The maternal predisposition to the syndrome of pre-eclampsia

By

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Abstract

Objectives

Pre-eclampsia, which is characterized by maternal hypertension, proteinuria, hypoperfusion of end organs and a systemic maternal innate inflammatory response, is a leading cause of maternal mortality and morbidity world-wide. When of early-onset, pre-eclampsia is associated with fetal intrauterine growth restriction (IUGR). IUGR can occur in isolation, so-called normotensive IUGR. What is poorly understood is that some women develop the maternal syndrome of pre-eclampsia whilst others have only the fetal syndrome (normotensive IUGR), despite the fact that the initiating event in both is believed to be reduced uteroplacental perfusion.

In this thesis I have asked two questions:

First, could the maternal innate inflammatory response of pre-eclampsia predict an increased lifetime risk for developing the systemic inflammatory response syndrome (SIRS), as it is with later atherosclerosis (another disease of inflammation)? My hypothesis was the maternal syndrome of pre-eclampsia is a form of the systemic inflammatory response syndrome.

Second, could it be that the maternal syndrome of pre-eclampsia is triggered by a reactivation of chronic infections common in the community, mainly infection with *Chlamydia pneumoniae* (*Chl pneumoniae*) or cytomegalovirus (CMV) infections? This is especially pertinent as these infectious agents have been implicated in the development of atherosclerosis. We wanted to determine whether or not the dichotomous response of pre-eclampsia and normotensive IUGR could be explained by reactivation of chronic infection with *Chl pneumoniae* or CMV during pre-eclampsia, but not normotensive IUGR.

My hypothesis was, could reactivated infectious trigger explain the differential maternal response to the shared placental pathology of pre-eclampsia and
normotensive growth restriction? This would provide a link between pre-eclampsia and its attendant lifelong risk of atherosclerosis.

**Study designs**

SIRS.

Cases were selected from women admitted to the intensive care unit (ICU) of St. Paul’s Hospital with the diagnosis of SIRS. Controls were women without SIRS, admitted to general medical/surgical wards with the same primary diagnosis and same underlying problem (eg: pneumonia, bowel resection) but unremarkable in-hospital course (NO SIRS). The controls were matched for both age (±5 years) and ethnicity.

*Chl pneumoniae* and CMV.

Seroprevalence and levels of anti-CMV and *Chl pneumoniae* IgG were compared in a nested case-control study. We compared between women with early-onset pre-eclampsia (<34 weeks'; n=9), late-onset pre-eclampsia (≥34+0 weeks'; n=29); normotensive IUGR (birthweight <3rd centile; n=33), and matched normal pregnancy (n=113, up to 2 per case).

**Results**

SIRS.

Most women in the ICU were too critically ill to be interviewed. Therefore, this study was deemed not to be feasible and was abandoned because of the low frequency of female admissions to the St Paul’s ICU and, insufficient obstetric information.

*Chl pneumoniae* and CMV.

There was a significant difference in both anti-CMV and *Chl pneumoniae* EB antibodies between groups (Kruskal-Wallis p<0.05). Women with early-onset pre-eclampsia had higher anti-CMV levels (median: 79 [95% confidence interval 47, 164]) than women with late-onset pre-eclampsia (26 [22, 82], p<0.05), normotensive IUGR (40 [31, 72], p<0.05), and normal pregnancy (49 [45, 70], p<0.05). Women with
normotensive IUGR had significantly lower anti-\textit{Chl pneumonai}e antibodies (0.10 [0.08, 0.38]) than did normal pregnancy controls (0.21 [0.20, 0.28], p<0.05).

**Conclusions**

SIRS.

We were unable to test the hypothesis as the study, as designed, was not feasible.

\textit{Chl pneumonai}e and CMV.

Women with early-onset pre-eclampsia had higher levels of IgG against \textit{Chl pneumonai}e and anti-CMV anti-bodies in their serum than in late-onset pre-eclampsia, normotensive IUGR, and normal pregnancy. This may provide a pathophysiological link between pre-eclampsia and the known increased risk for subsequent atherosclerosis.

Could a reactivated infectious trigger explain the differential maternal response to the shared placental pathology of pre-eclampsia and normotensive intrauterine growth restriction? In addition, could the association between the maternal IgG response to \textit{Chl pneumonai}e and CMV provide a link between pre-eclampsia (especially of early onset) and its attendant lifelong risk of atherosclerosis.

Early-onset pre-eclampsia may truly be the 'toxaemia' of pregnancy.
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Chapter 1

General introduction:

Maternal predisposition to the syndrome of pre-eclampsia

Inflammation in itself is not to be considered as a disease ... and in a disease, where it can alter the diseased mode of action, it likewise leads to a cure; but where it cannot accomplish that salutary purpose, it does mischief.

John Hunter; Treatise on the blood, Inflammation and Gunshot Wound, London, 1794
Background

1. Pre-eclampsia

1.1 Clinical description

Pre-eclampsia is a disorder specific to pregnancy. Pre-eclampsia/eclampsia is the main cause of maternal mortality and is associated with a five-fold increase in perinatal mortality in developing countries (1). It remains one of the two commonest causes of maternal mortality in the developed world (2-7). Pre-eclampsia is estimated to affect 7% to 10% of all pregnancies in the United States (8). Despite being one of the leading causes of maternal death and a major contributor of maternal and perinatal morbidity and its importance for the public health, the aetiology of pre-eclampsia has not yet been clearly established.

According to the International Society for the Study of Hypertension in Pregnancy, pre-eclampsia is present when a previously healthy woman develops hypertension combined with proteinuria after the 20th week of gestation (9). In the USA, pre-eclampsia is defined by the National High Blood Pressure Education Program (NHPBEP) criteria, as a pregnancy-specific increase in blood pressure (diastolic blood pressure (dBP)>90mmHg, or systolic blood pressure (sBP)>140mmHg) associated with >0.3g proteinuria/d (or protein: creatinine ratio >30mg protein/mmol creatinine on random sampling (10)). Pre-eclampsia resolves after delivery (11).

There are two syndromes in pre-eclampsia (Figure 1.1): the first is maternal, characterized clinically by hypertension and proteinuria and, pathologically by endothelial cell activation (12) and systemic inflammation (13). The second is fetal, manifested most commonly, by intrauterine growth restriction (IUGR) (14;15).
The maternal clinical signs and symptoms defines pre-eclampsia, given that incomplete placental implantation is also a feature of normotensive IUGR (17). IUGR can occur in isolation ('normotensive IUGR'), which is the fetal syndrome of pre-eclampsia in isolation (18) and shares the same underlying placental pathology (17), but lacks the maternal response in terms of either hypertension and proteinuria or systemic inflammation (19). The origins of pre-eclampsia and normotensive IUGR lie in a mismatch between fetoplacental demands and the maternal vascular supply ('uteroplacental mismatch' (20)), but result in dichotomous clinical syndromes (21).

Pre-eclampsia is associated with an increased incidence of IUGR. Infants who survive IUGR have an increased risk of both physical and mental handicap, and an increased risk of mortality in adult life from cardiovascular (22) and endocrine diseases (23;24).
The pathology of pre-eclampsia is expressed at three levels (25). The primary pathology is not yet known but is at least associated with the presence of trophoblast. Gestational trophoblastic disease, such as hydatidiform mole, is an abnormality in which the pregnancy contains very little or no fetal tissue with the products of conception being almost exclusively hyperplastic trophoblast. It is associated with an increased risk of pre-eclampsia. The secondary pathology of pre-eclampsia is the maternal adaptation to the impaired endovascular trophoblast invasion and it includes the defining signs of pre-eclampsia: hypertension and proteinuria. Under certain circumstances, the peripheral disturbances of pre-eclampsia can become so severe that they themselves initiate new or tertiary pathology. The most significant manifestation of tertiary pathology are the convulsions of eclampsia, cerebral haemorrhage, renal failure and the HELLP (haemolysis, elevated liver enzymes, low platelets) syndrome (26).

1.2 Pathophysiology of pre-eclampsia

The mechanisms responsible for the pathogenesis of pre-eclampsia are unclear. Hypertension associated with pre-eclampsia develops during pregnancy and resolves after delivery, implicating the placenta as a central culprit in the disease (27). It has been suggested that release of factors from the placenta in response to ischaemia results in endothelial dysfunction of the maternal circulation (12;28;29). The vascular endothelium has many important functions, including control of smooth muscle tone through release of vasoconstrictor and vasodilatory substances, and regulation of anticoagulation, antiplatelet, and fibrinolysis functions via release of different soluble factors. Evidence of endothelial dysfunction as an early event in pre-eclampsia suggests that it is a possible cause (12).

Additionally, in women who develop pre-eclampsia, preexisting maternal factors such as chronic hypertension, diabetes, and hyperlipidaemia may predispose the maternal
endothelium to further damage. Increased sensitivity to pressor agents and activation of the coagulation cascade occur early in the course of pre-eclampsia (12). Many markers of endothelial dysfunction have been reported in women who develop pre-eclampsia, suggesting that pre-eclampsia is an endothelial cell disorder. Circulating levels of fibronectin, plasma thrombomodulin and von Willebrand factor are significantly elevated in women with pre-eclampsia (30). Platelets also appear to play an important role in the etiology of pre-eclampsia. Enhanced platelet activation, and increased levels of platelet endothelial cell adhesion molecule-1 (PECAM-1), intercellular cell adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) also occur in women who develop pre-eclampsia (12;28;29). Many markers of endothelial dysfunction may function as predictors of the syndrome in women who develop pre-eclampsia as many are significantly elevated weeks before observing of clinical manifestations.

Studies over the past decade have provided a better understanding of the potential mechanisms responsible for the pathogenesis of pre-eclampsia. The initiating event in pre-eclampsia has been postulated to be reduced uteroplacental perfusion. This results in abnormal cytotrophoblast invasion of spiral arterioles leading to placental ischaemia. This ischaemia is widely cited as a key factor (29;31-33) and, with the placenta becoming increasingly ischaemic as gestation progress, it is thought to lead to widespread activation/dysfunction (Figure 1.2) of the maternal vascular endothelium (34). It is unclear what triggers this endothelial disturbance in pre-eclampsia, but neutrophil activation can result in release of a specific elastase, capable of mediating vascular damage by destroying the integrity of endothelial cells (35;36).

Recent finding are more suggestive of an inappropriate maternal inflammatory response within the framework of placentation. The innate immune system appears to be primarily
involved. Natural killer cells could be regulatory cells pushing maternal immune response toward a Th2 profile, beneficial for fetal survival, or toward a Th1 type of immune response, which acts in synergy (37). The exaggeration of this maternal intravascular inflammatory reaction to the invading trophoblast leads to the manifestation of pre-eclampsia in the mother and feto-placental unit (16). The most popular model for the pathogenesis of the maternal syndrome of pre-eclampsia describes a process by which the injured placenta communicates with the maternal endothelium, causing a syndrome of systemic endothelial cell dysfunction (Table 1.1) and end organ hypoperfusion (12) which leads to multiorgan dysfunction.

Inadequate delivery of blood to the pregnant uterus can cause a consistent rise in blood pressure and hypoxia could initiate the placental injury (38). Likewise, the renal morphologic changes present in women with pre-eclampsia are not found in any other form of hypertension. Furthermore, other pathophysiological changes, activation of coagulation cascade, increased sensitivity to pressors, reduced plasma volume, and abnormalities of renal proximal tubular function, all antedate increased blood pressure (12).

Table 1.1: Biochemical and morphological evidence supporting the concept of endothelial cell injury in pre-eclampsia Modified From Bolte et al. (39).

<table>
<thead>
<tr>
<th>Biochemical</th>
<th>Morphological</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VIII R:Ag ↑</td>
<td>Glomerular endotheliosis</td>
</tr>
<tr>
<td>Fibronectin ↑</td>
<td>Ultrastructural changes in placental Bed and uterine boundary vessels</td>
</tr>
<tr>
<td>PAI-1/PAI-2 ratio ↑</td>
<td>Subendocardial necrosis</td>
</tr>
<tr>
<td>Disturbance of tPA/PAI-1 balance</td>
<td></td>
</tr>
<tr>
<td>Disturbance of PGI₂/TXA₂ balance</td>
<td></td>
</tr>
<tr>
<td>Endothelin ↑</td>
<td></td>
</tr>
<tr>
<td>Thrombomodulin ↑</td>
<td></td>
</tr>
<tr>
<td>Growth factor activity ↑</td>
<td></td>
</tr>
</tbody>
</table>

PAI: plasminogen activator-inhibitor; PGI₂: prostacyclin; R:Ag: related antigen; tPA: tissue plasminogen activator; TXA₂: thromboxane
Figure 1.2: A model for the pathogenesis of pre-eclampsia. In this model of pre-eclampsia, 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 caused, in part, by 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 pre-eclampsia. 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 (not illustrated). Activated protein C could modify both the inflammatory and coagulation consequences of the endothelial cell activation. PBLs, peripheral blood leukocytes; PGs, eicosanoids; ROS, reactive oxygen species. Modified From von Dadelszen et al. (20).

Peripheral blood neutrophils may represent a link between the syncytiotrophoblast of the intervillous space and the systemic vascular endothelium (40;41), either by stimulating the endothelium in response to syncytiotrophoblast fragments or in response to prior endothelial cell activation by syncytiotrophoblast fragments themselves. There is evidence of neutrophil activation in pre-eclampsia superimposed on the neutrophilia (42-44) and alterations in neutrophil function which imply that normal pregnancy is a proinflammatory state (36;45-48).
As stated, incomplete placental implantation is also a feature of normotensive IUGR (17). What is it that causes one woman to develop the maternal syndrome of pre-eclampsia; another has a pregnancy complicated solely by IUGR? In part, this may be determined by a differential neutrophil-mediated maternal inflammatory response which characterises both normal pregnancy (42;43;45;47) and pregnancy complicated by normotensive IUGR (19), but is accentuated in pre-eclampsia (49-51). Neutrophil activation may be involved in the development of acute atherosis (52), as it is in atherogenesis (53;54).

There is increasing evidence to support the hypothesis that elements of the syncytiotrophoblast, which lines the intervillous space of the placenta, may be a trigger of the maternal response modulated by the endothelium. When neutrophils are activated, they synthesize and release proteases, such as a specific neutrophil elastase, capable of mediating vascular damage by destroying the integrity of endothelial cells, the vascular basement membrane, and the subendothelial matrix (35). Toxic oxygen species and leukotrienes are also released and can produce membrane lipid peroxides, lysis of endothelial cells, and increased vascular permeability and reactivity (35;55). Both hepatocellular necrosis and ARDS are characterized by neutrophil infiltration, and are believed to be related to neutrophil-mediated damage (56).

2. **Systemic inflammatory response syndrome (SIRS) and pre-eclampsia**

SIRS is clinically defined as: tachycardia, tachypnoea, hyperthermia and leukocytosis. It is the beginning of the inflammatory continuum that includes sepsis. It represents an acute alteration from baseline homeostasis in the absence of other known causes. There is strong epidemiologic evidence that SIRS, sepsis, severe sepsis, and septic shock represent a hierarchical continuum of an inflammatory response to infection (57). A confirmed infectious
process is required for diagnosing sepsis. If SIRS becomes excessive, it means the injury is overwhelming or is continuing because of inadequate therapy or lack of control of injured organs or systems and the patient may die. Thus, SIRS is sick, multiple organ dysfunction syndrome (MODS) is sicker, multiple organ failure (MOF) is sickest.

A systemic inflammation, with the release of multiple cytokines, plays an important role in the pathophysiology of SIRS (Figure 1.3).

SIRS is associated with most ICU morbidity (58). SIRS shares many clinical and laboratory characteristics with pre-eclampsia, including a hyperdynamic state, leukocytosis, and organ dysfunction (this comparison is described in greater detail in Chapter Two). Pre-eclampsia may persist, often deteriorating transiently, following the delivery of the placenta, much as SIRS develops following the resolution of trauma, burns, sepsis or other inflammatory events. In both conditions there is a systemic response with inflammatory mediator release, endothelial cell dysfunction, end organ hypoperfusion and neutrophil activation.

Two decades ago, hypertension-induced cerebral haemorrhage was the most common cause of maternal death associated with pre-eclampsia. However, since that time, women most commonly died from pre-eclampsia/eclampsia (40.5% of maternal deaths ascribed to hypertensive diseases of pregnancy) either from hepatocellular necrosis or the acute respiratory distress syndrome (ARDS) (6). Thus the clinical picture of pre-eclampsia is reminiscent of SIRS as it progress to evolve into MODS.
An initial toxic stimulus (e.g., endotoxin) triggers the production of proinflammatory monokines (e.g., tumor necrosis factor and interleukin-1). These cytokines, in turn, result in neutrophil–endothelial-cell adhesion, activation of clotting, and generation of numerous secondary inflammatory mediators, including other cytokines, prostaglandins, leukotrienes, and proteases. Antiinflammatory compounds, such as interleukin-6 and interleukin-10, that may serve as negative feedback to the inflammatory process, are also released. Modified from Wheeler A et al (59).

A question to be asked is whether a woman who develops pre-eclampsia is at increased risk for other conditions mediated by the innate immune system, due to intrinsic variations in her leukocyte function. Such another condition would be SIRS, which is most prevalent during the 6th and 7th decades of life (60;61). We have postulated that a history of pre-eclampsia will predispose women to the later development of SIRS, as has already been established for atherosclerotic disease. The hypothesis is that pre-eclampsia is associated with increased risk of systemic inflammation later in life, as it is possible that there are innate variations in neutrophil (or other inflammatory mediator) function that determine whether or
not a woman is more likely to develop pre-eclampsia that would predispose that woman to SIRS following sufficient pro-inflammatory provocation (details in Chapter 2).

3. Could subclinical infection be the differentiating trigger between pre-eclampsia and normotensive IUGR?

Because of the association between pre-eclampsia and subsequently increased risk for atherosclerosis (section 3.3), we have postulated that women may be more likely to develop the maternal syndrome of pre-eclampsia in the presence of subclinical infection with either *Chlamydia pneumoniae* (*Chlamydia pneumoniae*) or cytomegalovirus (CMV), than women whose pregnancies are either uncomplicated or complicated by IUGR in isolation (21). *Chlamydia pneumoniae* and CMV have been linked to atherogenesis (section 3.2). Both *Chlamydia pneumoniae* and CMV are especially credible potential pathogens given chronic carrier status in the general population and the susceptibility of pregnant women to disease reactivation due to reduced cell-mediated immunity in pregnancy (37). Periodontal, gastrointestinal and genitourinary tract disease are also prevalent, and the presence of clinical infection delays the resolution of the disease in women with severe HELLP (62). What makes this hypothesis particularly interesting is the potential to intervene with agents that are safe in pregnancy. Perhaps pre-eclampsia is truly the 'toxaemia' of pregnancy.

3.1 Human evidence for an infectious trigger in the pathogenesis of pre-eclampsia

Infection with *Chlamydia pneumoniae* is suspected to contribute to the pathogenesis of human atherosclerosis. Epidemiological studies have raised the question of whether bacterial infection especially with *Chlamydia pneumoniae* may contribute to this inflammatory process (63;64). Histopathologic studies show a higher prevalence of *Chlamydia pneumoniae* in diseased versus normal coronary arteries (65). Ohkuchi *et al* found that CMV infection in pregnancy
was certainly a mimicker of pre-eclampsia, if not a precipitant of pre-eclampsia (a plausible alternative explanation) (66).

Case reports have linked pre-eclampsia to chronic gastrointestinal infestation with parasites such as *Schistosomiasis japonica* (67) and *Strongyloides stercoralis* (68), and case-control studies have found associations between urinary and/or lower genital tract infection with *Ureaplasma urealyticum* and *Gardnerella vaginalis* (69;70). In a nested, case-control study, using multiple logistic regression, any "urinary tract infection" (bacteria not specified) in pregnancy increased the risk for the development of pre-eclampsia by 2.5 fold (95% confidence interval (CI) 1.3 – 5.0), particularly among primigravidae (OR 5.5 (95% CI 2.9 – 9.7)) (71).

In a more recently described cohort, the presence of periodontal disease at <26 weeks' gestation increased the risk for subsequent pre-eclampsia (OR 2.3 (95% CI 1.0 – 5.4)(72). Periodontal disease, which causes chronic inflammation, has been linked, in turn, with atherosclerosis (73).

Could the maternal syndrome have been both potentiated and sustained by a product of the infectious process, such as Gram negative-derived endotoxin? Cytokines are powerful mediators of the inflammation and sepsis that accompany endotoxin toxaemia. Consistent with a potential role of cytokine activation in pre-eclampsia is a study demonstrating that an intravenous infusion of a very low dose of the endotoxin lipopolysaccharide (LPS) resulted in significant and long-term increases in blood pressure and urinary albumin excretion and significant platelet aggregation in conscious pregnant rats (74). Decreased renal and hepatic blood flow with pre-eclampsia-like histological changes followed exposure to LPS in pregnant rabbits (75).
3.2 **Infection in atherosclerosis**

Increasingly, there is evidence that chronic infection may be an important co-factor in initiating atherogenesis (54). The epidemiological associations between atherosclerotic cardiovascular disease and infection with CMV and, particularly, *Chl pneumoniae* (54;76-78) (Figure 1.4) imply an infectious element to atherogenesis. CMV and *Chl pneumoniae* have been identified within atheromatous plaques (54;79;80). Potential mechanisms include direct local effects on endothelium (on vascular smooth muscle cells and/or on macrophages within the atherosclerotic lesion), or amplification of the systemic inflammatory response.

CMV has a seroprevalence of 60-85%, can persist in the human body for several years and can cause recurrent infection, also specifically in pregnancy (81). CMV is associated with the accelerated atherosclerosis complicating cardiac transplantation (82) and direct endothelial damage in systemic sclerosis (83).

Antibodies to *Chl pneumoniae* have a population prevalence of 50% by the age of 20, rising to 55% among women at 40 (84). These epidemiological data support the premise that chronic infection could link pre-eclampsia with later atherosclerosis, especially given the increased susceptibility to chronic infection due to reduced Th1 responses (cell-mediated immunity) in pregnancy (37).

Recently, increased seroprevalence of anti-*Chl pneumoniae* IgG (not IgA or IgM) has been described in pre-eclampsia pregnancy when compared with normal pregnancy (85). These changes were specific to *Chl pneumoniae* and not to either *Chl trachomatis* or *Chl psittici*. That this association between chronic infection and atherosclerosis exists is supported by the fact that macrolides, effective against *Chl pneumoniae*, may reduce the risk of recurrent coronary events (86;87), and that prophylactic post-transplant ganciclovir, active against cytomegalovirus, reduces the risk of accelerated post-transplant atherosclerosis (88).
A compatible local immune balance has been shown to be of considerable importance for successful pregnancy. The fetoplacental unit is known to secrete a wide range of cytokines and other immunomodulatory substances important for this immune balance (89). Th1 responses are thought to be detrimental to pregnancy whereas Th2 responses are beneficial. There is a compensatory upregulation of Th2 immunity in normal pregnancy (37). The percentage of Th1 and Th2, and the ratios of Th1:Th2 correlate with cytokine (IFN-α and IL-4) secretion level. The type 1 cytokine IFN-α and the type 2 cytokine IL-4, as well as the immunosuppressive cytokine IL-10, have been reported to be present locally in the uterus during human pregnancy (90-92). Up-regulation of Th1 responses and down-regulation of Th2 responses occur in the early phases of pre-eclampsia, thereby inhibiting placentation. The profile of secreted cytokines shifts in favour of Th1 activity (extremely high IFN-γ and low IL-6 and IL-10 secretion). Changes in NK and T lymphocyte subsets followed with Th1 cytokine IFN-γ over-activity, and Th1/Th2 imbalance could affect local immunoregulatory mechanisms in third trimester of women with pre-eclampsia.
The sensitized monocytes and granulocytes of a generalized Th2 response, in later pregnancy, would be the target of the reactivated infection and that would trigger the excessive Th2 upregulation and systemic inflammation characteristic of pre-eclampsia (13;93), especially early-onset pre-eclampsia (13), which differs in degree from that found in normotensive IUGR (21). Recently, Cunningham et al assessed maternal immune status in patients with the HELLP syndrome (62). The results of their study showed a depression of both T and B cell potential and impaired monocyte handling of intracellular pathogens (up to 33%, 11% and 17% of control values, respectively), and that the risk of opportunistic infections may therefore be increased in the patient with the HELLP syndrome because of the generalized immunosuppression and profound decrease in monocyte phagocytic and bactericidal activity.

### 3.3 Pre-eclampsia and atherosclerosis

The development of pre-eclampsia predicts later cardiovascular morbidity and mortality through atherosclerosis (94-98), and one of the pathognomonic findings in the pre-eclampsia placenta is that of acute atherosis (99). The arterial lesion observed in pre-eclampsia which is characterized by focal endothelial disruption, fibrinoid necrosis of the arterial wall, the infiltration of perivascular spaces by mononuclear cells and endovascular accumulation of lipid-laden macrophages. Acute atherosis shares with atherosclerosis (Figure 1.5) similar pathology, pathogenesis (which includes inflammation (13;100)), clinical settings in which it occurs (endothelial cell damage (12;54)), and an association with adverse long-term cardiovascular outcomes in the mother (94). Both acute atherosis and atherosclerosis occur in the presence of hyperlipidaemia (Figure 1.5) (especially the presence of oxidized lipoproteins and increased plasma cholesterol (101-104)) and involve a role for monocytes/macrophages (105-109). Can these similarities provide us with a clue to the pathogenesis of pre-eclampsia?
There is an epidemiological link between atherosclerosis and chronic infection and it is possible that such a link exists between pre-eclampsia and chronic infection. Could an infectious trigger explain the differential maternal response to the shared placental pathology of pre-eclampsia and normotensive intrauterine growth restriction? We believe that infection may be important in the pathogenesis of pre-eclampsia, both in terms of its initiation (by increasing the risk of acute uteroplacental atherosis) and/or its potentiation (by amplifying the maternal systemic inflammatory response).
intimal deposition of lipoprotein
• native / modified LP
  phospholipids
  cholesterol
  apoprotein B
  triglycerides
  lipid peroxides
• albumin

transport

HYPERLIPIDAEMIA
lipoconts, lipoproteins, cholesterol,
  lipid peroxides

surface

biosynthesis

foam cell formation

HYPERLIPIDAEMIA
lipoconts, lipoproteins, cholesterol,
  lipid peroxides

vascular smooth muscle cell

surface

biosynthesis

Figure 1.5: Atherosclerosis and acute atherosis s.is. (a) Atherosclerosis; (b) acute atherosis.
4. **General purpose for research project**

We attempted to investigate the relationship between a woman’s immune system and vulnerability to develop pre-eclampsia.

First, we attempted to investigate whether or not a personal history of pre-eclampsia increases the risk for later SIRS. This linked the postulated inflammation resulting from defective or excessive innate immunity that may occur in both syndromes. This initial study indicated that to test this hypothesis a very high number would be required, and is described in Chapter Two.

Second, using a nested case-control design, we have tested the *hypothesis* that infection may be important in the pathogenesis of pre-eclampsia, both in terms of its initiation (by increasing the risk of acute uteroplacental atherosis) and/or its potentiation (by amplifying the maternal systemic inflammatory response) (21). The corollary to that is that women whose pregnancies are affected solely by normotensive IUGR would be less likely to have a pregnancy associated with reactivation of a chronic infection. This project will be described in Chapter Three.
Chapter 2

Systemic Inflammatory Response Syndrome (SIRS)

"The study of problems which fall on the borderline between diverse specialties...affords unique opportunities to study the pathophysiology of disease".

-Soma Weis, 1941
**Background:**

Pre-eclampsia is a pregnancy-specific condition, it is the principal hypertensive disorder of human pregnancy (110). Death from pre-eclampsia most commonly follows the onset of neutrophil-mediated complications, hepatocellular necrosis and the acute respiratory distress syndrome (ARDS) (111). Hepatocellular necrosis and ARDS are both features of established systemic inflammatory response syndrome (SIRS) (112).

Pre-eclampsia may persist, often deteriorating transiently, following the delivery of the placenta, much as SIRS persists following the resolution of trauma, burns or sepsis. In both conditions there is a systemic response with inflammatory mediator release, endothelial cell dysfunction, a hyperdynamic state, end organ hypoperfusion, and neutrophil activation, resulting in the clinical syndromes. Both pre-eclampsia and SIRS result from differential responses to initiating factors shared with other clinical outcomes.

Pre-eclampsia results from incomplete placentation, which also underlies the development of intrauterine growth restriction in isolation (the fetal syndrome of pre-eclampsia without the associated maternal syndrome). Similarly, sepsis, trauma and burns also occur in patients who have unremarkable recoveries rather than developing SIRS. Is pre-eclampsia a form of SIRS?

2.1 **Terminology and epidemiology of the systemic inflammatory response (SIRS)**

There is now general agreement that sepsis and the systemic inflammatory response (SIRS) are accompanied by the inability to regulate the inflammatory response. The systemic response to infection has been termed sepsis (57;113). Human sepsis is associated with a generalized activation and systemic expression of the host's inflammatory pathways. This
activation occurs via stimulation of the host immune effector cells that subsequently 
synthesize and release potent mediators of cell inflammation (56). Thus, it has been referred 
to as the systemic inflammatory response syndrome (114). SIRS is the systemic 
inflammatory response to variety of severe clinical insults (115), and it is believed to be the 
final common pathogenic link between a number of disorders (113). Sepsis has been defined 
as the presence of various pathogenic organisms and their toxins in the blood or tissues (116).

The causes of the perturbations of SIRS are still unknown. Despite more than 20 years of 
extensive research, sepsis and SIRS remain the chief cause of death in the intensive care unit, 
with mortality rates between 30-70% (117). The incidence of sepsis has increased 139% over 
the past ten years despite progression in management and therapy for sepsis (118). The 
existence of resistant microorganisms may contribute to the increased incidence of sepsis as 
well as increased numbers of immuno-incompetent patients.

Sepsis is an increasingly common cause of morbidity and mortality, particularly in 
elderly, immunocompromised, and critically ill patients. It has been estimated to occur in 1% 
of all hospitalized patients, affecting more than 40,000 people in Canada every year. There 
are approximately 751,000 cases (3.0 cases per 1,000 population and 2.26 cases per 100 
hospital discharges) each year in the United States (119).

The incidence of sepsis and septic shock has not been clear because in the past there was 
no uniform term for definition of sepsis and septic shock. This also affects the statistical and 
edemiological issues in this matter (120). To address some of the above issues, the 
American College of Chest Physicians and the Society of the Critical Care Medicine proposed 
new definitions for sepsis (Table 2.1). They recognized that sepsis was the systemic 
inflammatory response to a documented infection, but acknowledged that this response also 
may be observed in a number of other clinical conditions. They concluded that sepsis was a
continuum of injury response, ranging from sepsis to septic shock, ultimately leading to the multi-organ dysfunction syndrome (MODS) (121). The end result of the host response to infection may be the development of diffuse endovascular injury, microvascular thrombosis, organ ischemia, multiorgan dysfunction, and death (122) (Figure 2.1).

Figure 2.1: The systemic inflammatory, procoagulant, and fibrinolytic host responses to infection: The inflammatory and procoagulant host responses to infection are intricately linked. Infectious agents and inflammatory cytokines such as tumour necrosis factor (alpha) (TNF-α) and interleukin-1 activate coagulation by stimulating the release of tissue factor from monocytes and the endothelium. The presentation of tissue factor leads to the formation of thrombin and a fibrin clot. Inflammatory cytokines and thrombin can both impair the endogenous fibrinolytic potential by stimulating the release of plasminogen-activator inhibitor 1 (PAI-1) from platelets and the endothelium. PAI-1 is a potent inhibitor of tissue plasminogen activator, the endogenous pathway for lysing a fibrin clot. In addition, the procoagulant thrombin is capable of stimulating multiple inflammatory pathways and further suppressing the endogenous fibrinolytic system by activating thrombin-activatable fibrinolysis inhibitor (TAFI). Endothelial injury results in decreased thrombomodulin levels. The end result of the host response to infection may be the development of diffuse endovascular injury, microvascular thrombosis, organ ischemia, multiorgan dysfunction, and death. Modified from Bernard et al. (122).
**Table 2.1: Definition of SIRS, sepsis, septic shock, and MODS. Modified from Balk A (120)**

**ACCP/SCCM Consensus Conference Definitions of Sepsis, Severe Sepsis, and Septic Shock**

**Systemic Inflammatory Response Syndrome (SIRS).** The systemic inflammatory response to a wide variety of severe clinical insults, manifested by two or more of the following conditions:

1. Temperature > 38 °C or < 36 °C
2. Heart rate > 90 beats/min
3. Respiratory rate > 20 breaths/min or PaCO2 < 33 mm Hg
4. White blood cell count > 12,000/mm3, < 4000/mm3, or > 10% immature (band) forms.

**Sepsis.** The systemic inflammatory response to a documented infection. In association with infection, manifestations of sepsis are the same as those previously defined for SIRS. It should be determined whether they are a direct systemic response to the presence of an infectious process and represent an acute alteration from baseline in the absence of other known causes for such abnormalities. The clinical manifestations would include two or more of the following conditions as a result of a documented infection:

1. Temperature > 38°C or < 36°C
2. Heart rate > 90 beats/min
3. Respiratory rate > 20 breaths/min or PaCO2 < 32 mm Hg
4. White blood cell count > 12,000/ml, < 4000/ml, or > 10% immature (band) forms.

**Severe Sepsis/SIRS.** Sepsis (SIRS) associated with organ dysfunction, hypoperfusion, or hypotension. Hypoperfusion and perfusion abnormalities may include, but are not limited to, lactic acidosis, oliguria, or an acute alteration in mental status.

**Sepsis (SIRS)-Induced Hypotension.** A systolic blood pressure < 90 mm Hg or a reduction of ≥ 40 mm Hg from baseline in the absence of other causes for hypotension.

**Septic Shock/SIRS Shock.** A subset of severe sepsis (SIRS) and defined as sepsis (SIRS)-induced hypotension despite adequate fluid resuscitation along with the presence of perfusion abnormalities that may include, but are not limited to, lactic acidosis, oliguria, or an acute alteration in mental status. Patients receiving inotropic or vasopressor agents may not longer be hypotensive by the time they manifest hypoperfusion abnormalities or organ dysfunction, yet they would still be considered to have septic (SIRS) shock.

**Multiple Organ Dysfunction Syndrome (MODS).** Presences of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention.

Based on the hypothesis that excessive levels of inflammation during infection can mediate the organ dysfunction, the pathogenesis of septic shock, and lethality of sepsis and septic shock, different therapies have been proposed (117). Various anti-inflammatory agents have been used in human clinical trials back to 1963. Some of those anti-inflammatory agents are: a high doses corticosteroid (123), and mediator-specific non-glucocorticoid anti-inflammatory agents, interleukin-1 receptor antagonist (IL-1ra) (124), soluble tumour necrosis factor-α (TNF-α) receptors (125), anti-TNF-α antibodies (126), platelet-activating factor (PAF) antagonists (127;128), and arachidonic acid metabolites (prostaglandin E1 and thromboxane) (129;130). Unfortunately, the history of clinical trials in sepsis has confirmed that even extremely successful results form animal models of sepsis cannot necessarily be translated into the clinical settings in humans and, have been disappointing and failed to show significant beneficial effect on outcome until now. It seems in the pathogenesis of systemic inflammatory response and septic shock, other important mechanisms are involved.

2.2 Pathogenesis of sepsis

The mechanisms of sepsis and septic shock start with infection and/or endotoxin production (131) (Figure 2.2). Endotoxin or products from bacterial cells activate the host defence system.

During the onset of sepsis, the inflammatory system becomes hyperactive, involving both cellular and humoral defence mechanisms (Figure 2.3) (117)

Humoral factors play an important role in initiating a systemic inflammatory response and in determining outcome (132). The primary effector organ for cell injury in SIRS is the activated immunocyte, including neutrophils, monocytes, macrophages, and lymphocytes. In septic patients with liver failure, Rosenbloom et al. (133) demonstrated that all circulating
leukocytes, including neutrophils, lymphocytes and monocytes, are activated and that the levels of TNF-α, IL-6, IL-1, IL-8 are increased (TNF-α and IL-6 are increased in severe pre-eclampsia). Simultaneously, robust production of acute phase proteins, such as C-reactive protein and humoral defence mechanisms such as the complement system are activated (117), resulting in production of pro-inflammatory mediators, including C5a. It is possible that polymorphisms in the genes encoding these mediators of inflammation could vary between those patients who develop either SIRS and/or pre-eclampsia, and those who do not.

The circulating immune effector cell activation was proportional to mean circulating IL-6 levels. These cells can induce organ system dysfunction across different vascular beds and at sites remote from the initiating stimuli. Endothelial and epithelial cells, as well as neutrophils, macrophages and lymphocytes, produce powerful pro-inflammatory mediators. In particular, the neutrophil is a central cellular defence type in the mediation of the inflammatory response. Its recruitment, activation, and cytotoxic capability are essential aspects of the body's ability to ward off infection (56). Classically, leukocyte activation is characterized by loss of the constitutively expressed cell surface protein L-selectin (as in pre-eclampsia). This is necessary for the initial weak cell adhesion, followed by an increasing display of both CD11b and CD18, the β2 integrins essential for firm adhesion of circulating neutrophils to endothelial cells, and CD35, a complement receptor (132).
Figure 2.2: Scheme for preventing and treating sepsis. Before sepsis develops, augmentation of immunologic defense mechanisms offers the best opportunity for protection. However, if host defense mechanisms are impaired, brief administration of agents that inhibit proximal mediators of inflammation may help block multiple-organ failure. SIRS denotes systemic inflammatory response syndrome. Adapted from Bernard et al. (134).

![Scheme for preventing and treating sepsis](image)

Figure 2.3: Excessive inflammatory mediator production during sepsis. Various stimuli can cause activation of different cell types and serum proteins, as well as the coagulation and complement systems, leading to excessive production of pro-inflammatory cytokines and chemokines and upregulation of adhesion molecules on endothelial cells and polymorphonuclear leukocytes (PMNs). Monocytes, PMNs and other phagocytes release large amounts of granular enzymes and generate ROS in response to the original stimulus in the early (hyperreactive) phase of sepsis. As result of excessive pro-inflammatory mediator production, vascular permeability increases, tissue damage and organ failure occur and crucial innate immune functions become defective, resulting in increased susceptibility toward infection in the later (hyporeactive) phase of the immune response, often along with immune paralysis. DIC, disseminated intravascular coagulopathy. Modified from Riedemann N et al. (117).
Adhesion and transmigration of leukocytes across the endothelial cell layer is a multi step process involving sequential interaction of specific adhesion molecules on the endothelial surface with counter receptors on the leukocyte surface. The first step is rolling of the leukocyte on the endothelial surface, the second step is activation of the neutrophil, which is achieved by triggering molecules such as selectins, platelet activating factor, C5a, chemokines and other inflammatory mediators. The third step is firm adhesion. The final step is transmigration (Figure 2.4).

**Figure 2.4**: Sequential activation of normal circulating PMNs and their adherence to the vascular endothelium. The first step is constituted by the activation of a constitutively expressed L-selectin leading to the initial weak cell adhesion with endothelial cellular adhesion modelules (ICAM-1) in the post-capillary venule. Secondarily, there is an activation of the b2 integrins, including CD11b/CD18, leading to a firm adhesion. Activated PMNs can now release their toxic reactive species into the micro-environment between the two cells, causing highly localized damage and microbe killing. Finally, if chemokines have established a concentration gradient, subsequent transendothelial migration of PMNs into the interstitium occurs. Modified from Adrie *et al.* (132).

### 2.3 Pre-eclampsia and the systemic inflammatory response syndrome compared

As stated in the General Introduction (Chapter 1), SIRS shares many clinical and laboratory characteristics with pre-eclampsia, including a hyperdynamic state, leukocytosis
and organ dysfunction. While discussed briefly in the Introduction to this thesis, I will now describe the similarities in greater detail (Table 2.2).

Table 2.2: Pre-eclampsia and SIRS share clinical and laboratory features.

<table>
<thead>
<tr>
<th>Clinical Finding</th>
<th>Laboratory Finding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute respiratory distress syndrome</td>
<td>Endothelial function and inflammation</td>
</tr>
<tr>
<td>Hepatic necrosis</td>
<td>↑plasma TNF-α</td>
</tr>
<tr>
<td>Disseminated intravascular coagulation</td>
<td>↑plasma IL-6</td>
</tr>
<tr>
<td>Microvascular thrombosis and haemolysis</td>
<td>↑PAI-1:PAI-2</td>
</tr>
<tr>
<td>Acute renal failure</td>
<td>↑thromboxane: prostacyclin ratio</td>
</tr>
<tr>
<td>Cardiomyopathy</td>
<td>↑endothelin</td>
</tr>
<tr>
<td>Hyperdynamic state</td>
<td>↑caerulopasmin</td>
</tr>
<tr>
<td>End organ hypoperfusion</td>
<td>↑α- Antitrypsin</td>
</tr>
<tr>
<td>↑vascular permeability</td>
<td>Complement Activation</td>
</tr>
<tr>
<td>Differential response to initiating agent</td>
<td>↓plasma transferrin</td>
</tr>
<tr>
<td>(placenta/ trauma/infection)</td>
<td>Hypoalbuminaemia</td>
</tr>
<tr>
<td>Persistence following removal of initiating agent</td>
<td>↓activated protein C</td>
</tr>
</tbody>
</table>

Neutrophil activation

<table>
<thead>
<tr>
<th>Neutrophil activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑plasma neutrophil elastase</td>
</tr>
<tr>
<td>↑neutrophil CD11b expression</td>
</tr>
<tr>
<td>↑basal intracellular ionized calcium</td>
</tr>
<tr>
<td>↑oxidative stress</td>
</tr>
</tbody>
</table>

Table 2.2: Pre-eclampsia and systemic inflammatory response syndrome share clinical and laboratory features. [up arrow]: increased compared with normal; TNF: tumour necrosis factor; IL: interleukin; PAI: plasminogen activator inhibitor; [down arrow]: decreased compared with normal. Modified from von Dadelszen et al (20).

At first glance, the statement that pre-eclampsia resembles SIRS is counter-intuitive, as SIRS is considered to be 'characterized' by hypotension, and pre-eclampsia 'characterized' by hypertension. However, hypotension is not an obligatory feature of SIRS, but of severe sepsis, septic shock or the multiple organ dysfunction syndrome (MODS); 96% of patients...
who fulfil the diagnostic criteria for SIRS do not require positive inotrope support (57). Also, pre-eclampsia can occur in the absence of hypertension, 21% of women having no documented hypertension prior to their first eclamptic seizure (135). Additionally, pre-eclampsia can also be complicated by MODS, a hypotensive disorder. In the Collaborative Eclampsia Trial, many of the 456 women who either died or suffered serious morbidity related to eclampsia had one or more findings consistent with MODS: pulmonary oedema (40 women), renal failure (125 women), liver failure (43 women), and coagulopathy (213 women) (136).

2.3.1 Clinical

What remains clear is the similarity between the two clinical entities. For example, SIRS can be characterized by hepatic failure and ARDS, which, as was stated, are both neutrophil-mediated and the most common causes for maternal mortality in women with pre-eclampsia (3-5). Both disorders persist following the removal of the initiating agent, which is poor placentation (pre-eclampsia) or gram negative sepsis (SIRS), but do not occur uniformly in the presence of that respective initiating agent. Disordered activation of the clotting cascade occurs in both syndromes, resulting in disseminated intravascular coagulation and microvascular thrombosis and haemolysis in pre-eclampsia (111) and SIRS (56;112;133;137). Renal failure can complicate both syndromes (111;112;137), although proteinuria is not seen characteristically in SIRS. In addition, cardiomyopathy may complicate both pre-eclampsia (138) and SIRS (139). Increased vascular permeability, most clearly presenting as oedema, is prominent in both conditions (111;112;137), although it may be difficult to differentiate from the physiological oedema of pregnancy in pre-eclampsia, increased vascular permeability is related to altered endothelial cell function (140).
2.3.2 **Endothelial dysfunction and inflammatory changes**

As in pre-eclampsia, SIRS is characterized by systemic endothelial dysfunction, which may persist long after resolution of the inciting event. Examples of this dysfunction are the elevated concentration of factor VIII-related antigen (141), plasminogen activator inhibitor-1 (56;112;137), and endothelin (142), as well as an increased thromboxane: prostacyclin ratio, all of which also occur in pre-eclampsia (143-145). Endothelial cell activation and dysfunction are felt to underlie the coagulopathy characteristic of both syndromes. Many clinical manifestations of pre-eclampsia and SIRS are related to end-organ hypoperfusion, secondary to endothelial cell-induced vasoconstriction and microvasculur coagulation (12;56).

That pre-eclampsia is a form of systemic inflammation is supported by the changes in the acute phase reactants, caeruloplasmin (146), α1-antitrypsin (147), complement (148), transferrin (149;150) and albumin (151) concentrations that parallel changes noted in SIRS (56;112;137;152-154). The plasma concentrations of the inflammatory cytokines, IL-6 and TNF-α, are increased in both pre-eclampsia (155-157) and SIRS (56;112;137). TNF-α invoking a possible role for peripheral blood monocytes in the development of both clinical syndromes.

As stated, in pre-eclampsia, it has been postulated that peripheral blood neutrophils may provide an important link between the poorly perfused intervillous space of the pre-eclampsia placenta and the endothelium (145).

2.3.3 **Neutrophil activation**

The current model for the development of organ dysfunction in patients with SIRS includes a central role for activated neutrophils *(Table 2.2)*. In SIRS, it is felt that stimuli,
such as bacterial endotoxin, injured tissue and ischaemia, result in disseminated activation of the innate immune system. SIRS and its sequelae, result from the action of host-derived mediators on host tissues. Measures of neutrophil activation present in both pre-eclampsia and SIRS include increased plasma concentration of neutrophil elastase (36;56), increased oxidative stress (158-161), increased surface expression of CD11b (49;50;162), increased concentration of basal intracellular free-ionized calcium (163;164), and, in vitro, increased chemoattractant-induced neutrophil superoxide production (165;166).

Neutrophilia is a prominent component of normal pregnancy, pre-eclampsia and SIRS. In normal pregnancy spontaneous neutrophil apoptosis is significantly retarded with respect to nonpregnant values, that explains the neutrophilia of normal pregnancy (19). The delay in spontaneous neutrophil apoptosis is significantly greater in neutrophils from women with pre-eclampsia than in neutrophils from women with normal pregnancies, and in normotensive IUGR (19). Pre-eclampsia is associated with both a relative neutrophilia when compared with normal pregnancy and greater delays in spontaneous neutrophil apoptosis than in either normal pregnancy or normotensive IUGR (19). Delayed spontaneous neutrophil apoptosis may explain the neutrophilia of both pre-eclampsia (19) and SIRS (162). This delay in neutrophil apoptosis may explain, in part, the persistence of the clinical syndrome, be it either pre-eclampsia (111) or SIRS (112;137), following elimination of the initiating agent, be that a placental stimulus in pre-eclampsia or either an infectious or traumatic insult in SIRS.

Therefore, in both pre-eclampsia and SIRS, there is a transient, usually localized, inciting stimulus, leading to a differential systemic response with inflammatory mediator release, endothelial cell dysfunction and neutrophil activation, resulting in the phenotypic organ dysfunction of the clinical syndrome. As trauma, burns and bacteria are to neutrophils in SIRS, so may placental debris be to neutrophils in pre-eclampsia. However, the clinical and
laboratory pictures of both pre-eclampsia and SIRS are further complicated by the intrinsic inter-individual differences in neutrophil response to the same initiating pathology.

2.4.1 Hypothesis

As it is believed that the maternal syndrome of pre-eclampsia is a form of the SIRS; we have postulated that a history of pre-eclampsia will predispose women to the later development of SIRS, as has already been established for atherosclerotic disease (see following chapters) (95;96).

2.4.2 Objectives

The primary objective of this prospective study was to determine whether pre-eclampsia is associated with increased risk of SIRS later in life, as it is possible that there are innate variations in neutrophil function that determine whether or not a woman is more likely to develop pre-eclampsia that would predispose that woman to SIRS following sufficient pro-inflammatory provocation.

The second objective was to determine whether specific polymorphisms in gene coding are more prevalent in patients with SIRS/pre-eclampsia. Pro-inflammatory cytokines, including TNF-α, IL-6 and IL-1, and the endotoxin receptor (CD-14), are believed to play a central role in the initiation and amplification of both SIRS and pre-eclampsia (36;56;167). Polymorphisms in the genes coding for these and other inflammatory mediators have been associated with a poor outcome (168). Establishing association between these polymorphisms and the clinical syndrome requires demonstration of increased level of the mediators that they code for.

2.5 Methods

This was a prospective case-control study.
2.5.1 Cases and controls

Cases were women admitted to the intensive care unit (ICU) of St. Paul’s Hospital with the diagnosis of SIRS. The women with SIRS were identified by a Research Associate and the student. All eligible subjects (or their proxy decision maker where the woman herself was obtunded) were approached to obtain informed consent.

Controls were women without SIRS, admitted to general medical/surgical wards with the same primary diagnosis and same underlying problem (e.g., pneumonia, bowel resection) but unremarkable in-hospital course (NO SIRS). The controls were matched for both age (+5 years) and ethnicity. Informed consent was obtained from the patients by the student.

2.6 Data collection

Ethics approval was obtained from the University of British Columbia.

A detailed obstetric history was taken that included: numbers of pregnancies, year(s) of pregnancy (ies), gestational age(s) at delivery, birth weight(s), gender(s), and history of 'toxaemia' of pregnancy (hypertension, proteinuria), or miscarriage. The obstetric history was taken either from the patient, the family, or from the medical chart by the student.

In the absence of an adequate obstetric history being obtained from either the patient or her next-of-kin, the patient’s general practitioner was contacted by telephone in an attempt to review the woman’s obstetric history.
2.7 Results

In a period of six months, we recruited only six cases and two controls including:

Cases: 39yo pneumonia, respiratory distress
        54yo diverticular abscess, bowel infection
        75yo perforated duodenal ulcer and colitis
        22yo pneumonia and respiratory distress
        66yo pneumonia, diabetic ketoacidosis, urosepsis
        70yo left lower lobe pneumonia and thoractomy

Controls: 72yo pneumonia and pulmonary oedema
          58yo pneumonia

Number of SIRS women patients admitted to ICU at our study period was 25 patients.

2.7.1 Limitations

Most women in the ICU were too critically ill to be interviewed, or when able to answer questions, were too confused to be able to remember details of their obstetric history. Two of the women were immigrants from the developing world without clear diagnoses, despite histories suggestive of pre-eclampsia; both came from environments in which the costs of accessing medical care were prohibitive. When contacted, none of these women’s general practitioners had medical records dating back to their obstetric history.

Therefore, this study was deemed to be not feasible and was abandoned because of the low frequency of female admissions to the St Paul’s ICU, the insufficient obstetric information available in the ICU setting, the poor quality data, and the fact that it was not
possible to go back to general practitioner charts as most women had delivered outside British Columbia.

2.8 Discussion

Clinical and Research Challenges

This study was abandoned in response to the reality of obtaining accurate obstetric histories in the intensive care unit setting. These women were either too unwell, too confused, or came from an environment in which accurate obstetric diagnoses were not made, to be able to provide accurate data. In addition, their next-of-kin, generally husbands or children, were not aware of specific diagnoses that were made.

These points argue for the population-based approach used in Scandinavia and Scotland, in which maternal-infant databases can be linked with general hospital databases to provide useful long term data (95;96).

Therefore, we were not able to test the hypothesis that the maternal syndrome of pre-eclampsia is a form of systemic inflammation, using this experimental design.

This was a useful experience for a scientist-in-training, as I identified some of the challenges involved in data collection, particularly prospective data collection rather than chart review. Especially important was the plan to launch a pilot study initially to determine the feasibility of the study design, and our decision to abandon this study once it was clearly not feasible.

Instead, the decision was made to pursue a related line of enquiry, that being the relationship between chronic infection and pre-eclampsia. That study will be the topic of the next chapter of this thesis.
CHAPTER 3

A nested case-control seroepidemiological study of possible triggers of the maternal syndrome of pre-eclampsia
3.1. **Preamble:**

As stated in the Introduction to this thesis (Chapter One), it is plausible that the maternal syndrome of pre-eclampsia may be triggered by reactivated chronic infection of those agents linked to the development of later atherosclerosis (21). These agents are CMV and *Chl pneumonias*. We used three different experimental methods in this study. First, we linked two databases to identify cases and controls. Second, we undertook CMV serology. Third, we performed *Chl pneumonias* serology.

3.1.1 **Background**

The evidence for a causal link between maternal infection and pre-eclampsia is incomplete at best. This body of evidence is summarized in Chapter One. Of the plausible infectious triggers of the maternal syndrome of pre-eclampsia, we feel that CMV and *Chl pneumonias* are of particular interest because of their putative role(s) in atherogenesis.

CMV-related chronic villitis (typified by an inflammatory infiltrate dominated by maternal leukocytes within fetal chorionic villi) is associated with both pre-eclampsia (169), normotensive IUGR (170), and stillbirth (21). Placental CMV DNA can be identified in the setting of chronic villitis and intrauterine fetal death, without immunohistochemical evidence of infection (171). In addition, women suffering from recurrent spontaneous abortion have a markedly impaired lymphocyte proliferative response to CMV (172). Recurrent spontaneous abortion is probably part of the spectrum of disease that includes pre-eclampsia (18).

In pregnant woman, diagnosis of CMV infection is, most of the time, performed indirectly by detecting CMV antibodies. Detection of primary infection is based on the observation of a seroconversion or the detection of both IgG and IgM which is rarely observed, and the presence of IgM antibody is sometime very hard to interpret (173).
Accumulating evidence suggests that infection by \textit{Chl pneumoniae} may be involved in the endothelial injury that marks atherosclerosis. \textit{Chl pneumoniae} may contribute to atherosclerosis by bringing monocytes to areas of arterial damage to form foam cells, leading to plaque, and thrombosis (174). Similarly, past, persistent, or recurrent infection with \textit{Chl pneumoniae} may contribute to abnormal vascular function, atherosis, and the abnormal placental perfusion that marks the first stage of pre-eclampsia. The IgG seroprevalence to \textit{C pneumoniae} was more common among women with pre-eclampsia than among women with unaffected term pregnancies (85). There is no association to pre-eclampsia for IgG seroprevalence to \textit{C trachomatis} or \textit{C psittaci} suggests a specific association between pre-eclampsia and the particular species of \textit{Chlamydia} that has been associated with atherosclerosis. An association to pre-eclampsia was not found for IgA or IgM seroprevalence to \textit{C pneumoniae}.

Both of these findings suggest that it is past, persistent, or chronic active infection and not acute infection or reinfection with \textit{C pneumoniae} that is associated with pre-eclampsia. Acute infection or reinfection would cause an elevation in IgM titers (85). In animal models, the induction of atherosclerosis has required persistent or chronic active infection, rather than a single past exposure to \textit{C pneumoniae}. Repeated respiratory infection with \textit{C pneumoniae}, and not a single, acute infection, is needed to promote the growth of atherosclerotic plaque in rabbits. Antibiotic treatment can prevent or reduce atherosclerotic changes in animals being reinfected with \textit{C pneumoniae} (175).
3.2. **Methods**

3.2.1 **Research design**

The study was a nested case-control study of prospectively collected serum (Provincial maternal rubella status screening programme). The *primary outcome* was the level of maternal immunoglobulin G (IgG) antibodies (persistent / reactivation of infection) against CMV and *Chl pneumoniae* in maternal serum.

The nested case-control study is an efficient epidemiological design whereby a case-control approach is employed within an established cohort. It is a solution to the problem of the small sample sizes; it starts with a group of people with the disease, the cases, and comparing them to a second group without the disease, the controls. In this case, both groups were similar with the respect of the time of the delivery.

3.2.2 **Identification of cases and controls**

Subjects were identified from the maternal database at the Children's and Women's Health Centre of British Columbia (CWHCBC). Diagnoses were confirmed by hand search of patient files, using the criteria established by the National High Blood Pressure Education Program (NHPBEP) in the USA (2000) (see Section 3.4). There was no direct patient contact. Research data were unlinked from patient identifying data by anonymous unlinked seroprevalence methods, this method having been used with similar sampling techniques in British Columbia in recent years (176) and having proven acceptable under the laws of the Province. Ethics approval was granted by both the University of British Columbia and the Children's and Women's Health Centre of BC Ethics Committees.

The cases and controls were identified from the ICD 9 codes in Health Records, BC Women's Hospital, and each chart was manually reviewed to determine matching for
selection criteria, the relevant data abstracted, and identifiers forwarded to UBCCDC. Due to
coding delays (12 months) and the time between rubella serology (usually 12-16 weeks'
gestation) and delivery, only a three month period was available for study. At the UBCCDC,
the serum was identified from the banked rubella serum, and all analyses were performed
unlinked to identifying maternal data. Laboratory staff personnel were blinded to pregnancy
outcome diagnoses.

3.2.2.1 Cases and controls

The cases were women with early-onset pre-eclampsia, late-onset pre-eclampsia, and
normotensive (IUGR), and up to two matched controls per case. (i) women with early-onset
pre-eclampsia (meeting the NHBPEP criteria (Section 2.4, below))(177) with clinical onset
prior to 34 weeks' gestation, (ii) women with late-onset pre-eclampsia (same criteria), with the
disease onset at ≥34+0 weeks' gestation, and (iii) women with normotensive IUGR (defined
as a birth weight <3rd centile for gestational age and gender). The controls were women with
normal pregnancy outcomes matched for maternal age (±5y) and parity (0, 1, ≥2). The
division of pre-eclampsia samples into two groups is designed to discriminate between those
women whose disease is more homogeneous and clinically significant (early-onset pre-
eclampsia) from those whose disease may be a physiological variant at term in primigravid
pregnancies (late-onset pre-eclampsia)(178).

3.2.2.2 Definitions

1. Pre-eclampsia: was defined by the NHBPEP criteria, namely: a pregnancy-specific
increase in blood pressure (dBP≥90mmHg, or sBP≥140mmHg) associated with >0.3g
proteinuria/d or ≥2+ dipstick proteinuria. Two or more episodes of dipstick proteinuria ‘+’
was accepted in the presence of other evidence of pre-eclampsia (e.g. hyperuricaemia).
2. **Normotensive IUGR**: was defined as a birth weight <3rd centile for gestational age and gender, following pregnancies complicated by neither hypertension nor proteinuria.

3. **Normal pregnancies**: were defined as pregnancies associated with a normally grown fetus, delivering at term (37-42 weeks), without evidence of either pre-eclampsia or gestational diabetes. These women had no history of major significant medical problems that predated the pregnancy (e.g. systemic lupus erythematosus, chronic hypertension, and renal disease).

The Medical Records coding of cases and controls is described on (Table 3.1).

Matched controls were age-matched (within ± 5 years) and parity-matched (0, 1, >2). Control patients were women with normal pregnancy who had undergone rubella serology testing close to the time of their appropriate case. This was designed to reduce confounding due to both the pregnant state and seasonal variation in infectious diseases.

Only singleton pregnancies were examined, and cases related to fetal hydrops or gestational trophoblast diseases were excluded.

3.2.3 **Serum bank**

Cases and controls serum were identified from the University of British Columbia Centre for Disease Control (UBCCDC) serum bank of rubella serology. This contains 90% of all rubella tests performed in the Province of British Columbia, all samples being kept for 24 months.
Table 3.1: The field name and definitions as described by Health Records at CWHCBC.

<table>
<thead>
<tr>
<th>Field Name</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>chart_locatn</td>
<td>Chart Location. Records Processing Dept. will need this info to pull charts women with pre-eclampsia (ICD9-CM pre-eclampsia codes used: 642.4, 642.5, 642.6, 642.7)</td>
</tr>
<tr>
<td>M#</td>
<td>Mother's unit#</td>
</tr>
<tr>
<td>Term_dig</td>
<td>Terminal digit of mother's unit#, required for record pulling</td>
</tr>
<tr>
<td>Disch</td>
<td>Mother's discharge date</td>
</tr>
<tr>
<td>Age</td>
<td>Mother's age</td>
</tr>
<tr>
<td>Dob</td>
<td>Mother's date of birth</td>
</tr>
<tr>
<td>Name</td>
<td>Mother's name</td>
</tr>
<tr>
<td>Phn</td>
<td>PHN# (PHN# = 10000000000 or 00000000000 means the PHN# was unavailable)</td>
</tr>
<tr>
<td>Deliv_date</td>
<td>Delivery date</td>
</tr>
<tr>
<td>Deliv_hr</td>
<td>Baby's delivery hour. Delivery date/hour used to find control patients</td>
</tr>
<tr>
<td>Deliv_type</td>
<td>C/S, Vag</td>
</tr>
<tr>
<td>Nullip</td>
<td>Y, N</td>
</tr>
<tr>
<td>Parity</td>
<td># previous deliveries</td>
</tr>
<tr>
<td>Ga</td>
<td>Gestational age at time of delivery. GA of clinical onset of condition is not captured in Med2020 or BCRCP, therefore GA at delivery was used.</td>
</tr>
<tr>
<td>Smoker_code</td>
<td>Current, Former, Never, Blank = not documented on chart and can be interpreted as &quot;never&quot;. This field is abstracted from the Antenatal Record.</td>
</tr>
<tr>
<td>64271 Present</td>
<td>&quot;Yes&quot; means that the ICD10 code 642.71= Pre-eclampsia or eclampsia superimposed on pre-existing hypertension was present. &quot;No&quot; means that the code was not present. This field is present only for the pre-eclampsia &lt;34 weeks and &gt;=34 weeks data.</td>
</tr>
<tr>
<td>M#</td>
<td>Mother's unit# repeated so print-out of baby data can be matched with maternal data.</td>
</tr>
<tr>
<td>B_serv</td>
<td>Baby's patient service. NB = Newborn SB = Stillborn</td>
</tr>
<tr>
<td>B#</td>
<td>Baby's unit#</td>
</tr>
<tr>
<td>b-dob</td>
<td>Baby's date of birth</td>
</tr>
<tr>
<td>B_discharge</td>
<td>Newborn's discharge date from BCWH. Baby may have been transferred to BCCH or another hosp, and that discharge date has not been provided.</td>
</tr>
<tr>
<td>Sex</td>
<td>M=Male, F=Female</td>
</tr>
<tr>
<td>Wt</td>
<td>Newborn weight at delivery.</td>
</tr>
<tr>
<td>3%ile_wt</td>
<td>3rd percentile birthweight vs GA vs Gender. This field is only present on the Normotensive w IUGR data.</td>
</tr>
</tbody>
</table>

Excluded cases:
- congenital infection due to toxoplasmosis, rubella, cytomegalovirus
- any chromosomal abnormality involved
- hydatidiform mole
- fetal hydrops or gestational trophoblast disease
- multiple pregnancies
3.2.4 Data Collection

The data extracted retrospectively from the women’s charts and, if required, the charts of infants admitted to a nursery in the CWHCBC, were entered into an Access™ database, including maternal variables and outcomes (see Section 3.2.4.2), newborn variables and outcomes (see Section 3.2.4.4), and BCCDC laboratory results. The laboratory data entered from the day of admission, for the day of delivery until the day of discharge for the purpose of the analyses.

3.2.4.1 Maternal Variables

Data on the following maternal variables were collected by chart review: maternal age, gestational age on admission, pregnancy weight, pre-pregnancy body mass index (BMI)(Kg/m$^2$), weight gain during pregnancy, blood pressure (sBP and dBP), total leukocyte count, mean arterial pressure, proteinuria (24h urine, dipstick proteinuria), creatinine, urinary output, bilirubin, uric acid, aspartate aminotransferase (AST), lactate dehydrogenase (LDH), bilirubin, albumin, platelet count, mean platelet volume (MPV) (Figure 3.1).

3.2.4.2 Maternal Outcomes

We developed, by Delphic consensus, a combined adverse maternal outcome. The Delphi Consensus technique is consensus method used to determine the extent of agreement on an issue. The technique involves asking a panel of experts to take part in a series of rounds to identify, clarify, refine and finally to gain consensus on the particular issue (179). Each Delphi round comprised a questionnaire, an analysis, and a feedback report. It is concerned with deriving quantitative estimates through qualitative approaches. The consensus process begins with a predetermined objective in mind, and consensus is reached when objections to that predetermined position have been quieted. In this case, the Delphic consensus included
15 internal medicine, critical medicine, maternal-fetal medicine, and paediatric intensive care medicine specialists from Canada, the UK, the USA, Australia and New Zealand.

The maternal adverse outcome consisting of: mortality, hepatic failure, hepatic haematoma/rupture, Glasgow coma scale (GCS) <13, stroke, seizures, cortical blindness, renal dialysis, renal transplantation, positive inotropic support, infusion of 3rd antihypertensive, myocardial infarction, >50% O2 > 1 hour, intubation and transfusion of >10U in total of any combination of blood or blood products (Figure 3.2). This combined adverse outcome has received general support at scientific meetings since its derivation by our research group, in collaboration with the same Canadian, British, American, and Australasian experts.

3.2.4.3 Fetal Variables

Where available, we entered the following ultrasound data into the database: amniotic fluid index (AFI) as percentile for gestational age and the ultrasound estimated fetal weight (EFW) as percentile for gestational age into the database. The data were present for most cases and for only a minority of controls, as routine ultrasound evaluation of 'normal’ pregnancies is not associated with improved pregnancy outcomes (Cochrane review). Similarly, placental pathology was available for only a minority of controls, as routine placental evaluation is not recommended in the absence of clinical indications (Am College of Pathologists).

3.2.4.4 Fetal Outcomes

We also developed a combined adverse prenatal outcome, using the same Delphic consensus. Included in the definitions were: stillbirth, neonatal death, perinatal mortality, intraventricular haemorrhage (IVH, grade 3 or 4), retinopathy of prematurity (ROP), cystic
periventricular leukomalacia (cPVL), bronchopulmonary dysplasia (BPD) and necrotising enterocolitis (NEC) (Figure 3.3).

**Figure 3.1**: Maternal variables and laboratory information.
Figure 3.2: Maternal outcome

Figure 3.3: Fetal outcome
3.2.5 Laboratory techniques

A nested case-control study of serum from a population-based bank was performed. Seroprevalence and levels of anti-CMV and *Chl pneumonieae* IgG were compared between groups. Generally, these samples were taken at 12-20 weeks of pregnancy as part of routine clinical care. Performing rubella and syphilis serology (± HIV and/or hepatitis B), along with blood group typing and alloimmunization antibody screening, is routine at this time. Samples are saved so that serology can be reviewed at a later stage should clinical suspicion of congenital infection arise during the index pregnancy or within one of the expected data of delivery.

The specimens, frozen at -20°C were thawed. An aliquot of each specimen was separated from the original serum and delinked from identifying data after the baseline demographic data were recorded. Case-control pairs or trios were assayed in batches to minimise inter-assay variability.

3.2.6 Methods for cytomegalovirus using unlinked anonymous seroprevalence

3.2.6.1 Sample selection

Stored specimens collected during 2000-2001 for cases and controls were used. An aliquot of each specimen was separated from the original sera and the following were recorded:

- Age at time the blood was drawn
- City or town of residence
- New study specimen number (not linked to previous specimen).

No unique identifying data were recorded for study purposes.
Anti-CMV IgG serology was performed on a VIDAS analyser (BioMerieux, St Laurent, Québec), according to manufacturer’s instructions. It is an easy, rapid, and reproducible test. It was evaluated in a multicentre study to differentiate between primary CMV infections and past infections or reactivations (173). In immunocompetent patients, and especially in pregnant women (considered immunocompetent patients), diagnosis of CMV infection is, most of the time, performed indirectly by detecting CMV antibodies and not the antigens.

3.2.6.2 The assay

The assay combines an enzyme immunoassay method with a final fluorescent detection (ELFA). Results were classified as being negative (<4mU/ml), equivocal (4-6mU/ml), and positive (>6mU/ml). The lower threshold for detection was 4mU/ml, and the upper range for the scale is 400mU/ml. For data entry, values <4mU/ml were assigned the value '3', and values >400 were assigned the value '401'. All reagents were from BioMerieux.

VIDAS CMV IgG is an automated assay for the VIDAS system, which enables anti-CMV IgG in human serum to be measured quantitatively. All reaction steps are performed by the VIDAS instrument.

VIDAS kits are stored at 2-8°C. A kit contains: disposable solid phase receptacle (SPRs), reagent strips, a positive control serum, a negative control serum and one standard, which is also known as a ‘calibrator’. SPRs are like pipette-tip disposable devices, coated on the inside surface with inactivated virus, and serve both as a combination pipette tips and reaction vessels during the assay. All of the other reagents (mouse monoclonal anti-human IgG antibodies labelled with alkaline phosphatase, washing buffers, and substrate) are available in a 10-well foil-sealed strip.
Before beginning an assay, the kit must be brought to room temperature at least 30 minutes prior to use. Calibrator, controls and samples were mixed thoroughly with a vortex to obtain a better result reproducibility.

3.2.6.3 Procedure (see Table 3.2)

We entered patient and assay data on the VIDAS terminal to create a work list. The assay code for VIDAS CMVG is CMVG. We mixed the sera thoroughly with a vortex, then pipetted 100μl of each into the sample well of the reagent strips in the order shown in the work list. The CMVG SPR and CMVG reagent strip were placed in the positions indicated by the VIDAS work list and, we followed the procedure as directed in the VIDAS User's Manual. The assay was completed in approximately 40 minutes. Once the ELFA assay was physically completed by the VIDAS, the anti-CMV IgG were mathematically calculated by the VIDAS, and expressed in U/ml. The calculation involves comparing the fluorescence of each control and patient sample to the current calibration curve, and their antibody levels mathematically determined in relation to the calibration curve which is stored in the VIDAS’s memory.

3.2.6.4 Quality control

Every new lot number when used for the first time, and every 14 days thereafter, had a calibration curve made by running the CMVG calibrator assayed in duplicate (“according to the Manual Menu”). Calibrators are sera with a very accurately known antibody level. The VIDAS determines the amount of fluorescence for each calibrator, compares it to the expected fluorescence for that antibody level, and makes a mathematical calculation to construct a curve. The curve is a graph with concentration plotted against fluorescence. If the
calibrators are repeatedly out of acceptable range, the curve is invalid and the kit must be discarded.

Positive and negative controls are included in each VIDAS CMV IgG kit. Controls are similar to calibrators, in that they are sera with a known antibody level. These controls were performed once daily, at the beginning of the day, to verify the accuracy of the curve, the integrity of the kit component, and the proper operation of the VIDAS instrument, prior to analyzing patient specimens. If the control value deviated from the expected values, a new curve must be made by rerunning the calibrator, and then the controls must be rerun. If after repeated attempts, one or both of the controls remains out of range, then the kit must be discarded, and the process begun again with a new kit.
Table 3.2 CMV: Kit Composition (a), Description of the CMVG Reagent Strip (b), and Thresholds and Interpretation of Results (c).

**a: Kit Composition (60 tests):**

<table>
<thead>
<tr>
<th>Kit Composition</th>
<th>Ready-to-use</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 CMVG strips</td>
<td>Ready-to-use</td>
</tr>
<tr>
<td>60 CMVG SPRs</td>
<td>SPRs coated with CMV antigen (AD169). Each SPR is clearly marked. Only remove the required number of SPRs. Made sure the pouch is well closed after opening.</td>
</tr>
<tr>
<td>2 x 30</td>
<td>Human serum containing anti-CMVG IgG + 1 g/l of sodium azide. Title in arbitrary unit/ml: range given on vial label.</td>
</tr>
<tr>
<td>Positive CMVG control</td>
<td>Human serum containing anti-CMVG IgG + 1 g/l of sodium azide.</td>
</tr>
<tr>
<td>1 x 1.5ml (liquid)</td>
<td></td>
</tr>
<tr>
<td>Negative CMVG control</td>
<td>Human serum not containing anti-CMVG IgG + 1 g/l of sodium azide.</td>
</tr>
<tr>
<td>1 x 1.5 ml (liquid)</td>
<td></td>
</tr>
<tr>
<td>CMVG calibrator</td>
<td>Human serum containing anti-CMV IgG + 1 g/l of sodium azide.</td>
</tr>
<tr>
<td>1 x 2 ml (liquid)</td>
<td>Title in arbitrary unit/ml given on vial label.</td>
</tr>
</tbody>
</table>

**b: Description of the CMVG Reagent Strip:**

<table>
<thead>
<tr>
<th>Wells</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sample well.</td>
</tr>
<tr>
<td>2</td>
<td>Serum diluent: Phosphate buffer (10 mmol/l) pH 7.2 - Tween + protein and chemical stabilizers + 1 g/l of sodium azide (300μl)</td>
</tr>
<tr>
<td>3</td>
<td>Pre-washing solution: Phosphate (10 mmol/l) pH 7.2 - Tween + protein and chemical stabilizers + 1 g/l of sodium azide (600μl)</td>
</tr>
<tr>
<td>4 - 5 - 7 - 8</td>
<td>Washing solution: TRIS (50 mmol/l) pH 7.4 + 1 g/l of sodium azide (600μl)</td>
</tr>
<tr>
<td>6</td>
<td>Conjugate: Alkaline phosphatase labelled monoclonal anti-human IgG antibodies (mouse) + 1 g/l of sodium azide (400μl)</td>
</tr>
<tr>
<td>9</td>
<td>Empty well.</td>
</tr>
<tr>
<td>10</td>
<td>Cuvette with substrate: 4 methyl-umbelliferyl-phosphate + 1 g/l of sodium azide (300 μl).</td>
</tr>
</tbody>
</table>

**c: Thresholds and Interpretation of results:**

<table>
<thead>
<tr>
<th>Value (U/ml)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 4</td>
<td>Negative</td>
</tr>
<tr>
<td>From &gt;4 to &lt;6</td>
<td>Equivocal</td>
</tr>
<tr>
<td>&gt;6</td>
<td>Positive</td>
</tr>
</tbody>
</table>

For all assays with results between 4 and 6, samples were assayed a second time to better determine true seroprevalence and levels.
3.2.7 *Chl pneumoniae*

The enzyme-linked immunosorbent assays (ELISAs) were developed in the UBC CDC research laboratory to detect IgG antibodies against *Chl pneumoniae* AR39 elementary body (EB) using an ELISA method previously published(180).

In brief, ELISA plates (Corning, NY, USA) were coated with 100μL/well of EB (0.1μg/well or 1x10⁵ IFU/well), at 4°C overnight. The EBs were diluted in 0.1 M NaHCO₃ (Sigma, St Louis, MO), at pH 9.0. The plates were washed once with phosphate buffered saline (PBS)-Tween 20 (Sigma), 150μL 3% bovine serum albumin (BSA, Sigma, Mississauga, ON) in PBS was added to each well, and the plates incubated for 1.5hr at 37°C. Plates were washed with PBS-Tween (Sigma). Serum samples were added (diluted 1:200 in 0.5% BSA/PBS) 100μL to each well, and incubated overnight at 4°C. The plates were washed three times. 100μL of horseradish peroxidase-conjugated goat anti-human IgG (1:2000 dilution; PharmaGen, Ocala, FL) in 0.5% BSA/PBS was added to each well. Plates were incubated for 2 hour at 37°C and then washed three times. 100μL of 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS; Amersham Biosciences, Amersham, Bucks, UK) substrate was added to each well, and allowed to develop in the dark for 60min, and then the plates read at OD405. The substrate was prepared by adding 1ml ABTS stock to 20ml 0.01M citrate buffer and 20μL 30% hydrogen peroxide. ABTS stock was prepared by adding 160mg ABTS to 10ml H₂O, stored in 500-1000μL aliquots at -20°C. For *Chl pneumoniae*, seropositivity was defined as an OD₄₅₀ reading >0.2 arbitrary units at a 1:200 dilution of serum. Seropositive means that there is a significant level of antibody detected in the serum, above an arbitrary cut-off determined by non-specific binding in known negative controls. If the serum contains a high level of antibody, it will give higher OD values. Although in our
study we considered any positive OD value as seropositive. We also recorded the absolute amount of antibody (up to the upper limit of 400) for our analysis.

3.2.8 Data analysis

Data were entered into a database and analysed using Prism 3.0 (GraphPad, San Diego, CA) software, using $\chi^2$, Kruskal-Wallis, and Mann-Whitney U tests, as appropriate. P<0.05 was considered statistically significant. Baseline maternal and pregnancy outcome data are presented parametrically, as those data were normally distributed (Table 3.3). No statistical analyses were made between groups in this table.

The primary outcome of interest was the level of anti-CMV and anti-Chl pneumoniae IgG. The secondary outcome was the seroprevalence of anti-CMV and/or anti-Chl pneumoniae IgG.

For seroprevalence comparisons, the $\chi^2$ was used to test the hypothesis that the seroprevalence of anti-CMV and/or anti-Chl pneumoniae IgG would be greater in women with pre-eclampsia, especially pre-eclampsia of early onset.

For the comparisons of levels of anti-CMV and anti-Chl pneumoniae IgG, the Kruskal-Wallis test was used as a non-parametric analysis of variance between multiple groups. Where the Kruskal-Wallis test found statistically significant differences within a comparison, individual groups were compared using the Mann-Whitney U test to identify the group(s) underlying the significant Kruskal-Wallis result.

3.3 Results

From the Health Records Department, BC Women's Hospital and Health Centre, we identified 18, 55, and 50 cases of early-onset pre-eclampsia, late-onset pre-eclampsia, and normotensive IUGR, respectively. 246 controls were identified, up to two per case.
UBC CDC had adequate stored samples and matched controls for serology for 9 (13 controls), 29 (47 controls), and 33 (53 controls) cases of early-onset pre-eclampsia, late-onset pre-eclampsia, and normotensive IUGR, respectively. The three groups of control women did not differ from each other in terms of their pregnancy details or their antibodies, so were amalgamated into a single group for analyses.

Maternal and perinatal characteristics and outcomes of the matched cases and their controls are detailed in (Table 3.3). Women with pre-eclampsia were uniformly hypertensive, and more often suffered from either transaminitis and/or thrombocytopenia than did other groups. One woman with early-onset pre-eclampsia had trace proteinuria but suffered from the HELLP syndrome, and, therefore, fulfilled our definition.

3.3.1 Analyses

238 (78%) and 119 (39%) of the total population (n=304) were seropositive for CMV and *Chl pneumoniae*, respectively. The seroprevalence results for the matched cases and controls are shown in (Table 3.4). There was no significant difference between groups ($\chi^2 p\geq0.13$).

There were significant differences in both anti-CMV IgG and anti-*Chl pneumoniae* EB antibodies across the groups (Table 3.5), with the early-onset pre-eclampsia group showing increased antibodies compared with the other three groups (Kruskal-Wallis test, $p=0.03$). Women with early-onset pre-eclampsia had higher anti-CMV titres than women with late-onset pre-eclampsia (Mann-Whitney U test, $p=0.012$), normotensive IUGR ($p=0.023$), and normal pregnancy controls ($p=0.028$). While there was a trend for women with early-onset pre-eclampsia to have greater anti-*Chl pneumoniae* titres than women with normotensive IUGR (Mann-Whitney U test, $p=0.092$), normotensive women delivered of an IUGR infant had significantly lower anti-*Chl pneumoniae* antibodies than did normal pregnancy controls (Mann-Whitney U test, $p=0.010$). Although the median value for late-onset pre-eclampsia
cases was the same as that for normotensive IUGR (0.103), this group was not significantly
different from normal controls (Mann-Whitney U p=0.133).

Table 3.3: Maternal and perinatal demographics and outcomes (mean (SD) or N [%]).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal pregnancy (n=113)</th>
<th>EO PET (n=9)</th>
<th>LO PET (n=29)</th>
<th>nIUGR (n=33)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal outcomes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal age (y)</td>
<td>30.8 (5.4)</td>
<td>28.8 (8.1)</td>
<td>30.9 (6.1)</td>
<td>30.6 (4.9)</td>
</tr>
<tr>
<td>Nulliparous</td>
<td>94 [83]</td>
<td>8 [89]</td>
<td>22 [76]</td>
<td>27 [82]</td>
</tr>
<tr>
<td>Significant past history*</td>
<td>5 [4]</td>
<td>0 [0]</td>
<td>2 [7]</td>
<td>0 [0]</td>
</tr>
<tr>
<td>Gestational age at delivery (wk)</td>
<td>39.0 (2.4)</td>
<td>30.9 (2.7)</td>
<td>38.5 (1.8)</td>
<td>37.3 (4.7)</td>
</tr>
<tr>
<td>Combined adverse maternal outcome*</td>
<td>0 [0]</td>
<td>1 [11]</td>
<td>0 [0]</td>
<td>0 [0]</td>
</tr>
<tr>
<td>Highest mean arterial pressure (mmHg)</td>
<td>97.7 (8.5)</td>
<td>124.8 (14.3)</td>
<td>125.7 (10.8)</td>
<td>97.4 (9.9)</td>
</tr>
<tr>
<td>Proteinuria (≥')§</td>
<td>14 (12.4)</td>
<td>8 (88.9)</td>
<td>28 (100)</td>
<td>2 (6.1)</td>
</tr>
<tr>
<td>Highest AST (IU/L)</td>
<td>25.0 (7.7)</td>
<td>75.0 (84.8)</td>
<td>69.7 (158.2)</td>
<td>-</td>
</tr>
<tr>
<td>Lowest platelet count (x10⁹/L)</td>
<td>191.9 (56.4)</td>
<td>123.8 (51.9)</td>
<td>176.2 (63.7)</td>
<td>202.1 (56.9)</td>
</tr>
<tr>
<td>Highest uric acid (μM)</td>
<td>319.8 (21.6)</td>
<td>374.4 (93.4)</td>
<td>369.6 (80.3)</td>
<td>315.0 (93.0)</td>
</tr>
<tr>
<td><strong>Perinatal outcomes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>3342 (571)</td>
<td>1614 (319)</td>
<td>3270 (524)</td>
<td>2281 (467)</td>
</tr>
<tr>
<td>IUGR**</td>
<td>0 [0]</td>
<td>1 [11.1]</td>
<td>0 [0]</td>
<td>33 [100]</td>
</tr>
<tr>
<td>Perinatal loss††</td>
<td>0 [0]</td>
<td>0 [0]</td>
<td>0 [0]</td>
<td>0 [0]</td>
</tr>
<tr>
<td>Combined adverse perinatal outcome††</td>
<td>0 [0]</td>
<td>1 [11.1]</td>
<td>0 [0]</td>
<td>1 [3.0]</td>
</tr>
</tbody>
</table>

EO: early-onset (<34+0 weeks' gestation); LO: late-onset (≥34+0 weeks' gestation); nIUGR: normotensive intrauterine growth restriction; PET: pre-eclampsia. Matched normal pregnancy controls were analysed as individual results. *body mass index >25 (3 missing values for nIUGR); Significant past history: previous pre-eclampsia, recurrent miscarriage, thromboembolic disease, hypertension, renal disease, or heart disease; Combined adverse maternal outcome: maternal death; hepatic failure, hepatic haematoma or rupture; Glasgow coma scale <13, stroke, ≥2 seizures, or cortical blindness (transient or permanent); positive inotrope support, myocardial infarction, or infusion of any 3rd antihypertensive; dialysis, or renal transplantation; requirement of >50% O₂ for ≥1h, or intubation; and/or transfusion of ≥10U of blood products (in total). §proteinuria considered significant if ≥', despite risks of false positives; one early-onset pre-eclampsia case had only trace proteinuria, but had hypertension and hyperuricaemia. **IUGR: intrauterine growth restriction: birthweight <3rd percentile for gender and gestational age. ††Perinatal loss: stillbirth >20 weeks' gestation and neonatal death in the first 28d of postnatal life. †††Combined adverse perinatal outcome: bronchopulmonary dysplasia; grade III or IV intraventricular haemorrhage; cystic periventricular leukomalacia; retinopathy of prematurity, stage 3 or 4; necrotising enterocolitis.
Table 3.4: *Chlamydia pneumoniae* and CMV seroprevalence in women with normal pregnancies, early- and late-onset pre-eclampsia, and normotensive intrauterine growth restriction (n (%)).

<table>
<thead>
<tr>
<th></th>
<th>Normal pregnancy (n=113)</th>
<th>EO PET (n=9)</th>
<th>LO PET (n=29)</th>
<th>nlUGR (n=33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV seropositive*</td>
<td>92 (81)</td>
<td>9 (100)</td>
<td>21 (72)</td>
<td>26 (79)</td>
</tr>
<tr>
<td><em>Chlamydia pneumoniae</em></td>
<td>60 (53)</td>
<td>6 (67)</td>
<td>11 (38)</td>
<td>10 (30)</td>
</tr>
<tr>
<td>EB seropositive*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV &amp; <em>Chlamydia pneumoniae</em> seropositive for both*</td>
<td>46 (36)</td>
<td>6 (67)</td>
<td>8 (28)</td>
<td>10 (30)</td>
</tr>
</tbody>
</table>

*χ² p>0.05; EO: early-onset (<34+0 weeks' gestation); LO: late-onset (≥34+0 weeks' gestation); nlUGR: normotensive intrauterine growth restriction; PET: pre-eclampsia. Matched normal pregnancy controls were analysed as individual results.

Table 3.5: Levels of IgG against *Chlamydia pneumoniae* and CMV differ between women with normal pregnancies, early- and late-onset pre-eclampsia, and normotensive intrauterine growth restriction (median [interquartile range]).

<table>
<thead>
<tr>
<th></th>
<th>Normal pregnancy (n=71)</th>
<th>EO PET (n=9)</th>
<th>LO PET (n=29)</th>
<th>nlUGR (n=33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chlamydia pneumoniae</em>* EB titre</td>
<td>0.207 [0.105, 0.359]</td>
<td>0.354 [0.067, 0.659]</td>
<td>0.103 [0.046, 0.315]</td>
<td>0.103 [0.031, 0.216]</td>
</tr>
</tbody>
</table>

Kruskal Wallis *p=0.024, **p=0.038. EO: early-onset (<34+0 weeks' gestation); LO: late-onset (≥34+0 weeks' gestation); nlUGR: normotensive intrauterine growth restriction; PET: pre-eclampsia. Matched normal pregnancy controls were analysed as paired results when appropriate.
3.4. **Discussion**

Pre-eclampsia is a dangerous disease of human pregnancy, which affects both the mother and her baby. Pre-eclampsia is a disease of a major obstetric importance throughout the world. Nonetheless it remains enigmatic. The disorder takes several months during pregnancy to develop, remits spontaneously immediately, or within a few weeks after, delivery. It is a complex disorder that cannot be attributed to any one single cause.

Our preliminary study is the first to associate both CMV and *Chl pneumoniae* serological status with early onset pre-eclampsia. It adds support to the evidence for both a causal link between maternal infection and pre-eclampsia and for a link between pre-eclampsia and later atherosclerosis (21), and recently reported solely for *Chl pneumoniae* in pre-eclampsia compared with normal pregnancy (85). We also noted that a lower level of antibodies to *Chl pneumoniae* is associated with normotensive IUGR. This latter point may help to explain the conundrum of why women with the same intrauterine pathology, namely uteroplacental mismatch (20), develop a dichotomous maternal response, the first being the systemic disorder of pre-eclampsia, the second being the fetal syndrome of pre-eclampsia in isolation (21).

Pre-eclampsia and atherosclerosis share many common pathophysiological features and risk factors. One common feature is endothelial dysfunction, which may derive from inflammation. In atherosclerosis, injury-induced mononuclear cell accumulation, migration and proliferation of smooth muscles, and formation of fibrous tissue ultimately lead to plaque formation and vessel obstruction have suggested an inflammatory origin to the altered endothelial dysfunction seen in atherosclerosis. In pre-eclampsia, too, heightened intravascular inflammation has been suggested as the origin of the abnormal endothelial function (93).
Accumulating evidence suggests that infection by *Chl pneumoniae*, a common pathogen may be involved in the endothelial injury that marks atherosclerosis. *Chl pneumoniae* is prevalent in atherosclerotic lesions but generally absent in normal coronary arteries (181). It induces atherosclerotic lesions in mice and rabbits (175;182), particularly in association with hypercholesterolaemia. The role of infection as a possible trigger to the problem is consistent with an important animal model (pregnant rat) of pre-eclampsia (74). Faas *et al.* developed this model for pre-eclampsia in which a single very low dose endotoxin infusion induced a pregnancy-specific pre-eclampsia-like state, based on McKay *et al.*’s (183) observation that pre-eclampsia resembles the Shwartzman reaction. These changes were prevented by the administration of either low dose aspirin or superoxide dismutase. Certainly, subclinical endotoxaemia has been detected in those with the systemic inflammatory response syndrome (SIRS) (184), with which pre-eclampsia shares many characteristics (13). While Mittendorf *et al.* (71) have postulated that the use of antibiotic prophylaxis might reduce the risk of pre-eclampsia in primigravidae with a history of urinary tract infection, given the findings of Faas *et al.*, a single infectious event may be enough to trigger the inflammatory response of pre-eclampsia.

*Chl pneumoniae* is an intracellular pathogen that may reside within macrophages of atheromatous arteries in a chronic, persistent state with low metabolic activity. About 70% of persons with acute myocardial infarction show a seroresponse to chlamydial lipopolysaccharide epitope and elevated titres against *Chl pneumoniae* in sera from such patients point to an exacerbation in a chronic infection as does a change in the nature of immune complexes containing chlamydial LPS. The presence of antibodies to *Chl pneumoniae* proteins in immune complexes suggests an intimate association of the pathogen with the vascular system (185).
How might *Chl pneumoniae* increase the risk of pre-eclampsia? Pre-eclampsia can be considered a two-stage disorder, with the first stage involving abnormal placental implantation and the second being the abnormal maternal adaptation to the pregnancy that results in systemic dysfunction (186). In healthy pregnancies, the maternal spiral arteries undergo extensive remodelling, this remodelling is incomplete in pre-eclampsia (187) and, that the vascular change most commonly associated with normal pregnancies was physiological change and subintimal thickening of both segments of the spiral arteries. In pre-eclampsia, invasion of the uterine spiral arteries is limited to the proximal decidua, with 30% to 50% of the spiral arteries of the placental bed escaping endovascular trophoblast remodelling (8;31). Myometrial segments of these arteries remain anatomically intact and undilated, and adrenergic nerve supply to the spiral arteries is not affected. The mean external diameters of the uterine spiral arteries in women with pre-eclampsia are less than one half of the diameters of similar vessels from uncomplicated pregnancies (32). In pre-eclampsia medial disorganisation and hyperplasia in the myometrial arteries and acute atherosis in decidual arteries were common. The outcome of abnormal spiral artery remodelling, may contribute to reduced placental perfusion and, is considered to be the root cause of pre-eclampsia.

*Chl pneumoniae* may also contribute to the second stage of pre-eclampsia through endothelial dysfunction (188). This endothelial pathophysiology is shared by obesity, diabetes, hyperlipidaemia and hypertension, all of which are established risk factors for coronary heart disease and, when present in a women entering pregnancy, greatly enhance their likelihood of developing pre-eclampsia (189).

Saikku *et al.* (63) reported an association of IgG antibody to *Chl pneumoniae* and coronary heart disease among young Finnish men and subsequent seroepidemiologic studies
suggested an association of IgG antibody to *Chl pneumoniae* with both coronary and carotid atherosclerosis. These studies followed by mounting evidence from pathological, animal experimental, and molecular studies that supported a possible etiologic role of infection with *Chl pneumoniae* in atherosclerosis (65;76). Similarly, past, persistent, or recurrent infection with *Chl pneumoniae* may contribute to abnormal vascular function, atherosis, and the abnormal placental perfusion that marks the first stage of pre-eclampsia.

Antibodies against both CMV and *Chl pneumoniae* have been correlated with reactivation of chronic infection in a number of other clinical scenarios. Specifically, for CMV, antibody levels against CMV have been correlated with viral load and clinical state. During the Th1 downregulation of normal pregnancy (37), it is recognised that immune control over chronically carried CMV can be lost (190;191). It is uncertain whether the same will be noted for *Chl pneumoniae* as well.

We also tested for the presence of a maternal immune response (IgG) to CMV, which is found in 60-80% of the population. CMV is thought to be responsible of a large proportion of graft versus host disease in bone marrow transplantation (192). CMV is a member of the βherpesvirinae, a subfamily of the herpesviridae, and after infection the virus is generally not eliminated, may maintain latency in many tissues of the host, and is capable of infecting trophoblast (193).

Virus excretion after infection continues for a long time. CMV is the most frequent cause of congenital infections (1-2% children). A fetal infection can lead to a preterm labour. The diagnosis can be done on the basis of identification of specific antibodies, virus cultivation and PCR from urine, saliva, blood, and cerebrospinal fluid. An antiviral drug ganciclovir can be used for treatment. Anti-CMV vaccines are under development (194).
Several pieces of evidence suggest that vascular endothelium may be a site of latent herpetic viral infection, and that activation of such infection might cause or aggravate atherosclerosis.

Other viruses (Herpes simplex 1, Herpes simplex 11, Coxsackie B) have been identified as potential culprits in atherogenesis, but evidence is less compelling than for CMV.

The mechanisms are reported by which infected endothelium may be damaged by marginated inflammatory cells (195), and be transformed from an anticoagulant to a procoagulant tissue (196). This transformation would promote the adherence of neutrophils and platelets to the endothelium. Viral infection of the endothelium induces the expression of viral glycoproteins and adhesion molecules, which promote neutrophil and monocyte adhesion. Viral infection also induces the procoagulant molecule, tissue factor, in endothelial cells. These enhanced procoagulant effects are associated with the loss of anticoagulants, including thrombomodulin, prostacyclin and tissue plasminogen activator (196). These studies support the speculation that viral infection in vivo promotes vascular injury and thrombosis, which may contribute to disease states such as atherosclerosis and pre-eclampsia.

CMV–related chronic villitis has been associated with pre-eclampsia (169), IUGR (170), and intrauterine fetal death (171). In addition, there is an impaired lymphoproliferative response to CMV in women suffering from recurrent spontaneous abortion (172), which is probably part of the spectrum of disease that includes pre-eclampsia (18). Antibodies against both CMV and Chl. pneumoniae have been correlated with reactivation of chronic infection in a number of other clinical scenarios. Specifically, for CMV, antibody levels against CMV have been correlated with viral load and clinical state in conditions as varied as human immunodeficiency virus infection and extraterrestrial travel as an astronaut (197;198).
With *Chl pneumoniae*, serum IgG antibodies have been correlated with atherosclerotic disease activity. During the Th1 downregulation of normal pregnancy (37), it is recognised that immune control over chronically carried CMV can be lost (190;191). It is uncertain whether the same will be noted for *Chl pneumoniae* as well.

Although based upon a prospective population-based cohort, our study was limited in a number of ways. First, all cases were identified in a tertiary referral centre. Second, not all of the cases and controls identified within this centre had either enough or any serum sample in the rubella bank to permit its use in this study. As a consequence, not all cases were either fully matched (two controls per case) or matched at all (controls were identified for only 9 of 18 early-onset pre-eclampsia cases), which reduced the statistical power of the results of this preliminary study. Finally, the sample size for early-onset pre-eclampsia, in particular, was small, although we used a rigorous case definition for pre-eclampsia and well established methods for the detection of the infectious agents.

The seroprevalence for both CMV and *Chl pneumoniae* was similar to the published experience for women of reproductive age (81;84). The sample size reduced the power of the study to determine any true differences in seroprevalence between groups, although they did appear to be higher in early-onset pre-eclampsia. We achieved sufficient power to detect 27%, 45%, and 47% differences in the seroprevalence for CMV, *Chl pneumoniae* and both CMV and *Chl pneumoniae*, respectively, at 80% power. At 60% power, the difference between groups for seroprevalence became significant for all comparisons. These data would be consistent with those of Heine and colleagues (85), who found increased seroprevalence of anti-*Chl pneumoniae* IgG (and not IgM) in pre-eclampsia when compared with normal pregnancy and not to *Chl trachmatis* or *Chl psittaci*, which suggest a specific association between infection with *Chl pneumoniae* and pre-eclampsia (85).
In the presence of enough other predisposing factors for pre-eclampsia, leading to uteroplacental mismatch (20), the presence of a reactivated infection with either CMV and/or Chl pneumoniae could precipitate that excessive innate immune response and inflammation especially characteristic of early-onset disease (19;178;199).

Early-onset pre-eclampsia differed from normal pregnancy, while late-onset pre-eclampsia did not. This supports the conjecture that early-onset pre-eclampsia is a different condition from late-onset disease (178). Early-onset disease carries a far greater burden of maternal (200) and fetal (20;201) risks, and differs more from the, often gestational age-dependent, physiological adaptations of normal pregnancy than does late-onset pre-eclampsia (202), perhaps supporting (19;20;156) the conjecture that late-onset pre-eclampsia may be adaptive in evolutionary terms (203), proffering survival advantages for a fetus exposed to uteroplacental mismatch (203-205) while minimally increasing maternal risks (200).

The results here presented suggest that pre-eclampsia could be a multifactorial disease and our findings could explain why it has been impossible so far to point out a unique disease responsible gene and suggest that other infectious agents could have a similar influence on the aetiology of this disease. Also, the infectious agents included in this study may serve as indicators of one or more other infectious agents, spreading in the same manner which may contribute to the development of pre-eclampsia.

Given the findings of this preliminary study, which require prospective population-based confirmation of both serology and direct measurement of infectious particle DNA by reverse transcriptase polymerase chain reaction within buffy coat cells, it is possible that antibiotics and antivirals may develop a role in pre-eclampsia prophylaxis.

Early-onset pre-eclampsia may truly be the 'toxaemia' of pregnancy.
Chapter 4

Conclusion, general approach, future directions for the research
4.1 General approach

This thesis examined the role of immune adaptation to pregnancy in influencing the development of the maternal syndrome of pre-eclampsia (13;20;93;206). The first approach was a case-control study of women admitted to an ICU with the systemic inflammatory response syndrome (SIRS). This was ultimately unsuccessful.

The second approach was an examination of the seroepidemiology of two infectious agents believed to be involved in atherogenesis, namely cytomegalovirus (CMV) and Chlamydophila pneumoniae (Chl pneumoniae) (21). The development of pre-eclampsia during a woman’s reproductive career predicts later atherosclerotic morbidity and mortality (95;96;207).

We undertook these two investigations to test two hypotheses.

First, as it is believed that the maternal syndrome of pre-eclampsia is a form of the SIRS; we postulated that a history of pre-eclampsia will predispose women to the later development of SIRS, as has already been established for atherosclerotic disease. This experiment was described in Chapter Two.

Second, we postulated that women may be more likely to develop the maternal syndrome of pre-eclampsia in the presence of subclinical infection with either Chl pneumoniae or CMV, than women whose pregnancies are either uncomplicated or complicated by IUGR in isolation.

4.2 Summary of the results

There were insufficient data from the pilot study of women admitted to the ICU at St Paul’s Hospital to undertake any valid analyses. Over a six month period, I identified,
consented, and obtained data from only six cases and two controls. The data collected were incomplete at best. Therefore, the pilot project was abandoned.

We proceeded to undertake a nested case-control study of serum from a population-based bank. Seroprevalence and levels of anti-CMV and *Chl pneumoniae* IgG were compared (non-parametrically) between women with early-onset pre-eclampsia (<34 weeks'; n=9), late-onset pre-eclampsia (≥34+0 weeks'; n=29); normotensive IUGR (birthweight <3rd centile; n=33), and matched normal pregnancy (n=113, up to 2 per case). There was a significant difference in both anti-CMV and *Chl pneumoniae* EB antibodies between groups (Kruskal-Wallis p<0.05). Women with early-onset pre-eclampsia had higher anti-CMV titres (median: 79 [95% confidence interval 47, 164]) than women with late-onset pre-eclampsia (26 [22, 82], p<0.05), normotensive IUGR (40 [31, 72], p<0.05), and normal pregnancy (49 [45, 70], p<0.05). Women with normotensive IUGR had significantly lower anti-*Chl pneumoniae* antibodies (0.10 [0.08, 0.38]) than did normal pregnancy controls (0.21 [0.20, 0.28], p<0.05).

4.3 Conclusions

No firm conclusions can be drawn from the case-control study undertaken in the ICU. From the seroepidemiology study we concluded that the anti-CMV and anti-*Chl pneumoniae* antibodies were higher in early-onset pre-eclampsia than in late-onset pre-eclampsia, normotensive IUGR, and normal pregnancy. This may provide a pathophysiological link between pre-eclampsia and the known increased risk for subsequent atherosclerosis (186).

This information could lead to novel therapeutic manoeuvres to attenuate the inflammatory response of pre-eclampsia, thereby safeguarding against fetal growth restriction and iatrogenic prematurity. A reduction of the pro-coagulant environment would reduce the
incidence of occlusive placental events, thereby protecting both the maternal perfusion of the inter villous space and, consequently, fetal nourishment. Similarly, some fetuses of women with pre-eclampsia are frequently delivered whilst still thriving *in utero* because of the severity of the maternal syndrome. Any manoeuvre, such as macrolide / nitrofurantoin antibiotic prophylaxis or short course antiviral agents, that was able to reduce the rate of the development of the maternal syndrome would have a direct effect on prematurity and its consequences. As stated, pre-eclampsia is the commonest cause of iatrogenic prematurity. Early-onset pre-eclampsia may truly be the 'toxaemia' of pregnancy.

### 4.4 Future directions

From the first, unsuccessful, study, it may be possible to answer the questions raised by the hypothesis using a number of different approaches. First, a long term follow-up study designed to investigate the inflammatory sequelae of having developed pre-eclampsia, including both atherosclerosis (an inflammatory condition) and SIRS. Second, using population-based data records, such as exist in Scandinavia and Scotland, to link maternal obstetric outcomes with later hospital admissions/death certification.

The results of the second study of *Chl pneumoniae* and CMV seroepidemiology require validation. First we must perform a prospective sample of cases and controls to determine if the seroprevalence is greater in pre-eclampsia. In particular we are interested in early-onset pre-eclampsia, than in other groups which control for disease severity (late-onset), abnormal placentation (normotensive IUGR), and pregnancy (normal pregnancy and non-pregnancy).

Second, we need to identify both *Chl pneumoniae* and/or CMV DNA in buffy coat (ie lymphocytes and monocytes) cells of women from the groups outlined above. This would
reveal that there was truly active infection at the time of disease diagnosis in pre-eclampsia. As opposed to the infection having been cleared previously this could be undertaken using molecular biological techniques such as semi-quantitative reverse transcriptase polymerase chain reaction.

Third, to investigate the expression of receptors handling these infectious agents, such as Toll-like receptors (TLR)-2 and -4, and polymorphisms in the genes encoding those receptors (tlr-2 and tlr-4).

Fourth, we require a population-based seroepidemiological study of *Chl pneumonias* and CMV timed to coincide with the triple marker screen (208) used to screen for aneuploidy and open neural defects, linked with pregnancy outcomes. This would identify if the change from adaptive to innate immunity in early pregnancy would permit the identification of a group of women subsequently at risk for developing pre-eclampsia, using levels of anti-*Chl pneumonias* and anti-CMV as a screening test.

Fifth using rubella serology as a control for prospective validation.

Finally, were these studies to prove successful, then intervention trials could be designed to prevent the onset of, especially early-onset, pre-eclampsia.

This line of enquiry has been embarked upon by the Vancouver Interdisciplinary Pre-Eclampsia and fetal growth Restriction group.
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