# MATERNAL INFECTIONS AND PREECLAMPSIA

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# ABSTRACT

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### By

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**Introduction:**

Preeclampsia (PE) is a pregnancy related health condition which affects maternal and newborn health significantly. No causative factor has been identified yet. Evidence suggests the role of *Helicobacter pylori,* cytomegalovirus and *Chlamydophila pneumoniae* in the development of PE. I conducted this research to explore the role of these microorganisms in the development of PE.

**Methods:**

I conducted a nested case-control study by using the ARCH dataset and matched cases with controls on: maternal age (±3 years), maternal race, parity and gestational age at blood withdrawal. My outcome variable was PE status and variable of PE was not present in the ARCH questionnaire; however, birth certificate provided this information. To find more cases, because we decided to review medical records (MR) of the ARCH study subjects. Case base for my study was obtained by reviewing medical records. I used conditional logistic regression to look for the association between IgGs of three microorganisms and other covariates of interest, and PE status by building three separate conditional logistic regression models.

**Results:**

Out of total sample of 73 subjects, 21 were cases. Two (9.5%) of the cases and three (5.8%) of the controls were found positive for anti *H. pylori* IgGs in their plasma. For CMV, 7 (33.3%) cases and 15 (28.9%) controls had anti CMV IgG in their plasma while thirteen (62%) cases and

25 (48%) of controls were positive for anti *C. pneumoniae* IgGs in their plasma. At the Univariable analysis, Smoking during pregnancy showed a protective effect for PE. The women who smoked during pregnancy were 40% less likely to have PE than women who did not smoke during pregnancy. Women whose pre-pregnancy BMI (PPBMI) was more than 30 were 8 times more likely to develop PE as compared to the women whose PPBMI was less than 25. Multivariable conditional analysis found that women who were positive for anti *H. pylori* IgGs in their plasma were 2.4 times more likely to develop PE (mOR: 2.4; 95% CI: 0.2-32.2) than those women whose plasma was negative for these antibodies after controlling for the effect of other variables in the model. Similarly, women with anti CMV IgGs in their plasma were 1.4 times more likely to have PE than women without any evidence of CMV infection (mOR: 1.4; 95% CI: 0.3-5.6) after controlling for the effect of other variables in the model. For *C. pneumoniae*, the odds of anti *C. pneumoniae* IgGs among PE cases is 2.3 times (mOR: 2.3; 95% CI: 0.6-9.1) the odds of anti *C. pneumoniae* IgGs after controlling for the effect of other variables in the model.

**Conclusions:**

Past infection, as depicted by anti *H. pylori* IgG, anti CMV IgG, and anti *C. pneumoniae* IgG, did not show any association with the development of PE in Lansing, Michigan. No evidence of recent or current infection with CMV as measured by IgM, identified as a risk factor for the development of PE in Lansing, Michigan. To explore this relationship further, similar studies should be carried out in future with a larger sample size especially in populations where prevalence of these infections is high. Future studies should include serology for anti-CagA antibodies for the exploration of the role of *H. pylori*. Exploration of the infectious agents DNA from placental tissue may support the immunological cause of PE.

This dissertation is dedicated to my parents who
raised their children against all odds.

# ACKNOWLEDGMENTS

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# KEY TO ABBREVIATIONS

| | |
|---|---|
| ACOG | The American College of Obstetrics & Gynecology |
| ACROBAT-NRSI of Interventions | A Cochrane Risk of Bias Assessment Tool for Non-Randomized Studies |
| ACS | Acute Coronary Syndrome |
| adjOR | Adjusted Odds Ratio |
| $AT_1R$ | Type-1 angiotensin II receptors |
| ARCH | The Archive for Child Health |
| AT-AA | Autoantibodies against $AT_1R$ |
| BMI | Basal Metabolic Rate |
| BP | Blood Pressure |
| CagA | Cytotoxin-associated protein |
| CC | Case control |
| CDC | The Center for Disease Control and Prevention |
| CI | Confidence interval |
| CMV | Cytomegalovirus |
| CO | Cardiac output |
| CP | *Chlamydophila pneumoniae (C. pneumoniae)* |
| DAG | Direct Acyclic Graph |
| DNA | Deoxyribonucleic Acid |
| ELISA | The enzyme-linked immunosorbent assay |
| EMBASE | Excerpta Medica dataBASE |
| EVT | Extra-villous Tissue |

| | |
|---|---|
| HDP | Hypertensive disorders of pregnancy |
| HLA | Human Leukocyte Antigen |
| HP | *Helicobacter pylori (H. pylori)* |
| Hsp | Heat Shock Proteins |
| IgA/IgG/IGM | Immunoglobulin A/ Immunoglobulin G/Immunoglobulin M |
| IL | Interleukins |
| IMT | Intima-media thickness |
| INF | Interferon |
| JA Kids | Jamaican National Children Study |
| LRT | Likelihood Ratio Test |
| MCH | Major Histocompatibility Complex |
| MeSH | Medical Subject Headings |
| MIF | Micro-immunofluorescent |
| mm Hg | Millimeter of Mercury |
| mmol | Milli mole |
| mOR | Matched OR |
| NHBPEP | The National High Blood Pressure Education Program |
| NK | Natural Killer |
| NS | Non-significant |
| OR | Odds Ratio |
| PCR | Polymerase Chain Reaction |
| PE | Preeclampsia |

| | |
|---|---|
| PlGF | Placental Growth Factor |
| RoB | Risk of Bias |
| RR | Risk Ratio |
| sEng | soluble Endoglin |
| SES | Socioeconomic status |
| sFlt-1 | soluble fms-like tyrosine kinase 1 |
| Th1/Th2 | T Helper cell 1/ T Helper cell 2 |
| TNF | Tissue Necrotic Factor |
| TVR | Total vascular resistance |
| UreC | Urease subunit C of *H. pylori* |
| UreE | Urease subunit E of *H. pylori* |
| USD | US Dollar |
| VacA | Vacuolating cytotoxin protein |
| VEGF | Vascular Endothelial Growth Factor |
| VT | Villous Tissue |
| WGO | The World Gastroenterology Organization |
| WHO | THE World Health Organization |

# 1 Chapter 1: Introduction

## 1.1 Hypertensive disorders of pregnancy

High blood pressure (systolic BP $\geq$ 140 or diastolic BP $\geq$ 90 mmHg) in pregnancy can present in a variety of ways, as a sole problem or accompanied by some associated features. Because of the multiple features associated with hypertension, the condition has been termed as the hypertensive disorders of pregnancy (HDP). Due to this variability in the presentation of this condition, the American College of Obstetrics and Gynecology (ACOG) developed and introduced a classification of hypertensive disorders of pregnancy in 1972. Afterwards, this classification was revised in the years 1990 and 2000 and has been documented in the reports of the National Health Blood Pressure Education Program Working Group (1).

**Table 1.1 Hypertensive disorders of pregnancy**

| Type of Hypertension | Description |
|---|---|
| Chronic hypertension | A situation when a woman is already diagnosed as hypertensive before conception or pregnancy or develops hypertension in pregnancy but before 20$^{th}$ week of pregnancy. |
| Gestational hypertension | Recording of high blood pressure after 20$^{th}$ weeks of pregnancy in previously non-hypertensive woman and normalization of the blood pressure within 12 weeks after delivery |
| Preeclampsia-eclampsia | A pregnant woman who presents with a new onset of hypertension along with proteinuria (or some other features) after the 20$^{th}$ week is diagnosed as preeclamptic and if she also experiences convulsions then she progresses a more severe stage of eclampsia |
| Chronic hypertension with superimposed preeclampsia | A pregnant woman who with chronic hypertension also develops new proteinuria during current pregnancy. |

Based on ACOG classification (1), four main groups have been categorized: 1) Chronic hypertension is a situation when a woman is already diagnosed as hypertensive before

conception or pregnancy. If a woman develops hypertension in pregnancy but before the 20th week of pregnancy she is still considered as diagnosed with chronic hypertension; 2) Gestational hypertension is  the recording of high blood pressure after the 20th week of pregnancy in a previously non-hypertensive woman and the normalization of the blood pressure within 12 weeks after delivery; 3) Preeclampsia-Eclampsia is a condition when a pregnant woman starts presenting with a new onset of hypertension along with proteinuria after the 20th week of gestation. If she also experiences convulsions, then it reflects the progression of preeclampsia to a more severe stage of eclampsia; and 4) Chronic hypertension with superimposed preeclampsia is the diagnosis made when a pregnant woman who has chronic hypertension also develops new proteinuria during current pregnancy.

The most common presentation of preeclampsia (PE) is a new onset of elevated blood pressure (systolic BP $\geq$ 140 or diastolic BP $\geq$ 90 mmHg, measured at least at two occasions with a minimum interval of 4 hours between the two measurements), which occurs after the 20th week of gestation and is accompanied by proteinuria (300 mg or more of protein in a 24-hour urine sample or 1+ when measured using dipstick method). In situations when proteinuria is absent, the diagnosis of PE can be made when hypertension is accompanied by one or more of the following conditions: thrombocytopenia, renal or hepatic failure, pulmonary edema, and cerebral or visual symptoms (1,2).

Among different categories of HDP, preeclampsia presents as a systemic problem affecting multiple organ systems, but the central component is always the placenta.  In the majority of the cases, the condition improves with the delivery of the fetus and placenta.  Very rarely, women may continue to have preeclamptic status in the postpartum period or develop hypertension and other accompanying features after the delivery of the fetus and placenta(3,4).

## 1.2 Effects of preeclampsia on human health

Preeclampsia belongs to the group of women's health conditions which have significant impact on the perinatal morbidity and mortality across the world (5). Regardless of the geographic region, about 10-15% of direct maternal deaths are attributed to preeclampsia or its complications (6–9). This may suggest that once the chain of events related to PE starts it becomes difficult to stop and to reverse the pathologic cascade of events, especially in the more severe categories of disease.

In addition to maternal mortality, preeclampsia has also been found to be responsible for 25% of stillbirths occurring worldwide (10). Besides its effects on mortality, there is substantial morbidity resulting from preeclampsia. Some women may develop seizures, which signal progression to eclampsia. Additionally, it may lead to stroke, renal and hepatic failure, and coagulopathy. These conditions usually require intensive care.

Apart from the immediate consequences, even when the woman apparently recovers uneventfully from a recent preeclamptic state, there are instances when both short and long term health complications could develop. Women may be at an elevated risk for hypertension, cardiovascular disease, and renal problems in later life(11), and newborns may suffer growth retardation and have higher risks of cerebral palsy and other neurodevelopment disorders later in life(12).

## 1.3 Epidemiology of preeclampsia

Globally, the burden of preeclampsia varies across different regions. As estimated by the World Health Organization (WHO), the incidence of PE around the world ranges from 2-10% of all pregnancies (13). The frequency is almost always low in high income countries as compared to

low income countries.  Again, the prevalence ranges from 1.0-4.5% in high income countries as shown in the Figure 1(14).
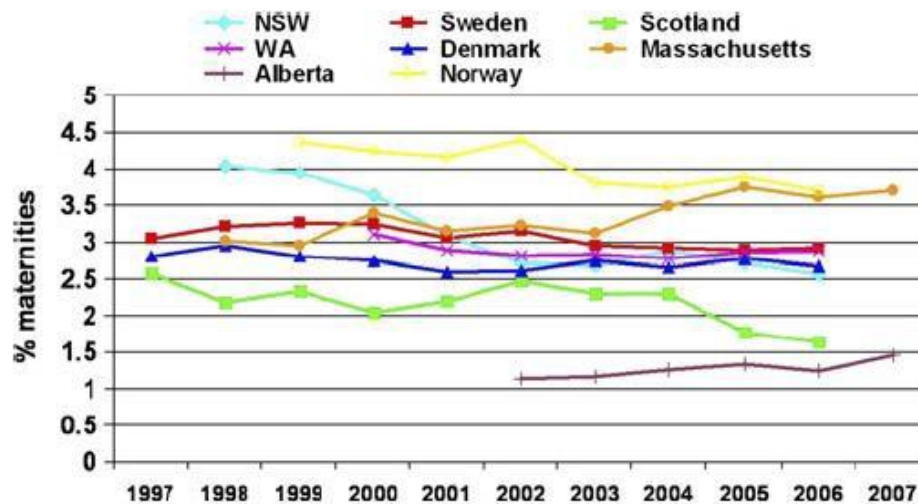


**Figure 1.1 Trends in preeclampsia incidence across high income countries**

*Source:* (14).

A WHO report found that PE incidence was seven times higher in low income countries compared to high income countries (13). For example, in Africa, PE incidence ranges from 1.8-7.8% of pregnancies, excluding Nigeria where preeclampsia presents with the highest frequency approaching 16.7% (15). Among countries from the middle income group (16), Jamaica shows a high incidence of preeclampsia at 5% (17).  About 25% of maternal deaths are attributable to preeclampsia in the Caribbean (7).

Comparatively, evidence is lacking on the trends of preeclampsia, especially in low income countries. Information is also not available for a majority of high income countries. Figure 1 provides some information on time trends for PE incidence for a selective group of countries. This figure shows that over the previous two decades the frequency of preeclampsia remained almost constant in Sweden, Denmark, and Western Australia but fluctuated for others. Since 2003, the rates have been fluctuating in different countries. Rates went down in Scotland,

minimally increased in Alberta, Canada, while rates significantly increased in Massachusetts, USA from 2.5 % in 1987 to 3.2 % in 2004 (18).
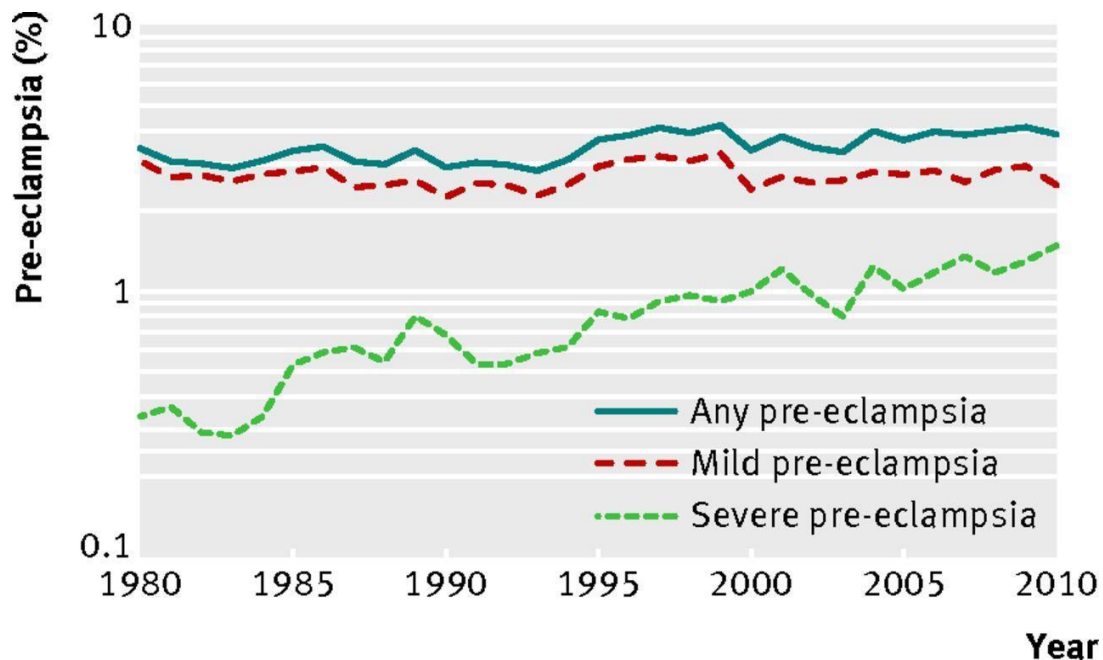


**Figure 1.2 Temporal changes in prevalence of pre-eclampsia: United States, 1980 to 2010**
*Source:* (18).

Figure 2 above further explains the trends in the US over three decades. There was a fluctuation in 'all/any type' and 'mild type' of preeclampsia with a small increase in 'all type' (3.4 to 3.8%) while there was a small dip in 'mild type' (3.1 to 2.5%). However, a remarkable change is reflected in the 'severe type' where it increased by more than 300%. This significant increase in severe type and decrease in mild type may also explain some of the mild type cases progressing towards severe type.

This is a dilemma that we, as a scientific community, lack information on the causative factors or agents for the development of PE. A large body of evidence has been produced, in a majority of the cases from high income countries, for the exploration of the causative factors in

the development of preeclampsia.  In this regard, research work has been done to find out genetic, nutritional and dietary, metabolic, environmental, and cardiovascular factors (19–21) without any remarkable success. The WHO Global Survey on Maternal and Perinatal Health has identified some risk factors which include: older age (more than 30 years), low literacy, high body mass index, nulliparity, chronic hypertension, diabetes, and cardiac and renal diseases (22).

Over the past few decades, efforts have been made for investigating the possible role of infections in the development of preeclampsia. However, the scientific and health communities have yet to produce sufficient evidence to establish a clear link between infections and the development of preeclampsia. For my dissertation, I propose the following aims and hypotheses for the exploration of the role of maternal infections in the development of PE.

Initially, I had planned to analyze the datasets from Lansing, MI and Jamaica. Unfortunately, our work has not yet finished in Jamaica. So, I am now analyzing and presenting my analysis and write up only on Lansing, MI dataset i.e.; the ARCH study data, however, the hypotheses still mention Jamaica.

## 1.4   Hypotheses

### 1.1.1   AIM 1: Exploring hypotheses about infection and PE

Hypothesis 1.1: Women with preeclampsia will have higher levels of antibodies (IgM or IgG) against *H. pylori.*

Hypothesis 1.2: Women with preeclampsia will have higher levels of antibodies (IgM or IgG) against CMV.

Hypothesis 1.3: Women with preeclampsia will have higher levels of antibodies (IgM or IgG) against *C. pneumoniae.*

## 1.1.2 AIM 2: Exploring the social context of PE

Hypothesis 2.1: After controlling for social circumstances, the associations of PE to antibodies to *H. pylori* will remain significantly elevated.

Hypothesis 2.2: After controlling for social circumstances, the associations of PE to antibodies to CMV will remain significantly elevated.

Hypothesis 2.3: After controlling for social circumstances, the associations of PE to antibodies to *C. pneumoniae* will remain significantly elevated.

# 2 Chapter 2: Background

In this chapter, a brief note on the types of PE (preeclampsia) is provided, followed by a description of the pathophysiology of PE. The section on pathophysiology is divided into stages, corresponding to the development of PE:

- Core Problems in the Development of PE
- Effect of Defective Placentation
- Clinical Manifestations of PE.

Following the description of pathophysiology, the role and types of microorganisms which affect the vascular system and take part in the development of atherosclerosis are discussed. The same section also elaborates on infection in pregnancy along with different infectious agents which have been hypothesized to have a role in the development of PE.

## 2.1 Types of preeclampsia

PE has been classified into two groups based on the gestational age at which clinical features develop (23). If a woman presents with PE at some time between the $20^{th}$ week and the 34th week of pregnancy then it is called early PE; when a woman develops symptoms of PE after the 34th week of pregnancy, she is said to have developed late PE. Early PE is also termed placental in origin due to more frequent involvement of pathology of placental vessels which results in a state termed low cardiac output-high total vascular resistance (low CO-high TVR). In contrast, late PE is viewed as maternal in origin because of the association of late PE with maternal constitutional causes including diabetes mellitus and pre-pregnancy BMI; the late PE is also termed high CO-low TVR (23,24).

## 2.2 Pathophysiology of preeclampsia

PE is a disorder that affects multiple organ systems; however, the central organ for the development of PE is the placenta. After placental delivery, the condition of a woman with PE improves in almost all cases (19). Typically, PE starts with poor blood supply to a placental bed, which results in placental ischemia; however, what causes this to occur is unknown. Regardless of the cause, the pathophysiologic mechanisms of PE may be described in three stages. The first stage is the defective development of the placenta. This disrupts the homeostasis of the mother's body. The first stage consists of two processes: first, invasion of the decidual stroma by trophoblast cells and second, remodeling of the spiral arteries. These two processes are considered the primary steps in the development of the placenta in a normal pregnancy. A placental defect during the first stage (to be described in more detail below) leads to an imbalance in many biomarkers in the body during the next step, the intermediate stage. The final stage is characterized by a cascade of events resulting from the disturbed homeostasis which affects multiple organs and systems in the body. This final stage is responsible for the clinical features that a case of preeclampsia may present (25,26).

### 2.2.1 First stage: Core problems in the development of the placenta

In a majority of cases, the pathologic mechanisms for the development of PE start during the very early stages of pregnancy when development of the placenta starts. Two mechanisms are usually proposed for the explanation of the development of PE. These are, in fact, the primary steps in this developmental process of the placenta (invasion of trophoblast cells and remodeling of spiral arteries). When these steps do not proceed normally, defects in the placenta result. Moreover, other mechanisms, which usually initiate as a factor, external to the placenta, have been proposed for the explanation of the development of PE (19–21). Two such external

mechanisms are related to recent maternal infections or immunological response to maternal infections in the past (27–30). In the following sections, a summary of these mechanisms is presented.

Incomplete invasion of maternal decidual stroma by trophoblast cells:

Out of the two types of trophoblast cells (the villous and extravillous), the extravillous are invasive in their character and are further divided based on the area they invade (31). The extravillous trophoblast cells which invade the interstitial layers of the uterus are called the interstitial trophoblast cells. Invasion of the decidual stroma by the trophoblast cells is considered the first step in the development of the placenta. Normally, this process starts approximately two weeks after egg fertilization when the trophoblast cells start invading the maternal uterine wall as deep as the muscle layer. Eventually, these cells approach the newly developing spiral arteries (31–33). Invasion by the trophoblast cells is not uniform. The central portion of the placenta has the deepest invasion and as the distance increases from the center to the periphery the invasion correspondingly reduces (33). This invasion by the trophoblast cells facilitate the dilatation of the spiral arteries as these cells disorganize the muscular layer of the arteries. Deeper invasion by the trophoblast cells leads to lengthening and dilatation of the spiral arteries. During early pregnancy, infiltrating macrophages and uterine natural killer cells also play a role mainly by two processes: secreting angiogenic factors and inducing apoptosis for a portion of muscle and endothelial cells (34,35). In a preeclamptic placenta, however, these trophoblast cells are not able to go beyond a superficial layer of the maternal decidual stroma. The inability of trophoblast cells to penetrate deeply results in underdeveloped blood channels, which are insufficient to meet the demands of the developing fetus.

Lack of remodeling of spiral arteries:

In normal pregnancy, the endovascular trophoblast is responsible for the widening of the lumen
of spiral arteries in the placenta by invading these vessels and replacing the endothelial layer
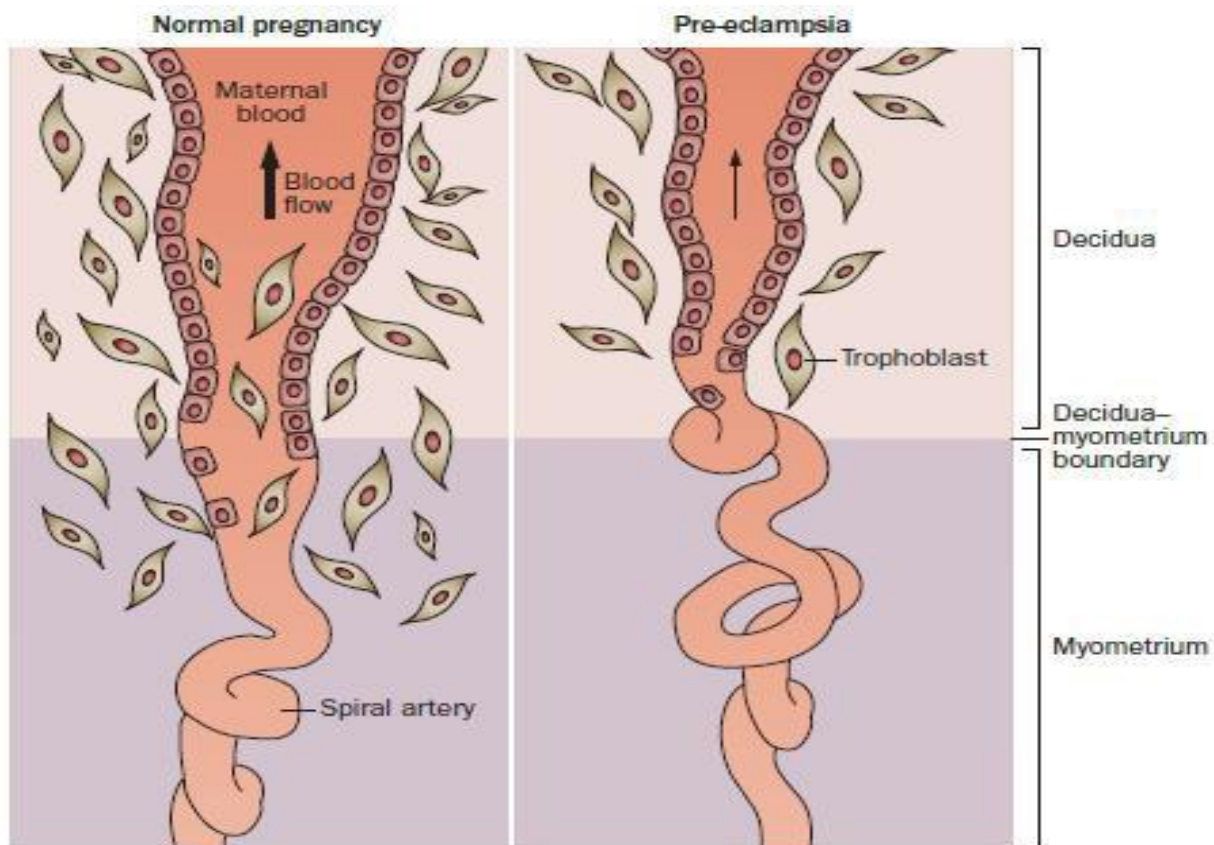


**Figure 2.1 Physiological transformation of spiral arteries in normal pregnancy and preeclampsia.**
*Source*: (26)

 (31–35). This invasion of spiral arteries by the trophoblast cells results in low-resistance wide

channels. This change allows for vasodilation and a marked increase of blood flow to the

placenta and to the growing fetus (36). In PE, however, the endovascular trophoblast cells do not

invade spiral arteries deeply enough to convert these arteries to the dilated vessels, which are

necessary for the maintenance of uninterrupted blood supply to the fetus (37). Failure of the

endovascular trophoblasts to properly remodel the spiral arteries is attributed to various factors: the inability of endovascular trophoblast cells to show vascular phenotype, trophoblasts at increased risk of apoptosis, inadequate changes in the decidua and poor immunological response as shown in Figure (38–40). It has been found that poorly-transformed spiral arteries are at an increased risk of developing atherotic plaques, which are similar to that of early atherosclerotic changes (41). This results in the fibrinoid necrosis of the arterial wall, perivascular infiltration of mononuclear cells along with deposition of lipid-laden macrophages in the lumen (41). Defective placentation results in placental and fetal hypoxia and under-nutrition of the fetus.

External factors inhibiting the normal development of the placenta: the role of maternal infections and immunity

A growing amount of evidence shows that the inhibition of trophoblast invasion may be due to the presence of infectious agents (27,28,42). These studies isolated infectious agents from the trophoblast tissue and found that the viability of trophoblast cells was reduced with poor capacity to invade through the extracellular matrix. A significantly higher proportion of cases of PE had *C. pneumoniae* DNA than controls (28).

Similarly, evidence shows that an episode of previous infection may also contribute to the development of PE, either as a reactivation of infection or as an immunological response to the circulating antibodies to some infectious agents (29,30,43). It is suggested that antibodies cross-react with the endothelial cells and trophoblast cells to initiate the cascade of steps towards the development of PE.

## 2.2.2      Intermediate stage: Effect of defective formation of Placenta

Th1 and Th2 imbalance and other immunological factors:

Different kinds of cytokines and other biomarkers are released in the human body depending upon the demands of the body as a result of PE. Cytokines, small proteins secreted by cells, have a main task of communication between cells (44). These are, in the majority of the cases, secreted by CD4-positive T helper cells in the body, which are an important component of the body's immune system.

Two variants of the T helper cells, Th1 and Th2, are involved as immune mediators in pregnancy. In normal pregnancy, Th2 predominates and is responsible for the production of different types of interleukins (ILs), such as IL-4, IL-5, IL-6, IL-10, IL-13, and antibodies. Th2 and the interleukins produced promote normal pregnancy. In contrast, Th1 cells, which are pro-inflammatory, produce IL-2, tissue necrotic factor (TNF)-$\beta$, interferon (INF)-$\gamma$, and induce cellular immunity, dominate the situation in women with PE (45). This change in the ratio of Th1 and Th2 and the resulting secretions of cytokines and other factors reflect an evidence of intravascular inflammation which produces the features of PE (46).

In PE, the up-regulation of Th1 results in cytotoxic activity due to the stimulation of natural killer (NK) cells and CD-8 positive T cells (47). Cultures of peripheral blood mononuclear cells prepared in the blood of preeclamptic women, showed high concentrations of, Th1 cytokines such as, TNF-$\beta$, IL-2, and INF-$\gamma$ which stimulate CD-8. The blood of normal pregnant women does not show any increase in these factors (48). This experiment supports the association between PE and the factors produced by Th1 cells. Additionally, Th1 has been found to be associated with an increase in endothelin-1, a potent vasoconstrictor with which has a protective effect pro-inflammatory properties, and a reduction in plasminogen activator inhibitor-2, on the body as far as viral infections and tumor cells are concerned (49,50).

Apart from the direct role of T cells, some evidence indicates a relationship between autoimmunity and the development of PE. Normally, pregnant women show a low responsiveness to angiotensin II, an octapeptide which plays a significant role in the hemostasis of cardiovascular system (51,52); however, women with preeclampsia show high affinity for angiotensin-II. Women with preeclampsia have been found to carry agonistic autoantibodies (AT-AA) against type-1 angiotensin II receptors (AT$_1$R); these autoantibodies in turn cause stimulation of these receptors, AT$_1$R, in vascular smooth muscle cells, endothelial cells and mesangial cells (53,54).
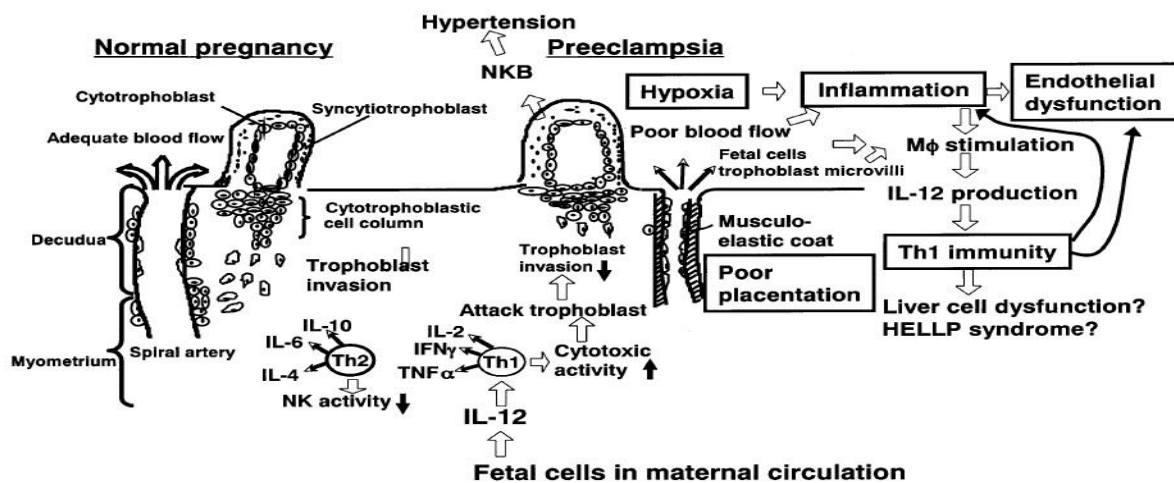


**Figure 2.2 Pathological changes in Preeclampsia with Inflammatory cells**

Source: (47)

Moreover, in an animal model, AT$_1$-AA were injected in rats which produced hypertension, edema, increased glomerular response and production of preeclampsia-syndrome producing biomarkers (55).

Change in the concentration of placental factors:

A variety of biomarkers are found in pregnant women. A balance in their concentration ensures a safe and successful pregnancy. Some important factors in this regard are placental growth factor

(PlGF) and vascular endothelial growth factor (VEGF). As the names suggest they are responsible for the optimum growth of placenta and the associated vasculature. Apart from issues related to the development of spiral arteries and endothelial injury, these biomarkers, PlGF and VEGF, have also been extensively studied and are hypothesized to play important roles in the causation of PE. These are derived from the placenta and are present in normal pregnancies in high concentrations to maintain blood flow and placental condition within normal limits. But in PE, these factors are lacking (56–58).

Additional factors such as soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble Endoglin (sEng) are also placental in origin. They are potent antagonists of PlGF and VEGF and thus their concentrations correspond positively to the existence and severity of PE. In fact, these factors bind to PlGF and VEGF and thus reduce the concentration of free PlGF and VEGF circulating in the blood. Deficiency of PlGF and VEGF result in poor vascularization and ischemia which causes endothelial dysfunction. Endothelial dysfunction leads to reductions in prostacyclin and nitric oxide concentrations causing vasoconstriction. At the same time, there is increased production of endothelin which disturbs the clotting mechanism in the body in addition to causing vasoconstriction (49).

Maternal endothelial response and coagulopathy:

Based on the discussion of the previous paragraphs, PE seems to have a clear association with maternal endothelial response. Several markers of endothelial injury are found during the intermediate phase of PE These markers are normally found in pregnant women (45). However, when PE develops, an abnormality in the concentration of these factors appears. Factors, which are favorable for pregnancy, like prostacyclin, are reduced in concentration, while factors which

promote vasoconstriction or hyper-coagulation, such as endothelin-1, von Willebrand factor, circulating cellular fibronectin, and thromboxane are increased (49,59).

### 2.2.3   Final stage: Manifestations of PE

In the final stage, the biomarkers which are produced and appear during the intermediate phase act on different organs in the body, creating the clinical features that characterize PE.  PE is a multi-organ problem, as seen by its pathophysiologic features. Different features of PE reflect the variety of organs affected and the type of pathophysiologic mechanism involved. The endothelial injury seems to be a central target of different biochemical changes in the development of PE, leading to its clinical features. The endothelial reaction results in increased vascular permeability, which explains the proteinuria and edema found in preeclamptic women.

The changes in vascular tone can underlie the development of hypertension. Another feature of PE is the production of pro-coagulants, which promotes hyper-coagulopathy (60,61). The probability of coagulopathy is increased as a result of enhanced concentrations of clotting factors. More severe situations result in high consumption of platelets ending up in thrombocytopenia (59).

## 2.3.   Microorganisms with predilections for the vascular system

Research on the origins of preeclampsia, primarily from developed countries, has explored genetic, dietary, metabolic, environmental, and cardiovascular risk factors for the development of PE (19–21). For over more than a decade, efforts have been made to explore the possible role of infections in the development of PE. However, the scientific and health communities are still waiting for evidence that could provide causal links between infections and the development of preeclampsia. One motivation to study the role of maternal infections in the development of PE is that it is analogous to the role of microorganisms in the development of atherosclerosis as

discussed below. Multiple microorganisms have been found to show an affiliation with vascular system. Specifically, the role of *Chlamydophila pneumoniae, Helicobacter pylori,* and cytomegalovirus (CMV) in the development of atherosclerosis has been especially well-documented (62–70).

### 2.3.1 Chlamydophila pneumoniae:

*Chlamydophila pneumoniae* has been extensively studies for its association with atherosclerosis. In one study where the coronary artery specimens of 40 subjects were explored for the evidence of infection, it was found that 22 specimens with acute coronary syndrome (ACS) had significantly higher concentrations of *C. pneumoniae* as compared to 18 specimens without ACS (62).

Another study of 76 patients with unstable angina found that the atherosclerotic plaques of 75% of the patients showed presence of *C. pneumoniae* DNA (65). One other study attempted to correlate the presence of serum antibodies against *C. pneumoniae* with the presence of C. *pneumoniae* DNA in the coronary artery atherosclerotic plaque and found that higher proportions of atherosclerotic plaques had *C. pneumoniae* DNA (63).

### 2.3.2 Helicobacter pylori:

The research on the relationship between *H. pylori* and vascular diseases has shown positive results. To determine the role of *H. pylori* in the development of atherosclerosis, one study looked for an association between inflammatory markers such as IL-8 and *H. pylori* in the process of atherosclerosis development in carotid arteries and found that the subjects with carotid intima-media thickness (IMT) had significantly higher levels of IL-8 than those without IMT (64). A follow-up study of *H. pylori* infected and non-infected individuals found a significant association between infection and carotid artery plaque formation (66). A meta-analysis of 13

studies reported a significantly higher risk of ischemic stroke in people infected with *H. pylori* (67).

### 2.3.3 Cytomegalovirus:

The role of CMV in the development of atherosclerosis has been supported in animals and humans. In an animal model, it was found that a higher proportion of CMV-infected mice had atherosclerotic plaques than uninfected controls (68). Other studies, carried out in humans, have found higher rates of CMV infection either assessed by antibodies or by finding the virus in atherosclerotic plaque in patients with coronary artery disease as compared to the controls (69,70).

As can be seen the existing literature shows a positive relationship between infections and the process of atherosclerosis. In this regard, the literature, supports the role of *H. pylori*, CMV and *C. pneumoniae* infections in the development of atherosclerotic process. As previously mentioned, since both the atherosclerotic process of PE and that of coronary and cerebral vessels have some common features of inflammatory response, these two processes may be considered analogous to each other.

## 2.4 Infection with H. pylori, CMV and C. pneumoniae and pregnancy

For this dissertation, the focus is on *H. pylori*, CMV and *C. pneumoniae* infections, not only because some studies have shown a link to PE but also because they are implicated in the development of atherosclerosis. Additionally, these infections have a high frequency of chronic infection or re-infection in the population, and for some women, an increase is especially seen around childbearing age. *C. pneumoniae* IgG antibodies, for example, are found in less than 10% of the population up to age 10 years, but then increase abruptly to a level of 50% seropositivity by age 40 and afterwards a steady increase results in prevalence reaching up to 80% (71,72). In

the US, up to 4% of the pregnant women get CMV(73). In comparison to this value, seroprevalence in some low-income countries, such as Nigeria, reaches up to more than 92%. CMV is documented as having a deleterious effect on pregnancy and the newborn. Each year, more than 25,000 children are born with CMV and about 150 die because of the infection; among the survivors, more than 55,00 would develop some sort of developmental problems, including: healing loss, visual impairment, psychomotor disability, behavioral problems and cerebral palsy (74,75). An analysis of the National Health and Nutrition Examination Survey (NHANES) data reflected that black females had the highest prevalence reaching upto 90% in their 40s followed by Mexican females indicating role of ethnicity in CMV infections (76) Also noteworthy is the prevalence of *H. pylori* in developing countries where it reaches up to 85-90%, while across the globe it is 50% (77), paralleling the epidemiology of PE.

While reviewing the related epidemiological studies on the role of *H. pylori*, CMV and *C. pneumoniae* infections in the development of PE, it was found that results from individual studies were mixed. Some show an association (78–80), while others do not (56,81). To look for the evidence of infections in the development of PE, I reviewed epidemiological studies on this topic (more details on this review is provided in Chapter 3). Based on this context of ambiguous findings related to infections and PE, I conducted my own updated systematic review on the associations of maternal infection with the three organisms of interest and PE (82). During my review, I also found two systematic reviews that looked at the role of maternal infections and PE (83,84). One review found no association (84) while the second was suggestive of a role of infection in the development of PE (83). With six additional studies published since the two earlier reviews, the research shows a trend of an association between maternal infections and PE,

but further research to investigate the role of these organisms was warranted to increase the body of evidence.

## 2.5   Determining the evidence of infection

For the determination of an evidence of infection, usually two laboratory methods are utilized. Either serological tests are performed which provide evidence of the existence of an infectious agent, current infection or past (85), or actual DNA of the infectious agent is isolated by employing the polymerase chain reaction (PCR) test (86). The PCR test provides a stronger evidence about the existence of an infectious agents but it may not be found applicable in all situations.

### 2.5.1   Serological tests

Serological tests use the antigen-antibody reactions to diagnose various health conditions. As the name suggests these tests mostly utilize the serum or liquid portion of blood where these antibodies are found. There are generally two types of serological tests: Direct and Indirect. In the direct serological test, an unknown antigen or microorganism is detected by using preparations of known antibodies. However, in the indirect method the known antigen is used to isolate antibodies in the serum of an individual which are being developed against that specific antigen (87,88).

An antigen-antibody reaction is the hallmark of serological tests. Usually, the serological tests are named following the type of antigen-antibody reaction/mechanism (89). Serological tests utilize a variety of testing strategies. Below is a brief description of the tests usually utilized for the detection of *H. pylori, C. pneumoniae,* or CMV.

Enzyme-Linked Immunosorbent Assay (ELISA) is one of the serological testing techniques; it follows the principle of antigen-antibody reactions. Antigens from the serum of

patients are allowed to attach to a surface where an antibody that has the sole purpose of trapping the antigen is already added. Once the antigen is trapped, known antibodies which are already chemically attached to an enzyme are allowed to come in contact with the same surface where the antigen is trapped and thus these antibodies create a reaction with the trapped antigens. Further, a substrate is added for the enzyme. The quantity of antigen-antibody complex formed in this way is directly proportional to the enzyme-substrate reaction and this is reflected by a change in the color (87).

Another test which utilizes serological techniques is the Micro-immuno Fluorescence (MIF) Test. It is a specific type of serological test utilized for some micro-organisms including *C. pneumoniae* (90,91). This test uses an indirect fluorescent antibody (FA) technique, which helps in observing the binding of an antigen to an antibody. This is facilitated by anti-globulin, which is fluorescein-conjugated, and corresponds to specific antibody molecules. The antigen used in this test comes from whole elementary body of C*hlamydophila* organisms. These elementary bodies are grown in a cell culture, purified, and then treated with 0.05% formalin. This preparation of antigens can be stored in a refrigerator for many years (91).

Interpretation of serological tests

Apart from indicating the evidence of an infection, the level of antibody titer also provides information on the status of infection. Immunoglobulin A (IgA) is more frequently found in the elderly and in individuals who have chronic infections. IgM is the first antibody to appear in the serum in response to infection due to bacteria, viruses, or toxins as shown in Figure 2.3.
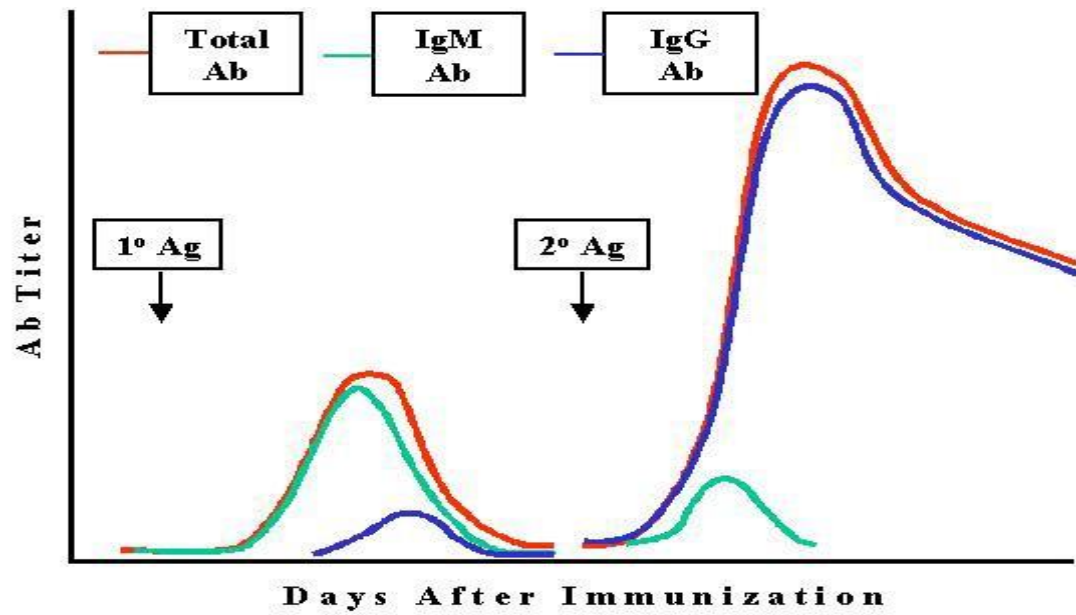
**Figure 2.3 Antibody response to antigen**

Source: (92)

Usually, IgG appears in the body when IgM levels start decreasing, and then it persists for a longer duration. IgG also appears as a response to chronic infection (93).

# 3 Chapter 3: Review of related studies

The existing body of epidemiological literature on the role of *H. pylori*, CMV and *C. pneumoniae* in the development of preeclampsia is not very large. To the best of my knowledge, one systematic review has been conducted exclusively on the role of these microorganisms in the development of preeclampsia (82). Apart from this review quite a few individual studies have been published ranging from three each for *H. pylori* (94–96) and CMV (78,80,97),  and eight studies have been published on the role of *C. pneumoniae* (28,42,79,98–102). Additionally, two studies investigated two microorganisms; one explored *H. pylori* and *C. pneumoniae* (81) and the other CMV and *C. pneumoniae* (103). In the following paragraphs, a summary of review of these studies is presented by the microorganism type.

## 3.1. Helicobacter pylori (H. pylori) and preeclampsia

Most studies have focused on using serological methods to identify various types of antigenic structures found in *H. pylori* (Appendix A). There is a long list of  antigenic structures associated with *H. pylori,* however, the most commonly used for diagnostic purpose include: cytotoxin-associated protein (CagA), vacuolating cytotoxin protein (VacA), urease subunits, flagellin subunits and heat-shock proteins (HspA and HspB) (96).

As mentioned above, out of a total of four studies, only one study used the PCR test for isolating *H. pylori* DNA in addition to exploring the antibodies produced in response to different antigens of *H. pylori* by using serological methods (94). The rest of the three studies looked for only antibodies (81,95,96). Of these latter studies, one looked at antibodies produced in response to both *H. pylori* and *C. pneumoniae* (81) while the other two for *H. pylori* only (95,96). All studies found a statistically significant association between antibodies and *H. pylori* (81,95,96) but the PCR test results for the extraction of DNA from placenta were found to be negative (94).

As far as information on confounders is concerned, only two (94,96) of the four studies mentioned controlling for confounders. One study (96) controlled for maternal age, pre-pregnancy BMI, parity, maternal and family risk factor by carrying out multivariable analysis while the other study (94) matched on parity. The studies which did not share adjustment for confounding factors were found at serious or critical risk of bias (81,95).

## 3.2. Cytomegalovirus (CMV) and preeclampsia

Three of the four studies investigated the relationship between CMV and PE by looking at the association between anti-CMV antibodies and PE (78,80,103). Two of the four studies between CMV and PE (80,97) found a significant association. Additionally, one of these three studies looked at the relationship of PE with antibodies against both *C. pneumoniae* and CMV (103). The fourth one explored the association between the presence of CMV DNA in peripheral blood and PE (97).

The study that explored the association of PE with C. *pneumoniae* and CMV simultaneously assessed the association across the two categories of PE, i.e.; early onset PE (diagnosed during 20-34 weeks gestation) and late onset PE (diagnosed after 34 weeks gestation) (103). Both the microorganisms were found to be risk factors for the early onset PE while the two microorganisms were protective for late onset PE. However, for neither category it was a significant association.

With regards to the adjustment for confounders, two studies shared information on controlling for confounding factors (78,103); one adjusted for maternal age, parity and smoking (78) while the other matched cases with controls on maternal age, and parity (103). Again, the studies which did not adjust for confounding factors were assessed to be at serious risk of bias (80,97).

### 3.3. Chlamydophila pneumoniae (C. pneumoniae) and preeclampsia

Ten studies investigated the evidence of infection with *C. pneumoniae* and development of PE (28,42,79,81,98–103). Out of the ten, eight studies attempted to find the role of only *C. pneumoniae* infection in the development of PE (28,42,79,98–102). One of the remaining two studies examined the association of *C. pneumoniae* along with CMV and PE (103) and the final study investigated *C. pneumoniae* along with *H. pylori* and PE (81). Five studies found a significant association between *C. pneumoniae* and PE (28,42,79,98,101). Similarly, eight of the studies used serological methods to discover this evidence, one study utilized DNA extraction and one study used both serological tests and DNA extraction to establish evidence of this relationship. All studies were conducted using a case-control design except one, which used a cohort design.

The eight studies which utilized only serological tests varied in their choice of antibodies against *C. pneumoniae*. Six of them looked for all three antibodies-IgA, IgG and IgM (42,98–102), one study investigated IgG and IgM (81) and the remaining two explored IgG only (79,103). Out of the two studies which looked for IgG only, just one found a significant association between IgG and PE (79) while for the other study, which assessed early (diagnosis of PE between 20-34 weeks gestation) and late onset PE (diagnosis of PE after 34 weeks of gestation), the association was not significant (103). The one which looked for IgM and IgG found significance with neither of them (81).

Of the six studies which looked for three immunoglobulins, IgA, IgM and IgG by using the serological tests, two showed an association with IgG only; one was significant (98) and the other was not (42). Two more were at a borderline for a significant association (99,101). One of the remaining two studies did not report the estimates but mentioned the significance status as

'NS', which means there was not a significant association (100), another was found to be non-significant with protective effects (102).

The two studies which detected *C. pneumoniae* by identifying DNA both found supportive evidence. One study tested the correlation between gDNA copy numbers and anti-*C. pneumoniae* which was found to be strongly correlated (42), the other study explored *C. pneumoniae* in placental tissue by using PCR. This study explored both the villous tissue (VT) and extra-villous tissue (EVT); a significant association was found for the combination of VT and EVT with PE but not for EVT only (28).

Four of the ten studies did not share any information on controlling for the confounders (28,81,100,101). Out of the remaining six studies, three adjusted for confounding factors: BMI, smoking and family history of PE (79), age and gestational age (98), age, parity and seropositivity (99); while three matched cases with controls on: age, parity and gestational age (102), maternal age, parity, and maternal age at blood sampling (42), maternal age and parity (103). Six studies were either assessed to at serious risk (28,103) or at critical risk of bias (81,99–101).

While some research has been conducted on the relationship of *H. pylori,* CMV and *C. pneumoniae* and the development of PE, it is limited and warrants more. The available research has some limitations; research design of these studies could be improved. This draws towards inconclusive overall evidence and suggests for a need of more research on this topic with rigorous design and implementation.

# 4 Chapter 4.    Methods

## 4.1. Source population – the ARCH study

To test the hypotheses of my dissertation, I drew a sub-sample from the Archive for Child Health (ARCH) data. The ARCH study was started in 2008 and since then it is an ongoing cohort study of unselected pregnant women assembled in three clinics in Lansing, MI, whose aim is to provide a comprehensive biological, clinical and epidemiological database without a large investment of funds. These clinics are the Sparrow Hospital Residency Clinic, the Sparrow Hospital Faculty Clinic, and the Ingham County Health Department Clinic. The first clinic listed above has supplied about half of the cohort, which is somewhat below average in socio-economic circumstances. The sample so far includes more than 800 women with more than 450 infants. All women recruited are informed about the research project, its advantages, and the role of participants. The mean gestational age (GA) at recruitment is 13.1 weeks. The study has approval from the appropriate IRBs for collection of interview data and sample collection. The participants were invited to participate in the study, and provided written informed consent

**Table 4.1 Data collection in the ARCH cohort**

| Timing in pregnancy | Types of behavioral questionnaires used | Bio-samples obtained |
|---|---|---|
| Prenatal | Maternal interview: socio-demographics; medical history; height; weight; diet; physical activity; depression scale; access to medical records | Urine specimens: $1^{st}$, $2^{nd}$, $3^{rd}$ trimester<br>Blood specimens: $1^{st}$, $2^{nd}$ trimester |
| Birth | Birth certificate<br>Maternal & infant hospital discharge abstracts | Placental sample;<br> access to screening blood spots |
| Postnatal | Maternal telephone interview at 1 month, 1 year, then annually: child health; developmental milestones; infant feeding practices | Consent to re-contact women for further study |

before starting the in-person interview or collection of specimens. The cohort is 25% African-American and slightly less than 10% Hispanic. About 70% of women approached in their first prenatal visit for obtaining consent to participate in ARCH. Nearly every woman in the study had successful first trimester blood archived, and most had an early third trimester sample as well. The research protocol of the ARCH study focuses on four key points:

1. Getting consent and enrolling study subjects with completion of interview at the first prenatal visit.

2. Obtaining permission for accessing state-archived data on pregnancy and birth:

• The confidential portion of the birth certificate (100 perinatal variables)

• Maternal/infant hospital discharge abstracts (diagnoses, procedures, length of stay)

• The newborn blood spot archived after genetic screening.

• Medical records, but we reserve these for amplification of diagnoses and for sub-studies.

3. Storing the biological specimens. The laboratory serving the clinics sets aside an extra red top (plain) and lavender top (k2EDTA) tube at the first prenatal blood draw and at the routine 24-28 week glucose tolerance test. Blood and urine samples are divided into multiple 250-μL aliquots for repeated retrieval and analysis; mean N of aliquots stored per collection is 11.3 for serum, 11.0 for plasma and 29.7 for urine. Maternal serum, plasma, blood lysate, filter paper (5 x 0.5 cm2 spots) and urine aliquots are frozen at -80°C. Having the clinical laboratory obtain blood is both less costly and more acceptable to participants than arranging separate phlebotomies for research. A sample of placental cord, parenchyma and membrane is stored in formalin, whenever possible (n = 185). Urine collections were the first collection initiated in ARCH, with blood collections only starting after some 6-9 months of recruitment. Thus blood collections are less complete than urine.

4. Following-up the families is by telephone at one month, and annually (re-consented for five more years at age 5. with assessment of child development, and recording of new illnesses and diagnoses.

Several of the procedures of this research which uses 'convenience sample of clinics in Lansing, MI,' enhance "robust and unbiased results", including:

1. The clinics from which study subjects are recruited are general population clinics not targeted to women at high risk.

2. All forms of payment are accepted in the clinics of where recruitment of the study subjects is carried out, so there is limited selection of patients for socio-economic status.

3. The only exclusion criteria are for age below 18, and not-speaking English (<5% of our sample).

4. The enrollment process is completed at first prenatal visit whenever it occurs, even if late in gestation

5. A descriptive log, of women approached in the clinic who refuse enrollment, including also the reasons for refusal, is maintained. The acceptance rate (women enrolled divided by women approached for participation) has ranged between 60-70% over the duration of the study.

6. The post-partum follow-up   contacts with the families reach to 85.2% of the ARCH cohort after birth, most multiple times. Contacts are kept up to date. 88.2% of women followed have been interviewed at least once since 2014; nearly a third of all participants have had a follow up interview so far in 2016.

7. The sample size is large enough to provide power for many important exposure-outcome relationships.

8. The design of the study focuses on prospective, real-time data collection. The information obtained about pregnancy exposures is not dependent on the memory of the mother.

9. Much of the data (e.g. birth certificates, hospital abstracts) are obtained from a single statewide source and thus less subject to the variability of different data collection systems.

### 4.1.1 Collection of Biological Samples in ARCH

Biological samples (Table 4.1) for the ARCH study are leftover clinical specimens. After recruitment of study subjects, interview data were collected by well-trained and supervised volunteer MSU undergraduates. As mentioned earlier, women consent at their first prenatal visit, at 12.7 weeks of gestation on average to allow their samples in the study. Multiple aliquots of each biological sample, which for blood includes plasma, serum, and filter paper blood spots, are prepared at the ARCH laboratory to permit repeated analysis without refreezing and stored at -80 degrees C. Post-partum follow-up rates are 74%, 67%, and 62% at 1 month, 1 year, and 2 years, respectively, without investment as yet of any special resources.

## 4.2. Study subjects for dissertation (a sub-sample of the ARCH study)

As a first step, I identified women who developed PE irrespective of the timing of PE development (early onset: 20-34 weeks gestation; late onset more than 34 weeks gestation) from among the subsets of the cohorts with archived blood. Next, I selected matched controls from the same dataset of the ARCH study. So, a woman was labeled as a 'case' if she had PE and labeled a 'control' if she did not have PE.

### 4.2.1 Case definition for the dissertation:

A woman is diagnosed as having PE if she had the following features:

- Blood pressure more than 139/89 mm Hg after 20th week of gestation; and

- Proteinuria (proteins detected in urine) of equal to or more than 300 mg in 24 hours.

- If proteins were not found, then presence of at least one of the following conditions is needed:

  - thrombocytopenia (platelets less than 100,000/ml);

  - renal insufficiency (serum creatinine: > 1.1 mg/dl or a doubling of the serum creatinine concentrations in the absence of other renal disease);

  - impaired liver functions (elevated blood concentration of liver transaminases to twice normal concentrations), pulmonary edema; and cerebral/visual symptoms;

  - pulmonary edema; and

  - cerebral/visual symptoms

- Eclampsia; or

- Maternal death was post mortem diagnosis of signs of pre-eclampsia

### 4.2.2   Control definition for the dissertation:

A woman without high blood pressure or if she had high blood pressure but none of the following features after the 20$^{th}$ week of gestation is labelled as a control:

- Proteinuria, Thrombocytopenia (platelets less than 100,000/ml);

- Renal insufficiency (serum creatinine: > 1.1 mg/dl or a doubling of the serum creatinine concentrations in the absence of other renal disease);

- Impaired liver functions (elevated blood concentration of liver transaminases to twice normal concentrations), Pulmonary edema and Cerebral/visual symptoms;

- Impaired liver functions (elevated blood concentration of liver transaminases to twice normal concentrations);

- Pulmonary edema; or

- Cerebral/visual symptoms; or

- Maternal death undergoing post mortem, without diagnosis of signs of PE; or

- Matching criteria to controls,

### 4.2.3 Selection of Study Participants (women with PE and controls)

The ARCH primary dataset does not contain information on two key variables, blood pressure and proteinuria, for making the diagnosis of PE. As the diagnosis of PE is made when the diagnostic features (high blood pressure, proteinuria and/or others) appear in a pregnant woman after the 20th week of gestation, determination of the timing of high blood pressure and proteinuria or any other accompanying feature were required to declare a pregnant woman as a case of PE. Therefore, it was planned to conduct a review of medical records (MR) of the study participants who had already agreed to share the required information, for research purposes, from their medical records.

I conducted MR review at Sparrow hospital for the ARCH dataset. Before conducting the review, an independent approval was obtained from the Institutional Review Boards (IRB's) of: Michigan State University, East Lansing, MI and Sparrow Hospital, Lansing MI. For this purpose, a set of five abstraction forms was developed (one form was added at the time of renewal for IRB application). Along with the abstraction forms, a Microsoft Excel sheet was used to identify and finalize the sample. The overall number of subjects was 784 at the time the selection of cases and controls was undertaken, and they were the source for identifying PE cases through the MR process.

### 4.2.4 MR Review Process

Determining a case of PE

Initially, it was planned to follow the American College of Obstetrics and Gynecology (ACOG) guidelines (Table 4.3) as the main diagnostic criteria for determining a case of PE (1). However,

the process of reviewing MR and its initial findings convinced me to expand my diagnostic

criteria by adding physicians' notes, with or without the documentation of the International

Classification of Disease (ICD) 9 or 10 codes (104,105). So, after discussing with my advisor

and a maternal-fetal medicine (MFM) committee member, it was decided that I would review

MR and

**Table 4.2 Definition of a case and a control of PE**

| Case Definition | Control Definition |
| --- | --- |
| A woman was diagnosed as having PE if she had the following features:<br>• Blood pressure more than 139/89 mm Hg after 20<sup>th</sup> week of gestation; and<br>• Proteinuria (proteins detected in urine) of equal to or more than 300 mg or more in 24 hours.<br>  • If proteins were not found, then presence of at least one of the following conditions:<br>    ○ thrombocytopenia (platelets less than 100,000/ml;<br>    ○ renal insufficiency (serum creatinine: > 1.1 mg/dl or a doubling of the serum creatinine concentrations in the absence of other renal disease)<br>  ○ impaired liver functions (elevated blood concentration of liver transaminases to twice normal concentrations), pulmonary edema; and cerebral/visual symptoms.<br>  ○ pulmonary edema; and<br>  ○ cerebral/visual symptoms<br>• Eclampsia<br>• Maternal death was post mortem diagnosis of signs of pre-eclampsia | A woman without high blood pressure and none of the following features was labelled as a control:<br>• Proteinuria, Thrombocytopenia (platelets less than 100,000/ml,<br>• Renal insufficiency (serum creatinine: > 1.1 mg/dl or a doubling of the serum creatinine concentrations in the absence of other renal disease)<br>• Impaired liver functions (elevated blood concentration of liver transaminases to twice normal concentrations), Pulmonary edema and Cerebral/visual symptoms<br>• Impaired liver functions (elevated blood concentration of liver transaminases to twice normal concentrations)<br>• Pulmonary edema; and<br>• Cerebral/visual symptoms<br>• Maternal death undergoing post mortem, without diagnosis of signs of pre-eclampsia |

declare a pregnant woman as a case of PE based on at least one of the following criteria:

1. Physicians' notes documenting the diagnosis of PE and using the ICD-9 or 10 code,

2. Physicians' notes documenting the diagnosis of PE but not using the ICD-9 or 10 code, and

3. ACOG guidelines.

I used the following strategy based on case definition as provided in Table 4.2 (section 4.2.2) to declare a pregnant woman as a case of PE or not:

1. After reviewing the delivery date, gestational age at delivery, and the mode of delivery, I looked at blood pressure readings in the record before and after the 20$^{th}$ week of gestation.

2. If the blood pressure was found to be high (systolic > 139 or diastolic > 89 mm Hg), the next step was looking for at least one more reading of high blood pressure at an interval of six hours or more; these two readings of high blood pressure at least 6 hours apart declared a woman as hypertensive. If the blood pressure was found within normal limits, the woman was labeled as 'without PE' because high blood pressure is the cardinal feature of PE, and if that is within normal range, a woman cannot be declared as a case of PE.

3. Then I looked for the next component, which is the presence of proteinuria, either it was tested after the collection of a 24 hour urine sample ($\geq$ 300 mg) or through the dipstick method ($\geq$+1). Following the ACOG guidelines, both high blood pressure and proteinuria in a pregnant woman constitutes a case of PE, so the woman was labeled as a 'case of PE'.

4. Next, I looked for the remaining laboratory tests and clinical features as mentioned in the AOCG guidelines (Table 4.3) even if the diagnosis of PE was made. In the event that a

**Table 4.3 Recommendation of ACOG for the diagnosis of PE**

| | |
|---|---|
| Blood Pressure | • Greater than or equal to 140 mm Hg systolic or greater than or equal to 90 mm Hg diastolic on two occasions at least 4 hours apart after 20 weeks gestation in a woman with previous normal blood pressure. <br> • Greater than or equal to 160 mm Hg systolic or greater than or equal to 110 mm Hg diastolic, hypertension can be conformed within a short interval (minutes) to facilitate timely hypertensive therapy. |
| And | |
| Protein | Greater than 300 mg/24 hours urine collected (or this amount extrapolated from a timed collection). <br> Or <br> Protein/creatinine ratio greater than 0.3 (both measured in mg/dl). <br> Dipstick recording of 1+ (useful only if other quantitative methods are not available). |
| Or in the absence of proteinuria, new - onset hypertension with the new onset of any of the following | |
| Thrombocytopenia | Platelet count less than 100,000/ml |
| Renal Insufficiency | Serum creatinine concentration more than 1.1 mg/dl or a doubling of the serum creatinine concentration in the absence of other renal disease. |
| Impaired Liver Function | Elevated blood concentration of liver transaminases to twice normal concentration. |
| Pulmonary Edema | |
| Cerebral/Visual symptoms | |

woman was not found as a case of PE, I still completed my data collection procedure and documented values for other laboratory tests, such as proteinuria, serum creatinine, etc. If she had high blood pressure before the 20th week of gestation, the pregnant woman was labeled as having a pregnancy with gestational hypertension or pregnancy-induced hypertension (PIH) with PE.

I also looked for any co-morbidity conditions, such as diabetes, chronic hypertension, kidney disease, and/or other hormonal problems. I stopped MR process once I found 25 PE cases

which also had blood samples. The remaining subjects (excluding 25 cases) constituted the potential control base for the sample of this dissertation, which was 760.

### 4.2.5. Inclusion criteria

I included all women who participated in the ARCH study, and agreed to provide biosamples and behavioral information for the research purpose.

### 4.2.6. Exclusion criteria

I excluded the women who delivered twins or multiples.

Selection of controls

The case base for dissertation sample was 25 women with PE while the potential control base was 760 subjects without PE. For each case, three controls were matched; both cases and controls were drawn from the same main cohort who gave the biosamples. Matching increased the statistical power and improved study efficiency (106). The controls were matched with cases on

 1. maternal age (±3 years),

2. parity (0, ≥ 1),

3. race, and

4. gestational age at blood withdrawal (±2 weeks).

Other variables were allowed to vary and were the subject of detailed statistical analysis. Finally, study sample included 21 cases, as for three cases I could not find matched controls and one case had to be removed because of insufficient quantity of blood sample, along with 52 matched controls making a total sample of 73. As reflected by the total sample size of 73, not all cases had three controls. Rather, 3 cases each had one matched control, 5 cases each had two matched controls and the rest of the cases were matched with three controls.

## 4.3. Study Design

I utilized the nested case-control research design to test hypotheses. The key response variable was PE (PE) which was measured by the review of the MR (medical records) at Sparrow hospital, Lansing, Michigan. A woman was labeled as a case of PE following the case definition as mentioned in Table 4.2 above. The exposure was defined as evidence of infection caused by at least one of the three infectious agents, *C. pneumoniae* (*Chlamydophila pneumoniae*), *H. pylori* (*Helicobacter pylori*), or CMV (cytomegalovirus). The exposure status was determined based on the presence of immunoglobulins (Igs) against these infectious agents as investigated with the help of the serological tests carried out on the plasma of the study subjects. These tests were performed in the laboratory of the Institute of Virology at the University of Minnesota (107). The other covariates of central interest were maternal age, socio-economic status of the mother, parity, race of mother and father, marital status, physical activity, obesity, previous history of PE, family history of PE, smoking during pregnancy, and sex of the newborn.

### 4.3.1 Power of the study

Assuming a two-tailed α of .05, three controls per case, with exposure prevalence in controls of about 25% and in cases about 60%, a sample size of 73 calculated a power of about 80% (108).

**Table 4.4 Calculation of sample size**

| Pc | Pi | Power ≥ 80% | | |
|----|----|----|----|----|
| | | Cases | Controls | Total |
| 0.25 | 0.5 | 15 | 63 | 78 |
| 0.3 | 0.55 | 24 | 72 | 96 |
| 0.35 | 0.6 | 30 | 87 | 117 |

Pc=proportion in controls; Pi=proportion in index case

### 4.3.2   Sources of Data and Measurement of Variables

In addition to the archived blood samples and medical records, additional data were available from questionnaires. The questionnaires focused the following areas: socio-demographics, pregnancy, delivery, diet, physical activity, family history, and comorbidities like, diabetes mellitus, depression. The birth certificate provided information on mother and father race, parity, newborn sex, duration of pregnancy, personal habits, such as smoking and alcohol use.  . Information on all variables was obtained from the questionnaire. Maternal age was calculated in years based on date of birth, and parity was calculated by adding the number of alive and deceased babies; information on the race of the mother and father was provided by the mother. The information on the timing of blood withdrawal was determined as the week of gestation when blood was collected. The information on income and education status of the women was taken as proxy variables for the determination of socioeconomic status. Physical activity was assessed by asking about the involvement of the woman in different activities ranging from household chores to exercise, and obesity was calculated by the weight and height of the respondent.

## 4.4. Statistical Analysis

I used JMP (109) and the Statistical Analysis System (SAS) (110) for cleaning and analyzing the data.

### 4.4.1   Data Management:

The ARCH dataset was managed by the Biomedical Research Information Core (BRIC) at the College of Human Medicine, Michigan State University (MSU), East Lansing, MI.  After the completion of interviews the questionnaire was reviewed for any missing/incorrect information by the interviewers and their supervisors. Then the questionnaires were moved to the data

management center for thorough editing and data entry. The questionnaires are kept in a secure place for storage after the data entry was completed to maintain privacy and full confidentiality. BRIC is responsible for storing and archiving the ARCH data.

Matching Controls for Cases of PE

As mentioned earlier, I selected 21 cases of PE following the strategy presented in the previous section (section: 4.2.4). The rest of the pregnant women constituted a potential control base. Each case was matched with three controls on: maternal age ($\pm 3$ years), parity ($0, \geq 1$), race and date of blood sample withdrawal ($\pm 2$ weeks). As the primary dataset did not have maternal age so it was calculated by utilizing JMP software (109) followed matching of cases with controls for which SAS software (110) was used. Once the cases were matched with controls, I attempted to review them in the records to ensure their control status. However, I was able to review almost half of the controls due to limited access to records. As a result of this review, I found one control high/abnormal proteinuria but could not review the BP for these subjects due to non-availability of information on vitals. This control were removed to avoid misclassification.

### 4.4.2 Analysis

Analysis for this dissertation was at two levels: descriptive analysis and logistic regression. I used SAS software (110) for the analyses of this dataset.

Descriptive analysis:

The proportions and means for antibody status and other variables were examined at the first step and compared them, separately for both groups. I also examined the distribution of many other factors:

- Maternal age
- Education of mother

- Race of mother

- Race of father

- Household income

- Newborn sex

- Parity of women

- Season at birth

- Smoking during pregnancy

- Pre-pregnancy BMI, and

- Depression during pregnancy

Unadjusted Analysis:

As this is a matched study so I carried out conditional logistic regression to identify associated factors including the confounding factors and interaction terms. For this purpose, I looked for the association between each variable of interest and the PE status (case=1, control=0) separately; this provided me unadjusted conditional odds ratio (condOR). This OR was unadjusted since it provided the relationship between the covariate of interest and outcome variable (PE status) separately. For interpretation, we used 95% CI along with OR. The key variables of interest were: maternal age, educational level, income status, race of the mother and race of the father.

Adjusted Analysis:

I built a conditional logistic regression model to look for the simultaneous effect of different variables with the outcome variable, PE. At this stage, I also looked for the confounders, effect modifiers and variables coming in the path of the exposure (infections) to the outcome (PE). Based on the unadjusted OR estimated in the previous section, adjusted OR was calculated. I included all the variables, for the final model building, which showed a p-value of 0.25 or less

for their unadjusted analysis. Finally, I constructed a parsimonious model. Before I could

develop a final model, I constructed the Directed Acyclic Graph (DAG) to show relationship of

my outcome variable, PE with the exposure of interest and other covariates. The DAG was

completed using (111).                .
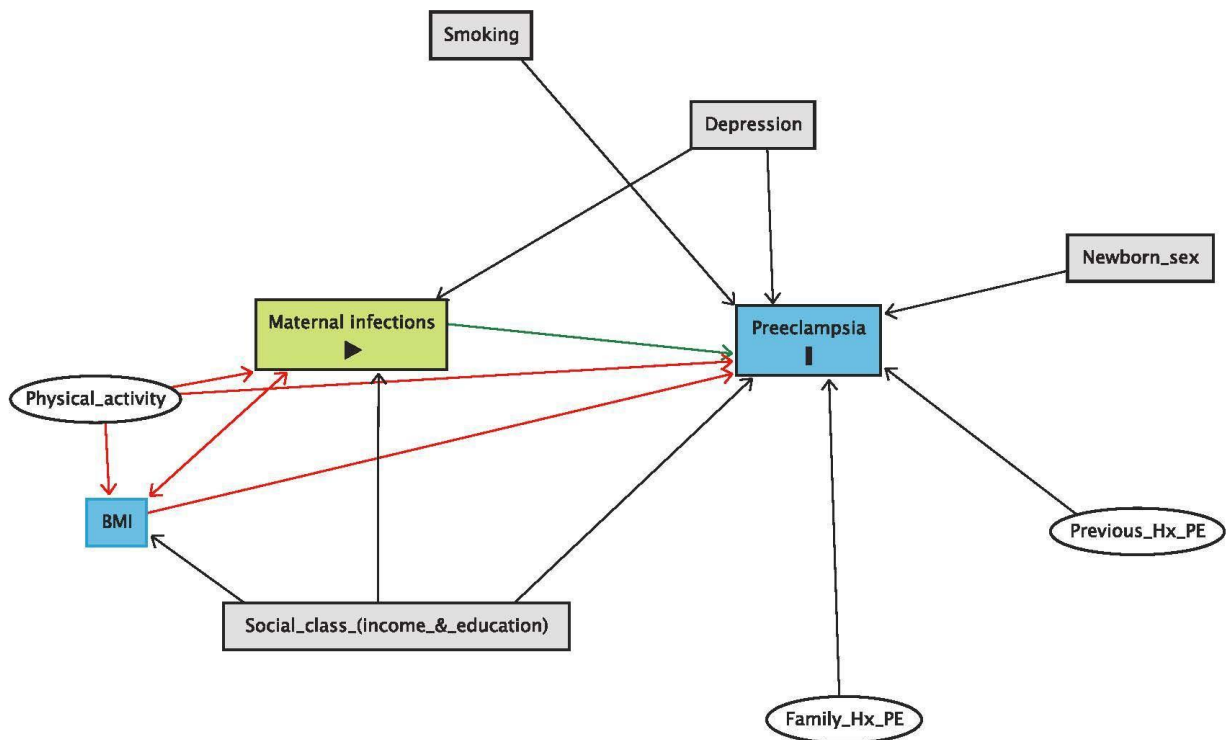


**Figure 4.1 Causal diagram of PE for the current study**

# 5   Chapter 5.   Results

## 5.1. Characteristics of the study participants

The overall sample for this study was 73 subjects; 21 were cases of PE matched with 53 controls on maternal age (±3 years), maternal race, parity (0, ≥1) and gestational age at blood withdrawal(± 3 weeks). The age of the participants range from 22-40 years with mean 29 (±3.4). Women with PE were younger than the control group (Table 5.1). About one-fifth (19%) of the women in PE group were below 25 years of age as compared to 6% of the controls. A similar proportion of the women (80%) in both the groups were either living with their partners or alone. The women's educational background and household income were used as proxy variables for socio-economic status (SES) which was found to be lower for the cases as shown in Table 5.1.

More women with PE (29%) did not receive a high school diploma as compared to the controls (19%). Similarly, 75% of the cases as compared to 68% of the controls had an annual household income which was less than $25,000. With regards to the race of the study participants, more than two-thirds (67%) of the cases and three-fourths (75%) of the controls were white. Among the African-Americans, a higher proportion was preeclamptic as compared to the controls (29% vs. 23%). The distribution of the race of the husband/partner was not very much different (Table 5.1). Slightly more than half of the cases in comparison to 75% of the husbands from the control group were white. Similar to the race of the woman, more husbands belonged to the African-American group (33% vs. 16%).

**Table 5.1 Socio-demographic characteristics of participants by PE status.**

| Variables | PE Status | | | |
| | Cases=21 | | Controls=52 | |
| | Number | Percentage | Number | Percentage |
|---|---|---|---|---|
| Woman age | | | | |
|   20-24 years | 4 | 19.1 | 3 | 5.8 |
|   25-29 years | 9 | 42.9 | 28 | 53.9 |
|   30-34 years | 7 | 33.3 | 20 | 38.5 |
|   35 years and above | 1 | 4.8 | 1 | 1.9 |
| Mean age (SD) | 28.9 (±4.1) | | 29. (±3.2) | |
| Marital status | | | | |
|   Unmarried | 17 | 81 | 41 | 78.9 |
|   Married | 4 | 19.1 | 11 | 21.2 |
| Woman education | | | | |
|   Less than high school | 6 | 28.6 | 9 | 18.8 |
|   High school diploma | 7 | 33.3 | 15 | 31.3 |
|   More than high school | 8 | 38.1 | 24 | 50 |
| Household income (USD) | | | | |
|   Less than 25,000 | 15 | 75 | 34 | 68 |
|   25,000 and above | 5 | 25 | 16 | 32 |
| Woman race | | | | |
|   White | 14 | 66.7 | 39 | 75 |
|   Black or African American | 6 | 28.6 | 12 | 23.1 |
|   Asian Indian | 1 | 4.8 | 1 | 1.9 |
| Husband/partner race | | | | |
|   White | 10 | 55.6 | 28 | 75.7 |
|   Black or African American | 6 | 33.3 | 6 | 16.2 |
|   Others | 2 | 11.1 | 3 | 8.1 |

With regards to the obstetrical characteristics, almost half of the study participants from both groups were nulliparous as shown in Table 5.2. Out of the participants who had children, more cases had one living child (33%) than the controls (27%). A majority of the babies (38%), among cases, were delivered during fall (October-December) as compared to a quarter (25%) delivered among controls. Among the newborn babies, cases had a higher proportion of males (57%) as compared to the controls (46%).

With reference to non-obstetrical characteristics, about one-fifth of the cases (19%) as compared to one-third of the controls (31%) reported smoking during pregnancy As far as pre-pregnancy BMI is concerned, about half of the cases than approximately 85% of the controls had BMI 25 or higher. About 10% of the women with PE reported having episodes of depression during

**Table 5.2 Obstetrical and medical characteristics of participants by PE status.**

| Variables | PE Status | | | |
|---|---|---|---|---|
| | Cases=21 | | Controls=52 | |
| | **Number** | **Percentage** | **Number** | Percentage |
| Parity of woman | | | | |
|   Nulliparous | 10 | 47.6 | 26 | 50 |
|   Primiparous or multiparous | 11 | 52.4 | 26 | 50 |
| Mean (SD) | 0.71 (±0.78) | | 0.85 (±1.1) | |
| Number of children living | | | | |
|   None | 10 | 47.6 | 26 | 50 |
|   One | 7 | 33.3 | 14 | 26.9 |
|   Two or more | 4 | 19.1 | 12 | 23.1 |
| Season at birth | | | | |
|   Winter | 4 | 19.1 | 10 | 19.2 |
|   Spring | 4 | 19.1 | 16 | 30.8 |
|   Summer | 5 | 23.8 | 13 | 25 |
|   Fall | 8 | 38 | 13 | 25 |
| Newborn sex | | | | |
|   Male | 12 | 57.1 | 24 | 46.2 |
|   Female | 9 | 42.9 | 28 | 53.8 |
| Smoking during pregnancy | | | | |
|   Yes | 4 | 19.1 | 16 | 31.4 |
|   No | 17 | 80.9 | 35 | 68.6 |
| Pre-pregnancy BMI | | | | |
|   Less than 25 | 6 | 28.6 | 22 | 43.1 |
|   25-29.9 | 5 | 23.8 | 22 | 43.1 |
|   30 or above | 10 | 47.6 | 7 | 13.7 |
| Depression during pregnancy | | | | |
|   Present | 2 | 9.5 | 10 | 19.2 |
|   Absent | 19 | 90.5 | 42 | 80.8 |
| No infection reported during pregnancy | | | | |
|   Yes | 15 | 71.4 | 41 | 80.4 |
|   No | 6 | 28.6 | 10 | 19.6 |

pregnancy while almost double the proportion of the controls (19%) had episodes of depression. A higher proportion of cases reported infection (non-specific) during pregnancy. This question was asked in negative sense as 'no infection reported during pregnancy'; so 71% of the cases as compared to 80% of the controls reported yes.

**Table 5.3 Distribution of serological test results by PE status.**

| Microorganisms | Cases 21: n (%) | Control 52: n (%) | IgG status after revised analysis | |
| --- | --- | --- | --- | --- |
| | | | Cases: n (%) | Control: n (%) |
| CMV | | | | |
| IgG + | 3 (14.3) | 3 (5.8) | 7 (33.3) | 15 (28.9) |
| Indeterminate | 0 | 9 (17.3) | | |
| IgG - | 18 (85.7) | 40 (74.4) | 14 (66.7) | 37 (71.2) |
| H. pylori | | | | |
| IgG + | 1 (4.8) | 2 (3.9) | 2 (9.5) | 3 (5.8) |
| Indeterminate | 1 (4.8) | 1 (1.9) | | |
| IgG - | 19 (90.4) | 49 (94.2) | 19 (90.5) | 49 (94.2) |
| C. pneumoniae | | | | |
| IgG + | 7 (33.3) | 28 (53.9) | 13 (61.9) | 25 (48.1) |
| Indeterminate | 3 (14.3) | 10 (19.2) | | |
| IgG - | 11 (52.4) | 14 (26.9) | 8 938.1) | 27 (51.9) |

As far as the antibody status of three micro-organisms is concerned, a higher proportion of the women with PE (14%) were found to have IgG antibodies against CMV in their plasma as compared to the controls (6%) as shown in Table 5.3. None of the cases had an indeterminate result as compared to about 17% of the controls were found in this category. Our laboratory consultant repeated the analysis and found significant changes in the status. Majority of the indeterminate and some of the negative plasma at the first analysis turned positive and this is not a new phenomenon. So, revised results showed that about one-third of the cases (33%) were found positive for CMV IgG as compared to 29% of the controls.

For *H. pylori,* IgG antibodies were found positive in the plasma of 5% of the cases and 4% of the controls. More than 90% of the women, irrespective of the PE status, were found to be negative for the *H. pylori* IgG antibody status in their plasma. After merging positive with indeterminate (per recommendation of our laboratory expert), the status slightly changed. Approximately 10% (n=2) of PE cases as compared to 6% (n=3) of the controls were found to be IgG positive.

With regards to *C. pneumoniae* about one-third of the cases and slightly more than half of the controls were found IgG positive. After adjustment by merging the positive with indeterminate, the status changed remarkably. More than 60% of the cases and less than 50% of the controls were IgG positive.

## 5.2. Univariable analysis

Univariable analysis was the preliminary step for the final conditional logistic model building for each variable separately (Table 5.4). As the covariates of interest (other than the main exposure category of micro-organisms) were same so univariable analysis is the same for all three papers/microorganisms.

The odds of household income of less than $25,000 among PE cases was 1.3 times the odds of income of less than $25,000 among controls. The education of mother showed an association with PE with different categories. In comparison with women having more a than high school diploma, the women with education of less than a high school diploma were 2.4 times more likely to be cases of PE than controls. For the category of education, with a high school diploma, the women were 1.8 times more likely to preeclamptic as compared to control.

Smoking during pregnancy and diagnosis of depression during pregnancy showed a protective effect for PE. The odds of developing PE in women who smoked during pregnancy was 0.6 times the odds of developing PE in controls. Similarly, the odds of developing PE in women who had depression during pregnancy was 0.4 times the odds of developing PE in controls. Season of birth showed a varying effect; women who delivered during spring and summer as compared to winter showed a protective effect and were

30% and 10% respectively less likely to develop PE. While women who delivered in fall as compared to women with delivery in winter were 1.4 times more likely to develop PE.

**Table 5.4 Univariable analysis of potential factors associated with PE in Lansing area, MI with conditional odds ratios and 95% confidence intervals.**

| Variables | Cases | Controls | mOR | p-value |
|---|---|---|---|---|
| Household income | | | | |
| Less than 25,000 | 15 (75) | 34 (68) | 1.3 (0.3-4.9) | 0.6974 |
| More than 25,000 | 5 (25) | 16 (32) | 1 | |
| Marital status | | | | |
| Unmarried | 17 (81) | 41 (78.9) | 1.2 (0.3-5.0) | 0.8521 |
| Married | 4 (19) | 11 (21.1) | 1 | |
| Education of mother | | | | |
| Less than high school | 6 (28.6) | 9 (18.8) | 2.4 (0.6-10.0) | 0.4705 |
| High school diploma | 7 (33.3) | 15 (31.2) | 1.8 (0.5-7.1) | |
| More than high | 8 (38.1) | 24 (50) | 1 | |
| Smoking during | | | | |
| Yes | 4 (19) | 16 (31.4) | 0.6 (0.2-2.0) | 0.3934 |
| No | 17 (81) | 35 (68.6) | 1 | |
| Pre-pregnancy BMI | | | | |
| 30 or above | 10 (47.6) | 7 (13.7) | 7.9 (1.6-39.5) | 0.0076 |
| 25-29.9 | 5 (23.8) | 22 (43.1) | 1.4 (0.3-6.2) | |
| Less than 25 | 6(28.6 | 22 (43.1) | 1 | |
| Newborn sex | | | | |
| Male | 12 (57.1) | 24 (46.2) | 1.8 (0.6-5.5) | 0.2651 |
| Female | 9 (42.9) | 28 (53.8) | 1 | |
| Infection reported | | | | |
| Yes | 15 (71.4) | 41 (80.4) | 0.6 (0.2-1.9) | 0.442 |
| No | 6 (28.6) | 10 (19.6) | 1 | |
| Depression during | | | | |
| Present | 2 (9.5) | 10 (19.2) | 0.4 (0.1-2.1) | 0.256 |
| Absent | 19 (90.5) | 42 (80.8) | 1 | |
| Season at birth | | | | |
| Spring | 4 (19) | 16 (30.8) | 0.6 (0.1-2.8) | 0.7042 |
| Summer | 5 (24) | 13 (25) | 0.9 (0.2-3.9) | |
| Fall | 8 (38) | 13 (25) | 1.4 (0.4-5.9) | |
| Winter | 4 (19) | 10 (19.2) | 1 | |

## 5.3 H. pylori and development of preeclampsia in Michigan

Abstract

Introduction:

Preeclampsia (PE) is a pregnancy related health condition which affects maternal and newborn health significantly. No causative factor has been identified yet. Evidence suggests the role of *H. pylori* in the development of PE. We conducted this research to explore the role of *H. pylori* in the development of PE.

Methods:

I conducted a nested case-control study and matched cases with controls on: maternal age ($\pm 3$ years), race, parity and gestational age at blood withdrawal. I used conditional logistic regression to look for the association between *H. pylori* IgGs and other covariates of interest, and PE status.

Results:

Out of total sample of 73, 21 were cases. Two (9.5%) of the cases and three (5.8%) of the controls were found positive for *H. pylori* IgGs in their plasma. Multivariable conditional analysis found a strong but non-significant association of *H. pylori* IgGs with PE status (mOR: 2.4; 95% CI: 0.2-32.2) after controlling for the effect of other variables in the model.

Conclusions:

*H. pylori* did not show an association with PE in this cohort from Michigan. Large-sample studies are needed especially in populations with higher prevalence of *H. pylori* infection to explore this relationship further. Adding anti-CagA IgG to the serological test may enhance the strength of association to a significant level.

### 5.3.1 Methodology

Statistical analysis

As the serological test showed almost all study subjects as negative or indeterminate except three, so it was planned to perform multivariable conditional logistic analysis by taking *H. pylori* IgG status as binary (positive=1; negative + indeterminate=0) and continuous to develop two separate models. In the meanwhile, I receive a recommendation from the serological laboratory expert that I could merge indeterminate with positive laboratory results instead of negative to keep consistency as majority of the indeterminate turned out to be positive in other analyses. Then I develop a third model, again taking *H. pylori* IgG status as binary but with a different combination, positive + indeterminate =1 and negative = 0.

The previous two models are attached at the end as Appendix B. Below narrate the strategy and steps involved in the development of the final model which was built to look for the association between *H. pylori* IgG status and PE status in the presence of other variables.

Purposeful strategy of model building

I followed the purposeful strategy of model building as described in Applied Logistic Regression by Hosmer and Lemeshow 3rd edition (112). We built our final model as a parsimonious model by purposeful selection of the variables based on two principles: 1) variables showing significant association with p-value not more than 0.25 as assessed by Wald test for that variable; 2) a variable with biological importance or plausibility.

The first step of this strategy, the Univariable analysis, had already been carried out this analysis (Table 5.4) and built the final model based on this Univariable analysis. As *H. pylori* IgG is the main exposure variable, we started with this variable as shown in the Model 1 in Table 5.5. For a better understanding about the relationship between covariates of interest and PE status

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in the presence of the main exposure variable, *H. pylori* IgG status, I also performed a Bivariable

analysis. In this analysis, all covariates of interest were added one-by-one to the model which

had only main exposure variable in it, this helped building our final model. Model-2 through

Model 5 show these Bivariable models.

Next, I choose the Bivariable model with pre-pregnancy BMI, as this was the covariate

which had the lowest p-value in Univariable analysis; in this case it is shown in Model 2 in Table

5.5. At this point I assessed three components of the model: 1) change in the coefficients of the

existing model, a change more than 15-20% would suggest that the variable which caused this

change (by adding or removing) should be kept in the model as its effect shows that this is a

confounder and it is necessary to control for its effect by keeping it in the model; 2) p-value of

the individual variables in the model by using Wald statistics; and 3) p-value of the Likelihood

Ratio Test (LRT) to compare the reduced with the full model. Here I saw the *H. pylori* IgG mOR

dropped from 1.5 to 0.9 without any change in the p-value while pre-pregnancy BMI was

affected very little.

A closer look at Table 5.4 revealed that pre-pregnancy BMI is the only covariate of

interest which satisfied the condition of p-value of 0.25. Onwards, more variables were added

following the second principle of the biological plausibility. Social class (education and income)

is always an important index which needs to be controlled to assess the effect of main exposure

variable. So, I added mother's education (Model 6) which did not change the mOR of the two

variables already present in the model but 95%CI of BMI further widened from 58 to 85 on the

upper side. Mother's education category of 'less than high school diploma' increased with no

improvement in p-value. At this time, I added second component of Social class, the household

income (Model 7) which slightly changed mOR of all variables including its own; however, the

major significant change was upper bound of 95% confidence level of BMI which went up to 104. At this point, I further evaluated the literature and our DAG; I found that BMI is a collider variable. It is highly recommended that a collider variable should not be controlled otherwise it would introduce bias. So, I removed it from the model (Model 8); it improved the mOR and turned the 95% CI more precise. At the last step, I added depression during pregnancy to the model (Model 9) which further improved the estimates.

**Table 5.5 Univariable analysis of main exposure (H. pylori) & Bivariable analysis of covariate of interest with PE status.**

| Variables | Model-1 mOR (95%CI) | p-value | Model-2 (LRT p: 0.0206) mOR (95%CI) | p-value | Model-3 (LRT p: 0.5879) mOR (95%CI) | p-value | Model-4 (LRT p: 0.8856) mOR (95%CI) | p-value | Model-5 mOR (95%CI) | p-value |
|---|---|---|---|---|---|---|---|---|---|---|
| H. pylori IgG | | | | | | | | | | |
| Positive | 1.5 (0.1-13.4) | >.9999 | 0.9 (0.1-11.2) | >.9999 | 1.9 (0.1-27.4) | 0.8724 | 1.4 (0.1-13.2) | >.9999 | | |
| Negative | 1 | | 1 | | 1 | | 1 | | | |
| Pre-pregnancy BMI | | | | | | | | | | |
| 30 or above | | | 7.1 (1.3-58.0) | 0.0156 | | | | | | |
| 25-29.9 | | | 1.4 (0.2-9.9) | 0.9635 | | | | | | |
| Less than 25 | | | 1 | | | | | | | |
| Education of mother | | | | | | | | | | |
| < high school | | | | | 2.2 (.4-13.5) | 0.4401 | | | | |
| High school dipl | | | | | 1.7 (0.3-9.3) | 0.671 | | | | |
| > high school | | | | | 1 | | | | | |
| Household income | | | | | | | | | | |
| <$25,000 | | | | | | | 1.2 (0.3-6.4) | >.9999 | | |
| >$25,000 | | | | | | | 1 | | | |
| Depression | | | | | | | | | | |
| Yes | | | | | | | | | 0.4 (0.1-2.1) | 0.256 |
| No | | | | | | | | | 1 | |

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**Table 5.6 Final conditional model building steps and final model of exposure and covariates of interest with PE status.**

| Variables | Model-6 (LRT p: 0.0423) mOR (95%CI) | p-value | Model-7 (LRT p: 0.1660) mOR (95%CI) | p-value | Model-8 mOR (95%CI) | p-value | Model-9 mOR (95%CI) | p-value | Model-5 mOR (95%CI) | p-value |
|---|---|---|---|---|---|---|---|---|---|---|
| H. pylori IgG | | | | | | | | | | |
| Positive | 0.9 (0.04-16.2) | >.9999 | 0.8 (0.03-15.1) | >.9999 | 2.0 (0.1-29.1) | 0.8610 | 2.4 (0.2-38.2) | 0.7217 | | |
| Negative | 1 | | 1 | | | | | | | |
| Pre-pregnancy BMI | | | | | | | | | | |
| 30 or above | 7.6 (1.3-85.0) | 0.0167 | 6.8 (0.9-104.2) | 0.0526 | | | | | | |
| 25-29.9 | 2.0 (0.3-20.6) | 0.7135 | 2.5 (0.3-26.8) | 0.5669 | | | | | | |
| Less than 25 | 1 | | 1 | | | | | | | |
| Education of mother | | | | | | | | | | |
| Less than high school | 3.4 (0.5-34.3) | 0.2847 | 3.4 (0.5-38.6) | 0.2832 | 2.7 (0.3-23.2) | 0.3853 | 2.9 (0.5-24.6) | 0.3157 | | |
| | | | | | | | | | | |
| High school diploma | 1.8 (0.3-12.2) | 0.7381 | 1.7 (0.2-16.5) | 0.8426 | 2.0 (0.3-17.0) | 0.6697 | 2.0 (0.3-17.7) | 0.6775 | | |
| More than high school | 1 | | 1 | | 1 | | 1 | | | |
| Household income | | | | | | | | | | |
| Less than $25,000 | | | 1.5 (0.2-13.9) | >.9999 | 0.8 (0.1-5.0) | >.9999 | 0.7 (0.1-4.6) | >.9999 | | |
| More than $25,000 | | | 1 | | 1 | | 1 | | | |
| Depression during pregnancy | | | | | | | | | | |
| Yes | | | | | | | 0.2 (0.4-2.5) | 0.3607 | | |
| No | | | | | | | 1 | | | |

<u>Multivariable analysis</u>

I built multivariable model based on the Univariable analysis. I used purposeful selection to reach the final models and, in this regard, built two final models; both models were similar in the choice of covariates except one model had sex (Model 8) while the other model had depression (Model 9). According to Model 8, after controlling the effects of all covariates, the odds of *H. pylori* IgG among PE cases is 1.9 times the odds of *H. pylori* IgG among controls.

In contrast, Model 9 states that the odds of *H. pylori* IgG among PE cases is 2.4 times the odds of *H. pylori* IgG among controls after adjusting the effect of all other variables in the model..

### 5.3.2   Discussion

This manuscript from the dissertation provides research on the role of *H. pylori* in the development of PE in Michigan. The level of IgG (immunoglobulin-G) against *H. pylori* measured in the blood of the study participants provided the frequency of this microorganism in two groups. This study did not find a higher percentage of cases than controls with IgG in their blood. This finding does not provide an evidence which supports the hypothesis that a higher percentage of PE cases would have *H. pylori* IgG in their blood as compared to the controls.

Biology of H. pylori

*H. pylori* is a Gram-negative bacteria which primarily targets gastric mucosa cells (113) and has been found to be associated with immune response (113,114). The immune response works through pattern recognition receptors, the Toll-like receptors (TLRs). Multiple TLRs exist such as, TLR-2 and TLR-4. Usually, TLR-4 is involved in immune response but in case of *H. pylori,* TLR-2 has more affinity for lipopolysaccharides (LPS) of *H. pylori* than TLR-4. When *H. pylori* attaches to the gastric mucosa cells, gastric cells express TLRs which initiate the innate immune response (114).  Additionally, *H. pylori* has also been found to suppress TLR-4 and stimulate interleukin (IL)-8 and 12, a mechanism proposed for a possible association between the micro-organism and chronic inflammation induced as a result of infection with *H. pylori* (115).

The severity of inflammation caused by *H. pylori* depends upon the pathogenicity of a strain. Among different strains, the one with cytotoxin-associated gene A (CagA), which is responsible for coding hydrophilic, surface-exposed proteins (116), causes local and systematic inflammation (117). *H. pylori* strains possessing CagA has been found with more severe inflammatory response in gastric mucosa than *H. pylori* without this gene (118). Because of the

same characteristic of stimulating an immune response in the gastric mucosa, *H. pylori* has been found to play a role in the inflammatory processes in organs outside the stomach. This is true especially for vessels and other tissues originating from endothelial cells (119). The key feature is that anti-Cag A antibodies cross react with antigens present on the surface of endothelial cells (120,121).

Cytotrophoblast cells also originate from the endothelial cells. The proteins actin, more specifically β-actin, are present on the surface of cells to maintain their cytoskeleton and keep cells together through intercellular adhesions (43,122,123). As discussed above, anti-CagA antibodies attach to antigens present on the surface of the cells, in this case the antibodies attach to β-actin and initiate an inflammatory response. This inflammatory response has been proposed as the key feature in the pathogenesis of PE in women who were infected with *H. pylori* in the past.

Emerging evidence supports this hypothesis as investigators have found that anti-CagA antibodies of *H. pylori* react with β-actin on cytotrophoblast and reduce their invasive ability in the process of remodeling of spiral arteries and proper development of the future placenta (14,17). Ponzetto et al, found a serological evidence of *H. pylori* infection but did not find DNA of *H. pylori* in the placental tissue of women with PE (94) further supporting the hypothesis that PE was a result of an immunological response and not a result of recent episode of infection. Following discussion in the above paragraphs, it seems anti-CagA IgG is also an important biomarker to explore the involvement of *H. pylori* in the development of PE and should be serologically assessed in the blood of the study participants. The current study did not investigate the levels of anti-CagA IgG. It is expected that if these antibodies had been assessed, a higher

level of IgGs would have been found. Some of the earlier studies assessed these IgGs and found levels either similar to *H. pylori* IgGs or even higher (94,96).

<u>Serological test and interpretation</u>

For *H. pylori,* the serological test utilized an indirect ELISA where the target was *H. pylori* antigen. A majority of the study subjects were found negative while very few turned out to be positive or indeterminate, I show all combinations in Table. The results did not correspond to what earlier studies have found (81,94–96). There could be more than one explanation for this discrepancy. First, one study used IgA serological test (81) as compared to this study, I used IgG so I could not make any comparison. The remaining three studies found 50-81% of the preeclamptic women positive for *H. pylori* IgG while the current study found less than 10 % of PE cases as positive. Ponzetto et al, found more than half of the women with PE (94) and Cardaropoli et al, found 70%-85% (among different PE groups) women with PE (96) as positive for *H. pylori* IgG. However, their method of determination of positive test was different from this study. Ponzetto et al, used cut off based on percentage of their controls values while Cardaropoli et al, used manufacture instructions, however, these instructions could not be found in the text or on webpage.

Apart from providing an evidence of infection, the type of immunoglobulin also guide on the duration of infection. Whether it is a recent episode, or occurred in the past or a recurrent episode of infection; IgM is the type of antibody which appears first in the blood after an episode of infection. Its concentration increases over the next couple of days and then gradually drops. While IgM is disappearing, IgG starts to appear in the blood to remain there for longer period of time.  So, IgM reflects a recent or acute infection and IgG shows that the infection had appeared in the past. In a situation when IgG is there but not in high concentration and IgM is also present,

then it represents a reinfection with the same micro-organism. In the current study, IgM was not found positive for even a single participant suggesting that all subjects which were found positive were most likely represented the infection in the past. One single main factor is very low sensitivity (less than 7%) of IgM as compared to IgG which shows a sensitivity of at least 85% (124). In very rare situations, there may be a narrow window, when IgM has disappeared but IgG has not yet started to appear. In such situations, it is recommended to assess IgG avidity. This is a phenomenon which is described by the affinity or binding of an antibody to the antigenic epitopes on the surface a protein. During early days and weeks of infection, IgG avidity is low but over the next couple of months the avidity increases, in other words, due to increase in the affinity between IgG and antigen the binding site gets saturated and this shows an episode of infection which was occurred about six months before. This has been studied more frequently for other microorganisms than *H. pylori* (125).

Conditional logistic regression and associated factors

The Univariable analysis in Table 5.4 shows protective effect of smoking on PE. The estimates show that women who smoked during pregnancy were 0.6 times more likely to have PE as compared to women who did not smoke. In other words, the pregnant women with smoking history during pregnancy were at 40% less risk of developing PE than pregnant women who did not smoke. These results are consistent with the existing literature (126,127). Also, women with high pre-pregnancy BMI were at higher risk of developing PE as compared to pregnant women with lower pre-pregnancy BMI. This finding also agrees with the available information on this relationship (128,129)

In the pursuit of evaluating the hypothesis of association of various covariates with PE status, I built our conditional logistic regression final models based on the Univariable analysis.

These results show that after matching on four variables, maternal age, maternal race, parity and time of blood withdrawal for serological test at design phase, I adjusted for three more covariates at analysis phase: mother education, household income and sex of the newborn (Model 8) or depression during pregnancy (Model 9) to look for the risk of developing PE among women whose blood found *H. pylori* IgGs. The direction of the effect of *H. pylori* IgGs was the same which I had hypothesized, however, it was not statistically significant. In fact none of the covariates could reach to a level of significant association; the main reason seems the small sample size. The hypothesis stated that controlling for the social class (education and income), *H. pylori* will be associated with PE. Previously, two studies controlled for the covariates by adjusting at the analysis stage (94,96). One adjusted only for parity (94) while the second study adjusted for multiple variables, including: maternal age, parity, pre-pregnancy BMI, maternal family risk factors (96). In this analysis, pre-pregnancy BMI was dropped because it was found as a collider variable. Direct Acyclic Graphs (DAGs) are graphical representation of the causal assumptions made when one designs a study and generates data. In DAGs when two arrows on a path point to a variable representing that they are the causes of that variable, that variable is termed as a collider variable (130–132). As shown in Figure 4.1, physical activity (133,134), social class (education and income) (135,136) and maternal infection (137,138) are found to be associated with pre-pregnancy BMI making it a collider variable.

As data for this study come from an ongoing study, this study had limitations in getting some important variables such as, family history of preeclampsia and previous history of preeclampsia. However, I managed to get that information through medical review process. Unfortunately, I was unable to get access to all records or once I found the record, it was difficult to get the full information. This also resulted in my inability to verify the status of the controls. I

feel that this might have resulted in misclassification of outcome. However, there would not be more 2-3 such controls which could become cases at a prevalence of 5-8% of PE. In an effort to avoid any chance of misclassification, I removed one potential control from the dataset who had high proteinuria but I could not verify the BP status.

Although, this study had small sample size especially the cases were not very large; however, I tried to address this issue by matching three controls for each case. In addition to controlling the effects of confounders by matching controls with cases on variables which are considered strong confounders, matching has also been found to increase the statistical efficiency of the study and reduces the variance in the parameters of interest (139).

It is important to note that the earlier studies found high prevalence for the seropositivity of *H. pylori* IgG. (81,94–96), however, only two studies attempted to adjust for the confounders (94,96) and out of these two studies, only one adjusted the potential confounders appropriately (96). I attempted to adjust the effects of confounders at both design phase by matching cases with three controls and then at analysis level when different variables were assessed through conditional logistic regression.

## 5.4      Cytomegalovirus and development of preeclampsia in Michigan

## Abstract

Introduction:

Preeclampsia (PE) is a pregnancy related health condition which affects maternal and newborn health significantly. No definitive cause has been identified yet. Emerging evidence suggests that CMV is associated with f PE. This research was conducted to explore the role of CMV in the development of PE.

Methods:

A nested case-control study was conducted and cases were matched with controls on: maternal age ($\pm 3$ years), race, parity and gestational age at blood withdrawal. ELISA tests were carried out on the plasma to identify IgG and IgM antibodies. For statistical analysis, I used conditional logistic regression to look for the association between anti CMV antibodies and other covariates of interest, and PE status.

Results:

Out of the total sample of 73 study subjects (cases: 21 and controls: 52) 7 (33.3%) cases and 15 (28.9%) controls were found positive for anti CMV IgG. None of the study subjects was found positive for anti CMV IgM. Multivariable conditional analysis found a strong but non-significant association between anti CMV IgGs and PE (mOR: 1.4; 95% CI: 0.3-5.6) after controlling for the effect of other variables in the model.

Conclusions:

This study did not find an association between anti CMV IgGs and PE status. Large-sample studies are needed especially in populations with higher prevalence of CMV infection to explore this relationship further.

### 5.4.1 Methodology

<u>Statistical analysis</u>

As CMV serological test showed three types of results: positive, indeterminate and negative. Initially, I built the Multivariable conditional model by taking anti CMV IgG status as binary (positive=1; negative + indeterminate=0). In the meanwhile, I received the revised set of results on the same plasma which improved the findings as the new set showed that about 33% of the cases were positive for anti CMV IgG as compared to the earlier results where only 14% of the cases were anti CMV IgG positive. So, I built another model based on the revised results. Below are the steps involved in the final model which was built to look for the association between anti CMV IgG status and PE status in the presence of other variables.

<u>Purposeful strategy of model building</u>

The purposeful strategy of model building was followed as described in Applied Logistic Regression by Hosmer and Lemeshow 3$^{rd}$ edition (112). The final model was built as a parsimonious model by purposeful selection of the variables based on two principles: a) variables showing significant association with p-value not more than 0.25 as assessed by Wald test for that variable at Univariable analysis stage; b) a variable of biological importance or plausibility.

The first step of this strategy, the Univariable analysis, had already been carried out (Table 5.4) and the final model was built based on this Univariable analysis. As anti CMV IgG is main exposure variable, I started with this variable as shown in the Model-1 of in Table 5.6-A.

For the better understanding about the relationship between covariates of interest and PE status in the presence of main exposure variable, anti CMV IgG status, I also performed Bivariable analysis. In this analysis, all covariates of interest were added one-by-one to the model which had only main exposure in it, this helped building the final model. Model-2 through Model-5 show these Bivariable models.

Next, I chose the Bivariable model with pre-pregnancy BMI, as this was the covariate which had the lowest p-value at Univariable analysis; in this case it is reflected in Model-2 in Table 5.7. At this point I assessed three components of the model: 1) change in the coefficients of the existing model, a change more than 15-20% would suggest that the variable which caused this change (by adding or removing) should be kept in the model as its effect shows that this is a confounder and it is necessary to control for its effect by keeping it in the model; 2) p-value of the individual variables in the model by using Wald statistics; and 3) p-value of the Likelihood Ratio Test (LRT) to compare the reduced with the full model. Here, CMV IgG mOR dropped from 1.3 to 0.7 without any change in the p-value while pre-pregnancy BMI was affected very little.

A closer look at Table 5.3 revealed that pre-pregnancy BMI is the only covariate of interest which satisfied the condition of p-value of 0.25. Onwards, more variables were added following the second principle for the conditional logistic model building, the biological plausibility. Education and income representing the social class is always an important variables which needs to be controlled to assess the effect of main exposure variable. So, next added mother's education (Model 6) which slightly changed the mOR of CMV IgG from 0.7 to 0.9 and that of BMI 8.3 to 9.8 with further widening of the 95%CI. Mother's education category of 'less than high school diploma' increased slightly. At this time, the household income (Model 7) was

added which slightly changed mOR of all variables. At this time, I removed BMI from the model

being a collider variable based on the literature and casual diagram presented in this dissertation

(Figure 4.1). It is highly recommended that a collider variable should not be controlled otherwise

it would introduce bias. So, BMI was removed from the model (Model 8); it improved the mOR.

At the last step, I added the variable depression during pregnancy to the model (Model 9) which

further improved the estimates.

**Table 5.7 Univariable analysis of main exposure (CMV IgG) & Bivariable analysis of covariate of interest with PE status.**

| Variables | Model-1 (LRT p⊗0.6975) | | Model-2 (LRT p: 0.0189) | | Model-3 (LRT p: 0.6707) | | Model-4 (LRT p: 0.9272) | | Model-5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | mOR (95%CI) | p-value | mOR (95%CI) | p-value | mOR (95%CI) | p-value | mOR (95%CI) | p-value | mOR (95%CI) | p-value |
| CMV IgG | | | | | | | | | | |
| Positive | 1.3 (0.4-3.9) | >.6981 | 0.7 (0.2-2.3) | >.6548 | 1.1 (0.3-3.8) | 0.8370 | 1.0 (0.3-3.3) | >.9995 | 1.5 (0.4-4.9) | 0.5323 |
| Negative | 1 | | 1 | | 1 | | 1 | | 1 | |
| Pre-pregnancy BMI | | | | | | | | | | |
| 30 or above | | | 8.3 (1.6-43.2) | 0.0119 | | | | | | |
| 25-29.9 | | | 1.3 (0.3-6.1) | 0.7771 | | | | | | |
| Less than 25 | | | 1 | | | | | | | |
| Education of mother | | | | | | | | | | |
| | | | | | | | | | | |
| Less than high school | | | | | 2.4 (0.6-10.0) | 0.2413 | | | | |
| High school diploma | | | | | 1.7 (0.4-7.1) | 0.4467 | | | | |
| More than high school | | | | | 1 | | | | | |
| Household income | | | | | | | | | | |
| Less than $25,000 | | | | | | | 1.3 (0.3-5.0) | >.7026 | | |
| More than $25,000 | | | | | | | 1 | | | |
| Depression during pregnancy | | | | | | | | | | |
| Yes | | | | | | | | | 0.4 (0.1-2.0) | 0.2376 |
| No | | | | | | | | | 1 | |

**Table 5.8 Final conditional model building steps and final model of exposure and covariates of interest with PE status.**

| Variables | Model-6 (LRT p: (0.7587) mOR (95%CI) | p-value | Model-7 (LRT p: 0.5682) mOR (95%CI) | p-value | Model-8 (LRT p: 0.8191) mOR (95%CI) | p-value |
|---|---|---|---|---|---|---|
| CMV IgG | | | | | | |
|   Positive | 1.0 (0.3-3.4) | 0.9367 | 1.4 (0.3-5.6) | 0.6368 | 0.9 (0.3-3.3) | 0.9157 |
|   Negative | | | | | 1 | |
| Education of mother | | | | | | |
|   Less than high school | 2.8 (0.5-14.9) | 0.2167 | 3.3 (0.6-17.3) | 0.1633 | 2.5 (0.4-13.9) | 0.2970 |
|   High school diploma | 2.0 (0.4-10.8) | 0.4163 | 1.9 (0.3-10.1) | 0.4683 | 1.9 (0.3-10.4) | 0.4644 |
| | | | | | | |
|   More than high school | 1 | | 1 | | 1 | |
| Household income | | | | | | |
|   Less than $25,000 | 0.9 (0.2-4.3) | >.9421 | 0.9 (0.2-4.1) | >.9032 | 1.0 (0.2-4.6) | 0.9910 |
|   More than $25,000 | 1 | | 1 | | 1 | |
| Depression during pregnancy | | | | | | |
|   Yes | | | 0.2 (0.01-2.4) | 0.2037 | | |
|   No | | | 1 | | | |
| Newborn sex | | | | | | |
|   Male | | | | | 1.4 (0.4-4.8) | 0.5641 |
|   Female | | | | | 1 | |

<u>Multivariable analysis</u>

I built Multivariable model based on Univariable analysis. By using the purposeful selection strategy, the final model was developed. According to Model 9, after controlling the effects of all covariates, the odds of CMV IgG among PE cases is 1.4 times the odds of CMV IgG among controls.

I also checked for the interaction term, however, no interaction term emerged as significant.

### 5.4.2 Discussion

This manuscript explores the role of CMV in the development of PE in Lansing, Michigan. The Enzyme-Linked Immunosorbent Assay (ELISA) assessed the level of IgM (immunoglobulin-M) and IgG (immunoglobulin-G) in the plasma of the study participants for the evidence of CMV infection. The test did not find any evidence of acute infection; however, IgG levels were slightly higher in cases than controls which supported our hypothesis without any statistical difference.

Biology of CMV

Human cytomegalovirus is herpesvirus type 5 and it is the largest among the group. It is double-stranded linear DNA core covered by an icosahedral nucleocapsid which is further by matrix. The diameter of a mature virus ranged from 200-300 nanometers (140). Important feature in the pathogenicity of CMV is that its primary infection is usually does not produce symptoms in a health and immune-competent individual. Thus after getting access to human body through different modes such as oral, sexual, placental, breastfeeding etc, it acquires a latency status in the body. It may reactivate in situations when its host encounters immunocompromised condition.

With regards to the mechanisms through which it may induce events leading to the development of PE, more than one paths or mechanism have been proposed. It has been found that CMV has some role in directly affecting the arterial walls with platelet dysfunction and causing atherosis (141) which is a unique feature in placental vessels and results in narrowing of the vessels inducing ischemic changes. Moreover, CMV also has ability to affect indirectly through immunological pathways ending up in the production of pro-inflammatory cytokines eventually causing injury to the placental tissue(142,143).

<u>Serological test and interpretation</u>

For CMV, IgM and IgG both were assessed. None of the study participant was found positive for IgM while more than one third of the participants had IgG in their plasma; cases with positive results were slightly higher in percentage than controls. Earlier studies found higher proportion of study participants with positive results (78,80,103) . One study was prospective in design and found about half of the cases with positive results while controls were even higher in proportion (61%) (78). The mean age of the participants were similar to our data; however, that study had higher proportion of nulliparous women than our study. Out of the remaining two studies, one showed 100% positive results for PE cases, although their PE cases were 9 (103). In comparison to our less than half of nulliparous women, their study had more than 90% nulliparous women.

In addition to providing the evidence of infection, the immunoglobulins of serological tests also provide information on the chronicity of the infection. In this regard, IgM is considered a marker of acute or recent infection as compared to IgG which reflects past infection. The antibody which appears first of all in response to an infectious agent, is IgM. It gradually increases in its concentration over the next few days and then declines. There is a paradoxical relationship between IgM and IgG; when IgM starts decreasing the levels of IgG start to increase in the blood and remain there for longer period of time.  So, IgM reflects a recent or acute infection and IgG shows that the infection had appeared in the past. In a situation when IgG is there but not in high concentration and IgM is also present, then it represents a reinfection with the same micro-organism. In our study, IgM was not found positive for even a single participant suggesting that all subjects which were found positive were most likely presented with the past infection.

As discussed above, IgM cannot be a reliable marker for the detection of primary infection, although it identifies acute infection. The reason is that it also appears whenever there is reinfection. To assess for the presence of primary infection, a trend of the IgG levels provides the required information. IgG avidity is a phenomenon which is described by the affinity or binding of antibody to the antigenic epitopes on the surface of a protein. During early days and weeks of infection, IgG avidity is low but over the next couple of months the avidity increases, in other words, due to increase in the affinity between IgG and antigen the binding site get saturated and this shows an episode of infection which was occurred about six months before (125). CDC recommends looking for IgG trends in two samples taken approximately three months apart. If the first shows low avidity and the second shows high avidity it means primary infection with CMV has occurred (93).

Conditional logistic regression and associated factors

Univariable analysis of this study shows protective effect of smoking on PE (Table 5.4). The estimates show that women who smoked during pregnancy had 40% low risk of developing PE as compared to women who did not smoke. Earlier studies also found a similar relationship (126,127). Also, women with high pre-pregnancy BMI were at higher risk of developing PE as compared to pregnant women with lower pre-pregnancy BMI. This finding is also consistent with what is already known (128,129).

For evaluating the hypothesis of association of various covariates with PE status, I built the conditional logistic regression final models based on the Univariable analysis. The results show that after matching on four variables, maternal age, maternal race, parity and time of blood withdrawal for serological test, model also adjusted for three more covariates: mother education, household income and depression during pregnancy (Model 8) to look for the risk of developing

PE among women whose blood found anti CMV IgGs. The direction of the effect of CMV IgGs was the same which I had hypothesized; however, it was not statistically significant. In fact none of the covariates could reach to a level of significant association which I think is due to the small sample size. The hypothesis stated that controlling for the social class (education and income), CMV will be associated with PE. Previously, only one study provided information on controlling for the covariates at the time of analysis (78). This study controlled for maternal age, parity and smoking during pregnancy. In the current analysis, the variable pre-pregnancy BMI was dropped because it was found to be a collider variable. In DAGs when two arrows on a path point to a variable representing that they are the causes of that variable, that variable is termed as a collider variable (130–132). As shown in Figure 4.1, physical activity (133,134), social class (education and income) (135,136) and maternal infection (137,138) are found to be associated with pre-pregnancy BMI making it a collider variable.

As data for this study come from an ongoing study, this study had limitations in getting some important variables such as, family history of preeclampsia and previous history of preeclampsia. However, I managed to get that information through medical review process. Unfortunately, I was unable to get access to all records or once I found the record, it was difficult to get the full information. This also resulted in my inability to verify the status of the controls. I feel that this might have resulted in misclassification of outcome. However, there would not be more 2-3 such controls which could become cases at a prevalence of 5-8% of PE. In an effort to avoid any chance of misclassification, I removed one potential control from the dataset who had high proteinuria but I could not verify the BP status.

Although, this study had small sample size especially the cases were not very large; however, I tried to address this issue by matching three controls for each case. In addition to

controlling the effects of confounders by matching controls with cases on variables which are considered strong confounders, matching has also been found to increase the statistical efficiency of the study and reduces the variance in the parameters of interest (139).

It is important to note that the earlier studies found high prevalence for the seropositivity of anti CMV IgG or other related antibody (78,80,103), however, only one study attempted to adjust for the confounders (78). In the current study, all efforts were made to adjust the effects of confounders at both design phase by matching cases with three controls and then at analysis level when we assessed different variables through conditional logistic regression.  In this study, I matched on maternal race taking that as a strong confounder. One earlier study found higher seroprevalence of CMV in non-Hispanic black children and Mexican children as compared to non-Hispanic white (76). As the age advanced the seroprevalence further increased reaching to 90% in the fifth decades of their lives

## 5.5 *Chlamydophila pneumoniae and development of preeclampsia in Michigan*

Abstract

Introduction:

Preeclampsia (PE) is a pregnancy related health condition which affects maternal and newborn health significantly. No causative factor has been identified yet. Emerging evidence suggests association of *C. pneumoniae* in the development of PE. We conducted this research to explore the role of *C. pneumoniae* in the development of PE.

Methods:

I conducted a nested case-control study and matched cases with controls on: maternal age (±3 years), race, parity and gestational age at blood withdrawal. Serological tests were carried out on the plasma to identify antibodies. For statistical analysis, I used conditional logistic regression to look for the association between *C. pneumoniae* IgGs and other covariate of interest, and PE status.

Results:

This study had 21 cases and 52 matched controls. Thirteen (62%) cases and 25 (48%) of controls were positive for anti *C. pneumoniae* IgGs in their blood. Multivariable conditional analysis found a strong but non-significant association of anti *C. pneumoniae* IgGs with PE status (mOR: 2.3; 95% CI: 0.6-9.1) after controlling the effect of other variables in the model.

Conclusions:

This study did not find association between anti *C. pneumoniae* IgG and PE status. However, Large-sample studies are needed especially in populations with higher prevalence of *C. pneumoniae* infection to explore this relationship further.

### 5.5.1 Methodology

<u>Statistical analysis</u>

The serological test results for *C. pneumomiae* were the last among the three microorganisms reviewed. By that time, I have received a recommendation from our serological laboratory experts that I could merge indeterminate results with positive to keep consistency as majority of the indeterminate turned out to be positive in other analyses. Thus, the final model was developed taking *C. pneumoniae* IgG status as binary, taking positive + indeterminate =1 and negative = 0.

Below is a narration of the strategy and steps involved in development of the final model to look for the association between anti *C. pneumoniae* IgG status and PE status in the presence of other variables?

<u>Purposeful strategy of model building</u>

The purposeful strategy of model building was followed as described in Applied Logistic Regression by Hosmer and Lemeshow 3rd edition (112). I built the final model as a parsimonious model by purposeful selection of the variables based on two principles: a) variables showing significant association with p-value not more than 0.25 as assessed by Wald test for that variable at Univariable analysis stage; b) a variable with biological importance or plausibility.

The first step of this strategy, the Univariable analysis, had already been carried out (Table 5.4) and the final model was built based on this Univariable analysis. As anti *C. pneumoniae* IgG is the main exposure variable, I started with this variable as shown in the Model-1 of in Table 5.7-A. For the better understanding about the relationship between covariates of interest and PE status in the presence of main exposure variable, *C. pneumoniae* IgG status, I also performed a Bivariable analysis. In this analysis, all covariates of interest were added one-by-one to the

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model which had only main exposure in it, this helped building the final model. Model-2 through Model-5 show these Bivariable models.

Next, I chose the Bivariable model with pre-pregnancy BMI, as this was the covariate which had the lowest p-value at the Univariable analysis; in this case it is shown in Model 2 in Table 5.7-B. At this point I assessed three components of the model: 1) change in the coefficients of the existing model, a change more than 15-20% would suggest that the variable which caused this change (by adding or removing) should be kept in the model as its effect shows that this is a confounder and it is necessary to control for its effect by keeping it in the model; 2) p-value of the individual variables in the model by using Wald statistics; and 3) p-value of the Likelihood Ratio Test (LRT) to compare the reduced with the full model. Here, *C. pneumoniae* IgG mOR dropped from 1.9 to 1.6 without any change in the p-value while pre-pregnancy BMI was affected very little.

A closer look at Table 5.3 revealed that pre-pregnancy BMI is the only covariate of interest which satisfied the condition of p-value of 0.25. Onwards, more variables were added following the second principle for model building, the biological plausibility. Social class (education and income) is always an important index which needs to be controlled to assess the effect of main exposure variable. So, we added mother's education (Model 6) which changed the mOR of *C. pneumoniae* IgG from 1.6 to 2.1 and that of BMI 7.5 to 9.4 with further widening of the 95%CI. Mother's education category of 'less than high school diploma' increased with no improvement in p-value. At this time, the second component of Social class, the household income (Model 7) was added which slightly changed mOR of all variables except BMI of more than 30. At this point, I removed BMI from the model being a collider variable based on the literature and casual diagram presented in this dissertation (Figure 4.1). It is highly

recommended that a collider variable should not be controlled otherwise it would introduce bias. So, BMI was removed from the model (Model 8); it improved the mOR. At the last step, we added depression during pregnancy to the model (Model 9) which further improved the estimates.

**Table 5.9 Univariable analysis of main exposure (C. pneumoniae IgG) & Bivariable analysis of covariate of interest with PE status.**

| Variables | Model-1 (LRT p:0.2405) | | Model-2 (LRT p: 0.0157) | | Model-3 (LRT p: 0.3293) | | Model-4 (LRT p: 0.5572) | | Model-5 (LRT p: 0.3145) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | mOR (95%CI) | p-value | mOR (95%CI) | p-value | mOR (95%CI) | p-value | mOR (95%CI) | p-value | mOR (95%CI) | p-value |
| C. pneumoniae IgG | | | | | | | | | | |
| Positive | 1.9 (0.6-5.6) | 0.2455 | 1.6 (0.5-5.0) | 0.4386 | 2.4 (0.7-8.4) | 0.8132 | 1.8 (0.6-5.4) | 0.3149 | 1.7 (0.6-5.2) | 0.3153 |
| Negative | 1 | | 1 | | 1 | | 1 | | 1 | |
| Pre-pregnancy BMI | | | | | | | | | | |
| 30 or above | | | 7.5 (1.5-38.0) | 0.0059 | | | | | | |
| 25-29.9 | | | 1.3 (0.3-6.3) | 0.2597 | | | | | | |
| Less than 25 | | | 1 | | | | | | | |
| Education of mother | | | | | | | | | | |
| Less than high school | | | | | 3.2 (0.7-14.6) | 0.1753 | | | | |
| High school diploma | | | | | 1.7 (0.4-7.0) | 0.9191 | | | | |
| More than high school | | | | | 1 | | | | | |
| Household income | | | | | | | | | | |
| Less than $25,000 | | | | | | | 1.2 (0.3-4.6) | 0.7505 | | |
| More than $25,000 | | | | | | | 1 | | | |
| Depression during pregnancy | | | | | | | | | | |
| Yes | | | | | | | | | 0.4 (0.1-2.4) | 0.3531 |
| No | | | | | | | | | 1 | |

**Table 5.10 Final conditional model building steps and final model of exposure and covariates of interest with PE status.**

| Variables | Model-6 (LRT p: (0.0269) | | Model-7 (LRT p: 0.1203) | | Model-8 (LRT p: 0.5714) | | Model-9 (LRT p: 0.3987) | | Model-10 (LRT p: 0.5714) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | mOR (95%CI) | p-value | mOR (95%CI) | p-value | mOR (95%CI) | p-value | mOR (95%CI) | p-value | mOR (95%CI) | p-value |
| C. pneumoniae IgG | | | | | | | | | | |
| Positive | 2.1 (0.5-8.1) | 0.2929 | 2.0 (0.5-8.4) | 0.3234 | 2.4 (0.6-9.4) | 0.1971 | 2.3 (0.6-9.1) | 0.2383 | 2.3 (0.6-9.1) | 0.2192 |
| Negative | 1 | | 1 | | 1 | | | | 1 | |
| Education of mother | | | | | | | | | | |
| Less than high school | 4.7 (0.7-30.5) | 0.1164 | 5.1 (0.7-36.5) | 0.1022 | 4.1 (0.7-24.0) | 0.1431 | 4.3 (0.7-24.9) | 0.1232 | 3.6 (0.6-23.2) | 0.1973 |
| | | | | | | | | | | |
| High school diploma | 1.7 (0.3-8.6) | 0.7136 | 1.7 (0.3-11.6) | 0.7344 | 2.0 (0.4-11.0) | 0.9664 | 1.9 (0.3-10.9) | 0.9336 | 1.9 (0.3-10.8) | 0.9855 |
| More than high school | 1 | | 1 | | 1 | | 1 | | 1 | |
| Household income | | | | | | | | | | |
| Less than $25,000 | | | 1.3 (0.2-7.1) | 0.7601 | 0.9 (0.2-3.9) | 0.8931 | 0.9 (0.2-3.9) | >.8985 | 0.9 (0.2-4.2) | 0.9424 |
| More than $25,000 | | | 1 | | 1 | | 1 | | 1 | |
| Depression | | | | | | | | | | |
| Yes | | | | | | | 0.3 (0.02-2.7) | 0.2743 | | |
| No | | | | | | | 1 | | | |
| Newborn sex | | | | | | | | | | |
| Male | | | | | | | | | 1.3 (0.4-4.6) | 0.6900 |
| Female | | | | | | | | | 1 | |
| Pre-pregnancy BMI | | | | | | | | | | |
| 30 or above | 2.2 (0.3-14.5) | 0.6514 | 2.6 (0.4-18.9) | 0.9392 | | | | | | |
| 25-29.9 | 9.4 (1.5-57.6) | 0.0084 | 7.6 (1.2-49.7) | 0.0307 | | | | | | |
| Less than 25 | 1 | | 1 | | | | | | | |

<u>Multivariable analysis</u>

I built Multivariable model based on Univariable analysis. By using the purposeful selection strategy, the final model was developed. At the end, there are two models, one with the variable depression in pregnancy (Model 9) and the other with the variable newborn sex (Model 10) in addition to social class. According to Model 9, after controlling the effects of all covariates, the odds of *C. pneumoniae* IgG among PE cases is 2.3 times the odds of *C. pneumoniae* IgG among controls.

Model 9 and Model-10 have similar *C. pneumoniae* IgG mOR, however, individual variable p-values as measured by the Wald test and the LRT p-value are better for Model 9 as compared to Model 10.

### 5.5.2 Discussion

This manuscript is about the research on the role of *C. pneumoniae* in the development of PE in Lansing, Michigan. The level of IgG (immunoglobulin-G) against *C. pneumoniae* measured in the blood of the study participants provided the frequency of this microorganism in two groups. A higher percentage of PE cases were found to be *C. pneumoniae* IgG positive in their plasma than controls. This finding, although not statistically significant, provides an evidence which supports the hypothesis that a higher percentage of PE cases would have *C. pneumoniae* IgG in their blood as compared to the controls.

Biology of C. pneumoniae

*Chlamydophila pneumoniae* is a species of the genus *Chlamydophila* which belongs to the family *Chlamydiaceae*. The scientific classification of the micro-organism suggests that it originates from the kingdom Bacteria. This is an obligate intracellular mircro-organism and passes through two phases of its developmental cycle of replication; one is extracellular phase and the other is intracellular. The microorganism behaves differently during these two phases. The objective is to survive for itself and to infect other cells. It is infectious when it is in extracellular phase and this is beneficial for the spread of the micro-organism. Additional benefit is that the micro-organism is resistant to antibiotic in this phase while it is sensitive to antibiotic during intracellular phase; however, it is very difficult for the antibiotic to gain access into the cell.

The bi-phasic nature of *C. penumoniae* helps in attaining chronicity. It remains in body tissues for a longer duration of time and may induce an inflammatory response when the circumstances are favorable. In contrast to *H. pylori* and CMV, who have been found to facilitate PE development via an immunological pathway, *C. pneumoniae* has been found to infect

monocytes which get access from the tissues to the blood vessel walls through lumen of the vessel. At this point they initiate an inflammatory process and pass through various steps of plaque formation as stated by Campbell et al (144). As it is known that PE is a disorder whose mechanism of development and pathophysiology has been explained from different aspects (19–21). *C. pneumoniae* mechanism of developing PE is not very well known. However, the closest analogy is the tendency of *C. pneumoniae* to infect monocytes which then get access to blood vessel wall and pass through next steps of plaque formation in the wall of the vessel.

Serological test and interpretation

Enzyme Linked Immunosorbent Serological Assay (ELISA) utilized for the detection of *anti-C. pneumoniae* Ig detect and IgG. IgM is reflective of a recent infection while IgG may suggest an infection in the past or a recurrent infection. Unlike *H. pylori* and CMV, the utilization of avidity for the determination of primary infection has not been discussed under this microorganism.

In the current study, comparatively higher proportion of cases had anti *C. pneumoniae* positive IgG than controls. The findings of this study are consistent with the previous ones (42,79,101,102). Proportion of cases with positive IgG was higher in this study as compared to the earlier one except one (42).

Conditional logistic regression and associated factors

The Univariable analysis of this study shows protective effect of smoking on PE (Table 5.3). The estimates show that women who smoked during pregnancy had 40% low risk of developing PE as compared to women who did not smoke. Earlier studies also found a similar relationship (126,127). Also, women with high pre-pregnancy BMI were at a higher risk of developing PE as compared to pregnant women with lower pre-pregnancy BMI. This finding is also consistent with what is already known (128,129).

Multivariable conditional logistic regression model was built to look for the association of various covariates with PE status based on the Univariable analysis (Table 5.3). These results show that after matching on four variables, maternal age, maternal race, parity and time of blood withdrawal for serological test at design phase, three more covariates were adjusted for at analysis phase: mother education, household income and depression during pregnancy (Model 9) or sex of the newborn (Model 10) to look for the risk of developing PE among women whose blood found *C. pneumoniae* IgGs. The direction of the effect of *C. pneumoniae* IgGs was the same which I had hypothesized, however, it was not statistically significant. In fact none of the covariates could reach to a level of significant association which I think is due to the small sample size. The hypothesis stated that controlling for the social class (education and income), *C. pneumoniae* will be associated with PE.

The literature shows variability in CMV seroprevalence across different ethnic groups (76). The authors found high prevalence in non-Hispanic black and Mexican children as compared to non-Hispanic white children. Over the years, the prevalence further increased reaching upto 90% in non-Hispanic black.

Previously, only one study controlled for the covariates by adjusting at the analysis stage (79). The authors controlled for BMI, smoking during pregnancy and family history of preeclampsia. In the current analysis, the variable pre-pregnancy BMI was dropped because I found it as a collider variable. Direct Acyclic Graphs (DAGs) are graphical representation of the causal assumptions made when one designs a study and generates data. In DAGs when two arrows on a path point to a variable representing that they are the causes of that variable, that variable is termed as a collider variable (130–132). As shown in Figure 4.1, physical activity (133,134), social class (education and income) (135,136) and maternal infection (137,138) are

found to be associated with pre-pregnancy BMI making it a collider variable. As data for this study come from an ongoing study, this study had limitations in getting some important variables such as, family history of preeclampsia and previous history of preeclampsia. However, I managed to get that information through medical review process. Unfortunately, I could not get access to all records or in some cases when I found the record, it was difficult to get the full information. This also resulted in my inability to verify the status of the controls. I feel that this might have resulted in misclassification of outcome. However, in my opinion there would not be more 2-3 such controls which could become cases at a prevalence of 5-8% of PE. In an effort to avoid any chance of misclassification, I removed one potential control from the dataset who had high proteinuria but its reading could not be verified.

Although, the current study had small sample size especially the cases were not very large; however, I tried to address this issue by matching three controls for each case. In addition to controlling the effects of confounders by matching controls with cases on variables which are considered strong confounders, matching has also been found to increase the statistical efficiency of the study and reduces the variance in the parameters of interest (139).

It is important to note that the earlier studies found high prevalence for the seropositivity of *C. pneumoniae* IgG.: however, only two studies attempted to adjust for the confounders (94,96) and out of these two studies, only one adjusted the potential confounders appropriately (96). We attempted to adjust the effects of confounders at both design phase by matching cases with three controls and then at analysis level when we assessed different variables through conditional logistic regression.

# 6        Conclusions and future directions

1.  Past infection with *H. pylori*, CMV, or *C. pneumoniae* in the form of *H. pylori* IgG, CMV IgG, and *C. pneumoniae* IgG, did not show any association are  with the development of PE in Lansing, Michigan.

2.  No evidence of recent or current infection with CMV as measured by IgM, identified as a risk factor for the development of PE in Lansing, Michigan.

3.  To explore this relationship further, similar studies should be carried out in future with a larger sample size especially in populations where prevalence of these infections is high.

4.  Future studies should include serology for anti-CagA antibodies for the exploration of the role of *H. pylori*.

5.  Exploration of the infectious agents DNA from placental tissue may support the immunological cause of PE.

**APPENDICES**

# Appendix A: Initial models with H. pylori

**Table 7.1 Types of antigens of H. pylori**

| Cag | Cytotoxin-associated protein |
|---|---|
| Vac | Vacuolating cytotoxin protein |
| UreA | Urease subunits |
| UreB | |
| UreC | |
| HspA | Heat-shock protein |
| HspB | |
| Flagellin subunits | |
| Catalase | |
| lipopolysaccharide | |

**Table 7.2 Initial model H. pylori 1**

| Exact method | | | Without exact method *H. pylori* IgG status as a continuous variable | |
|---|---|---|---|---|
| Variables | mOR (95%CI) | p-value | mOR (95%CI) | p-value |
| *H. pylori* IgG status | | | | |
| Positive | 0.6 (0.0081-16.5) | >.999 | 0.7 (0.-2.5) | 0.697 |
| Negative | 1 | | ■■■■■■ | |
| Household income | | | | |
| Less than 25,000 | | | | |
| More than 25,000 | | | | |
| Education of mother | | | | |
| Less than high school | | | | |
| High school diploma | | | | |
| More than high school | | | | |
| Pre-pregnancy BMI | | | | |
| 30 or above | 7.4 (1.4-60.6) | 0.0123 | 8 (1.5-42.4) | 0.015 |
| 25-29.9 | 1.4 (0.2-9.8) | 0.9888 | 1.3 (0.3-6) | 0.7321 |
| Less than 25 | 1 | | 1 | |

**Table 7.3 Initial model H. pylori 2.**

| Exact method | mOR (95%CI) | p-value | Without exact method *H. pylori* IgG status as a continuous | |
|---|---|---|---|---|
| Variables | mOR (95%CI) | p-value | mOR (95%CI) | p-value |
| *H. pylori* IgG status | | | | |
| Positive | 0.5 (0.006-13.6) | >.999 | 0.6 (0.2-2.4) | 0.4985 |
| Negative | 1 | | | |
| Household income | | | | |
| Less than 25,000 | | | | |
| More than 25,000 | | | | |
| Education of mother | | | | |
| Less than high school | 3.5 (0.5-34.9) | 0.2697 | 4.2 (7-26.5) | .1588 |
| High school diploma | 1.8 (0.3-12.3) | 0.7318 | 1.8 (0.4-9.3) | .641 |
| More than high school | 1 | | 1 | |
| Pre-pregnancy BMI | | | | |
| 30 or above | 7.8 (1.4-86.1) | 0.0124 | 11 (1.6-73.6) | .0072 |
| 25-29.9 | 2.1 (0.3-21.5) | 0.7031 | 2.2 (0.3-14.2) | .5091 |
| Less than 25 | 1 | | 1 | |

**Table 7.4 Initial model H. pylori 3.**

| Exact method | | | Without exact method *H. pylori* IgG status as a continuous variable | |
|---|---|---|---|---|
| Variables | mOR (95%CI) | p-value | mOR (95%CI) | p-value |
| *H. pylori* IgG status | | | | |
| Positive | 0.4 (0.005-10.9) | 0.9544 | 0.6 (0.1-2.5) | 0.4682 |
| Negative | 1 | | ███████ | |
| Household income | | | | |
| Less than 25,000 | 1.7(0.2-15.1) | 0.8068 | 1.8 (0.3-12.1) | 0.5331 |
| More than 25,000 | 1 | | 1 | |
| Education of mother | | | | |
| Less than high school | 3.5 (0.5-39.7) | 0.2746 | 4.2 (0.7-27.2) | 0.1276 |
| High school diploma | 1.6 (0.2-15.4) | 0.9708 | 1.6 (0.3-10.6) | 0.7673 |
| More than high school | 1 | | 1 | |
| Pre-pregnancy BMI | | | | |
| 30 or above | 7.8 (1.1-117.6) | 0.0315 | 10.6 (1.4-81.3) | 0.0224 |
| 25-29.9 | 2.9 (0.3-38.3) | 0.4966 | 3.2 (0.4-23.4) | 0.9745 |
| Less than 25 | 1 | | 1 | |

# Appendix B: Initial models CMV

**Table 7.5 Initial model CMV 1**

| Variables | mOR (95%CI) | p-value |
|---|---|---|
| CMV IgG status | | |
|   Positive | 1.3 (0.1-15.4) | 1.000 |
|   Negative | 1 | |
| Household income | | |
|   Less than 25,000 | | |
|   More than 25,000 | | |
| Education of mother | | |
|   Less than high school | | |
|   High school diploma | | |
|   More than high school | | |
| Pre-pregnancy BMI | | |
|   30 or above | 7.2(1.3-61.3) | 0.0164 |
|   25-29.9 | 1.3(.2-9.3) | 0.9996 |
|   Less than 25 | 1 | |

**Table 7.6 Initial model CMV 2.**

| Variables | mOR (95%CI) | p-value |
|---|---|---|
| CMV IgG status | | |
|   Positive | 1.91 (0.2-27.1) | 0.8439 |
|   Negative | 1 | |
| Household income | | |
|   Less than 25,000 | | |
|   More than 25,000 | | |
| Education of mother | | |
|   Less than high school | 3.9(0.5-42.2) | 0.2403 |
|   High school diploma | 2(0.3-14.3) | 0.6493 |
|   More than high school | 1 | |
| Pre-pregnancy BMI | | |
|   30 or above | 7.3(1.3-79.1) | 0.0178 |
|   25-29.9 | 2.1(0.3-22.2) | 0.6862 |
|   Less than 25 | 1 | |

**Table 7.7 Initial model CMV 3**

| Variables | mOR (95%CI) | p-value |
|---|---|---|
| CMV IgG status | | |
| Positive | 1.6 (0.1-30.6) | 1.000 |
| Negative | 1 | |
| Household income | | |
| Less than 25,000 | 1.4(0.2-9.9) | 0.9381 |
| More than 25,000 | 1 | |
| Education of mother | | |
| Less than high school | 3.6 (0.5-41.5) | 0.2844 |
| High school diploma | 1.7(0.2-15.8) | 0.8323 |
| More than high school | 1 | |
| Pre-pregnancy BMI | | |
| 30 or above | 6.4 (1.1-71.7) | 0.0373 |
| 25-29.9 | 2.4 (0.3-21.6) | 0.5809 |
| Less than 25 | 1 | |

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