ABSTRACT

Objectives:
The aim of this study was to further investigate the role of pro-inflammatory cytokines in the pathogenesis of fetal cerebral white matter injury associated with chorioamnionitis by characterizing the time course of the cytokine response in the pregnant guinea pig following a maternal inflammatory insult.

Rationale of Hypothesis:
Chorioamnionitis increases the risk for fetal brain injury. In the guinea pig, a threshold maternal inflammatory response must be reached for significant fetal brain injury to occur. However, a previous study demonstrated that, by seven days after an acute maternal inflammatory insult, cytokine levels in both maternal and fetal compartments are not different from controls. The purpose of this study, therefore, was to test the hypothesis that a significant cytokine response occurs within the first seven days following an acute maternal inflammatory response.

Methodology:
Pregnant guinea pigs (n=34) were injected intraperitoneally with 100µg/kg lipopolysaccharide (LPS) at 70% gestation and euthanized at 24 hours, 48 hours or 5 days following endotoxin exposure. Control animals were euthanized at 70% gestation without exposure. Concentrations of interleukin-6, interleukin 1-β and tumour necrosis factor-α (IL-6, IL-1β, TNF-α) were quantified in the maternal serum and amniotic fluid by enzyme-linked immunosorbent assay.
**Results:**

IL-6 and IL-1β concentrations were elevated in the maternal serum at 24 hours and returned to control levels by five days. In the amniotic fluid, IL-6 peaked at 48 hours and IL-1β at 24 hours. TNF-α levels were not significantly increased.

**Conclusions:**

A single maternal LPS injection produces transient increases in cytokine concentrations in the maternal serum and amniotic fluid. This further implicates the cytokines as potential mediators of fetal white matter damage. Although this response might not be sufficient to produce the brain injury itself, it may initiate harmful pro-inflammatory cytokine cascades, which could even continue to harm the fetus following delivery. A human diagnostic protocol was developed to assess the use of serial serum biomarkers, including IL-6 and TNF-α, in the prediction of histological chorioamnionitis. Preliminary analysis of the pilot study suggests that certain biomarkers might be worthy of further investigation in a larger-scale study.
CO-AUTHORSHIP

The author performed all experiments described in this thesis, with the supervision of Dr. Graeme Smith and the assistance of Richard Casselman, Erica Harnett and Carolina Venditti for the animal experiments. Richard Casselman provided technical support for all protein tests. Clinical subjects were recruited with the help of Dr. Graeme Smith, Lizy Kodiattu, Heather Ramshaw, and Melissa Swansburg. Human serum samples were drawn by research nurses at Kingston General Hospital.
ACKNOWLEDGEMENTS

I want to thank all of you who have given your time, assistance and patience so generously during my graduate studies. I cannot thank my family enough for their unwavering support throughout my extensive and expensive studies. I extend deepest thanks to Dr. Graeme Smith for your consistent trust and support as I made my way through this arduous process. With unparalleled encouragement and understanding, your mentorship has guided me towards a most promising career. I would also like to express gratitude to my fellow members of the Smith lab, past, present or honorary, for their expertise and assistance: Richard Casselman, Lindsay Patrick, Shannon Bainbridge, Laura Moore, Erica Harnett, Carolina Venditti, Erin Bell and Elizabeth Sidle. Richard, you are an indispensable resource of knowledge and I thank you for your extensive contributions to my projects. Lab rats, you have made the entire experience an entertaining and fulfilling one. I am deeply appreciative of your friendship and support. Thank you to Heather Ramshaw, Lizy Kodiattu, and Melissa Swansburg for their help with my clinical study and for their tolerance of my numerous questions. I am grateful for the opportunity I was given to work alongside such exceptional professors, students and staff in the Department of Anatomy and Cell Biology especially, but also in the Queen’s and KGH community as a whole.

I also wish to recognize financial support from the Physician’s Services Incorporated Foundation, an Ontario Graduate Scholarship, and a Queen’s Graduate Scholarship from the School of Graduate Studies and Research.
TABLE OF CONTENTS

ABSTRACT........................................................................................................................ ii
ACKNOWLEDGEMENTS....................................................................................................... v
TABLE OF CONTENTS................................................................................................... vi
LIST OF TABLES................................................................................................................ ix
LIST OF FIGURES AND ILLUSTRATIONS....................................................................... x
Glossary of abbreviations ............................................................................................ xii
Glossary of definitions .................................................................................................. xiv

CHAPTER 1: Introduction and Literature Review............................................................. 1

Chorioamnionitis............................................................................................................. 1
Periventricular Leukomalacia ......................................................................................... 5
Cerebral Palsy ............................................................................................................... 6
Hypothesis: Prematurity causes white matter damage................................................. 9
Hypothesis: Hypoxia/ischemia causes white matter damage ..................................... 10
Hypothesis: Chorioamnionitis and pro-inflammatory cytokines cause white matter
damage ......................................................................................................................... 10
Research Problem: Characterization of the cytokine response ................................ 16
Research Problem: Diagnosis of chorioamnionitis .................................................... 18
Objectives .................................................................................................................... 20

CHAPTER 2: Transient lipopolysaccharide-induced cytokine responses in the maternal
and fetal guinea pig........................................................................................................... 22

INTRODUCTION ........................................................................................................ 22
MATERIALS AND METHODS.................................................................................... 24
LIST OF TABLES

Table 1: Common microorganisms involved in chorioamnionitis ................................. 4
Table 2: Diagnostic value of single samples of biomarkers ........................................... 45
Table 3: Characteristics of recruited subjects ............................................................... 50
Table 4: Sensitivity, specificity of serum biomarkers. .................................................. 71
LIST OF FIGURES AND ILLUSTRATIONS

Figure 1: Cytokine hypothesis for the development of cerebral palsy ............................. 15
Figure 2: Uterine horns exposed by midline laparotomy.................................................. 27
Figure 3: Outcome of the litters ........................................................................................ 29
Figure 4: IL-6 concentrations in the maternal serum and amniotic fluid ......................... 32
Figure 5: IL-1β concentrations in the maternal serum and amniotic fluid ....................... 33
Figure 6: TNF-α levels in the maternal serum and amniotic fluid..................................... 34
Figure 7: Human chorionic gonadotropin concentrations according to gestational age... 53
Figure 8: C-reactive protein concentrations according to gestational age......................... 54
Figure 9: Serum intracellular adhesion molecule-1 concentrations according to gestational age................................................................................................................... 55
Figure 10: Interleukin-6 concentrations according to gestational age ............................. 56
Figure 11: Tumour necrosis factor-α concentrations according to gestational age .......... 57
Figure 12: Matrix metalloproteinase-9 concentrations according to gestational age ....... 58
Figure 13: Insulin-like growth factor binding protein-1 concentrations according to gestational age................................................................................................................. 59
Figure 14: Placental growth factor concentrations according to gestational age.............. 60
Figure 15: Endoglin concentrations according to gestational age .................................... 61
Figure 16: Scatter plot of human chorionic gonadotropin concentrations ...................... 62
Figure 17: Scatter plot of C-reactive protein concentrations ............................................. 63
Figure 18: Scatter plot of intracellular adhesion molecule-1 concentrations .................... 64
Figure 19: Scatter plot of interleukin-6 concentrations ..................................................... 65
Figure 20: Scatter plot of tumour necrosis factor-α concentrations .................................. 66
Figure 21: Scatter plot of matrix metalloproteinase-9 concentrations ............................. 67
Figure 22: Scatter plot of insulin-like growth factor binding protein-1 concentrations ... 68
GLOSSARY OF ABBREVIATIONS

ANOVA: analysis of variance
BBB: blood brain barrier
cChA: clinical chorioamnionitis
ChA: chorioamnionitis
CI: confidence interval
CP: cerebral palsy
CRP: C-reactive protein
ELISA: enzyme-linked immunosorbent sandwich assay
Eng: endoglin
hCG: human chorionic gonadotropin
hChA: histologic chorioamnionitis
IGFBP-1: insulin-like growth factor binding protein-1
IL-1β: interleukin-1β
IL-6: interleukin-6
IgA: immunoglobulin-A
IgG: immunoglobulin-G
IVH: intraventricular hemorrhage
LPS: lipopolysaccharide
MDD: minimum detectable dose
MMP-9: matrix metalloproteinase-9
MRI: magnetic resonance imaging
NPV: negative predictive value
OL: oligodendrocytes
P/GF: placental growth factor
PROM: premature rupture of the membranes
PPROM: preterm premature rupture of the membranes
PPV: positive predictive value
PVL: periventricular leukomalacia
SD: standard deviation
SEM: standard error of the mean
sFlt-1: soluble fms-like tyrosine kinase-1
sICAM-1: soluble intracellular adhesion molecule-1
SSTs: serum separator tubes
TGF: transforming growth factor
TNF-α: tumour necrosis factor-α
UTI: urinary tract infection
WBC: white blood count
WMD: white matter damage
GLOSSARY OF DEFINITIONS

Clinical chorioamnionitis: Symptomatic later stages of intrauterine infection involving the placental membranes and the amniotic fluid and producing clinical symptoms including maternal fever, uterine tenderness, maternal leukocytosis, malodorous amniotic fluid discharge, and maternal or fetal tachycardia.

Histologic chorioamnionitis: Asymptomatic, earlier stages of intrauterine infection involving leukocyte infiltration into the placental membranes.

Periventricular leukomalacia: A sequence of focal and diffuse neuropathologic changes in the cerebral white matter of premature infants.

Cytokine: A group of soluble regulatory proteins that are released by cells of the immune system and act as intercellular mediators of the inflammatory response.

Cerebral palsy: A static condition in which cerebral white matter damage occurs before brain development is completed, characterized by a heterogeneous group of motor and postural disturbances which manifest early in life.
CHAPTER 1: Introduction and Literature Review

Chorioamnionitis

Chorioamnionitis (ChA) is a polymicrobial intrauterine infection involving the placental membranes and the amniotic fluid. It is a significant complication present in 0.5%-10% of all pregnancies and has been associated with acute neonatal morbidity, including neonatal sepsis, respiratory distress and mortality, as well as a number of maternal morbidities such as thrombosis of pelvic vessels and a potential for pulmonary emboli. It is believed to contribute to a number of preterm births producing very ill low birth weight babies and is generally considered to be one of the leading causes of fetal and neonatal death.

The clinical diagnosis of ChA differs widely from one medical center to the next. The presence of maternal fever, uterine tenderness, maternal leukocytosis, malodorous amniotic fluid discharge, and maternal or fetal tachycardia are used as diagnostic criteria, alone or in combination, for clinical chorioamnionitis (cChA). Positive amniotic fluid cultures collected via amniocentesis are also used as an indication of intrauterine infection in women with intact membranes. Due to the frequency of false-positive reports of cChA, these criteria have ultimately been recognized as having poor sensitivity and specificity for placental inflammation. Nevertheless, a gold standard for the diagnosis of ChA has yet to be resolved.

What further confounds the diagnosis of ChA is the fact that it frequently presents as a subclinical condition. Histologic, or subclinical, chorioamnionitis (hChA), the
maternal polymorphonuclear leukocyte invasion of the amniotic membranes, chorionic plate or umbilical cord \(^7\), and is therefore retrospectively diagnosed upon pathologic examination of the placental tissues following delivery \(^10\). Histologic ChA occurs more frequently than clinically diagnosed intrauterine infection, therefore it has been suggested that hChA represents a less advanced stage in the continuum of infection \(^2;3;12\). Because placentas only undergo microscopic examination if there is suspected pathology, many cases of hChA may be missed altogether \(^2\). The lack of explicit symptoms in many cases of hChA further supports the hypothesis that subclinical chorioamnionitis is less advanced than the clinically evident stages of intrauterine infection.

Chorioamnionitis is believed to occur most frequently as an ascending infection, when endogenous cervical and vaginal flora invade the uterine cavity and gain access to the placental tissues \(^11\). Pathogens can enter and infect the uterus by haematogenous spread through the placenta, or via iatrogenic routes (eg. amniocentesis) \(^11\). Many different microorganisms have been implicated in this infection, most of low virulence \(^3\), and the spectrum of which closely parallels the composition of the cervico-vaginal tract \(^13\)(Table 1). Group B streptococci are commonly isolated from the placenta or amniotic fluid of women with ChA, as well as other facultatively anaerobic bacteria such as *Escherichia coli*, *Hemophilus influenza* and *Listeria monocytogenes* \(^1;13\). Viruses including hepatitis B, hepatitis C, cytomegalovirus (CMV), human immunodeficiency virus (HIV) and herpes simplex virus can also contribute to placental inflammation through haematogenous or ascending routes \(^11\). It is important to note that not all cases of ChA are confirmed by positive cultures in either the amniotic fluid or the placenta \(^2\).
Microorganisms are only isolated in approximately 70% of placentas with histologic evidence of ChA. Even so, the general consensus among researchers suggests that inflammation does not itself guarantee the sustained presence of a pathogen. The degree and the nature of the inflammatory response to an initial infectious stimulus is thought to be more important in the development of the clinical signs of ChA and its sequelae than the presence of the microbe itself.
**Table 1:** Common microorganisms involved in chorioamnionitis and their corresponding route of entry into the uterine cavity. Adapted from references 3,11,14,15.

<table>
<thead>
<tr>
<th>Route of Infection</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascending</td>
<td><em>Ureaplasma urealyticum</em></td>
</tr>
<tr>
<td></td>
<td><em>Mycoplasma hominis</em></td>
</tr>
<tr>
<td></td>
<td><em>Klebsiella pneumoniae</em></td>
</tr>
<tr>
<td></td>
<td><em>Bacteroides species</em></td>
</tr>
<tr>
<td></td>
<td><em>Fusobacterium species</em></td>
</tr>
<tr>
<td></td>
<td><em>Streptococcus species</em></td>
</tr>
<tr>
<td>Haematogenous</td>
<td><em>Listeria monocytogenes</em></td>
</tr>
<tr>
<td></td>
<td><em>Treponema pallidum</em></td>
</tr>
<tr>
<td>Iatrogenic</td>
<td>Skin flora (via amniocentesis)</td>
</tr>
<tr>
<td></td>
<td>Vaginal flora (via chorionic villus sampling)</td>
</tr>
<tr>
<td>Intrapartum</td>
<td>Group B streptococci</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td></td>
<td><em>Chlamydia trachomatis</em></td>
</tr>
<tr>
<td></td>
<td><em>Neisseria gonorrhoeae</em></td>
</tr>
</tbody>
</table>
Women most at risk for ChA include those with prolonged cervical dilation, prolonged labour and prolonged rupture of membranes (PROM) because their endogenous barriers against ascending infection are compromised. Exogenous opportunistic microorganisms may be introduced into the cervico-vaginal tract through multiple vaginal examinations, increasing the risk for intrauterine infection. To address the risk of infection in these cases, maternal antibiotics have been tested; however the amniotic cavity is largely inaccessible to the treatment. Antibiotic treatment is unable to delay delivery and does not reduce the risk of preterm delivery or improve neonatal outcome. Most often correlated to the development of intrauterine infection, however, is preterm labour. In fact, ChA is present in as many as 45% of pregnancies complicated by preterm labour.

The sole treatment for ChA presently involves expedited delivery. Immediate removal of the fetus from the hostile infected or inflamed environment reduces the risk of harmful sequelae of ChA. Subsequent treatment with antibiotics for mother and baby may follow if sepsis is suspected. Corollaries of ChA in the surviving infants include neonatal sepsis, chronic lung disease and thymic involution. Arguably the most detrimental potential sequelae of ChA are a broad spectrum of devastating neurologic impairments such as periventricular leukomalacia and cerebral palsy.

Periventricular Leukomalacia

Periventricular leukomalacia (PVL) is described as a sequence of focal and diffuse neuropathologic changes in the cerebral white matter of premature infants.
Focal necrosis and liquefaction generally occur deep in the cerebral white matter with subsequent cyst formation. Diffuse injury is cell-specific and triggers damage to oligodendrocyte (OL) precursors. The OL precursors are destined to develop into mature myelinating cells; therefore PVL is often accompanied by myelin deficiency in the central nervous system. The rare focal lesions are easily detected by cranial ultrasonography, while the common diffuse lesions are not. Diffuse white matter damage (WMD) may be detected by diffusion-weighted magnetic resonance imaging (MRI).

Infants of very low birth weight (500g-1500g) born prematurely have an incidence of 3-15% of PVL. These infants are presumably most at risk for brain injury due to their underdeveloped blood brain barrier (BBB). In preterm infants, the consequences of PVL can be grave; neurocognitive abnormalities are diagnosed by school age and persist into adulthood. Common afflictions include mental retardation, learning disorders, motor abnormalities and problems with vision and hearing. Almost 10% of very low birth weight infants will develop cerebral palsy, and 90% of these cases correlate directly to the presence of PVL. PVL remains the most common cause of cerebral palsy.

Cerebral Palsy

Cerebral palsy (CP) is a chronic disability of the central nervous system caused by lesions in the cerebral white matter occurring before development of the brain is complete. Because cerebral development continues throughout the first 2 years of...
life, brain damage causing CP may occur in the prenatal, perinatal or postnatal periods, although 70-80% of cases are acquired in utero; a great proportion of these causes are largely unexplained. Birth asphyxia accounts for only 6% of cases of CP, and nearly 10-20% of cases occur postnatally as a consequence of bacterial or viral infections, accident-related brain injury or child abuse. Historically, many definitions have been suggested to describe the clinical condition we currently recognize as CP. Some definitions encapsulate a broader range of motor deficits, while others restrict the concept to a more distinct pathology of movement. Present in each of the definitions is the notion that CP exists as a heterogeneous group of motor and postural disturbances which manifest early in life. Also included is the stipulation that CP must remain a static condition. As such, when previously-learned skills are lost or if reflexes disappear, a neurodegenerative condition may be precipitating the symptoms rather than CP.

The cerebral white matter lesions that underlie CP are associated with damage to upper motor neurons, resulting in aberrant control of movement. Among the symptoms leading to a diagnosis of CP are delays in motor development, observations of unusual posture and limb favouring, and abnormal muscle tone. Muscle tightness, tremors, involuntary movement, difficulties with gross or fine motor skills, and difficulties with perception and sensation are common. Laboratory tests generally follow a suspicion of CP to elucidate the symptoms and to eliminate any progressive, hereditary or metabolic causes. Cerebral imaging such as MRI and ultrasound are invaluable tools as the lesions detected during the neonatal period can support an ultimate diagnosis of CP.
Neuroimaging can also define the origins of CP by distinguishing lesional patterns and may give insight into the timing of the insult $^{25}$.

In addition to the severe motor dysfunction experienced, a higher incidence of neurologic dysfunction, comprising seizure disorders, ataxia, intellectual impairment, growth problems and visual deficits, is observed in the afflicted individuals $^{1,20}$. Almost two thirds of children with CP will have some degree of cognitive impairment, while half of pediatric patients will have seizures $^{20}$. As such, CP is an extremely debilitating disorder, and tremendously costly both economically and emotionally for affected families. It is now estimated that in the United States of America, the economic cost of CP reaches five billion dollars per annum $^{26}$. CP currently occurs in approximately 1.4 to 2.7 of 1000 live births, making it one of the most prevalent causes of early childhood disability $^{21;25;27}$. This translates into over 9,500 infants and children being diagnosed every year in the United States of America alone $^{20}$. What’s more, many reports describe a recent rise in the prevalence of CP, perhaps due to the increase in survival of very preterm or low birth weight infants $^{20;21;25}$.

Although the etiology of this syndrome is poorly understood, many risk factors have been identified. Increasing the risk for the development of CP are prenatal factors such as preterm birth, birth asphyxia, maternal fever, prenatal strokes and maternal intrauterine infection, namely ChA $^{1;4;28}$. Multiple pregnancies increase the risk for CP at term, especially with the death of a co-twin $^{25}$. Because these factors are generally non-modifiable and by no means account for all the cases of CP, there currently exists no
prevention for this disability. Very little is known about the pathogenesis of the disorder and as a result, appropriate animal models for CP are currently lacking. Nevertheless, many hypotheses for the development of CP have been explored.

**Hypothesis: Prematurity causes white matter damage**

Prematurity and low birth weight are the most common causes of CP. As the brain undergoes rapid fetal development, it is most vulnerable to injury. The prevalence of CP increases as gestational age decreases. Accordingly, the occurrence of CP in infants delivered at term is 0.6-1.2 cases per 1000 live births, whereas in infants born before 27 weeks gestation this figure rises to 44-82 cases per 1000 live births. Very preterm infants (less than 32 weeks gestational age) often suffer from types of cerebral WMD which are rare among term newborns. Even the incidence of PVL increases with decreasing gestational age. Although very preterm infants represent only 2% of the infants born each year, they represent 25% of all children with CP. The elevated threat of WMD associated with prematurity is related to vulnerabilities in developmentally-regulated endogenous fetal protection systems. Initially, protection against the entry of toxins into the brain is ineffective as the BBB is not completely regulated until week 27 of gestation. In addition, myelination processes are vulnerable to harm as developing oligodendrocytes (OLs), which sheathe the axons of the white matter in myelin, can be targeted by various inflammatory molecules which induce cell death. Endogenous protector molecules such as neurotrophins and some hormones are not readily available in the very preterm brain, and therefore cannot promote its proper development nor protect oligodendrocytes or neurons from cell death.
Hypothesis: Hypoxia/ischemia causes white matter damage

The cerebral white matter of the developing fetus is prone to ischemia-reperfusion injury due to the presence of arterial end zones in the periventricular tissues 32. A premature neonate may exhibit pressure-passive circulation, leading to oxidative stress and the generation of free radicals 26. In an ovine model of partial umbilical cord occlusion, lipid peroxidation was observed in the white matter of near-term fetuses 33. Another study noted that the reperfusion following occlusion was the most important source of free radical formation 34. Because OL-progenitors are susceptible to harm or death through glutamate uptake in oxidative stress, myelination becomes disrupted and causes the white matter lesions observed in PVL and CP 26. Fetal systemic hypotension causes a reduction in blood flow to structures of the cerebral white matter, and ultimate encephalopathy 26;35. Vasculopathy in the placenta and clot formation through pre-existing prothrombotic conditions can cause focal infarctions in the fetus, a common cause of CP 35. Perinatal events of oxygen deprivation have also been implicated in the development of CP, but seem to play a minor role as birth asphyxia and prolonged labour have decreased with advancements in obstetrical care, but a decline in the rate of CP has not been observed 4,23.

Hypothesis: Chorioamnionitis and pro-inflammatory cytokines cause white matter damage

One widely accepted hypothesis for the development of CP concerns intrauterine infection and the release of pro-inflammatory cytokines during pregnancy (Figure 1).
Chorioamnionitis greatly increases the risk for fetal WMD, leading to a roughly 2- to 12-fold higher risk of CP in term infants.\textsuperscript{4,12,35-37} It is believed that when the maternal inflammatory response to infection is accompanied by a fetal inflammatory response, ChA is associated with significantly higher neonatal morbidity, mortality, and greater resource use.\textsuperscript{38}

Supporting this theory is a number of reports from research conducted on animals, \textit{in vitro}, and \textit{in vivo}. In a model completed by Yoon and colleagues, cervical inoculation of \textit{E. coli} to pregnant rabbits at 70\% gestation produced histologic evidence of ascending intrauterine infection and fetal WMD was observed.\textsuperscript{39} Work completed by Bell and colleagues determined that intracervical injection of lipopolysaccharide (LPS; endotoxin released by \textit{E. coli} to which the body mounts an inflammatory response) in pregnant rats was associated with dose-dependent mortality, activated astrocytes, and increased apoptotic cell death in the cerebral tissues.\textsuperscript{40} A previous guinea pig model of ChA has shown that intracervical inoculation of \textit{E. coli} results in fetal brain injury in the exposed pups, regardless of the presence of bacteria infection of the amniotic fluid.\textsuperscript{41} Control levels of amniotic fluid TNF-\textalpha{} and IL-6 were significantly lower than those from inoculated sows.\textsuperscript{41} In an ovine model of intrauterine infection by Nitsos and colleagues, whereby pregnant ewes received chronic intra-amniotic LPS infusion, from 80 days of gestation, fetal leukocytosis was observed as well as the infiltration of activated astrocytes and microglia into the subcortical white matter, causing extensive focal damage.\textsuperscript{42} In a study by Debillon and colleagues closely mimicking human ChA, intrauterine \textit{E.coli} inoculation of pregnant rabbits at 80\% gestation was associated with
increased survival of fetuses when maternal antibiotics were administered. Nevertheless, all live pups showed some evidence of programmed cell death in areas of the periventricular white matter \(^{43}\).

As products and mediators of the immuno-inflammatory response, pro-inflammatory cytokines are presumed to represent the link between ChA and fetal brain injury. Cytokines are soluble markers that mediate immunity \(^{44}\). Most importantly, they regulate the amplitude and the time-course of the inflammatory response \(^{44}\). Among those most often implicated in the etiology of fetal brain injury are IL-6, IL-1\(\beta\) and TNF-\(\alpha\). Interaction of these cytokines occurs in a cascade system where synergism amplifies inflammation \(^{44}\). TNF-\(\alpha\) is an acute-phase pyrogenic and cytotoxic cytokine that initiates the earliest steps in the inflammatory cascade \(^{45}\). By activating a variety of signal transduction pathways, TNF-\(\alpha\) can induce pro-inflammatory cytokine gene transcription and translation \(^{44}\). IL-6 is a cytotoxic cytokine that can also enable B cell differentiation, antibody production and T cell activation in an immuno-inflammatory response \(^{44}\). The biological activity of the cytotoxic and pyrogenic IL-1\(\beta\) consists of chemotaxis and induction of macrophages for cytokine production \(^{44}\).

Higher expression of pro-inflammatory cytokines in brains with PVL \(^{46}\) and induced cytokine production and WMD in fetal rat brains after maternal LPS administration \(^{24}\) corroborates the premise that cytokines are capable of harming the developing brain tissue of a fetus. When pre-treated with TNF-\(\alpha\) in vitro, only 2.6% of OL-progenitors in culture survive after 9 days, whereas in the untreated cultures the
majority of the cells undergo differentiation into the myelinating phenotype. Therefore not only can TNF-α induce apoptosis, but it can also inhibit the differentiation of OL-progenitors. Both IL-6 and IL-1β are thought to have direct cytolytic properties against neurons and developing OLs.

Research has shown that TNF-α, produced only by the amnion, can be released directly into the amniotic fluid; whereas IL-1β, produced only by the chorion, must diffuse across a greater distance to reach the amniotic fluid during intrauterine infection. IL-6 itself is produced by both the amnion and the chorion and therefore appears in greater concentrations in the amniotic fluid in models of ChA. Each of these cytokines is produced in elevated concentrations upon LPS stimulation in the amniochorion. Pro-inflammatory cytokines are elevated in the maternal circulation during infection and are produced by the amniochorion. The ability of cytokines to cross the placenta is still unclear, with only IL-6 shown to be capable of bi-directional transfer across the placenta, gaining access to both the maternal and fetal circulation and ultimately the fetal brain.

Inflammatory mediators, including IL-6, IL-1β, TNF-α, and various coagulation factors, are found in higher concentrations in the dried neonatal blood of infants with spastic CP when compared to controls. Because the inflammatory and coagulation pathways interact, they may both play a role in the etiology of CP. In neonates born with white matter lesions associated with PVL, umbilical vein plasma IL-6 levels are elevated, supporting the hypothesis that PVL can occur through cytokine-mediated pathways. In one clinical study, concentrations of IL-6 in umbilical vein plasma over 400pg/mL
identified PVL with a sensitivity of 72% and a specificity of 74% \(^{54}\). In a study by Duncan and colleagues \(^{55}\) intravenous injections of LPS administered to ovine fetuses at 70% gestation and repeated over five days caused hypoxemia and hypotension. Elevated circulating IL-6 concentrations were noted in the first two days of exposure, which then declined and resolved following subsequent LPS injections. Each exposed ovine fetus sustained neuropathologic injury, with features resembling PVL and CP \(^{55}\). It is presumed that a combination of the pro-inflammatory cytokine damage and the tissue hypoxemia both contributed to the cerebral WMD observed.

Inflammation of the placental tissues can lead to interruption of blood flow to the fetus as well as obstruction of gas exchange at the maternal-fetal interface. As such, the cytokine hypothesis of fetal WMD does not exclude that of hypoxia/ischemia. Nor does it exclude the predilection to harm caused by prematurity, when it is considered that ChA and prematurity are frequently comorbid.
Figure 1: Cytokine hypothesis for the development of cerebral palsy in the fetus following intrauterine infection. The uterus, the fetal circulation and the fetal brain exist as three compartments, with the placenta and the blood brain barrier (BBB) as boundaries. Pro-inflammatory cytokines, produced in the immunoinflammatory response, gain access to all three compartments. *Pro-inflammatory cytokines originate in the uterus and the amniotic fluid and may cross the placenta, but they may also be produced by the placental tissues themselves. Therefore, the cytokines in the fetal circulation are of both maternal and fetal origin. These cytokines increase the permeability of the BBB. Adapted from references 12, 18, 26, 50.
Research Problem: Characterization of the cytokine response

Many facets of the cytokine response remain unclear in its association to ChA and fetal brain injury. Speculation exists that length of exposure to an inflammatory process directly affects the severity of its sequelae. Presumably, the earlier silent stages of ChA pose less harm to the developing fetus than the acute clinical phase. For infants born at less than 32 weeks gestation with intrauterine infection, Locatelli and colleagues found no evidence that a longer duration of active labour was associated with an increase in risk for white-matter damage. Their results also suggested that the duration of cChA was not associated to the risk for fetal cerebral injury, although the researchers recognized the limited power of their study 58. Because this issue has implications in an obstetrician’s decision to expedite delivery subsequent to a suspicion of ChA, it must be investigated further before standards of practice are altered.

Because the earlier stages of ChA do not produce clinically evident symptoms, it is believed that low levels of inflammation responding to the initial infectious stimulus may not cause any harm to the developing fetal white matter. Only more severe responses and more prolonged robust inflammatory reactions are presumed to produce cChA and cause the fetal brain injury typically observed in PVL and CP. This scenario might not be restricted solely to intrauterine infections if the inflammation is extreme: three case studies of acute appendicitis published in the United States suggested that major extrauterine infections during pregnancy may also play a role in the occurrence of fetal WMD such as PVL 59. In a dose-response model of maternal LPS exposure at 70% gestation and fetal WMD in the guinea pig, high doses beyond an identified threshold
resulted in significant apoptosis in the periventricular regions of the fetal brain, observed seven days after the inflammatory insult \(^{60}\). Maternal LPS doses below the threshold did not produce observable brain injury to the fetus, suggesting that the severity of the inflammatory response governs the outcome. In the same study, cytokine concentrations in the maternal serum and in the amniotic fluid were quantified at seven days. Levels were not different from controls, even for those doses producing demonstrable WMD, therefore preventing the authors from drawing associations between the brain injury and the actions of the pro-inflammatory cytokines. In a guinea pig model of ChA and fetal neurologic injury developed by Patrick and colleagues, intracervical inoculation of pregnant animals with \(E. coli\) at 70% gestation produced significant apoptosis in regions of the central fissure and in periventricular areas \(^{41}\). Levels of TNF-\(\alpha\) and IL-6 quantified in the amniotic fluid two to three days after maternal inoculation were significantly higher in the exposed animals when compared to controls, again involving the cytokines in the inflammatory white matter damage and suggesting an early transient response. Unpublished work by the same author, in a cytokine time-course after high-dose maternal LPS administration, showed peaks in IL-6 and IL-1\(\beta\) levels in the amniotic fluid within 48 hours of intraperitoneal injection of LPS, while TNF-\(\alpha\) levels remained virtually unchanged. If cytokines are indeed the major players in the etiology of cerebral white matter injury, it is important to characterize the time-course that they follow in an immuno-inflammatory response in order to further determine their involvement in the pathogenesis of fetal brain injury and CP and to identify possible targets that may be time limited for the future prevention of fetal WMD.
**Research Problem: Diagnosis of chorioamnionitis**

Due to the heterogeneity in the diagnostic criteria for cChA, studies that have reviewed the associations between intrauterine infection and fetal brain injury often reach conflicting conclusions. A meta-analysis published in 2000 reviewed a number of studies examining the relationship between ChA and CP\(^4\). Although the articles that were evaluated generally reported different associations, the meta-analysis found that both the clinical and subclinical forms of ChA pose a risk for adverse perinatal neurologic outcome in preterm infants\(^4\). In term infants, a positive correlation was made between cChA and CP\(^4\). According to this study, both cChA and hChA can induce a fetal inflammatory response\(^4\). Both are also significantly associated with cystic PVL\(^4\). A significant association was found between cChA and CP, however this relationship was stronger in the studies where more stringent diagnostic criteria were implemented\(^4\). Conversely, subclinical ChA was significantly associated to CP in only one of the 5 studies examined\(^4\). Few of the studies used the same diagnostic criteria for cChA and only 2 of the reports diagnosed hChA according to the same pathologic findings\(^4\).

In a large retrospective cohort study, Smulian et al. reported that 38.1% of the clinical cases of ChA had negative placental histology\(^2\). Partly responsible for this discrepancy was the fact that most of the signs and symptoms for ChA could also be brought upon by non-inflammatory causes\(^2\). In effect, a number of studies have reported that maternal fever may be produced by factors such as epidural analgesia, a number of other infections including urinary tract infections (UTI), and dehydration during labour\(^2\)-\(^4\). Epidural analgesia may also trigger maternal and fetal tachycardia to further confound
the diagnosis. In addition, antipyretics prevent rising maternal temperatures and may therefore conceal potential cases of ChA. Also responsible were the inconsistencies in the evidence used to diagnose cChA. In almost 14% of the cases studied by Smulian et al., none of the standard diagnostic criteria for cChA were present, suggesting a subjective diagnosis on behalf of the clinicians involved. Despite the use of more stringent diagnostic criteria (using the presence of maternal fever and at least two other clinical symptoms consistent with cChA), an overdiagnosis of cChA has nevertheless been described. Investigators revealed that the available clinical evidence for ChA identified true hChA with a sensitivity of only 24% and a specificity of 87%.

To assist in the diagnosis of ChA, serial maternal serum white blood cell counts (WBC) have been used in obstetric practice. Generally, a cut-off value of 16,000 or a 30% increase in white blood cells is used to predict the presence of intrauterine infection. These tests carry sensitivities ranging from 85% to 95% depending on the cut-off values used. However, the specificity of the tests remain low, from 23% to 45%. Clinicians therefore frequently make use of additional non-invasive tests such as biophysical profiles and daily non-stress testing with a fetal heart rate monitor to predict ChA. Unfortunately, the sensitivity/specificity of these tests is also poor (39.1%/84.6% and 25%/92.6%, respectively).

The above research highlights the need for standardization of the diagnostic criteria for ChA. Furthermore, it becomes clear that diagnoses of cChA must be confirmed with pathologic evidence of hChA in order to investigate the true associations.
between intrauterine infection and both PVL and CP. After all, inaccurate clinical diagnoses of ChA could lead to inappropriate pregnancy management, which could potentially increase the risk of maternal and neonatal morbidity. In preterm premature rupture of the membranes (PPROM), for example, where the risks associated with preterm delivery could outweigh those of sustained exposure to infection/inflammation in utero, an accurate, standardized diagnosis of ChA is vital for proper clinical management. Because cChA might represent a more advanced stage of infection, it might pose a greater risk for both the mother and the infant. Hence, early detection of the presence or the development of hChA would aid in improving clinical management of the patients, which would expectantly decrease the risk of maternal and neonatal infectious morbidity and perinatal brain injury.

**Objectives**

1. To determine the cytokine time course in the pregnant guinea pig in the first five days following maternal inflammation. The observation of changes in cytokine concentrations in the maternal serum and the amniotic fluid will offer insight into the maternal and fetal animals’ responses to acute inflammation. Hypotheses will be drawn from this information to describe the cytokine response in human ChA. Significant cytokine responses will provide valuable evidence for the role of cytokines in fetal brain injury.

2. To carry out a pilot study to determine whether serial serum biomarkers are useful for the diagnosis of subclinical ChA. The diagnosis of ChA is based on clinical findings; these alone have relatively poor specificity and sensitivity and only
relate to more advance clinical ChA. A protocol of bi-weekly blood sampling in pregnant inpatients will be developed for the eventual use in larger-scale biomarker studies. Ultimately, when informative biomarkers are ascertained, not only will the association between ChA and CP be better delineated, but also the obstetrician will be better informed in the case-by-case decision to deliver. The incidence of fetal cerebral WMD resulting from inflammatory injury could be significantly reduced.
CHAPTER 2: Transient lipopolysaccharide-induced cytokine responses in the maternal and fetal guinea pig

INTRODUCTION

Cerebral palsy is a heterogeneous group of motor and postural disturbances manifesting early in life 21. Prematurity and low birth weight are the most common risk factors for the development of neurologic damage in newborns, including CP 30;36;66. Also increasing the risk for the development of CP are prenatal factors such as birth asphyxia, intrapartum fever, and intrauterine infection, namely ChA 1;4;28;66.

Chorioamnionitis is an infection/inflammation of the placental membranes as well as the amniotic fluid 35. Clinical ChA is present in 0.5-10.5% of all pregnancies 1;2;14 and may increase the risk of CP in both preterm and term infants 2- to 12-fold 4;12;35-37. Evidence has shown that maternal infection and inflammation gives rise to a fetal inflammatory response, which itself may play a role in perinatal brain injury 48;67-69. What remains unclear is what duration of exposure to infection/inflammation increases the risk of adverse neonatal neurologic outcome. It is also unknown to what degree the severity of the infection/inflammation plays a role in the development of fetal brain injury, or why frequent minor maternal infections do not have the same deleterious effects on the developing fetus.

Animal studies have provided much insight into the etiology of brain injury following intrauterine infection. Harnett and colleagues recently demonstrated in
pregnant guinea pigs that there exists an endotoxin-dose fetal brain-injury response following single maternal injections of *Escherichia coli* endotoxin (lipopolysaccharide (LPS)) at 70% gestation, the peak period of fetal brain development. Low doses of LPS were not associated with demonstrable apoptotic/necrotic brain injury in the fetus, but there was a threshold LPS dose beyond which significant fetal cerebral white matter damage was observed. It is therefore presumed that low levels of inflammation resulting from most cases of ChA and minor systemic infections in pregnancy may be insufficient to cause neonatal neurologic injury. However, brain injury may arise following more robust maternal immuno-inflammatory responses to ChA or to acute systemic infections. The severity of the inflammatory response in both the mother and the fetus appears to dictate the fetal outcomes in maternal infections during pregnancy.

Because pro-inflammatory cytokines control the duration and the amplitude of the inflammatory response, they are thought to represent the link between infection and brain injury. They are generated in response to invasive bacteria and their concentrations are higher in the amniotic fluid of women with ChA, as well as in the fetal circulation and the fetal brain. Elevated levels of these cytokines in the amniotic fluid were associated with an increased risk for the infant’s development of CP. In the Harnett dose-response study the cytokine response in guinea pigs was only evaluated at seven days following the maternal endotoxin administration at which point the cytokines were not found to be different from control.
The present study aims to further investigate the time-course of this cytokine response. Using a similar pregnant guinea pig animal model, the pro-inflammatory cytokine time-course in the amniotic fluid and in the maternal serum was quantified following single-dose maternal LPS administration. It is hypothesized that peaks in cytokine concentrations, representing a significant inflammatory response, would be seen in both mother and fetus within five days of the maternal LPS injections. The presence of this response would support the involvement of the pro-inflammatory cytokines in the pathogenesis of demonstrated fetal brain injury.

**MATERIALS AND METHODS**

**Animal breeding**

Virgin female and male Dunkin-Hartley albino guinea pigs (*Cavia porcellus*) of breeding age were ordered from Harlan Sprague-Dawley Inc. (Indianapolis, IN, USA) and were housed in the Animal Care Facility at Queen’s University. The animal protocol was approved by the Queen’s University Animal Care Committee (protocol Smith-2006-005-OR). All guinea pigs were maintained under constant temperature and humidity, and a 12-hour light/dark cycle, standard to environmental requirements. Animals were housed in breeding cages each containing three nulliparous females and one male. *Ad libitum* access to commercial guinea pig diet and water was provided. Guinea pigs were handled daily and the health of each assessed by the Animal Care staff. Palpation of the fetus in the uterus at day 25 of gestation was used to confirm pregnancy. Pup weight upon laparotomy was later used to verify the accuracy of dating.
**Endotoxin injection**

Lipopolysaccharide (serotype 55:B5; Sigma Chemical Co., St. Louis, MO, USA) from *Escherichia coli* was diluted with sterile 0.9% saline to concentrations of 100µg/200µL and stored at –80°C. Experimental sows (n=27), at approximately 70% gestation (between gestational days 45 and 55), were injected intraperitoneally with a dose of 100µg LPS per kilogram of maternal body weight. This dose was chosen because it was associated with a small degree of brain injury in guinea pig pups while allowing virtually 100% maternal and fetal survival in the dose-response study by Harnett and colleagues.  

**Post-injection procedure**

Spontaneous abortions occurring post-injection were noted and excluded from the study. Maternal guinea pigs were sacrificed at time-points of 24 hours, 48 hours, or 5 days following the LPS injection. These were chosen as regular intervals between the time of injection and the seven-day time-point examined in the Harnett study. Of particular interest were the first 48 hours due to unpublished work by Patrick et al. who found transient peaks in cytokine concentrations in the amniotic fluid of guinea pig sows exposed to a dose of 500µg/kg LPS within 2 days of the intraperitoneal injection. Time-zero control sows (n=8) were not given an LPS injection but were sacrificed at 70% gestation. Euthanasia was achieved by intraperitoneal injection of 1mL Euthanyl (240mg/mL pentobarbital) from Bimeda-MTC Animal Health Inc. (Cambridge, ON, CA), followed by a 1mL intracardiac injection of Euthanyl once effective sedation was
observed. Uterine horns were exposed by midline laparotomy and pups were removed and weighed separately (Figure 2). Approximately 3mL of maternal blood was collected from the ventricles and allowed to clot. One millilitre of amniotic fluid was collected from each gestational sac. All samples were centrifuged at 3000g for 3 minutes. Amniotic fluid and serum samples were stored at -80°C until further analysis.

**Cytokine quantification**

Pro-inflammatory cytokine concentrations in each of the amniotic fluid and maternal serum samples were measured by standard enzyme-linked immunosorbent assay (ELISA). Murine cytokine detection kits for each IL-6, IL-1β, and TNF-α were obtained from R&D Systems (Minneapolis, MN, USA; see kit information in Appendix I). The sensitivities of these kits are 1.6pg/mL, 3.0pg/mL and 5.1pg/mL, respectively. Samples were assayed in triplicate. ELISA kit standards and controls were performed for each assay.

**Statistical analysis**

One-way analysis of variance (ANOVA) was performed with Tukey’s Multiple Comparison post-hoc tests to compare differences between the mean cytokine concentrations at each time-point, including the time-zero control. All analyses were performed with GraphPad Prism 4.03 software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was established at 95% confidence (p<0.05).
**Figure 2:** Uterine horns exposed by midline laparotomy. Three pups (black arrows) and their placentas (white arrows) can be seen.
RESULTS

Endotoxin injection outcome

Thirty-five maternal animals were used in this experiment; 27 received a dose of endotoxin and 8 animals served as controls. Spontaneous abortion is known to occur naturally in 3% of guinea pig pregnancies. Three spontaneous abortions (8.57% of pregnancies) were noted in this study, each within 24 hours of the endotoxin injection. In addition, 3 further litters were rejected from the analysis because their mean pup weight did not correspond to the appropriate weight for expected gestational age. Fetal resorption was found upon laparotomy in 9 separate litters (10.74% of pups). In one case, the sow was a control animal. These litters were excluded from the final data analysis which included the remaining 20 litters (Figure 3). Mean litter size was 3.44 pups per litter, which was not different from controls.
Figure 3: Outcome of the litters following single maternal endotoxin injections for each experimental time-point. Miscarriages occurring before surgery, litters dated incorrectly and those with partially resorbed fetuses found upon laparotomy were excluded from the analysis. Included in the final analysis were 20 litters.
Cytokine quantification

Many of the cytokine concentrations determined by ELISA were below the lower limit of sensitivity for the assay. As such, the value of the lower limit of sensitivity of the particular ELISA kit used was assigned to each of these samples for inclusion in the analysis. It is recognized that such an adjustment could conceal a subtle difference that may exist in the results. When the data were analysed by ANOVA, mean maternal serum IL-6 levels were found to be significantly increased over the control group (p<0.01, 95% confidence interval (CI) of difference: 2.874-20.00 pg/mL), the 48 hour group (p<0.05, 95% CI of difference: 2.056-20.82 pg/mL) and the 5-day group (p<0.05, 95% CI of difference: 2.382-19.51 pg/mL, Figure 4) following the LPS injection. Concentrations of IL-1β in the maternal serum were also significantly increased at 24 hours compared to the time-zero control (p<0.01, 95% CI of difference: 1.808-10.33 pg/mL), the 48 hour time-point (p<0.01, 95% CI of difference: 1.578-10.56 pg/mL) but not the 5-day time-point (p>0.05, 95% CI of difference: -0.9056-7.291 pg/mL, Figure 5). No significant change was seen in the TNF-α concentrations in maternal serum following the endotoxin injection, although the highest mean concentration was also found at the 24-hour time-point (18.70 pg/mL, Figure 6). According to a Bartlett’s test, the variances between the mean amniotic fluid IL-6 concentrations for each tested time-point differed significantly (p<0.0001). Because the ANOVA assumes equal variances across groups, a low Bartlett’s P value indicates that the groups are different, regardless of the conclusions from a one-way ANOVA. Nevertheless, according to the ANOVA and the subsequent Tukey Multiple Comparison Test, IL-6 amniotic fluid levels at the 48-hour time-point were significantly increased compared to the mean concentration at 24 hours (p<0.05,
95% CI of difference: 0.0009-3.416 pg/mL, Figure 4). Amniotic fluid IL-1β levels were significantly elevated at 24 hours following maternal LPS injections when compared to control (p<0.05, 95% CI of difference: 0.6901-17.49) and the 5-day time-point (p<0.05, 95% CI of difference: 0.3001-17.59, Figure 5). As in the maternal serum, no changes were seen in the amniotic fluid TNF-α concentrations, yet the highest mean level was again seen at 24 hours (8.034 pg/mL, Figure 6).
Figure 4: IL-6 concentrations in the maternal serum and amniotic fluid with respect to time from maternal endotoxin exposure. Data are presented as mean with standard error of the mean (SEM). The asterisk indicates that maternal serum IL-6 levels at 24 hours are significantly different from control (p<0.01).
Figure 5: IL-1β concentrations in the maternal serum and amniotic fluid with respect to time from maternal endotoxin exposure. Data are presented as mean with standard error of the mean (SEM). The asterisks indicate that both the amniotic fluid and maternal serum IL-1β levels at 24 hours are significantly different from control (P<0.01).
Figure 6: TNF-α levels in the maternal serum and amniotic fluid with respect to time from maternal endotoxin exposure. No significant differences were seen (P>0.05) between the TNF-α levels at each time-point. Data are presented as mean with standard error of the mean (SEM).
COMMENT

Maternal immune responses in the presence of acute infection and inflammation may contribute to damage of the developing fetal brain. Notably, cytokine production following ChA has been implicated in the etiology of neonatal cerebral white matter injury. This study aimed to elucidate the cytokine time-course response following a maternal inflammatory insult. In the first 24 hours following the maternal LPS injection, IL-6 and IL-1β concentrations in the maternal serum were significantly elevated. These concentrations returned to control levels within 5 days. In the amniotic fluid, much the same was observed; IL-6 levels peaked at 48 hours and IL-1β concentrations peaked at 24 hours after maternal endotoxin exposure. TNF-α levels did not reach significance but followed the same observed trend. Similar, more robust, cytokine responses were also found in unpublished work by Patrick et al. following maternal administration of a 500µg/kg dose of LPS. Peaks in IL-6 and IL-1β concentrations occurred in the amniotic fluid of guinea pig sows within 48 hours of the intraperitoneal injection, while TNF-α levels remained unchanged. The transient cytokine responses observed in these studies, in both the maternal serum and the amniotic fluid, provide evidence of acute maternal and fetal inflammatory responses that may account for the brain injury that was subsequently demonstrated; even at a time when the cytokine concentrations had essentially returned to normal. Maternal and fetal cytokine responses appear to be rapid and transient, occurring within the first 48 hours of the maternal inflammatory insult likely paralleling the elimination of LPS from the maternal system. Although a marked cytokine response for TNF-α was not reported, this cytokine cannot be excluded in the pathogenesis of neonatal cerebral white matter damage. It is
presumed that TNF-α is one of the first cytokines to appear in a pro-inflammatory cascade \(^6;44\) and it is therefore plausible that the TNF-α concentrations peaked and dissipated in the maternal serum and the amniotic fluid between the first sampled time-points. In the work by Patrick et al., cytokines were quantified at intervals of 4 hours until the 48-hour time-point and suggested that a TNF-α response may occur within the first 24 hours of a maternal inflammatory insult. Clinical research conducted by Shalak et al. revealed elevated cord blood levels of IL-6 at 6 hours after delivery in infants exposed to ChA, followed by a decline in these levels at 30 hours \(^37\). There is evidence that increased levels of cytokines in the amniotic fluid and in the fetal circulation are resolved rapidly in the days following an early maternal inflammatory response and may therefore not be seen upon delivery \(^77\). Cytotoxic cytokines undeniably play a role in the etiology of CP following ChA, yet the exact mechanism by which this occurs remains unclear \(^26\). The processes responsible for the fetal inflammatory response and resulting brain injury appear to begin in utero and may continue following delivery \(^77\). Because cytokine concentrations rapidly peak and resolve, they are unlikely to be directly causative of fetal brain injury, but may initiate a series of cascades including cytokine induction in the fetal brain, which are responsible for the demonstrable brain injury.

Cytokines, whether of maternal or fetal origin \(^50;56\), increase the permeability of, and are able to cross, the immature fetal blood brain barrier \(^18;50\). They are also produced by astrocytes and microglia in the fetal brain when stimulated by inflammation \(^18;24;50\). Through both of these pathways, the cytokines are thought to gain access to the fetal cerebral white matter where they may mediate both direct and indirect injury, leading to
systemic hypotension, causing blood vessel obstruction and ultimately inducing ischemic damage to the fetal cerebral white matter\textsuperscript{16,50}. Previously, Patrick et al. demonstrated that intracervical inoculation with \textit{Escherichia coli} results in an ascending infection and produces ChA in maternal guinea pigs\textsuperscript{41}. In these experiments it was shown that the maternal and fetal inflammation was also associated with fetal brain injury regardless of the presence of bacteria in the amniotic fluid\textsuperscript{41}. In a study by Cai et al. in 2000, maternal intraperitoneal endotoxin injections induced an elevated expression of IL-1\textbeta and TNF-\textalpha mRNA in the fetal rat brains\textsuperscript{24}. In 2003, Pang et al. showed that direct introduction of LPS into neonatal rat brains resulted in damage to developing oligodendrocytes, hypomyelination and white matter injury\textsuperscript{78}. Local inflammation and the resulting induction of pro-inflammatory cytokines were presumed to mediate this response\textsuperscript{78}. Together, these animal studies provide further evidence for a link between maternal/fetal inflammation and a cytokine-mediated cascade which harms the developing fetal brain.

While it is understood that this guinea pig model of inflammation may not reflect the precise pathology of human ChA, it allows insight into the complex cytokine responses that are seen in true infection. The animal model represents acute inflammation, following a single injection of LPS, whereas in true human ChA the infection is progressive\textsuperscript{10,38}. In this study a transient cytokine response was seen and was rapidly resolved, once LPS was likely cleared from the system. In humans, this response may be drawn out due to the prolonged exposure to the causative micro-organisms. Cytokine levels, although possibly lower than concentrations seen in acute inflammation, could also remain elevated for longer periods of time before being cleared. Thus chronic
infection/inflammation may therefore represent a situation of higher risk for negative outcome due to a constant immuno-inflammatory assault on the maternal and fetal systems, including a continuous activation of the cytokine-mediated cascade.

The current study suggests that the processes responsible for neonatal cerebral white matter damage are initiated rapidly following a maternal infectious or inflammatory insult of adequate severity *in utero* and may progress until, or even continue after, delivery. Because clinically evident ChA poses a greater risk for the development of fetal brain injury than do the subclinical stages of ChA⁴⁻⁵, biomarkers for subclinical ChA could better predict infants at risk and allow obstetricians to better manage each pregnancy. Investigation into more appropriate and sensitive/specific markers of the early stages of ChA remains a priority in this field of research. Given the sensitivity of single samples, serial biomarker assessments may be more valuable in the diagnosis of subclinical infection.
CHAPTER 3: Pilot study: the use of serial serum biomarkers in the detection of subclinical chorioamnionitis

INTRODUCTION

Chorioamnionitis increases the risk for abnormal fetal cerebral white matter development. A meta-analysis by Wu and Colford published in 2000 including 26 studies on the relationships between ChA and fetal brain injury revealed that significant associations to fetal white matter damage could be drawn from cChA in both term and preterm infants. Interestingly, few of these studies defined ChA using the same clinical criteria and six of these articles provided no specific clinical criteria at all. Likely due to the insufficient specificity and sensitivity of clinical criteria in the diagnosis of underlying intrauterine inflammation, other studies report that only hChA, the retrospective microscopic evaluation of polymorphonuclear leukocyte invasion into the amnion and chorion, is a significant predictor of adverse fetal neurologic outcomes including PVL and CP. In work by De Felice and colleagues in 2001, cChA could not be associated to abnormal brain sonography including echodensities, echolucencies and interventricular hemorrhage (IVH), perhaps because some of the clinical cases of intrauterine inflammation were not supported by leukocyte infiltration into the placental tissues. Other studies reached opposite conclusions; that cChA was significantly related to abnormal brain development in the preterm population and in the term or near-term population. A systematic review conducted in 2002 revealed updated information for many of the previously examined studies and reiterated a significant association between cChA and both PVL and CP. This relationship was especially strong in those studies with the most rigorous diagnoses. The discrepancies between reports highlight a need
for more stringent clinical diagnostic criteria for ChA, and for all cases of ChA to be corroborated with histological evidence of inflammation. Only then can researchers properly determine the impact of intrauterine infection and inflammation on neurodevelopment and take the necessary steps towards improving obstetric and neonatal care.

The value of biomarkers of infection and inflammation has been extensively investigated for the prediction of ChA. Amniotic fluid glucose\textsuperscript{65}, WBC\textsuperscript{65,80}, IL-6\textsuperscript{65,81} and matrix metalloproteinase-9 (MMP-9)\textsuperscript{81,82} have all been proposed as markers for the diagnosis of intrauterine infection, among a score of others. White blood counts are part of the standard of care in most hospitals for obstetric inpatients, specifically for those at high risk for ChA as leukocytosis represents an immune response to infectious stimuli. Amniotic fluid WBC greater than 30/mm\textsuperscript{3} has a sensitivity of 57\% and a specificity of 78\% in the detection of positive amniotic fluid culture\textsuperscript{65}. Studies assessing maternal blood serum WBC, however, report dissimilar sensitivities and specificities\textsuperscript{80,83} (Table 2). The increased production of pro-inflammatory cytokines such as IL-6 and TNF-alpha, by activated polymorphonuclear leukocytes and monocytes infiltrating the decidua and the membranes, also indicates the initiation and propagation of an infectious and inflammatory cascade\textsuperscript{83}. One study reports that IL-6 over 114.ng/mL in a single amniotic fluid sample can predict the presence of intra-amniotic infection with a sensitivity of 73\% and a sensitivity of 79\%\textsuperscript{81}. As a mediator of extracellular matrix remodelling, MMP-9 acts as a zymogen to degrade matrix proteins and can be regulated by inflammation. MMP-9 has been implicated in the mechanism of preterm prelabour membrane rupture\textsuperscript{84}. 
Levels of the enzyme are elevated in the amniotic fluid in ChA and are reported to diagnose intra-amniotic infection with a sensitivity of 83% and a specificity of 95% \(^{82}\).

The main drawback for the majority of these studies is that the proposed biomarkers have been examined in the amniotic fluid. Therefore, to obtain the sample for analysis women must undergo ultrasound-guided transabdominal amniocentesis, an invasive procedure that carries potential risks to both maternal and fetal health \(^{85}\). The procedure is not readily available and even in tertiary centres, may not be available 24 hours a day and seven days a week \(^{86}\). Many of the proposed tests in amniotic fluid report low sensitivity and specificity in the detection of ChA (Table 2). However, the most practical clinical tests for the prediction of the development of ChA would require less invasive, less expensive and ultimately safer procedures. As such, maternal serum samples have been suggested as a more reliable and cost-effective choice in the assessment of diagnostic biomarkers for hChA.

Fewer single-sample serum tests have been investigated than those requiring amniocentesis. Among these are serum WBC \(^{80,83}\), C-reactive protein (CRP) \(^{8,80,83}\) and intercellular adhesion molecule-1 (ICAM-1) \(^{83,87}\). These tests still report poor sensitivities and specificities in the prediction of ChA, perhaps because of the difficulties in differentiating the serum biomarkers specific to hChA as opposed to those reflecting more generalized systemic infection or inflammation (Table 2). A few studies report the use of serum CRP in identifying those with ChA. CRP is an acute-phase protein synthesized by hepatocytes in response to inflammation \(^{88}\). In intrauterine infection, the
inflammatory reaction begins in the amniotic fluid and then proceeds into the maternal
circulation where the cytokines produced can stimulate CRP synthesis in the liver \(^{88}\).
Thus, elevated CRP in both the amniotic fluid and in the maternal circulation may
provide evidence of infection and inflammation. However, CRP is normally elevated in
pregnancy. CRP in umbilical cord blood has been assessed to determine the risk of
funisitis, with a sensitivity and specificity of 62% and 83%, respectively for a cut-off of
CRP value of 200ng/mL \(^{89}\). A range of sensitivities and specificities is reported when
serum CRP is used to predict hChA \(^{8;80;83}\), but these studies do suggest that the biomarker
has potential clinical value (Table 2). A study by Greig and colleagues in 1997, for the
detection of asymptomatic ChA, reported that women in preterm labour with ChA had
significantly elevated IL-6 serum concentrations compared to those without intrauterine
infection \(^{90}\). In one report, serum from women in preterm delivery showed significantly
elevated levels of ICAM-1 if they also had evidence of hChA when compared to those
without inflammation \(^{83}\). As such, sICAM-1 levels greater than 106 ng/mL proved to be
better predictors of hChA than CRP over 1.1 mg/dL or WBC greater than 11.8 \(x\) \(10^3\)/µL,
both with levels significantly elevated in subclinical hChA \(^{83}\). With a sensitivity of 98%
and a specificity of 93.8% \(^{83}\), serum sICAM-1 shows the most promise as an accurate
indicator of hChA. As an important mediator in the mechanisms of cell lysis by immune
cells, ICAM-1 plays a role in the modulation of inflammatory reactions and its expression
is upregulated by cytokines including IL-6 and TNF-alpha when inflammation is present
\(^{87}\). ICAM-1 is thought to be produced by cells of the amniotic fluid in response to acute
inflammation of the fetal membranes in a local response to infection. Elevated serum
ICAM-1 thus reflects the maternal systemic response to the intrauterine infection \(^{87}\).
Chorioamnionitis has been shown to reduce human placental endocrine function. Stimulation with LPS caused decreased human chorionic gonadotropin (hCG) protein levels in placental explants. This suppression may be paralleled in the maternal serum during intra-uterine infection. As a readily available and rapid screening test commonly used in hospitals, hCG has great potential as a biomarker for ChA. Insulin-like growth factor binding protein-1 (IGFBP-1) has been previously involved in the diagnosis of intrauterine infection. Proteomic profiles developed by Gravett et al. showed elevated expression of amniotic fluid IGFBP-1 in a primate model of ChA. This study also implicated calgranulin B and a small proteolytic fragment of the IGFBP-1 protein as preferentially expressed in intrauterine infection. In pooled human maternal serum, these proteins were again preferentially expressed in the infected cohort. Placental growth factor (P/GF) and endoglin (Eng) have both been recently investigated in pre-eclampsia, but have not yet been implicated in ChA. P/GF, an angiogenic protein, is necessary for the maintenance of endothelial health during pregnancy. In pre-eclampsia, higher P/GF levels lower a woman’s risk for adverse perinatal outcome. In ChA however, local inflammation may cause endothelial damage and blood vessel constriction via cytokine actions, which may therefore require elevated P/GF expression to maintain adequate vascular function. Soluble Eng, elevated in the serum of pre-eclamptic women, modulates angiogenesis, cell growth, cell differentiation and apoptosis through transforming growth factors (TGF) including TGF-β1 and TGF-β3. It appears to play a major role in vascular homeostasis. Therefore, in pre-eclampsia, elevated Eng may increase vascular permeability. In ChA, Eng may become elevated to counterbalance the blood vessel constriction induced by inflammation.
More research is required to evaluate the efficacy of such tests in a broad range of patients in order to generate comparable, reproducible results between studies. Considering the potential of single maternal serum sample for the prediction of hChA, serial biomarker assessments could prove to be even more informative. Because asymptomatic ChA represents less advanced stage of infection, diagnosing histological, rather than cChA will provide the physician with an earlier, more educated, approach in the decision to deliver. This pilot study will develop a protocol to retrospectively evaluate the use of the biomarkers described above in serial serum samples for the detection of hChA. When sensitive and specific serial biomarkers are discovered, their use in the rapid detection of subclinical ChA could contribute to a reduction in the incidence of fetal cerebral WMD resulting from inflammatory injury.
Table 2: Diagnostic value of single samples of biomarkers in the amniotic fluid (AF) and maternal serum for the detection of intrauterine infection. sICAM-1 stands out as the most promising biomarker for serial serum samples. ‘N’ refers to the sample size used in each study. * Refers to a meta-analysis of 6 studies.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Biomarker</th>
<th>Study</th>
<th>N</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF</td>
<td>WBC</td>
<td>Yoon et al., 1996</td>
<td>90</td>
<td>77</td>
<td>96</td>
<td>96</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>Harirah et al., 2002</td>
<td>84</td>
<td>73</td>
<td>79</td>
<td>61</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>MMP-9</td>
<td>Harirah et al., 2002</td>
<td>84</td>
<td>77</td>
<td>100</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Locksmith et al., 1999</td>
<td>44</td>
<td>83</td>
<td>95</td>
<td>71</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>WBC</td>
<td>90</td>
<td>40</td>
<td>82</td>
<td>74</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yoon et al., 1996</td>
<td>90</td>
<td>63.3</td>
<td>61.2</td>
<td>62</td>
<td>62.5</td>
</tr>
<tr>
<td></td>
<td>CRP</td>
<td>Yoon et al., 1996</td>
<td>90</td>
<td>54</td>
<td>86</td>
<td>83</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Steinborn et al., 2000</td>
<td>97</td>
<td>75.5</td>
<td>71.4</td>
<td>72.6</td>
<td>74.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wiwanitkit et al., 2005</td>
<td>466*</td>
<td>72.8</td>
<td>76.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>sICAM-1</td>
<td>Steinborn et al., 2000</td>
<td>97</td>
<td>98</td>
<td>93.8</td>
<td>97.8</td>
<td>94.1</td>
</tr>
</tbody>
</table>
MATERIALS AND METHODS

Subject recruitment

Approval for the study was obtained from the Queen’s University and Kingston General Hospital (KGH) Research Ethics Boards in September 2005. Subjects were recruited at KGH in Kingston, Ontario, Canada between November 2005 and August 2007. Written informed consent was obtained from each participant (Appendix II and III). Medical charts were reviewed to ensure that protocols were being adhered to consistently. Maternal age of all subjects and their relevant medical histories including parity, previous complications of pregnancy and gestational age were recorded.

Case recruitment

This study included n=16 pregnant women diagnosed with preterm premature rupture of membranes (PPROM) between 24 and 34 weeks of pregnancy. Confirmation of PPROM was documented clinically by positive nitrazine and ferning tests. Exclusion criteria for all subjects and controls consisted of multiple gestations, fetal anomalies or any additional severe complication of pregnancy. Subjects with PPROM were managed conservatively according to routine practice at KGH: administration of antenatal corticosteroids and broad spectrum antibiotics, daily fetal non-stress testing (NST), maternal temperature and clinical examinations (including assessments for evidence of cChA), twice-weekly biophysical profile (BPP) and white blood counts (WBC). Clinical ChA was defined as a combination of two or more of the following symptoms: maternal fever above 38°C, uterine tenderness, purulent amniotic fluid, maternal or fetal tachycardia and maternal leukocytosis. Bi-weekly analyses for WBC counts, human
chorionic gonadotropin (hCG) and c-reactive protein (CRP) were carried out in the core lab of KGH. Maternal blood samples were collected bi-weekly at the same time as routine blood-work, from the time of admission until induction or onset of labour. Blood was collected in two 5mL serum separator tubes (SSTs) and sent to the core lab of the hospital where it was processed. Samples were centrifuged and the serum was stored in approximately 1mL aliquots at -80°C. Freeze-thaw cycles were avoided before the analysis of the samples. Upon delivery, placentas from all subjects were sent to the pathology department of KGH for the microscopic determination of ChA. Histological ChA was defined as the invasion of neutrophils in the subchorionic space and associated chorion.

Controls

An additional n=6 control women without PPROM were recruited as gestational-age matches (±1 week) through the obstetric clinics at KGH. At each clinic visit, blood was collected for WBC counts, hCG, and CRP analyses. Serum from two SSTs was also processed, aliquoted and frozen for further analysis, according to the same protocol as described for the case subjects. Following delivery, placentas from the controls were also sent to the pathology department of KGH for the microscopic determination of ChA.

Serum analysis

Samples were coded before removal from hospital premises to ensure confidentiality of the subjects. All frozen serum samples collected from cases and controls were assayed for sICAM-1, IL-6, TNF-α, MMP-9, IGFBP-1, P/GF, and Eng.
Excluded from analyses were all samples collected within 24 hours of corticosteroid administration, and within 24 hours of induction or onset of labour. All biomarkers were quantified using commercially-available enzyme-linked immunosorbent sandwich assays (ELISA; R&D Systems, Minneapolis, MN, USA; Diagnostics Biochem Canada Inc., London, ON, CA, see Appendix I). Samples were diluted according to the assay specifications of each kit with the provided diluents solutions. Each sample was analysed in triplicate.

**Statistical Analysis**

One-way analysis of variance (ANOVA) was performed with Tukey’s Multiple Comparison post-hoc tests to compare differences in the serum biomarker concentrations between the two infected groups (clinical and subclinical) and the two control groups (ruptured cases and non-ruptured controls). All analyses were performed with GraphPad Prism 3.00 software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was established at 95% confidence (p<0.05).
RESULTS

Subject outcome

Twenty-two pregnant women were enrolled in this study: 16 with PPROM and 6 without. Of the 16 women with ruptured membranes, 7 developed subclinical ChA (diagnosed by pathologic examination of the placenta after delivery), 3 women were diagnosed with cChA based on fever and leukocytosis and 6 women showed no signs of infection at all. The overall incidence of hChA in the subjects with PPROM was 62.5% (10 of 16), similar to values reported by a large-scale study on PPROM published in 2005. Of those 9 women who ruptured prior to 28 weeks, the incidence of hChA was 66.7%. After 30 weeks, the incidence dropped to 25%. An average of 3 (range 1-12) serum samples were collected from the 22 pregnant study subjects. No significant differences were found in maternal age and parity among the four different study groups (Table 3). There was no significant difference observed in the duration of rupture of those women with PPROM, regardless of infection or inflammation. As expected, there was a difference in the gestational age at delivery of the non-ruptured controls who delivered significantly later as opposed women with PPROM who all delivered prior to, or at 34 weeks (Table 3).
Table 3: Characteristics of recruited subjects according to membrane rupture status and infection status. PPROM = preterm premature rupture of the membranes. The asterisk * indicates a significant difference over the three other study groups, p<0.001.

<table>
<thead>
<tr>
<th>Characteristics: mean (range)</th>
<th>PPROM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Chorioamnionitis (n = 6)</td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>26 (19-33)</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)</td>
<td>31 (27-34)</td>
</tr>
<tr>
<td>Duration of PPROM (days)</td>
<td>18 (3-44)</td>
</tr>
<tr>
<td>Parity</td>
<td>0.83 (0-2)</td>
</tr>
</tbody>
</table>
Serum analysis

Biomarker concentrations from subjects with 2 or more available samples were graphed according to the gestational age at the time of serum sampling (Figures 7-15). High variability was observed in the biomarker concentrations, from subject-to-subject as well as in the biomarker profile of a single subject over time. When the concentrations were grouped according to infection and rupture status, regardless of gestational age, the mean levels could be compared between study groups (Figures 16-24). The mean hCG concentration in the non-rupture control group was 19504 IU/L, similar to the reported control levels in 3rd trimester pregnancies of normotensive women with a median concentration below 20000 \(^9\). Levels of hCG in ChA had the highest mean at 22549 IU/L but were not significantly different from the non-rupture controls, nor the ruptured non-infected group. The average CRP concentration in the non-rupture control group was 7.1 mg/L, comparable to previously established control levels (3-7 mg/L) \(^80,83,89\). Levels of CRP in ChA had the highest mean at 15.77 mg/L and were significantly elevated over the ruptured non-infected group (p<0.05), but were not significantly different from the non-rupture controls. The mean sICAM-1 concentration in the non-rupture control group was 272.8 ng/mL, higher than the previously reported control levels in 3rd trimester pregnancies of 70-188 ng/mL, in 3 separate studies \(^83,87,100\). Again, the ChA group reported a higher mean of 321.2 ng/mL, although this finding was non-significant. The mean IL-6 concentration in the non-rupture control group was 2.059 pg/mL. Other studies have reported dissimilar control levels in 3rd trimester pregnancies ranging from 2 pg/mL to upwards of 10 pg/mL \(^51,90,101\). Women who developed ChA in this study had an average serum IL-6 concentration of 2.495 pg/mL. Non-ruptured controls possessed a
mean TNF-α concentration of 2.070 pg/mL, the highest average concentration between the 3 study groups. These values are much lower than control averages reported as high as 50.5 pg/mL one study. Average MMP-9 for the non-ruptured control group was 1601 ng/mL, the highest average concentration among the study groups. There were no previously reported figures to compare this value against as studies have only examined MMP-9 levels in amniotic fluid and fetal plasma. The mean IGFBP-1 concentration in the non-rupture control group was 72.41 µg/L. The ChA group presented the lowest average at 52.23 µg/L. Second trimester control IGFBP-1 levels have been reported as ranging from 70-200 µg/L, third trimester levels can reach 400 with a mean of 249.3 µg/L. Mean PI GF for the non-ruptured controls was higher than both other groups at 729.3 pg/mL, although no appropriate comparisons could be drawn from previous studies. Normal soluble Eng concentrations in serum are 20 ng/mL or less. In our study, the non-ruptured control average concentrations was 6.995 ng/mL, and the group of women with ChA had an average concentration of 22.26 ng/mL, over-represented due to two samples with Eng concentrations over 100 ng/mL. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for each serum biomarker, with selected cut-off values set at the 85th and 95th percentile of all samples collected. Concentrations exceeding those values represented a positive diagnosis of ChA (Table 4). Because IGFBP-1 appeared to follow an inverse trend, the cut-off values were set at the 15th and 5th percentile, and concentrations below those values represented a positive diagnosis of ChA (Table 4).
Figure 7: Human chorionic gonadotropin concentrations in subjects with 2 or more samples collected before onset or induction of delivery, graphed according to gestational age at the time of sample collection. Red lines indicate levels in subjects with PPROM who developed clinical chorioamnionitis, yellow lines indicate levels in subjects with PPROM who developed subclinical chorioamnionitis, green lines represent levels from subjects with PPROM who did not develop infection, and purple lines represent levels from the non-ruptured controls.
Figure 8: C-reactive protein concentrations in subjects with 2 or more samples collected before onset or induction of delivery, graphed according to gestational age at the time of sample collection. Red lines indicate levels in subjects with PPROM who developed clinical chorioamnionitis, yellow lines indicate levels in subjects with PPROM who developed subclinical chorioamnionitis, green lines represent levels from subjects with PPROM who did not develop infection, and purple lines represent levels from the non-ruptured controls.
Figure 9: Serum intracellular adhesion molecule-1 concentrations in subjects with 2 or more samples collected before onset or induction of delivery, graphed according to gestational age at the time of sample collection. Red lines indicate levels in subjects with PPROM who developed clinical chorioamnionitis, yellow lines indicate levels in subjects with PPROM who developed subclinical chorioamnionitis, green lines represent levels from subjects with PPROM who did not develop infection, and purple lines represent levels from the non-ruptured controls.
Figure 10: Interleukin-6 concentrations in subjects with 2 or more samples collected before onset or induction of delivery, graphed according to gestational age at the time of sample collection. Red lines indicate levels in subjects with PPROM who developed clinical chorioamnionitis, yellow lines indicate levels in subjects with PPROM who developed subclinical chorioamnionitis, green lines represent levels from subjects with PPROM who did not develop infection, and purple lines represent levels from the non-ruptured controls.
**Figure 11:** Tumour necrosis factor-α concentrations in subjects with 2 or more samples collected before onset or induction of delivery, graphed according to gestational age at the time of sample collection. Red lines indicate levels in subjects with PPROM who developed clinical chorioamnionitis, yellow lines indicate levels in subjects with PPROM who developed subclinical chorioamnionitis, green lines represent levels from subjects with PPROM who did not develop infection, and purple lines represent levels from the non-ruptured controls.
**Figure 12:** Matrix metalloproteinase-9 concentrations in subjects with 2 or more samples collected before onset or induction of delivery, graphed according to gestational age at the time of sample collection. Red lines indicate levels in subjects with PPROM who developed clinical chorioamnionitis, yellow lines indicate levels in subjects with PPROM who developed subclinical chorioamnionitis, green lines represent levels from subjects with PPROM who did not develop infection, and purple lines represent levels from the non-ruptured controls.
Figure 13: Insulin-like growth factor binding protein-1 concentrations in subjects with 2 or more samples collected before onset or induction of delivery, graphed according to gestational age at the time of sample collection. Red lines indicate levels in subjects with PPROM who developed clinical chorioamnionitis, yellow lines indicate levels in subjects with PPROM who developed subclinical chorioamnionitis, green lines represent levels from subjects with PPROM who did not develop infection, and purple lines represent levels from the non-ruptured controls.
Figure 14: Placental growth factor concentrations in subjects with 2 or more samples collected before onset or induction of delivery, graphed according to gestational age at the time of sample collection. Red lines indicate levels in subjects with PPROM who developed clinical chorioamnionitis, yellow lines indicate levels in subjects with PPROM who developed subclinical chorioamnionitis, green lines represent levels from subjects with PPROM who did not develop infection, and purple lines represent levels from the non-ruptured controls.
Figure 15: Endoglin concentrations in subjects with 2 or more samples collected before onset or induction of delivery, graphed according to gestational age at the time of sample collection. Red lines indicate levels in subjects with PPROM who developed clinical chorioamnionitis, yellow lines indicate levels in subjects with PPROM who developed subclinical chorioamnionitis, green lines represent levels from subjects with PPROM who did not develop infection, and purple lines represent levels from the non-ruptured controls.
Figure 16: Scatter plot of human chorionic gonadotropin concentrations from all maternal serum samples collected.
Figure 17: Scatter plot of c-reactive protein concentrations from all maternal serum samples collected.
Figure 18: Scatter plot of intracellular adhesion molecule-1 concentrations from all maternal serum samples collected.
Figure 19: Scatter plot of interleukin-6 concentrations from all maternal serum samples collected.
Figure 20: Scatter plot of tumour necrosis factor-α concentrations from all maternal serum samples collected.
Figure 21: Scatter plot of matrix metalloproteinase-9 concentrations from all maternal serum samples collected.
Figure 22: Scatter plot of insulin-like growth factor binding protein-1 concentrations from all maternal serum samples collected.
Figure 23: Scatter plot of placental growth factor concentrations from all maternal serum samples collected.
Figure 24: Scatter plot of endoglin concentrations from all maternal serum samples collected.
Table 4: Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of serum biomarkers for the diagnosis of chorioamnionitis. Concentrations above the cut-off values indicate a positive diagnosis of chorioamnionitis. *Due to an observed inverse trend for IGFBP-1, values below the established cut-offs indicate a positive diagnosis of chorioamnionitis.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th># samples</th>
<th>Cut-off Value</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
<th>PPV %</th>
<th>NPV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCG</td>
<td>56</td>
<td>95th percentile: 45129 IU/L</td>
<td>15.79</td>
<td>100.00</td>
<td>100.00</td>
<td>69.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>85th percentile: 36438 IU/L</td>
<td>21.05</td>
<td>86.49</td>
<td>44.44</td>
<td>68.09</td>
</tr>
<tr>
<td>CRP</td>
<td>52</td>
<td>95th percentile: 37.5 mg/L</td>
<td>10.00</td>
<td>96.88</td>
<td>66.67</td>
<td>63.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>85th percentile: 16.7 mg/L</td>
<td>35.00</td>
<td>96.88</td>
<td>87.50</td>
<td>70.45</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>59</td>
<td>95th percentile: 481.65 ng/mL</td>
<td>5.26</td>
<td>95.00</td>
<td>33.33</td>
<td>67.86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>85th percentile: 395.86 ng/mL</td>
<td>21.05</td>
<td>87.50</td>
<td>44.44</td>
<td>70.00</td>
</tr>
<tr>
<td>IL-6</td>
<td>58</td>
<td>95th percentile: 4.2062 pg/mL</td>
<td>11.11</td>
<td>100.00</td>
<td>100.00</td>
<td>71.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>85th percentile: 3.1590 pg/mL</td>
<td>22.22</td>
<td>87.50</td>
<td>44.44</td>
<td>71.43</td>
</tr>
<tr>
<td>TNF-α</td>
<td>57</td>
<td>95th percentile: 3.7204 pg/mL</td>
<td>5.56</td>
<td>94.87</td>
<td>33.33</td>
<td>68.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>85th percentile: 2.3370 pg/mL</td>
<td>27.78</td>
<td>89.74</td>
<td>56.56</td>
<td>72.92</td>
</tr>
<tr>
<td>MMP-9</td>
<td>58</td>
<td>95th percentile: 2100 ng/mL</td>
<td>5.26</td>
<td>92.31</td>
<td>25.00</td>
<td>66.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>85th percentile: 1981.67 ng/mL</td>
<td>21.05</td>
<td>87.18</td>
<td>44.44</td>
<td>69.39</td>
</tr>
<tr>
<td>IGFBP-1*</td>
<td>57</td>
<td>5th percentile: 23.966 µg/L</td>
<td>15.79</td>
<td>100.00</td>
<td>100.00</td>
<td>70.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15th percentile: 34.946 µg/mL</td>
<td>21.05</td>
<td>86.86</td>
<td>44.44</td>
<td>68.09</td>
</tr>
<tr>
<td>PIGF</td>
<td>45</td>
<td>95th percentile: 1050 pg/mL</td>
<td>10.53</td>
<td>80.77</td>
<td>28.57</td>
<td>55.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>85th percentile: 996.678 pg/mL</td>
<td>10.53</td>
<td>80.77</td>
<td>28.57</td>
<td>55.26</td>
</tr>
<tr>
<td>Eng</td>
<td>47</td>
<td>95th percentile: 16.2665 ng/mL</td>
<td>18.18</td>
<td>97.22</td>
<td>66.67</td>
<td>79.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>85th percentile: 10.1756 ng/mL</td>
<td>18.18</td>
<td>86.11</td>
<td>28.57</td>
<td>77.50</td>
</tr>
</tbody>
</table>
COMMENT

Because of extensive changes in biomarker levels over the natural course of pregnancy and due to natural variation in levels from one woman to the next, the clinical study lacked predictive power. A study such as this would require a large sample size before any significant conclusions can be drawn. A larger sample size (n value) leads to increased precision in analyses of various properties of the population. A larger sample size will better account for normal variability and prevent any bias arising from over-representation of the more extreme samples in the groups, therefore reducing type I error (false positives) and type II error (false negatives) in the results. Essentially, a larger n value increases the chances of a biomarker test reaching optimal sensitivity and specificity for its use in a clinical setting.

Nevertheless, in a pilot study, it is important to develop a working protocol where issues that undoubtedly occur can be dealt with on a smaller scale before it could be applied to larger-scale studies. Examples of issues that arose in the early stages of the pilot study included problems with the coordination of research blood collection with routine blood sampling, missed sample collection, and insufficient blood volume drawn at sampling. It also is understood that our criteria for selection of the PPROM cases may have eliminated those women at highest and lowest risk for ChA. By only recruiting those women before 34 weeks of gestational age improved chances of obtaining more than one serum sample before induction or onset of labour. Yet, these women who rupture after 34 weeks are at the lowest risk for ChA and therefore the true pregnant population is not completely represented. By excluding those women rupturing earlier
than 24 weeks, who might actually rupture due to infection itself, we are eliminating the women at highest risk for ChA. Recognizing this bias is necessary to the proper interpretation of any conclusions that will be drawn from studies adopting a similar protocol, and only improves the scientific credibility of an investigation.

It is understood that the results of this study do not prove the value of one biomarker over another in the prediction of asymptomatic ChA. What can be inferred from the data is that certain biomarkers exhibit patterns that suggest they might be worthy of further investigation. Useful biomarkers may be gestational age dependent, with different clinical value at different points in pregnancy. In examining the concentrations of CRP in relation to gestational age for example, it is possible that elevated CRP values, between 26 and 28 weeks especially, could be indicative of the development of infection. Whereas an increase in CRP levels could indicate the presence of an inflammatory response, preliminary data suggest that perhaps a decline in IGFBP-1 is most informative for predicting ChA. Still, at this point in time, none of the biomarkers examined can be excluded from future research into the development of ChA due to the lack of power of the study.
CHAPTER 4: General Discussion and Future Directions

Results obtained from the cytokine time-course show the transient nature of the pro-inflammatory cytokines. Often missed in single-sample tests, when quantified over time cytokines become much more informative and a pattern of their actions can be determined. Even before the threshold of inflammation is reached, when the severity of maternal infection becomes significant for the production of fetal brain injury, cytokines levels are increasing as they respond to the maternal inflammatory insult. Cytokines are responsible for fighting off the microorganisms causing infection and inflammation. Unfortunately, this maternal protection can itself cause harm to the fetal brain tissue. It is therefore likely that both the duration of this cytokine response as well as its intensity determines whether the fetus will be involved. Although studies have not yet been performed to examine the relationship between the duration of inflammation, results from a guinea pig LPS exposure model has shown that the severity of the inflammatory response and the resulting fetal brain injury is dose-dependent \(^\text{70}\). Perhaps a constant and slow propagation of the inflammatory response, triggered by chronic infection or prolonged pathogen presence can trigger a cytokine cascade to eventually lead to fetal involvement and cerebral WMD. It is also likely that the most severe acute infections and the most robust responses to infection represent an alternative method of fetal brain injury.

Not all pregnant women develop ChA, nor do all women with ChA have children with neurologic impairment. Perhaps certain innate features within the cytokine response of both mother and child predispose them to, or protect them from, such harm.
Gestational age at exposure definitely affects the risk for fetal brain injury, with the greatest risk existing in early gestation when a fetus’ blood brain barrier is underdeveloped\(^\text{30}\). Another possible answer to this quandary lies in one’s genetic makeup. A study by Nguyen and colleagues reported ethnic variations in polymorphisms in the genes of the innate immune system and those encoding the pro-inflammatory cytokines\(^\text{105}\). For example, the presence of an IL1RN*2 allele is associated with a greater degree of inflammation in the immune response as opposed to the IL1RN*1 allele and is much less common in people of black descent rather than Hispanics or Caucasians. Maternal IL1RN*2 carriage has been associated with elevated levels of IL-1\(\beta\) and chronic inflammatory disorders. This could reasonably include a greater inflammatory response if intra-uterine infection did occur\(^\text{105}\). In another report, carriage of a TNFA2 allele, an A to G polymorphism in the promoter region of the gene for TNF-\(\alpha\) at position -308, resulted in high TNF-\(\alpha\) production and therefore a more vigorous inflammatory response following an infectious stimulus\(^\text{45}\). As such, carriage of the allele is associated with a three-fold elevated risk for cChA\(^\text{45}\). Newborn infants carrying the TNFA2 polymorphism, or those with a mannose-binding lectin polymorphism impairing their ability to defend against invading pathogens are all at increased risk for CP, regardless of gestational age\(^\text{106}\). Each of the biomarkers evaluated can be influenced and mediated by a number of conditions including pregnancy, disease states, and genetics. Because there exists important genetic variation in the polymorphisms for genes encoding pro-inflammatory cytokines, the same genetic variation could certainly be found in the genes encoding the other markers studied. Similar polymorphisms affecting the biomarkers examined in this pilot study could have created the disparities in concentrations that were
observed within the study groups. These polymorphisms might influence a woman’s response to infection and inflammation and identify which women would be more at risk for developing a stronger inflammatory response to intrauterine infection. Thus, the risk for fetal brain injury could be indirectly ascertained through a maternal genetic test.

Proteins can also provide insight into the various biochemical processes occurring in normal and diseased states \(^{92}\). As such, the value of proteomic profiling for the diagnosis of ChA has begun to interest researchers \(^{92;107}\). Recent advances in proteomic technologies and profiling methods have better adapted their use for clinical diagnostics and disease prevention research, making it easier to isolate and identify novel biomarkers \(^{92;107}\). Hence, the detection of certain proteins preferentially expressed in maternal serum during the earlier, subclinical stages of ChA could provide another approach in the accurate diagnosis of intrauterine infection (Appendix IV).

The transient nature of cytokines in this study may represent more closely the elimination of LPS from the maternal system, analogous to the suppression of causative microorganisms in ChA. In progressively increasing infection, cytokines may in fact be present in elevated concentrations. However, as potential markers of infection, it is clear that the cytokines present important problems in the clinical detection of ChA. Nevertheless, as major players in the pathogenesis of PVL and CP, it will be of great value to further investigate the cytokine cascades for the development of potential targets in the prevention of fetal brain injury. The cytokine responses occur rapidly following infection. Therefore, measures for the timely diagnosis of infection and for prompt
clinical action must be in place to prevent any negative outcome. If the cytokine cascade can be interrupted before fetal involvement occurs, without affecting the maternal immune system long-term, the cytokines might not gain access to the developing cerebral white matter, where the damage they mediate is irreversible. Any investigations into the roles of the cytokines once they have accessed the fetal circulation and the fetal brain will allow both PVL and CP to be better understood so future treatments and preventions can be developed.

Future animal experiments should include the investigation of the cytokine response at hourly intervals within the first 48 hours of the cytokine response to target the initiation of the TNF-α response. In addition, repetitive administration of low-dose LPS in the guinea pig model should be performed to ascertain the cytokine responses and the resultant effects on the fetal brain. As opposed to our acute model of inflammation, this experiment would provide a model for chronic inflammation to better represent the conditions arising in true ChA. A TNF-α blocking drug might provide protection from adverse perinatal outcome in ChA, but it must be first tested in an animal model of intrauterine inflammation. Risks of using such a drug, including the threat of developing serious systemic infection, must be investigated. Placental perfusion studies would first be required to determine whether the drug is even capable of crossing the placenta and reaching the fetus where its actions would be most essential.
Serum proteomics methods should be optimized for their use in the diagnosis of the development of human ChA. Issues of depleting the high abundant serum proteins while retaining proteins of interest must be addressed. Any finding from a proteomics study must be readily translated for rapid and simple use in a clinical setting. Of course, a large study across a range of gestational ages in a representative population is required before any proteomic or serum biomarker study to determine normal ranges of proteins and biomarkers during pregnancy and to allow for reasonable cut-off values to be chosen for the sensitive and specific diagnosis of ChA.
SUMMARY AND CONCLUSIONS

The guinea pig model of intraperitoneal injection of LPS at 70% gestation adds support to the hypothesis of cytokine involvement in the pathogenesis of fetal brain injury, and provides a reasonable model with which to investigate the brain injury associated with ChA. A cytokine time-course using this model has therefore reliably represented processes initiated following maternal intra-uterine infection, albeit in a more acute situation than true ChA. This study has provided important evidence for cytokine responses in a model where more severe inflammatory insults lead to cell death in periventricular regions of the cerebral white matter. It has been shown that cytokines are time-sensitive and exhibit transient responses following maternal inflammatory insult. Responses in both the amniotic fluid and maternal serum implicate both mother and fetus in cytokine responses, as the cytokines measured may have been produced by the placenta, in the maternal circulation and in the fetal circulation. The cytokines are capable of not only signalling that inflammation is occurring, but likely mediate the inflammatory insult in the maternal and fetal circulation, and in the fetal brain. As biomarkers of ChA, they haven’t proven to be sensitive or specific enough, especially in single samples. This is likely due to their transient nature, whereby certain critical intervals of cytokine concentration peaks can easily be missed. Thus, a pilot study was undertaken to develop a reliable protocol for the assessment of the value of multiple serial serum biomarkers in the prediction of ChA. Serial samples reduce the risk of a missed critical interval, and may help to predict the earlier stages of infection before a higher risk for brain injury is imparted onto the fetus. The methods of subject recruitment, sample collection and sample analysis were straightforward and easily
replicated and therefore could be applied to much larger-scale biomarker studies in the future. Future experiments using the guinea pig model to replicate conditions of chronic infection and to ascertain the value of TNF-α blocking drugs in preventing fetal brain injury must be performed. Large-scale studies establishing normal levels of biomarkers and typical protein profiles in the maternal serum would definitely advance the search for a sensitive and specific marker of hChA.
REFERENCE LIST


78. Pang Y, Cai Z, Rhodes PG. Disturbance of oligodendrocyte development, hypomyelination and white matter injury in the neonatal rat brain after


89. Yoon BH, Romero R, Shim JY, Shim SS, Kim CJ, Jun JK, C-reactive protein in umbilical cord blood: a simple and widely available clinical method to assess the


97. Luft FC. Soluble endoglin (sEng) joins the soluble fms-like tyrosine kinase (sFlt) receptor as a pre-eclampsia molecule. Nephrol.Dial.Transplant 2006;21:3052-54.


APPENDIX I: ELISA Kit Information

Mouse IL-6 Quantikine Kit:
R&D Systems, Minneapolis, MN, USA
Catalogue # M6000B
Mean minimum detectable dose (MDD) of 1.6 pg/mL
Inter-assay and intra-assay CV of 7.5% and 4.8%

Mouse IL-1β/IL-1F2 Quantikine Kit:
R&D Systems, Minneapolis, MN, USA
Catalogue # MLB00B
MDD of 3.0 pg/mL
Inter-assay and intra-assay CV of 4.2% and 2.7%

Mouse TNF-α/TNFSF1A Quantikine Kit:
R&D Systems, Minneapolis, MN, USA
Catalogue # MTA00
MDD of 5.1 pg/mL
Inter-assay and intra-assay CV of 7.5% and 6.6%

Human IL-6 Quantikine Kit:
R&D Systems, Minneapolis, MN, USA
Catalogue # D6050
MDD of 0.70pg/mL
Inter-assay and intra-assay CV of 4.5% and 2.6%

Human TNF-α/TNFSF1A Quantikine HS Kit:
R&D Systems, Minneapolis, MN, USA
Catalogue # HSTA00C
Mean MDD of 0.12 pg/mL
Inter-assay and intra-assay CV of 13.4% and 6.7%
Human sICAM-1 Quantikine Kit:
R&D Systems, Minneapolis, MN, USA
Catalogue # BBE1B
MDD of 0.35ng/mL
Inter-assay and intra-assay CV of 7.4% and 4.4%

Human MMP-9 Quantikine Kit:
R&D Systems, Minneapolis, MN, USA
Catalogue # DMP900
MDD 0.156ng/mL
Inter-assay and intra-assay CV of 7.5% and 2.3%

Human PlGF Quantikine Kit:
R&D Systems, Minneapolis, MN, USA
Catalogue # DPG00
MDD of 7 pg/mL
Inter-assay and intra-assay CV of 11.2% and 5.4%

Human Endoglin/CD105 Quantikine Kit:
R&D Systems, Minneapolis, MN, USA
Catalogue # DNDG00
Mean MDD of 0.007 ng/mL
Inter-assay and intra-assay CV of 6.5% and 3.0%

Human IGFBP-1 Direct ELISA Kit:
Diagnostics Biochem Canada Inc., London, ON, CA
Catalogue # CAN-IGF-4140
Lower detection limit of 0.5 µg/L
Inter-assay and intra-assay CV of 6.2% and 2.8%
APPENDIX II: Informed consent document for subject recruitment (for pregnant women with PPROM)

Diagnosing the development of subclinical chorioamnionitis in women with Preterm Premature Rupture of the Membranes (PPROM).

You are being invited to participate in a study carried out by Dr. Graeme Smith from Queen’s University/ Kingston General Hospital and by Michelle Dickinson, a graduate student from Queen’s University. The study is funded by the Physicians’ Services Incorporated (PSI) Foundation.

The following information describes the study. The study will be discussed with you. Feel free to ask any questions you may have.

Purpose of the Study

We know that there is a higher risk of infection in the uterus if your water breaks early (premature rupture of the membranes). We also know that babies exposed to chorioamnionitis (infection inside the uterus) during pregnancy are at a 2- to 12-fold higher risk for developing cerebral palsy (CP) than babies who are not. CP is caused by brain damage early in development and results in disorders of body movement.

Currently, obstetricians diagnose chorioamnionitis when a number of physical symptoms such as maternal fever, increased heart rate and uterine tenderness are present. This usually results in urgent delivery of the baby because of the increased risk to mother and baby. However, if this infection could be detected at an earlier stage, the baby’s risk of developing CP may be decreased and the obstetrician could make a more informed decision in the management of the pregnancy.

Approximately 36% of women with premature rupture of the membranes develop chorioamnionitis. In these cases, an accurate early diagnosis of infection is especially important because the obstetrician will have to weigh the risks of premature delivery with those of the infection.

The aim of the study is to determine if a certain substance or a combination of substances in the blood can detect the earlier stages of maternal infection in the uterus (i.e before
physical symptoms can be seen). If this is found to be true, in the future it may be possible to better predict which babies are at a higher risk for CP.

This is exploratory research and will have no effect on your usual care. No action will be taken as a result of the study blood work.

**Who is Eligible?**

You will be considered for this study if you have been diagnosed with preterm premature rupture of the membranes (PPROM) between 24 and 34 weeks of this pregnancy and your pregnancy is otherwise uncomplicated.

**Details of the study**

A nurse will take two tubes of blood (approximately two teaspoons) from you during your regular clinic visits until you deliver your baby. The samples will be analyzed to look for signs of infection. After the delivery, the placenta will be examined to tell us if your baby was exposed to infection before it was born. Blood and placenta will be destroyed following the analysis.

**Benefits of the study**

If you participate in this study, the information we gather may help, in the future, to better diagnose infection in the uterus. With this knowledge, obstetricians will be able to make more informed decisions in the management of women with PPROM, to prevent advanced infection and potentially brain damage to the baby. You and your baby may be eligible for future studies relating to this study.

**Risks of the study**

There is a risk of slight discomfort with blood drawing. After we take your blood you may develop a small bruise at the site of the blood test that should disappear after a few days.

**Voluntary Participation and Withdrawal**

You are being invited to participate but you are under no obligation to do so. You may choose not to participate without any reason. You may withdraw yourself at any time without explaining your decision. Your decision to not participate, or to withdraw, will not affect the care you receive at KGH now or in the future.

**Withdrawal from the Study**

The study physician may decide to withdraw you from this study if there is concern that you are too ill to participate.
Confidentiality

We respect your confidentiality. No information that has your name will be released or published without your written consent. All study records will be kept locked with restricted access in the office of Dr. Smith. Representatives of the Queen’s University-Kingston General Hospital Research Ethics Board may review your records for audit purposes. If information leaves the hospital, it will be coded and you will not be identified by name.

Questions about the Study

It is important to us to answer any concerns you may have. If at any time you have further questions, problems or adverse effects, you can contact

Dr. Graeme Smith (Principal Investigator) at 548-2405 or
Dr. Michael McGrath (Head, Department of Obstetrics and Gynaecology) at 548-1372.

If you have any questions about your rights as a research subject please contact Dr. Albert Clark, Chair of Queen’s University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board at (613) 533-6081.

Subject Statement

I have read and understand the information sheet and consent form for this study. I have had the purpose, procedures and technical language of this study explained to me. I have been given sufficient time to consider the above information and to seek advice if I chose to do so. I have had the opportunity to ask questions which have been answered to my satisfaction. I am voluntarily signing this form. I will receive a copy of this consent form for my information.

By signing this consent form, I am indicating that I agree that I will participate in this study.

X ___________________ ___________________ ___________________
Name of Participant Signature of Participant Date

X ___________________ ___________________ ___________________
Name of Investigator or Delegate Signature of Investigator or Delegate Date
APPENDIX III: Informed consent document for control recruitment
(for pregnant women without PPROM)

Diagnosing the development of subclinical chorioamnionitis in women with
Preterm Premature Rupture of the Membranes (PPROM).

You are being invited to participate in a study carried out by Dr. Graeme Smith from
Queen’s University/Kingston General Hospital and by Michelle Dickinson, a graduate
student from Queen’s University. The study is funded by the Physicians’ Services
Incorporated (PSI) Foundation.

The following information describes the study. The study will be discussed with you. Feel free to ask any questions you may have.

Purpose of the Study

We know that there is a higher risk of infection in the uterus if your water breaks early
(premature rupture of the membranes). We also know that babies exposed to
chorioamnionitis (infection inside the uterus) during pregnancy are at a 2- to 12-fold
higher risk for developing cerebral palsy (CP) than babies who are not. CP is caused by
brain damage early in development and results in disorders of body movement.

Currently, obstetricians diagnose chorioamnionitis when a number of physical symptoms
such as maternal fever, increased heart rate and uterine tenderness are present. This
usually results in urgent delivery of the baby because of the increased risk to mother and
baby. However, if this infection could be detected at an earlier stage, the baby’s risk of
developing CP may be decreased and the obstetrician could make a more informed
decision in the management of the pregnancy.

Approximately 36% of women with premature rupture of the membranes develop
chorioamnionitis. In these cases, an accurate early diagnosis of infection is especially
important because the obstetrician will have to weigh the risks of premature delivery with
those of the infection.

The aim of the study is to determine if a certain substance or a combination of substances
in the blood can detect the earlier stages of maternal infection in the uterus (i.e before
physical symptoms can be seen). If this is found to be true, in the future it may be possible to better predict which babies are at a higher risk for CP.

This is exploratory research and will have no effect on your usual care. No action will be taken as a result of the study blood work.

**Who is Eligible?**

You are being asked to participate in this study as a control subject because you do not have preterm premature rupture of the membranes (PPROM) and your pregnancy is otherwise uncomplicated.

**Details of the study**

A nurse will take two tubes of blood (approximately two teaspoons) from you during your regular clinic visits until you deliver your baby. The samples will be analyzed to look for signs of infection. After the delivery, the placenta will be examined to tell us if your baby was exposed to infection before it was born. Blood and placenta will be destroyed following the analysis.

**Benefits of the study**

If you participate in this study, the information we gather may help, in the future, to better diagnose infection in the uterus. With this knowledge, obstetricians will be able to make more informed decisions in the management of women with PPROM, to prevent advanced infection and potentially brain damage to the baby. You and your baby may be eligible for future studies relating to this study.

**Risks of the study**

There is a risk of slight discomfort with blood drawing. After we take your blood you may develop a small bruise at the site of the blood test that should disappear after a few days.

**Voluntary Participation and Withdrawal**

You are being invited to participate but you are under no obligation to do so. You may choose not to participate without any reason. You may withdraw yourself at any time without explaining your decision. Your decision to not participate, or to withdraw, will not affect the care you receive at KGH now or in the future.

**Withdrawal from the Study**

The study physician may decide to withdraw you from this study if there is concern that you are too ill to participate.
Confidentiality

We respect your confidentiality. No information that has your name will be released or published without your written consent. All study records will be kept locked with restricted access in the office of Dr. Smith. Representatives of the Queen’s University-Kingston General Hospital Research Ethics Board may review your records for audit purposes. If information leaves the hospital, it will be coded and you will not be identified by name.

Questions about the Study

It is important to us to answer any concerns you may have. If at any time you have further questions, problems or adverse effects, you can contact

Dr. Graeme Smith (Principal Investigator) at 548-2405 or
Dr. Michael McGrath (Head, Department of Obstetrics and Gynaecology) at 548-1372.

If you have any questions about your rights as a research subject please contact Dr. Albert Clark, Chair of Queen’s University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board at (613) 533-6081.

Subject Statement

I have read and understand the information sheet and consent form for this study. I have had the purpose, procedures and technical language of this study explained to me. I have been given sufficient time to consider the above information and to seek advice if I chose to do so. I have had the opportunity to ask questions which have been answered to my satisfaction. I am voluntarily signing this form. I will receive a copy of this consent form for my information.

By signing this consent form, I am indicating that I agree that I will participate in this study.

X ______________________ ______________________    _____________________
Name of Participant  Signature of Participant  Date

X ______________________ ______________________    _____________________
Name of Investigator or Delegate  Signature of Investigator or Delegate  Date
APPENDIX IV: Serum protein investigation

Protein studies were initiated to attempt to replicate interesting results obtained in a study by Gravett et al., where calgranulin-B, IGFBP-1 and a proteolytic fragment of IGFBP-1 were all preferentially expressed in maternal serum samples from pregnant women with intra-amniotic infection in comparison to non-infected controls. These same proteins were found to be upregulated in amniotic fluid samples of primates with intra-amniotic infection. The methods used by Gravett et al. provided effective determination of protein in the serum samples, although the human serum used was pooled before analysis. The approach to this protein endeavour was to test the methods in the Gravett report using single maternal samples obtained from infected and non-infected patients and to examine the value of these tests in the determination of ChA. The proteins of interest in our serum samples were the same; IGFBP-1, with a molecular weight of 36kDa, and calgranulin-B, with a molecular weight of 14kDa.

The first step in our protein pilot study was to attempt to eliminate the top six highly abundant serum proteins (albumin, immunoglobulin-G (IgG), immunoglobulin-A (IgA), transferrin, haptoglobin and antitrypsin) which constitute 80% of the serum proteins and could obscure any potential protein biomarkers. Equipment specifically developed for the depletion of the major abundant serum proteins is expensive and the required methods involve a risk of also depleting proteins of interest from a serum sample. It was important to first ascertain how much these large proteins were obscuring the smaller proteins of interest in the 50-3 kDa range and whether more simple methods could be used to eliminate them. Using a large polyacrylamide gel, various volumes of
test serum (from a consenting pregnant, non-infected subject) from 26ul to 0.1ul were run through gel electrophoresis. Human serum albumin, present in every lane, obscured any proteins in the smaller fraction (Figure 25). Loading less serum (from 0.8 to 0.0125ul) provided a cleaner gel, although little protein was observed the region of interest (Figure 26).

As such, Microcon centrifugal filter devices (Millipore, Billerica, MA, US) were tested. Serum was first run through a 50kDa cutoff filter. The >50kDa retentate was collected as well as the <50kDa filtrate. The filtrate was then run through a 3kDa filter. Each of the >50kDa, <50kDa, 3-50kDa and <3kDa fractions were run on a large 12% gel. The human serum albumin, with a molecular mass of 67 kDa, was nevertheless able to leak through the 50kDa filter. Proteins of importance between 3-50kDa did not appear to be concentrated in the retained filtrate (Figure 27). Various alterations to the protocol, including pre-rinsing the filters and reducing centrifugation times did prevent some albumin from leeching through, but did not concentrate the lower fraction. In fact, it appeared that the smaller proteins of interest were either bound to albumin, or were prevented from crossing the filter as albumin blocked their passage. The >50kDa fraction contained the greatest quantity of the smaller proteins, most of which were not seen in the 3-50kDa fractions (Figure 28).

The next experiments performed were western blots for IGFBP-1 (rabbit polyclonal anti-human antibody; Santa Cruz Biotechnology, Inc.; Santa Cruz, CA, US) and calgranulin-B (rabbit polyclonal anti-human antibody; Santa Cruz Biotechnology,
Inc.; Santa Cruz, CA, US) in those test serum samples prepared with the centrifugal filters. Neither protein was identified, despite having loaded pure concentrated filtrates. Using the same technique and twice as much filtrate, the same negative result was obtained. Denaturing the proteins with urea to increase their solubility and therefore to better exclude albumin had no effect on the result. Boiling samples and removing the polymerized albumin removed a large portion of the albumin and did retain the lower fraction. This method was therefore used to run western blots on test serum. Calgranulin-B was identified in the test serum; however IGFBP-1 was still not appearing in the blots. These methods were repeated on serum from a consenting pregnant subject with ChA, with western blots for IGFBP-1, and calgranulin-B. Interestingly, in two separate western blots, calgranulin-B protein was only identified in the serum of the non-infected patient, as well as the control lane where no primary antibody was added (Figure 29 A and B). The other proteins were not visible in the blots for either sample.

Immunoprecipitation was performed on the sample of the infected subject, for IGFBP-1. The antibody proved to be non-specific as various proteins appeared in the western blot, but none representing accurately the protein of interest (Figure 30 A). With a new anti-human antibody for IGFBP-1 (mouse monoclonal antibody, Diagnostic Systems Laboratories Inc; Webster, TX, US), these experiments were repeated. Unfortunately, the same results were observed (Figure 30 B).

The limitations of this methodology are apparent; a large volume of protein, therefore a large initial serum sample, must be obtained for any difference to be seen in
biomarker levels. Furthermore, the removal of the six major abundant proteins is difficult and can remove biomarkers of interest from a sample. The experiments are time consuming, and do not have the accuracy to produce reproducible results. The lack of accurate and specific antibodies for use in these tests adds further error to the result. Micro-chip arrays allowing the rapid detection of a number of proteins in a sample, with rapid automated comparison between samples, may be more valuable in serum proteomic analysis for the purpose of detecting novel biomarkers for the development of ChA.
**Figure 25:** Gel electrophoresis of serum samples on a polyacrylamide gel. At the highest volumes, abundant human serum albumin distorts the lanes and obscures proteins in the 3-50kDa range. The marker lane is also distorted. The volumes above indicate the volume of serum in each lane. M= marker, B= buffer.
**Figure 26:** Gel electrophoresis of serum samples on a polyacrylamide gel. Lower serum volumes allow visualisation of the 3-50kDa range without distortion, where very little protein is observed at serum concentration below 0.4µl. The volumes above indicate the serum in each lane. M= marker, B= buffer.
Figure 27: Gel electrophoresis of molecular weight-filtered serum samples on a polyacrylamide gel. Expected molecular weight range is shown from >50 to <3 kDa. Volume of serum run in each lane is also indicated. M= marker, B= buffer, S= unfiltered serum.
Figure 28: Gel electrophoresis of molecular weight-filtered serum samples on small polyacrylamide gels, with filters that have been previously rinsed with distilled water (A, B) and with optimized centrifugation periods (B). Expected molecular weight range is shown from >50 to <3 kDa. Volume of serum run in each lane is also indicated. M= marker, B= buffer, S= unfiltered serum.
Figure 29 A and B: Western blots for calgranulin-B (14kDa molecular weight) using filtered serum samples run through gel electrophoresis. The lane with serum from a patient with chorioamnionitis shows no calgranulin-B, whereas the lane with serum from a non-infected patient and the control lane (which did not receive primary antibody) indicate the presence of the protein. M=marker, ChA=chorioamnionitis Ab=antibody.
Figure 30: Western blots for IGFBP-1 (36kDa molecular weight) following immunoprecipitation, using filtered serum samples run through gel electrophoresis. Both anti-human IGFBP-1 antibodies (from Santa Cruz Inc. in A, Diagnostic Systems Laboratories Inc. in B) lack specificity as various proteins are observed. The molecular weight ladder is shown at the left. M=marker, ChA=chorioamnionitis Ab=antibody, V=villus extract (control), B=buffer.