MAGEE-WOMENS RESEARCH INSTITUTE & FOUNDATION

THE MAGEE-WOMENS RESEARCH INSTITUTE CLINICAL TRAINEE RESEARCH AWARD

FACE PAGE (must be typewritten)

APPLICANT INFORMATION		
NAME (Last, f rst, m dd e) [redacted]		DEGREE(S): DO, MS ARE YOU A RESIDENT OR FELLOW: Fellow
POSITION TITLE: [redacted]		OFFICE MAILING ADDRESS (bu d ng, room, street, c ty, state, z p code) [redacted]
YEAR(S) IN TRAINING: [redacted] YEAR(S) IN CURRENT PROGRAM: [redacted]		
DEPARTMENT [redacted]		
TEL: [redacted]	FAX:	E-MAIL ADDRESS [redacted]

APPLICATION TITLE: **Placental nitric oxide effects on GBS chorioamnionitis.**

HUMAN SUBJECTS RESEARCH	☒ No ☐ Yes	IRB APPROVAL DATE:
VERTEBRATE ANIMALS	☐ No ☒ Yes	IACUC APPROVAL DATE:
TOTAL FUNDS REQUESTED	$5,000	

FACULTY SPONSOR	DEPARTMENT CHAIR OR DIRECTOR OF FELLOWSHIP/RESIDENCY PROGRAM
Name [redacted]	Name [redacted]
T t e [redacted]	T t e [redacted]
SIGNATURE [redacted]	SIGNATURE [redacted]

APPLICANT SIGNATURE	DATE
[redacted]	

PROPOSED BUDGET

Personnel – List **effort** for **all** personnel to be involved in carrying out the proposed research, whether or not salary is requested, **beginning with P.I.**

Name	Role	% Effort	Salary Requested	Fringe Benefits*	Total
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
		Subtotals	$ [redacted]	$ [redacted]	$ [redacted]

Equipment N/A	Subtotal	$ 0.00
Supplies (list) Bacterial culture media and disposable materials Molecular biology reagents Antibodies for immunohistochemistry RNA extraction kit	Subtotal	$1,200.00
Other Expenses Experimental animals (wild type and NOS knockout mice) Tn-seq fees	Subtotal	$3,800.00
	Total	$ $5,000.00

*MWRIF Staff fringe benefit rate is 25.0%

BUDGET JUSTIFICATION

Bacterial culture media, including tryptic soy (TS) broth/agar, and disposable materials, including plastic culture plates and tubes, are required to grow bacterial cultures for all experiments listed in Aim 1 and to grow bacteria for the murine chorioamnionitis model in Aim 2. The molecular biology reagents include PCR master mix that will be used for the experimental condition in Aim 2. RNA extraction kits will be used for Aim 2. Primary and secondary antibodies will be purchased for immunohistochemistry in Aim 2. Commercially available wild type mice (C57/BL6), and NOS knockout mice (B6;129P2-NOS2tm1Lau/J) will be used to set up the murine GBS chorioamnionitis model in Aim 2. Tn-seq is performed off-site by the University of Maryland Institute for Genomics and is needed for Aim 1b. All supplies and other expenses not covered by this grant will be afforded by Dr. Hooven's funding.

ABSTRACT
Group B *Streptococcus* (GBS) remains a major cause of neonatal infectious morbidity and mortality. Vaginal colonization and subsequent uterine invasion can lead to chorioamnionitis prior to delivery. Chorioamnionitis increases the risk of miscarriage, preterm birth, and early-onset sepsis. GBS possesses virulence factors that allow it to bypass the maternal-fetal defenses of the placenta. One such defense is nitric oxide (NO), a component of innate immunity, which induces microbial death. NO is produced by nitric oxide synthase (NOS), and NOS has been found in human trophoblast cells and placental macrophages. This study aims to elucidate the mechanisms that GBS uses to survive NO exposure in vitro and to understand the role of placental NO in host-pathogen interactions in a murine GBS chorioamnionitis model. We hypothesize that placental NOS activity is increased in chorioamnionitis and that exposure to NO results in GBS up-regulation of pathways to enhance survival in a NO-rich environment.

TRAINING GOALS

Through participation in the research described in this proposal, Dr. [redacted] will develop a varied and versatile set of scientific skills that will include recombinant genetics, bioinformatics, microbiology, animal modeling, and aspects of cell biology. She played an active role in **planning her project** and **designing experiments** to answer questions about GBS host-pathogen interactions that interest her.

She will be primarily responsible for conducting the experiments, troubleshooting problems, and interpreting results. **Her explicit goal is to complete a draft manuscript describing this research by the end of her fellowship.** I believe this paper will be impactful and will mark an important milestone in her academic medical career. She will also have **opportunities to publicly present her work**, including at the upcoming ESPR conference for which her research was selected for a platform presentation.

In the approximately six months that she has spent in the lab so far, Dr. [redacted] has already made exceptional progress:

- She has thoroughly tested the influence of nitric oxide on growth and survival of a panel of GBS isolates representing all 10 capsular serotypes.
- She has learned to perform bacterial RNA extraction and to interpret genome-wide RNA-seq data.
- She has nearly completed whole-genome fitness screening using a GBS saturated transposon mutant library (Tn-seq) in the presence of nitric oxide.
- She has mastered our murine model of chorioamnionitis and is in the process of assessing the influence of GBS placental invasion on host nitric oxide synthase expression.

In our conversations about her career goals, Dr. [redacted] has expressed an interest in pursuing translational neonatal research at an academic center. While this is a challenging course to pursue, I recognize in Dr. [redacted] a rare combination of scientific curiosity, rigorous attention to experimental detail and critical thought, and a sustained desire to make scientific discoveries with the potential to improve her patients' clinical outcomes. I believe that she has the attributes that will allow her to succeed in her goals, and that this project will be an important stepping stone toward achieving them. This award from MWRI will provide valuable recognition and support, and will help her bridge the crucial and often daunting gap between training and research independence.

Thank you for your consideration.

SPECIFIC AIMS: We hypothesize that GBS has evolved mechanisms of NO resistance that promote its survival in the placenta and that exaggerated placental NO release in the presence of GBS chorioamnionitis contributes to decidual injury, increasing the risk of stillbirth or preterm delivery.

Aim 1. We will use whole-genome molecular screens and targeted knockdown approaches to elucidate the adaptive mechanisms that allow GBS to survive in NO-rich environments.

a. We will perform whole-genome transcriptomic analyses in two clinically relevant strains of GBS to determine what genes are significantly upregulated or downregulated in the presence of NO.
b. Using an existing saturated GBS transposon mutant library, we will perform next-generation sequencing of transposon-genome junctions (Tn-seq) to determine candidate genes that confer fitness under NO selective pressure.
c. Using a CRISPRi gene expression knockdown system, we will generate targeted knockdowns of at least 10 GBS genes identified in Aims 1a and 1b that were upregulated in a NO environment and that conferred protection from NO by Tn-seq.

Aim 2. Using an established murine GBS chorioamnionitis model with wild type and NOS deficient dams, we will examine the interplay between GBS placental invasion, host NO response, and pregnancy outcomes

a. To characterize expression of NOS in the placenta during GBS invasion, we will perform qPCR and immunohistochemistry on placental tissue from a murine model of chorioamnionitis (and sham controls).
b. To understand the effect of NO production on pregnancy outcomes during GBS chorioamnionitis, we will assess rates of ascending infection, fetal demise, and preterm birth using wild type and inducible NOS knockout dams.

SIGNIFICANCE: Group B *Streptococcus* (GBS) is an important global cause of perinatal morbidity and mortality[1]. GBS vaginal colonization during pregnancy is a risk factor for ascending chorioamnionitis, which can lead to neonatal sepsis, premature labor, miscarriage, stillbirth, and maternal complications[2 3].

Chorioamnionitis is an infection of the placenta, fetal membranes, and amniotic fluid usually caused by vertical ascension of bacteria from the vaginal tract. In order to survive in the pregnant uterus, GBS must employ defense mechanisms to circumvent the host innate immune response. One key placental immune response is generation of nitric oxide (NO) by nitric oxide synthase (NOS). NO is produced by multiple placental and fetal cell types, including resident phagocytes[4]. NO has multiple downstream effects, including induction of further cytokine signaling, vasodilation, and conversion to reactive oxygen species and reactive nitrogen intermediates that lead to bacterial cell death[5].

Bacteria differ in their abilities to withstand NO exposure. Multiple human pathogenic bacteria, which face elevated NO concentrations at infection sites, encode stress response genes that are activated by NO-mediated oxidative damage and which function to detoxify the cell, promoting prolonged bacterial survival and further invasion[6]. While the bactericidal effects of NO are initially adaptive, overexpression of NO can affect host tissue viability as a result of blood flow dysregulation and direct oxidative damage. In the placenta, high concentrations of NO in the setting of chorioamnionitis are thought to contribute to placental cell apoptosis, potentially leading to preterm labor or fetal demise[7].

In the studies proposed here, we will use a set of unbiased, bacterial genetic tools, combined with clinically relevant animal models of ascending GBS chorioamnionitis, stillbirth, and preterm delivery, to examine GBS molecular pathways that promote evasion of placental NO and the role of NO on GBS-related adverse pregnancy outcomes.

EXPERIMENTAL DESIGN

Aim 1. We will use whole-genome molecular screens and targeted knockdown approaches to elucidate the adaptive mechanisms that GBS possesses to survive in nitric oxide-rich environments.

Aim 1a. We will perform whole-genome transcriptomic analyses in two clinically relevant strains of GBS to determine what proteins are significantly upregulated or downregulated in the presence of NO.

Experimental plan: For this aim, which is largely complete, we exposed two clinically relevant GBS strains—CNCTC 10/84 (capsular serotype V) and A909 (capsular serotype Ia)—to DETA, a nitric oxide donor, or a diluent control in culture. We then purified whole RNA from stationary phase experimental and control GBS cultures. Following rRNA depletion and reverse transcription, we generated sequencing libraries and performed next-generation sequencing on an Illumina NextSeq platform. We used DESeq to compare expression profiles between NO and control conditions for the two GBS strains.

Aim 1b. Using an existing saturated GBS transposon mutant library, we will perform next-generation sequencing of transposon-genome junctions (Tn-seq) to determine candidate genes that confer fitness under NO pressure.

Experimental plan: These experiments are complete except for sequencing and bioinformatics. An existing saturated transposon mutant library in GBS strain A909 (also used in Aim 1A) was grown to stationary phase with sublethal NO exposure or a diluent control, after which DNA was extracted and processed to isolate transposon-genome junctions as previously published by our group[8]. Transposon junction library sequencing (Tn-seq) is underway using the Illumina NovaSeq system. Once sequencing is complete, we will perform bioinformatic analysis using ESSENTIALS, a publicly available Tn-seq analysis package, to identify statistically significant differences in transposon junction detection between experimental and control conditions.

Aim 1c. Using a CRISPRi gene expression knockdown system, we will generate targeted knockdowns of at least 10 GBS genes identified in Aims 1a and 1b that were upregulated in a NO environment and that conferred protection from NO by Tn-seq.

Experimental plan: We will choose at least 10 GBS genes that showed statistical significance in our RNA-seq and Tn-seq experiments. We will then use the Broad Institute's Genetic Perturbation Platform to generate protospacer sequences targeting the corresponding genes in GBS strains CNCTC 10/84 and A909 with constitutive dCas9 expression. Synthetic protospacer oligonucleotides will be cloned into the CRISPRi shuttle plasmid 3015b, after which the plasmid will be transformed into competent *Escherichia coli*. Successful cloning will be confirmed by colony PCR and Sanger sequencing, after which the plasmid will be purified from *E. coli* and used to transform competent GBS by electroporation. We will perform qPCR of each targeted GBS gene to confirm successful expression knockdown. The new knockdown GBS strains will then be exposed to DETA, the nitric oxide donor used in Aim 1a and 1b, or diluent control in culture. We will use growth curves and CFU quantification to determine altered NO resistance between control and knockdown GBS strains.

Aim 2. Using an established murine GBS chorioamnionitis model with wild type and NOS deficient dams, we will examine the interplay between GBS placental invasion, host NO response, and pregnancy outcomes.

Aim 2a. To characterize expression of NOS in the placenta during GBS invasion, we will perform qPCR and immunohistochemistry on placental tissue from a murine model of chorioamnionitis (and sham controls).

Experimental plan: Wild type C57BL/6J mice will be mated to establish timed pregnancies. Pregnant dams will be vaginally colonized with approximately GBS CNCTC 10/84 or sham PBS on day 13 of gestation and will be sacrificed and dissected on day 16 to give sufficient time for development of chorioamnionitis.

A vaginal swab and homogenized samples of placentas and pups will be plated on chromogenic agar plates to confirm presence of GBS and the homogenized tissues will be used for whole RNA purification. Intact samples will be paraformaldehyde-fixed for immunohistochemistry. cDNA will be reverse transcribed from the RNA samples (along with appropriate negative controls) and then qPCR will be performed to quantify the level of endothelial (eNOS) and inducible (iNOS) expression (normalized to the mouse housekeeping gene Polr2a). Fixed tissue samples will be sectioned and stained for GBS capsule, eNOS, and iNOS using commercially available primary and secondary antibodies. Quantitative fluorescence microscopy will be performed, comparing localization and expression of GBS and NOS in infected mice. We will also assess baseline NOS expression in samples from the sham control mice. Together with qPCR results, these data will establish an initial understanding of how GBS in the placenta influences NOS expression.

Aim 2b. To understand the effect of NO production on pregnancy outcomes during GBS chorioamnionitis, we will assess rates of ascending infection, fetal demise, and preterm birth using wild type and inducible NOS knockout dams.

Experimental plan: GBS vaginal colonization will be performed on pregnancy day 13 as described above in commercially available iNOS knockout mice (B6;129P2-NOS2^{tm1Lau}/J) or wild type controls. Colonized mice will be weighed and observed twice daily through pregnancy day 16, at which point they will be sacrificed and dissected. Key outcome measures will be 1) weight change, 2) preterm delivery, 3) persistence of vaginal colonization, 4) presence and density of GBS in placental tissue, amniotic fluid, and fetuses, and 5) IUFD.

TIMELINE: Experiments for this project began in August 2020, at the start of my second year of fellowship. I have completed all but one month of clinical service time leaving the majority of the next 12 months dedicated solely to research.

	Months 1-3	Months 4-6	Months 7-9	Months 10-12
Aim 1				
Aim 2				

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2. Patras, K.A. and Nizet, V. (2018) Group B Streptococcus Maternal Colonization and Neonatal Disease: Molecular Mechanisms and Preventative Approaches. *Front Pediatr,* 6(27).

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4. Eis, A.L.W., et al. (1997) Immunolocalization of the Inducible Nitric Oxide Synthase Isoform in Human Fetal Membranes. *AJRI,* 38, 289-294.

5. Chakravortty, D. and Hensel, M. (2003) Inducible nitric oxide synthase and control of intracellular bacterial pathogens. *Microbes and Infection,* 5(7): 621-627.

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7. Nakatsuka, M., et al. (1999) Generation of peroxynitrite and apoptosis in placenta of patients with chorioamnionitis: possible implications in placental abruption. *Human Reproduction,* 14(4): 1101-1106.

8. Hooven, T.A., et al. (2016) The essential genome of Streptococcus agalactiae. *BMC Genomics*, 17: 406.