

Peptidoglycan Recognition Proteins in Pathogenesis of
Preeclampsia and Periodontal Disease

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Introduction

Infections and responses to infections are one proposed cause of preeclampsia. Infections can originate from distant sites e.g., mouth. Pattern recognition receptors (PRRs) are a group of molecules that identify essential parts of microbes, e.g., peptidoglycan, and help respond to infections. Peptidoglycan can trigger responses at the placenta, resulting in secreted proteins, from placenta into the blood, leading to preeclampsia. Peptidoglycan recognition proteins (PGRP) are newly discovered group of PRRs that kill bacteria and can digest peptidoglycan, thereby potentially influencing disease processes like preeclampsia. Through this study we propose to investigate the role of PGRP in preeclampsia. Infections and subsequent host responses activated through pattern recognition receptors (PRRs), e.g., TLR-2 & TLR-4, can cause preeclampsia. PRRs recognize essential bacterial patterns, like cell-wall components, e.g., peptidoglycan & lipopolysaccharide, and trigger trophoblast apoptosis and robust pro-inflammatory responses at the maternal-fetal interface. Microbes/microbial patterns can originate from distant sites e.g., infected gingiva, and activate placental responses. Peptidoglycan recognition proteins are a newly discovered group of PRRs that are bactericidal and are thought to modulate inflammatory responses. The role of PGRPs in the pathogenesis of preeclampsia will be elucidated through this project.

Preeclampsia (PE) is a pregnancy related disease and is one of the complications of human pregnancy [1]. It occurs in at least 3-14% of all pregnancies with a risk of recurrence between 7.5 to 65% [2]. It originates in the placenta and remains a leading cause of maternal and fetal morbidity and mortality [2]. There are over 76,000 maternal and 500,000 fetal deaths, annually due to PE [2]. PE is commonly understood as a hypertensive disorder of pregnancy, characterized by increased blood pressure

accompanied by proteinuria, both occurring after 20 weeks gestation and may also cause intrauterine growth restriction of the fetus [2]. PE also poses a threat of increased risk for development of future cardiovascular disease [3]. The causes for the development of PE are largely unknown and reflects the limited understanding of the immunobiology and inflammatory pathways in normal and abnormal pregnancies[1]. Despite decades of intense research, there is no early pregnancy-screening test to recognize those at risk for PE [2]. Symptomatic treatment is the only option for this condition and the only known cure is delivery [4].

Different hypotheses have been proposed for the pathogenesis of PE. Failure of physiological transformation of myometrial spiral arteries leads to inadequate placentation which results in PE [5]. The exact mechanisms that cause inadequate trophoblast invasion and consequent abnormal placentation are not known. Recently, altered immune-inflammatory responses at the placental level have been suggested to be involved in pathogenesis of PE. Interestingly, PE is characterized by intravascular inflammation and endothelial cell dysfunction [6, 7]. These immune-inflammatory responses at the placental level are thought to be in response to infectious agents.

Chronic infections at distant sites could be a potential sources for such infectious agents, e.g., infectious diseases of the oral cavity like chronic periodontitis and various types of infections like periapical abscesses, granuloma etc. [11]. Oral infections, particularly periodontal diseases are near ubiquitous and affect 70%-90% of the adult US population. The frequent bacteremic episodes, detection of circulatory bacterial cell wall components, and the potential spillover effects of local inflammatory mediators originating from diseased gingiva are thought to contribute to an increase in systemic

inflammation [33]. Oral pathogens e.g., *Porphyromonas gingivalis*, *Fusobacterium nucleatum* etc., and their components have been detected in chorioamnionitis. Favorable conditions may permit the survival of selective bacterial species from oral cavity to enable it to travel through the circulation and become enriched at the placental bed [8]. In fact periopathogenic bacteria have been identified in greater frequency in the placenta of women with PE as compared to control subjects [9]. Oral bacterial antigens like peptidoglycan (PGN) and their fragments by themselves can reach the placental bed [10]. Additional clinical associative links point to a connection between oral infections and PE. For example, several studies have suggested that periodontal disease may be associated with increased risk for PE [11]. Periodontitis is a chronic immuno-inflammatory disease initiated and propagated by host response to dental biofilm and is one of the most prevalent chronic diseases in humans[12]. Periodontal disease is reported to affect 20-50% of pregnant women especially of lower socio-economic status[11] Maternal periodontal disease is shown to be associated with increased risk for development of PE, independent of the effects of maternal age, race, smoking, gestational age at delivery, and insurance status[13]. Additionally, since the pathogenesis of periodontal disease occurs through an infective-inflammatory axis, it has been proposed to potentially cause an increase in the levels of endotoxins, bacterial components, inflammatory cytokines and oxidative stressors in the systemic circulation. This increase may have consequences at the maternal fetal interface i.e., placenta in pregnant women. Therefore it has been proposed that periodontitis may be a vascular stressor that plays a role in the development of PE[11] .

The infectious-inflammatory axis is initiated by innate recognition of microbes. Innate microbial recognition occurs through evolutionarily conserved host receptors called pattern recognition receptors [14]. Examples of PRRs include a group of proteins called toll like receptors (TLR) [14] and nuclear oligomerization domain (NOD) like receptor proteins[15],[16].These PRRs recognize molecular patterns of components that are essential for survival of the microbes. These patterns are collectively called microbial associated molecular patterns (MAMPs). Each of the MAMPs have specific cognate PRRs; for example, TLR-4 recognizes a gram negative bacterial cell wall component i.e., lipopolysaccharides [14] while TLR-2, NOD-1 and NOD-2 are thought to sense another cell wall component of both gram positive and gram negative bacteria, i.e., peptidoglycans[15],[16]. NOD receptors are primarily intra-cellular receptors and therefore recognize peptidoglycans of phagocytized microbes or its components. Among the MAMPs PGN is particularly interesting because the polymeric PGN and each of its enzymatically digested fragments have different sets of cognate PRRs producing diversity in how the host responds to PGN. Gram-negative and Gram-positive bacteria have PGN as integral part of their cell wall but have significant differences in their structural make up that the host cells can differentiate.

PGN can be released into the systemic circulation in a number of ways: a) during PGN turnover as part of bacterial division, b) through host or other microbial hydrolases or c) bacterial lysis. PGN thus released has been found in distant sites, e.g., from gut to systemic circulation and found in brain. As mentioned earlier PGN and its breakdown products have different cognate PRRs. Polymeric PGN is recognized extracellularly by TLR-2, and CD14. Intracellularly fragments of PGN namely, mesoDAP-containing

tripeptides (TriDAP) found predominantly in Gram- Negative bacteria, and muramyl dipeptides (MDP) found in both Gram-negative and Gram positive species are recognized by NOD1 and NOD2 respectively. PGN and its fragments have been proposed to instruct the development of host immune response especially PGN fragments released from the gut into systemic circulation. It is conceivable that such a similar instruction takes place at the maternal – fetal interface to establish homeostasis during pregnancy. Disruption of this instruction can potentially lead to disorders like preeclampsia.

A new class of PRRs called peptidoglycan recognition proteins (PGRP) constituting four distinct molecules PGRP-1, PGRP2, PGRP3 and PGRP4, each with distinct functions, are emerging as key player in modulating host responses to PGN and its breakdown products [17]. Binding of PGRPs to peptidoglycans and bacteria suggests their direct role in recognition of bacteria. However, the consequences of this binding and, thus, the exact role of PGRPs in innate immunity to bacteria in mammals, are largely unknown[18]. Constitutive PGRP expression has been demonstrated in various tissues including oral mucosal tissues and the tongue [17]. PGRPs are mostly stored in vesicles and/or secreted. Therefore, they are likely to act in different cellular or tissue compartments as compared to NODs [19]. Mammalian PGRP-1 is expressed in high levels in the bone marrow, specifically in neutrophils and their precursors. In neutrophils, PGRP-1 is almost exclusively found in tertiary granules from where it can be released by exocytosis. It is also expressed in low quantities in oral epithelial cells and fibroblasts and can be inducibly up-regulated in these cells in response to peptidoglycans[17]. PGRP-2 is constitutively expressed in liver, which secretes it into blood. It is also expressed in lower levels in oral epithelial cells. Its expression is up-regulated in these cells and fibroblasts

by bacteria and cytokines in a NOD-2 dependent manner [17]. PGRP-2 is also known to enzymatically cleave peptidoglycan. PGRP-3 and PGRP-4 are found in salivary glands, specifically submandibular glands and in other oral tissues including tongue and throat. Their expression is also up regulated by bacteria in keratinocytes, fibroblast and other cells through TLR-2, TLR-4, NOD-1 and NOD-2 [17].

PGRP1, 3 & 4 are bactericidal and act by activating the two component bacterial lethal stress response signal transduction systems. PGRP-2 is an amidase that can hydrolyze PGN and some of its breakdown products [20]. In addition, PGRPs have been shown to modulate the microbiome as well as the inflammatory response. PGRP1 has pro-inflammatory effects; while PGRP3 & PGRP4 are thought to suppress inflammatory cytokine expression in response to TLR/NOD signaling [21]. For example, knock out of PGRP-1 protects mice from experimental asthma. Knock out of PGRP-2, 3 and 4 results in inflammatory diseases like arthritis, psoriasis etc. in mice [17]. These experimental findings also suggest a modulatory effect on inflammation by various PGRPs independent of their bactericidal/enzymatic effects [22].

This evidence further suggests a role for PGRPs in the pathogenesis of various microbial triggered inflammatory diseases like psoriasis, atherosclerosis, arthritis, ulcerative colitis, and Crohn's disease [17]. PGRP-1 is shown to be associated with hypertension in a population based multidisciplinary cardiovascular study [23]. PGRP-2 is particularly intriguing as it breakdowns PGN and depending on the context may serve to reduce pro-inflammatory effect of PGN, shift the response from anti-inflammatory NOD2 signaling to NOD1 signaling in addition to enhancing bacterial killing by antibacterial peptides.

Not much is known about the role of PGRP at the maternal –fetal interface, particularly during pathogenesis of PE. Innate immune responses, specifically TLR and NOD mediated responses at the level of trophoblasts, specialized fetal epithelial cells of the placenta, and placenta have been implicated in the pathogenesis of PE. For example, TLR-4 protein expression is increased in trophoblasts in the placental samples of patients with pre-eclampsia [24]. Proinflammatory cytokines that are released from macrophages, dendritic cells, and T cells through activation of TLRs can result in inhibition of trophoblast migration. These cytokines can also be directly cytotoxic to trophoblasts. It has been demonstrated that TLR-4 expression results in inhibition of trophoblast migration and TLR-2 expression causes apoptosis of trophoblasts[24]. Activation of NOD has also been implicated in inflammatory reactions at the fetal-maternal interface.

Interestingly PGRPs are expressed downstream of TLR-2 and NOD like proteins [21]. Human oral epithelial cells exhibit enhanced PGRP expression in response to synthetic ligands targeting TLRs and NODs in both cases through activation of nuclear factor- κ B. This activation interestingly did not result in increased inflammatory cytokine expression. This suggests a possible immune modulatory effect of PGRPs on TLR and NOD signaling.

Dysregulation of this immune modulation, especially in the placenta may result in adverse pregnancy outcomes like PE. Proteomics based studies provide tantalizing hints on the importance of PGRP in the context of pregnancy. Cervical mucous plugs show elevated levels of PGRPs. Granulocyte associated PGRP was found to be increased in conceptus fluid of bovine miscarriages, which were a result of infections. The authors of that study speculated that increased levels of PGRPs is probably a direct response to an

immuno-inflammatory episode caused by either microbial invasion of the uterine/conceptus space or as a consequence of an inflammatory event [25].

The immune-inflammatory responses to microbes are mediated by recognition of bacterial components called microbial associated molecular patterns (MAMPs) through conserved cognate receptors called pattern recognition receptors (PRRs). One such critical MAMP, bacterial cell wall component peptidoglycan (PGN) and cognate receptors are of particular interest in the context of homeostasis at the maternal-fetal interface. A new class of PRRs called peptidoglycan recognition proteins (PGRP) constituting four distinct molecules PGRP 1-4 is emerging as key player in modulating host responses to PGN and its breakdown products.

Not much is known about the expression and function of these proteins in the maternal-fetal interface. Proteomics based studies provide tantalizing hints of their importance in the context of pregnancy. It is well known that PGN can contribute to preeclampsia by either apoptosis of trophoblasts and subsequent release of damage associated molecular patterns (DAMPs) into the circulation or through increase of proinflammatory cytokines into the systemic circulation. Considering that, PGRP can modulate PGN response; the precise role of PGRP at the maternal fetal interface needs further exploration.

Taken together, we propose that a critical knowledge gap exists on the role of PGRPs in the innate immune responses that occur at the maternal-fetal interface in response to pathogens and their components that may be present in maternal circulation secondary to chronic infections like gingivitis and periodontitis. It is conceivable that PGRPs may

be differentially regulated in healthy pregnancies and during preeclampsia. Therefore, the aim of this project is:

To evaluate the expression of PGRPs in pre-eclamptic human placental tissue in comparison with patients without pre-eclampsia

The overall goal of this project was to establish the role of PGRPs in the pathogenesis of preeclampsia. An additional goal was explore the potential link between oral pathogens and innate immune responses at the maternal-fetal interface. Considering that PGRPs are secreted proteins and therefore are quantifiable, the results of this study could lead to development of salivary and/or plasmatic biomarkers for early detection of pre-eclampsia. Understanding the role of PGRPs at the maternal-fetal interface may also provide potential new therapeutic targets for treating pre-eclampsia.

Preliminary Data:

Since no data was available on the expression of PGRP in the human placenta, we decided to investigate the mRNA expression of various PRRs in pooled placental sample from different subjects and compared it with three different unstimulated trophoblast cell lines, namely SW71, 3a-Sub-E and HTR8. We noted that PGRP1 and PGRP3 were expressed constitutively in all the cell lines. The pooled placental sample had elevated mRNA expression of PGRP1 (30 fold) and PGRP3 (400 fold) when compared with cell lines (Figure 1). Similarly, TLR2, TLR4 and NOD2 mRNA were elevated in placental samples. (Figure 1, *unpublished data*) We also noted differences in expression for the

various PRRs between the trophoblast cell lines (Figure 1 *unpublished data*).

Surprisingly, we didn't detect any PGRP4 in either the cells lines or in the pooled placental sample. As expected, PGRP2 was not expressed in placenta, consistent with its role as a circulatory scavenger expressed by liver into the systemic circulation.

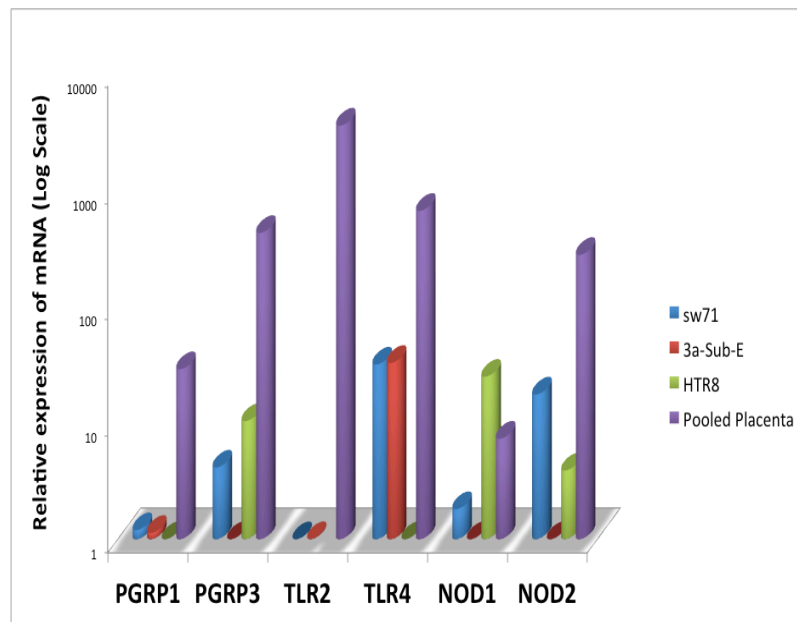


Figure 1: Relative mRNA expression of various PRRs in Placenta and in trophoblasts normalized to beta actin mRNA.

The role of TLR and NOD mediated immune responses in the pathogenesis of periodontitis has been extensively documented. As discussed previously, periodontal pathogens have also been implicated in adverse pregnancy outcomes. The presence of periodontal pathogens and their components in the maternal circulation, secondary to chronic infection in the oral cavity, can result in activation of immune response at the maternal fetal interface, which may involve PGRPs. We have noted significant increase in

PGRP 1, 2 and 3 in gingival tissue of subjects with chronic periodontitis when compared to health (Figure 2 *unpublished data*). The robust elevation of PGRP2 in periodontal tissues might be secondary to elevated levels in systemic circulation in response to oral infection or alternatively PGRP2 maybe elevated locally in periodontal tissue. Therefore, oral infections may elevate PGRP2 in systemic circulation, having consequences for distant sites when there is presence of coexisting infectious agents in the circulation.

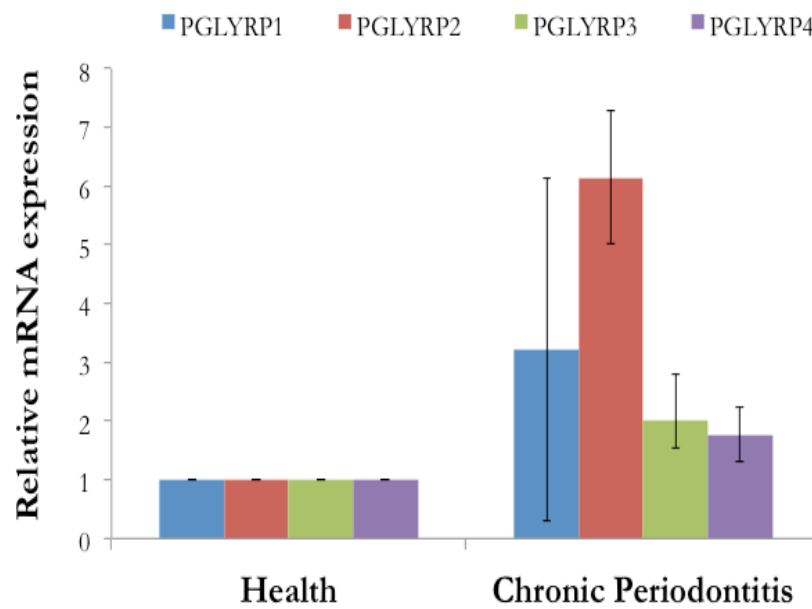


Figure 2: Relative mRNA expression of PGLYRPs in health and disease.

We also included immunohistochemically quantifying expression of different PGRPs in gingival tissues and categorizing their inflammatory status by histological analysis and correlating it back to the mRNA levels.

The overall goal of the research project was to understand the role of innate immunity in the pathogenesis of PE and in addition to explore the contribution of

periodontal diseases in the pathogenesis of PE. Specifically, the current study will investigate the expression of a group of innate immune pattern recognition receptors (PRRs), called Peptidoglycan Recognition Proteins (PGRP), in the placenta of pre-eclamptic women.

Hypothesis: PGRPs are expressed in the human placenta and differ in their expression in pre-eclampsia compared with normal controls.

Materials and Methods

Study design & Sample size determination

A case control study was conducted to examine the expression pattern of PGRP-1, 2, 3, & 4 in placental biopsy specimens through real time RT-PCR. Originally, the study design was adapted from Kim et al 2005[24]. The sample size for the proposed study was estimated based on differences in levels of TLR-4 as published in the study by Kim et al[24]. Their study, with 15 placental samples per test group, and 20 samples in control group noted differences in TLR expression between health and disease in human placental samples, with effect sizes ranging from 3.4 (significant) to 0.4 (non-significant) depending on the TLR. With a sample size of 20 subjects in the test group (pre-eclamptic samples) and 20 in control group (normal samples), the proposed study was designed to have 80% power to detect an effect size of 1.0, assuming two-sided tests each conducted at a 5% significance level.

Experimental Groups:

The pilot study groups consisted of subjects with: (1) uncomplicated term pregnancies (n=7) (2) pre-eclamptic group (n=7). Pregnancies were considered normal when there was no evidence of medical and/or obstetric complications and the birth weight appropriate for gestational age (term, ≥ 37 weeks of gestation). Preeclampsia was defined as hypertension (systolic blood pressure of ≥ 140 mm Hg or diastolic blood pressure of ≥ 90 mm Hg on at least 2 occasions, 4 hours to 1 week apart) and proteinuria (≥ 300 mg in a 24-hour urine collection or one dipstick measurement of $\geq 2+$). Only patients who delivered via Cesarean- section were included. Patients with diabetes, chorioamnionitis (bacterial infection of the amniotic fluid and the membranes surrounding the fetus) and multiples (twins, triplets) were excluded from the study. The cases and

controls were matched according to the maternal age, gestational age, BMI, race and ethnicity. The placental samples were obtained from an existing collection of tissues in Dr. Jill Reiter's lab and the IUPUI Building Blocks Pregnancy Biobank. Study approval was obtained from the Institutional Review Board of Indiana University Purdue University, Indianapolis.

This project was done in collaboration with the Department of Obstetrics and Gynecology at the Indiana University School of Medicine. The project was carefully discussed and reviewed from its inception with research as well as clinical faculty from the Dept. of OB/GYN. The collaborators of the project include Dr. Jill Reiter (Director, Perinatal Epigenetics Research Laboratory, Assistant Professor of Obstetrics and Gynecology, Maternal Fetal Medicine Division) and Dr. David Haas (Associate Professor of OB/GYN, Vice Chair for Research, Dept. of OB/GYN).

Sample Collection:

All the samples used for the study purposes were de-identified and assigned a study number which could be linked back to their hospital records by authorized personnel only if need be, for any additional information regarding their health history.

Some of the placental samples were available in Dr. Reiter's lab. Dr. Reiter had been collecting placental samples for research purposes for the last three to four years. Information of a potential delivery would be sent to the research personnel at the lab who would go to the IU Hospital delivery room to secure the placenta in a timely manner. Typically 4-5 core samples of the placenta would be taken with a specialized metal biopsy tool as shown in the picture below (Figure 3). The secured samples would then be stored

in - 80° C freezer for RNA extraction at a later time.

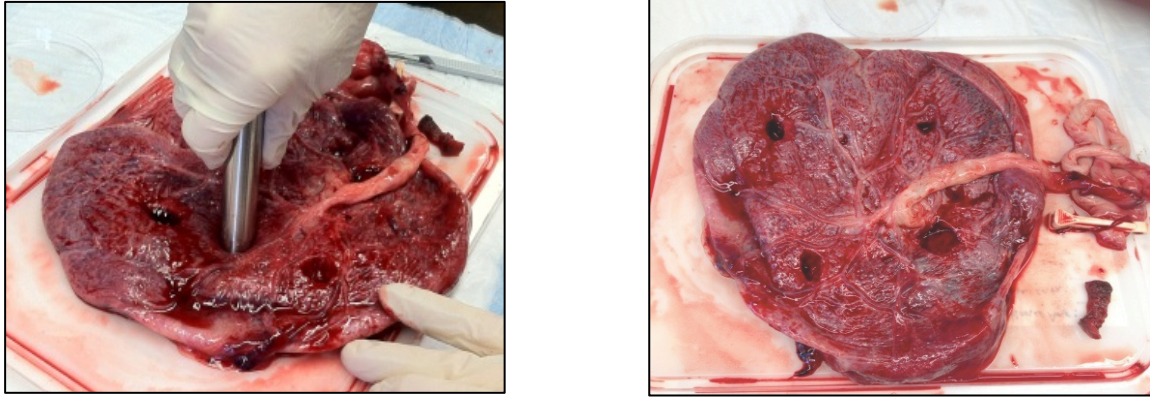


Figure 3: Securing placental samples

The inclusion and the exclusion criteria of the cases and controls were submitted to the coordinator of the IU Building Blocks Pregnancy Biobank. Once the list of possible samples were returned to us, selection of the appropriate samples from that list would be made. The placental samples were then delivered to Dr. Reiter's lab from the storage facility on dry ice.

Once the samples were delivered to the lab, they were weighed and about 100µg of the sample was secured for the study purposes and the rest of the specimen was returned to the Biobank.

Human RNA isolation & cDNA conversion

Total RNA was isolated from placental tissues using RNeasy Mini Kit (Qiagen Sciences, Valencia, CA) as per the manufacturer's protocol and previously described [26].

Tissue disruption and homogenization was carried out with a MagNA Lyser shaking-type bead mill (Roche) using 1.4 mm ceramic beads. RNA isolation was carried out according to the manufacturer's instructions (Ambion TRIzol and PureLink RNA mini kit). DNase I treatment was performed on column before elution of the RNA.



Figure 4: MagNA Lyser

Source: UMass.edu



Figure 5: Ceramic Beads for tissue homogenization

Tissue Homogenization

Before

After

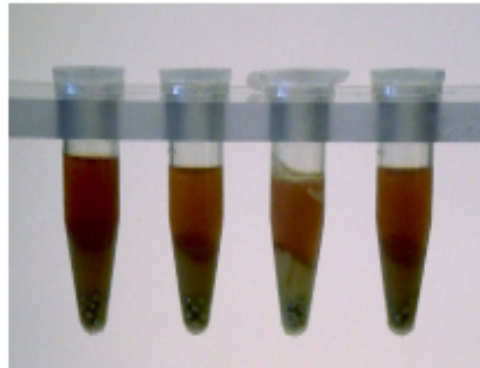
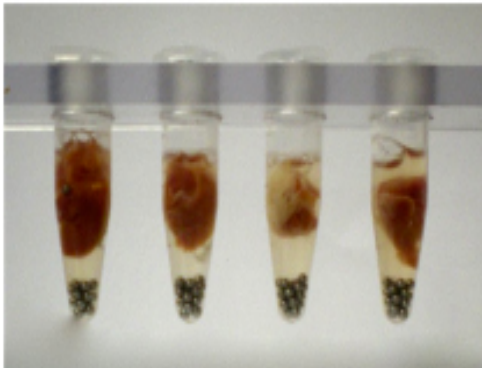


Figure 6: Pre and Post tissue homogenization

Source: Homogenizers.net

Total RNA extracted was quantified and integrity was determined by measuring the RNA quality index (RQI) using RNA StdSens chips on an Experion automated electrophoresis station (Bio-Rad Laboratories). Random hexamer primed reverse transcription will be performed using an cDNA synthesis kit (Qiagen Sciences, Valencia, CA) per manufacturer instruction. The reaction mixture was incubated under the following conditions: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C [26]. Genomic DNA elimination was done per manufacturer instructions. Once the samples are loaded RQI- an algorithm which estimates the level of degradation in total RNA samples.

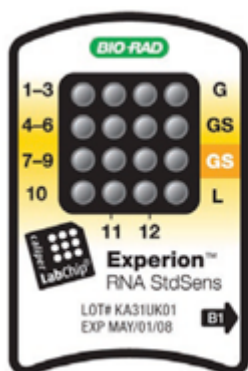


Figure 7: This is a RNA Chip where the samples are loaded into each well, which gives an output that looks the image below

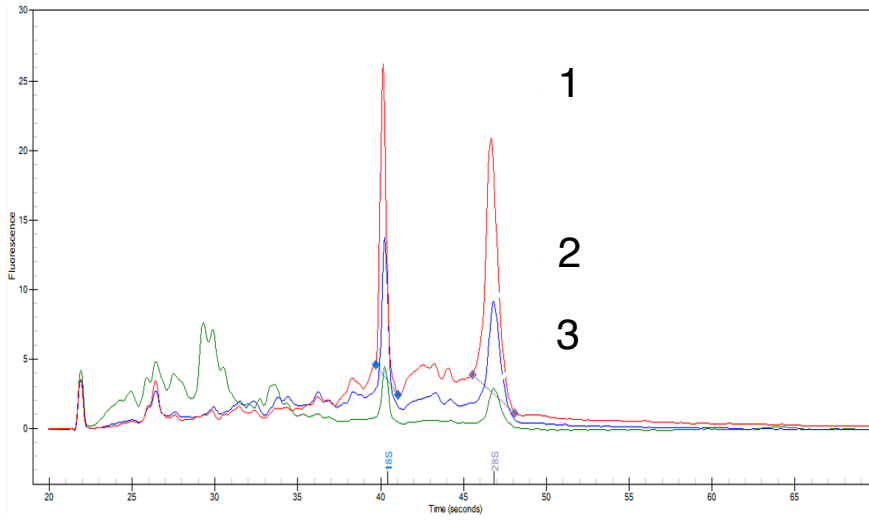


Figure 8: Electropherogram

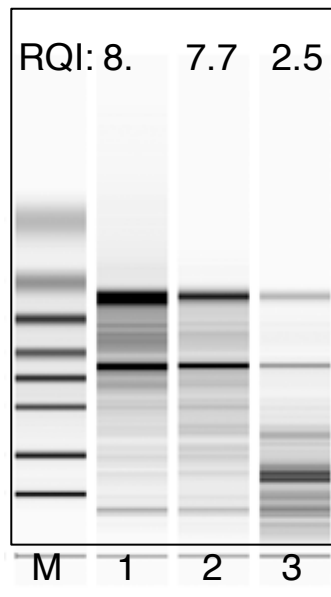


Figure 9: Virtual Gel Image

RQI of 10 indicates intact RNA and 1 indicates degraded RNA

RQI of 7-10 indicates acceptable quality RNA.

PCR array:

A custom PCR array (Catalog # CAPH11996; Qiagen, Frederick, MD) was used to analyze relative mRNA expression of the following pattern recognition receptors TLR2, TLR4, NOD1, NOD2, PGLYRP1, PGLYRP2, PGLYRP3, and PGLYRP4. B-actin and GAPDH were used as house keeping genes. The Array had appropriate controls to evaluate for human genomic DNA contamination, reverse transcription control and a positive PCR control. The array was validated with periodontal tissue specimens. Below image illustrates the plate layout with a total of 96 wells with a single sample loaded across the row in each of the 12 wells.

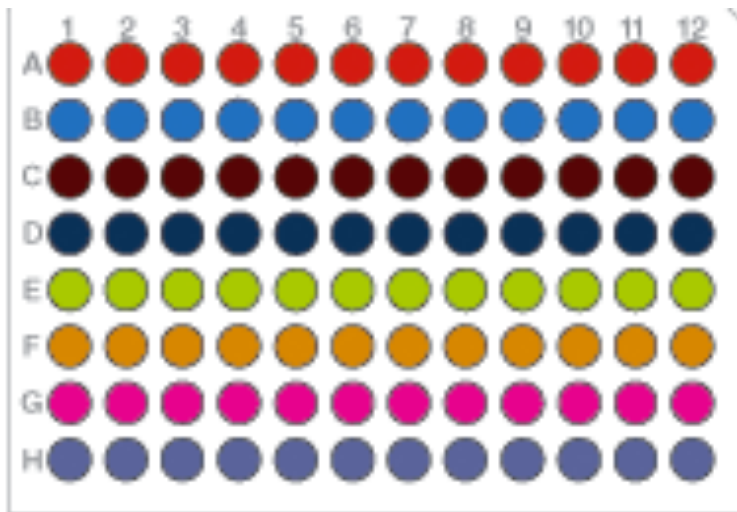


Figure 10: PCR plate layout

Source: Qiagen.com

Quantitative real-time polymerase chain reaction

Per manufacturer instruction 500ng of cDNA for each placental sample was mixed with appropriate amounts of SYBR green (per manufacturer instruction) and loaded into

the PCR array wells. CDNA was amplified using SYBR Green super mix with an ABI Prism 700 real-time PCR machine (Applied Bio systems, Foster City, CA). Melting curve analysis was performed to confirm that the detected signal is that of SYBR Green binding to the expected amplification product and not to the possible primer-dimers. Duplicate reverse transcriptase reactions and a single PCR amplification were performed and expressed as relative amounts of housekeeping gene b-actin and GAPDH through the Pfaffl method [27].

Immunohistochemistry (IHC):

Antibodies made in rabbits immunized with the indicated KLH-conjugated peptides and purified by peptide-affinity chromatography, frozen in 50% glycerol were used for the IHC. The antibodies used were obtained from Dr. Roman Dziarski's lab at IUSM, Gary, Indiana as well as commercially available antibodies (Novus, Cloud Clone Corp.) were used. All the antibodies were polyclonal, PGRP1 antibody was obtained from goat and the remaining (PGRP 2,3,4) were rabbit antibodies. The staining part of the IHC was done by the lab personnel at IU Pathology Lab.

Statistical Analysis:

Summary statistics (mean, standard deviation, range, 95% confidence interval for the mean) were calculated for PGLYRP 1-4 expression for each group. Two sample t-tests were used to test for group differences in PGLYRP 1-4 expression. Correlation coefficients were calculated between the expression levels of different receptors to examine possible association of expression.

Results:

A total of 14 samples were tested in a pilot study, 7 pre-eclamptic cases and 7 controls. Upon examination of the partial data from this study to verify the assumptions used in the calculations, we determined that the study would need to detect an effect sizes of 0.3 to 0.5, which would require sample sizes of 64 to 176 per group. Having such a large sample size would be a good long-term study but for the purposes of this pilot resident project, a total of 14 samples were used.

The cases and controls were matched as best as practically possible minimizing the risk for bias. The average age of the sample population was 27.6 ± 8.7 years. The mean age of the cases was 27.8 ± 5.1 years and the mean for the controls was 31.4 ± 3.9 years. The average BMI of the population was 31.3 ± 10.4 , the average BMI for the cases was 33.1 ± 5.1 and for the controls the average BMI was 34.2 ± 6.7 . Out of the 7 cases, 6 were Caucasian, 1 was African American. For the control group, 5 samples were Caucasian, 1 African American and 1 was unknown.

Sample Population	PE	CONTROLS
Number of Subjects	7	7
Age Range (years)	27.8 ± 5.1	31.4 ± 3.9
BMI	33.1 ± 5.1	34.2 ± 6.7
Race: Caucasian/African American/Unknown	6/1/0	5/1/1

Table 1: Demographics

RT- PCR Analysis: We wanted to confirm the expression of PGRPs in placenta before assessing their expression in the normal and diseased specimens. In order to do that, we perform PCR analysis with placental samples and positive controls known to express these protein molecules. The positive controls used were tissues from human brain, liver, skin, T-cells (Jurkat cell lines). The results from this analysis showed expression of PGRPs 1, 3 and 4 (purple curve) in the placental samples. However, the expression levels were low compared with the positive controls. Figure 11 is an amplification curve generated from the PCR data demonstrating the expression of PGRPs 1, 3 and 4. The PCR reaction theoretically doubles the DNA amount after completing each cycle, so it is an exponential reaction. The reaction, however, finally tails off and reaches a plateau, which is represented by the red arrow on the top of the image and is the end point of data collection at plateau (gel analysis). The center arrow is a representation of the reactions that start varying due to reagent depletion & decreased PCR efficiencies (enzyme activity, more product competing for primer annealing) and the bottom arrow demonstrates that the real time PCR does early phase detection at the exponential state. The x-axis is the cycle number where the curve crosses and the y-axis ΔR_n is the fluorescence signal with baseline subtracted (background signal subtracted). This step also enabled us to confirm that the assays were working, therefore reducing the potential for any false positives and/or negative results.

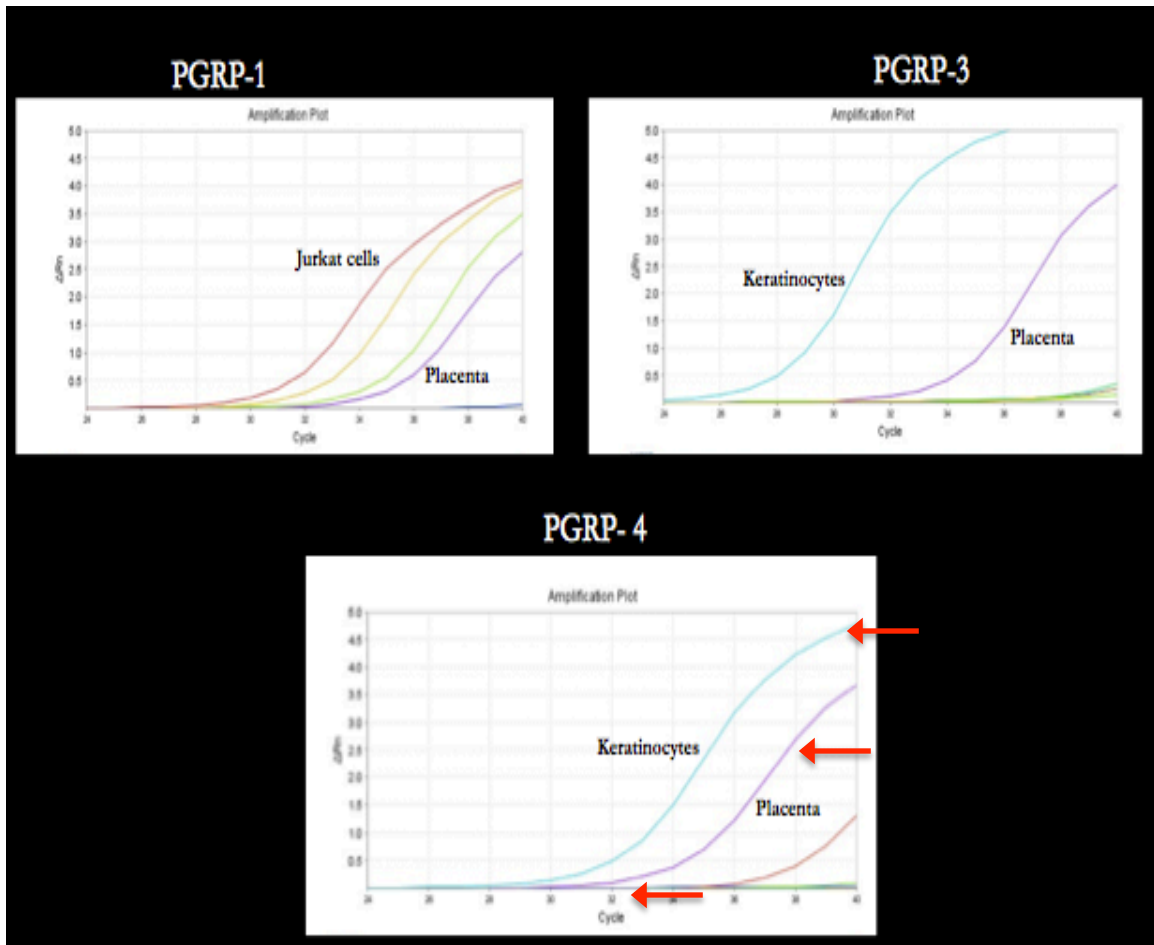


Figure 11: PGRP expression in placenta and positive controls (Amplification Plots)

The PCR data showed the expression of PGRPs 1, 3 and 4 in the pre-eclamptic samples. PGRP1, PGRP3, and PGRP4 expression was not significantly different between the three groups ($p \text{ value} \geq 0.05$). However, the clinical significance of this difference is yet to be determined. The fold changes in expression were calculated by the ΔCt method. Ct (Threshold cycle) is the cycle number at which the fluorescence signal crosses threshold. So a fold change of expression (up or down regulation) = Difference between the target gene and the reference gene. Here the target gene(s) is PGRP and the reference gene is NOD-1. A decrease in CT value increases expression. There was an up-regulation

of PGRP-1 (1.4 fold) and down regulation of PGRP-3 (1.3 fold) and PGRP-4 (1.6 fold) TLR2, TLR4 and NOD2 mRNA were also elevated in the placental samples. However, these changes were not statistically significant for this small sample size.

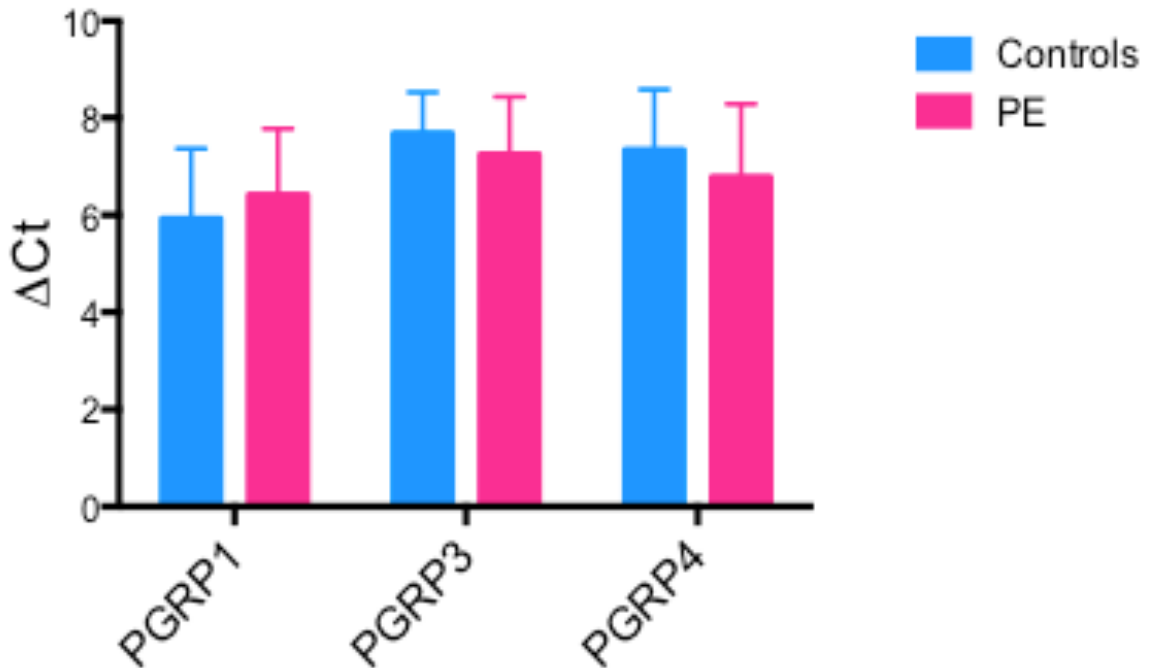


Figure 12: PGRP expression- Fold changes between cases and controls

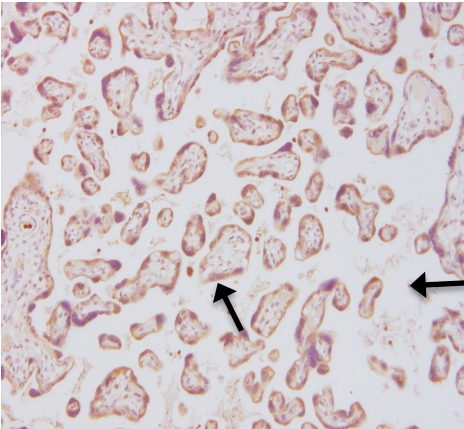
Immunohistochemistry demonstrated positive staining for PGRPs 1, 3 and 4 in the syncytial trophoblasts. No positive staining was noted for PGRP 2. The IHC confirms our PCR results. We had to make sure we were using good positive controls, which included:

- Acute appendicitis for PGRP 1 since neutrophils were known to express PGRP 1
- Hepatocytes in the liver for PGRP 2
- Keratinocytes for both PGRP 3 and PGRP 4 since they are known to be expressed in

skin by keratinocytes. The below slides are a cross-section through chorionic villi. The outer ring of cells, which are the syncytial trophoblasts appear to be staining.

Immunohistochemistry Images:

Figure 13: **PGRP-1** Placenta



Acute Appendicitis (+ve Control)

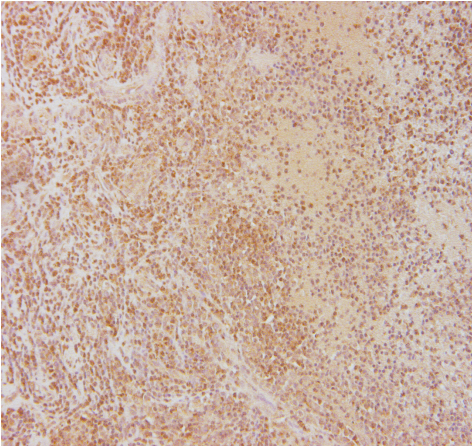
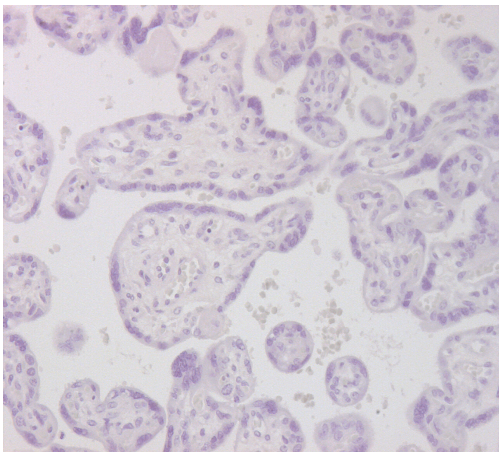


Figure 14: **PGRP- 2** Placenta



Liver (+ve Control)

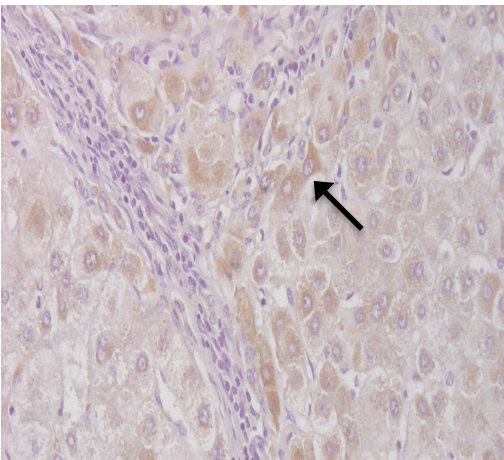
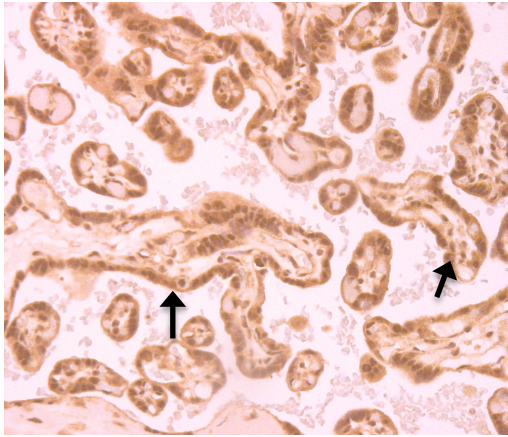


Figure 15: **PGRP-3** Placenta



Keratinocytes (+ve Control)

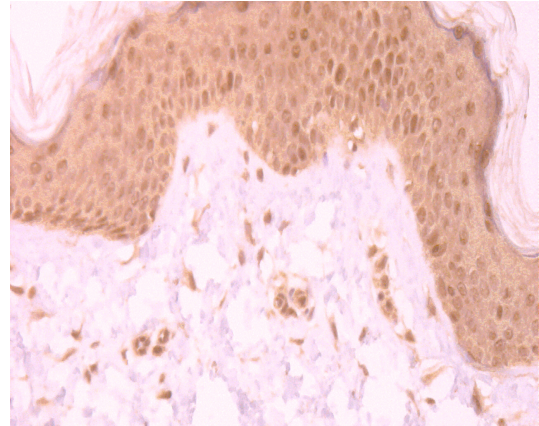
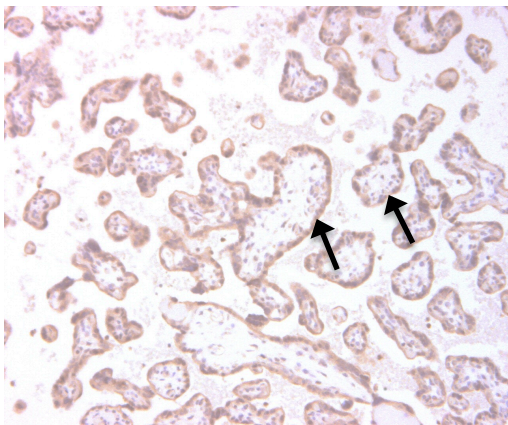
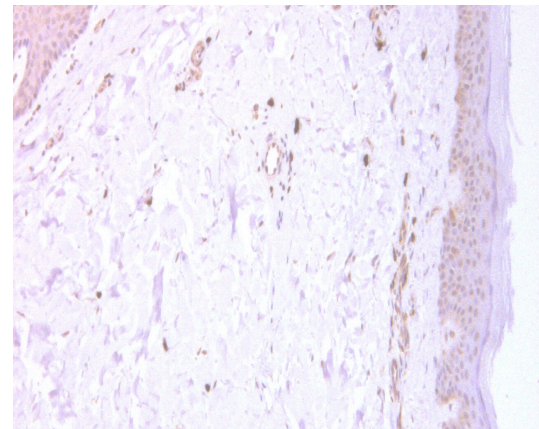


Figure 16: **PGRP-4** Placenta



Keratinocytes (+ve Control)



Discussion:

Periodontal disease has been linked to various adverse pregnancy outcomes including pre-eclampsia, premature birth, low birth weight, intra uterine growth restriction, still birth etc. [31]. However, only two meta-analyses specifically investigating periodontal disease and preeclampsia have been performed which concluded that periodontal disease during pregnancy is associated with increased risk of pre-eclampsia (OR 1.76, CI- 1.43-2.18) [32] and periodontal disease appears to be a possible risk factor for pre-eclampsia (OR 2.17, CI- 1.38–3.41) [33]. However, there is a knowledge gap in understanding the underlying mechanisms, many of which could be attributed to the inherent limitations of the studies, including a high degree of variability in study populations, recruitment, assessment, disparities in data collection methods, inconsistent case definitions, which may under or over estimate the disease prevalence and not all the studies were investigating the same outcome variable. As a result, the studies included in the meta-analyses show a high degree of heterogeneity [34]. Also a majority of the studies in the literature are observational studies whose strength is modest and generalizability is an issue. There is a lack of studies investigating the biological mechanisms explaining the pathophysiology and risk profile of this association between periodontal disease and adverse pregnancy outcomes [31].

Four types of human PGRPs (PGRP1, PGRP2, PGRP3 and PGRP4) have been recognized [13]. Constitutive PGRP expression has been demonstrated in various tissues including oral mucosal tissues and the tongue [13]. Mammalian PGRP1 is expressed in high levels in the bone marrow specifically in neutrophils and their precursors. In neutrophils, it is almost exclusively found in tertiary granules from where it can be

released by exocytosis. It is also expressed in low quantities in oral epithelial cells and fibroblasts and can be inducibly up-regulated in these cells in response to PGN [13]. PGRP2 is constitutively expressed in liver, which secretes it into blood. It is also expressed in lower levels in oral epithelial cells. Its expression is up-regulated in these cells and fibroblasts by bacteria and cytokines in NOD2 dependent manner [13]. PGRP3 and PGRP4 are found in salivary glands specifically submandibular glands and in other oral tissues including tongue and throat. Their expression is also up regulated by bacteria in keratinocytes, fibroblasts and other cells through TLR 2, TLR4, NOD1 and NOD2 [13]. Initially, PGRPs were considered as anti-microbial PRR proteins [15]. However, recent evidence indicates that they can also modulate inflammation and immune responses independent of their bactericidal and enzymatic activities [16]. PGRPs are speculated to be involved in the pathogenesis of various inflammatory diseases like psoriasis, atherosclerosis, arthritis, ulcerative colitis, and Crohn's disease [13].

Considering their role in various chronic inflammatory conditions, we hypothesized that PGRPs may play a significant role in the pathogenesis of pre-eclampsia. Inflammatory diseases appear to have common themes in their pathogenesis and are often found in combination with other co morbidities. Periodontitis is one such inflammatory disease, which appears to be associated with many systemic diseases such as diabetes, hypertension, obesity etc. There is evidence showing an association between periodontal disease and adverse pregnancy outcomes such as pre-eclampsia. We are interested in focusing on the role of innate immune mechanism in these inflammatory diseases. Pattern recognition receptors are a part of the innate immune mechanism, which have different classes of protein molecules of which PGRPs are one. Interestingly,

PGRPs are secreted proteins. Therefore they are quantifiable and measurable as a fluid biomarkers along with other clinical finding and in addition may exert effects on different organs.

This is a pilot project specifically exploring the expression of PGRPs in relation to pre-eclampsia. Little is known about the role of PGRP in the immuno-modulation that is necessary to maintain homeostasis at the placental level. The studies that have been done so far are mainly epidemiological studies, which are mainly observational. More studies investigating the biological mechanisms involved in these inflammatory diseases are needed to understand the pathophysiological processes involved in order to design better therapeutic programs and identify individuals who are at risk and would actually benefit from them.

The sample size had to be reduced compared to the initially proposed number of 20 samples in each group due to unforeseen issues related to the time in securing the samples, the cost involved as well as the limitations associated with the inclusion and exclusion criteria. Based on the preliminary data, our statistician stated that, in the initial proposal with 15 cases we would be able to detect an effect size of 1.1. The effect sizes with the data we had for PGRP1, PGRP4, and TLR4 were 0.27, 0.46, and 0.30, respectively. To have 80% power to detect an effect size of 0.50, we needed 64 samples per group, and to detect an effect size of 0.3 we needed 176 samples per group.

We included women, who were non-smokers, BMI falling in the 'normal' range of (18.5-24.9) without any pathology associated with the pregnancy. We excluded smokers because of its inflammatory effects on both the periodontium and pregnancy and its associated profound confounding effect. There are reports of an association between

periodontal disease and inflammatory conditions such as diabetes and obesity, and they are known to negatively affect the pregnant women as well, hence the exclusion. We included births via Cesarean section only because the process of natural birth could trigger a separate inflammatory cascade, which would confound our results.

Chorioamnionitis is an inflammation of the fetal membranes (amnion and chorion) due to a bacterial infection, which is also a confounding factor because potentially any expression of PGRPs could be in effect of the infection(s) and not pre-eclampsia. Since smoking and obesity rates are high in Indiana, it was not practical for us to procure ‘ideal’ samples, hence we decided to include overweight and slightly obese patients based on their BMI and obtain matching controls in order to control for bias to a certain extent.

This is the first study on reporting the expression of PGRP1, PGRP3 and PGRP4 in the human placenta; however, the mRNA levels were not significantly different between healthy samples and pre-eclamptic samples. Our results were consistent with earlier reports of higher expression of TLR-2, TLR-4 and NOD-2 [24] in pre-eclampsia. The human placenta expresses transcripts for TLRs 1 through 10 and the proteins for many of these TLRs activate innate immune responses at the maternal–fetal interface [35]. TLR ligand-exposed trophoblasts secrete more proinflammatory cytokines and chemokines to enhance immune cell migration [35]. Among them, TLR-2 is known to recognize peptidoglycan from Gram-positive bacteria and TLR-4 is said to recognize lipopolysaccharide (LPS) from Gram-negative bacteria. TLR-4 expression was also reported increased in placental trophoblasts from women with pre-eclampsia. A study using pregnant rats has shown that low doses of lipopolysaccharide acting via TLR-4 induced pre-eclampsia like symptoms [36]. Many studies have stated an altered cytokine

profile in women with pre-eclampsia. To summarize, pre-eclamptic women showed increased TNF- α , IL-6 and IL-8 and decreased levels of IL-10 [35]. IL-10 is known to inhibit matrix metalloproteinases, which is critical for the trophoblast invasion during placentation. Also bacterial insult from distant sites could trigger release of pro-inflammatory cytokines at the placental interface, which are directly cytotoxic to trophoblasts and prevent their migration into the uterine arteries and lead to their apoptosis leading to hypoxia and consequently pre-eclamptic events. Since similar events and inflammatory factors are causal for periodontal events, biologically the association between the pre-eclampsia and periodontal disease seems plausible. Another interesting aspect to the inflammatory process in pre-eclampsia is that treatment of bacteriuria with sulfadimidine or sulfamethoxydiazine did not reduce the risk of pre-eclampsia. Additionally, Michalowicz et al. demonstrated that periodontal treatment during pregnancy did not significantly alter pre-eclampsia [36] may indicate that pre-eclampsia may not be a direct consequence of infection but due to the influence of pro-inflammatory cytokines secondary to infection which makes the investigation of PGRPs more valid.

This is the first study reporting on the expression of PGRP 1, 3 and 4 in human placental tissue. This was different from the result reported by Dziarski et al, where one placental sample was analyzed for PGRP expression, which did not show expression [30]. The clinical significance of this expression needs further investigation through longitudinal studies. Long-term data with a large sample size could help the obstetrics community better understand the pathogenesis of pre-eclampsia. It will also help bridge the gap in knowledge linking oral diseases to systemic conditions.

Clinical Significance:

Considering that PGRPs are secreted proteins and therefore are quantifiable, the results of this study can lead to development of salivary and/or plasmatic biomarkers for early detection of pre-eclampsia. Understanding the role of PGRPs at the maternal-fetal interface may also provide potential new therapeutic targets for treating pre-eclampsia in the future and this is the first step in that direction.

References:

1. Taylor, R.N., *Review: immunobiology of preeclampsia*. Am J Reprod Immunol, 1997. **37**(1): p. 79-86.
2. Ahn, H., et al., *Immunologic characteristics of preeclampsia, a comprehensive review*. Am J Reprod Immunol, 2011. **65**(4): p. 377-94.
3. Xie, F., et al., *Toll-like receptor signaling and pre-eclampsia*. Am J Reprod Immunol, 2010. **63**(1): p. 7-16.
4. Duley, L., S. Meher, and E. Abalos, *Management of pre-eclampsia*. BMJ: British Medical Journal, 2006. **332**(7539): p. 463.
5. Brosens, I.A., W.B. Robertson, and H.G. Dixon, *The role of the spiral arteries in the pathogenesis of preeclampsia*. Obstet Gynecol Annu, 1972. **1**: p. 177-91.
6. Roberts, J.M., et al., *Preeclampsia: an endothelial cell disorder*. Am J Obstet Gynecol, 1989. **161**(5): p. 1200-4.
7. von Dadelszen, P., T. Wilkins, and C.W. Redman, *Maternal peripheral blood leukocytes in normal and pre-eclamptic pregnancies*. Br J Obstet Gynaecol, 1999. **106**(6): p. 576-81.
8. Fardini, Y., et al., *Transmission of diverse oral bacteria to murine placenta: evidence for the oral microbiome as a potential source of intrauterine infection*. Infection and immunity, 2010. **78**(4): p. 1789-1796.
9. Barak, S., et al., *Evidence of periopathogenic microorganisms in placentas of women with preeclampsia*. Journal of periodontology, 2007. **78**(4): p. 670-676.
10. Arce, R.M., et al., *Increased TLR4 expression in murine placentas after oral infection with periodontal pathogens*. Placenta, 2009. **30**(2): p. 156-162.

11. Sgolastra, F., et al., *Relationship between periodontitis and pre-eclampsia: a meta-analysis*. PLoS One, 2013. **8**(8): p. e71387.
12. Chaparro, A., et al., *Increased inflammatory biomarkers in early pregnancy is associated with the development of pre-eclampsia in patients with periodontitis: a case control study*. J Periodontal Res, 2013. **48**(3): p. 302-7.
13. Boggess, K.A., et al., *Maternal periodontal disease is associated with an increased risk for preeclampsia*. Obstet Gynecol, 2003. **101**(2): p. 227-31.
14. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition*. Annu Rev Immunol, 2002. **20**: p. 197-216.
15. Girardin, S.E., et al., *Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan*. Science, 2003. **300**(5625): p. 1584-7.
16. Girardin, S.E., et al., *Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection*. J Biol Chem, 2003. **278**(11): p. 8869-72.
17. Dziarski, R. and D. Gupta, *Review: Mammalian peptidoglycan recognition proteins (PGRPs) in innate immunity*. Innate Immun, 2010. **16**(3): p. 168-74.
18. Dziarski, R., et al., *Defect in neutrophil killing and increased susceptibility to infection with nonpathogenic gram-positive bacteria in peptidoglycan recognition protein-S (PGRP-S)-deficient mice*. Blood, 2003. **102**(2): p. 689-97.
19. Dziarski, R. and D. Gupta, *The peptidoglycan recognition proteins (PGRPs)*. Genome Biol, 2006. **7**(8): p. 232.
20. Lu, X., et al., *Peptidoglycan recognition proteins are a new class of human bactericidal proteins*. J Biol Chem, 2006. **281**(9): p. 5895-907.

21. Uehara, A., et al., *Chemically synthesized pathogen-associated molecular patterns increase the expression of peptidoglycan recognition proteins via toll-like receptors, NOD1 and NOD2 in human oral epithelial cells*. *Cell Microbiol*, 2005. **7**(5): p. 675-86.
22. Saha, S., et al., *PGLYRP-2 and Nod2 are both required for peptidoglycan-induced arthritis and local inflammation*. *Cell Host Microbe*, 2009. **5**(2): p. 137-50.
23. Victor, R.G., et al., *The Dallas Heart Study: a population-based probability sample for the multidisciplinary study of ethnic differences in cardiovascular health*. *Am J Cardiol*, 2004. **93**(12): p. 1473-80.
24. Kim, Y.M., et al., *Toll-like receptor 4: a potential link between "danger signals," the innate immune system, and preeclampsia?* *Am J Obstet Gynecol*, 2005. **193**(3 Pt 2): p. 921-7.
25. Riding, G.A., et al., *Differential proteomic analysis of bovine conceptus fluid proteins in pregnancies generated by assisted reproductive technologies*. *Proteomics*, 2008. **8**(14): p. 2967-82.
26. Srinivasan, M., K.N. Kodumudi, and S.L. Zunt, *Soluble CD14 and toll-like receptor-2 are potential salivary biomarkers for oral lichen planus and burning mouth syndrome*. *Clin Immunol*, 2008. **126**(1): p. 31-7.
27. Pfaffl, M., *A new mathematical model for relative quantification in real-time RT-PCR*. *Nucleic acids research*, 2001. **29**(9): p. e45.

28. Zhong, G., *Regulatory effect of peptidoglycan on the expression of toll-like receptor 2 mRNA and proteins in trophoblast cell line TEV-1 cells*. ISRN obstetrics and gynecology, 2010. **2011**.
29. Abrahams, V.M., et al., *TLR6 modulates first trimester trophoblast responses to peptidoglycan*. The Journal of Immunology, 2008. **180**(9): p. 6035-6043.
30. Liu, C., Dziarski, R., et al., *Peptidoglycan Recognition Proteins: A novel family of four human innate immunity pattern recognition molecules*. The Journal of Biologic Chemistry, 2001. 276 (37), Issue of September 14, pp. 34686–34694.
31. Sanz M, Kornman K, and on behalf of working group 3 of the joint EFP/AAP workshop. *Periodontitis and adverse pregnancy outcomes: consensus report of the Joint EFP/AAP Workshop on Periodontitis and Systemic Diseases*.
32. Conde- Agudelo, A., et al, 2008. *Maternal infection and risk of preeclampsia*- American Journal of Obstetrics & Gynecology.
33. Sgolastra, F., et, 2013. *Relationship between Periodontitis and Pre-Eclampsia: A Meta-Analysis*. PLOS ONE 8(8): e71387.
34. Ide, M., and Papapanou, P. 2013. *Epidemiology of association between maternal periodontal disease and adverse pregnancy outcomes – systematic review*. April 2013, Vol. 84, No. 4-s, Pages S181-S194 , DOI 10.1902/jop.2013.134009
35. Ahn H, Park J, Gilman-Sachs A, Kwak-Kim J. Immunologic characteristics of preeclampsia, a comprehensive review. Am J Reprod Immunol 2011; 65: 377–394

36. Michalowicz BS, Hodges JS, DiAngelis AJ, Lupo VR, Novak MJ, Ferguson JE, Buchanan W, Bofill J, Papapanou PN, Mitchell DA, Matseoane S, Tschida PA: Treatment of periodontal disease and the risk of preterm birth. *N Engl J Med* 2006; 355:1885–1894.

Abstract

Background: Pre-eclampsia a potentially life threatening hypertensive disorder occurring in 3-14% of pregnancies. Its etiology is multifactorial involving the placenta. The only “cure” that currently exists is the delivery of the baby, which is often pre-term. There is no early pregnancy screening test to recognize those at risk. Recently, an altered immune-inflammatory responses at the placental level in response to infectious agents (*eg.*, periodontal pathogens) have been proposed to be etiological for this pregnancy complication. A new class of Pattern Recognition Receptors called Peptidoglycan Recognition Proteins (PGRPs) constituting 4 distinct molecules PGRP 1-4 is emerging as a key player in modulating host responses to peptidoglycan and its breakdown products. A critical knowledge gap exists on the role of PGRPs in the innate immune responses that occur at the maternal-fetal interface in response to pathogens and their components that may be present in maternal circulation secondary to chronic infections. Aim: The aim of this pilot study is to investigate the expression PGRPs in the placenta of pre-eclamptic women. The overall goal is to better understand the association of periodontal disease and adverse pregnancy outcomes.

Methods and Materials: This case control study consisted of subjects with: (1) normal term pregnancies (n=7) (2) pre-eclampsia (n=7). Preeclampsia was defined as hypertension (systolic blood pressure of ≥ 140 mm Hg or diastolic blood pressure of ≥ 90 mm Hg on at least 2 occasions, 4 hours to 1 week apart) and proteinuria (≥ 300 mg in a 24-hour urine collection or one dipstick measurement of $\geq 2+$). A real time quantitative PCR array was used to analyze

the relative mRNA expression of TLR2, TLR4, NOD1, NOD2, PGRP1, PGRP2, PGRP3, and PGRP4. Immunohistochemistry was performed to determine the cell type(s) expressing the PGRP proteins in the placental tissue. Summary statistics (mean, standard deviation, range, 95% confidence interval for the mean) were calculated for PGRP 1-4 expression for each group.

Results and conclusions: The PCR data showed the expression of PGRPs 1, 3 and 4 in the placental samples. There was an up-regulation of PGRP-1 (1.4 fold) and down regulation of PGRP-3 (1.3 fold) and PGRP-4 (1.6 fold). TLR2, TLR4 and NOD2 mRNA were also elevated in the placental samples. Immunohistochemistry demonstrated positive staining for PGRPs 3 and 4 in the trophoblasts. The results from this novel research could lead to development of salivary and/or plasmatic biomarkers for early detection of PE and warrants further investigation

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