A Role for RAGE System Activation in Preterm Birth

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A ROLE FOR RAGE SYSTEM ACTIVATION IN PRETERM BIRTH

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Margaret Ann Baumbusch

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ABSTRACT

A ROLE FOR RAGE SYSTEM ACTIVATION IN PRETERM BIRTH. Margaret A. Baumbusch, Catalin S. Buhimschi, Guomao Zhao, Emily A. Oliver, Irina A. Buhimschi. Department of Obstetrics, Gynecology and Reproductive Endocrinology, Yale University, School of Medicine, New Haven, CT.

The receptor for advanced glycation end products (RAGE) is a multiligand pattern recognition receptor involved in transducing endogenous damage associated stimuli into inflammatory responses. Advanced glycation end products (AGEs), HMGB1 (amphoterin) and S100 proteins, such as S100B, are prototype RAGE ligands, while soluble RAGE (sRAGE), a product of RAGE activation, acts as a ligand scavenger and RAGE inhibitor. We propose that RAGE may play important yet distinct pathogenic roles in both inflammation-induced preterm birth and preeclampsia and that, while inflammation-induced preterm birth associates with fetal RAGE activation, preeclampsia induces a maternal state of RAGE activation. We sought to identify the putative stimuli and implications for the opposing types of RAGE activation in these two leading causes of prematurity. Biological samples from two cohorts of prospectively enrolled women were analyzed. In women with symptoms of preterm birth who were assessed to rule out intra-amniotic infection, we measured amniotic fluid for HMGB1 and cord blood for HMGB1 and S100B. For ex vivo validation of our findings, HMGB1 was also measured in tissue explants subjected to endotoxin. From women assessed clinically to rule out preeclampsia, blood and urine of three subgroups (severe preeclampsia, healthy controls, and chronic hypertension but no preeclampsia) were used to determine levels of AGE and HMGB1 as potential stimuli for RAGE activation. Immunohistochemistry on reproductive tissues was used on select cases in both cohorts for cellular localization and validation of
immunoassay findings. We found that HMGB1 levels are increased in amniotic fluid of women with intra-amniotic inflammation and preterm birth, and the likely source is the damaged amniochorion, as demonstrated by explant experiments and immuno-histochemistry. Cord blood levels of HMGB1 correlate with the extent of fetal inflammation and S100B but not with either the level of intra-amniotic infection or amniotic fluid HMGB1, indicating that other RAGE axis modulating molecules may play a role. We confirmed that severe preeclampsia is associated with elevated sRAGE, a product of RAGE activation, but that neither serum AGE nor HMGB1 levels mirrored the changes in sRAGE. In contrast, women with severe preeclampsia and especially those with HELLP and/or eclampsia had significantly elevated HMGB1 excretions that correlated directly with circulating sRAGE.

In conclusion, this research further provides evidence for a biologically relevant role of HMGB1 in driving the distinct types of RAGE activation in two major obstetrical complications leading to prematurity. Heightened fetal HMGB1-RAGE axis activation may play a role in intra-amniotic inflammation while a maternal HMGB1-RAGE system may contribute to pathogenesis of preeclampsia.
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The original research presented in this Thesis is based on results derived from experiments in which I have directly participated in whole or in part. These are included in the following materials as of February 2009:

**Abstracts presented at Society for Maternal Fetal Medicine 2009, San Diego, CA**


**Articles in press, submitted for publication or in preparation:**


Oliver E, Dulay AT, Zhao G, Jing S, Cackovic M, Baumbusch MA, Buhimschi CS, Buhimschi IA. Evidence for receptor for advanced glycation end-products (RAGE) activation in women with severe preeclampsia. *Manuscript in preparation*.

Baumbusch MA, Buhimschi CS, Zhao G, Thung S, Oliver EA, Buhimschi IA. A study of advanced glycation end-products (AGEs) and amphoterin as putative stimuli of RAGE activation in women with severe preeclampsia. Manuscript in preparation.

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7. RESULTS

7.1. Specific Aim 1. Identify putative stimuli driving fetal RAGE activation in pregnancies complicated by preterm birth and intra-amniotic inflammation

HMGB1 levels are increased in amniotic fluid of women with intra-amniotic inflammation and preterm birth, and the source may be the damaged fetal membranes.

HMGB1 is released by human fetal membranes ex vivo in response to endotoxin and oxidative stress.

Cord blood HMGB1 levels do not correlate with the extent of intra-amniotic inflammation but rather with the extent of fetal inflammation and S100B.

7.2. Specific Aim 2. Identify putative stimuli driving RAGE activation in pregnancies complicated by preeclampsia

RAGE activation in severe preeclampsia occurs independent of levels of advanced glycation end-products (AGEs).

Women with preeclampsia have higher HMGB1 urine levels and higher HMGB1 fractional excretion.

8. DISCUSSION

8.1. Spontaneous preterm birth: infection and inflammation

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1. PRETERM BIRTH

1.1. Classification and general considerations

Preterm birth, defined as birth before 37 completed weeks of gestation, is a significant public health problem and the leading cause of perinatal mortality worldwide [1, 2]. Preterm birth is generally classified as either spontaneous, following preterm labor with intact membranes or preterm premature rupture of membranes (PPROM), or medically indicated, when physicians artificially interrupt the course of pregnancy for maternal or fetal indications. Medically indicated preterm birth accounts for approximately 30 to 35 percent of preterm births, while 40 to 45 percent follow preterm labor, and 25 to 30 percent follow PPROM [3].

In 2006 the prevalence of preterm birth rose to an unprecedented 12.8 percent in the United States, a 21 percent increase since 1990, despite advances in medical care [4, 5]. Other developed countries report rates of preterm birth between five and nine percent [3]. Five percent of preterm births occur at less than 28 weeks (extreme prematurity), approximately 15 percent at 28 to 31 completed weeks (severe prematurity), about 20 percent at 32 to 33 completed weeks (moderate prematurity) and 60 to 70 percent at 34 to 36 completed weeks (near-term) gestation [3]. In the United States, over 500,000 premature infants are born annually [4], 60,000 of which have a birth weight less than 1,500 grams [6]. While early preterm birth (less than 34 weeks gestation) has increased 10 percent since 1990, late preterm birth (34 to 36 completed weeks gestation) has increased 25 percent [4, 5]. Much of this increase in preterm birth has been attributed to medically indicated preterm birth [7]. Multiple births, which tend
to deliver earlier than singletons, have contributed to the rise in preterm birth, however substantial increases in preterm birth rates are also seen in singleton births [4]. Assisted reproductive techniques have also contributed to the overall increase in preterm births in two ways: increased multiple gestations and increased preterm birth in singleton pregnancies after in vitro fertilization [3].

Preterm birth is impacted by significant racial disparity, and understanding the underlying causes of this disparity has been a major public health concern. As of 2003, preterm birth occurs in 11.7 percent of non-Hispanic white births, 18.4 percent of non-Hispanic black births and 12.2 percent of Hispanic births [5]. The preterm birth rate for non-Hispanic blacks declined during the 1990s but has risen six percent since 2000, and the substantial racial gap in infant mortality has been linked to the higher rate of infants born at very short gestation in minorities [5]. Black women are three to four times more likely to have a very early preterm birth than women from other racial and ethnic groups, even when controlling for socioeconomic and maternal behavior characteristics [3].

A previous preterm delivery is a major risk factor for preterm birth in a subsequent pregnancy, and for unknown reasons this relationship holds true for both spontaneous and medically indicated preterm birth [8]. Interestingly, women who have a medically indicated preterm birth in their first pregnancy are at greater risk to deliver preterm subsequently, for either medical indications or spontaneously [7,8].

Clinically predicting preterm birth is for obvious reasons a topic of great interest. While a history of preterm birth is a reasonable indicator of increased risk of subsequent preterm birth, this information is not available to women during their first pregnancy. This challenge has spurred research into factors that may be able to predict with
reasonable accuracy preterm birth in nulliparous women, who comprise about 40 percent of pregnancies in the United States [4]. A retrospective study in a large cohort of nulliparous women confirmed that the risk of preterm birth in this population is about 12 percent. The same study also found that none of the current models using maternal characteristics or screening tests already implemented in practice is sufficiently informative to allow case selection to mitigate interventions in nulliparous women [9].

1.2. Sequelae of prematurity
The first premature nurseries existed and the first books on the care of the newborn were written as early as the 1930s and 1940s in the United States. But it was not until the late 1960s and early 1970s that major changes in clinical care occurred, including miniaturization of blood samples needed for analysis and the ability to provide intravenous nutritional support and maintain normal body temperature. By 1975, neonatology-perinatology had become a subspecialty board of the American Board of Pediatrics. Prior to the widespread use of assisted ventilation in the 1970s, few neonates survived birth before 28 gestational weeks. The increasing and earlier use of antenatal corticosteroids, postnatal surfactant administration and assisted ventilation, as well as the evolution of neonatal intensive care, dramatically improved the survival rates by the mid-1990s for very preterm births, especially those before 28 weeks. In 1960 a 1,000 gram infant (approximately 28 weeks) had a mortality rate of 95 percent [10]. By 2000 that statistic had flipped to a 95 percent chance of survival [11]. Infants born at less than 28 weeks gestation in 1960 were considered previable [10]. Today over 50 percent of infants born at 24 weeks gestation survive, and the limit of viability is considered 23 to 24 weeks [12].
Improvements in neonatal intensive care have led to increased survival of preterm infants but also increased disability and a heightened awareness of the need to improve neonatal outcomes associated with prematurity [13]. Today preterm birth accounts for 75 percent of perinatal mortality and more than half of long-term perinatal morbidity [3]. Gestational age has the greatest impact on outcomes of preterm birth, followed by birth weight. Infants born at less than 32 weeks of gestation comprise approximately one-third of deaths in the perinatal period [14], and 67 percent of deaths occur in infants less than 2,500 grams (about 36 weeks) [11]. Thus, while prematurity is a harbinger of poor outcomes, it is the smallest and youngest neonates who are at the greatest risk and thus pose the greatest challenge to the clinical decision-maker.

Conventional wisdom holds that the primary causes of the high neonatal morbidity and mortality attendant preterm birth are complications of immature organ systems, such as respiratory distress syndrome, intraventricular hemorrhage, necrotizing enterocolitis, bronchopulmonary dysplasia and white matter disorders. In 2005 the cost of caring for premature infants in the United States was estimated at $26.2 billion per year, a figure that excludes long-term care costs [15]. The societal burden of prematurity is further underscored by the fact that medical and other supportive care expenses exceed the estimated lifetime earnings per survivor with a birth weight of less than 900 grams (approximately 27 weeks of gestation) [16].

Fifty percent of premature infants have long-term neurological handicaps, including blindness, deafness, developmental delay and cerebral palsy [17,18,19]. The prevalence of cerebral palsy is inversely related to gestational age. Approximately five percent of the nearly 55,000 preemies who survive the newborn period exhibit cerebral
palsy, and 25 to 50 percent have cognitive and behavioral deficits [6]. The consequences of prematurity, however, are not restricted to severe neurological handicaps. Even premature infants with relatively uncomplicated neonatal courses are at risk for developmental delays in motor skills, lower cognitive performance and learning difficulties, as well as behavioral, psychological and social impairments [20,21]. Very preterm infants born at less than 32 completed weeks gestation without major neurological deficits have a two- to three-fold increase in behavioral problems, such as hyperactivity, conduct problems and emotional symptoms [22]. Premature infants also attain slightly but significantly lower rates of educational achievement, employment and independent living [21].

Prematurity is a major cause of infant death, and survivors are at significant risk of serious long-term disability. Preterm birth rates continue to rise despite advances in prevention and treatment, and preterm birth remains one of the most complicated research and public health issues in the fields of obstetrics and pediatrics. Understanding the etiologies of preterm birth and identifying specific biomarkers to identify at-risk patients are keys to developing targeted interventions to prevent prematurity and its attendant problems.
2. SPONTANEOUS PRETERM BIRTH

2.1. Spontaneous preterm birth, a heterogeneous syndrome

From a clinical standpoint spontaneous preterm birth debuts as either increased uterine activity accompanied by cervical ripening or PPROM. The current view is that regardless of its clinical manifestation spontaneous preterm birth results from the clinical convergence of multiple yet distinct pathophysiological pathways, including intrauterine inflammation, uterine over-distention, utero-placental circulatory disturbances (ischemia or hemorrhage) and stress, including oxidative (redox) imbalance [23]. An increased level of activation of the maternal or fetal hypothalamic pituitary axis has been quoted as responsible for a subgroup of preterm births characterized by increased concentration of corticotropin releasing hormone (CRH) and decreased CRH binding protein in maternal circulation [24]. Furthermore, systematic placental histological studies identified fetal eosinophilic gradients in nearly one-fifth of the cases of spontaneous preterm birth, pointing to fetal allergy as an associated component, especially in instances where acute inflammation was also found [25]. Because of the multitude of pathways leading to preterm birth and different levels of intricacy among effectors, the initial triggers remain for the most part unknown. A schematic representation of the current understanding of the Figure 1: Proposed view of how multiple etiologies and pathogenic pathways converge into the clinical manifestations of spontaneous preterm birth.
etiologic, pathogenic and clinical circumstances associated with spontaneous preterm birth is shown in Figure 1.

2.2. Relevance of infection as a pathogenic pathway to preterm birth

The heterogeneous nature of preterm birth is substantiated by the findings that not all preterm births are equal with respect to their contribution to prematurity sequelae. Evidence suggests that the poor outcomes of many children delivered prematurely are not entirely dependent on their gestational age at birth and birthweight [26,27]. When adjusted for gestational age at birth, it appears that intrauterine infection and subsequent inflammation contributes disproportionately to neonatal mortality and morbidity compared to other etiologies, suggesting that the associated intrauterine processes may adversely affect the fetus in utero prior to birth [28]. The implication of this finding is important: the intrauterine environment may be harming instead of protecting the fetus. Therapeutic interventions solely aimed to extend gestation or unilaterally to treat infection without addressing the consequent host response, therefore, may have more deleterious consequences than prematurity alone [29]. This conundrum has been substantiated by the 2008 childhood outcome results of the ORACLE I and II clinical trials that show antibiotics given to women in threatened preterm labor or with PPROM increase rather than decrease the risk of cerebral palsy [30,31]. These results suggest that while antibiotics induce suppression of bacteria they fail to quell the inflammatory processes already underway and the causes of fetal cellular damage.

Evidence from humans [32] and animal models [33] suggests that intrauterine inflammation in response to infection may evolve silently. The fetus is therefore adversely affected by host defense mechanisms before the onset of signs and symptoms
of preterm birth [34]. In such cases, prematurity may be inappropriately cited as the only cause of perinatal mortality or morbidity [35].

The prevalence of infection as a cause of preterm birth is higher the younger the gestational age at the onset of clinical manifestations [36]. Simply put, infections are responsible for most severe forms of prematurity. One study showed that 67 percent of patients delivering at 21 to 24 weeks gestation had chorioamnionitis, as compared to 23 percent at 33 to 36 weeks gestation. Further, the severity of chorioamnionitis was greater with preterm compared to term delivery [37]. Evidence of intra-amniotic infection appears to be more common in preterm births presenting with PPROM than in cases presenting with uterine contractions and intact membranes, suggesting that cellular destruction within vital fetal tissues and organs may silently mirror the processes leading to dissolution of the amnion and chorion in PPROM [38,39]. Overwhelming evidence supports the conclusion that intra-amniotic inflammation superimposed on prematurity can lead to more devastating consequences for the neonate than prematurity alone [33,40,41,42,43].

That approximately only 25 to 40 percent of preterm births are the result of infection is likely underestimated given the difficulty in detecting intrauterine infection with conventional culture techniques [3,44]. Using DNA-based techniques, a recent study showed that the process of intra-amniotic infection involves uncultivated or difficult-to-cultivate bacterial species that induce a potent host response [44].

In addition to its impact prior to birth, intrauterine infection is a risk factor for early-onset neonatal sepsis (EONS) [45], which occurs in approximately 15 to 19 per 1,000 live births in infants weighing less than 1,500 grams [46]. Prematurity itself
directly predisposes to commensal infections due to prolonged intravascular access and mechanical ventilation, explaining why both early- and late-onset sepsis occur with increased frequency in premature neonates [13,47,48,49]. For the same reasons, in statistics published by Yale-New Haven Hospital, which holds the longest running, single-center database of neonatal sepsis started in 1928, mortality attributable to sepsis remains at 11 percent despite advances in neonatal care [45].

2. 3. Intrauterine infection, inflammation and fetal damage

As detailed above, preterm infants are at substantial risk of developing neonatal infections that, combined with their immaturity, lead to poor outcomes. The exact mechanisms translating intrauterine infection into cellular damage, however, are not well understood and seem to involve particularities of the fetal and neonatal response to infection that cause pathology unique to the premature fetus, including heightened inflammatory and oxidative states that act synergistically with microbial insult.

Host defense mechanisms can be grouped into innate and adaptive processes. Innate immunity includes the inflammatory reactions of neutrophils and monocytes, while adaptive immunity refers to lymphocytes responses that recognize specific microbial antigens. Both innate and adaptive immunity have evolved phylogenetically to counteract and clear pathogens to prevent tissue injury [50] and rely on the host’s ability to sense when potentially dangerous microorganisms intrude. Several classes of microbial sensing proteins have been described, the most intensely studied of which are Toll-like receptors (TLRs) that detect microbes in the extracellular compartments [51]. More recently other classes of sensing proteins have been discovered. These include NOD-like receptors (NLRs), with roles in intracellular cytosolic surveillance, and the membrane-
associated C-type lectins (CLRs), which bind glycosylated ligands [52,53]. Irrespective of class or location, however, these protein sensors mitigate host defense through engagement of ubiquitous and conserved bacterial constituents known as pathogen-associated molecular patterns (PAMPs) [54]. Upon engagement of PAMP epitopes, sensing receptors elicit the release of inflammatory chemokines and cytokines primarily through TLRs, including interleukin (IL)-8, IL-1β and tumor necrosis factor (TNF) α, which in turn stimulate release of other inflammatory mediators and recruit inflammatory cells, such as neutrophils, monocytes and macrophages, to the respective sites of injury [51]. These inflammatory cells produce a variety of bacteriostatic and bactericidal molecules along with free radicals, reactive oxygen species and matrix degrading enzymes (matrix metalloproteases) aimed primarily to neutralize bacteria.

For obvious reasons mounting an adequate inflammatory response is critical for intact survival of the mother and fetus. To be successful this effort has to assure the correct discrimination between self (host) and microorganisms, and the inflammatory reaction must cease once the intruding germs have been annihilated. Evidence suggests that in pregnancies complicated by infection-induced preterm birth the checks and balances of the innate immune response may be imperfect and result in an inappropriate trigger and control of the inflammatory course [55,56,57].

Even after adjustment for gestational age at delivery, strong correlations exist between the level of intrauterine and/or fetal inflammation (as reflected by elevated cytokine levels in the fetal circulation) and histological chorioamnionitis and the incidence of perinatal complications [57,58,59]. Numerous inflammatory cells are found in the lungs of neonates born to women with chorioamnionitis, and the resulting
pneumonitis is considered an important contributor to bronchopulmonary dysplasia in these infants [60,61,62]. Other studies have established synergistic relationships between intrauterine inflammation and important adverse neonatal outcomes, including intraventricular hemorrhage, necrotizing enterocolitis, retinopathy of prematurity and long-term handicaps, such as cerebral palsy [13,59,63,64].

Fetal systemic and central nervous system inflammation are critical to the genesis of brain injury in the neonate [65]. A growing body of evidence supports the notion that umbilical cord vasculitis (funisitis), elevated neurotoxic cytokines and sepsis in children with cerebral palsy are preceded by a robust intra-amniotic inflammatory response [13,57,62,66,67]. Bacterial endotoxins are known to cause white matter injury in preterm animal models [68]. In humans, extremely low birth weight infants (401 to 1,000 grams at birth) with sepsis have 50 to 100 percent higher rates of cognitive deficits, cerebral palsy and other neurodevelopmental disabilities compared to non-infected infants [69]. Links among sepsis, brain injury and adverse outcomes in preterm infants have long been proposed [70,71]. The mechanisms mediating periventricular leukomalacia, the most common ischemic brain injury in preterm infants, in premature neonates with sepsis are postulated to include injury to pre-oligodendrocytes induced by free radicals, reactive oxygen species and inflammatory cytokines [70]. Injuries to endothelial cells induced by similar mechanisms add to the damage, leading to multisystemic organ dysfunction [72]. Taken together, these observations suggest that, whereas an adequate innate and adaptive immune response is critical for the survival of mother and child, excessive inflammation is associated with adverse outcomes in newborns [73].
Recent evidence from adults indicates that not only pathogen PAMPs can trigger an inflammatory reaction but also endogenous molecules described collectively as damage-associated molecular pattern molecules (DAMPs) [74], including high mobility group box protein-1 (HMGB1), S100 proteins, heat-shock proteins, uric acid and DNA. DAMPs are abundant intracellular proteins that when released in excess in the extracellular compartment as a result of cell death or injury become “danger signals” that activate immune cells and induce further damage to host cells [75]. Owing to their similarity to cytokines and their release by activated or damaged cells under conditions of cell stress, these endogenous pro-inflammatory molecules have also been termed alarmins [76]. A common feature of DAMPs is the ability to engage the receptor for advanced glycation end products (RAGE), which results in uncontrolled activation of inflammation. An important feature of the RAGE receptor is that its expression is low in normal tissues but increases transcriptionally in environments where RAGE ligands, such as DAMPS, accumulate, escalating tissue damage through positive feedback [77]. As will be described in the following sections, fetal RAGE activation may represent the elusive stimulus for the transition from the adequate inflammation aimed to protect the fetus to the maladaptive phenomenon characterized by cellular destruction.
3. PREECLAMPSIA AND INDICATED PRETERM BIRTH

3.1. Preeclampsia, an elusive pregnancy-specific disorder

Preeclampsia is a pregnancy-specific hypertensive disorder of unknown etiology and a major cause of maternal and fetal morbidity and mortality. Preeclampsia complicates approximately six to eight percent of pregnancies and accounts for 15 percent of all preterm births [78,79]. This disease is responsible for 20 percent of pregnancy-related maternal deaths in the United States [80] and more than 76,000 deaths worldwide each year [81]. Importantly, preeclampsia is the leading cause of medically indicated preterm birth [7,8,82].

Preeclampsia is characterized by heterogeneous clinical and laboratory findings and can be classified as a maternal disorder, fetal disorder (growth restriction) or both. While currently defined as the onset of hypertension (≥140/≥90 mmHg) and proteinuria (≥0.3g in 24 hours) after 20 weeks gestation in a previously normotensive woman, preeclampsia is primarily a clinical diagnosis and should also be considered when hypertension and proteinuria are associated with persistent cerebral symptoms, epigastric or right upper-quadrant pain with nausea or vomiting, or thrombocytopenia and abnormal liver enzymes [83]. Severe preeclampsia is diagnosed in the presence of severe hypertension (≥160/≥110 mmHg), proteinuria or multiorgan involvement, such as pulmonary edema, seizures (eclampsia), oliguria, thrombocytopenia, abnormal liver enzymes associated with persistent epigastric or right upper-quadrant pain, or persistent and severe neurological symptoms, such as altered mental status, headaches, blurred vision or blindness [83]. In addition, preeclampsia is also considered severe if
accompanied by symptoms of end-organ damage, such as HELLP syndrome (defined as hemolysis, elevated liver enzymes or low platelet counts) or fetal growth restriction.

Prior to the 19th century, the term eclampsia referred to the observation of convulsions occurring in a pregnant woman that ceased after delivery [84]. In 1840, the French pathologist Pierre Rayer demonstrated proteinuria in three edematous pregnant women. In 1843 John Charles Lever of London, struck by the resemblance of eclamptic patients to those with nephritis, published the observation that nine out of 10 convulsing women had albuminuria as a “precursor of puerperal fits” [85]. In the same month (November 1843), Sir James Young Simpson recognized that “patients attacked with puerperal convulsions had almost invariably aluminous urine […] in some rather preceding dropsical complication” [85]. In observing albuminuria, Lever and Simpson are thus credited with the first recognition of a subclinical abnormal state preceding eclampsia. Albuminuria thus represented the first tool that could be used to predict toxemia and prevent convulsions. The realization that hypertension was also present in these cases did not occur until the invention of the sphygmomanometer with inflatable armband by the Italian physician Scipione Riva-Rocci in 1896 and its subsequent application to obstetrics after 1910. Our obstetrics predecessors found an incidence of eclampsia of approximately one in 500 pregnancies, with a maternal mortality of 20 to 30 percent and an even higher perinatal mortality.
3.2. Challenges in diagnosis and management of preeclampsia

Preeclampsia, as it is defined today, is diagnosed only after the assessment of urinary proteins and blood pressure, proteinuria and hypertension thus replacing the prior diagnostic gold standard of “convulsions in a pregnant woman.” To this day the goal is to be able to predict who is going to develop preeclampsia as early as possible, and research efforts have intensified to find the best combination of altered markers before a woman becomes hypertensive or proteinuric. Even today, despite advances in diagnosis, the only definitive cure for preeclampsia remains delivery. Identifying new markers may aid clinicians with case management when contemplating early interruption of the pregnancy course when clinical signs and symptoms are less clear-cut. On the other hand, if the markers are pathogenically related to the cause of preeclampsia, perhaps they will point us to new ways to treat preeclampsia and avoid its progression to the clinically manifest syndrome. Figure 2 is a representation of how the delineation of the preeclampsia syndrome has and perhaps will continue to evolve historically along with our progress in elucidating the factors responsible for this condition. As this progress is markedly influenced by technological advances in diagnostic tools, it is expected that future developments in fields like genomics and proteomics may allow identification of

[Figure 2: Proposed view of how our ability to detect an abnormal subclinical state preceding manifest disease has and likely will further change terminology and management of preeclampsia syndrome. * by ACOG guidelines]
biomarkers reflective of an abnormal state that precedes the onset of clinical manifestations as defined today (proteinuria and hypertension) or the gestational cut-off of 20 weeks. Such a state will then require new terminology, perhaps “pre-preeclampsia.”

The current difficulty in diagnosing preeclampsia lies in part in the fact that many women present with a constellation of signs and symptoms inconsistent with the current diagnostic criteria. In fact, some women who develop severe gestational hypertension without proteinuria have higher maternal and perinatal morbidities than those with mild preeclampsia [86,87]. Furthermore, hypertension and/or proteinuria are absent in 10 to 20 percent of women who develop HELLP syndrome [88] and in 38 percent of women who develop eclampsia [89], two complications of preeclampsia that are associated with significantly higher rates of maternal and perinatal morbidities than mild preeclampsia.

About one to five percent of pregnant women have chronic hypertension, defined as sustained hypertension present before conception or prior to 20 weeks gestation [90], and rates are increased with obesity, diabetes and advancing maternal age, as well as in African Americans. The current criteria for preeclampsia diagnosis are unreliable in women with pre-gestational hypertension or proteinuria or in those women presenting for their first prenatal care assessment after 20 weeks gestation [83]. In such cases markers other than proteinuria and hypertension are needed to guide practitioners in their diagnosis and with regard to the appropriate timing of intervention. Although at this time there is no etiologically or pathogenically targeted therapeutic arsenal for preeclampsia, practitioners would like to postpone delivery as long as safely possible while balancing the degree in clinical worsening of the maternal condition with the degree of prematurity or fetal distress. In addition, antenatal steroid administration has clearly been shown to
reduce the risk of respiratory distress, the need for respiratory assistance and overall neonatal mortality [91]. This, however, requires obstetricians to precisely initiate corticosteroid prophylaxis at least 48 hours but not more than one week prior to birth, a difficult task in the current clinical practice. Given the devastating consequences of preeclampsia, any sign of maternal decompensation rapidly prompts toward delivery even in the absence of a complete steroid course.

The only definitive treatment for preeclampsia is delivery of the fetus and of the placenta, a decision that is particularly concerning for both medical practitioners and pregnant women at young gestational ages. The task becomes even more challenging when a pre-existing hypertensive or renal disorder makes differentiating preeclampsia from similar clinical syndromes difficult, thus clouding the clinical picture and further complicating the decision to deliver preterm. For example, while chronic hypertension predisposes to preeclampsia and placental abruption, thus increasing neonatal morbidity and mortality, chronic hypertension uncomplicated by preeclampsia is usually a benign pregnancy complication.

In the United States, where preterm infants are cared for in advanced neonatal intensive care units, obstetricians are less concerned with mandated delivery beyond 34 weeks of gestation. Mandated delivery before 34 weeks gestation, however, is unanimously considered a poor pregnancy outcome. Given the finality of treatment by delivery, it is vitally important for research to uncover methods to definitively diagnose preeclampsia and thus correctly identify those cases that mandate delivery.

Because of the high rates of maternal and fetal morbidity and mortality associated with preeclampsia and high neonatal survival beyond 34 weeks of gestation, it is
generally agreed that patients should be delivered if disease develops at greater than 34 weeks of gestation [92,93]. This shift in management has led to a sharp rise in the incidence of late preterm births (33 to 36 weeks of gestation). Although the true consequences of this interventional management for neonatal and childhood outcomes has yet to be determined, some recent studies have suggested an increased risk of mortality and morbidity in late preterm newborns compared with infants born at term. In a large population cohort perinatal, neonatal and infant mortality rates were significantly higher in the late preterm group. Infants in this group needed resuscitation more often, had a significantly higher incidence of respiratory morbidity and infections and had a significantly longer duration of hospital stay than newborns in the term group [94]. Furthermore, children born late preterm are over three times more likely to be diagnosed with cerebral palsy than children born at term [95]. In this context obstetricians need to weigh very carefully the risks versus benefits when opting for an indicated preterm delivery even at near-term. The real challenge, however, remains in determining the best clinical course in pregnancies less than 34 weeks of gestation, when severe preeclampsia alone is associated with high rates of perinatal morbidity and mortality [96]. Temporization of delivery may lead to in utero fetal death or asphyxial damage, as well as an increase in maternal morbidity, while immediate delivery will unambiguously lead to high neonatal morbidity and mortality due to prematurity, as well as prolonged hospitalization in the neonatal intensive care unit. Two randomized clinical trials conducted since 1990 have attempted to address this issue, both of which support expectant management (glucocorticoid therapy for lung maturation followed by delivery only for specific maternal or fetal indications) in a select group of patients (stable
maternal and fetal conditions) with severe preeclampsia at 28 to 34 weeks of gestation [92,93]. The goal of such management is to improve perinatal outcome by prolonging gestation and reducing neonatal morbidities without increasing maternal morbidity. Several recent retrospective and observational studies have extended the recommendation for expectant management for severe preeclampsia at 24 to 34 weeks of gestation [96]. For gestational ages less than 23 completed weeks of gestation, expectant management is associated with high maternal morbidity and limited perinatal benefit [96]. But again, the clinical criteria to determine the point where maternal and fetal conditions are stable are far from precise and vary widely with parameters unrelated to the disease process, such as individual clinician experience or availability of intensive care resources at the respective hospital site. There is great research interest, therefore, in identifying from the general population the women who ultimately are likely to develop preeclampsia so that measures to improve maternal and fetal outcomes are put into place in a timely manner.

3.3. Preeclampsia risk factors

Many pre-pregnancy risk factors are associated with dramatically increased rates of preeclampsia, including hypertensive disorders, diabetes, cardiovascular disease, connective tissue disorders, thrombophilias and obesity [97]. In addition, limited sperm exposure, prior paternal conception of a preeclamptic pregnancy and assisted reproductive technologies are also thought to increase the risk of preeclampsia [97]. Furthermore, a history of preeclampsia, eclampsia or HELLP in a prior pregnancy increases the risk in a current pregnancy by 20 to 50 percent and up to 65 percent [97]. Several pregnancy-related factors also increase the risk of preeclampsia, including hydrops (seen in triploidy), multifetal gestation, unexplained fetal growth restriction,
gestational hypertension and infections [97]. Nulliparous women, for example, have rates of preeclampsia ranging only from two to seven percent, which is substantially lower than the 14 percent rate in women with twin gestations and the 17 percent rate in women with previous preeclampsia [78]. Although the majority of cases of preeclampsia still tend to occur in healthy nulliparous women, 75 percent of these cases occur after 37 weeks gestation. Conversely, the incidence is substantially higher (20 percent) in women with previous preeclampsia and/or preexisting chronic hypertension, and, as the majority of these women deliver preterm [90,98], they represent a true high-risk population that needs to be targeted for future research.

3. 4. Relevance of preeclampsia to the mother and newborn

Maternal and fetal complications of preeclampsia are substantial and a major cause of maternal and fetal morbidity and mortality. Moreover, evidence suggests that the consequences of preeclampsia may extend beyond the immediate pregnancy and may predispose a patient to complications later in life [99,100,101,102].

Acute maternal complications in a pregnancy complicated by preeclampsia include eclampsia (seizure), stroke, placental abruption, disseminated intravascular coagulation, HELLP syndrome, rupture of the liver, pulmonary edema, adult respiratory distress syndrome, acute renal failure and even death. Fetal complications include growth restriction, hypoxia, stillbirth and of course preterm birth with its array of long-term consequences resulting from prematurity, such as chronic lung disease, retinopathy of prematurity, cerebral palsy and mental retardation.

Preeclampsia also seriously impacts long-term maternal health, with complications such as chronic hypertension, diabetes, chronic renal failure, coronary
artery disease, stroke and premature death [99,100,101,102]. Consistent with the finding that preeclampsia is associated with key components of the metabolic syndrome, women with preeclampsia have in postpartum higher circulating concentrations of fasting insulin, cholesterol and coagulation factors compared to controls [103,104]. A genetic component to preeclampsia has also been proposed based on the finding that girls born in the context of a preeclamptic pregnancy have a higher chance of developing preeclampsia themselves [105]. In addition, an intergenerational study from the Walker cohort, a database of over 48,000 births that took place in Scotland between 1952 and 1966 and allows linkage of records across siblings and generations, found that infants of preeclamptic pregnancies had an increased risk of developing diabetes later in life, although birthweight seemed to be a more important determinant of such risk [106].

Given the challenges in diagnosing and managing patients with preeclampsia and the substantial risks to the mother and fetus, better tools are needed to definitively diagnose patients. Improved diagnostic tools in coordination with an improved understanding of the etiology of preeclampsia may lead to important discoveries regarding effective treatments to prevent and/or treat preeclampsia.

3. 5. Socio-economic impact of preeclampsia

The most current 2005 database of health care costs quotes 402,539 maternal hospital admissions with a diagnosis of hypertensive disorders complicating pregnancy at a median cost of $3,472 per maternal case (compared to $2,124 for a normal delivery) [107]. With preeclampsia responsible for approximately 90,000 preterm births per year and a median hospitalization cost of $15,385 per preterm newborn case delivered (compared to $717 per normal term live-born), healthcare expenses attributable to
preeclampsia in 2005 exceeded $13 billion [107]. These estimates understate the true costs as they do not take into account the costs of direct medical care accrued beyond the initial infant hospitalization, nor do they incorporate the indirect costs of medical and educational interventions, disability and lost individual and family productivity. In the most comprehensive analysis to date on the economic cost of prematurity, the Institute of Medicine estimated that the societal economic burden associated with preterm birth in the United States in 2005 was $51,600 per infant born preterm [15].

Much of the research focus in reducing the rate of preterm birth has been to search for strategies to prevent spontaneous preterm birth, despite the fact that the rate of spontaneous preterm birth has been steadily decreasing in the past two decades [8] while medically indicated preterm birth is increasing [7]. Although it remains plausible that prolongation of gestation might be beneficial for the neonate in selected cases of very preterm labor where fetal compromise and infection have been ruled out, data from randomized trials suggests tocolytics cannot prolong pregnancy more than 48 hours [108,109,110,111]. Furthermore, their use alone had no meaningful impact on neonatal morbidity and mortality [112,113]. The prophylactic use of 17 alpha-hydroxy-progesterone caproate is currently advocated to reduce the rate of a subsequent preterm birth in women with a prior history [114,115]. Results of recent studies aimed to extend its use to other categories of high risk women, however, have shown the treatment to be ineffective in women pregnant with twins, and it failed to improve the outcome of the neonates delivered by mothers with short cervix [116,117]. Nevertheless, it is advocated that even the predicted reduction in the preterm birth rate by two percent [118] might render the strategy cost effective by saving approximately $2 billion annually [119] if all
30,000 pregnant women with a prior spontaneous preterm birth in the United States were universally treated with 17 alpha-hydroxy-progesterone caproate.

We believe that strategies to reduce the rate of or prevent mandated preterm delivery for preeclampsia would be more efficient and cost-effective in reducing the rate of preterm birth than strategies aimed at preventing spontaneous etiologies. Such strategies should have at the forefront: 1) identification of novel biomarkers to improve the differential diagnosis of cases in true need of early delivery, 2) accurate prediction of such cases so that antenatal steroid courses can be completed, and 3) research on novel pathophysiologic pathways leading to preeclampsia and focused on identifying new therapeutic targets. The first two strategies are important because they can be implemented without delay in the current state of clinical practice. The potential for the third strategy to postpone or even prevent maternal and fetal deterioration, favoring fetal development in utero rather than in neonatal intensive care unit, makes its challenging undertaking worthwhile.

3. 6. Pathophysiology of preeclampsia

The etiology of preeclampsia is yet unknown, but the syndrome is known to involve multiple organ systems and generalized vasoconstriction, metabolic changes, endothelial dysfunction (thought to be induced by oxidative stress), coagulation dysfunction and inflammation. Compelling evidence suggests that key disturbances at the fetal-maternal interface play a central role in the etiology and pathogenesis of the clinical manifestations of this syndrome, leading to progressive deterioration of the maternal and fetal condition [120]. Proposed pathophysiologic mechanisms include impaired trophoblast differentiation and invasion, placental and endothelial dysfunction, immune
maladaptation to paternal antigens and an exaggerated maternal systemic inflammatory response characterized by aberrant lymphocyte Th1/Th2 cytokine balance [121,122,123,124].

One of the difficulties in studying preeclampsia has been to separate initiating events related to etiological factor(s) from those that could be attributed to compensatory mechanisms aimed to maintain maternal and fetal homeostasis. For instance, the increase in blood pressure seen in the mother may develop consequent to poor placental perfusion, although the reverse scenario is also plausible. In other words, both the poor placental invasion and trophoblast hypoxia could be secondary rather than primary events. Therefore, because the etiology of preeclampsia is poorly understood, interventions directed at risk factors or at normalizing low-end homeostatic disturbances, such as antihypertensives, antioxidants and low-dose aspirin, have had minimal impact in preventing poor outcomes related to preeclampsia and medically indicated preterm birth [97,125].

Despite the various controversies on the initial trigger for preeclampsia, researchers seem to agree at least on two ominous pathophysiologic characteristics: generalized activation of the maternal endothelium and the presence of toxic humoral factor(s) that circulate in plasma of patients afflicted with preeclampsia and can perturb endothelial cell function in vitro [126]. Although experimental evidence from many different laboratories has repeatedly demonstrated marked alterations in cell behavior and metabolic processes when cultured endothelial cells or blood vessels are incubated with serum or plasma samples from women with preeclampsia as compared to control pregnant women, the search for the elusive toxemia factor has proven difficult, perhaps
due to its non-singular nature [127,128,129]. For instance, the factor(s) stimulating nitric oxide production in endothelial cells appear to be lipoprotein or lipoprotein aggregates and different from what stimulates prostacyclin production, which is induced by a small molecular weight aqueous fraction [129] that is heat and acid labile and activated by mild proteolytic digestion [130].
4. A ROLE FOR RAGE ACTIVATION IN PRETERM BIRTH

4.1. RAGE activation, a link between chronic inflammation and injury

RAGE is a multiligand transmembranar receptor and a member of the immunoglobulin superfamily with crucial roles in various pathophysiologic processes, including inflammation [131]. RAGE is present on numerous cell types, including endothelial cells, macrophages, neurons and smooth muscle cells. More recently, RAGE has also been identified in the fetal membranes and placenta [132]. RAGE was initially identified by its ability to engage advanced glycation end-products (AGEs). In time, however, it became clear that a wide range of ligands can bind to RAGE and that RAGE activation is a generalized feature of many chronic inflammatory conditions, including diabetes, rheumatoid arthritis, arteriosclerosis and Alzheimer’s disease [133,134,135]. Known ligands include HMGB1, S100 proteins, heat shock proteins and β-amyloid [133,131,134,135,136].

Like TLRs, RAGE is a pattern recognition receptor that is engaged by three-dimensional structures rather than specific amino acid sequences. Expression of RAGE is constitutively increased during embryonal development, especially in the cerebral cortex, hippocampus and cerebellum, but downregulated in adults [137]. In adults, downregulated cells can be induced to express RAGE, however, when inflammatory mediators and RAGE ligands accumulate [77].

Binding of a ligand to RAGE activates key signaling pathways, such as the proinflammatory NF-κB pathway, to promote cytokine production, including TNF, IL-6, and IFN-γ, and cell survival. NF-κB transactivation plays a pivotal role in parturition via
induction of pro-inflammatory cytokines, prostaglandins and matrix metalloproteases [138]. In addition, RAGE has been shown to activate the MAP-kinases ERK and p38, which also result in the downstream activation of NF-κB. RAGE-mediated NF-κB activation is unique because of its self-perpetuating and sustained nature, which overwhelms the auto regulatory inhibitory feedback loops [139]. An oxidant environment has been demonstrated to play a key role in fuelling RAGE activation, and ligand binding to RAGE is known to generate intracellular reactive oxygen species and consequently deplete antioxidant defense mechanisms [133,140,141,142]. Because RAGE-mediated cellular stimulation promotes increased expression of the receptor itself, RAGE functions as a perpetuation relay factor that propagates in a positive feedback fashion a destructive host response leading to a state of chronic inflammation rather than resolution.

A number of studies have shown that RAGE signaling is abrogated by an endogenous soluble truncated form of the RAGE (soluble RAGE, or sRAGE), which acts as a decoy by binding RAGE ligands [133]. sRAGE derives from proteolytic cleavage of the membrane-bound RAGE [143]. The stimulus for RAGE shedding seems to involve HMGB1 binding to RAGE and the sheddase ADAM10 [143,144]. In patients with diabetes, elevated plasma sRAGE levels have been shown to act as markers of increased cellular RAGE expression and activation [145].

Recent evidence from Dr. Irina A. Buhimschi’s laboratory at the Yale University School of Medicine has indicated that RAGE activation may play important pathogenic roles in preterm birth and preeclampsia [132,146,147]. In point, it has been shown that preeclampsia is associated with a state of RAGE activation on the maternal side characterized by increased maternal circulating levels of soluble RAGE [146]. This
occurs in contrast to fetal RAGE activation and low fetal soluble RAGE levels observed in inflammation-induced preterm birth [147,132]. This disparity spearheaded our search for putative stimuli and for implications for the opposing types of RAGE activation in the two major obstetrical syndromes leading to preterm birth and perinatal morbidity: intra-amniotic infection and preeclampsia. The results of these findings are included in Section 7 of this Thesis.

4.2. Particularities of RAGE activation in inflammation-induced preterm birth

Using proteomic mapping of amniotic fluid, Dr. Buhimschi’s laboratory has found that one of the putative RAGE ligands, S100A12, is present in the amniotic fluid of women with intra-amniotic infection in direct relationship to the degree of inflammation and

Figure 3. (A) Representative SELDI-TOF mass spectrometry profiles of amniotic fluid samples from three different women with varying degrees of intra-amniotic inflammation (MR 0: no inflammation; MR 2: mild inflammation; MR 3-4: severe inflammation). P3 (marked with red) denotes the biomarker corresponding to S100A12. (B) Survival analysis for the 131 patients, illustrating the shorter duration from amniocentesis-to-delivery in women with MR scores 3 or 4. Originally published in PLoS Med. 2007 Jan 16; 4(1):e18. Republication herein permitted by authors under Creative Commons Attribution License.
severity of histological chorioamnionitis [146,148]. Moreover, of the four proteomic biomarkers with diagnostic value for intra-amniotic inflammation, it was the appearance of the peak corresponding to S100A12 that best predicted impending preterm birth [149,150]. Figure 3 illustrates representative proteomic profiles of amniotic fluid from women with and without inflammation obtained using surface enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry for the four biomarkers of the Mass Restricted (MR) score, a composite proteomic profile with biological and clinical significance [149,150]. The MR score is calculated by summing the number of peaks identified at SELDI-TOF analysis and thus varies from 0 (no biomarkers present) to 4 (all four biomarkers present). In this way the proteomic information is digitized in a numerical variable that then can be manipulated using regular statistics. The biomarker peaks were determined to correspond to P1 (neutrophil defensins-2), P2 (neutrophil defensins-1), P3 (S100A12, calgranulin C) and P4 (S100A8, calgranulin A), all proteins with important roles in innate immunity. An important observation from a prospective study of 169 samples of amniotic fluid was that the biomarkers of the MR score appear in an ordered temporal sequence from P1 to P4, which corresponded biologically to the transition of the process of intra-amniotic inflammation from absent (M=0) to acute (MR 1 or 2, 93% of cases represented by the presence of defensins peaks) to chronic (MR 3 or 4). The transition from acute to chronic inflammation was rendered in all but one case of MR=3 by the appearance of the biomarker corresponding to S100A12 [150]. Reasoning that in addition to being biomarkers the proteins of the MR score may participate directly in modulating the intra-amniotic inflammation process and its consequent preterm birth
and fetal injury, our group sought to understand the pathophysiological implication of the observation of the elevated amniotic fluid S100A12.

S100A12 (also known as calgranulin C) is a member of the low-molecular weight EF-hand S100/calgranulin family [151] known to be expressed in granulocytes [152], monocytes [153] and select epithelial cells [154] and that characteristically accumulates at sites of chronic rather than acute inflammation [155]. S100A12 protein has recently gained increased attention as a ligand for RAGE [131], a property that led it to be named ENRAGE (extracellular newly identified RAGE-binding protein) [133]. In the first study to assess the presence and regulation of the S100A12/RAGE axis in human pregnancy [132] Buhimschi’s group found that 1) S100A12 is present in amniotic fluid of women with intra-amniotic infection in direct relationship with the degree of inflammation and severity of histological chorioamnionitis and funisitis, 2) S100A12 localized primarily to infiltrating inflammatory cells, 3) histological chorioamnionitis was associated with elevated S100A12 mRNA levels in fetal membranes but not placenta, and 4) amnion epithelial, decidual and extravillous trophoblastic cells in fetal membranes and placenta are potential sites of signaling via RAGE, which is ubiquitously expressed in these cell types. The group thus proposed that the low levels of endogenous sRAGE earlier in pregnancy (less than 30 weeks) may predispose to enhanced RAGE activation in the event of a microbial challenge, while the excess of sRAGE later in gestation (greater than 30 weeks) may act as a natural blocker of the NF-κB activation. This gestational age dependence of the inhibitor molecule sRAGE may be one potential explanation for the higher incidence of infection-related preterm deliveries and mostly PPROM at earlier gestational ages [132].
In a subsequent study [147], Buhimschi’s group found significantly lower sRAGE in human fetuses that mounted robust inflammatory responses. Moreover, in addition to S100A12 and S100A8, our group found that human amniotic fluid from pregnancies with intra-amniotic inflammation-induced preterm birth also contain elevated levels of HMGB1, a prototype DAMP and well-recognized RAGE ligand. Furthermore, in an animal model of endotoxin-induced fetal damage and preterm birth, we determined that inflammation induces a significant change in expression of RAGE and HMGB1 at sites of tissue injury localized in vital fetal organs. These results, some of which are included in Section 7 of this Thesis, indicate that RAGE activation fuelled by accumulation of HMGB1 may be an important mediator of the antenatal cellular damage in fetuses delivered in the setting of inflammation-induced preterm birth.

4.3. Particularities of RAGE activation in preeclampsia

The role of RAGE signaling in pregnancy-associated pathology has not been well studied. Recent research from Buhimschi’s laboratory showed that women with severe preeclampsia have increased serum and amniotic fluid levels of sRAGE as compared to gestational age-matched controls [146]. These findings are consistent with a state of RAGE pathway activation but in contrast to the findings of decreased sRAGE levels in fetuses exposed to a chronic state of intra-amniotic inflammation [147]. Since preeclampsia is known to associate with oxidative stress, Buhimschi’s group explored and found that sRAGE is released from amnion and maternal decidua in response to a free radical challenge ex vivo. In contrast, placental villous tissue (obtained at elective C-section at term) did not secrete detectable sRAGE or respond to either an oxidative or inflammatory challenge with elevated sRAGE, suggesting that the source of the elevated
sRAGE in preeclampsia may be in the fetal membranes rather than in the placenta [146]. Moreover, since the cord blood sRAGE levels of fetuses born in the context of severe preeclampsia did not appear significantly elevated, it was reasoned that the fetus is probably not affected and that the preeclampptic RAGE activation is likely confined to the maternal compartment. Regardless of its source, however, identifying the stimulus responsible for the maternal RAGE activation in preeclampsia was of particular interest to our laboratory and my studies, the findings of which are incorporated in Section 7 of this Thesis. Briefly, we explored whether prototype RAGE ligands, such as advanced glycation end-products (AGEs) or HMGB1, are differentially expressed in relationship with presence or severity of preeclampsia as well level of inflammation or oxidative stress, two RAGE-related pathogenic pathways known to be activated in preeclampsia in relationship with symptom severity.

4.4. Excess advanced glycation end-products (AGEs) as a stimulus for RAGE

Advanced glycation end products (AGEs) are a pleiotropic group of molecules formed from the nonenzymatic reaction of reducing sugars with free amino groups of proteins, lipids and nucleic acids. This reaction occurs often in cooking processes when sugars are heated together with fats or proteins (browning) and is referred to as the Maillard reaction after the French chemist Louis-Camille Maillard, who first described it chemically in 1912 as part of his PhD Thesis. The reaction between sugars and amino groups was previously described in 1908 by two Englishmen, Walter John Skyes and Arthur R. Ling, who while studying beer brewing processes theorized that the amber color of the beer results from a chemical reaction of sugars and proteins [156]. It was Maillard, however, who realized the implications of the reaction for other areas, including medicine, and
made the insightful statement that the reaction may lead to “better utilization of sugars by diabetics” [157].

Today it is well established that AGEs also form endogenously as a byproduct of normal metabolism and contribute to the normal processes of aging that affect tissue proteins. Under certain pathologic conditions, such as diabetes, Alzheimer’s disease, rheumatoid arthritis, systemic lupus erythematosus or other states characterized by oxidative stress, the formation of AGEs occurs at an extremely accelerated rate [158,159]. In such situations or in renal failure, when the clearance of AGEs is impaired, excess AGEs deposit in various tissues, as is the case with the well known hemoglobin A1c in diabetes, which has been linked to diabetic vascular complications [158]. Excess AGEs have also been implicated in the progression of neurodegenerative diseases and cancers [160]. Ingesting excess food AGES has been found to play an important role in the pathogenesis of AGE-related disorders as well [161].

Evidence suggests that the pathogenic role of accumulated AGEs is a consequence of their ability to stimulate RAGE, eliciting oxidative stress and subsequently altering gene expression in various types of cells. Furthermore, it has been demonstrated that excess AGEs generate auto-antibodies against the modified amino acid residues in an organism’s effort to clear the excess AGEs. \(\text{Ne-carboxymethyl-lysine (CML)}\) is a prototype AGE and a major antigenic structure \textit{in vivo} that has been closely linked to oxidative stress and chronic inflammatory conditions via RAGE activation and anti-CML antibody formation [162]. CML, which was the first AGE to be identified \textit{in vivo}, can be formed by pathways alternative to the Maillard reaction (such as auto-oxidation). In fact, CML has received great interest in non-hyperglycemic diseases
characterized by oxidative stress and inflammation, such as cardiovascular hypertensive disorders and atherosclerosis, where it has been linked to endothelial dysfunction [163].

Given that preeclampsia unanimously exhibits oxidative stress, inflammation and endothelial dysfunction in the placenta and maternal vascular system, we sought to determine whether elevated maternal serum AGEs could explain these pathophysiologic findings. Prior to our studies, one report found that CML levels appeared elevated in preeclamptic women compared to healthy pregnant and non-pregnant women, and preeclamptic placentas presented higher CML staining intensity compared to controls [164]. Furthermore, skin autofluorescence, a non-invasive measure of tissue AGE accumulation, was also increased in women recently diagnosed with preeclampsia [165]. Neither study, however, included a group of pregnant hypertensive but non-preeclamptic women to delineate whether the increased AGEs represent a primary event related to preeclampsia pathogenesis or secondary to hypertension. Moreover, as the renal clearance parameters change in pregnancy, serum CML levels would have to be interpreted together with the kidney’s ability to clear CML, and prior to our investigation no published study examined these aspects in relationship to the level of RAGE activation.

Pregnancy is known to produce dramatic changes in volume and composition of the body fluids consistent with an increase in plasma volume of up to 40 percent and a dilutional fall in hematocrit and plasma albumin concentration, as well as increased renal blood flow and glomerular filtration rate up to 50 percent by the end of the first trimester. In preeclampsia, however, the rise in glomerular filtration rate observed in normal pregnancy is virtually absent [166]. Furthermore, the increase in urinary protein excretion
in preeclampsia that occurs secondary to alterations in the size and/or charge selectivity of the glomerular filter, increase in glomerular capillary pressure and compromise of proximal tubular reabsorption may also non-specifically affect blood levels of analytes with renal excretion [166]. Calculation of fractional excretion (clearance ratios) or analyte levels after correction for proteinuria, therefore, may help better elucidate whether any increased serum values are due to increased production or decreased excretion [167,168].

4.5. **HMGB1 as a stimulus of RAGE**

Originally described as a DNA-binding protein that stabilizes nucleosomes and facilitates transcription [169], HMGB1 is expressed in all cells and preferentially localized in the cell nucleus. Recently, HMGB1 has also been found to have extracellular roles as a potent cytokine and delayed mediator of inflammation. Damaged and necrotic cells release HMGB1 into the extracellular space, exhibiting cytokine-like activity and triggering inflammatory responses. In contrast, apoptotic cells retain HMGB1 within the intracellular space and consequently do not promote inflammation. Activated macrophages, mature dendritic cells and natural killer cells are able to secrete HMGB1 in response to injury, infection and inflammatory stimuli and, in contrast to non-immune cells, are protected against its toxic effects [74,75,76,77]. After it has been released in the extracellular space, HMGB1 engages pattern recognition receptors, such as RAGE, TLR-2 and TLR-4, to activate innate immunity via NF-κB transactivation, thus acting as a prototype DAMP.

Recently it was discovered that HMGB1 is a delayed mediator of endotoxin lethality [170]. Unlike TNFα, which is produced by macrophages within hours after
activation, HMGB1 is released for days after endotoxin exposure. Injection of recombinant HMGB1 into mice is lethal and causes clinical signs of sepsis and multiple organ failure [171]. HMGB1 detection in serum is delayed by 12 to 18 hours after peak levels of the classical early pro-inflammatory mediators TNFα and IL-1, and LPS, TNFα and IL-1 all have been demonstrated to cause extracellular release of HMGB1 [170, 171]. Oxidative stress is another known trigger of HMGB1 release [74].

To date, there has been no published systematic study of HMGB1 in pregnancy complications. One immunohistochemistry study examined placentas from a group of normal pregnancies at term compared to a group of preeclamptic women (term and preterm) and found that HMGB1 was present in both tissues at similar levels, although visually the decidua of preeclamptic women appeared more intensely stained [172]. Prior to our studies, there are no reports in the literature related to HMGB1 levels in relationship to HMGB1 in intra-amniotic infection, inflammation or processes leading to fetal cellular damage.

4. 6. The S100 protein, S100B, as a stimulus for RAGE

S100B is a member of the S100 family of proteins, calcium-binding proteins that contain two canonical EF-hand structural motifs [173], and is abundantly expressed in the central nervous system [174]. In general, relatively high concentrations of S100 proteins are required for activation of RAGE in responsive cells. Compared to other S100 proteins, S100B is unique in that it can engage RAGE in neurons at both low and high concentrations with divergent trophic or toxic effects. In the human brain S100B is mainly synthesized by astroglial cells and its physiological roles are important in regulating cell cycle and modulating long-term synaptic plasticity [175]. Changes in brain homeostasis
consistent with cell damage or cell stress trigger secretion and release of S100B in the extracellular space, where its action depends remarkably on its concentration [174,176]. At nanomolar levels S100B is neuroprotective while micromolar concentrations of S100B promote apoptosis and cellular damage via overproduction of reactive oxygen species [174]. Typically S100B is referenced as the homodimer of two S100β chains. Higher order S100β assemblies (hexamers, tetramers, octamers), however, have been observed in human brain extracts and are thought to act as high affinity extracellular RAGE activators through receptor multimerization, which could represent a distinct and higher order regulatory level of S100B’s RAGE-dependent activity aside from its concentration [177]. Important for neuro-inflammation is the co-release of HMGB1 with S100B, resulting in a shift in the effects of physiological S100B concentrations from homeostatic to cell damaging [178]. Thus, S100B-HMGB1-RAGE interactions may have important consequences during development as well as in inflammatory and oxidative states with a central nervous system component.

Transgenic mice over-expressing S100B have been shown to exhibit increased vulnerability to perinatal hypoxic-ischemic injury, with heightened glial activation, neuro-inflammation and increased mortality [179]. S100B increases in cerebrospinal fluid (CSF) of infants affected by perinatal asphyxia and post-hemorrhagic brain damage [180, 181]. In this setting, S100B levels in CSF correlate with the extent of brain lesions, long-term neurological outcome and neurological impairment or death at one year of age [180,181]. Under the assumption that S100B is released from the damaged tissue during active brain injury, previous studies attempted to determine the usefulness of measuring S100B in blood as a predictor of hypoxic-ischemic encephalopathy [182]. Increased
concentrations of S100B have been detected 48 to 72 hours before clinical, laboratory or ultrasound signs of cerebral bleeding in preterm infants [183]. An animal model demonstrated that intravenous endotoxin administration to pregnant sheep caused fetal white matter damage, inflammation and an increase in S100B release in fetal blood at one and three hours after administration [184]. The studies exploring the relationships between S100B and fetal inflammation, however, are limited. One report examined the levels of S100B in amniotic fluid and found that although intra-amniotic infection elevated the S100B concentration, the elevation appeared unrelated to neonatal outcome and thus concluded that amniotic fluid S100B may reflect intra-amniotic inflammation but not necessarily fetal neurological damage [185]. An alternative explanation may be that tissues other than the fetal brain also contribute to the pool of amniotic fluid S100B, making it difficult to determine which portion of S100B is of fetal neural origin. No prior study has attempted to determine relationships between severity of intrauterine inflammation and release of S100B in fetal circulation or to correlate levels of S100B to fetal levels of other late-phase cytokines (e.g., HMGB1) or to indicators of morbidity in premature newborns. As detailed in Section 7 of this Thesis, we found that fetal S100B levels are elevated in relation to cord blood HMGB1 in intrauterine infection.
5. STATEMENT OF PURPOSE

The hypothesis that stays at the basis of the research presented in this Thesis is that the difference in the types of RAGE activation that occur preterm fetuses born in the context of intrauterine infection and inflammation and in women with preeclampsia is driven by differences in the stimuli that engage RAGE in these two very different clinical scenarios. If this hypothesis holds true, agents targeted against RAGE pathway activation either by binding the excessive specific stimuli or blocking downstream RAGE signaling elements may have potential therapeutic value for preventing occurrence or exacerbation of preeclampsia symptoms or fetal damage. A detailed knowledge of the nature, source and levels of molecules capable of activating the maternal or fetal RAGE axis and resulting in pregnancy complications leading to spontaneous or indicated preterm birth is, therefore, necessary and the purpose of the research presented herein.

5.1. Specific Aims

Specific Aim 1. Identify putative stimuli driving fetal RAGE activation in pregnancies complicated by preterm birth and intra-amniotic inflammation.

To achieve this aim in case control study design our objectives were as follows:

a) To determine the relationships between severity of intra-amniotic inflammation and amniotic fluid levels of HMGB1.

b) To determine the relationships between severity of fetal inflammation (as reflected by cord blood levels of the acute phase cytokine IL-6) and fetal cord blood HMGB1 and S100B concentrations.

c) To determine the relationships between cord blood levels of the late phase cytokine
HMGB1 and S100B concentration.

d) To determine levels and patterns of HMGB1 expression in placenta, amniochorion and umbilical cord in relationship to histological evidence of chorioamnionitis and funisitis.

e) To examine the pattern of HMGB1 release in response to endotoxin challenge *ex vivo*.

**Specific Aim 2. Identify putative stimuli driving RAGE activation in pregnancies complicated by preeclampsia.**

To achieve this aim in case control study design our objectives were as follows:

a) To determine whether blood and urine levels of conventional RAGE ligands, such as the prototype advanced glycation end-product Nε-carboxymethyl-lysine (CML), and anti-CML auto-antibodies, a reflection of excess CML antigenicity, are elevated in women with severe preterm preeclampsia as compared to gestational age matched controls.

b) To determine whether blood and urine levels of HMGB1 (amphoterin) are elevated in women with severe preterm preeclampsia as compared to gestational age matched controls.

c) To determine whether fractional excretion indicators of CML, anti-CML auto-antibodies and HMGB1 are altered in women with severe preeclampsia and gestational age matched controls.

d) To determine whether significant changes in either blood or urine levels or fractional excretion indicators of the above analytes are either gestational age- or hypertension-related rather than preeclampsia-related and could be thus deemed nonspecific. The above results were therefore interpreted relative to those of pregnant normal women at term or women with chronic hypertension who did not develop preeclampsia.
e) To determine the relationships between levels of HMGB1, CML and anti-CML auto-antibodies and levels of systemic inflammation or RAGE activation as reflected by maternal blood concentrations of TNFα and sRAGE, respectively.

f) To determine levels and cellular sites of expression of CML and HMGB1 in placenta and amniochorion of women with severe preterm preeclampsia as compared to non-preeclamptic women with either a term elective C-section delivery or spontaneous idiopathic preterm birth (i.e. absence of clinical or histological evidence of infection, inflammation or abruption).
6. METHODS

All procedures and experiments described in this section were conducted by me unless otherwise noted.

6.1. Patient population, biological samples, clinical definitions and clinical laboratory analyses.

Biological samples (maternal blood, maternal urine, amniotic fluid and cord blood) were collected from women recruited at Yale-New Haven Hospital (YNHH) following admission to Labor and Birth or assessment in high- or low-risk antepartum units. Patient enrollment was performed depending on clinical presentation in two prospective cohorts, “rule-out preeclampsia” or “rule-out preterm birth,” pursuant to protocols approved by the Human Investigation Committee of Yale University. For both enrollment cohorts patients were followed until delivery or discharge, and medical record data was entered prospectively in a de-identified perinatal research database that links the medical record information (including laboratory results and neonatal assessments for the newborns admitted to Newborn Special Care Unit) with the codes on the stored biological samples. This database is updated continuously by specific research staff. Patients included in the analyses herein were recruited from May 2004 to October 2007. The decision to recommend admission, any clinically indicated laboratory tests or procedures, including amniocentesis or delivery of the fetus, was made by the primary physician, independent of the research protocols. Patient recruitment, sample processing and abstraction of data from medical records were performed by medical or research staff as appropriate and were independent of me.
After collection, samples for research were transported to the laboratory where they were spun at 3000g and 4°C for 20 minutes, aliquoted in polypropylene cryotubes and stored at -80°C until analysis.

Maternal serum and urine samples were collected contemporaneously as previously described [167,168] and selected based on availability from women originally enrolled in the “rule-out preeclampsia cohort.” The urine sample (5-10 mL) was collected in a sterile container (“clean catch” method or bladder catheterization if performed for clinical indications). Blood samples were collected by venipuncture prior to intravenous administration of fluids and allowed to clot.

Gestational age was established based on last menstrual period and/or early ultrasound evaluations (less than 20 weeks of gestation) in all cases [186]. Severe preeclampsia was defined based on the American College of Obstetricians and Gynecologists criteria [187] as gestational age greater than 20 weeks, blood pressure of 160 mm Hg systolic or higher or 110 mm Hg diastolic or higher on 2 occasions at least 6 hours apart, and/or proteinuria of at least 5 g in a 24-hour urine specimen or 3+ or greater on 2 random urine samples collected at least 4 hours apart [187]. Other elements of the diagnosis included: intrauterine growth restriction (less than 10th percentile), cerebral or visual disturbances (headache, visual changes), epigastric or right upper-quadrant pain, pulmonary edema or cyanosis, oliguria (urinary output less than 500 mL in 24 hours) or elements of HELLP syndrome, such as impaired liver function tests (greater than two times normal) and/or thrombocytopenia (less than 100,000 cells/µL). Chronic hypertension was defined as a sustained elevation in blood pressure greater than 140/90 mmHg before pregnancy or before 20 weeks gestation. Since preeclampsia is a
progressing disease and by definition a clinical diagnosis for which no acceptable gold standard is yet available, our data was also analyzed based on an outcome measure (the need for delivery for preeclampsia) rather than solely by clinical classification at enrollment. Similar to our prior study, we reasoned that an indication for mandated delivery belongs to a team, is the last management resort when all other strategies have failed and its resulting outcome is final, cannot be revoked and is thus less subject to bias [188].

Selection of samples from the biological repository and assignment to either control or study groups for the purpose of the analyses included herein was based on clinical diagnoses at sample collection and/or outcome and was done not by me and prior to my evaluation of analytes of interest or statistical analysis. None of the originally selected samples was excluded from the final analysis.

Amniotic fluid was collected from women pregnant with singletons originally enrolled in the “preterm birth cohort” who had a clinically indicated amniocentesis to rule-out infection as deemed necessary by managing physicians and independent of the research protocol. Amniotic fluid was collected by ultrasound-guided amniocentesis, and each woman was followed prospectively until delivery.

Eligible women had a gestational age at delivery greater than or equal to 23.1 weeks, preterm labor contractions refractory to tocolysis, preterm premature rupture of membranes (PPROM) or advanced cervical dilatation (greater than 3 cm). Exclusion criteria were the presence of anhydramnios, human immunodeficiency or hepatitis viral infections, congenital anomalies, abnormal karyotype or any known maternal medical conditions. Preterm labor was defined as the presence of regular uterine contractions and
documented cervical effacement and/or dilatation in patients less than 37 weeks of gestation. PPROM was confirmed by vaginal amniotic fluid pooling, nitrazine, ferning or an amniocentesis-dye positive test. Corticosteroids and antibiotics were recommended as clinically indicated. The neonatology resuscitation team was present at the time of delivery for all neonates.

Clinical laboratory tests performed for the purpose of diagnosing infection or inflammation included glucose, lactate dehydrogenase (LDH), white blood cell (WBC) count, Gram stain and amniotic fluid cultures. For clinical management, an amniotic fluid glucose cut-off of less than or equal to 15 mg/dL and LDH levels greater than or equal to 419 U/L were considered suggestive of intra-amniotic infection [189,190].

Amniotic fluid cultures were performed by the microbiological laboratory. Briefly, the fluid was centrifuged and the sediment re-suspended in 0.25 ml of supernatant and cultured for aerobic bacteria, including *Ureaplasma* and *Mycoplasma spp*, using laboratory media such as Chocolate, Martin Lewis, MacConkey, Azido benzoic acid, Thioglycollate, Bacteroides Bile Esculin/Laked Blood Kanamycin Vancomycin (BBE/LKV), Columbia CNA agar, *Ureaplasma* broth and agar. In addition, amniotic fluid was cultured in an anaerobic chamber (Forma Anaerobic System, Thermo Electron Co, Waltham, MA) at 37°C. The results of the microbiological tests were available for case management and were reported as final after five days of culturing. Presumptive bacterial identification was based on standard microbiological criteria of colonial morphology, medium reaction, Gram stain and the use of a VITEK 2 automated card system (bioMérieux, Hazelwood, MO, http://www.biomerieux-usa.com) for bacterial identification based on biochemical tests and antibiotic susceptibility. Amniotic
fluid not used for clinical purposes was transported to the research laboratory and processed as described earlier.

In addition to the amniotic fluid from the women enrolled in the “preterm birth cohort,” we analyzed two groups of normal pregnant women with either early gestational age (2nd trimester) amniocentesis to rule out chromosomal abnormalities or near-term (3rd trimester) amniocentesis for lung maturity testing. These two groups of women represent virtually the only ethically acceptable circumstances to retrieve amniotic fluid from women without clinical symptoms of preterm birth and are necessary to include in analyses when testing for potential gestational age regulation of sought analytes.

Cord blood specimens were obtained for clinical assessment of the fetal acid-base status at birth and/or research purposes. Within 10 minutes of delivery, cord blood samples (umbilical artery and vein) were collected in pre-heparinized 1 cc. syringes, capped and transported to the laboratory. The acid-base status was determined with the ABL 800 FLEX blood gas analyzer (Radiometer Medical A/S, Denmark). Umbilical cord blood for research was obtained by aseptic puncture of the clamped umbilical vein, allowed to clot, centrifuged as described above and serum aliquoted in sterile polypropylene tubes and stored at –80°C.

Histological evaluation of the placenta is routinely performed for all preterm birth cases at Yale-New Haven Hospital by staff affiliated with Department of Pathology. Hematoxylin and eosin stained sections of extraplacental membranes (amnion and chorio-decidua), chorionic plate, chorio-decidua and umbilical cord were examined systematically for inflammation. Three histological stages of chorioamnionitis [191] (stage I: intervillositis, stage II: chorionic inflammation, and stage III: full-thickness
inflammation of both chorion and amnion) were complemented by the described histological grading system derived by Salafia et al., which includes four grades of inflammation of the amnion, chorio-decidua and umbilical cord [192].

6. 2. Evaluation of presence and severity of intra-amniotic inflammation by SELDI-TOF mass spectrometry and Mass Restricted (MR) scoring

Evaluation of amniotic fluid samples by mass spectrometry for determination of the MR score was conducted by laboratory personnel other than me. The method for generation of the MR score has been previously described [23,149,150]. The MR score ranges from 0 to 4, depending upon the presence or absence of each of the four protein biomarkers [149,150]. A value of 1 was assigned if a biomarker peak was present and 0 if absent. Based on our previous results, we stratified the study population based on the severity of inflammation (MR 0: no inflammation; MR 1-2: minimal inflammation; MR 3-4: severe inflammation) [150].

6. 3. Immunoassay procedures

ELISA for CML. 50 µL of undiluted urine samples and serum samples diluted 1:4 were mixed with 50 µL anti-CML-adduct monoclonal antibody and plated into wells pre-coated with CML-BSA (Circulex, MBL International, Woburn, MA) and incubated for one hour at room temperature. After washing and a one hour incubation with an HRP conjugated polyclonal antibody specific for mouse IgG, plates were washed, and substrate solution was added to the wells, and color development was allowed to proceed for 15 minutes, at which time stop solution was added and the plates read at 450nm with background correction at 650 nm using a VERSAmax™ microplate reader with Softmax
Pro 3.1.1 software (Molecular Devices, Sunnyvale, CA). The minimum detectable concentrations reported by manufacturers and validated in our laboratory is 0.13 ng/mL.

**ELISA for anti-CML antibody.** 50 µL of undiluted urine samples and serum samples diluted 1:10 were plated into wells pre-coated with CML-BSA or BSA (Circulex, MBL International, Woburn, MA) and incubated for one hour at room temperature. Plates were washed and incubated for one hour with an HRP conjugated polyclonal antibody specific for rat IgG. Plates were washed, and substrate solution was added to the wells, and color development was allowed to proceed for 15 minutes, at which time stop solution was added and the plates read at 450nm using a microplate reader. The readings from the BSA-coated plate were subtracted from the readings from the CML-BSA-coated plate.

**ELISA for HMGB1.** 10 µL of urine, maternal serum, amniotic fluid or cord blood serum samples were plated into wells pre-coated with a polyclonal antibody specific for HMGB1 (Shino-Test, Kanagawa, Japan) and incubated for 22 hours at 37°C. Plates were washed and incubated for 2 hours with a POD-conjugate anti-HMGB1,2 monoclonal antibody. After washing, substrate solution was added to the wells, and color development was allowed to proceed for 30 minutes, at which time stop solution was added and the plates read as described above. The minimum detectable concentration is 1 ng/mL.

**ELISA for S100B.** 100 µL of cord blood serum samples diluted 1:4 with sample dilution buffer were plated into wells pre-coated with polyclonal anti-cow S100B antibody (BioVendor, Candler, NC) and incubated for 90 minutes at room temperature. Plates were washed, and, after a 90 minute incubation with a monoclonal anti-human S100B antibody labeled with HRP and another wash, substrate solution was added to the wells,
and color development was allowed to proceed for 15 minutes, at which time stop solution was added and the plates read at 450nm using a microplate reader. The minimum detectable concentration was 20 pg/mL.

**ELISA for sRAGE.** 50 µL of undiluted maternal serum samples were plated into wells pre-coated with a monoclonal antibody specific for RAGE (R&D Systems, Minneapolis, MN) and incubated for 2 hours at room temperature. Plates were washed then incubated for 2 hours with an enzyme-linked polyclonal antibody specific for the extracellular domain of RAGE. Plates were washed, and substrate solution was added to the wells, and color development was allowed to proceed for 30 minutes, at which time stop solution was added and the plates read at 450nm using a microplate reader. The minimum detectable concentration was 4 pg/mL.

**ELISA for TNFα.** 200 µL of undiluted maternal serum samples were plated into wells pre-coated with a polyclonal antibody specific for mouse TNFα (R&D Systems, Minneapolis, MN) and incubated for 3 hours at room temperature. After washing and a 2-hour incubation with an enzyme-linked polyclonal antibody specific for mouse TNFα, plates were washed, and substrate solution was added to the wells and incubated for one hour. Amplifier solution was added, and color development was allowed to proceed for 30 minutes, at which time stop solution was added and the plates read at 450nm using a microplate reader. This assay detects the total amount of TNFα in the sample composed of free TNFα and that bound to soluble receptors. The mean minimal detectable concentration in the assays for TNFα reported by the manufacturer was 0.106 pg/mL. The intra- and inter-assay coefficients of variation were <3.1% and 7.1%, respectively.
**ELISA for IL-6.** Immunoassays for human IL-6 (Pierce-Endogen, Rockford, IL) was performed on amniotic fluid or cord blood in duplicate according to manufacturer’s instructions by laboratory research staff excluding me. The minimal detectable concentration was 1 pg/mL and the inter- and intra-assay coefficients of variation <10%. Samples were diluted appropriately to assure that the final values fall inside the standard curve and are interpolated rather than extrapolated.

6.4. **Immunohistochemical procedures and Western blotting**

Five µm paraffin sections were deparaffinized in xylene and rehydrated with graded ethanol to potassium-phosphate-buffered saline solution, pH 7.2. Following antigen retrieval with citrate buffer (for AGEs) or basic solution buffer pH 10 (for HMGB1), the sections were pretreated with 1% hydrogen peroxide for 15 minutes followed by one-hour incubation in 5% donkey serum. The sections were then incubated overnight at 4°C with a primary antibody. Detection was performed with biotinylated donkey anti-rabbit or goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, 1:600 dilution) as appropriate followed by avidin-biotin staining (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA) and incubated with 3,3’-diaminobenzidine/nickel sulfate as chromogen solution. Specificity of staining was confirmed by omitting the primary antibodies. Specific staining was evaluated semi-quantitatively in a blinded fashion by examining six fields per slide and subjectively scoring on a scale from 0 (no staining) to 5 (intense blue-black staining) the intensity of the chromogen deposited in the amnion epithelium, chorio-decidua, placental villous trophoblast, stromal and endothelial cells. Slides stained immunohistochemically purposefully were not counterstained so that morphological changes were hidden to the examiner. For illustration purposes select
sections were developed using Vector NovaRed (Vector) chromogen and were
counterstained with hematoxylin.

Primary antibodies used for immunohistochemistry included anti-AGE antibody
(Clone 6D12, CosmoBio, Tokyo Japan, 1:8000 dilution), rabbit polyclonal anti-HMGB1
antibodies (Shino-Test, Kanagawa, Japan 1:1000 dilution) and goat polyclonal anti-
RAGE (R&D Systems, 1:100 dilution). A substantial amount of time was spent
identifying a specific anti-HMGB1 antibody. Other HMGB1 antibodies (obtained from
Abcam, Boston, MA or from Millipore, Billerica, MA) were rejected by us based on
concerns regarding specificity at Western blot analysis. The Shino-Test antibody was
determined to be the only antibody specific for HMGB1 and had to be shipped from
Japan, requiring USDA approval for importation.

Western blot for HMGB1 was necessary to confirm antibody specificity as
described above. Gel electrophoresis was carried out on 10% SDS-PAGE gels using a
BioRad Miniprotean II (Bio-Rad, Richmond, CA) gel apparatus. Ten microliters of
amniotic fluid were diluted 1:2 volume/volume with electrophoresis sample buffer
(BioRad, Richmond, CA) and reduced by boiling for 5 minutes. After electrophoretic
transfer to a PVDF membrane (BioRad) at 100 V for 60 minutes, blots were incubated in
3% hydrogen peroxide for 15 minutes then blocked with 5% milk and incubated
overnight at 4°C with different rabbit anti-HMGB1 primary antibodies diluted 1:500 or
1:1000. Blots were incubated with conjugated F(ab’)2 goat anti-rabbit IgG 1:1000 in 5%
milk as secondary antibody followed by streptavidin-biotinylated HRP 1:8000. Blots
were subsequently subjected to ECL using a Western blotting detection system
(Amersham, Arlington Heights, IL). Autoradiography film was applied to the blot until
satisfactory exposure was achieved. Antibody specificity was confirmed by omitting the primary antibody.

6. 5. Urine and serum protein, creatinine quantification and calculations of fractional excretion indicators

For total protein measurements, urine or maternal serum samples were diluted 1:12 or 1:60, respectively, with deionized water and 10 µL of the diluted sample plated in duplicate. 200 µL of 1:50 bicinechonic acid reagent mixture (BCA protein assay, Thermo Scientific, Rockford, IL) was added and incubated 20 minutes at 37°C then read at 500nm using a microplate reader against human serum albumin standards.

For creatinine measurements, urine samples were diluted 1:100 and serum samples 1:8 with picric acid (Stanbio Laboratory, http://www.stanbio.com) and spun at 12,000 rpm for 30 minutes. 1:4 dilutions of 3% NaOH and sample supernatant were plated in duplicate and incubated at room temperature for 10 minutes then read at 500 nm using a microplate reader against standard curves derived from known creatinine concentrations.

Fractional excretion indicators (clearance ratios) were calculated using the formula: (urine/plasma analyte concentration) ÷ (urine/plasma creatinine concentration) x 100. The fractional excretion of a substance represents the proportion of the substance excreted in the urine compared to that filtered by the glomeruli and is generally reported relative to creatinine clearance since creatinine is neither reabsorbed nor significantly secreted.
6.6. Placental explant experiments

Placentas were obtained from healthy women without any significant past medical history undergoing scheduled, elective cesarean delivery in the absence of labor (GA: 38-40 wks). Indications for abdominal delivery included elective repeat or primary cesarean delivery for fetal malpresentation (e.g., frank breech). No patient had abnormal placentation (placenta previa, abruption). All infants were appropriately grown for gestational age and had reassuring fetal heart rate patterns prior to surgery. Placental cotyledons from the central part of the placenta and amniochorion at distance from the membrane rupture site were removed under sterile conditions within 30 minutes of delivery and dissected in a Petri dish into pieces of similar weight and washed thoroughly with ice-cold saline. Four pieces (approximately 100 mg wet weight) were cultured freely suspended in 24-well plates in 1.5 mL RPMI 1640 medium (Gibco, Grand Island, NY) containing 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco). Cultures were maintained at 37°C in a humidified gas mixture of 5% CO2-95% air in the presence and absence of 1 µg/mL lipopolysaccharide from E. Coli (LPS). After 18 hours of incubation, the supernatants were collected, centrifuged to remove cellular debris and stored at –80°C. The incubated tissue was immediately homogenized in 1 mL cell extraction buffer (20-mmol/L Tris-HCl, 150-mmol/L NaCl, 1% Triton X-100, 1-mmol/L phenylmethylsulfonyl fluoride, and Complete protease inhibitor cocktail [Roche, Indianapolis, IN]). Specimens were spun at 1000 g at 4°C for 15 minutes and protein quantification in incubated tissue performed using the BCA protein assay as described above. The analytes’ explant medium concentration was normalized to total protein in tissue extract to correct for variations in tissue incubated per each well. For each
experimental condition, values were derived by averaging normalized values from duplicate wells either without (untreated) or with LPS. Values were further interpreted as fold change from the untreated level. All chemicals were from Sigma Chemical Co. (St Louis, MO) unless otherwise specified. The time of incubation (18 hours) was selected to assure tissue viability as assessed by a non-significant change in release of the intracellular enzyme LDH into the incubation medium LDH activity was measured using LDH Liqui-UV® Assay (Stanbio, Boerne, TX).

The explant experiments were set up by laboratory personnel other than me. I analyzed the stored medium for level of HMGB1.

6. 7. Statistical analysis

Data were tested for normality using the Kolmogorov-Smirnov test and reported as mean and standard error or median and ranges, depending on the type of data distribution. Comparisons between two groups were performed using Student t tests or Mann-Whitney rank-sum tests, as appropriate. Multiple comparison procedures were performed using one-way or Kruskal-Wallis analysis of variance (ANOVA) followed by Student-Newman-Keuls or Dunn’s post-hoc comparisons, as appropriate. Relationships between variables (correlations) were explored using Pearson’s product moment or Spearman’s rank-order correlations. Comparison between correlations was achieved based on z statistic. Multivariate linear analysis was performed using a stepwise model with variables eliminated based on \( P > 0.1 \). MedCalc (Broekstraat, Belgium) or SigmaStat 2.03 (Systat Inc. Chicago, IL) statistical softwares were used as aids for analysis. A \( P < 0.05 \) was judged to denote statistical significance.

For Specific Aim 1, sample size calculations were based on our prior data with IL-6
and severity of inflammation by the MR score [150]. It was estimated that 28 women in each group would be necessary to detect differences of 85 percent of the standard deviation in a 3 group ANOVA. For Specific Aim 2, sample size calculations were based on our prior data of serum levels of TNFα [168]. It was estimated that 27 patients in each group would be necessary to detect differences of 78 percent of the standard deviation. Additional enrolled subjects will facilitate a finer comparison between groups required to account for confounders and possible effect modifiers.
7. RESULTS

7.1. Specific Aim 1. Identify putative stimuli driving fetal RAGE activation in pregnancies complicated by preterm birth and intra-amniotic inflammation

HMGB1 levels are increased in amniotic fluid of women with intra-amniotic inflammation and preterm birth, and the source may be the damaged fetal membranes.

We first asked the question whether amniotic fluid HMGB1 concentration exhibits gestational age regulation in the absence of infection or inflammation. For this purpose, we analyzed only amniotic fluid from women with normal outcomes who had an amniocentesis for either genetic indications (n=25), lung maturity (n=25) or suspicion of intra-amniotic infection that was ruled out and who eventually delivered at term (n=25). We found that although detectable by immunoassay the amniotic fluid HMGB1 concentration did not vary significantly across gestation (one-way ANOVA \( P=0.400 \); Figure 4).

To provide evidence that HMGB1, a prototype DAMP, is a key component of the process of intra-amniotic inflammation, we evaluated its levels in 132 samples of amniotic fluid retrieved from women whose pregnancies were complicated by preterm birth, had a clinically indicated amniocentesis to rule out infection and/or inflammation and resulted in a newborn admitted to the Newborn Special Care Unit. The median
gestational age at amniocentesis was 29 [range: 18-34] weeks and at delivery was 30 [23-34] weeks. Thirty-one percent of the newborns had a birthweight less than 1,000 grams, and 58 percent were less than 1,500 grams, illustrating the clinical relevance of this prospective cohort. We determined that women with severe intra-amniotic inflammation by proteomic analysis (MR 3-4) had significantly increased amniotic fluid HMGB1 concentration compared to women with absent and mild inflammation (Kruskall Wallis ANOVA P<0.001; Figure 5A). There was a direct and significant correlation of amniotic fluid HMGB1 to severity of intra-amniotic inflammation by MR score (P<0.001). In multivariate regression analysis amniotic fluid HMGB1 levels were predicted by the combination of an MR 3-4 and low amniotic fluid sRAGE inhibitor concentration, independent of gestational age at amniocentesis (P<0.001) or membrane status (ruptured or intact). Amniotic fluid HMGB1 also correlated significantly with amniotic IL-6 levels (R=0.702; P<0.001; Figure 5B).

**Figure 5:** (A) HMGB1 levels in amniotic fluid (AF) of women with preterm birth (GA at delivery 30 [23-34] wks, n=132) and absent (MR 0), mild (MR 1-2) or severe (MR 3-4) intra-amniotic inflammation (IAI); (B) Relationship of amniotic fluid acute phase cytokine (IL-6) to the late phase cytokine (HMGB1) in the same patient population.
We next sought to determine the source of amniotic fluid HMGB1 in pregnancies complicated by severe intra-amniotic inflammation. By immunohistochemistry we found that HMGB1 was highly expressed in fetal membranes and cord vessels and even more so in women with severe intra-amniotic inflammation (Figure 6). HMGB1 staining was particularly intense in necrotic choriodecidual areas. Placental villi had low HMGB1 expression irrespective of inflammatory status (not shown).

**Figure 6**: HMGB1 staining in fetal membranes (a & b) and umbilical cord (c) in a patient with preterm birth and no inflammation (MR=0) as compared to a patient with severe intra-amniotic inflammation (MR 3-4), histological chorioamnionitis (b) and funisitis (d & e). Note the intense positive staining in necrotic zones of chorio-decidua (b: arrowheads). Bar: 50 µm (a & b) or 25 µm (c-e). Lu: vessel lumen; UA: umbilical artery; UV: umbilical vein. Chromogen: Vector NovaRed. Hematoxylin counterstain.

**Figure 7**: HMGB1 (a) and RAGE (b) staining in fetal membranes in a patient with preterm birth, severe intra-amniotic inflammation and necrotizing chorioamnionitis. (c) Negative control slide with omitted primary antibody. Note the intense positive staining of both HMGB1 and RAGE in the amnion. Bar: 50 µm. Chromogen: (a) Vector NovaRed and Hematoxylin counterstain; (b & c) Ni-DAB, no counterstain.
Importantly, we observed that staining for the RAGE receptor in amniotic membranes of women with histological chorioamnionitis localizes to the same cellular injury areas as HMGB1 (Figure 7), suggesting that the HMGB1-RAGE axis may play a role in the damage of the amniochorion in the context of intra-amniotic inflammation.

**HMGB1 is released by human fetal membranes ex vivo in response to endotoxin and oxidative stress.**

To further determine whether the amniochorion contributes to the amniotic fluid pool of HMGB1 as a source or whether the highly hydrophobic HMGB1 formed elsewhere simply infiltrates the damaged amnion and deposits within the chorio-decidua, we challenged explants of either human fetal membranes or placental villous tissue obtained from elective Cesearean sections at term (n=4) with endotoxin *ex vivo* (LPS, 1 mg/mL). We observed a significant elevation in HMGB1 released in amniochorion medium (Figure 8, *P*=0.008), while release by the placental villous explants remained virtually unchanged (not shown, *P*>0.05).

![Figure 8: (A) HMGB1 release was measured by ELISA in the medium of amniochorion explants at 18 hours of incubation. Data was normalized for total protein in the incubated tissue. Tissues were obtained under sterile conditions from 4 women undergoing elective C-sections at term. Statistical analysis: Mann Whitney test.](image-url)
Cord blood HMGB1 levels do not correlate with the extent of intra-amniotic inflammation but rather with the extent of fetal inflammation and S100B.

We next sought to determine whether the level of intra-amniotic inflammation and amniotic fluid HMGB1 translate into an increased HMGB1 concentration in umbilical cord blood. For this purpose we analyzed available cord blood samples of newborns of mothers where we also measured amniotic fluid HMGB1 (n=121). Interestingly, we did not determine that cord

![Graphs showing relationships between cord blood HMGB1 and amniotic fluid cytokines and S100B](image)

**Figure 9:** (A) Relationships of cord blood HMGB1 with amniotic fluid levels of the acute phase cytokine IL-6 and (B) amniotic fluid HMGB1. (C) Relationships of cord blood HMGB1 to fetal inflammatory status as assessed by cord blood levels of IL-6. (D) Relationship of cord blood HMGB1 and cord blood levels of S100B, another DAMP.
blood HMGB1 levels are impacted by amniotic fluid inflammation as assessed either by the MR score ($P=0.232$) or by levels of amniotic fluid IL-6 (Figure 9A). Cord blood HMGB1 also did not correlate with amniotic fluid HMGB1 (Figure 9B). This lack of significant relationships maintained even when data was analyzed in multivariate analysis and other parameters were included in the model (gestational age, severity of histological chorioamnionitis, funisitis, status of the membranes and interval amniocentesis-to-delivery).

HMGB1 level in cord blood of preterm newborns was, however, significantly correlated with the level of fetal inflammation, as determined by cord blood IL-6 (Figure 9C) and highly correlated with cord levels of S100B protein (Figure 9D), the astroglial-derived DAMP. Cord blood HMGB1 level was the sole predictor of elevated S100B protein, independent of gestational age at delivery, histological chorioamnionitis, PPROM, race or cord blood IL-6 level (F-ratio 108.6, $P<0.0001$). Importantly, S100B did not vary significantly ($P>0.05$) with any other maternal or fetal variable tested except for the cord level of HMGB1.

Our data shows that cord blood sRAGE also increases with gestational age ($R=0.42$, $P=0.007$). Importantly, cord blood HMGB1 levels are significantly higher compared to both sRAGE and S100B, identifying HMGB1 as an important signaling molecule for the fetus (Figure 10).
In summary, these findings suggest that amniotic fluid HMGB1, likely a reflection of the damaged amniochorion in the context of severe intra-amniotic fluid inflammation, does not directly translate into an elevation in fetal HMGB1. Another checkpoint therefore must exist to prevent the accumulation of amniotic fluid HMGB1 in the fetus, perhaps amniotic fluid sRAGE. Alternatively, unlike the amniochorion, the fetus may have the ability to metabolize HMGB1, thus controlling the HMGB1 level within the fetal compartment independent from that within the amniotic cavity.

7. 2. Specific Aim 2. Identify putative stimuli driving RAGE activation in pregnancies complicated by preeclampsia

RAGE activation in severe preeclampsia occurs independent of levels of advanced glycation end-products (AGEs).

We first searched for the presence of the prototype AGE Nε-carboxymethyl-lysine (CML) and CML auto-antibodies in a case control study of time-matched samples of blood and urine retrieved from 118 women with preterm severe preeclampsia (sPE, n=79) and indicated preterm birth as compared to women with uncomplicated pregnancies who delivered at term (CRL, n=39). There was no statistical difference in gestational age at sample collection between the two groups (mean ± SEM gestational age sPE: 29.7 ± 0.6wks vs. CRL: 30.7 ± 0.6 wks, t-test P=0.171). As demonstrated in Figure 11, we found CML and CML auto-antibodies were present in the blood and urine of both severe preeclamptic women and normal controls, but no differences were seen between groups.

As illustrated in Figure 12, however, calculation of fractional excretion indicators demonstrated that women with severe preeclampsia appear to have lower fractional
excretions of CML when normalized for proteinuria (sPE vs. CRL, \(P=0.003\)), suggesting that proteinuria of preeclampsia contains a decreased concentration of AGE-modified proteins relative to normal pregnancy. Alternatively, the kidneys of preeclamptic women may preferentially retain AGE-modified proteins that may participate in kidney tissue injury or aging later in life.

Circulating sRAGE and TNF\(\alpha\) are considered markers of the extent of maternal RAGE activation and/or systemic inflammation, respectively. We found that women with severe preeclampsia had increased circulating levels of sRAGE, thus confirming the state of RAGE system activation (Figure 13, \(P<0.001\)).

In a linear regression analysis that included all 118 women in this study, sRAGE
levels were significantly but weakly correlated with TNFα (Spearman $R=0.261$; $P=0.004$). This relationship did not maintain when the analysis was restricted to preeclamptic women alone (n=79, $R=0.116$; $P=0.307$).

Next we were interested in identifying if any factors or combination of factors might influence levels of CML. For this purpose we performed a multivariate linear regression with CML and CML autoantibody concentrations or fractional excretions as dependent variables and maternal age, parity, gestational age, race and serum levels of sRAGE and TNFα as independent variables. The only relationship revealed was that women of non-Caucasian race appeared to have higher CML serum levels ($P=0.008$).
There was no significant correlation between CML levels in either blood or urine and extent of RAGE activation or inflammation. Similarly, the levels of CML auto-antibodies did not appear to be determined by any of the above-listed maternal demographical variables or by systemic levels of sRAGE or TNFα.

Lastly, we employed immuno-localization for CML in placental tissue sections from women with severe preeclampsia (n=6). We used as control placental tissues from gestational age matched women with idiopathic preterm birth and no histological chorioamnionitis (n=6). While we identified clumpy deposits of CML immunoreactive proteins in decidua, villous stroma and maternal vascular spaces, there were no discernable differences between the groups (Figure 14).

In summary, although we confirmed that maternal systemic inflammation and an elevation in sRAGE activation are phenomena characteristic of preeclampsia, it does not appear that an increase in circulating or deposited AGEs is a determinant mechanism.

Figure 13: (A) Serum TNFα, a reflection of the maternal level of systemic inflammation and (B) levels of sRAGE, a reflection of maternal RAGE activation in women with severe preeclampsia (sPE, n=79) as compared to normal women (n=39). Statistical analysis was performed using Mann-Whitney tests.
Women with preeclampsia have higher HMGB1 urine levels and higher HMGB1 fractional excretion.

We further sought to identify whether HMGB1 is a possible stimulus driving RAGE activation and the increased maternal serum sRAGE levels in preeclampsia. This case control study included three groups of time-matched blood and urine samples from 136 women with either preterm severe preeclampsia and indicated preterm birth (sPE, n=79), uncomplicated pregnancies delivered at term (CRL, n=44) or chronic hypertension but no preeclampsia (crHTN, n=13). There was no statistical difference in gestational age at sample collection between the three groups (mean ± SEM gestational age sPE: 30.6 ± 0.4 wks vs. CRL: 30.1 ± 0.7 vs. crHTN: 31.1 ± 1.4 wks, one-way ANOVA $P=0.295$). We measured serum levels of sRAGE as a marker of RAGE activation and TNFα as a marker of TLR activation (Figure 15). We found that only
sRAGE upregulation is a phenomenon specific for preeclampsia while the increase in

\[ \text{TNF}\alpha \]

is not specific to preeclampsia and rather similarly elevated in chronic hypertension.

We next analyzed serum and urine levels of HMGB1 and found that urine but not

serum HMGB1 levels were significantly elevated in severe preeclampsia \((P<0.001)\), independent of gestational age. The chronic hypertension group was not statistically different from either severe preeclampsia or controls \((P<0.001)\). Furthermore, we found that urinary excretion of HMGB1 correlated with circulating sRAGE \((r=0.426, P<0.001)\), proving that the increased excretion of HMGB1 in preeclampsia directly correlates with the extent of RAGE activation.

We next calculated the fractional excretion of total protein and the fractional excretion of HMGB1. The fractional excretion of HMGB1 was significantly increased in

\[ \text{Serum TNF}\alpha, \text{Serum sRAGE} \]

\(\text{Serum TNF}\alpha, \text{Serum sRAGE} \)
severe preeclampsia as compared to controls and women with chronic hypertension (Figure 17A, $P<0.01$).

We then asked whether presence or absence of end-organ disease, defined as eclampsia ($n=5$), HELLP ($n=20$) or intrauterine growth restriction (IUGR $n=18$),

Figure 16: (A) Serum HMGB1 and (B) urine HMGB1 concentrations in women with severe preeclampsia (sPE, $n=79$), normal women (CRL, $n=44$) and women with chronic hypertension (crHTN, $n=13$). Statistical analysis was performed using one-way ANOVA and Student-Newman-Keuls post-hoc tests.

Figure 17: (A) Fractional excretion of HMGB1 in women with severe preeclampsia (sPE, $n=79$), normal women (CRL, $n=44$) and women with chronic hypertension (crHTN, $n=13$). (B) Analysis with the severe preeclampsia group divided into women with end-organ disease as defined by eclampsia and/or HELLP (sPE+: $n=23$) or without end-organ disease (sPE−: $n=26$). Statistical analysis was performed using one-way ANOVA. * denotes $P<0.05$ vs. all other groups.
impacted the levels of HMGB1 in severe preeclampsia. Analysis of the data using these categories showed that women with eclampsia and/or HELLP had elevated urine HMGB1 concentrations ($P=0.026$) and fractional excretions ($P<0.001$) compared to those with severe preeclampsia but no end-organ disease or IUGR. This difference maintained in a one-way ANOVA that included controls and chronic hypertensive women (Figure 17B).
8. DISCUSSION

This study is the first to compare and contrast two modes and putative stimuli of RAGE activation as related to two important causes of prematurity: intra-amniotic inflammation, an important pathogenic pathway for spontaneous preterm birth and antenatal fetal injury, and preeclampsia, the most important cause of mandated preterm delivery.

8.1. Spontaneous preterm birth: infection and inflammation

Our research supports a role for fetal HMGB1-RAGE system activation in intra-amniotic infection and inflammation. We found that women with severe intra-amniotic inflammation had significantly increased amniotic fluid HMGB1 compared to women with absent and mild inflammation. We further showed that cord blood HMGB1 significantly correlated with the levels of cord blood IL-6 and S100B protein. In fact, cord blood HMGB1 level was the sole predictor of elevated S100B protein, independent of gestational age at delivery, histological chorioamnionitis, PPROM, race or cord blood IL-6 level, and, importantly, S100B did not vary significantly with any other maternal or fetal variable tested, except for the cord level of HMGB1. In addition, we found that cord blood sRAGE also increases with gestational age. Notably, cord blood HMGB1 levels are significantly higher compared to both sRAGE and S100B, identifying HMGB1 as an important signaling molecule for the fetus.

The high level of association between S100B and HMGB1 independent of gestational age is compelling evidence for a role of alarmins as mediators of perinatal complications. It is important to note that both HMGB1 and S100B are ligands of RAGE, and both are antagonized by the decoy receptor sRAGE [133]. Previous studies
investigating the presence of the RAGE axis in pregnancy demonstrated that sRAGE is present in amniotic fluid and increases with gestational age, which may account for the higher incidence of inflammation-related preterm deliveries at earlier gestational ages [132].

Despite many studies linking blood and CSF levels of S100B with brain injury, several authors have argued against the potential of cord blood S100B to serve as predictor of brain damage in newborns. This reluctance was based on the assumption that S100B immunoreactivity was also detected in the placenta [193]. It was therefore postulated that placental damage may also contribute to the S100B measured in cord blood. While there is no published study to date that validates this assumption, our data argue to the contrary. Since cord blood S100B has no relationship with the presence, absence or severity of histological chorioamnionitis, it is unlikely that S100B transfers to cord blood from a damaged placenta. Moreover, cord blood S100B levels in normal pregnancy measure higher than those in amniotic fluid and maternal blood [194]. The concentration gradient therefore favors transfer from the fetus to the amniotic fluid or maternal compartment and not vice versa [57]. This contrasts with IL-6, where the concentration gradient favors transfer to the fetus and is highly dependent on a damaged placenta, as we showed in an analysis of the same cohort of preterm neonates [57]. Due to the biological implications of an elevated S100B level for the fetus, we further argue that the source may be unimportant. Regardless of the origin, S100B remains a potent activator of RAGE and mediator of cytotoxicity.

Intrauterine infection is an important cause of preterm birth and is thought to be a chronic process leading to adverse outcomes that eclipse the effects of prematurity
alone. Intrauterine infection and subsequent inflammation contributes disproportionately to neonatal mortality and morbidity. The results of the ORACLE I and II clinical trials showed an increased risk of cerebral palsy when antibiotics were given to women in threatened preterm labor or with PPROM, suggesting that antibiotics fail to suppress the processes leading to fetal cellular damage despite inducing suppression of bacteria. Prolonging fetal exposure to such a damaging environment by delaying labor in this context, therefore, may cause further harm to the fetus. Thus, identifying markers of inflammation that may appear prior to the onset of clinical signs and symptoms of intrauterine infection is crucial to improving outcomes.

8. 2. Medically indicated preterm birth: preeclampsia

Our research further supports a role for a different type of HMGB1-RAGE axis activation in preeclampsia that is characterized by elevated maternal compartment sRAGE. We identified HMGB1 as a potential stimulus driving this RAGE activation given that 1) urine but not serum HMGB1 levels were significantly elevated in severe preeclampsia, independent of gestational age, 2) the fractional excretion of HMGB1 was significantly increased in severe preeclampsia as compared to controls and women with preexisting hypertension but no preeclampsia, and 3) HMGB1 urinary excretion correlated with circulating soluble RAGE. Importantly, we also found that the increased fractional excretion of HMGB1 relates to disease severity and end-organ involvement. Severe preeclamptics with end-organ involvement, as defined by HELLP or eclampsia, have higher fractional excretions of HMGB1 than those without such manifestations. These results suggest that the DAMP HMGB1 may play a role in fueling RAGE activation in women with severe preeclampsia.
HMGB1 is known to be a hydrophobic, “sticky” protein, and thus its elevated excretion but lack of detectable change in serum may be the result of its adherence to vascular endothelium or preferential elimination by the preeclamptic kidney. Alternatively, it is possible that HMGB1 has increased nephritic production that leads to increased excretion without detectable serum changes.

In contrast to HMGB1, however, our data does not support a role for excess AGEs in RAGE system activation, as evidenced by the lack of increase in CML or CML auto-antibody levels in severe preeclamptics as well as the lack of correlation between CML or CML auto-antibody levels with soluble RAGE.

Preeclampsia is the leading cause of a medically indicated preterm birth and a major cause of maternal and fetal morbidity and mortality. Preeclampsia, which accounts for 15 percent of preterm births [78,79] and is responsible for 20 percent of pregnancy-related maternal deaths in the United States [80], is a disease with high medical and economic impact. And yet preeclampsia remains a poorly understood disorder. While clinical signs and symptoms often make the diagnosis relatively simple, no diagnostic tools exist to definitively diagnose preeclampsia. Given the substantial risks to the mother and fetus, it is crucial that research uncover an improved understanding of the etiology of preeclampsia, which may lead to effective prevention and/or treatment of this serious disease.

Previous research has shown that severe preeclampsia is characterized by RAGE system activation, which leads to inflammation, oxidative stress and endothelial injury. This RAGE pathway activation may be involved in fueling some of the pathophysiologic manifestations of preeclampsia, and its further study is warranted.
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