CHARACTERIZING THE HOFBAUER CELL RESPONSE TO PARENTAL PHYSICAL ACTIVITY DURING PREGNANCY

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PREFACE TO THIS THESIS

When this thesis was proposed, an original aim was to compare the polarization response of Hofbauer cells to environments mimicking physical activity. The experimental plan encompassed the use of primary cultures exposed to hypoxic conditions and activity-induced myokines. Due to persistent mycoplasma contamination (January 2022 – May 2022), reagent shortages amid the COVID-19 pandemic, and restrictions in scheduling shared equipment (May 2022 – August 2022), these experiments could not be completed. In preparation for these experiments, I obtained a seed grant from the Amnion Foundation (North Carolina, USA) providing Hofbauer cells and reagents and optimized a flow cytometry protocol to examine the proportions of differentially polarized cells. In addition, a protocol was optimized to mitigate mycoplasma contamination. My supervisor Dr. Kristi Adamo and committee members Drs. Michael de Lisio and Katey Rayner supported the modifications to my thesis in the face of these challenges.

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Last, but certainly not least, to the heroes who participated in the PLACENTA study. You shared with us your time and excitement during a monumental period in your life to support future parents and children everywhere. Science and healthcare as we know it would without magnanimous spirits such as yours. From the bottom of my heart – thank you.

ABSTRACT

Background: Pregnant individuals who participate in physical activity throughout gestation have been shown to experience a wide spectrum of health benefits, along with the fetus. In non-pregnant populations, PA influences the polarization state of tissue resident macrophages, resulting in increased regulatory and decreased inflammatory profiles. The effects of PA on placenta-resident macrophages, or Hofbauer cells (HBCs), remains unknown. My thesis aimed to explore this novel area.

Methods: The first objective of my thesis was to identify any associations between gestational PA and HBC polarization. PA was objectively measured in both mid (24-28 weeks) and late (34-38 weeks) pregnancy using accelerometry. Immunofluorescent localization of the pan-macrophage marker CD68 and the anti-inflammatory macrophage marker CD206 was used to assess polarization states. Protein and gene expression of CD68 and CD206 were assessed using Western blot and qPCR, respectively. The second objective was to explore the relationships between gestational PA, HBC polarization, and angiogenic factors in the placenta. Western blot measured the relative protein expression of FGF2 and SPRY2, and the localization of FGF2, SPRY2, and VEGF within HBCs was explored using immunofluorescent colocalization in term placenta tissue and primary HBC cultures.

Results: While there were no differences in the absolute numbers of total or $CD206^+$ HBCs, the proportion of $CD206^+$ HBCs was elevated in active individuals. There were no significant differences in the gene expression of CD68 or CD206, nor in the gene expression of CD206; however, CD206 protein expression was observed to be lower in active participants. Both $CD206^+$ and $CD206^-$ HBCs expressed VEGF. Active individuals had significantly higher low molecular

weight-FGF2. There were no differences in the protein expression of SPRY2, total FGF2, or high molecular weight FGF2 based on PA. HBCs both *in vitro* and *in vivo* of all polarizations expressed VEGF, SPRY2, and FGF2, and were observed to create intracellular junctions and multi-nucleated giant cells.

Conclusions: In conclusion, PA was associated with a higher proportion of $CD206^{+}$ HBCs and reduced levels of CD206 protein. In combination with the lack of significant difference in CD206 mRNA based on PA levels, this suggests a potential effect mediated by PA on the transcriptional regulation of CD206. HBCs were seen to express SPRY2, VEGF, and FGF2, identifying them as potential players in angiogenesis regulation in the placenta. The elevated levels of low molecular weight FGF2 in active individuals suggests the PA may play a role in the modulation of placental angiogenesis. Future research should continue to explore the relationships between PA, HBC polarization, and angiogenesis.

RÉSUMÉ

Contexte : Il a été démontré que les femmes enceintes qui pratiquent une activité physique tout au long de la gestation bénéficient d'un large éventail de bienfaits pour leur santé et celle de leur fœtus. Dans les populations non enceintes, l'AP influence l'état de polarisation des macrophages résidant dans les tissus, ce qui se traduit par une augmentation des profils régulateurs et une diminution des profils inflammatoires. Les effets de l'AP sur les macrophages résidant dans le placenta, ou cellules de Hofbauer (HBC), restent inconnus. Ma thèse visait à explorer ce nouveau domaine.

Méthodes : Le premier objectif de ma thèse était d'identifier toute association entre l'AP gestationnel et la polarisation des cellules de Hofbauer (n = 21). L'AP a été mesurée objectivement au milieu (24-28 semaines) et à la fin (34-38 semaines) de la grossesse à l'aide de l'accélérométrie. La localisation par immunofluorescence du marqueur pan-macrophagique CD68 et du marqueur macrophagique anti-inflammatoire CD206 a été utilisée pour évaluer les états de polarisation. L'expression de la protéine et du gène CD68 et CD206 a été évaluée par Western blot et qPCR, respectivement. Le deuxième objectif était d'explorer les relations entre l'âge gestationnel, la polarisation HBC et les facteurs angiogéniques dans le placenta. Le Western blot a mesuré l'expression protéique relative du FGF2 et du SPRY2, et la localisation du FGF2, du SPRY2 et du VEGF dans les CBH a été étudiée par colocalisation immunofluorescente dans le tissu du placenta à terme et dans les cultures primaires de CBH.

Résultats : Bien qu'il n'y ait pas de différences dans les nombres absolus de CBH totaux ou CD206+, la proportion de CBH CD206+ était élevée chez les personnes actives. Il n'y avait pas de

différences significatives dans l'expression génétique de CD68 ou CD206, ni dans l'expression génétique de CD206 ; cependant, l'expression de la protéine CD206 était plus faible chez les participants actifs. Les CBH CD206+ et CD206- exprimaient tous deux le VEGF. Les individus actifs avaient un taux significativement plus élevé de FGF2 de faible poids moléculaire. Il n'y avait pas de différences dans l'expression protéique de SPRY2, du FGF2 total ou du FGF2 de haut poids moléculaire en fonction de l'âge. Les CBH in vitro et in vivo de toutes les polarisations ont exprimé le VEGF, le SPRY2 et le FGF2, et on a observé qu'ils créaient des jonctions intracellulaires et des cellules géantes multinucléées.

Conclusions : En conclusion, l'AP a été associé à une proportion plus élevée de CBH CD206+ et à des niveaux réduits de protéine CD206. En combinaison avec l'absence de différence significative dans l'ARNm CD206 en fonction des niveaux d'AP, cela suggère un effet potentiel médié par l'AP sur la régulation transcriptionnelle de CD206. On a constaté que les CBH exprimaient SPRY2, VEGF et FGF2, ce qui les identifie comme des acteurs potentiels de la régulation de l'angiogenèse dans le placenta. Les niveaux élevés de FGF2 de faible poids moléculaire chez les personnes actives suggèrent que l'AP pourrait jouer un rôle dans la modulation de l'angiogenèse placentaire. Les recherches futures devraient continuer à explorer les relations entre polarisation HBC et l'angiogenèse.

ABBREVIATIONS

Canadian Society for Exercise Physiology (CSEP)

Chemokine Ligand 2 (CCL2)

Developmental Origins of Health and Disease (DOHaD)

Enzyme-linked immunosorbent assays (ELISA)

Fibroblast growth factor 2 (FGF2)

Fluorescence-activated cell sorting (FACS)

Formalin-fixed, paraffin-embedded (FFPE)

Fraction of tissue coverage (FTC)

Hofbauer cells (HBCs)

Immunofluorescence (IF)

Macrophage fusion receptor (MFR)

Moderate PA (MPA)

Moderate physical activity (MPA)

Moderate to vigorous physical activity (MVPA)

Oxidized low-density lipoprotein (OxLDL)

PhysicaL Activity and diEtary implicatioNs Throughout pregnAncy study (PLACENTA)

Polyvinylidene difluoride (PVDF)

Research Ethics Board (REB)

Room temperature (RT)

Society of Obstetricians and Gynecologists (SOGC)

Sprouty 2 protein (SPRY2)

Tissue resident macrophages (TRM)

Tmour associated macrophages (TAMs)

Tris-buffered saline (TBS)

Appropriate for gestational age (AGA)

Body mass index (BMI)

Brown adipose tissue (BAT)

Fibroblast growth factor 2 (FGF2)

Gestational diabetes mellitus (GDM)

Gestational hypertension (GH)

Gestational weight gain (GWG)

High molecular weight fibroblast growth factor (HMW-FGF2)

Immediate adaptive responses (IARs)

Large-for-gestational-age (LGA)

Low molecular weight fibroblast growth factor (LMW-FGF2)

Mitogen-activated protein kinase (MAPK)

Moderate-to-vigorous PA (MVPA)

Multinucleated giant cells (MGC)

Natural killer (NK

Physical activity (PA)

Pre-eclampsia (PE)

Predictive adaptive responses (PARs)

Receptor tyrosine kinase (RTK)

Resident cardiac macrophages (RCM)

Soluble CD206 (sCD206)

Sprouty 2 (SPRY2)

Subcutaneous white adipose tissue (scWAT)

Total fibroblast growth factor 2 (T-FGF2)

Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF)

CHAPTER 1: THESIS OVERVIEW

1.1 INTRODUCTION AND LITERATURE OVERVIEW

1.1.1 DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE

The association between maternal behaviours throughout pregnancy and the short- and long-term health of the developing fetus has been well documented in the literature. Originating as the Barker Hypothesis in the 1980s, the theory was renamed as the Fetal Origin of Adult Diseases in the 1990s, and has since evolved to the current model, entitled the Developmental Origins of Health and Disease (DOHaD) (1,2). The DOHaD framework posits that various exposures *in utero* may significantly affect both the short- and long-term health of the developing fetus through adaptive responses (1,3). The alteration of developmental trajectory can occur through immediate adaptive responses (IARs) when there is an imminent threat to the fetus' survival, or through predictive adaptive responses (PARs) when the acquisition of an alternate phenotype that offers future improved fitness (3).

As the affected tissues or structure are in critical stages of development *in utero*, there is a strong possibility that they will be permanently altered by the adaptive responses (4). How these adaptations affect the fitness of the offspring in environments throughout the lifespan can have significant health effects. When the acquired phenotype is not advantageous or "mismatched," it can pose serious health effects throughout the lifespan (3).

Much of the evidence supporting the DOHaD model has been obtained through experimental animal research or observational human trials and has focused largely on nutritional alterations and exposure to teratogenic agents (3). Both maternal over- and under-nutrition during pregnancy can result in metabolic, immunological, reproductive, and mental

health disorders (2). While the literature contains extensive documentation of how IARs and PARs can reduce offspring fitness, there is a growing emphasis on how other exposures, particularly maternal behaviours, can result in improved fitness and overall health throughout their life.

1.1.2 PARENTAL PHYSICAL ACTIVITY

In the clinical sciences, physical activity (PA), defined within this thesis as any bodily movement produced by skeletal muscles that results in energy expenditure (5), has been shown to be a substantial component of a healthy lifestyle. The benefits of habitual PA include the primary and secondary prevention of cardiovascular disease, cancer, obesity, and osteoporosis, while also contributing to the improvement of mental health and cardiorespiratory fitness (6). Despite the demonstrated benefits in non-pregnant populations, there has persisted a misconception that PA during pregnancy should be avoided to mitigate the risk of low birth weight, preterm delivery, and miscarriage (7–9). Despite extensive research, these concerns have remained largely unsupported. In fact, a thorough investigation in recent years has shown that regular PA throughout gestation is not only safe but associated with numerous health benefits for both the mother and fetus (10–12).

1.1.2.1 GUIDELINES FOR PHYSICAL ACTIVITY THROUGHOUT GESTATION

In order to create recommendations for pregnant individuals and obstetric care providers, the Society of Obstetricians and Gynecologists (SOGC) and the Canadian Society for Exercise Physiology (CSEP) collaborated in evaluating gestation PA and its associated outcomes in maternal, fetal, and neonatal health. The resulting *2019 Canadian guideline for PA throughout pregnancy* provides the evidence based recommendation that pregnant persons without contraindications should undertake a minimum of 150 minutes of moderate PA (MPA) per week,

accumulated over at least three days (13). In the case of contraindications or care provider instructions to limit PA, these recommendations should be modified on a per case basis.

1.1.2.2 GESTATIONAL PHYSICAL ACTIVITY AND FETAL HEALTH OUTCOMES

Weight gain during pregnancy is not solely comprised of the mass of the developing fetus and placenta; a body must change to support a pregnancy, including increases in amniotic fluid, blood volume, breast and uterine tissue, and increases in nutrient storage (14). Specific weight gain ranges are recommended for individuals based on their pre-pregnancy body mass index (BMI) (15). Gaining outside of these recommendations increases the risk of several maternal and fetal morbidities and pregnancy-related disorders (14). Excessive gestational weight gain (GWG) early in pregnancy can contribute to the risk of developing gestational diabetes mellitus (GDM), gestational hypertension (GH), and pre-eclampsia (PE) (16,17). Individuals who gain excessively during pregnancy are more likely to deliver neonates that are large-for-gestational-age (LGA) (18). There are multiple morbidities associated with LGA newborns, including lower APGAR scores, respiratory disorders, hypoglycemia, and hyperbilirubinemia (i.e., an excessive production of bilirubin as a byproduct of red blood cell breakdown) (19,20). Children from pregnancies with excessive GWG are also more likely to have increased adiposity and BMI throughout the lifespan, thereby perpetuating the cycle of obesity (21). By supporting gestational weight management, PA mitigates the risk of developing the previously described conditions.

1.1.2.3 GESTATIONAL PHYSICAL ACTIVITY AND MATERNAL HEALTH OUTCOMES

While many studies that have focused on the DOHaD paradigm have examined the importance of maternal behaviors and their downstream effects on the short- and long-term health of the offspring, there is also a significant body of research that documents how PA during

pregnancy affects the mother. In uncomplicated cases, PA has been associated with numerous benefits, including improved mental health and reduced risk for urinary incontinence, hypertensive disorders, and post-partum weight retention (13). Through modulating GWG, PA also reduces the risk of delivering by caesarian section, prolonged labour, and delivery complications (13).

1.1.3 THE TWO BRANCHES OF THE HUMAN IMMUNE SYSTEM

The maintenance of homeostasis within the human body requires the constant monitoring of internal conditions. When there is a disturbance to this optimal state of functioning, namely through genetic factors, lifestyle conditions, or environmental exposure, homeostatic mechanism across the network of biological systems act to return the body to its homeostatic balance. The immune system is an essential part of the complex network, detecting and defending the body from environmental threats (e.g., bacteria, viruses, toxins, allergens) through a two-fold approach. The first, innate immunity, acts through perpetually activated elements that constantly monitor for and defend against foreign invaders. The innate immune system is able to identify harmful stimuli but possess no specificity against different pathogens. Once a threat is identified, the effectors of innate immunity initiate an inflammatory response in order to fight it off (22). Through the process of inflammation, the slower acting second branch, adaptive immunity, is able to launch highly specific defenses against individual pathogens through a cellular memory that possesses a catalogue of previous infections. While inflammation is a critical process of the immune system, if it is prolonged or wrongfully initiated, it can harm or interrupt the functioning of other vital systems (23). Autoimmune disorders can develop when inflammation is activated in the absence of a foreign body, and chronic inflammation has been

linked to cancer, kidney disease, diabetes mellitus, cardiovascular disease, and neurodegenerative disorders (24). Because of these risks, it is crucial to have a mechanism in place to regulate inflammation.

1.1.3.1 TYPE I AND TYPE II IMMUNE RESPONSES

Much like the innate and adaptive immune systems work together to protect against harmful agents, both are involved in regulating inflammation and repairing injuries. Various immune components across both branches can adopt a spectrum of diverse phenotypes with different functionalities in response to their surrounding microenvironment (25). These phenotypes can be generally classified into two categories. Type I (i.e., classical phenotype) immune responses work through pro-inflammatory mechanisms to fight against infections. In contrast, type II (i.e., alternative phenotype) immune responses are characterized by their anti-inflammatory actions, which promote regulation and repair (26). Together, type I and type II responses form a simplified paradigm that protects the body from foreign invaders while mediating inflammation and promoting the repair of autologous tissue (27). Several effectors of both innate and adaptive immunity possess the plasticity to shift between inflammatory or regulatory states based on the surrounding tissue microenvironment. Examples of these include T cells, which can polarize to either Th1 or Th2 (corresponding to Type I and Type II phenotypes), and macrophages, which shift between M1 and M2 (corresponding to Type I and Type II phenotypes) (28,29).

1.1.3.2 THE SPECTRUM OF MACROPHAGE POLARIZATION

Phagocytic cells of the innate immune system, macrophages can adopt various polarizations along the inflammatory spectrum broadly classified as either M1 or M2 states. These classifications are named to reflect the pro- and anti-inflammatory characteristics of type I and

type II immune responses, respectively. Though the M1-M2 paradigm is an overly simplified view, it can be said that M1 macrophages typically are involved in promoting pro-inflammatory mechanisms, while M2 macrophages balance these responses through the mediation and resolution of inflammation (28). M2 phenotypes can be further divided into M2a, M2b, M2c, and M2d subtypes that possess distinct characteristics, as illustrated in Figure 1.

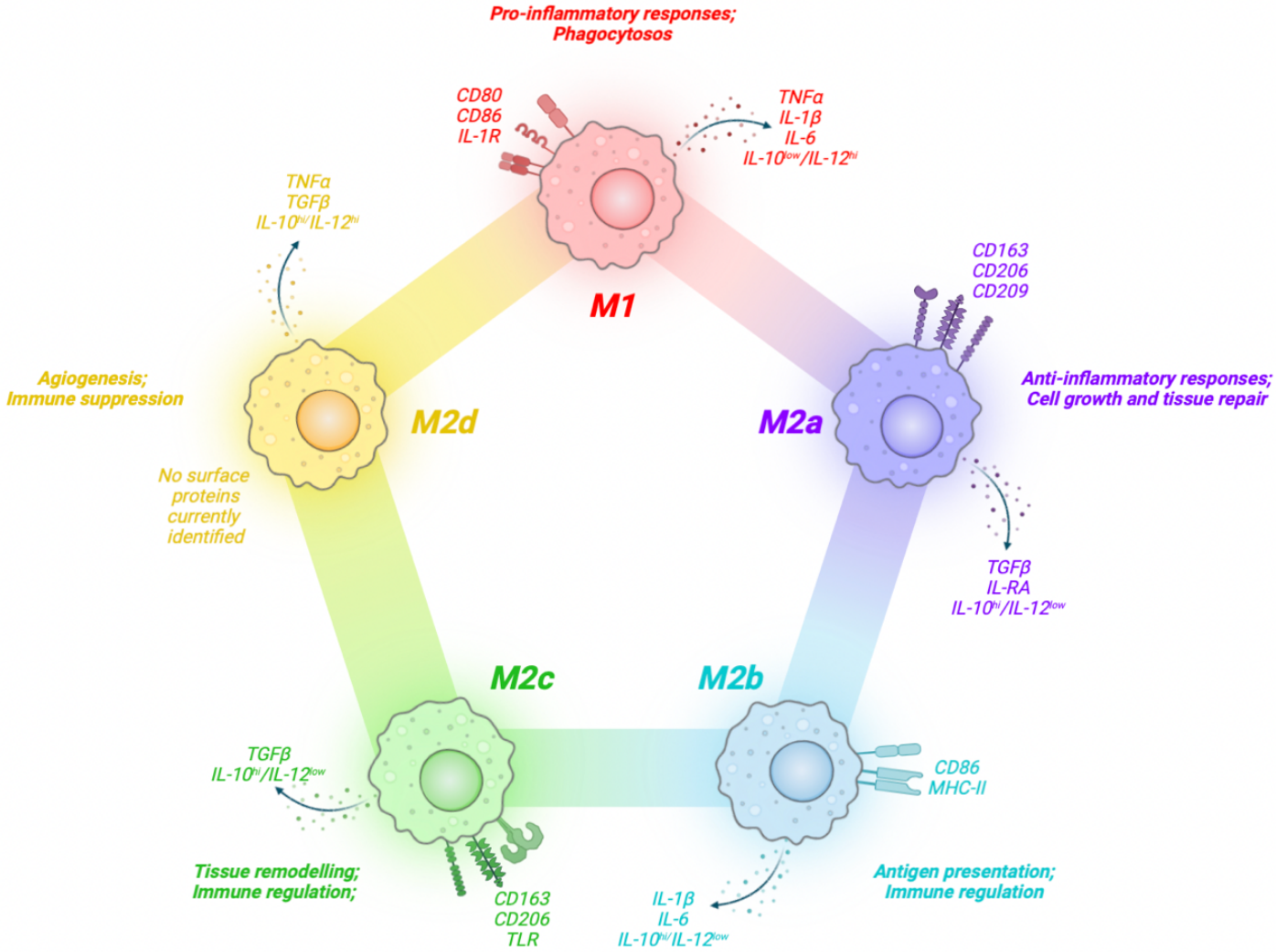

Figure 1. Markers and secreted factors of the predominant subtypes in the spectrum of macrophage polarization. Created with BioRender.

1.1.3.3 THE IMMUNOMODULATORY EFFECTS OF PHYSICAL ACTIVITY

It has been well documented in the literature that in non-pregnant populations, both habitual training and acute bouts of PA are able to influence changes across the immune system. Cells from both the adaptive immune system (including T cells and B cells) and the innate immune system (including natural killer (NK) cells, neutrophils, and macrophages) demonstrate adaptations when the body participates in PA (30). T cells experience decreased proliferation and a shift towards an anti-inflammatory Th2 phenotype (31). B cells, known as memory cells due to their ability to produce antigens that act specifically against individual pathogens, do not respond with any shift in polarization (30). However, PA serves as a suppressive force against B cell production of immunoglobulins, and, subsequently, against symptoms of allergies or autoimmune disorder (30). In a regularly physically active individual without the presence of toxins or infection, the number of regulatory NK cells and neutrophils increase and adopt a state of decreased cytotoxicity, which poses a risk to surrounding autologous cells and tissues (32,33). Similarly, PA is associated with a shift from M1 to M2 phenotypes in macrophages (34). This shift can be observed in both circulating and tissue-resident macrophage populations, including resident cardiac macrophages (RCM), microglia, brown adipose tissue (BAT) and subcutaneous white adipose tissue (scWAT) macrophages, Kupffer cells, alveolar macrophages, and skeletal muscle-resident macrophages (35–41).

1.1.3.4 PLACENTA RESIDENT MACROPHAGES: HOFBAUER CELLS

The placenta is a transient organ growing alongside the fetus as gestation progresses, serving as the interface between mother and fetus. After the birth of the neonate, hormonal increases in oxytocin causes the placenta to detach from the uterine wall and be delivered, its purpose

being completed. Acting as a surrogate of the baby's lungs, liver, and kidneys before delivery, it is responsible for the exchange of oxygen and carbon dioxide, as well as nutrients and waste between the mother and fetus (42). It also serves a protective function by creating an immune barrier to shield the uterine environment from toxins and pathogens. While there are multiple immune cell populations in the deciduous tissue of the uterus, macrophages compose the only immune cell population in the placenta. Distinct from the parental macrophages in the decidua, placenta resident macrophages, or Hofbauer cells (HBCs), are of fetal origin and reside in the stromal core of the chorionic villi (43). HBCs are known to possess the same phenotypic plasticity associated with pro- and anti-inflammatory functionalities as other tissue-resident and circulating macrophages (43). They are thought to play a role in maintaining homeostasis in the placenta, thereby contributing to the development and maintenance of a healthy pregnancy (43). Uncomplicated pregnancies are characterized by a heterogenous population of HBCs containing M2a, M2b, and M2c phenotypes (44). In the current literature, there is no evidence that HBCs can adopt an M2d polarization. The presence of M1 polarizations has been associated with detrimental conditions, including low birth weight, pre-eclampsia, and early pregnancy loss (45). As the interface between parent and fetus, the placenta possesses a distinct vasculature, comprising two separate networks. Parental and fetal circulations are contained in the uteroplacental and fetoplacental systems, respectively. While the circulations themselves never mix, their proximity to each other within the chorionic villus enables vital nutrient, gas, and waste exchange (46). Forming this complex system requires vasculogenesis, the de novo creation of new blood vessels that branch into smaller branches to form the capillary beds during angiogenesis (47). HBCs have been implicated in both vasculo- and angiogenic placental

mechanisms due to their secretion of crucial factors, including fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF) (46–49). Of note, FGF2 comes in two isoforms: high molecular weight (HMW) FGF2, exhibiting mitogenic properties in the nucleus, and low molecular weight (LMW) FGF2, which fosters angiogenesis upon external secretion (50). Besides expressing pro-angiogenic factors, it has been observed in recent years that HBCs express SPRY2, known to inhibit angiogenic pathways that are mediated by FGF2 (51). By secreting both pro- and anti-angiogenic factors, HBCs have attracted attention as likely regulators of placental blood vessel development. The precise impact of HBC polarization on SPRY2 secretion remains enigmatic. While existing knowledge points to M2 macrophages as secretors of both VEGF and FGF2, with CD206+ macrophages primarily responsible for FGF2 production (52), the specific contribution of HBC subtypes in the secretion of VEGF and FGF2 necessitates further exploration.

1.2 STUDY RATIONALE

As reviewed above, there are multiple health benefits for both the mother and fetus associated with habitual PA throughout gestation; however, the mechanisms through which these benefits are achieved remain to be fully understood. In non-pregnant populations, PA has been associated with a shift in the balance of type I and type II responses through increased anti-inflammatory cells and reduced pro-inflammatory cells. This shift can be seen in macrophages, as PA increases the ratio of M2 to M1 phenotypes across multiple tissue types. As the only immune cell present in the placenta, HBCs are thought to act as an immune barrier for the developing fetus, promoting an anti-inflammatory fetal environment, and regulating angiogenesis in the placenta (43,48). Due to their critical functions throughout gestation, they present a point of interest in investigating the mechanisms of PA-associated health benefits. This

thesis explores the relationship between habitual PA throughout gestation and HBC subtype populations in term placenta and the related expression of angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2), and sprout 2 (SPRY2).

1.3 THESIS AIMS

AIM 1: Compare the polarization state of Hofbauer cells in term placenta tissue of physically active and inactive participants.

Hypothesis: The placentas of physically active individuals have fewer Hofbauer cells with an augmented proportion of $CD206^{+}$ polarization state at term.

AIM 2: Examine the expression of select angiogenic factors (e.g. FGF2, VEGF, and SPRY2) in *in vivo* and *in vitro* models.

Hypothesis: $CD206^{+}$ HBCs produce VEGF and FGF2 and PA is positively correlated with the protein expression of FGF2 and its isoforms. Due to the limited literature on the effects of either PA or macrophage subtypes on the expression of SPRY2, the investigation of SPRY2 expression and localization was considered exploratory.

CHAPTER 2: CHARACTERIZATION OF HOFBAUER CELL POLARIZATION AND VEGF EXPRESSION IN HUMAN TERM PLACENTA FROM ACTIVE AND INACTIVE PREGNANT INDIVIDUALS

PREAMBLE TO MANUSCRIPT 1

The manuscript entitled: "*Characterization of Hofbauer cell polarization and VEGF expression in human term placenta from active and inactive pregnant individuals*" was submitted to Physiological Reports on the 20th of January, 2023 in accordance with the requirements stipulated by the Journal. The manuscript was revised as requested on the 19th of May, 2023, and published on June 4th, 2023. This manuscript covers the first objective of this thesis as it investigates the polarization states in placenta tissue from physically active and inactive participants.

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ORIGINAL ARTICLE

Physiological Reports

Characterization of Hofbauer cell polarization and VEGF localization in human term placenta from active and inactive pregnant individuals

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Abstract

Physical activity (PA) during pregnancy is associated with parental and fetal health benefits; however, the mechanisms through which these benefits arise are yet to be fully understood. In healthy pregnancies Hofbauer cells (HBCs) comprise a heterogenous population containing $CD206^+$ and $CD206^-$ phenotypes. In healthy pregnancies, $CD206^+$ represent the majority, while dysregulations have been associated with pathological conditions. HBCs have also been identified as potential drivers of angiogenesis. As PA induces changes in macrophage polarization in non-pregnant populations, this novel study examined the relationship between PA and HBC polarization and to identify which HBC phenotypes express VEGF. Participants were classified as active or inactive, and immunofluorescence cell-labelling was used to quantify total HBCs, $CD206^+$ HBCs, and the proportion of total HBCs expressing CD206. Immunofluorescent colocalization assessed which phenotypes expressed VEGF. Protein and mRNA expression of CD68 and CD206 were measured in term placenta tissue using Western blot and RT-qPCR, respectively. Both $CD206^+$ and $CD206^-$ HBCs expressed VEGF. The proportion of $CD206^+$ HBCs was elevated in active individuals; however, CD206 protein expression was observed to be lower in active participants. Combined with a lack of significant differences in CD206 mRNA levels, these findings suggest potential PA-mediated responses in HBC polarization and CD206 translational regulation.

KEYWORDS

angiogenesis, Hofbauer cells, macrophage polarization, physical activity, placenta, pregnancy

1 | INTRODUCTION

Habitual maternal physical activity (PA) reduces the risk of developing pregnancy-related complications such as gestational diabetes (GDM), hypertension, preeclampsia, and pre-term birth, while promoting appropriate birth weight (Mottola et al., 2018). As the placenta plays a key part in supporting and maintaining a healthy pregnancy,

PA throughout gestation may provide a downstream regulatory influence over fundamental biological processes of placental function. Tissue-resident macrophages can be identified in most organs throughout the body, and comprise significantly heterogeneous populations to accommodate the different functionalities required in their tissue-specific niches (Davies et al., 2013). Placenta-resident macrophages of fetal origin, termed Hofbauer cells (HBCs), perform various functions during pregnancy, including antigen presentation, phagocytosis, and cytokine secretion (Zulu et al., 2019). As is characteristic of macrophages, HBCs possess the ability to polarize between functional states by adapting to their microenvironment (Yao et al., 2019). These phenotypes can generally be divided into M1 and M2 classifications, a pattern characteristic of Type 1 pro-inflammatory and Type 2 anti-inflammatory responses, respectively (Rőszer, 2015). Broadly speaking, M1 macrophages are pro-inflammatory effectors of the immune system, while their anti-inflammatory M2 counterparts contribute to the regulation and repair of tissues (Zulu et al., 2019). The M2 branch can be further divided into subtypes M2a, M2b, M2c, and M2d (Figure 1). Despite the widespread practice of categorizing macrophages within these discrete subtypes, there is a recognition that it is an oversimplified paradigm. Due to the extensive adaptability and plasticity of macrophages, polarization exists on a continuous spectrum, with a wide range of intermediates that often possess overlapping characteristics (Murray, 2017). Moreover, macrophages develop different functional capabilities and morphologies based on their tissue location (Italiani & Boraschi, 2015). As such, it is important to recognize the pitfalls of strictly classifying macrophages into defined categories with distinct roles.

Bearing in mind the limitations of such classifications, healthy pregnancies are characterized by HBC populations composed of subtypes that most closely resemble M2a, M2b, and M2c phenotypes, with M2a and M2c subtypes comprising the majority of the population (Zhang et al., 2022). The literature clearly indicates that healthy pregnancies are characterized by a lack of M1 HBCs (Reyes & Golos, 2018; Schliefsteiner et al., 2017; Zulu et al., 2019). While M2 macrophages are often generally regarded as anti-inflammatory, M2b macrophages share several characteristics with their M1 counterparts, including the expression of the surface marker CD86 and secretion of pro-inflammatory cytokines, such as TNFα, IL-1β, IL-6, and IL-12 (Wang et al., 2019). Moreover, a shift toward a higher proportion of M2b phenotypes has been observed in pregnancies with inflammatory pathologies such as GDM (Schliefsteiner et al., 2017). The cause of this shift has yet to be identified; however, M2b macrophages and their strong immunosuppressive abilities have been linked to the promotion of infections (Wang et al., 2019). The evidence that individuals who develop GDM during their pregnancy are more susceptible to chorioamnionitis, the infection of the placenta and amniotic membranes, lends support to this theory (Bohiltea et al., 2019).

In non-pregnant human and animal populations, PA has been associated with phenotypic changes in macrophage polarization states (Murray, 2017). Zhang et al. (2020) found that treating macrophages with lactate concentrations corresponding to the physiological levels associated with PA induce a switch from M1 to M2

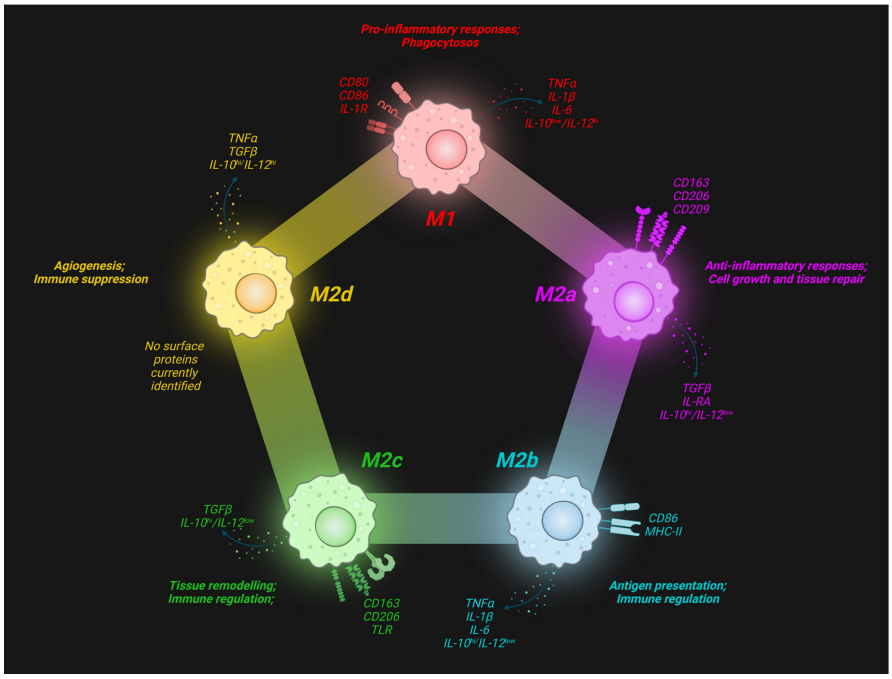

FIGURE 1 Surface markers and secreted factors of predominant subtypes in the macrophage polarization spectrum. Created with BioRender.com.

polarization states (Zhang et al., 2020). Furthermore, M2 tissue-resident macrophages in non-placental tissue have been linked to the expression of vascular endothelial growth factor (VEGF) and the promotion of pro-angiogenic responses. Based on the knowledge of the polarization response of tissue-resident macrophages to PA and the associated increase in angiogenic properties, it is possible that HBCs provide a similar response in the placenta.

Placental vascularization is necessary to maximize nutrient transfer to the growing fetus and promote proper development. Much like a skeletal muscle responds to PA, an increase in placental parenchymal volume, vascular volume, and villous surface area has been linked to maternal aerobic activity over gestation (Prior et al., 2004). Similar to other tissue-resident macrophages, such as microglia (Dudiki et al., 2020), HBCs possess angiogenic properties (Loegl et al., 2016; Seval et al., 2007; Zhao et al., 2018). These angiogenic properties include the expression of VEGF, a signaling protein promoting the growth of new blood vessels (Melincovici et al., 2018). While in most macrophage populations, the M2d subtype is the primary driver of VEGF secretion, this phenotype has not been observed in HBCs. The subtype that is the main driver of VEGF production in HBC populations has yet to be identified. A previous study by our lab has shown that as maternal PA increases, so does the protein expression of VEGF and its receptor VEGF receptor-1 in term placenta (Bhattacharjee, Mohammad, Goudreau, & Adamo, 2021). This finding suggests that PA over gestation may enhance placental pro-angiogenic factors, promoting placental vascularization.

The primary aim of this preliminary study was to investigate the effect of PA throughout pregnancy on HBC presence and polarization in previously banked tissue samples from term placenta. Given that the previously published research by Bhattacharjee, Mohammad, and Adamo (2021) and Bhattacharjee, Mohammad, Goudreau, and Adamo (2021) illustrated a higher level of VEGF expression in the term placenta of active participants, we also endeavored to examine which HBC phenotypes expressed the angiogenic factor VEGF.

2 | METHODS

2.1 | Ethics statement

Participants provided informed written consent to participate in the PhysicaL Activity and diEtary implicatioNs Throughout pregnAncy (PLACENTA) study, at the University of Ottawa. The PLACENTA study was approved by the Research Ethics Board (REB) of the University of Ottawa (file number: H11-15-29) and conformed to all aspects of the Declaration of Helsinki.

2.2 | Participant recruitment

Participants were recruited as a part of the PLACENTA study from the Ottawa region (Ottawa, Ontario) via flyers posted at health clinics, community centers, universities, and social media. Interested individuals were pre-screened by phone to assess their eligibility. The inclusion criteria for the pregnant individuals were as follows: 18–40 years old; able to communicate in either English or French; less than 28 weeks gestation; weight stable for at least six months before conception (±2 kg); carrying a singleton fetus; and having a self-reported pre-pregnancy body mass index (BMI) of normal or overweight (18.5–29.9 kg/m^2). Exclusion criteria for study recruitment were having contraindications to exercise; being diagnosed with pre-pregnancy diabetes; or having untreated thyroid disease. Individuals participated in an in-lab visit twice during their pregnancy; once during mid-gestation (between 24 and 28 weeks of gestation) and once during late gestation (between 34 and 38 weeks).

2.3 | Physical activity analysis

After completing the assessments at both gestational timepoints, participants were given a take-home package that included an omnidirectional Actical® accelerometer (Philips Respironics). Participants were instructed to wear the accelerometer around their waist for waking hours over seven consecutive days to record periods of free-living PA. To be included in further analyses, participants were required to have three valid days with a minimum wear time of ten hours per day (da Silva et al., 2021). Data analyses were performed as previously described (Tremblay & Connor Gorber, 2007) using SAS version 9.4 as per the Canadian Health Measures Survey procedures. Data output from the accelerometers were used to measure the minutes of PA accumulated during the week. Participants were classified as active or inactive based on the evidence-based 2019 Canadian guideline for physical activity during pregnancy recommendation that pregnant individuals should perform at least 150 min of moderate PA a week, or an average of 21.4 min per day (Mottola et al., 2018). Individuals who averaged 21.4 min per day of moderate-to-vigorous PA (MVPA) or more at both mid and late gestation were classified as active. Conversely, participants that did not reach this average at both gestational time points were classified as inactive.

2.4 | Sample collection

Term placentae were sampled within an hour of delivery to preserve specimen integrity. Large tissue biopsies (approximately 2.5 cm^3) were dissected from central and peripheral cotyledons. Tissue samples were further dissected into small pieces (~1 cm^3), then placed in cryovials and flash-frozen in liquid nitrogen. Frozen placenta tissue was powdered on ice and homogenized in radioimmunoprecipitation assay (RIPA) buffer (BioRad**)** using Powergen 125 homogenizer (Fisherbrand). The protein lysate was centrifuged at 1000 *g* for 10 min at 4°C and stored at −80°C until further analysis. Full-thickness histological samples were taken from healthy cotyledons of the placenta, avoiding areas of necrosis and calcification. Sections were rinsed in non-sterile phosphate-buffered saline (PBS). For formalin-fixed, paraffin-embedded samples (FFPE), the dissected sections were placed in 10% formalin for 48 h, then rinsed three times in non-sterile PBS and subsequently preserved in paraffin blocks. Additional sections were placed into plastic cryomolds containing O.C.T embedding compound. Cryomolds were wrapped in aluminum foil and flash-frozen in liquid nitrogen.

2.5 | Western blotting

Twenty to forty μg of total placental protein were loaded on Mini-PROTEAN® TGX gel (Bio-Rad) and resolved at 150 volts for 1 h. The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and blocked with 5% powdered milk in tris buffered saline solution with 0.05% tween-20 (TBST) for 1 h at room temperature (RT). Membranes were incubated overnight at 4°C with mouse monoclonal anti-CD68 (1:500, Abcam Cat# ab201973, RRID:AB_2936513) and rabbit polyclonal anti-CD206 (1:800, Abcam Cat# ab64693, RRID:AB_1523910). The following day, blots were washed with TBST and incubated with 1:5000 dilutions of horse radish-peroxidase conjugated secondary antibodies (Goat Anti-Mouse IgG Bio-Rad Cat# 1706516, RRID:AB_2921252; Goat Anti-Rabbit IgG Bio-Rad Cat# 1706515, RRID:AB_2617112) for 1 h at RT. The blots were developed using Clarity ECL Western Substrate (Bio-Rad) and imaged using ChemiDoc™ XRS+ Imaging System (Bio-Rad). Membranes were permanently stained with 1% Amido Black for total protein lane quantification. Band expression and total protein expression were analyzed by densitometry (ImageJ, Bio-Rad). Placental protein expression for CD68 and CD206 was standardized to total participant pooled protein lysate samples.

2.6 | Immunofluorescence

Placental FFPE tissue was processed into slides by the Louise Pelletier Histology Core at the University of Ottawa. Tissue was sectioned at 4 μm thickness for analysis. Slides were then deparaffinized and rehydrated using xylene and a graded series of ethanol dilutions before being rinsed in double-distilled water. Heat-mediated antigen retrieval was performed using sodium citrate buffer (10 mM, pH 6.0). Tissue sections were permeabilized in 0.2% Triton-X in TBS for 20 min at RT. Sections were then incubated in 10% bovine serum albumin (BSA) for 1 h at RT to block non-specific binding, then incubated in 0.1% Sudan Black in 75% EtOH (wt/vol) for 10 min at RT to quench tissue autofluorescence. The primary antibodies for CD68 (mouse monoclonal Abcam Cat# ab201973, RRID:AB_2936513) and CD206 (rabbit polyclonal Abcam Cat# ab64693, RRID:AB_1523910) were diluted in TBST at 4 and 2 μg/mL, respectively, before being applied to the sections, and then incubated overnight at 4°C. This step was omitted in negative controls (Figure 2). Following the application of the primary antibodies, slides were washed three times in TBST, then incubated in 1:250 and 1:1000 dilutions of Alexa 488 goat anti-mouse secondary antibody (Thermo Fisher Scientific Cat# A28175, RRID:AB_2536161), and Alexa 594 goat anti-rabbit secondary antibody (Thermo Fisher Scientific Cat# A-11012, RRID:AB_2534079) respectively for 1 h at RT in the dark. Slides were washed three times for five minutes each in TBST in the dark, then mounted with Prolong Gold with DAPI. Edges were sealed with nail polish.

Immunofluorescence was also used to localize VEGF in relation to HBCs. Frozen histological tissue samples were brought to room temperature and rinsed in PBS, then fixed in 4% paraformaldehyde at RT for 5 min. After being washed in PBST (0.2% Triton-X), sections were blocked in 10% BSA in PBST for 1 h and quenched with 0.1% Sudan Black in 75% EtOH for 10 min at RT. CD68 (mouse monoclonal Abcam Cat# ab201973, RRID:AB_2936513) and CD206 (rabbit polyclonal Abcam Cat# ab64693, RRID:AB_1523910) primary antibodies were diluted in PBST at 4 and 2 μg/mL, respectively. On day one, sections were incubated in the diluted primary antibodies overnight at 4°C. On day two, slides were washed of primary antibodies thoroughly with PBST and the secondary antibodies were applied (Thermo Fisher Scientific Cat# A28175, RRID:AB_2536161; Thermo Fisher Scientific Cat# A-11012, RRID:AB_2534079) at 1:1000 dilution for 1 h at RT. After thoroughly washing the secondary antibodies 3 × 5 min with TBST, the anti-VEGF primary antibody (mouse monoclonal; Novus Cat# NB100-664, RRID:AB_10001947) was diluted at 1:50 in TBST and applied to the slides overnight at 4°C. On day three, slides were washed three times in TBST and incubated in a 1:500 dilution of Alexa 647 goat anti-mouse

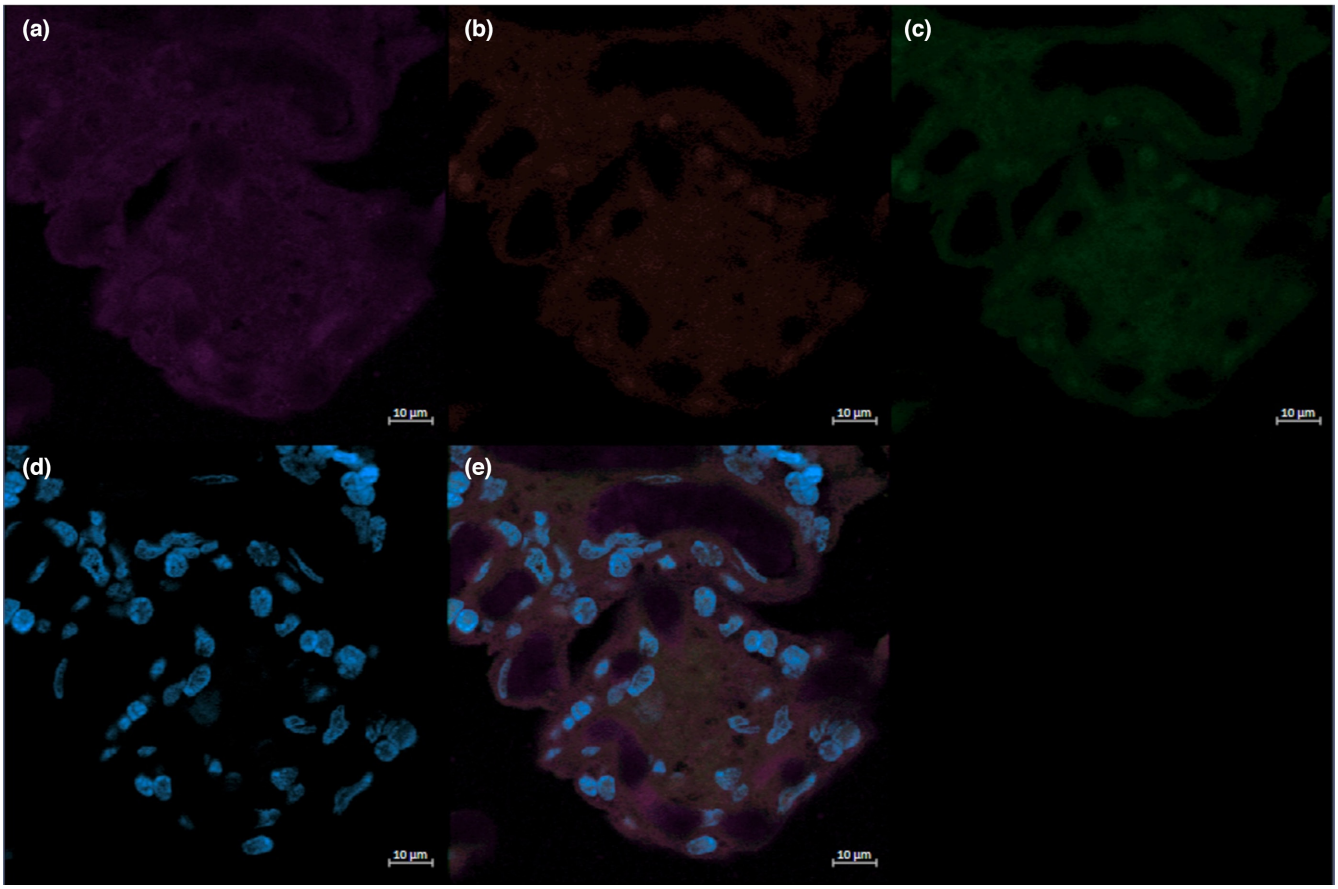

FIGURE 2 The negative control for immunofluorescent staining of VEGF (a, purple), CD206 (b, red), CD68 (c, green), and DAPI (d, blue) and the corresponding merged image (e) is shown.

secondary antibody (Thermo Fisher Scientific Cat# A32728, RRID:AB_2633277) for 1 h at RT. This three day protocol ensured that anti mouse secondary antibodies recognized their primary antibody targets. Slides were then washed and mounted as described above.

2.7 | Cell quantification and analysis

Slides were imaged using a fluorescent microscope (Zeiss AxioObserver M2) equipped with blue (excitation 390/22 nm, emission 460/50 nm), green (excitation 470/40 nm, emission 525/50 nm) and red (excitation 560/40 nm, emission 630/75 nm) filters to visualize the cells of interest. Eight representative images of each section from all 22 participant samples were acquired to count the CD68 and CD206 positive cells. Bearing in mind the limitations of classifying HBCs into discrete categories, as well as the M2b-like characteristic of secreting pro-inflammatory cytokines, HBCs were identified as either $CD68^{+}CD206^{+}$ (indicative of an M2a- or M2c-like state) or $CD68^{-}CD206^{-}$ (indicative of an M2b-like state). The number of cells stained with the pan-macrophage marker CD68 represented total number of Hofbauer cells. Cell numbers were normalized to the fraction of tissue coverage (FTC) in the image using Image J (NIH). To do so, images were converted to a binary color scheme where black pixels represented tissue and white pixels represented intervillous space. Black pixels were quantified by the program and divided by the number of total pixels to obtain the FTC. Cell counts were divided by the FTC to calculate the adjusted values, which were used for statistical analyses.

2.8 | RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was isolated from term placenta tissue as previously described (Bhattacharjee, Mohammad, Goudreau, & Adamo, 2021). From the isolated RNA, 1 µg was reverse transcribed into cDNA using iScript™ cDNA Synthesis Kit (1708891; Bio-Rad Laboratories). The Roto-Gene RG-3000 system (Corbett Research) was used to amplify cDNA through a real-time quantitative polymerase chain reaction (qPCR). Predesigned qPCR 20X probes for CD68 (Hs.PT.58.2488447) and CD206 (Hs.PT.58.15093573) were purchased from Integrated DNA Technologies. For all samples, YWHAZ (Hs.PT.39a.22214858) was used as an endogenous control (Meller et al., 2005). Threshold cycle

(TC) values were obtained for each sample, and used to analyze the relative gene expression via the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001). Gene expression values were normalized to the endogenous control, and the relative gene expression of physically active participants was determined in comparison to the gene expression of their physically inactive counterparts.

2.9 | Statistical analysis

All data are presented as mean ± standard deviation. The gene expression analysis software Rotor-Gene 6 (version 6.1; Corbett Research) was used to analyze qPCR data and GraphPad Prism software (version 9.0.0, GraphPad Software Inc.) was used for all statistical analyses. The Shapiro–Wilks test was used to assess normality, and the ROUT method employed to test for outliers. Unpaired *t*-tests or Mann–Whitney *U* tests, where appropriate, were used to assess the statistical difference of participant demographics, Western blot, immunofluorescence, and qPCR data between active and inactive participants. Pearson correlations were also used to analyze the number of $CD68^+$ and $CD68^+CD206^+$ cells in comparison to the average minutes of MVPA/day obtained by each participant. Statistical significance was defined as $p \leq 0.05$.

3 | RESULTS

3.1 | Participant demographics

Maternal demographic information and newborn outcomes are described in Table 1 according to PA status throughout gestation. By study design, physically active participants had significantly higher MVPA (min/day) than their inactive counterparts in both mid- and late-gestation ($p < 0.001$). Maternal age, height, pre-pregnancy weight, or pre-pregnancy BMI did not differ significantly between active and inactive groups. Similarly, there were no significant differences in newborn birth weight or birth length between physically active and inactive individuals.

3.2 | Placental CD206 expression is significantly higher in physically inactive individuals

Placental CD68 and CD206 protein expression were analyzed to determine the difference in protein levels between active and inactive participants by parametric and non-parametric *t*-test, where applicable. No significant differences in the expression of CD68 were found between active and inactive individuals ($p > 0.05$); however, the protein expression of CD206 was lower in active pregnancies compared to inactive ($p = 0.050$; Figure 3a,b).

3.3 | CD206 and CD68 mRNA expression did not differ based on activity status

The mRNA levels of CD206 and CD68 (Figure 3c,d) in the placenta was investigated by qPCR using RNA isolated from term placental tissue using non-parametric *t*-tests. No significant differences in CD206 ($p = 0.720$) or CD68 ($p = 0.604$) mRNA were observed.

TABLE 1 Study participant maternal demographics and newborn outcomes ($n = 21$).

	Active ($n = 11$)	Inactive ($n = 10$)	*p*-Value
Maternal demographics			
Maternal age (years)	32.45 ± 2.98	32.90 ± 2.69	0.7239
Height (cm)	165.37 ± 6.57	168.10 ± 7.26	0.3775
Pre-pregnancy weight (kg)	64.25 ± 10.33	67.57 ± 13.28	0.5275
Pre-pregnancy BMI (kg/m^2)	23.45 ± 2.67	23.66 ± 3.00	0.8641
Gestational age at birth (weeks)	41.27 ± 3.08	40.54 ± 1.04	>0.9999
Mid gestation MVPA (min/day)	46.03 ± 10.03	6.03 ± 3.96	<0.0001
Late gestation MVPA (min/day)	34.27 ± 9.10	3.7 ± 3.54	<0.0001
Newborn outcomes			
Birth weight (kg)	3.28 ± 0.38	3.58 ± 0.43	0.1075
Birth length (cm)	49.85 ± 2.52	51.78 ± 1.98	0.0672
Sex, *n*			
Male	7	4	
Female	4	6	

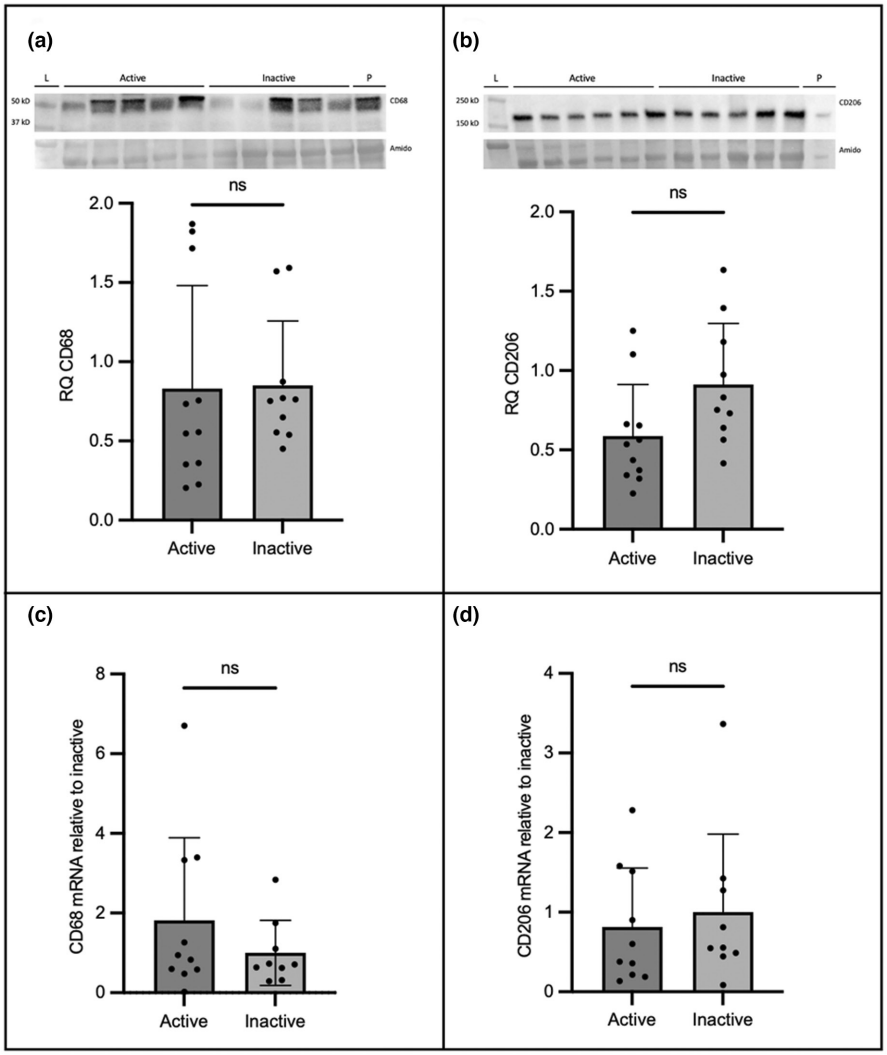

FIGURE 3 Representative immunoblots and the corresponding semi-quantitative densitometric analysis are shown for CD68 (a) and CD206 (b). The relative quantification of mRNA for CD68 and CD206 are illustrated in panels (c) and (d), respectively. All data are represented as mean ± SD. *$p < 0.05$.

3.4 | Physically active individuals have higher proportions of M2 HBCs

Immunofluorescence studies identified total HBCs ($CD68^+$) and $CD68^+CD206^+$ HBCs in the term placenta of active and inactive participants (Figures 4–6). Total HBCs and $CD206^+$ HBCs were counted to determine the differences in polarization between groups. Using the ROUT method, one outlier in the active group of both the absolute numbers of total HBCs and $CD206^+$ HBCs data was identified and excluded from further analysis. No significant differences were found in the absolute number of total HBCs ($p = 0.405$) and $CD206^+$ HBCs ($p = 0.600$). However, the number of $CD206^+$ HBCs as a proportion of the total was significantly higher in physically active individuals ($p = 0.024$). There was a significant correlation between the proportion of $CD206^+$ HBCs and the average minutes of MVPA achieved per day in both the mid ($r = 0.514$, $p = 0.017$) and late ($r = 0.457$, $p = 0.037$) gestation, with large and medium effect sizes, respectively. No significant correlations between the absolute numbers of total HBCs or $CD206^+$ HBCs and MVPA (min/day) were observed at either timepoint (data not shown).

3.5 | Colocalization of VEGF and HBC markers

Figure 7 displays images from the immunofluorescence studies examining the localization of VEGF, the pan-macrophage marker CD68, and M2a/M2c macrophage marker CD206. In term placenta, VEGF was colocalized within cells expressing both CD68 and CD206, indicative of an M2a- or M2c-like phenotype. Interestingly, VEGF was also found in cells that only expressed CD68, indicative of an M2b-like phenotype.

4 | DISCUSSION

Previous research has shown that PA can polarize macrophages from a pro-inflammatory to an

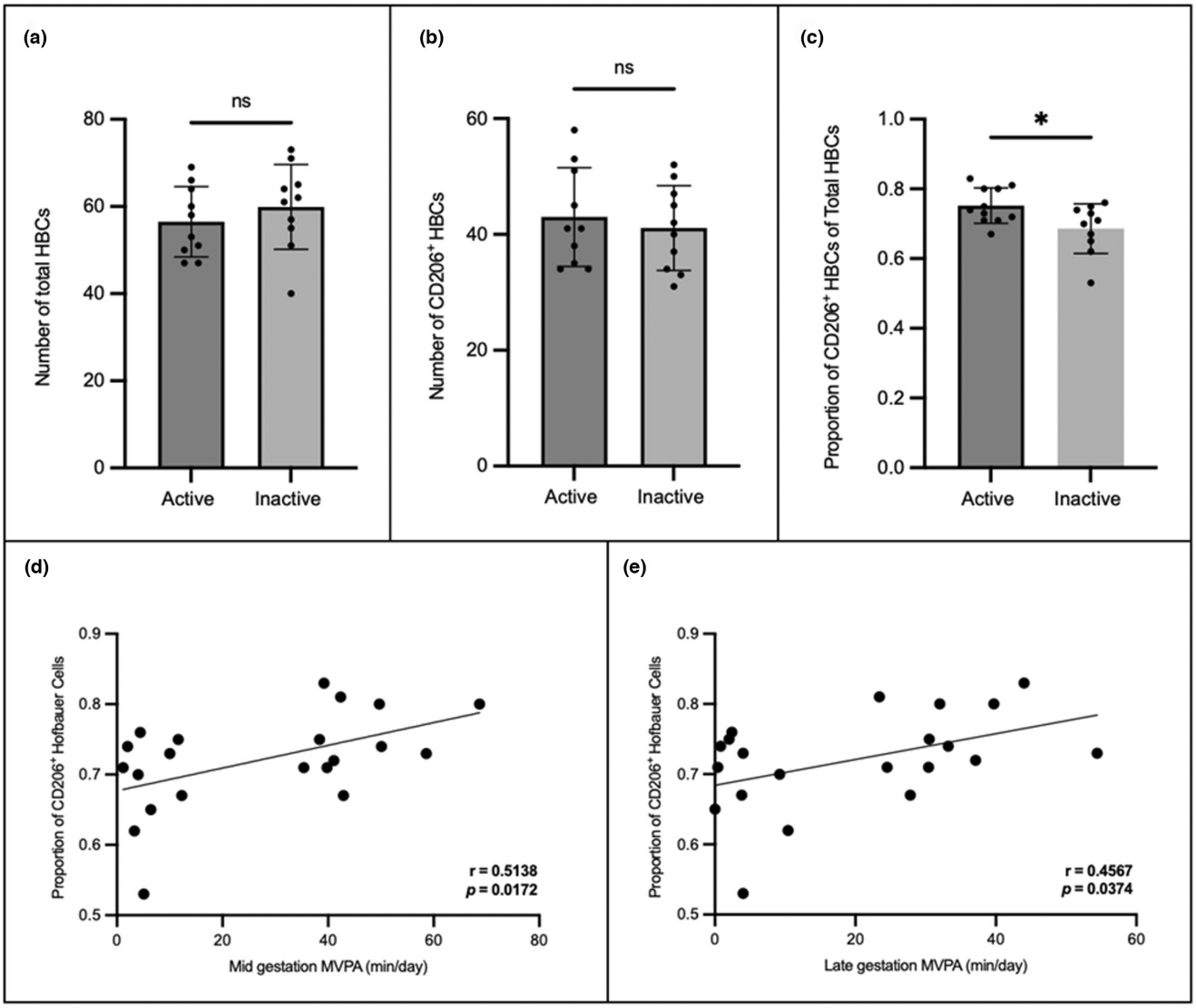

FIGURE 4 The (a) number of total Hofbauer cells and (b) number of CD206+ Hofbauer cells did not significantly differ between active and inactive individuals; however, the proportion of CD206+ Hofbauer cells (c) was significantly higher in active participants. a–c data are represented as mean ± SD. There were significant correlations between the proportion of CD206+HBCs and average minutes of moderate to vigorous physical activity (MVPA) in mid (d) and late (e) gestation. *$p < 0.05$.

anti-inflammatory state in circulation, as well as in adipose, lung, and central nervous system tissues (Blanks et al., 2020; Mee-Inta et al., 2019; Rentz et al., 2020; Shi et al., 2020; Silveira et al., 2016). However, this study is the first to explore the association between gestational PA and the polarization of HBCs. While there were no differences in the absolute numbers of $CD206^+$ HBCs or total HBCs between physically inactive and active participants, active individuals had significantly higher levels of $CD206^+$ HBCs as a proportion of the total. Due to variations in the intervillous space, absolute cell count numbers were divided by the percentage of tissue coverage in the image. By doing so, the risk of observing differences in cell counts attributable to differences in tissue coverage was mitigated. In observing significant differences in the $CD206^+$ HBCs as a proportion of the total HBCs only, it is possible that looking at absolute numbers of polarized and total HBCs is an insufficient method of analysis. Additionally, there were large and significant correlations between the proportion of $CD206^+$ HBCs and minutes of MVPA per day in both mid and late gestation. Our findings suggest that PA engagement in pregnancy is a potential determinant of HBC polarization during pregnancy.

The function of the pan-macrophage marker CD68 has not been extensively investigated; however, it has been shown that the protein can be shuttled to the cell surface, where it is involved in the binding of oxidized low-density lipoprotein (OxLDL; Chistiakov et al., 2017). The ability of CD68 to bind to OxLDL may play a role in the development of foam cells, which have been observed in preeclampsia. No differences were found in the number of

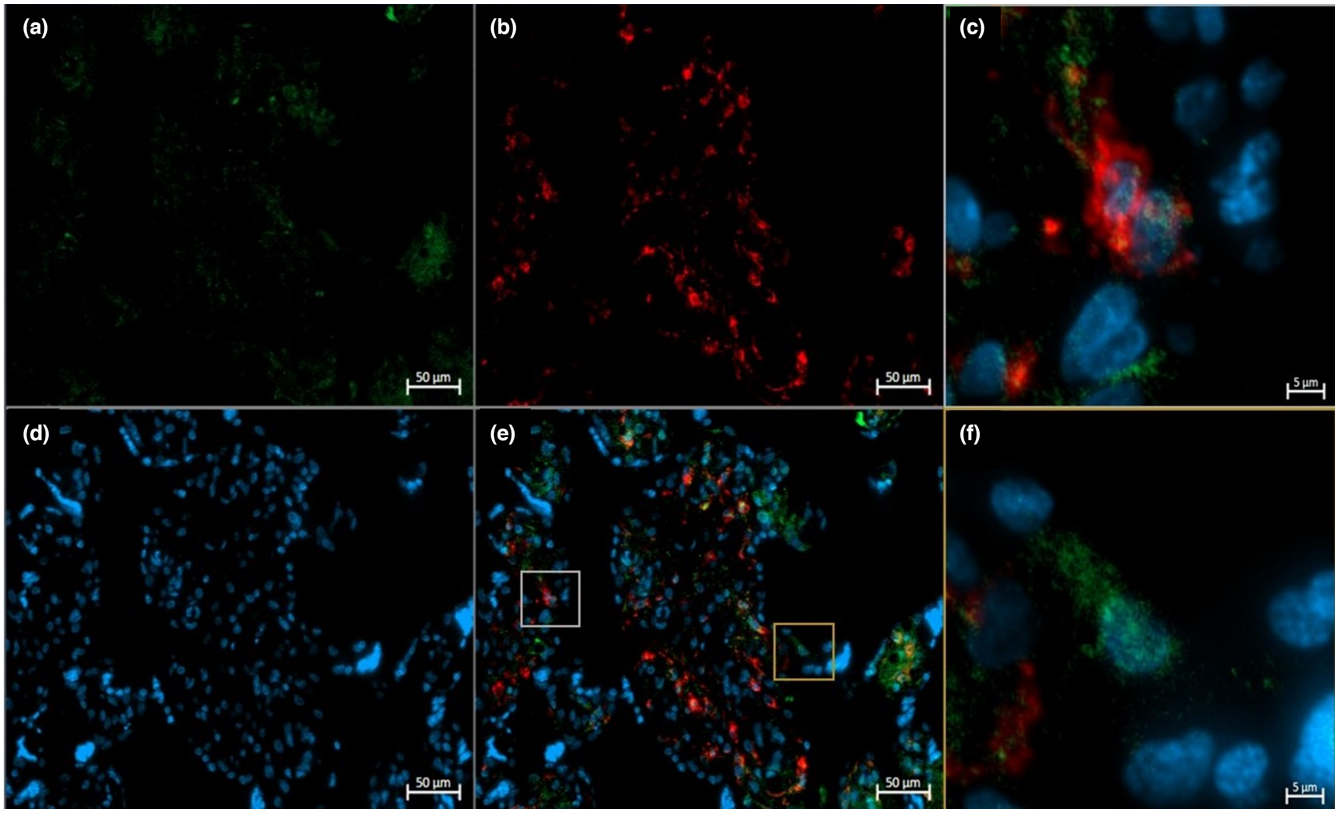

FIGURE 5 Representative immunofluorescent staining of CD68 (a, green), CD206 (b, red), and DAPI (d, blue) in the term placenta of an active participant. The yellow box highlights a CD206+ (M2a/M2c) Hofbauer cells, while the gray box highlights a CD206− (M2b) Hofbauer cells on the merged (e) image. Magnified images of CD206+ (c) and CD206− (f) Hofbauer cells are shown.

$CD68^+$ cells, CD68 protein expression, or mRNA levels, indicating that PA likely does not influence the expression CD68 in term placenta.

CD206 is expressed predominantly by macrophages and monocytes, along with dendritic, lymphatic, and endothelial cells (Azad et al., 2014). Upregulation of CD206 has been observed in a multitude of conditions, including cancers and inflammatory diseases (Choi et al., 2017; Nielsen et al., 2020). Tumor-associated macrophages (TAMs), thought to contribute to the progression of cancer through immunosuppression and matrix remodeling, have enhanced levels of CD206 (Choi et al., 2017; Martinezpomares, 2001; Nielsen et al., 2020). Elevated expression of CD206 contributes to mannose-dependent endocytosis, as well as the uptake of collagen, contributing to matrix deposition, fibrosis, and inflammation (Arlt et al., 2020; Choi et al., 2017; Nielsen et al., 2020). In the current study, while the proportion of $CD206^+$ HBCs was statistically higher in active individuals, the protein expression of CD206 was significantly higher in inactive individuals. It is possible that other cells expressing CD206, such as endothelial cells, may be responsible for the higher levels of placental CD206 protein observed in those characterized as inactive. However, acute bouts of PA are thought to result in a temporary hypoxic state in the placenta; such conditions have been shown to promote endothelial cell proliferation (Bhattacharjee, Mohammad, & Adamo, 2021; Wong et al., 2017). This proliferation would result in higher CD206 levels, which is opposite to what our study observed. Additionally, to the best of our knowledge, there is no literature suggesting that PA, hypoxia, or inflammation differentially regulates the expression of CD206 within placental endothelial cells. Therefore, it is improbable that the higher level of CD206 observed in inactive individuals is due to endothelial cells; for these reasons, we posit that the difference in CD206 is due to an HBC response. As a significant difference was observed between active and inactive participants in placental CD206 protein expression, but not in mRNA levels, the potential PA-mediated downregulation of CD206 likely occurs during translation. Therefore, we posit that PA may downregulate placental CD206 expression at a translational level in HBCs, potentially leading to decreased inflammation and fibrosis. This hypothesis, as well as the effects of PA-mediated changes in CD206, need to be explored in future research and functional studies.

The literature shows that in uncomplicated pregnancies, HBCs do not express an M1 phenotype (Schliefsteiner et al., 2017). Due to this observation, it is plausible that cells identified as $CD206^-$ HBCs in the current study possessed

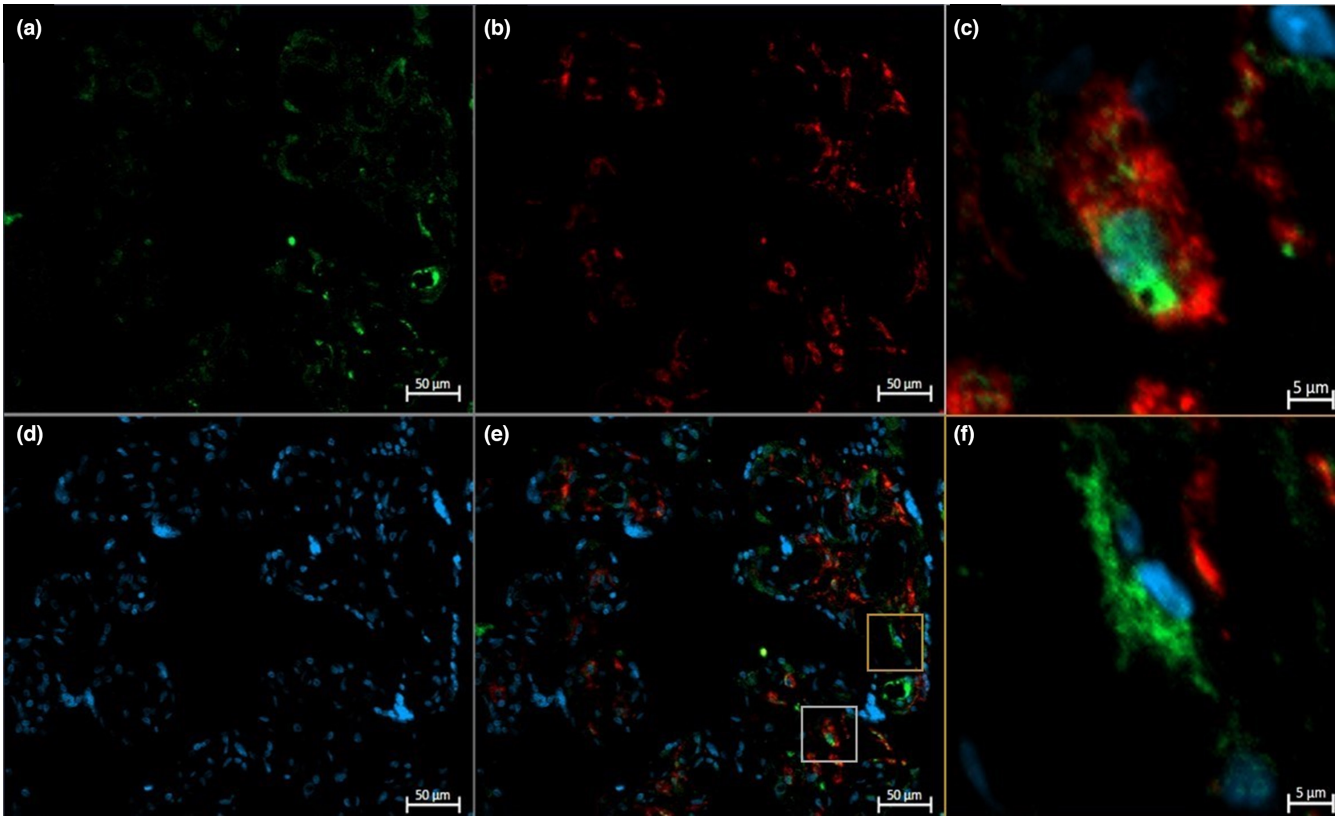

FIGURE 6 Representative immunofluorescent staining of CD68 (a, green), CD206 (b, red), and DAPI (d, blue) in the term placenta of an inactive participant. The yellow box highlights a CD206+ (M2a/M2c) Hofbauer cells, while the gray box highlights a CD206− (M2b) Hofbauer cells on the merged (e) image. Magnified images of CD206+ (c) and CD206− (f) Hofbauer cells are shown.

an M2b phenotype. Interestingly, while M2b macrophages have been demonstrated to share characteristics with M1 macrophages, including the secretion of TNF-α, IL-1β and IL-6, they also possess regulatory characteristics and are involved in immunosuppression. As mentioned previously, an increase in M2b phenotypes has been associated with GDM (Schliefsteiner et al., 2017). While, to the best of our knowledge, there is no current literature describing the role of M2b macrophages in pregnancies complicated by infections or conditions such as preeclampsia, M2b macrophages in other tissues can exacerbate bacterial, viral, and fungal infections by blunting the immune response (Wang et al., 2019). Furthermore, cytokines secreted by the M2b phenotype, including TNF-α, IL-1β, and IL-6 have been associated with preeclampsia. Based on the current knowledge, increased presence of M2b phenotypes may contribute to the development of preeclampsia. While it has been demonstrated that healthy pregnancies are characterized by a lack of M1 HBCs, the expression of these cytokines can also be attributable to the M1 phenotype. Przybyl et al. discovered that in the presence of preeclampsia, HBCs downregulate the expression of CD74, a human leukocyte antigen class II histocompatibility antigen-γ chain (Przybyl et al., 2016). The Przybyl team posited that this downregulation induces a phenotypic switch in HBCs from an M2 to an M1 state (Przybyl et al., 2016). HBC populations in pregnancies complicated by pathologies should be studied to determine whether complications induce an M1 phenotype, as well as the potential effects this would have on the expression of secreted factors, including cytokines.

As a previous study by our lab identified that physically active individuals had an increased level of VEGF expression when compared to inactive individuals (Bhattacharjee, Mohammad, Goudreau, & Adamo, 2021), we aimed to investigate if this difference related to expression within different HBC subtypes. In examining the colocalization of VEGF and CD206, VEGF was distinctly localized with both $CD206^{+}$ HBCs and $CD206^{-}$ HBCs. This specific localization indicates that the potential angiogenic action of HBCs is likely not restricted to M2a- and M2c-like phenotypes but is a characteristic of all M2 HBCs in healthy pregnancies. Future research should utilize fluorescence-activated cell sorting (FACS) to isolate subtypes of HBCs, including M1, M2a, M2b, and M2c, as well as enzyme-linked immunosorbent assays (ELISA) to determine which phenotype is the main driver of VEGF production in both healthy and complicated pregnancies.

It is important to note that inherent limitations are associated with the current study. The sample size is small and

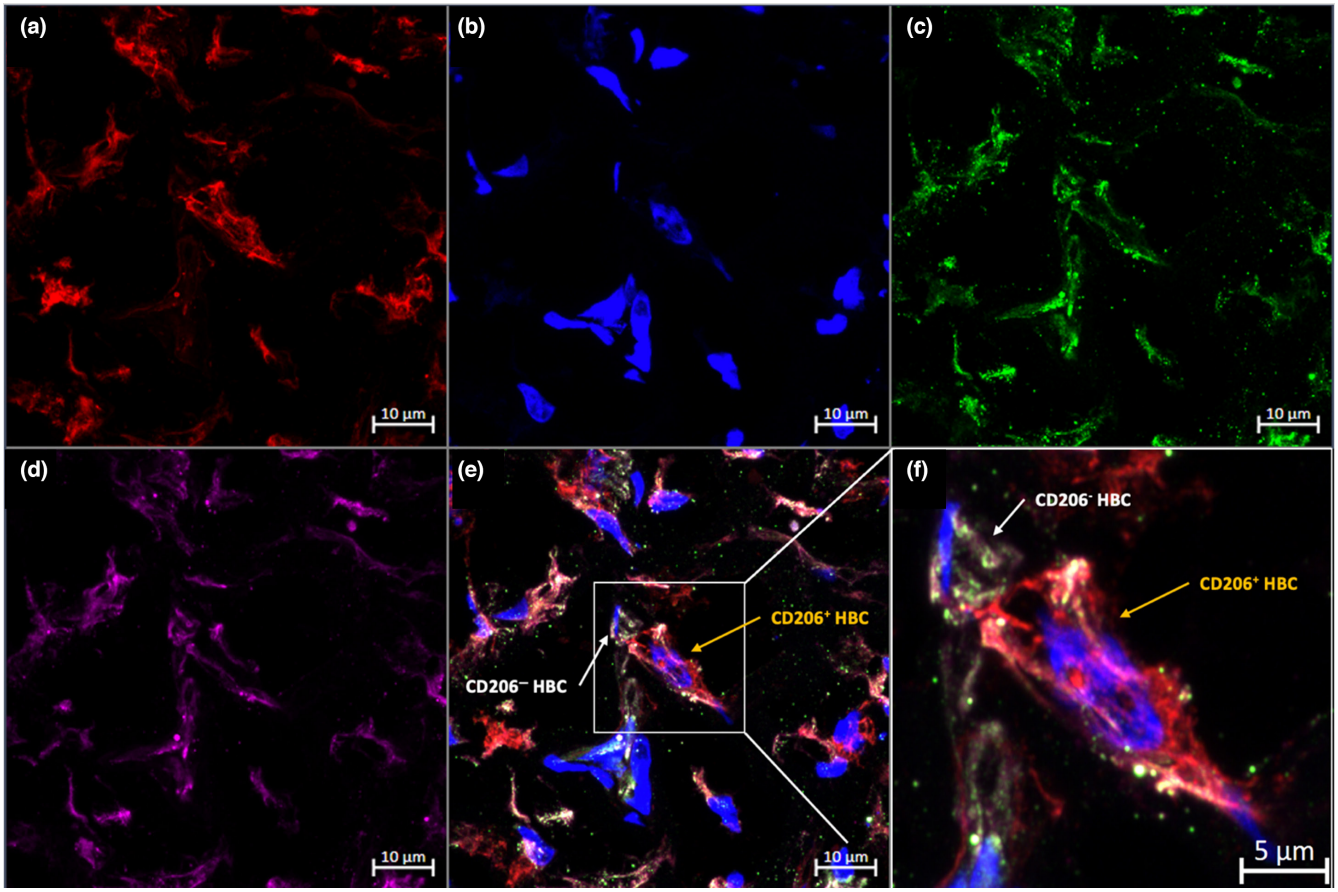

FIGURE 7 Representative immunofluorescent staining of CD206 (a, red), DAPI (b, blue), CD68 (c, green), and VEGF (d, purple) in term placenta. The white box highlights a CD206+ (M2a/M2c) Hofbauer cells and a CD206− (M2b) Hofbauer cells on the merged (e) image. Magnified images of CD206+ (f) Hofbauer cells are shown.

relatively homogenous; therefore, it is possible that results may not be representative of the population. As this investigation was carried out with frozen, banked tissue samples, experiments requiring fresh tissue, such as the isolation of primary HBC cultures to identify HBC polarization with flow cytometry, could not be conducted. Due to a lack of non-invasive methods available for studying the human placenta, tissue collected from term deliveries was used for the quantification of mRNA, protein, and cell types. The results from term tissue may not be generalizable to other gestational timepoints. Additionally, immunofluorescence is a semi-quantitative and subjective method. We attempted to control for the subjective nature of the technique by defining an objective set of parameters to be followed before commencing cell counting, as well as having one researcher conduct cell counting, thus decreasing interobserver variability. Blinding of the images also reduced any potential researcher bias. Another possible risk of immunofluorescence is bleed-through of the secondary antibodies, potentially resulting in false positive staining. To mitigate this risk, fluorophores with distinct spectra were selected. In performing immunofluorescence, we used a previously published protocol enabling the utilization of two primary antibodies from the same species (Tzaneva et al., 2014; Tzaneva & Perry, 2016). While the use of two primary antibodies from the same species could potentially lead to cross-reactions, by incubating the first round of secondary antibodies overnight, they were allowed to saturate the binding sites available for the mouse anti-CD68 antibody. Following this, the sections were thoroughly washed to remove any unbound goat anti-mouse secondary antibody. This mitigates the risk of binding between the mouse anti-CD68 primary antibody and goat anti-mouse Alexa 649 antibody, and between the mouse anti-VEGF primary antibody and the goat anti-mouse Alexa 488 secondary antibody. The staining patterns of CD68 and VEGF were assessed and found to have different staining patterns (Figure 8).

Our study also possesses multiple strengths, including the use of accelerometry as a validated objective measure of PA; measuring PA at multiple time points throughout gestation; controlling for potential confounding factors such as maternal age, BMI status, and gestational age at delivery; and having a standardized protocol for placenta collection. Additionally, we use a tissue homogenate of multiple placental locations to account for tissue heterogeneity throughout the placenta.

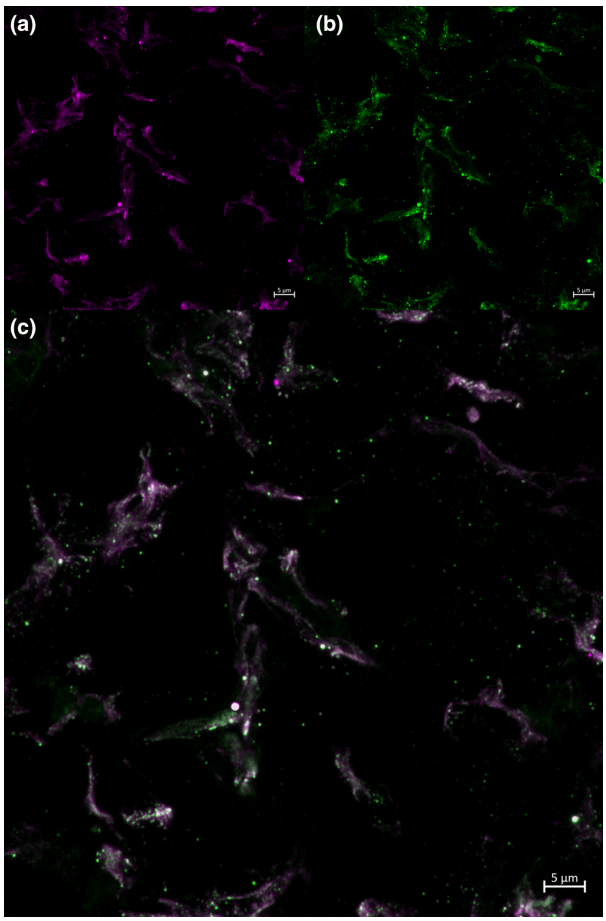

FIGURE 8 This image illustrates the distinct staining pattern of CD68 and VEGF of Hofbauer Cells with mouse anti CD68 and mouse anti VEGF using 3-day sequential staining protocol. (a) mouse anti VEGF, (b) mouse anti CD68, (c) merged digitally magnified images. Panel c demonstrates distinct patterns of staining for VEGF and CD68 within cells. This is a maximum projection from a z stack of Hofbauer cells captured using Zeiss LSM880 with AirScan Processing confocal microscope at 20× magnification. Scale bar is 5 µm.

5 | CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, VEGF was seen to be expressed in M2a, M2b, and M2c phenotypes. PA was observed to be positively correlated with M2a and M2c HBCs as a proportion of the total in term placenta, with a large effect size seen relative to PA levels mid-gestation ($r > 0.5$), and a moderate effect size seen in late gestation ($r > 0.3$). While flow cytometric analyses were not possible in this pilot study due to a lack of fresh tissue, these results provide a strong basis for future research to examine polarization differences using fluorescence-activated cell sorting. Placental CD206 protein expression was lower in individuals who were physically active throughout pregnancy compared to ones who were inactive. Placental CD206 mRNA levels were not significantly different between active and inactive individuals, suggesting a potential PA-mediated downregulation of CD206 during translation. The cause of this hypothesized protein downregulation and potential downstream consequences should be explored in further studies. Future research should also ensure that the proportions of cells are examined in conjunction with absolute numbers to avoid overlooking potentially significant discoveries. Finally, the amount of VEGF secreted from distinct HBC subtypes should be investigated in healthy and complicated pregnancies to determine if changes in polarization may affect the amount of VEGF secreted.

AUTHOR CONTRIBUTIONS

ADG, VT, and KBA contributed to study conception, design and data analysis. ADG, CE, LT, and VT carried out primary data collection and interpretation. ADG and CE performed data analysis. ADG wrote the manuscript with KBA as the corresponding author. All authors revised, edited, and approved the final version of the manuscript. All authors revised and edited the manuscript and have approved the final version.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, KBA, upon reasonable request.

ETHICAL STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The PLACENTA study was approved by the Research Ethics Board (REB) of the

University of Ottawa (file number: H11-15-29) and conformed with all aspects of the Declaration of Helsinki.

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CHAPTER 3: EXAMINING THE EFFECTS OF GESTATIONAL PHYSICAL ACTIVITY AND HOFBAUER CELL POLARIZATION ON ANGIOGENIC FACTORS

PREAMBLE TO MANUSCRIPT 2

The manuscript entitled: "*Examining the effects of gestational physical activity and Hofbauer cell polarization on angiogenic factors*" was submitted to the International Journal of Environmental Research and Public Health (IJERPH) on the 27th of February, 2023 in accordance with the requirements stipulated by the Journal. The manuscript was revised as requested on the 21st of June, 2023, and published on July 4th, 2023. This manuscript covers the second objective of this thesis as it investigates the expression and localization of angiogenic factors in relation to Hofbauer cell polarization and gestational physical activity.

International Journal of
Environmental Research and Public Health

MDPI

Article

Examining the Effects of Gestational Physical Activity and Hofbauer Cell Polarization on Angiogenic Factors

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Abstract: While gestational physical activity (PA) has demonstrated health benefits for both birthing parent and fetus, the mechanisms still need to be fully understood. Placental macrophages, or Hofbauer cells (HBCs), comprise a heterogenous population containing inflammatory (CD206-) and anti-inflammatory (CD206+) phenotypes. Similar to other tissue-resident macrophages (TRMs), HBCs are potential mediators of angiogenesis due to their secretion of both pro- and anti-angiogenic factors, including FGF2, VEGF, and SPRY2. While PA is associated with an increase in the proportion of VEGF- and FGF2-producing CD206+ macrophages in other tissues, the phenotypes producing FGF2, VEGF, and SPRY2 in the placenta and the associated relationships with gestational PA have not been studied. Using accelerometry, pregnant participants were classified as physically active or inactive in mid- and late-gestation. Term placenta tissue was collected at delivery and used for Western blotting and immunofluorescence to examine the protein expression of FGF2 and SPRY2, and to localize FGF2 in histological samples, respectively. Primary cultures of HBCs were used to examine the phenotypic differences in FGF2, SPRY2, and VEGF production. While no differences in the placental expression of SPRY2, total FGF2, or high-molecular-weight FGF2 were observed based on PA status, active individuals had significantly reduced levels of low-molecular-weight FGF2. Additionally, HBCs of all polarizations produce VEGF, FGF2, and SPRY2, and can form intercellular junctions and multinucleated giant cells. These findings suggest a possible relationship between PA and HBC-driven angiogenesis, providing an avenue for future exploration.

Keywords: physical activity; placenta; Hofbauer cells; macrophage polarization; angiogenesis; pregnancy

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1. Introduction

It has been thoroughly documented in the literature that habitual physical activity (PA) throughout gestation can contribute to the development of a healthy pregnancy and reduce the risk of complications, including pre-eclampsia, gestational hypertension, and gestational diabetes mellitus (GDM) [1]. Individuals who are consistently physically active throughout their pregnancy are also less likely to develop urinary incontinence or experience post-partum weight retention [1]. Through modulating GWG, PA also reduces the risk of delivering by caesarian section, prolonged labour, and complications requiring instrumental delivery interventions with forceps or vacuums [1]. Despite the illustrated benefits of PA throughout pregnancy, the mechanisms through which they arise are not fully understood.

The human placenta is unique, as both a transient and multifunctional organ. Serving as the interface between the gestational parent and developing fetus, it carries nutrients and oxygen to the fetus, removes waste products, and has a protective role by forming an immunological barrier. As the only immune cells in the villous chorion, Hofbauer cells (HBCs) are placenta resident macrophages of fetal origin that remain present throughout gestation [2–4]. Similar to other macrophage populations, HBCs can adopt different

phenotypes based on their microenvironment [2,5]. Generally speaking, the spectrum of polarization can be divided into classically activated, pro-inflammatory M1 subtypes, and alternatively activated, anti-inflammatory M2 subtypes. M1 macrophages are typically involved in pathogen resistance, while M2 macrophages act as mediators of the inflammatory response and contribute to tissue growth and repair. M2 populations can be further diversified, containing M2a, M2b, M2c, and M2d phenotypes. In keeping with other tissue-resident macrophage populations, HBCs possess heterogenous states of polarization [2,5]. Healthy pregnancies are characterized by a lack of an M1 phenotype, with M2a and M2c (CD206+) HBCs comprising the majority. M2b phenotypes are present in healthy pregnancies; however, greater expression of the M2b phenotype has been associated with detrimental conditions, such as gestational diabetes mellitus (GDM) [6].

HBCs have been shown to play a part in the development of the placental circulatory system. The vasculature of the placenta is special in the fact that it contains two distinct vascular networks. The uteroplacental and fetoplacental circulatory systems carry parental and fetal blood, respectively, to the gestational interface in order to facilitate nutrient and gas exchange [7]. To develop this vasculature, the growing placenta undergoes two critical processes. Vasculogenesis, or the de novo formation of new blood vessels, and angiogenesis, the development of the smaller vessels that form the capillary beds (Figure 1) [8]. HBCs have been implicated in both vasculo- and angiogenesis in the placenta through their secretion of angiogenic factors, such as fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF) [5,9]. Interestingly, in recent years, the FGF2-mediated angiogenesis inhibitor Sprouty 2 protein (SPRY2) has also been shown to be secreted by HBCs [10]. Isoforms of FGF2 include high molecular weight (HMW) and low molecular weight forms [11]. HMW-FGF2 is a mitogenic factor in the nucleus, while LMW-FGF2 is secreted to promote angiogenesis [11]. The expression of both pro- and anti-angiogenic factors by HBCs makes them likely regulators of blood vessel development. It is currently unclear if HBC polarization leads to differences in SPRY2 secretion, and while M2 macrophages have been shown to secrete both VEGF and FGF2, with CD206+ macrophages being the main producers of FGF2 [12], the role of HBC subtypes in the secretion of VEGF and FGF2 has not been well studied. In previous research conducted by our lab, physically active individuals were shown to express higher levels of VEGF [13], and have augmented proportions of CD206+ cells, indicative of an M2a or M2c phenotype [14]. As such, this study aimed to explore which HBC subtypes expressed FGF2, VEGF, and SPRY2, as well as to examine the potential associations between PA and the expression of FGF2 and SPRY2.

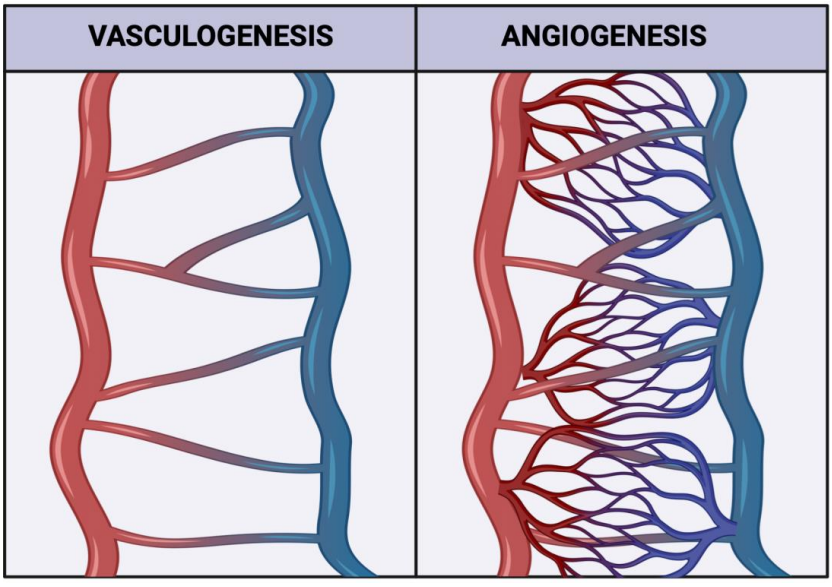

Figure 1. Representative schematic illustrating how both vasculogenesis and angiogenesis contribute to forming a mature vascular network. Created with biorender.com (accessed on 11 September 2022).

2. Materials and Methods

2.1. Participant Recruitment and Ethical Approval

Pregnant individuals from Ottawa, Ontario, were invited to participate in the PhysicaL Activity and diEtary implicatioNs Throughout pregnAncy (PLACENTA) study. Par-

2. Materials and Methods

2.1. Participant Recruitment and Ethical Approval

Pregnant individuals from Ottawa, Ontario, were invited to participate in the PhysicaL Activity and diEtary implicatioNs Throughout pregnAncy (PLACENTA) study. Participants were pre-screened by phone to assess study edibility by the a priori inclusion and exclusion criteria (Table 1). Informed written consent was obtained before enrollment into the PLACENTA study. The PLACENTA study was approved by the Research Ethics Board (REB) of the University of Ottawa (file number: H11-15-29) and conformed to all aspects of the Declaration of Helsinki.

Table 1. Inclusion and exclusion criteria for participation in the PLACENTA study.

Inclusion Criteria	Inactive
• Between 18 and 40 years of age • Proficiency in English and/or French • Gestational age below 28 weeks • Stable pre-pregnancy weight (± 5 lbs) • Pregnant with a singleton fetus • Normal or overweight BMI status (18.5–29.9 kg/m^3)	• Contraindications to physical activity • Pre-pregnancy diabetes • Untreated thyroid disease

2.2. Accelerometry Data Capture

Individuals completed two in-person assessments in mid (24 to 28 weeks gestation) and late (34 to 38 weeks gestation) pregnancy. At the end of each visit, a take-home package was provided that contained an omnidirectional Actical® accelerometer (Philips Respironics, Bend, OR, USA), which was to be worn around the waist during waking hours over a period of seven consecutive days to record periods of free-living PA. A minimum of three completed days with ten or more wear hours per day was required to be included in further analyses [15]. Accelerometry data were analyzed to yield the minutes of PA accumulated over the seven days. As per the Canadian Health Measures Survey procedures, data analysis was performed as previously described [16] using SAS version 9.4. PA status was assessed based on the 2019 Canadian guideline for physical activity during pregnancy recommendation to accumulate a total of 150 min of moderate PA (MPA) each week, or an average of 21.4 min per day [1]. Participants in the physically active group averaged 21.4 or more minutes per day of moderate-to-vigorous PA (MVPA). Participants in the physically inactive group averaged less than 21.4 MVPA minutes per day. The most and least physically active participants were chosen for future analysis.

2.3. Collection of Term Placenta Samples

Within an hour of vaginal delivery or caesarian section, term placentae were collected and the umbilical cord and chorioamniotic membranes were removed. All sampling was performed on ice and avoided areas of excess calcifications, abruptions, or necrosis. Tissue samples of approximately 2.5 cm^3 were taken from both central and peripheral cotyledons and then divided into smaller pieces of approximately 1 cm^3. Dissected samples were placed into cryovials and flash-frozen in liquid nitrogen for transport, then stored at −80 °C. To isolate placenta protein lysate, frozen samples were powdered on ice and homogenized with the Powergen 125 homogenizer (Fisherbrand, Pittsburg, PA, USA) in radioimmunoprecipitation buffer (BioRad, Hercules, CA, USA). Lysate underwent 1000× *g* centrifugation at 4 °C for 10 min to remove debris. In addition to tissue samples, full-thickness histological samples were taken from the fetal side of healthy cotyledons. Sections were fixed in formalin for 48 h before being embedded in paraffin blocks.

2.4. Western Blotting

The total protein content of placenta tissue lysate was determined using a DC protein assay (Bio-Rad Laboratories, Mississauga ON, Canada). Thirty and forty µg of protein were

loaded onto Mini-PROTEAN® TGX gel (Bio-Rad Laboratories, ON, Canada) for Western blot analysis of SPRY2 and FGF2 protein expression, respectively. The protein content was resolved using SDS-page electrophoresis in reducing conditions at 150 volts for 1 h. Following electrophoresis, the protein was transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, ON, Canada) and blocked with 5% non-fat dry milk in tris buffered saline solution (TBST) for 1 h at room temperature (RT). The membranes were incubated at 4 °C overnight in primary antibodies diluted at 1:1000 in 5% milk. The primary antibodies used were rabbit monoclonal recombinant anti-FGF2 antibody (1:1000, ab92337, Abcam, Toronto ON, Canada) and rabbit monoclonal recombinant anti-SPRY2 antibody (1:1000, ab180527, Abcam). Thereafter, blots were washed with TBST before a 1 h incubation at RT with the horseradish-peroxidase conjugated secondary antibody, goat anti-mouse IgG (1:5000, Bio-Rad Laboratories, ON, Canada). After washing with TBST, the blots were developed with Clarity ECL Western Substrate (Bio-Rad Laboratories, ON, Canada) and imaged using the ChemiDoc™ XRS+ Imaging System (Bio-Rad Laboratories, ON, Canada). Amido black at a 1% concentration was used to permanently stain the blots for total protein lane quantification. Densitometric analysis (Image J, Bio-Rad Laboratories, ON, Canada) was used to quantify the relative expression of the target band and total protein. Protein expression of FGF2 and SPRY2 were standardized to total participant pooled lysate samples.

2.5. Hofbauer Cell Culture

Primary cultured isolated HBCs were purchased from the Amnion Foundation (Winston-Salem NC, USA). Preliminary experiments were conducted to determine the optimal culture time. As per manufacturer instructions, cells were cultured for a maximum of 7 days before examination. It was determined that by reducing the total culture time to 5 days, cell viability at the end of the period was moderately improved.

Cells were seeded in wells 1–7 (Figure 2) on iBidi 8-well slides (WI, USA), while well 8 served as a contamination control. Cultures were maintained in Gibco RPMI Media (Thermo Fisher Scientific, Nepean, ON, Canada), supplemented with 5% fetal bovine serum (FBS) and 25 mM HEPES, at 37 °C and 5% CO_2. To mimic the chronic physiological normoxic conditions of the term placenta, an oxygen concentration of 8% was used [17]. HBC complete media was filtered using a Stericup quick release-VP sterile vacuum filtration system with a 0.1 µm pore size to mitigate the risk of mycoplasma contamination. For all experiments, cells were seeded and incubated overnight to allow for adhesion. Media was changed at 24 and 72 h post-seed, before fixation and staining at 120 h post-seed.

Figure 2. A schematic of the iBidi slide layout relative to HBC culture and application of primary antibodies.

2.6. Immunofluorescence Staining

2.6.1. In Vitro Immunofluorescence Staining

Immunofluorescent staining was performed to localize FGF2, SPRY2, and VEGF within cultured HBCs. After being cultured for 5 days in normoxic conditions, the media were removed from each iBidi well, and cells were washed three times in sterile PBS. The protocol for cell fixation and permeabilization was performed at RT as follows. Cells were fixed with 4% paraformaldehyde diluted in sterile PBS for 15 min, then washed three

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2.6.2. In Vivo Immunofluorescence Staining

Immunofluorescent staining was again performed to localize FGF2 in relation to HBCs in collected placenta tissue. Formalin-fixed paraffin-embedded (FFPE) tissue was processed into 4 µm thick sections and mounted on slides by the Louise Pelletier Histology Core at the University of Ottawa. Slides were deparaffinized and rehydrated using xylene and ethanol in a graded series of dilutions and rinsed in double-distilled water. Sodium citrate buffer (10 mM pH 6.0) was utilized during heat-induced epitome retrieval before the tissue was permeabilized in 0.2% Tiron-X in TBS for 20 min at RT. To block non-specific binding and to quench tissue autofluorescence, the slides were incubated at RT for 1 h in 10% bovine serum albumin, then for 10 min in 0.1% Sudan Black in 75% EtOH (w/v). Primary antibodies for CD68 (mouse monoclonal; Abcam ab201973) and CD206 (rabbit polyclonal; Abcam ab64693) were diluted in TBST at 4 µg/mL and 2 µg/mL, respectively. Slides were incubated in the diluted antibodies overnight at 4 °C. Once the incubation was complete, slides were washed in TBST and then incubated in the dark for 1 h with a secondary antibody solution containing a 1:250 dilution of Alexa 488 goat anti-mouse and a 1:1000 dilution of Alexa 594 goat anti-rabbit antibodies (Thermofisher). Slides were rinsed three times for five minutes in TBST then incubated overnight at 4 °C in a 1:1000 dilution of rabbit monoclonal recombinant anti-FGF2 antibody (1:200, ab92337, Abcam). Following the overnight incubation, a 1:1000 dilution of the secondary antibody Alexa 647 goat anti-rabbit (Thermofisher) was applied for 1 h at room temperature. After the slides were rinsed again three times for five minutes in TBST, Prolong Gold with DAPI was used to mount the slides, and the edges were sealed with nail polish. The application of primary antibodies

was omitted in negative controls. Slides were imaged using a fluorescent microscope (AxioObserver M2, Zeiss) equipped with blue (excitation 390/22 nm, emission 460/50 nm), green (excitation 470/40 nm, emission 525/50 nm), and red (excitation 560/40 nm, emission 630/75 nm) filters to visualize the cells of interest.

2.7. Statistical Analysis

GraphPad Prism software (version 9.0.0, GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analyses of data, which are presented herein as mean ± standard deviation. The normality of data was tested with Shapiro–Wilks tests. Unpaired *t*-tests or Mann–Whitney U tests, where appropriate, were used to analyze participant demographics, Western blot protein expression, and cell counts between active and inactive participants. Pearson correlations were also used to analyze the protein expression and cell counts in comparison to the average minutes of MVPA/day accumulated by each participant. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Participant Demographics

Maternal demographic information and newborn outcomes are described in Table 2 according to PA status throughout gestation. By study design, physically active participants had significantly higher MVPA (min/day) than their inactive counterparts in both mid and late gestation ($p < 0.001$). Maternal age, height, pre-pregnancy weight, or pre-pregnancy BMI did not differ significantly between active and inactive groups. On average, neonates born to physically inactive participants had significantly higher birth weights and lengths when compared to their counterparts born to active individuals.

Table 2. Study participant maternal demographics and newborn outcomes (n = 17). Statistically significant p-values ($p < 0.05$) are indicated in bold.

	Active (n = 10)	Inactive (n = 7)	p-Value
Maternal demographics			
Maternal age (years)	32.50 ± 3.14	32.29 ± 2.69	0.8854
Height (cm)	165.80 ± 6.77	167.19 ± 8.68	0.7163
Pre-pregnancy weight (kg)	64.44 ± 10.87	67.64 ± 15.87	0.6271
Pre-pregnancy BMI (kg/m^2)	23.39 ± 2.80	23.94 ± 3.48	0.7219
Gestational age at birth (weeks)	40.47 ± 0.90	41.02 ± 0.49	0.1658
Mid gestation MVPA (min/day)	46.79 ± 10.23	6.44 ± 4.01	**<0.0001**
Late gestation MVPA (min/day)	34.65 ± 9.50	3.34 ± 3.43	**<0.0001**
Newborn outcomes			
Birth weight (kg)	3.31 ± 0.38	3.75 ± 0.23	**0.0169**
Birth length (cm)	50.13 ± 2.46	52.69 ± 1.39	**0.0260**
Sex, *n*			
Male	6	3	
Female	4	4	

3.2. Expression of FGF2 and SPRY2 in Term Placenta

The protein expression of placental CD68 and CD206 were analyzed to determine the difference in expression between active and inactive participants by *t*-test or Mann–Whitney U test where applicable. No significant difference in the protein expression of total FGF2, HMW FGF2, or SPRY2 was found between active and inactive women ($p > 0.05$). However, the protein expression of LMW FGF2 was significantly lower in active women when compared to their inactive counterparts (Figures 3 and 4).

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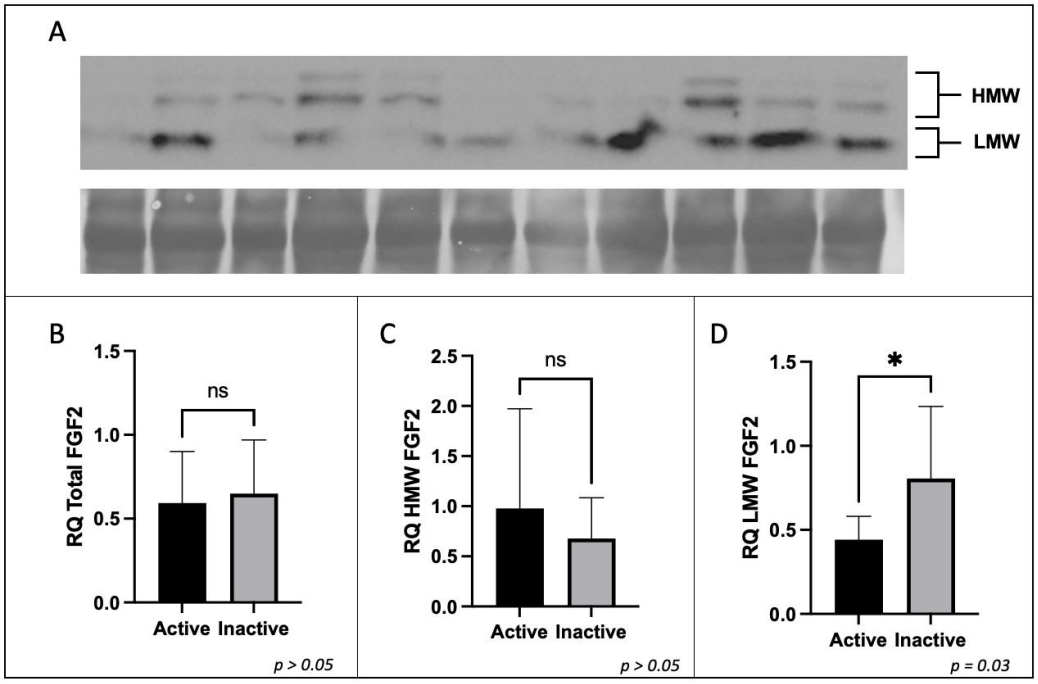

Figure 3. Expression of FGF2 protein by Western immunoblotting in term placenta (2:1 ratio of tissue from the central and peripheral regions of the placenta, respectively) from physically active ($n = 10$) and inactive ($n = 7$) participants. A representative immunoblot for (**A**) FGF2 is shown. The corresponding semi-quantitative densitometric analysis is shown for (**B**) total FGF2 expression, (**C**) high molecular weight FGF2 expression, and (**D**) low molecular weight FGF2 expression. All data are represented as mean ± SD. * $p \leq 0.05$. ns: not significant.

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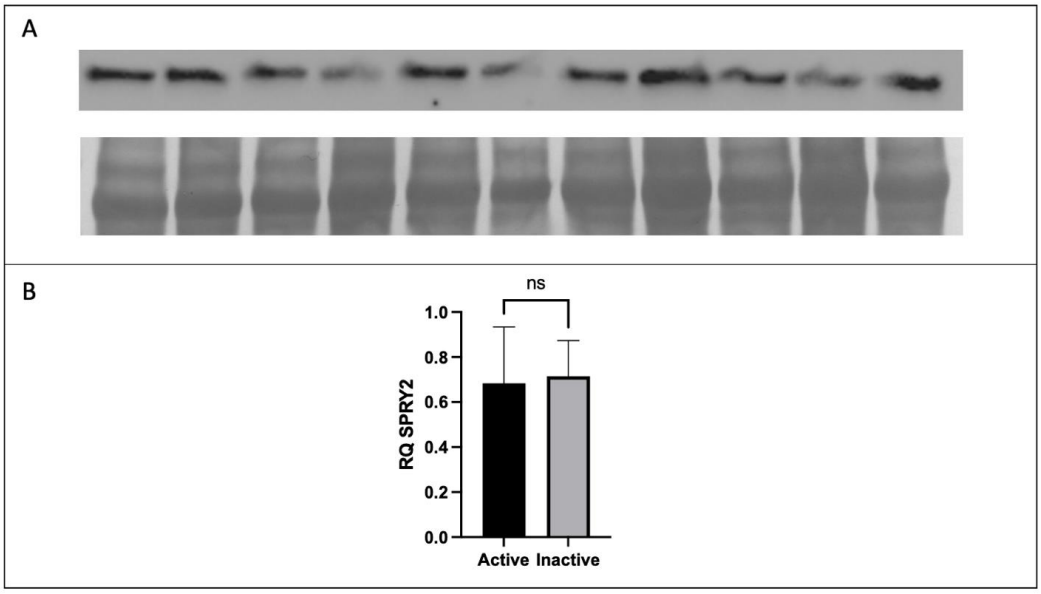

Figure 4. Expression of SPRY2 protein by Western immunoblotting in term placenta (2:1 ratio of tissue from the central and peripheral regions of the placenta, respectively) from physically active ($n = 10$) and inactive ($n = 7$) participants. A representative immunoblot for (**A**) SPRY2 is shown. The corresponding semi-quantitative densitometric analysis is shown for (**B**) SPRY2 expression. All data are represented as mean ± SD. * $p \leq 0.05$. ns: not significant.

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3.3. Expression of FGF2 in Term Placenta

In vivo immunofluorescence studies showed the localization of FGF2, the pa
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3.3. Expression of FGF2 in Term Placenta

In vivo immunofluorescence studies showed the localization of FGF2, the pan-macrophage marker CD68, and M2a/M2c macrophage marker CD206 in both active (Figure 5) and inactive (Figure 6) participants. In term placenta, it can be observed that FGF2 is colocalized within cells that express CD68, regardless of the presence or absence of CD206, indicative of FGF2 expression in M2a, M2b, and M2c phenotypes.

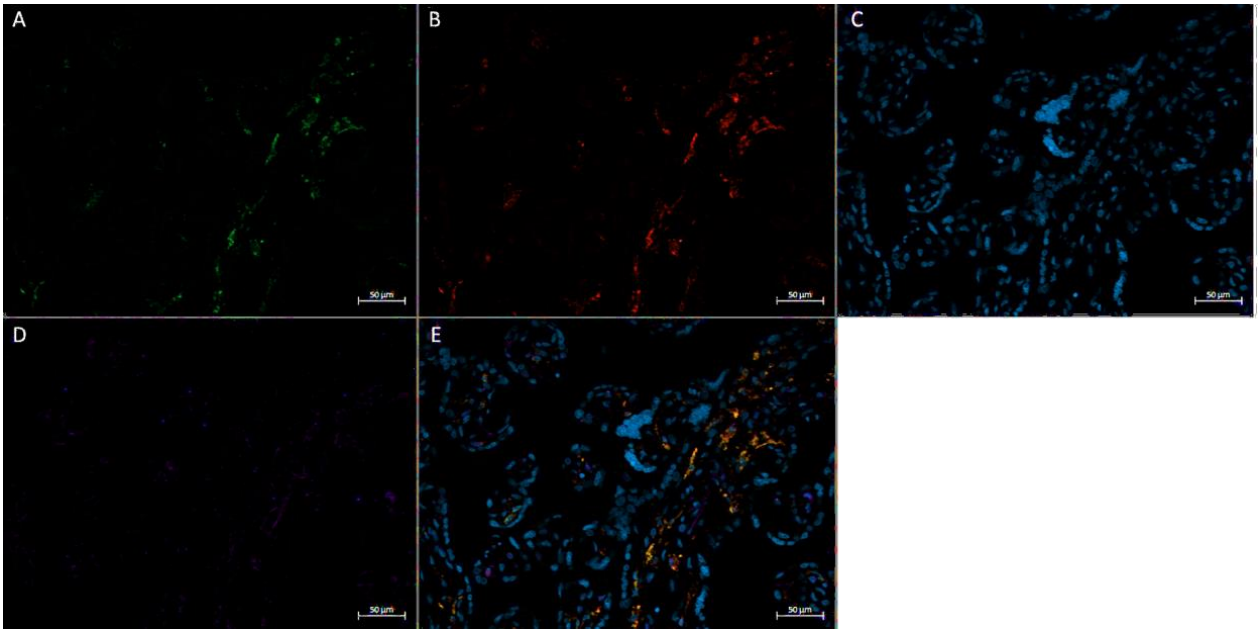

Figure 5. Immunofluorescence staining of CD68, CD206, and FGF2 of a physically active participant. FFPE placenta tissue slide imaged with an Axio Imager M2 epifluorescent microscope. (**A**) CD206; (**B**) FGF2; (**C**) DAPI (cell nuclei); (**D**) CD68; (**E**) merged image. Scale bar is 50 µm.

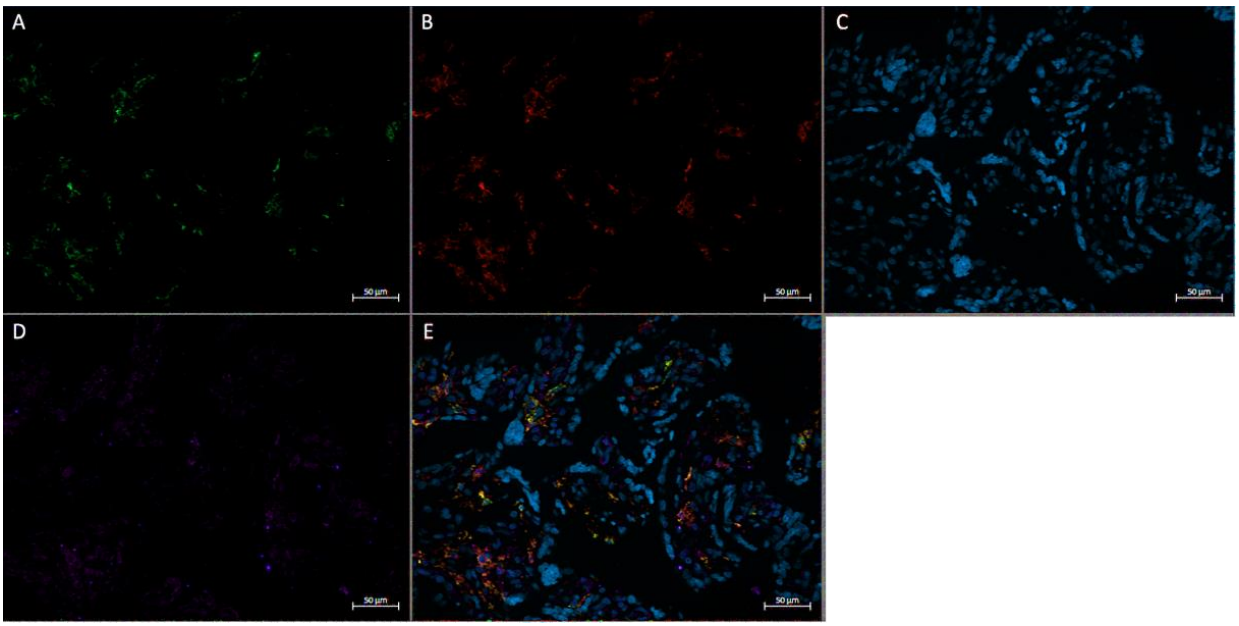

Figure 6. Immunofluorescence staining of CD68, CD206, and FGF2 of a physically inactive participant. FFPE placenta tissue slide imaged with an Axio Imager M2 epifluorescent microscope. (**A**) CD206; (**B**) FGF2; (**C**) DAPI (cell nuclei); (**D**) CD68; (**E**) merged image. Scale bar is 50 µm.

3.4. Expression of Angiogenic Markers in Cultured Hofbauer Cells

Consistent with our findings stated herewithin, as well as previous work by our lab [14], it was seen that both FGF2 (Figures 7 and 8) and VEGF (Figure 9) were colocalized within cells expressing CD68 with or without the expression of CD206. In a similar manner to both FGF2 and VEGF, SPRY2 was also found in all CD68+ cultured HBCs (Figures

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Morphologically, HBCs in culture were observed to possess the high degree of adhesive properties that is often observed in macrophage populations. This is demonstrated through macrophage aggregates (Figures 8 and 9), and intercellular connections (Figures 10 and 11).

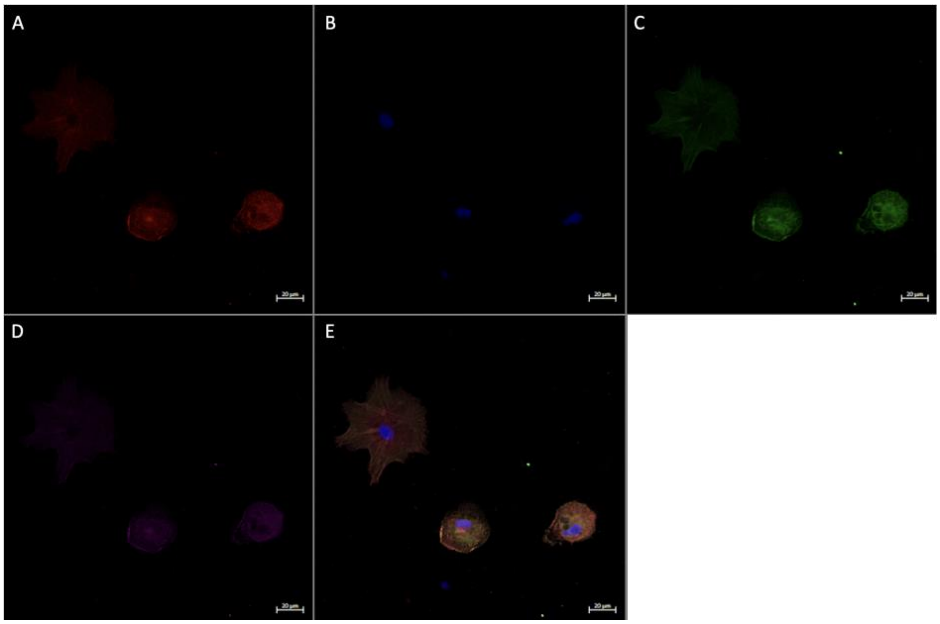


Figure 7. Immunofluorescence staining of CD68, CD206, and FGF2 in cultured Hofbauer cells imaged at 20× magnification with the Zeiss LMS880 AxioObserver Z1 confocal microscope. (**A**) CD206; (**B**) DAPI (cell nuclei); (**C**) CD68; (**D**) FGF2; (**E**) merged image. Scale bar is 20 µm.

Figure 7. Immunofluorescence staining of CD68, CD206, and FGF2 in cultured Hofbauer cells imaged at 20× magnification with the Zeiss LMS880 AxioObserver Z1 confocal microscope. (**A**) CD206; (**B**) DAPI (cell nuclei); (**C**) CD68; (**D**) FGF2; (**E**) merged image. Scale bar is 20 µm.

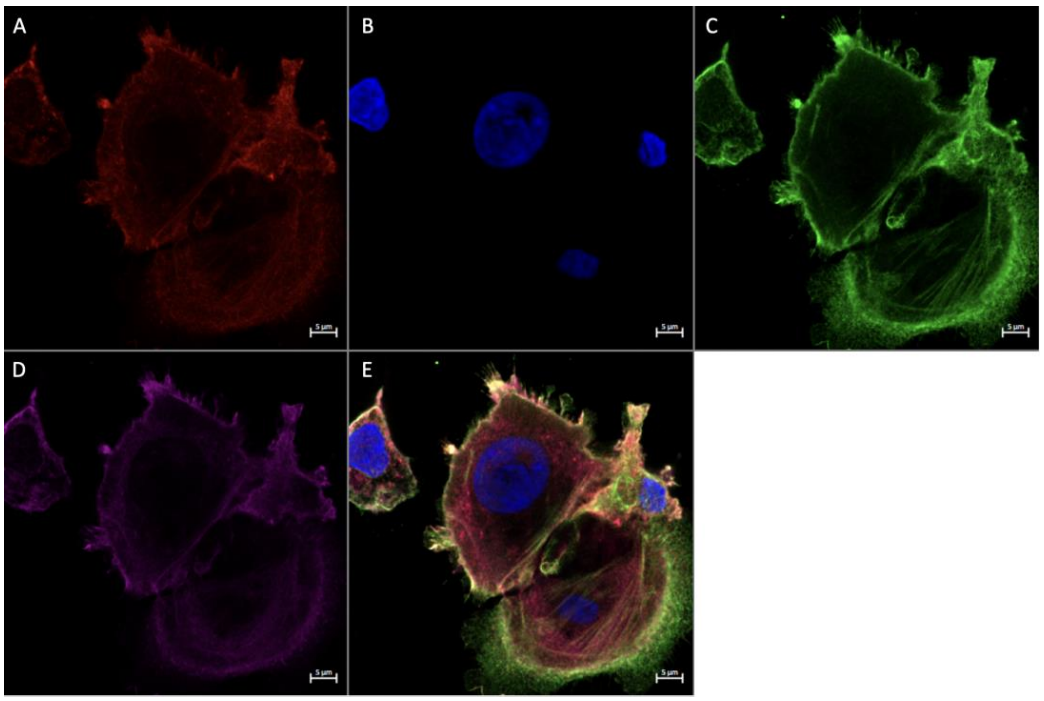
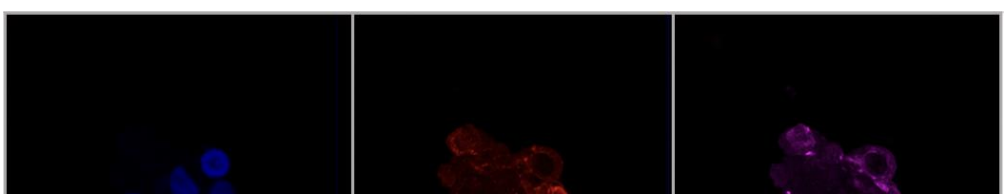


Figure 8. Immunofluorescence staining of CD68, CD206, and FGF2 in cultured Hofbauer cells imaged at 63× magnification with the Zeiss LMS880 AxioObserver Z1 confocal microscope. (**A**) CD206; (**B**) DAPI (cell nuclei); (**C**) CD68; (**D**) FGF2; (**E**) merged image. Scale bar is 5 µm.

Figure 8. Immunofluorescence staining of CD68, CD206, and FGF2 in cultured Hofbauer cells imaged at 63× magnification with the Zeiss LMS880 AxioObserver Z1 confocal microscope. (**A**) CD206; (**B**) DAPI (cell nuclei); (**C**) CD68; (**D**) FGF2; (**E**) merged image. Scale bar is 5 µm.



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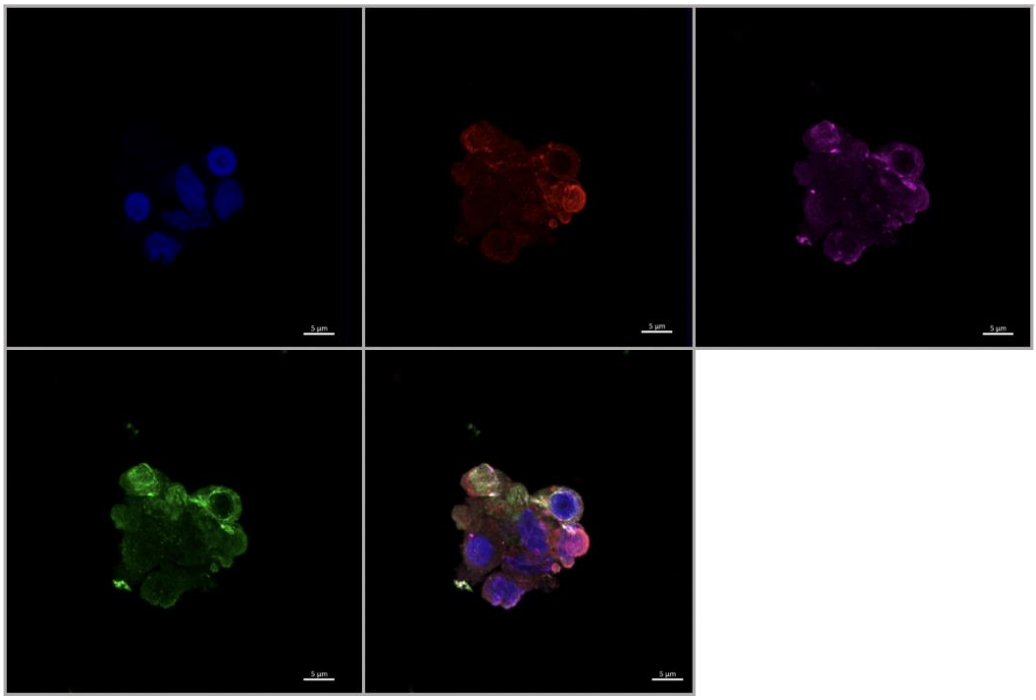


Figure 9. Immunofluorescence staining of CD68, CD206, and VEGF in cultured Hofbauer cells imaged at 63× magnification with the Zeiss LMS880 AxioObserver Z1 confocal microscope. (**A**) CD206; (**B**) DAPI (cell nuclei); (**C**) CD68; (**D**) VEGF; (**E**) merged image. Scale bar is 5 μm.

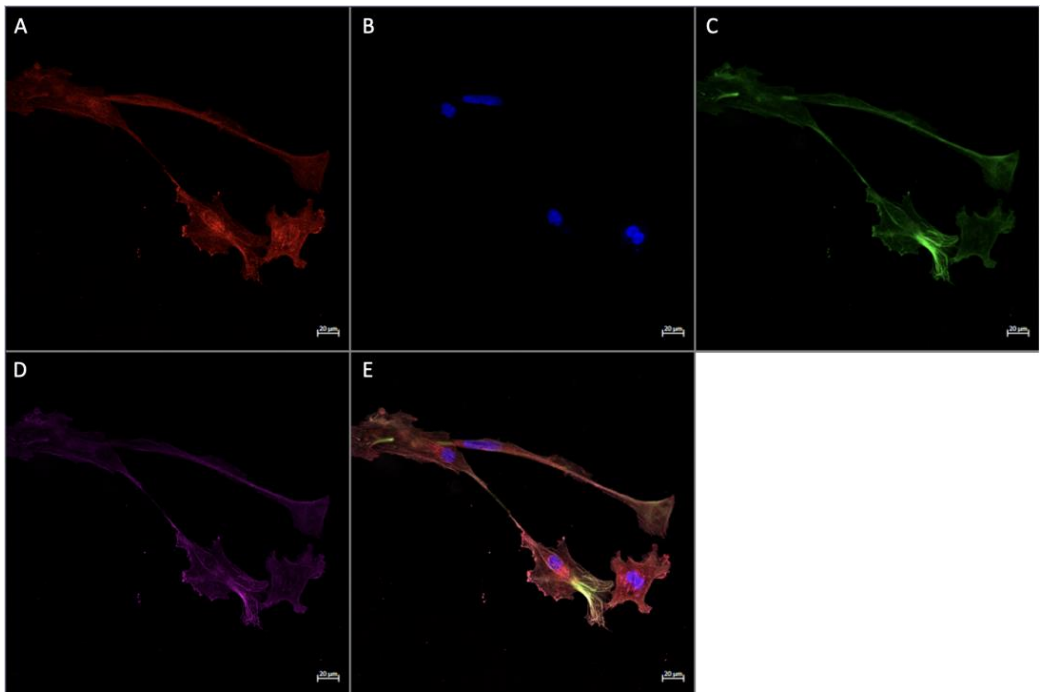
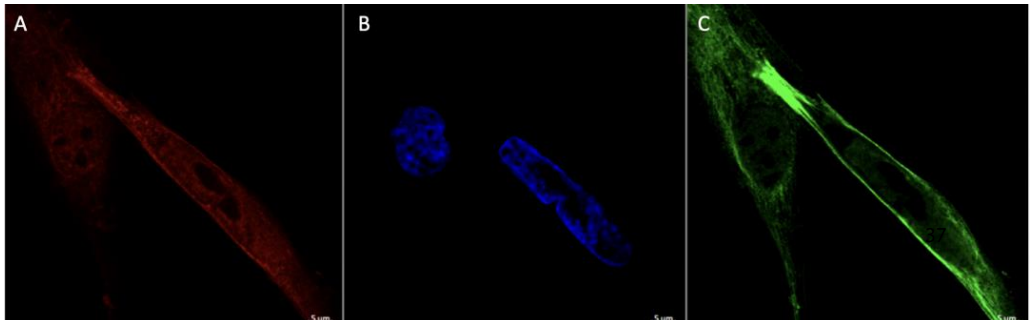


Figure 10. Immunofluorescence staining of CD68, CD206, and SPRY2 in cultured Hofbauer cells imaged at 20× magnification with the Zeiss LMS880 AxioObserver Z1 confocal microscope. (**A**) CD206; (**B**) DAPI (cell nuclei); (**C**) CD68; (**D**) SPRY2; (**E**) merged image. Scale bar is 20 μm.



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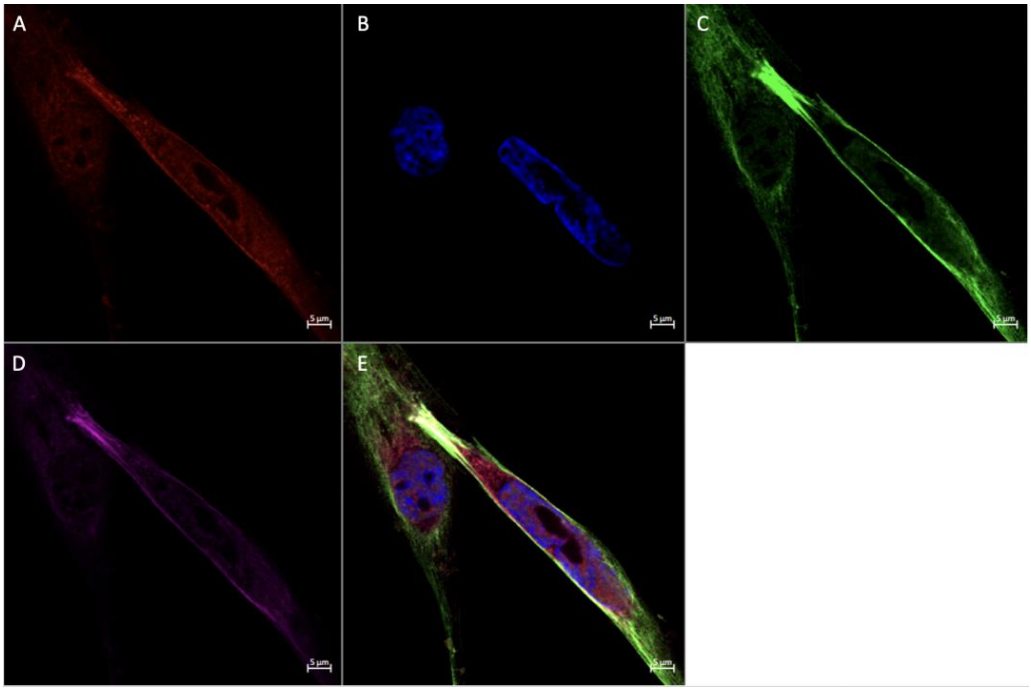

Figure 11. Immunofluorescence staining of CD68, CD206, and SPRY2 in cultured Hofbauer cells imaged at 63× magnification with the Zeiss LMS880 AxioObserver Z1 confocal microscope. (**A**) CD206; (**B**) DAPI (cell nuclei); (**C**) CD68; (**D**) SPRY2; (**E**) merged image. Scale bar is 5 µm.

4. Discussion

Morphologically, HBCs in culture were observed to possess the high degree of adhesive properties that is often observed in macrophage populations. This is demonstrated through macrophage aggregates (Figures 8 and 9) and intercellular connections (Figures 10 and 11).

4. Discussion

It has been demonstrated in past research that HBCs express both the pro-angiogenic factor FGF2 and anti-angiogenic factor SPRY2 [3,7,8], marking them as potential contributors to the development of vasculature in the placenta. Recent findings from our lab showed that physically active participants have elevated proportions of CD206+ subtypes (Goudreau, unpublished). In other tissue-resident macrophages, these phenotypes have been identified as the drivers of FGF2 [18], while SPRY2 expression in different macrophage polarizations has not been determined. As such, this novel study aimed to elucidate the role of PA and macrophage polarization in the production of FGF2 and SPRY2 within the placenta. Though there were no observed differences in the protein expression of SPRY2, total FGF2, or HMW FGF2 between active and inactive participants, active individuals had significantly lower levels of LMW FGF2 in term placenta. We also report for the first time that HBCs of all polarizations produce VEGF, FGF2, and SPRY2. Additionally, we observed that HBCs demonstrate the ability to form filopodia and cell aggregates, marking them as potential candidates to develop intercellular junctions and multinucleated giant cells (MGC).

The establishment of a vascular network within the placenta is a crucial process that allows it to act as a multi-organ surrogate for the developing fetus. As the placenta develops, the chorionic villi containing fetal capillaries expand into the intervillous space to intermingle with parental vessels [19]. The transport of gases, nutrients, and waste at this interface allows the placenta to act as the lungs, liver, and kidneys of the fetus, while also providing nutrients from parental circulation [20]. HBCs are a mononuclear phagocytic cell family member, which also includes other tissue-resident macrophages such as osteoclasts, alveolar macrophages, Langerhans cells, and microglia [21–24]. Such macrophages, including HBCs, have been implicated in the development of vasculature through the expression of pro- and anti-angiogenic factors, including SPRYs, FGFs, and

VEGF. While there were significant differences observed in LMW-FGF2 protein expression in the term placenta between active and inactive participants, no differences were seen in the expression of HMW-FGF2, T-FGF2, or SPRY2. A previous study from our lab found that VEGF protein and mRNA levels were significantly higher in active individuals [13]. Interestingly, the opposite effect was associated with LMW-FGF2, with active participants expressing significantly less of the protein. One explanation for this may be that because VEGF-driven angiogenesis results in higher vascular permeability than FGF2, the combination of higher VEGF and lower LMW-FGF2 levels may lead to capillary beds with higher vascular permeability, increasing the efficacy of gas and nutrient exchange [25]. Additionally, in contrast to FGF2, VEGF has anti-inflammatory effects, potentially protecting developing tissues from the implantation-induced inflammation and aiding the subsequent shift to an anti-inflammatory environment for the remainder of gestation [26–28].

As for other contributors to the development of placental vasculature, SPRYs are membrane-associated proteins that regulate receptor tyrosine kinase (RTK) signalling [8]. The SPRY homologs act as negative regulators for several RTK-induced pathways, including the mitogen-activated protein kinase (MAPK), P13K/Akt, PLC γ pathways, which are stimulated by growth factors such as VEGF and FGF2 [29–33]. While there were differences between PA groups in FGF2 and VEGF protein levels, SPRY2 remained unchanged based on PA. The literature clearly demonstrates that dysregulations in RTK pathways can have deleterious effects. While underactive pathways lead to insufficient angiogenesis and decreased cell survival [34], overexpression has been linked to proliferative disorders and vascular anomalies [35,36]. Thus, it is possible that SPRY2 expression is highly conserved in order to prevent deleterious dysregulations of RTK-induced pathways, thereby adding a level of protection to the development of the vascular network.

As both CD206+ and CD206- HBCs expressed SPRY2 and FGF2 in in vitro and in vivo models, it is logical to posit that all subtypes contribute to the production of the angiogenic factors, reinforcing the recent focus on HBCs as mediators of placental angiogenesis. The quantity of FGF2 isomers secreted from distinct HBC subtypes in both healthy and complicated pregnancies, as well as the potential significance FGF2 expression has on fetal outcomes, should be explored in future research.

Using immunofluorescence to visualize HBCs in culture, we observed their tendency to interact with each other, demonstrated by the formation of filopodia and cellular aggregates. Interestingly, both aforementioned processes are critical steps in the formation of multinucleated giant cells (MGCs) [37]. It has been shown that M2 macrophages, which form the majority of the HBC population, express chemokine ligand 2 (CCL2) [38]. Under the influence of CCL2, macrophage motility increases and membrane-protruding filopodia are formed [37]. Interacting filopodia form intercellular connections that facilitate the genesis of cell aggregates, within which macrophages adhere and rearrange their cytoskeletons, extracellular membranes, and organelles [37]. In this manner, MGCs are formed. To the best of our knowledge, the study by Kesson et al. (1993) provides the only other evidence of this characteristic in cultured HBCs, defined as placental macrophages of fetal origin and distinct from macrophages in the decidua [39]. Historically, the role of MGCs has been linked to cytopathic effects, such as those seen by viral infections [40]. While the inference that MGC formation is indicative of adverse conditions was consistent with the literature at the time, a recent review by Miron et al. (2018) suggested that MGC formation in and of itself may represent neither a healthy nor detrimental environment, and points to the necessity of characterizing the polarization states of such cells [40]. Further research is needed to characterize the profiles of multinucleated giant HBCs and their associated attributes. This research should include immunofluorescence experiments to identify known fusogens (molecules that fuse membranes) and junction proteins present within the process of MGC formation. Electron microscopy could be used to provide more insight into the morphology of these potential placental structures.

There are inherent strengths and limitations associated with the study. It is strengthened by the objective measurement of PA through accelerometry at multiple gestational

time points and following a standardized protocol for placenta collection and sample preparation, including using homogenized tissue from multiple placental locations to account for tissue heterogeneity. We also controlled for maternal age, BMI status, and gestational age at delivery. Although the neonates of physically inactive individuals had statistically higher weights and lengths at delivery, all of the measurements were within the appropriate gestational age (AGA) range. Western blotting and immunofluorescence are limited due to their semi-quantitative or qualitative natures. Though flow cytometric analyses were not possible in this pilot study due to a lack of fresh tissue, these results provide a strong basis for future research to examine polarization differences using fluorescence-activated cell sorting in both in vivo and in vitro models. While ensuring that placenta samples from all participants were obtained at term meant it was possible to control for a potential confounding variable, the interactions between activity, HBCs, and angiogenic factors should be examined at multiple gestational time points. As angiogenesis is a cross-gestational process that occurs as placenta vasculature develops and remodels, the results obtained from term tissue may not be generalizable to other gestational time points. To reduce the risk of secondary antibody bleed-through in immunofluorescence experiments resulting in false positive staining, fluorophores with distinct spectra were selected, and single stain controls of each antibody were performed. As the sample size contained within the study is small and relatively homogenous, it is possible that results may not be representative of the larger population. The observations recorded here should be examined in heterogenous cohorts and larger sample sizes.

5. Conclusions

In conclusion, while there were no significant differences in the protein expression of SPRY2, total FGF2, or HMW FGF2 based on participant activity status, LMW was lower in active individuals. Combined with previous data observing increased levels of VEGF in physically active participants, there may be a difference in the mechanism of angiogenesis in the placenta to facilitate optimized nutrient and gas exchange between parent and fetus. Furthermore, HBCs of all polarizations both in vivo and in vitro produce VEGF, FGF2, and SPRY2, which strengthens the previous literature implying the role of placental macrophages in gestational angiogenesis. Finally, the capabilities of HBCs in vitro to form intracellular junctions and MGCs provide points of interest for future research into the possible polarizations of MGCs and the interactions of macrophagic networks. The results contained herewithin should drive further exploration of the roles of HBCs in angiogenesis, and the potential effects of gestational PA.

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CHAPTER 4: DISCUSSION AND GENERAL CONCLUSIONS

4.1 KEY FINDINGS

This thesis endeavored to explore the relationship between physical activity, HBC polarization, and the expression of select angiogenic markers in the term placentas of uncomplicated pregnancies. Through this investigation, it was observed that physically active individuals had higher proportions of $CD206^+$ HBCs and a lower protein expression of CD206 and the LMW FGF2 isoform than inactive individuals.

The manuscript entitled *Characterization of Hofbauer cell polarization and VEGF expression in human term placenta from active and inactive pregnant individuals* which formed the second chapter of this thesis described the associations between PA and HBC polarization. We hypothesized that physically active individuals have fewer Hofbauer cells with an augmented proportion of $CD206^+$ polarization state in the placenta at term. Neither the absolute counts of total HBCs nor $CD206^+$ HBCs were affected by physical activity status. Active individuals were found to have higher proportion of $CD206^+$ cells in the total HBC population, and there were correlations between the proportion of $CD206^+$ HBCs and the average minutes of MVPA per day in both mid and late gestation, with a higher effect size being observed in mid-pregnancy. There were no changes between activity status in CD68 mRNA or protein expression, nor in CD206 mRNA; however, CD206 protein was significantly lower in active individuals.

The third chapter of this thesis reported the relationship between PA, HBC polarization, and the expression of angiogenic factors, specifically VEGF, SPRY2, and FGF2, in the article *Examining the effects of gestational physical and Hofbauer cell polarization on angiogenic factors.* We hypothesized that PA would be associated with an increased expression of FGF2 and its isoforms,

and that VEGF and FGF2 would be produced by $CD206^+$ HBCs. Due to the limited literature on the effects of either PA or macrophage subtypes on the expression of SPRY2, the investigation of SPRY2 expression and localization was considered exploratory. In active individuals, SPRY2, T-FGF2, and HMW-FGF2 were unchanged when compared to the inactive population; however, a relationship between PA and the expression of LMW-FGF2 was identified, with lower levels of the protein found in the placentae of active participants. VEGF, SPRY2, and FGF2 were identified in all HBC subtypes in both *in vitro* and *in vivo* models.

4.2 CONNECTING PHYSICAL ACTIVIY, HOFBAUER CELLS, AND ANGIOGENIC FACTORS

The potential of maternal lifestyle behaviours during pregnancy to affect the short- and long-term health of both mother and fetus has been well documented throughout the literature. As the interface between maternal and fetal systems, the health and function of the placenta is imperative for a successful pregnancy. Due to the effects of PA on other TRM populations, we aimed to investigate whether habitual PA throughout gestation could influence the polarization and functions of HBCs. In the first study of this thesis, detailed in the second chapter, we hypothesized that physically active individuals would have a lower number of Hofbauer cells that contained a higher proportion of $CD206^+$ cells in term placenta. Contrary to our hypothesis, we did not observe a difference in the absolute number of total HBCs. Inflammatory states, such as the ones observed in sedentary populations (24,53–55), are often characterized by an increased number of macrophages (56–63). It is possible that, as both the inactive and active populations included in the study had uncomplicated pregnancies, there was not a discernible difference in the inflammatory profiles based solely on PA. Another potential explanation is that HBC functions are of such importance in the development of a healthy pregnancy that their numbers are highly

conserved, and that dysregulations may result in pathologies that would not have been included in the current study. While there were no differences in the numbers of total, $CD206^+$, or $CD206^-$ HBCs, active participants had a significantly higher proportion of anti-inflammatory $CD206^+$ HBCs than their inactive counterparts. This supports the current literature, which shows evidence of PA increasing $CD206^+$ macrophages in several populations, including skeletal muscle (64,65), adipose tissue (66), and systemic circulation (66). Interestingly, though the number of $CD206^+$ macrophages was higher in the active population, the expression of CD206 protein was lower. While the difference in protein expression could possibly be attributable to a change in endothelial cells, hypoxic environments mimicking PA have been shown to increase endothelial cells (67), with no evidence that PA or a surrogate thereof increases the expression of CD206 within endothelial cells. In contrast, upregulation of CD206 protein has been demonstrated in multiple inflammatory conditions, including colitis, Crohn's disease, Kawasaki disease, and rheumatoid arthritis (68–70). Of note, inflammatory conditions such as those previously listed have been shown to exacerbate the shedding of the soluble form of CD206 (sCD206) in particular (70). By modulating inflammation, PA in physical activity may downregulate the production and subsequent shedding of sCD206. No significant differences were observed when comparing CD206 RNA levels between the inactive and active groups. As such, we postulate that PA may reduce CD206 protein expression, either through a down-regulation of CD206 protein at a translational level within HBCs, or by mitigating sCD206 shedding, thereby providing further inflammation regulation.

Given the differences in HBC polarization and CD206 expression based on PA status, indicating a reduced inflammatory profile in active participants, our next study was designed to examine if PA also affected the protein expression of FGF2 and SPRY2 in term placenta, respectively pro- and anti-angiogenic factors. As a study published by our lab in 2020 revealed that active participants had higher expression in term placenta of the pro-angiogenic factor VEGF that is expressed by HBCs, we also aimed to investigate which subtypes of HBCs produced VEGF, as well as FGF2 and SPRY2. We hypothesized that active individuals would have higher levels of FGF2 and its isoforms, and that $CD206^{+}$ HBCs would be the subtype responsible for the production of VEGF and FGF2. Given that FGF2 has been demonstrated to induce the expression of SPRY2 the objective to examine SPRY2 expression in relation to HBC polarization was considered exploratory due to a lack of relevant literature.

While protein expression of SPRY2, T-FGF2, and HMW-FGF2 were not significantly different based on activity levels, LMW-FGF2 protein in term placenta was observed to be lower in physically active participants. We also reported that HBCs in culture of all polarizations produce VEGF, FGF2, and SPRY2, and are able to form intercellular junctions and multi-nucleated giant cells (MGC).

A recent publication from our lab found that physically active participants had higher levels of VEGF protein expression in term placenta (71). As such, we hypothesized that a similar effect would be seen in T-FGF2 and its isoforms. While there were no differences in T-FGF2 or HMW-FGF2, it was interesting to observe that LMW-FGF2 was in fact lower in active participants. A potential explanation for this is that angiogenesis triggered by VEGF rather than FGF2 leads to vessels with an elevated number of fenestrations, increasing the level of vascular permeability

and, subsequently, gas and nutrient exchange (72). While FGF2 has been shown to induce the secretion of pro-inflammatory molecules, VEGF has demonstrated anti-inflammatory effects (73,74). It is possible that having an increased ratio of VEGF to FGF2 may protect developing tissues from the inflammation brought about during implantation, and assist the shift to the anti-inflammatory environment that is present until the beginning of labour (75).

When comparing the SPRY2 protein expression between individuals who were habitually active throughout their pregnancy with those who were not, there was no significant difference in the expression of SPRY2. Regulation of RTK pathways is crucial, as improper functioning can create harmful results. Suboptimal expression of the pathways can lead to reduced cell survival and impaired angiogenesis; at the other extreme, hyperactivation may bring about proliferative disorders including cancer, as well as vascular defects (76–78). Bearing this in mind, SPRY2 protein expression may be tightly regulated to keep the activation of RTK pathways within healthy limits.

Given that we observed that both $CD206^{+}$ and $CD206^{-}$ HBCs in *in vivo* formalin samples and *in vitro* cell cultures expressed VEGF, FGF2, and SPRY2, it is reasonable to put forth the notion that all HBCs of all polarizations contribute to the production of angiogenic factors. This provides support for the hypothesis that they may be critical players in the development of placental vasculature (48). The quantity of said factors secreted from the specific HBC subtypes in both the presence and absence of pregnancy pathologies, as well as the potential association between expression levels and fetal outcomes, are important questions to pursue in future research.

While examining the localization of VEGF, FGF2, and SPRY2 *in vitro*, we found evidence that HBCs in culture formed intercellular interactions that are characteristic of many tissue-resident

macrophage populations (37). This process was demonstrated by the formation of filopodia and cellular aggregates; two critical steps in the formation of multinucleated giant cells (MGCs) (37). Through the expression of chemokine ligand 2 (CCL2), $CD206^{+}$ macrophages extend filopodia and increase motility to form aggregates (37,38). Such aggregates can develop into MGCs through membrane fusion and rearrangement of organelles and cytoskeletal components (37). It is currently unclear whether the observed aggregates were indeed MGCs that had undergone cellular fusion or simply cellular clusters that are not uncommonly observed in culturing conditions. Future research should investigate the possibility of placental MGCs formed from fusing HBCs. Immunofluorescence studies can be utilized to identify the expression of fusogens, molecules that are involved in membrane fusion. As an example of a fusogenic protein, the macrophage fusion receptor (MFR) is highly induced at the onset of macrophage fusion (79). Immunofluorescence studies can characterize the expression of MFR and other fusogens in lone and aggregated HBCs. If fusogens are identified and observed to be higher in aggregates, co-precipitation targeting the receptor and specific ligands can provide both evidence of MGC presence and preliminary insights into the mechanism of HBC-specific MGC formation. To the best of our knowledge, the research conducted by Kesson *et al*. (1993) is the sole source of any evidence of cultured HBCs containing MGCs (80). As MGCs have demonstrated associations with cytopathic effects and are often observed in the presence of pathological conditions (81–83), Kesson *et al.* suggested that adverse conditions induced MGC formation in the placenta, an assumption that was consistent with the available literature at the time. The 2018 review by Miron *et al*. challenges this assumption, positing that MGC presence cannot inherently be associated with a diseased or adverse state (84). Instead, the review underscores the necessity

of examining the polarization states of these cells (84). Unraveling the true nature of MGCs requires a comprehensive exploration of their characteristics and behaviors.

4.3 FUTURE DIRECTIONS

The results of this thesis provides avenues for future research that should be done in the field of HBCs and physical activity. The PA-mediated effects on CD206 protein and mRNA expression within macrophages in various tissue types should be further investigated. It is currently unclear if PA surrogates (such as exposure to myokines, intermittent hypoxia, etc.) influence HBC polarization *in vitro*. This should be studied by flow cytometry of isolated HBCs from both HBC cell culture and placenta tissue culture. A flow cytometry protocol for such an investigation has been developed and optimized by our lab (Appendix D). It also remains to be determined if PA surrogates effects the production and/or secretion of angiogenic factors. Secreted products can be measured using ELISAs in HBC cell culture and placenta tissue culture media, while intra-cellular concentrations can be assessed by cell sorting, lysis, and western blotting.

Questions that arose throughout this thesis also pose interesting topics to be considered and explored. The risk of falsely attributing differences in CD206 expression in tissue samples to TRM populations can be mitigated by examining how independent variables, such as PA, affect the expression of CD206 protein in endothelial cells. We observed that MGC could form from HBCs; in order to derive conclusions from the observation of these cells, it is imperative that they are characterized based on the polarizations they can adopt. While the current literature characterizes HBC populations to be composed of M2a, M2b, and M2c subtypes, without M1 or M2d polarizations, there were features observed within the population that are not characteristic of the suggested polarization framework (e.g., the pan-HBC expression of VEGF,

typically an M2d feature). While the current paradigm of macrophage polarization is not without flaws in other tissues, it may be that the current model is not accurate for HBCs. As such, exploratory research to identify the phenotypes of placental macrophages and their associated characteristics (i.e., markers, morphologies, secreted products, influence on surround structures, etc.) should be conducted. The research of this thesis on HBCs should be investigated in pregnancies with complications, such as gestational diabetes and preeclampsia. It is also of critical importance that future explorations of HBCs should be conducted in both healthy and complicated pregnancies.

4.4 SIGNIFICANCE

The findings from this thesis support the current literature that PA during pregnancy may result in health benefits for both mother and fetus through HBC polarization and angiogenesis. The investigation of HBC polarization remains an important facet when considering PA-mediated responses throughout gestation. As the only leukocytes in the placenta and critical mediators of immune regulation, placental morphogenesis, and placental homeostasis, any effect that PA has on HBCs may also have a significant effect on the intrauterine environment. There are many potential clinical applications of utilizing PA-induced changes in HBC form and function. By modulating HBC polarization and their expression of crucial proteins, PA may exercise a protective effect against conditions that are characterized by an increase of inflammatory macrophage subtypes.

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APPENDIX A: ELUCIDATING THE INTERATCION BETWEEN MATERNAL PHYSICAL ACTIVITYAND CIRCULATING MYOKINES THROUGHOUT GESTATION: A SCOPING REVIEW

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REVIEW ARTICLE

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Elucidating the interaction between maternal physical activity and circulating myokines throughout gestation: A scoping review

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Abstract

Physical activity (PA) during pregnancy provides both maternal and fetal health benefits. It has been theorized that myokines, peptides secreted by contracting skeletal muscle, may play an important mechanistic role in facilitating the health benefits obtained from prenatal exercise. The objective of this review was to synthesize the current literature on the relationship between maternal PA and myokine response. A search strategy was developed using the terms pregnancy, PA, IL-6, IL-10, IL-13, and TNF-α. A systematic search was completed in July 2020, in Medline, SPORTDiscus, EMBASE, CENTRAL, and in November 2020 for unpublished dissertations (grey literature; Proquest). Both human- and animal-based studies of any design were included, while commentaries and editorial articles were excluded. Data were extracted by two independent reviewers and summarized narratively. Data were thematically summarized based on the myokine and whether findings were from human or animal studies. Ten studies were included in this review. Findings from studies that examined IL-6, IL-10, and TNF-α suggest a trimester-specific interaction between PA and myokine levels; no studies evaluated IL-13. Future research should investigate the PA-myokine relationship throughout all stages of gestation.

KEYWORDS
gestation, IL-10, IL-13, IL-6, physical activity, pregnancy, TNF-alpha

1 | INTRODUCTION

Throughout gestation, maternal and fetal immune systems play an interconnected role in maintaining a healthy pregnancy. The maternal immune system undergoes complex adaptations and modifications to accept the semi-allogenic fetus during the implantation period.[1] Like the non-pregnant population, a wide variety of proteins and hormones can balance a pro- and anti-inflammatory state during pregnancy.[1] These factors, including cytokines, hormones, and signaling molecules, can affect the immune system based on their microenvironment.[2] Broadly stated, immune responses are categorized into two groups: type I and type II. Type I, the classical immune response, triggers a pro-inflammatory state to fight infections, whereas type II, the alternative immune response, fosters anti-inflammatory measures to promote regulation and repair.[3] The polarization between these two responses is initiated by physiological stressors,[4,5] such as acute or chronic exercise.[6]

The recently updated *2019 Canadian Guideline for Physical Activity throughout Pregnancy* recommends that pregnant individuals without contraindications to physical activity (PA) should engage in a minimum

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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of 150 min of moderate PA (MPA) per week.[7] Meeting these PA recommendations has shown to improve maternal and fetal outcomes, including the reduced risk of several prenatal complications,[7] such as gestational diabetes mellitus (GDM), gestational hypertension, and pre-eclampsia.[8] Despite the known benefits of PA during pregnancy for maternal and fetal health, limited investigations have sought to examine potential mechanistic pathways that may explain the role of PA in strengthening maternal and fetal immune responses.

1.1 | Physical activity and the non-pregnant immune system

In both human and animal non-pregnant populations, the immune system polarizes toward a regulatory state in response to PA by modulating myokine levels[9]—cytokines synthesized and released by muscle tissue (ie, myocytes) in response to muscle contraction. While over 600 myokines have been identified to date, this review will specifically focus on four myokines that have been shown to influence macrophage polarization.[10] By increasing anti-inflammatory myokines such as interleukin (IL)-6, IL-10, and IL-13, and decreasing pro-inflammatory myokines such as tumor necrosis factor alpha (TNF-α),[6,10] this shift in polarization mediated by PA confers many benefits throughout the body by modulating the effects associated with both pro- and anti-inflammatory cytokines (Table 1). IL-6, IL-10, and IL-13 act on macrophages to polarize them toward an M2 phenotype, corresponding to a Type II anti-inflammatory response.[11-13] The M2 phenotype is associated with widespread benefits throughout the body (Table 2), including the secretion of agents that influence the polarization of other immune cells toward a regulatory state. The immune system polarizes toward an anti-inflammatory state in response to PA by increasing the levels of select myokines, including IL-6, IL-10, and IL-13.[6] This shift toward an anti-inflammatory profile influences macrophages and other innate immune cells to polarize toward an M2 phenotype, further promoting type II responses and lessening systemic inflammation.[11] Thus, IL-6, IL-10, and IL-13 possess extensive protective properties that act throughout the body, as well as the potential ability to shift macrophages at the maternal-fetal interface toward an M2 polarization, a process that may be beneficial. Due to their ability to cross the placental barrier, these myokines have the potential to interact with Hofbauer cells, placenta resident macrophages of fetal origin that have been implicated in pregnancy regulation, angiogenesis, and fetal tolerance, thereby indirectly affecting the fetal environment.[14]

While IL-6, IL-10, and IL-13 are released in response to muscle contraction, TNF- α is a myokine that is typically released in response to muscle damage to clear away debris and necrotic tissue before the reparative actions of anti-inflammatory myokines and cytokines become predominant.[15] Outside of skeletal muscle, TNF- α initiates the trans-IL-6 cascade by binding to the nuclear factor kappa B (NF-κB) binding site of the IL-6 promoter.[16] However, TNF- α is not involved in IL-6 secretion from skeletal muscles as a response to PA.[17,18] The classical IL-6 signaling mechanism accompanied by an increase in TNF- α has been shown to shift macrophage polarization toward an M1 state,[19,20] while habitual PA is linked with lower basal levels of TNF- α, indicating that any PA-associated increases in IL-6 arise from a TNF-α independent mechanism. Given its role in PA, TNF-α can be assessed as an agent of M1, pro-inflammatory macrophage polarization, and a surrogate marker of the mechanism of IL-6 release.

1.2 | The pregnant immune system and select myokines

The maternal-fetal interface consists of several populations of immune cells, such as decidual natural killer cells, macrophages, B and T cells, and dendritic cells.[21] Several adaptations occur during decidualization, including the increase of leukocyte populations in the uterine environment and shifting maternal T-cell phenotypes to accommodate the semi-allogenic fetus.[22,23] It has become evident that these changes in maternal and placental immune cells are important for a successful pregnancy. For example, fewer regulatory T cells in the maternal decidua are linked to an increase in unexplained, recurrent, spontaneous miscarriages,[24] while dysregulations in the number and polarization state of Hofbauer cells have been associated with chorioamnionitis, GDM, pre-eclampsia, intrauterine growth restriction, Zika virus, and human immunodeficiency virus infection.[11,25] It is hypothesized that Hofbauer cells, the only immune cell in the placenta throughout gestation, may be critical for regulating pregnancy and maintaining a homeostatic uterine environment.[11] Therefore, immune system regulation in pregnancy is a pivotal matter for both maternal and fetal health outcomes.

Myokines such as IL-6, IL-10, and IL-13 are crucial for proper placental development. IL-6 is linked to an increased fatty acid uptake in trophoblast cells, a function necessary for nutrient transfer between mother and fetus.[26,27] Increased IL-10 levels are thought to be a key regulator in positive pregnancy outcomes by suppressing type I inflammation through the inhibition of the NF-κB signaling pathway in the second and third trimester while the fetus is rapidly developing.[28,29] Deficiencies in IL-10 have been associated with excess systemic inflammation, leading to preterm birth and pre-eclampsia.[29] While the role of IL-13 in pregnancy remains to be defined, both excessive and insufficient circulating concentrations of this myokine have been linked to adverse fetal outcomes such as atopic disease and asthma.[30-32] Increased levels of TNF-α during early pregnancy contribute to the inflammatory conditions required for successful implantation[33]; however, if TNF-α levels remain increased after the first trimester, insulin resistance can be exacerbated and lead to the development of GDM.[34]

1.3 | Physical activity throughout the immunological phases of pregnancy

There are distinct immunological profiles that accompany the different stages of pregnancy (Figure 1). A local pro-inflammatory *milieu* dominates the period from implantation until the beginning of the

TABLE 1 Summary of the associated effects of selected myokines (ie, IL-6, IL-10, IL-13, and TNF-α) and M2 macrophages on various organ systems in non-pregnant populations

Factor	Organ system	Effects
IL-6 Myokine	Cardiovascular	↑ Cardiomyocyte size and survival[69]; ↑ cardiac regeneration and proliferation[69]; ↑ cardiac function after myocardial infarction[70]; ↓ fibrosis[70]; ↓ scar tissue[70]
	Immune	↓ Basal IL-6 levels[71]; ↓ basal TNF-α levels[71]
	Integumentary	↑ Lipolysis[71]
	Musculoskeletal	↑ Skeletal muscle per unit of body weight[70]; ↑ fiber cross-sectional area[70]; ↑ satellite cell proliferation[9]; ↓ satellite cell differentiation[72]
	Metabolic	↑ Hepatic glucose production[71]; ↑ insulin secretion[71]; ↑ fatty acid oxidation[71]
IL-10 Myokine	Cardiovascular	↑ Cardiomyocyte survival[73]; ↓ cardiac inflammation[73]; ↓ lipid metabolic responses in cardiomyocytes[73]; ↑ cardiac function[74]; ↓ infarction size[74]
	Musculoskeletal	↑ Chondroprotective action[75]
	Nervous	↑ Neuron survival[76]; ↑ axon regeneration[76]; ↓ neuronal damage[76]
	Metabolic	↓ Hepatic fibrosis[77]
IL-13 Myokine	Cardiovascular	↑ Cardiac regeneration[78]; ↑ cardiomyocyte proliferation and survival[78]
	Nervous	↑ Functional performance[79]; ↓ neuroinflammation[79]; ↓ Type 1 microglia[79]; ↓ Type 1 macrophages[79]
	Metabolic	↑ Fatty acid oxidation[80]; ↑ mitochondrial respiration[80]; ↑ pancreatic beta-cell survival[81]
TNF-α Myokine	Cardiovascular	↑ Atherosclerosis[82]; ↑ risk of myocardial infarction[82]; ↑ progression of cerebrovascular disease[82]; ↑ cardiomyocyte apoptosis[83]
	Musculoskeletal	↑ Muscle atrophy[84]; ↑ proteolysis[85]; ↓ bone mineral density[86]
	Nervous	↑ Type 1 microglia[37]; ↑ Type 1 macrophages[37]; ↑ neurodegeneration[87]
	Metabolic	↑ Dysregulation of gut microbiome[88]; ↑ hepatocyte apoptosis[89]; ↑ insulin resistance[90]
M2 Macrophage	Cardiovascular	↓ Atherosclerosis[91]; ↑ cardiac regeneration[92]; ↑ vascularization[93]
	Immune	↓ Chronic inflammation[94]
	Integumentary	↑ Lipolysis[95]; ↑ brown adipose tissue[96]
	Musculoskeletal	↑ Skeletal muscle regeneration[97]; ↑ myoblast differentiation[98] ↑ tissue repair[99]
	Metabolic	↓ Decreases insulin resistance[100]; ↓ liver fibrosis[101]; ↓ hepatocyte apoptosis[102]
	Nervous	↑ Neurogenesis[103]; ↓ neuroinflammation[103]; ↓ neurodegeneration[104]

second trimester, when placentation is complete.[35,36] As the blastocyst breaks through the uterine epithelial lining and damages the epithelial tissue to implant, the uterine environment resembles an open wound.[37] The result is a highly pro-inflammatory condition necessary to facilitate repair of uterine epithelium and clearance of debris. The pro-inflammatory state established to facilitate implantation is further necessary to allow for the development of the placenta.[36,38] This includes the clearance of debris, the replacement of damaged epithelium with trophoblasts, and the development of spiral arteries.[39] As gestation progresses into the second and early third

TABLE 2 Characteristics of studies including observational and intervention methods of physical activity and myokines measured

Author (year), country	Study type	Sample size (n)	Methodology	Exercise protocol	Myokines measured	Main outcomes
Acosta-Manzano et al. (2020), Spain	Non-randomized control trial	Pregnant: $n = 50$	Maternal venous serum was taken from a fasted state from overweight and obese participants	Participants wore an accelerometer for 9 consecutive days, 24 h/day, requiring a minimum of 7 days with ≥10 h/day wear time	TNF-α, IL-6, IL-10	No significant differences were found in the myokine levels of pregnant individuals meeting PA guidelines and those who do not
Acosta-Manzano et al. (2019), Spain	Cross-sectional	Exercise group: $n = 26$ Control group: $n = 26$	Fasting maternal venous serum samples were taken from overweight and obese participants throughout gestation and immediately after delivery. Umbilical venous and arterial serum was taken at delivery	Combination aerobic-resistance exercise program of moderate-vigorous intensity, 3 days/week, for 60 min/session throughout gestation	TNF-α, IL-6, IL-10	Participants who underwent the intervention had lower levels of TNF-α at 35 weeks and lowered circulating levels of IL-10 at delivery. Additionally, the umbilical arterial serum had a lower level of IL-6, and the umbilical venous serum had lower levels of TNF-α
Bae-Hartz et al. (2016), Germany	Longitudinal animal study	Pregnant mice: $n = 56$	Diet-induced obese mouse dams were divided into a sedentary obese or an obese intervention group	Intervention groups performed voluntary wheel running throughout gestation	IL-6	There was a significant decrease in circulating IL-6 serum levels in the intervention obese dams
Clapp and Kiess (2000), USA & Germany	Longitudinal	Exercise group: $n = 31$, Lifestyle PA group: $n = 16$, Stopped exercise group: $n = 17$	Maternal venous serum samples were obtained 2–4 h postprandially.	Participants underwent bimonthly tests, including respiratory calorimetry, portable heart rate monitors, and completed rate of perceived exertion and exercise logs	TNF-α	Participants who exercised during pregnancy had lower levels of TNF-α. Individuals who stopped exercising experienced rising levels of TNF-α
Dubé et al. (2017), Canada	Cross- sectional	Active group: $n = 14$ Inactive group: $n = 16$	Circulating levels of myokines were analyzed in plasma of lean, overweight, and obese pregnant individuals during the 25–28th week of gestation	Participants wore an accelerometer and were characterized as active or inactive based on Canadian Physical Activity Guidelines	IL-6, IL-10, IL-13	No significant differences were found in the myokine levels according to activity status

(Continues)

mechanism accompanied by the rise in TNF-α has been shown to shift macrophage polarization toward an M1 state.[19,20] Of the nine studies included in this review that examined IL-6 levels in response to PA, only five looked at the accompanying levels of TNF-α in conjunction.[49-53,56] Interestingly, all three of the studies that had significant results also measured and accounted for circulating levels of TNF-α.[50,52,53] By controlling for levels of TNF-α, researchers will be better suited to assess the tissue of IL-6 origin. It is likely that to determine the effect of PA on IL-6 levels, TNF-α levels must also be assessed, ensuring that an inflammatory condition, such as obesity or muscle damage, is not confounding the myokine response to PA.

To the best of our knowledge, this scoping review is the first to summarize the available published literature on the relationship between maternal PA and circulating levels of IL-6, IL-10, IL-13, and TNF-α throughout gestation. In conjunction with Arksey and O'Malley's framework for scoping reviews, PRISMA guidelines were followed to ensure a thorough and systematic search. The scarcity of currently available research on this topic rendered conducting a systematic review and meta-analysis premature. Future research with objectively measured prenatal PA with larger and representative populations is vital. This research should include randomized control trial exercise interventions and the associated effects on maternal myokines; the examination of this relationship within the distinct inflammatory phases of gestation; and the impact of maternal PA on myokines produced by the fetus, determined by the levels within the umbilical arterial serum.

5 | CONCLUSION

The literature available on the response of IL-6, IL-10, IL-13, and TNF-α to maternal PA throughout gestation is limited. This review provides preliminary evidence of a trimester-specific relationship between PA and myokine levels during pregnancy. More research is needed to understand the interaction between maternal PA and myokine levels throughout gestation, including the levels of fetal-produced myokines in umbilical arterial serum at delivery.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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decrease. This preliminary evidence suggests that research done in mice may be a poor predictor of the response of myokines to PA in humans. Research done by Masopust et al.[57] postulates that the pristine conditions kept for laboratory mice prevents the maturation of their immune system, and thus, the results from mouse studies are not generalizable to human models. Furthermore, murine models can be more extensively controlled in terms of genetic background, nutrition, and the exclusion of pre-existing conditions. This is not to say that murine models are not valuable; on the contrary, they have many advantages, including the control of confounding variables, a shorter gestation, and a genome that has many conserved elements when compared to that of humans.[58] When comparing the murine model of myokine responses to PA in pregnancy to that of humans, further research is necessary.

Interesting to note, of the 10 studies included in this review, the ones that found significant differences in myokine levels in pregnant individuals were longitudinal or nested case–control studies. Research has shown that as gestation progresses, levels of PA change, with most individuals decreasing activity levels from the second to the third trimester.[59] Due to this effect, PA measurements obtained once in pregnancy are unlikely to be indicative of PA throughout gestation. As discussed above, pregnancy is characterized by different immunological profiles at each stage, with a pro-inflammatory state predominating during implantation, and an anti-inflammatory state being necessary from the beginning of the second trimester until the induction of delivery.[35] While habitual PA generally elicits anti-inflammatory responses,[60] these responses would likely be overpowered by the "open wound" state required during implantation and placentation.[35] As the implantation-characteristic inflammatory state resolves, it is possible that the effects of PA would be more evident. Evidence of the emerging effects of PA can be seen in the studies that examined TNF-α. In studies on early pregnancy, there were opposing results when comparing TNF-α levels to PA, with different studies finding increased levels, decreased levels, or non-significant changes.[49,51-53,56] Studies done in mid-pregnancy recorded mostly non-significant results when comparing TNF-α levels to PA.[49,50,52,53,56] However, in late pregnancy, the majority of studies reported significant decreases in TNF-α as PA increased.[50,52,53,56] Therefore, we posit that there may be a trimester-specific effect of PA on myokines during pregnancy, influenced by the corresponding inflammatory profile at the time of measurement.

There were pronounced discrepancies between the study designs of the eligible articles, including differences in the length of intervention, level of activity, and variations in demographics which may speak to the contrasting findings. Additionally, there was a wide variety of PA measurement reporting between the studies. Of the seven human studies that looked at habitual PA, four studies used accelerometers, while the remaining three used a variety of different self-report questionnaires, bimonthly indirect calorimetry, heart rate monitors, and intervention session logging. Prior studies in the field have found significant discrepancies between PA measures, with particularly high errors in self-report measures.[61] The variety of measurement techniques used in the included studies may have contributed to the inconsistencies in the results. Thus, studies are needed to assess the effects of habitual maternal PA on the myokine response in each of the immunological phases during gestation, to compare the observed levels of myokines to the baseline of the corresponding phase. Studies should include the use of accelerometry as the gold standard technique of PA assessment or doubly labeled water for a direct measure of energy expenditure, evaluate PA throughout gestation, and use evidence-based intervention programs.

The limitations of observational studies in humans can also contribute to discrepancies between study results. Observational studies are at risk for selection bias, measurement errors, self-report biases, and confounding variables.[62] Those who volunteer for a study on PA in pregnancy may be more inclined to participate as they may already follow an active lifestyle—this can lead to a sampling bias where the study cohort is not a true representation of the intended population. By using surveys to assess lifestyles and pre-existing conditions, studies can be further misled by accidental or purposeful misreporting.[63] Where possible, intervention studies that control for confounding variables should be used.

Of the studies included, only one examined the levels of myokines at delivery.[50] Delivery represents a unique opportunity to obtain umbilical serum, thereby investigating the link between mother and fetus. Acosta-Manzano et al.[50] found significantly increased concentrations of IL-6 in umbilical arterial serum but discovered no significant differences in umbilical venous serum or maternal venous serum. Where the umbilical vein carries blood from the placenta to the fetus, the umbilical artery carries blood from the fetus to the placenta. Thus, the authors postulated that PA might regulate fetal production of IL-6. While increased levels of IL-6 are necessary for the successful induction and completion of labor, IL-6 levels that are too high may pose a risk in the form of adverse conditions.[64-66] However, most of the said adverse conditions, including chorioamnionitis and fetal anemia, are also associated with a rise in TNF-α levels.[64-66] As there was no such elevation in TNF-α levels in either umbilical arterial serum or umbilical venous serum in this study,[50] PA may provide a mechanism for increasing the fetal production of IL-6 but not TNF-α. This anti-inflammatory increase in IL-6 would thereby increase the chances of a successful, uncomplicated delivery, but not of inflammatory conditions that predispose both mother and child to poorer outcomes. This hypothesis should be explored in future studies, as well as the fetal production of other myokines as it relates to maternal PA.

IL-6 is a complex molecule that has pleiotropic effects, acting either as a pro-inflammatory or anti-inflammatory agent.[67,68] In trans-IL-6 signaling, the cytokine is principally defined as a pro-inflammatory molecule.[68] Inflammatory stimuli upregulate the pro-inflammatory cytokines IL-1 and TNF-α, which initiates the trans-IL-6 cascade by binding to the NF-κB-binding site on the IL-6 promoter.[16] However, when secreted from skeletal muscles as a response to PA, IL-6 increases are observed without an increase in TNF-α,[17,18] promoting anti-inflammatory effects, which can influence the M2 macrophage response. The classical IL-6 signaling

TABLE 3 (Continued)

Myokine	Study	Design	Early pregnancy	Mid pregnancy	Late pregnancy	Delivery
TNF-α	Acosta-Manzano et al. (2020)	Cross-sectional	No significant differences	-	-	-
	Acosta-Manzano et al. (2019)	Non-randomized control trial	-	No significant differences	**↓ TNF-α in intervention group**	**↓ UVS TNF-α in intervention group.** No significant differences in UAS or MS
	Clapp and Kiess (2000)	Longitudinal	**↓ TNF-α in exercise groups**	**↓ TNF-α in exercise groups**	**↓ TNF-α in exercise groups**	-
	van Poppel et al. (2014)	Longitudinal	**↑ TNF-α in high MVPA group**	No significant differences	No significant differences	-
	Nayak et al. (2016)	Longitudinal	No significant differences	No significant differences	**↓ TNF-α with decreased sedentary time**	-
	Steckle et al. (2018)	Nested case–control	No significant differences	No significant differences	-	-

Bold text indicates that a significant difference was identified in the indicated reviewed publications.

Abbreviations: IL, interleukin; LPA, light physical activity; MS, maternal serum; MVPA, moderate to vigorous physical activity; TNF-α, tumor necrosis factor alpha; UAS, umbilical arterial serum; UVS, umbilical venous serum.

positively associated with sedentary time in late pregnancy, yet no differences were observed in early and mid-pregnancy.[52,53] While high MVPA was associated with higher circulating IL-10 levels, a nested case–control study found that IL-10 levels were lower as light PA increased.[49]

3.3 | IL-13

Of the two studies that investigated the levels of IL-13, one did not find any significant correlation between IL-13 and PA levels in mid pregnancy,[47] while the other was unable to detect circulating IL-13 levels in maternal serum in early and mid-pregnancy.[49]

3.4 | TNF-α

Six studies assessed the relationship between PA and TNF-α levels, with contradictory findings. In one cross-sectional study, there was no significant difference between PA levels and TNF-α during early pregnancy.[51] Similarly, no relationship with PA was observed in early or mid-pregnancy levels of TNF-α in participants who underwent spontaneous preterm labor or delivered at term.[49,51] In an exercise intervention, no significant difference was found in TNF-α levels mid -pregnancy, yet as gestation progressed, individuals in the intervention saw a decrease in TNF-α levels.[50] This decrease in TNF-α was not seen at term, as no difference was found in maternal serum or umbilical arterial serum; however, lower levels of TNF-α were observed in the umbilical venous serum in the intervention group.[50] Of the three longitudinal studies, only one study found consistently reported lower TNF-α levels in each stage of gestation in participants categorized as active.[56] Similarly, Nayak et al.[52] found a lower circulating TNF-α level as sedentary time decreased during late pregnancy. Contrary to these findings, van Poppel et al.[53] found an increase in TNF-α levels in the early pregnancy high MVPA group, yet observed no difference as gestation progressed.

4 | DISCUSSION

In this review, we explored the currently available published literature on circulating IL-6, IL-10, IL-13, and TNF-α in the context of prenatal exercise. While there is no apparent association between PA and the levels of circulating myokines during gestation at this time, there is emerging evidence of a trimester-specific effect.[49,50,52,53] Animal studies on this topic were limited; however, the results of the two mice studies found a negative relationship between IL-6 levels in late pregnancy as it related to PA. This finding directly opposes the results of studies done in humans during the equivalent gestational period, which found either a positive relationship or no significant associations. While the animal studies in this review only investigated the response in mice with obesity, the results of the studies done in human pregnancies with obesity did not reflect the observed

TABLE 3 The relationship between maternal physical activity and levels of IL-6, IL-10, IL-13, and TNF-α throughout gestation

Myokine	Study	Design	Early pregnancy	Mid pregnancy	Late pregnancy	Delivery
IL-6	Acosta-Manzano et al. (2019)	Non-randomized control trial	-	No significant differences	No significant differences	↑ **UAS IL-6 in intervention group.** No significant differences in UVS or MS
	Acosta-Manzano et al. (2020)	Cross-sectional	No significant differences	-	-	-
	Hutchinson et al. (2019)	Cross-sectional	-	No significant differences	-	-
	Dube (2017)	Cross-sectional study	-	No significant differences	-	-
	Nayak et al. (2016)	Longitudinal	No significant differences	↑ **IL-6 with decreased sedentary time**	No significant differences	-
	Steckle et al. (2018)	Nested case–control	No significant differences	No significant differences	-	-
	van Poppel et al. (2014)	Longitudinal	↑ **IL-6 in high MVPA group**	↑ **IL-6 in high MVPA group**	↑ **IL-6 in high MVPA group**	-
IL-6	Bae-Gartz et al. (2016)	Longitudinal animal study	-	-	↓ **IL-6 in exercise group.**	-
	Zhu et al. (2019)	Longitudinal animal study	-	-	↓ **IL-6 in obese-exercise.** No significant differences between lean groups	-
IL-10	Acosta-Manzano et al. (2020)	Cross-sectional	No significant differences	-	-	-
	Acosta-Manzano et al. (2019)	Non-randomized control trial	-	No significant differences	No significant differences	↑ **MS IL-10 in intervention group.** No significant differences in UVS or UAS
	van Poppel et al. (2014)	Longitudinal	↑ **IL-10 in high MVPA group**	No significant differences	No significant differences	-
	Nayak et al. (2016)	Longitudinal	No significant differences	No significant differences	↓ **IL-10 with decreased sedentary time**	-
	Steckle et al. (2018)	Nested case–control	↓ **IL-10 with increased LPA**	No significant differences	-	-
	Dube (2017)	Cross-sectional	-	No significant differences	-	-
IL-13	Dube (2017)	Cross-sectional	-	No significant differences	-	-
	Steckle et al. (2018)	Nested case–control	Not detectable	Not detectable	-	-

(Continues)

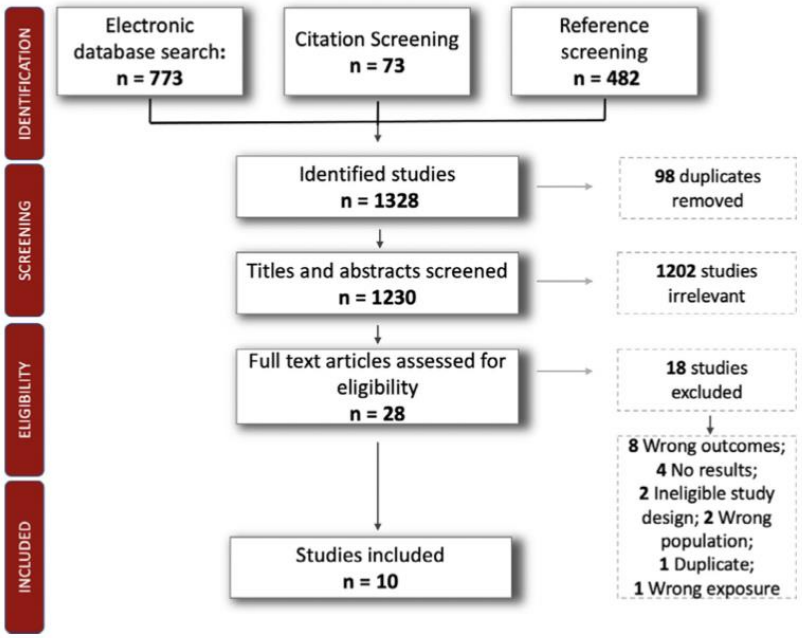

FIGURE 2 PRISMA flow diagram for study selection

studies were conducted in human populations,[47-53,56] and two used mouse models.[54,55] Five studies were longitudinal observational designs,[52-56] three were cross-sectional designs,[47,48,51] one was a non-randomized control trial,[50] and one was a nested case–control study.[49] Nine studies examined myokine response under chronic PA conditions,[47,49-56] while one investigated the response after an acute bout of exercise.[48] Of the 10 studies included, with several studies examining more than one myokine, nine measured IL-6,[47-55] six measured TNF-α,[49-53,56] six measured IL-10,[47,49-53] and one measured IL-13.[47] The study characteristics are detailed in Table 2, and the main study results relevant to the markers assessed are summarized in Table 3.

3.1 | IL-6

3.1.1 | Human studies

Seven studies provided data on the effects of prenatal PA on IL-6 response, one being a cross-sectional observational study that examined the response of circulating IL-6 after an acute bout of exercise. There were no significant differences in IL-6 levels after an acute bout of exercise, nor at baseline in active vs. inactive groups in early or mid-pregnancy in cross-sectional studies.[47-49,51] One intervention study provided an aerobic and resistance exercise program to pregnant individuals who had overweight or grade I obese BMI classifications. No difference in IL-6 concentrations in maternal serum throughout gestation was found; however, a statistically significant increase in IL-6 in the umbilical arterial serum of the intervention group was reported.[50] Two longitudinal studies that followed participants with lean, overweight, or obese BMI classifications throughout their pregnancy found IL-6 levels to be significantly increased in each stage of gestation in those with high moderate-to-vigorous PA (MVPA) levels. These studies also found a significant negative association with sedentary time; however, the latter was only observed in mid-pregnancy.[52,53]

3.1.2 | Animal studies

Two mouse studies observed the longitudinal effect of an exercise intervention on maternal IL-6 levels, with one using voluntary swimming and the other voluntary wheel running. Both studies found a decreased levels of circulating IL-6 in the mice with obesity that underwent the exercise intervention.[54,55] While Zhu et al.[54] found a decrease in circulating IL-6 concentrations in mice with obesity who underwent the intervention, no difference was found in lean mice between exercise and control groups.

3.2 | IL-10

Six studies assessed the relationship between IL-10 levels and PA. Three studies that examined circulating IL-10 levels throughout pregnancy did not observe any significant difference between participants who met PA guidelines and those who did not.[47,50,51] Individuals who underwent a combined aerobic-resistance exercise program did not have any significant differences in maternal IL-10 levels after completing the intervention; however, the intervention group had higher circulating IL-10 at delivery compared to control.[50] Contrary to these findings, IL-10 concentrations were higher in each stage of pregnancy in individuals with high MVPA.[51] IL-10 was also

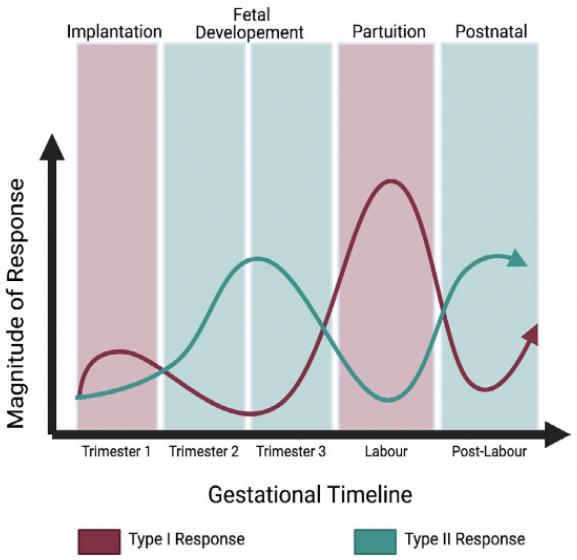

FIGURE 1 The inflammatory profile throughout pregnancy. Implantation, placentation, and early fetal development are accompanied by a strong pro-inflammatory response. As the fetus develops, the mother's immune system shifts toward an anti-inflammatory state for pregnancy maintenance and optimal fetal development. During the final immunological phase, increased pro-inflammatory responses are required to trigger parturition and induce labor. Created with BioRender.com

trimesters, fetal development accelerates, and an anti-inflammatory state takes over.[35] In the last immunological phase, pro-inflammatory agents are recruited to the maternal-fetal interface to trigger delivery.[35] This pro-inflammatory climate promotes uterine contractions and subsequently the delivery of the baby and placenta.[40]

An exacerbated pro-inflammatory environment following the period of implantation and placentation can be detrimental for the fetus. Systemic inflammation in the mother is capable of producing fetal inflammatory response syndrome (FIRS), which increases the risk for preterm birth,[41] the development of autism, schizophrenia, and neurosensory dysfunction.[35,42-44] Through the action of myokines, PA may play an important role in modulating the inflammatory environment and conferring maternal and fetal benefits, both *in utero* and later in life. Given the limited studies that have investigated the role of PA in modulating myokines during pregnancy, we completed a scoping review to map the available literature on the relationship between maternal PA and the levels of IL-6, IL-10, IL-3, and TNF-α. Importantly, findings from this scoping review identify critical research gaps and provide recommendations for future studies to conduct high-quality prenatal PA investigations and assess myokine response.

2 | MATERIALS AND METHODS

A scoping review was completed in compliance with Arksey and O'Malley's proposed framework,[45] following the 2020 PRISMA-ScR checklist.[46] Our search encompassed two core concepts: (1) Prenatal exercise or PA, and (2) Myokines, specifically IL-6, IL-10, IL-13, or TNF-α. The following databases were screened on July 15, 2020: Medline, SPORTDiscus, EMBASE, and the Cochrane Central Register of Controlled Trials (CENTRAL). Our search strategy consisted of keywords and MeSH headings for Pregnancy AND "Physical Activity OR Exercise" combined with IL-6 OR IL-10 OR IL-13 OR TNF-α. The search strategy for Medline is presented in Table S1. In addition, relevant studies available in unpublished dissertations (grey literature; Proquest) were reviewed on November 15, 2020, using identical search terms. Our searches did not include date or language restrictions. Retrieved titles and abstracts were transferred to Endnote software (Version X5). Following the removal of duplicates, all remaining articles were transferred to Covidence review software (Veritas Health Innovation) for further screening.

The following inclusion criteria were applied: (1) pregnant population; (2) exposure to prenatal exercise or PA; and (3) measurement of IL-6, IL-10, IL-13, or TNF-α in response to exercise or PA. Given the expected scarcity of literature that has evaluated these specific markers in relation to prenatal PA, we included evidence from both animal and human-based studies, with no restriction on study design. Commentaries and editorial articles were excluded. Two independent reviewers screened all titles and abstracts. Studies that appeared to meet the inclusion criteria were further assessed in full text. If there were any disagreements between the two reviewers throughout the screening process, a third reviewer was consulted, and a final decision was made. Additionally, reference and citation lists of all included studies were screened for potential inclusion. Relevant data were extracted from the selected studies and charted onto a standard Excel spreadsheet.

From the eligible studies, the following characteristics were extracted: author and publication details, country, study design, PA/exercise description, control group (if applicable) description, and any reported study limitations. If applicable, we extracted study population characteristics, including age, pre-pregnancy body mass index (BMI), and parity. PA intervention details (frequency, intensity, time, type; if applicable), participant PA levels, and outcome data (IL-6, IL-10, 1L-13, TNF-α) were extracted as reported by authors. Data were extracted by one reviewer and checked by a second reviewer. To map the data, we narratively synthesized the main findings for each of the four myokines and further categorized the results as evidence from animal or human studies. Finally, based on the available evidence, we proposed mechanistic frameworks to elucidate the potential impact prenatal PA may have on the selected myokines and offer future research recommendations.

3 | RESULTS

The initial search and supplementary searches retrieved 1328 studies, of which 10 met the inclusion criteria (Figure 2). Of the 10 studies included, three were completed in Canada,[47-49] two were conducted in Spain,[50,51] two were performed in the Netherlands,[52,53] one was carried out in China,[54] one was completed in Germany,[55] and one was a joint study between the USA and Germany.[56] Eight of the

TABLE 2 (Continued)

Author (year), country	Study type	Sample size (n)	Methodology	Exercise protocol	Myokines measured	Main outcomes
Hutchinson et al. (2019), Canada	Cross-sectional	Pregnant: $n = 13$ Control: $n = 17$	Fasted maternal venous serum was taken from lean and overweight individuals. Participants were given a snack of ~340 kcal before undergoing the exercise protocol. Following completion, maternal venous serum was taken from a non-fasted state	30-min moderate-intensity (4%0–60% heart rate reserve) treadmill exercise protocol	IL-6	No change was found in circulating IL-6 levels pre- and post-exercise study or between pregnant and non-pregnant populations
Nayak et al. (2016), Netherlands	Longitudinal	Pregnant: $n = 46$	Maternal venous serum was taken during a fasted state from overweight and obese participants	Participants wore an accelerometer for 4 days, 24 h/day, requiring a minimum of 8 h/day wear time	TNF-α, IL-6, IL-10	Decreased sedentary time was associated with increased IL-6 in mid-pregnancy and decreased IL-10 and TNF-α in late pregnancy
Steckle et al. (2018), Canada	Nested case–control	Pregnant: $n = 80$	Cytokines at 16- and 27-week gestational age were measured in peripheral plasma of patients who had spontaneous preterm labor and those delivered at term	Physical activity patterns were determined using IPAQ	TNF-α, IL-6, IL-10, IL-13	A negative relationship was found between the intensity of light physical activity and IL-10 levels early pregnancy
van Poppel et al. (2014), Netherlands	Longitudinal	Low MVPA: $n = 46$ High MVPA: $n = 23$	Maternal venous serum samples were taken during a fasted state from overweight and obese participants	Participants wore an accelerometer for 3 days, requiring a minimum of 8 h/day wear time	TNF-α, IL-6, IL-10	TNF-α, IL-6, and IL-10 levels were increased in the active group at 15 weeks; however, only IL-6 remained at an increased concentration in active participants throughout pregnancy
Zhu et al. (2019), China	Longitudinal animal study	Pregnant mice: $n = 56$	Female mice were subdivided into four groups based on a standard chow diet and high- fat diet. On gestation days ~17–19, indirect calorimetry was performed for energy metabolism and body composition analysis. Liver and serum tissues were taken for analysis	The intervention group swam 5 days a week and 2 days over the weekend	IL-6	Swimming intervention significantly decreased circulating maternal IL-6 levels in intervention obese dams compared to sedentary obese group. No significant difference in lean dams

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APPENDIX B: SCHOLARY ACHEIVEMENTS

B1. PUBLICATIONS

Refereed

Everest C, Goudreau A, Valko E. Prenatal cannabidiol administration on mice growth and development: A preliminary study for use during human labour [Abstract]. In: Scinapse 2018-2019 Undergraduate Science Case Competition: Cannabis, harm or health? March 2019; Ottawa, ON. Abstract 34.

Nagpal T, Everest C, Goudreau A, Manicks M, Adamo K. To HIIT or not to HIIT? The question pregnant women may be searching for online: a descriptive observational study. Perspectives in Public Health. 2021;141(2):81-88. doi:10.1177/1757913920985898 Bhattcharjee J, Mohammad S, Goudreau AD, Adamo KB. Maternal physical activity is associated with differential expression of VEGF, PlGF and their receptors in the human term placenta. *Physiological Reports*

Goudreau AD, Everest C, Nagpal TS, Puranda JL, Bhattacharjee J, Vasanthan T, Adamo KB. Elucidating the interaction between maternal physical activity and circulating myokines throughout gestation: A scoping review. Am J Reprod Immunol. 2021 Nov;86(5):e13488. doi: 10.1111/aji.13488. Epub 2021 Aug 19. PMID: 34331363.

Everest C, da Silva DF, Puranda J, Souza SCS, Goudreau AD, Nagpal TS, Edwards CM, Gupta R, Adamo KB. Physical Activity and Weight Gain Throughout Pregnancy Are Associated With Umbilical Cord Markers. J Obstet Gynaecol Can. 2022 Dec;44(12):1262-1270. doi: 10.1016/j.jogc.2022.09.012. Epub 2022 Oct 8. PMID: 36216221.

Submitted

Goudreau AD, Everest C, Tzaneva V, Adamo KB. Does physical activity the influence polarization state of placenta-resident macrophages? *(Revisions requested, Physiological Reports March 2023)*

Goudreau AD, Tanara L, Tzaneva V, Adamo KB. Characterization of Hofbauer cell polarization and VEGF localization in human term placenta from active and inactive pregnant individuals *(Submitted to IJERPH, March 2023).*

B2. CONFERENCE ABSTRACTS AND PRESENTATIONS

Canadian National Perinatal Meeting Research, Virtual Conference

Poster Presentation 2021

"Circulating myokines as they relate to physical activity throughout gestation."

Society for Reproductive Investigation, Virtual Conference

Poster Presentation 2021

"Maternal physical activity differentially regulates CD206 in term placenta."

B3. COMPETITIONS AND COMMUNITY INVOLEVEMNT

2020/11 – 2022/15	Site Coordinator	Twice Upon a Time
2020/01 – 2022/04	Volunteer Tutor	Students Offering Support – University of Ottawa Chapter
2019/09 – 2020/03	Children's Literacy Volunteer	Twice Upon a Time
2021/09 – 2022/04	VP of Marketing	Students Offering Support – University of Ottawa Chapter
2020/10 – 2020/11	Workshop Facilitator	IgNITE Case Competition
2018/10 – 2020/12	VP of Design and Promotion	Ottawa Integrated Cancer Centre – University of Ottawa Chapter

uOttawa

Faculté des sciences
Faculty of Science

CERTIFICATE OF PARTICIPATION

Presented by the Faculty of Science to

Alexandra Goudreau

For competing in the 2019 Scinapse USCC

B4. AMNION SEED GRANT

Amnion Foundation
Seed Grant Application

OPPORTUNITY DESCRIPTION

Grantor: Amnion Foundation
Address: 111 N. Chestnut St., Ste 100
City, State ZIP: Winston-Salem, NC 27103
Phone: 336-955-2241
Email: info@amnionfoundation.org
Project name: Primary Cell Access, Placenta / Umbilical Cord

APPLICANT INFORMATION

Applicant: Alexandra D Goudreau
Institution: University of Ottawa
Address: 200 Lees Avenue
City, State ZIP: Ottawa, ON, K1S 5S9
Phone: [redacted]
Email: [redacted]
If Student / Trainee, Principal Investigator: kristi.adamo@uottawa.ca

PROGRAM DETAILS

The Amnion Foundation is a registered 501(c)3 organization specializing in the repurposing of otherwise discarded birth tissues (placenta and umbilical cord) for the generation of primary human cells to support advancements in regenerative medicine and drug discovery. Available cell types include umbilical vein-derived endothelial cells, placental tissue-derived endothelial cells, cytotrophoblasts, macrophages, classical umbilical cord-derived MSC, and pericyte-like placental stem/stromal cells. Recipients of Primary Cell Access Seed Grants will be eligible to receive primary cells and supporting reagents valued at up to $10,000.00 to support an Investigator's ongoing efforts toward the development of *in vitro* models or regenerative medicine products. Technical support will also be provided to the recipients for their selected cell type(s).

Eligibility: Selected applicants will have an established, active program of applied research in placental or umbilical cord cell biology within one or more of the following disciplines: toxicology, pathogenesis, pharmacology, developmental biology, or regenerative medicine. Applicants must have a lab in which they conduct mammalian cell culture routinely and an interest in transitioning from non-human or cancer-derived cell lines to primary human cell strains. Applicants must be an employee of a North American registered academic institution or business. Graduate students or postdoctoral trainees may apply in conjunction with the support of their primary mentor.

IMPACT STATEMENT

Please include a statement that articulates the overall impact of completing the proposed project in the context of your field (i.e., toxicology, drug development, regenerative medicine). <150 words

Physical activity (PA) during pregnancy is associated with health benefits for both mother and fetus. While the mechanisms producing these benefits have yet to be fully elucidated, research in other fields has shown beneficial changes in immune cell populations may be at play. For example, research in other organ systems has shown that PA results in a phenotypic shift in macrophages from an M1 inflammatory state to an M2 regulatory state. Hofbauer cells (HBCs) are the most predominant immune cell in the placenta at term. These HBCs have been associated with various functions, including placental vasculogenesis and angiogenesis, promoting maternal tolerance of the semi-allogenic fetus, and maintaining a homeostatic uterine environment. As such, they present a point of interest as possible mediators of the benefits of PA in pregnancy. This project will explore the potential associations between maternal PA and phenotypic shifts in HBC populations in term placenta in hopes of providing novel insight into possible mechanisms through which PA imparts health benefits during pregnancy. The overreaching aim of this project is to characterize the Hofbauer cell response to habitual maternal PA through three main goals: 1. examining the polarization state in vivo in term placenta from physically active and inactive participants; 2. analyzing the effect of PA proxies (i.e., exposure to hypoxia or PA-induced myokines) on polarization in vitro; and 3. investigating the impact of polarization on the expression of inflammatory and angiogenic factors. In the field of preventative medicine, characterizing the polarization of HBCs remains an important aspect of examining the effect of chronic PA on the placental immune response. By modulating the polarization of HBCs, PA may have a protective role against conditions characterized by an increase in pro-inflammatory HBCs, such as pre-eclampsia, metabolic disorders, and birth complications. Additionally, an intrauterine environment optimized for pregnancy maintenance and homeostatic regulation could decrease the incidence of delivery complications for the fetus and promote long-term health. Research in this relatively understudied field may provide mechanistic support for exercise guidelines in pregnancy and subsequently inform prenatal healthcare workers of the best practices to guide their patients. Characterizing how HBC polarization contributes to establishing healthy pregnancies opens the door for future research into technologies and techniques designed to influence the polarization of these cells as intervention methods in unhealthy pregnancies.

PROPOSAL

Lay Abstract: (Please provide a <500 word description of the project that the requested cells will support)

As Dr George R Saade said, "There is nothing in medicine that can return so much on an investment as a healthy pregnancy and delivery, because that has years and years of impact later. And placental health is critical to the health of a pregnancy." The placenta is a temporary organ which provides the fetus with oxygen and nutrients during pregnancy. Physical activity during pregnancy supports a healthy mom, baby, and placenta. How these benefits arise is yet to be fully understood. In the non-pregnant population, regular PA leads to changes in the immune system to an anti-inflammatory profile. Placenta-resident immune cells, known as Hofbauer cells, reflect the ability of other macrophage subtypes to polarize between pro- and anti-inflammatory states. While Hofbauer cells exist in a predominantly anti-inflammatory state, changes in polarization state have been associated with adverse health outcomes such as pre-eclampsia, gestational diabetes, and pre-term birth. The purpose of this novel study is to explore how substitutes for physical activity affect the polarization and gene expression of Hofbauer cells. It is necessary to fully understand these mechanisms before effective clinical strategies can be developed to protect against pregnancy complications and downstream/future health effects.

Project Description

Please attach a 1-page description of the studies that will includes the following sections:
1) Cell type(s) requested
2) Technical description of the planned studies
3) Timeline for completion

Investigator

Please attach the Principal Investigator's CV. If the applicant is a post-doctoral trainee or graduate student, please include a CV from the trainee / student as well as a CV and letter of support from the Principal Investigator of the lab where the work will be completed.

Applicant Signature [redacted] **Date** 26 November 2021

(printed name) Alexandra Goudreau

AMNION FOUNDATION USE ONLY

Approved? ☐Yes ☐No Approved Amount: $_____ (in-kind)

Cells / Reagents Provided:_____

_____

_

Amnion Foundation Authorized Signature: _____ Date: _____

Project Description

The proposed study will utilize primary culture Hofbauer cells (HBCs) isolated from the placenta of healthy women by the Amnion Foundation. **AD Goudreau** will receive training in experimental techniques from established and qualified members of the University of Ottawa and will have access to state-of-the-art equipment and wet lab spaces through supervisor **KB Adamo** and collaboration with core facilities, including the **Cell Biology and Image Acquisition (CBIA)** and the **Flow Cytometry and Virometry (FCV)** core facilities.

Introduction: Macrophages are a critical component of the immune system that present different physiological phenotypes in response to their microenvironment. These phenotypes can be broadly characterized into M1 pro-inflammatory macrophages or M2 anti-inflammatory macrophages. As the primary immune cell in the placenta at term, HBCs are thought to play a crucial role in the regulation of pregnancy and the maintenance of a homeostatic environment. Polarization towards an M1 pro-inflammatory state has been associated with many detrimental conditions, including pre-eclampsia, early pregnancy loss, and low birth weight. In physically active non-pregnant participants, peripheral and tissue-resident macrophages polarize towards a regulatory state (M2), presumably attenuating the development of inflammatory diseases. It is currently unclear whether maternal physical activity influences the polarization of HBCs, thereby contributing to protective mechanisms for mother and fetus.

Methods: To examine the effects of PA on HBC polarization, cultured HBCs will be exposed to environments of intermittent hypoxia as a proxy for PA, as well as myokines (e.g., FGF21, EPO, IL-15, SPARC, fractalkine and BDNF) previously identified by our lab as having higher expression in pregnant women after an acute bout of exercise. HBCs will be seeded at 80% confluency, and i) exposed to hypoxia (3% O_2), ii) treated with myokine concentrations determined from previously published studies, or iii) a combination of the two. Cells exposed to normoxia without myokine or just myokine treatment will serve as control groups.

Aim 1: In collaboration with the FCV core facility, flow cytometry will be used to quantify the proportion of $CD206^+$ and $CD68^+$ cells. The number of cells containing $CD68^+$ will represent the total HBC population, while those expressing both $CD68^+$ and $CD206^+$ will represent the M2 population.

Aim 2: Cells treated as described above will be used for expression analysis. Cells will be prepared for both Western blot and RT-PCR analysis to examine protein and mRNA levels, respectively, to analyze the expression of secreted angiogenic factors, including TGF-β and VEGF. Immunofluorescence will be performed and analyzed in collaboration with the CBIA to examine the proportion of $CD68^+CD206^-$ and $CD68^+CD206^+$ cells.

Aim 3: Finally, supernatant will be collected, and cells will be harvested immediately post-treatment for downstream analysis. The supernatant will be collected for ELISA to quantify the amounts of M1 and M2 macrophage characteristic proteins; for example, the M1-associated cytokines TNF-α and IL-1β, and the M2-associated cytokines IL-10, and IL-12.

Our multi-method study will be completed over an eight-month period. **Aim 1** will examine the secreted factors from HBCs exposed to hypoxia and physical activity-induced myokines; **Aim 2** will explore the expression of proteins within HBCs; and **Aim 3** will characterize the proportion and absolute numbers of HBCs from hypoxic and myokine conditions.

Significance: Characterizing the polarization of HBCs remains an important aspect of examining the effect of habitual PA on the placental immune response. As the primary immune cell of the term placenta, any effect PA has on HC polarization may also have a significant effect on the environment of the placenta. By modulating the polarization of HBCs, PA may play a protective role against conditions characterized by an increase in M1 HBCs, such as pre-eclampsia, metabolic disorders, and various birth complications.. Characterizing how HBC polarization contributes to establishing healthy pregnancies opens the door for future research designed to influence the polarization of these cells as intervention methods in unhealthy pregnancies.

APPENDIX C: TRAINING CERTIFICATES

C1. UNIVERSITY OF OTTAWA BIOSAFETY TRAINING

University of Ottawa

This certifies that

Alexandra Goudreau

has completed the following workshop

Biosafety Training - For Users (RG2/3)

ORM, Radiation and Biosafety

Saturday June 2, 2018

[redacted]

uOttawa

C2. UNIVERSITY OF OTTAWA RADIATION SAFETY TRAINING

APPENDIX D: HOFBAUER CELL POLARIZATION FLOW CYTOMETRY PROTOCOL

Flow Cytometry Protocol – Hofbauer Cell Polarization

A. HARVESTING CELLS

1. Remove media from flask
   ⇒ Save media for future experiments if needed
2. Add 2 mL TrypLE™ to flask and place in incubator for 10 minutes
   ⇒ After incubation, tap flask vigorously for 1 minute to disrupt cell adhesion
   ⇒ Confirm cell detachment under microscope
3. Add 10 mL media to flask; thoroughly wash to inactivate TrypLE™
4. Transfer media to 15 mL falcon tube
5. Centrifuge for 10 minutes at 500 xg
6. Decant supernatant
7. Resuspend pellet in 300 uL FACS buffet
   ⇒ Mix thoroughly with wide bore pipette
8. Transfer 100 uL of cell suspension to 1.5 mL Eppendorf tube
   ⇒ Set aside on ice to be used as the unstained control **(i)**
9. Centrifuge for 10 minutes at 500 xg
   ⇒ FACS buffer (PBS with 1% BSA) can be added as needed for balancing purposes
10. Decant supernatant

B. VIABILITY STAINING

11. Resuspend pellet in 1 mL PBS
12. Centrifuge for 10 minutes at 500 xg
    ⇒ Prepare 1:500 dilution of reconstituted Zombie NIR™ dye
13. Decant supernatant
14. Resuspend pellet in 100 uL diluted Zombie NIR™ dye
15. Incubate for 30 minutes at room temperature, protect from light
16. Add 2 mL FACS buffer
17. Centrifuge for 10 minutes at 500 xg
18. Decant supernatant
19. Resuspend in 200 uL FACS buffer
20. Split contents into two 1.5 mL Eppendorf tubes (100 μL per tube)
    ⇒ Set aside one tube on ice to be used as the viability only control **(ii)**
    ⇒ Continue protocol in remaining tube to be used as the all-stain control **(iii)**

C. FIXATION AND PERMEABILIZATION (cytofast)

21. Dilute the Cyto-Fast™ Perm Wash solution (10X) to 1X using deionized water
22. Add 150 μl of Cyto-Fast™ Fix/Perm Buffer to tube iii and mix
23. Incubate for 20 minutes at room temperature
24. Add 1 ml of 1X Cyto-Fast™ Perm Wash solution
25. Centrifuge at 350 xg for 5 minutes, discard supernatant
26. Repeat steps 24-25
27. Resuspend in 100 μL of 1X Cyto-Fast™ Perm Wash Solution

D. FcR BLOCKING (tru stain)

28. Add 5 μL of Human TruStain FcX™ to tube iii
29. Incubate for 10 minutes at RT
    ⇒ Proceed directly to antibody staining without washing

E. ANTIBODY STAINING

30. Add 5 µL of each antibody 333807 and 321113 to tube iii
31. Incubate for 20 minutes at RT protected from light
32. Wash cells with 1 ml of 1x Cyto-Fast™ Perm Wash solution
33. Centrifuge at 350 xg for 5 minutes and discard supernatant
34. Add 1 mL of FACS buffer
35. Centrifuge at 350 xg for 5 minutes and discard supernatant
36. Resuspend in 300 µl of FACS buffer
    ⇒ Set aside on ice as an all-stained control (**iii**)
37. Prepare single stain controls by adding 5 µL of each antibody to one tube of compensation beads (**Iv** and **v**)
38. Acquire samples **i-v** on a flow cytometer

Flow Cytometry Reagents – Hofbauer Cell Polarization

All reagents sourced through BioLegend®. Contact company representative Karen Morley for inquiries.

Antibodies

PE anti-human CD68 antibody

- Cat. #: 333807
- https://www.biolegend.com/en-us/products/pe-anti-human-cd68-antibody-4845?Clone=Y1/82A

Alexa Fluor® 488 anti-human CD206 antibody

- Cat. #: 321113
- https://www.biolegend.com/en-us/products/alexa-fluor-488-anti-human-cd206-mmr-antibody-2998?Clone=15-2

Kits

Zombie NIR™ Fixable Viability Kit

- Cat. #: 423105
- https://www.biolegend.com/en-us/products/zombie-nir-fixable-viability-kit-8657?Clone=

Other Reagents

Human TruStain FcX™

- Cat. #: 422301
- https://www.biolegend.com/en-us/products/human-trustain-fcx-fc-receptor-blocking-solution-6462?Clone=

Cyto-Fast™ Fix/Perm Buffer Set

- Cat. #: 426803
- https://www.biolegend.com/en-us/products/cyto-fast-fix-perm-buffer-set-16765?Clone=

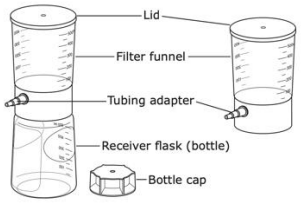
APPENDIX E: TROUBLESHOOTING PERSISTENT CONTAMINATION IN CELL CULTURE

1. REAGENT PREPARATION

A. HBC COMPLETE MEDIA FORMULATION

- ***Perform under sterile conditions (BSC)***
- *RPMI Media + 5% FBS + 25 mM HEPES*
- *Stock media*: 500 mL RPMI media (preheat to 37°C)
- Component preparation
  - 5% FBS (preheat to 37°C in water bath)
    - FBS V = (500.0 mL)(0.050)
    - FBS V = 25.00 mL
  - 25 mM HEPES
    - HEPES M = (238.3 g/mol)(0.025 mol)
    - HEPES M = 5.966 g
- HBC Complete Media production
  - Aspirate and discard 25.00 mL RPMI media
  - Replace volume with 25.00 mL 37°C FBS
  - Dissolve 5.966 g HEPES in 5% FBS-RPMI

B. FILTERING MEDIA



Stericup Quick Release-VP Sterile Vacuum Filtration System – Diagram

B. FILTERING MEDIA *(continued)*

- ***Perform under sterile conditions (BSC)***
- Utilize **0.1 µm pore size** *Stericup Quick Release-VP Sterile Vacuum Filtration System*
  - *Omit filtration for negative controls*
- Decant up to 250* mL of media (variable based on product size) into filter funnel
- Connect sterilized aspirator to tubing adaptor
- Apply suction until media flows into receiver flask
- *Storage*: remove filter funnel and seal receiver flask with bottle cap

C. CONTAMINANT GROWTH & MONITORING

- ***Perform under sterile conditions***
- Incubator #1: Hera Cell
  - Pipette 5 mL ***filtered*** media to T25 ***flask #1***
  - Pipette 5 mL ***unfiltered*** media into T25 ***flask #2***
- Incubator #2: Thermofisher
  - Pipette 5 mL ***filtered*** media to T25 ***flask #3***
  - Pipette 5 mL ***unfiltered*** media into T25 ***flask #4***
- Place in incubator (37°C – 5% CO_2 – 21% O_2)
  - Maintain for a minimum of 48 hours
- Image using Adamo Lab transmitted light microscope every 24 hours to screen for visual contamination

2. METHOD OF STAINING

OPTION #1

LIVE STAINING BY DIRECT DAPI ADDITION[1]

D1. PREPARATION FOR STAINING

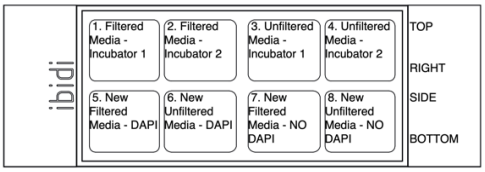
- ***Perform under sterile conditions for each flask***
- *Final DAPI concentration*: 10 µg/mL in flask
- Prepare 100 µg/mL DAPI solution (10X solution) in sterile PBS
- ***Do not*** remove media from flask (risk of cell and/or contaminant loss)
- Transfer enough 100 µg/mL DAPI solution into T25 flask for a final concentration of 10 µg/mL DAPI (1X) in total volume
  - *Flask starting volume*: **5 mL**
  - *100 µg/mL DAPI volume:* **0.556 mL**
  - *Total flask volume:* **5.56 mL**
- **Transport 8 flasks (T25) covered in tinfoil to protect fluorophore from light exposure**
  - 4 flasks with incubated media
    - 2 Filtered (1 per incubator)
    - 2 Unfiltered (1 per incubator)
  - 2 flasks with new filtered media
    - 1 with DAPI
    - 1 without DAPI
  - 2 flasks with new unfiltered media
    - 1 with DAPI
    - 1 without DAPI

OPTION #2

FIXATION AND DAPI ADDITION[1, 2, 3]

D2. PREPARATION FOR STAINING

- ***Perform under sterile conditions for each flask***
- Decant media and wash flask with sterile PBS
- Add 1 mL TrypLE™ and place in 37°C incubator for 5 – 10 minutes
- Thoroughly wash flask with HBC media to inactive TrypLE™ and collect possible contaminants
- Aspirate media and transfer to a 15 mL sterile falcon tube
- Centrifuge at 400 *x*g for 5 minutes
- Decant supernatant off while conserving pellet (may not be visible)
- **1 FLASK = 1 WELL**
  - Resuspend each pellet in 500 µL HBC media
  - Pipette 400 µL of each resuspension into the corresponding well
  - Add 1 drop pf DAPI + Prolong Gold to each well



- **Transport iBidi slide sealed in Paraffin and covered in tinfoil**

3. MICROSCOPIC ANALYSIS

- Image flasks using an *inverted epifluorescent microscope*
  - ***DAPI spectrum information***
    - *Excitation:* 358 nm
    - *Emission:* 461 nm
  - ***Objectives***
    - *Air:* 4X, 10X, 20X
    - *Oil:* 40X, 60X

- ***RECOMMENDED SYSTEM:***

- *EVOS FL Auto 2 Cell Imaging System (RGN3164A*

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* *Millipore provides Stericup filtration systems with receptor flasks volumes ranging from 150 mL – 1000 mL*

[1-3] *Sources for* ***Section 2. Option 1 – Section 2. Option 2*** *from which protocols were adapted are hyperlinked as superscript*