust or matching	Moment of evaluation of exposure	Main findings
Prior to pregnancy						
Goulis, 2005(11)	London, United Kingdom	Cohort	37 women with previous PET / 37 controls	None	Prior pregnancy	Parous women with previous PET had higher level of IgG antibody to Cp than parous women with no previous PET.
< 20wk of pregnancy						
Karnen, 2008(12)	Oulu, Finland	Case-control	99 PET/ 1479 NPC	None	First trimester (mean, 10.4 wks of gestation)	No association
Teran, 2003(13)	Quito, Ecuador	Cohort	11 LOPET/ 73 Controls	Age	16 wks' gestation	No association
von Dadelszen, 2003(9)	Vancouver, Canada	Case-control	38 PET/ 113 NPC	Age, parity, body mass index	12-16 wks' gestation	No association between seroprevalence of IgG antibodies to Cp; EOPET had higher levels of Cp IgG than controls
> 20wk (Diagnosis of disease)						
Aral, 2006(15)	Turkey	Case-control	69 PET/ 47 without PET	None	At delivery	PET had higher seroprevalence Cp IgG than controls.
Goulis, 2005(11)	London, United Kingdom	Cohort	32 PET/ 22 controls	None	>20 wks of gestational age.	No association
Heine, 2003(14)	Pittsburgh, USA	Case-control	37 PET/ 37 without PET	Ethnicity	Admission for labor and delivery	PET had higher seroprevalence of IgG antibodies to Cp than controls.
Xie, (this study)	Vancouver, Canada	Case-control	50 PET/ 57 without PET	Age, parity, gestational age	>20 wks of gestational age.	No *Cp* IgG seroprevalence among groups. PET had higher *Cp* gDNA loads than normal pregnancy controls.

Note: *Cp*: *Chlamydia pneumonia*; PET: preeclampsia; EO: early-onset; LO: late-onset; NPC: normal pregnancy controls; wks: weeks; GA: gestational age

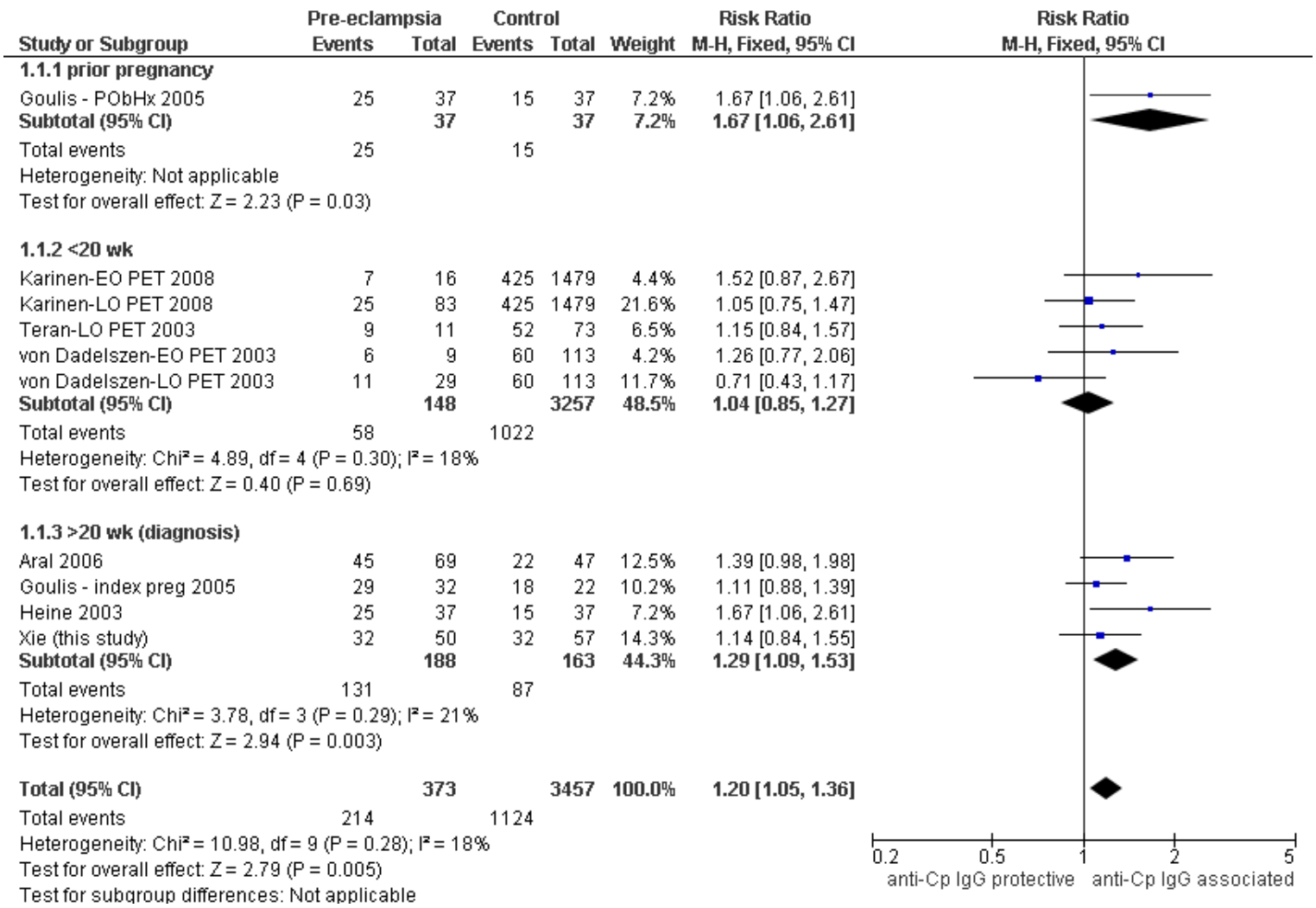

Figure 2.1 Data synthesis: the association between anti-*C. pneumoniae* IgG seroprevalence and the risk for developing preeclampsia

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CHAPTER 3: An association between cytomegalovirus infection and preeclampsia: a case-control study and data synthesis[1]

3.1 Introduction

Several parallels exist between preeclampsia and atherosclerosis. Factors that place women at increased risk for both diseases include obesity, insulin resistance, lipid abnormalities, inflammation and angiogenic factors (1-5). In addition, women who had preeclampsia more frequently develop cardiovascular disease later in life than those who remained normotensive (6, 7).

The origins of preeclampsia are heterogeneous (8) and appear to lie in a mismatch between fetoplacental demands and uteroplacental supply (9), a situation that can also arise in normotensive intrauterine growth restriction (IUGR) (4). When of early onset, preeclampsia is more frequently associated with IUGR (10) and increased risk of premature cardiovascular disease (11); whereas when appearing near term, there is a predominance of fetal macrosomia (10). The maternal physiology of normotensive IUGR and preeclampsia, particularly in early-onset preeclampsia, differ in terms of clinical phenotype, neutrophil activation (12), circulating placental debris (13), biochemical risk factors (14), putative infectious triggers (9, 15, 16), and neonatal outcomes (17). Why there are different responses to similar underlying pathology remains unclear. As increasing evidence indicates an association between preeclampsia and chronic infectious states (15, 18), chronic infection-associated inflammation may trigger the maternal response towards preeclampsia, rather than the stable maternal state of normotensive IUGR (19).

Cytomegalovirus (CMV), an intracellular pathogen, has been associated with cardiovascular disease (20) and gestational hypertension in previous epidemiological observations (9, 16, 21). Some studies reported that preeclampsia might be linked with increased CMV IgG seroprevalence and antibody level (9, 22), although a recent review did not confirm this relation (15).

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[1] "A version of this chapter has been published. **Xie F**, Hu Y, Magee LA, Money DM, Patrick DM, Thomas E, von Dadelszen P. An association between cytomegalovirus infection and preeclampsia: a case-control study and data synthesis. *Acta Obstetetrica et Gynecologica Scandanavica*. 2010;89(9):1162-7."

In this study, we sought to investigate whether, like atherosclerosis, there is a potential link between CMV infection and preeclampsia. A case-control study was preformed to examine CMV serology among women with preeclampsia, normotensive IUGR and normal pregnancies. Furthermore, given the uncertainty surrounding the relation between anti-CMV IgG seroprevalence and preeclampsia risk (15), we preformed systematic literature review and undertook data synthesis combining published findings (9, 16, 18, 22) and our results.

3.2 Methods

Ethics approval was provided by the University of British Columbia and the Children's and Women's Health Centre of British Columbia (C&W). Informed consent was obtained. The laboratory staff was blinded to the clinical characteristics of the women during the experiments.

Preeclampsia was defined as hypertension (systolic blood pressure (BP) ≥140 mmHg and/or diastolic BP ≥90 mmHg, taken twice a day more than 4 hours apart) after 20 weeks' gestation, and the presence of proteinuria (≥0.3 g/day, ≥30mg protein/mmol creatinine, or ≥2+ dipstick proteinuria). Blood pressures were taken in a semi-recumbent position, with arm supported and appropriately-sized cuff, using a manual mercury sphygmomanometer, with Korotkoff V used to determine diastolic BP.

Women with normotensive IUGR had singleton pregnancies and were identified by an antenatal ultrasound measurement of the fetal abdominal circumference below the 5^{th} percentile for gestational age (23), or birth weight below the 5^{th} percentile (24), or birth weight below the 10^{th} percentile (24) with either uterine artery notching at 22-24 weeks, antenatal oligohydramnios, and/or absent/reversed end diastolic flow on umbilical artery Doppler, excluding all cases of aneuploidy and congenital infections.

The subjects included preeclampsia (n=78), normotensive IUGR (n=30) and normal pregnancy controls (n=109) (at least one matched control for each case). Cases of preeclampsia or normotensive IUGR were first identified prospectively as they presented with clinical complications, and then the normal pregnancy women were recruited from the family practice maternity service of the hospital. The normal pregnancy controls were matched for maternal age (± 5 years), gestational age at sampling (± 2 weeks), and parity (0, 1, ≥2) who delivered healthy grown infants at term without any known medical

complications of pregnancy.

3.2.1 Clinical specimens

16ml of venous blood was taken antenatally. Serum was prepared by centrifugation and frozen at -80 ℃ until analysis. Case-control pairs were batch assayed to minimize any effect of inter-assay variability.

3.2.2 Detection of IgG and IgM antibodies against CMV

Enzyme-linked immunosorbent assays (ELISA) were preformed to detect anti-CMV IgG and IgM (Calbiotech, Sprint Valley, CA, USA) in maternal serum samples according to the manufacturers' instructions. Serum samples for IgG and IgM antibodies were screened directly against CMV. Briefly, we prepared a 1:21 dilution of tested serum samples, by adding 10 µl of the sample to 200 µl of sample diluents. 100 µl of diluted sera-calibrator and controls were added into wells and coated with purified CMV antigen (CMV IgG, IgM ELISA, Calbiotech Inc., Spring Valley, CA, USA). After the wells were washed, enzyme conjugate and substrate were added to bind antibody-antigen complexes. The plate was incubated to allow the hydrolysis of the substrate by the enzyme, 100 µl of stop solution was added and the optical density (OD) read at 450 nm using ELISA reader within 15 minutes. For the CMV IgG and IgM ELISAs, optical densities corresponded to IgG and IgM concentrations. Antibody indices were calculated by dividing the OD of each sample by a calibration factor. CMV IgG or IgM antibody indices greater than 1.10 were considered positive.

3.2.3 Detection of IgA antibody against CMV

Maternal serum samples for CMV IgA antibodies were measured directly against CMV in serum samples. Briefly, we prepared 1:40 dilution of tested serum samples by adding 5 µl of the sample to 200 µl of sample diluents. 100 µl of diluted sera, calibrator and controls were dispensed into wells coated with purified CMV antigen (CMV IgA ELISA, Diagnostic Automation Inc, CA, USA). After the wells were washed, enzyme conjugate and substrate were added to bind antibody-antigen complexes. The plate was incubated to allow the hydrolysis of the substrate by the enzyme. 100 µl of stop solution was added and finally O.D. read at 450 nm using ELISA reader within 15 minutes. For CMV IgA ELISA,

OD corresponded to IgA concentrations, and antibody indices were calculated by dividing the OD of each sample by a calibration factor. CMV IgA antibody indices greater than 1.0 were considered positive.

3.2.4 Statistical analysis

Data were analyzed using Prism 4.0 software (GraphPad, San Diego, CA, USA) comparing results among groups. For categorical variables, Fisher's exact and Chi-squared tests were used; and for continuous variables, Kruskal-Wallis ANOVA and Mann-Whitney U-tests were applied as appropriate. A p value <0.05 was considered significant for all tests, other than for Mann-Whitney U tests, where $p<0.01$ was used to adjust for multiple comparisons.

The relative contribution of anti-CMV IgG seroprevalence and/or positive CMV PCR to the development of preeclampsia was estimated using Review Manager 5.0 (RevMan, Nordic Cochrane Centre, Copenhagen, The Cochrane Collaboration, 2008) and presented as risk ratio (RR) (95% confidence interval (CI)]. Data synthesis was preformed by combining our data with published findings (9, 16, 18, 22, 25, 26) using RevMan. The exposure (independent) variable was the prevalence of anti-CMV IgG/ CMV gDNA positivity in the included studies. The outcome (dependent) variable was the presence or absence of preeclampsia.

3.3 Results

3.3.1 Clinical characteristics

The maternal clinical characteristics are shown in Table 3.1. Women with preeclampsia had higher mean artery pressure ($p< 0.0001$), lower platelets ($p< 0.0001$), more frequently delivered preterm birth ($p< 0.0001$) and low birth weight babies ($p< 0.0001$) than normal pregnant controls.

3.3.2 Cytomegalovirus serology

CMV seropositivity and antibody indices are shown in Table 3.2. We observed that CMV-IgG seropositivity was more prevalent in preeclampsia than in normotensive IUGR (53% (41/78) versus 40% (12/30), Chi-squared test: $p< 0.01$) and normal pregnancy controls (53% (41/78) versus 27% (29/109), Chi-squared test: $p<0.01$). In addition,

preeclampsia had higher anti-CMV IgG antibody index than normal pregnancy controls (median [range]: 1.06 (0.09, 4.52) versus 0.27 (0.06, 2.88), Mann-Whitney U test: $p<0.0001$). No difference was observed on CMV-IgG, IgM and IgA seroprevalence as well as antibody index level between normotensive IUGR and normal pregnancy controls.

3.3.3 Data synthesis

The characteristics of studies included in this systematic review were summarized in Table 3.3. For presentation of the data synthesis (Figure 3.1), we divided the studies into those with CMV-IgG seroprevalence tested prior to 20 weeks' gestation and later development of preeclampsia (9, 22), IgG seroprevalence at the time of diagnosis of the maternal syndrome of preeclampsia (18) including this study and CMV serology detection of babies delivered from women with preeclampsia (16, 26). The data synthesis revealed that CMV infection was more prevalent in women with preeclampsia than normal pregnancy controls (RR: 1.5; 95% CI: 1.2, 1.9). No heterogeneity was observed among study groups. The heterogeneity observed among the three groups (I^2 >50%) could be explained by the difference noted between women tested prior to the onset of clinical disease (sub-analysis 3.2.1 in Figure 1) and women and babies tested in association with clinical preeclampsia (sub-analysis 3.2.2 and 3.2.3). Excluding the first sub-analysis data results in homogeneous observation (I^2 =19%), whereby anti-CMV IgG seropositive women were more likely to develop preeclampsia (RR: 2.0; 95% CI: 1.6, 2.5).

3.4 Discussion

The results in our study suggest that CMV IgG seropositivity is more prevalent in women with preeclampsia than in normal pregnancy controls. This finding was supported by the systematic review limited to women after the clinical presentation of preeclampsia as well as to the babies delivered by women with preeclampsia (RR: 2.0, 95% CI: 1.6, 2.5)

To our knowledge, this is the first case-control study to examine three classes of CMV specific serum antibodies IgG, IgM and IgA among normal pregnancies, normotensive IUGR and women with preeclampsia. IgG indicates previous infection; anti-CMV IgM suggests acute or primary CMV infection; and anti-CMV IgA is associated with primary, chronic, or recurrent infections (27), but it is not a specific indicator for CMV infection. In chronic or recurrent infection, detectable IgM is uncommon, while IgG levels

may rise quickly (27). Previously, we observed increased anti-CMV IgG in women who later developed early-onset preeclampsia (9); the blood was drawn at the time of rubella testing in the late first and early-to-mid second trimester, prior to the onset of clinical disease. In this study, we have examined CMV antibodies in women after the clinical presentation of preeclampsia, and observed similar results.

Also, our findings suggested that CMV IgG seroprevalence was increased in preeclampsia compared with normotensive IUGR. No differences in CMV serology were observed between normotensive IUGR and normal pregnancy controls. This observation has expanded the investigation of the differential maternal response (8, 9, 12) observed in women with preeclampsia and normotensive IUGR who are believed to share the same intrauterine pathology, namely uteroplacental mismatch. It supports our previous hypothesis that CMV infection act as an immune stimulus that contributes to this differential response.

No infants exhibited clinical evidence of congenital CMV, which would be consistent with the observation that the placenta and membranes act as barriers to significant vertical transmission (28).

There are several mechanisms potential mechanisms by which CMV infection might contribute to the pathogenesis of preeclampsia. It has been reported that preeclampsia was associated with Toll-like Receptor (TLR) up-regulation (5, 29, 30), and CMV infection can activate inflammatory cytokine responses via CD14 and TLR2 (31). This binding may contribute to the stimulation of immune pro-inflammatory cytokines observed in preeclampsia (30, 32). Secondly, CMV can augment the expression of macrophage-colony-stimulating factor (33) (34) and result in pro-coagulant activation through altering the coagulation balance of endothelial cells (35, 36), all of which could trigger the maternal syndromes of preeclampsia. Thirdly, CMV infection, as well as other "infectious agents", may be involved in lowering the threshold level at which the clinical phenotype of preeclampsia is triggered (15, 19). However, the specific mechanisms by which CMV infection might contribute to triggering clinical preeclampsia remain to be further clarified.

There are some limitations to this study. Our sample size was still relatively small. In addition, all cases were identified in a single tertiary referral centre. The patient population served by our center consisted of three predominant ethnic groups, Caucasian,

East Asian, and South Asian and 40% of the adult population was born outside Canada. Thus, we were unable to determine if either ethnic background or country of birth might modify the relation between apparent CMV infection and preeclampsia risk. In addition, CMV avidity testing has not been included in this study, which is a useful method to distinguish CMV primary infection from non-primary infection. Adding this information in the future will be particularly important in guiding the treatment decision regarding to the application of antiviral therapy during pregnancy.

In summary, this study indicated that CMV infection may influence the occurrence of preeclampsia. Taken together with other risk conditions such as pre-pregnancy obesity and insulin resistance, CMV infection might act as a co-factor that lowers the threshold for developing this clinical disorder. To attain sufficient statistical power, our findings need to be confirmed in a larger, more representative obstetric population followed longitudinally.

Table 3.1: Maternal and perinatal clinical characteristics in women with preeclampsia, normotensive IUGR and normal pregnancy controls.

Characteristic	Preeclampsia (n=78)	normotensive IUGR (n=30)	Normal Pregnancy Control (n=109)	p value (Fisher's Exact or MWu)
Maternal age (years)	34 [21, 44]	34 [25, 42]	33 [24, 45]	0.47
Nulliparous	46 (59)	15 (50)	60 (52)	0.62
Gestational age at delivery (weeks)	34 [25, 41]	36 [25,39]	39 [36, 41]	<0.0001
Mean artery blood pressure (mmHg)	127 [110, 139]	95 [78,107]	85 [80, 92]	<0.0001
Uric acid (μM)	393 [249, 595]	271 [197, 343]	-	<0.001
AST (μM)	48.5 [20, 2475]	21 [18, 30]	-	<0.01
Proteinuria (≥ '++')	75 (96)	1 (3)	0 (0)	<0.0001
Platelets	159 [37, 277]	211 [125, 346]	236 [137, 272]	<0.0001
Birth weight (g)	1720 [395, 4400]	1733 [360,2645]	3481 [2730, 4615]	<0.0001

Note: GA, gestational age; MWu test: *Mann-Whitney U test*: versus preeclampsia; AST: aspartate transaminase. Values are given as n(%) or median [range].

Table 3.2: Anti-CMV seroprevalence and antibody index (n (%) or median [range])

Variable	Preeclampsia (n=78)	Normotensive IUGR (n=30)	Normal pregnancy controls (n=109)
CMV Seroprevalence			
IgG	41 (53)	12 (40) **	29 (27) **
IgM	8 (10)	2 (7)	7 (6)
IgA	22 (28)	4 (13)	21 (19)
CMV-antibody index			
IgG	1.06 [0.09, 4.52]	0.50 [0.03, 2.29]	0.27 [0.06, 2.88] ***
IgM	0.41 [0.09, 2.69]	0.25 [0.09, 0.57]	0.36 [0.10, 2.13]
IgA	0.51 [0.02, 4.70]	0.30 [0.03, 3.66]	0.24 [0.03, 3.32]

Note: CMV: cytomegalovirus; PET: preeclampsia. Using a case-control design, maternal serum samples were taken from preeclampsia, normotensive IUGR and normal pregnancy controls. CMV antibody indices were measured by enzyme-linked immunosorbent assay. Data were compared to calculate *Chi-squared* or *Mann-Whitney U* test.
** $p < 0.01$; *** $p < 0.001$ (*Chi-squared* or *Mann-Whitney U test*: versus preeclampsia).

Table 3.3: Characteristics of studies included in the systematic review according to CMV infection in preeclampsia

First Author, Year	Region, Country	Design	Participants	Adjust or matching	Moment of evaluation of exposure	Main findings
Prior to preeclampsia						
Trogstad, 2001(22)	Norway	Cohort	33 women with PET/ 945 NPC	Age, parity	10 wks' gestation	No association
von Dadelszen, 2003(9)	Vancouver, Canada	Case-control	38 women with PET/ 113 NPC	Age, parity, body mass index	12~ 16 wks' gestation	No association
Women with preeclampsia						
Carreiras, 2002(26)	Caracas, Venzuela	Case-control	26 women with PET/ 29 NPC	Ethnicity; No history of autoimmune disorder	At delivery	No association of HCMV infection between women with PET and controls; The presence of maternal alleles HLA-G*0104, DRB1*07/06 associated with PET risk.
Rustveld, 2004(25)	USA	Cohort study	48 PET/ 140 controls	Maternal age; parity Race; BMI; Smoking	3rd trimester	Association between primary CMV infection and PET
Xie, (this study)	Vancouver, Canada	Case-control	78 PET/ 109 NPC	Maternal age; parity	>20 wks; after clinic presentation of PET	PET had increased CMV IgG seropositivity compared with NPC.
Neonatal CMV DNA PCR + positive						
Carreiras, 2002(26)	Caracas, Venzuela	Case-control	25 neonates delivered from mother with PET/ 23 neonates controls	Ethnicity; No history of autoimmune disorder	After delivery;	The presence of HCMV sequences and fetal inheritance of maternal G*0104 associated with PET risk.
Gibson, 2008(16)	Caucasian, Australia	Case-control	23 neonates delivered from PIHDs/ 587 controls	Ethnicity	After delivery; newborn screening	Fetal exposure to CMV is associated with PIHDs.

Note: PET: preeclampsia; NPC: normal pregnancy controls; wks: weeks; hCMV: human cytomegalovirus; PIHDs: pregnancy induced hypertension disorders)

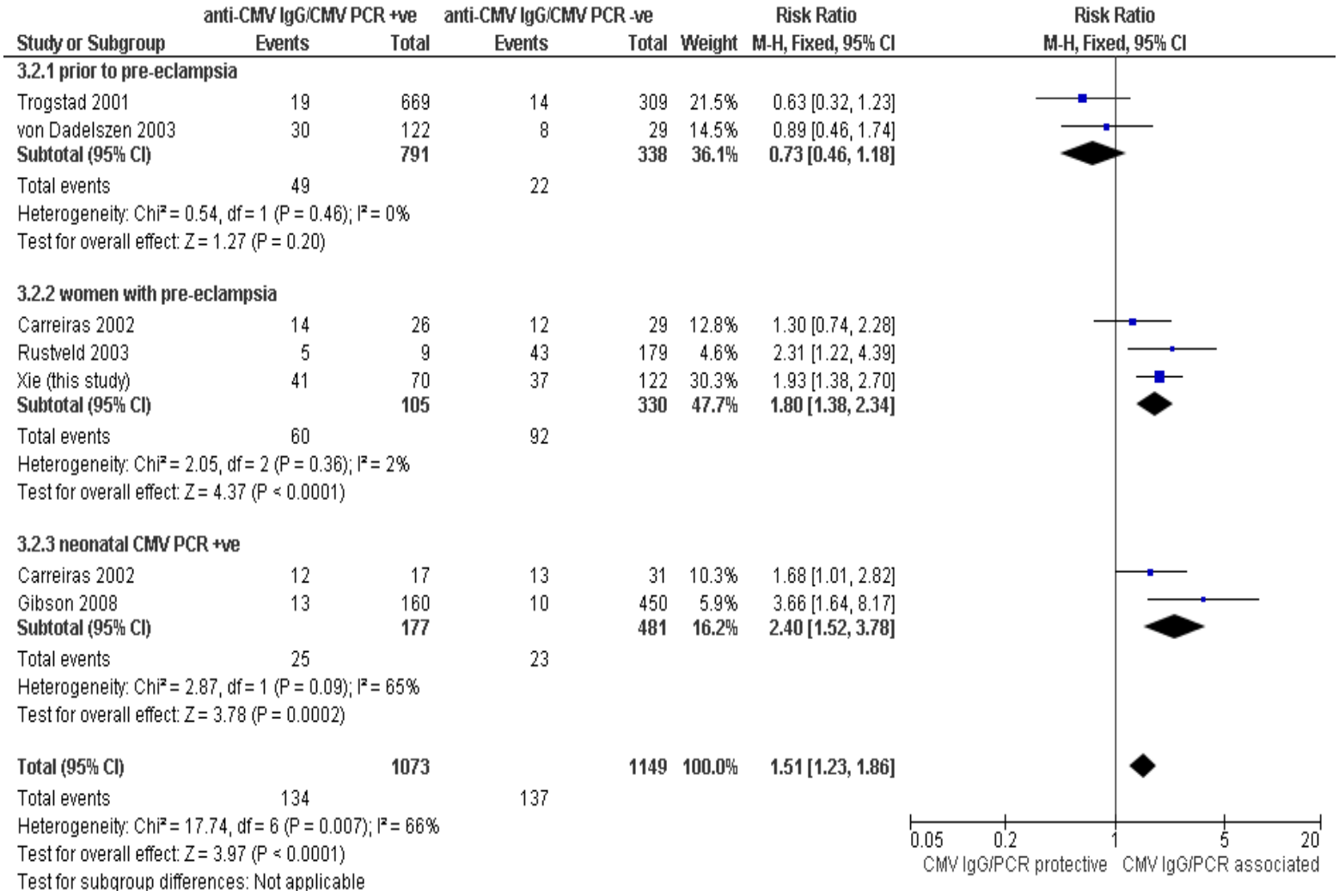

Figure 3.1 Data synthesis: the association between CMV infection and the risk for developing preeclampsia.

3.5 References

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CHAPTER 4: Toll-like receptors 2 and 4 and the cryopyrin inflammasome in normal pregnancy and preeclampsia[1]

4.1 Introduction

Preeclampsia is not only a major cause of maternal morbidity and mortality but also places the mother at increased risk for developing cardiovascular disease later in life (1, 2). It is a placenta-dependent disorder and characterised by proteinuric gestational hypertension after 20 weeks of gestation (3, 4). The activation of the innate immune response found in normal pregnancy is exaggerated in preeclampsia (5, 6).

The origins of preeclampsia are complex and appear to lie in a mismatch between fetoplacental demands and uteroplacental supply (7), a situation which also occurs in normotensive intrauterine growth restriction (IUGR) (8). Why there is a different maternal response to the similar underlying pathology remains unclear. When preeclampsia occurs early in pregnancy, it is particularly dangerous and more frequently associated with IUGR (9); whereas, at term, there is a predominance of fetal macrosomia (9).

Increasing evidence suggests that the inflammatory system plays a pivotal role in the pathogenesis of preeclampsia and intrauterine growth restriction (5) (10, 11). Inappropriate inflammatory patterns have been associated with abnormal trophoblast invasion (12), endothelial damage (11), and renal dysfunction (13). Endotoxin or lipopolysaccaride (LPS)-mediated inflammation is believed to be of critical importance in systemic infectious and non-infectious disorders (14, 15). In an animal model, Fass *et al* demonstrated that infusion of low-dose of LPS induces a preeclampsia-like syndrome, including hypertension, proteinuria and glomerular endotheliosis (16). LPS leads to release of pro-inflammatory cytokines through activation of two major pattern recognition receptors (PRRs) present on innate immune cells, including the extra-cellular Toll-like receptor (TLR)4 and TLR2 (17), and the intracellular cryopyrin, a nucleotide-binding oligomerisation domain (NOD) protein, also known as the caspase-activating recruitment domain. Cryopyrin modulates LPS-induced caspase-1 activation and IL-1β secretion (18).

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[1] "A version of this chapter has been published. **Xie F**, Hu Y, Turvey SE, Magee LA, Brunham RM, Choi KC, Krajden M, Leung PCK, Money DM, Patrick DM, Thomas E, von Dadelszen P. *BJOG*. 2010;117: 99-108."

The induction of signal transduction pathways is necessary for the activation of the innate immune response in preeclampsia (21). One of the most likely pathways is the NF-κB pathway. NF-κB exists as a heterodimer composed of p50 and p65 subunits bound to IκB in the unstimulated state. Upon activation, IκB is phosphorylated and degraded causing the release of p50/p65 components of NF-κB (22). The active p50/p65 heterodimer translocates to the nucleus and initiates the transcription of genes involved in the regulation of leukocyte responses, such as pro-inflammatory cytokines and cell surface adhesion molecules (23). However, the expression of NF- κB subunits in preeclampsia has not been previously investigated.

In the present study, we hypothesized that up-regulation of TLRs and cryopyrin in neutrophils would relate to systemic inflammation in women with preeclampsia. A case-control study was designed to examine the TLR2, TLR4 and cryopyrin expression, to assess transduction nuclear factor (NF)-κB subunit changes, and to detect inflammatory cytokine profiles (IL-6, TNF-a, IFN-γ, IL-10 and IL-2) among women with early-onset preeclampsia, late-onset preeclampsia, normotensive IUGR, normal pregnancy and non-pregnancy controls.

4.2 Methods

In this case-control study, we collected blood from women following informed consent. Samples were collected between September 2004 and March 2008. Ethics approval was granted by the University of British Columbia and the Children's and Women's Health Centre of British Columbia (C&W). Research laboratory staff was blinded to the clinical characteristics of the cases and controls.

Preeclampsia was defined as hypertension, blood pressure (BP) ≥140/90 mmHg, taken twice a day more than 4 hours apart and proteinuria (≥0.3 g/day, ≥2+ dipstick reading for proteinuria, or ≥30mg protein/mmol creatinine) after 20 weeks of gestation (24). Blood pressures were taken in a semi-recumbent position, with a supported arm and appropriately-sized cuff, using a manual mercury sphygmomanometer, with Korotkoff V used to determine diastolic BP (5).

Cases were (i) 25 women with early-onset preeclampsia (clinical onset $<34^{+0}$ weeks' gestation), (ii) 25 women with late-onset preeclampsia (same criteria, clinical onset $\geq 34^{+0}$ weeks' gestation), and (iii) 25 women with normotensive IUGR (identified by an

antenatal ultrasound measurement of the fetal abdominal circumference < 5^{th} centile for gestational age and gender).

The controls were composed of 75 women who ultimately delivered after normal pregnancies at term (>37 weeks without documented concerns about hypertension, proteinuria, gestational diabetes, or IUGR during their pregnancy) and 25 non-pregnant women. Normal pregnancy controls were matched for maternal age (± 5 years), gestation age (± 2 weeks), and parity (0, 1, ≥2). There are 25 matched women with normal pregnancies for women with each of (i) early onset preeclampsia, (ii) late onset preeclampsia and (iii) normotensive IUGR (i.e., 25 per group). All pregnancies were singleton gestations. Non-pregnancy controls were women aged between 20 to 40 years old, not using systemic hormonal contraception. Many of the observations in this present study had not been examined previously in either normal or complicated pregnancies. The effects of pregnancy *per se* on these aspects of innate immune activation were considered important (25).

4.2.1 Clinical specimens

Venous blood was taken antenatally. 200 μL of whole blood was used for genomic DNA (gDNA) extraction. Serum was prepared by centrifugation and secimens were frozen at -80 ℃ until analysis. Case-control pairs were batch assayed to minimize any effect of inter-assay variability.

4.2.2 Neutrophil isolation

In brief, 25ml blood was transferred to 50ml polypropylene tubes (BD Falcon) with Ca^{2+} and Mg^{2+} free Dulbecco PBS (Gibco, Grand Island, NY; total volume 40ml). 6% Dextran sedimentation was preformed for 30 minutes, and the resulting leukocyte-rich plasma (LRP) transferred into a new Falcon 50ml tube for centrifugation (Eppendorf Centrifuge 5810R, Swing-Bucker Rotor, Hamburg of Germany), re-suspension in 20ml of

PBS layered onto 10ml of Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden), and differential centrifugation (700 × g, room temperature, 20min, with brake). The resulting neutrophil-enriched cell pellet was washed twice in PBS, and red cell lysis preformed in 5ml of 1 × RBC Lysis Buffer (0.155M Ammonium Chloride, 0.01M Potassium Bicarbonate and 0.1M EDTA) for 5 minutes prior to flooding with PBS. Two further washes in PBS were preformed and the number of cells counted using a heamacytometer.

4.2.3 RNA preparation and synthesis of first-strand cDNA

Neutrophil RNA was extracted with an RNeasy minikit (Qiagen) within 60 minutes of phelbotomy. Intact total RNA extracts (0.5-5μg) were reverse transcribed into cDNA according to the manufacturer's instructions (ThermoScript RT-PCR System, Invitrogen, Carlsbad, CA, USA).

4.2.4 Relative quantitative SYBR Green real-time polymerase chain reaction (PCR)

The sequences of the primer pairs for TLR2, TLR4, cryopyrin, IL-1β, NFκBp50, and NFκBp65 are listed in Table 4.1. The mRNA expressions were detected by quantitative SYBR Green real-time PCR on a Sequence Detection System (ABI Prism 7300, Applied Biosystems). Reactions were prepared in a 96-well MicroAmp optical plate (Applied Biosystems) by adding the addition of a 5μL of cDNA to 20μL of a PCR master mixture consisting of SYBR Green universal PCR master mix and primers. Primers were titrated to check for amplification efficiency. The relative level of the TLR2, TLR4, cryopyrin, NFκBp50, NFκBp65, IL-1β gene expression in clinical samples was compared with a calibrator. The calibrator used in all subjects was the same cDNA generated as the same way as test samples and pooled. All quantitations are also normalized to an endogenous control 18s rRNA to account for variability in the initial concentration and quality of the total RNA and in the conversion efficiency of the reverse transcription reaction. The calculation formula was shown in the following.

For each clinical sample, the TLR2/TLR4/cryopyrin/ NFκBp50/ NFκBp65/ IL-1β C_T value was normalized using the formula $\Delta C = C_T$ (TLR2/ TLR4/cryopyrin/ NFκBp50/ NFκBp65/ IL-1β) - C_T (18s). This relative expression was determined using formula: $\Delta\Delta C_T = \Delta C_T$ (1) sample – ΔC_T (1) calibrator. The value used to plot relative

TLR2/TLR4/cryopyrin/ NFκBp50/ NFκBp65/ IL-1β expression was calculated using the expression $2^{-\Delta\Delta CT}$.

4.2.5 Flow cytometry

Neutrophil surface TLR2 and TLR4 protein expression was examined by flow cytometry (BD FACS Calibur System, Mississauga, ON, Canada). Phycoerythrin (PE)-labelled antibodies against TLR2 and TLR4 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and isotype control (PE Mouse IgG2a, k, BD Biosciences Pharmingen, Mississauga, ON, Canada) were used for neutrophil staining. To minimise data variation caused by instrument fluctuation, Quantum PE molecular equivalents of soluble fluorochrome (MESF) kits (Bangs Laboratories, Warrington, PA, USA) were run simultaneously with each sample. TLR2 and TLR4 protein expression intensity was determined by a standard calibration curve by plotting the MESF (Y axis) against the peak channel (X axis).

4.2.6 Cytokine measurements using Luminex® beads

Circulation inflammatory (TNF-α, IL-2, IL-6, and IFNγ), and anti-inflammatory IL-10 cytokine profiles were measured by a multiplexed fluorescent microsphere immunoassay using the Luminex 100 System (Luminex Corporation, Austin, TX, USA). Multiplex bead kit-based assays were preformed in duplicate according to the manufacturer's protocol (LINCO Research, Inc., St. Charles, MO, USA). Thresholds for detection were 0.1pg/ml for all assays.

4.2.7 Statistics

Data were analyzed using Prism 4.0 software (GraphPad, San Diego, CA, USA), comparing results across all groups, and then between individual groups. In most analyses, early onset preeclampsia was set as the comparator group for presentation (see tables and figures). For continuous variables, Kruskal-Wallis ANOVA, Dunn's post-test, and *Mann-Whitney U* were used, as appropriate. A p value <0.05 was considered statistically significant.

4. 3 Results

4. 3. 1 Clinical characteristic

The maternal clinical characteristics are shown in Table 4.2. Women with early onset preeclampsia more frequently delivered preterm birth ($p< 0.0001$) and more small-for-gestational age babies ($p< 0.0001$) than matched normal pregnancy controls.

4. 3. 2 Neutrophil TLR2, TLR4, cryopyrin, IL-1β, NFκBp50 and NFκBp65

Preeclampsia (as a single combined group of early and late onset) had increased TLR2 (median 0.669 [interquartile range (IQR) 0.390, 1.621] vs 0.348 [0.173, 0.625]; Mann-Whitney U (MWu) $p<0.0001$) and TLR4 (0.921 [0.571, 1.572] vs 0.502 [0.245, 0.697]; MWu $p< 0.0001$) mRNA expressions, TLR2 (1633 [1063, 2950] vs 1205 [928, 1069]; MWu $p= 0.001$) and TLR4 (0.921 [0.501, 1.572] vs 0.502 [0.245, 0.697]; MWu $p=0.0389$) protein expressions as well as elevated cryopyrin mRNA (0.182 [0.083, 0.581] vs 0.040 [0.018, 0.115]; MWu $p= 0.0003$) expression compared with combined matched normal pregnancy controls, respectively. These differences were more marked in early-onset preeclampsia (Figures 4.1 and 4.2). We have also observed that mRNA expressions of cryopyrin and IL-1β varied among study groups and were elevated in early onset preeclampsia (Figure 4.2). In addition, compared with normotensive IUGR, women with early onset preeclampsia had up-regulated mRNA levels of TLR2, TLR4, cryopyrin and NFκBp65 (TLR2: MWu $p<0.05$; TLR4: $p<0.001$; cryopyrin: $p<0.01$; NFκBp65: $p<0.05$). Furthermore, when combined into a single group, normal pregnancy women had higher mRNA expressions of cryopyrin (median [IQR]: 0.048 [0.028, 0.154] vs 0.032 [0.015, 0.050]; MWu $p= 0.0118$ and NFκBp50 (7.08 [3.64, 11.32] vs 2.81 [2.10, 4.87]; MWu $p=0.0097$) than non-pregnancy controls.

4. 3. 3 Sérum TNF-α/IL-10 and IL-6/IL-10

The ratios of serum TNF-α/IL-10 and IL-6/IL-10, which differed among groups, were increased in early-onset preeclampsia (Table 4.3). No significant difference was detected in ratios of serum IL-2/IL-10 and IFN-γ/IL-10 among study groups (Table 4.3). For individual cytokine, IL-6, IL-10 and TNF-α varied among groups (Kruskal-Wallis (KW) $p=0.0089$, 0.0186, 0.0192 respectively), but not IL-2, and IFN-γ (KW $p=0.6221$, 0.3034, respectively). Preeclampsia (as a single combined group of early- and late- onset)

had increased serum IL-6 level (24.2pg/ml [10.6, 37.9] vs 9.35pg/ml [5.78, 15.580; MWu p= 0.0018) compared with combined normal pregnancy controls.

4.4 Discussion

To our knowledge, this is the first study to report an association between early-onset preeclampsia and up-regulated expressions of neutrophil TLR2, TLR4 and cryopyrin, increased mRNA expressions of NF-κB subunit p50 and p65, as well as imbalanced pro-inflammatory and anti-inflammatory cytokine expression, all in a single case-control series. These differences were not noted between late onset preeclampsia and normotensive IUGR. New observations were made of neutrophil TLR2, TLR4, and cryopyrin upregulation in normal pregnancy.

Women with early- and late-onset preeclampsia and normotensive IUGR share the same intrauterine pathology, namely uteroplacental mismatch based on either incomplete placentation (early-onset preeclampsia and most cases of normotensive IUGR (8, 9)) or exuberant fetoplacental growth (i.e., late-onset disease complicating macrosomia or multiple pregnancy (9)). Uteroplacental mismatch can evolve into either the systemic disorder of early and late onset preeclampsia (with or without IUGR) or the isolated fetal syndrome normotensive IUGR (25-27). These data reinforce the conjecture that both the origins (i.e, amount of circulating placental debris) and maternal inflammatory response of early onset preeclampsia may explain the differential maternal response and risk of early onset versus late onset preeclampsia or normotensive IUGR (25, 28).

We have chosen to study neutrophils because of the key role that they play in innate immune defence (29) and of the evidence for their role in preeclampsia (4, 25, 30). In addition, neutrophils express extra-cellular receptors of TLR2 and TLR4, and the intracellular cryopyrin inflammasome, which are crucial to inflammatory cytokine production (31) (32) (33).

TLRs play a critical role in the induction of the immune response to invading pathogens. To date, ten human TLRs have been identified and designated, TLR 1–10 (34). Although each extra-cellular TLR is distinct in its specificity, all receptors signal to a common intracellular pathway. Following ligation, TLRs signal through the adapter molecule MyD88 to activate the NF-κB pathway; this results in an immune response characterized by the production of cytokines, antimicrobial products, and the regulation of

costimulatory molecules (35). TLR2 identifies specific cell-wall components of Gram-positive and Gram-negative bacteria, fungi and viruses (34). In addition, TLR2 interactions with trophoblast at the fetomaternal interface are responsible for infection-induced immune responses (36). TLR4 recognizes specific components of Gram-negative bacterial lipopolysaccharide (LPS) (34). Up-regulated TLR4 protein expression was reported in interstitial trophoblasts in women with preeclampsia (37). In addition to the increased expression of neutrophil TLR2 and TLR4 we observed in this study for women with early-onset preeclampsia, we have recently determined that the presence of TLR2 (RR 2.57, 95%CI 1.31-5.05) and TLR4 (RR 2.06, 95%CI 1.16-3.57) single nucleotide polymorphisms aggregated with early onset (n=42), but not late onset (n=52) preeclampsia compared with women with normal pregnancy outcomes (n= 176) (38). Through synthesis of these and the relevant published data (38-40), TLR2 and TLR4 polymorphisms appear to lower thresholds for early-onset and severe preeclampsia, but not late-onset or mild disease (38). Data from this cohort of women contributed to that case-control series. Therefore, the current body of evidence suggests that TLR2 and TLR4 may contribute to the neutrophil activation of preeclampsia.

Cryopyrin (NALP3), a member of the nucleotide binding and oligomerisation domain-like receptor (NLR) family, serves as an activator in response to specific toxins, endogenous danger signals, or microbial pathogens (41). Independent of TLR signalling, crypyrin has been shown to form the assembly of the inflammasome, a crucial part of the innate immune response and a cytosolic complex of proteins that activates caspase-1 to process the pro-inflammatory cytokine IL-1β, with NALP1 and NALP2 (42). IL-1β activation may lead to delayed neutrophil spontaneous apoptosis (43), which has been reported in early-onset preeclampsia (25). Our study also suggests a role for cryopyrin in preeclampsia.

Within leukocytes, nuclear translocation of NF-κB results in elevated synthesis of inflammatory cytokines, such as IL-6, and alterations in cell surface adhesion molecule expression (22). We have found early onset preeclampsia was associated with an increased mRNA expression of NF-κB as compared with non-pregnancy. Similarly, there was elevated NF-κBp65 as compared with matched normal pregnancy. Although we were unable to detect neutrophil NF-κB activation, preeclampsia may be characterised by NF-

κB pathway activation in peripheral blood mononuclear cells, when compared with non-pregnancy, and with women with uncomplicated pregnancies at term (21).

We examined serum cytokine profiles in women with established, but different onset of preeclampsia and observed an association between increased serum ratios of TNF-α/IL-10 and IL-6/IL-10 and early onset preeclampsia. For the individual cytokines, we observed increased TNF-α and IL-6 and reduced IL-10 in the combined preeclampsia cohort compared with matched normal pregnancy controls; these are consistent with other published reports (44-48). The imbalance between pro-inflammatory and anti-inflammatory cytokines probably contribute to endothelial dysfunction (49), and are thus linked with the pathogenesis of preeclampsia.

In this study, we did not detect any difference in IL-2, or IFN-γ levels between preeclampsia-complicated and normal pregnancies, which are consistent with other published observations (46, 50-52). As the present study was undertaken in women with established preeclampsia, it is not possible to determine whether the altered cytokine levels represent a cause or consequence of the disease. It would be interesting, therefore, to determine whether serum levels of cytokines are altered prior to the emergence of the maternal syndrome of preeclampsia. Further studies are required to determine whether measurement of serum cytokines in the first and second trimesters could predict preeclampsia.

Our data reinforce the view that early-onset preeclampsia differs more from normal pregnancies of similar gestation than does late-onset preeclampsia (28). Clinically, early-onset preeclampsia represents additional maternal risk, as maternal mortality is some 20-fold higher when preeclampsia develops at less than 32 weeks of gestation than when preeclampsia develops near term (53). Our findings add further evidence that preeclampsia is a form of systemic inflammation (6), and is particularly dangerous when it occurs early in pregnancy (25, 26).

Our study has several limitations. First, all cases were identified in a single tertiary referral centre, and our sample size is relatively small. Second, although we tested several sources of commercial cryopyrin antibody, cryopyrin protein expression could not be detected in study groups. To our knowledge there are no previous reports of the use of cryopyrin protein antibodies in the studies of either human or animal neutrophils. Thus, neutrophil cryopyrin protein expression is under-explored. Third, the patient population

served by our centre consists of three predominant ethnic groups, Caucasian, East Asian, and South Asian, and 40% of the adult population was born outside Canada. Also, approximately 20% of our maternity population is of mixed ethnicity, which confounds any interpretation of the role of ethnicity in our centre. Thus, we were not able to determine whether either ethnic background or country of birth might modify the association between TLRs and the cryopyrin inflammsome and the maternal syndrome of early onset preeclampsia. Fourth, our normal pregnant population is not a random sample. This is an intrinsic problem with a case-control study design that could be addressed through a future prospective, population-based, nested case-control cohort. Therefore, our findings need to be confirmed in a study with a larger sample size and more separation of ethnic groups.

In summary, we reported an association between early onset preeclampsia and neutrophil TLR2, TLR4 and cryopyrin. In addition, we observed that early-onset preeclampsia is related to an imbalance between circulation inflammatory and anti-inflammatory patterns. These changes were not noted for women with either late onset preeclampsia or normotensive IUGR. These findings provide new insights into possible roles for TLRs and cryopyrin in the host innate immune defence mechanisms and the pathogenesis of systemic inflammation in both normal pregnancy and, in an exaggerated form, earl-onset preeclampsia. Through further investigation of the immune system receptors and transcription pathways (Figure 4.3), it may be possible to identify potential anti-inflammatory targets for preeclampsia intervention.

Table 4.1 The sequences of the primer pairs used in real-time PCR

Primer	Sequence
human TLR2-sense:	5'-GAATCCTCCAATCAGGCTTCTCT-3'
human TLR2-antisense:	5'-CCTGAGCTGCCCTTGCA-3'
human TLR4-sense:	5'-GGCATGCCTGTGCTGAGTT-3'
human TLR4-antisense:	5'-GGACCGACACACCAATGATG-3'
human cryopyrin-sense:	5'- GAAGCCGTCCATGAGGAAGA-3'
human cryopyrin-antisense:	5'-TGCCCCGACCCAAACC-3'
human IL-1β-sense:	5'-TCAGCCAATCTTCATTGCTCAA-3'
human IL-1β-antisense:	5'-TGGCGAGCTCAGGTACTTCTG-3'
human NFκBp50-sense:	5’-AAGTCACATCTGGTTTGATTTCTGAT-3’
human NFκBp50-antisense:	5’-AAGTGCAAGGGCGTCTGGTA-3’
human NFκBp65-sense:	5’-GCCGGGATGGCTTCTATGA-3’
human NFκBp65-antisense:	5’-TGGATTCCCAGGTTCTGGAA-3’

Table 4.2 Maternal and perinatal clinical characteristics

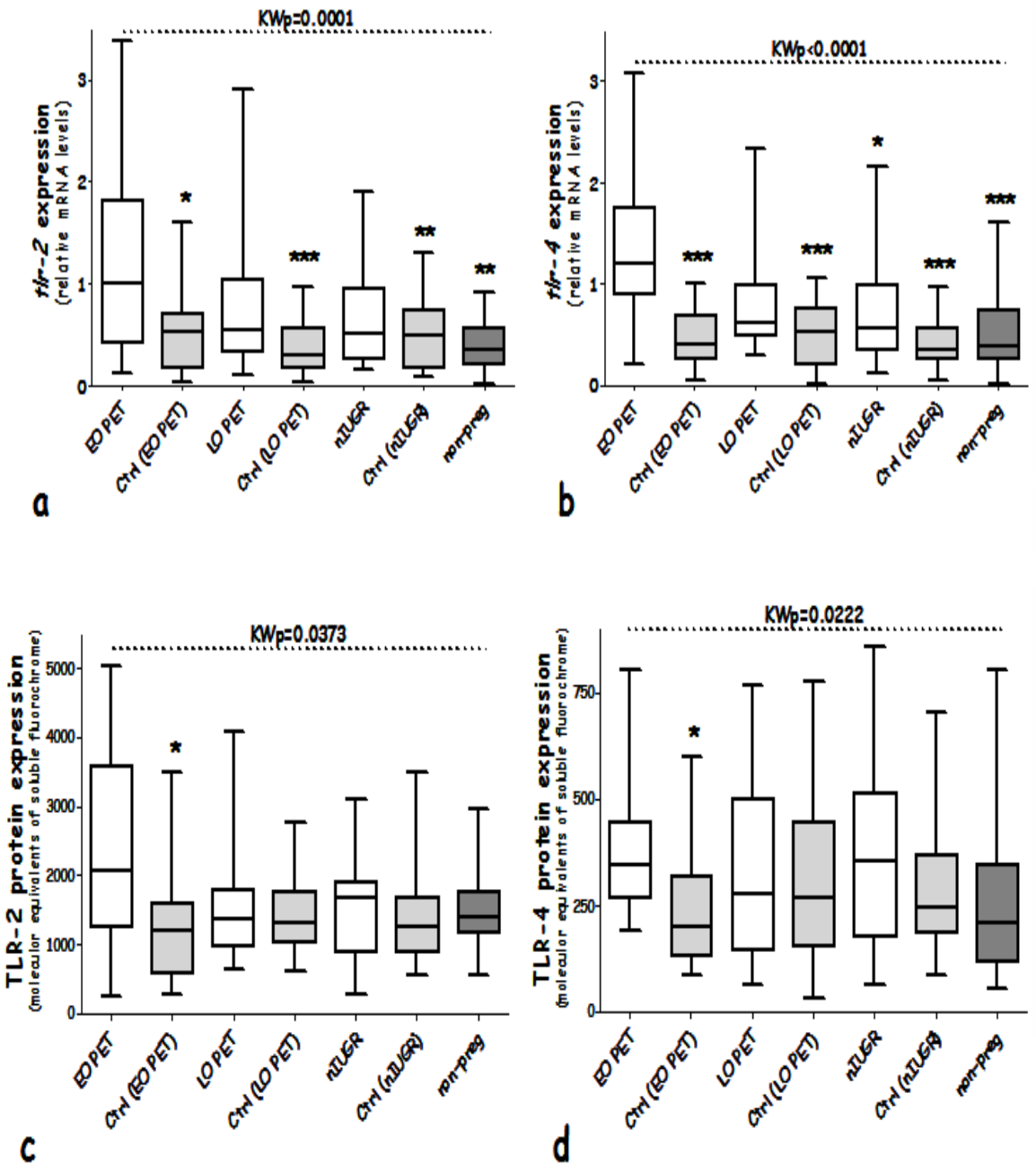
Characteristic	EOPET (n=25)	Ctrl (EOPET) (n=25)	LOPET (n=25)	Ctrl (LOPET) (n=25)	nIUGR (n=25)	Ctrl (nIUGR) (n=25)	Nonpreg (n=25)	P value (χ^2 or KW)
Maternal								
Maternal age (year)	35 [19, 43]	33 [24, 41]	34 [23, 41]	33 [24, 39]	34 [29, 42]	33 [29, 43]	32 [21, 43]	0.486
GA at sampling (weeks)	32.1** [22.7, 33.8]	30.3 [21, 33.7]	37.1 [34.4, 40]	36.7 [34.1, 39.6]	32.7** [21.4, 39]	32 [21, 38.3]	-	<0.0001
Nulliparous	16 (64)	15 (60)	21 (84)	22 (88)	15 (60)	15 (60)	-	0.05
GA at delivery (weeks)	32.9 [26.3, 37.1]	38.7††† [36.4, 41]	37.6††† [34.4, 40.1]	39.9 [37.9, 40.9]	35.7†† [24.6, 39.1]	39.1 [37.3, 40.9]	-	<0.0001
MAP (mmHg)	127 §§ [110, 139]	83†† [80, 92]	118 §§ [84, 139]	83** [81, 103]	95 [78, 107]	101 [100, 116]	-	<0.0001
Uric acid (µM)	347 §§ [249, 521]	-	384 §§§ [298, 464]	-	271 [194, 343]	-	-	<0.001
Platelets ($x10^9$/L)	195 [88, 263]	148 [137, 170]	176 [77, 277]	215* [151, 269]	211 [125, 346]	-	-	0.033
AST (µM)	32.5 §§ [27, 133]	-	29 § [20, 237]	-	21 [18, 30]	-	-	0.0035
Proteinuria	25 (100) §§	0 (0)	25 (100) §§	0 (0)	1 (4)	0 (0)	-	0.0002
Perinatal								
Birth weight (g)	1480 [395, 3385]	3318††† [2730, 4615]	2702† [1480, 4400]	3448 [2885, 4270]	1733 [360, 2645]	3500 [2750, 4420]	-	<0.0001
SGA (<5th centile)	11 (44)	0 (0)	8 (32) §§	0 (0)	16 (64)	0(0)	-	<0.0001

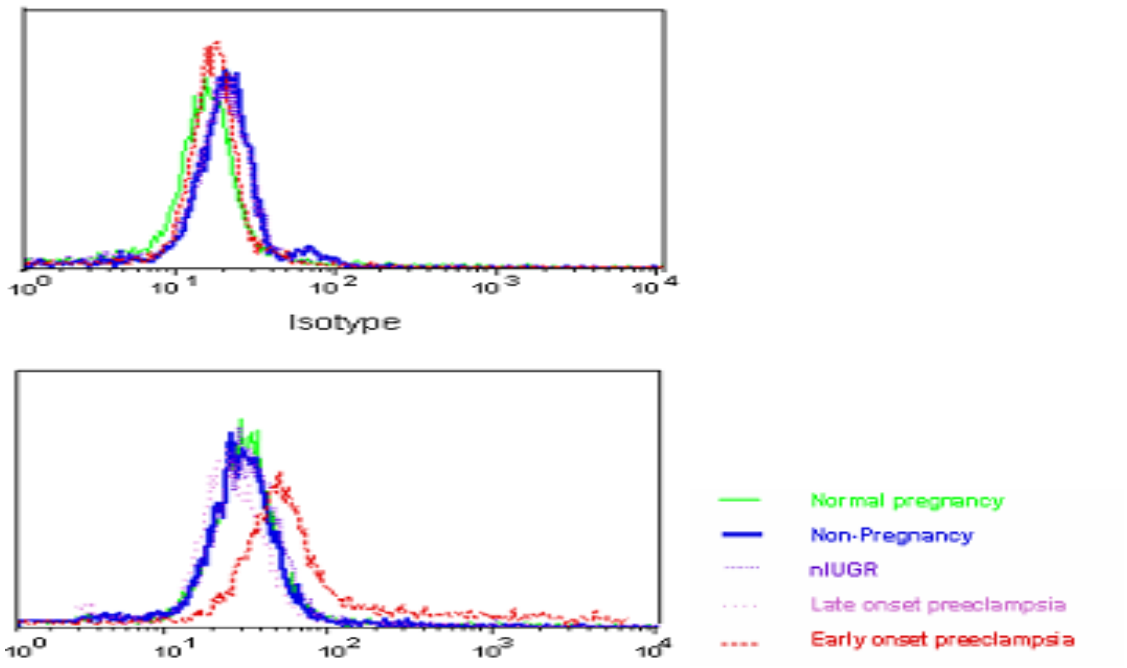
AST: aspartate transaminase; **Ctrl**: matched normal pregnancy control; **EO**: early onset (<34^{+0} weeks); **GA**: gestational age; **KW**: Kruskal-Wallis ANOVA; **LO**: late onset (≥34^{+0} weeks); **MAP**: mean arterial pressure = diastolic blood pressure + (pulse pressure/3); **nIUGR**: normotensive intrauterine growth restriction; **PET**: preeclampsia; **SGA**: small for gestational age.
For SGA, the pregnancy was deemed to have achieved the outcome if any one fetus was born less than fifth percentile for gestational age and gender using multiethnic Canadian birth weight charts (54).
Data expressed as median [range] or n (%).
Data were first compared across all columns to calculate the Kruskal-Wallis ANOVA or χ^2 p. Between-column comparisons were preformed with early onset pre eclampsia, late onset preeclampsia, and nIUGR as the comparator groups (as outlined below). * $p<0.05$; ** $p<0.001$ (Dunn's test, vs late onset preeclampsia); † $p<0.05$; †† $p<0.01$; ††† $p<0.001$ (Dunn's test, vs early onset preeclampsia); § $p<0.05$; §§ $p<0.01$; §§§ $p<0.001$ (Dunn's and Fisher's exact tests, vs nIUGR)

Table 4.3 Ratio of pro-inflammatory and anti-inflammatory cytokines

Variable	EOPET (n=25)	Ctrl (EOPET) (n=25)	LOPET (n=25)	Ctrl (LOPET) (n=25)	nIUGR (n=25)	Ctrl (nIUGR) (n=25)	Nonpreg (n=25)	P value (χ^2 or KW)
IFN-γ/IL-10 ratio	1.45 [0.17, 2.70]	1.51 [0.61, 10.1]	0.94 [0.14, 2.27]	1.41 [0.54, 3.44]	1.31 [0.41, 3.86]	1.23 [0.11, 13.38]	1.44 [0.08, 2.45]	**0.3175**
IL-2/IL-10 ratio	0.75 [0.37, 1.37]	0.62 [0.23, 0.88]	0.54 [0.09, 1.59]	0.66 [0.28, 1.72]	0.99 [0.15, 1.29]	0.71 [0.11, 3.03]	0.68 [0.03, 1.89]	**0.1089**
IL-6/IL-10 ratio	1.95 [0.32, 4.49]	0.40 *** [0.02, 2.09]	1.17 * [0.49, 3.28]	0.64 [0.28, 3.28]	0.66 [0.23, 3.17]	0.75 [0.22, 1.66]	0.64 [0.34, 3.23]	**0.0035**
TNF-α/IL-10 ratio	0.43 [0.11, 0.76]	0.19 ** [0.10, 0.64]	0.20 * [0.05, 0.52]	0.26 [0.11, 0.68]	0.33 [0.06, 1.2]	0.19 * [0.03, 0.5]	0.25 [0.09, 0.63]	**0.0022**

Ctrl: control; **EO**: early-onset ($<34^{+0}$ weeks of gestation); **IFN-γ**: interferon- γ; **IL**: interleukin; **KW**: Kruskal-Wallis ANOVA; **LO**: late onset ($\geq 34^{+0}$ weeks of gestation); **nIUGR**: normotensive intrauterine growth restriction; **PET**: preeclampsia; **TNF-α**: tumour necrosis factor-α. Data are expressed as median [range]. Using a prospective cross-sectional, case-control design, peripheral blood samples were taken from cases (preeclampsiaand nIUGR) and both matched normal pregnancy and non-pregnancy controls. Cytokines were measured by multiplex immunoassays. Data were first compared across all columns to calculate the Kruskal-Wallis ANOVA p. Between-column comparisons were preformed with early onset preeclampsia as the comparator group. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ (Dunn's test, vs early onset preeclampsia)


KWp=0.0001
tlr-2 expression
(relative mRNA levels)
EO PET
Ctrl (EO PET)
LO PET
Ctrl (LO PET)
nIUGR
Ctrl (nIUGR)
non-preg
a
KWp<0.0001
tlr-4 expression
(relative mRNA levels)
b
KWp=0.0373
TLR-2 protein expression
(molecular equivalents of soluble fluorochrome)
c
KWp=0.0222
TLR-4 protein expression
(molecular equivalents of soluble fluorochrome)
d



e

Figure 4.1: TLR2 and TLR4 mRNA and protein expression on neutrophils

Using a cross-sectional, case-control design, peripheral blood samples were taken from cases (preeclampsia and nIUGR) and both matched normal pregnancy and non-pregnancy controls. Both TLR2 and TLR4 mRNA (a & b, respectively; quantitative real-time PCR) and protein (c & d, respectively) expressions varied significantly among groups and were increased in women with early onset preeclampsia compared with normal pregnancies and non-pregnancy (gene expression) or matched normal pregnancy controls (protein expression by flow cytometry).
The flow cytometry data are summarised in c & d, with representative individual patient TLR2 data shown in e. The upper panel of e shows the staining profile of immunoglobulin isotype controls, the lower panel the TLR2 surface protein staining in the same women, characterised by study group. Box and whisker plots; whiskers: data range; box: interquartile range, with horizontal line at the median.
Ctrl: control; EO: early-onset ($<34^{+0}$ weeks of gestation); KW: Kruskal-Wallis ANOVA; LO: late-onset ($\geq 34^{+0}$ weeks of gestation); nIUGR: normotensive intrauterine growth restriction; non-preg: nonpregnancy; PET: preeclampsia; *tlr*: Toll-like receptor gene. Data were first compared across all columns to calculate the Kruskal-Wallis ANOVA p. Between-column comparisons were preformed with early onset preeclampsia as the comparator group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Dunn's post-test: vs early-onset preeclampsia).

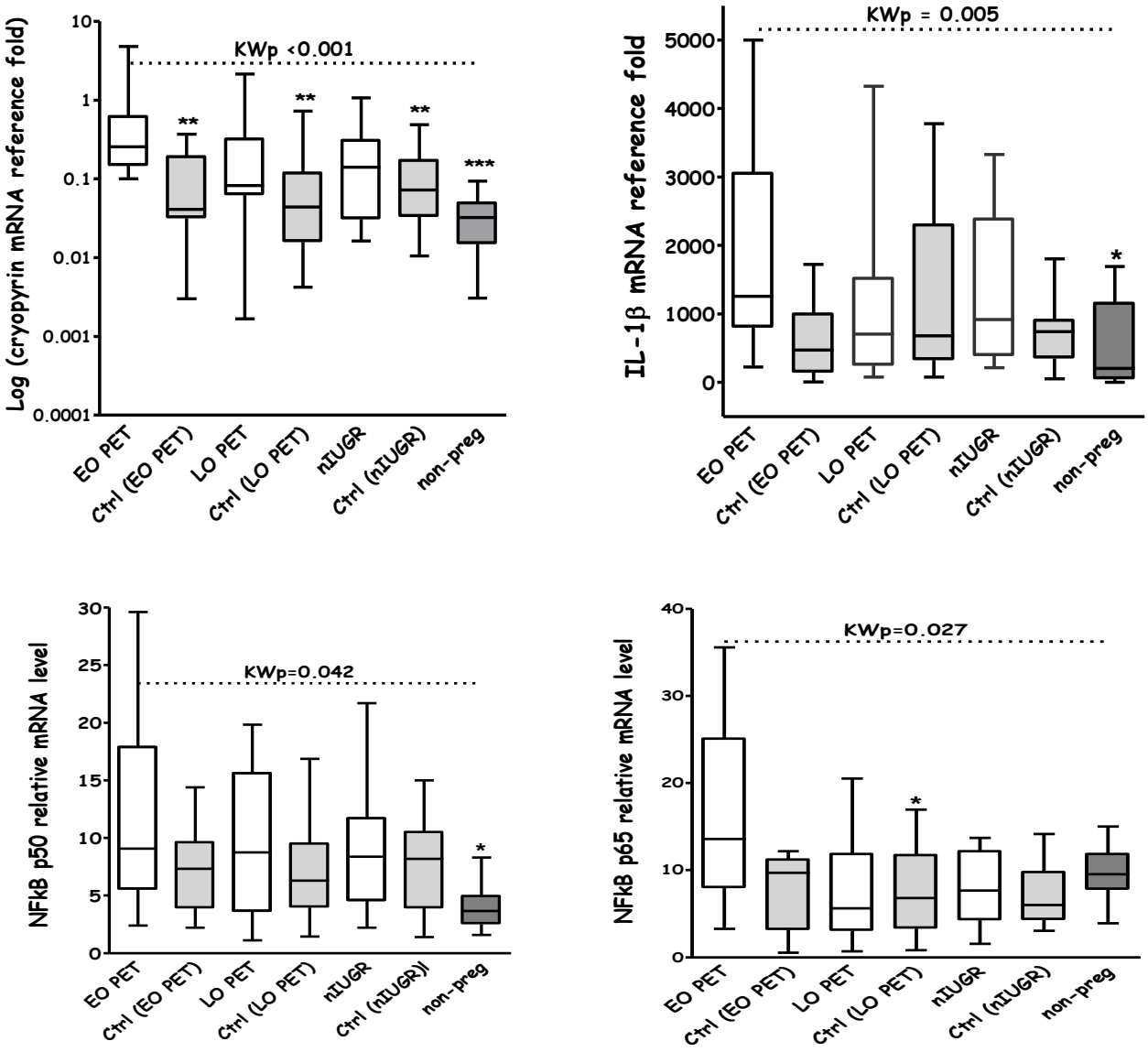


Figure 4.2: Neutrophil cryopyrin, IL-1β, NFκBp50, and NFκBp65 mRNA expression
Cryopyrin (a), IL-1β (b), NFκBp50 (c) and NFκBp65 (d) mRNA expressions varied significantly among groups and were increased in women with early-onset preeclampsia compared with normal pregnancies (cryopyrin) and nonpregnancy (cryopyrin and IL-1β). Box and whisker plots; whiskers: data range; box: interquartile range, with horizontal line at the median. Ctrl: control; EO: early onset ($<34^{+0}$ weeks of gestation); KW: Kruskal-Wallis ANOVA; LO: late-onset ($\geq 34^{+0}$ weeks of gestation); nIUGR: normotensive intrauterine growth restriction; non-preg: nonpregnancy; PET: preeclampsia. ** $p < 0.01$; *** $p < 0.001$ (Dunn's post-test: vs early-onset preeclampsia); ¶$p<0.01$ (*Mann-Whitney U* test: vs early-onset preeclampsia)

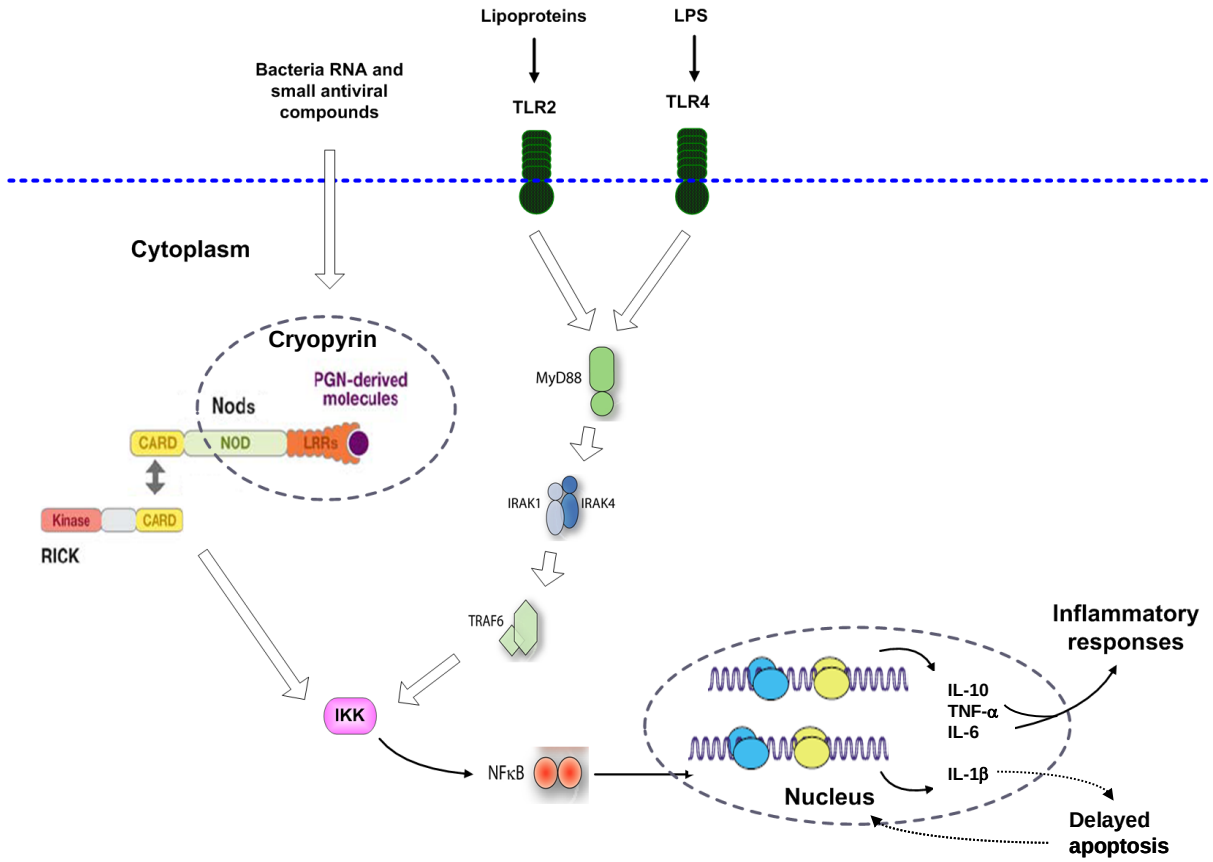


Figure 4.3 Activation of the innate immune system through TLR2, TLR4 and the cryopyrin inflammasome.

In this schematic overview, cryopyrin inflammasome, one of Nods protein, is involved in the recognition of bacterial PGN-derived molecules, resulting in recruitment of a downstream kinase protein RICK, through CARD-CARD interaction. Once RICK is recruited, Nods activate transcriptional factors NF-κB. TLR4 is essential for the recognition of LPS and TLR2 is for recognition of lipoporoteins at the cell surface, resulting in recruitment of MyD88-dependent pathway. Taken together with Toll-like receptors, Nods appear to play important roles in innate and acquired immunity as sensors of bacterial components. Once these downstream factors are recruited, several kinase complexes (IRAK1/IRAK4, TRAF6) are formed and activated which result in IKKs. These signaling events lead to the production of pro-inflammatory cytokines (such as TNF-α and IL-6), down regulation of anti-inflammatory cytokines (e.g., IL-10), and up-regulation of the anti-apoptotic IL-1β.

CARD: caspase-recruitment domain; IRAK: interleukin-1 receptor-associated kinase; IKK: IκB kinase; LPS: lipopolysaccharide; LRRs: leucine-rich repeats; MyD: myeloid differentiation primary response gene; NOD: nucleotide-binding oligomerisation domain; PGN: peptidoglycan; RICK: a CARD-containing protein kinase; TRAF: TNF receptor-associated factor

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CHAPTER 5: Toll-like receptor gene polymorphisms and preeclampsia risk: a case-control study and data synthesis [1]

5. 1 Introduction

Preeclampsia, a major cause of maternal and perinatal morbidity and mortality, is characterised by hypertension, proteinuria, and systemic inflammation presenting after 20 weeks' gestation. When of early-onset (usually defined as $<34^{+0}$ weeks), it is associated with greater maternal risks than arising at term (1). It is unlikely that the multifactorial syndrome of preeclampsia has a single inciting cause, but that it is, rather, a phenotype that requires a series of triggers that may vary widely between women. Such a trigger may be genetic predisposition (2).

The rapid recognition of pathogens by the innate immune system is mediated through Toll-like receptor (TLRs) and binding of a microbe to a TLR results in activation of several inflammatory pathways (3, 4). Upregulation of and signalling by TLRs may be one of the mechanisms by which intrauterine inflammation triggers both parturition and preeclampsia (4). We have recently observed that neutrophil expression of both TLR2 and TLR4 mRNA and protein is increased in early-onset, but not late-onset, preeclampsia compared with the upregulation noted in normal pregnancy (5). Previously, we had observed that women later to develop early-onset preeclampsia had higher concentrations of anti-*Chlamydia pneumoniae* (*C. pneumoniae*) and anti-cytomegalovirus (CMV) IgG than women who had unremarkable pregnancies (6). In that study, we examined antibody levels in samples taken from rubella serology testing in the late first and early second trimesters. We have recently extended these observations and have evidence of both TLR4-binding *C. pneumoniae* (7) and TLR2-binding CMV (8) activation in women with preeclampsia, particularly early-onset disease.

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[1] "A version of this chapter has been published. **Xie F**, Hu Y, Speert D, Turvey SE, Peng G, Money DM, Magee LA, von Dadelszen P. (2010) Toll-like receptor gene polymorphisms and preeclampsia risk: a case-control study and data synthesis. *Hypertension in pregnancy*. 2010;29(4):390-8."

Therefore, specific gene polymorphisms in TLR genes, which are associated with altered inflammatory responsiveness, may alter thresholds for the development of preeclampsia. However, data from a single study of a TLR2 SNP (9) and two studies of TLR SNPs (10, 11) have observed variable associations between TLR SNPs and the predisposition to develop preeclampsia.

In this study, we examined whether single nucleotide polymorphisms (SNPs) in the TLR2 and TLR4 genes were associated with the development of maternal risk of preeclampsia, especially that of early onset. In addition, with the intention of examining the inconsistency observed between published studies, we conducted a systematic review and preformed a data synthesis in combining our TLR2 and 4 SNP data with the published studies (9-11).

5. 2 Methods

5. 2. 1 Patient population

In a case-control study, following informed consent, we drew and stored a portion of whole blood samples collected for experimental purposes at BC Women's Hospital, a tertiary perinatal centre. DNA collection was approved by the University of British Columbia's Clinical Research Ethics Board and the Children's and Women's Health Centre of the BC Research Review Board.

Preeclampsia was defined as the development of hypertension (blood pressure ≥ 140/90 mmHg) and proteinuria (≥ 300mg/24h, ≥ 30mg protein/mmol creatine, or ≥ 2+ dipstick proteinuria) after 20 weeks' gestation (1). Women with preeclampsia were subclassified as having had either early-onset (clinical diagnosis < 34^{+0} weeks' gestation) or late-onset (≥ 34^{+0} weeks' gestation), as previously proposed in response to the increased maternal risks associated early-onset disease and consistent findings of greater deviation from normal pregnancy in terms of inflammatory biomarkers (reviewed in (12)).

5. 2. 2 Genomic DNA isolation

Genomic DNA (gDNA) was extracted from 200 µl of EDTA anti-coagulated whole blood by the use of DNA mini kits (Qiagen, Mississauga, ON) according to the manufacturer's instructions. gDNA was eluted from the Qiagen columns in a final volume of 80 µl of distilled water.

5. 2.3 TLR2 and TLR4 genotyping by Taqman-based quantitative real-time polymerase chain reaction (qPCR)

Genotyping of the TLR2 allelic variant, Arg753Gln (rs5743708), and the TLR4 allelic variants, Asp299Gly (rs4986790) and Thr399Ile (rs4986791), were preformed by qPCR, as described (11). Briefly, probe pairs for each SNP had either FAM (Arg753, Asp299, and Thr399) or VIC (753 Gln, 299Gly, and 399Ile) fluorescent labels on the 5'-end, and non-fluorescent quenchers on the 3'-end. Alleles were discriminated by measuring fluorescence signal released by FAM (492nm excitation; 520nm emission) or VIC (520nm excitation; 550 nm emission). The sequences of TLR2 Arg753Gln, and TLR4 Asp299Gly and Thr399Ile pairs used in real-time PCR are listed in Table 5.1.

Allele-specific probes were constructed by dual-labelling with a fluorescent label and a non-fluorescent quencher conjugated to a minor groove binder (MGB). Probes to detect Arg753 (FAM) and 753Gln (VIC) were 5'-[FAM]-AGCTGCGGAAGAT-[MGB]-[quencher]-3' and 5'-[VIC]-AGCTGCAGAAGAT- [MGB]-[quencher]-3'. Probes to detect Asp299 (FAM) and 299Gly (VIC) were 5'-FAM- CCTCGATGATATTATT-MGB-[quencher]-3' and 5'-[VIC]-CCTCGATGGTATTATT-3' MGB-[quencher]-3', while probes to detect Thr399(FAM) and 399Ile (VIC) were 5'-[FAM]-AGGCTGGTTGTCC- [MGB]-[quencher]-3' and 5'-[VIC]-AGGCTGATTGTCC-[MGB]-[quencher-]-3'. PCRs (25µl) were run in 96-well optical reaction plates (Applied Biosystems, Foster City, CA) and consisted of genomic DNA 5µl (200ng), SNP-specific primers (300nM) and probes (200nM), 2 × TaqMan Universal PCR Master Mix (Applied Biosystems) and distilled water. The fluorescence was detected in real time during PCR thermal cycling by the use of an ABI 7300 Sequence Detection System (Applied Biosystems).

5.2.4 Statistical analysis

The relative contributions of TLR2 and TLR4 genotypes to the development of preeclampsia were estimated using Review Manager 5.0 (RevMan, Nordic Cochrane Centre, Copenhagen, The Cochrane Collaboration, 2008) and are presented as risk ratio (RR) with 95% confidence intervals (CI). We systematically reviewed current published paper of TLR2 and/or TLR4 SNP in preeclampsia and preformed data synthesis by combining our data with those from Fraser *et al* (9), Molvarec *et al* (10) and van Rijn *et al* (11), respectively, using RevMan. The characteristics of studies included in this systematic review were summarized in Table 5.4. For

the van Rijn study (11), we calculated the rates of preeclampsia in the TLR4 SNP and wild type groups by imputation using the published unadjusted Peto odds ratio (and 95% CI) as the analytical method inside RevMan. The exposure (independent) variable was the presence or absence of any individual SNP. The outcome (dependent) variable was the presence or absence of early-onset preeclampsia or late-onset preeclampsia.

Separate analyses were conducted for TLR2 and TLR4 data. The basic data used in the unadjusted analyses consisted of a series of 2x2 tables defined by the dichotomous SNP/wild type and early- or late-onset, or mixed, preeclampsia/normal pregnancy controls for each study. The RR was used as the measure of the relation between SNPs and preeclampsia. Results from different reports were combined to produce a pooled RR [95% CI], according to the Mantel–Haenszel method. We pooled results from individual studies using a fixed-effects model.

5.3 Results

We examined the TLR2 and TLR4 genotype distribution in 94 women with preeclampsia (early-onset, n=42; late-onset, n=52) and compared them with 176 healthy pregnancy controls. Clinical and allelic frequency details are presented in Tables 5.2 and 5.3, respectively. Women with preeclampsia had higher mean artery pressure ($p<0.05$), more frequently delivered pertem birth ($p<0.05$) and low birth weight babies ($p< 0.001$) than normal pregnant controls (Table 5.2). No significant difference of allele frequencies of the TLR2 and TLR4 SNPs Asp299Gly, Thr399Ile and Arg753Gln was observed between preeclampsia and normal pregnancy controls.

The TLR2 SNP (Arg753Gln) aggregated in women with early-onset, but not late-onset preeclampsia compared with normal pregnancy controls (Figure 5.1 (a)). All allelic frequencies in groups were found to be in Hardy-Weinberg equilibrium. When included in a data synthesis, these SNP data are consistent with those of Fraser et al (9), who examined the same TLR2 SNP, in a population of women with preeclampsia of mixed gestational age (median gestational age: 35.6 weeks).

The presence of the two co-segregating TLR4 SNPs (Asp299Gly and Thr399Ile) aggregated in women with early-onset, but not late-onset, preeclampsia (Figure 5.1 (b)). Hardy-Weinberg equilibrium was observed. When included in a data synthesis, these SNP data are consistent with the apparently disparate findings of Molvarec et al (10) and van Rijn et al (11), and explain the heterogeneity observed between these studies ($I^2 = 77\%$).

5. 4 Discussion

We observed that allelic variants of the extracellular pattern-recognition receptors, TLR2 and TLR4, aggregate with a lower threshold for the development of early-onset, but not late-onset, preeclampsia. Individual TLRs recognise a distinct, but limited, repertoire of conserved microbial products. TLR4 is a central component of the lipopolysaccharide (LPS or endotoxin) sensor. TLR2 is essential for the recognition of microbial lipopeptides and may be involved in some LPS-mediated signalling (13). Our findings suggest a potential role for endotoxin responsiveness in the susceptibility to preeclampsia.

Endotoxin can induce pregnancy-specific inflammatory and endothelial cell disturbances in rats, including hypertension, proteinuria, and glomerular endotheliosis, that resembles the clinical syndrome of preeclampsia (14). Taken together with human data showing elevated circulating biomarkers of the soluble and cellular components of the innate immune system in women with pregnancies complicated by early-onset preeclampsia (12), we wondered whether or not genetically encoded inter-individual differences in the inflammatory response to LPS could influence maternal predisposition to develop preeclampsia.

These data also reinforce the impression that additional knowledge can be gained through the subclassification of preeclampsia into that of early- ($<34^{+0}$ weeks') and late-onset ($\geq 34^{+0}$ weeks') (12). Such differences include evidence of chronic infection in women destined to develop preeclampsia with agents that bind to TLRs (*C pnemoniae* and CMV) (15), and the neutrophil expression of TLR2 and TLR4 (5). In addition to TLR2 and TLR4, we have previously identified that the neutrophil NALP3 cryopyrin inflammasome was upregulated in early-onset preeclampsia (5). Uric acid, which is increased in preeclampsia (16), can stimulate acute lung injurey through TLR2, TLR4 and the cryopyrin inflammasome (17). Acute lung injury is a hallmark of severe preeclampisa (1).

Together, these data support the conjecture that chronic infection, acting, at least in part, through TLRs, may alter the threshold for the development of the maternal syndrome of preeclampsia (15, 18). Infection-induced TLR activation, or TLR activation by components of the intervillous soup of preeclampsia (1), may trigger TLR autoamplification feed-forward loops invoving circulating leukocytes. The interaction between the maternal endothelium and activated peripheral blood leukocytes (19) would either further reduce, or cross, the threshold for the development of clinical preeclampsia (20).

By sub-classifying our preeclampsia cohort, we identified a possible role for TLR2 SNPs in altering the susceptibility to developing preeclampsia, and, through the process of data synthesis, have altered the interpretation of the excellent study of Fraser *et al*, who studied a population of women with 'mixed' preeclampsia (i.e., early- and late-onset), and failed to find an association between the same TLR2 SNP and preeclampsia risk (9).

In addition, the combination of sub-classification and data synthesis may help to explain, in part, the apparent divergence of results observed between Molvarec *et al* and van Rijn *et al* (Figure 5.1) (10, 11). Molvarec *et al* studied women with largely late-onset preeclampsia and did not find an association between TLR4 SNPs and preeclampsia, while van Rijn *et al* found an association between TLR4 SNPs and either early-onset pre-eclampsia and/or HELLP (Hemolysis, Elevated Liver enzymes and Low Platelet count) syndrome.

The primary limitation of our study is that we were underpowered to determine whether or not ethnic background might modify the relationship between TLR SNPs and preeclampsia risk. Our sample size was relatively small, and in light of the low population prevalence of the examined SNPs (21), our results should be confirmed in larger studies; data synthesis is retrospective and should best be considered to be hypothesis-generating. Another limitation was that we solely examined SNPs in the sequences of TLR2 and TLR4, and it is possible that other TLR alleles and cytokine polymorphisms (as reviewed in (9)) could alter susceptibility to the development of preeclampsia.

In summary, we have observed an association between TLR2 and TLR4 SNPs and early-onset preeclampsia, and have helped to explain some previously unresolved differences in the literature. These findings suggest that TLR2 and TLR4 SNPs, as well as others observed that relate to the many triggering pathways that lead to the maternal phenotype, might have value as early screening markers for susceptibility to early-onset preeclampsia.

Table 5.1 The sequences of TLR2 Arg753Gln, and TLR4 Asp299Gly and Thr399Ile primer pairs used in real time-PCR

Primer	Sequence
TLR2 Arg753Gln forward primer	5’-GGAGCCCATTGAGAAAAAAGC-3’
TLR2 Arg753Gln reverse primer	5’-TCCAGGTAGGTCTTGGTGTTCA-3’
TLR4 Asp299Gly forward primer	5’-GGCCTGTGCAATTTGACCAT-3’
TLR4 Asp299Gly reverse primer	5’-TCACCAGGGAAAATGAAGAAACA-3’
TLR4 Thr399Ile forward primer	5’-AGGTTGGCTGTTCTCAAAGTGATTTT-3’
TLR4 Thr399Ile reverse primer	5’-TCTAGTTGTTCTAAGCCCAAGAAGTTT-3’

Table 5.2 Maternal clinical characteristics

Variables	Preeclampsia (n= 94)	Normal Pregnancy (n= 176)	p value
Maternal Age (year)	33 [19, 43]	32 [24, 42]	p>0.05
Gestational age at delivery (week)	35.2 [26.3, 40.1]	38.7 [26.4, 40.9]	p<0.05
Nulliparous	55 (68%)	105 (60%)	p>0.05
MAP (mmHg)	122 [84, 139]	90 [80, 116]	p<0.05
Proteinuria	94 (100%)	-	
Birth weight (g)	1794 [359, 4400]	3422 [2730, 4615]	p<0.05
Small for Gestational Age (<5th centile)	33 (35%)	6 (3.4%)	p<0.01

Note: Data expressed as median [range] or n (%). Proteinuria defined as >300mg/24h, protein: creatinine ratio >30mg/mmol, or ≥++ dipstick proteinuria. MAP: mean arterial pressure = diastolic blood pressure + (pulse pressure/3)

Table 5.3 Allele frequencies of the TLR2 and TLR4 SNPs Asp299Gly, Thr399Ile and Arg753Gln were determined by quantitative PCR.

	Preeclampsia (n=94)	Normal pregnancy (n=76)	p-value (Chi-square)	Odds ratio (95% CI)
TLR2 Arg753Gln				
Genotype				
Arg/Arg	86 (91.5)	168 (95.5)		
Arg/Gln	8 (8.5)	8 (4.5)	0.094	1.95 (0.71-5.38)
Gln/Gln	0 (0)	0 (0)		
Total	94	176		
Allele				
Arg	180 (95.7)	344 (97.7)		
Gln	8 (4.3)	8 (2.3)	0.098	1.91 (0.71-5.18)
Total	188	352		
TLR4 Asp299Gly				
Genotype				
Asp/Asp	81 (86.2)	158 (89.8)		
Asp/Gly	12 (12.8)	18 (1.2)	0.31	1.41 (0.66-3.02)
Gly/Gly	1 (1.0)	0 (0)		
Total	94	176		
Allele				
Asp	174 (92.6)	334 (94.8)		
Gly	14 (7.4)	18 (5.2)	0.14	1.49 (0.73-3.07)
Total	188	352		
TLR4 Thr399Ile				
Genotype				
Thr/Thr	86 (91.5)	162 (92.0)		
Thr/Ile	8 (8.5)	14 (8.0)	0.44	1.07 (0.43-2.67)
Ile/Ile	0 (0)	0 (0)		
Total	94	176		
Allele				
Thr	180 (95.7)	338 (96.0)		
Ile	8 (4.3)	14 (4.0)	0.44	1.07 (0.44-2.61)
Total	188	352		

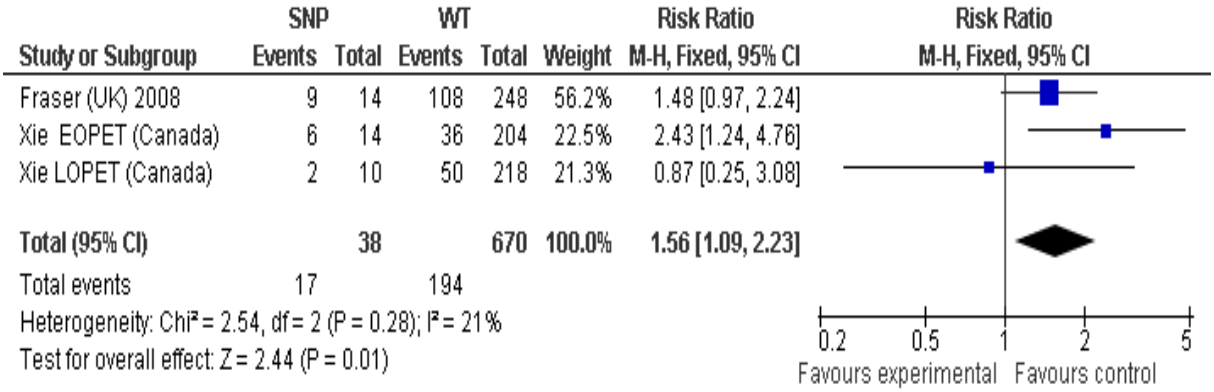
Note: No significant difference of allele frequencies of the TLR2 (Arg753Gln) and TLR4 SNPs (Asp299Gly, Thr399Ile) were observed between preeclampsia and normal pregnancy controls.

Table 5.4 Characteristics of studies included in the systematic review according to TLR2/TLR4 gene polymorphisms in preeclampsia.

First Author, Year	Region, Country	Cases and Controls	Adjust or matching	Main findings
TLR2 (Arg753Gln)				
Fraser, 2008(9)	United Kingdom	117 PET/ 146 controls	Maternal Age	No association
Xie, EOPET (this study)	Canada	42 EOPET/ 176 NPC	Maternal Age, parity	There is association between EOPET and TLR2 (Arg753Gln) gene polymorphisms.
Xie, LOPET (this study)	Canada	52 LOPET/ 176 NPC	Maternal Age, parity	No association.
TLR4 (Asp299Gly and Thr399Ile)				
EOPET				
Van Rijin, 2008(11)	Holland	58 PET/ 172 NPC	Ethnicity, smoking, height	An association of common TLR4 gene variants with history of EOPET and HELLP syndromes.
Xie, EOPET (this study)	Canada	42 EOPET/ 176 NPC	Maternal Age, parity	An association of common TLR4 gene variants with EOPET.
LOPET				
Molvarec, 2008(10)	Hungary, Caucasian	180 PET/ 172 NPC	Ethnicity	No association between TLR4 Asp299Gly and Thr399Ile gene polymorphisms and PET.
Xie, LOPET (this study)	Canada	52 LOPET/ 176 NPC	Maternal Age, parity	An association of common TLR4 gene variants with LOPET.

Note: LO: late-onset preeclampsia; EO: early-onset; PET: preeclampsia; NPC**:** normal pregnancy control; TLR: Toll-like receptor

(a)



(b)

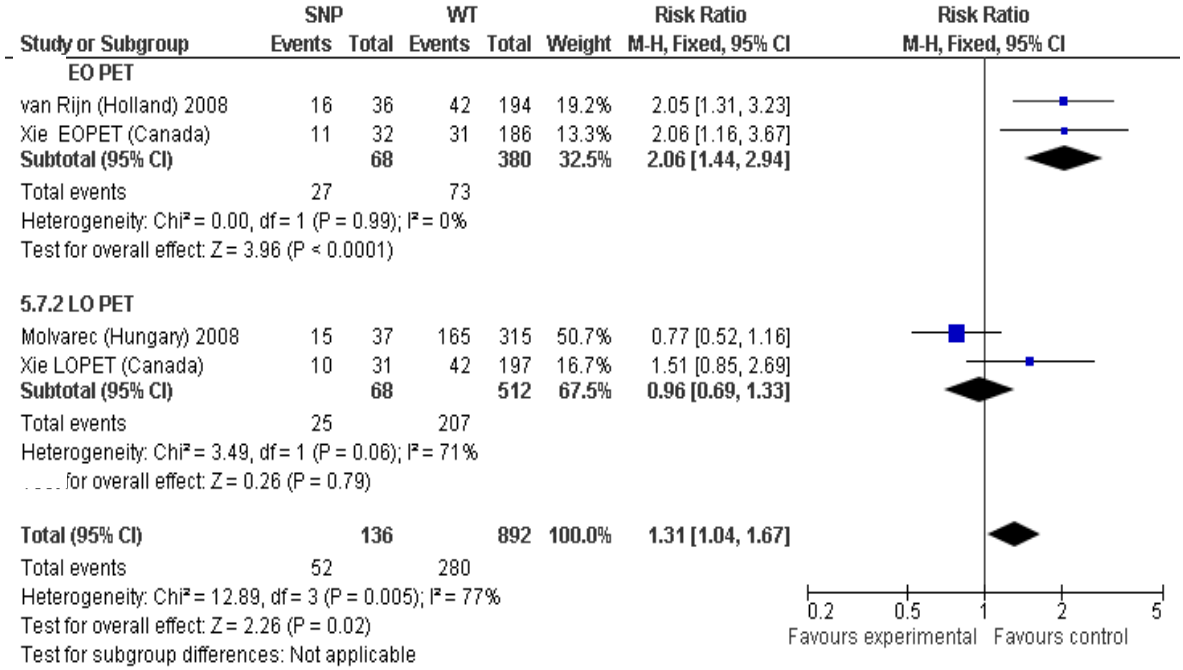


Figure 5.1 Data synthesis for the effect of TLR2 (a) and TLR4 (b) single nucleotide polymorphisms on the incidence of preeclampsia.
EO: early-onset; LO: late-onset; PET: preeclampsia

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CHAPTER 6: Toll like receptor signaling and preeclampsia

6.1 Introduction

Preeclampsia, complicating at least 5-8% of all pregnancies, is not only a major cause of obstetric mortality and morbidity but also increased risk of later cardiovascular and metabolism disorders (1, 2). The clinical findings of preeclampsia can manifest as either a maternal syndrome (hypertension, proteinuria, or various symptoms), or a fetal syndrome (growth restriction), or both (3). Despite recent progress toward understanding the cause of preeclampsia, the etiology of this serious disorder remains elusive. Current theories include abnormal placentation, cardiovascular maladaptation to pregnancy, genetic and immune mechanisms, an enhanced systemic inflammatory response, and angiogenic factors (4). It seems probable that multiple factors are involved (5).

Although the placenta has been shown to play a crucial role in the development of preeclampsia, recent investigations have increasingly suggested that inflammatory and immune mechanisms, activated by infectious or non-infectious agents, and even host-derived molecules, might be important in the pathogenesis of preeclampsia. Now, exciting discoveries related to signaling via Toll-like receptors (TLRs) have rekindled intense interest in the relationship between immune defense mechanisms and the established maternal systemic inflammatory response seen in preeclampsia. In this review, we discuss the background leading to these discoveries, highlight emerging evidence, and present the outlook for new interventions for preeclampsia.

6. 2 Toll like receptor signaling and the innate immune response

In 1997, vertebrate homologs of the *Drosophila* spp. transmembrane 'pattern recognition receptors' (PRR) 'Toll' were identified and termed the 'Toll-like receptors' (6). To date, 11 human and 13 mouse TLRs have been cloned (7). They are a family of pattern-recognition receptors and have been shown to be central to the innate immune response (8).

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[1] "A version of this chapter has been published. **Xie F**, Turvey SE, Williams MA, Mor G, von Dadelszen P. Toll-like receptor signalling and preeclampsia. *Am J Reprod Immunol*.2010; 63:7- 16. "

6.2.1 TLRs associated pathogen molecular patterns

TLRs recognize conserved pathogen-associated molecular patterns (PAMPs) on microorganisms. Different TLRs recognize different classes of PAMPs (9). TLR1, 2, 4 and 6 recognize lipopetides. The major ligands for TLR2 include the following: specific cell-wall components of Gram-positive and Gram-negative bacteria; mycobacteria; fungi; parasites; and viruses. TLR4 recognizes specific components of Gram-negative bacterial lipopolysaccharide (LPS). In contrast to these extracellular-localized TLRs, the so-called antiviral TLRs (TLR3, 7, 8, 9) have their recognition domains in an endosome within the cell (e.g. double-stranded viral RNA in the case of TLR3) (10). Another class of TLRs binds protein ligands; flagellin, for example, is detected by TLR5 (11). The human TLRs and their ligands are summarized in Table 6.1.

6.2.2 TLR activation and the "Danger" model

It is commonly accepted that TLRs are activated only by microbial patterns; however, a "danger model" has been proposed by several studies (12). Matzinger reported that the presence of potentially infectious PAMPs does not necessarily trigger an immune response unless there is evidence of host tissue injury signified by so-called 'alarm' signals (13). In support of this hypothesis, Matzinger and his colleagues have demonstrated that, in the absence of any foreign pathogens, resting dendritic cells can be activated by virally infected or necrotic cells, but not by healthy cells, or cells undergoing programmed apoptosis (14). Although debate surrounds these studies and theories, there is increasing evidence to indicate that TLRs can also detect host-derived molecules and non-infectious agents under certain circumstances. For instance, TLR4, recognized the chemotherapeutic agent paclitaxel (15). In addition, heat shock protein 60, a molecular chaperone conserved in both invertebrates and vertebrates, can activate nuclear factor kappaB (NFκB) by binding to either TLR2 or TLR4 (16). Moreover, the extracellular matrix can stimulate the innate immune response via TLRs when it is altered after tissue destruction, even in the absence of pathogens (17).

Recognition and activation of host-derived molecules by TLRs may be an important link between preeclampsia and the activation of the immune system. Indeed, investigations indicate that following the ligation of TLR-3 by the viral ligand, poly(I:C), or TLR-4 by bacterial LPS, trophoblast secretion of chemokines is significantly increased and this in turn results in elevated

monocyte and neutrophil chemotaxis (18) . Abrahams et al found that trophoblast cells, when treated with poly (I:C), produce and secrete high levels of RANTES (Regulated on Activation Normal T expressed and Secreted) and interferon genes (19, 20). In addition, Koga *et al* (21) have shown that injection of low doses of poly (I:C) induces a strong pro-inflammatory response by trophoblast cells leading to changes on the distribution and activation of immune cells at the implantation site. This response leads to damage of placenta and consequent preterm delivery and fetal death. All these data suggest that TLRs function as important sensors for the trophoblast, allowing it to coordinate the local immune response and promote cell invasion and placental formation (20). TLRs may also provide the bridge for placental recognition of danger signals (22), and a subsequent shift in the type of response generated may have harmful consequences for the pregnancy, like preeclampsia.

6.2.3 TLR signaling

Activation of TLRs causes an immediate defensive response, including the production of an array of pro-inflammatory cytokines and antimicrobial peptides. Accumulating evidence has shown that individual TLRs can activate overlapping as well as distinct signaling pathways, ultimately giving rise to distinct biological effects (11).

Each TLR activates a number of signaling pathways, some of which are common to all TLRs and some of which are specific to particular TLR types. Almost all TLRs use a Toll/Interleukin-1 Receptor (TIR)-containing adaptor myeloid differentiation primary response protein (MyD88) to activate a common signaling pathway that results in the activation of NF-κB to express cytokine genes relevant to inflammation (11). TIR- containing adaptors including TIRAP (TIR domain-containing adaptor protein), TRIF (TIR-domain-containing adaptor inducing interferon β), and TRAM (TRIF-related adaptor molecule) have been identified and shown to selectively interact with several TLRs. In particular, activation of the TRIF-dependent pathway through TLR3 confers antiviral responses by inducing anti-viral genes including that encoding interferon-β (11).

6.2.4 MyD88-dependent and -independent pathways in TLR signaling

MyD88 was the first adaptor protein found to be critical to TLR signaling, and signaling pathways that act through this adaptor are termed "MyD88-dependent pathways" (23). MyD88 can associate with all TLR types except TLR3. Activation of MyD88-dependent pathways promotes NFκB translocation to the nucleus and induces gene transcription of proinflammatory cytokines and chemokines (24).

The MyD88-independent pathway can involve the adaptor protein TRIF or TRAM. TLR3 and TLR4 can activate TRIF-dependent pathways without MyD88 association (11). Studies have revealed that the MyD88-independent pathway activates the transcription factor, interferon regulatory factor 3 (IRF-3) (25). Following viral infection or LPS treatment, IRF-3 undergoes phosphorylation and translocates from the cytoplasm to the nucleus, resulting in the production of interferon and co-stimulatory molecules (7) (See Figure 6.1).

6. 3 Immune mechanisms and preeclampsia: evidence and controversies

6.3.1 Infectious pathogens in preeclampsia

Epidemiologic studies have shown that maternal infection and preeclampsia are connected, and have suggested that the link between these could be through TLR activation (18, 26, 27).

A recently published systemic review and meta-analysis has reported that urinary tract infection were significantly associated with an increased risk of preeclampsia (5). Inspired by the increasing evidence that infectious agents, such as *Chlamydia pneumoniae* and cytomegalovirus (CMV) significantly contribute to clinical cardiovascular disease, researchers have started to examine serologic evidence linking *Chlamydia pneumoniae* and CMV antibodies to preeclampsia (28). Several studies detected increased *Chlamydia pneumoniae* antibodies (28-31) in preeclampsia; the evidence came from enzyme linked immunosorbent assays and microimmunofluorescence studies (32, 33). In our research group, we have found both elevated cytomegalovirus antibodies level (28) and gDNA loads in women with early onset preeclampsia (34, 35). Furthermore, a genetic study reported that the presence of CMV increased the risk of developing preeclampsia in women carrying specific HLA-DRB1 alleles (36).

In addition, a number of other infectious agents have been evaluated. These have included *Helicobacter pylori* (37), Adeno-associated virus-2 (AAV-2) (38), HIV (39), malaria

(40), mycoplasma hominis (41), Epstein-Barr (42), and herpes simplex virus 2 (42). Since there are conflicting results, current research findings indicated that preeclampsia is related more closely to total "infectious burden" than to specific pathogens (5).

One agent of potential importance in activation of the inflammatory response is LPS. Low doses of LPS infused into pregnant rats produce pathologic changes similar to those observed in preeclampsia (43). This suggests that inflammatory cytokines released by monocytes and macrophages are related to preeclampsia. In addition, following the ligation of bacterial LPS, trophoblast secretion of chemokines is significantly increased; this in turn results in increased monocyte and neutrophil chemotaxis, which might contribute to the pathogenesis of preeclampsia (18). These observations suggest that systemic proinflammatory mediators such as LPS through TLR signaling may be pathogenically linked to the development and progression of preeclampsia. It is also possible that the effects are indirectly mediated, for example, by producing a generalized proinflammatory condition in which preeclampsia might be facilitated. Indeed, recent studies have provided *in vivo* evidence for a direct mechanistic link between TLR4 signaling and innate immune system activation and preeclampsia (18) (see later "Emerging Discoveries: TLRs and Preeclampsia").

The reduced placenta debris and other release of molecular may further stimulate TLRs ligands and upregulation of TLR expression, which will cause positive feed-back loop to propagate a clinical course and slow resolution past postpartum.

6.3.2 Clinical trials with antibiotics

Three clinical trials evaluated the effect of antibiotic treatment for bacteriuria during pregnancy on the risk of preeclampsia. One randomized controlled trial noted that treatment with sulphamethoxydiazine or sulphadimidine, as compared to placebo, did not affect the risk of preeclampsia (44). Two nonrandomized clinical studies from Germany (45) and Croatia (46) reported that, compared to women with non-treated bacteriuria, antibiotic treatment for bacteriuria was associated with a statistically significant reduction in the incidence of preeclampsia. Results from a nonrandomized clinical study in Italy (47) indicated that pregnant women treated with spiramycin (because of seroconversion for *Toxoplasma gondii*) had a lower incidence of preeclampsia than low risk women who had not taken any antibiotic during pregnancy.

However, the difference was not statistically significant. Recently, Michalowica *et al* (48) reported the in results of a multi-center trial that periodontal treatment during pregnancy did not significantly alter the risk of preeclampsia. An explanation for these results may be its association with timing. Once the damage is made because of the early response to the infection, the treatment with antibiotics may not reverse the process. Therefore, there is a need to develop therapies that not only that target the bacteria but could also regulate the inflammatory process prior to pregnancy or during early pregnancy. In the future, observational studies should be large enough and adjust for indicators of potential confounding factors in order to reconcile these discordant results.

6.4. Emerging discoveries

6.4.1 Clinical evidence: TLR in preeclampsia

The theory that TLRs are linked to preeclampsia is supported by correlations seen in clinical studies. Mazouni *et al* reported that TLRs mediate the imbalance between inflammatory and anti-inflammatory patterns of monocytes in patients with preeclampsia (27). Another study found that TLR4 protein expression is increased in interstitial trophoblasts of patients with preeclampsia (26). In addition, TLR4 positive trophoblasts from preeclampsia patients were frequently immunoreactive to activated nuclear factor-kappaB, tumor necrosis factor-alpha, and M30 (a specific apoptosis antigen for trophoblast) (26). Kim *et al* have suggested that, in preeclampsia, "danger signals" at the feto-maternal interface, recognized by trophoblasts through TLR4, may play a key role in the creation of a local abnormal cytokine milieu (26). This indicates a novel mechanism that links preeclampsia to activation of the innate immune system through TLR4 (26).

Preeclampsia is associated with circulating neutrophil activation (49). Our recent data found that, as compared to normal pregnancy controls, preeclampsia was associated with elevated neutrophil TLR2 and 4 mRNA and protein expressions (50).

6.4.2 Experimental evidence: TLR in trophoblast miration and apoptosis

A wealth of evidence suggests that activated TLR signaling could affect preeclampsia in multiple ways. TLRs play a central role in determining the Th1/Th2 balance of immune responses. TLR activation promotes the generation of a Th1-dominated immune response, and

inhibits Th2 cytokine production (51). Th1 response up-regulation occurs at the feto-maternal interface in preeclampsia, and it is closely related to poor placentation and endothelial dysfunction (26).

A recent study has shown that TLRs also play a role in the regulation of immune cell migration by first trimester trophoblast cells (18). Following the ligation of TLR3 by the viral ligand, poly(I:C), or TLR4 by bacterial LPS, trophoblast secretion of chemokines is significantly increased; this in turn results in elevated monocyte and neutrophil chemotaxis (18). In addition, TLR3 stimulation induces trophoblast cells to secrete RANTES (Regulated on Activation, Normal T Expressed and Secreted). which is also known as CCL5) (18). These results suggest a novel mechanism by which first trimester trophoblast cells may differentially modulate the maternal immune system both during normal pregnancy, and in the presence of an intrauterine infection (18). Some studies have indicated that, in first trimester trophoblast cells, the apoptotic pathway may be activated through a heterodimer of either TLR-2/TLR-6 (52) or TLR-2/TLR-1(18).

6.4.3 Genetic evidence: TLR polymorphisms and susceptibility to preeclampsia

The genetic variation in the population results some individuals having a "subtle" but specific immunodeficiency. Increasing evidence has demonstrated that two common TLR4 polymorphisms and TLR2 polymorphism impair TLRs signaling and are associated with susceptibility to cardiovascular disease (53) and preterm birth (54). Recently, several direct studies of TLR gene polymorphisms in preeclampsia have been described. TLR2 (Arg753Gln) and TLR4 (Asp299Gly/ Thr399Ile) single nucleotide polymorphisms (SNPs) appear to alter susceptibility to developing the maternal syndrome of preeclampsia. van Rijn *et al* suggested that maternal TLR4 polymorphisms alter susceptibility to early-onset preeclampsia and elevated liver enzymes and low platelets (HELLP) syndrome (55). Additionally, we have found the presence of TLR-2 Arg753Gln and two TLR4 SNPs (Asp299Gly and Thr399Ile) was associated with early-onset preeclampsia as compared to normal pregnancy controls (56).

We preformed a data synthesis of our findings with those in published reports, and summarized it in Figure 2. Through synthesis of these data, TLR4 and TLR2 polymorphisms appear to lower thresholds for early-onset and severe preeclampsia, in particular (Figure 6.2). A concern in all these studies is the wide range of allele frequencies in control groups. Inferences

from these early studies are limited in part because of their relatively small sample size and by the fact that population stratification (confounding by participants race/ethnicity) have not been adequately addressed. Future larger studies that allow for stratification by race/ethnicity and which may also consider gene-gene and gene-environment interactions are needed to move this literature forward. Therefore more extended studies are needed to establish whether there is an association between TLR gene polymorphisms and the risk of preeclampsia, and conclusions must be guarded at this time. A definitive and fully powered cohort study is required.

The relative contributions of TLR2 and TLR4 genotypes to the development of preeclampsia were estimated and presented as risk ratio (RR) with [95% Confidence Interval (CI)]. The TLR2 and 4 SNP data synthesis was preformed by combining our data with those from Fraser *et al* (57), Molvarec *et al* (58), and van Rijn *et al* (55), respectively. For van Rijn (55) the rates of preeclampsia in the TLR-4 SNP (single nucleotide polymorphism) and WT (wild type) groups were calculated by imputation. The exposure (independent) variable was presence or absence of any individual SNP. The outcome (dependent) variable was the presence or absence of EOPET (early-onset preeclampsia) or LOPET (late-onset preeclampsia). Separate analyses were conducted for TLR2 and TLR4 data. The basic data used in the unadjusted analyses consisted of a series of 2× 2 tables defined by the dichotomous SNP/WT and early- or late-onset, or mixed, preeclampsia/ normal pregnancy controls for each study. The risk ratio was used as the measure of the relation between SNPs and preeclampsia. Results from different reports were combined to produce a pooled risk ratio (95% CI), according to the M–H method. We pooled results from individual studies using a fixed-effect's model, and expressed our data as first author (study population's country), risk ratio, and expressed our data as first author study (modified from Xie et al.(56)).

6.5 Conclusions

Recent research in the field of TLR shows that these receptors play many important roles in pregnancy complications, especially in preeclampsia. Exciting evidence supporting the theory that TLR activation contributes to the development and progression of preeclampsia has come from epidemiological, clinical and basic science studies. Previously, most of the evidence favoring a link between immunity and preeclampsia pathology has been indirect. We now require more data from experimental animal models, which might directly implicate the

signaling pathways associated with innate immunity. Before we can begin to develop effective therapies, it will be necessary to expand our understanding of these links.

Table 6.1 Human TLRs and Their Ligands

TLR	Ligands	Origin	Reference
TLR 1	Lipoproteins/triacylated lipopeptides	Bacteria, Mycobacteria	(59)
TLR 2	Bacterial Lipoproteins (BLPs)	Bacteria	(60)
	Peptidoglycans	Bacteria	(61)
	Glycosylphosphatidylinositol (GPI)	Trypanasoma cruzi	(62)
	Iipotechoic acid	Gram-positive bacteria	(63)
	Atypical LPS	Gram-negative bacteria	(64)
TLR 3	Double-stranded RNA (dsRNA)	Viruses	(65)
TLR4	R-Lipopolysaccharides (LPS)	Gram-negative bacteria	(66)
	Lipid A	Gram-negative bacteria	(67, 68)
	Heat Shock Proteins (HSPs)	Host	(69, 70)
TLR 5	Flagellin	Gram-negative bacteria	(71)
TLR 6	Diacylated Lipopeptides	Bacteria	(72)
	Zymosan	Fungi	(73)
	Soluble Tuberculosis Factor (STF)	Mycobacteria	(74)
TLR 7	R-848 (Resiquimod and Imiquimod)	Synthetic	(23, 75)
	Loxoribine	Synthetic	(76)
	Single-stranded RNA (ssRNA)	Viruses	(77)
	siRNA	Synthetic	(78)
TLR 8	R-848 (Resiquimod)	Synthetic	(75)
	Single-stranded RNA (ssRNA)	Viruses	(76, 77)
TLR 9	CpG DNA	Bacterial/Viral	(79)
	CpG ODNs	Synthetic	(80)
	Hemozoin	Protozoa (Malaria)	(81)
TLR 10	Unknown		

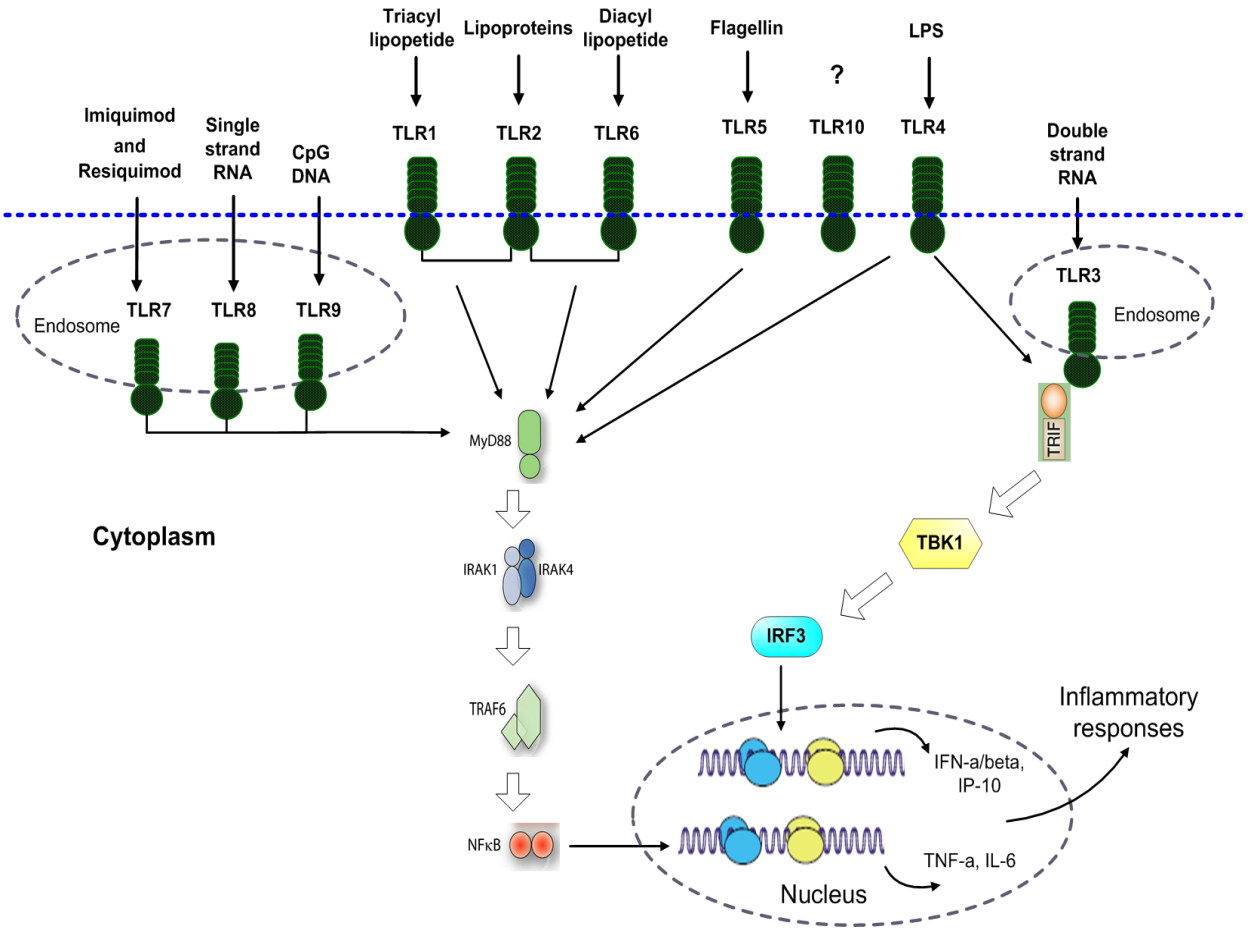
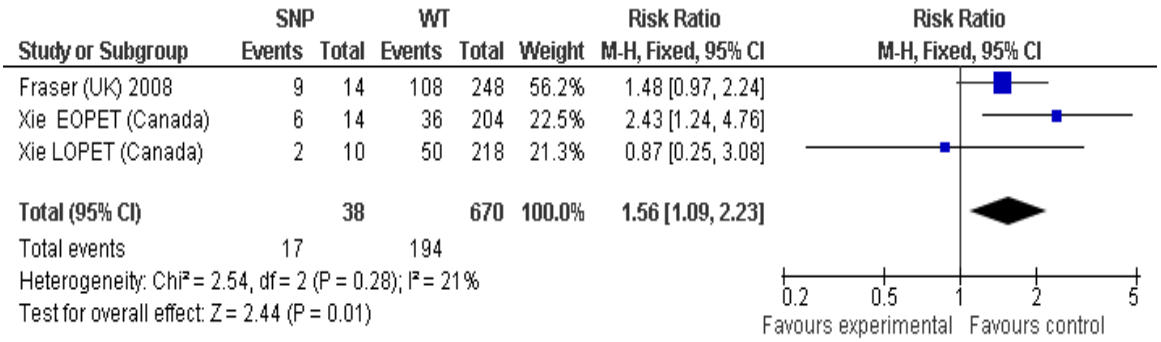
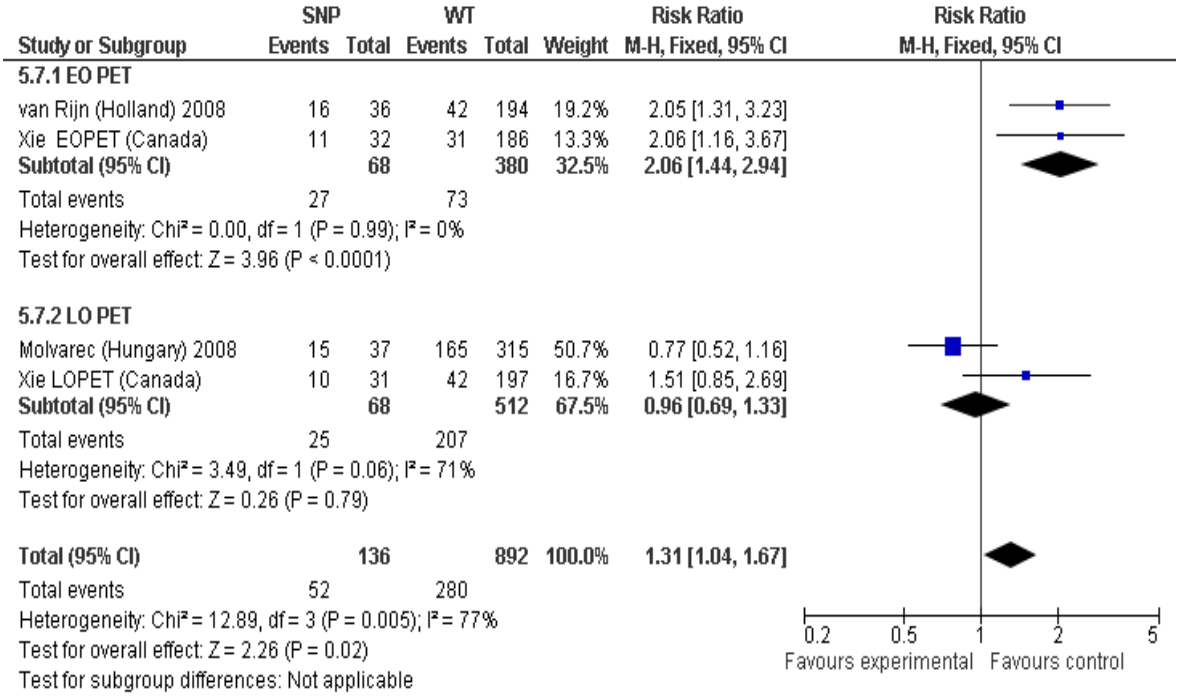


Figure 6.1 Toll like receptor signaling pathway.

TLRs recognize specific patterns of microbial components that are conserved among pathogens family. At present, there are 10 TLR family members that have been identified in humans. The binding of a microbial molecule to its TLR, transmits a signal to the cell's nucleus through a series of downstream cascade, ultimately culminate in the increased expression of immune and inflammatory response. All TLRs, except for TLR3, are thought to signal through a MyD88-IRAK-TRAF6 pathway to induce NF-κB kinases.



A



B

Figure 6.2 Data synthesis for the effects of TLR2 (A) and TLR4 (B) single nucleotide polymorphisms on the incidence of preeclampsia.

The effects of TLR2 (Arg753Gln) (a) and TLR4 (Asp299Gly/Thr399Ile) (b) single nucleotide polymorphisms on the incidence of pre-eclampsia. EO, early-onset; LO, late-onset; PET, pre-eclampsia; SNP, single nucleotide polymorphism; WT, wild type; Fixed: fixed effects model; CI, confidence interval; M-H, Mantel-Haenszell

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CHPATER 7: Summary and future directions

The immune mechanisms that contribute to the progression of preeclampsia are not completely understood. Previous studies observed an inflammatory response both in normal and preeclampsia pregnancy; and inflammation seems excessive in preeclampsia (1, 2). In addition, inflammation appears to play a critical role in modulating the endothelial dysfunction seen in the preeclampsia disease process (3). In this thesis, we examined the association between infection with *C. pneumoniae* and CMV and the differential presentation of preeclampsia (ie, early-onset versus late-onset and preeclampsia versus normotensive IUGR). Furthermore, the immune cellular receptors TLR2, TLR4 and cryopyrin, as well as down-stream effectors and inflammatory cytokine responses were investigated in normal and preeclampsia-complicated pregnancies. Furthermore, we have preformed experiments to identify TLR gene polymorphisms associated with the progression of preeclampsia. At each stage, systematic review and data synthesis methodology have been applied to place our findings with the context of current published studies in the area and to clarify apparent discrepancies among these studies.

7.1 *C. pneumoniae* and CMV Infection in preeclampsia

It has been postulated that infection may activate the maternal inflammatory process of preeclampsia, either through initiation and/or its enhancement (4) , or through direct effects on trophoblast cells (5). These hypotheses are supported by both clinical and epidemiologic data (6). In this present work, we applied both ELISA and quantitative real-time PCR methods to reveal that preeclampsia was associated with elevated *C. pneumoniae* genomic DNA loads as well as increased anti-CMV IgG antibody level compared with matched normal pregnancy controls. Additionally, systematic review suggested that preeclampsia was associated with increased anti-*C. pneumoniae* IgG seroprevalence. For CMV, the relevant data synthesis found a similar conclusion for anti-CMV IgG and/or positive CMV PCR. The completed data for our original design for *C. pneumoniae* and CMV serology and gDNA loads in early-onset preeclampsia, late-onset-preeclampsia, normotensive IUGR, matched normal pregnancy controls and non-pregnancy controls are listed in table 7.1 and 7.2.

Recently, the potential infectious mechanisms in preeclampsia have been studied extensively. First, preeclampsia is associated with a distinct pathologic lesion of the decidual

arterioles known as acute atherosis, which closely resembles atherosclerotic lesions of coronary arteries (7). Infectious agents such as *C. pneumoniae* and CMV can exert direct effects on atherogenesis by residing in the vascular wall, after being delivered to the vessel wall by circulating monocytes (8). Secondly, preeclampsia and atherosclerosis share similar risk factors, including dyslipidemia, insulin resistance, obesity, hypertension (9), and endothelial dysfunction, all of which involve inflammation. Maternal infections with *C. pneumoniae* and CMV may trigger release of pro-inflammatory cytokines into the maternal circulation, which may further enhance the already heightened level of inflammation observed in women with preeclampsia, resulting in endothelial cell dysfunction and oxidative stress (2, 3, 10). Third, as suggested by previous research findings (11, 12), *C. pneumoniae* and CMV infection can reduce trophoblast invasion into the uterine wall and this is associated with preeclampsia.

There are some limitations to these two related studies. First, all cases were identified in a single tertiary referral centre and our sample size was relatively small. The second limitation is that of heterogeneity. The patient population served by our centre consisted of three predominant ethnic groups, Caucasian, East Asian, and South Asian and 40% of the adult population was born outside Canada. Third, instead of a single pathogen, multiple pathogens may lead to the activation of inflammatory cells and genesis of cytokines that could exacerbate preeclampsia processes. This role of "pathogen burden" needs to be further studied. Therefore, to attain sufficient statistical power, our findings need to be confirmed in a larger, more representative obstetric population followed longitudinally, and address the additional confounders.

7.2 Immune pattern recognition receptors investigation in preeclampsia

The appropriate influx of innate immune cells into the endometrium is thought to be a prerequisite for successful implantation and pregnancy. However, elevated leukocyte infiltration and inappropriate activation may be an underlying cause of preeclampsia. Studies have demonstrated that following the ligation of TLRs by bacterial or viral products, trophoblast expression and secretion of chemokines is significantly increased and differentially modulated, and this in turn results in elevated and differential leukocyte chemotaxis (13).

The innate immune system detects the presence of microbial infection through germ line encoded PRRs. TLRs, retinoic acid-inducible gene-I-like receptors (RIG-1) and nucleotide-binding oligomerization domain (NOD) -like receptors (NLRs) serve as pattern recognition

receptors (PRRs) that recognize different but overlapping microbial components. They are expressed in different immune cellular compartments (such as the cell surface, endosome, lysosome or cytoplasm) and various immune cell types activate specific signaling pathways that lead to expression of genes that tailor immune responses to microbes (14).

In our study, we found preeclampsia was associated with elevated maternal circulatory neutrophil expression of PRRs including TLR2, TLR4 and cryopyrin, and increased pro-inflammatory cytokine responses. Our data supported the previous view that TLR signaling, the bridge for recognition of danger signals and a subsequent shift in the type of response generated may have harmful consequences for the pregnancy (13). Moreover, we are the pioneer study to examine the inflammasome, cryopyrin, finding a potential role in the immune systemic activation in this disorder.

In this present work, TLR2 and TLR4 expression have been first time tested on maternal neutrophils; however, other virus pattern receptor such as TLR3, 7, 9 and other important circulatory immune cell types are under investigation. In addition, caspases regulate apoptosis following activation of the cryopyrin inflammasome, and given the previous work of our group (15, 16), this needs to be further explored to determine the mechanistic insights into the origins of preeclampsia.

In the future, comprehensive examination of PRR expression and related inflammatory pathways in different immune cell types (such as monocytes, macrophage, NK-cells, and dendritic cells) may provide the additional data to allow identification of treatment targets for infectious agents, danger signals recognition, and the subsequent maternal inflammatory responses to prevent the harmful consequences for the pregnancy. Additionally, we need investigate other co-factors such as obesity or other co-infectious agents and variables in this thesis, as well as examine the interaction between those factors. Moreover, we hope to extend our study to examine the role of infection with *C. pneumoniae* and CMV in preterm birth in the future.

7.3 Inflammatory gene polymorphisms and preeclampsia

An altered inflammatory activity due to functionally relevant polymorphisms of the innate immune system may influence pregnancy outcomes, therefore, impact on the frequency of preeclampsia (17, 18). Some inflammatory genes proposed for a possible involvement in

placental abruption may be related to preeclampsia, which includes inflammatory gene polymorphisms on tumor necrosis factors (TNF) α (19, 20), interleukin (IL) 1β (21, 22), and CD14 (the receptor for lipopolysaccharide) (23, 24).

In this thesis, we reported an association between gene polymorphism in TLR2/ TLR4 and early-onset preeclampsia. This finding provided new evidence that alterations in the TLRs genetics of inflammatory system may modify the risk of preeclampsia, and are supported by the associated data synthesis through systematic reviewing of published literatures.

The SNPs of TLR2 and TLR4 that we chose to investigate appear to be functional (25, 26), although the pattern and direction of functional change is uncertain from published literatures (27). We reported an association between gene polymorphism in TLR2 and TLR4 and early-onset preeclampsia. Early-onset preeclampsia is more closely associated with inadequate placentation (28) than in late-onset disease. Our data indicated that TLR SNPs may not only alter leukocyte function, but also potentially influence the interaction between trophoblast and maternal immune surveillance in the first and early second trimester decidual (29, 30).

While we recognize that this study was limited by our inability to investigate the role of ethnicity in the TLR SNP preeclampsia relationship and the population prevalence of the genotype, in our study the mixed ethnicity was similar to two other studies, one in a Vancouver-based population (31) and another in a Caucasian population (32). Therefore, we are reassured that TLR2 and TLR4 genotype might be likely to be generalizable, but recognize that a larger, ethnically-defined, cohort is required.

With the advent of DNA microchips, which allow a simultaneous and rapid assessment of multiple genetic variants (33, 34), future larger sample-sized studies on the functional relevance of gene polymorphisms may draw a more accurate profile of the inflammatory gene variants involved in preeclampsia.

7.4 The increased likelihood of developing cardiovascular disease later in life following a preeclampsia pregnancy

C. pneumoniae, CMV, TLR, cryopyrin and TLR SNPs are potential contributors to the clinical phenotype, but not individually responsible for the pathogenesis of preeclampsia and cardiovascular disease. It widely accepted the clinical phonotype of preeclampsia is likely to be a

"multi-hit" phenomenon, the origins of which lie in many separate pathways converging to trigger the disease (6).

Maternal systemic inflammation and innate immune responses during pregnancy contribute to the medical conditions that not only predispose to preeclampsia, but also affect the long term prognosis of women with preeclampsia (35). They are, for example, several times more likely to develop cardiovascular diseases, making pregnancy an important way of screening for individual susceptibility to these conditions (36). It has been proposed that pregnancy is a metabolic stress test (37) that reveals constitutive tendencies, which become manifest in women's later life (Figure 7.1).

7.5 Conclusion

In conclusion, we found that infection with *C. pneumoniae* and CMV may play a role in the pathogenesis of preeclampsia. Activation of innate immunity PRRs through the NF-κB pathway may be responsible for the involvement of systemic inflammation in the development of preeclampsia. Moreover, TLR2 and TLR4 gene polymorphisms are correlated with early-onset of preeclampsia, which suggests the inflammatory gene mutations, may regulate the maternal inflammatory response in preeclampsia. We preformed this translational research to provide a pathophysiological link between infection, inflammation and innate immune mechanisms of preeclampsia, which may be linked with women cardiovascular disease in later life. Through a greater understanding the inflammatory and immunological facets of preeclampsia, we hope to spur improvements in prediction and prevention of this disorder and the subsequent cardiovascular disease.

Individual retrospective, some case-control and prospective studies have reported on the relationship between *C. pneumoniae* and CMV in association with preeclampsia. Our findings and combined results confirm these study findings. Although the exact cause of inflammation is not completely understood, *C. pneumoniae* and CMV infection are known to stimulate the innate immune system and may provide a biologically plausible explanation for preeclampsia-related inflammation. Further investigation of the effect of potential therapeutic agents and identification of anti-inflammatory targets will help with treatment of preeclampsia and lead to improved health care for both mother and fetus.

Table 7.1 Anti- *Chlamydia pneumoniae* seropositivity, antibody index and genomic DNA bacterial loads in early-onset, late-onset preeclampsia, nIUGR, normal pregnancy and non-pregnancy controls.

Variable	EOPET (n=25)	EOPET ctrl (n=28)	LOPET (n=25)	LOPET ctrl (n=29)	nIUGR (n=25)	nIUGR ctrl (n=27)	Non-Pregnancy (n=25)	P value (χ^2 or KW)
Anti- *C. pneumoniae* seropositivity								
IgG	15 (60%)	13 (48%)	17 (68%)	19 (66%)	15 (60%)	17 (63%)	19 (76%)	0.47
IgM	0 (0%)	0 (0%)	1 (4%)	0 (0%)	0 (0)	0 (0)	0 (0)	NA
IgA	18 (72%)	17 (61%)	20 (80%)	19 (66%)	17 (68)	18 (67)	15 (60)	0.85
Anti- *C. pneumoniae* antibody index								
IgG	0.56 [0.39, 2.17]	0.45 [0.14, 2.21]	0.57 [0.13, 1.59]	0.64 [0.15, 1.76]	0.59 [0.14, 1.90]	0.54 [0.11, 1.93]	0.60 [0.12, 1.58]	0.37
IgM	0.13 [0.03, 0.43]	0.12 [0.002, 0.31]	0.12 [0.01, 1.18]	0.13 [0.02, 0.38]	0.13 [0.03, 0.36]	0.12 [0.02, 0.51]	0.17 [0.01, 0.44]	0.39
IgA	2.00 [0.54, 4.51]	1.30 [0.45, 3.83]	1.88 [0.34, 4.11]	1.34 [0.43, 4.39]	1.60 [0.36, 4.56]	1.61 [0.35, 4.07]	1.16 [0.39, 3.54]	0.24
***Chlamydophila pneumoniae* gDNA**								
Measurable copies (n (%)	6 (24%)	7 (25%)	7 (28%)	6 (21%)	4 (16%)	5 (19%)	5 (20%)	0.96
gDNA load (copies/ml WB)	4650 [800, 36000]	2100* [600, 30000]	3800 [1000, 26000]	2500 [1020, 8000]	3000 [700, 6000]	2400 [600, 7000]	800* [600, 5400]	0.33

Note: EO: early-onset (<34^{+0} weeks); LO: late-onset (≥34^{+0} weeks); PET: preeclampsia; ctrl: control; nIUGR: normotensive intrauterine growth restriction; gDNA: genomic DNA; WB: whole blood; KW: Kruskal-Wallis ANOVA. Data are expressed as median [range] or n (%). Data were compared to calculate *Chi-squared* or *Mann-Whitney U* or Kruskal-Wallis ANOVA tests. * p< 0.05 (*Chi-squared* or *Mann-Whitney U test*: versus early-onset preeclampsia).

Table 7.2 Anti- cytomegalovirus seropositivity, antibody index and genomic DNA viral loads in early-onset, late-onset preeclampsia, nIUGR, normal pregnancy and non-pregnancy controls.

Variable	EOPET (n=25)	EOPET ctrl (n=28)	LOPET (n=25)	LOPET ctrl (n=29)	nIUGR (n=25)	nIUGR ctrl (n=27)	Non-Pregnancy (n=25)	P value (χ^2 or KW)
Anti- Cytomegalovirus antibody seropositivity								
IgG	17 (68)	10 (35)*	9 (36)*	9 (33)	12 (48)	10 (36)	8 (32)*	0.09
IgM	2 (8)	1 (3)	2 (8)	0 (0)	0 (0)	1 (4)	0 (0)	0.396
IgA	7 (28)	1 (4)*	2 (8)	1 (3)	1 (4)*	1 (4)	1 (4)*	0.007
Anti- Cytomegalovirus antibody index								
IgG	1.36 [0.1, 4.52]	0.40 [0.12, 2.87]	0.50 [0.09, 2.77]	0.53† [0.06, 2.56]	1.12 [0.03, 2.29]	0.53 [0.09, 2.27]	0.32 [0.08, 2.76]	0.048
IgM	0.35 [0.2, 1.32]	0.31 [0.2, 1.21]	0.22 [0.11, 1.12]	0.21 [0.15, 0.46]	0.25 [0.09, 0.57]	0.26 [0.10, 1.11]	0.22 [0.07, 0.78]	0.489
IgA	0.34 [0.07, 1.71]	0.16 [0.03, 1.52]	0.14† [0.02, 1.35]	0.11†† [0.03, 1.28]	0.13 [0.03, 0.56]	0.12†† [0.03, 0.55]	0.11† [0.02, 1.11]	0.002
Cytomegalovirus gDNA								
Measurable copies (n (%)	7 (28)	6 (20)	6 (24)	6 (21)	5 (20)	5 (19)	5 (20)	0.988
gDNA load (copies/ml WB)	15000 [5100, 24000]	3000 [2800, 3600]	10000 [4800, 16800]	4950†† [3200, 6600]	2700 †† § [1800, 3000]	3000 † [1000, 4800]	1300 ††† §§ [600, 3300]	<0.001

Note: EO: early-onset (<34^{+0} weeks); LO: late-onset (≥34^{+0} weeks); PET: preeclampsia; ctrl: control; nIUGR: normotensive intrauterine growth restriction; gDNA: genomic DNA; WB: whole blood; KW: Kruskal-Wallis ANOVA. Data are expressed as median [range] or n (%). * p<0.05 (Fisher's exact test, versus early-onset preeclampsia); † p<0.05; †† p<0.01; ††† p<0.001 (Dunn's test, versus early-onset preeclampsia); § p<0.05; §§ p<0.01 (Dunn's test, versus late-onset preeclampsia).

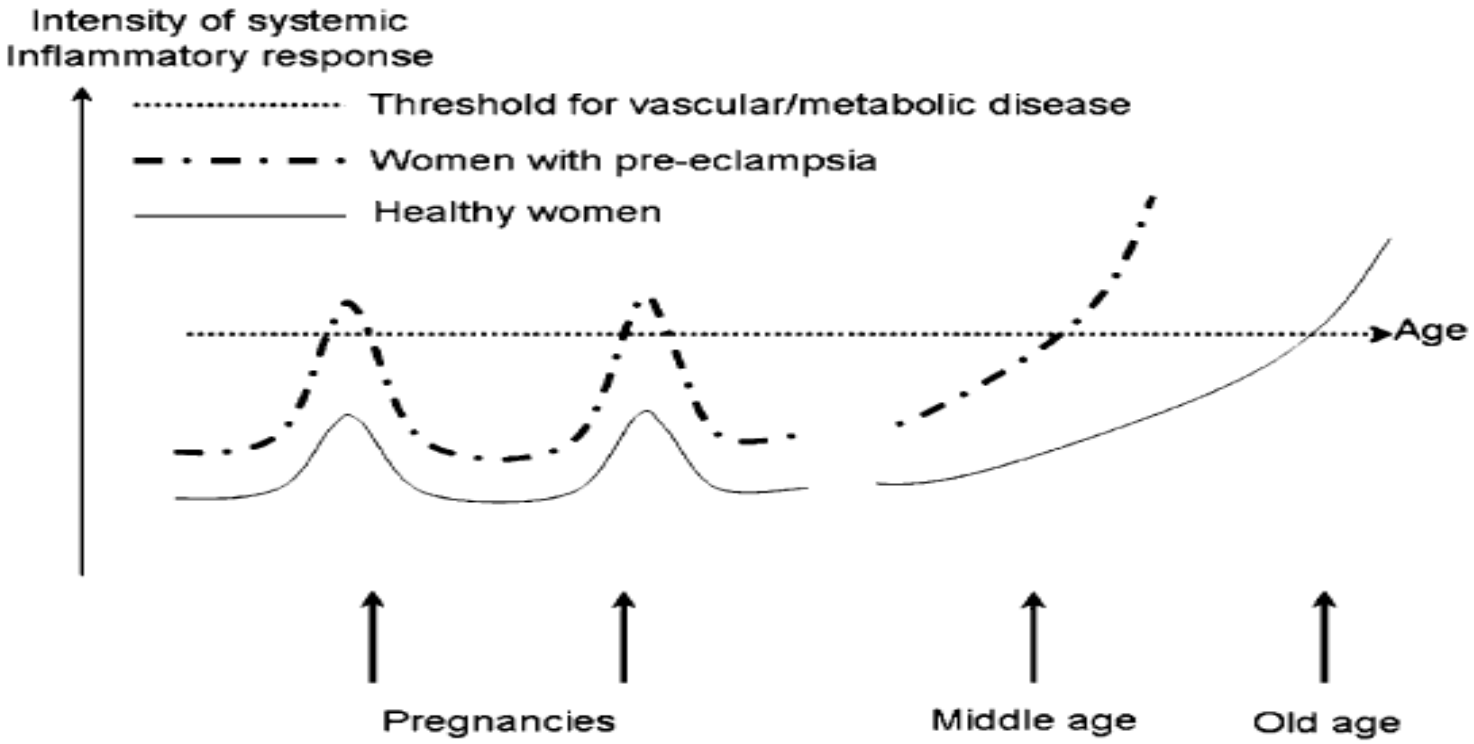

Figure 7.1 Pregnancy is a stress test revealing a propensity to medical conditions that will occur later in life.

The figure shows each pregnancy generates an inflammatory response that, in those with underlying medical conditions, may cause maternal responses to cross the thresholds for overt diseases which otherwise become apparent later in life. Adapted from Sattar and Greer (2002) (37).

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Appendix 1:

Consent form for collection of preeclampsia and intrauterine growth restriction (IUGR) patient blood samples

CHILDREN'S & WOMEN'S HEALTH CENTRE OF BRITISH COLUMBIA

INFORMATION SHEET AND CONSENT FORM

Patients with Early Onset of Pre-Eclampsia, Late Onset of Pre-Eclampsia and Intrauterine Growth Restriction (IUGR)

Project Title: Inflammation in Pregnancy and the Puerperium

Investigators:	Principal Investigator:	Dr. Peter von Dadelszen
	Co-Investigators	Dr. R. Liston Dr. L. Magee Dr. J. Russell Dr. D. Speert Dr. K. Walley Dr. D. Money

Emergency Contact: Dr. Peter von Dadelszen, Tel. 604-875-2424 ext 3054

Sponsor: Partial funding through Canadian Institute of Health Research (CIHR)

What are Pre-eclampsia and Intrauterine Growth Restriction (IUGR)?

You are being invited to take part in this research study because your pregnancy has been complicated with pre-eclampsia. Pre-eclampsia (i.e. high blood pressure, swelling and the detection of protein in the urine) is a common and sometimes dangerous complication of pregnancy. There are two syndromes in pre-eclampsia: maternal and fetal. The maternal syndrome defines the disease and may persist following the delivery of the baby and the placenta. Pre-eclampsia can have an early onset (less than or equal to 34 weeks gestation), or late onset (greater than or equal to 34 weeks gestation). The fetal syndrome is manifested by Intrauterine Growth Restriction (IUGR). IUGR means that by ultrasound, a baby is smaller than 95% of other babies at a particular stage in pregnancy. IUGR and pre-eclampsia share a problem with the formation of the placenta (afterbirth). We wonder why some women get high blood

pressure, while other women only have a small baby. We have some preliminary evidence to say that reactivated chronic infection with bacteria and viruses that are linked with heart disease and stroke may be important. Decisions about timing of delivery are hampered by incomplete knowledge of both maternal and fetal risks on a day to day basis.

What is the Study Design?

This study is designed to clarify how white blood cells and the inflammatory system function during pregnancies that are either normal or complicated by pre-eclampsia. The results of this investigation will be important to understand better both the reasons for and the extent of the disturbances that affect women with pre-eclampsia.

What will Participate in the Study Involve?

We are approaching you because you have either early onset of pre-eclampsia (less than or equal to 34 weeks gestation), late onset of pre-eclampsia (greater than or equal to 34 weeks gestation), or your pregnancy is at increased risk of being complicated by IUGR. If you choose to participate a blood sample (up to 30 ml, 2 Tablespoons) will be taken from an arm vein in the normal way, initially during pregnancy. Measurements would then be made on the responses of the white blood cells and the inflammatory system. The blood will also be tested for evidence of reactivated chronic infection with either cytomegalovirus (CMV) and/or Chlamydia pneumoniae (the bacterial implicated in cardiovascular disease). There will be 1 blood test prior to delivery. Having blood drawn can produce some discomfort, redness or bruising at the site where the blood was drawn. Where possible these blood tests will be taken at the same time as clinically indicated blood tests, to reduce your discomfort and inconvenience.

Your confidentiality will be respected. No information that discloses your identity will be released or published without your specific consent to the disclosure. However, research records and medical records identifying you may be inspected in the presence of the Investigator or his designate by representatives of Health Canada, and the UBC Research Ethics Board for the purpose of monitoring the research. However, no records which identify you by name or initials will be allowed to leave the Investigators offices. It is unusual to include your name, date of birth, or initials on research records and material forwarded to others. Most studies submit information identified by code numbers or letters only.

Please keep this information sheet until your pregnancy is completed so that you can show it to anyone who needs to know more about who has been taking blood tests from you, and why.

Contact

If you have additional enquiries please contact Dr. Peter von Dadelszen, Maternal Fetal Medicine, at 604-875-2424 ext. 3054. If you have any concerns about your treatment or rights as a research subject, please call the "Research Subject Line", University of British Columbia (UBC) Office of Research Services, at (604) 822-8598.

Consent Form:

- I acknowledge that the research study described on the attached form, and of which I have been provided with a **signed and dated** copy, has been fully explained to me, and that any questions or concerns have been answered to my satisfaction.
- I know that I may ask now, or in the future any questions that I may have about the study or the research procedures.
- I have been assured that all records will be kept confidential, as study numbers only will be utilised with no patient names, initials or hospital numbers. No information will be released or printed that would disclose my identity.
- I understand that I am free to refuse to enter as well as withdraw from the study at any time. I also understand that if I refuse to enter or withdraw from the study, my medical care will not be affected.
- I do not waive any of my legal rights by signing the consent.
- I will not be offered any monetary compensation.

I hereby consent to participate in the study.

Name of Patient (please print)

Signature of Patient **Date:**

Name of Witness (please print)

Signature of Witness **Date:**

Name of Principal Investigator or the designee

Signature of Principal Investigator or the designee **Date:**

Appendix 2:

Consent form for collection of normal pregnancies and non- pregnant women blood samples

INFORMATION SHEET AND CONSENT FORM

(Women with Normal Pregnancies and Non-Pregnant Women)

Project Title: **Inflammation in Pregnancy and the Puerperium**

Investigators:	Principal Investigator:	Dr. Peter von Dadelszen
	Co-Investigators:	Dr. M-F. Delisle Dr. A. Gagnon Dr. R. Liston Dr. L. Magee Dr. G. Quamme Dr. J. Russell Dr. D. Speert Dr. K. Walley

Emergency Contact: Dr. Peter von Dadelszen, phone 604 875-2424 ext 7913, pager # 41-01505

Clinical Trial Coordinator: Pamela Lutley, phone 604-875-2424 ext 6359, pager # 41-01339

Sponsor: Partial funding through Canadian Institute of Health Research (CIHR)

What are Pre-eclampsia and Intrauterine Growth Restriction (IUGR)?

You are being invited to take part in this research study as a control subject. Pre-eclampsia (i.e. high blood pressure, swelling and the detection of protein in the urine) is a common and sometimes dangerous complication of pregnancy. There are two syndromes in pre-eclampsia: maternal and fetal. The maternal syndrome defines the disease and may persist following the delivery of the baby and the placenta. Pre-eclampsia can have an early onset (less than or equal to 34 weeks gestation), or late onset (greater than or equal to 34 weeks gestation). The fetal

syndrome is manifested by Intrauterine Growth Restriction (IUGR). IUGR means that by ultrasound, a baby is smaller than 95% of other babies at a particular stage in pregnancy. IUGR and pre-eclampsia share a problem with the formation of the placenta (afterbirth). We wonder why some women get high blood pressure, while other women only have a small baby. We have some preliminary evidence to say that reactivated chronic infection with bugs that are linked with heart disease and stroke may be important. Decisions about timing of delivery are hampered by incomplete knowledge of both maternal and fetal risks on a day to day basis.

What is the Study Design?

This study is designed to clarify how white blood cells and the inflammatory system function during pregnancies that are either normal or complicated by pre-eclampsia. The results of this investigation will be important to understand better both the reasons for and the extent of the disturbances that affect women with pre-eclampsia. In pre-eclampsia, we will develop a severity score to help clinicians make management decisions.

What will Participating in the Study Involve?

1. For Women with Normal Pregnancies:

We are approaching you because we have recently investigated a woman with either pre-eclampsia and/or IUGR who is your age, is also having her first/second/third/fourth child, and at the time of her test, was a similar number of weeks pregnant.

2. For Non-Pregnant Women:

We are approaching you because you are in good health and you are not taking the oral contraceptive pill which would confer a pseudopregnant state.

For pregnant women, the blood samples of up to 30 ml (up to 2 Tablespoons) will be taken from an arm vein in the normal way once during pregnancy, and once whilst you are in the hospital following delivery (1, 2, or 3 days after delivery). Measurements would then be made on the responses of the white blood cells and the inflammatory system. The blood will also be tested for evidence of reactivated chronic infection with either Cytomegalovirus (CMV) and/or Chlamydia pneumoniae (the bugs implicated in cardiovascular disease). There will be 1 blood test prior to delivery and 1 blood test after delivery (day 1, 2 or 3 after delivery). Having blood drawn can produce some discomfort, redness or bruising at the site where the blood was drawn.

For the non-pregnant women, there will be one blood sample of up to 30 ml, (up to 2 Tablespoons) taken from an arm vein in the normal way. Having blood drawn can produce some discomfort, redness or bruising at the site where the blood was drawn.

Your confidentiality will be respected. No information that discloses your identity will be released or published without your specific consent to the disclosure. However, research records and medical records identifying you may be inspected in the presence of the Investigator or his designate by representatives of Health Canada, and the UBC Research Ethics Board for the

purpose of monitoring the research. However, no records which identify you by name or initials will be allowed to leave the Investigators offices. It is unusual to include your name, date of birth, or initials on research records and material forwarded to others. Most studies submit information identified by code numbers or letters only.

Please keep this information sheet until your pregnancy is completed so that you can show it to anyone who needs to know more about who has been taking blood tests from you, and why.

Contact:

If you have additional enquiries please contact Dr. Peter von Dadelszen, Maternal Fetal Medicine, at 875-2424 ext 7913 or Pamela Lutley, Clinical Trial Coordinator at 875-2424 ext 6359 or page 41- 01339.

If you have any concerns about your treatment or rights as a research subject, please contact the "Research Subject Information Line", University of British Columbia (UBC) Office of Research Services, at (604) 822-8598.

Consent Form:

- I acknowledge that the research study described on the attached form, and of which I have been provided with a copy, has been fully explained to me; and that any questions or concerns have been answered to my satisfaction.

- I know that I may ask now, or in the future, any questions that I may have about the study or the research procedures.

- I have been assured that all records will be kept confidential as study numbers only will be utilised with no patient names, initials or hospital numbers. No information will be released or printed that would disclose my identity.

- I understand that I am free to refuse to enter as well as withdraw from the study at any time. I also understand that if I withdraw from the study my medical care will not be affected.

- I do not waive any of my legal rights by signing the consent.

- I will not be offered any monetary compensation.

- I hereby consent to participate in the study.

Signature of Subject **Date:**

Name of Subject (please print)

Signature of the PI or the Designee **Date:**

Name of Principal Investigator or the Designee (please print)

Appendix 3:

Consent form for collection of patients blood samples for gene polymorphism study

CHILDREN'S & WOMEN'S HEALTH CENTRE OF BRITISH COLUMBIA

CONSENT FOR GENETIC RESEARCH
(addendum to information and consent forms for non-genetic pre-eclampsia, normotensive intrauterine growth restriction, normal pregnancy, and non-pregnancy study participants)

Title: Do gene polymorphisms, confined placental mosaicism, or chronic infection predispose to the maternal syndrome of pre-eclampsia?

Investigators:	Principal Investigator:	Dr. Peter von Dadelszen
	Co-Investigators:	Dr. R. Liston Dr. L. Magee Dr. W. Robinson Dr. S. Langlois Dr. D. Speert Dr. C.D. MacCalman Dr. A. Currie Dr. E. Thomas

24 hour Contact: Dr. Peter von Dadelszen, Tel. (604) 875-3054

Introduction:

You are being invited to take part in this research study. In addition to the main objectives of this study, we would like your permission to store indefinitely samples of genetic material extracted from your blood and/or your baby's(ies') afterbirth ('placenta'), if you are pregnant. You are still eligible to take part in other aspects of this research study, even if you do not want genetic testing preformed on your blood sample.

We have recently found evidence that pre-eclampsia may be related to either inappropriate inflammation in either the mother's bloodstream or in the bed of the placenta, an abnormal number of chromosomes limited to the placenta, or the presence of chronic infection in some white blood cells. We wish to continue with these lines of inquiry, and hope that you will be able to help us.

Purpose of the study:

One purpose of this study is to examine the effects of inherited genes on why some women develop pre-eclampsia and/or the systemic inflammatory response syndrome at some time in their life, and whether the two conditions are related. Another purpose of the study is to find if there is an association between the number of genes (chromosomal trisomy) and pre-eclampsia. A third purpose of the study is to determine if living with a long-term infection with either of two infectious organisms could increase a woman's risk for developing pre-eclampsia. As a result, this study will help us to understand which genes are protective and which ones are harmful when people are exposed to a particular biological stress, such as pregnancy, infection, trauma or a surgery.

Your participation is voluntary

Your participation is entirely voluntary, so it is up to you to decide whether or not to take part in this study. Before you decide, it is important for you to understand why the research is being done and what it will involve. This consent form will tell you about the study and why the research is being done, what it will involve, and the possible benefits, risks and discomforts to help you decide whether or not you wish to take part. If you wish to participate, you will be asked to sign this form. If you do decide to take part in this study, you are still free to withdraw at any time without giving any reasons for your decision. If you do not wish to participate, you do not have to provide any reason for your decision not to participate nor will you lose the benefit of any medical care to which you are entitled or are presently receiving. Please take time to read the following information carefully and to discuss it with your family and your doctor before you decide.

Who is conducting the study?

The study is being conducted by the Pre-Eclampsia Research group at Children's and Women's Health Centre of BC. Parts of this research have been funded by the Canadian Institutes of Health Research (CIHR).

Description of the research:

If you agree to participate in the study, 25 ml (two tablespoons) of blood will be drawn in the normal way ('venepuncture'). We will always attempt to time the research blood taking at the same time of routine blood tests. Your blood sample will be used as a source of genetic material (DNA) for study.

If you agree, the placenta will be collected at the time of delivery. Two samples of 1-2cm diameter from the baby's ('fetal') side of the placenta will be required. It is routine for the placenta to be examined by a pathologist after birth. The placental tissue will not be used for anything else other than for the proposed experiments. DNA will be extracted and used for the purpose of screening for chromosome aneuploidy (extra or missing chromosomes). RNA, a code that determines the proteins that are formed by cells, will be extracted and used to test the expression of a variety of proteins involved in cell growth and function. All these tests are meant

to identify features which may be associated with pre-eclampsia placentas. The information obtained from your genetic material will be used together with your clinical information using a code system to protect your confidentiality. Insurance companies, their agents and employee will have NO access to this information. Scientists will then be able to conduct studies on genes (human, viral, and bacterial) that may either increase or decrease a person's lifetime risk of developing an illness due to inappropriate inflammation.

Potential harms and informational risks:

Only genes thought to be related to either the systemic inflammatory response syndrome or pre-eclampsia will be studied. Some people are understandably concerned that genetic information acquired about them can be misused by third parties. These concerns include things like being denied access to employment and insurance.

The samples will be coded with a number upon receipt and the list of names will be kept under lock and key in the office of the principal investigator. A unique ID code will be used to identify each patient's current and past medical history (phenotypic data) and gene testing results (genotypic data). Once genotypic and phenotypic data are linked, all personal identifiers will be removed from all paper and computer records associated with the study other than for the single master list. Therefore, the samples are identifiable only to the investigators. The names of the subjects will not be used in any discussion or correspondence about the data. Some of the information obtained from this study will be used in scientific publications, but the identity of the subjects will not be revealed. The findings of this study will not appear in medical records or patient charts. As it is often difficult to anticipate future advances in science that open up new research questions, leftover DNA may be banked for further genetic research related to the general objectives of this proposal that may arise in the future. DNA will be stored in the Genetic Sub-study Laboratory, located in the BC Research Institute for Children's and Women's Health, located in Vancouver, BC, labelled with the code, until it is used entirely or until such DNA is withdrawn. The laboratory is in a secure building accessible by key card only. No further consent will be sought for these future studies.

The risks associated with this blood withdrawal are minimal and include discomfort, bruising or infection at the site of needle insertion. This procedure is not associated with any long-term pain or complications.

Benefits:

Participating in this study will not benefit you directly. However, your participation in this research may help people with septic shock (low blood pressure associated with infection) or preeclampsia in the future.

Confidentiality:

Your confidentiality will be respected. No information that discloses your identity will be released or published without your specific consent to the disclosure. All information that is obtained, either directly from you or from your hospital chart will be dealt with in a strictly confidential manner. The information will be entered into a special file. This file will be identified by a code-number instead of by your name. Only the researchers will have access to the code. However, research records and medical records identifying you may be inspected in the presence of the Investigator or his or her designate by representatives of Health Canada and the UBC Research Ethics Board for the purpose of monitoring the research. However, no records which identify you by name or initials will be allowed to leave the Investigators' offices.

Sample Storage:

Your blood samples and placental samples will be labelled with a random bar code number and stored in a secure storage freezer. No genetic information will be returned to your doctor, and your medical records will not include results of genetic assessments of the samples you contributed.

Processing of personal data:

Either your doctor or a research nurse will record your study information on forms that he/she will send to the central data centre. When the central data centre receives your study information, it will be stored and analysed using a computer. The data centre will forward the clinical information, stripped of your identification along with your genetic material to the Genetic Sub-study Laboratory. In this way, your genetic results cannot be linked or traced to you. Your name will not be in any publication.

Commercial issues:

The results of this genetic research might be valuable for commercial and/or intellectual property (patent) purposes. For example, your genetic information may be used as part of an effort to develop a drug, or to develop a diagnostic test. In so doing, the scientists involved in the Genetic Sub-study may cooperate with other research companies for the same reasons. You will not receive any compensation with respect to any commercial activities related to your genetic material including any interest in or share of any profits derived from the sale of any commercial test or treatment made possible by your genetic material. The Genetic Sub-study scientists retain sole ownership of the research results, and of any use or development of the research records (including your sample).

Voluntary participation / right to withdraw your permission for genetic testing:

You are free to withdraw from the study at any time and your medical care will not be affected. Your blood and placenta samples will be identified and destroyed. When this study is completed, the codes that link the samples to you will be destroyed at which point there is no way your sample can be identified and hence retrieved.

Questions about the study or your rights:

If you have any questions or concerns about this study, please contact Dr. Peter von Dadelszen at (604) 875-3054. If you have any questions regarding your treatment as a research subject, please call the Research Subject Information Line in the University of British Columbia (UBC) Office of Research Services, at (604) 822-8598.

Costs:

There are no costs related to this research study.

New Findings:

If, during the study period, any new information becomes available that may affect your willingness to remain in this study, you will be informed in a timely manner.

Subject Consent:

- I have read and understood the patient information and consent form.
- I have had sufficient time to consider the information provided and to ask for advice necessary.
- I have had the opportunity to ask questions and have had satisfactory responses to my questions.
- I understand that all of the information collected will be kept confidential and that the result will only be used for scientific objectives.
- I understand that my participation in the study is voluntary and that I am completely free to refuse to participate or to withdraw from this study at any time without changing in any way the quality of care that I receive. All DNA samples will be destroyed at that time.
- I understand that I am not waiving any of my legal rights as a result of signing this consent form.
- I understand that there is no guarantee that this study will provide any benefits to me (if applicable).
- I have read this form and I freely consent to participate in this study.
- I have been told that I will receive a dated and signed copy of this consent from for my records.
- I hereby consent to participate in the study.

I consent for the DNA banking blood	**Initials:**
I consent for the banking of the placental tissue	**Initials:**

Subject Signature **Date**

Subject print name

Witness signature **Date**

Witness: print name

Investigator's signature **Date**

Investigator: print name

Appendix 4: Ethical approval form

June 7, 2004

Certificate of Approval

PRINCIPAL INVESTIGATOR	DEPARTMENT	NUMBER
Von Dadelszen, Peter		W01-0035

CO-INVESTIGATORS:

Magee, Laura; Liston, Robert; Wilson, Douglas; Speert, David; Delisle, Marie-France; Gagnon, Alain; Quamme, Gary

C&W DEPARTMENTS, PATIENT BASED PROGRAMS AND ADMINISTRATIVE JURISDICTIONS IMPACTED BY THIS STUDY:

Pathology; Records Management; Birthing Program; Diagnostic/Ambulatory Program; Decision Support Services;

SPONSORING AGENCIES:

BCRICWH Establishment Award

TITLE

Inflammation in pregnancy and puerperium

TERMS OF RENEWAL APPROVAL	AMENDMENT:	AMENDMENT APPROVED:
June 7, 2004 - May 27, 2005	Consent form dated April 20, 2004	June 7, 2004

CERTIFICATION:

The protocol for the above-named project have been reviewed by the Research Review Committee and has been found to be appropriate with respect to ethics, methodology, patient impact and availability of C&W resources

Approval of the C&W Research Review Committee
Dr. M. Levine, Chair

This Certificate of Approval is valid for the above term provided there is no change in the research protocol

CHILDREN'S & WOMEN'S HEALTH
CENTRE OF BRITISH COLUMBIA
Research Review Committee

Room 202, 950 West 28th Avenue
Vancouver, BC V5Z 4H4
Phone: 604-875-3103
Fax: 604-875-2496

May 25, 2007

Certificate of Approval
-- Renewal --

PRINCIPAL INVESTIGATOR	Department	Certification Number
Von Dadelszen, Peter	Obstetrics & Gynecoloty	CW01-0035

CO-INVESTIGATORS:
Speert, David; Magee, Laura A.; Liston, Robert; Wilson, Douglas R. D.; Quamme, Gary A.;

SPONSORING AGENCIES:
BC Research Institute for Children and Women's Health;

CW DEPARTMENTS, PROGRAMS AND ADMINISTRATIVE JURISDICTIONS IMPACTED BY THIS STUDY
Pathology and Laboratory Medicine; Records Management and Patient Registration; Birthing Program; Diagnostic/Ambulatory Program; Decision Support;

PROJECT TITLE
Inflammation in pregnancy and puerperium

TERMS OF RENEWAL
May 25 2007 - May 17 2008

CERTIFICATION:

The protocol for the above-named project has been reviewed by the Research Review Committee and has been found to be appropriate with respect to ethics, methodology, patient impact and availability of C&W resources

Approval of the C&W Research Review Committee
Dr. M. Levine, Chair
Dr. M. Bond, Associate Chair

This Certificate of Approval is valid for the above term provided there is no change in the research protocol