Characterizing the Effect of Intermittent Hypoxia on Hofbauer Cell Polarization and Placenta Vascularization, *in vitro*.

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Thesis submitted to the University of Ottawa in partial fulfillment for the requirements for the degree of Master of Science, Human Kinetics

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Preface to this Thesis

When this thesis was proposed, we originally sought to elucidate the downstream role of HBC polarization induced by intermittent hypoxia on cytotrophoblast differentiation. The experimental plan for the original Aim 2 was to create a colour fusion assay to quantify the amount of syncytialization, the main step in syncytiotrophoblast formation. After 1 year of optimizing this method (May 2023 – May 2024), these experiments were deemed unfeasible, and we pivoted to using immunofluorescence to measure markers of syncytialization. After 3 months (May 2024 – July 2024) of optimizing the antibodies and trying numerous companies, two of our required antibodies for measuring cell fusion did not perform as advertised. We therefore switched to using western blot methods and optimized the required antibodies (July 2024 – September 2024). Due to the resource limitation of having one tuneable incubator, only one experimental condition can be completed at a time and so these experiments could not be completed. In the attached appendix of this thesis, the methods of the colour fusion assay, immunofluorescence and western blots are provided. My supervisor Dr. Kristi Adamo and thesis committee members Drs. Micheal De Lisio and Keir Menzies support the modifications to my thesis due to these challenges.

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Acknowledgements

At the forefront, I would like to express how profoundly thankful I am for my supervisor Dr. Kristi Adamo. With your unwavering guidance throughout my journey in academia, I have achieved many milestones that I had at one point thought were unattainable. Your exemplary passion for deconvoluting the unknown has inspired me to dive into the wonderful world of placenta biology and I am forever grateful for you and the lab you lead. Another pillar behind these projects is Dr. Velislava Tzaneva. There are not enough words in the dictionary to express my appreciation for your guidance, support and patience with me while I was learning the numerous techniques in the wet lab. You made the many late nights and early mornings at the hospital all worth it!

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learning the reins of primary cell culture. Although, I will forever second guess how I, and other Canadians say words with a “u”, and I will think of you when I say them.

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Abstract

Background: Gestational parent (gesP) physical activity (PA) is associated with a plethora of health benefits that also provides an advantage to the fetus. Previous work from the Adamo Lab has found that the placenta-resident macrophage, termed Hofbauer cell (HBC), favours the anti-inflammatory phenotype compared to the pro-inflammatory phenotype in the placenta from physically active gesPs. A theorised effect of gesP PA is intermittent hypoxia (IH), but we do not know if this effect will polarize HBCs in a similar manner. Additionally, the implications on the downstream roles in the placenta are not known.

Methods: The first objective of this thesis was to determine if IH influences HBCs *in vitro*. Primary human HBCs were cultured under two conditions: 5% O_2 as a control and IH. Flow cytometry analysis was conducted to quantify pro- and anti-inflammatory HBCs. CD68 was used as a pan-macrophage marker and double positive $CD86^+/CD68^+$ or $CD206^+/CD68^+$ were identified as pro-inflammatory or anti-inflammatory, respectively. To assess the impact of IH on the functional purpose of HBCs, we performed a phagocytosis assay. HBC conditioned media was collected and analyzed with a cytokine array to determine the components of the media. The second aim was to use the HBC conditioned media and determine the independent or combined effect with IH on placenta angiogenesis. Human umbilical vein endothelial cells (HUVECs) were used in tube formation and migration assays under the following conditions: 8% O_2 (control), 8% O_2 with HBC conditioned media, IH, or combined (HBC conditioned media and IH).

Results: No differences were found in the absolute number of pro-inflammatory ($p = .832$) or anti-inflammatory ($p = .614$) HBCs. The proportion of anti-inflammatory HBCs between the conditions did not differ significantly ($p = .543$) with a medium effect size, $d = 0.55$. Similarly, the proportion of pro-inflammatory HBCs had a negligible effect size, $d = -0.143$, and did not differ significantly ($p = .752$) between the culture conditions. Phagocytosis percentage was also not distinct between the two culture conditions ($p = .651$). We detected differential regulation of cytokines depending on the oxygen tension. Identification of many pro-angiogenic, anti-angiogenic, and pleiotropic factors were identified in the conditioned media. Tube formation assays showed significantly shorter segments in the IH ($p = .034$) and IH/HBC ($p = .026$)

condition compared to the HBC condition. There was also a lower total mesh area in the IH ($p = .038$) and IH/HBC ($p = .015$) conditions compared to the HBC condition, along with less master segments in the IH ($p = .039$) condition compared to the HBC condition. Lastly, the master segments were shorter in both the IH ($p = .038$) and IHHBC ($p = .028$) conditions compared to the HBC condition. No significant differences were found between the control condition and the experimental conditions. IH and HBCs did not impact endothelial cell migration.

Conclusions: In summary, IH may not be the main driver of differences in HBC polarization as previously noted by Goudreau *et al.* Our culture conditions maintained the heterogenous HBC population with some favouring of the pro-inflammatory phenotypes, suggesting that the culture conditions may not be fully representative of the *in-vivo* environment, a continued shortfall of *in-vitro* models. However, treatment with IH lead to different secretions from HBCs compared to the control. Therefore, IH appears to regulate HBC behaviour, subsequently influencing angiogenesis. While the impact of IH and HBCs on tube formation did not deviate significantly from baseline, our study shows that HBCs are master regulators of angiogenesis that do not override the protective mechanisms for vasculature formation under hypoxic conditions.

Abbreviations

B-lymphocyte Chemoattractant (BLC)

Brain-Derived Neurotrophic Factor (BDNF)

Developmental Origins of Health and Disease (DOHaD)

Epidermal Growth Factor (EGF)

Epithelial Neutrophil-Activating Peptide (ENA)

Erythropoietin (EPO)

Fibroblast Growth Factor (FGF)

Gestational Diabetes Mellitus (GDM)

Gestational Parent (gesP)

Glial Cell Line-Derived Neurotrophic Factor (GDNF)

Granulocyte Chemotactic Protein (GCP)

Granulocyte Colony Stimulating Factor (G-CSF)

Granulocyte Macrophage Colony Stimulating Factor (GM-CSF)

Hematopoietic Stem Cells (HSCs)

Hepatocyte Growth Factor (HGF)

Hypoxia-inducible Factors (HIF)

Hofbauer Cell (HBC)

Homologous to **l**ymphotoxin, exhibits **i**nducible expression and competes with HSV **g**lycoprotein D for binding to **h**erpesvirus entry mediator, a receptor expressed on **T** lymphocytes (LIGHT)

Human Chorionic Gonadotropin (βhCG)

Insulin-like Growth Factor (IGF)

Insulin-like Growth Factor Binding Protein (IGFBP)

Interleukin (IL)

Interferon gamma (IFN-γ)

Interferon Gamma-induced Protein 10 (IP-10)

Intermittent Hypoxia (IH)

Lipopolysaccharides (LPS)

Macrophage Colony -Stimulating Factor (M-CSF)

Macrophage Derived Chemokine (MDC)

Macrophage Inflammatory Protein (MIP)

Membrane Cofactor Protein (MCP)

Migration Inhibiting Factor (MIF)

Mitogen-Inducible Gene (MIG)

Neurotrophin (NT)

Osteopontin (OPN)

Partial Pressure of Oxygen (pO_2)

Physical Activity (PA)

Placental Growth Factor (PIGF)

Platelet-Derived Growth Factor (PDGF)

Pulmonary and Activation-Regulated Chemokine (PARC)

Regulated Upon Activation, Normal T Cell Expressed and Secreted (RANTES)

Secreted Protein Acidic and Rich in Cysteine (SPARC)

Sprouty (Spry)

Stem Cell Factor (SCF)

Stromal Cell-Derived Factor (SDF)

Thymus and activation-regulated chemokine (TARC)

Tissue Inhibitors of Metalloproteinases (TIMP)

Transforming Growth Factor (TGF)

Tumour Necrosis Factor (TNF)

Vascular Endothelial Growth Factor (VEGF)

Preamble to Chapter 1:

The manuscript titled *Breaking Boundaries: A Chronology with Future Directions of Women in Exercise Physiology Research, Centred on Pregnancy,* was submitted to *Advanced Exercise and Health Science* on March 1st, 2024, as an invited review. The manuscript was revised as requested on April 25th, 2024, and was accepted on April 26th, 2024. This manuscript is added to the thesis to establish the history of exercise physiology research focused on pregnancy and highlight the ongoing gaps in this novel field.

Chapter 1: The History of Exercise Physiology in Pregnancy

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Breaking boundaries: A chronology with future directions of women in exercise physiology research, centred on pregnancy

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ABSTRACT

Historically, females were excluded from clinical research due to their reproductive roles, hindering medical understanding and healthcare quality. Despite guidelines promoting equal participation, females are underrepresented in exercise science, perpetuating misconceptions about female physiology. Even less attention has been given to exercise in the pregnant population. Research on pregnancy and exercise has evolved considerably from the initial bedrest prescriptions but concerns about exercise risks during pregnancy persisted for many decades. Recent guidelines endorse moderate-intensity physical activity during pregnancy, supported by considerable evidence of its safety and benefits. Mental health during pregnancy, often overlooked, is gaining traction, with exercise showing promise in reducing depression and anxiety. While pregnancy guidelines recommend moderate-intensity physical activity, there remains limited understanding of optimal frequency, intensity, type and time (duration) for extremes like elite athletes or those with complications. Female participation in elite sport and physically demanding jobs is rising, yet research on their specific needs is lacking. Traditional practices like bed rest for high-risk pregnancies are being questioned, as evidence suggests it may not improve outcomes. Tangible neglect of gestational parents in research perpetuated stereotypes of female frailty, though recent years have seen a shift towards recognizing the benefits of an active pregnancy. Closing knowledge gaps and inclusivity in research are crucial for ensuring guidelines reflect the diverse needs of gestational parents. Therefore, the purpose of this review is to summarize the evolution of exercise physiology and pregnancy research along with future directions for this novel field.

1. Introduction

Historically, females have been viewed as reproductive vessels and the existence of reproductive organs led to their exclusion from clinical decision-making research. This exclusion was concomitant with a pervasive status quo that places a lower value on females as a result of conventional, cultural and systemic forces that stigmatize their participation in scientific research.[1,2] Owing to hormonal and reproductive organ complexities, it is common for researchers to adopt a very cautious approach to including females in clinical trials and scientific research. Since important medical research did not include representation of females and people with menstrual cycles, the quality of healthcare accessible to them has suffered.[3]

In recent decades, it has become apparent that excluding female participants from scientific research has hindered our understanding of their disease risk and progression as well as responsiveness to medications and other therapeutic treatments (e.g., medical devices and natural health products). Many of the breakthroughs in medicine stem from research primarily conducted on male cells and animals with the results of these studies being subsequently extrapolated to females. Consequently, the practice of applying knowledge gathered on males to females has impacted the pharma industry, with eight out of ten approved drugs being withdrawn from the market due to unforeseen health implications in female users.[4] Another example refers to the key differences between sexes surrounding cardiovascular disease (CVD) risk. Evidence of this disparity goes back to the late 1950's.[5] It is well

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documented now that females die from CVD more frequently than men, yet it remains understudied, under-recognized, underdiagnosed, and undertreated in the female population.[6]

Not only have males predominantly filled the roles of doctors, scientists, and researchers, but they have been the focus of the majority of the medical science studies on significant health issues that affect all sexes, like hypertension and diabetes. While clear lifestyle, environmental and behavioural proclivities between males and females are reflected in biological differences at the molecular and cellular level, it was not until 1994 that the US National Institutes of Health (NIH) created a guideline for the evaluation of sex-based differences in clinical trials to assess the safety and efficacy of drugs to treat the masses.[7] In Canada, concerns raised about a deficit of research on breast and gynecological cancers, led to the development of the "Canadian Guidance Document on The Inclusion of Women in Clinical Trials" recommending the inclusion of females at each stage of scientific research so that the impact of new drugs on specific sexes can be better understood.[8]

The issue of female representation is not limited to medical or clinical research. Despite guidelines aimed at promoting equal participation by both sexes, inclusion of females in the exercise sciences remains inadequate. Cowley *et al.* drew attention to the disproportions in their examination of the ratio between male and female participants in sport science research; 34% of the study population were female, and only 6% of total publications focused exclusively on females.[9] Furthermore, a recent systematic review examining the prevalence of female participants in exercise studies focusing on vascular endothelial function noted that only 36% of participants were female.[10] The scarce literature on how females respond to exercise, exercise training, and other physical activity (PA) interventions has led to a lack of understanding on this topic relative to men,[11] as visualized in Fig. 1. The factors leading to the low participation of females in exercise and physiology research are akin to those preventing them from participating in medical trials. Namely, concerns and uninformed beliefs about female physiology such as hormonal fluctuations during the menstrual cycle, hormonal contraceptives, and historical beliefs that females are not capable or interested in exercise. Whilst the common assumption that females are smaller versions of males continues to be the subject of debate in the peer-reviewed literature, with the publication of her book - *Up to Speed* - Christine Yu[12] is shedding light in the public sphere on the detrimental impact of applying exercise physiology and nutrition research results from males to females in athletics. The lack of evidence-informed practices with female athletes has led to the disruption of menses, stress fractures, drop-out and much more.[13]

While females, in general, make up the minority of research subjects, an even greater research pariah, especially in exercise science, are pregnant females. Subsequently, our knowledge and understanding of physiological responses to exercise in this population is lagging. The fear of studying pregnancy is, in part, due to the therapeutic tragedies of the past (e.g., the use of Thalidomide in the 1960s and offspring limb deformities) [14] and the perception of vulnerability. Undoubtedly, certain cautions should be applied when engaging with the pregnant population, but the sizeable underrepresentation of gestational parents (gesPs) in exercise and sports sciences has been fueled by the fear that gesPs are not capable of engaging in exercise without impairing fetal growth and development. While today we know these assumptions are not factual, the inclusion of gesPs in exercise physiology research is still low. Moreover, publications regarding pregnancy and exercise physiology may be marginalized due to the underrepresentation of females on sports science editorial boards, and fewer holding first and senior authorship positions in publications of randomized controlled trials compared to men.[15] Consequently, exercise physiology research focused on females may not be prioritized by some journals nor prioritized by male researchers.

As can be gathered from any exercise physiology or sports science textbook, research advancements in exercise science have been monumental in the last two centuries, with the male population and their responses being well characterized. While our understanding of females

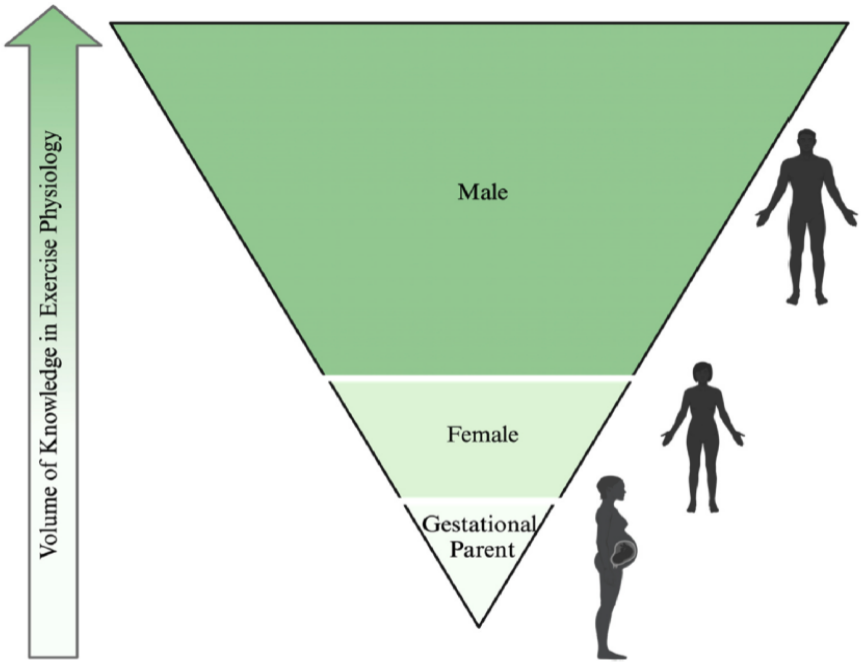

Fig. 1. Visualization of the Approximate Volume of Knowledge in Exercise Physiology Research of the Biological Sexes and Gestational Parents. Created using BioRender.com.

in exercise physiology research is gathering speed, the research remains significantly behind what we know about males, and our knowledge about the exercising pregnant population is still in the starting blocks. Therefore, this review aims to summarize the evolution of exercise physiology research on gesPs and offer potential future directions in this unique population.

2. Timeline

2.1. 1850s – Early 1900s: A time for bedrest

For centuries, bedrest was a common pregnancy prescription touted by medical professionals. In 1858, a midwifery textbook, written by Fleetwood Churchill, described gestational rest as "the most powerful prophylactic means we possess".[16] It was believed that the risk of pre-term birth, which poses a health threat to both the woman[2] and fetus, could be decreased through the implementation of bedrest.[17] An upright posture was thought to exert excess pressure on the cervix, thus, increasing the likelihood of pre-term delivery.[18] This belief served as justification for the near-unanimous prescription of bedrest in the years to come during pregnancies with multiple fetuses, where cervical pressure is even further amplified. Interestingly, bedrest was a frequently advised intervention at the first sign of any pregnancy complication despite an overwhelming lack of supporting evidence.[19]

The rationale for the implementation of bedrest was also partially justified by observations of pregnancy success rates in relation to socioeconomic status. Lower social class often correlated with poorer pregnancy outcomes; thus, it was proposed that higher levels of PA in lower class females were associated with these adverse outcomes.[20] While higher class females often had the means to assume bedrest in late gestation, lower class pregnant females worked until the end of their pregnancies, rendering PA and upright posture unavoidable.[20] PA redirects blood flow to the working musculature, exposing the placenta to a theoretical state of decreased blood flow and therefore depriving the fetus from nutrients and oxygen.[20] Accordingly, it was widely believed that PA was the culprit of many pregnancy complications, and prenatal bedrest was deemed a worthy intervention. This class-based divide in the practice of and access to bedrest was also seen in the postpartum period, where it was a socially encouraged part of Victorian society. Upper class females would endure what was known as the *lying-in* period, where remaining in bed post-birth was strictly enforced, whose duration could be up to several weeks.[21] In reality, the disparity in complication-free pregnancy rates based on socioeconomic status is a complex matter with many confounding variables, including but not limited to maternal nutrition, environmental stressors, smoking and substance use, and social support.[22]

Prescribing medical professionals rarely considered the detrimental impacts of bedrest. Inactivity for extended periods of time has harmful effects on health, implicated with increased muscle atrophy and weight loss, decreased cardiovascular function, disrupted hormones, and negative mental health outcomes (see Section 2.2).[19,23] Based on surveys performed by Maloni *et al.* in 1998,[23] physicians prescribing bedrest were largely unaware of its associated side effects. Consequently, females who had endured this largely unhelpful and unfounded intervention were too often left post-treatment without proper recovery, education, or guidance for their newly deconditioned bodies.

Today, we know that there is little to no supporting evidence for bedrest as a successful pregnancy intervention, and often does more harm than good.[24] Though historically it was an acceptable practice, research in the last century has made slow progress to uncover the truth about maternal bedrest, PA, and pregnancy, rendering unsupported recommendations for inactivity in healthy pregnancies a thing of the past.

[2] The inclusive term used by our team is gestational parent (gesP), however, we have chosen to use the term woman/women if it was the wording in the original historical text

2.2. Early 1900s: Emphasis on rest and housework

Following the bed rest and *lying-in* period, emerging practices of pregnancy "hygiene" began in the early 1900s. While much of the literature and the expected behaviours during this time period were purely opinion-based, medical professionals recommended that women should rest as much as they could, including a minimum 30-minute nap per day in addition to their regular chores.[25,26] The aspect of rest was heightened during the later portions of pregnancy. It was thought that pregnancy fatigued the body, especially the heart, and that strenuous exercise may lead to cardiac insufficiency.[27] Moreover, the expanding uterus was thought to misplace the heart and impair the diaphragm such that exercise could not be sustained.[28,29]

Throughout the early 20th century, the prevailing belief remained that the pregnant body cannot maintain exercise; as such, very light activities, such as walking outdoors, were recommended.[25,26,30,31] However, since women were responsible for the household at this time, doctors presumed that daily chores would be acceptable.[26] As such, many of the recommendations at this time included quitting all sports to conserve energy not only to support the growing fetus, but to continue performing chore work and upkeep the household. Only a few doctors recommended more "strenuous" activities, such as gymnastics during pregnancy, in addition to walking outdoors.[25] There were no specific guidelines for the duration or volume of exercise at this time, with some literature suggesting no limits as to walking and gymnastics engagement.[25]

Of the exceedingly limited scientific research that included women during the early 1900s, little attention was paid to pregnant women, and much of the pregnancy research was focused on the implications of pregnancy and heart disease.[28,29,31] A study completed by Reid in 1930[28] looking at heart disease during pregnancy compared necropsy files from male and female participants. This study divided the female participants based on marital status, not parity status; representing a bias that all married females bear children. After evaluating the mitral orifice and cause of death, the author concluded that pregnancy may cause cardiac insufficiency.[28] Since the participants were not actively pregnant at the time of death, nor were they divided by the number of pregnancies and time after birthing children, many confounding variables could explain the increase in cardiac insufficiency observed in the married group. Additionally, Newell (1912)[31] remarked that when pregnant women who did not engage in exercise came into labour, acute left ventricular dilation was often observed. Therefore, Reid's findings of cardiac insufficiency may not have been the result of pregnancy, but potentially due to the lack of engagement in exercise and PA throughout pregnancy. In support of exercise, other doctors observed a decrease in the prevalence of toxemias (preeclampsia as we know it today) and other diseases when women were considered "fit".[25,31,32] Interestingly, it was suggested that exercise and careful dieting during pregnancy may reduce the risk for obstetrical and surgical interventions during labour.[30]

In summary, research from the early 1900s supported engagement in light exercise and chores; however, prioritizing rest throughout pregnancy remained the standard practice. The expectations for pregnant women at this time were heavily routed in social norms and gender roles to support the household. Accordingly, researchers may have been influenced by these cultural standards and the worry of harming the fetus, stymieing further exploration of the benefits of exercise during pregnancy.

2.3. 1950s - late 1970s: Training for labour era

In the 1950s, the publication of Helen Headman's book, *A Way to Natural Childbirth*, represents the first time exercise classes focused on labour training appeared in the literature. The popularity of this concept led doctors to recommend exercises and classes that physically prepared and educated pregnant women for pregnancy and labour.[33] The physical exercise portion of this training for labour program was

two-fold: (1) to increase the tone and efficiency of the core muscles and the pelvic floor and (2) to promote relaxation. Similar training programs emerged during this era to help prepare for labour.

As the view of women being responsible for the house was still commonly held in the mid-1900s, exercise engagement in the form of walking outdoors was still recommended to ensure women could continue to fulfill their housekeeping duties during pregnancy, despite some of the literature noting that housework was no longer a substitute for exercise.[34] These training programs were not simply aimed to assist in labour; but rather it was thought that if abdominal strengthening began during the second trimester and into the post-partum period, women would more rapidly regain their figure and experience a reduction in the risk of uterine prolapse.[34] The recognition of the importance of the pelvic floor, and what are known today as Kegel exercises, emerged through the work of Arnold Kegel. He described a set of pelvic floor exercises that can be done with a *Perineometer* to restore function and tone to the pelvic floor during the post-partum period.[35] The maintenance of proper posture during pregnancy was also highlighted to reduce the pressure on the pelvis, support proper circulation, and reduce lower back pain[36]; but this literature was not based on research evidence, just medical opinion.

In addition to strengthening the core and pelvic floor, exercise classes termed “ante-natal” classes or “Lamaze” classes gained popularity and were the outcome of the exercise boom in the 1970’s.[37,38] These classes still maintained the common theme for this era of preparing for birth, but added the aspect of breathing techniques and strengthening the entire body. In a hallmark study conducted by Gunter (1956) [38] that looked at the use of ante-natal classes and the implications for birth, the exercise group laboured on average 6 hours less, experienced fewer episiotomies, half the number of birth complications and less post-partum hemorrhage compared to the control group.[38] Later in the 1960s and '70s, additional studies supported the benefits of exercise, demonstrating that fit women had shorter labour times and fewer complications than unfit women.[39,40]

Outside of the training for labour framework that was commonly denoted in the literature during the '70s, researchers started to examine the physiological responses of exercise in the pregnant population.[41,42] Pregnant women were shown to have increased ventilation, heart rate, and cardiac output per unit increase in work as compared to non-pregnant controls [41]; thus illustrating that pregnant women can sustain exercise. Moreover, data emerged linking exercise during pregnancy to better management of pre-pregnancy diabetes.[43]

Popular medical opinion from the late 19th and into the first decades of the 20th century was that pregnant women should use extreme caution to avoid fatigue and overexertion. The majority of the early published guidelines for gesPs were unscientific and reinforced the notion that females were weak and frail. As true scientific research began to emerge in the '70s, the ability to sustain exercise during pregnancy, along with the potential benefits, started to come into view.

2.4. 1980s – Present day: Evaluating the risks and benefits

While cardiopulmonary responses to exercise in pregnant females had been reported in the 1970s, the 1980s represented a boom in exercise physiology research in pregnancy. As much remained unknown, there were persistent concerns surrounding exercise risks in pregnancy; including the threat of a decrease in oxygen and nutrient delivery to the fetus, a fear of hyperthermia, and increased stress on the fetus initiating preterm labour and potential fetal mortality.[20,44,45] Albeit, the risks were a cause for concern at the time, the literature commonly espoused that the benefits of exercise outweighed the risks.[44] Thus, exercise with moderation (heart rate monitoring) was promoted with the caveat that exercise was not appropriate for those carrying more than one fetus or with a predisposition to heart disease.[44]

One study that contributed immensely to the concerns of fetal mortality with exercise was by Briend (1980),[20] whose group examined pregnant females working in physical jobs that required a lot of standing. They found that maternal mortality and stillbirth rates were higher in working pregnant women compared to those who did not work.[20] It is important to note that this study can be criticized for sampling bias and confounding variables as previously discussed in section 2.1.

Many historical myths surrounding the dangers of exercise in pregnancy have been disproven, with evidence showing no increase in neonatal mortality and obstetrical complications, no detrimental changes in oxygen and nutrient availability, and thus, no negative implications on fetal growth and development with gestational exercise.[46,47] There was also very little evidence that exercise led to changes in the fetal metabolism or blood catecholamines, signifying no changes to fetal stress with exercise.[47] Concerns of hyperthermia, fetal hypoxia and deprivation of nutrients and pre-term labour were also valid due to the lack of knowledge regarding exercise in pregnancy. Some researchers viewed these gaps in the literature as an opportunity to dive into the field of exercise physiology focused on pregnant females. Among these scientists was James F. Clapp III, who undertook a suite of hallmark studies that advanced exercise in pregnancy research. One integral study in the early 1990s performed by Clapp (1991)[48] provided evidence that core temperature increases during exercise were not detrimental to the embryo and fetus due to the physiological adaptations during pregnancy. Other studies also supported Clapp’s findings that the changes in core temperature experienced during exercise were very low and did not jeopardize the fetus.[47]

There was a common trend that emerged with some of Clapp’s research showing decreased weight gain during pregnancy and lighter weight offspring compared to non-exercising individuals.[49–51] Specifically, a study by Clapp and Capeless (1990) [49] found significant reductions in birth weight, fetal weight and size percentiles along with decreased adiposity without differences in crown-to-heel length and head circumference in the exercise group compared to non-exercising controls. The main differences (~70%) in weight reduction were thought to be equated to the reduced adiposity of the offspring.[49] While these changes in birth weight did reach statistical significance, this study has been criticized in that the reduction in weight was within normal ranges with no concern for preterm birth. Contrarily, when pregnant women who did not previously exercise were randomized to a weight-bearing exercise group for 8 weeks, their offspring were found to be heavier and longer than non-exercising controls.[52] This difference in weight was thought to be caused by an increase in both the offspring's lean mass and fat mass.[52] Interestingly, this study also found a greater placental growth rate and indexes of placental function during mid-pregnancy in the exercise group.[52] With these results in mind, it was concluded that aerobic exercise was safe for pregnant females and the fetus.

Additional benefits of exercise during pregnancy unearthed in the early '90s included reduced blood pressure, decreased risk of CVD, and the management of pre-pregnancy diabetes.[44] With the emergence of a greater volume of research with the transition to the 21st century, it became more apparent that exercise during pregnancy posed no threat for low-risk pregnancies, showed no detrimental effects and, in many cases, was beneficial for the pregnant woman and fetus. There was a continuation from the other decades in the observations of lower incidence of vaginal and abdominal surgeries along with shorter labour times experienced by pregnant women who exercised.[49] Adding on to these findings, less fetal stress was observed in women who exercised compared to those who did not exercise throughout pregnancy.[49]

Later in the 1980s, the publication of the Fetal Origins Hypothesis (FOH) paradigm by Sir David Barker, known today as the Developmental Origins of Health and Disease (DOHaD) paradigm, opened the door to examine PA and exercise as an *in-utero* exposure that may impact the health of the fetus. While Dr. Barker's interests did not lie with exercise, his seminal work examining geographical and temporal patterns of disease unveiled a relationship between heart disease

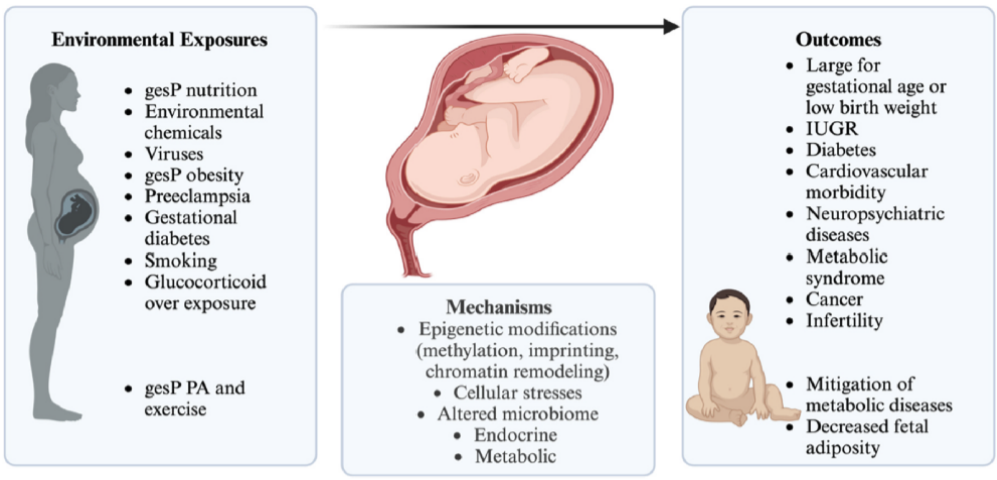

Fig. 2. Current findings supporting the DOHaD paradigm. gesP; gestational parent: PA; PA: IUGR; Intrauterine growth restriction. Created using BioRender.com Sources.[54–62]

and neonatal and post-neonatal mortality in the same locations 70 years prior.[53] He asserted that both intrauterine and early life factors influenced the risk of disease development in later life.[53] Much of the current work on the DOHaD paradigm has focused on factors such as nutrition, exposure to chemicals, smoking, and disease status of the pregnant female[54] (Fig. 2); there has been limited research on the prevention of these adverse outcomes through environmental exposure like prenatal PA and exercise.

Guidelines for exercise during pregnancy also began to be featured in the literature from the American College of Obstetrics and Gynecologists (ACOG) starting in 1985. The ACOG promoted that most aerobic exercise is safe for pregnancy and that pre-conception exercise can be maintained during pregnancy and the post-partum period.[63,64] These largely opinion-based recommendations were carried over to the early 2000s [65] when it was thought that exercise had not yet conclusively shown to be beneficial for improving perinatal outcomes.[63] Believing previous guidelines to be too conservative, and a desire to highlight the abundance of research over the last two decades showing no increases of early pregnancy loss, pregnancy complications, abnormal fetal growth, or negative fetal outcomes, [66] a Canadian team undertook the task of creating the first set of evidence-based clinical guidelines for PA in pregnancy. With the collaboration of the Society of Obstetrics and Gynaecologists Canada and the Canadian Society of Exercise Physiology (CSEP), the *2019 Canadian Guideline for Physical Activity Throughout Pregnancy* was developed using a vigorous methodological approach.[67] Recommendations put forth in the updated guidelines were supported by a set of 12 systematic reviews aimed at identifying the characteristics of exercise (i.e., frequency, intensity, duration, type, and volume) that were favourably associated with maternal, fetal, and neonatal health outcomes (summarized in Fig. 3).[67] The recommendations are that all women without contraindications, including those with GDM, were previously inactive, or are classified as a person living with obesity or overweight, should engage in a minimum of 150 minutes of moderate-intensity PA each week over a minimum of 3 days per week, although activity is encouraged every day.

There were vast advances in research on pregnancy and exercise during the 1980s to early 2000s to evaluate the risks and benefits of exercise during pregnancy along with hallmark studies that are the basis for current-day studies and advances in clinical and research obstetrics (Fig. 4). While the field of exercise and pregnancy has grown immensely since the 1850s leading to science-informed exercise during pregnancy guidelines,[67] there are still gaps in knowledge that need to be filled. While researchers have observed the fruitful benefits of PA during pregnancy, the mechanisms through which these benefits are accrued are not yet fully elucidated. Some of these mechanisms may include changes to oxygen tension in the placenta, remodelling of structures at the gesP-fetal interface, or changes in nutrient transport, cytokines and myokines. For a more in-depth review of the physiological responses to PA in pregnancy, please see other research work by the Adamo team.[68–70]

3. Future directions

3.1. Mental health and pregnancy

It is increasingly clear that the mental health of the gesP plays an integral role in long-term outcomes for both the birthing parent and the fetus. Prenatal depression is a strong predictor of postpartum depression,[71] a condition impacting approximately 17% of gesPs globally.[72] As such, gestational depression, anxiety and stress can have lasting effects on fetal development. Offspring from birthing parents with high levels of prenatal stress have a greater risk of decreased cognition, emotional problems such as depression and anxiety, sleep problems, as well as physical and physiological impairments (the reader is referred to Glover (2014)[73] for a more encompassing review). One study comparing the lipidome of placentas of gesPs with and without prenatal depression found several long-chain polyunsaturated fatty acids (LC-PUFA), including docosahexaenoic acid (DHA), to be down-regulated in depression.[74] DHA is critical for fetal brain development, and reduced levels in offspring at birth have been associated with decreased cognition at seven years of age.[75] It has also been theorized that the high cortisol levels that are often associated with poor mental health contribute to its negative impacts on offspring.[73,76] While the physiological alterations resulting from poor mental health during pregnancy are not yet understood, it is evident that suboptimal mental health fosters a mutually harmful environment for gesP and the fetus.

Despite their frequent prescription in the past, bedrest and sedentary behaviour have been associated with a plethora of negative mental health implications in pregnancy. Studies ranging from prolonged bedrest in postoperative patients to sedentary university students consistently demonstrate higher rates of anxiety, stress and depression.[19,77,78] Specific to pregnancy, gesPs have been shown to experience several psychological postpartum symptoms following antepartum bedrest, with the length of rest significantly correlated to the number of postpartum symptoms expressed.[79] Bedrest is further implicated with

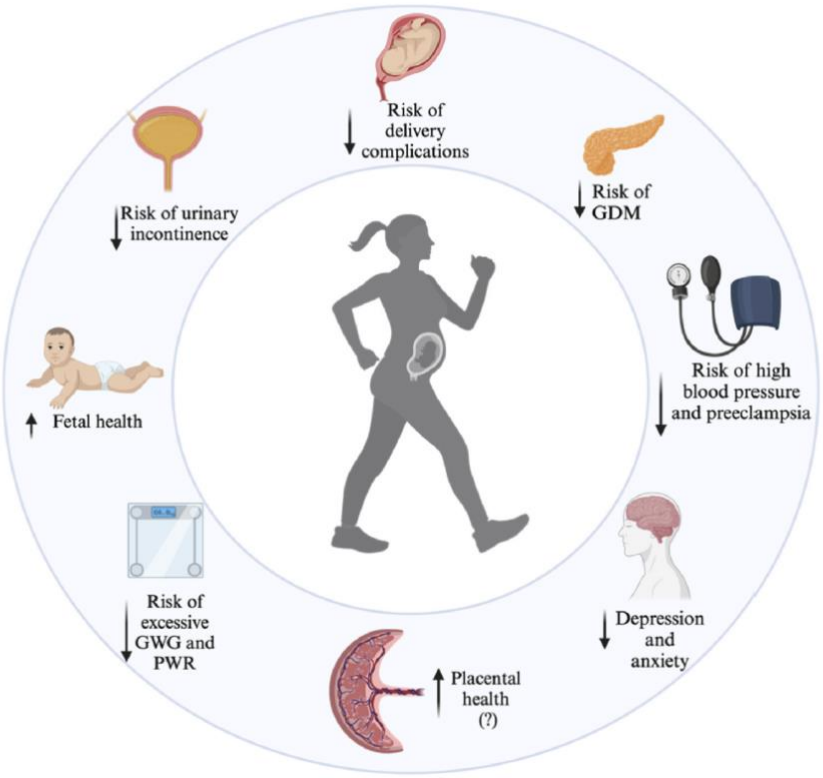

Fig. 3. Benefits of Gestational Parent PA. GDM; gestational diabetes mellitus: GWG; Gestational weight gain: PWR; postpartum weight retention. Created with BioRender.com.
Adapted from Bhattacharjee et al. (2021).[68] Created with BioRender.com.

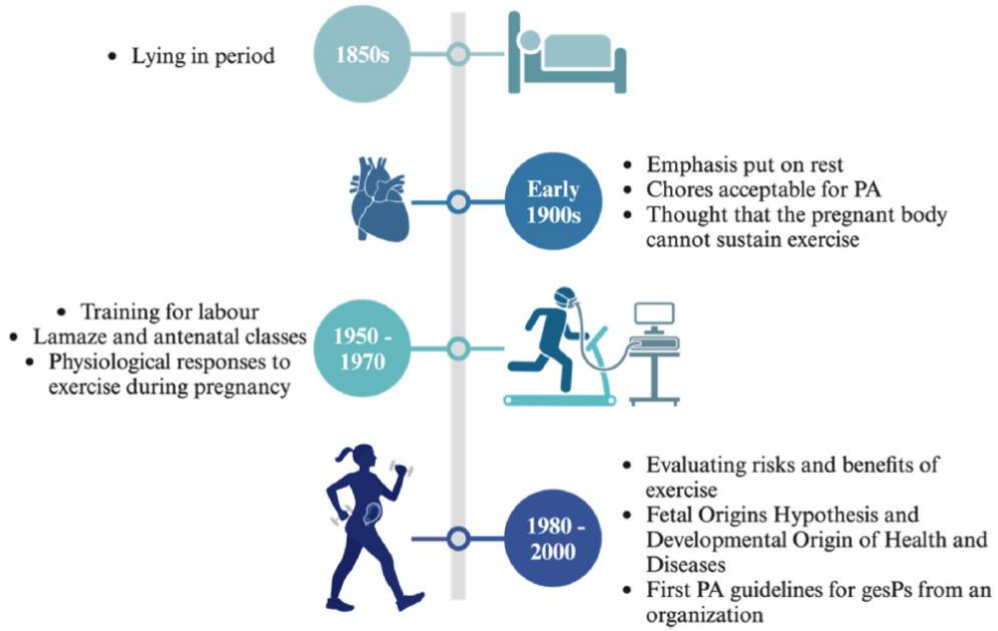

Fig. 4. The Evolution of Exercise Physiology Research in Pregnant Populations. PA: PA; gesP: gestational parent. Created with BioRender.com.

high depressive symptoms and antepartum stressors during multifetal pregnancies, continuing into the first several weeks of the postpartum period.[80] While antenatal mental health should be of critical importance, it has not frequently been highlighted or appropriately addressed in the literature. As a fitting reflection of the historically prominent bias toward men as study participants in scientific literature, some of the first papers to address the mental health implications of bedrest in pregnancy focused on the male partner, and the stressful environment they endured while the gesP could not contribute to the household.[81,82]

Recently, PA has been shown to positively affect mental health in the general population and as research progresses, similar patterns are emerging for gesPs. In 2019, a systematic review found significantly reduced postpartum depression scores in physically active gesPs. The effect sizes varied greatly across the included studies, suggesting confounding effects of frequency, intensity, type and duration of exercise.[83] Therefore, exercise guidelines should be explored to optimize mental health during pregnancy. Similarly, a 2022 systematic review of observational studies pertaining to PA and gesP mental health found PA to be associated with lower rates of prenatal depression, anxiety and stress, and decreased risk of postnatal depression and anxiety.[84] Interestingly, the same review noted no association of PA prior to pregnancy on these outcomes, though only a small number of studies exist on this topic.

While multiple umbrella and systematic reviews support PA as favourably impacting gesP mental health, many agree that additional high quality evidence is required to elucidate this relationship fully.[83–86] As science progresses, we have transitioned to a stage where bedrest, formerly a standard pregnancy recommendation, is now understood to be harmful to mental health. The literature is uncovering factors that affect gesP mental health and exploring PA as a viable intervention. Future work should continue to analyze the repercussions of PA in this context and examine the role of potential moderators such as PA variables (frequency, intensity, time and type of activity) and pre-pregnancy activity levels.

3.2. Specialty populations

While international guidelines endorse engaging in at least 150 minutes/week of moderate-intensity PA across pregnancy for uncomplicated pregnancies, we have limited knowledge about the upper and lower boundaries of exercise frequency, intensity, type and time (duration). Pregnant individuals can inhabit extreme categories: high-performance expectations (i.e., elite athletes) and extremely low performance (i.e., hospitalization for complications), and there are research gaps to fill on both ends of this activity spectrum.

3.2.1. Athletes and arduous occupations

Given that females constitute half of the global population, there is a pressing need to include, and encourage female participation in sports and exercise science research.[87] As the number of females participating in a wide variety of training modes and modalities (e.g., HIIT, Pilates, CrossFit, weightlifting) continues to increase, adequate research funding must be allocated to ensure equity in understanding the unique aspects of female physiology and performance across exercise disciplines. Female athlete representation at elite levels has grown exponentially, exemplified by their record-breaking presence at the recent Tokyo Olympics.[88] Notably, the peak performance years for female athletes often align with their prime reproductive years, making it inevitable that pregnancies will intersect with training and competitive periods. Despite this knowledge, there remains a dearth of research and frameworks specifically tailored to address the needs of female athletes, particularly in the antenatal period.[89] As there is limited evidence available regarding pregnant athletes who engage in high-intensity exercise, current clinical recommendations[90,91] as well as the consensus statement of the International Olympic Committee[92] rely heavily on expert opinions and best practices.

In parallel, the representation of females in arduous occupations (e.g., military, law enforcement, firefighting, manual labour) is increasing and, like athletes, many of these individuals are in their reproductive years. Occupational exposures (e.g., chemicals, stress, physical assault, shift work, biological agents) combined with the physiological, anatomical, and biomechanical changes associated with female reproduction result in numerous health and safety concerns for pregnant, post-partum, and parous females employed in arduous occupations.[93] Females returning to military service postpartum are known to be at an increased risk of musculoskeletal injuries,[94] and female servicemembers who have given birth sustain more repetitive strain injuries than nulliparous peers.[95] Yet, when provided support to overcome the potential challenges associated with childbearing, pre-pregnancy physical performance can be regained and surpassed. For example, elite runners who continue training over pregnancy are able to return to or exceed pre-pregnancy performance 1–3 years post pregnancy.[96] Similarly, if military members are able to overcome the fitness deficits and increased health risk seen in the first 12–18 months postpartum,[97] they are capable of attaining equal or better physical fitness than nulliparous matched peers. Significant knowledge gaps persist due to the insufficient research on pregnancy, postpartum recovery, and resumption of high physical exertion among both elite athletes and those employed in arduous occupations (e.g., military service members, police officers, firefighters, manual labourers).[90] While exercise guidelines include specific recommendations for the general population[67] and recreational athletes,[98] guidance for pregnant athletes engaging in high volume or high-intensity training remains scarce. There are, however, studies indicating that women can sustain high performance levels during pregnancy[99] and regain their pre-pregnancy fitness following childbirth,[96] but further research with larger sample sizes examining the limitations and required obstetrical management in these populations will enable a safer and more prompt return to work or competition.

3.2.2. High risk pregnancies - is activity restriction and bedrest the only option?

Steeped in tradition, activity restriction and in severe cases, bedrest or hospitalization are common approaches for managing contraindications such as preterm premature rupture of membranes, vaginal bleeding with or without placenta previa, multiple gestation, hypertensive disorders of pregnancy, short cervical length, and fetal growth restriction. This practice flies in the face of increasing evidence, reviewed by Palacio and Mottola,[100] indicating that restricting activity does not necessarily prevent negative perinatal outcomes, and might worsen both physical and psychosocial risks.

Knowing that a physically active pregnancy (accruing 150 minutes/week of moderate-intensity PA) offers health benefits to both the gesP and their offspring and that evidence indicates that excessive sedentary behaviour during pregnancy increases chronic disease risk and may impair birth outcomes,[101–103] how do we treat those who experience what are deemed absolute contraindications? In brief, few interventions have been tested to reduce the deconditioning impacts of activity restriction in pregnancy[24] and thus we are presently unsure. What we do know is that severe activity restriction is not the answer. Future research should evaluate the use of low intensity activity routines to lessen the effects of activity-restriction, ensuring gesPs are prepared to undertake newborn care responsibilities when returning home following childbirth.

4. Conclusion

For many years females have been neglected from medical and exercise physiology research, a trend exacerbated when considering pregnancy. Popular medical opinions from the late 19th century into the first decades into the 20th century were that gesPs should use extreme caution when engaging in PA and exercise to avoid fatigue and

overexertion. The bulk of early published guidelines were unscientific, thus hardening the belief that females were weak and frail. These guidelines remained an unquestioned dogma for decades while reinforcing gender norms. Since the 2000s, exercise and PA have consistently shown positive associations with the health of pregnant parents and the fetus. However, large knowledge gaps remain. The deliberate exclusion of pregnant and postpartum females and athletes, along with the inadequate representation of females overall, is unacceptable. With record levels of females entering arduous occupations and participating in elite sporting events, it is imperative that concerted efforts are undertaken across various levels to ensure that research participants accurately reflect the demographics of the population to which the findings are intended to apply.

CRediT authorship contribution statement

Abbey E. Corson: Writing – review & editing, Writing – original draft, Visualization, Project administration, Data curation. **Meaghan L. MacDonald:** Writing – review & editing, Writing – original draft, Data curation. **Velislava Tzaneva:** Writing – original draft. **Chris M. Edwards:** Writing – original draft. **Kristi Bree Adamo:** Writing – review & editing, Writing – original draft, Conceptualization, Supervision.

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Chapter 2: Introduction to the Placenta – The Conduit of Exercise Benefits

2.1 Benefits of Gestational Parent Physical Activity

As exercise physiology research on pregnancy has advanced since the 1800s, our knowledge surrounding the safety and benefits of gesP PA has led to the development of rigorous, evidence-based guidelines for pregnancy. With Canadians leading the charge, the *2019 Canadian Guideline for Physical Activity Throughout Pregnancy* states that all gesPs without contraindications to exercise should engage in PA throughout pregnancy for a minimum of 150 minutes of moderate-intensity PA over a minimum of 3 days, but movement every day is strongly recommended [1].

For the gesP, the risk of pregnancy complications is significantly reduced with habitual engagement in PA. Specifically, PA decreases the likelihood of developing gestational hypertension and preeclampsia, gestational diabetes mellitus (GDM), urinary incontinence, pregnancy loss, anxiety and depression [1–5]. There is also a reduction in excessive gestational weight gain, subsequently lowering the risk of delivering large for gestational-age babies [6]. A meta-analysis examining the effectiveness of exercise interventions found that gesP PA prevented excessive gestational weight gain and postpartum weight retention [7]. At the time of labour, c-sections are reduced with those who engaged in PA throughout pregnancy, as well as potentially shorter active labour times [8–10].

Inadequate growth of the fetus is often a concern with gesP PA; but, evidence shows that PA promotes appropriate size for gestational-age and prevents macrosomia, the growth of the fetus beyond a specific threshold [11,12]. In early life, offspring from a physically active gesP have lower adiposity and a decreased risk of neural tube defects [13,14]. As highlighted in Chapter 1, the beneficial effects of gesP PA on the fetus are accrued through *developmental plasticit*y or *fetal programming*, according to the DOHaD framework [15]. Fetal programming shows that the risk of developing future chronic diseases later in life is reduced with *in-utero* exposure to PA.

GesP PA not only lowers the prevalence of obesity, type 2 diabetes and cardiovascular diseases for the pregnant individual but also for the offspring into adulthood [3].

While the benefits of gesP PA have been observed at an epidemiological level, the mechanisms behind them are not yet fully known. Since the placenta is not innervated and is the conduit between the gesP and fetus, all signalling must be mediated through the placenta. Therefore, it is probable that the placenta is the likely affecter of these benefits. The mechanistic role of the placenta in gesP PA is supported by changes at this organ's tissue and cellular levels. Previously reviewed by Bhattacharjee, Mohammad and Adamo [5], gesP PA positively alters the placenta blood flow and hormone secretion and may improve placental efficiency. At the cellular and molecular level, gesP PA has been associated with changes in transcriptional, metabolomic, and protein expression, eliciting differences in the immune system, nutrient transporters, angiogenesis, secreted factors and the mitochondria of the placenta [16]. Changes within the genotypic and phenotypic aspects of the placenta suggest that the benefits of gesP PA are accrued through the placenta, but we do not yet know the mechanisms that elicit these differences.

As proposed by Clapp [17], changes in oxygen tension, such as intermittent hypoxia (IH), may be one of the mechanisms of action behind the changes in the placenta. As the gesP engages in PA, the uterine artery constricts, and blood is diverted to the working muscles, leading to a transient hypoxic state within the placenta. Supported by a study using ewes, uteroplacental blood flow is reduced during PA [18,19]. Utilizing Doppler ultrasound, the literature has documented an increase in uteroplacental vascular resistance after 5 minutes of exercise, suggesting a temporary decrease in uteroplacental blood flow [20]. The fetus is still adequately oxygenated since erythropoietin (a hypoxia marker) is unchanged in cord blood and amniotic fluid from physically active gesPs [17,21–23]. Therefore, reduced blood flow during PA likely leads to a redistribution of the blood, favouring the placenta and fetus since fetal hemoglobin has a higher affinity for oxygen [19,24]. Once the activity has concluded, the uterine artery will dilate to accommodate an influx of oxygen-rich blood that rapidly reoxygenates the placenta [5]. We know that the hypoxic state induced by gesP PA is not permanent, as PA improves the chronically hypoxic conditions in the placenta induced by obesity [25]. Additionally, the mitigation of chronic hypoxia in the placenta with obesity suggests that gesP PA promotes

angiogenesis and subsequently improves uteroplacental and placental fetal circulations [5]. There is not yet primary research that has quantified the IH effect of PA on the placenta in humans, but it is commonly referred to as a probable mechanism of action for gesP PA [17,26,27].

2.2 The Immune System, Physical Activity and Pregnancy

The immune system is made up of innate and adaptive immunity arms, comprised of many cells that regulate inflammation, protect against invading pathogens and cancer, and regulate tissue repair. The different types of immune cells, along with which immunity arm they are associated with, are summarized in Figure 1. Macrophages are critical players in the innate immune system, orchestrating first-line responses to pathogens and activating the adaptive immune system through antigen presenting capabilities. Derived from blood monocytes or yolk-sac progenitors, macrophages carry out phagocytosis, cytokine secretion and antigen presentation [29,30]. Macrophages have the immune-characteristic ability to respond to their tissue microenvironment, polarizing to pro- or anti-inflammatory phenotypes as required [28,30]. Often woefully oversimplified in the literature, macrophages can be classically activated to the pro-inflammatory M1-like phenotype or alternatively activated to the anti-inflammatory M2-like phenotype. Macrophage polarization occurs on a spectrum of activation; therefore, the polarization of macrophages cannot be classified as absolute [31]. For example, research has suggested the further division of M2 macrophages into M2a, M2b, and M2c subtypes, with an additional M2d phenotype recently identified in primarily tumorigenic environments [32,33].

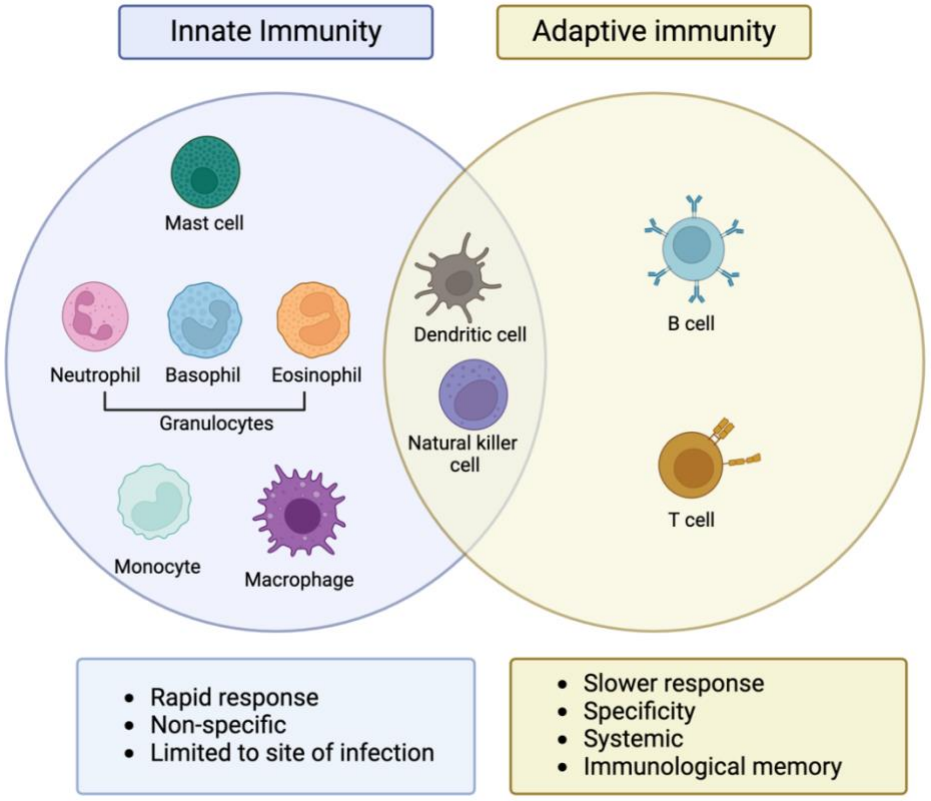

Figure 1. Cell Composition of the Innate and Adaptive Immune Arms.

Regulation of macrophage polarization occurs through three tightly regulated pathways: the external cytokine pathway, the internal pathway and the external non-cytokine pathway [34]. The external cytokine pathway is the most characterized pathway of macrophage polarization, utilizing cytokine stimuli to mimic the *in-vivo* T cell activation of macrophages [34]. Pro-inflammatory macrophages are stimulated by IFN-γ, LPS and TNF to acquire surface markers CD80, CD86 and IL-1R [28,34]. The pro-inflammatory characteristics of these macrophages are supported by their secretions of pro-inflammatory cytokines such as IL-1, TNF, and IL-12. As their name suggests, pro-inflammatory macrophages promote inflammation, impair tissue regeneration and perform phagocytosis to remove pathogens [28,31,34]. On the other end of the spectrum, anti-inflammatory M2a and M2c macrophages are activated by IL-4 and IL-13 or IL-10, respectively, with CD206 and CD163 expressed as surface markers [30,34,35]. These anti-inflammatory subtypes promote the regulation and resolution of inflammation, tissue remodelling, and matrix deposition [30,35]. M2b macrophages represent an intermediate polarization on the overall spectrum of activation, displaying and contributing to both pro- and

anti-inflammatory characteristics [36]. M2b macrophages share similar characteristics to M1 macrophages by expressing CD86 and secreting pro-inflammatory cytokines (IL-6 and TNF-α) [36]. The newly discovered M2d macrophage phenotype is less understood, but is thought to be activated by TGF-β and TLR agonists and characterized by secreting high levels of IL-10 and VEGF [37].

The ontogeny, or the internal pathway defined by macrophage origin, is a minor regulator of macrophage polarization [34]. Some macrophages are derived from blood monocytes from the bone marrow and display pro-inflammatory characteristics, whereas tissue-resident macrophages are derived from embryo yolk-sac progenitor cells and are typically more anti-inflammatory [34]. The ontogeny of the macrophages may not be as crucial as the effects of tissue microenvironment [34]. Results from macrophage transplant studies show that bone marrow-derived macrophages can differentiate and take on the tissue-resident macrophage characteristics and roles [34,38]. Therefore, the tissue microenvironment may play a more critical role in defining macrophage polarization.

The tissue microenvironment can be influenced by characteristics other than pro- and anti-inflammatory cytokines, such as oxygen tension. Hypoxia induces anti-inflammatory polarization, increasing growth and angiogenic factor secretions [37,39]. The literature regarding the effect of IH on macrophage polarization is much less clear. Some studies have found that the cycling between normoxia and hypoxia induces pro-inflammatory signalling with increases in IL-6 and promotes pro-inflammatory (M1-like) polarization of macrophages [40–42]. On the contrary, some researchers have found that IH induces the anti-inflammatory phenotype and tissue micro-environment, consistent with the observations of chronic hypoxia [43,44]. Our understanding of the non-cytokine external pathway is limited compared to our knowledge of the cytokine external regulation pathway.

During pregnancy, the immune system goes through complex changes to support the maintenance of pregnancy and the development of the fetus while still being able to fight off infection [45]. Previous research from the Adamo Lab revealed that exerkine (cytokines stimulated and released by exercise) levels are different between pregnant and non-pregnant

individuals [46]. The study found that at resting levels, erythropoietin (EPO) and oncostatin were higher in the pregnant group than in the control with increases in FGF21, EPO, IL-15, secreted protein acidic and rich in cysteine (SPARC), fractalkine and BDNF after exercise [46]. Additionally, the balance between pro- and anti-inflammatory cytokines may have a trimester-specific response for IL-6, IL-10 and TNF-α with gesP PA [47]. As such, the differences in the gesP immune system and cytokine levels may lead to differential macrophage polarization with PA.

PA has an immunomodulatory effect in the non-pregnant population that regulates the inflammatory profile [48]. Shifts in anti-inflammatory cytokines induced by PA can inhibit the production of pro-inflammatory cytokines, promoting the polarization of macrophages towards the M2 phenotypes [49]. Research regarding the role of PA on macrophage polarization consistently shows a favouring towards the anti-inflammatory phenotypes in healthy and disease models [50–55]. Promoting this phenotype with PA has led to the clinical practice of using PA to moderate the development of inflammatory diseases [49]. Therefore, gesP PA could lead to improved pregnancy outcomes and a healthier fetus due to a reduction in the inflammatory environment of the placenta. However, we do not know if the macrophage of the placenta experiences the same shifts in polarization with PA, as seen with other organs.

2.3 The Elusive Hofbauer Cell

The fetal-derived tissue-resident macrophage of the placenta, termed Hofbauer cell (HBC), are the only immune cell in the chorion and can be observed as early as 18 days post-conception [56,57]. Often located near fetal capillaries in the villous stroma alone or in groups of two or three, these 10-30 μm cells have a highly vacuolated appearance with membrane blebs, lamellipodia, and funnel-like structures [56]. HBCs have typical macrophage roles such as phagocytosis and cytokine secretion, but they also have unique roles in supporting the homeostasis and development of the placenta [58]. Often regarded for their abilities to protect the semi-allogenic fetus, HBCs also regulate stromal water balance, villous development, placental vasculogenesis and angiogenesis [58–63]. Additionally, HBCs in the placenta have been linked to anti-inflammatory roles such as tissue regeneration, villi development and angiogenesis [58,59,64].

As previously discussed in section 2.2, the ontogeny of macrophages can impact their polarization. It is debated in the literature if HBCs are derived from blood monocytes or blood islands of the yolk sac [58,65]. It is known that HBCs are derived from the fetus, as indicated by sex chromatin staining [66]. Specifically, these innate immune cells are derived from yolk-sac primitive hematopoietic stem cells (HSCs). Mice studies have observed the emergence of HSCs from the yolk sac to the fetal capillaries, entering the villous stroma to ingest stromal materials, completing their differentiation to become HBCs [65]. Although, as gestation continues, the ontogeny of the HBC population shifts. During the second and third trimesters, transitional cells between monocytes and macrophages have been observed in the placenta, suggesting that HBCs in the latter stages of pregnancy are derived from fetal monocytes [67].

Potentially due to the ontogeny of HBCs, the polarization of these cells is the M2a, M2b and M2c phenotypes [59,68]. When characterizing the phenotypes of HBCs in the placenta, Swieboda *et al.* [68] found that HBCs lack M1 polarizations and are mostly comprised of M2a and M2c characteristics, with M2b macrophages being identified only in healthy placentas at term. As previously discussed, M2b macrophages are an intermediate phenotype for macrophage polarization. Owing to the absence of the classical M1 polarization in the placenta, M2b polarized macrophages have been identified as the pro-inflammatory phenotype in the placenta. The pro-inflammatory role of M2b macrophages is carried out by the expression of CD86 and the secretion of TNF, IL-6 and IL-1 [36]. The pro-inflammatory role of M2b HBCs is supported by their association with being augmented in the inflammatory state of GDM [69]. GDM induces a shift towards the pro-inflammatory phenotype with higher IL-6 and IL-1β compared to non-effected placenta [69].

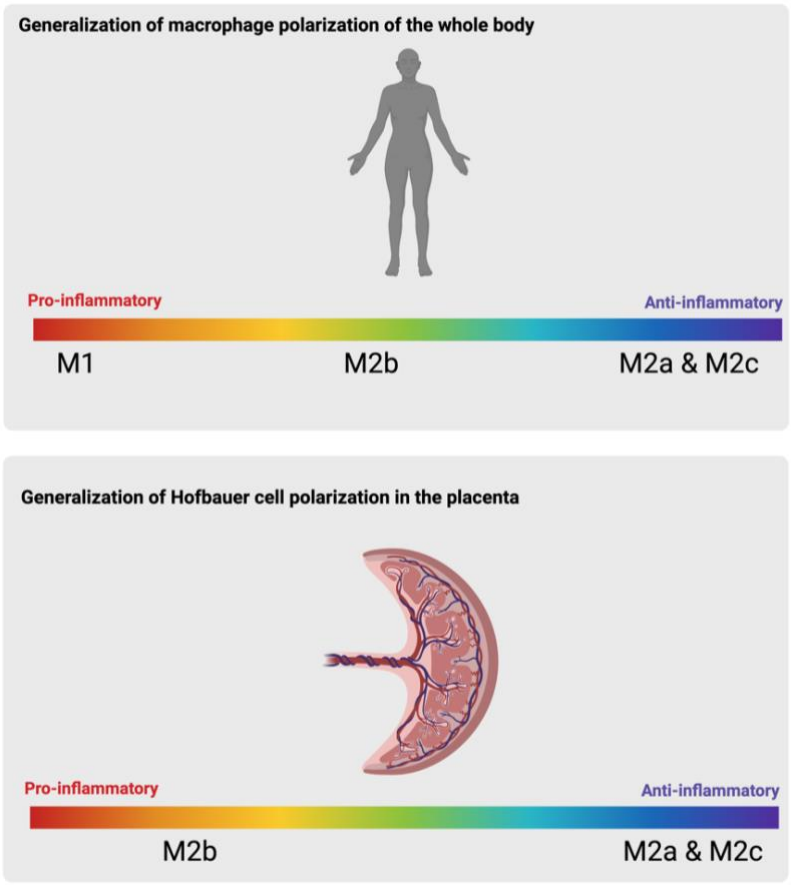

Figure 2. Generalization of Macrophage Polarizations of the Whole Body and Placenta.

In addition to GDM, environmental exposures during pregnancy, such as toxins, viruses, gesP obesity, and pregnancy pathologies, impact HBC characteristics [70]. As pregnancy progresses, the HBC population decreases throughout a healthy gestation [68]. This process is exacerbated with chorioamnionitis and advanced gestational age [71]. Low HBC numbers are also associated with early pregnancy loss [72], suggesting the requirement of HBCs to support the homeostasis of the placenta. Additionally, shifts in the pro- and anti-inflammatory HBCs in the placenta have been associated with Zika virus, *toxoplasma gondii*, HIV, obesity, and pollution [58,70,73]. Interestingly, previous work from the Adamo lab suggests that gesP PA may be another environmental factor that impacts HBC polarization. Placenta from physically active gesPs showed a greater proportion of the anti-inflammatory $CD206^+$ HBCs than those who were physically inactive [74]. The number of $CD206^+$ HBCs was also correlated to the number of moderate-to-vigorous PA minutes in mid and late gestation [74]. It is speculated that the greater

proportion of $CD206^+$ HBCs in physically active placentas could be due to the theorized IH effect of gesP PA, but this has yet to be tested. The modifying effects of diseases, toxins and gesP PA on HBC number and polarization allows us to speculate that environmental perturbations may regulate HBCs and potentially impact their downstream roles in the placenta. Although, the impact of enhanced anti-inflammatory HBCs on the development of the placenta has not been classified.

2.4 The Influence of Hofbauer Cells on Placenta Development

Within the developed villi of the placenta, HBCs are located near the fetal vasculature, indicating potential cell-to-cell interactions and symbiotic relationships. Previous research shows that placental vasculature is impacted by HBCs, as they are known promoters of angiogenesis - the process of forming new vasculature from already established vessels. Loegl *et al.* [59] previously identified that the M2a, M2b and M2c polarized HBCs regulate tube formation and migration of fetal placental endothelial cells. Due to the secretions of VEGF, TNF-α and FGF-2, tube formation was enhanced when exposed to HBC-conditioned media compared to the controls [59]. Interestingly, this study found that HBCs decreased endothelial cell migration, likely due to MIF, suggesting that these tissue-resident macrophages do not influence the direction of vascular growth in the placenta [59]. However, the study by Loegl *et al.* (2016) study only examined the secretion of FGF-2, VEGF, IL-6, TNF-α, PLGF and IL1RN, providing a limited insight into the regulatory role of HBCs on angiogenesis. The variety of assays employed by this 2016 study is a strength as it better classifies the multifaceted and complex process that is angiogenesis- albeit the tube assay evaluation was confined by the shortfall of variables that were assessed by the angiogenesis analyzer. Not including more variables that are provided by the angiogenesis analyzer contribute a finite overview of the impacts of HBCs on the complexity of tube formation; thus, reducing the overall applicability of the results. Maximizing the analyzed variables for the tube formation assay would provide a greater overview of the regulatory role of HBCs in angiogenesis. All phenotypes of HBCs can produce the pro-angiogenic factors FGF-2, VEGF and the anti-angiogenic factor Spry2, indicating their roles in tissue regulation and angiogenesis [75]. Specifically, low molecular weight FGF-2 within the placenta differs depending on gesP PA status, theorized to impact the permeability of fetal capillaries and subsequently enhance the efficiency of nutrient and gas exchange [75]. Differences in angiogenic

factors with gesP PA insinuate that PA is pro-angiogenic in the placenta, as seen with other organs [76,77]. Despite that, we do not know if the exact mechanisms apply to the placenta. Placentas from physically active individuals have been shown to have a larger vascular volume and greater capillary surface area supported by more significant placental growth and larger villi [26,27,78]. Compared to sedentary counterparts, a 24% increase in mid-pregnancy placenta growth has been observed with physically active gesPs [17]. These observed differences in placental development increase the surface area, thus optimizing the delivery of nutrients and gasses to the fetus. Moreover, the placenta from physically active gesPs has higher levels of pro-angiogenic VEGF and PIGF compared to placentas from gesPs who were physically inactive [79]. These results allow researchers to speculate that the pro-angiogenic and growth-stimulating effect of gesP PA could be due to differences in angiogenic factor secretion. For example, myokines and exerkines secreted during gesP PA (e.g. IL-15, IL-6, and BDNF) have been linked to differences in placenta development and growth [26].

Another proposed mechanism accounting for the differences in the vasculature and development of the placenta could be the IH effect of gesP PA. As stipulated by Jackson *et al.* [27], differences in the placenta vascularity with gesP PA may also be stimulated by changes in oxygen tension. Placenta vasculature is regulated through the activation and stabilization of HIF, leading to downstream secretion and signalling of VEGF [80–82]. We do not know if IH will regulate the same pathways, eliciting differences in markers of angiogenesis that have been observed in tumour models [83]. Changes in oxygen tension are normal throughout gestation as the beginning of pregnancy is a hypoxic environment before the gesP blood supply is established [84,85]. Once the gesP spiral arteries begin dumping blood into the villous spaces, oxygen tension is thought to peak in the placenta around weeks 12-17 of gestation [85–88]. The intervillous space of the placenta varies depending on the gestational stage, with the lowest at the beginning of pregnancy at a partial pressure of oxygen (pO_2) of 20 mmHg (~2-3% O_2) and peaking at 60mmHg (~6% O_2) from weeks 12-15 of gestation as it slowly declines to 30 mmHg pO_2 until term [87,88]. For context, atmospheric oxygen tension is at a pO_2 of 159 mmHg (21% O_2), with the lowest oxygen tension in the non-pregnant body at the end capillaries and veins with a pO_2 of 40mmHg [87,88]. In summary, IH and HBCs moderate angiogenesis: though the implication of the combination of these factors is not yet known.

2.5 Thesis Aims

These studies aim to delineate the interaction between HBCs and IH and determine their downstream effects on placenta vasculature. To address this overarching aim, the research project will be broken down into two aims:

Aim 1: Compare the polarization state of HBCs in culture when exposed to IH.

Hypothesis: HBCs exposed to IH have a higher ratio of anti-inflammatory to pro-inflammatory phenotypes than HBCs that are maintained at physiological normoxic levels.

Aim 2: Determine the impact of HBCs and IH on placenta angiogenesis.

Hypothesis: HUVECs treated with IH and HBC-conditioned media have faster migratory abilities and more expansive vessel formation.

2.6 Study Rationale

Exercise physiology research falls short in classifying the effects of PA within the female population, which is further exacerbated with the pregnant population. Historically, females have been treated as if they were smaller males with complex hormone profiles, resulting in cautious approaches to exercise research that is exacerbated in the pregnant population. To advance our overall understanding of exercise, researchers must focus on identifying the benefits and clarifying the mechanisms specific to women and pregnant individuals. This project will help fill the overarching knowledge shortfall regarding PA and exercise in pregnant individuals, helping close the gender gap in exercise physiology.

The benefits of gestational PA for both the gesP and the fetus have been well established in the literature, but there is a significant information deficit regarding the mechanism(s) behind these benefits. The field of exercise physiology is comparatively well-versed in the effect(s) and mechanism(s) of PA on other organs, such as the bones, skeletal muscles and kidneys, while the placenta is neglected. Compared to the previously characterized organs, there are inherent challenges in studying the placenta, a temporary organ that is not innervated, is composed of gesP and fetal tissues and can only examined at one time point (delivery). Much of the research regarding placenta biology is with pregnancies impacted by pathologies, leading to a greater understanding of placenta dysfunction vs. healthy function. This proposed research is crucial for

advancing our understanding of the molecular interactions among cells in the healthy placenta, significantly enhancing our insights into the cell-to-cell relationships. Moreover, this project will strengthen the current understanding of the roles and regulations of HBCs and endothelial cells. Of the limited research on healthy placentas, the cells are studied in solitude, which is not representative of the *in vivo* conditions. With the placenta comprising heterogeneous cell populations, delineating the cell-to-cell interactions will help developmental biologists understand the paracrine roles and symbiotic or commensalism relationship between these cells.

This set of studies are groundbreaking as it will be among the first to investigate how intermittent hypoxia (IH) affects HBC polarization and placental angiogenesis. Current research primarily focuses on the impact of chronic hypoxia in disease models, which are not directly applicable to placental conditions or gesP PA. This project aims to evaluate whether IH elicited the observed variations in HBC polarization reported by the Adamo Lab. Given the rising prevalence of metabolic syndromes and cardiovascular diseases in society, deconvoluting the mechanisms of gesP PA could offer valuable insights into its potential as a preventive measure throughout pregnancy.

Previous research has shown that HBC polarization in the physically active placenta favours the M2-like polarization, but we do not know what elicited these differences. HBCs are the most abundant immune cells present in the placenta, but we do not know what, other than cytokines, regulate these cells. While it is commonly accepted that IH is a consequence of gesP PA, we do not yet know if this effect regulates HBC polarization. Characterizing the polarization response of HBCs when exposed to IH will provide insight into the under researched non-cytokine external regulation pathway. Furthermore, previous research regarding the immunomodulatory effects of PA is focused on macrophages derived from monocytes or other tissue-resident macrophages; thus, we do not know if the results apply to the placenta. Therefore, determining how IH regulates HBC polarization will contribute to immunology and exercise physiology research, getting one step closer to determining the mechanism behind gesP PA.

Historically, primary cells were cultured at atmospheric oxygen tension (21% O_2); however, these *in vitro* models are no longer physiologically relevant. The study that this thesis will be

recreating by Loegl *et al*. used atmospheric oxygen tension and HBC conditioned media to mimic the effect of HBCs on angiogenesis. If similar results are found with physiological oxygen tensions, recreating these studies will strengthen our knowledge regarding the role of HBCs and further increase the reliability of these studies. Overall, this study addresses the significant gaps in the literature regarding the regulation and role of HBCs and how IH, an effect of gesP PA, impacts the interactions with endothelial cells.

Preamble to Chapter 3:

The manuscript entitled: *Hofbauer Cell Polarization is not Impacted by Intermittent Hypoxia* is formatted for the *Physiological Reports* as a follow up to previous work by Goudreau *et al.* Retrospectively, we have determined that the study was underpowered. Since the flow cytometry antibody panel needed to be optimized, our HBC bank was used up and due to resource limitations, we are not able to meet the required sample size of 5. This manuscript covers the first objective of this thesis as it investigates the regulatory role of IH on HBC polarization.

Chapter 3: Determining the Regulatory Role of Intermittent Hypoxia on Hofbauer Cell Polarization

Research Article

Title: Hofbauer Cell Polarization is not Impacted by Intermittent Hypoxia.

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Abstract: The tissue-resident macrophage of the placenta, termed Hofbauer cell (HBC), has roles in placental angiogenesis, promoting homeostasis, protecting the semi-allogenic fetus and expected macrophage roles such as phagocytosis and cytokine secretion. These vast roles are fulfilled through the plastic ability of HBCs to respond to the tissue microenvironment, leading to polarization. Cytokine external regulation pathways are commonly the focus of macrophage activation research; however, other non-cytokine factors, such as intermittent hypoxia (IH), an effect of gestational parent physical activity, may regulate polarization. We exposed primary Hofbauer cells to a control 5% O_2 condition or bouts of IH and quantified their polarization using flow cytometry. To observe the impact on their functionality, we analyzed phagocytosis and cytokine secretion by performing phagocytosis assays and cytokine arrays, respectively. IH did not significantly affect the number of $CD68^+/CD86^+$ ($p = .832$) or $CD68^+/CD206^+$ macrophages ($p = .614$). IH also did not alter the proportion of anti-inflammatory to pro-inflammatory macrophages ($p = .287$). We observed similar phagocytotic activity ($p = .651$) and differential cytokine secretions between the 5% O_2 and IH conditioned mediums. In conclusion, IH may not be the main mechanism of action for promoting an anti-inflammatory phenotype for HBCs but elicits changes to the secretome.

Key words: Hofbauer cells; Macrophage regulation; placenta; physical activity; intermittent hypoxia; pregnancy.

1 | Introduction

Making up about 70% of the immune cells found in the placental villi (Megli & Coyne, 2020), the fetal-derived tissue-resident macrophage, termed Hofbauer Cell (HBC), has a plethora of roles that support the growth and development of the placenta and subsequently support the growth of the fetus (Megli & Coyne, 2020; Zulu et al., 2019). Specifically, HBCs are involved in cytokine secretion, phagocytosis, maintenance of placental homeostasis, promoting tolerance of the semi-allogenic fetus, angiogenesis and more (Loegl et al., 2016; Swieboda et al., 2020; Zulu et al., 2019). The ability of HBCs to exhibit these vast roles is due to their plastic ability to respond to changes in the tissue microenvironment through macrophage polarization. Often oversimplified in the literature, macrophage polarization is thought to be a spectrum of activation through cytokines and microbes leading to two different phenotypes: M1-like macrophages, which are "pro-inflammatory" through classical pathway activation, and M2-like macrophages, which are thought to be "anti-inflammatory" through the alternative pathway activation. Pro-inflammatory macrophages have been characteristically shown to be activated with IFN-y with LPS or TNF and GM-CSF, leading to the expression of CD80, CD86, and IL-1R surface proteins, and the secretion of pro-inflammatory cytokines (e.g. IL-1, IL,12, TNF, IL-23) (Lendeckel et al., 2022; Martinez et al., 2008; Martinez & Gordon, 2014; Murray, 2017). This phenotype has a high capacity to present antigens, high chemokine ligand 9 production and secretes low levels of IL-10 (Martinez et al., 2008; Mosser & Edwards, 2008). Moreover, its roles are to remove foreign pathogens through phagocytosis, promote inflammation and impair wound healing and tissue regeneration (Martinez & Gordon, 2014; L. Wang et al., 2019). However, M1-like macrophages are not found in the placenta (Swieboda et al., 2020).

M2a, M2b and M2c macrophages are the anti-inflammatory phenotypes on the other end of the spectrum and found in the placenta. Additionally, there is the newly discovered M2d macrophage, but it is poorly characterized and is not identified in the placenta. Anti-inflammatory macrophage polarization is activated through many forms of stimulus for each subtype, such as IL-10, glucocorticoids, M-CSF, IL-4, and immune complexes, expressing surface markers CD206 and CD163 (Gordon & Martinez, 2010; Martinez & Gordon, 2014), as summarized in Figure 1. These anti-inflammatory macrophages promote roles of wound healing, matrix deposition and phagocytosis (Chinetti-Gbaguidi et al., 2011; Gordon & Martinez, 2010; Lendeckel et al., 2022;

Martinez & Gordon, 2014). Within the placenta at early and mid-gestation, HBC polarization mainly comprises the anti-inflammatory M2a and M2c phenotypes (Swieboda et al., 2020). At term, a small proportion of HBCs are the M2b phenotype (Swieboda et al., 2020). It is important to note that the M2b polarized macrophages do not fit into the typical anti-inflammatory characteristics. M2b polarized macrophages contribute to pro-inflammatory M1-like polarization characteristics such as the expression of CD86 along with the secretion of pro-inflammatory cytokines like TNF-α, IL-1β, IL-16, and IL-12 (Martinez & Gordon, 2014; L. Wang et al., 2019). Inflammatory diseases such as gestational diabetes mellitus (GDM) have been linked to an increased presence of $CD86^{+}$ M2b macrophages (Schliefsteiner et al., 2017). Therefore, M2b HBCs represent the pro-inflammatory population of macrophages in the placenta.

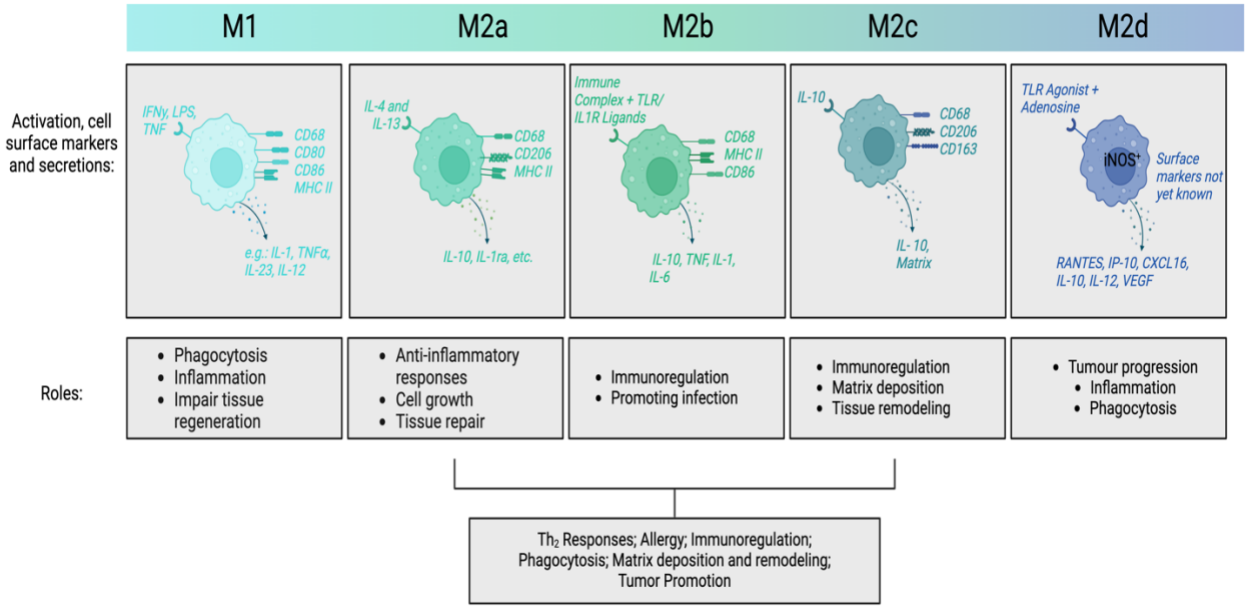

Figure 1. Generalization of Macrophage Polarization. (Gharavi et al., 2022; Lendeckel et al., 2022; Martinez & Gordon, 2014; Murray, 2017; Schaefer et al., 2017; Shrivastava & Shukla, 2019; L. Wang et al., 2019; Wu et al., 2012)

Macrophage polarization occurs through tightly regulated mechanisms that are the extrinsic (cytokines), non-cytokine external, and intrinsic pathways (ontogeny of the macrophage) (Murray, 2017). We have previously explored the role of the extrinsic pathway through the cytokines above (shown in Figure 1), but the role of non-cytokine external regulation is less known within the literature. Environmental factors such as pollution, disease state of the pregnant individual and diet

have been shown to impact macrophage parameters such as macrophage number, pro- and anti-inflammatory balance, and cytokine secretion (Chambers et al., 2021). Additionally, viral infection of HBCs with HIV, Zika virus and diseases such as chorioamnionitis have been linked to changes in HBC numbers, shifts in polarization and modifications to cytokine secretion (Zulu et al., 2019).

Interestingly, a behavioural factor like gestational parent (gesP) physical activity (PA) may influence HBC polarization. In previous work by our lab, Goudreau *et al.* (Goudreau et al., 2023) examined the impact of gesP PA throughout pregnancy on HBC polarization. This novel study collected placenta samples from physically active and inactive participants and found that those who engaged in habitual PA throughout pregnancy had a significantly greater proportion of $CD206^{+}$ HBCs. This observed change is beneficial as increased anti-inflammatory HBCs associated with gesP PA may support placental development, decrease inflammation and potentially mitigate the risk of inflammatory diseases (Goudreau et al., 2023; Loegl et al., 2016; Zulu et al., 2019). In the non-pregnant population, PA is also linked to the promotion of an anti-inflammatory *milieu* and the promotion of M2-like polarization (Baek et al., 2020; Blanks et al., 2019; Luo et al., 2020; Oliveira et al., 2013). However, the mechanism(s) of the observed favouring of M2-like polarization of HBC polarization with gesP PA has not yet been investigated.

It is speculated that the mechanisms behind the promotion of the anti-inflammatory phenotype with gesP PA could be due to the exerkine response or intermittent hypoxia (IH) (Goudreau et al., 2021; Hutchinson et al., 2019). As proposed by Clapp (Clapp, 2003) and reviewed by Bhattacharjee *et al.* (Bhattacharjee et al., 2021), IH is thought to occur as the pregnant individual engages in PA. During PA, oxygen-rich blood is diverted to the working muscle, leading to a transient mild state of hypoxia and decreased placental perfusion that is rapidly replenished when PA is ended (Clapp, 2003; Skow et al., 2017). Rapid increases in oxygen tension, a theorized event occurring after hypoxia during PA, are linked to M2-like polarization (Escribese et al., 2012). Moreover, a review by Murray (Murray, 2017) stipulated that oxygen tension may be a major non-cytokine external regulator by activating the HIF pathways. Studies that have looked at the impact of chronic hypoxia or ischemic injury on tissue-resident macrophages of other organs show a shift in the pro- and anti-inflammatory balance, often promoting the anti-inflammatory response due to changes in energy metabolism, and similarly concluded that HIF pathways become activated

(Díaz-Bulnes et al., 2020; Escribese et al., 2012; Ke et al., 2019; N. Wang et al., 2014; Yakupova et al., 2022). Oxygen tension regulates macrophage polarization through the HIF-PHD- NF-κB axis (Escribese et al., 2012).

While the impact of continuous hypoxia on macrophage polarization has been documented, the literature on IH is limited and has provided mixed results. For example, there are data suggesting that IH promotes a pro-inflammatory environment (Fitzpatrick et al., 2021; Schaefer et al., 2017; Zhou et al., 2018), while other studies suggest that IH is a stimulus for anti-inflammatory phenotypes (Almendros et al., 2015; Campillo et al., 2017; Hou et al., 2022). The current IH literature often uses whole-body IH, utilizes tumour models or looks at monocyte infiltration and activation. It is not yet known how IH within an organ impacts tissue-resident macrophage polarization, especially HBCs. Therefore, this study aimed to characterize the impact of intermittent hypoxia, an effect of gesP PA, on HBC polarization. We hypothesize that when exposed to IH, HBCs will favour an anti-inflammatory profile and demonstrate polarization towards $CD206^+$ M2a and M2c populations, with a decrease in $CD86^+$ M2b HBCs.

2 | Materials & Methods

2.1 | HBC culture

Primary human HBCs were isolated from 3 healthy placentas (Amnion Foundation, USA). Demographic information of the donors can be found in Table 1.

Table 1

Hofbauer Cell Donor Characteristics

Gestational Parent Age (years)	33.3(4.51)
Gestational Age (weeks)	39.4(1.050)
Fetal weight (g)	3633(852.7)
Fetal sex (Male/Female)	1/2
Mode of delivery (Vaginal/C-section)	2/1

Note. Data are presented in mean (standard deviations). n = 3

HBCs were cultured in tissue culture-treated 6-well plates (FisherBrand, PA, USA) at a density of $1.0x10^5$ cells/cm^2 in RPMI-1640 media (Gibco, NY, USA) supplemented with 5% FBS, 25mM

HEPES (Sigma-Aldrich, MA, USA) and 1x Pen/Strep (Gibco, NY, USA) for 48 hours at 37°C and 5% CO_2 with the following oxygen tensions: 5% O_2 or IH. The oxygen tension of 5% O_2 was selected to reflect a physiologically relevant oxygen tension within the placenta and is thus the control condition (Huppertz, 2023; Keeley & Mann, 2019). HBCs in the IH condition were exposed to an oxygen tension of 3% O_2 for three three-hour bouts (to mimic the effects of gestational parent PA) after the cells were in culture for 24 hours. After 48 hours, HBCs and spent media were collected. The spent media was spun at 5000g for 10 minutes at 4°C then stored at -20°C for future analysis. Cultured HBCs were washed three times with DPBS before dissociating with TrypleE (ThermoFisher, MA, USA) for 7-10 minutes
at 37°C. Cells were then pelleted and resuspended in ice-cold FACS buffer (10% FBS, 3mM EDTA and PBS) to be stained for flow cytometry.

2.2 | Sample preparation

Harvested HBCs were stained with primary antibodies to distinguish the anti-inflammatory and pro-inflammatory macrophage polarizations (Liu et al., 2022) for 20 minutes at room temperature (RT) in the dark. Antibody concentration and conjugations are described in Table 2. Cells were then centrifuged at 400g for 7 minutes at 4°C, resuspended in ice-cold FACS buffer, and strained with a 100 μm cell strainer (Corning, NY, USA). 7AAD (ThermoFisher, MA, USA) was added as a viability dye and the cells were kept on ice until flow cytometry analysis.

Table 2

Flow Cytometry Antibodies

Antibody	Concentration	Conjugate	Marker	Supplier
Anti-CD68	1:50	PE	Pan-macrophage	Miltenyi, Germany
Anti-CD86	1:20	Brilliant Violet 421	Pro-inflammatory (M2b)	BioLegend, USA
Anti-CD206	1:20	Alexa Fluor 488	Anti-inflammatory (M2a & M2c)	BioLegend, USA

2.3 | Polarization quantification

For all antibodies, fluorescence minus one (FMO) and single stained controls (Supplemental Material 1) were used for compensation and to establish gates (Figure 1). The fluorescence signal was compensated for each antibody using UltraComp eBeads Plus compensation beads (ThermoFisher). Attune™ NxT Acoustic Focusing Flow Cytometer System (ThermoFisher Scientific). See Collao *et al.* (Collao et al., 2023) for more detailed methods. Cells were identified as pro-inflammatory macrophages ($CD68^+/CD86^+$) or anti-inflammatory macrophages ($CD68^+/CD206^+$). FCS files were processed in FlowJo™ Software (BD Life Sciences; v.10.8).

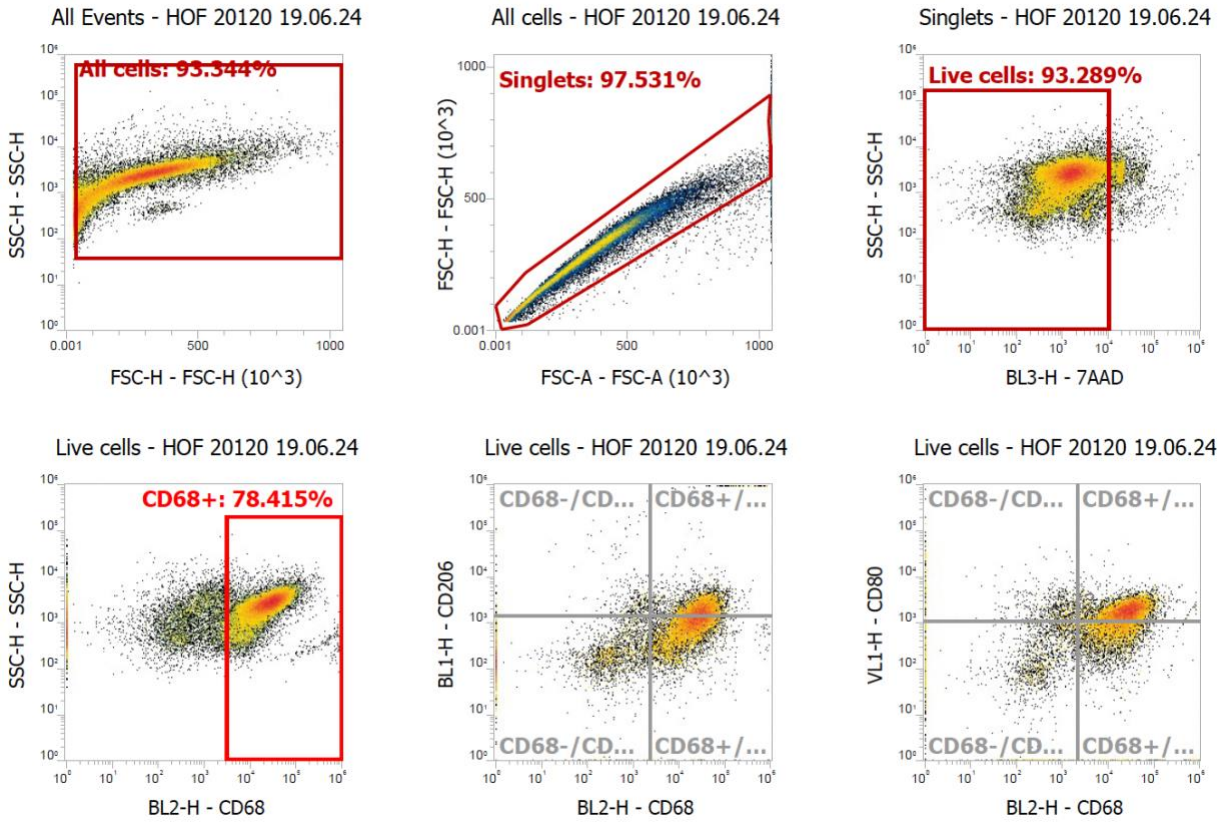

Figure 1. Flow cytometry gating strategy.

2.3 | Phagocytosis assay

We conducted a phagocytosis assay to quantify the functional phagocytotic activity in the control (5% O_2) and the intermittent hypoxia conditions. Following the manufacturing guidelines of a phagocytosis assay kit (Cayman Chemical; 500290), HBCs were cultured under the same

conditions as previously described in section 2.1 but were cultured in u-Slide 8 well ibiditreat chambered coverslips (Ibidi; 80826) for 48hours. Rabbit IgG-FITC Complex coated latex beads (Cayman Chemical; 400291) were diluted to a ratio of 1:200 in prewarmed supplemented RPMI 1640 media and added to the cells for 2 hours at their respective culture conditions. After the assay, any unbound beads and media were removed by washing the cells three times with the cell-based assay buffer (Cayman Chemical; 10009322). The cells were then fixed in 4% PFA for 10 minutes at RT and washed thrice with the cell-based assay buffer. Quenching was performed with 1x Trypan Blue to prevent autofluorescence (Cayman Chemical; 400292) for 2 minutes at RT. The cells were then washed three more times with the cell-based assay buffer, at which point Prolong Glass Antifade Mountant with NucBlue (ThermoFisher) was used to mount and visualize the cell nuclei. Ibidi slides were then imaged using an inverted fluorescence microscope (AxioObserver Z1, Toronto, Canada) equipped with blue and green filters at the Cell Biology and Image Acquisition Core Facilities at the University of Ottawa. Each sample was imaged using the tiling function (5 x 5) for 25 images. Negative controls omitted the addition of the latex beads. Phagocytotic activity was represented as the percentage of cells with beads (Sharma et al., 2014). The assay was completed once per donor for each condition.

2.4 | Cytokine array

Cytokines and chemokines secreted by HBCs were analyzed by performing a cytokine array (Abcam; ab133998) on conditioned media previously collected and stored from the 5% O_2 and IH conditions. As a negative control, we performed the array on the basal RPMI-1640 media to quantify the baseline levels of cytokines in the media. The array was completed following the manufacturer's instructions. We performed overnight incubations at 4°C for the media, biotin-conjugated cytokine antibodies, and the HRP-Streptavidin steps. The array was imaged with the ChemiDoc Imaging System (Bio-Rad). The integrated density of the grey value was measured for each spot using ImageJ (2.14.0/1.54f). The negative control was then subtracted from each spot to remove the background. The pixel density was normalized to the average density of the positive controls from the reference array (RPMI complete media) using the manufacturer's equation of $X(Ny) = X(y) * P1/P(y)$; where $X(Ny)$ is the normalized signal density of the spot, $X(y)$ is the mean signal density of the spot, $P1$ is the mean signal density of the positive control spots on the reference array, and $P(y)$ is the mean signal density of the positive control spots of the array. Data

were then normalized using log2-fold change versus the control condition. Cytokines with log2-fold changes greater than 2 or less than -2 were considered biologically relevant.

2.5 | Statistical analysis

R-studio software (version 4.4.0) was used for statistical analysis. Mean and standard error cell counts of pro- and anti-inflammatory macrophages and percentage phagocytosis were calculated. The effect size was measured using Cohen's d. Differences in cell counts and phagocytotic activity were compared between culture conditions using paired-sample t-tests. Statistical significance was set at $p < 0.05$. Heatmap analysis was completed for the cytokine array using the "ggplot2" package in R.

3 | Results

3.1 | HBC Polarization does not change with IH

To determine the effect of IH on HBC polarization, we compared the number and ratio of pro- and anti-inflammatory HBCs between the control condition (5% O_2) and IH with a paired samples t-test. Means and standard errors for the number of $CD68^+/CD206^+$ (anti-inflammatory HBCs) and $CD68^+/CD86^+$ (pro-inflammatory HBCs) are presented in Figure 2A and Figure 2B, respectively. The analysis revealed no significant effect of culture condition, t(2) = 0.591, $p = .614$, on the number of anti-inflammatory HBCs between the 5% O_2 (M = 6374, SD = 8744) and IH conditions (M = 4753, SD = 5660) with a small effect size, $d = 0.30$, 95% CI [-1.155, 1.763]. Additionally, the number of pro-inflammatory HBCs for the 5% O_2 (M = 8656, SD = 7605) and IH conditions (M = 8154, SD = 7564) were not statistically significant, t(2) = 0.240, $p = .832$ and had a negligible effect size, $d = 0.07$, 95% CI [-0.70, 0.83]. The relative percentage of anti-inflammatory and pro-inflammatory HBCs within the separate conditions are presented in Figure 2c. The proportion of anti-inflammatory HBCs was not significantly different, t(2) = 0.726, $p = .543$, between the 5% O_2 (M = 37.3 % , SD = 24.0%) and the IH condition (M = 26.1%, SD = 16.0%) with a medium effect size, $d = 0.55$, 95% CI [-1.700, 2.795]. Similarly, the relative proportion of pro-inflammatory HBCs in the 5% O_2 (M = 47.5%, SD = 24.2%) and IH (M = 50.9%, SD = 14.0%) conditions did not differ significantly , t(2) = -0.362, $p = .752$, with a negligible negative effect size, $d = -0.143$, 95% CI [-1.245, 0.959].

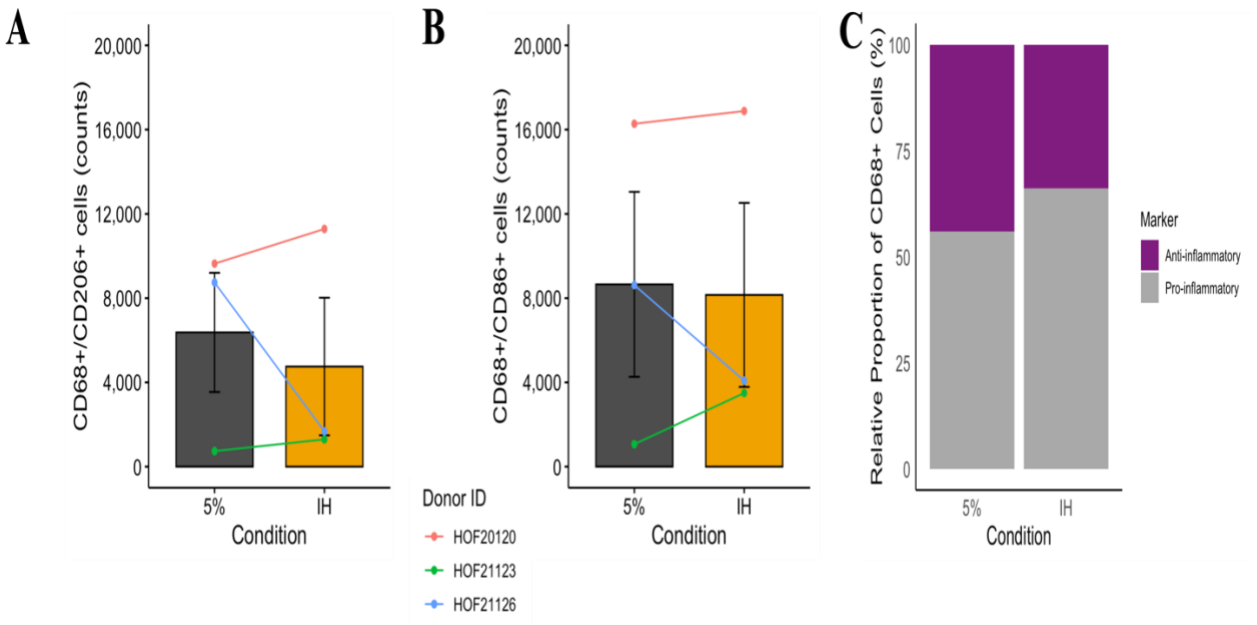

Figure 2. Hofbauer Cell Counts and Proportions Between Culture Conditions. Data are presented in mean + standard error. HBC counts of CD68+/CD206+ (A) and CD68+/CD86+ (B) for each culture condition. The data from the 5% condition are shown in the dark grey bars with the intermittent hypoxia condition shown in the gold bars. Individual data points correspond to values from each cell donor, with each donor being represented by a unique colour. The relative contributions of anti-inflammatory (purple) to pro-inflammatory (light grey) HBCs relative to all CD68+ cells (C). IH: Intermittent hypoxia. No significant differences were identified. $n = 3$.

3.2 | Phagocytosis activity is unchanged

Phagocytic activity was determined by calculating the percentage of cells that contained beads. The mean percent phagocytosis per culture condition with standard error of the mean is visualized in Figure 3. Upon analysis, no significant differences in phagocytotic activity were revealed, $t(2) = 0.527$, $p = .651$, between the 5% O_2 (M = 35.0%, SD = 5.06%) and IH (M = 33.4%, SD = 7.73%). Representative images are presented for the 5% O_2 condition in Figure 4, and the IH condition is presented in Figure 5. The negative control (no beads added) is shown in Figure 6.

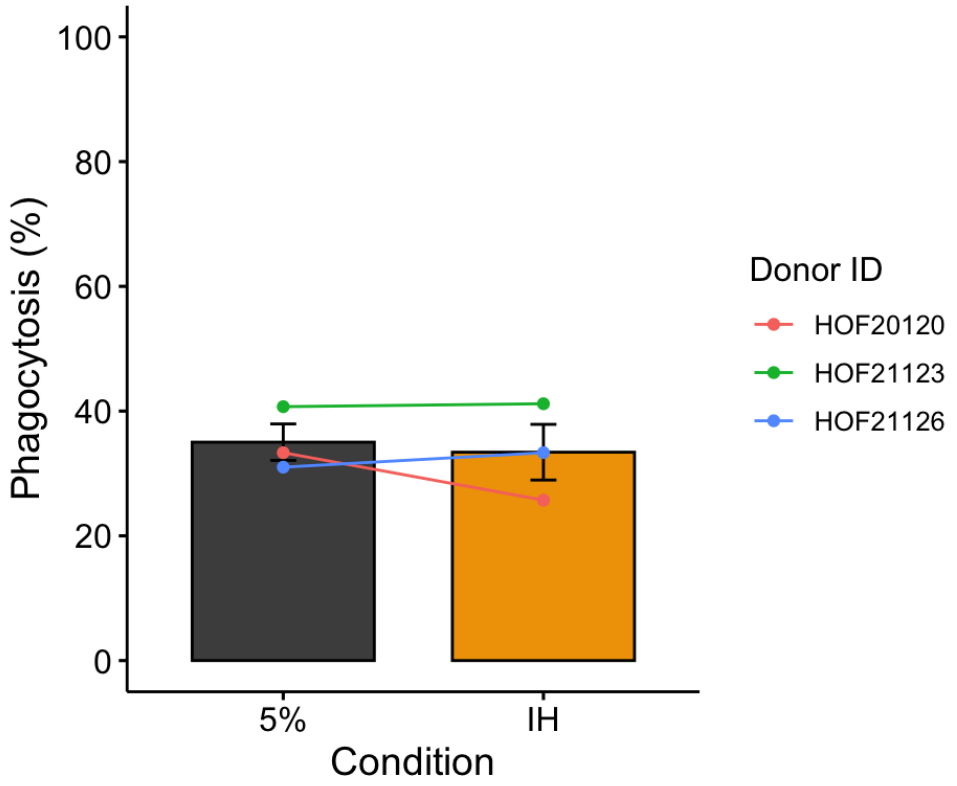

Figure 3. Comparison of Phagocytic Activity for the Control and Intermittent Hypoxia Conditions. Data are presented as mean and standard error of the mean. The percent phagocytosis is presented on the y-axis, with the culture condition on the x-axis. The 5% O_2 and intermittent hypoxia conditions are visualized in the dark grey and gold bars, respectively. Individual data points correspond to values from each cell donor, with each donor being represented by a unique colour. IH: Intermittent hypoxia. No significant differences were identified. *n = 3.*

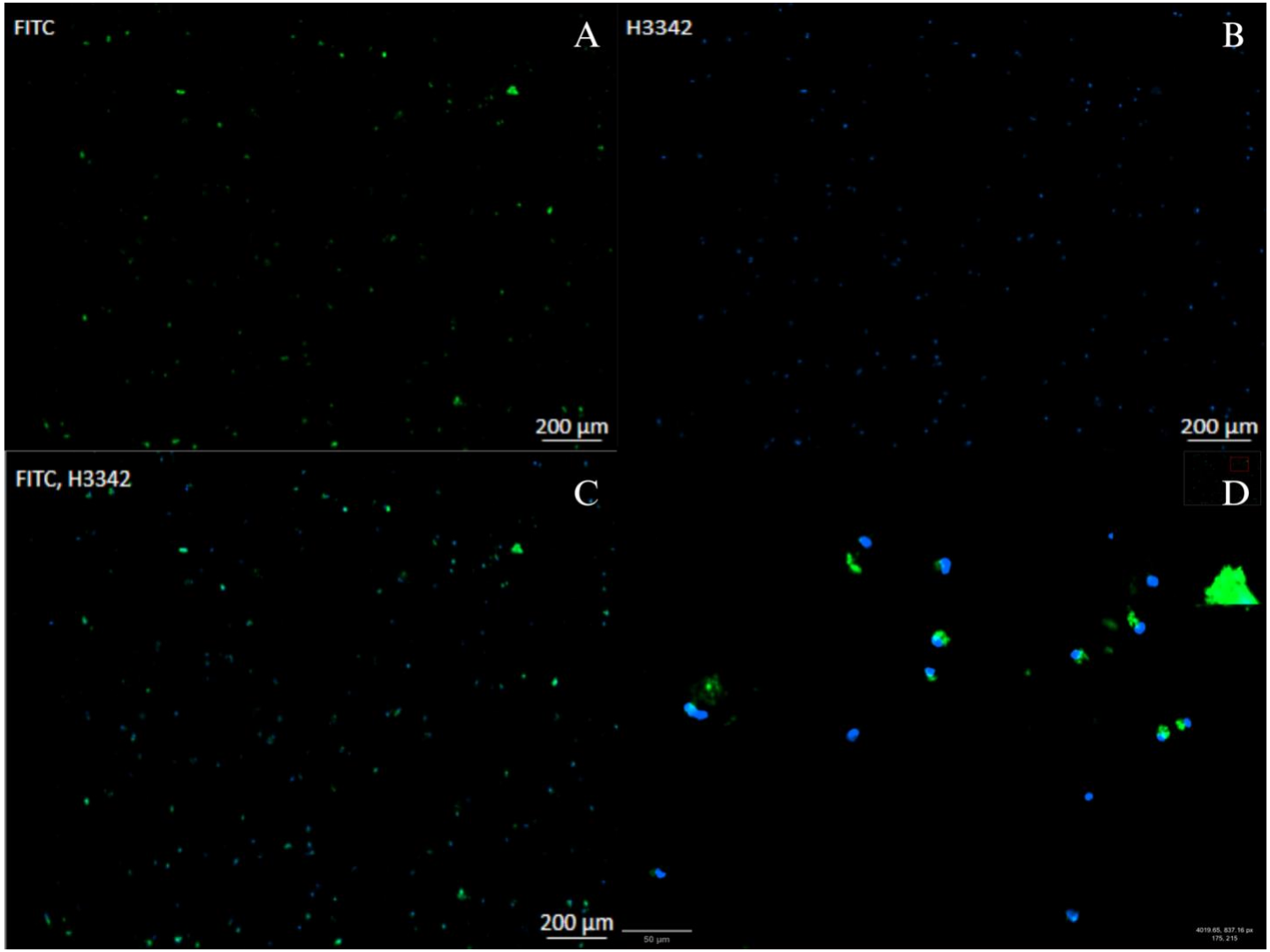

Figure 4. Phagocytosis Assay for the 5% O_2 Condition. FITC channel (A) shows the phagocytized beads. Nuclei stained with Hoechst (H3342) (B). Merged image (C). Images taken with the 20x objective with the tiling function (5 x 5). Scale bar is 200 µm. Zoomed in picture to show colocalization (D), with a scale bar of 50 µm.

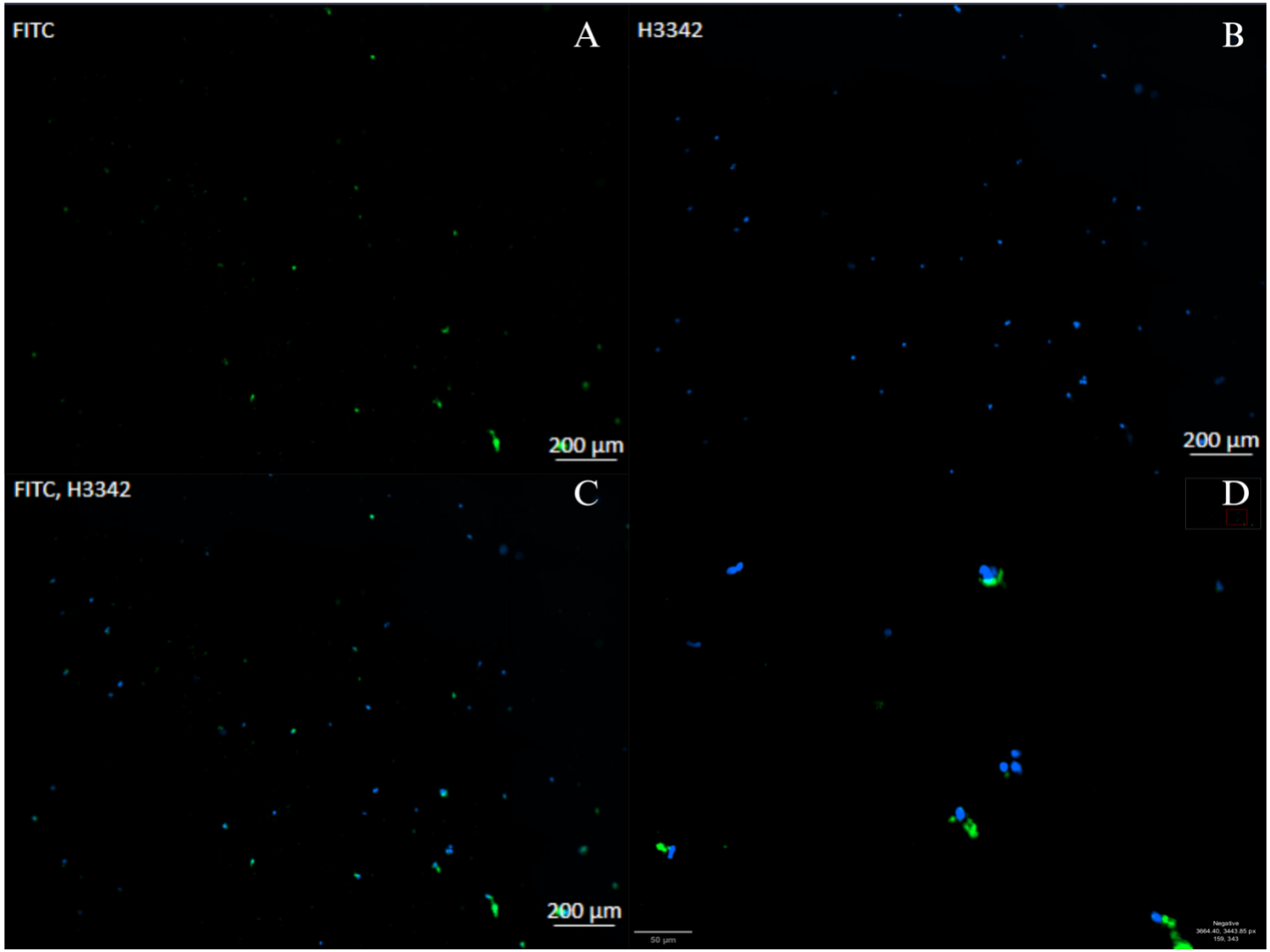

Figure 5. Phagocytosis Assay for the Intermittent Hypoxia Condition. FITC channel (A) shows the phagocytized beads. Nuclei stained with Hoechst (H3342) (B). Merged image (C). Images taken with the 20x objective with the tiling function (5 x 5). Scale bar is 200 µm. Zoomed in picture to show colocalization (D), with a scale bar is 50 µm.

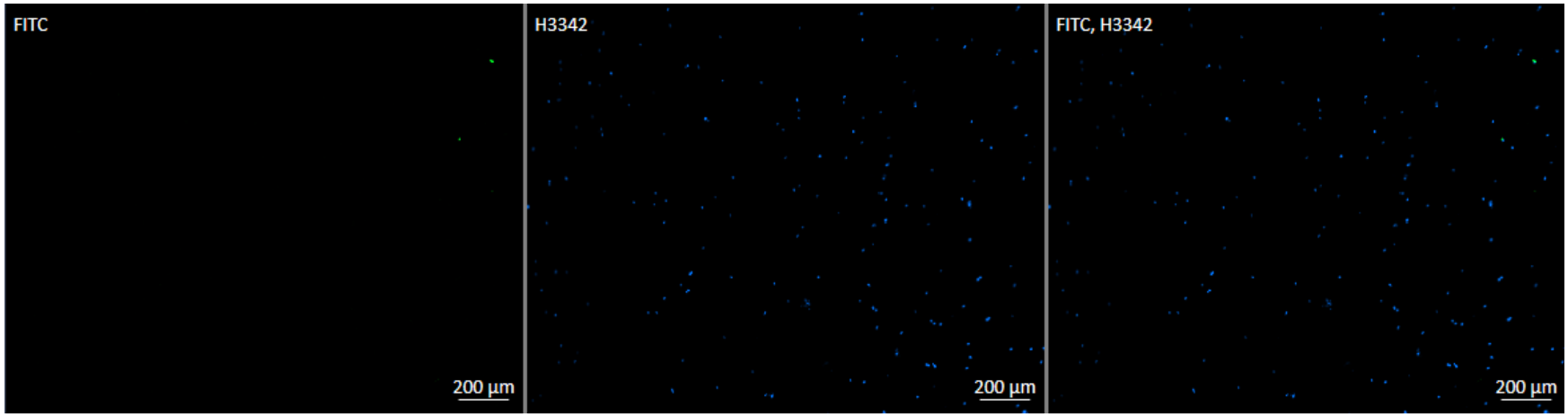

Figure 6. Negative Control for the Phagocytosis Assay. FITC channel (left) shows the phagocytized beads. Nuclei stained with Hoechst (H3342) shown in the middle. Merged image shown on the right. Images taken with the 20x objective with the tiling function (5 x 5). Scale bar is 200 µm.

3.3 | Differential secretome

The log2-fold changes for the cytokines in the media were normalized to the control for the conditioned medias from the 5% O_2 and IH conditions shown in Figure 7. Relative to the control, 5% O_2 had 52 (27 up- and 25 downregulated) and IH had 57 (11 up- and 46 downregulated) differentially expressed cytokines (Figure 7A). Comparing the two conditions, 25 of the differentially expressed cytokines were affected in the same direction, while 7 were influenced in opposite directions (Figure 7B). Specifically, opposing regulations of IGF-1, HGF, IGFBP-4, MCP-4, MIF, OPG and TIMP-1 were identified. There were 37 cytokines that were differentially expressed in one condition only. There were 15 up- and 4 downregulated cytokines in the 5% condition with 2 up- and 16 downregulated cytokines in the IH condition. The most up-regulated change compared to the control was IL-8, with a 21.53 and 20.85 log2-fold change in the 5% and IH conditions, respectively. The most down-regulated cytokine was MCP-3 in the IH condition at -19.84 log2-fold change compared to the control.

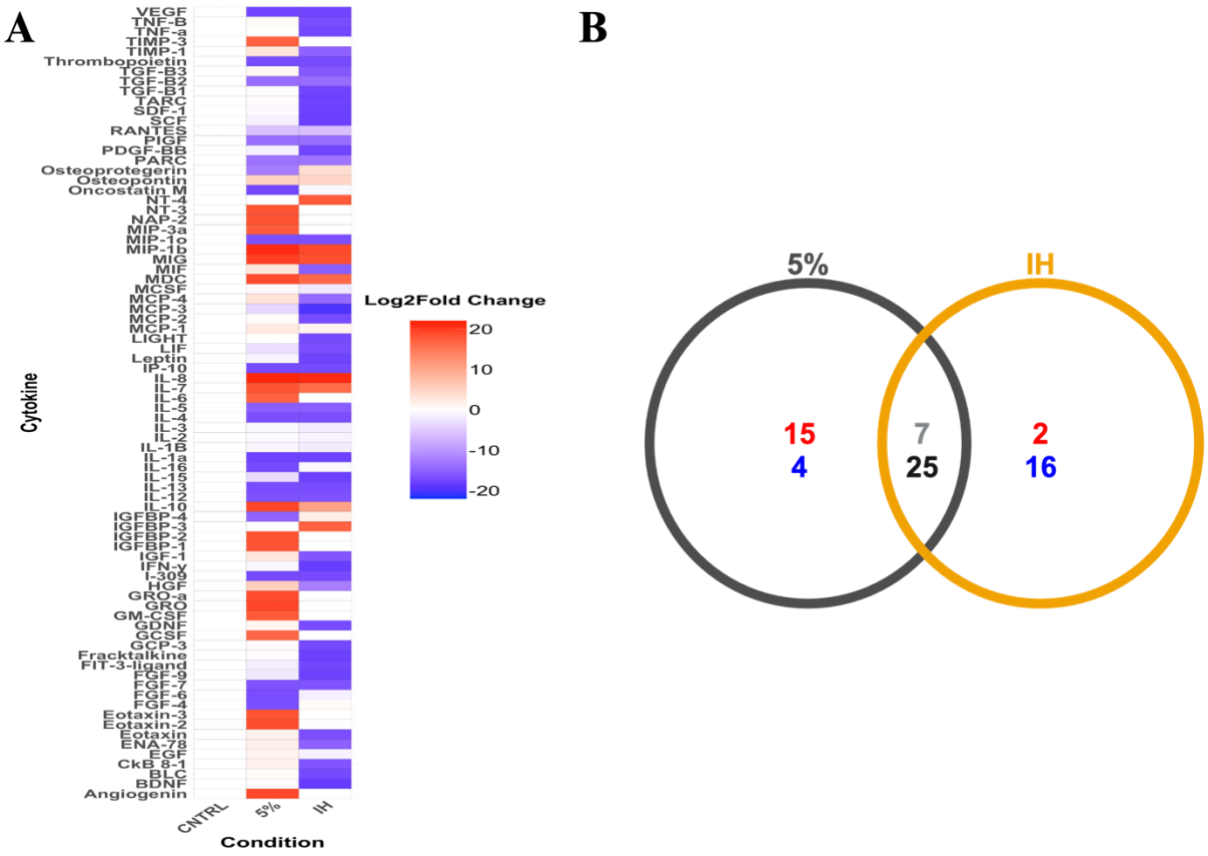

Figure 7. Oxygen tension influences cytokine secretion from Hofbauer cells. Data were normalized to the densitometry of the control condition. Fold changes greater than 100% (log2fold >2 or <-2) were identified as biologically relevant. A heat map comparing the log2fold changes normalized to the control condition for all cytokines (A). A Venn diagram with the number of cytokines that were relatively up-regulated (red) or down-regulated (blue) for the 5% and intermittent hypoxia conditions, shown in dark grey and gold circles, respectively (B). Cytokines that had the same directional effect for both conditions are shown in the black text in the overlapping circles. The number of cytokines that had relative differences in opposing regulation for both conditions are shown in light grey in the overlapping circles. CNTRL: Control; IH: Intermittent Hypoxia.

4 | Discussion

Previously, placenta from physically active gesPs was shown to have a significantly higher proportion of anti-inflammatory HBCs compared to placenta from those who were physically inactive (Goudreau et al., 2023). IH is a theorized effect of gesP PA and a potential mechanism;

therefore, this study sought to test if IH contributed to the differences observed by Goudreau *et al.*(Goudreau et al., 2023). We hypothesized that IH would promote greater polarization of anti-inflammatory HBCs. We did not see differences in the absolute number of $CD206^+$ or $CD86^+$ HBCs at varying oxygen tensions. Unlike the previous study, we did not identify any statistically significant differences in the relative proportions of HBC polarization with IH. However, there was a medium effect size with the impact of oxygen tension on the relative contribution of anti-inflammatory HBCs. The effect of IH favoured the pro-inflammatory phenotypes, which is the opposite of our hypothesis and the findings from our previous work. Therefore, IH may not be the main driver of the changes in the HBC polarization observed by Goudreau *et al.* (Goudreau et al., 2023).

In chronic hypoxia studies, less than 1% O_2 has been shown to activate HIF-1α, leading to downstream metabolic changes such as switching to the glycolytic metabolism and initiating the inflammatory response (Díaz-Bulnes et al., 2020). However, this switch does not occur as rapidly in M2a and M2c macrophages, as they can utilize fatty-acid oxidation for extended periods to support their roles (Díaz-Bulnes et al., 2020; Viola et al., 2019). Moreover, while M2b macrophages offer some metabolic flexibility, they are less metabolically flexible than the anti-inflammatory M2a and M2c phenotypes (Fuchs et al., 2024). Therefore, the lack of significant shifts in the polarizations may be due to their metabolic flexibility, leading to statistically preserved proportions in the IH condition. Additionally, oxygen tension is thought to regulate macrophage polarization through epigenetic enzymes such as histone demethylases, but the exact mechanisms are not yet known (Díaz-Bulnes et al., 2020). Therefore, future studies should quantify changes in these enzymes to elucidate any differences at the metabolic and molecular level of HBCs with IH.

Healthy pregnancies contain a majority of the anti-inflammatory HBC phenotypes at term (Swieboda et al., 2020); however, we observed a greater absolute amount of pro-inflammatory HBCs and a lower proportion of anti-inflammatory phenotypes, independent of the oxygen tension. Specifically, Swieboda *et al.* (2020) determined that ~85% of the term HBCs were of the M2a and M2c anti-inflammatory phenotypes while the pro-inflammatory M2b phenotype only accounted for ~10% of the total term HBCs. We did not observe similar proportions as previously reported, which may be due to confounding variables of fetal-sex and the culture media. In this

study, we have reported multiple characteristics regarding our donors, which strengthens the results of this study. Interestingly, all characteristics were relatively similar, but one participant had much higher proportions of pro-inflammatory HBCs and was the only participant that had a male fetus. Previous research in placenta biology suggests that there may be sex-specific differences in the placental inflammasome (Arthurs et al., 2022; Aiken and Ozanne, 2013), which could justify the outlying data. More research is required to confirm the hypothesis on the sex-differences in HBC polarization, therefore reporting fetal sex in studies that utilize primary cells for study placental biology should be required.

Secondly, we can posit that the HBCs cultured in this study did not maintain their innate polarization in culture due to the composition of the media. FBS could impact macrophage polarization in cell culture media as it can introduce cytokines and other signalling molecules involved in the external regulation pathways. In the supplemented RPMI-1640 media, we detected cytokines that are involved in stimulating macrophage polarization. Specifically, we identified VEGF, TGFβ, M-CSF, IL-4, IL-5, and EGF, known stimulators of anti-inflammatory polarizations (Casella et al., 2016; Kerneur et al., 2022; Lin et al., 2015; Murray, 2017; Xu et al., 2022). On the other hand, we discerned IFN-γ, a potent pro-inflammatory stimulator, along with RANTES, IL-12, TNF, IP-10, and IL-6 (Kerneur et al., 2022; Murray, 2017). The role of IL-6 in macrophage polarization may not be as pro-inflammatory as some of the literature suggests (Chen et al., 2022; Nishimoto & Kishimoto, 2006). IL-6 is often labelled pro-inflammatory, but this cytokine can also enhance the polarization of alternatively activated macrophages (Fernando et al., 2014), potentially due to the upregulation of IL-4 receptor α (Braune et al., 2017). The presence of these pro-inflammatory cytokines could elicit the pro-inflammatory phenotype that we observed in both conditions, which is not truly representative of the *in-vivo* polarizations.

Since there were no differences in macrophage polarization, our findings of similar phagocytic activity are consistent. We could speculate that the phagocytotic ability of these cells becomes saturated at around 30% when performing this assay. When pro-inflammatory macrophages are activated by IFN-γ, Fc-mediated phagocytosis (the process for phagocytizing the IgG-coated beads) is downregulated (Frausto-Del-Río et al., 2012). This decrease in phagocytosis is thought to be due to downregulation in the Fc receptor FcγR (Frausto-Del-Río et al., 2012) or limited by

the available surface area of the cell membrane (Cannon & Swanson, 1992). Due to the presence of IFN-γ in the RPMI-1640 media, it is plausible that our HBCs could have been activated by this cytokine, leading to a decrease in their phagocytic ability and favouring the pro-inflammatory polarization. As reported in the literature, exposure to hypoxia is thought to boost phagocytosis by activating p38 MAP Kinase-HIF-1α link (Anand et al., 2007), but, to the best of our knowledge, there are no reports available on how IH impacts tissue-resident macrophage phagocytosis. This study suggests that IH does not impact phagocytosis for HBCs.

Along with phagocytosis, another major role of macrophages is their ability to secrete cytokines and chemokines after being polarized to facilitate their various roles. While IH may not be the primary regulator of HBC polarization, we identified numerous biologically relevant cytokines that were differentially secreted between the two conditions; suggesting that oxygen tension can influence HBC behaviour. Overall, there was four times more down-regulation and about seven times less up-regulation of cytokines compared to control levels when comparing the IH and 5% conditions. These data suggest that IH may have a downregulatory effect on select cytokines compared to control conditions. There were 25 cytokines that were similarly regulated, while seven cytokines were regulated in opposite directions. Some of the differentially regulated cytokines include an up-regulation of IGF-1, HGF, MCP-4, MIF and TIMP-1 in the 5% condition with all being down-regulation in IH condition. The down-regulation of IGF-1 with exposure to IH may have ramifications on the uptake and metabolism of glucose and amino acids, as this growth factor has roles in nutrient transport in the skeletal muscle (Kaur et al., 2021). Placental growth and development, including angiogenesis and the maintenance of membrane integrity, may also be impacted by IH as the down-regulation of HGF, MIF, TIMP-1 and IGF-1 all have roles in these complex processes (Bach, 2015; Cartwright et al., 1999; Hellström et al., 2016; Ietta et al., 2018; Kaur et al., 2021; Kim et al., 2012; Todros et al., 2021; Vincent et al., 2015). While we can speculate on the effects of the observed down-regulation of these cytokines, the epidemiological research regarding the consequences of gesP PA are not consistent with our findings. The current literature suggests that gesP PA is linked to increased placental growth and angiogenesis with no detrimental effects on nutrient transport and the metabolism within the placenta (Adamo et al., 2024; Jackson et al. 1995). Moreover, MIF has been shown to be increased with hypoxia-reoxygenation (Ietta et al., 2018), but we observed the opposite effect. The different findings with

MIF levels could be due to varying methods with oxygen tensions used, suggesting that the chosen IH condition may not have been as extreme as the previous study, or that the cells did not experience hypoxia-reoxygenation cycles. In hindsight, experiments should have been completed to confirm that the cells experienced hypoxia in this study. Moreover, we do not know what oxygen tensions are experienced by the placenta during gesP PA; thus, the differential cytokine levels observed in this study may not be meaningful *in-vivo*. Taken together, these results reiterate our findings that IH may not be a main mechanism responsible for the benefits of gesP PA. Additionally, classifying and comparing the cytokines that we have observed within placenta from physically active and inactive placentas could allow us to make connections or unveil relationships that might help justify our current results.

Furthermore, the differential regulation of cytokines between the culture conditions leads us to speculate that there could be implications on the downstream roles of HBCs. We identified many cytokines that are involved in the development and growth of the placenta. Some of the contrasting cytokines of interest are IL-10, Angiogenin, G-CSF, NT-4, MIP-1β, MCP-1, and more. These cytokines are responsible for tissue remodelling, immune responses, trophoblast function, and angiogenesis (Briana et al., 2007; Furmento et al., 2014; Hartung, 1998; Kim et al., 2023; Lee et al., 2014; Miyake et al., 2015; Pala et al., 2023; Pavlov et al., 2014).The cytokine displaying the greatest up-regulation was IL-8 in both the HBC-conditioned mediums, with more in the 5% O_2 condition. This cytokine is responsible for activating other immune cells to help protect the fetus from invading bacteria (Shimoya et al., 1992) and participates in the signalling of maternal spiral artery remodelling, implantation and maintenance of pregnancy (Vilotić et al., 2022). IL-8 also has roles in angiogenesis, trophoblast migration, and differentiation (Brkić et al., 2018; Jovanović et al., 2010; Martin, 2001). We also observed an upregulation of IL-6 only in the 5% O_2 condition, which is often secreted from pro-inflammatory macrophages to support their pro-inflammatory roles (Baay et al., 2011). However, IL-6 can also be secreted from anti-inflammatory macrophages when stimulated with IL-4 (Casella et al., 2016), which we identified in the RPMI media. IL-6 is integral for establishing pregnancy, gesP spiral artery remodelling and may be a key molecule in the initiation of parturition (Vilotić et al., 2022). Implantation and placentation may be further aided by HBC secretion of Osteopontin (OPN), an extracellular matrix molecule and cytokine in the conditioned mediums (Johnson et al., 2003). As such, the secretions of HBCs are involved in

the development of the placenta and the progression of pregnancy. Our results suggest that oxygen tension may have indirect effects on these processes due to variability in cytokine secretion.

Of interest, we also found up-regulation of IGFBP-3 and IGFBP-4 in the IH condition, with a downregulation of IGFBP-4 for the 5% condition. On the other hand, IGFBP-1 and IGFBP-2 were up-regulated in the 5% culture condition only. Within this family, IGFBP-1, -3 and -4 are pleiotropic promoting both pro- and anti-angiogenic while IGFBP-2 is thought to be only pro-angiogenic (Slater et al., 2019). Another factor that we observed to be down-regulated to a lesser extent in the 5% compared to the control was FGF-4, which is thought to promote wound closure, stimulate trophoblast proliferation and be positively related the development of gestational diabetes mellitus (GDM) (Devi et al., 2020; Fan et al., 2022; Sun et al., 2023; Tanaka et al., 1998). Similarly, we detected differential patterns of regulation between the conditions for osteoprotegerin, a factor that is involved in glucose homeostasis during pregnancy (Huang et al., 2020). The IH condition, a proxy for the effect of gesP PA, resulted in upregulated osteoprotegerin secretion while the 5% condition induced a downregulation of osteoproetegrin compared to control. These results allow us to hypothesize that the observed decrease in the prevalence of GDM with gesP PA could be due to the influence of oxygen tension on HBC secretions of factors that regulate glucose homeostasis. While the role of HBC number and inflammatory profile in GDM has been elucidated (Sisino et al., 2013; Zulu et al., 2019), the involvement of secretions from HBCs may be a potential mechanism to be investigated. Detecting these factors with the cytokine array reinforces the importance of characterizing macrophage polarization as a dynamic spectrum and avoiding the oversimplified M1/M2 paradigm (Katkar & Ghosh, 2023; Martinez & Gordon, 2014; Murray et al., 2014). Overall, the identified changes in cytokine secretions suggest that oxygen tension may impact the downstream role of HBCs in regulating the development of the placenta, the maintenance of pregnancy and potentially the pathogenesis or progression of placental diseases.

When oversimplified in the literature, IL-10 and IL-12 are thought to be the main secretions of anti- and pro-inflammatory macrophages, respectively (Zulu et al., 2019). We detected an up-regulation of IL-10 compared to the control, with more in the 5% condition. While both polarizations of macrophages can secrete IL-10, anti-inflammatory macrophages can secrete

comparatively higher amounts (Murray, 2017). Therefore, we could postulate that the HBCs exposed to IH had a lower anti-inflammatory effect than the 5% O_2 condition. Our flow cytometry data partially confirm this finding of lower anti-inflammatory contributions after exposure to IH, although they were not statistically different. Interestingly, we detected the same downregulation of IL-12 in both conditions compared to the control. IL-12 is a main secretion for pro-inflammatory M1 macrophages (Kerneur et al., 2022), therefore, we can speculate that the identified $CD86^+$ macrophages are of the M2b phenotype as they secrete IL-10 at high levels compared to low levels of IL-12 (L. Wang et al., 2019). These findings are consistent with the literature that the classical M1 pro-inflammatory macrophages are not found in the placenta and that the M2b macrophages are responsible for carrying out the pro-inflammatory roles in the placenta.

A continuing shortfall of *in vitro* models is that they cannot directly represent the *in vivo* conditions, potentially leading to the observed promotion of the pro-inflammatory phenotype. Conditions such as cell density, media contents and pH have previously been shown to impact macrophage polarization (Ruder et al., 2023; Wu et al., 2019). With this in mind, the exposure time of IH to the HBCs may have been too short as these primary cells do not proliferate and are only viable in culture for three to five days. Previous studies have shown that the duration, timing and degree of difference in the hypoxic conditions of the placenta can differentially regulate cell behaviour and disease outcome (Siragher & Sferruzzi-Perri, 2021). Exploring the effect of IH on explant cultures could sustain HBCs in culture for more prolonged periods, allowing us to look more at the chronic effect of IH and sustain their innate polarizations observed in healthy placentas. Additionally, a *post-priori* power calculation using our large effect size for the ratio of HBCs revealed that we were underpowered. Reaching a sample size of 5 would increase the probability of detecting an effect of oxygen tension on HBC polarization (Supplemental Table 1) and thus, adequately powered future studies should explore this topic further.

5 | Conclusion

In conclusion, our results from the inflammatory profile of the cytokines and flow cytometry confirm that our cultured HBCs contain a heterogeneous population. We can also speculate that IH, an effect of gesP PA, may not be the primary mechanism behind the observed differences in HBC polarization by Goudreau *et al.*(Goudreau et al., 2023). However, changes in oxygen tension

did impact the secretome, thus potentially changing their downstream roles in angiogenesis, GDM risk, and placenta development. The cytokine external regulation pathways, metabolic changes and epigenetic regulation often impact macrophage polarization. Previous studies have characterized the secretions of exerkines and cytokines throughout pregnancy and are suspected of eliciting differences in HBC polarization (Goudreau et al., 2021; Hutchinson et al., 2019); therefore, future research should elucidate the role of these factors on HBC polarization with gesP PA.

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Supplemental Files

Table S1

Input parameters for G*Power.

Variable	Value
Effect size f	1.479
α error of probability	0.05
Power (1- β error probability)	0.8
Statistical Test	Means: difference between two dependent means

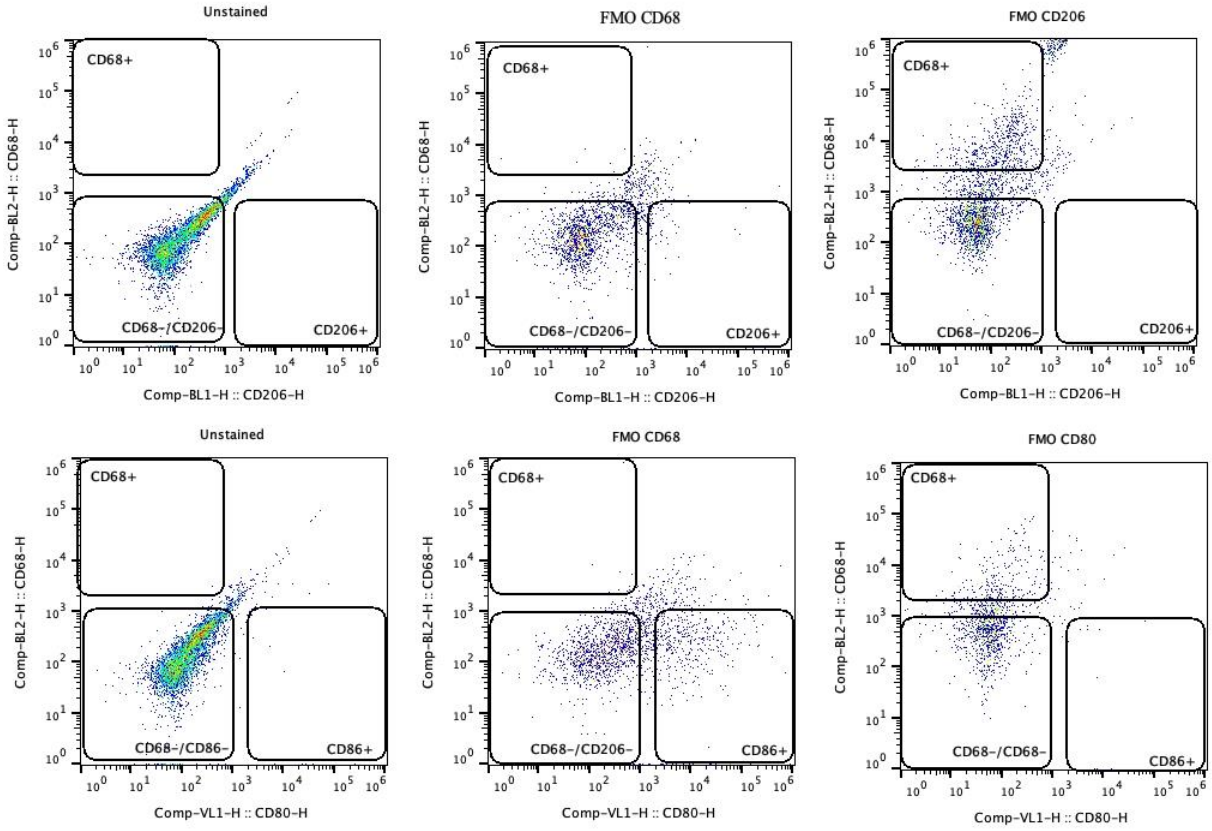

Figure S1. Fluorescence minus one (FMO) staining to establish the gaiting strategy.

Preamble to Chapter 4:

The manuscript entitled: *Implications of Hofbauer Cells and Intermittent Hypoxia on Placental Angiogenesis* formatted for *Placenta*. This manuscript covers the second objective of this thesis as it investigates the regulatory role of IH and HBCs on placental angiogenesis.

Chapter 4: Implications of Hofbauer Cells and Intermittent Hypoxia on Placental Angiogenesis

Research Article

Title: Hofbauer Cells and Intermittent Hypoxia: Potential Regulators for Angiogenesis in the Placenta.

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Highlights

- Hofbauer cells are overall regulators of angiogenesis.
- Pro and anti-angiogenic factors were detected in Hofbauer cell conditioned media.
- Hofbauer cells secrete angiogenin.
- Tube assays treated with conditioned media have more expansive and dense networks than intermittent hypoxia.
- The migration of HUVECs is not impacted by intermittent hypoxia and Hofbauer cells.

Abstract:

The role of Hofbauer cells (HBCs) in placental angiogenesis has been well documented in the literature. Mechanisms such as intermittent hypoxia (IH) - a theorized effect of gestational parent physical activity, have been linked to regulating angiogenesis in other organs but not the placenta. In this study, we aimed to evaluate the role of IH on placental angiogenesis and investigate if there are any interactive or additive effects with the secretions from HBCs. Utilizing tube formation and migration assays, human umbilical vein endothelial cells (HUVEC) were exposed to HBC-conditioned media and/or IH to investigate their effect(s) on indicators of angiogenesis. A cytokine array revealed that HBCs secrete pro- and anti-angiogenic factors, providing evidence of their role in the overall regulation of angiogenesis. Analysis of the tube

formation assay did reveal significantly shorter segments in the IH ($p = .034$) and IH/HBC ($p = .026$) condition compared to the HBC condition. The total mesh area was also lower in the IH ($p = 0 .38$) and IH/HBC ($p = .015$) conditions compared to the HBC condition, along with less master segments in the IH ($p = .039$) condition compared to the HBC condition. Lastly, the length of the master segments was shorter in both the IH ($p = .038$) and IHHBC ($p = .028$) conditions compared to the HBC condition. No significant differences were revealed for the migration assay. In conclusion, we can speculate that IH is less angiogenic than the secretions from HBCs, but more research is required to confirm.
Keywords: Hofbauer cells; angiogenesis; intermittent hypoxia; physical activity.

1. Introduction

Physical activity (PA) throughout gestation has been linked to improved health outcomes for the gestational parent (gesP) and the fetus [1–3]. While the exact mechanisms behind the plethora of evidence-based benefits are not yet known, the placenta is likely a mediator of these benefits, as it is the conduit between the gesP and the fetus. Previous research regarding the impact of gesP PA on the placenta suggests that PA is linked to improved endothelial function, increased placental angiogenesis, and a reduced risk of vascular dysfunction (hypertension and preeclampsia) throughout pregnancy [4,5]. Exposure to PA has been shown to improve vascular function with gesP PA through greater placental growth and increased placental vascularity [6]. These changes optimize the delivery of nutrients and oxygen to the fetus, supporting fetal growth and development. Previously, Jackson *et al*. [7] observed increased total vascular volume and greater capillary surface area and volume in the villi of the placenta from those who engaged in PA compared to non-physically active controls. Changes in the placenta vasculature when exposed to PA are likely due to angiogenesis– the sprouting of new vessels from previously established vessels [8]. Dubé *et al*. [9] further speculated that gesP PA is pro-angiogenic on the developing placenta. Data from the vitamin D and lifestyle intervention for gestational diabetes mellitus prevention (DALI) lifestyle study suggests that PA throughout pregnancy does lead to greater density of villi, thus leading to a larger surface area for gas and nutrient exchange [10]. However, these changes are independent of increased vessel density of the placenta [10]. Therefore, more research is needed to provide robust evidence regarding the role of PA on the placenta vascular system.

Angiogenesis occurs through numerous processes, such as endothelial cell proliferation, migration, enzymatic degradation of the capillary basement membrane, tube formation and vessel fusion [8]. A predominant angiogenic cell in the placenta is the tissue-resident macrophage, termed the Hofbauer Cell (HBC). Found only in the placenta, HBCs are responsible for cytokine secretion, phagocytosis, protecting the semi-allogenic fetus, and promoting homeostasis [11]. HBCs have consistently been found near fetal capillaries [12], implicating their role in angiogenesis through paracrine effects. Specifically, the number of HBCs is correlated to the number of vascular structures in the placenta [13]. In contrast, a lower number of HBCs is associated with blighted ovum and defective placental vasculature [14], indicating their critical role in regulating angiogenesis.

While often oversimplified in the literature, this highly plastic innate immune cell can become polarized over a range of phenotypes, most notably, the pro-inflammatory (M1-like) or the anti-inflammatory (M2-like) phenotypes. This plasticity allows HBCs to not only participate in angiogenesis [15] but also in maintaining stromal water balance, promoting tolerance of the semi-allogenic fetus, phagocytosis, and cytokine secretion, among other functions.[11,13,16]. Interestingly, our group has shown that increases in the proportion of anti-inflammatory phenotypes have been positively associated with increases in PA minutes throughout gestation [17]. Precisely, identified HBCs do not adopt the M1 phenotypes but were polarized to the M2a, M2b and M2c phenotypes, which stimulate placental angiogenesis through the secretion of VEGF and FGF2 *in vitro* [13,15]. Additionally, HBCs secrete Spry2, an angiogenic regulator, reiterating their participation in angiogenesis [18]. Other angiogenic factors and their roles are summarized in Figure 1.

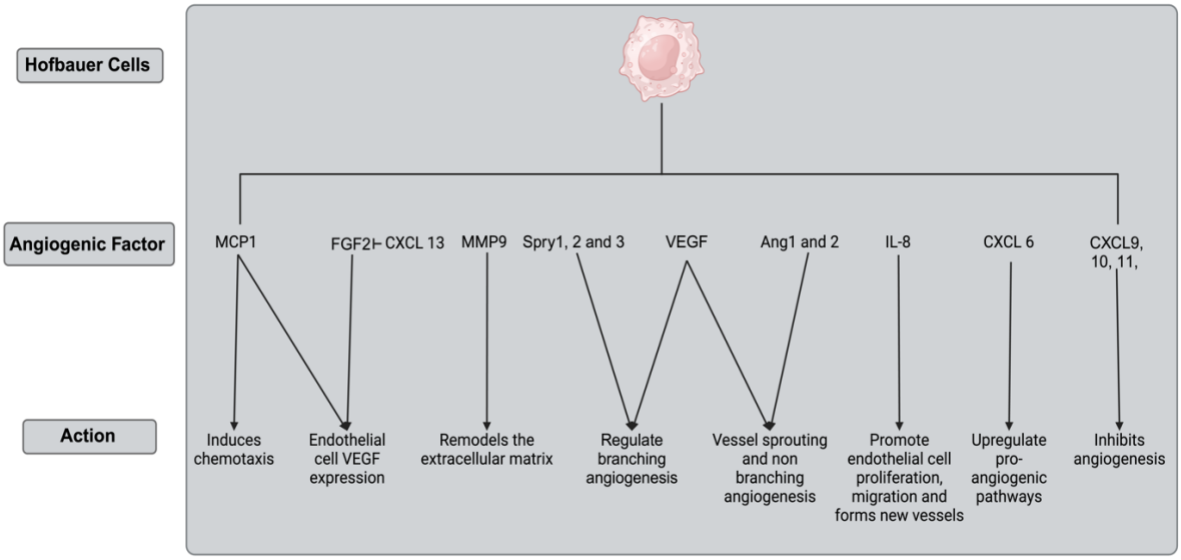

Figure 1. Angiogenic factors secreted by Hofbauer cells and their actions in angiogenesis [16,19–29].

The literature also suggests that the observed vascular changes within the placenta may be an adaptive response to a transient decrease in oxygen tension caused by gesP PA [9]. During PA, it has been widely accepted that blood is diverted to the working muscles, leading to a transient state of hypoxia within the placenta [30], which has been speculated to stimulate placental angiogenesis [7]. It is thought that the placenta can respond to moderate changes in oxygen availability to prioritize the development of the fetus [31]. The theorized intermittent hypoxic (IH) state that occurs as a result of gesP PA initiates angiogenesis through the activation and stabilization of hypoxia-inducible factor (HIF), leading to downstream upregulation of VEGF [8,31–33]. While much of the research has been focused on the detrimental impacts of chronic hypoxia on the placenta and, subsequently, the fetus [31], there is very little research regarding the impact of IH on the healthy placenta. In tumours, IH has been shown to increase the formation of tubes and migration of endothelial cells compared to normoxia [34]. However, no studies have examined how the placenta would respond to IH. More research is needed to fill the gap in knowledge regarding the impact of IH on placental angiogenesis and if there is an interactive effect with the pro-angiogenic secretions from HBCs. Therefore, this study aims to determine the independent or additive effect(s) of HBCs and IH on placental angiogenesis *in vitro*. We hypothesize that when human umbilical vein endothelial cells (HUVECs) are exposed

to HBCs and IH, more expansive tube networks and faster migration will be observed compared to the controls.

2. Methods

2.1 Hofbauer Conditioned Media

Primary human HBCs isolated from healthy pregnancies (Amnion Foundation, USA) were seeded in a cell-culture-treated 6-well plate (ThermoFisher). HBCs remained in culture at 37°C with 5% CO_2 for 48 hours at a density of 5.0 x 10^5 cells/well. HBCs were cultured in RPMI 1640 media (ThermoFisher) supplemented with 5% FBS, 25 mM HEPES (Sigma-Aldrich) and 1x Pen/Strep (Gibco). To reflect the physiologically normoxic conditions of the placenta, an oxygen tension of 5% O_2 was selected [35]. Media was collected after 48 hours in culture, spun at 5000g for 10 minutes to clear any debris, aliquoted, and then stored at -20°C for future use and analysis.

2.2 Analysis of Conditioned Media

We analyzed the cytokines and chemokines from the collected HBC-conditioned media with a cytokine array (Abcam; ab133998). A negative control blot was run with RPMI complete media (described in section 2.1). The array was completed following the manufacturer's instructions. We performed overnight incubations at 4°C for the media, biotin-conjugated cytokine antibodies, and the HRP-Streptavidin steps. The array was imaged with the ChemiDoc Imaging System (Bio-Rad). The integrated density of the grey value was measured for each spot using ImageJ (2.14.0/1.54f). The negative control was then subtracted from each spot to remove the background. The array data was normalized to the average density of the positive controls from the reference array (RPMI complete media) using the manufacturer's equation of $X(Ny) = X(y) * P1/P(y)$; where $X(Ny)$ is the normalized signal density of the spot, $X(y)$ is the mean signal density of the spot, $P1$ is the mean signal density of the positive control spots on the reference array, and $P(y)$ is the mean signal density of the positive control spots of the array. Data were then normalized using log2-fold change versus the control condition. Cytokines with log2-fold changes greater than 2 or less than -2 were considered biologically relevant.

2.3 Cell Culture Conditions and Assay

To test the potential independent or additive impact of HBCs and IH on angiogenesis in the placenta, human umbilical vein endothelial cells (HUVECs) were cultured under the following four conditions: Endothelial Cell Growth Media (PromoCell) at 8% O_2 (control); 30% HBC conditioned media at 8% O_2 (test the independent effect of HBCs); Endothelial Cell Growth Media with IH (bouts of 3% O_2; test the independent effect of IH); and 30% HBC conditioned media with IH (test the potential additive or interactive effect(s)). HBC-conditioned media was optimized to be diluted to 30% in Endothelial Cell Media. An oxygen tension of 8% was selected as a control to reflect the physiologically relevant oxygen tension of the placenta blood vessels [35].

2.4 Tube Formation Assay

Assays were performed to assess the tube-forming ability [36] of HUVECs while exposed to HBC-conditioned media and/or IH. HUVECs were expanded for 24-48 hours with Endothelial Cell Growth Media. Glass bottom 24-well plates (CELLTREAT) were coated with 250 µL of reduced growth factor phenol red-free Matrigel (Corning) on ice, then put in a humidified chamber at 37°C for 30 minutes to polymerize. HUVECs were harvested, counted and spun at 400g for 5 minutes at 4°C. Harvested HUVECs were resuspended at $1.0x10^6$ cells/mL with Endothelial Cell Growth Media. Cells were then seeded in the Matrigel-coated wells at a density of $1.0x10^5$ cells/well. The density of cells was optimized to ensure there were enough cells to form tubes, but not monolayers. Assays were performed for the optimized time of 4 hours. The IH condition for this assay was 2 hours at 8% O_2, followed by 2 hours at 3% O_2. This assay was completed with three technical replicates for each individual replicate for a sample size of $n = 3$.

After 4 hours, phase contrast images were taken with a Zeiss Axiovert 40 C microscope equipped with Lumenera INFINITY2-2C Colour CCD Camera. The wells were divided into four quadrants to minimize bias. One image was taken in the centre and one from each quadrant, for a total of five images per well. Images were then uploaded to ImageJ and converted to 8-bit RGB images for tube formation analysis. Using the Angiogenesis Analyzer by Carpentier *et al.* [37], we quantified the number of nodes, junctions, meshes, total mesh area, number of segments, total

length, branching length, branching interval, mesh index, mean mesh size and number of branches. A description of the variables quantified in the tube assay is presented in Table 1.

Table 1.

Description of Tube Assay Variables

Measured Variable	Description
Number of Nodes	Total count of connecting points where multiple tubes intersect. Showing potential branching points.
Number of Junctions	The intersection of two connecting branches. Showing complex branching points.
Number of Meshes	Total count of enclosed loops that are formed by tubes.
Total Mesh Area	Calculated combined area of all meshes. Indicates network density and extent.
Number of Segments	Number of tube structures connecting nodes or junctions. Segments are the building blocks of the network.
Total Length	The sum of all tube lengths in the network.
Number of Branches	The total count of tubes that connect nodes and junctions.
Branching Length	The sum of all the branches that extend outwards.
Branching Interval	The average length between branches. Indicates the pattern and regularity of the branches.
Mesh Index	Ratio of number of meshes to the total number of nodes or junctions. Measure reflects the complexity of the network.
Mean Mesh Size	The average size of the enclosed loops.

2.3 Wound Closure Assay

Wound closure assays were completed to assess the impact of HBCs and IH on HUVECS migration and proliferation, as described by Justus *et al*. [38]. HUVECs were cultured in cell-culture-treated 24-well plates (Fisher) and grown to confluency in Endothelial Cell Growth Media. A sterile 200μL pipette tip was used to gently press against the bottom of the well in a straight line from left to right to create a wound in the cell monolayer. Following the aspiration of cell media, each well was washed with DPBS and gentle swirling to remove any loose cells in the wound. DPBS was then aspirated, followed by adding freshly warmed media according to the experimental conditions. The IH condition for this assay was two 3-hour bouts of 3% O_2 separated by 3 hours at normoxia (8% O_2). The assay was completed for each condition with three technical replicates for three individual replicates, $n = 3$. Phase contrast images (Zeiss Axiovert 40 C with Lumenera INFINITY2-2C Color CCD Camera) of the wound were taken in the centre of the well at the initial time of the wound and every three hours after for 12 hours total. Wound width was analyzed by ImageJ, using the measure tool to measure the width (in pixels) of three spots of the wound (left, centre, and right) per time point. The average wound width was then calculated and normalized to the initial wound size to allow for comparisons between and within the culture conditions.

2.4 Data Analysis

Data are presented as mean and standard error. Statistical analyses were conducted on R-studio (4.4.0). Differences between the culture conditions for the independent variables from the tube assay were assessed using multiple one-way analysis of variances (ANOVA), while the differences for the wound closure assay were assessed through a repeated measures ANOVA. Post-hoc analyses were performed using a Bonferroni correction to determine where differences were significant. Significance was set at $p < .05$.

3. Results

3.1 Cytokine Array of HBC Media.

The log2fold change in the HBC conditioned media compared to the RPMI control media is presented in Figure 2. Only cytokines that were detected in the HBC conditioned media were

included in the analysis. The secretion of cytokines was considered biologically relevant when there was a 100% difference (log2fold change >2 or <-2) compared to the control RPMI media. The analysis revealed that 30 cytokines were upregulated in the HBC conditioned media compared to the control, with none downregulated. The greatest difference was 20.78 log2fold change with IL-8. The lowest biologically relevant upregulation was MCP-1 with a 2.09 fold increase from the control.

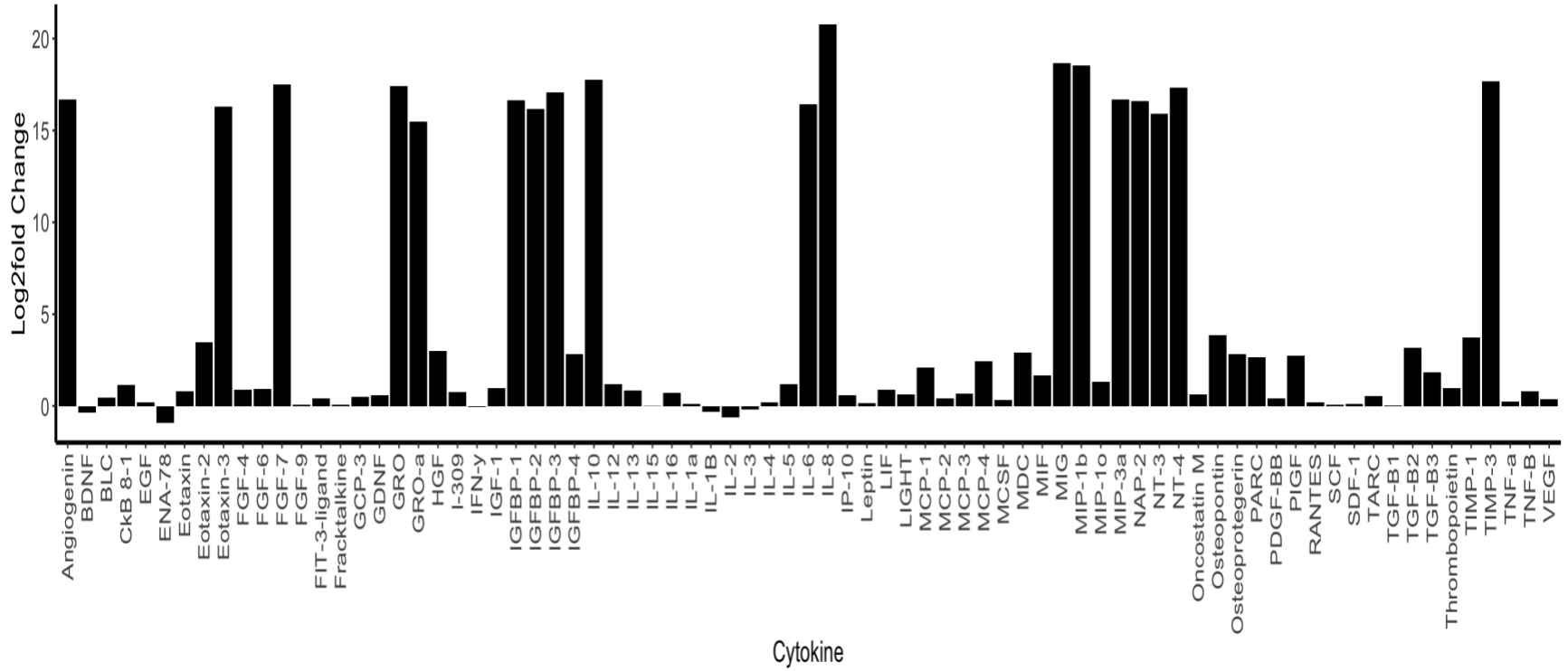

Figure 2. Angiogenic Factor Secretion from Hofbauer Cells. Data were normalized to the control condition. Fold changes greater than 100% (>2 or <-2) were considered biologically relevant. A bar graph with the log2fold changes on the y-axis and cytokines that were present in the conditioned media are shown on the y-axis.

3.2 Wound Closure Assay

Representative images of wound closure for all culture conditions are presented in Figure 3.

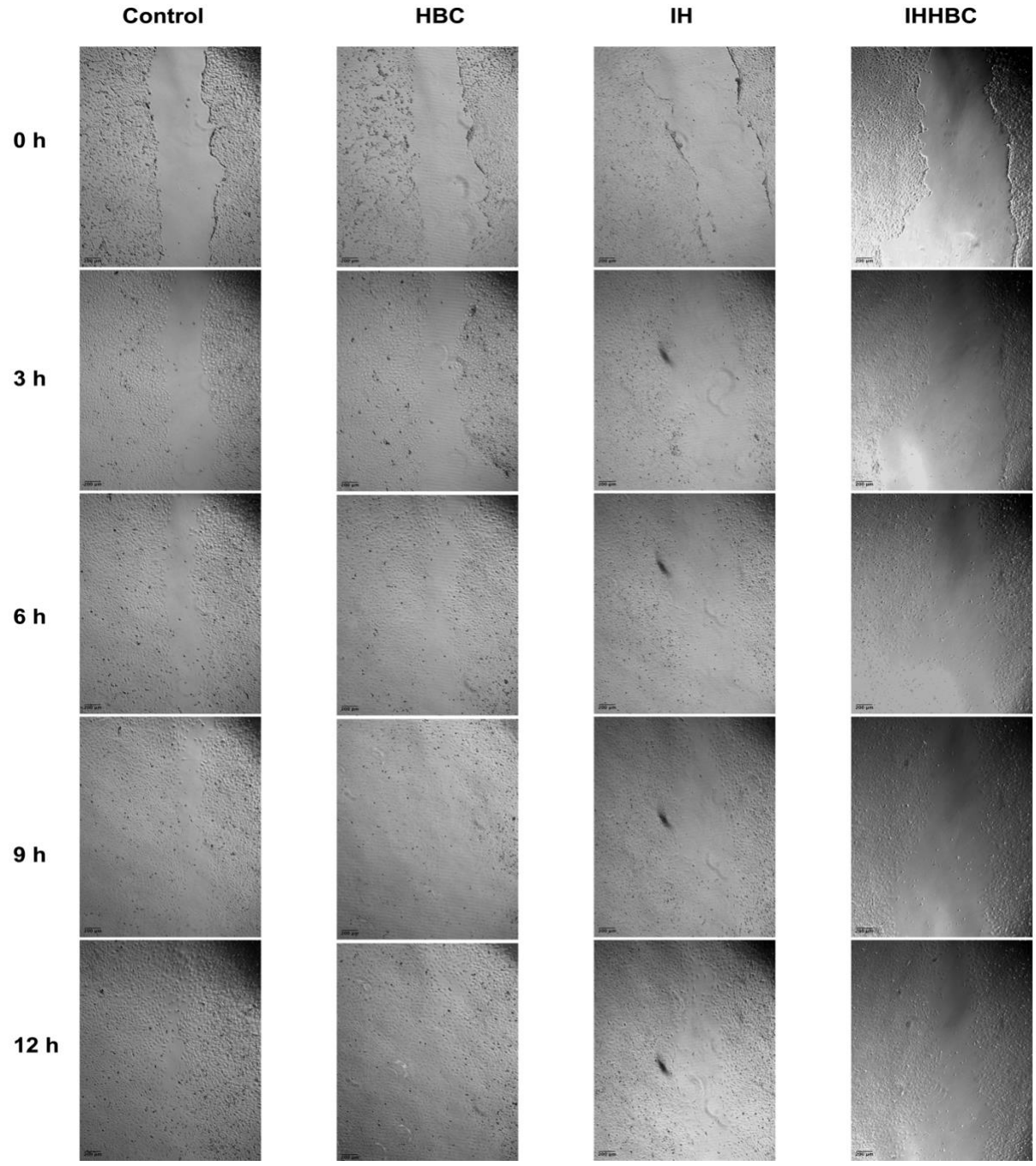

Figure 3. Representative wound closure over time per condition. Images were taken with the 5x objective. The culture conditions are labelled on the top of the figure, and the time points that the images were taken are labelled on the left. Images were taken of the initial wound (0h) and every three hours until 12h. HBC: Hofbauer cells; IH: Intermittent hypoxia; IH/HBC: Intermittent hypoxia and Hofbauer cell.

Mean wound closure ratio of all culture conditions is presented in Figure 4. A repeated measures ANOVA was run to determine the effect of culture condition on wound closure. The analysis revealed a significant main effect for time, $F(4, 32) = 194.75$, $p < .001$, $\eta_p^2 = 0.96$. Post-hoc pairwise comparisons with Bonferroni corrections indicated that wound closure significantly differed at all time points ($p < .001$). The analysis did not reveal a significant main effect for cell culture condition, $F(3, 8) = 2.26$, $p = .158$, $\eta_p^2 = 0.459$. We also did not observe an interactive effect between time and cell culture condition, $F(12, 32) = 1.64$, $p = .130$, $\eta_p^2 = 0.38$.

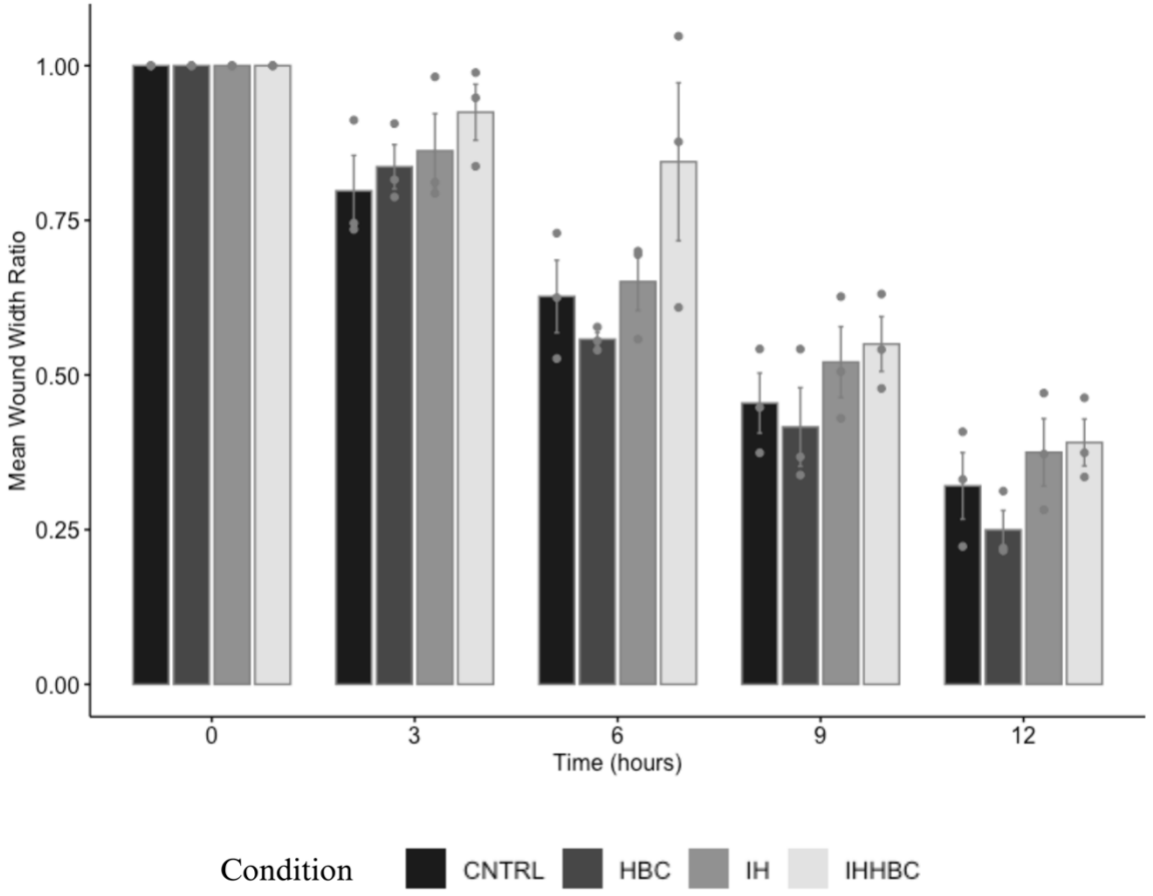

Figure 4. Mean wound size ratio with SEM over time. The mean wound size was normalized to the initial wound size; the ratio is shown on the y-axis. The time, in hours, is on the x-axis. The black = control, dark grey = HBC condition, IH = medium grey and IHHBC wound closures = light grey lines. No significant differences were identified. HBC: Hofbauer cells; IH: Intermittent hypoxia; IH/HBC: Intermittent hypoxia and Hofbauer cell.

3.3 Tube Assay

Representative images of the tube formation assay for each condition are shown in Figure 5.

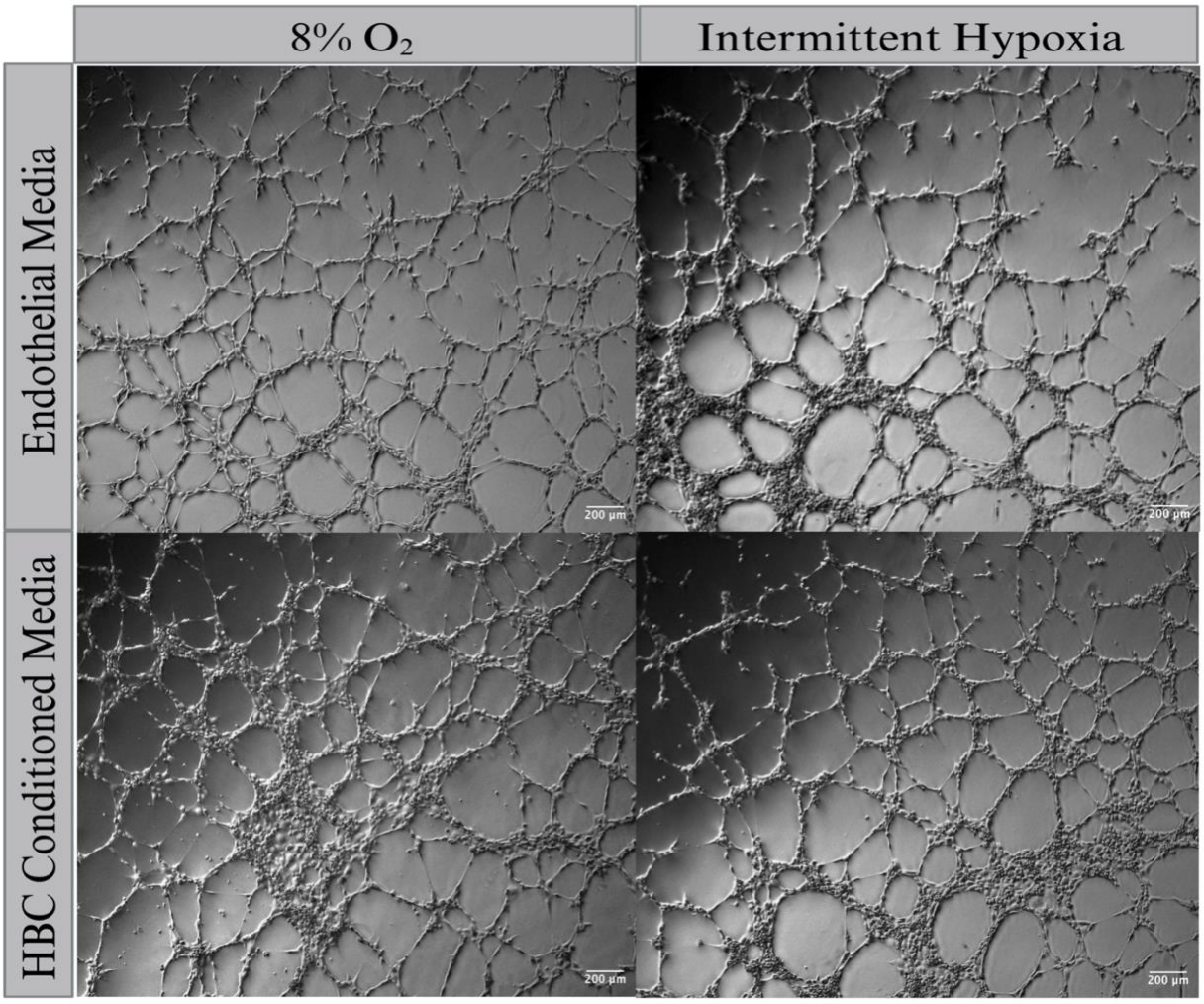

Figure 5. Representative images of tube formation assay for each culture condition. Images were taken with the 5x objective. The media type is presented on the left side of the figure and the oxygen tension is shown on the top. HBC; Hofbauer cells.

To have a comprehensive understanding of tube formation, we calculated the number of nodes, junctions, meshes, segments, total segment length, number of master segments, total master segment length, total tube length, mesh area, branching length, branching interval, mesh index, mean mesh size, and number of branches. We conducted a series of one-way ANOVAs to compare the effect of cell culture condition on the variables of tube formation. The results of the ANOVAs are summarized in Table 2.

Table 2.

ANOVA Results for Tube Formation Assay

Variable	$SS_{Between}$	$df_{Between}$	$MS_{Between}$	SS_{Within}	df_{Within}	MS_{Within}	F value	P value	η2
Number of Nodes	31221	3	10407	17000	8	2125	4.898	.032*	0.65
Number of Junctions	2581	3	860.2	1448	8	180.9	4.754	.035*	0.64
Number of Segments	4055	3	1351.5	2347	8	293.4	4.607	.037*	0.63
Total Segment Length	$1.549x10^7$	3	$5.164x10^6$	$6.242x10^6$	8	780264	6.618	.015*	0.71
Number of Master Segments	1421.40	3	473.8	693.4	8	86.68	5.466	.024*	0.67
Total Master Segment Length	$1.668x10^7$	3	$5.561x10^6$	$6.954x10^6$	8	869306	6.397	.016*	0.71
Total Length	25278931	3	8426310	12348671	8	1543584	5.459	.025*	0.67
Number of Branches	726.8	3	242.28	462.7	8	57.83	4.189	.047*	0.61
Total Branches Length	2033305	3	677768	2120361	8	265045	2.557	.128	0.49
Branching Interval	376.8	3	125.59	469.9	8	58.74	2.138	.174	0.44
Number of Meshes	207.0	3	69.00	142.3	8	17.79	3.879	.056	0.59
Total Meshes Area	$5.713x10^{10}$	3	$1.904x10^{10}$	$2.079\ x10^{10}$	8	$2.599x10^9$	7.327	.011*	0.73
Mean Mesh Size	339866	3	113289	20989475	8	2623684	.043	.987	0.02
Mesh Index	170.4	3	56.79	1921.5	8	240.2	0.236	.869	0.08

Note. SS: Sum of Squares; df: degrees of freedom; MS: Mean Squares; η2: Eta Squared.

** $p < 0.05$*

ANOVAs with significant results moved onto post-hoc pairwise comparisons with Bonferroni corrections. The post-hoc analysis revealed that the HBC condition had a significantly higher total mesh area than the IH ($p = .038$) and IH/HBC ($p = .015$) conditions. Additionally, the total segment length was significantly longer in the HBC condition than in the IH ($p = .015$) and IH/HBC ($p = .026$) conditions. The HBC condition was found to have a significantly higher number of master segments than the IH/HBC ($p = .039$) condition. The total master segment length was also longer in the HBC condition than in the IH ($p = .038$) and IH/HBC ($p = .028$) conditions. There were no significant effects between the control condition and the culture conditions for all variables. The means and standard errors for each dependent variable of tube formation are presented in Figure 6.

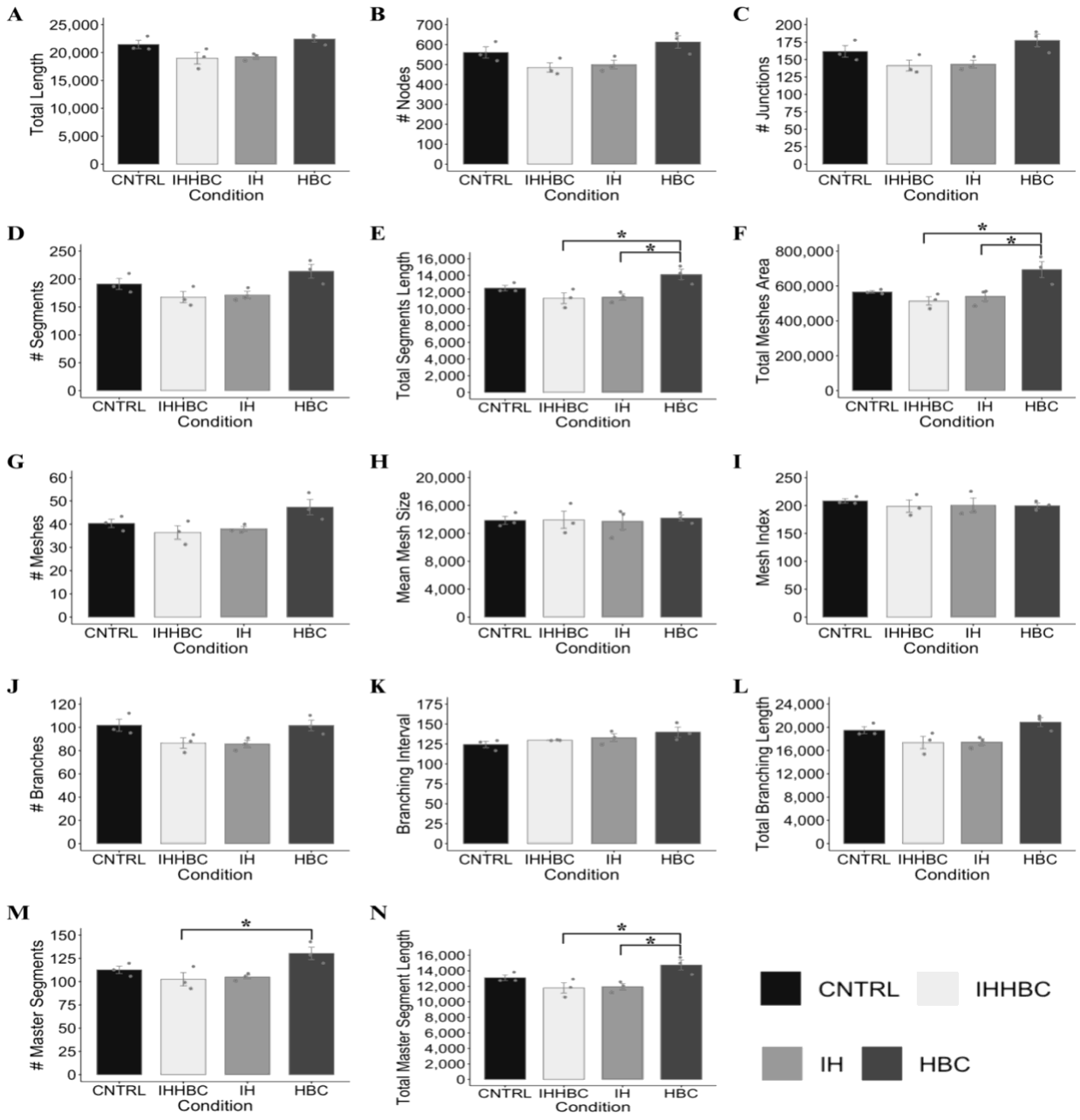

Figure 6. Tube Formation Characteristics. The mean and standard errors are presented for the total length (A), number of nodes (B), number of junctions (C), number of segments (D), total segment length I, total mesh area (F), number of meshes (G), mean mesh size (H), mesh index (I), number of branches (J), branching interval (K), total branching length (L), number of segments (M) and total master segment length (N). The dependent variables are on the y-axis with the condition on the x-axis. The control condition is depicted with the black bars. The HBC condition is shown with the dark grey bars. The medium and light grey bars present the IH and the IH/HBC conditions, respectively. CNTRL: Control; HBCs: Hofbauer cells; IH: Intermittent hypoxia; IH/HBC: Intermittent Hypoxia and Hofbauer Cell.

4. Discussion

This study aimed to determine if HBCs and IH have an independent or additive effect on placental angiogenesis, measured through HUVECs tube formation and migration. It was hypothesized that tube formation and wound closure would be greater in the combined HBC and IH condition. Current research suggests that IH increases tube formation and migration with endothelial cells [34,39]. However, much of the IH research uses large variations in oxygen tensions, potentially eliciting these differences in angiogenic markers that may not be physiologically relevant. Acute changes in oxygen tension are considered primary stimuli for angiogenesis through the activation of HIF-1, which subsequently induces angiogenic factor secretion in endothelial cells [40,41]. Specifically, hypoxia-reoxygenation cycles with endothelial cells have been shown to regulate HIF-1 NF-κB and Nrf2, potentially corresponding to the observed increase in tube formation and cell migration in the literature [34]. An increase in the expression of genes involved in angiogenesis, such as IL-15, BDNF, VEGF, FGF2 and EPHB, has been observed in Eahy926 endothelial cells when exposed to IH [34]. However, IH may also elicit anti-angiogenic effects since it downregulates pro-angiogenic genes such as IL-8, EPOR, NRG1 and PDGFβ [34]. Downregulation of these genes could have led to the observed shorter master segment length, lower total mesh area, and shorter segment lengths in the tube formation assay for the IH and IH/HBC conditions compared to the HBC condition. Decreases in these values indicate that IH may lead to less intricate and dense networks which may have implications for nutrient and gas transport efficiency compared to the HBC alone condition. We cannot confirm that IH leads to an overall decrease in vessel network extent and branching as there were no significant differences from control. Although, the IH and IH/HBC conditions did trend towards lower tube formation values than the control, whereas the HBC conditions did trend towards the same or higher tube formation values than the control, the differences were not statistically different (see Supplemental Table 1). The placenta, our organ of interest, experiences physiological shifts in oxygen tension during its development, subsequently regulating the HIF pathways and angiogenesis [32]. Therefore, we would anticipate that the placenta vasculature would similarly respond to IH. The observed null effect may be due to the IH treatment time being too short, not having a large enough difference in oxygen tensions, or not timing the hypoxia correctly since the duration, intensity, and timing of hypoxia have been linked to differential changes in the placenta [31].

Angiogenesis is a complex process comprising many steps with various pathways (e.g., PI3K/AKT and MAPK/ERK) that differences in VEGF, FGF, and OPN can regulate. Precisely, HBC polarization is thought to play a role in promoting angiogenesis in the placenta. M2a, M2b, and M2c HBCs have previously been linked to regulating angiogenesis *in-vitro*, measured through tube formation and migration assays [15]. While M1 macrophages secrete VEGF [17], research suggests that they have less angiogenic potential than the anti-inflammatory counterparts due to FGF2 and PIGF signalling [42]. Culture conditions such as cell density and pH of the media have been shown to impact the polarization of macrophages [43,44], and thus potentially change their downstream roles. Since we identified pro-inflammatory cytokines IFN-γ and TNF-α in the RPMI media, we may have stimulated the HBCs towards a pro-inflammatory polarization [45]. Additionally, some of the insignificant results could be equated to the low sample size. To determine if our study was underpowered, we performed a retrospective power analysis using G*power (Supplemental Table 1). The analysis determined that we need to include five independent samples per condition when using the average effect size from these experiments. An increase in sample size will provide more power to the study as the HBC condition was close to statistical significance compared to the control for the tube assay (Supplemental Table 1).

As expected, many factors detected in the HBC-conditioned media have roles in immune regulation, responses, and placental development [46–51]. Additionally, results from the cytokine array show that HBCs regulate angiogenesis by secreting pro- and anti-angiogenic factors. We detected an up-regulation of FGF-7, GRO, NAP-2, Eotaxin-2, Eotaxin-3, and PIGF, which have been shown to promote angiogenesis [52–56]. It was previously identified that all polarizations of HBCs expressed VEGF [18], and in this study, we have shown that HBCs secrete VEGF, but we do not know if the secretion depends upon HBC polarization. Importantly, this study has also identified that HBCs can secrete Angiogenin. A previous study by Pavlov and colleagues [57] suggested that Angiogenin was only secreted by trophoblasts, mesenchymal stem cells, and decidual macrophages, but not from HBCs when using $CD45^+$ as a marker. However, in this study, we have detected Angiogenin in the HBC-conditioned media and not in the control media,

showing that HBCs can secrete this pro-angiogenic factor, which induces new vessel formation and endothelial cell proliferation [58].

The highest up-regulated cytokine from the control was IL-8, which has been consistently linked to promoting angiogenesis, endothelial cell survival, migration and proliferation [29,59]. IL-8 is thought to directly interact with HUVECs and impact tube organization through potentially binding to the CXCR2 receptor [29]. In human intestinal microvascular endothelial cells, IL-8 binding to the CXCR2 receptor led to downstream effects such as phosphorylation of extracellular signal-regulated protein kinase ½ (ERK ½) [60], a known angiogenic pathway [61]. Additionally, IL-8 stimulates the production of matrix metallopeptidase 2 (MMP)2 and MMP9, which degrade the extracellular matrix to allow for endothelial cell migration, invasion, and tube formation [29,62]. Furthermore, the secretion of IL-8 from skeletal muscle is regulated by muscle contractions [63], so this cytokine may be a regulator of the observed changes in placenta vasculature with exposure to gesP PA. MCP-1 and Osteopontin (OPN) were other pro-angiogenic factors detected at high levels in the HBC-conditioned media. Through the receptors CCR2 and MCP-induced-protein (MCPIP), MCP-1 binds to endothelial cells, inducing the upregulation of VEGF and HIF-1α gene expression, subsequently inducing the secretion of VEGF-A leading to new vessel formation [25,27,56]. MCP-1 secreted from HBCs also regulates cytotrophoblast differentiation and low levels are associated with intrauterine growth restriction [64,65]. Secretions of OPN by HBCs at high amounts have previously been identified and are thought to increase angiogenesis via CD44 and integrin complexes and activation of ERK1/2 and PI3K/AKT pathways [66,67]. OPN also impacts pregnancy outcomes by regulating signal transduction at the uterine-placental interface, contributes to decidualization, and regulates other immune cell behaviour and cytokines as a product of immune cells [68]. The high levels of MCP-1 and OPN recapitulate the multifaceted regulatory role of HBCs in placental development.

A balance between pro-angiogenic and anti-angiogenic factors is integral to mitigating the risk of developing pathologies like preeclampsia [69]. Anti-angiogenic factors such as IL-10 and NT-4, were biologically relevant in the conditioned media [70–72]. The pleiotropic cytokine IL-6 was also detected, which exerts both pro- and anti-angiogenic effects and regulates endothelial cell

migration [59,73,74]. Moreover, the insulin-like growth factor-binding protein (IGFBP) family helps maintain the balance of pro- and anti-angiogenic regulation [75]. IGFBP1 and IGFBP2 are pro-angiogenic, while IGFBP4 is believed to be anti-angiogenic; however, the mechanisms are not yet known [75]. At the same time, IGFBP3 can act as a promoter and inhibiter of angiogenesis by inducing the expression of VEGF or inhibiting endothelial cell adhesions, respectively. Similarly, TIMP-1 is another pleiotropic factor identified and can inhibit or promote angiogenesis. Depending on the tissue environment, TIMP-1 can inhibit angiogenesis by suppressing MMPs or promote angiogenesis by stimulating endothelial cell proliferation and survival [76,77]. These findings are validated by the discovery of the anti-angiogenic Sprouty (Spry) proteins from HBCs [18,20]. Spry-2 protein expression in the placenta was similar between physically active and inactive gesPs, indicating that HBCs maintain their regulatory role [18]. The regulation of vasculature with anti-angiogenic factors is an integral aspect of establishing healthy vessels to maintain homeostasis of the tissues. In response to tissue demands, capillaries can respond to changes in the tissue environment by growing or regressing [8]. Too much angiogenesis can lead to pathologies such as age-related macular degeneration, rheumatoid arthritis and endometriosis [78–80]. Therefore, anti-angiogenic factors are integral for balancing pro-angiogenic factors, maintaining the balance of new vessel formation, thus, reiterating the regulatory role of HBCs on angiogenesis through their secretions.

Hypoxia is consistently shown to stimulate angiogenesis; however, the vessels formed from this stimulus may have faulty vessel integrity. The induction of high VEGF secretion by hypoxia elicits disorganized and structurally immature vessels, leading to impaired oxygen and nutrient delivery [81,82]. Our findings of lower markers of tube formation for the IH and the IH/HBC conditions compared to the HBC condition may be due to a temporary decrease in angiogenesis when the cells experience hypoxia. A temporary decrease in angiogenesis during hypoxia could be a protective mechanism to maintain the integrity of the already established vasculature. Moreover, hypoxic environments are important stimuli for trophoblast development [83]; therefore, the transient decrease in angiogenesis during this time could protect vessel integrity while allowing the trophoblasts to optimally develop the villus spaces for the expanding vasculature. Since the combined condition showed similar tube formation values to the IH alone condition, these results suggest that HBCs do not override this potentially protective mechanism,

as there were no differences with the control. The lack of significant differences from the control shows that the adaptations in the tube formation with different environments do not cause significant changes from baseline, indicating a minor overall regulatory effect of IH and HBCs. HBCs are often referenced as pro-angiogenic; however, our results from the tube formation assay and cytokine array strongly suggest that HBCs can also be anti-angiogenic, indicative of overall master regulators of angiogenesis in the placenta.

The use of migration assays in our model allowed us to determine that secretions from HBCs do not impact the migratory abilities of HUVECs. Loegl and colleagues [15] similarly utilized conditioned media from HBCs on fetoplacental endothelial cells and observed a decrease in migration compared to the controls. They had equated these results to the presence of migration inhibiting factor (MIF), which we also detected. Our lack of differences in the migration assay of this study may be due to a neutralization between factors that promote (e.g., IL-8, OPN, and Angiogenin) or inhibit (e.g., TIMP-3) endothelial cell migration [29,53,58,59,84–86]. To confirm this speculation, we would need to enhance our analysis of the mechanistic roles of these factors and perform more sensitive assays.

In conclusion, our data suggest that IH, an effect of gesP PA, does not have a compounding effect on the angiogenic abilities of HBCs. However, further adequately powered research using more sensitive assays is required to confirm the independent roles of HBCs and IH. Factors secreted by HBCs found in the conditioned media promoted tube formation more than IH and IH/HBC conditions. Previous IH studies have used large extremes in oxygen tensions that may not be physiologically relevant. To ensure physiologically relevant conditions, primary research is required to quantify the changes in oxygen tension within the human placenta to provide informed cell culture models. Our results show that HBCs exhibit a regulatory paracrine effect on angiogenesis by secreting pro-angiogenic, anti-angiogenic and pleiotropic factors. Specifically, we detected high levels of IL-8, Angiogenin, OPN, TIMP-1 and MCP-1. Elucidating the mechanisms behind HBC regulation of these pathways will allow us to deconvolute the angiogenic dysregulations linked to preeclampsia and fetal growth restriction.

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Supplemental Files

Table S1

Input parameters for G*Power.

Variable	Value
Effect size f	0.546[a]
α error of probability	0.05
Power (1- β error probability)	0.8
Number of Groups	4
Statistical Test	ANOVA: Repeated measures, between factors

[a]effect size was calculated by taking the average effect size that was calculated from each variable of the tube formation assay.

Table S2

Results of Post-hoc Pairwise Comparisons with Bonferroni Corrections

Outcome	Comparisons	Mean Difference	SE of Difference	95% CI	T-value	Adjusted p-value
	CNTRL-HBC	-52.16	37.64	-172.69, 68.37	-1.386	1.00
	CNTRL-IH	61.17	37.64	-59.36, 181.70	1.625	.857
Number of	CNTRL-IHHBC	75.77	37.64	-44.76, 196.30	2.013	.473
nodes	HBC-IH	113.33	37.64	-7.197, 233.9	3.011	.101
	HBC-IHHBC	127.93	37.64	7.403, 248.5	3.399	.056
	IH-IHHBC	14.600	37.64	-105.93, 135.13	0.388	1.00
	CNTRL-HBC	-15.778	10.98	-50.95, 19.393	-1.437	1.00
	CNTRL-IH	18.222	10.98	-16.949, 53.39	1.659	.814
Number of	CNTRL-IHHBC	20.18	10.98	-14.993, 55.35	1.837	.621
junctions	HBC-IH	34.00	10.98	-1.171, 69.17	3.096	.089
	HBC-IHHBC	35.96	10.98	0.785, 71.13	3.274	.068
	IH-IHHBC	1.956	10.98	-33.22, 37.13	0.178	1.00

*$p < .05$

Table S2 Continued

Outcome	Comparisons	Mean Difference	SE of Difference	95% CI	T-value	Adjusted p-value
	CNTRL-HBC	-22.83	13.99	-67.61, 21.96	-1.632	.847
	CNTRL-IH	19.593	13.99	-25.19, 64.38	1.401	1.000
Number of	CNTRL-IHHBC	23.42	13.99	-21.37, 68.20	1.674	.796
segments	HBC-IH	42.42	13.99	-2.36, 87.21	3.033	.097
	HBC-IHHBC	46.24	13.99	1.460, 91.0	3.307	.065
	IH-IHHBC	3.822	13.99	-40.9, 48.6	0.273	1.000
	CNTRL-HBC	-1629.3	721.2	-3939, 680.4	-2.259	.323
	CNTRL-IH	1069.2	721.2	-1240.4, 3379	1.482	1.000
Total segments	CNTRL-IHHBC	1213.7	721.2	-1095.9, 3523	1.683	.785
length	HBC-IH	2698	721.2	388.8, 5008	3.741	.034*
	HBC-IHHBC	2843	721.2	533.3, 5153	3.942	.026*
	IH-IHHBC	144.53	721.2	-2165, 2454	0.200	1.000
	CNTRL-HBC	-17.711	7.602	-42.05, 6.632	-2.330	.289
	CNTRL-IH	7.667	7.602	-16.676, 32.01	1.009	1.000
Number of	CNTRL-IHHBC	10.067	7.602	-14.276, 34.41	1.324	1.000
master segments	HBC-IH	25.38	7.602	1.035, 49.72	3.338	.062
	HBC-IHHBC	27.78	7.602	3.435, 52.12	3.654	.039*
	IH-IHHBC	2.400	7.602	-21.94, 26.74	0.316	1.000

*$p < .05$

Table S2 Continued

Outcome	Comparisons	Mean Difference	SE of Difference	95% CI	T-value	Adjusted p-value
Total master segments length	CNTRL-HBC	-1637.1	761.3	-4075, 800.8	-2.150	.382
	CNTRL-IH	1154.6	761.3	-1283.3, 3592	1.517	1.000
	CNTRL-IHHBC	1311.2	761.3	-1126.7, 3749	1.722	.740
	HBC-IH	2792	761.3	353.8, 5230	3.667	.038*
	HBC-IHHBC	2948	761.3	510.4, 5386	3.873	.028*
	IH-IHHBC	156.64	761.3	-2281, 2595	0.206	1.000
Total length	CNTRL-HBC	-977.2	1014.4	-4226, 2271	-0.963	1.000
	CNTRL-IH	2190	1014.4	-1058.2, 5439	2.159	.377
	CNTRL-IHHBC	2458	1014.4	-790.1, 5707	2.423	.250
	HBC-IH	3168	1014.4	-80.99, 6416	3.123	.085
	HBC-IHHBC	3436	1014.4	187.10, 6684	3.387	.057
	IH-IHHBC	268.1	1014.4	-2980, 3517	0.264	1.000
Number of branches	CNTRL-HBC	0.178	6.209	-19.707, 20.06	0.029	1.000
	CNTRL-IH	16.022	6.209	-3.862, 35.91	2.580	.196
	CNTRL-IHHBC	15.267	6.209	-4.618, 35.15	2.459	.236
	HBC-IH	15.844	6.209	-4.040, 35.73	2.552	.204
	HBC-IHHBC	15.089	6.209	-4.795, 34.97	2.430	.247
	IH-IHHBC	-0.756	6.209	-20.64, 19.129	-0.122	1.000

*$p < .05$

Table S2 Continued

Outcome	Comparisons	Mean Difference	SE of Difference	95% CI	T-value	Adjusted p-value
	CNTRL-HBC	-128175	41627	-261478, 5128	-3.079	.091
	CNTRL-IH	24775	41627	-108528, 158078	0.595	1.000
Total meshes	CNTRL-IHHBC	51675	41627	-81628, 184979	1.241	1.000
area	HBC-IH	152950	41627	19646, 286253	3.674	.038*
	HBC-IHHBC	179850	41627	4657, 313154	4.321	.015*
	IH-IHHBC	26900	41627	-106403, 160203	0.646	1.000

*$p < .05$

Chapter 5: Discussion & General Conclusions

5.1 Key Findings

This thesis sought to determine the impact of IH, a theorized effect of gesP PA, on HBC polarization and the subsequent implications for placental angiogenesis. We discerned that IH may not be a primary mechanism behind HBC polarization; however, we did find that IH led to differences in the secretome of HBCs and subsequently impacted HUVEC tube formation.

In Chapter 3 of this thesis, we endeavoured to establish if IH evokes similar differences in HBC polarization, as seen in previous work by the Adamo Lab [74]. We hypothesized that when HBCs were exposed to IH, there would be a favouring of the $CD206^{+}$ phenotype. We did not observe any difference in the absolute or ratio values for the pro-inflammatory and anti-inflammatory phenotypes between the culture conditions. We did identify distinctions in the regulation of cytokines and chemokines secreted between the two groups. There were 25 similarly regulated cytokines from the control media, with 7 cytokines that were regulated in opposing directions between the two culture conditions. We measured a greater number of upregulated cytokines in the 5% condition (n = 15) compared to the IH condition (n = 2) while more cytokines were downregulated in the latter condition (n = 16) than the former (n = 4). The RPMI media contained IFN-γ, IL-6, and IL-4, which may have impacted the polarization of the HBCs.

The manuscript titled *Hofbauer Cells and Intermittent Hypoxia: Potential Regulators for Angiogenesis in the Placenta,* comprising Chapter 4, aimed to explore the independent and combined effects of HBC-conditioned media and IH on markers of angiogenesis. We hypothesized that the combined treatment group would have more significant HUVEC tube formation and cell migration compared to the controls. No differences were found between all conditions for the migration assay, showing that IH and HBCs independently and coordinately do not impact the motility of HUVEC. Interestingly, we found that the IH and IH/HBC conditions had lower total mesh area, significantly shorter segments and fewer master segments than the HBC condition. Additionally, there were shorter master segments in the IH groups compared to the HBC condition. Using a cytokine array, we classified the angiogenic secretions from HBCs.

We detected angiogenin, a pro-angiogenic cytokine previously not thought to be secreted from HBCs. In addition, we noted pro-angiogenic (PIGF and FGF-7), anti-angiogenic (MIF and NT-4) and pleiotropic factors (IL-6, IGFBP) in the HBC conditioned media. Overall, the study in Chapter 4 alludes to HBCs being master regulators of angiogenesis in the placenta and not just pro-angiogenic.

5.2 Hofbauer Cells & Placental Angiogenesis with Implications of Gestational Parent Physical Activity

The healthy development of the placenta is integral to a successful pregnancy as it provides the foundation for fetal life through its roles of nutrient and gas transport, hormone secretion and protection against infection. The benefits of gesP PA are well documented in the literature [1,89–91], but the mechanisms of action are not yet determined. The placenta likely mediates these benefits as the conduit between the gesP and the fetus. Previous work from the Adamo Lab found a significantly greater ratio between the anti- and pro-inflammatory HBCs in the placenta from physically active vs. inactive gesPs [74]. In the first study of this thesis, we hypothesized that the differential ratios in HBC polarization from Goudreau *et al.* [74] were elicited by IH, a theorized effect of gesP PA [17,27]. Contrary to our hypothesis, we did not observe a difference in the HBC polarization ratio after exposing the cells to IH. There were also no changes in the absolute number of HBC phenotypes or percent phagocytosis between the culture conditions.

The baseline polarization of the HBCs in culture may have influenced the response to IH. There was a more prominent presence of pro-inflammatory HBCs in both conditions compared to what is reported by the literature [68]. In the healthy population, HBCs are primarily of the M2a and M2c anti-inflammatory phenotypes, suggesting an overall role of tissue regulation and regeneration in the placenta [58,68]. In contrast, inflammatory states, such as GDM, are associated with an increased presence of the pro-inflammatory M2b HBCs [69]. Since the HBCs were isolated from healthy placentas, we would not anticipate a marked participation of the pro-inflammatory phenotype. This suggests that the cells did not maintain their innate polarization in culture. Therefore, we speculate that our null findings - IH exposure not inducing differences in HBC polarization - could be equated to a differential composition of phenotypes, which may have been resistant to the stimuli.

Differences in the innate polarization of HBCs could be due to the confounding factor of FBS in the RPMI-1640 media. Known stimulators of pro-inflammatory polarization such as IFN-γ, RANTES, IL-12, TNF, IP-10 and IL-6, were measured in the media [34,92]. IFN-γ is a potent pro-inflammatory stimulus that is well-classified in the literature [93]. Secreted from T helper 1 cells, cytotoxic T lymphocytes and group 1 innate lymphoid cells, IFN- γ binds to its receptors, inducing activation of the Janus kinase (JAK)-signal transducer which subsequently activates signal transducer and activator of transcription 1 (STAT1) pathways [93]. The activation of STAT1 influences transcriptional, chromatin and metabolic mechanisms, thereby polarizing the macrophage towards the pro-inflammatory phenotype [93]. IFN-γ mediated polarization increases the sensitivity to other pro-inflammatory stimuli and resists anti-inflammatory stimuli, which is thought to result in *super-activation* [93]. Although, the concentration, duration and balance with other cytokines will influence the pro-inflammatory repercussions of IFN-γ on macrophage polarization [34]. For example, IL-6 is often regarded as a robust pro-inflammatory cytokine, which may be more regulatory than researchers first expected, as IL-6 upregulates the IL-4 receptor α, leading to alternative activation of macrophages [39,94]. In the media, we also detected anti-inflammatory stimulating cytokines such as VEGF, TGFβ, M-CSF, IL-4, IL-5, and EGF. The presence of these cytokines in the media may have influenced HBC polarization through the external cytokine pathways instead of our intended stimulus of IH. Overall, the cytokine *milieu* of the media could lead to the adoption of a more pro-inflammatory phenotype that is not seen in the healthy placenta. FBS in culture media may be a confounding factor that researchers should consider when studying macrophage polarization. The integral need of FBS to maintain the cells in culture makes it challenging to eliminate this reagent, as starve media is not physiologically relevant to a healthy tissue environment, and cells cannot thrive in these conditions long term.

Moreover, the initial polarization of HBCs before culture may have been impacted by the delivery method, as the majority of isolated HBCs were from placentas delivered vaginally. Parturition is thought to be an inflammatory event in multiple reproductive tissues, including the placenta [95]. Swienboda *et al.* [68] only noted the pro-inflammatory HBC phenotype in term placenta, not in early- and mid-gestation. The incurred presence of M2b HBCs at term suggested

that this phenotype may be involved in regulating and initiating labour-induced inflammation [68]. The induction of the inflammatory phenotype during labour processes may pose a factor to consider when culturing these cells. Although, parturition likely has a minor effect since the placenta samples from Goudreau *et al.* [74] were delivered vaginally and did not observe a majority pro-inflammatory phenotype. To determine the extent of the pro-inflammatory impact of parturition on these cells, the literature should compare first-trimester and term HBCs to study their *in-vitro* regulation.

Consequently, our findings suggest that IH may not be the primary mechanism behind the results from Goudreau *et al.* [74]; however, variations in oxygen tension may have caused changes in the tissue microenvironment. The influence of the microenvironment vs. ontogeny is heavily debated in the literature [34]. While HBCs naturally adopt the anti-inflammatory phenotypes in a healthy placenta, we observed that IH decreased the proportion of anti-inflammatory to pro-inflammatory HBCs. These results allow us to speculate that changes in the tissue microenvironment have a greater impact on HBC polarization than ontogeny. Although the difference was not statistically significant, there was a large effect size, suggesting that changes in oxygen tension influence macrophage behaviour, regardless of their inherent polarization. These findings are consistent with transplant studies. For example, after exposure to radiation, chemotherapy or systemic infection, researchers have learned that tissue macrophage populations in other organs can be replaced with bone marrow-derived cells [34]. The transplanted macrophages acclimate, leading to the characteristic tissue-macrophage polarizations for that organ [34]. In the context of pregnancy pathology, the inflammatory state and high glucose microenvironment with GDM promote pro-inflammatory HBCs [69], further supporting the pivotal role of tissue microenvironment over ontogeny. As such, macrophage polarization may not be entirely dependent on the origin of the macrophage.

The coupling of flow cytometry with the markers CD206 and CD86 allowed us to quantify the anti- and pro-inflammatory phenotypes, respectively, but did not provide an in-depth analysis of the sub-phenotypes. By analyzing the secretions of the HBCs, we can speculate on the presence of the M2a, M2b and M2c proportions. IL-12 and IL-10 are often characterized as the main secretions of pro- or anti-inflammatory macrophages, respectively [34,36,58]. In the IH

condition, there was a much lower up-regulation of IL-10 than the 5% O_2 condition and detected a down-regulation of IL-12 in both conditions compared to the control. While all macrophage phenotypes secrete IL-10, anti-inflammatory macrophages are the main secretors of this cytokine [34]. For example, M2b macrophages secrete IL-10 with very low levels of IL-12 and express $CD86^+$ as a surface marker. Therefore, the pro-inflammatory macrophages we identified in this study are likely the M2b polarization, not the M1 phenotype, which is consistent with the macrophage composition of the placenta. Moreover, the levels of these cytokines suggest that the IH condition contained more pro-inflammatory HBCs, as confirmed by our flow cytometry data.

Many cytokines in the conditioned mediums are expected to affect immune regulation and tissue remodelling [96–103]. It is important to note that the large up-regulation of IL-8, found in the HBC-conditioned media, which has roles in immunity by protecting the fetus from invading bacteria, implantation, maintenance of pregnancy and placental development [104–108]. Interestingly, this chemokine is pro-inflammatory and is thus thought to come from pro-inflammatory macrophages [109]. Despite this cytokine being regarded as pro-inflammatory, IL-8 in the conditioned media was higher in the 5% O_2 condition, which had fewer $CD86^+$ HBCs. Therefore, IL-8 may not be exclusively secreted from pro-inflammatory HBCs, reiterating the fluidity of macrophage polarization. A prime example of this fluidity is IL-6, which was only up-regulated in the 5% O_2 condition. Often regarded for its pro-inflammatory nature, this cytokine can display pleiotropic characteristics, resulting in its ability to promote homeostasis of the amniotic cells, stimulate trophoblast invasion and migration, spiral artery remodelling, regulate placental hormone synthesis and parturition [104,110]. Interestingly, the balance between IL-8 and IL-6 in the placenta may have implications for the development of GDM, preeclampsia, pregnancy loss, embryo implantation and trophoblast invasion [104]. Since we did not identify any IL-6 in the IH condition, gesP PA may have ramifications on the IL-8/IL-6 balance and subsequently influence pathology risk.

We also discerned biologically relevant up-regulation of OPN, MCP-1 and macrophage inflammatory protein beta (MIP-1β) in the 5% O_2 condition compared to the IH condition. OPN is secreted from many cell types, including macrophages, and mediates inflammation and extracellular matrix proteins [111], a major role for M2c macrophages [112]. As such, this

secretion helps confirm that a portion of the identified anti-inflammatory HBCs in both conditions were of M2c phenotype, with potentially more in the 5% O_2 condition. MCP-1 is involved in trophoblast invasion, endothelial cell migration, immune system modulation and an angiogenic factor [96], reiterating the purpose of HBCs in placenta development through paracrine effects. Similarly, MIP-1β has functions in maintaining the immunosurveillance aspect of macrophages and may help mediate inflammatory responses [113]. Overall, the classified cytokines and chemokines in the conditioned media reaffirm the regulatory position of HBCs in placenta development and recapitulate that HBCs are a heterogeneous cell population.

Given the differences in the secretory profile of HBCs when exposed to IH, our subsequent study in Chapter 4 aimed to classify the downstream impact on placental angiogenesis. Previous studies have shown that HBC secretions of VEGF, FGF2, and Spry2 regulate the processes of angiogenesis [59,74,75], but most studies often focus on the pro-angiogenic capabilities of these cells [58]. Additionally, the IH effect of gesP PA is thought to be a pro-angiogenic stimulus that induces differences in the density of placenta vasculature [26,27]. Hypoxia stabilizes HIF proteins, leading to downstream upregulation of VEGF and the promotion of angiogenesis in other organs [80–82,114]. Interestingly, gesP PA has been associated with higher levels of pro-angiogenic factors VEGF and PIGF, along with their receptors in the placenta [79]. These prior investigations lend credence to the hypothesis that HBC secretions and IH may have a concomitant impact on angiogenesis. Independently, IH and HBCs are pro-angiogenic, but we do not know how these two regulators interact. In Chapter 4 of this thesis, we hypothesized that IH and HBCs would synergize, leading to more expansive HUVEC tube formation and faster cell migration.

While interpreting our findings on the effects of IH and HBCs on angiogenesis, we can speculate that these factors may have opposing effects on tube formation. At physiological normoxia, the HBC condition had more expansive and complex vasculature that did not differ significantly from baseline levels. Greater tube formation values in the HBC condition reinforce the literature's findings that these macrophages lead to more expansive vessel networks [59]. Markers of the complexity and vastness of tube networks were markedly lower in the IH and IH/HBC conditions than in the HBC conditions. The deviations from baseline tube formation

allow us to speculate that IH results in a temporary reduction in angiogenesis as a protective mechanism to prevent the formation of faulty or leaky vasculature [115,116] as the complexity and vastness of the vessels were lower. HBCs do not override this protective mechanism, as the IH/HBC condition had similar variables to the IH-alone condition. We speculate that HBCs conserve their pro-angiogenic role to protect the integrity of the vasculature being formed in the placenta when exposed to the IH effect of gesP PA. Overall, these results suggest HBC secretions differentially affect vascular formation, depending on the environment.

Our results strongly suggest that HBCs are global master regulators of angiogenesis of the placenta. Consistent with the literature, we identified many pro-angiogenic factors such as NAP-2, FGF-7, Eotaxin and PIGF in the HBC-conditioned media [117–121]. As seen in Aim 1, we detected the most up-regulation with IL-8 in the conditioned media. This cytokine promotes angiogenesis by enhancing endothelial cell proliferation, migration and survival, impacting tube formation [122,123]. The mechanism of action for IL-8 is through the activation of the ERK1/2 angiogenic pathway and stimulates the production of MMPs, which break down the extracellular matrix to promote endothelial migration and tube formation [123–126]. Also acting through the ERK1/2 pathways and shown to be upregulated compared to the control in the conditioned media was OPN, a pro-angiogenic factor that has roles in the placentation and decidualization [127–129], supporting the early regulatory roles of HBCs. We can speculate that HBC secretions of MCP-1 further enhance the angiogenic processes in the placenta. It is thought that MCP-1 binds to the CCR2 receptor on endothelial cells, upregulating HIF-1 and VEGF gene expression, which leads to new vessel formation [121,130,131].

A compelling novelty of this thesis is the discovery that angiogenin is secreted from HBCs. Previous classification of angiogenin secretion from the placenta revealed that this factor was only secreted by trophoblasts, mesenchymal stem cells and decidual macrophages [132]. When using $CD45^{+}$ as a marker for HBCs, Pavlolv *et al.* [132] did not identify secretions of this pro-angiogenic factor from the placenta resident macrophage. Angiogenin promotes angiogenesis by binding to endothelial cells and then internalized to the nucleus, regulating many pro-angiogenic genes, such as MMPs [101]. Interestingly, angiogenin levels in the placenta have been shown to be regulated by gesP PA [133]. Specifically, gesP PA was associated with a 10-fold increase in

this protein expression, along with increases in placental angiogenesis and no associations with markers of endoplasmic reticulum or oxidative stress [133]. The previous study did not determine the origin of angiogenin secretion, so future studies should determine the direct role of angiogenin from HBCs on the placenta vasculature and the regulatory effect of gesP PA. When exposed to IH, the response of angiogenin did not differ from control, suggested that the previously observed results of increased angiogenin in the placenta with gesP PA was not due to changes in oxygen tension. Once again, reaffirming the common theme that IH may not be the main driver for differences in the biology of the placenta associated with gesP PA. Although, our findings support the common theme in the literature that HBCs are potent pro-angiogenic stimulators through paracrine effects.

The literature often emphasizes the pro-angiogenic role of HBCs and neglects their anti-angiogenic role. Pro-angiogenic processes are frequently regarded as beneficial, ignoring the fact that too much angiogenesis is linked to endometriosis, rheumatoid arthritis and age-related macular degeneration [134–136]. Therefore, it is integral to have modulation of this complex process to maintain homeostatic conditions. Regulation in the placenta may come from the known anti-angiogenic IL-10 and TIMP-1 [137–141], which were up-regulated in the HBC-conditioned media. There were also numerous pleiotropic factors in the conditioned media that can exhibit a regulatory effect on angiogenesis. Noteworthy factors include the IGFBP regulator family, with IGFBP-3 and IGFBP-4 that we found to be up-regulated in the IH condition, while IGFBP-1 and IGFBP-2 were up-regulated in the 5% O_2 condition. Although the mechanisms are not yet known, IGFBP-2 promotes angiogenesis, while IGFBP-1, -3 and -4 are pleiotropic, displaying pro- and anti-angiogenic properties [142]. Therefore, we can posit that a homeostatic angiogenic equilibrium is achieved by HBCs, ensuring a steady-state production of new vasculature through the secretion of pro- and anti-angiogenic factors, which may be altered depending on oxygen tension.

The balance between angiogenic factors likely led to no differences in HUVEC migration in the HBC and IH/HBC conditions. It seems that the path of vessel growth was not affected by HBCs. MIF in the HBC-conditioned media has previously been observed to have an inhibiting effect on the migration of fetal endothelial cells [59]. The secretions of pro-migratory (e.g. IL-8, OPN,

Angiogenin and FGF) and anti-migratory (TIMP-3) factors in the conditioned media further support our findings that HBCs are master regulators of angiogenesis in the placenta [101,118,122,123,143–145]. Overall, the different secretions of angiogenic factors between the culture conditions confirm the findings that IH impacts HBC behaviour and may subsequently influence their downstream roles.

While the results of this thesis have provided significant insight into the roles of HBCs in the placenta, it is crucial to recognize the limitations of these projects. The sample size of this thesis presents a notable limitation as, retrospectively, we determined that the studies were underpowered. Increasing the sample size would improve the power, thus enhancing the generalizability, reliability and validity of these studies. Additionally, a continual shortfall of *in-vitro* models is the limited external validity. HBC polarization and angiogenesis are complex processes that involve numerous regulators and stimuli [28,30,82]. We recognize that our examination was only a subset of the potential regulatory pathways in these processes.

We have attempted to increase the external validity of these projects by using physiologically relevant oxygen tensions. Historically, researchers cultured cells with atmospheric oxygen tension (21% O_2; 159mmHg), which does not reflect the *in-vivo* oxygen tension of the placental environment. Within the non-pregnant human body, the lowest oxygen tension is found in the end capillaries and veins with a partial pressure of oxygen (pO_2) of 40mmHg [87]. Oxygen tension in the placenta varies depending on the gestational stage, beginning with a low pO_2 of 20mmHg in the intervillous spaces of the placenta [87]. When the maternal spiral artery blood flow is established during the 12^{th}- 15^{th} week of pregnancy, placental oxygenation peaks at 60mmHg [87]. After that, oxygen tension decreases to a pO_2 of 34mmHg and 30mmHg in the 24-36 week of pregnancy and 37 weeks until labour, respectively [87].

Consequently, culturing primary cell lines from the placenta at atmospheric oxygen tension is not physiologically relevant to this organ. The inappropriate use of atmospheric oxygen tensions has complicated the literature regarding the effect of hypoxia and IH in the placenta, as the models are not reflective of the true environment and have large differences in oxygen tensions; thus, these results are not as generalizable. Justifiably, changing oxygen tension from atmospheric

partial pressures to hypoxia (< 1% O_2) would trigger detrimental effects on cell behaviour and lead to the current incorrect conclusions regarding the repercussions on the developing fetus, as this is a 20% difference. Physiologically, the shifts in oxygen tension during gesP PA are not this large since the literature shows that the fetus does not experience hypoxia with gesP PA [17,23]. Moreover, the timing, duration and intensity of hypoxia differentially affect placental cell behaviours; therefore, hypoxia studies require future standardization to allow for comparable results [80].

5.3 Future Directions

The studies in this thesis have provided ample material for future projects. Despite the pioneering work in the field of exercise physiology in pregnancy by Clapp [7], who theorized the IH effect of gesP PA, these events have not yet been quantified. Therefore, we do not have a clear picture of the biological impact of gesP PA on the placenta. Furthermore, we are not able to accurately mimic the IH effect in *in-vitro* models to expand our mechanistic understanding of the benefits associated with gesP PA. Given the overall trend of cytokine down-regulation observed with IH, future research should aim to categorize these cytokines in placentas from physically active and inactive gesPs. This approach would help determine whether similar patterns of regulation are present and enable further evaluation of the theorized IH effect.

Since HBCs are located near the trophoblast cell populations, they probably exhibit a paracrine or cell-to-cell effect on trophoblast differentiation. Previous research has examined the impact of HBC secretions on cytotrophoblast differentiation into extra-villous trophoblasts. There is minimal literature characterizing the outcomes of syncytiotrophoblast development, an integral cell population that carries out many roles. Similarly, culturing individual cell lines from the placenta does not genuinely represent the *in-vivo* environment. While solo culture models are required to control confounding variables and pinpoint cellular mechanisms, HBC polarization and their downstream roles are likely impacted by cell-to-cell communications and paracrine effects in a symbiotic nature between other structures in the placenta. In culture, HBCs do not proliferate and can only survive three to five days, which provides significant time restraints on experiments. Utilizing cotyledon explant culture could allow researchers to classify these mechanisms and preserve the HBCs long enough to expose the cells to more chronic IH.

Future projects should continue to explore the mechanisms behind the differences in HBC polarization with gesP PA by Goudreau *et al.* [74]. While we theorized these distinctions in HBC polarization were due to IH, other mechanisms like the exerkine response to PA should be examined. Previous work from the Adamo lab has characterized the exerkine response during PA during pregnancy [46,47] but has not directly tested the relationship on HBC polarization. Other regulators may be the shear stress forces exerted after IH or a combination of all three. Outside of pregnancy, the mechanisms involved in PA rarely act in solitude; therefore, we should look at classifying the whole-body responses to gesP PA on HBCs. Due to these studies being slightly underpowered and the biologically relevant differences noted in cytokines and chemokines with changes in oxygen tension, we cannot rule out the polarization stimulus of IH on HBCs. Therefore, adequately powered studies are required to confirm the involvement of oxygen tension on HBC polarization and behaviour. Moreover, IH may still elicit differences in other aspects of placental development as the mechanisms of gesP PA are likely multifaceted and cell-specific.

Additionally, researchers should critically evaluate how we address macrophage polarization nomenclature. For the ease of studying macrophage phenotypes, the nomenclature is oversimplified to the M1 and M2 phenotypes. Currently, researchers are highlighting the need for revising these oversimplifications of polarization that have led to a compounding effect of mis-guided information on macrophage polarization. In reality, macrophage polarization is a highly complex set of processes that the harsh borders of M1 or M2 classifications cannot bind. As we have demonstrated in the first study of this thesis, macrophage polarization is a spectrum with an amalgamation of both pro- and anti-inflammatory characteristics. A prime example of this confluence of qualities is the M2b macrophage, which displays many characteristics and assumes many roles. Macrophage polarization research should aim to consolidate the markers of polarization and nomenclature, as suggested by Murray *et al.* [31] and Martinez and Gordon [112]. Consistent methods and terms will facilitate consistency in the literature and allow for more comparisons between studies.

Lastly, future projects should look at the mechanistic role of the cytokines in placenta development. These cytokines could include angiogenin, IL-8, MCP-1, OPN, and IGFBPs. These cytokines have previously been thought to have roles in trophoblast differentiation and survival, angiogenesis and macrophage polarization, but the exact mechanisms have not been classified. Classifying any differences in these cytokines from the placentas of physically inactive and active gesPs could provide preliminary evidence of their roles in mediating the differences observed in other studies from the Adamo Lab.

6.4 Significance

As shown in the literature, this thesis supports the thought that HBCs are multifaceted innate immunity cells with a regulatory role in placenta development. These are some of the first projects that have looked at the downstream roles of HBCs within the healthy placenta and examined how gesP PA may modify the behaviour of this transient organ. The novelty of this project is enhanced by the use of primary cell lines that can be traced back to donor characteristics. We have determined that IH, an effect of gesP PA, may not be the primary regulator in HBC polarization, but it does regulate HBC behaviour. Therefore, this project has opened new avenues of inquiry to classify the mechanism(s) responsible for the results found by Goudreau *et al.* [74]. To our knowledge, this work is the first to have identified that HBCs secrete angiogenin. Our elucidation of the regulatory role of HBCs in angiogenesis will help researchers have a more comprehensive picture of the factors involved in the healthy placenta and the development of pregnancy pathologies.

In the context of exercise physiology, we know a great deal about how other tissues and organs respond to PA, yet little is currently known about the placenta- a critical organ for the appropriate development of the fetus. Knowing that PA is beneficial during pregnancy, much remains unanswered regarding its impact on the placenta. These projects aimed to identify how cells in the placenta respond to IH, a theorized mechanism of action for gesP PA. Based on the current literature, we believe that IH is a sensible proxy for the impact of gesP PA on the placenta. However, our results suggest that this theorized effect is not the preliminary independent mechanism of gesP PA for influencing differences in HBC polarization and placental angiogenesis. Future research should investigate additional mechanisms that may act

synergistically with IH. Studies focusing on the influence of gesP PA on the placenta are necessary to narrow the gender gap in research and to provide evidence supporting informed policies and practices across pregnancy.

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Appendices

Appendix A: Cytotrophoblast Methods

A1. Colour Fusion Assay

Timeline:

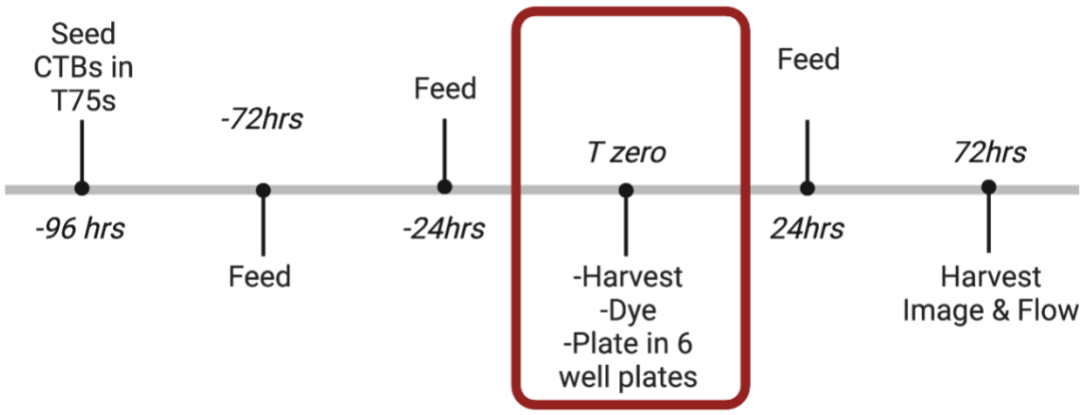

Figure A1. Timeline for colour fusion assay.

Reconstitution of Dyes:

CellTracker Green CMFDA Dye (*ThermoFisher; C7025*)

1. Bring to room temp
2. Briefly centrifuge/vortex vial to get all particles to the bottom of the vial
3. Add 215.1 uL of DMSO to make a 10mM stock solution.
4. Aliquot into 10uL aliquots.
5. Stock is 1000x concentration

CellTracker Orange CMTMR Dye (*ThermoFisher; C2927)*

1. Bring to room temp
2. Briefly centrifuge/vortex to get all the particles to the bottom of the vial
3. Add 361uL DMSO to make a 10mM stock solution
4. Aliquot into 10uL aliquots
5. Stock is 1000x concentration

Protocol:

0. Grow CTBs to confluency in collagen IV coated T75(s)

1. Trypsinize cells from flasks and count

2. Reconstitute cell pellet at 1 x 10^6 cells/1mL in Adv DMEM/F12

3. In y-irradicated FBS coated 15mL conicals, add the following volumes

    3.a CMFDA dyed cells (for one well):

        0.5mL resuspended cells + 1.85mL Basal Media + 3uL CMFDA dye

    3.b CMTMR dyed cells (for one well):

        0.5 mL resuspended cells + 1.85mL Basal Media + 3uL CMTMR dye

**Note: If you have more than one well, multiply each value by the number of wells.*

Ex: if you have 6 wells total:

*0.5mL resuspended cells (*6) + 1.85mL Basal Media (*6) + 3uL CMTMR dye*

3mL resuspended cells + 11.1mL Basal Media + 18uL CMTMR dye

3mL resuspended cells + 11.1 mL Basal Media + 18uL CMFDA Dye

4. Stain cells at 37C with rocking for 30 mins

5. Pellet cells at 40xg for 5 mins

6. Resuspend in 15mL of DPBS and spin for a total of three washes

7. Resuspend cells in 1mL/well of basal media

8. Add 1mL of cell suspension to each well of a collagen IV coated 6 well plate per colour (1mL CMTMR dyed cells + 1mL CMFDA dyed cells per well)

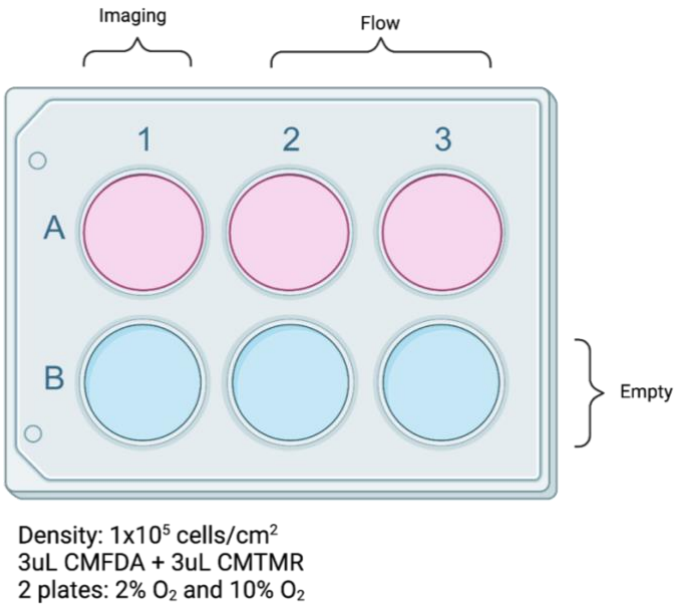

Figure A2. 6-well Plate Map.

9. Add one plate to a 2% O_2 incubator and the other to a 10% O_2 incubator

10. Feed plates 24hrs after seeding with fresh warmed basal media

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Fixing, Imaging and Flow

1. After 72hrs post-seeding in 6-well plates, remove from incubator and aspirate spent media

2. Gently wash cells 3x with RT DPBS

    2.a With 1mL DPBS, put down the side of the place to not disrupt the cells

3. For cells that will be imaged, fix cells in 4% formaldehyde for 15-20 minutes with gentle rocking

    3.b Remove spent formaldehyde and dispose in the respective waste receptacle

    3.c Wash cells gently 3x with RT DPBS

    3.d Stain nuclei with 1ug/mL Hoescht's stain in DPBS for 10 mins at RT

    3.e Remove spent stain

    3.f Wash cells gently 3x with RT DPBS

    3.g Add 2mLs of DPBS to each well for imaging. If no wells are being used for flow, seal plate and image. If you are doing flow, go to step 4.

4. For flow cytometry, remove spent media

    4.b Wash cells gently 3x 1mL RT DPBS

    4.c Add 1mL TrypleE to each well and immediate aspirate spent TrypleE

4.d Add 1mL TrypleE and incubate at 37C for 10 mins

4.e Observe detachment under microscope

4.f Aggressively wash the bottom of the well to detach all cells

4.g Put 2mL DPBS to a labeled 15mL conical

4.h Add the cell suspension to the conical with DPBS to neutralize

4.i Put 1mL of TrypleE to each well again, observe under the microscope and decide if incubation is required to get the rest of the cells. If the cells have lifted, wash the bottom of the well and add to the rest of the cell suspension

4.j Spin at 400xg for 5 mins

4.k Remove supernatant

4.l Resuspend cell pellet with 500uL running buffer

4.m Run Flow

A2. Immunofluorescence

Immunofluorescent staining of cytotrophoblasts was performed to quantify the extent of syncytialization. After being culture for 4 days in normoxic, intermittent hypoxic and/or exposed to HBC conditioned media, spent media was removed from the ibidi well and the cells were washed with DPBS. Fixation of the cells was performed using 4% Paraformaldehyde at RT for 10 minutes. The fixed cells were then washed with DPBS and permeabilized with 0.1% Triton X in DPBS. Samples were blocked with 5% BSA for 1 hour at RT to prevent non-specific binding and washed with DPBS. Probing with primary antibodies for cytokeratin, e-cadherin and syncytin 2, diluted in 1% BSA in DPBS occurred as indicated in Table A1, overnight at 4°C. The following day, samples were washed with DPBS and incubated in the dark for 1hr with the respective secondary antibodies (see Table A1) and washed again. The e-cadherin and syncytin 2 sampels were mounted with ProLong Glass Antifade mountaint with NucBlue (ThermoFisher) to visualize the cell nuclei. To differentiate between cytotrophoblast and syncytiotrophoblasts, cytokeratin-stained cells were double stained for βhCG, following the same protocol with the respective antibody. The application of primary antibodies was omitted for the negative controls. Slides were imaged using an inverted fluorescence microscope (AxioObserver Z1, Toronto, Canada).

Table A1.

Markers of CTB Differentiation with Antibody Concentrations

Antibody	Company (cat #)	Marker	Primary	Secondary (species)
Anti-wide spectrum Cytokeratin Antibody	Abcam (ab9377)	Expressed in all trophoblasts	1: 500	1: 1000 (Alexa Fluor 647 goat anti rabbit)
Anti-E Cadherin antibody	Abcam (ab40772)	Intracellular junction marker	1:100	1: 300 (Alexa Fluor 488 goat anti rabbit)
Syncytin 2 Polyclonal Antibody	ThermoFisher (# BS-15466R)	Trophoblast cell fusion	*N/A	*N/A
Anti-hCG Antibody	Abcam (ab9582)	Hormone secreted by STB	*N/A	*N/A

Note: * N/A indicative of antibody concentrations are not yet optimized.

A3. Immunoblots

Fourty µg of total cell lysate were loaded on Mini-PROTEAN® 4-15 % TGX gel (Bio-Rad) and resolved at 120V for 1 hour. The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) at 100v for 90 minutes. The membrane was then blocked with 5% powdered milk (MILK) in tris-buffered saline solution with 0.05% tween-20 (TBS-T) for 1 hour at room temperature (RT). Membranes were incubated overnight at 4°C with anti-β-hCG antibody diluted in 5% MILK at the respective concentration summarized in Table 1. The following day, blots were washed with TBS-T and incubated with diluted (see Table 1) horse radish-peroxidase conjugated secondary antibodies (Goat-anti-rabbit; Bio-Rad; # 1706516) for 1 hour at RT. The blots were developed using Clarity ECL Western Substrate (Bio-Rad) and imaged on the ChemiDoc™ XRS+ Imaging System (Bio-Rad). After the blots were visualized,

the blots were washed with TBST and stripped with stripping buffer (2mM NaOH and 1:1000 Betamercaptoethanol) to be probed with the next antibody. The blots were washed with TBS-T and blocked with 5% MILK in TBS-T for 1 hour at RT. The steps for probing the blot with primary antibody, the respective secondary antibody (See Table A2), visualizing along with stripping again were repeated thrice. Once the blot was probed for all the markers of CTB differentiation, the membranes were permanently stained with 1% Amido Black for total protein lane quantification. Band expression and total protein expression were analyzed by densitometry (ImageJ). Cell lysate protein expression for β-hCG, syncytin-2 (Sync-2), E-cadherin (E-cad) and cytokeratin were standardized to pooled cell lysate samples.

Table A2.

Markers of CTB Differentiation with Antibody Concentrations

Antibody	Company (cat #)	Marker	Primary	Secondary (species)
Anti-wide spectrum Cytokeratin Antibody	Abcam (ab9377)	Expressed in all trophoblasts	1: 1000	1: 7000 (goat anti rabbit)
Anti-E Cadherin antibody	Abcam (ab40772)	Intracellular junction marker	1:1000	1: 7000 (goat anti rabbit)
Syncytin 2 Polyclonal Antibody	ThermoFisher (# BS-15466R)	Trophoblast cell fusion	1:500	1:5000 (goat anti rabbit)
Anti-hCG Antibody	Abcam (ab53067)	Hormone secreted by STB	1:500	1:5000 (goat anti rabbit)

Appendix B: Cytokine Array Map

	A	B	C	D	E	F	G	H	I	J	K
1	pos	pos	pos	pos	neg	neg	ENA-78	GCSF	GM-CSF	GRO	GRO-a
2	I-309	IL-1a	IL-1B	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
3	IL-12	IL-13	IL-15	IFN-y	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1b
4	MIP-1o	RANTES	SCF	SDF-1	TARC	TGF-B1	TNF-a	TNF-B	EGF	IGF-1	Angiogenin
5	OncostatinM	Thrombopoietin	VEGF	PDGF-BB	Leptin	BDNF	BLC	Ck B 8-1	Eotaxin	Eotaxin-2	Eotaxin-3
6	FGF-4	FGF-6	FGF-7	FGF-9	Flt-3 Ligand	Fracktalkine	GCP-2	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3a	NAP-2	NT-3
8	NT-4	Osteopontin	Osteoprotegerin	PARC	PIGF	TGF-B2	TGF-B3	TIMP-1	TIMP-3	Pos	Pos

Figure A3. Cytokine Array Map.

Appendix C: Academic Achievements

C1. Selected Publications

1. Adamo, K. B., Goudreau, A. D., **Corson, A.,** MacDonald, M. L., O'Rourke, N., & Tzaneva, V. (2024). Physically active pregnancies – insights from the placenta. *Physiological Reports*.

2. **Corson, A. E**., MacDonald, M., Tzaneva, V., Edwards, C. M., & Adamo, K. B. (2024). Breaking Boundaries: A Chronology with Future Directions of Women in Exercise Physiology Research, Centred on Pregnancy. *Advanced Exercise and Health Science*. https://doi.org/10.1016/j.aehs.2024.04.001

C2. Conference Presentations

1. **Corson, A.E**., Tzaneva, V., & Adamo, K.B. (2024). *Physical Activity and Hofbauer Cells: Enhancers of Angiogenesis in the Placenta*. [Poster Presentation]. International Biochemistry of Exercise Conference 2024. Limerick, Ireland.

2. **Corson, A.E**., V. Tzaneva & Adam, K.B. (2023). *A proposal for determining the independent or additive effects of Hofbauer cells and parental physical activity in the placenta.* [Poster Presentation]. 2023 Scientific Meeting of the Canadian DOHaD Society. Montebello, QC.

C3. Awards

2023 Ontario Graduate Scholarship – University of Ottawa, $15000
2022 Michael Smith Foreign Study Supplement – Canadian Institute of Health Research, $6000
2022 Amnion Seed Grant – Amnion Foundation, $5000 USD
2022 CGS-M – Canadian Institute of Health Research, $17500