ASSESSING THE IMPACT OF MATERNAL PHYSICAL ACTIVITY ON SMALL EXTRACELLULAR VESICLES AND PLACENTAL VASCULARIZATION DURING PREGNANCY

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A thesis submitted in partial fulfillment of the requirements for the Doctorate in Philosophy Degree in Human Kinetics

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Dedication

This thesis is dedicated to all of my Afghan sisters (خواهران) who continue to fight for their right to receive an education. This work is dedicated to all of the women who came before me and paved the way for my unhindered curiosity. I wish for all Afghan women to be able to fulfill their greatest dreams.

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To my family who has supported me since day one, I would not be here without you. My parents came to this country seeking refuge and the opportunity for their children to have a chance at a bright future. They wholeheartedly supported my long journey here. To my partner, Eric Jones, who now touts the benefits of exercise in pregnancy to friends and colleagues that know pregnant people, I am ever grateful for your patience and support. Thank you to one of my biggest cheerleaders, Jennifer Macklem, who I am privileged to have in my life.

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Preface to the Thesis

In early March of 2020, I was off to share my findings from *Project 1* at one of the largest and most prestigious conferences in the field of Reproduction. Little did we know the impact that the COVID-19 pandemic would have on academic and research activities moving forward, let alone its impact on society and the world.

Research activities were stopped for a period of three months from March to June 2020. The first phase of research remobilization was initiated on June 12th, 2020, where permission and access to campus and research facilities could not exceed 33% and was limited to only approved physically-distanced work. This was followed by the second phase of research remobilization from July 22nd to September 25th, 2020, where capacity increased to 66%. Capacity limits were then increased slowly to resume research activities to 100%. Even as research ramped up to 100% personnel capacity, research involving human participants considered vulnerable groups (i.e., individuals at risk of more severe disease or outcomes from COVID-19 infection, including those who are pregnant) was not permitted. Luckily, I had collected the majority of samples and data from human participants needed for the work presented in this thesis before COVID-19 restrictions were in effect. However, access to key facilities and equipment essential for my data collection was functionally-restricted.

The projects originally proposed and approved by my thesis advisory committee in the year preceding the COVID-19 pandemic relied heavily on collaboration with other laboratories in the Faculty of Health Sciences and Faculty of Medicine at the Roger Guindon Campus of uOttawa (our lab is located at the Lees Campus of uOttawa). Due to building capacity limits and prioritization of limited space and laboratory resources/personnel, I could no longer access critical resources needed for specialized data collection. Systems in place to access core facilities were

cumbersome in light of COVID-19 restrictions coupled with challenges related to inter-faculty and inter-campus access. All of these factors heavily hindered and delayed my data collection. In light of these obstacles, the scope of my thesis work and data collection techniques were changed to allow for the completion of my studies and degree in a timely manner.

Pivoting research projects and data collection methods was incredibly challenging during this stressful time but was supported by my mentor and supervisor, Dr. Kristi Adamo. The research projects presented herein reflect efforts to uncover the impact of physical activity on the mother and fetus during pregnancy. It is my hope that contributions made during this difficult time move the needle forward (however slightly) on our understanding of the complex world of physical activity and pregnancy. I greatly appreciate the support I received from my supervisor, thesis advisory committee, and lab members to complete my doctoral research projects during this uncertain time.

List of Abbreviations

6-FAM; 6-carboxyfluorescein

ANCOVA; Analysis of covariance

ANOVA; Analysis of variance

ASA24; Automated Self-Administered 24-h Dietary Assessment Tool

BFRE; Blood flow-restricted resistance exercise

b-hCG; beta-human chorionic gonadotropin

BMI; Body mass index

bpm; beats per minute

BSA; Bovine serum albumin

cAMP; cyclic adenosine monophosphate

CBIA core; Cell Biology and Image Acquisition core

cDNA; Complimentary DNA

CIHR; Canadian Institutes of Health Research

cpm; counts-per-minute

CT; Threshold cycle

DAB; 3,3' Diaminobenzidine

DAPI; 4′,6-diamidino-2-phenylindole

DMSO; Dimethyl sulfoxide

dsDNAs; double-stranded DNAs

EDTA; Ethylenediaminetetraacetic acid

eNOS; Endothelial nitric oxide synthase

EVs; Extracellular vesicles

FBS; Fetal bovine serum

FGR; Fetal growth restriction

Flot-1; Flotillin-1

GDM; Gestational diabetes mellitus

GWG; Gestational weight gain

HR; Heart rate

HRR; Heart rate reserve

HUVECs; Human umbilical vein endothelial cells

IHC; Immunohistochemistry

ISEV; The International Society for Extracellular Vesicles

KDR/Flk-1; Kinase insert domain receptor

lEVs; large extracellular vesicles

LPA; Light physical activity

MISEV2018; Minimal information for studies of extracellular vesicles 2018 position statement

MPA; Moderate physical activity

MS; Mass spectrometry

MVBs; Multivesicular bodies

MVPA; Moderate-to-vigorous physical activity

mRNA; messenger RNA

miRNAs; microRNAs

nano-UHPLC-MS/MS; nano-ultra-high-performance liquid chromatography-tandem mass spectrometry

NA-seq; Nucleic acid sequencing

NTA; Nanoparticle tracking analysis

NSERC; Natural Sciences and Engineering Research Council of Canada

OHSN-REB; Ottawa Health Science Network Research Ethics Board

PA; Physical activity

PBS; Phosphate buffered saline

PBST; Phosphate buffered saline with TWEEN® 20

PE; Preeclampsia

PLACENTA; Physica**L AC**tivity and di**E**tary implicatio**N**s **T**hroughout pregn**A**ncy trial

PLAP; Placental alkaline phosphatase

PlGF; Placenta growth factor

PVDF; polyvinylidene fluoride

PWR; Postpartum weight retention

qPCR; Quantitative real-time polymerase chain reaction

RIPA buffer; Radioimmunoprecipitation assay buffer

RPM; Revolutions per minute

SD; Standard deviation

sEVs; small extracellular vesicles

TEM; Transmission electron microscopy

TBS; Tris buffered saline

TBST; Tris buffered saline with TWEEN® 20

tRNAs; transfer RNAs

TSG-101; Tumor susceptibility gene-101

VEGF; Vascular endothelial growth factor

VEGFR-1; Vascular endothelial growth factor receptor-1

VEGFR-2; Vascular endothelial growth factor receptor-2

VPA; Vigorous physical activity

Thesis Abstract

Physical activity (PA) reduces the risk for deleterious outcomes in both mother and fetus during pregnancy and improves health across the lifespan. How these benefits are bestowed remains poorly understood but may involve the placenta, the critical interface responsible for fetal growth and survival during pregnancy. This thesis first aims to determine whether small extracellular vesicles (sEVs), potential biological mediators of cell-to-cell communication, are released into circulation after acute exercise during pregnancy and how this compares in the non-pregnant state. Pregnant women were found to have greater circulating sEVs levels compared to non-pregnant controls after a moderate-intensity treadmill walk. Since exercise-associated sEVs are proposed to mediate tissue cross talk in response to exercise, exercise-associated sEVs were examined for their ability to influence trophoblasts (specialized placental cells) *in vitro* using the BeWo choriocarcinoma cell line. Exercise-associated sEVs from pregnant and non-pregnant women interacted with trophoblast-like cells but did not alter their proliferation, gene expression of angiogenic growth factors, or production of the pregnancy hormone, human chorionic gonadotropin. Finally, the relationship between differing intensities of maternal PA and fetoplacental vascular density in a cohort of healthy pregnant women followed prospectively from 24 weeks of gestation until term delivery. Using traditional histopathological point-counting techniques, there was no difference in the fetoplacental vascular density of individuals meeting or exceeding recommended 150 min of moderate-to-vigorous intensity PA. However, the analysis revealed unexpected associations between fetoplacental vascular density and lower intensities of PA, and sedentary time. Together, the work presented in this thesis highlight the potential for exercise-associated sEVs to communicate the benefits of PA to mother and fetus and the need to investigate the effects of varying PA intensities on placental vascular development.

CHAPTER 1: INTRODUCTION

1.1 Physical Activity during Pregnancy

Engagement in regular physical activity (PA) is associated with significant health benefits, including improvements in both psychological and physiological well-being, and decreased risk of chronic disease and all-cause mortality (Medicine 2013; Warburton et al. 2006). PA is also an important component of a healthy pregnancy. The recent *2019 Guideline for PA Throughout Pregnancy* recommends that women without contraindication engage in at least 150 minutes of moderate-intensity PA per week to attain clinically-relevant benefits and reduce the risk of pregnancy complications (Mottola et al. 2018). These evidence-based guidelines also apply to those who were previously inactive, diagnosed with gestational diabetes mellitus (GDM), or categorized as overweight or obese [body mass index (BMI) $\geq$25 kg/m^2] before pregnancy. While the guidelines indicate that greater engagement in PA (i.e., duration, frequency, and volume) is associated with more substantial benefits (i.e., a dose-response relationship), PA below recommendations was still found to produce some benefits (Mottola et al. 2018). The combination of aerobic exercise and resistance training versus aerobic exercise alone was associated with the most appreciable improvements in health outcomes (Davenport et al. 2018b; Skow et al. 2019). The benefits of maternal PA are numerous; they include decreased risk for delivery by caesarian section, lower incidences of pregnancy complications including GDM, preeclampsia, gestational hypertension, excessive gestational weight gain, and depressive symptoms during pregnancy (Davenport et al. 2018a; Davenport et al. 2018c; Davenport et al. 2019b; Di Mascio et al. 2016; Ruchat et al. 2018). Furthermore, prenatal PA has been associated with the development of appropriate for gestational age-sized newborns (Davenport et al. 2018b; Ferraro et al. 2012; Juhl et al. 2010; Owe et al. 2009; Wiebe et al. 2015), and reduced fat mass at birth (Clapp 1996; Clapp

et al. 1998). The benefits of maternal PA for both mother and offspring are shown schematically in **Figure 1**.

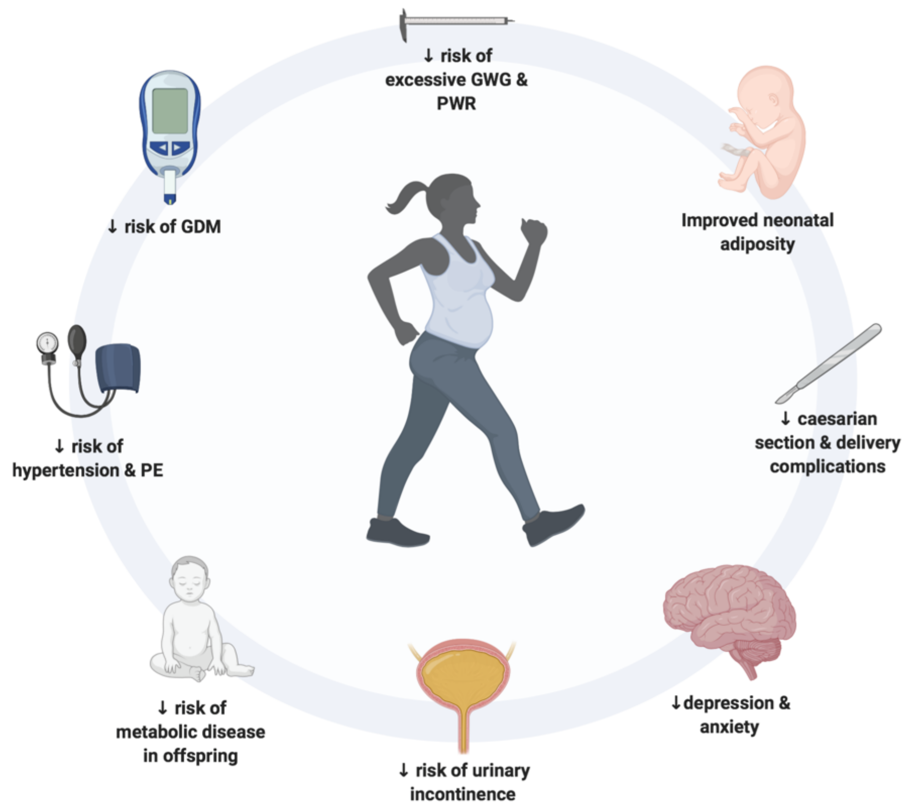

Figure 1. The benefits of physical activity during pregnancy. Maternal PA bestows benefits on both mother and fetus and are not limited to only those included in this illustration. GDM, gestational diabetes mellitus; GWG, gestational weight gain; PE, preeclampsia; PWR, postpartum weight retention. Figure is original work published by (Bhattacharjee et al. 2021a) created using Biorender.com.

Contrary to popular myths, the safety of moderate-to-vigorous intensity PA is well accepted and is not associated with increased risk for congenital anomalies (Davenport et al. 2019c), pregnancy loss (Davenport et al. 2019a), or preterm birth (Davenport et al. 2018b; Davenport et al. 2019b; Di Mascio et al. 2016; Huang et al. 2019). Additionally, both acute and chronic exercise during pregnancy (at moderate-intensity) does not negatively impact

uteroplacental blood flow metrics (and resulting fetal oxygenation) or fetal heart rate (Clapp 2003; Clapp et al. 1995; Kennelly et al. 2002; Skow et al. 2019). Although specific recommendations could not be made regarding the safety or additional benefit of exercising at higher intensities than suggested by the *2019 Guideline for PA Throughout Pregnancy*, evidence from a meta-analysis conducted by Beetham and colleagues (2019) concluded that vigorous-intensity exercise into the 3rd trimester appeared to be safe for most healthy pregnancies (Beetham et al. 2019). Therefore, regular maternal PA is a therapeutic strategy to optimize maternal-fetal health acutely and across the lifespan.

Although a breadth of epidemiological evidence supports the notion that PA optimizes health during pregnancy, the biological mechanisms involved in communicating these health benefits to mother and fetus remain unclear. Many changes in maternal physiology accompany habitual PA across pregnancy, ranging from improving metabolism and glycaemic control, autonomic nervous system regulation, cardiovascular (i.e., increased heart rate, stroke volume, cardiac output, etc.), and musculoskeletal accommodations all of which are reviewed in great detail elsewhere (Davenport et al. 2018d; Dietz et al. 2016; Dubé et al. 2017; Ferraro et al. 2012; Reyes and Davenport 2018). Our laboratory, along with others, has hypothesized that the critical organ of pregnancy, the placenta, may be positively impacted by PA, thereby mediating health benefits while attenuating the risk of disease development for both mother and fetus (Bhattacharjee et al. 2021a). The work presented in this thesis aims to characterize some of the biological mechanisms that may be contributing to changes in placental physiology in response to maternal PA. This will be achieved through the investigation of extracellular vesicles (EVs) (specifically, small EVs), novel molecular conduits implicated in the systemic response to exercise (Brahmer et al. 2020; Estébanez et al. 2021; Nederveen et al. 2020). Additionally, I will seek to examine the effect of

maternal PA on placental vascularization in a cohort of healthy pregnant individuals as a part of the Adamo Lab's PLACENTA (**P**hysica**L** **AC**tivity and di**E**tary implicatio**N**s **T**hroughout pregn**A**ncy) observational trial. **The central focus of this thesis is to enhance our understanding of the biological consequences of maternal PA on the placenta by:**

1. *Characterizing the circulating small EV profile of pregnant versus non-pregnant women after acute moderate-intensity exercise*
2. *Assessing the influence of exercise-associated small EVs on trophoblasts (specialized placental cells) in vitro*
3. *Examining markers of fetoplacental vascularization in a cohort of healthy pregnant individuals of varying levels of PA exposure across pregnancy*

Results from the studies mentioned above will help to clarify i) whether small EVs are released into circulation in response to exercise during pregnancy, ii) the potential role of these small EVs in placental function, including the production of angiogenic growth factors, and iii) how fetoplacental vascularization is affected by differing intensities of PA. Given the paucity of research on PA and placental biology, these studies are critical for clarifying the molecular mechanisms implicated in modifying the placental environment to optimize maternal-fetal health.

1.2 Overview of Extracellular Vesicles

Extracellular vesicles (EVs) are a heterogeneous group of lipid bilayer membrane nanoparticles secreted by cells into extracellular space (Colombo et al. 2014; Raposo and Stoorvogel 2013). EVs are loosely classified into three main groups based on their size and mode of release or biogenesis: (1) small EVs are typically 20-120 nm in size (herein referred to as 'sEVs' and historically referred to as 'exosomes' among other names) formed through intracellular

endocytic pathways and released through multivesicular bodies (MVBs) (Harding et al. 1983; Pan et al. 1985), (2) large EVs of approximately 100-1000 nm in size (herein referred to as lEVs and historically called ‘microparticles’ or ‘microvesicles’ in addition to other names) budding directly from plasma membranes (Mathieu et al. 2019; Tricarico et al. 2017; van Niel et al. 2018), and (3) apoptotic bodies of >1000 nm in size formed during membrane disintegration (Akers et al. 2013; Colombo et al. 2014; Gould and Raposo 2013). Properties of differing EV subtypes often overlap, leading to some ambiguity in the literature when describing EV populations based solely on designations of size and mode of release/biogenesis (Booth et al. 2006; Gould and Raposo 2013; Jeppesen et al. 2019).

EVs allow for intercellular communication over long distances as they contain and facilitate the transfer of biologically active molecules, including nucleic acids [mRNAs and other small RNAs, i.e., microRNAs (miRNAs)], proteins, lipids, and small metabolites from donor cells to recipient or target cells (Kowal et al. 2014; Mathieu et al. 2019; Ratajczak et al. 2006; Skog et al. 2008; Valadi et al. 2007; Vallabhaneni et al. 2015). The biological topology of EVs is similar to that of cells (with the exception of a functional nucleus), with cytoplasmic proteins and RNAs on the inside and extracellular membrane-bound receptors and ligands on the outside (Maas et al. 2017). Thus, EVs interact with target cells through cell membrane-mediated signaling (Mathivanan et al. 2010; Zhang et al. 2019) and/or through internalization or uptake via clathrin or caveolin-mediated endocytosis, phagocytosis, or macropinocytosis (Escrevente et al. 2011; Feng et al. 2010; Nakase et al. 2015; Tian et al. 2014; Vargas et al. 2014). The biological composition of EVs (and thus, biological function) largely depends on the cell of origin and its physiological state. It is hypothesized that EVs may selectively package or capture cell-specific RNAs, proteins, and lipids to become a part of their membrane or cargo (Akers et al. 2013; van

Niel et al. 2018). Whether EV contents, especially RNAs, are actively selected for or are merely captured from the intracellular microenvironment of the donor cell is a contentious topic (Mateescu et al. 2017; Pegtel et al. 2010; Pegtel and Gould 2019; Veziroglu and Mias 2020).

EVs can be found in a wide range of biological fluids *in vivo*, including blood, lymph, urine, saliva, milk, lavages, cerebrospinal, synovial, and amniotic fluids, and *in vitro* in cell culture media (Tkach and Théry 2016). They are involved in a multitude of physiological and pathophysiological processes and thus have become an emerging field of great interest (Colombo et al. 2014; Tkach and Théry 2016). In addition to their paracrine and endocrine roles in intercellular communication, EVs have gained considerable interest in clinical applications as diagnostic and prognostic biomarkers of health and disease, potential therapeutic agents, and drug delivery tools (Barile and Vassalli 2017; Murphy et al. 2019; Zhang et al. 2019; Zhou et al. 2021). Although the study of EVs has gained exponential notoriety over the past two decades, the concept of EVs can be traced back to the 19th century and Charles Darwin, who proposed that cells could produce 'gemmules' (particles of 'minute' size) containing molecules acting as messengers for cell-to-cell communication (Darwin 1868; Liu and Chen 2018; Margolis and Sadovsky 2019). The first published works recognizing the functional roles of EVs in mammals emerged at least 50 years ago when two groups concurrently reported EVs in epiphyseal cartilage matrix calcification (Anderson 1969) and neuronal signaling at the neuromuscular junction (Grillo 1970). sEVs were first described in the maturation of reticulocytes (Harding et al. 1983; Johnstone et al. 1987; Pan and Johnstone 1983), and have subsequently been observed to be released by several cell types *in vitro*, including cells of the maternal-fetal interface (Donker et al. 2012; Luo et al. 2009) and those involved in exercise such as myotubes (Le Bihan et al. 2012; Romancino et al. 2013; Whitham et al. 2018), in addition to numerous bodily fluids *in vivo*. In this thesis, the term 'EVs' specifies any

type of released vesicles; however, the work herein focuses on sEVs in exercise and pregnancy.

Figure 2 illustrates a simplified sEV release and uptake model in response to exercise.

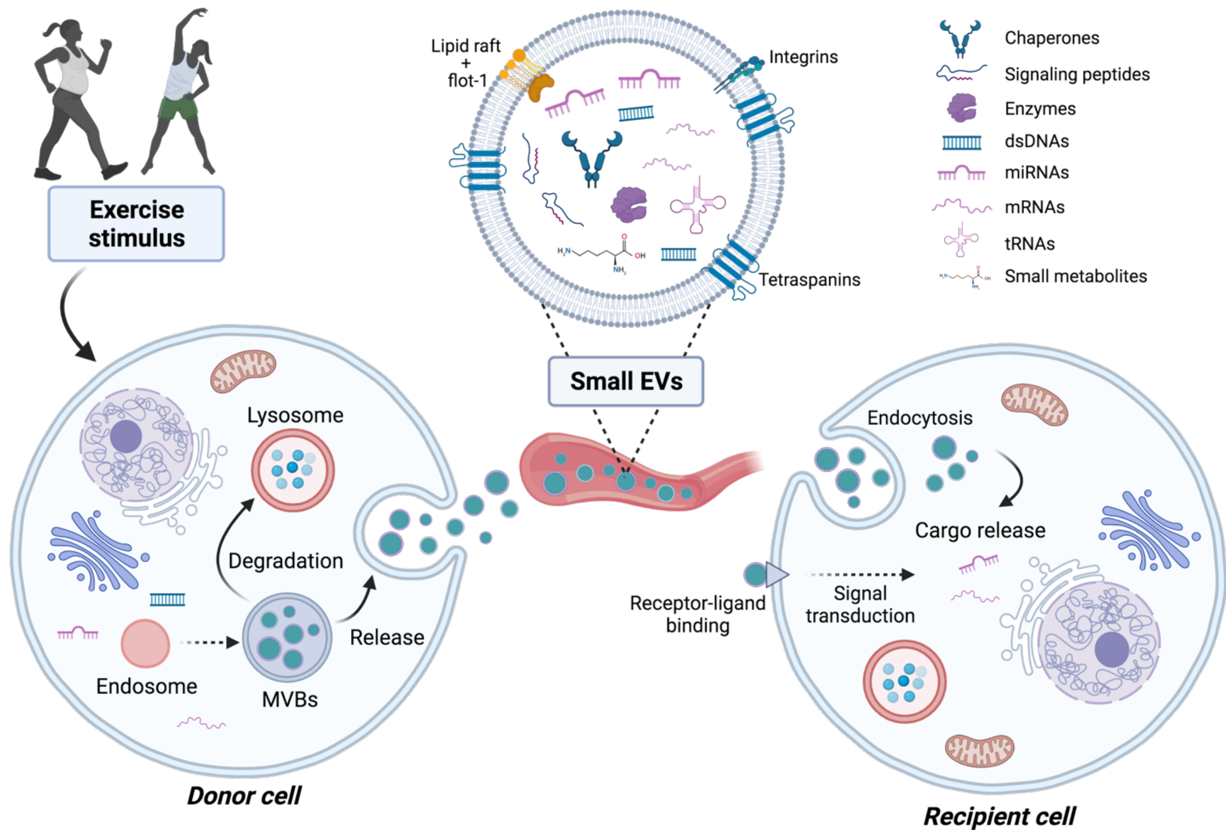

Figure 2. Simplified schematic representation of sEVs released and taken up by cells in response to exercise. In donor cells, sEVs (~30-120 nm) are formed in endosomes which then mature into multivesicular bodies (MVBs). MVBs can either fuse with lysosomes for degradation or with the plasma membrane for release into extracellular space. It is theorized that sEVs are released into circulation to reach recipient cells, where they can interact via receptor-ligand binding or internalization by endocytosis (simplified for this diagram but can occur by a variety of mechanisms). After sEV internalization or binding, cargo are released or signal transduction pathways are stimulated. The magnified centre panel shows the range of observed sEV contents and features including transmembrane receptors and proteins, enzymes, signaling peptides, nucleic acids, and small metabolites. dsDNA, double-stranded DNAs; EVs, extracellular vesicles; Flot-1; flotillin-1; miRNAs, microRNAs; mRNAs, messenger RNAs; miRNA, MVBs, multivesicular bodies; tRNAs, transfer RNAs. Figure adapted from (Estébanez et al. 2021; Nederveen et al. 2020) and created using Biorender.com.

1.2.1 Small extracellular vesicle isolation, measurement, and contents

sEVs are commonly purified from biofluids by a series of high-speed centrifugation and ultracentrifugation steps culminating at 100,000 x *g* (Gardiner et al. 2016; Théry et al. 2006). Further purification can be achieved through ultracentrifugation using sucrose density gradients (Raposo et al. 1996). Other separation techniques include filtration, precipitation, immunoisolation, size exclusion chromatography, or a combination of the techniques above (Gardiner et al. 2016; Théry et al. 2018). No single optimal separation method has been established to isolate sEVs, yielding proper characterization of sEV isolates essential for their study. The *Minimal information for studies of extracellular vesicles 2018* (MISEV2018) position statement guidelines of the International Society for Extracellular Vesicles (ISEV) [updated from the 2014 guidelines (Lötvall et al. 2014)] recommends sEV characterization by multiple complementary techniques to confirm and validate sEV isolation and not co-isolation of other materials (Théry et al. 2018). The guidelines state that sEV characterization should include (1) enumeration and quantification of EV abundance, with the source volume, tissue, or cell numbers expressly noted, (2) testing for the presence of classical sEV markers (i.e., tetraspanins and proteins involved in MVB formation, lipid rafts, and chaperones) and absence of non-sEV markers (i.e., nuclear proteins), and (3) the characterization of single vesicles by two complementary techniques that estimate the biophysical features of sEVs (i.e., optical visualization by electron or atomic force microscopy and single-particle analysis via particle tracking methodologies) (Théry et al. 2018). It must be noted that there are over 100 putative sEV biomarkers and no consensus has been reached on a single marker to identify sEV populations (Barile and Vassalli 2017; Keerthikumar et al. 2015). The MISEV2018 guidelines also provide recommendations for the functional analysis of sEVs, including

appropriate controls and processes to assess the influence of non-sEV soluble components or co-isolated factors (Théry et al. 2018). These are essential factors to consider when interpreting sEV literature or designing sEV experiments.

Insights into the cellular origins and functional roles of sEVs can be gleaned by characterization of their contents, aided by high-throughput ‘omics’ technologies. Proteomics can be used to identify sEV protein contents through either global or targeted quantitative approaches by chromatography coupled with mass spectrometry (MS) (Schey et al. 2015). MS can also be used to assess lipid contents of sEVs (*via* lipidomics), which make up a large portion of total sEV volume (Kreimer et al. 2015). Metabolomics has been used to profile metabolite contents of sEVs (Eylem et al. 2020; Tao et al. 2019; Vallabhaneni et al. 2015), but is hindered by the quantity of EVs necessary to reach MS detection limits (Veziroglu and Mias 2020). Differences in sEV isolation protocols have led to significant challenges in the comparison and interpretation of sEV proteomics and lipidomics data between research groups, further highlighting the need for the standardization and optimization of sEV purification methods (Abramowicz et al. 2016; Taylor and Shah 2015). Leveraging emerging technologies with high sensitivity like next-generation nucleic acid sequencing (NA-seq), we are now capable of profiling sEV RNA cargo (McCombie et al. 2019). Numerous RNA species have been identified in sEVs, including mRNAs, miRNAs, tRNA-derived small RNA fragments, Y-RNAs, long-non-coding RNAs, and circular RNAs [reviewed by (Veziroglu and Mias 2020)]. Combining next-generation NA-seq methodologies with computational approaches could lead to the identification of sEV RNA signatures associated with a given cellular origin in complex biofluids (Veziroglu and Mias 2020). Antibody-based techniques are beneficial in more targeted studies to characterize sEV surface proteins, aiding in identifying sEV cellular origins in complex biofluids and potential biological functions.

Challenges arise in the fluorescent labeling of particles in the typical size range of sEVs. Still, these methodologies have benefitted from recent advances in high-sensitivity and nano-resolution flow cytometry with multiplexing capabilities (Ji et al. 2019; Morgan 2018; Tian et al. 2018).

The excitement and potential in the burgeoning field of EV science has led to a high degree of diversity in assessment methodologies and nomenclature among EV studies, with notable issues in the reproducibility and validity of published results (Veziroglu and Mias 2020). Coupled with the experimental and technical complexity of sEV isolation, characterization, and evaluation of functional roles, it can be challenging to appraise and therefore execute efficacious EV work. Nevertheless, EVs represent an exciting avenue for investigating complex systemic physiological processes, such as those involved in the exercise response. It is worth noting that the limitations and challenges arising in EV research are an opportunity for novel explorations and discoveries. The following discussion frames our current understanding of sEVs in the context of exercise and identifies key gaps in the sEV exercise literature that will be addressed through this thesis.

1.2.2 Small extracellular vesicles and exercise

Given that the multi-systemic benefits of PA are likely mitigated in part through paracrine and endocrine mediators like myokines (peptides produced and released by skeletal muscle) (Hoffmann and Weigert 2017; Pedersen 2011), EVs may represent another biological mechanism by which the effects of exercise are communicated throughout the body. Indeed, evidence has implicated the involvement of sEVs in facilitating tissue cross-talk and intercellular communication in response to exercise at a systemic level (Brahmer et al. 2020; Frühbeis et al. 2015; Nederveen et al. 2020; Whitham et al. 2018). One of the first studies examining the effects of an acute exercise bout on circulating sEV abundance was conducted by Frübeis and colleagues in 2015 (Frühbeis et al. 2015). sEV response was evaluated in 12 healthy, physically active young

men after an acute bout of exhaustive cycling (N=8) or treadmill running (N=4). The cycling exercise consisted of an incremental test using an ergometer, starting at 50 W and increasing power every three minutes until exhaustion. The treadmill running exercise was also conducted via an incremental test with a starting speed of 6 km/h and increasing velocity by 2 km/h every three minutes until exhaustion, at a constant incline of 1.5%. Plasma was collected pre- and immediately post-exercise, and 90 min after the exercise session. sEVs were isolated from fresh plasma by differential ultracentrifugation and filtration, then quantified by western blotting (N=5-6) and nanoparticle tracking analysis (NTA) (descriptive data only, N=2). They found an acute release of sEVs (<140 nm in size) in response to cycling in an intensity-dependent manner, returning to baseline levels 90 min post-exercise (Frühbeis et al. 2015). Analysis of sEV plasma isolates collected at the end of each incremental stage of the cycling exercise showed that the release of sEVs occurred early in the exercise bout before the anaerobic threshold was reached (Frühbeis et al. 2015). sEV concentration after the treadmill exercise did not increase to the extent that was observed after cycling, but statistical comparisons were not conducted due to the low sample size (N=2 evaluated by NTA for sEV size and number). These results should be interpreted with caution as sEV concentrations were compared using semi-quantitative methods (immunoblotting) of unvalidated sEV markers instead of single-particle techniques like NTA. Further, the mean vesicle size after the treadmill exercise was ~164 nm when the upper limit is ~140 nm (Harding et al. 2013), indicating the presence of either lEVs or contaminating co-factors such as lipoproteins or chylomicrons (Karimi et al. 2018; Mørk et al. 2017). Verification of sEV morphology by optical visualization methods like electron microscopy was also missing. Nonetheless, the results showed that exercise modality might influence sEV release and kinetics.

Another study by Whitham *et al.* (2018) employed quantitative proteomics to test the hypothesis that exercise may stimulate the secretion of sEVs, providing a signaling mechanism important for tissue cross-talk (Whitham et al. 2018). In this study, 11 healthy male subjects cycled for 1 h (30 min at 55%, 20 min at 70%, and approximately 10 min at 80% of $VO2_{max}$ until exhaustion), and blood samples were collected by femoral artery catheterization pre- and immediately post-exercise, and 4 h after exercise. sEVs were isolated by high-speed centrifugation (20,000 x *g*) and contents were identified using nano-UHPLC-MS/MS (nano-ultra-high-performance liquid chromatography-tandem mass spectrometry). sEVs were further quantified by size and number using NTA and visualized by cryo-electron microscopy. After a 1 h bout of cycling, there was an increase in the number of EVs ranging from 50-350 nm in size, which declined to baseline levels by 4 h after exercise. Although the authors showed that EV isolates were positive for some classical sEV markers, they likely also contained lEVs due to the lack of ultra-centrifugation or other more refined isolation methods for separating circulating sEVs. A total of 322 unique proteins were significantly different between the exercise and baseline EV fractions, including 35 novel candidate myokines. Gene ontology analysis revealed a significant enrichment of proteins involved in signal transduction, regulation of immune cell proliferation, G-protein and GTPase signaling, and glycolysis. Animal model evidence illustrated the uptake and accumulation of fluorescently-labeled EVs in the liver. Finally, *in vitro* data demonstrated the uptake of sEVs and corresponding cargoes from C2C12 myotubes or exercising mouse plasma into AML12 hepatocytes.

Since the publication of these first two human studies on circulating sEVs in the context of acute exercise, the field has rapidly expanded to include differing exercise modalities [i.e., plyometric jumping and downhill running (Lovett et al. 2018), ischemic resistance exercise (Just

et al. 2020), eccentric resistance exercise (Annibalini et al. 2019; Kyriakidou et al. 2021), high-intensity interval exercise (D'Souza et al. 2018)], training status [i.e., untrained males (Hou et al. 2019; Lovett et al. 2018), aerobically-trained males (Brahmer et al. 2019; Hou et al. 2019), resistance-trained males (Annibalini et al. 2019)], and populations [i.e., older men (Kyriakidou et al. 2021; Nair et al. 2020), men and women with obesity (Rigamonti et al. 2020), young females (Silver et al. 2020)], with the majority testing high intensity, sustained exercise until volitional exhaustion. Some studies reported an immediate increase in the number of sEVs in circulation after acute exercise (Frühbeis et al. 2015; Whitham et al. 2018), while others observed no change (Brahmer et al. 2019; Just et al. 2020; Kyriakidou et al. 2021; Lovett et al. 2018) or a decrease post-exercise (Rigamonti et al. 2020). Vesicle size is commonly reported in sEV and exercise research with comparisons of mean particle size before and after exercise. To date, only qualitative observations show a difference in vesicle size pre- vs. post-exercise, due to limited sample size (Frühbeis et al. 2015; Whitham et al. 2018). Work from animal models reflects observations from human studies, with some reporting an increase in the release of sEVs after acute exercise (Barone et al. 2016; Oliveira et al. 2018). The majority of animal model exercise sEV studies involve chronic exercise interventions showing higher concentrations of circulating sEVs in trained vs. untrained or sedentary animals (Bei et al. 2017; Bertoldi et al. 2018; Ma et al. 2018), or no difference (Hou et al. 2019). Upon review of the sEV exercise literature, it was evident that **there were no studies on the circulating sEV profile after acute exercise in the pregnant population, in either humans or animal models**. When experiments for *Project 1* were designed and conducted, no published works existed involving the female sex. Since then, works by (Rigamonti et al. 2020) and (Silver et al. 2020) have been published, but the latter likely isolated lEVs and did not quantify nor characterize sEVs. Further, all acute exercise sessions currently examined in the

literature thus far were either at a high intensity (i.e., 80% $VO2_{max}$) or endurance exercise until exhaustion, both of which do not fall within the recommendations for PA during pregnancy. **Therefore, *Project 1* was designed to describe the circulating sEV profile in pregnant and non-pregnant women after an acute bout of moderate-intensity treadmill walking for 30 min [(as per the *PA and Pregnancy Guidelines* (Mottola et al. 2018)], following the MISEV2018 guidelines (Théry et al. 2018) for reporting and characterizing sEVs (see section 1.4 for specific aims).**

While not the focus of this thesis, several groups have attempted to characterize the contents of circulating sEVs and their potential cellular origins. A short summary of the main findings of studies is discussed here, but excellent and in-depth reviews can be found elsewhere (Estébanez et al. 2021; Nederveen et al. 2020). Protein contents of exercise-associated sEVs are involved in signaling pathways, including immune signaling, angiogenesis, coagulation, and glycolysis (Brahmer et al. 2019; Bryl-Górecka et al. 2018; Just et al. 2020; Whitham et al. 2018). However, it must be noted that some of these studies likely included a mixture of both sEVs and lEVs based on reported size estimations and isolation methods (Bryl-Górecka et al. 2018; Whitham et al. 2018). Several groups have examined the RNA contents of exercise-associated sEVs, primarily focusing on miRNAs [reviewed by (Estébanez et al. 2021)]. Of those, miRNAs predicted to be involved in processes such as cell cycle control (Just et al. 2020), metabolism (Sadovska et al. 2021), protein folding (Sadovska et al. 2021), endothelial function (D'Souza et al. 2018; Ma et al. 2018), muscle and cardiac hypertrophy (Nair et al. 2020), ischemia/reperfusion injury and apoptosis of cardiomyocytes (Hou et al. 2019), and the IGF-I (Nair et al. 2020) and MAPK signaling pathways (Oliveira et al. 2018) have been identified. Further, miRNAs associated with skeletal muscle, referred to as ‘myomiRs’, have been described in exercise-associated sEVs

(Annibalini et al. 2019; D'Souza et al. 2018; Lovett et al. 2018; Silver et al. 2020; Yin et al. 2019), hypothesized to play key roles in muscle development, differentiation, growth, and repair (Kirby et al. 2015). With-respect-to the complex cellular origins of circulating sEVs released in response to exercise, evidence implicates cardiomyocytes (Bei et al. 2017), platelets (Brahmer et al. 2019; Frühbeis et al. 2015), leukocytes (Brahmer et al. 2019), endothelial or endothelial progenitor (Brahmer et al. 2019; Hou et al. 2019; Ma et al. 2018; Wang et al. 2020), and muscle cells (Guescini et al. 2010; Rigamonti et al. 2020; Whitham et al. 2018) as contributors to the exercise-associated sEV pool. Identification and deep molecular characterization of sEV contents and origins may help elucidate their functional roles in the exercise response.

Although several groups have described the release of sEVs into systemic circulation following either acute or chronic exercise, few have investigated the ability of sEVs to produce functional consequences in target cells. Most of the work on the physiological effects of exercise-associated sEVs is in animal models, where their interactions have been shown to delay prostate cancer progression (Sadovska et al. 2021), provide sustained cardioprotection *via* miR-342-5p (Hou et al. 2019), and elicit beneficial effects to the brain in ischemic stroke (Wang et al. 2020), suggesting exercise-sEVs may play a regenerative role in disease. In humans, sEVs obtained after blood flow-restricted resistance exercise (BFRE) in young, healthy men were found to increase the proliferation of fibro-adipogenic progenitor cells (Just et al. 2020). Plasma was collected from participants immediately before and 1 h after performing a single BFRE bout consisting of five sets of knee extensions under partial blood flow restriction until volitional exhaustion. sEVs were isolated by immunoisolation methods (kit-based) and size-exclusion chromatography for miRNA analysis and functional *in vitro* studies, respectively. Next-generation sequencing revealed 12 differentially expressed miRNAs after BFRE, with several predicted gene targets involved in cell

cycle control and proliferation (Just et al. 2020). Since BFRE is known to affect skeletal muscle, the authors examined the effect of sEVs obtained post-BFRE on muscle stem cells. Fluorescently labeled post-BFRE sEVs accumulated within muscle stem cells and fibro-adipogenic progenitor cells after incubation for 24 h *in vitro*. Proliferation increased significantly after exposure to post-BFRE sEVs to fibro-adipogenic progenitor cells only, with no differences compared with pre-BFRE sEVs vs. non-sEV controls (PBS only). Exposure to post-BFRE sEVs did not alter the differentiation of muscle stem cells into multinucleated myotubes compared to non-sEV controls (Just et al. 2020). Although this field is still in its infancy, these studies demonstrate that sEVs released in response to exercise have the potential to mediate physiological changes.

The impact and the exact function of these bioactive molecules remain unknown but may play a role in the benefits received from engagement in PA. sEVs are known to be released and taken up by the placenta as a means of maternal-fetal cross-talk (Adam et al. 2017; Mitchell et al. 2015; Salomon and Rice 2017; Sheller-Miller et al. 2019; Tong and Chamley 2015). Therefore, exercise-associated sEVs should be explored as a potential avenue for exercise-mediated biological changes in the placenta. **I hypothesize that cells making up the maternal-fetal interface, the trophoblasts, are target or recipient cells of circulating exercise-associated sEVs amenable to alterations in their physiology, leading to benefits for both mother and fetus during pregnancy (*Project 2*, refer to section 1.4 for specific aims).**

1.3 The Placenta

The placenta is the critical interface between mother and fetus, responsible for exchanging gases, nutrients, and waste while providing endocrine and immune support. The placenta plays essential roles in supporting the growth, development, and survival of the fetus and,

correspondingly, the health of the mother. Defective placental development can result in significant pregnancy complications, including FGR, preeclampsia, stillbirth, and preterm birth (Aplin et al. 2020; Brosens et al. 2011; Kaufmann et al. 2003; Woods et al. 2018). The mature placenta is a discoid organ embedded into the maternal uterus consisting of chorionic villi bathed in maternal blood. **Figure 3** shows an illustration of the term human placenta, with the juxtaposed maternal and fetal circulations and components of the maternal decidua (transformed uterine endometrium) displayed. The maternal-fetal interface is comprised of the maternally-derived decidua and the fetally-derived placenta, attached physically by anchoring villi. The actions of the maternal-fetal interface are coordinated by several unique cell types, including decidual stromal cells, decidual immune cells, and trophoblast cells of differing subtypes (Turco and Moffett 2019; Yang et al. 2019).

The placenta is truly a fascinating and unique organ; its transient yet vital role in sustaining and nourishing life *in utero* is unmatched. Of its distinctive features, the invasive nature of the placenta is remarkable as its entire surface is in direct contact with maternal blood, with an area of at least 11-13 m^2 available for maternal-fetal exchange at term (Mayhew 2014). Recent work on the basal membrane covering the chorionic villi has revealed that the surface area available for exchange is likely much more extensive than appreciated (Tashev et al. 2022). The placenta is uniquely positioned to mediate biological communication between the mother and the fetus, with its impact on health across the lifespan widely recognized (Barker 1992). Therefore, its study is essential to understand the biological mechanisms underpinning the benefits of PA during pregnancy.

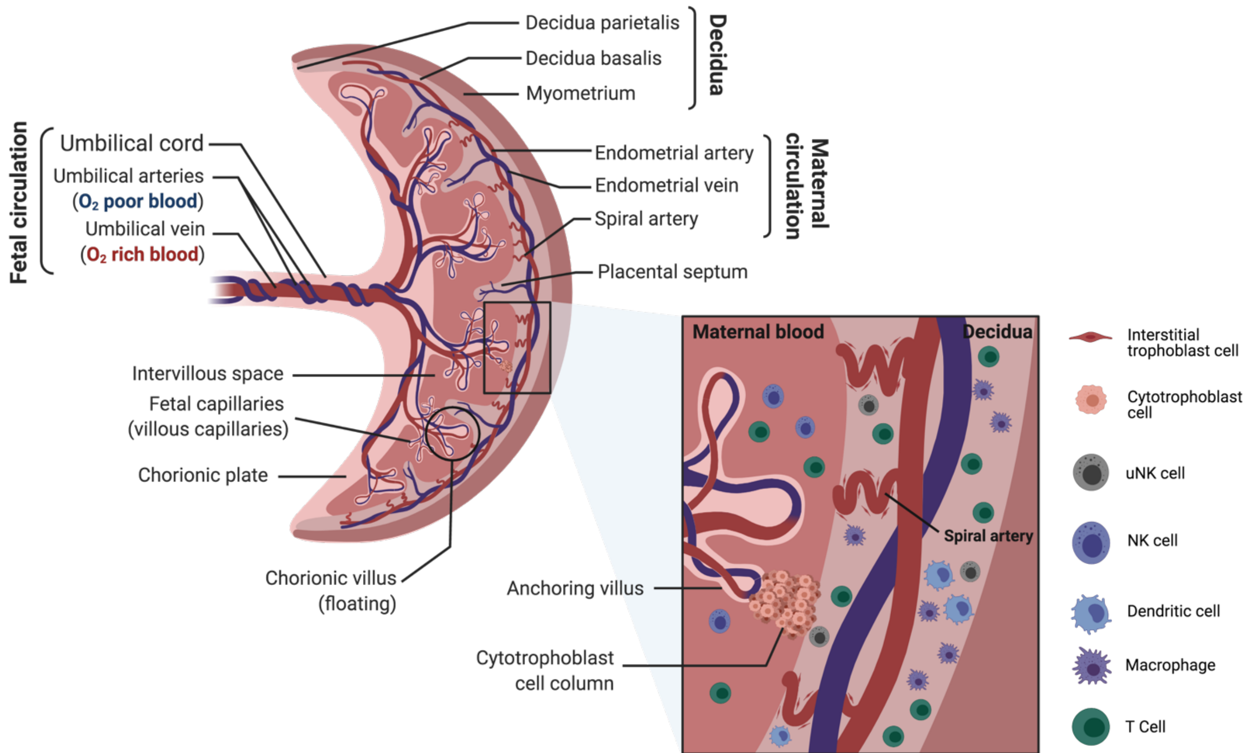

Figure 3. Diagram of the gross structure of the human placenta. The major anatomical structures of the human placenta are shown, with the fetal circulation on the left and the maternal circulation on the right of the top panel. The inset (right) panel highlights the placental bed with various cell populations from the fetal placenta and maternal transformed endometrium (referred to as the decidua) comprising the maternal-fetal interface. Within the decidua are distinct maternal immune cells, including uterine natural killer (uNK) cells, dendritic cells, macrophages, and T cells. These immune cells are thought to play key roles in spiral artery remodeling and pregnancy maintenance by interacting with invasive trophoblast populations including cytotrophoblasts, extravillous trophoblasts, and interstitial trophoblast cells. The intervillous space contains maternal blood with peripherally-circulating immune cells [i.e., natural killer (NK) cells, and T cells] that are in direct contact with the chorionic villi. Figure is original work modified from (Bhattacharjee et al. 2021a) and created with BioRender.com.

1.3.1 Placental development and cell types

A brief summary of placental development follows, emphasizing placental cells and structures pertinent to this thesis, including the fetoplacental vasculature and associated trophoblast populations responsible for maternal-fetal exchange. Many comprehensive reviews and book chapters are available for a more detailed account of placental development (Benirschke

et al. 2012b; Bhattacharjee et al. 2021a; Bischof and Irminger-Finger 2005; Castellucci and Kaufmann 2006; Hamilton and Boyd 1960; Turco and Moffett 2019).

The placenta originates from the outer layer of the blastocyst or pre-implantation embryo, called the trophectoderm. The trophectodermal cells invade the endometrium (then becoming the transformed maternal decidua) at implantation and differentiate into mononuclear cytotrophoblasts and multinucleated syncytiotrophoblast, giving rise to the primary villi (Benirschke et al. 2012b). The primary villi are invaded by mesenchymal cells (then referred to as secondary villi) from the embryo, generating vascular capillaries through vasculogenesis, thus forming the tertiary villi. Over the course of gestation, these villi experience transformation and maturation, which includes: (1) further proliferation of cytotrophoblasts and differentiation into syncytiotrophoblast, (2) branching angiogenesis of capillaries and growth of the vascular tree, and (3) non-branching angiogenesis resulting in the longitudinal growth and maturation of the vasculature (Demir et al. 2006; Demir et al. 2007; Herr et al. 2010; Jackson et al. 1992). As pregnancy progresses towards fetal viability (approximately 24-26 weeks gestation), terminal villi start to form as short outgrowths from the larger branches of the growing fetoplacental vascular tree (namely, the stem and mature intermediate villi), approximately 80 µm in diameter and up to 100 µm in length (Kaufmann et al. 1979; Kingdom et al. 2000). Terminal villi are the most critical physiological components of the fetoplacental vascular tree as they are the primary site of maternal-fetal exchange, with 50% of their cross-sectional area occupied by vessels (Kaufmann et al. 1979). At term, the vasculo-syncytial membrane (distance between maternal blood in the intervillous space and a villus capillary) of terminal villi is reduced to a thickness of 1-2 µm (Burton and Tham 1992). A cross-section describing the structure of the chorionic villi and terminal villus with corresponding cell layers is shown in **Figure 4**. The structure of chorionic villi consists of a stromal

cell core containing fetal blood vessels and their respective endothelial cells, mesenchymal stromal cells, Hofbauer cells, and fibroblasts surrounded by an epithelial trophoblast layer in direct contact with maternal blood (Castellucci and Kaufmann 2006; Wang and Zhao 2010).

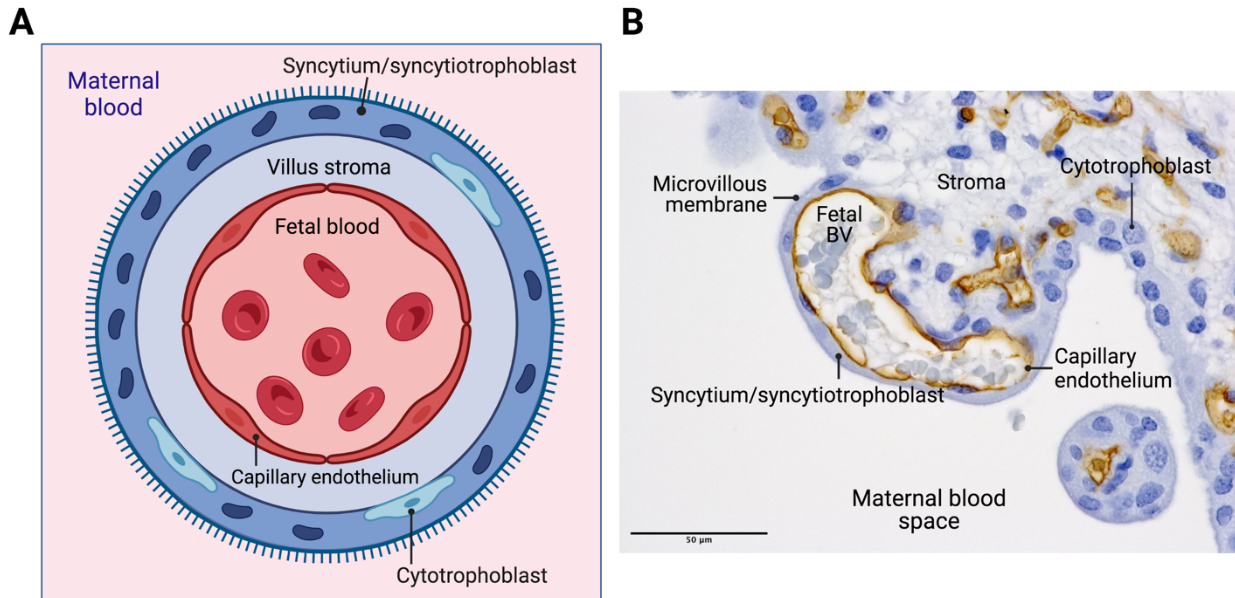

Figure 4. Schematic and histological cross-section chorionic villi and terminal villus in the human placenta. (A) shows a diagram depicting the cell layers between maternal and fetal blood. On the outside is the continuous and multinucleated syncytiotrophoblast cell layer (often referred to as the 'syncytium') with a microvillous membrane or brush border. The underlying cytotrophoblasts act as a stem cell population contributing to the syncytiotrophoblast layer. Beneath the trophoblast layers is the villus stroma, containing fibroblasts, placental macrophages, and fetal blood vessels lined with a thin capillary endothelium. (B) is an original histological image showing a formalin-fixed, paraffin-embedded term placenta tissue cross-section stained using anti-CD34 immunohistochemistry (IHC) to highlight the fetal capillary endothelium (brown) and counterstained with hematoxylin. Figure inspired by (Holder et al. 2021) and created with Biorender.com. Scale bar = 50 µm. BV, blood vessel.

Trophoblast cells carry out the primary functions of the placenta. A variety of trophoblast subtypes have been described (Liu et al. 2018); however, there are three main subtypes: syncytiotrophoblast, cytotrophoblasts, and extravillous trophoblasts. Cytotrophoblast cells are mitotically-active, polarized mononuclear cells that line the stromal core of chorionic villi and are the source of replenishment for syncytiotrophoblast and extravillous trophoblasts (Bischof and Irminger-Finger 2005). Extravillous trophoblast cells proliferate and migrate from the tips of

anchoring placental villi to invade the maternal uterine wall and remodel existing maternal vasculature, ensuring sufficient blood flow to the intervillous space (Genbacev et al. 1993; Harris et al. 2009). This thesis focuses on the villous trophoblast populations, the syncytiotrophoblast and cytotrophoblasts.

The syncytiotrophoblast is a contiguous, terminally-differentiated, multinucleated cell layer covering the entirety of the chorionic villi, formed through the differentiation and fusion of the underlying villous cytotrophoblasts (Castellucci and Kaufmann 2006; Mayhew 2014). At term, the syncytiotrophoblast layer contains approximately 58 billion nuclei (Burton and Jauniaux 1995) and serves many functions essential to fetal growth and survival. Given that the syncytiotrophoblast is in direct contact with maternal blood, it is the conduit whereby gases and nutrients are exchanged and correspondingly has a dense layer of microvilli, increasing its surface area five- to seven-fold (Teasdale and Jean-Jacques 1985). Both the apical microvillous and basal basement membrane have an abundance of receptors and protein transporters to mediate the transfer or action of growth factors, hormones, and metabolites, and facilitate the efflux of xenobiotics (Robinson et al. 2009; Turco and Moffett 2019; Vähäkangas and Myllynen 2009). Other vital functions of the syncytiotrophoblast layer include the production of hormones and peptides crucial for the maintenance of pregnancy, protection from pathogens through physical barriers and antibody transfer (Gaunt and Ockleford 1986; Roopenian and Akilesh 2007), and as an immunological barrier between mother and fetus due to its lack of human leukocyte antigen expression (Moffett and Loke 2006). Historically the syncytiotrophoblast was presumed to be the primary driver of placental metabolism, but work by Kolahi and colleagues (2017) revealed that the metabolic rate of cytotrophoblasts exceeds that of the syncytiotrophoblast (Kolahi et al. 2017).

Villous trophoblasts also are major players in placental vascular development, essential for

the formation of placental circulation and, consequently, normal fetal growth and development. Villous cytotrophoblasts produce various angiogenic growth factors, including those of the vascular endothelial growth factor (VEGF) family (Benirschke et al. 2012a; Herr et al. 2010). The VEGF family consists of five members [VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF)] and their receptors; VEGF receptor-1 (VEGFR-1 or Flt-1), VEGFR-2 (or Flk-1/KDR), and VEGFR-3], and neuropilin-1 and -2 (Wang and Zhao 2010). VEGF mRNA transcripts and protein have been localized in villous trophoblasts and other placental cell types including Hofbauer cells and villous fibroblasts (Clark et al. 1998; Sharkey et al. 1993; Shiraishi et al. 1996; Vuorela et al. 1997). VEGF mainly exerts its effects on endothelial cells, stimulating non-branching angiogenesis in early to mid-pregnancy (Charnock-Jones and Burton 2000). VEGFR-1 and -2 have also been localized in the BeWo choriocarcinoma cell line, modeling villous cytotrophoblasts, indicating roles in the regulation of trophoblast functionality (Charnock-Jones et al. 1994). Another critical mediator of placental angiogenesis produced by villous trophoblasts is PlGF (Vuorela et al. 1997). PlGF performs its actions through binding to VEGFR-1 resulting in endothelial tube formation (Ahmed et al. 2000). Other growth factors and hormones produced by villous trophoblasts, including insulin-like growth factor II (IGF-II) (Herr et al. 2003) and human chorionic gonadotropin (hCG) (Zygmunt et al. 2002), also mediate placental angiogenesis.

Although PA is known to affect placental growth, expression of angiogenic mediators, and vascularization [summarized by (Bhattacharjee et al. 2021a)], the mechanisms by which these changes occur is not understood. The impact of PA on placental growth, structure, and function is discussed further below.

1.3.2 Physical activity and the placenta

Nearly all body organs and systems benefit positively from engagement in PA, including the transient organ of pregnancy, the placenta. Our lab recently summarized the current state of the literature on the impact of PA on structures and organs of the maternal-fetal interface, ranging from effects on implantation, embryogenesis, the immune response, endocrine production to both utero- and fetoplacental blood flow (Bhattacharjee et al. 2021a). Habitual engagement in PA throughout pregnancy has been associated with the altered placental expression of nutrient transporters (Brett et al. 2015a; Hutchinson et al. 2020), endothelial nitric oxide synthase (eNOS) (Ramírez-Vélez et al. 2013), proteins involved in oxidative stress (Kusuyama et al. 2021), and angiogenic growth factors (Bhattacharjee et al. 2021a; Gilbert et al. 2012; Hardy et al. 2021). Our lab reported increased placental expression of VEGF and VEGFR-1 at the mRNA and protein levels in physically active vs. inactive study participants (Bhattacharjee et al. 2021b). However, it is unknown whether the enhanced expression of key players responsible for placental angiogenesis due to maternal PA translates to improved or optimized placental vascularization.

Data from randomized control trials and observational trials in humans show that PA during pregnancy positively influences placental growth and vascularization. In 1995, Jackson *et al.* were the first to report that physically active (referred to by the authors as 'fit') women who continued to exercise at their periconceptual levels at moderate to high-intensity across pregnancy had greater placental villous volumes when compared to sedentary control pregnancies (Jackson et al. 1995). The authors conducted detailed histomorphometric measurements of term placental tissues through traditional hematoxylin and eosin staining to estimate placental volumes and vascular surface areas. Significant differences between groups were confined to the larger stem villi at the most proximal level of the villous trees and not the distal terminal villi, the primary site

of maternal-fetal exchange (Jackson et al. 1995). Following up on this study, the same group conducted a randomized control trial involving aerobic PA restricted to only moderate-intensity beginning in early pregnancy (Clapp et al. 2000). The exercise intervention incorporated 20 min of moderate-intensity aerobic exercise 3-5 times per week from week 8 of gestation until term delivery. The control group was instructed to avoid performing any recreational weight-bearing exercise. The authors reported significantly greater terminal villous volumes in those of the exercise vs. the control group and surmised that the differing findings were likely related to a lower volume and intensity of exercise compared to their previous study (Clapp et al. 2000).

Continuing the study of PA volume and timing during pregnancy, this same research group carried out a randomized controlled trial where participants were divided into three exercise intervention groups at week 8 of gestation: (1) 'Lo-Hi' (low volume PA in early pregnancy then increasing to high volume from 24 weeks to term), (2) 'Mod-Mod' (consistent moderate volume PA throughout pregnancy), and (3) 'Hi-Lo' (high volume PA early decreasing to low volume from 24 weeks until term) (Clapp et al. 2002). Those in the 'Hi-Lo' group were found to have the greatest terminal villous volume of the three exercise groups, suggesting that a reduction in PA volume into the third trimester enhanced fetoplacental growth. However, it is important to consider that this study did not include a control 'non-exercising' group. Still, the findings highlight critical periods of placental growth in early pregnancy ideal for PA intervention. Finally, an observational cohort study of habitual runners engaging in higher volumes of sustained weight-bearing PA reported greater villous vascular densities compared to healthy controls (Bergmann et al. 2004). Runners (N=11) ran ≥4 times per week for 40-60 min per session at 55-65% of their periconceptual maximal aerobic capacity, while the controls (N=11) only engaged in recreational, low-intensity PA, as determined from self-reported logs. Unlike previous histological examinations to date,

immunohistochemistry (IHC) against CD34, a marker of endothelial cells in term placenta (Benirschke et al. 2012a), was used to highlight the fetoplacental vasculature. Evidence from exercise in pregnant animal models reaffirms observations of altered placental vascularization in human clinical studies. In pregnant rats, exercise was found to attenuate hypertension-induced pathologies and improve placental angiogenesis and vascularization (Abate et al. 2012; Gilbert et al. 2012).

From the studies above, it is clear that habitual PA is associated with differences in placental vascularization gleaned from the histological examination of term placental tissues. It is presumed that increased placental vascularity or perfusion may result in enhanced or optimized transfer function. A significant gap in the PA and placenta literature to date is the use of objective measurement to ensure unbiased quantification of PA in terms of volumes, frequencies, and intensities (Brett et al. 2015b; Guérin et al. 2018). Therefore, no assessments have examined the effect of differing PA intensities and their relationship with placental vascularization. **For *Project 3*, term fetoplacental vascular densities were quantified and compared in a cohort of healthy pregnant women who met the *PA and Pregnancy Guidelines* (Mottola et al. 2018) vs. those who did not, as determined *via* objective measurement of PA prospectively. I hypothesized that there would be a dose-response relationship between moderate-to-vigorous intensity PA and fetoplacental vascular density.**

1.4 Specific Aims and Hypotheses

Although the benefits of maternal PA are extensive and well-known, the physiological mechanisms mediating these benefits remain unclear. Considering that evidence implicates circulating sEVs as a potential mechanism contributing to the systemic response to exercise, this

thesis will first determine whether sEVs are released after a suggested bout of acute exercise in pregnant women. Since trophoblasts are responsible for the major functions of the placenta and are in direct contact with maternal blood, exercise-associated sEVs will be investigated for their potential to influence trophoblast physiology. Finally, given the importance of placental vascularization for fetal health, the relationship between differing intensities of PA and fetoplacental vascular density will be examined in a cohort of healthy individuals. The specific aims for each project and accompanying hypotheses are as follows:

1. **Project 1:** To characterize the circulating sEV profile of pregnant and non-pregnant women after an acute bout of moderate-intensity exercise.
   - *Sub-Aim 1:* Isolate and validate plasma sEVs of pregnant and non-pregnant women obtained pre- and immediately post- a 30 min treadmill walk of moderate intensity.
   - *Sub-Aim 2:* Quantify and compare the circulating exercise-associated sEV profile by particle size (diameter) and number between groups (pregnant vs. non-pregnant) and time points (pre- vs. post-exercise).
   - *Hypothesis:* I hypothesize that sEV size will remain unchanged but that the number will increase post-exercise in both groups.
2. **Project 2:** To evaluate whether exercise-associated sEVs from a bout of acute exercise (those from Project 1) can influence trophoblasts *in vitro*. Since trophoblasts make up the maternal-fetal interface and are major contributors to placental function, it is imperative to assess whether sEVs released into circulation in response to exercise can impact placental cell function.
   - *Sub-Aim 1:* Determine whether exercise-associated sEVs from pregnant and non-pregnant women can interact with cells representing cytotrophoblasts and syncytiotrophoblast *in vitro*.

- *Sub-Aim 2:* Investigate the potential of exercise-associated sEVs to alter trophoblast cell proliferation, gene expression of key angiogenic growth factors VEGF and PlGF, and the secretion of the pregnancy hormone b-hCG.
- *Hypotheses:* I hypothesize that exercise-associated sEVs will be found within both cytotrophoblast and syncytiotrophoblast-like cells. I also hypothesize that exposure of trophoblast-like cells *in vitro* to exercise-associated sEVs will increase proliferation, gene expression of *VEGF* and *PlGF*, and secretion of b-hCG compared to treatment with non-sEV controls. I surmise that the difference will be more pronounced upon exposure to exercise-associated sEVs from pregnant compared to non-pregnant individuals.

3. **Project 3:** To determine if fetoplacental vascular density differs according to PA status (active vs. inactive) or differing intensities of free-living PA.
   - *Aim 1:* Use IHC to quantify fetoplacental vascular density and compare among 10 healthy PLACENTA study participants who were followed prospectively to obtain objective measurements of PA during their 2nd and 3rd trimesters.
   - *Hypotheses:* I hypothesize that those meeting or exceeding 150 min of moderate-intensity PA or greater would have greater fetoplacental vascular densities compared to those who did not meet PA recommendations. I also hypothesize that fetoplacental vascular density will be positively associated with moderate-to-vigorous PA across pregnancy.

CHAPTER 2

Project 1 manuscript: Circulating small extracellular vesicles increase after an acute bout of moderate-intensity exercise in pregnant compared to non-pregnant women

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Contributions: SM performed the experiments and drafted the manuscript. SM and KH primarily performed data collection and designed the acute exercise study. DDS helped with statistical analysis and data interpretation. KM secondarily performed data collection including blood processing and nutritional analysis of the snack given pre-exercise. JB helped with western blotting to confirm EV markers. DB contributed to data collection (transmission electron microscopy) analysis, interpretation, and technical expertise. KBA lead the study (including providing funding for the study). All authors contributed to the design of the study, revised, edited, read and approved the final version of the manuscript.

Abstract

The physiological and molecular mechanisms linking prenatal physical activity and improvements in maternal-fetal health are unknown. It is hypothesized that small extracellular vesicles (EVs, ~20-120 nm) are involved in tissue cross-talk during exercise. We aimed to characterize the circulating small EV profile of pregnant versus non-pregnant women after an acute bout of moderate-intensity exercise. Pregnant (N=10) and non-pregnant control (N=9) women performed a single session of moderate-intensity treadmill walking for 30 min. Plasma was collected immediately pre- and post-exercise, and small EVs were isolated by differential ultracentrifugation. EV presence was confirmed by western blotting for the small EV proteins TSG-101 and flotillin-1. Small EVs were quantified by size and concentration using nanoparticle tracking analysis and transmission electron microscopy. All EV fractions were positive for TSG-101 and flotillin-1, and negative for calnexin. Mean vesicle size at baseline and percent change in size post-exercise were not different between groups. At baseline, pregnant women had higher levels of small EVs compared to controls (1.83E+10 ± 1.25E+10 particles/mL vs. 8.11E+09 ± 4.04E+09 particles/mL, respectively; $p=0.032$). Post-exercise, small EVs increased significantly in the circulation of pregnant compared to non-pregnant women after correcting for baseline values (64.7 ± 24.6% vs. -23.3 ± 26.1%, respectively; $F=5.305$, $p=0.035$). Further research is needed to assess the functional roles of exercise-induced small EVs in pregnancy.

Introduction

Engagement in regular physical activity is associated with significant health benefits, including improvements in both psychological and physiological well-being and decreased risk of chronic disease and all-cause mortality [1]. Physical activity is also an important component of a healthy pregnancy. The *2019 Guidelines for Physical Activity Throughout Pregnancy* recommend that women without contraindication to exercise, engage in a minimum of 150 minutes of moderate-intensity physical activity per week, with engagement every day being preferable [2]. Physical activity during pregnancy is associated with both short- and long-term benefits for the mother and fetus, which include lowering the risk for adverse pregnancy complications such as gestational diabetes mellitus, preeclampsia, gestational hypertension, excessive gestational weight gain, and macrosomia (large for gestational-age neonates) [3-6]. Although the benefits of chronic prenatal physical activity are extensive and well-known, the physiological mechanisms involved in response to exercise in pregnancy leading to these benefits remain largely unclear. The benefits sustained from engagement in chronic exercise are derived from acute physiological changes promoted by a single bout of exercise experienced over time [7,8]. During exercise, several molecules are released into circulation, including catecholamines [9,10], myokines [11,12], and small EVs [7,13], which seem to mediate the adaptations caused by the cumulative effect of daily bouts of 'acute' physical activity (i.e., the chronic effects of exercise) [14].

In the non-pregnant population, small extracellular vesicles (EVs) (20-120 nm in diameter, often referred to as exosomes) are one of the potential mechanisms proposed to modulate the systemic benefits of exercise [7,14,15]. EVs are a heterogeneous group of lipid bilayered membrane nanoparticles secreted by cells into extracellular space [16,17]. EVs are believed to be involved in intercellular communication over long distances as they may facilitate the transfer or interaction

of biologically active molecules, including nucleic acids (mRNAs and other small RNAs, i.e., microRNAs), proteins, and lipids from donor cells to target cells [18,19]. EVs are found in a wide range of biological fluids, including blood, urine, saliva, milk, and cells types in culture [20]. Their involvement in a multitude of physiological and pathophysiological processes has led to their emergence as particles of interest in the complex realm of the physiological response to exercise.

Work from our lab has shown that the acute physiological response to exercise, relating to the release of soluble factors like cytokines into circulation, is different in pregnant compared to non-pregnant women [21]. It remains unknown whether acute prenatal exercise results in a change in the levels of circulating small EVs, and whether the response differs depending on pregnancy status. It is known that plasma concentrations of small EVs are significantly higher in pregnant versus non-pregnant women [22]. It is thus posited that the major contributor accounting for this difference is the placenta, the critical organ of pregnancy [22,23]. Evidence supports the beneficial impact of both acute and chronic prenatal exercise on the placenta [21,24-30]; however, the mechanisms are currently unknown. Small EVs are involved in maternal-fetal communication [23,31], and therefore, warrant further investigation in the context of exercise during pregnancy.

In males, small EVs are released rapidly into circulation after an acute bout of sustained, vigorous-intensity exercise [7,13], and are thought to be involved in mediating intercellular communication that can contribute to the chronic adaptations of exercise [7]. However, there are no studies on the impact of acute exercise on the levels of small EVs in the circulation of pregnant or non-pregnant women. We aimed to characterize the plasma small EV profile and concentration after a single bout guidelines-based of moderate-intensity exercise in pregnant women, compared to their non-pregnant counterparts. To our knowledge, this is the first study to examine small EVs in response to acute exercise in pregnant and non-pregnant women.

Methods

Ethical approval

All experimental procedures were approved by the University of Ottawa Research Ethics Board (file number: H-06-18-634) and conducted following the ethical principles outlined in the Declaration of Helsinki. Informed written consent was obtained from all study participants after an explanation of study procedures and objectives.

Study participants

Pregnant and non-pregnant women were recruited from the Ottawa region (Ontario, Canada), and eligibility was assessed by the research team. Healthy women between the ages of 18-40 years with no contraindications to exercise were included. Participants were weight-stable (± 5 kg) for at least six months before the study, with a self-reported pre/non-pregnant body mass index (BMI) of 18.5-29.9 kg/m^2. Those with untreated thyroid disease, hypertension, diabetes (pre/non-pregnant or gestational diabetes), or other chronic health conditions, and frequent users of drugs, tobacco, or alcohol were excluded. Moreover, pregnant participants needed to be between 13-28 weeks of gestation and carrying a singleton fetus to be included. In total, 10 pregnant and 9 non-pregnant women met inclusion criteria and were included in this study.

Experimental procedure

The experimental protocol followed methodologies outlined by Hutchinson *et al.* (2019) [21]. Briefly, study participants were asked to abstain from exercise for 12 hours and food for 8 hours before the experimental session. Body weight and height were measured using standard methods. Participants were provided with a standardized snack of approximately 340 kCal before the exercise session. Then, resting heart rate (HR) was measured using a Polar V800 HR monitor (Polar Electro, Lachine, QC) for 10 min while participants were comfortably seated. The values

obtained during the last 5 min of measurement (recorded at 1-min intervals) were averaged to determine resting HR. A target of 40-59% of HR reserve (HRR) was used to specify moderate-intensity exercise [2,32] was calculated using the following equation [33]:

Target HR range = [(Maximal HR – resting HR)* %intensity] + resting HR.

Maximal HR=220-age

A 3-min warm-up at 2.0 miles per hour (mph) at an incline of 2.0% was followed by an increase to 6.0% incline, where the speed increased by 0.2 mph at 1-min intervals until the upper range of the target moderate-intensity HR was reached (approaching 59% HRR). Once this range was met, the participants continued to exercise for 30 min, adjusting the speed accordingly to ensure that the target HR range was maintained. HR values were recorded at 1-min intervals during the 30 min of continuous moderate-intensity exercise. Approximately 14-20 min after the snack, immediately before and after the exercise session, 10 mL of blood was drawn from the median cubital vein using a BD Vacutainer® Safety-Lok™ blood collection set (#367281; BD Biosciences, Mississauga, ON) and potassium EDTA (#367863; BD Biosciences) collection tubes. To obtain plasma, whole blood was immediately centrifuged at 1700 × *g* for 15 min at 4°C. Plasma samples were stored at -80°C until further analysis.

Small EV isolation

Isolation of small EVs was achieved by differential ultracentrifugation as previously described [34,35]. Frozen plasma samples (1.0 mL) were thawed at 37°C. After thawing, plasma samples were always kept on ice. Samples were centrifuged at 20,000 × *g* for 20 min at 4°C to remove apoptotic bodies and large EVs. The resulting supernatant was centrifuged at 100,000 × *g* using a Beckman Coulter Optima MAX ultracentrifuge (Beckman Coulter Inc., Brea, CA, USA) with a TLA-55 rotor (Beckman Coulter) for 90 min at 4°C. The resulting small EV pellet was washed with 1.0

mL of 0.1 μm-filtered phosphate-buffered saline (PBS) and then centrifuged at 100,000 × *g* as described above. The final small EV pellet was resuspended in 100 μL of 0.1 μm-filtered PBS. Aliquots of 10 μL were separated and frozen at -80°C for further analysis.

Western blot analysis

Western blotting was performed as described by Bhattacharjee *et al.* (2010) [36], with some modifications, to test for the presence and absence of small EV markers. Immediately following isolation, 1 μL of 10X radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail (#P8340, MilliporeSigma Canada Co, Oakville, ON, Canada) was added to 10 μL of small EV isolates in PBS. Samples were then sonicated for 1 min to achieve protein lysis. An equal volume of protein lysates (5 μL) from each sample was subjected to SDS-PAGE electrophoresis under reducing conditions using a 4-15% mini-protean TGX precast gel (Bio-Rad Laboratories, Mississauga, ON, Canada) at 120 V for 60 min. Separated proteins were blotted onto a PVDF membrane (Bio-Rad Laboratories) at 100 V for 90 min. Blots were then blocked in 5% non-fat dry milk in TBS with 0.1% TWEEN®20 (TBST) for 60 min at room temperature. After blocking, blots were incubated with primary antibodies for markers of small EVs, flotillin-1 (mouse anti-flotillin-1, 1:1000; #610820; BD Biosciences, CA, USA) and tumor susceptibility gene 101 (TSG101) (mouse anti-TSG101, 1:3000; #ab83; Abcam Inc, Toronto, ON, Canada), and for the negative control, calnexin (rabbit anti-calnexin, 1:10,000; #AB2301; Millipore Sigma), in 5% milk in TBST overnight at 4°C. Following overnight incubation, the blots were washed three times with TBST and incubated with goat anti-mouse (1:7000; #1706516; Bio-Rad Laboratories) or goat anti-rabbit secondary antibody coupled with HRP (1:7000; #1706515; Bio-Rad Laboratories) diluted in 5% milk in TBST for 60 min at room temperature. After washing three times with TBST, the peroxidase-labeled blots were incubated with Clarity Western ECL Substrate (#1705060; Bio-Rad

Laboratories) and visualized using a ChemiDoc XRS+ system (Bio-Rad Laboratories). Protein lysates from BeWo choriocarcinoma cells and term human placenta tissue were used as positive controls for the calnexin immunoblots.

Nanoparticle tracking analysis

Particle size and concentration were determined using nanoparticle tracking analysis (NTA) as described previously [35,37]. Dilutions of small EV isolates (in 0.1 μm-filtered PBS ranging from 1:250 – 1:1000) were analyzed using the ZetaView PMX 110 Multiple Parameter Particle Tracking Analyzer (Particle Metrix, Meerbusch, Germany) in size mode with Zetaview software (version 8.03.08). Small EVs were captured at 11 camera positions at 21°C with the following pre-acquisition parameters: shutter speed of 70, 30 frames per second. The following post-acquisition settings were used: minimum brightness of 30, minimum tracelength of 15, minimum size of 20, and maximum size of 1000.

Transmission electron microscopy

Small EVs were examined by transmission electron microscopy (TEM) as previously described [38]. Briefly, small EVs were pelleted by ultracentrifugation at 100,000 x g for 90 min at 20°C. The supernatant was aspirated, and the small EV pellet was fixed with 2.5% glutaraldehyde in PBS for four hours at room temperature. The pellet was then washed in 0.1M Na cacodylate buffer, post-fixed in 2% OsO4, and dehydrated in a series of graded ethanols. Samples were then embedded in Spurr Resin, and 60 nm sections were prepared on copper grids. Samples were visualized using a JEOL JEM-1400 Plus electron microscope.

Statistical analysis

All statistical analyses were conducted using SPSS version 23.0 (IBM Corp, Armonk, NY, USA), and graphs were created using GraphPad Prism version 8.4.2 (GraphPad Software, La Jolla, CA,

USA). All data are shown as mean ± standard deviation. Normality was assessed using the Shapiro-Wilk test. Study participant demographics and baseline (pre-exercise) characteristics (size and concentration) of small EVs were compared between pregnant and non-pregnant women using independent *t*-tests. The change in HR during the 30 min continuous exercise session was assessed using a two-way mixed ANOVA with Bonferroni's multiple comparisons post-test. The change in small EV size and concentration post-exercise was calculated using the following equation:

$$\text{Change in small EV size or concentration} = (\text{post-exercise} - \text{pre-exercise})/\text{pre-exercise} * 100$$

An independent *t*-test was used to compare the change in small EV size in pregnant versus non-pregnant women. Due to the differences observed in the baseline levels of small EVs, an analysis of covariance (ANCOVA) with Bonferroni correction for *post hoc* comparisons was used to compare the change in small EV concentration, controlling for baseline values, in pregnant versus non-pregnant women. Adjusted means and standard errors are presented where applicable. Statistical significance was considered when $p<0.05$.

Results

Study participant demographics

All study participant demographics are presented in Table 1. By design, pregnant and non-pregnant women did not differ in anthropometric variables, including age, height, pre/non-pregnant weight, pre/non-pregnant BMI and resting HR. Also, the time to reach the moderate intensity HR range (approaching 59% HRR) was not different between groups, nor was the total exercise duration (Table 1). Further, the average speed achieved and the change in HR during the moderate-intensity exercise session were not different between groups (Table 1 & Figure 1).

Table 1 Study participant demographics

	Pregnant (N=10)	Non-pregnant (N=9)	p-value
Age (years)	31.1 ± 2.7	32.7 ± 3.2	0.68
Gestational age (weeks)	20.6 ± 5.4	-	-
Height (cm)	167.0 ± 5.3	163.8 ± 7.2	0.28
Pre/non-pregnant weight (kg)	62.7 ± 8.6	59.8 ± 8.6	0.50
Pre/non-pregnant BMI (kg/m²)	23.4 ± 3.3	22.3 ± 2.8	0.43
Resting heart rate (bpm)	77.9 ± 10.9	69.2 ± 6.6	0.052
Time to reach moderate intensity heart rate range (min)	10.4 ± 2.2	11.8 ± 1.9	0.16
Heart rate during continuous exercise session (bpm)	139.4 ± 7.5	137.6 ± 5.0	0.55
Average speed during continuous exercise session (mph)	3.4 ± 0.5	3.6 ± 0.4	0.18
Total exercise duration (min)	40.4 ± 2.2	41.8 ± 1.9	0.16

Data are presented as mean ± standard deviation. BMI, body mass index; bpm, beats per minute; mph, miles per hour; SD, standard deviation. Data were analyzed using independent *t*-tests

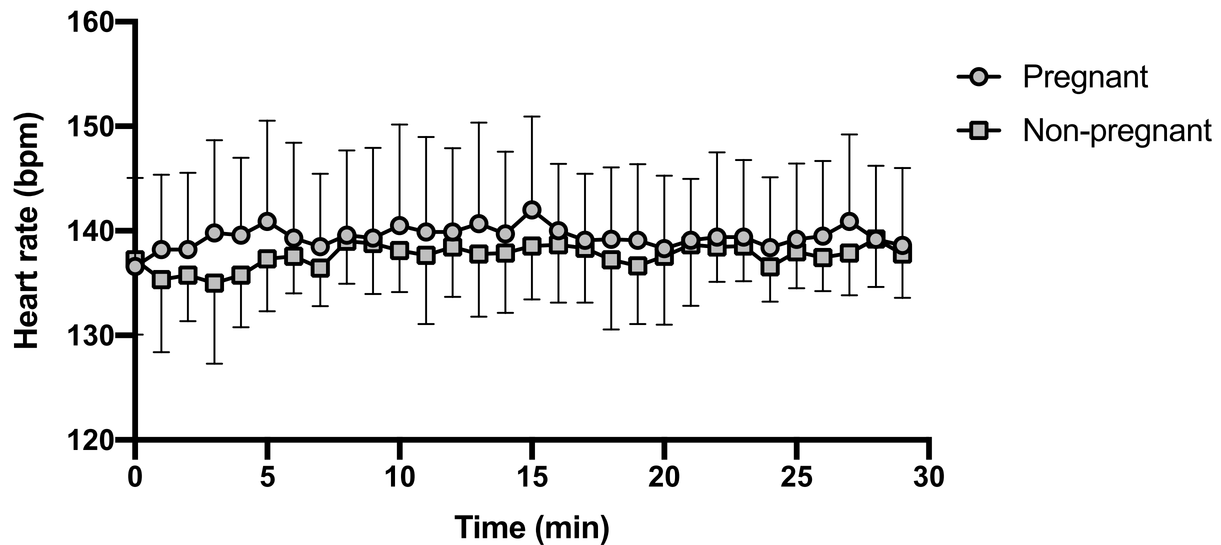

Fig. 1 Heart rate during the continuous moderate-intensity exercise session. The change in heart rate was not different between pregnant and non-pregnant women across the 30 min moderate-intensity exercise session**.** The main effects of time and pregnancy were not significantly different (F=1.1, p=0.38 and F=0.50, p=0.50, respectively), nor was the time × pregnancy interaction (F=0.55, p=0.97). Data are shown as mean ± standard deviation. Bpm, beats per minute

Small EV isolates exhibit classical characteristics

Western blotting was used to qualitatively assess the presence of small EV protein markers, flotillin-1, and TSG101. Positive signals for flotillin-1 and TSG101 were found in protein lysates from small EV isolates of pregnant and non-pregnant women, both pre- and post-exercise (Figure 2a). Calnexin, a calcium-binding protein found in the membrane of endoplasmic reticulum, was absent in small EV isolate fractions but positive in BeWo choriocarcinoma and human term placenta tissue lysates (Figure 2a). Further, TEM analysis showed the presence of small particles (~100 nm in size) with distinct, intact membranes (Figure 2b). Together, these data confirmed the presence of small EVs in the plasma samples of our study participants.

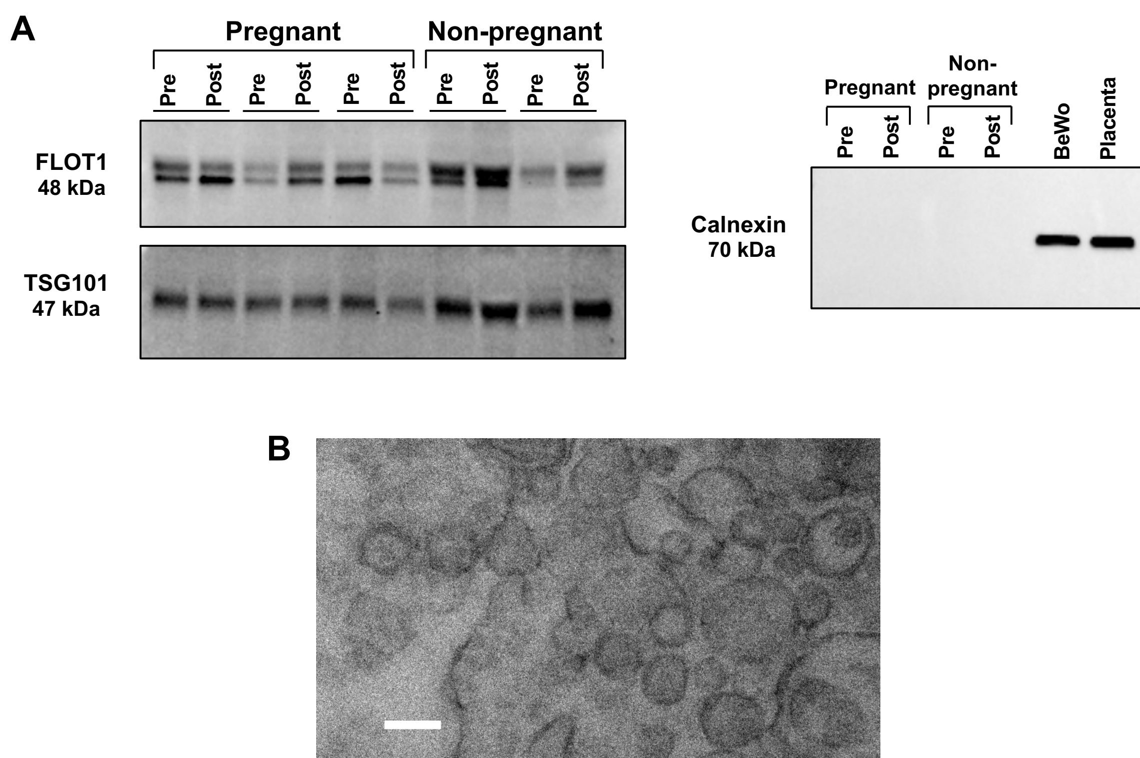

Fig. 2 Circulating small EV isolates both pregnant and non-pregnant women expressed markers of small EVs both pre- and post-exercise. (A) Cropped representative western blot images show the positive expression of flotillin-1 (FLOT1) and tumor suppressor gene-101 (TSG101) in small EV isolates from pregnant and non-pregnant women, both pre- ('Pre') and post-exercise ('Post'). Calnexin, a negative control marker for small EVs, was not detected in small EV isolates from either group. Positive controls for calnexin expression included BeWo choriocarcinoma cell lysates ('BeWo') and term human placenta tissue lysates ('placenta'). Each lane represents a sample from a single individual, while each pair of lanes were obtained from the same participant. Full length blots are presented in Supplementary Figure 1. (B) Shows a representative transmission electron microscopy image of small EV pellets where round particles (~100 nm in size) with distinct, intact membranes can be seen (scale bar = 100 nm), from a representative pregnant sample. EVs, extracellular vesicles

Small EV isolates did not differ by size

Characterization of the mean vesicle size of small EV isolates revealed no differences in the baseline (pre-exercise) plasma samples of pregnant versus non-pregnant women (101.7 ± 11.7 nm and 110.8 ± 14.1 nm, respectively; p=0.141) (Figure 3a). There was no significant difference in vesicle size post-exercise in either pregnant or non-pregnant individuals (percent change of pregnant= 0.33 ± 7.9% and non-pregnant= 2.48 ± 14.1%; p=0.683) (Figure 3b). The size

distributions of small EV isolates and the percentage of particles from 30-120 nm are shown in Figure 4.

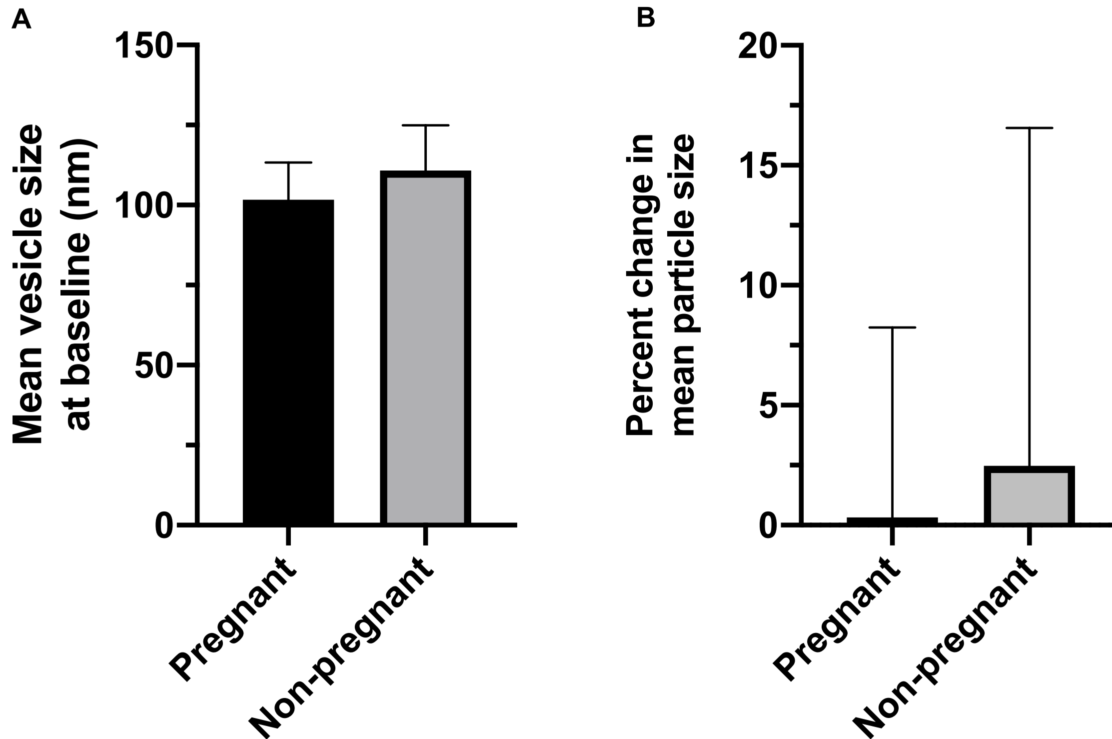

Fig. 3 Small EVs did not differ in size at baseline or post-exercise in pregnant versus non-pregnant women. Using nanoparticle tracking analysis the mean size of circulating small EVs were not different at baseline (pre-exercise) in pregnant compared to non-pregnant women (p=0.141) (A). Furthermore, the change in small EV size post-exercise was not different between groups (p=0.683) (B). Data are shown as mean ± standard deviation. EVs, extracellular vesicles

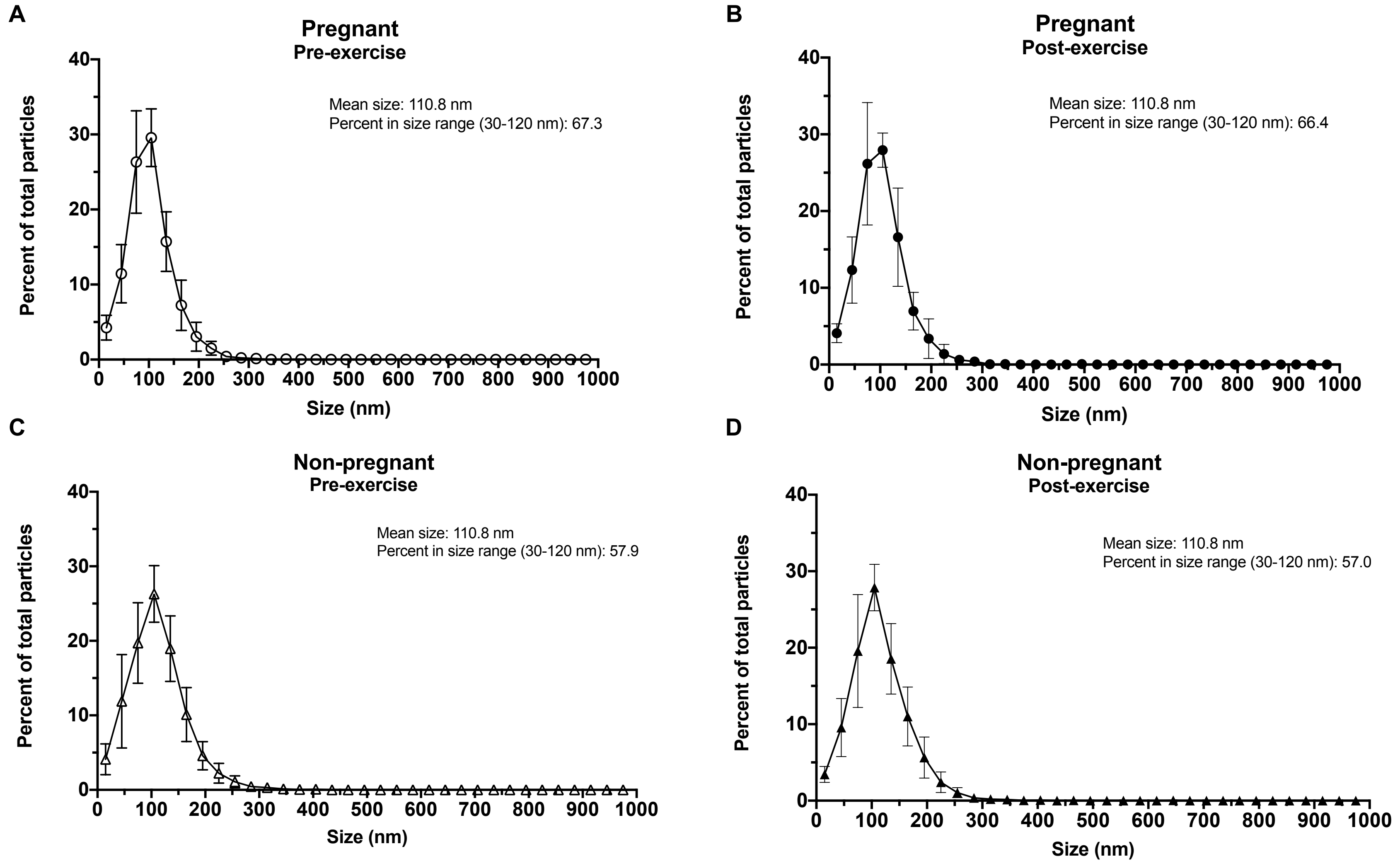

Fig. 4 Size distribution of small EV isolates from pregnant and non-pregnant women both pre- and post-exercise. The size distribution of small EV isolates obtained from pregnant (A&B) and non-pregnant (C&D) obtained via nanoparticle tracking analysis are shown. All data points are displayed as mean ± standard deviation. EVs, extracellular vesicles

Pregnant women display higher levels of small EVs before and after exercise compared to non-pregnant controls

First, the baseline (pre-exercise/rest) levels of small EVs were compared between pregnant and non-pregnant women. At rest, pregnant women had higher levels of small EVs in circulation compared to their non-pregnant counterparts (1.83E+10 ± 1.25E+10 particles/mL and 8.11E+09 ± 4.04E+09 particles/mL, respectively; p=0.032) (Figure 5a). When correcting for differences in baseline levels, small EVs increased significantly in the circulation of pregnant versus non-pregnant women post-exercise (adjusted means ± standard error: 64.7 ± 24.6% vs. -23.3 ± 26.1% for pregnant and non-pregnant women, respectively; F=5.305, p=0.035)(Figure 5b).

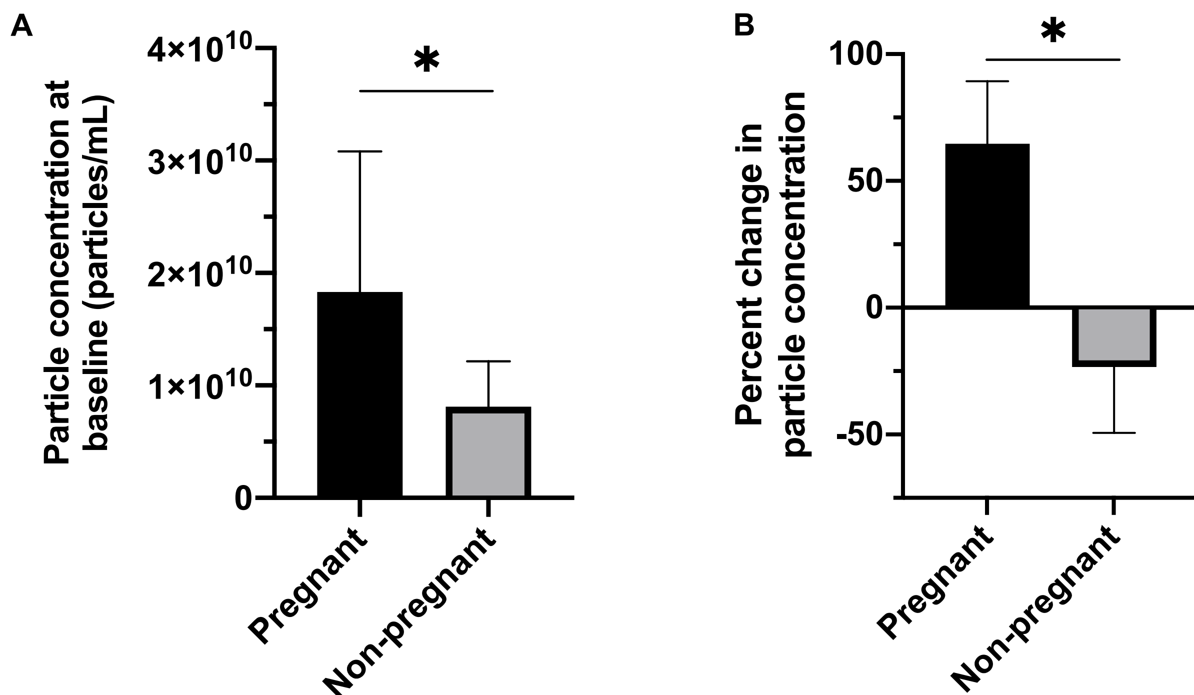

Fig. 5 Pregnant women had higher levels of circulating small EVs at baseline and post-exercise compared to non-pregnant women. Nanoparticle tracking analysis revealed that pregnant women had higher levels of small EVs in plasma at baseline (pre-exercise) (p=0.032) (A). (B) Furthermore, when accounting for baseline levels of small EVs, pregnant women were found to have significantly higher levels on average of small EVs post-exercise compared to non-pregnant women (F=5.305, p=0.035). Data are presented as mean ± standard deviation. *= $p<0.05$. EVs, extracellular vesicles

Discussion

To the best of our knowledge, this is the first report characterizing small EVs in response to exercise in pregnant and non-pregnant women. We compared the circulating small EV profile of pregnant compared to age- and pre-pregnant BMI-matched non-pregnant women after an acute bout of moderate-intensity treadmill exercise. Small EV isolates from the plasma of pregnant and non-pregnant women expressed protein markers indicative of small EVs before and after an acute bout of exercise. The results also indicate that while there is no change in the size of small EVs in circulation post-exercise in healthy pregnant and non-pregnant women, pregnant women had significantly higher levels of small EVs in plasma after exercise.

Previous studies involving male subjects reported a rapid release of small EVs into circulation after sustained, high-intensity cycling, or running exercise until physical exhaustion [7,13]. We only found a significant difference in the number of small EVs post-exercise when comparing between groups after controlling for the baseline differences between pregnant and non-pregnant women, not within groups (data not reported). There may be a few reasons for this discrepancy. Frühbeis *et al.* (2015) quantified small EVs by NTA in a small subset of participants (N=2 per exercise modality) and, therefore, could not be used to compare with the results in our study. Furthermore, the reported increase in small EVs after exercise was based on data from the semi-quantitative analysis of protein markers by western blotting [13], which was not used to quantify the absolute number of small EVs of our study. Also, Whitham *et al.* (2018) observed an increase in particles in the size range attributed to small EVs, but it is not clear how many samples were quantified by NTA (only a 'subset of samples' is described) or what statistical tests were employed, and if significance was observed. In a study assessing the plasma small EV profile after exercise in human patients deemed 'at-risk' for cardiometabolic disease, an increase in the number of EVs

without a difference in size was observed [39]. Our findings support exercise studies in animals, where differences were detected in the number of small EVs only and not in size [40]. We believe that the discrepancy between our results and those of the current human literature may be due to differences in the study population and exercise intensity, duration, and mode. Participants studied by Frühbeis and colleagues (2015) and Whitham *et al.* (2018) exercised at higher intensities or durations than our study participants (moderate-intensity exercise). Since we studied pregnant women, we opted to use a physiologically-relevant exercise stimulus, guided by *2019 Guidelines for Physical Activity Throughout Pregnancy* [2], to more appropriately examine the potential changes in the small EV profile after exercise.

Small EVs are theorized to contain biologically-active materials involved in long-distance intercellular communication throughout the body. Regarding exercise, quantitative proteomic analysis of small EV cargo showed that they contained myokines [7], peptides produced by skeletal muscles and thought to mediate cross-talk between muscles and other tissues during exercise [11,12]. Critical studies involving animals revealed that exercise-induced small EVs localized to the liver, possibly regulating metabolism in response to exercise [7]. Furthermore, it has been hypothesized that the systemic and long-term benefits of exercise are mediated in part by small EVs [14]. The potential bioactivity of exercise-induced small EVs is not fully understood, but were found to be cardioprotective in a cardio-ischemic reperfusion injury mouse model [39]. Although the aim of the current study was not to characterize the biological contents of small EVs in circulation after exercise, further research is warranted to determine the physiological impact of small EVs specifically in the context of exercise in pregnancy given that we observed remarkable differences depending on pregnancy status in a healthy population of women.

Our results show that the physiological response to acute exercise may be different in pregnant versus non-pregnant women. Likewise, we have recently demonstrated that myokine profiles were different in pregnant and non-pregnant women after a matched-intensity exercise bout [21]. It has been hypothesized that the release of small EVs is triggered by an acute stress response in the body prompted by exercise [13]. It is, therefore, conceivable that the physiological stress response to exercise is heightened during pregnancy, which may help to explain our findings. It is not known whether the increase in small EV number observed post-exercise in pregnant women was due to *de novo* secretion or decreased clearance/uptake when compared to their non-pregnant counterparts, and requires further study. Interestingly, we found that on average, levels of small EVs decreased in non-pregnant women post-exercise whereas the opposite result was observed in pregnant women. Small EVs have been shown to be cleared from circulation in-part by members of the innate immune system, namely, macrophages [41,42]. It is well known that immunity in pregnancy shifts from a Th-1 or innate immunity-biased profile (cell-mediated immunity/pro-inflammatory) to that of one that is Th-2 or adaptive immunity-biased (humoral immunity/anti-inflammatory) response to tolerate the semi-allogeneic fetus [43,44]. This may explain why we observed a decrease in the concentrations of EVs in non-pregnant women after exercise, when immunity is Th-1-biased and macrophages may be playing a greater role in the clearance of small EVs from circulation. However, it is currently unknown whether the clearance of small EVs is impacted by immune status during pregnancy.

The differing responses to exercise-induced small EVs depending on pregnancy status is not surprising given that healthy pregnant women displayed a 50-fold increase in the concentration of small EVs in plasma compared to non-pregnant women [22]. We confirmed the increase in small EVs in pregnant versus non-pregnant women but failed to observe the same dramatic differences.

This lack of validation may be due to differences in sample collection, processing, and analysis, but we noted the same trend. Nevertheless, we postulate that the placenta may contribute to the overall increase in small EVs observed after acute exercise, but necessitates further assessment. The placenta is known to release small EVs vital for mediating maternal-fetal communication [31,45-47], and it is hypothesized that the placenta releases the majority of EVs in circulation during pregnancy, namely, from trophoblast cells [22,23]. Some reports estimate that between 12-25% of small EVs in maternal circulation have placental origins [48]. It has also been proposed that oxygen tension is inversely correlated with the release of small EVs by various placental cell types [22,49-51]. This evidence could provide an explanation for the differences in small EV levels after exercise, as it has been hypothesized that the placenta may experience intermittent hypoxia in response to exercise [52-54]. Studies have linked small EVs released by varying placental cell types in promoting vascular smooth muscle cell migration [50,55] as well as endothelial cell migration and vasculogenesis [51], critical processes involved in promoting proper angiogenesis of the feto-placental vasculature. Exercise is known to improve placental perfusion and vascular surface volume [27,28,56]. Therefore, it is plausible that small EVs could provide a mechanistic contribution to help understand how the benefits of exercise during pregnancy are bestowed upon the mother and fetus. However, further research is needed to establish the functional role of exercise-induced small EVs in healthy pregnancies.

Our study is not without limitations. Although our sample number was equivalent to or greater than reported in previous work (ranging from N=4-11 per group [7,13]), the sample size is still small. An *a priori* sample size calculation could not be performed due to differences in the study population, exercise, and quantitative techniques used by other studies published in this field (i.e., differing study populations, sustained vigorous-intensity exercise, quantification by semi-

quantitative methodologies). Despite our small sample size, we believe our exercise protocol was well-controlled due to the application of a matched-intensity exercise bout guided by HRR. Unlike the other studies involving acute exercise and small EVs, we did not evaluate differing exercise intensities/modes or collect samples after exercise recovery or at multiple timepoints. We believe that a strength of our study was the type, duration, and intensity of the exercise selected for examination. Since walking is the preferred exercise for pregnant women [57,58], we surmise our study presents strong applicability and generalizability. It is unknown whether the small EV profile could be affected by volume, intensity or mode of exercise in pregnant women. It is known that exercise modality impacted small EV release and/or clearance in men [13]. Future studies should evaluate differing exercise durations, intensities, and types of exercise (i.e., aerobic versus resistance training, weight-bearing versus non-weight bearing exercise). Another strength was that our groups were matched for age and pre/non-pregnant BMI to reduce potential confounding factors allowing for more robust comparisons to be made between groups. Further, we used differential ultracentrifugation to isolate small EVs, a technique known to produce low, yet highly specific yields [59,60], allowing for further analysis using proteomic research methodologies. We did not investigate the molecular contents, cellular origin, or bioactivity of exercise-induced small EVs, a limitation of our current study, but is the subject of ongoing studies in this population. As of yet, the role of small-EVs induced by acute exercise on the chronic adaptations to maternal exercise have yet to be determined. The origins and molecular contents, and, therefore, the resulting bioactivity is likely complex; nonetheless, our study provides a rationale for the continued exploration of exercise-induced small EVs in pregnancy to not only understand the potential impacts of small EVs on maternal-fetal health, but also the maternal physiological response to

exercise. Further research is needed to clarify the kinetics and role of small EVs in the context of both acute and chronic exercise during pregnancy.

Conclusions

We performed a novel examination of the exercise-induced small EV profile in pregnant women in comparison to non-pregnant controls. Although we found no differences in small EV size post-exercise, we found a significant increase in the concentration of small EVs in pregnant versus non-pregnant women after a matched-intensity treadmill exercise. The physiological relevance of small EVs in response to exercise in pregnancy warrants further examination. This study is a preliminary step to understand the role of small EVs in the physiological response to exercise in pregnancy.

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Author Contributions: SM drafted the manuscript. SM and KH primarily performed data collection and designed the study. DDS performed data analysis and interpretation. JB and KM secondarily performed data collection. DB contributed to data analysis, interpretation and technical expertise. KBA lead the study. All authors contributed to the design of the study, revised, edited, read and approved the final version of the manuscript.

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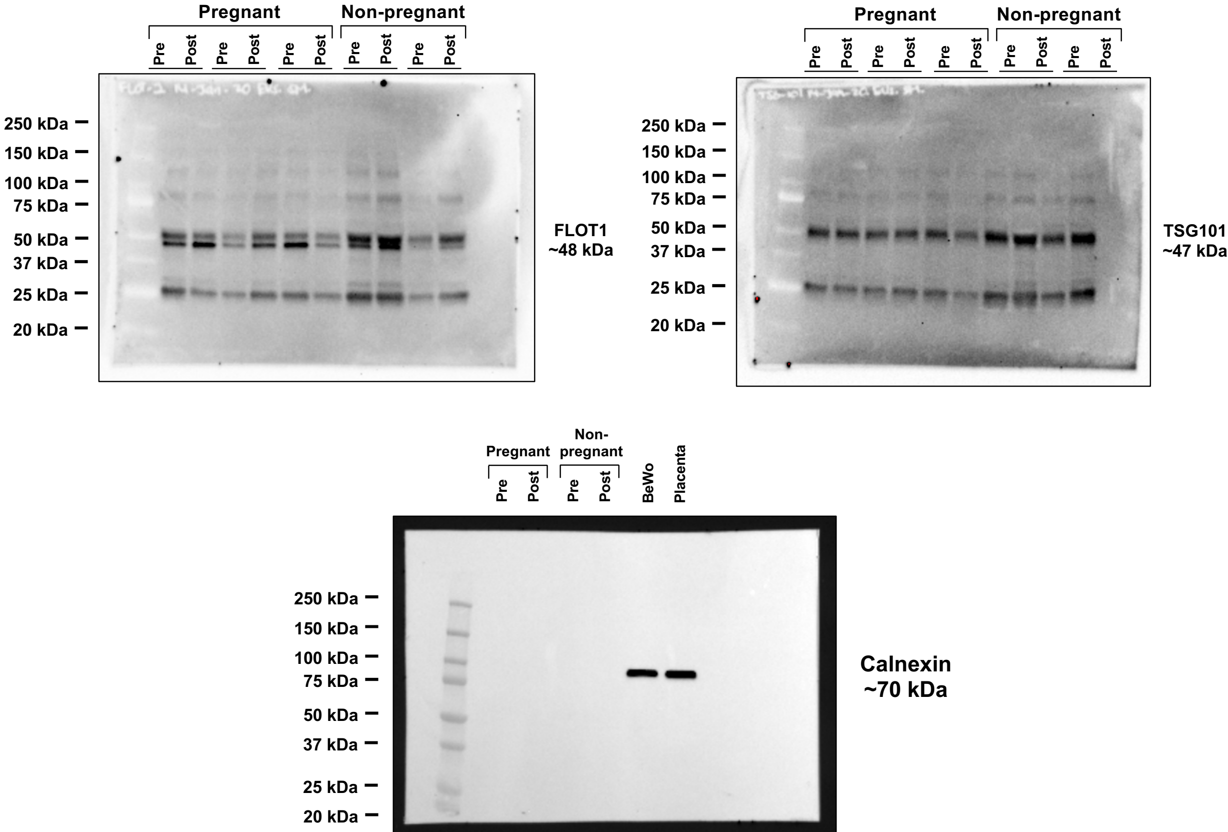
Supplementary Material



Supplementary Figure 1. Full-length western blots corresponding to Figure 2.

CHAPTER 3

Project 2 manuscript: The influence of exercise-associated small extracellular vesicles on trophoblasts *in vitro*

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Contributions: SM designed and performed the experiments, as well as drafting the manuscript. JB contributed to experimental design of work with trophoblasts *in vitro*. VT helped conduct the sEV visualization work with expertise in confocal microscopy. SM and KH designed and performed the acute exercise study from which samples were obtained for the *in vitro* trophoblast experiments. MS contributed to data collection of proliferation analysis by immunofluorescence. DB contributed to experimental design, analysis, and interpretation with expertise in EV analyses. KBA lead the study and held funding to support this work.

Abstract

It is established that exercise induces the release of small extracellular vesicles (sEVs) into circulation that are postulated to mediate tissue cross-talk during exercise. We previously reported

that pregnant individuals released greater levels of sEVs compared to matched non-pregnant controls, but their biological functions remain unknown. In this study, sEVs isolated from the plasma of healthy pregnant and non-pregnant participants after a single bout of moderate-intensity exercise were evaluated for their impact on trophoblasts *in vitro*. Exercise-associated sEVs were found localized within the cytoplasm of unsyncytialized BeWo choriocarcinoma cells, but positive signals were not different from background in syncytialized BeWo cells. Exposure to exercise-associated sEVs did not significantly alter BeWo cell proliferation, gene expression of angiogenic growth factors VEGF and PlGF, or the release of the hormone human chorionic gonadotropin. Treatment with plasma sEVs isolated at rest (pre-exercise) from pregnant and non-pregnant individuals decreased *VEGF* gene expression compared to PBS controls. *PLGF* expression in BeWo cells increased only after exposure to plasma sEVs obtained from non-pregnant participants pre-exercise when compared to PBS controls. These results support that exercise-associated sEVs can interact with trophoblasts in culture, and warrant further investigation to reveal their potential role in communicating the effects of exercise to the maternal-fetal interface.

Keywords: Exercise, physical activity, small extracellular vesicles, placenta, trophoblast, pregnancy

Statements & Declarations

The authors declare no conflicts of interest or competing interests.

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Introduction

Exercise during pregnancy is well-known to bestow benefits on both mother and fetus, with the potential to improve health across two generations. To promote maternal health and as a front-line therapy for mitigating the risk of pregnancy disorders, the 2019 *Canadian Guideline for Physical Activity throughout Pregnancy* recommends that those without contraindications should engage in at least 150 minutes of moderate-intensity physical activity per week [1]. Maternal physical activity is associated with decreased risk of pregnancy complications including preeclampsia, gestational diabetes mellitus and gestational hypertension [2], and improvement of prenatal depressive symptoms [3] while reducing blood glucose levels [4]. Prenatal physical activity is also associated with beneficial neonatal outcomes such as reduced odds of macrosomia [5] and fat mass at birth [6]. While the advantages are numerous and well-established, the biological mechanisms by which these benefits are communicated to the mother and fetus are not well understood.

Exercise has been posited to alter the structure and physiology of the placenta [7], the critical interface between mother and fetus chiefly responsible for maternal-fetal communication. The placenta supports fetal growth and survival by mediating the exchange of gases, nutrients, and waste, while providing endocrine and immune support. As a transient discoid organ anchored to the maternal uterus, the placenta is composed of a heterogenous population of cells organized in a manner to promote optimal maternal-fetal exchange between juxtaposed maternal and fetal circulatory systems. In the placenta, maternal exercise has been shown to impact oxidative stress [8], nutrient transporters [9, 10], and factors involved in angiogenesis [11, 12]. Data from exercise interventions also show beneficial effects on placental growth [13] and volume available for

maternal-fetal exchange [14, 15]. While it is evident that exercise impacts placental function, the biological mechanisms contributing to these changes are less clear.

The benefits of exercise are hypothesized to be mediated in part by the release of bioactive molecules into circulation after exercise including myokines (cytokines produced and released by skeletal muscle) [16, 17] and small extracellular vesicles (sEVs) [18, 19]. sEVs (historically referred to as 'exosomes' [20]) are lipid membrane-enclosed particles (~20-120 nm in diameter) secreted by cells into extracellular space and contain bioactive compounds such as nucleic acids (i.e., mRNAs and miRNAs), lipids, and proteins [21, 22]. sEVs are thought to facilitate long-distance intercellular communication by interaction or transfer of their biological cargo from donor to target cells [23, 24]. Data from both human and animal studies demonstrate that sEVs are released into circulation after exercise (reviewed by [19]), and contain biological molecules that are involved in tissue cross-talk in response to exercise [18]. We previously reported that pregnant women release greater levels of sEVs into circulation compared to non-pregnant women after a single bout of moderate-intensity exercise [25]. The potential biological function and cellular targets of circulating sEVs released in response to maternal exercise are unknown. Since the placenta releases and takes-up sEVs as a part of maternal-fetal communication [26, 27], exercise-associated sEVs could act on the placenta. Therefore, circulating sEVs may represent a potential mechanism involved in altering placental function in the context of maternal exercise.

In this study, we examined whether exposure to circulating sEVs obtained before and after acute exercise affected trophoblasts *in vitro*. Trophoblast cells are specialized epithelial cells that constitute the maternal-fetal interface and are responsible for hormone production and exchange of gases, nutrients, and waste between mother and fetus. Maternal-fetal exchange occurs in the floating chorionic villi which are in direct contact with maternal blood [28]. Floating chorionic

villi are covered in an outer multinucleated layer of syncytiotrophoblast, which is derived from the fusion of the underlying mononuclear cytotrophoblast cells (reviewed by [29]). In the present study, we employed the well-documented trophoblast cell line, BeWo choriocarcinoma cells, to model trophoblast *in vitro*. BeWo cells are thought to represent proliferating cytotrophoblasts and can be induced to differentiate or syncytialize to mimic syncytiotrophoblast *in vitro* [30-32]. We sought to determine whether sEVs obtained from the plasma of healthy pregnant and non-pregnant women before and after an acute bout of moderate-intensity exercise could interact with unsyncytialized and syncytialized BeWo cells. Further, we evaluated whether exposure to exercise-associated plasma sEVs could influence metrics of trophoblast biology *in vitro*, including proliferation, expression of angiogenic growth factors known to be altered in placenta of physically active vs. inactive women [11], and secretion of the major pregnancy hormone, human chorionic gonadotropin (b-hCG). Treatment with sEVs from healthy non-pregnant controls were used to determine whether the potential effects on trophoblast biology were associated with pregnancy status or exercise stimulus. We hypothesized that exercise-associated sEVs from pregnant women would elicit greater effects on trophoblast biology compared to exercise-associated sEVs from non-pregnant women.

Methods

Ethical approval & study participants

Experimental procedures were approved by the University of Ottawa Research Ethics Board (file number: H-06-18-634). All protocols were performed in fulfillment of guidelines described in the Declaration of Helsinki. Informed written consent was secured from participants after explanation of study procedures. Pregnant and non-pregnant individuals were recruited from the Ottawa area (ON, Canada) for participation in the study. Healthy women without

contraindication to exercise between the ages of 18-40 were eligible for inclusion, with a self-reported pre/non-pregnant body mass index (BMI) of 18.5-29.9 kg/m^2. Participants were required to be weight-stable (±5 kg) for approximately six months before the study. Pregnant participants needed to be between 13-28 weeks gestation carrying a singleton fetus to participate in the study. Those with chronic health conditions including hypertension, diabetes (pre/non-pregnant or gestational diabetes), untreated thyroid disease, and frequent users of tobacco, drugs, or alcohol were excluded from participation.

Acute exercise procedure

The acute exercise stimulus followed procedures outlined in detail by Hutchinson *et al.* (2019) [33] and Mohammad *et al.* (2021) [25]. Briefly, participants were requested to refrain from exercise and food for 8 hours before the acute exercise session. Participants were provided with a standardized snack, after which resting heart rate was determined as previously described [25, 33] . A target range of 40-59% of heart rate (HR) reserve was used to specify moderate-intensity exercise [34, 35], where HR reserve was calculated using the Karvonen equation [36] as described previously [25, 33]. The moderate-intensity acute exercise bout consisted of a brisk 30 min treadmill walk with continuous HR monitoring using a Polar V800 HR monitor (Polar Electro, Lachine, QC, Canada). A short warm-up (3 min at 2% incline and 2 miles per hour (mph)) was followed by an incremental phase (6% incline), where treadmill speed was increased by 0.2 mph every minute until the upper range of HR reserve (59%) was met. Once this range was met, the participants continued to exercise for 30 min. Blood was collected (10 mL) immediately before (at rest) and after the exercise session from the median cubital vein using potassium EDTA blood collection tubes (#367863; BD Biosciences, Mississauga, ON). Plasma was immediately

processed by centrifugation at 1700 × *g* for 15 min at 4°C, and samples were stored at -80°C until further analyses.

sEV isolation and labeling

Isolation of sEVs from plasma was performed using differential ultracentrifugation as described previously [25, 37, 38]. Plasma samples (1.0 mL) were thawed at 37°C, then kept on ice for all remaining procedures. Samples were first centrifuged at 20,000 × *g* for 20 min at 4°C to remove large EVs and apoptotic bodies. The remaining supernatant was centrifuged at a speed of 100,000 × *g* using a Beckman Coulter Optima MAX ultracentrifuge (Beckman Coulter Inc., Brea, CA, USA) equipped with a TLA-55 rotor (Beckman Coulter) for 90 min at 4°C. The resulting pellet of sEVs was washed with 1.0 mL of 0.1 μm-filtered phosphate-buffered saline (PBS) and then centrifuged at 100,000 × *g* as described above. The final residual pellet of sEVs was resuspended in 100 μL of 0.1 μm-filtered PBS. Aliquots of 10 μL of this suspension were separated and frozen at -80°C for further analysis. One 10 μL aliquot was used for protein extraction and quantification to standardize sEV treatment concentrations for subsequent functional assays. To extract protein, 1 μL of 10X radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail (MilliporeSigma Canada Co, #P8340, Oakville, ON, Canada) was added to a 10 μL sEV aliquot. Samples were sonicated for 1 min to achieve EV and protein lysis and subsequently were incubated on ice for 30 min before protein quantification using a DC protein assay (Bio-Rad Laboratories, #5000112, Mississauga, ON, Canada). sEVs from this cohort were previously validated to be the expected size (~100-120 nm), and displayed characteristics confirming presence of these particles (i.e., expression of classical sEV protein markers TSG-101 and flotillin-1, absence of non-sEV marker calnexin, and intact membrane integrity and characteristic size by transmission electron microscopy; see Fig. 2 from Mohammad *et al.* 2021) [25]. Where indicated,

sEVs were fluorescently labeled with PKH26, a lipophilic membrane dye, using the PKH26 Red Fluorescent Cell Linker Kit according to the manufacturer's instructions (Phanos Technologies, MilliporeSigma Canada Co, #MINI26-1KT). Following incorporation of the dye, the labeled sEVs were centrifuged at 100,000 × *g* for 90 min at 4°C, and the resulting sEV pellets were resuspended in 100 µL of 0.1 µm-filtered PBS and stored at -80°C for subsequent sEV internalization and interaction fluorescence assays.

Cell culture

BeWo choriocarcinoma cells were obtained from the American Type Culture Collection (ATCC® CCL-98™, Manassas, VA) and grown in Ham's F-12K (Kaighn's) medium (Gibco™, Thermo Fisher Scientific, #21127022, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C and 5% CO_2 in a humidified environment. For all sEV treatment experiments, cells from passages 7-12 were used in the presence of F-12K medium supplemented with 10% EV-depleted FBS. EVs were depleted from FBS by ultracentrifugation at 100,000 × *g* for 90 min at 4°C and retention of the resulting supernatant [39]. Cells were manipulated experimentally 48 h post-seeding.

BeWo cell syncytialization

BeWo cells were syncytialized for the sEV internalization and interaction fluorescence assays only, to evaluate whether plasma sEVs can interact with a model of syncytialized trophoblast *in vitro*. Syncytialization was promoted by exposure to the cyclic AMP (cAMP) agonist, forskolin, a known inducer of BeWo cell fusion and differentiation [30, 31, 40]. BeWo syncytialization through cAMP stimulation decreases the expression of the cell junction protein, E-cadherin [41], in addition to increasing the production of the hormone human chorionic gonadotropin (b-hCG) [30, 31]. Therefore, we conducted E-cadherin immunofluorescence on cells

and measured b-hCG levels in cell culture media to confirm syncytialization after forskolin stimulation. For E-cadherin immunofluorescence, BeWo cells were seeded at a density of 7.5×10^5 cells in 6-well dishes on 22mm square glass coverslips (Fisherbrand™, Fisher Scientific Company, #125-541B, Ottawa, ON, Canada), and 48 h later, fresh media was added with a final concentration of 50 µM forskolin [42] (MilliporeSigma Canada Co, #F3917) or 0.1% dimethyl sulfoxide (DMSO) vehicle control (Fisher BioReagents™, Fisher Scientific Company, #BP231-100). Media was exchanged every 24 h over the course of 72 h with the addition of fresh forskolin or vehicle control. At the end of the experiment, cell culture supernatant was removed and wells were washed three times using PBS with 0.1% TWEEN 20® (PBST), then cells were fixed with 10% buffered formalin for 10 min at room temperature. All subsequent steps were carried out at room temperature unless specified otherwise. After three washes with PBST, cells were permeabilized for 5 min using 0.1% Triton X-100 in PBS. Cells were washed again three times with PBST then blocked for 30 min using BlockAid™ Blocking Solution (Invitrogen™, Thermo Fisher Scientific, #B10710), after which cells were incubated overnight at 4°C with rabbit monoclonal anti-E-cadherin [EP700Y] antibody (1:500; Abcam Inc., #ab40772, Cambridge, MA, USA) in PBST or negative control (PBST only). After three washes with PBST, cells were incubated with AlexaFluor 647 goat anti-rabbit IgG (H+L) secondary antibody (Invitrogen™, Thermo Fisher Scientific, #A-21245) for 60 min. Then after three washes with PBST, cells were incubated with phalloidin-iFluor 488 Reagent (1:1000; Abcam Inc, #ab176753) in 0.1% BSA in PBS for 20 min, as a background stain to visualize actin filaments in the cytoskeleton. Cells were washed, then mounted using ProLong™ gold antifade mountant with DAPI (Invitrogen™, Thermo Fisher Scientific, #P36931). The immortalized first trimester trophoblast cell line, HTR-8/SVneo, was used as a negative cellular control for E-cadherin expression as these cells do not express E-

cadherin [43]. Growth conditions for HTR-8/SVneo cells are described in the supplementary methods. Images were taken using a Zeiss AxioObserver Z1 inverted epifluorescent microscope with Zen Blue (v. 2.3, Carl Zeiss Microscopy GmbH, Köln, Germany). A total of 10 images per condition were acquired at 10X magnification for immunofluorescent analysis of E-cadherin expression. A ratio of E-cadherin staining intensity to cell area by phalloidin staining was compared between forskolin- treated and vehicle control (DMSO) using the open-source imaging processing software Fiji (v. 2.3.0/1.53f) [44]. The image analysis workflow steps are described in the supplementary methods and Fig. S1.

To measure b-hCG levels in cell culture media after forskolin stimulation, the same experiment was conducted as described above, without the square glass coverslips. At the end of the experiment, cell culture supernatant was collected and stored at -80°C for downstream b-hCG analysis, while the cells were washed twice with cold PBS, then lysed for protein extraction using 300 µL of 1X RIPA buffer with protease inhibitor cocktail (MilliporeSigma Canada Co, #P8340). Cells were kept on ice for 30 min, then centrifuged for 15 min at 14,000 RPM (Sorvall ST 16R, Thermo Fisher Scientific) at 4°C. Total protein lysate concentration was quantified using a DC protein assay (Bio-Rad Laboratories, #5000112). Cellular protein concentration was used to normalize b-hCG concentrations in cell media for syncytialization confirmation experiments only. A DRG b-hCG ELISA kit (DRG® International, #EIA-1911) was used to measure b-hCG in cell media to confirm syncytialization, and was performed according to the manufacturer's instructions. Serum from a pregnant person in their second trimester recruited in the PLACENTA study [11] was used as a positive control for b-hCG.

sEV localization by fluorescence confocal microcopy

BeWo cells (2.5×10^4) were plated in 8-well chamber slides (ibidi USA, #80826, Fitchburg, WI, USA) and incubated with 2.5 µg/mL of PKH26-labeled sEVs (or PBS control) overnight (16 h) at 37°C and 5% CO_2. The cell culture supernatant was removed, and wells were vigorously washed five times with PBST, then fixed with 10% buffered formalin for 10 min at room temperature. All subsequent steps were conducted at room temperature unless noted otherwise. Wells were washed 3 times with PBST to remove fixative, then cells were permeabilized using 0.1% Triton X-100 in PBS for 5 min. Wells were washed three times with PBST then incubated with phalloidin-iFluor 488 Reagent (1:1000; Abcam Inc, #ab176753, Cambridge, MA, USA) in 1% BSA in PBS for 25 min. Wells were washed three times with PBST then incubated with 1 drop of NucBlue™ Fixed Cell ReadyProbes™ Reagent (DAPI) (Invitrogen™, Thermo Fisher Scientific, #R37606) per well for 5 min. Mounting media (ibidi USA, #500001) were added to each well before imaging using an inverted Zeiss LSM 880 AxioObserver Z1 laser-scanning confocal microscope with Airyscan FAST detector equipped with Zen Black software (v. 2.3, Carl Zeiss Microscopy GmbH). Images were taken using a 63X oil-immersion objective lens (Zeiss, Plan Apochromat 63/1.4 NA oil) with optical slices (z-stacks) at a thickness of 0.20 µm. The confocal was equipped with lasers emitting at 405, 488, and 561nm and were used for the excitation of each fluorophore: dapi (Ex/Em 405/450nm), phalloidin (Ex/Em 488/516nm), and PKH26 (Ex/Em 561/579nm). Each confocal microscopy image was acquired using the same imaging parameters. Images were subjected to linear unmixing of the measured spectral profiles for each fluorophore (DAPI, phalloidin, and PKH26) using Zen Black software to account for signal crossover between spectral channels. Representative maximum intensity projections were acquired from a subset of z-stacks corresponding to middle of the cells. To account for background autofluorescence, manual intensity thresholding relative to vehicle control treatment images (PBS only) by visual inspection

(unsyncytialized displayed minimum value = 4091 and syncytialized displayed minimum value = 5444) was conducted using Fiji software. For each condition, a minimum of three random fields-of-view were selected and examined for sEV localization. To increase the quality of the images for display purposes, we set lookup tables for the phalloidin channel image to 'Magenta' and for the PKH26 channel image to 'Green' using Fiji software.

For the localization experiments, a range of PKH26-labeled sEV concentrations were initially tested (1, 2.5, 5, and 10 µg/mL) for visualization by confocal microscopy. The concentration of 2.5 µg/mL of sEVs incubated overnight (16 h) was found to be the exposure with the best signal-to-noise ratio.

Proliferation assessment by Ki67 immunostaining

To assess the influence of exercise-associated plasma sEVs on BeWo cell proliferation *in vitro*, Ki67 immunostaining was used. BeWo cells (2.5×10^4) were seeded onto 8-well chamber slides (ibidi USA, #80826) and incubated with 10 µg/mL sEVs (or PBS control) for 24 h at 37°C and 5% CO_2 in duplicate. Then, the cell culture supernatant was removed and wells were washed three times with PBST. All of the following steps were carried out at room temperature unless stated otherwise. Cells were fixed with 10% formalin for 10 min, then washed three times with PBST. Cells were then permeabilized using 0.1% Triton X-100 in PBS for 5 min followed by three washes with PBST. Cells were blocked for 30 min with BlockAid™ Blocking Solution (Invitrogen™, Thermo Fisher Scientific, #B10710), then incubated with recombinant anti-Ki67 rabbit monoclonal antibody [SP6] (1:250; Abcam Inc, #ab16667) at 4°C in PBST overnight. Negative controls omitted the primary antibody. The following day, wells were washed three times with PBST then incubated with goat AlexaFluor 488 anti-rabbit IgG (H+L) Superclonal™ recombinant secondary antibody (1:1000; Invitrogen™, Thermo Fisher Scientific, #A27034) in

PBST for 60 min. Wells were washed three times with PBST then incubated with 1 drop of NucBlue™ Fixed Cell ReadyProbes™ Reagent (DAPI) (Invitrogen™, Thermo Fisher Scientific, #R37606) per well for 5 min. Mounting media (ibidi USA, #500001) were added to each well before imaging using a ThermoFisher FL Auto 2 inverted automated epifluorescent microscope equipped with Auto2 software (Invitrogen™, Thermo Fisher Scientific). Each well was divided into four quadrants, with one image taken at 20X magnification per quadrant for a total of four images per well. All experiments were performed in triplicate. A ratio of Ki67 immunostaining intensity to nuclear area was measured using Fiji software. The image analysis workflow with detailed steps are outlined in the supplementary methods and Fig. S2.

RNA isolation and quantitative real-time polymerase chain reaction (qPCR)

BeWo cells were seeded at a density of 1.0×10^5 in 12-well dishes and 48 h later, were treated with 10 µg/mL plasma sEVs (or PBS control) for 24 h at 37°C and 5% CO_2. Cell culture supernatant was collected and stored at -80°C for downstream b-hCG analysis described below. Cells were washed twice with cold sterile PBS then lysed for total RNA isolation using an illustra™ RNAspin Mini isolation kit (Cytiva Life Sciences™, Fisher Scientific Company, #25050071) as per the manufacturer's instructions. Isolated total RNA was eluted in RNase-free water and were analyzed for concentration and purity using spectrophotometry (Take3™, Gen5 software version 1.11.5, BioTek® Instruments Inc., Winooski, VT, USA). RNA integrity was verified using a 2% agarose gel stained with SYBR™ Safe DNA gel stain (Invitrogen™, Thermo Fisher Scientific, #S33102) and electrophoresis of bromophenol blue-labeled RNA aliquots at 100V for 30 min in TAE (Tris-acetate-EDTA) buffer. RNA bands were visualized by ultraviolet transillumination using a ChemiDoc XRS+ system (Bio-Rad Laboratories). Then, 0.5 µg of RNA was reverse transcribed into cDNA using an iScript™ cDNA synthesis kit (Bio-Rad Laboratories,

#1708891) and a Biometra® TPersonal Combi Thermocycler (Analytik Jena, Jena, Germany) according to the manufacturer's protocol, and then stored at -20°C until further analysis. 25 ng of cDNA was used for quantitative polymerase chain reaction (qPCR) using TaqMan® Advanced Master Mix (Applied Biosystems™, Thermo Fisher Scientific, #444557). Samples were loaded into a Rotor-Gene 3000™ real-time DNA detection system with Rotor-Gene software (Version 6.1.93, Corbett Research, Sydney, Australia). The expression of angiogenic growth factors *VEGF* and *PLGF* was measured relative to endogenous control, *GAPDH*. Taqman® gene expression assay probes labeled with 6-FAM (6-carboxyfluorescein) fluorescent dye were used for the detection of *VEGF* (Hs00900055_m1), *PLGF* (Hs00182176_m1), and *GAPDH* (Hs02786624_g1) were employed (Applied Biosystems™, Thermo Fisher Scientific, #4331182). The qPCR reaction was as follows: hold at 50°C for 2 min, then hold at 95°C for 2 min, followed by 40 cycles consisting of amplification at 95°C for 3 s and hold at 60°C for 30 s. All qPCR reactions were performed in duplicate with appropriate controls (no reverse transcriptase cDNA control and no template qPCR control), and all experiments were conducted in triplicate. Corresponding threshold cycle (CT) values were recorded, and relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method [45]. Data from the target genes (*VEGF* and *PLGF*) were expressed as a ratio to *GAPDH* gene expression for normalization. Gene expression values from PBS control treatment conditions were considered as control, and the relative gene expression of sEV treatment from pregnant and non-pregnant pre- and post-exercise was determined.

b-hCG assay

Cell culture supernatant collected from the gene expression analyses described above were used to determine the effect of sEV treatment on BeWo cell b-hCG production after 24 h of exposure using a DRG b-hCG ELISA kit (DRG® International, #EIA-1911) as per the

manufacturer's instructions. b-hCG concentration was normalized to mg of total RNA isolated from the corresponding gene expression experiments and are presented in mIU/mL.

Statistical analysis

All data are presented as mean ± standard deviation (SD) from three independent experiments. All statistical analyses and graphs were generated using GraphPad Prism version 9.3 (GraphPad Software, La Jolla, CA, USA). An unpaired *t*-test was used to compare E-cadherin immunofluorescence staining intensity for syncytialization validation experiments. For comparisons of BeWo cell proliferation, qPCR, and b-hCG levels after treatment with sEVs obtained from pregnant and non-pregnant groups pre- and post-exercise, a two-way repeated measures ANOVA (exercise × pregnancy status) with Bonferroni's multiple comparisons post-test was used. In the aforementioned experiments, a one-way ANOVA with Dunnett's post-test was used to compare whether no treatment (PBS control) was different to treatment with sEVs pre- or post-exercise in pregnant and non-pregnant groups, where indicated. Statistical significance was considered when $p<0.05$.

Results

Exercise-associated sEVs interact with unsyncytialized BeWo cells

We first examined whether circulating sEVs obtained from pregnant and non-pregnant women before (rest) and after a bout of acute moderate intensity exercise could be found within trophoblast-like cells *in vitro* after overnight incubation. Representative images obtained by confocal microscopy show that PKH26-labeled sEVs from both pregnant and non-pregnant women, pre- and post-exercise localized within the cytoplasm of unsyncytialized BeWo cells (Fig. 1).

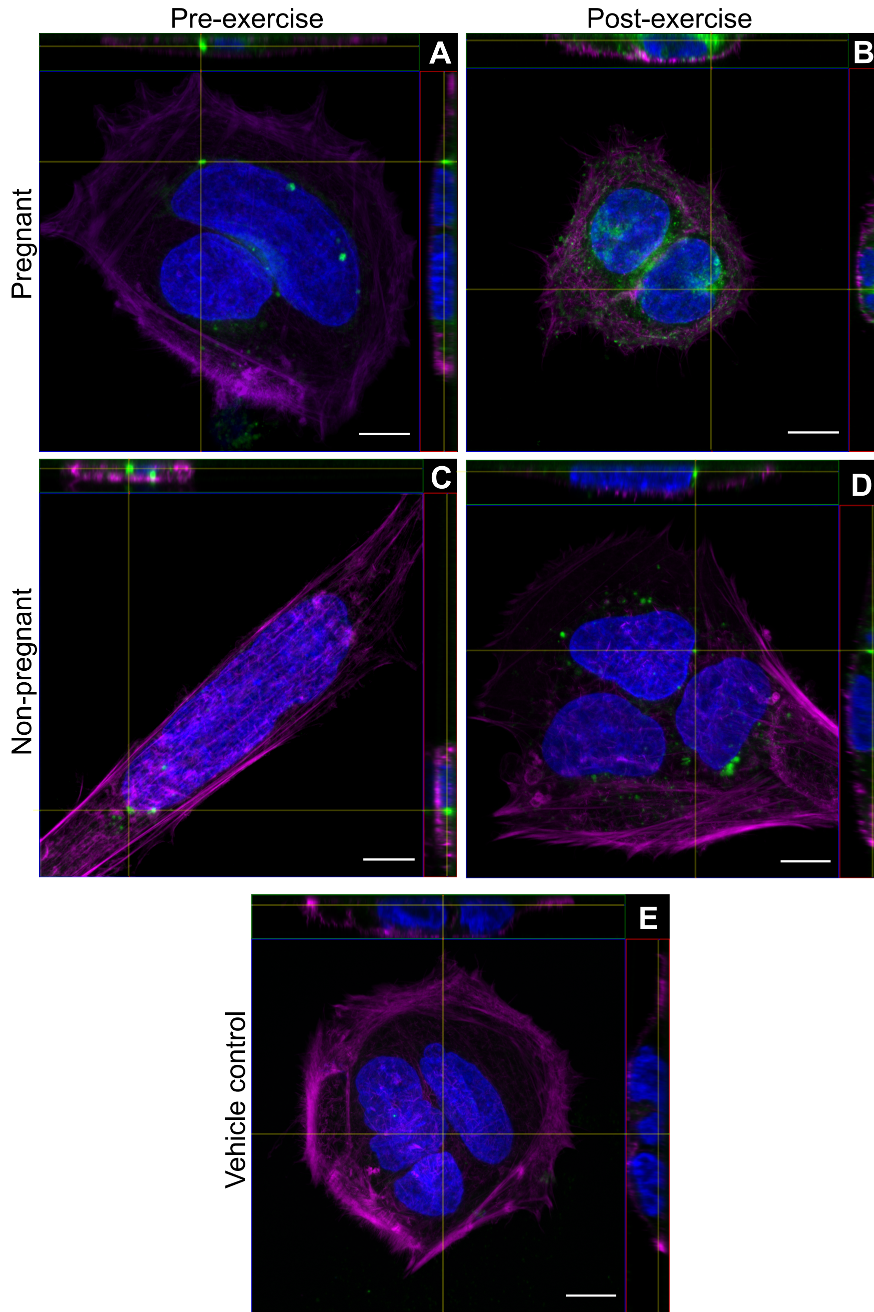

Fig. 1 Exercise-associated sEVs were found within unsyncytialized BeWo cells *in vitro*. Representative confocal microscopy images of unsyncytialized BeWo cells after overnight (16 h) incubation with 2.5 µg/mL PKH26-labeled sEVs from pregnant (A-B) and non-pregnant (C-D) plasma, both pre- and post-exercise. Panel E shows a representative vehicle control (PBS). Blue represents dapi staining for nuclei, magenta depicts phalloidin staining for actin filaments in the cytoskeleton, and green shows PKH26-labeled sEVs. 'Magenta' and 'Green' lookup tables were used to display phalloidin and sEV labeling, respectively. Orthogonal projections show the XY (main image), YZ (right of main image) and XZ (top image) planes, respectively. All images were taken using a 63X objective lens with oil immersion. Scale bar = 10 µm for all images.

Forksolin was used to induce syncytialization of BeWo cells and was validated through the examination of b-hCG release by cells into media and by investigation of E-cadherin expression levels. Measurement of b-hCG in cell culture media from forskolin-treated BeWo cells revealed 20.8-fold greater concentration when compared to vehicle control (DMSO) (Fig. S3). A significant decrease in E-cadherin expression was observed in forskolin-treated cells vs. DMSO control (Fig. S4). Together, these data confirm syncytialization of BeWo cells. Representative confocal images show that PKH26 positive signal could not be differentiated from background in syncytialized BeWo cells after exposure to PKH26-labeled sEVs from pregnant and non-pregnant women obtained pre- and post-exercise (Fig. 2A-D).

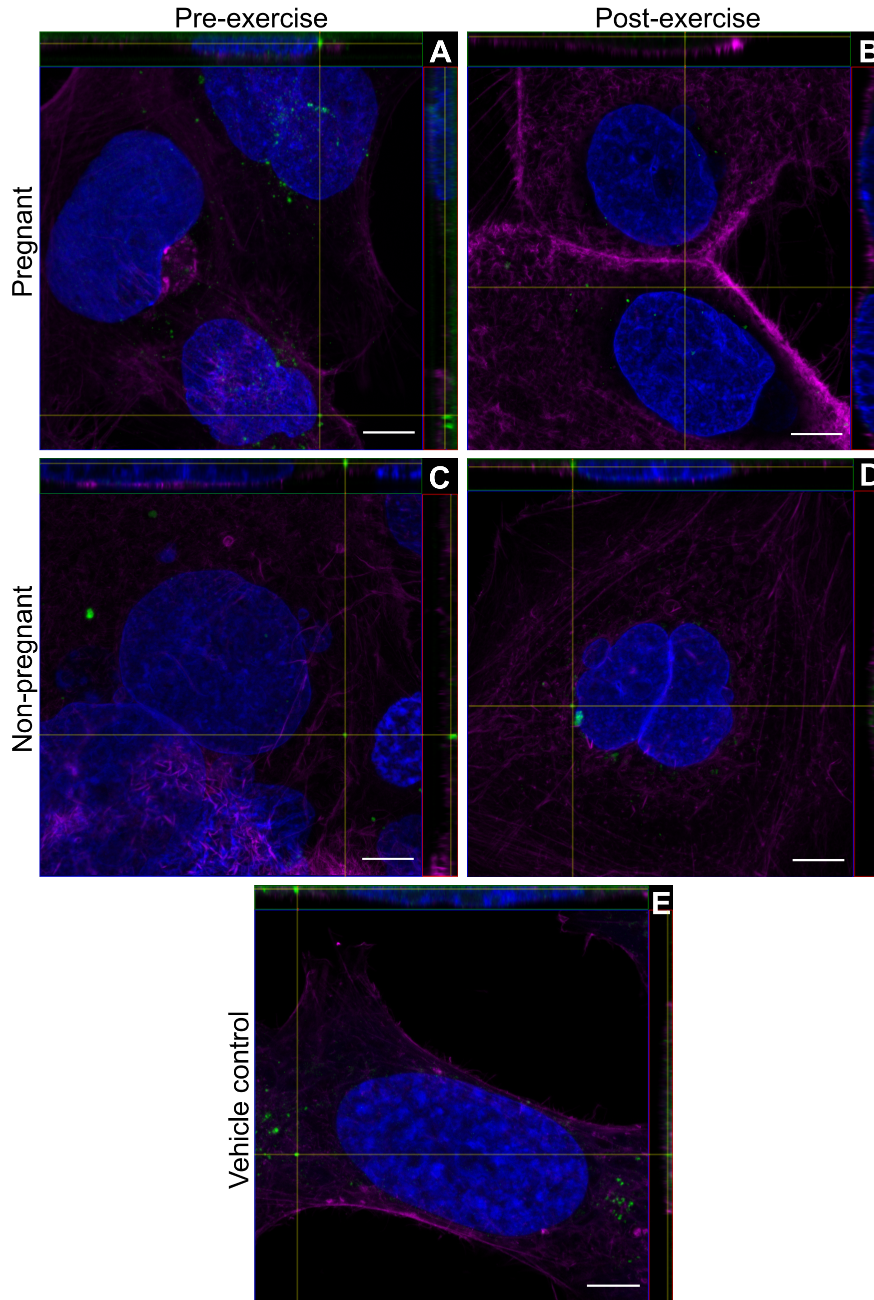

Fig. 2 Positive signal from PKH26-labeled exercise-associated sEVs were not different from background in syncytialized BeWo cells *in vitro*. Representative confocal microscopy images of syncytialized BeWo cells after overnight (16 h) incubation with 2.5 µg/mL PKH26-labeled sEVs from pregnant (A-B) and non-pregnant (C-D) plasma, both pre- and post-exercise. Panel E shows a representative vehicle control (PBS). Blue represents dapi staining for nuclei, magenta depicts phalloidin staining for actin filaments in the cytoskeleton, and green shows PKH26-labeled sEVs. 'Magenta' and 'Green' lookup tables were used to display phalloidin and sEV labeling, respectively. Orthogonal projections show the XY (main image), YZ (right of main image) and XZ (top image) planes, respectively. All images were taken using a 63X objective lens with oil immersion. Scale bar = 10 µm for all images.

BeWo cell proliferation was not affected upon exposure to exercise-associated sEVs

Immunostaining of the protein Ki67 was used to assess proliferation in BeWo cells after 24 h incubation with exercise-associated sEVs. Ki67 is a widely used marker of cell proliferation and has been used in the examination of trophoblast and BeWo cell proliferation and phenotype [46]. Exposure to sEVs obtained from pregnant women and non-pregnant controls before and after exercise did not influence BeWo cell proliferation after 24 h (Fig. 3A-E). There was no significant interaction between exercise (pre vs. post-exercise) and pregnancy status (pregnant vs. non-pregnant) ($F=0.365$, $p=0.579$) and no main effects of exercise ($F=7.40$, $p=0.053$) or pregnancy status ($F=0.689$, $p=0.453$) (Fig. 3A). Further, there were no differences in proliferation between no treatment (PBS control) and treatment with sEVs obtained pre- ($F=0.480$, $p=0.635$; Fig. 3B) or post-exercise ($F=0.469$, $p=0.642$; Fig. 3C) from either group.

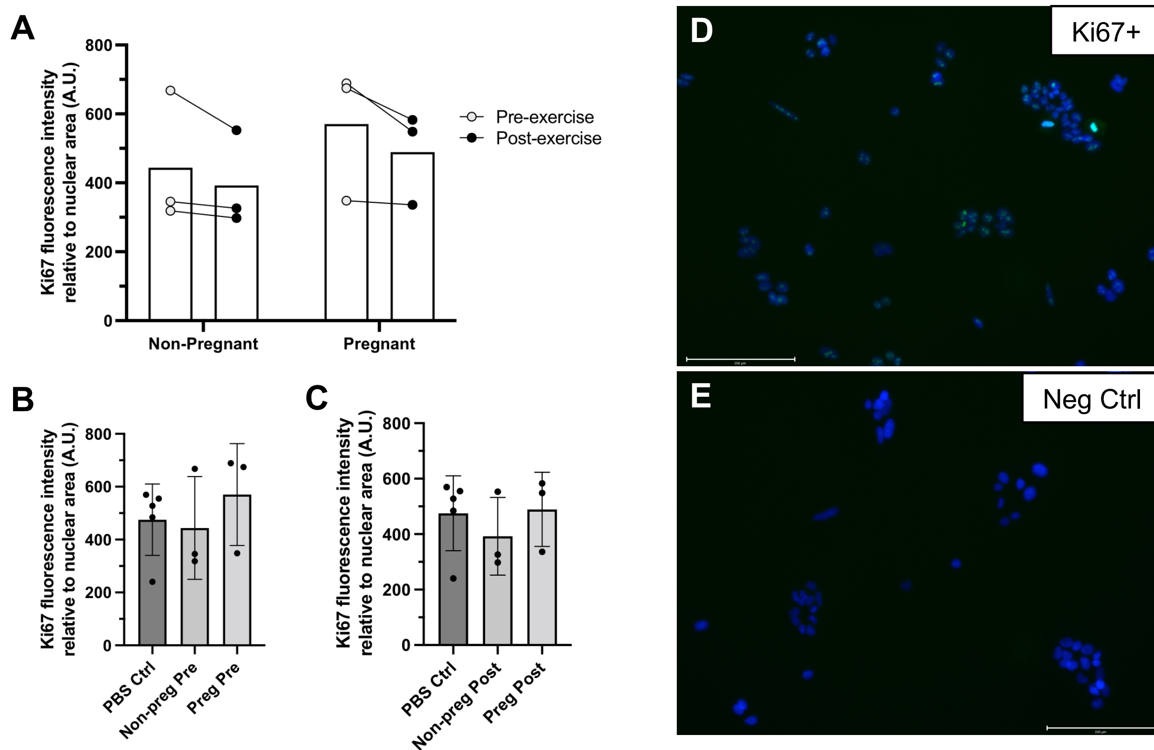

Fig. 3 Exercise-associated sEVs did not effect BeWo cell proliferation. Ki67 immunostaining image analysis of BeWo cells incubated with plasma sEVs (10 µg/mL for 24 h) obtained from non-pregnant and pregnant participants both pre- and post-exercise showed no significant main effects of pregnancy status (F=0.689, *p*=0.453) or exercise (F=7.40, *p*=0.053), and no significant interaction between pregnancy status and exercise (F=0.365, *p*=0.579) (A). There were no differences in proliferation when no treatment ('PBS Ctrl') was compared to treatment with sEVs obtained pre- (F=0.480, *p*=0.635) or post-exercise (F=0.469, *p*=0.642) from non-pregnant and pregnant individuals (B&C). Representative merged fluorescence image of a Ki67 positive image (D) and a negative control image (E) where primary antibody was omitted are shown, where blue depicts dapi staining for nuclei and green shows Ki67 positive signal. All images were taken at 20X magnification. Data in (A) were analyzed using a two-way repeated measures ANOVA and shows only the mean, with each line connecting pre-post values for an individual participant. Data in (B) and (C) are shown as mean ± SD and were analyzed using a one-way ANOVA test. All experiments were conducted in triplicate, with sEVs obtained from N=3 non-pregnant and N=3 pregnant participants, with corresponding PBS controls (N=5). Scale bar = 200 µm. Neg ctrl, negative control; PBS Ctrl, phosphate-buffered saline control; Pre, pre-exercise; Preg, pregnant; Post, post-exercise; Non-preg, non-pregnant.

Exposure to plasma sEVs altered the gene expression of angiogenic growth factors in BeWo cells

We previously reported that maternal exercise was associated with differential expression of angiogenic proteins in the placenta of women categorized as physically active vs. inactive during pregnancy [11]. We therefore determined whether exposure to circulating exercise-

associated sEVs from pregnant or non-pregnant women affected gene expression of *VEGF* and *PLGF* in BeWo cells.

For *VEGF*, there was no significant interaction between exercise (pre- vs. post-exercise) and pregnancy status (non-pregnant vs. pregnant) ($F=1.295$, $p=0.326$), nor main effects of exercise ($F=2.20$, $p=0.212$) or pregnancy status ($F=0.244$, $p=0.647$) (Fig. 4A). However, *VEGF* gene expression was significantly lower ($F=5.39$, $p=0.033$) upon treatment with sEVs obtained pre-exercise from both groups when compared to PBS control (PBS control vs. non-pregnant pre-exercise, $p=0.0462$, and PBS control vs. pregnant pre-exercise, $p=0.0497$) (Fig. 4B). *VEGF* gene expression did not differ between PBS controls vs. treatment with sEVs obtained post-exercise from both groups ($F=1.98$, $p=0.200$) (Fig. 4C).

With-respect-to *PLGF* gene expression, no significant interaction between exercise and pregnancy status was found ($F=3.58$, $p=0.132$), nor significant main effects of exercise ($F=2.92$, $p=0.163$) or pregnancy status ($F=1.89$, $p=0.241$) (Fig. 4D). Expression of *PLGF* was found to be significantly different when no treatment (PBS control) was compared to treatment with sEVs obtained pre-exercise ($F=4.56$, $p=0.048$), where sEVs from non-pregnant individuals pre-exercise resulted in significantly higher expression ($p=0.037$) (Fig. 4E). *PLGF* gene expression was not different in BeWo cells exposed to sEVs derived from either group post-exercise vs. PBS control ($F=0.726$, $p=0.513$) (Fig. 4F).

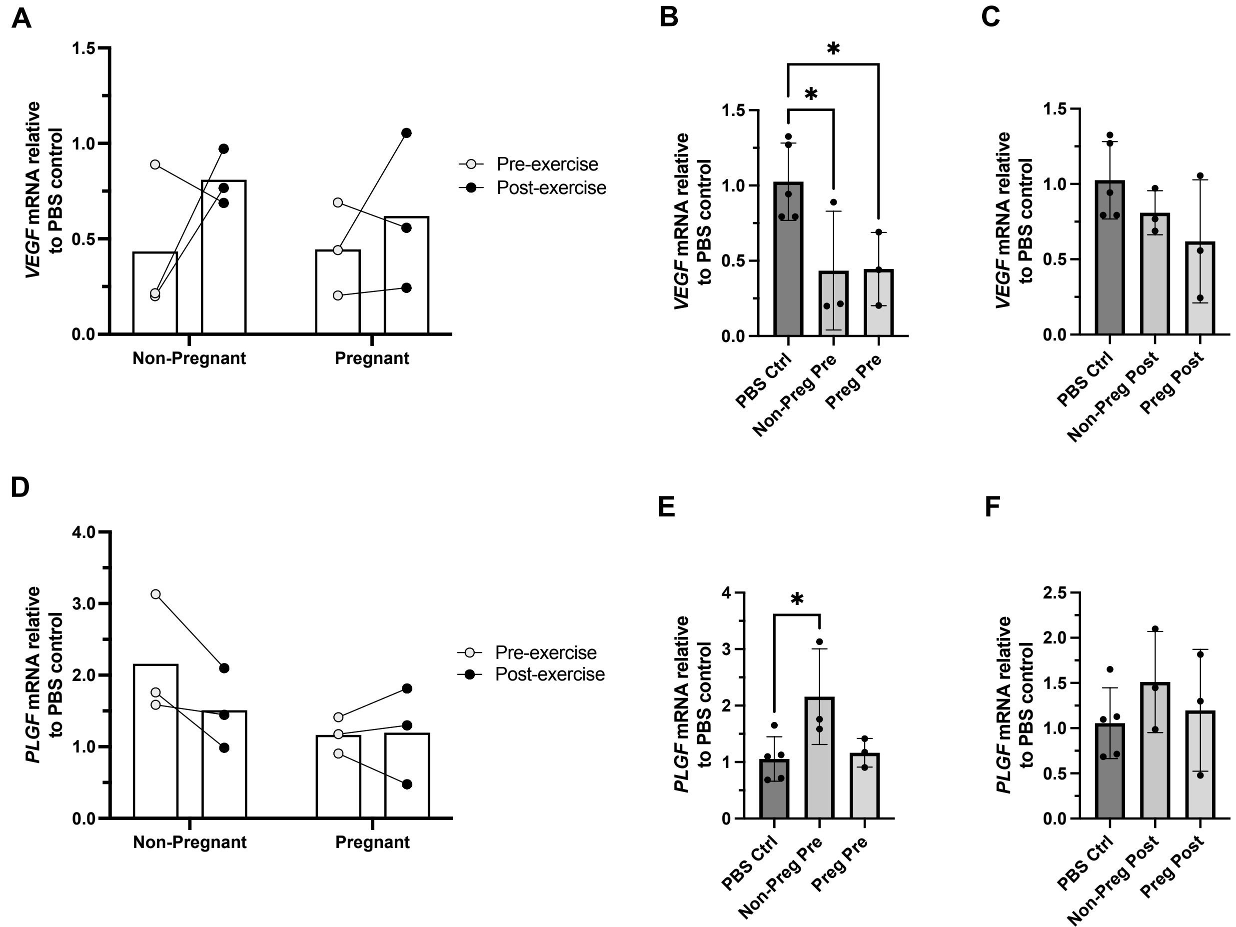

Fig. 4 The effect of exercise-associated sEVs on relative gene expression of angiogenic growth factors in BeWo cells. (A) *VEGF* gene expression was not altered upon exposure to circulating sEVs (10 µg/mL for 24 h) obtained from non-pregnant and pregnant individuals, pre- and post-exercise (main effect of exercise: F=2.20, *p*=0.212; main effect of pregnancy status: F=0.244, *p*=0.647; interaction of exercise × pregnancy status: F=0.294, *p*=0.617). (B) Gene expression of *VEGF* was significantly lower (F=5.39, p=0.033) upon treatment with plasma sEVs obtained pre-exercise from both groups (PBS control vs. non-pregnant pre-exercise, *p*=0.0462 and PBS control vs. pregnant pre-exercise, *p*=0.0497). (C) *VEGF* gene expression was not different between no treatment (PBS control) and exposure to sEVs obtained post-exercise from either group (F=1.98, *p*=0.200). (D) *PLGF* gene expression was not affected by treatment with sEVs pre- vs. post-exercise from either group, as there was no significant interaction between exercise × pregnancy status (F=3.58, *p*=0.132), nor main effects of exercise (F=2.92, *p*=0.163) or pregnancy status (F=1.89, *p*=0.241). (E) *Plgf* expression was found to be significantly different when no treatment (PBS control) was compared to treatment with sEVs acquired pre-exercise (F=4.56, *p*=0.048), where non-pregnant pre-exercise sEVs generated a higher expression of *PLGF* compared to PBS control (*p*=0.037). (F) There were no differences in *PLGF* expression when BeWo cells were treated with sEVs post-exercise from both groups vs. PBS control (F=0.726, *p*=0.513). Gene expression data were normalized to expression levels of PBS controls. Data in (A&B) were analyzed using a two-way repeated measures ANOVA and shows only the mean, with each line connecting pre-post values for an individual participant. Data in (B&C) and (E&F) are shown as mean ± SD and were analyzed using a one-way ANOVA test. All experiments were conducted in triplicate, with sEVs obtained from N=3 non-pregnant and N=3 pregnant participants, with corresponding PBS controls (N=5). * = *p*<0.05. PBS Ctrl, phosphate-buffered saline control; Pre, pre-exercise; Preg, pregnant; Post, post-exercise; Non-preg, non-pregnant.

Human chorionic gonadotropin levels were not affected upon exposure to exercise-associated sEVs

The hormone b-hCG is known to be produced and released from BeWo cells *in vitro* in the process of syncytialization, an essential part of trophoblast differentiation and function [47, 48]. We examined the levels of b-hCG in cell media after incubation with plasma sEVs from non-pregnant and pregnant women, pre- and post- acute exercise. There were no main effects of exercise (F=0.125, p=0.742) or pregnancy status (F=1.16, p=0.342), nor a significant interaction between exercise and pregnancy status (F=0.070, p=0.804) (Fig. 5A), indicating that exposure of BeWos to exercise-associated sEVs for 24 h did not alter release of b-hCG. Furthermore, exposure to sEVs obtained pre- or post-exercise from either group did not result in differing levels of b-hCG in cell media when compared to no treatment (PBS control exposure) (F=0.600, p=0.572 and F=0.885, p=0.450, respectively; Fig. 5B&C).

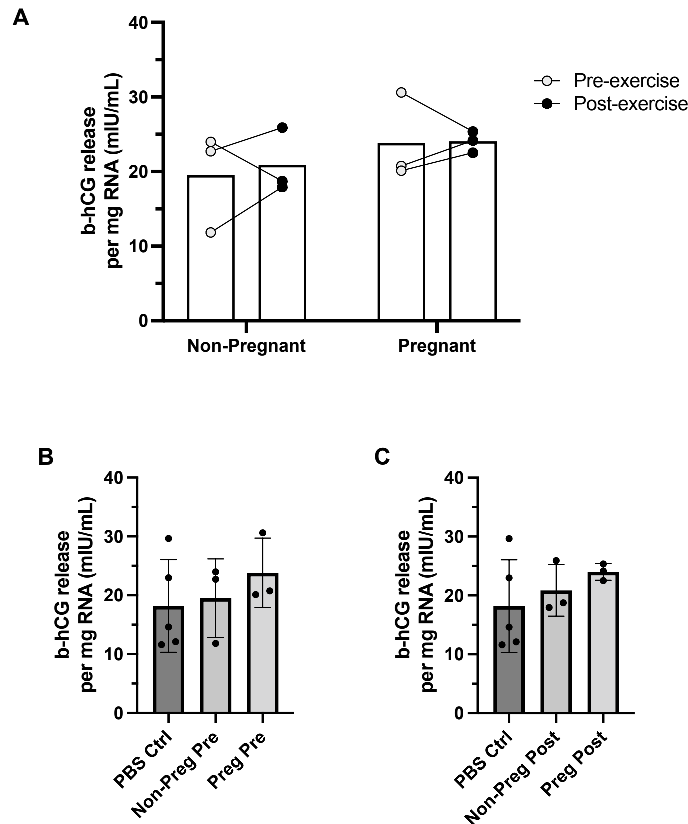

Fig. 5 Exercise-associated sEVs did not alter release of b-hCG by BeWo cells. b-hCG levels in cell media measured by ELISA after BeWo cells were exposed to plasma sEVs (10 µg/mL for 24 h) obtained from non-pregnant and pregnant participants both pre- and post-exercise showed no significant main effects of pregnancy status (F=1.16, *p*=0.342) or exercise (F=0.125, *p*=0.742), and no significant interaction between pregnancy status and exercise (F=0.070, *p*=0.804) (A). There were no differences in b-hCG cell media levels when no treatment ('PBS Ctrl') was compared to treatment with sEVs obtained pre- (F=0.600, *p*=0.572) or post-exercise (F=0.885, *p*=0.450) from non-pregnant and pregnant individuals (B&C). Data in (A) were analyzed using a two-way repeated measures ANOVA and displays only the mean, with each line connecting pre-post values for an individual participant. Data in (B) and (C) are shown as mean ± SD and were analyzed using an ANOVA test. All experiments were conducted in triplicate, with sEVs obtained from N=3 non-pregnant and N=3 pregnant participants, with corresponding PBS controls (N=5). PBS Ctrl, phosphate-buffered saline control; Pre, pre-exercise; Preg, pregnant; Post, post-exercise; Non-preg, non-pregnant.

Discussion

This study aimed to determine whether sEVs released after a single bout of acute moderate-intensity exercise in pregnant individuals and respective non-pregnant controls can be internalized by cells modeling placental trophoblasts *in vitro* and alter their function. We are the first to establish that sEVs released after exercise into circulation can interact with trophoblast *in vitro*,

but did not alter traditional indices of trophoblast biology including proliferation, gene expression of angiogenic markers, or production of the pregnancy hormone b-hCG. sEVs are postulated to be an important delivery mechanism in the adaptive response to exercise on a systemic level [18, 19, 49]. This study provides preliminary evidence that may facilitate our understanding of how exercise is communicated from the mother to the fetus during pregnancy via the maternal-fetal interface.

Central to the hypothesis that sEVs communicate the benefits of exercise systemically is their uptake into recipient tissues and cells, or surface interactions with activating receptors. Exercise-associated sEVs from both pregnant and non-pregnant women were localized within unsyncytialized BeWo cells. Few studies have evaluated whether sEVs released into circulation in response to exercise can be taken up by or interact with recipient cells. Notably, Whitham *et al.* (2018) showed that fluorescently-labeled EVs from mouse myoblasts could be localized within mouse liver hepatocytes and that cargo of plasma EVs from exercising mice incorporated into mouse liver hepatocytes [18]. In the context of exercise training and cardiomyocyte injury, PKH26 labeled-EVs isolated from the plasma of exercising rats were found to be internalized into cardiomyocytes *in vitro* after a 6-hour incubation period [50]. In a study conducted by Just *et al.* (2020), sEVs released after acute blood flow-restricted resistance exercise in healthy young men were localized within muscle stem cells and fibro-adipogenic progenitor cells *in vitro* [51]. Our proof-of-concept experiments show that exercise-associated sEVs can also interact with trophoblasts, specialized cells that are in direct contact with maternal blood and constitute the maternal-fetal interface. Since evidence suggests that EV uptake is not a passive process and involves a variety of energy-requiring endocytic pathways (reviewed by [52]), we postulate that

exercise-associated sEVs may play a role in modifying trophoblast function in the context of maternal exercise.

We observed differing localization of PKH26-labeled exercise-associated sEVs depending on whether BeWo cells were unsyncytialized in their native proliferative state representing villous cytotrophoblast, or induced to syncytialize and fuse to model syncytiotrophoblast. Labeled sEVs appeared to interact with unsyncytialized but not syncytialized BeWo cells. However, the sEV localization data in syncytialized cells must be interpreted with caution as we were not able to eliminate possible positive background staining in untreated controls, possibly due to treatment of BeWo cells with forskolin to induce syncytialization. The reason for the differences in sEV interaction between unsyncytialized and syncytialized cells is unknown, but could partially be explained by the permissibility of syncytiotrophoblast vs. cytotrophoblasts. Syncytiotrophoblast is a fully-differentiated, contiguous, multinucleated cell layer covering the surface of chorionic villi in direct contact with maternal blood. The syncytiotrophoblast layer functions as a structural and biochemical barrier between mother and fetus, while selectively taking up and transporting nutrients, hormones, growth factors, and cytokines from maternal blood [53]. The underlying proliferative cytotrophoblast layer acts as a reservoir for the overlying syncytiotrophoblast. Additionally, although many studies have reported the release of EVs from syncytiotrophoblast into maternal circulation (i.e., feto-maternal trafficking of EVs) [54-57], few have investigated the uptake of maternal sEVs into syncytiotrophoblast (i.e., materno-fetal trafficking of EVs). An elegant series of experiments conducted by Sheller-Miller *et al.* (2019) using a transgenic mouse model with a membrane-targeted red fluorescent protein and green fluorescent protein cyclic-recombinase reporter construct showed that maternal sEVs can indeed be trafficked across the placenta and produce functional changes in fetal tissues [58]. The molecular trafficking pathways

responsible for orchestrating the passage of maternal sEVs into syncytiotrophoblast and across the placental barrier are currently unknown. Moreover, modeling of syncytiotrophoblast *in vitro* poses significant challenges due to their terminally-differentiated nature and resulting limited lifespan [59, 60]. Further research is warranted to unravel the molecular mechanisms of exercise-associated sEV uptake and interaction in various trophoblast populations comprising the maternal-fetal interface.

Having observed that exercise-associated sEVs can interact with trophoblast-like cells *in vitro*, we sought to investigate their impact on trophoblast physiology. The secretion of b-hCG in cell culture media was investigated as a marker of BeWo cell and trophoblast differentiation. Impaired trophoblast differentiation and fusion are seen in pregnancy pathologies including pre-eclampsia [48], for which risk is mitigated by regular engagement in physical activity during pregnancy [2]. The release of b-hCG was not affected by exposure to exercise-associated sEVs from pregnant or non-pregnant individuals, nor was BeWo cell proliferation. Given that we previously reported differential expression of angiogenic growth factors VEGF, PlGF, and their respective receptors in term placenta of individuals categorized physically active or inactive [11], we aimed to determine whether gene expression of *VEGF* or *PLGF* were affected by exercise-associated sEVs. Regardless of pregnancy status, exposure to exercise-associated sEVs did not produce changes in *VEGF* or *PLGF* expression levels in BeWo cells. Interestingly, significant differences in *VEGF* or *PLGF* expression were seen when treatment with sEVs obtained at rest (i.e., 'pre-exercise') was compared to data from PBS controls. *VEGF* gene expression was significantly lower compared to PBS control exposure regardless of pregnancy status, while *PLGF* expression was significantly higher after exposure to sEVs obtained at rest from non-pregnant participants only. Trophoblasts produce VEGF and PlGF to promote branching and non-branching

placental angiogenesis, respectively [61, 62]. Our observations differ from data in human umbilical vein endothelial cells (HUVECs), where exposure of plasma sEVs isolated from pregnant and non-pregnant women at resting conditions was found to increase endothelial cell migration similarly to VEGF-induced migration [27]. It must be noted that the concentration of sEVs used by Salomon *et al.* (2014) was 10-fold higher than concentrations used to expose trophoblasts in our study (i.e., 100 µg/mL vs. 10 µg/mL EV protein, respectively), and did not examine gene expression of *VEGF*. Although the sample size used in our preliminary study was small (N=3 pregnant and N=3 non-pregnant, pre- and post-exercise), these data emphasize the importance of comparison to treatment controls to assess the full impact of sEVs in the context of exercise. It does not appear to be sufficient to only compare the treatment of pre- to post-exercise sEVs, appropriate treatment controls should always be included.

Relatively few studies to date have determined the physiological impact of exercise-associated sEVs on the biological functions of target cells, and none have been conducted on trophoblasts or cells constituting the maternal-fetal interface. The majority of work on the physiological consequences of exercise-associated sEVs involve animal models, where their interactions have been shown to delay prostate cancer progression [63], provide sustained cardioprotection [50], and elicit beneficial effects in ischemic stroke [64]. In humans, sEVs obtained after blood flow-restricted resistance exercise in healthy men were found to increase the proliferation of fibro-adipogenic progenitor cells [51]. The lack of biological impact on trophoblast biology demonstrated in the present study is likely due to a variety of factors. The bioactivity of sEVs is largely dependent on their diverse biological cargo, which is influenced by the cells of origin. Identifying the contents and origins of exercise-associated sEVs in pregnant and non-pregnant participants was beyond the scope of this preliminary study, but represents a critical

knowledge gap in this emerging field. Characterization of the biological contents and cellular origins of exercise-associated maternal sEVs will allow for the development of more targeted hypotheses regarding assessments of trophoblastic function. Exercise training status may influence the biological contents of exercise-associated sEVs, as Nair *et al.* (2020) reported that microRNA profiles differed in circulating sEVs obtained from sedentary vs. active older men [65]. In the present study, we were unable to objectively validate the habitual physical activity patterns of the study participants. Future studies should examine whether exercise training or chronic habitual exercise could alter the bioactivity of maternal sEVs, and evaluate their potential impacts on trophoblast. Currently, there is inconsistent evidence to suggest that exercise intensity or modality could affect sEV release and contents [19, 66-68]. It is unknown whether differing exercise intensities (i.e., moderate vs. vigorous-intensity) could impact sEV cargo, and whether a specific intensity or threshold is required to produce functional changes in trophoblasts.

Our study presents some strengths and limitations. As noted by others, caution must be exercised when extrapolating results obtained by transformed immortalized cell lines including BeWo choriocarcinoma cells in the modeling of normal trophoblast populations [29, 41, 69]. BeWo cells are readily available and accessible yet still provide invaluable insights into trophoblast function and syncytialization [29, 69, 70]. Future experiments deducing the potential function of sEVs in the context of maternal exercise should employ primary human trophoblasts or trophoblast populations obtained from the derivation and differentiation of trophoblast stem cells [71, 72]. A strength of the sEV localization analysis stems from the use of an Airyscan detector in conjunction with confocal microscopy allowing for enhanced detection not available in traditional confocal systems [73]. However, it is important to note that the confocal imaging studies presented here are qualitative in nature. Another strength is the duration and type of

exercise selected for the representation of acute exercise during pregnancy. The Canadian evidence-based physical activity guidelines recommend women engage in a minimum of 150 min of moderate-intensity physical activity to achieve benefits, within a recommended heart rate range based on age [1]. We therefore intentionally designed a physiologically-appropriate exercise session for pregnancy, when the majority of studies to date on exercise-associated sEVs involve sustained vigorous-intensity exercise until volitional exhaustion in men (reviewed by [19]). Low sample size was a major limitation of our study, but herein we provide pilot data to support continued exploration of maternal exercise-associated sEVs and their potential impact on placental function.

In summary, we demonstrated that exercise-associated sEVs could be localized within trophoblast-like cells *in vitro*. Whether they can produce biological changes sufficient to improve trophoblast biology or stimulate other intercellular signaling pathways that may transmit signals to the fetus remain unknown. Since the placenta is primarily responsible for maternal-fetal communication and fetal growth, further investigation is warranted to determine the biological impact and mechanisms linking maternal sEVs to the benefits sustained from engagement in exercise during pregnancy.

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Supplementary Material

Methods

HTR-8/SVneo cell culture

HTR-8/SVneo cells (herein referred to as 'HTR8') were purchased American Type Culture Collection (ATCC® CRL-3271™, Manassas, VA) and grown in RPMI-1640 medium (Gibco™, Thermo Fisher Scientific, #11875093, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C and 5% CO_2 in a humidified environment. For E-cadherin immunofluorescence experiments, HTR8 cells from passages 5-8 were seeded at a density of 7.5 $\times 10^5$ cells in a 6-well dish on square glass coverslips. Cells were grown for 48 h before E-cadherin immunofluorescence as described in the *'BeWo syncytialization'* methods section.

E-cadherin immunofluorescence image analysis workflow

The detailed image analysis workflow for comparison of E-cadherin immunofluorescence between forskolin-treated vs. vehicle control BeWo cells is described schematically in Figure S1. Fluorescence staining intensity of an entire field-of-view (FOV) for the E-cadherin channel image (far red filter; excitation 640/30nm, emission 690/50nm) was first quantified (Fig S1A). Then, total cell area was determined for an entire FOV for the corresponding phalloidin channel image (green filter; excitation 470/40nm, emission 525/50nm) guided by thresholding to delineate areas occupied by cells (Fig S1B) and create a binary mask allowing for particle measurement. A ratio of fluorescence intensity measurement (integrated density) from the E-cadherin channel image to the corresponding measured cellular area from the phalloidin channel image was calculated and used for statistics and plots. Before starting manual analysis of any images, the following measurement parameters ('Analyze>Set Measurements') were selected: Area, Standard deviation, Min & max gray value, Integrated density, Mean gray value.

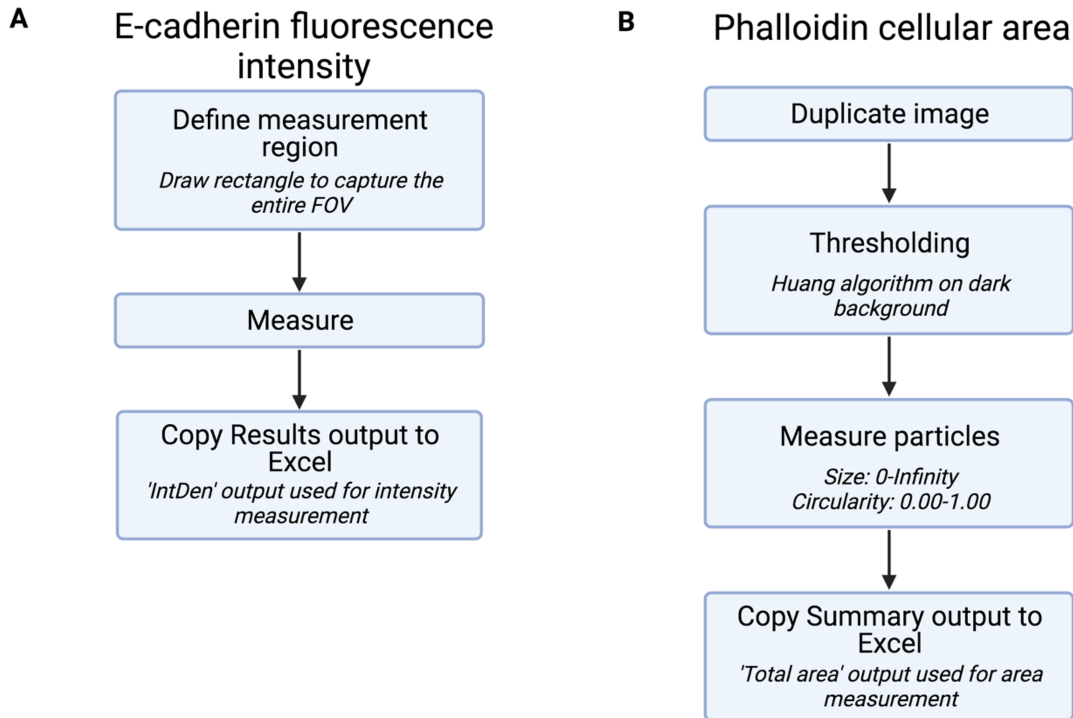

Figure S1 Overview of the E-cadherin immunofluorescence image analysis workflow in Fiji software to assess BeWo cell syncytialization. Flow chart diagrams show the step-by-step workflow to quantify (A) E-cadherin fluorescence intensity, and (B) total cellular area through phalloidin cytoskeletal staining using Fiji digital imaging software. Figure created using Biorender.com.

Ki67 proliferation immunofluorescence image analysis workflow

The step-by-step image analysis workflow for the estimation of BeWo cell proliferation upon exposure to exercise-associated sEVs from study participants is shown in Figure S2. Fluorescence staining intensity of an entire FOV for the Ki67 channel image (green filter; excitation 470/22nm, emission 510/42nm) was first determined (Fig S2A). Then, total nuclear area was quantified for an entire FOV for the corresponding dapi channel image (blue filter; excitation 357/44nm, emission 447/60nm) guided by thresholding to capture areas occupied by nuclei (Fig S2B) and create a binary mask. A ratio of fluorescence intensity measurement (integrated density) from the Ki67 channel image to the corresponding measured nuclear area from the dapi channel image was determined for statistical analysis and plots. At the start of manual image analysis, the following

measurement parameters ('Analyze>Set Measurements') were selected: Area, Standard deviation, Min & max gray value, Integrated density, Mean gray value.

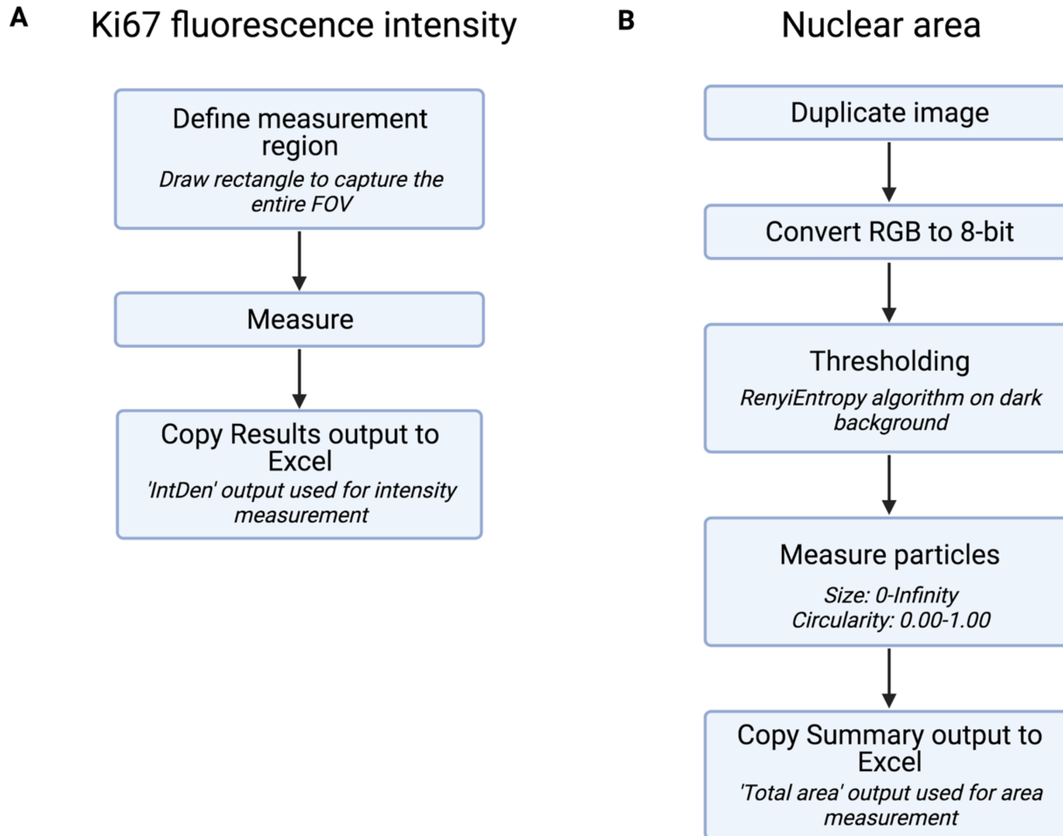

Figure S2 Overview of the Ki67 immunofluorescence image analysis workflow in Fiji software to assess cell proliferation. Flow chart diagrams show the step-by-step workflow to quantify (A) Ki67 fluorescence intensity, and (B) total nuclear area through dapi staining using Fiji digital imaging software. Figure created using Biorender.com.

Results

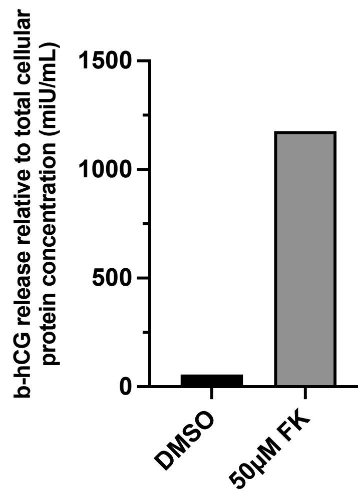

Figure S3 Validation of BeWo cell syncytialization by measurement of human chorionic gonadotropin (b-hCG) after forskolin exposure. b-hCG levels in cell culture media from BeWo cells exposed to 50 µM forskolin for 72 h were 20.8-fold higher than vehicle (DMSO) control. B-hCG levels in cell media were normalized to total cellular protein concentration from N=1 (descriptive data) experiments. DMSO, dimethylsulfoxide; FK, forskolin.

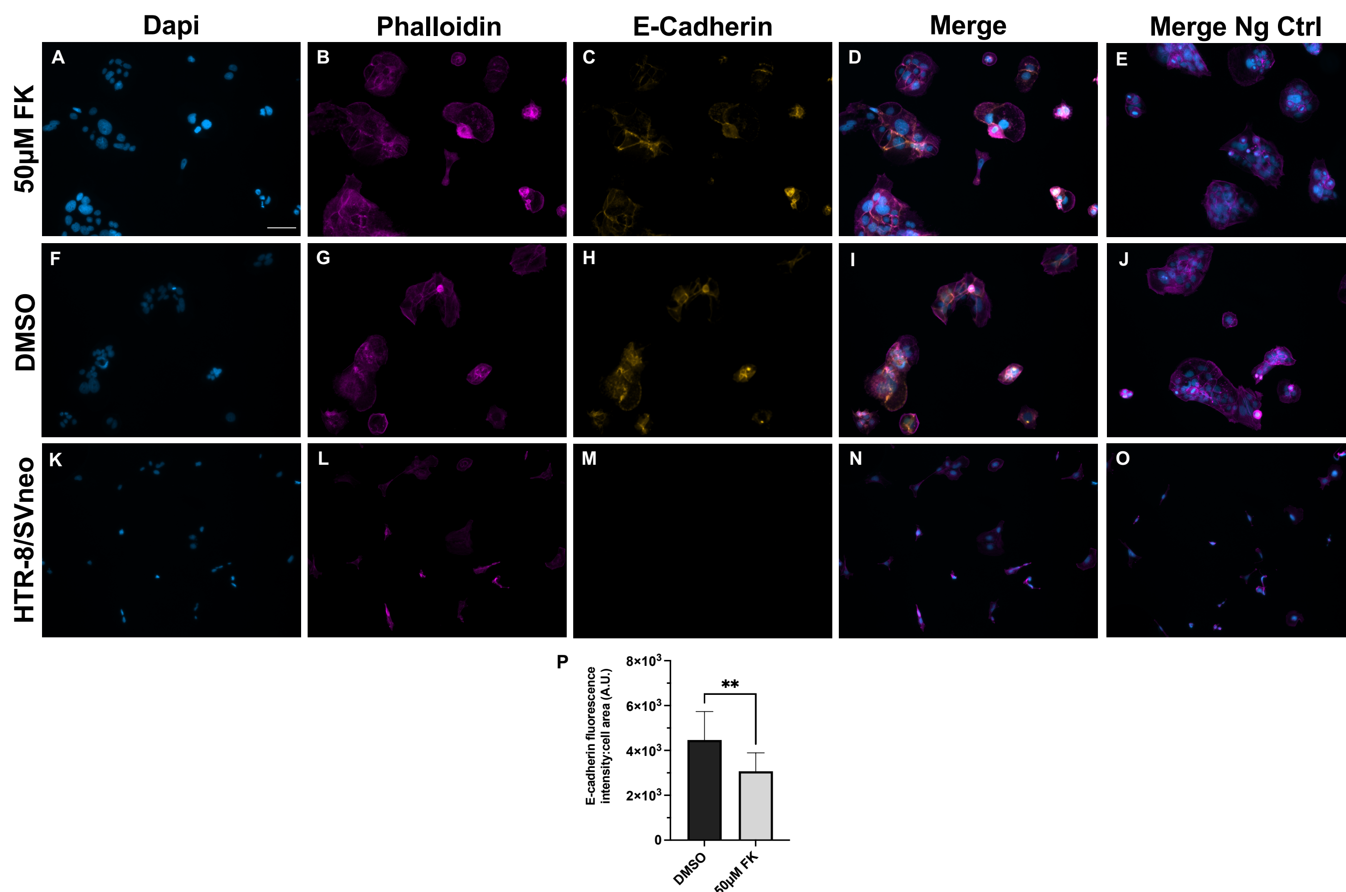

Figure S4 Decreased E-cadherin expression by immunofluorescence after BeWo cell syncytialization. BeWo choriocarcinoma cells were exposed to 50µM forksolin (FK) (A-E) or vehicle control DMSO (F-J) for 72 h to induce syncytialization, then probed for E-cadherin protein expression and localization by immunofluorescence. Blue shows nuclei stained with dapi, magenta represents phalloidin staining of cytoskeleton to delineate cellular area, while yellow displays E-cadherin expression. E-cadherin staining appeared absent from cell borders of cells appearing to undergo syncytialization. There was a significant reduction in E-cadherin immunofluorescence in cells exposed to 50µM forskolin vs. DMSO vehicle control (P, p=0.0090) as determined by fluorescence intensity pixel quantification relative to cell area using Fiji software and an unpaired t-test. HTR-8/SVneo cells do not express E-cadherin and thus were used as a negative cellular control (K-O), where no positive staining was observed for E-cadherin (M). Panels E, J, and O are representative negative antibody controls where primary antibody (anti-E-cadherin) was omitted. For display purposes only, the phalloidin channel images' lookup table (LUT) was set to 'Magenta', while the LUT for the E-cadherin channel images was set to 'Yellow Hot' using Fiji software. Scale bar in panel A = 100 µM for all images. A.U., arbitrary units; DMSO, dimethylsulfoxide; FK, forskolin; Ng Ctrl, negative control.

CHAPTER 4

Project 3 manuscript: Assessment of fetoplacental vascular alterations associated with prospective objectively-measured physical activity in pregnancy

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Contributions: SM and KBA generated the original idea for the manuscript. Placenta sampling was performed by SM and JB. SM designed and performed all histological and immunohistochemical experiments. SM also performed all data analysis and drafted the manuscript. SM, JB, VT, and KBA interpreted the results and critically revised the manuscript. KBA applied for an was granted ethical approval for the PLACENTA study, along with funding support from CIHR. All authors have approved the submitted version of the manuscript.

Abstract

Introduction: Proper placental vascularization is essential for fetal growth and survival. Physical activity has been shown to influence placental villous volumes and the expression of angiogenic growth factors in placental tissues. Studies examining placental vascularization and maternal

physical activity have used subjective measures rather than objective measures of free-living physical activity at differing intensities.

Methods: We compared fetoplacental vascular densities of individuals defined as 'active' (N=5) or 'inactive' (N=5) based on meeting recommendations for physical activity during pregnancy (≥150 min moderate or greater intensity physical activity per week). Accelerometry was used to quantify physical activity volume and intensity prospectively during the 2nd and 3rd trimesters (24-28 and 34-38 weeks gestation, respectively) and compared to fetoplacental vascular densities in CD34-stained tissue sections obtained by histopathology.

Results: When comparing active vs. inactive groups, no differences in fetoplacental vascular density were observed (31.40 ± 4.04% vs. 29.85 ± 6.45%, respectively; p=0.662). Further, no associations were found between 2nd trimester levels of moderate-to-vigorous intensity physical activity and fetoplacental vascular density (r=0.065). Correlations of moderate strength were noted between 2nd trimester sedentary time and fetoplacental vascular density, and 3rd trimester light physical activity and steps per day and fetoplacental vascular density, but did not reach statistical significance.

Discussion: Fetoplacental vascularity did not differ in those meeting physical activity guidelines, and there was no dose-response relationship between increasing moderate-to-vigorous intensity physical activity and placental vascularity when measured by histopathology at term. Further inquiry is required to reveal the effect of varying intensities of physical activity and sedentary behaviours on fetoplacental vascular density.

Keywords: Exercise, physical activity, placenta, pregnancy, vascularization

Introduction

During pregnancy, sufficient placental vascular development of the juxtaposed uteroplacental and fetoplacental circulatory systems is essential for the transfer of nutrients and gases between mother and fetus, and thereby crucial to fetal growth and survival [1]. Consequences of abnormal placental vascular development are associated with various pregnancy complications including fetal growth restriction (FGR) and preeclampsia [2-5]. Moreover, improper establishment of the placental vasculature is associated with increased future risk of cardiovascular disease later on in adult life [6]. Therefore, understanding strategies and maternal behaviours that support placental vascular development have the potential to optimize maternal-fetal health during pregnancy and across the lifespan.

Engagement in physical activity during pregnancy is a behaviour found to impact placental angiogenesis [7] and vasculature [8-11]. We previously reported that the expression of angiogenic growth factors VEGF and PlGF, and their associated receptors were differentially expressed in term placentas between physically active and inactive individuals during pregnancy [7]. Placental vascularization findings from observational studies and randomized controlled trials involving maternal physical activity are inconsistent. These discrepancies likely stem from differences in physical activity measurements (i.e., self-report or questionnaires) [9, 12], timing of physical activity during gestation (i.e., early vs. mid- vs. late-pregnancy vs. throughout pregnancy) [8-12], and duration, volume or intensity of physical activity (i.e., 20 vs. 60 min aerobic activity sessions; light vs. moderate vs. vigorous) [8, 9, 11]. Jackson and colleagues (1995) reported increased vascular placental volumes in those who exercised in early and mid-pregnancy, but did not result in significant changes at the level of the terminal villi [8], where the majority of maternal-fetal exchange occurs. A study conducted by Bergmann *et al.* (2004) involving sustained weight-

bearing exercise throughout pregnancy found that runners had greater villous vascular volumes at term compared to physically active controls who engaged in low-intensity recreational physical activity only [9]. Despite these results, few studies to date have examined free-living maternal physical activity in an objective, prospective manner, where intensities are captured using accelerometry to define physical activity patterns across pregnancy. Previous work from our lab demonstrated that self-reported physical activity was grossly overestimated compared to objective accelerometry measurement [13]. It is unknown how differing intensities of physical activity throughout pregnancy can influence placental vascular development.

The purpose of this study was to examine the morphology of the fetoplacental vasculature at term and determine whether objectively measured free-living physical activity was associated with alterations in fetoplacental vascularity at the level of the terminal villi. We hypothesized that those defined as physically active during pregnancy would display greater fetoplacental vascular densities compared to their physically inactive counterparts, and that there would be a dose-response relationship between greater intensities of physical activity and fetoplacental vascular density. To test this hypothesis, physical activity was measured objectively in a cohort of healthy women without contraindications to exercise as part of a prospective, longitudinal observational study with collection of term placenta samples for histomorphometric examination.

Methods

Study participant recruitment

Healthy pregnant people from the Ottawa region (Ontario, Canada) were recruited for participation in the PhysicaL ACtivity and diEtary implicatioNs Throughout pregnAncy (PLACENTA) research study. The PLACENTA study protocols were approved by the Ottawa Health Science Network Research Ethics Board (OHSN-REB; protocol number: 20160178-01H)

and the University of Ottawa Research Ethics Board (file number: H-11-15-29). All procedures were conducted in accordance with the ethical standards outlined in the Declaration of Helsinki. Informed written consent was obtained from all participants after explanation of study protocols. Study inclusion and exclusion criteria are described in Table 1.

Table 1. Study participant inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
• Maternal age of 18-40 years old • Self-reported pre-pregnancy body mass index (BMI) of 18.5-29.9 kg/m^2 (lean/overweight classification) • Weight stable (± 5 kg) at least six months before pregnancy • No contraindications to exercise during pregnancy • Singleton pregnancy	• Pre-pregnancy diabetes managed by insulin. • Untreated thyroid disease • Pre-pregnancy hypertension requiring medication • Diagnosis of hypertensive diseases or FGR • Unable to communicate in English or French. • Planning to have the infant adopted

Objective physical activity measurements and anthropometrics

Objective physical activity measurements were obtained during the 2nd (24-28 weeks of gestation) and 3rd (34-38 weeks of gestation) trimesters by accelerometry. Participants were instructed to wear an omnidirectional Actical® accelerometer (Phillips Respironics, OR, USA) on the right hip during all waking hours over seven days to measure free-living physical activity. Data were considered to be valid when worn for a minimum of 10 h per day, with three valid days of wear (weekdays or weekends) for data analysis per measurement period [14]. Data were downloaded and then analyzed using SAS software (version 9.4, SAS Institute, NC, USA) following the protocols described by the Canadian Health Measures Survey [15]. The cutoffs for the accelerometer counts specifying each range of physical activity intensity were as follows: <100 counts per minute (cpm) for sedentary time, 100 to <1535 cpm for light-intensity physical activity (LPA), 1535 to <3962 cpm for moderate-intensity physical activity (MPA), and ≥3962 cpm for

vigorous-intensity physical activity (VPA) [16]. Physical activity classification was based on meeting recommendations outlined in the evidence-based *Canadian Guideline for physical activity throughout pregnancy* of at least 150 min of MPA or greater (moderate-to-vigorous intensity physical activity; MVPA) per week (or 21.4 min per day) [17]. Participants were categorized 'active' if they met or exceeded an average of 21.4 min of MVPA per day during the 2nd trimester assessment period. During the same physical activity assessment periods, dietary intake data was collected using the Automated Self-Administered 24-h (ASA24) Dietary Assessment Tool (version ASA24-Canada-2018, National Cancer Institute, MD, USA) [18]. A minimum of 2 weekdays and 1 weekend day were required for valid dietary data analysis. Approximately 24-48 h after birth, offspring anthropometric assessments, including weight, height, and skinfold thickness measurements were carried out following standard protocols [19, 20]. Skinfold thickness measurements were used to estimate offspring body fat percentage as described by Schmelzle and Fusch, 2002 [20]. Fetal:placental birth weight ratio was calculated by dividing the recorded offspring weight by the trimmed placental weight (described below). Offspring birth weight percentiles were determined by comparing measured offspring weight and offspring sex to Canadian means and standard deviations for birth weight [21]. Finally, offspring weight-for-length z-scores were calculated using sex, birth weight and birth length and standard protocols outlined by the World Health Organization [22].

Placental sampling and histological processing

Placental sampling was performed as described previously [7]. Term placental tissues were collected immediately after vaginal birth or caesarian section. Trimmed placental weight was recorded after removal of the umbilical cord and fetal membranes. Two full-thickness biopsies were collected from the periumbilical region, at a distance of approximately 2-3 cm away from the

umbilical cord insertion site. Tissues were washed in phosphate buffered saline (PBS) then placed in histological cassettes for fixation in 10% buffered formalin for 48 h at room temperature. Tissues were rinsed three times with PBS, then placed in 70% ethanol until processing for wax embedding using standard methods at the Louise Pelletier Histology Core (University of Ottawa, ON, Canada). Paraffin-embedded full-thickness placental tissues were cut at a thickness of 5 µm and mounted on Superfrost Plus slides (#12-550-15, Fisher Scientific Company, ON, Canada). A total of five slides (1 full-thickness tissue section per slide) were prepared for each participant (N=5 active and N=5 inactive), cut at a distance of 50 µm between each slide.

Immunohistochemistry

Immunohistochemistry for the fetoplacental vasculature was achieved by staining for the endothelial cell marker CD34, as described previously [23]. Slides were deparaffinized using three changes of xylene and rehydrated using descending grades of ethanol (100%, 95%, and 70%) for 5 min each. Slides were then rinsed with ddH_2O after which sodium citrate (10 mM, pH 6.0) heat-induced antigen retrieval was performed to unmask epitopes for antibody binding. Slides were washed twice using tris-buffered saline with 0.05% TWEEN® 20 (TBST; #P1379, MilliporeSigma Canada Co, Oakville, ON, Canada), then blocked for endogenous peroxidase activity with 3% hydrogen peroxide (#H325-500, Fisher Scientific Company, ON, Canada) for 30 min at room temperature. After two washes with TBST, slides were incubated with 10% normal goat serum (#P8340, MilliporeSigma Canada Co, ON, Canada) for 60 min at room temperature to block protein. Slides were incubated with an anti-CD34 (QBEND/10) antibody (#MCA547GT, Bio-Rad Laboratories, ON, Canada) at a dilution of 1:1000 in TBST or TBST only (negative control, primary antibody omitted) overnight at 4°C. The following day, slides were washed three times with TBST and incubated with goat anti-mouse IgG (H + L)-HRP conjugated secondary

antibody (#1706516, Bio-rad Laboratories) at a dilution of 1:1000 for 60 min at room temperature. Slides were washed three times with TBST then exposed to diaminobenzidine (DAB) chromogen (#ab64238, Abcam Inc, ON, Canada) for 2 min at room temperature. The DAB reaction was stopped with ddH_2O, then slides were counterstained with Harris' hematoxylin (#26754, Electron Microscopy Sciences, PA, USA). Slides were dehydrated in ascending grades of ethanol (70%, 95%, and 100%), cleared using xylene, and mounted using Entellan™ mounting medium (#1079610100, MilliporeSigma Canada Co). Images were acquired using an AxioImager M2 upright microscope with an AxioCam mRc colour camera and Zen Blue software (version 2.3, Carl Zeiss Microscopy GmbH, Köln, Germany). First, areas containing terminal villi were identified and then three images were captured at random per slide at 20X magnification (15 images per participant, therefore, 150 images total for analysis). Fetoplacental vascular density was quantified using the point counting technique described by Junaid *et al.* 2014 [23] with some modifications and Fiji image processing software (version 2.3.0/1.53f) [24], as shown in Figure 1. The modifications are as follows. A 108-point square grid was applied to each blinded image, and at each intersection (i.e., grid point), the following were counted separately using Fiji's 'Cell Counter' plugin: (1) Villous profiles (i.e., tissue positive for hematoxylin (blue) including surrounding stroma with or without vessels), and (2) Vessel profiles (i.e., brown CD34 positive endothelial cells including vessel lumen). Fetoplacental vascular density was determined by the following equation:

Fetoplacental vascular density (%) = (number of vessel profile hits/number of villous profile hits + number of vascular profile hits) x 100

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 9.3.1, GraphPad Software, La Jolla, CA, USA). All data are presented as mean ± standard deviation (SD). Normality was assessed using the Shapiro-Wilk test. Study participant demographics were compared using the parametric independent *t*-test or the non-parametric Mann-Whitney test, where appropriate. A paired t-test was used to compare MVPA levels between the 2nd and 3rd trimester. Pearson's correlation was used to evaluate the association between physical activity variables and fetoplacental vascular density. Strength of the correlations were classified according to the thresholds proposed by Hopkins *et al.* (2009), as trivial (<0.1), small (0.1 to <0.3), moderate (0.3 to <0.5), large (0.5 to <0.7), very large (0.7 to <0.9), and almost perfect (≥0.9) [25]. Statistical significance was considered when $p<0.05$.

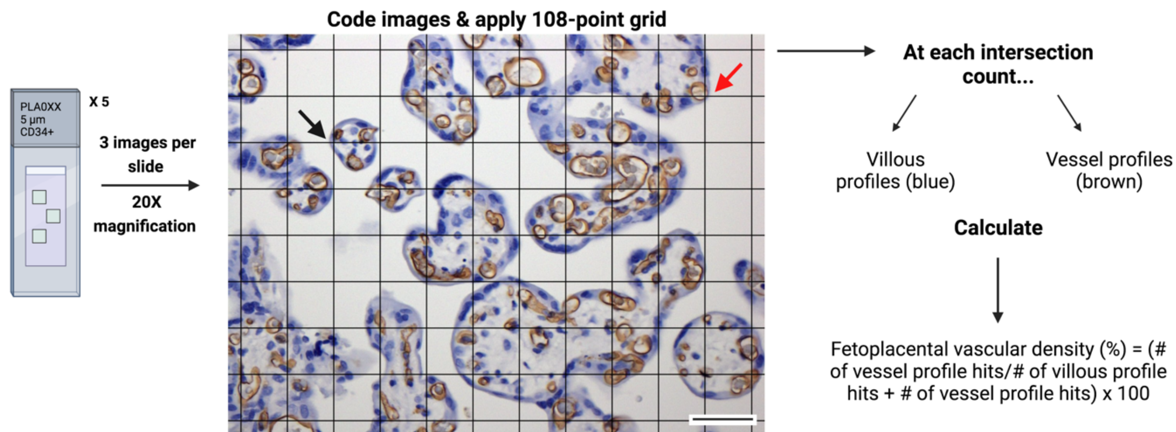

Figure 1. Schematic diagram describing immunohistochemical quantification of fetoplacental vascular density. Five full-thickness placental tissue slides per participant ('PLA0XX', N=5 active and N=5 inactive) were cut and stained by immunohistochemistry for CD34. Three images per slide (represented as boxes on the full-thickness tissue section) were captured by light microscopy with a 20X objective lens and coded for blinded unbiased analysis. Using Fiji software, a 108-point grid was applied to each image and at each grid point intersection, villous (blue; black arrow) or vessel (brown; red arrow) profiles were counted. Scale bar = 50 µm. Created with Biorender.com.

Results

All study participant demographics and clinical characteristics are described in Table 2. By design, those who met physical activity recommendations (i.e., 21.4 min MPA or greater/day) during the 2nd trimester had significantly higher levels of MVPA (p=0.0005), MPA (p=0.0005), and VPA (p=0.048) when compared to those who did not meet physical activity guidelines and were classified as 'inactive' during pregnancy. LPA, sedentary time, and steps per day were not significantly different between active compared to inactive groups. In the 3rd trimester, those defined as active displayed higher levels of MVPA when compared to the inactive group (p=0.031), but did not differ significantly with-respect-to other physical activity measures. MVPA levels decreased on average in all participants as pregnancy progressed, albeit not reaching significance (p=0.0502; Figure S1). Of those who were classified as 'active' during the 2nd trimester (N=5), N=4 data points were available for analysis in the 3rd trimester, and N=3 obtained ≥21.4 min of MVPA and thus met physical activity recommendations across both assessment periods. Offspring birth weight and birth weight percentile were noted to be significantly lower in the active vs. the inactive group (p=0.010 and p=0.0089, respectively). All other variables and clinical metrics did not differ significantly between groups.

Fetoplacental vascular density was not found to be significantly different between those defined as active vs. inactive during pregnancy (31.40 ± 4.04% vs. 29.85 ± 6.45%, p=0.662; Figure 2A). Further, there was no correlation between 2nd trimester MVPA and fetoplacental vascular density (r=0.065, p=0.859; Figure 2B). Although statistical significance was not achieved for any correlations between physical activity measures captured in the 2nd and 3rd trimesters and fetoplacental vascular density, some moderate strength correlations were noted (Table 3). Interestingly, 2nd trimester sedentary time was negatively correlated with fetoplacental vascular

density (r=-0.433; Figure 3), whereas in the 3rd trimester, LPA (r=0.376) and steps per day (r=0.318) were found to be positively correlated with fetoplacental vascular density.

Table 2. Study participant demographics and clinical characteristics.

	Active (N=5)	**Inactive (N=5)**	***p*-value**
Maternal age (years)	30.2 ± 1.6	32.4 ± 4.5	0.335
Height (cm)	166.8 ± 2.3	166.3 ± 5.0	0.843
Self-reported pre-pregnancy weight (kg)	69.8 ± 10.6	66.1 ± 8.4	0.554
Self-reported pre-pregnancy BMI (kg/m^2)	25.2 ± 3.8	24.2 ± 3.0	0.648
2nd trimester			
MVPA (min/day)	48.92 ± 15.74***	8.94 ± 3.03	0.0005
MPA (min/day)	45.14 ± 14.21***	8.92 ± 3.05	0.0005
VPA (min/day)	3.78 ± 4.75*	0.02 ± 0.04	0.048
LPA (min/day)	147.36 ± 44.34	218.54 ± 69.76	0.090
Sedentary time (min/day)	580.65 ± 59.33	548.80 ± 65.10	0.442
Steps per day	9584 ± 3410	5906 ± 1530	0.059
Total caloric intake (kcal/day) [#]	2570.5 ± 683.4	2284.0 ± 562.2	0.522
3rd trimester			
MVPA (min/day) [†]	34.53 ± 18.13*	6.55 ± 8.13	0.031
MPA (min/day) [†]	25.33 ± 13.30	6.45 ± 7.93	0.051
VPA (min/day) [†]	9.20 ± 18.27	0.10 ± 0.20	0.714
LPA (min/day) [†]	139.39 ± 28.43	199.25 ± 77.34	0.197
Sedentary time (min/day) [†]	581.88 ± 47.35	571.33 ± 51.87	0.774
Steps per day [†]	6609 ± 2395	5665 ± 2377	0.596
Total caloric intake (kcal/day) [‡]	3127.2 ± 402.0	2291.4 ± 655.5	0.062
Birth			
Gestational age at birth (weeks)	40.7 ± 0.8	40.7 ± 1.0	0.974
Delivery mode	V: 3 CS: 2	V: 3 CS: 2	-
Placental weight (g)	456.8 ± 108.7	580.4 ± 90.1	0.086
Offspring sex	F: 3 M: 2	F: 3 M: 2	-
Offspring birth weight (g)	3200 ± 283.8*	3698 ± 174.3	0.010
Offspring birth length (cm)	49.1 ± 3.0	51.9 ± 1.3	0.093
Fetal:placental weight ratio (g/g)	7.25 ± 1.3	6.52 ± 1.3	0.403
Offspring birth weight percentile	20.6 ± 11.0**	57.6 ± 21.4	0.0089
Offspring body fat percentage (%)	15.9 ± 2.8	18.4 ± 4.6	0.338
Offspring weight-for-length z-score	0.34 ± 1.4	-0.14 ± 1.2	0.571

Data are shown as mean ± SD. [#]N=4 inactive and N=5 active for 2nd trimester dietary intake data. [†]N=4 inactive and N=4 active for 3rd trimester physical activity data. [‡]N=5 inactive and N=4 active for 3rd trimester dietary intake data. *$p<0.05$, **$p<0.01$, ***$p<0.001$. BMI, body mass index; CS, caesarean section; F, female; M, male; LPA, light intensity physical activity; MPA, moderate-intensity physical activity; MVPA, moderate-to-vigorous-intensity physical activity; TPA, total physical activity; VPA, vigorous-intensity physical activity; V, vaginal.

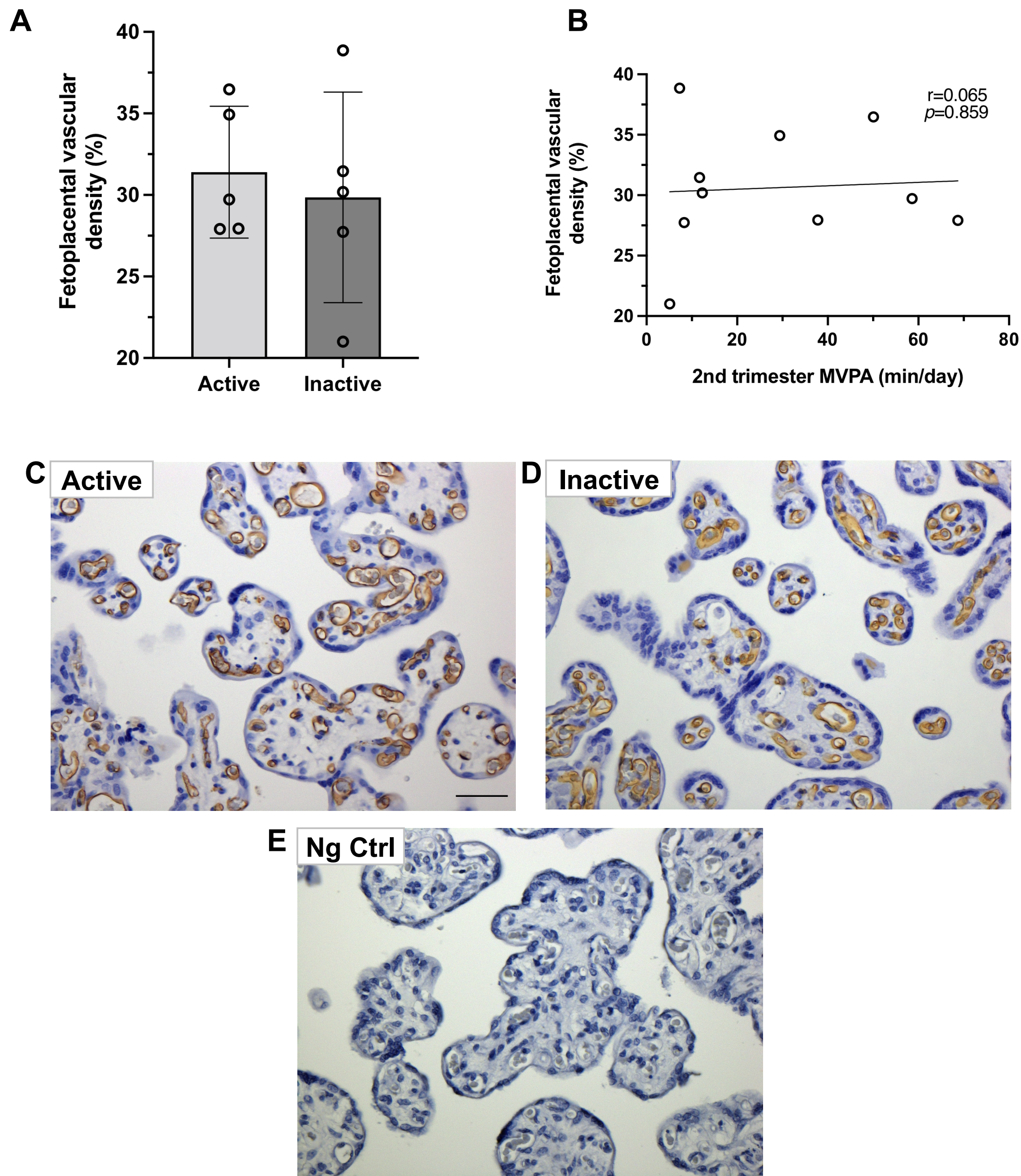

Figure 2. Physical activity in the 2nd trimester was not associated with alterations in fetoplacental vascular density at term. (A) Fetoplacental vascular density was not different in those categorized as active vs. inactive during the 2nd trimester (*p*=0.662). Each point represents fetoplacental vascular density from an individual study participant (N=5 active, N=5 inactive), plotted as mean ± SD. (B) There was no correlation between minutes of moderate-to-vigorous intensity physical activity (MVPA) and fetoplacental vascular density. Each point represents the average from all images from an individual study participant. Panels (C) and (D) show representative CD34 immunohistochemistry images (20X magnification) from each group, where brown delineates positive staining for fetal endothelium and blue shows counterstaining with hematoxylin. Panel (E) shows a representative negative control where the primary antibody was omitted. Scale bar = 50 µm for all images.

Table 3. Correlations between objective physical activity measures and fetoplacental vascular density.

Variable	r-value	*p*-value
2nd trimester (n=10)		
MVPA (min/day)	0.065	0.859
MPA (min/day)	0.098	0.788
VPA (min/day)	-0.151	0.676
LPA (min/day)	0.045	0.903
Sedentary time (min/day)	-0.433	0.211
Steps per day	-0.288	0.420
3rd trimester (n=8)		
MVPA (min/day)	0.023	0.957
MPA (min/day)	0.106	0.803
VPA (min/day)	-0.081	0.847
LPA (min/day)	0.376	0.359
Sedentary time (min/day)	-0.033	0.938
Steps per day	0.318	0.443

All r-values presented are Pearson's r.

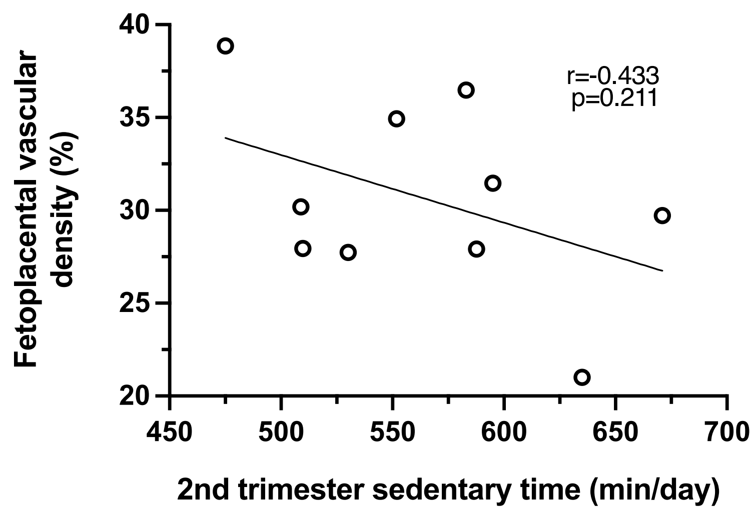

Figure 3. Sedentary time in the 2nd trimester was negatively associated with fetoplacental vascular density at term. Although not reaching statistical significance, 2nd trimester sedentary time was negatively correlated at moderate strength with fetoplacental vascular density at term. Each point represents the average from all images from an individual study participant.

Discussion

There is a paucity of studies describing fetoplacental vasculature in healthy pregnancies, with nearly all work focused on placental pathologies including FGR, gestational hypertension, and preeclampsia. Here we describe the first report involving the use of prospective, objective

measurement of free-living physical activity in a cohort of healthy pregnancies and their effect on fetoplacental vascular density at term. In individuals classified as active or inactive during pregnancy, no differences were noted in fetoplacental vascular densities using established point-counting techniques to estimate placental vascularization [9, 23]. Although we did not observe any associations between physical activity of moderate or greater intensity and fetoplacental vascular density, we identified a few interesting associations with lower-intensity physical activity (i.e., LPA) and sedentary time that require validation in a greater number of healthy pregnant individuals.

The evidence-based *Canadian physical activity guidelines throughout pregnancy* [17] and its associated systematic reviews identified a dose-response relationship between increasing intensities of physical activity and decreasing odds of gestational diabetes, gestational hypertension, preeclampsia, and a reduction in circulating maternal blood glucose and maternal depressive symptoms [26-31]. Furthermore, accumulating more physical activity (i.e., duration, frequency, or volume) was associated with more significant benefits [17]. Therefore, we hypothesized that those meeting or exceeding 150 min of moderate-intensity physical activity or greater would have greater fetoplacental vascular densities compared to those who did not meet physical activity recommendations. In this cohort, we did not detect differences between individuals classified as active vs. inactive, nor find an association between moderate-intensity physical activity or greater and fetoplacental vascular density at term. A total of four studies measuring fetoplacental vascular densities or villous volumes in the context of maternal physical activity have reported varying results, none of which measured free-living physical activity and all but one used objective measures of physical activity. Jackson and colleagues (1995) were the first to establish that fit women who continued their periconceptual levels of sustained moderate to

high-intensity exercise during pregnancy had greater placental villous volumes when compared to non-exercising control pregnancies, based on self-reported physical activity data [8]. Significant differences were limited to the larger stem villi at the most proximal level of the villous trees and not the terminal villi responsible for maternal-fetal exchange [8]. A follow-up randomized control trial by the same group involving aerobic moderate-intensity physical activity beginning in early pregnancy reported significantly greater terminal villous volumes in subjects enrolled in the exercise vs. the control group [11]. The exercise intervention consisted of moderate-intensity aerobic exercise for 20 min per session 3-5 times per week (therefore, 60-100 min per week) from week 8 of gestation until term delivery, whereas the control group did not perform any recreational weight-bearing exercise [11]. The authors postulated that the differing placental morphometric findings were likely related to a lower volume and intensity of exercise across pregnancy. To study the effect of both physical activity volume and timing during pregnancy, Clapp and colleagues (2002) conducted a randomized controlled trial where participants were randomized to three groups at week 8 of gestation: (1) 'Lo-Hi' (low volume of physical activity in early pregnancy then increasing to high volume from 24 weeks to term), (2) 'Mod-Mod' (consistent moderate volume across pregnancy), and (3) 'Hi-Lo' (high volume early in pregnancy then decreasing to low volume from 24 weeks until term) [10]. Those in the 'Hi-Lo' intervention group had the greatest terminal villous volume of the three groups, indicating that a reduction in the volume of physical activity in the third trimester enhanced fetoplacental growth [10]. Although exercise was quantified objectively via monitored exercise sessions, a control 'non-exercising' group was not included for examination. Nonetheless, the findings highlighted critical windows of placental growth in early pregnancy. Finally, an observational cohort study involving higher volumes of sustained weight-bearing physical activity reported greater villous vascular density in the placenta

of runners compared to healthy controls [9]. Runners (N=11) ran ≥4 times per week for 40-60 min per session (therefore, ≥160-240 min per week) at 55-65% of their periconceptual maximal aerobic capacity, while the controls (N=11) engaged in recreational physical activity (i.e., golf, gardening, hiking), as determined from self-reported physical activity logs [9]. A significant gap in the physical activity and placenta literature to date is the use of objective measurement to ensure unbiased quantification of physical activity in terms of volumes, frequencies, and intensities. Although we objectively measured physical activity in the 2nd and 3rd trimesters, future examinations should include assessments earlier in pregnancy (i.e., <24 weeks gestation) when the placental vasculature is first being established.

Quantification of differing intensities and volumes of free-living physical activity across pregnancy facilitated exploratory analyses yet to be investigated in the context of maternal physical activity and placental vascularization. Preliminary analyses revealed unique relationships between 2nd trimester sedentary time and 3rd trimester light physical activity and steps per day with fetoplacental vascular density. Although these relationships did not reach statistical significance (likely due to low sample size), indices such as sedentary time and light physical activity should be considered in future examinations of maternal physical activity and its effect on the placenta. Indeed, evidence suggests that lower-intensity physical activity has been shown to impart benefits [17]. In the current study, those who did not meet physical activity recommendations still appeared to attain some level of physical activity, albeit at lower intensities. The relationships between varying intensities, frequencies, and volumes of exercise and their effect on placental vascularization in healthy pregnant women are likely complex and heterogeneous and therefore necessitate further study in a larger population.

The preliminary work shown here presents strengths and limitations. It is abundantly clear that objective measures of maternal physical activity should be used as our group previously reported that pregnant individuals overestimated MVPA by 16 h per week [13]. Enumeration of free-living physical activity by accelerometry has yet to be assessed in the context of placental vascularization, a major strength of our study. However, limitations of accelerometry using the hip-worn Actical® monitor include the inability to detect activities including bicycling, swimming, skating, and weight training [32, 33]. A major limitation of the current study is the small sample size included for the examination of physical activity and fetoplacental vascular density at term. As a result of the low sample size, we found differences in offspring birth weight and birth weight percentile between active vs. inactive individuals that have not been shown in larger, more representative populations of healthy pregnant individuals [7, 34]. There was no correlation between neonatal morphometric outcomes and fetoplacental vascular density (data not shown). Despite the low sample size, morphological examination of the placenta enables essential descriptive analysis of human pregnancy under a range of normal physiological variations including maternal physical activity. Immunohistochemical analysis of vasculature in term placental provides a snapshot within a defined period (term delivery in this case) when the placenta is available for histomorphometric study. Future studies ought to employ *in vitro* methods to study placental vasculogenesis and angiogenesis in the context of maternal exercise. Also, more detailed and systematic examinations of placental vasculature using machine learning and artificial intelligence can be leveraged to improve the sensitivity of such analyses, as has been done in the context of placental villous maturation [35]. The investigation of placental vascularization in a larger subset of physically active vs. inactive individuals during pregnancy is currently underway using machine learning and digital pathology methodologies.

In conclusion, we did not observe differences in the fetoplacental vascular densities of individuals meeting physical activity recommendations during pregnancy vs. those who did not. We also did not observe a dose-response relationship between physical activity of moderate or greater intensity physical activity and fetoplacental vascular density. Other indices such as light physical activity and sedentary time should be studied in relation to placental vascular development.

Contribution to Authorship

SM and KBA generated the original idea for the manuscript. Data collection was performed by SM and JB. SM performed data analysis and drafted the manuscript. SM, JB, VT, and KBA interpreted the results and critically revised the manuscript. All authors have reviewed the data, and take full responsibility for the accuracy and integrity of the data contained herein. All authors have approved the submitted version of the manuscript.

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Declaration of Competing Interests

The authors declare no competing interests.

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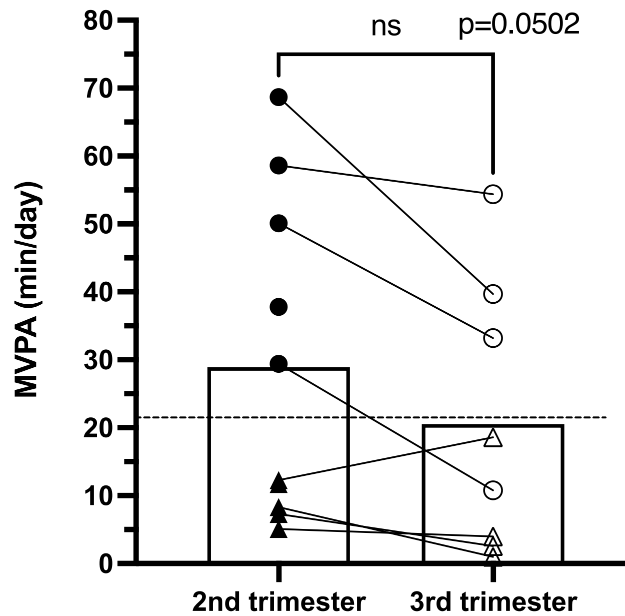
Supplementary Material



Supplementary Figure 1 MVPA levels decreased as pregnancy progressed. Circles represent those defined as ‘active’ while triangles represent those classified as ‘inactive’ based on 2nd trimester MVPA levels. Each data point represents a single individual, with a line connecting paired observations across the two assessment periods. N=10 and N=8 data points were available for analysis for the 2nd and 3rd trimester periods, respectively. Data were analyzed using a paired *t*-test and are shown as the mean only. The dotted line shows the threshold (≥21.4 min per day) for meeting physical activity guidelines. MVPA, moderate-to-vigorous intensity physical activity; ns, non-significant.

CHAPTER 5: DISCUSSION AND CONCLUSION

The purpose of the work presented in this thesis was to help uncover how the effects of PA are communicated between mother and fetus *via* the placenta, the most important mediator of fetal growth and development. Maternal PA has been touted as one of the most impressive interventions health care providers can recommend during pregnancy in its impact on a multitude of perinatal and maternal outcomes (Berghella and Saccone 2017). Indeed, expert opinion is strongly supported by the evidence-based PA guidelines (Mottola et al. 2018), yet little is known about the biological mechanisms that underlie the benefits of maternal PA. The gap in understanding the complex physiological processes surrounding PA and pregnancy is not surprising given the disparities in reproductive health research. Work by Mercuri and Cox (2021) identified that non-reproductive organs are researched 5-20 times more than reproductive organs, indicating a concerning knowledge gap in the field of reproductive sciences (Mercuri and Cox 2021). Furthermore, pregnant women are an underrepresented clinical population in research due to concerns regarding perceptions of safety for the mother and fetus (Heyrana et al. 2018). Research examining the physiological mechanisms linking health behaviours like PA and placental development, biology, and function are greatly needed for all the reasons noted above.

This thesis first determined whether sEVs, putative bioactive mediators posited to be released by various tissues and cells throughout the body in response to exercise, could be identified during pregnancy. In *Project 1*, circulating exercise-associated sEVs were assessed in pregnant women and their non-pregnant counterparts after an acute bout of moderate-intensity exercise. We designed the intervention to model an exercise modality and intensity recommended by the *2019 PA throughout pregnancy guidelines*, unlike most studies examining sEVs in exercise only in high-intensity endurance settings. We are the first to describe the presence of exercise-

associated sEVs within the circulation of pregnant and non-pregnant women and the first to report sEV release in response to moderate-intensity, non-exhaustive exercise. Although the proposed hypothesis was exploratory in nature, the results were still unexpected. The concentration of circulating sEVs increased immediately post-exercise in pregnant women compared to non-pregnant controls after a 30 min treadmill walk matched for intensity based on heart rate (Mohammad et al. 2021). It is difficult to compare these results considering the nature of other exercise interventions reported in the literature (i.e., high-intensity endurance exercise), but the decrease in sEV concentration observed after exercise in non-pregnant subjects is consistent with work presented by Rigamonti *et al.* (Rigamonti et al. 2020). The differences between the sEV response to exercise in pregnant vs. non-pregnant groups would likely be explained if the cellular origins were assessed. As posited in the discussion of *Project 1's* manuscript, it is plausible that the placenta may be releasing sEVs in response to exercise, adding to the circulating maternal sEV pool. Attempts were made to assess the placental contribution of circulating exercise-associated sEVs by western blotting using known membrane surface markers unique to placenta-derived sEVs [(i.e., syncytin-1 and placental-alkaline phosphatase (PLAP) (Dragovic et al. 2015; Salomon et al. 2017)]. However, the technique lacked the sensitivity to detect the presence of these markers reliably (data not shown). A significant obstacle in the advancement of sEV research is the paucity of techniques available to detect and profile the cellular origins of sEVs within complex biofluids like plasma (Veziroglu and Mias 2020). Conventional flow cytometry techniques used to identify placenta-derived sEVs are hindered by the submicron resolution of these particles, and thus, more technically-challenging techniques such as nano-scale high-resolution flow cytometry have been proposed (Morgan 2018). In the future, deep phenotyping of the cellular contributors to the

maternal sEV pool is warranted. The inclusion of a non-pregnant control group will be critical to deduce and compare the cellular origins in pregnant compared to non-pregnant states.

In a recent review highlighting investigative opportunities relating to placental sEVs, Sadovsky *et al.* propose that the field should move towards innovative mechanistic pursuits and away from weak biomarker screens (Sadovsky et al. 2020). *Project 2* presents an attempt to delve into the possibility of connecting exercise-associated sEVs mechanistically to the modulation of placental cell function. While we did not observe significant changes in traditional markers of trophoblast biology or gene expression of angiogenic growth factors known to be altered with habitual exercise during pregnancy, we were able to show that exercise-associated sEVs can interact and localize within trophoblast-like cells *in vitro*. Further investigation using primary human trophoblasts to generate syncytiotrophoblast *in vitro* is necessary to confirm whether exercise-associated sEVs interact with the cell layer in direct contact with maternal blood during pregnancy. Future work should also investigate the potential mechanisms of sEV interaction with trophoblasts using known inhibitors or antagonists of sEV internalization (Mulcahy et al. 2014), as flotillin-dependent endocytosis is less likely to be observed in syncytiotrophoblast compared to villous cytotrophoblast (Walton et al. 2013). As noted in *Project 2's* manuscript discussion, the potential functional impacts of exercise-associated sEVs on trophoblasts would be greatly aided by the phenotyping of their contents and cargoes. Indeed, work by Just *et al.* deftly illustrates the utility of evaluating the molecular contents found within exercise-associated sEVs to generate specific hypotheses on their functional roles within target cells (i.e., proliferation of muscle progenitor cells in this specific case) (Just et al. 2020). Characterization of the molecular contents of sEVs generated in *Project 1* was out of the scope of the work presented in this thesis.

Nonetheless, it is of the utmost importance to deeply phenotype the contents of exercise-associated sEVs in pregnancy to gain insight into their potential to affect placental physiology.

Although the concept of EVs is not a relatively new phenomenon, many aspects critical to EV function, including selective packaging of biological contents, the release of EVs into extracellular space, recognition by target or recipient cells, and delivery of biological cargo to recipient cells, remain elusive (Margolis and Sadovsky 2019; Sadovsky et al. 2020; Veziroglu and Mias 2020). In the context of maternal exercise and sEVs, this thesis has revealed the following unanswered questions, rife for further inquiry: (*1) Which tissues or cells participate in the liberation of sEVs during exercise, and is this different in pregnant vs. non-pregnant individuals? Does the source of sEVs differ depending on physical fitness, exercise intensity, duration, or type? (2) Do differing exercise modalities, intensities, or durations result in differing molecular contents? How does this differ between pregnant and non-pregnant individuals? (3) Does the placenta release sEVs in response to exercise? What are the kinetics of exercise-associated sEV released by the placenta, and is this affected by exercise intensity, duration, or modality? (4) How does the placenta recognize exercise-associated sEVs? Does this involve the interaction of specific cell surface molecules and receptors, and are these interactions influenced by placental developmental stage? (5) Are exercise-associated sEVs able to modify placental function, and if so, what are the potential mechanisms and signaling pathways involved? Finally, (6) are exercise-associated sEVs able to cross the placental barrier and produce meaningful functional changes in fetal tissues?* The work presented in this thesis provides evidence for further investigation of the role of exercise-associated sEVs in the maintenance of a healthy pregnancy.

It is also worth considering that the placenta is likely not the only target or recipient of exercise-associated sEVs during pregnancy. Indeed, many physiological changes are brought

about through participation in PA across pregnancy, including improvements in the autonomic nervous system, metabolism and glycaemic control, cardiovascular, and musculoskeletal systems (Davenport et al. 2018d; Dietz et al. 2016; Dubé et al. 2017; Ferraro et al. 2012; Reyes and Davenport 2018). It is possible that exercise-associated sEVs may elicit improvements in systems independent of the maternal-fetal interface. Hypotheses regarding potential organs and targets of exercise-associated sEVs could be bolstered using animal models to track their movement and fates *in vivo*. It is hoped that the work presented in this thesis will encourage more detailed examination of sEVs and their potential consequences in the field of maternal PA.

Results from *Project 3* were unexpected given the support for maternal PA of moderate- or greater intensity to positively influence placental vascularization when examining term tissues by histopathology (Bergmann et al. 2004; Clapp et al. 2000; Clapp et al. 2002; Jackson et al. 1995). A major strength of the study design in *Project 3* was capturing PA intensity using objective and prospective measurement *via* omni-directional accelerometry. A study conducted by Brett and colleagues in 2015 demonstrated that pregnant individuals overestimated their PA by over 16 hours per week when using the self-report Pregnancy PA Questionnaire compared to the concurrent measurement of free-living PA by accelerometry (Brett et al. 2015b). Upon comparing differing PA intensities and fetoplacental vascular density, we observed some unanticipated relationships surrounding lower intensity PA and sedentary time. Few studies exist assessing placental vascularization and sedentary time, and those few have focused on obesity during pregnancy (Son et al. 2019; Zafaranieh et al. 2021), rendering significant challenges when comparing to a ‘healthy’ weight population. Nonetheless, in a mouse model of high-fat, diet-induced obesity and maternal exercise, Son *et al.* reported reduced placental vascular density in obese dams stratified to the ‘no exercise’ group compared to those in the ‘exercise’ group (Son et al. 2019). Sedentary behaviours

during pregnancy have been associated with increased risk for macrosomic neonates, but no other deleterious maternal outcomes such as GDM (Fazzi et al. 2017). It is unclear whether the relationship between increasing fetoplacental vascularization and decreasing sedentary time has any impact on meaningful clinical outcomes. Thus, the results of *Project 3* need to be validated in a larger population of healthy pregnant individuals, including PA assessments before 24 weeks of pregnancy.

Histological examination of placental tissue post-delivery presents unique challenges. Since it is not possible to assess the structure of the placenta *in vivo*, we must rely on post-mortem examination by histological and molecular methods of samples presumed to represent the placenta as a whole. Post-mortem placental analysis is currently standard practice, but efforts are being made to develop new methods to assess the structure and function of the placenta in real time (Guttmacher et al. 2014). In addition to the application of non-invasive methodologies to visualize and estimate placental function *in vivo*, technologies such as deep learning and artificial intelligence are being leveraged to better quantitate and compare placental pathology from histological specimens. Work is currently underway with international collaborators to improve the accuracy and sensitivity of histopathological analyses of placental morphology in maternal PA using tissues from PLACENTA study participants. It is hoped that the combination of traditional placental histology with novel digital pathology techniques will reveal potential differences in placental structure, maturation, or health that may not be so readily discernable using currently available protocols.

In the study of maternal behaviours like PA in pregnancy, our work focuses on a population of 'healthy' women without contraindications to exercise. The majority of studies in the pregnancy literature compare a healthy or control population to diseased or abnormal states. Our studies

attempt to compare 'healthy' pregnancies to other 'healthy' pregnancies to detect noticeable differences in placental physiology in the absence of disease, a seemingly formidable challenge. This thesis aimed to define and describe behaviours that contribute to normal physiological limits that optimize and maintain maternal-fetal health during pregnancy and beyond.

In our laboratory's recent review of the impact of PA on structures of the maternal-fetal interface, we discuss the reigning hypotheses that are believed to contribute to PA-mediated changes in placental structure and function: (1) shear stress, (2) hypoxia-reoxygenation, and (3) the release of bioactive molecules into circulation (Bhattacharjee et al. 2021a). The original experiments proposed for this thesis included using *in vitro* systems to expose placental cells to conditions or stimuli likely experienced during exercise, a critical gap in the PA and placenta field. We proposed to expose placental cells to mechanical stretch and strain to evaluate sEV release and kinetics, but this was no longer feasible due to COVID-19 restrictions. We had also proposed studying the release of sEVs in hypoxia-reoxygenation to replicate the potential oscillations in oxygen tension likely experienced by trophoblast before, during, and after exercise. Although I could not conduct these experiments, the most critical next step would be to expose placental cells *in vitro* to stimuli modeling exercise (i.e., exposing trophoblasts to shear stress) to answer the fundamental question of how PA influences trophoblast biology at a mechanistic and molecular level.

In summary, this thesis demonstrates that pregnant women display greater levels of circulating sEVs after an acute bout of moderate-intensity exercise when compared to age- and BMI-matched non-pregnant controls (*Project 1*). These exercise-associated sEVs interact with trophoblast *in vitro*, with their physiological implications on placental function remaining to be fully understood (*Project 2*). Finally, our examination using IHC with traditional point-counting

techniques used in histopathology found that moderate-to-vigorous intensity PA does not appear to affect fetoplacental vascularization in a dose-response manner (*Project 3*). However, lighter intensities of PA and sedentary behaviours should be explored for their potential to impact the placental vasculature.

CHAPTER 6: REFERENCES FOR CHAPTERS 1 (INTRODUCTION) & 5 (DISCUSSION AND CONCLUSION)

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CHAPTER 7: APPENDICES

APPENDIX A: Ethics Approval for Myokine Study (Project 1 & 2)

23/08/2019

Université d'Ottawa
Bureau d'éthique et d'intégrité de la recherche

University of Ottawa
Office of Research Ethics and Integrity

CERTIFICAT D'APPROBATION ÉTHIQUE I CERTIFICATE OF ETHICS APPROVAL

Numéro du dossier / Ethics File Number	H-06-18-634
Titre du projet / Project Title	The potential role for exercise-induced myokines in the optimization of placental growth and function
Type de projet / Project Type	Thèse de maîtrise / Master's thesis
Statut du projet / Project Status	Renouvelé / Renewed
Date d'approbation (jj/mm/aaaa) / Approval Date (dd/mm/yyyy)	23/08/2019
Date d'expiration (jj/mm/aaaa) / Expiry Date (dd/mm/yyyy)	26/07/2020

Équipe de recherche / Research Team

Chercheur / Researcher	Affiliation	Role
Kelly Ann HUTCHINSON	École des sciences de l'activité physique / School of Human Kinetics	Chercheur Principal / Principal Investigator
Kristi ADAMO	École des sciences de l'activité physique / School of Human Kinetics	Superviseur / Supervisor
Shuhiba MOHAMMAD	École des sciences de l'activité physique / School of Human Kinetics	Étudiant-chercheur / Student-researcher
Nhung VUONG	École des sciences de l'activité physique / School of Human Kinetics	Coordonnateur de recherche / Research Coordinator

Conditions spéciales ou commentaires / Special conditions or comments

550, rue Cumberland, pièce 154
Ottawa (Ontario) K1N 6N5 Canada

550 Cumberland Street, Room 154
Ottawa, Ontario K1N 6N5 Canada

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www.recherche.uottawa.ca/deontologie I www.recherche.uottawa.ca/ethics

APPENDIX B: Myokine Study Informed Consent Form (Project 1 & 2)

Université d'Ottawa | University of Ottawa

PARTICIPANT INFORMED CONSENT

Faculté des sciences de la santé
Pavillon Roger-Guindon

Faculty of Health Sciences
Roger Guindon Hall

613-562-5800 (5603)
613-562-5437

sante.uOttawa.ca
health.uOttawa.ca

451 Smyth
Ottawa ON K1H 8M5
Canada

Title of Study: The potential role for exercise-induced myokines in the optimization of placenta growth and function

Researchers:

Kristi Adamo Ph. D.	Associate Professor, University of Ottawa
Nhung Vuong Ph. D.	Research Coordinator, Adamo Lab
Kelly Ann Hutchinson	Graduate Student, Adamo Lab

The Principal Investigator of this study is Kelly Ann Hutchinson, under supervision of Dr. Kristi Adamo. This research project is being conducted as part of Ms. Hutchinson's Master's thesis.

Funding Agency: Natural Sciences and Engineering Research Council (NSERC).

Participation in this study is voluntary. Please read this Participant Informed Consent Form carefully before you decide if you would like to participate. Please ask the Principal Investigator and the study team as many questions as you would like. We encourage you to discuss your options with family, friends or your health care provider.

Invitation to Participate:
You are being asked to participate in this research study because you are either a pregnant or a non-pregnant woman, are between 18 to 40 years old and have indicated that you are interested in learning more about our research study.

Purpose of the Study:
This study is designed to look at how physical activity during pregnancy can affect how nutrients move to the growing fetus. The placenta is an organ that forms during pregnancy to support the baby. The placenta controls the transfer of nutrients (sugar, protein and fat) and oxygen to the fetus, and removes waste. If the placenta is not working properly, the baby may receive too few or too many nutrients. It is not well known how physical activity affects the transfer of nutrients to the fetus.

The goal of this study is to observe whether myokines, which are peptide and cytokines, released from skeletal muscle when engaging in physical activity, affects certain measurements of placental growth and function. More specifically, we want to test whether myokines affect the ability of placental cells to survive and replicate in vitro (test-tube experiment), as well as whether they play a role in optimizing the

transfer of nutrients from the mother to the fetus. We hope that the information we learn in this study will be helpful in designing preventive health strategies for pregnancy.

We will be analyzing the pre- and post-exercise myokines in the blood samples collected. Further, we will be treating cultured placental cells with a synthetic mixture of myokines mimicking the profile detected in the blood samples. Placental metrics including cell proliferation, cell viability and nutrient transporter expression as well as function will be analyzed.

Participation:
If you consent to participate in this study, you will be asked to come the University of Ottawa-Lees Campus for one exercise session.

Exercise session details:
The visit will last 3 hours maximum

- We ask that you refrain from exercise 12 hours before the session.
- A small fasting (no food for 8 hours before) blood sample (15mL, ~1 tablespoon) will be taken. Since this blood sample requires being fasted, we recommend that we book the visit in the morning.
- After the blood sample, you be given a small snack, for example a granola bar and some juice.
- You will be asked to complete i) the International Physical Activity Questionnaire (IPAQ), ii) the Godin Leisure Time questionnaire so that we can get a better idea of how active you are on a regular basis and, iii) a Sociodemographic questionnaire.
- We will measure your height and weight.
- We will then ask you to put on a heart rate monitor and a face mask for indirect calorimetry measures. We will ask you to rest seated for 30 minutes to allow relaxation of your muscles and digestion of snack.
- After the relaxation phase, you will undergo a moderate-intensity steady-state treadmill walking session (60 minutes maximum duration).
- If your heart rate reaches 85% of your predicted heart rate maximum, the test will be stopped.
- Immediately following the cool-down, we will take another blood draw (15mL).
- Following the blood draw, we will ask you to remain seated for a second post-exercise resting phase of a 30-minute duration.

Risks:

1. **Pregnant participants**

There is little risk to you or your baby by participating in this study.

Blood drawing causes some pain and may cause bruising, bleeding or infections at the site of the needle stick. A nurse or certified phlebotomist has been trained in safely drawing blood. Care will be taken to avoid these complications.

The walking sesssion will occur in a safe environment and will incorporate the most recent evidence for exercise guidelines during pregnancy. CPR and first aid trained personnel, specially

trained to perform exercise testing, will coordinate and monitor the testing. Heart rate will be continually monitored to ensure that you do not reach an unsafe heart rate. Research staff will make sure the treadmill is adjusted properly and will conduct a proper warm-up and cool-down to prevent injuries.

The risk of an adverse event is minimized through the supervision of testing by qualified personnel. In the unlikely event that you experience an injury, medical or psychological crisis (i.e. chest pains, heart attack, panic attack, etc.) during the fitness test, a safety protocol is in place and the university emergency response team will be contacted immediately.

2. Non-pregnant participants

There is little risk to you by participating in this study.

Blood drawing causes some pain and may cause bruising, bleeding or infections at the site of the needle stick. A nurse or certified phlebotomist has been trained in safely drawing blood. Care will be taken to avoid theses complications.

The walking session will occur in a safe environment. CPR and first aid trained personnel, specially trained to perform exercise testing, will coordinate and monitor the testing. Heart rate will be continually monitored to ensure that you do not reach an unsafe heart rate. Research staff will make sure the treadmill is adjusted properly and will conduct a proper warm-up and cool-down to prevent injuries.

The risk of an adverse event is minimized through the supervision of testing by qualified personnel. In the unlikely event that you experience an injury, medical or psychological crisis (i.e. chest pains, heart attack, panic attack, etc.) during the fitness test, a safety protocol is in place and the university emergency response team will be contacted immediately.

Benefits:

1. Pregnant Participants

The results of these tests may not be directly beneficial to you and your baby, but the results will help define the potential role of physical activity in pregnancy. The knowledge gained from this study may benefit other pregnant women in the future. The group results from this study will be shared with health care professionals including general practitioners, obstetricians and gynecologists, exercise and nutrition professionals as well as policy makers and health care planners.

2. Non-pregnant participants

The results of these tests may not be directly beneficial to you. However, this study will help determine whether physical activity results in the release of different myokines by skeletal muscle in pregnant compared to non-pregnant women and how these potential differences may affect the development of the placenta and thereby the fetus. The group results from this study will be shared with health care professionals including general practitioners, obstetricians and gynecologists, exercise and nutrition professionals as well as policy makers and health care planners.

Anonymity, Confidentiality and Data Storage:

- If I decide later that I would like to withdraw my participation and/or consent from the study, I can do so at any time.
- I voluntarily agree to participate in this study.
- I will be given a copy of this signed Participant Informed Consent Form.

Please initial:

___ Yes __ No I realize that my participation is voluntary, and I am free to withdraw from the study at any time.

___ Yes __ No Having my blood samples stored and used for future ethics approved research on health behaviours (i.e. nutrition & physical activity), pregnancy outcomes and weight regulation.

___ Yes __ No To be contacted in the future for follow up studies

If I have any questions about the study, I may contact the researcher or her supervisor.

If I have any questions regarding the ethical conduct of this study, I may contact the Protocol Officer for Ethics in Research, University of Ottawa, Tabaret Hall, 550 Cumberland Street, Room 154, Ottawa, ON K1N 6N5
Tel.: (613) 562-5387
Email: ethics@uottawa.ca

There are two copies of the consent form, one of which is mine to keep.

Participant full name (Print):

Participant's signature: **Date:**

Investigator or Delegate Statement
I have carefully explained the study to the study participant. To the best of my knowledge, the participant understands the nature, demands, risks and benefits involved in taking part in this study.

Researcher's signature: **Date:**

APPENDIX C: PKH26 EV Fluorescence Staining Protocol (Project 2)

PKH26 Fluorescence

EVs labeled with PKH26 (Sigma; MINI26-1KT) according to manufacturer's protocol
8-well ibidi chambered coverslip slides used (ibidi; 80806)

1. **Wash** vigorously 5x with PBST (0.1% Tween-20)
2. **Fix** cells for ***10 min*** at room temp using 10% formalin (300uL/well)
3. Wash 3x with PBST
4. **Permeabilize** cells for ***5 min*** at room temp using PBS + 0.1% Triton X-100 (300uL/well)
5. **Wash** 3x with PBST
6. **Incubate** with Alexafluor-488 conjugated Phalloidin (Invitrogen; A12379) working solution (1:1000 in 1% BSA in PBS; 300uL per well) for ***25 min*** at RT
7. **Wash** 3x with PBST
8. **Stain** with NucBlue™ Fixed Cell ReadyProbes™ Reagent (DAPI) (Invitrogen; #R37606) 1 drop per well – incubate for ***5 min*** at RT
9. **Mount** with ibidi Mounting Medium (ibidi; 50001) with enough to cover entire well. Parafilm edges of lid

S. Mohammad
Summer 2021 – Version 1.0

APPENDIX D: Ki67 Immunofluorescence Protocol (Project 2)

Ki67 Immunofluorescence

DAY 1

1. **Wash** 3x with PBST (0.1% Tween-20)
2. **Fix** cells for ***10 min*** at room temp using 10% formalin
3. Wash 3x with PBST
4. **Permeabilize** cells for ***5 min*** at room temp using PBS + 0.1% Triton X-100
5. **Wash** 3x with PBST
6. **Block** for ***30 min*** at room temp with 300uL BlockAid™ solution (ThermoFisher, #B10710)
7. **Wash** 3x with PBST
8. **Incubate** with 1:250 Ki67 (Abcam #16667 rabbit monoclonal antibody) in PBS ***overnight*** at 4°C or just PBS for negative control

DAY 2

1. **Wash** 3x with PBST
2. **Incubate** with Alexafluor-488 conjugated goat anti-rabbit secondary antibody (1:1000; Inivtrogen #A27034) for ***1 hr*** at room temp
3. **Wash** 3x with PBST
4. **Stain** with NucBlue™ Fixed Cell ReadyProbes™ Reagent (DAPI) (Invitrogen; #R37606) 1 drop per well – incubate for ***5 min*** at RT
5. **Mount** with ibidi Mounting Medium (ibidi; #50001) with enough to cover entire well. Parafilm edges of lid

S. Mohammad
Summer 2021 – Version 1.0

APPENDIX E: E-Cadherin Immunofluorescence Protocol (Project 2)

E-Cadherin Immunofluorescence

*negative control=HTR8 cells.
Unsyncytialized BeWos should express E-Cadherin on their cell surface

DAY 1

1. **Wash** 3x with PBST (0.1% Tween-20)
2. **Fix** cells for ***10 min*** at room temp using 10% formalin
3. **Wash** 3x with PBST
4. **Permeabilize** cells for ***5 min*** at room temp using PBS + 0.1% Triton X-100
5. **Wash** 3x with PBST
6. **Block** for ***30 min*** at room temp with 300uL BlockAid™ solution (ThermoFisher, #B10710)
7. **Wash** 3x with PBST
8. **Incubate** with anti-E-Cadherin [EP700Y] @ 1:500 (Abcam #ab40772 rabbit monoclonal antibody) in PBST ***overnight*** at 4°C

DAY 2

1. **Wash** 3x with PBST
2. **Incubate** with Alexafluor-647 conjugated goat anti-rabbit secondary antibody @ 1:400 (Invitrogen #A21245) in PBST for ***1 hr*** at room temp ***(all subsequent steps protected from light)***
3. **Wash** 3x with PBST
4. **Incubate** with Phalliodin iFluor488 reagent @1:1000 in 0.1% BSA in PBS (Abcam #ab176753) for ***20 min*** at room temp
5. **Wash** 3x with PBST
6. **Mount** with Dapi + Prolong Gold mounting media & coverslip. Keep slides in a dark box until imaging

S. Mohammad
Summer 2021 – Version 1.0

APPENDIX F: PLACENTA Study Ethics Approval (Project 3)

Ottawa Health Science Network Research Ethics Board (OHSN-REB) / Conseil d'éthique de la recherche du réseau de science de la santé d'Ottawa (CÉR-RSSO)

Civic Campus, Box 675, 725 Parkdale Avenue, Ottawa, Ontario, K1Y 4E9 613-798-5555 extension 16719 Fax: 613-761-4311

http://www.ohri.ca/ohsn-reb

Monday, March 28, 2022

Dr. Mark Walker
Attn: Ruth Rennicks White
Ottawa Hospital - General Campus
Department of Obstetrics/Gynecology/Newborn Care
Center for Practice-Changing Research, Room L1241, Box 241
501 Smyth Road, Ottawa, ON K1H 8L6

Dear Dr. Walker:

RE: Protocol #20160178-01H - PhysicaL ACtivity and diEtary implicatioNs Throughout pregnAncy (PLACENTA)

Renewal Expiry Date - Tuesday, March 28, 2023

I am pleased to inform you that your Continuing Review Form was reviewed by the Ottawa Health Science Network Research Ethics Board (OHSN-REB) and is approved. No changes, amendments or addenda may be made in the protocol or the consent form without the OHSN-REB's review and approval.

Date of approval: March 28, 2022

Renewal is valid for a period of one year. If the study is to continue beyond the expiry date, a Continuing Review Form should be submitted to the REB, in hardcopy. All Continuing Review Forms, regardless of review type (i.e., full board or delegated), must now be submitted according to the full board meeting submission deadlines AND at least 30 days prior to the expiry date of the study to prevent a lapse in approval. If the study is completed by this date, a Study Closure Form should be submitted.

Protocol dated July 18, 2019 is currently approved by the REB.

The OHSN-REB operates in compliance with, and is constituted in accordance with, the requirements of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; Integrated Addendum to ICH E6 (R1): Guideline for Good Clinical Practice E6 (R2); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations; or with the definition in the Interim Order Respecting Clinical Trials for Medical Devices and Drugs Relating to COVID-19; and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. OHSN-REB is qualified through the CTO REB Qualification Program and is registered with the U.S. Department of Health and Human Services (DHHS) Office for Human Research Protection (OHRP).

Yours sincerely,

Raphael Saginur, M.D.
Chairperson
Ottawa Health Science Network Research Ethics Board

/dw

From: Kim Thompson
Sent: May 27, 2021 4:14 PM
To: Kristi Adamo; Velislava Tzaneva
Cc: jarnason@science.uottawa.ca; Shannon Bainbridge-Whiteside
Subject: uOttawa Ethics File #H11-15-29 - Renewed

Dear Researchers,

Thank you for submitting a copy of the renewal certificate for your research project entitled "**Physical activity and dietary implications throughout pregnancy (PLACENTA)**" (uOttawa ethics file # **H11-15-29**).

Your ethics approval has been renewed and is **valid until April 21, 2022** (same as OHSN REB). In order to help us keep your file up to date, please forward us a copy of any and all renewal, amendment and/or closure reports submitted to and approved by the primary REB, as they become available.

If you have a grant for your research, you must provide a copy of this email to Research Management Services (https://research.uottawa.ca/rms/about).

Please don't hesitate to contact us if you have questions.

Best regards,
Kim

From: Kim Thompson
Sent: May 27, 2021 12:25 PM
To: kadamo@cheo.on.ca; Kristi Adamo <Kristi.Adamo@uottawa.ca>
Cc: jarnason@science.uottawa.ca; Shannon Bainbridge-Whiteside <Shannon.Bainbridge@uottawa.ca>
Subject: uOttawa Ethics File #H11-15-29 - Expired

Dear Researchers,

This is a reminder that your ethics approval certificate for the following file has expired:

uOttawa Ethics File: **H11-15-29**
Title: **Physical activity and dietary implications throughout pregnancy (PLACENTA)**
Expiration date: **May 22, 2021**

If data collection continues, you need to resubmit an Annual Status Report Form in order to renew your certificate and ensure that your file is in good standing. If data collection is complete, please submit a Final Report Form in order to close the file. You will find both forms at the following link: https://research.uottawa.ca/ethics/forms. Should you choose to renew your certificate, please also append a renewed certificate from the OHSN-REB.

Please submit this document by June 8, 2021. If we do not hear from you by this date, your file will be closed and the file will be considered non-compliant in regards to TCPS 2 regulations. Please note that you must have a valid ethics certificate in order to collect data involving human participants. Proceeding without this is a violation of the University policies and of the *Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans* (TCPS2). Therefore, if data collection is ongoing, it must cease immediately.

Also, according to Article 6.14 of the TCPS it is the researcher's responsibility to report "**unanticipated issues** (see Article 6.15) or **changes** to the research (see Article 6.16)". If either of these instances applies to your research project you must inform the REB of this via the Office of Research ethics as soon as possible.

Finally, please note that non-compliance regarding TCPS 2 and REB requirements may affect future ethics approvals and ongoing monitoring requirements.

Much thanks in advance,
Kim

Responsable d'éthique en recherche / Protocol Officer for Ethics in Research
Bureau d'éthique et d'intégrité de la recherche / Office of Research Ethics and Integrity
Université d'Ottawa / University of Ottawa
613-562-5800 x 1379 https://research.uottawa.ca/ethics

APPENDIX G: PLACENTA Study Informed Consent Form (Project 3)

PARTICIPANT INFORMED CONSENT FORM

Title of Study: Physica**L AC**tivity and di**E**tary implicatio**N**s **T**hroughout pregn**A**ncy **(PLACENTA)**

Investigators:

Kristi Adamo, Ph.D.	Associate Professor, University of Ottawa 613-562-5800 x 1009
Dr. Laura Gaudet	Associate Scientist, Ottawa Hospital Research Institute 613-737-8899 x 76655
Martin Holcik, Ph.D.	CHEO Research Institute
David Grynspan, Ph.D.	CHEO Pathology
Shannon Bainbridge, Ph.D.	Ottawa Hospital Research Institute University of Ottawa
Jane Shearer, Ph.D.	University of Calgary
John T. Arnason, Ph.D.	University of Ottawa
Zach Ferraro, Ph.D.	University of Ottawa

Collaborators:

Dr. Natalie Gauthier	Hôpital Montfort
Jameason Cameron, Ph.D.	CHEO Research Institute
Dr. Liisa Honey	Queensway Carleton Hospital
Research Coordinator:	University of Ottawa 613-562-5800 x1012
Funding Agency:	Canadian Institute of Health Research (CIHR)

Participation in this study is voluntary. Please read this Participant **Informed** Consent Form carefully before you decide if you would like to participate. Please ask the principal investigator and the study team as many questions as you like. We encourage you to discuss your options with family, friends or your health care provider.

Why am I being given this form?

You are being asked to participate in this research study because you are pregnant and planning to deliver locally and have indicated that you are interested in learning more about our research study.

Why is this study being done?

The PLACENTA study is designed to look at how lifestyle behaviours during pregnancy can affect how nutrients move to the growing fetus. The placenta is an organ that forms during pregnancy to support the baby. The placenta controls the transfer of nutrients (sugar, protein and fat) and oxygen to the fetus, and removes waste. If the placenta is not working properly, the baby may receive too few or too many nutrients. It is not well known how health behaviours, like exercise or nutrition, affect the transfer of nutrients to the fetus.

The goal of this study is to see if there are differences in the placenta's ability to transfer nutrients in pregnant women who exercise regularly compared to women who do not exercise regularly. This study is an observational study, we do not want you to make changes to your lifestyle because you are part of this study. We are simply looking to observe the natural differences between people and the impact that those differences may have.

During pregnancy, the placenta plays an important role in the growth and/or overgrowth of the baby. Based on work done by our team and others we believe that healthy eating and physical activity will be linked to differences in:

A) The way that new blood vessels form from existing ones (in the way that your cells respond to oxygen)
B) In the way that nutrients and oxygen move across the placenta (both of which are key controllers of the baby's growth).

This study will compare samples of the placenta from women who were physically active throughout their pregnancy and those who were not. We will explore the impact of physical activity on the genes that control the transfer of nutrients across the placenta to the fetus. We will also analyze the nutritional, environmental, and genetic factors that may affect placenta function by studying the relationship between genes/DNA and natural processes that occur during pregnancy which determine how a baby grows while in the womb and after birth. Genes are the building blocks or instructions found inside cells, which contribute to the different features you have, such as the colour of your hair and eyes. We hope that the information we learn in this study will be helpful in designing preventive health strategies for pregnancy.

How is the study designed? What is expected of me?

If you consent to participate in this study, you will be asked to come to the University of Ottawa-Lees Campus to meet our research team once during each trimester at specific times and dates which will be arranged between you and the study's research coordinator. Our goal

is to recruit a group of women in their first trimester of pregnancy and should you attend your first visit during your first trimester, you will attend three (3) visits in total. However, many women do not know that they are pregnant or see their doctor before their second trimester. If you are recruited in your second trimester, you will be asked to come for two (2) visits.

The first trimester visit will occur between weeks 12-16 of your pregnancy. The second trimester visit will occur between weeks 24-28 of your pregnancy. The third and final visit will occur between weeks 34-38 of your pregnancy. The estimated length of each visit is 1.5 hours. Visits will take place during the week or on the weekend at a time that is convenient for you.

Visit details: first, second and third trimester

These visits will take approximately 1.5 hours each.

1) A small fasting (no food for 8 hours before) blood sample (2 teaspoons or 10 ml) will be taken. It is best if we book your appointments for first thing in the morning, before you have eaten breakfast. This blood sample will be used to measure metabolic markers associated with nutrient transport, fetal growth, and weight gain.
2) After the blood sample, you be given a small snack, for example a granola bar and some juice (or you can bring your own snack).
3) You will be asked to complete a few questionnaires:
    a. Socio-demographic information (first visit only)
    b. General health behaviours
    c. Diet History Questionnaire (first visit only)
    d. Edinburgh Postnatal Depression Score
4) We will measure your height and weight.
5) We will then ask you to put on a heart rate monitor, and skin temperature sensor stickers and rest seated for 20 minutes in order to collect your resting energy requirements and heart rate. After the period of seated rest, we will ask you to walk on a treadmill at a comfortable pace (2 mph, 3.2 kph). The incline of the treadmill will increase every 3 minutes for approximately 20-25 minutes, but you can choose to stop at any point. At the end of each stage, we will ask you how hard you feel that you are working (Borg scale of perceived exertion), how hot you feel (thermal sensation scale), and we will measure your body temperature using an ear thermometer. We will measure your heart rate and stop the test before you reach 85% of their maximum estimated heart rate.
6) You will be asked to complete three 24-hour dietary recalls (food and drinks) in the week following each visit (2 week days, 1 weekend day), this will take about 20

minutes/day. You can do this online with information that we will give you, or on paper if you don't have access to the internet.

7) You will be given a small physical activity monitor to wear for 1-week after each visit. It is a 'smart' pedometer that works like the motion sensor (counting steps) but also provides information on your speed and direction of movement. It is always on but it is activated only by movement. This will give us an idea of your typical physical activity habits. The monitor (1" square) is safe, relatively small, non-invasive and is worn on a soft belt around the waist either under or over clothing and will not impact day-to-day activities. While we hope that you treat your accelerometer responsibly, please note that in the case of loss or breakage of the physical activity monitor, you will not be responsible for costs of replacement.

8) In the week following the visit, you will be asked to collect a small stool sample using a collection kit that we will provide you. You will then mail the kit, the physical activity monitor, and the physical activity log sheet to our office in the pre-addressed, pre-stamped envelope that we will provide you.

Visit details: At birth and 24-48 hours post-birth

This visit will take approximately 10 minutes.

1) When you arrive at the hospital/birthing unit in labour, please mention that you are part of our study. We will already have given the hospital/birthing unit a note in your file (and a door magnet) to indicate that you are participating in our study and to remind the hospital/birthing unit staff to call the research team for placenta sampling if you present at or greater than 37 weeks gestation. If you arrive in labour less than 37 weeks gestation, you will be excluded from the placenta sampling and infant measurements. The note will include your name, date of birth, the phone number to reach our research team, and brief sample processing directions for blood samples and placenta and will be kept in your paper file but not in your electronic online hospital file. We will not interact with you while you are in labour. The Labour and Delivery clerk or Midwife will call the research team to visit the hospital/birthing unit/home to pick up your placenta for sampling.

2) Once the placenta is no longer attached to you or your baby, the nursing staff or midwife will provide us with your placenta so that we can take a blood sample (5 –10mL or 1 - 2 teaspoons) from the umbilical cord, weigh the placenta and take tissue samples from it. This will not involve any pain and will not involve sticking your baby with a needle. These samples will be used to assess markers associated with nutrient transport, growth and development. Our sampling may prevent you from taking part in

a private cord blood storage program. The blood sample that we store for research purposes will not be available to you for any other purposes.

3) We will return to the hospital 24 – 48 hours after you have your baby, to measure the infant's height, weight, and body composition (skin folds). If you have been released by the hospital by this time, we will visit your home for the very short assessment. We will confirm these details with you during your last visit at the Lees Campus.

4) Approximately one (1) week after you give birth, you will be asked to collect a small stool sample from yourself, as well as one from your baby, and place them into the special collection kits that we will have given to you. We will call you to remind you to send in your samples. You will then mail the kit to our office in the pre-addressed, pre-stamped envelope that we will provide you.

5) Access to your medical charts from your delivery will be made through the service of clinical archives of the Montfort hospital or the Ottawa Hospital to obtain information on a variety of measures that are conducted and recorded as part of your standard care unrelated to the study. We will collect information such as your child's sex, date of birth, birth weight, crown-heel length, if your baby was born premature or at term, and APGAR scores. We will also retrieve information about you, including your age, height, weight and gestational age at the time of delivery, index of parity, the weight of your placenta, level of labour pain, method of delivery, recorded alcohol and drug consumption during pregnancy, OGTT scores, pregravid weight, and any complications that arose during pregnancy or delivery.

Table 1: Study Visits and Procedures

Boxes marked with an X show what will happen at each visit.

Visit	**1st trimester visit (weeks 12-16) Sub-sample early recruits only**	**2nd trimester visit (weeks 24-28) ALL participants**	**3rd trimester visit (weeks 34-38) ALL participants**	**Birth & Post Birth**
Length of time needed	1.5 hours	1.5 hours	1.5 hours	10 minutes with baby
Maternal height and weight	X	X	X	
Questionnaires	X	X	X	
Blood Sample	X	X	X	
Fitness Test	X	X	X	
Placenta processing				X (birth)
Infant length, weight and body composition				X (24 hrs post-birth)
Stool samples (Maternal all visits and post-birth) & Baby post-birth	X	X	X	X (1 week post-birth) X (baby)

If you are interested, you can choose to allow us to contact you for future research studies done by our study team related to health behaviours and pregnancy outcomes. This is not a mandatory part of your participation for this study. The signature page at the end of this document allows you to indicate your preference. Also, you will be asked to sign a separate consent form for any future research projects that you may optionally be contacted about and wish to participate in.

Will my samples be used in future research studies?

If you accept this optional component, any leftover blood/DNA and placenta tissue as well as leftover samples of your baby's blood/DNA will be stored for future research studies related to placenta function. The study of biology is rapidly evolving, as is our understanding of genetics and more notably something called 'epigenetics'. This is a relatively new area of research and simply put, epigenetics is the study of biological mechanisms that can switch genes on and off. For example, what you eat, where you live, who you interact with, when you sleep, how you exercise, and even aging can change the way our genes are turned on or off over time. Some of these changes can be inherited and the different combinations of genes that are turned on or off are what makes each one of us unique. We would like to have the opportunity to examine

this further as it relates to behaviours in pregnancy. Research Ethics Board approval will be sought before any future research begins.

We will take the baby's sample from the umbilical cord once it is cut away from the baby after delivery and no longer attached to you or your baby. Similarly, the placenta tissue sample will be taken after it has been delivered and thus will not involve any pain whatsoever nor will these procedures involve sticking your baby with a needle.

All samples will be coded (they will not contain any of your personal identifying information) and will be kept in a locked –80°C freezer located Kristi Adamo's lab at the University of Ottawa-Lees Campus for an indeterminate period of time. All of your personal health information collected will be de-identified and associated with only a study ID number, however a password protected master list containing your name and study ID number will exist and be accessible only to the Clinical Research Coordinator conducting the study.

As recommended, all the samples will be owned and governed by the Principal Investigator, Kristi Adamo, Ph.D. In order to protect the biospecimen integrity, the samples will be kept in a locked –80°C freezer at the University of Ottawa, Lees Campus. Samples may be released to co-investigators to analyze with specialized equipment in their labs at CHEO, the University of Calgary, or other labs at the University of Ottawa. Further, samples may be released to external investigators conducting similar investigations as part of secondary analysis, under the guidance of Kristi Adamo, Ph.D. In the event of future research, samples will only be released following the approval of secondary analysis by the Research Ethics Board at the University of Ottawa. Kristi Adamo has been successfully funded for 8 years and will make every effort to ensure the long term sustainability of the biobank. After having completed all of the analyses and stored for the appropriate duration, the University of Ottawa will take all unused blood samples and will destroy them according to their usual method.

How long will I be involved in the study?

Your participation in the study will last approximately 6 months or until delivery. Over this time, you will be required to visit the University of Ottawa-Lees campus, once per trimester of pregnancy, up to a total of 3 times.

What are the potential risks I may experience?

There is little risk to you or your baby by participating in this study.

Blood drawing causes some pain and may cause bruising, bleeding or infections at the site of the needle stick. A nurse or certified phlebotomist has been trained in safely drawing blood. Care will be taken to avoid theses complications.

The walking fitness test will occur in a safe environment and will incorporate the most recent evidence for exercise guidelines during pregnancy. CPR and first aid trained personnel, specially trained to perform exercise testing, will coordinate and monitor the testing. Heart rate will be continually monitored to ensure that you do not reach an unsafe heart rate. Research staff will make sure the treadmill is adjusted properly and will conduct a proper warm-up and cool-down to prevent injuries.

The risk of an adverse event is minimized through the supervision of testing by qualified personnel. In the unlikely event that you experience an injury, medical or psychological crisis (i.e. chest pains, heart attack, panic attack, etc.) during the fitness test, a safety protocol is in place and the university emergency response team will be contacted immediately.

The accelerometer has been approved by Health Canada as a medical device, however, it is small in size and as a result although unlikely, could pose a risk as a choking hazard to children under the age of 3 years old if it were to become detached from its accompanying belt. For this reason, the accelerometer should remain attached to the belt at all times and be worn only by the study participant.

Questionnaires:
You might not like all of the questions that you are asked. You do not have to answer any questions that make you uncomfortable.

If this study uncovers information that might be helpful to your current or future health, the Research Coordinator would offer to discuss these findings with you. The Research Coordinator would first advise you of any risks and benefits of sharing this information with you. If necessary, the investigator will recommend consultation with an appropriate medical professional (e.g. Clinical Psychologist).

Can I expect to benefit from participating in this research study?

The results of these tests may not be directly beneficial to you and your baby but the results will help define the potential role for physical activity in pregnancy, and the knowledge gained from this study may benefit other pregnant women in the future. The results from this study will be shared with health care professionals including general practitioners, obstetricians and gynecologists, exercise and nutrition professionals as well as policy makers and health care planners. At the end of the research study, you are provided the option to receive a summary of your results if you wish. The entire duration of the project is 5 years, therefore it could take over 5 years to receive your results.

Would you like to receive a summary of research results (please check the appropriate box)?

☐ Yes ☐ No

Do I have to participate? What alternatives do I have?

You can choose not to participate in this study. The alternative to this study is not to participate.

Your participation in this study is voluntary. You may decide not to be in this study, or to be in the study now, and then change your mind later without affecting the medical care, education, or other services to which you are entitled or are presently receiving at this institution.

If I agree now, can I change my mind and withdraw later?

You may withdraw from the study at any time without any impact on your current or future care at the Ottawa Hospital, CHEO, Montfort Hospital, or Queensway Carleton Hospital.

- If you withdraw your consent, the study team will no longer collect your personal health information for research purposes and you will no longer be expected to attend the study visits.

Information and samples collected for the study before you cancel this consent may still be used unless you request for them to be destroyed. You have the right to request for them to be destroyed.

What compensation will I receive if I am injured or become ill in this study?

In the event of a study-related injury or illness, you will be provided with appropriate medical treatment and care. Financial compensation for lost wages, disability or discomfort due to an injury or illness is not available. You are not waiving any of your legal rights by agreeing to participate in this study. The Principal Investigator, the Ottawa Hospital, the Montfort Hospital, CHEO, the Queensway Carleton Hospital, the University of Ottawa and the University of Calgary still have their legal and professional responsibilities.

Will I be paid for my participation or will there be any additional costs to me?

You will not be paid to be a participant in the study; however, a parking voucher will be provided to you to cover bus or parking costs for all visits attended with the study team at the University of Ottawa-Lees Campus.

At the end of the study, you will be given the option to receive a general health fitness program developed by a certified exercise physiologist and supervised workout access at our private fitness facility at University of Ottawa – Lees Campus, Behavioural and Metabolic Research Unit. Access will be provided for 3 consecutive months post-partum, starting after you have recovered from the birthing process and within six months of the birth of your baby. Participation in this component is optional and only if you desire.

At the end of the study you will also be given a thank you card with a $50 gift certificate to either a grocery store, book store, or coffee shop (based on your preference) as a token of appreciation for your time to participate in the study visits.

How is my personal information being protected?

- All information and samples collected during your participation in this study will be identified with a unique study number, and will not contain information that identifies you, such as your name, address, etc.
- The link between your unique study number and your name and contact information will be stored securely, password protected and separate from your study records, at The University of Ottawa. The link will not leave the University of Ottawa.
- Any documents or samples leaving the University of Ottawa will contain only your unique study number. This includes publications or presentations resulting from this study.
- Your and/or your baby's medical record will be accessed. However, the information collected, as well as the placenta tissue and blood samples, which leave The Ottawa Hospital, CHEO, Montfort Hospital, or the Queensway Carleton Hospital, will only contain your unique study number.
- Information that identifies you will be released only if it is required by law.
- For audit purposes only, your and your baby's original medical records may be reviewed under the supervision of an investigator and/or their staff by representatives from:
    - the Ottawa Health Science Network Research Ethics Board (OHSN-REB)
    - the Ottawa Hospital Research Institute
    - CHEO Research Ethics Board
    - The Health Sciences and Science REB at the University of Ottawa
    - Montfort Research Ethics Board
    - University of Calgary Conjoint Health Research Ethics Board (CHREB)
    - The Queensway Carleton Hospital Research Ethics Board
- Research records will be kept for 10 years, as required by the OHSN-REB.
- At the end of the storage time, all paper records will be shredded and all electronic records will be securely deleted.

A description of this clinical trial will be available at http://www.ClinicalTrials.gov. This website will not contain any information that identifies you. At most, the Web site will provide a summary of results. You can search this Web site at any time.

Do the investigators have any conflicts of interest?

The investigators have no conflicts of interest to declare related to this study.

What are my responsibilities as a study participant?

It is important to remember the following things during this study:

- Ask the research team if you have any questions or concerns.
- Tell the research team if anything about your health has changed.
- You should not eat or drink (except for water) for 8 hours before each visit so that we can obtain fasting blood samples. A snack will be provided immediately following the blood draw.

Who do I contact if I have any further questions?

If you have any questions about this study, please contact Kristi Adamo, Ph.D., at 613-562-5800 x 1009, Dr. Laura Gaudet at 613-737-8899 x 7665, or the study staff at 613-562-5800 x1012 or x7367.

The Ottawa Health Science Network Research Ethics Board (OHSN-REB), CHEO Research Ethics Board (CHEO REB), Hôpital Montfort Research Ethics Board, Queensway Carleton Hospital Research Ethics Board, University of Ottawa Research Ethics Board and the University of Calgary Research Ethics Board have reviewed this protocol. If you have any questions about your rights as a study participant, you may contact the Chairperson of the Ottawa Health Science Network Research Ethics Board at 613-798-5555, extension 16719, the Chairperson of the CHEO Research Ethics Board at 613-737-7600, extension 3272, the Hôpital Montfort Research Ethics Board Manager at 613-746-4621, extension 2221, the Queensway Carleton Hospital Research Ethics Board at (613) 721-2000 extension 1019, or the Office of Research Ethics and Integrity at the University of Ottawa at 613-562-5387.

PhysicaL ACtivity and diEtary implicatioNs Throughout pregnAncy (PLACENTA)

Consent to Participate in Research

- I understand that I am being asked to participate in a research study about physical activity and diet during pregnancy.
- This study was explained to me by ___________________________.
- I have read, or someone has read to me, each page of this Participant **Informed** Consent Form.
- All of my questions have been answered to my satisfaction.
- If I decide later that I would like to withdraw my participation and/or consent from the study, I can do so at any time.
- I voluntarily agree to participate in this study.
- I will be given a copy of this signed Participant **Informed** Consent Form.

Consent for the baby's participation

You accept that your newborn participates in this research conducted by Principal Investigator Kristi Adamo Ph.D.'s research team. I was explained all relevant aspects of the research and my questions were answered to my satisfaction. I was informed that my newborn's participation to this project is voluntary and can cease at any time without any form of penalty. I was given enough time to discuss with my family about the nature and involvement of my newborn in this project. I authorize the archives service to give to the research team only the information from my newborn's medical record mentioned above. I can withdraw my newborn from this project without having to provide a reason.

Please initial:

___ Yes __ No I realize that my participation is voluntary and I am free to withdraw from the study at any time.

___ Yes __ No Having my blood/DNA and placenta tissue samples stored and used for future ethics approved research on health behaviours (i.e. nutrition & physical activity), pregnancy outcomes and weight regulation.

___ Yes __ No Have my child's cord blood/DNA samples banked and used for future, ethics approved research on health behaviours (i.e. nutrition & physical activity), pregnancy outcomes and weight regulation.

___ Yes __ No To be contacted in the future for follow up studies

Contact Information:

Daytime telephone number(s): ______________________________
Evening telephone number(s): ______________________________
Email address: ______________________________
Alternate Email address: ______________________________

______________________ ___________________________ ______________________

Name of Participant (Print) Signature of Participant Date

Version date: 13 – November – 2017

PhysicaL ACtivity and diEtary implicatioNs Throughout pregnAncy (PLACENTA)

Assistance Declaration

Was the participant assisted during the consent process? ❑ Yes ❑ No

❑ The consent form was read to the participant/substitute decision-maker, and the person signing below attests that the study was accurately explained to, and apparently understood by, and consent was freely given by the participant/substitute decision-maker.

❑ The person signing below acted as a translator for the participant/substitute decision-maker during the consent process. He/she attests that they have accurately translated the information for the participant/substitute decision-maker, and believe that the participant/substitute decision-maker has understood the information translated.

___________________________ ____________________________ _____________________

Name of Person Assisting (Print) Signature Date

Investigator or Delegate Statement

I have carefully explained the study to the study participant. To the best of my knowledge, the participant understands the nature, demands, risks and benefits involved in taking part in this study.

______________________________ ______________________________ __________________

Investigator/Delegate's Printed Name Investigator/Delegate's Signature Date

APPENDIX H: PLACENTA Study Sample Collection at Birth Protocol (Project 3)

PLACENTA Study: Sample Collection at Birth

During on-call period, ensure:

- OnPage is working & activated for yourself & your on-call teammate.
- Your cell phone is working, charged & volume is on loud for OnPage notification. Be aware of where your phone is at all times.
    - *If you encounter technical problems with your cell phone (i.e. malfunctioning, won't turn on, won't charge), please notify your on-call teammate ASAP & provide alternatives for communication (i.e. S/O's contact info etc.).*
- The PLACENTA delivery bag is prepared with all necessary reagents, equipment etc. (see Appendix for checklist).
- You know the Participant's due date, delivery details (OB vs. Midwife, location), **name**, & Study ID# (PLA0XX).
- The "PLACENTA Delivery Locations Contact Sheet" is readily accessible on your person.
- Your Lees & RGN key card access pass is in a known & readily accessible location.
- Your ID badge & uniform is ready & easily accessible. You must wear a badge to identify yourself & appropriate lab attire – close-toed shoes, long hair tied back, long pants (**no street clothes**).
    - *Delivery at Montfort requires a separate key card pass* – Shuhiba has one & Rose has one (in the front pocket of the delivery bag). They expire in February every year (must be renewed).

Pre-Delivery:

Responding to the OnPage Notification

1. Call your on-call teammate and coordinate who will call the call-back number (preferably someone who knows the Participant details & delivery location) – update your teammate as soon as information becomes available.
2. Call back the number provided & see which delivery location has paged – if it is a hospital, look up the correct extensions to get in touch with the labour & delivery unit if not included in the call-back number.
3. Answer the page with the following, *"Hello, my name is* ____________________ *with the PLACENTA Study. I was just paged from this number, is a study participant's delivery imminent?"*
4. Depending on the location & who answers, you may need to explain that there is a patient delivering as part of the PLACENTA Study & that we will be on our way to sample the placenta. Ask to speak with who paged us & confirm delivery location & Participant's name.
5. Ask about when birth is expected. If you cannot arrive just as the placenta is delivered please ask the birthing team to put the placenta on ice. Ask if they can take a venous cord blood sample for us using the tubes provided in the medical chart/pink or yellow folder and to put these on ice as well.
6. Proceed even if birth is not immediately imminent & use the time to set up all necessary equipment for sampling.
7. Head to the Wet Lab (E255) at Lees Campus to retrieve the PLACENTA Delivery Bag items.

Arrival at Lees Campus Wet Lab

**Doors are locked from 11pm – 7am daily, you may need to use your key card access pass to enter. Each team lead should have a key to E255 with their key card access pass.*

1. Gather all delivery bag supplies using the PLACENTA Study Delivery Bag checklist – but this should be fast & efficient since it will be mostly prepared beforehand. Main items to gather:
    a. Frozen ice packs in -20°C freezer
    b. Ice in styrofoam box
    c. Liquid nitrogen in portable canister
2. Dispense liquid nitrogen very carefully using the metal ladle found on the table next to the tank. Ensure sure you are wearing close-toed shoes & large blue cryo-gloves.
3. Double check the delivery bag contents with the checklist.
4. Make sure that the liquid nitrogen bag is placed securely on the floor of the backseat with the weigh scale stabilizing the bag.
5. Drive with a window down to ensure liquid nitrogen venting.

Arrival at the Delivery Location (Hospital/Birth & Wellness Centre/Home)

1. Double check the directions to the birthing unit using the "Delivery Locations Contact Sheet".
2. Check-in with clerk (or Birth Centre Aid) & nursing 'team-lead' to let them know you have arrived & will be collecting placenta samples post-birth.
    a. Say, *"Hello, my name is ____________________ from the PLACENTA Research Study. We were paged about (Participant's name)'s birth. May we speak with her nurse?"*
    b. Ask about how far long she is in her labour.
3. If possible, ask the nurse/midwife to take a venous cord blood sample for you. The blood tubes may be located in their medical charts in the Pink notification Folder. If not, there are extras in the delivery bag with syringes & needles.
4. Wait for the delivery of the placenta; Do one of the following:
    a. Leave your cell phone number with the nursing team & ask them to call you when it has arrived while you wait in the waiting room (when delivery is delayed/not imminent).
    b. Wait in the sampling area & ask the team to bring the placenta to you when it is ready post-birth.
    c. Wait outside the patient's room if arrival of placenta is **imminent.**
    **The placenta is often delivered just shortly after the delivery of the baby, but can take up to 30 minutes post-birth. You may need re-prompt the nursing team about the placenta.*
5. Make sure that they give you the placenta in a plastic labeled bag (double-bagged as per hospital protocol) and **double check that the name of the Participant matches the name on the bag.**
6. Ask if the placenta needs to be sent to pathology for examination – if so, you will need to send photos of the placenta and before and after sampling weights to CHEO pathologist, David Grynspan (dgrynspan@cheo.on.ca).

Placenta Sampling (on-site)

**Place the plasma (purple top – invert 8-10 times) blood tube on ice & keep the serum (red top – invert 5 times) tube at room temperature until after sampling is finished (then place on ice).*

1. Place flat gel ice pack on sampling surface. Tape one blue absorbent pad down over the flat ice pack to the table; place another loose one on top (not taped down).
2. Make sure tools & equipment are ready for sampling:
    a. **Metabolomics: CANNOT TOUCH ANYTHING OTHER THAN METHANOL & TISSUE.** Make sure one person just does the metabolomics sampling first before the rest.
    b. Other dissection tools need to be wiped with RNaseZap. If tools are autoclaved, proceed to wiping with RNaseZap. If not, wipe down all tools & glass dishes with 70% ethanol first.
    *Surgical blades are pre-sterilized & do not need to be RNaseZapped.
    c. Organize pre-labeled cryovials in the blue tube rack holder & **label with a date.**
    d. Place sterile PBS 50ml tube on ice
    e. Have all other supplies on-hand (i.e. clear ruler, ice packs, histology containers, O.C.T embedding compound, non-sterile PBS)
    f. Fill-out as much of the "PLACENTA – Birth" form that you can, but make sure you get all the pertinent details afterwards from the Birthing team.
3. Weigh the placenta.
    a. Tare the scale.
    b. Weigh the placenta on the loose absorbent pad **(~22 g – subtract this from the final weight**) - not in the bag & do not include 'extra pieces' of placenta or umbilical cord that are not attached to the placenta.
    c. Record the weight to the nearest gram.
4. Measure the umbilical cord length.
5. If placenta is being sent to pathology, take a photo of both the maternal and fetal sides of the placenta.
6. Cut membranes and the umbilical cord from the placenta & place in the plastic bag the placenta was received in.
7. Weigh the 'trimmed' placenta – subtract absorbent pad weight and record.
8. Flip the placenta so that the maternal side is facing you.
9. Carefully unwrap the metabolomics tools from the aluminum foil pouch & dissect 2 central and 1 peripheral sample – have your on-call teammate open the cryovials for you.
    a. Place the cryovials in the liquid nitrogen canister to flash freeze the sample.
    b. Wrap the tools back into the aluminum foil pouch – **DO NOT WASH.**
10. Dissect the remaining pieces according to the "Placental Sampling Guide" diagram – avoid the maternal surface (shiny decidua – top layer) & the fetal side (bottom layer).
    a. Cut 3 large (~2.5cm^3) central cotyledon pieces & place in cleaned glass petri dish on ice.
    b. Cut 2 large (~2.5cm^3) peripheral cotyledon pieces & place in cleaned glass petri dish on ice.
    c. Work together to cut the large pieces into smaller ones (size of an eraser on the end of a pencil) using curved scalpel blades (size 10).
    d. Wash samples with sterile PBS.
    e. Combine the appropriate pieces & flash freeze using liquid nitrogen (i.e. place tubes inside canister).
11. Once all cryovials are frozen, dissect the pieces for histology following the "Placenta Histology Sampling Methodology" guide.

a. Flip the placenta over to the fetal side & cut a rectangular piece (~1cm x 3cm) of full-thickness placenta using the straight scalpel blades (size 11 blade & cut at least 2cm away from umbilical cord insertion).
b. Use scissors to help remove the rectangular section.
c. Cut the rectangular piece in half (height-wise or sagitally) so you have 2 pieces ~1cm x 1.5cm.
d. Dip each piece in 50 ml non-sterile PBS tube and hold placenta piece with forceps.
e. Place each piece into the white histology container (PLA0XX A & B). Close the container and place in the 10% formalin-labeled container.
f. Slice an additional rectangular section immediately adjacent to the histology section you cut from the placenta. Make sure the thickness is <0.5cm.
g. Cut in half similar to above – try to maintain integrity of the tissue. Wash the tissue in non-sterile PBS as above.
h. Slowly place some O.C.T embedding compound into the bottom of the clear plastic frozen histology mold (no bubbles) – ensure a thin clear layer lines the bottom of the mold.
i. Place tissue onto the compound in the mold.
j. Cover the top of the tissue with embedding compound (carefully & slowly).
k. Carefully wrap the frozen histology sample in aluminum foil and place is horizontally onto the pile of cryovials in the liquid nitrogen canister.

12. Weigh the placenta to obtain the 'Post-Sample' weight (if being sent to pathology).
13. Return the placenta to its double-bagged state & place a "Sampled by Adamo Lab" sticker on the outside of the bag.
14. Clean up - use 10% bleach to wipe down all surfaces.
    a. Wash the tools with bleach but rinse well with water & dry before storing them in the delivery bag (**EXCEPT FOR METABOLOMICS TOOLS**).
15. Find whoever was in charge for disposal/processing of the placenta (nurses/midwives/birth care aids) and let them know you are done with the sampling. Thank them for all of their help before running away.

Return to Lees Campus Wet Lab

1. Start processing the cord blood in as per standard protocols.
    a. Follow same blood protocol steps as the visit blood sampling but use the tubes to balance each other (i.e. spin them both in the same run) – but spin at the higher speed of 1700xg for 15 minutes.
    b. Use the 'cord blood'-specific cryovials located above the lab bench (2nd shelf) with the appropriate dot labels.
2. Pour the contents of the liquid nitrogen tank into a large styrofoam container – use a plastic ladle (on table next to large liquid nitrogen tank) to fish out the samples onto the lab bench.
3. Place samples in the appropriate freezer boxes at -80°C – record the positions using the "PLACENTA – BIRTH" form.
4. The frozen histology samples are re-wrapped with pre-cut aluminum foil in cabinet above work bench (label the ID# on white sticker portion).

5. Clean the metabolomics tools with methanol & Kim wipes.
    a. Methanol is stored in the large yellow flammable cabinet in the inside room, bottom shelf – **do not aliquot into separate container.**
    b. Let the tools dry on paper towel; if tools are needed immediately proceed to PLACENTA Study Prep Protocol for how to clean the tools for the next delivery (protocol in the 'Wet Lab – Protocols' binder just above lab bench). There are an extra set of clean, autoclaved tools on the 2nd shelf (right side) ready to use.
6. Wash & clean the rest of the tools (surgical tools, ruler, glass petri dishes) with water. Leave them to dry until they can be autoclaved again or until they are needed for the next delivery.
7. Transfer the formalin-fixed placenta tissue to the larger plastic container sitting in the fume hood (~250ml volume).
    a. Pour more 10% formalin on the histology samples & leave in fume hood at room temperature for a total of 48 hours after collection (to total ~250ml).
    b. After 48 hours has elapsed, rinse the samples 3 times with PBS (non-sterile, kept in fumed hood).
        i. **YOU MUST WASH THE TISSUES IN THE FUME HOOD. FORMALIN IS TOXIC TO MUCOUS MEMBRANES.**
    c. Once washed 3 times with PBS, place the cassettes back into the smaller container and fill with ethanol (~100ml) – place in 4°C fridge & ensure proper labelling technique.
    d. To process the placenta tissue into paraffin blocks, please bring container containing labelled histology containers in 70% ethanol to RGN Pathology. Shuhiba takes care of this portion.
8. Return the "PLACENTA – BIRTH" form to the PLACENTA Study binder & fill out the "Case Report Form (CRF)" to reflect at birth data collection.

PLACENTA Study Delivery Bag Checklist

(*) needs to be prepared at the start of on call period, see "**PLACENTA Delivery Supplies Prep Protocol"**

REAGENTS (plastic Tupperware container)

- 70% ethanol*
- 10% bleach*
- RNAzap
- O.C.T. embedding compound
- 10% formalin (under fume hood) in labelled plastic histology container (across lab bench)
- (2) 50ml falcon tubes of sterile PBS* (with 40ml each) & (2) 50ml falcon tubes of non-sterile PBS

CONSUMABLES (duffle bag)

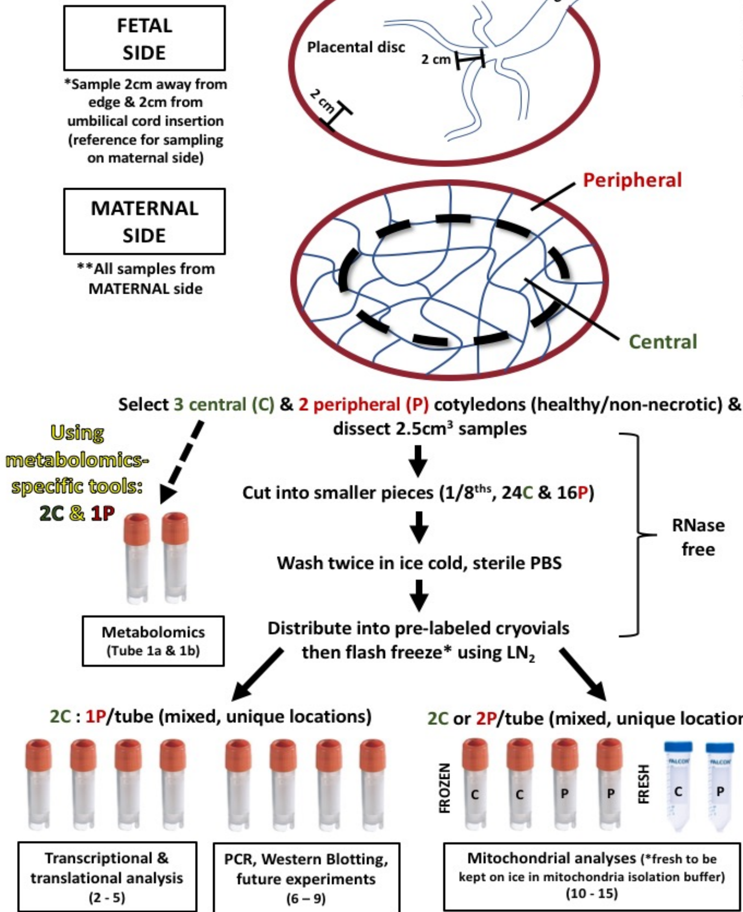
- Pre-labeled 2.0ml cryovials (orange, screw top) with appropriate dot labels (see legend above lab bench & PLACENTA sampling diagram) in Ziploc bag
- Extra 2.0ml cryovials (orange top)
- Ziploc bag of syringes & needles for cord blood collection
- (3) Ziploc bags with gloves (small, medium, large)
- Absorbent pads & large Kim Wipes
- Individual scalpel blades (#10 & #11)
- Face masks
- Extra, large Ziploc bags (min 3 extra)

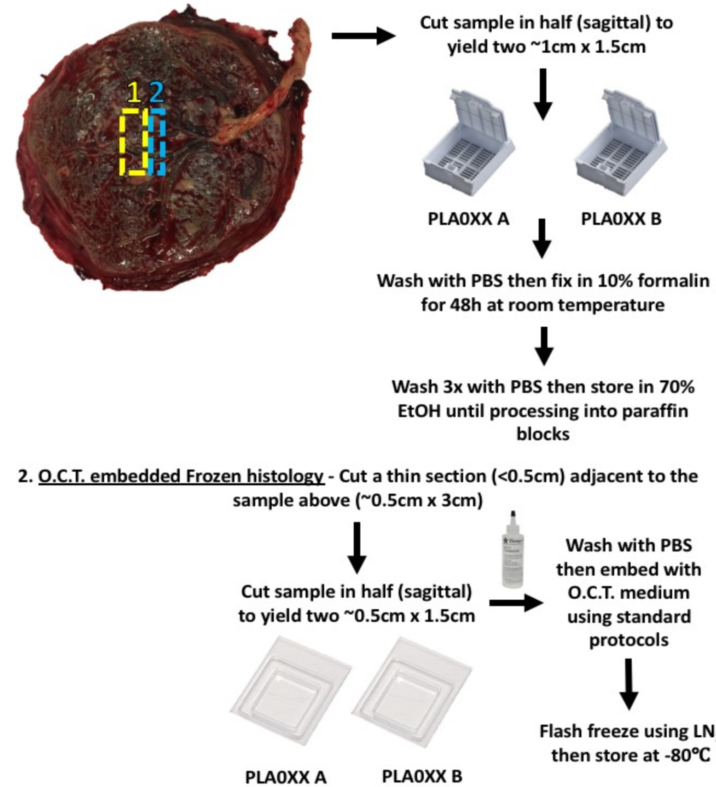
- Kim Wipes (small box)
- Paper towel roll
- Bandages (first aid supply with stationary)
- (2) White histology cassettes labeled with pencil – PLA0XX A or B
- (2) Clear plastic molds (frozen histo sample) labeled with permanent marker – PLA0XX A or B
- "Sampled by Adamo Lab" sticker labels to indicate was sampled by our lab
- Aluminum foil (pre-cut in bag)

<u>EQUIPMENT</u> (duffle bag & others)

- Baby scale (blue IKEA bag)
- Clear 30cm ruler
- Monfort Key card pass (front pocket of duffle bag)
- Dissection tools
  - <u>Autoclaved:</u> tooth forceps, scissors, blunt-ended forceps & (2) glass dissecting dishes*
  - <u>Non-autoclaved:</u> Rankin forceps (hemostat clamp) & (2) scalpel blade handles
- Metabolomics tools (separate tooth forceps & scissors washed with methanol)*
- (2) lab coats (in separate plastic bag)
- (1) portable, battery-powered lamp (in top, smaller zippered pouch)
- Stationary – permanent fine tip markers, pens, pencils, tape (front pocket of duffle bag)
- PLACENTA Delivery lab book with Blank Sampling Record sheets & printed protocols
- (2) ice packs (1 stored on top, door shelf in freezer & 1 large gel ice pack lying flat)
- Liquid nitrogen (LN_2) tank filled halfway (~500ml) with LN_2 **(DO NOT ALLOW TO TIP!) – carried in insulated blue patterned bag with cardboard dividers**
- (4) Extra "C" batteries
- Blue tube rack

APPENDIX I: PLACENTA Study Placenta Sampling Diagram (Project 3)





APPENDIX J: IHC Protocol for CD34 (Project 3)

Shuhiba Mohammad
April 2019 – Version 1.0

IHC-P: CD34 Staining Protocol

Negative control – Diluent only (TBST)

Day 1

1. **Deparaffinization:** Xylene 1 – **5 min**
   Xylene 2 – **5 min**
   Xylene 3 – **5 min**
2. **Rehydration:** EtOH 100% - **5 min**
   EtOH 95% - **5 min**
   EtOH 70% - **5 min**
3. **Rinse:** ddH2O – **2 min**
4. **Antigen Retrieval:** Sodium citrate (pH 6.0) HIER using microwave – bring slides and buffer almost to boiling then zap for 15 sec every 30 sec for 5 min then let slides cool for 15 min
5. **Rinse:** ddH2O then circle tissue sections with PAP pen
6. **Wash:** 2x with TBST (Tween 20 0.05%)
7. **Quenching:** 3% H_2O_2 room temp. – **30 min**
8. **Wash:** 2x with TBST
9. **Serum Block:** 10% normal goat serum in TBST room temp. – **60 min**
10. **Wash:** 2x with TBST
11. **Primary Antibody** – in TBST – **overnight at 4°C**
    - CD34 Antibody (QBEND/10) (BioRad – cat. no. MCA547GT) @ 1.0 mg/mL – 1:1000
    - Negative control – TBST only

Day 2

12. **Wash:** 3x with TBST
13. **Secondary Antibody** – Goat Anti-Mouse IgG (H L)-HRP Conjugate (BioRad – cat. no. 1706516) @ 0.8 mg/ml at 1:1000 in TBST at room temp. – **60 min**
14. **Wash:** 3x with TBST
15. **Apply** DAB chromogen (Abcam Inc – cat. no. ab64238) – **2 min**
16. **Rinse:** 2x ddH2O
17. **Counterstain**: filtered Harris Hematoxylin (Electron Microscopy Sciences - cat no. 26754) – **2 min**
18. **Rinse:** running tap water - **5 min**
19. **Dehydration:** EtOH 70% - **5 min**
    EtOH 95% - **5 min**
    EtOH 100% - **5 min**
20. **Clearing:** Xylene 4 – **3 min**
    Xylene 5 – **3 min**
21. **Mounting & Coverslipping:** with Entellan mounting medium (EMD Millipore/Sigma - cat. no. 1079610100)

APPENDIX K: Scholarships & Awards during Ph.D.

Ontario Graduate Scholarship ($15,000) 2019-2020

Oral Presentation Award 2019
Annual Human Kinetics Graduate Student Conference, University of Ottawa

Ontario Graduate Scholarship ($15,000) 2017-2018

Doctoral Fellowship for Advancement of Biological Perspectives for Exercise Interventions across Lifespan ($25,000/year) 2017-2019
Children's Hospital of Eastern Ontario & University of Ottawa

Graduate Admission Scholarship ($18,000/year) 2017-2021

Doctoral Incentive Scholarship ($10,000) 2017
School of Human Kinetics, University of Ottawa

APPENDIX L: List of publications during Ph.D.

Works-in-progress

1. **Mohammad S**, Bhattacharjee J, Tzaneva V, Hutchinson KA, Shaikh M, Burger D, Adamo KB. The influence of exercise-associated small extracellular vesicles on trophoblast biology. *Under review at Reproductive Sciences (RESC-S-22-00345), Apr 7th, 2022.*
2. **Mohammad S,** Bhattacharjee J, Tzaneva V, Adamo KB. Assessment of fetoplacental vascular alterations associated with physical activity in pregnancy. *Submitted to Placenta (PLAC-S-22-00199), March 29th, 2022.*

Published Manuscripts

1. **Mohammad S**, Bhattacharjee J, Vasanthan T, Harris CS, Bainbridge S, Adamo KB. Metabolomics to understand placental biology: Where are we now? *Tissue Cell.* 2021 Dec 1;73:101663. doi: 10.1016/j.tice.2021.101663
2. **Mohammad S**, Hutchinson KA, da Silva DF, Bhattacharjee J, McInnis K, Burger D, Adamo KB. Circulating small extracellular vesicles increase after an acute bout of moderate-intensity exercise in pregnant compared to non-pregnant women. *Sci Rep.* 2021 Jun 16;11(1):12615. doi: 10.1038/s41598-021-92180-5.
3. Bhattacharjee J, **Mohammad S**, Adamo KB. Does exercise during pregnancy impact organs or structures of the maternal-fetal interface? *Tissue Cell.* 2021 Apr 16;72:101543. doi: 10.1016/j.tice.2021.101543.
4. Shearer J, Klein MS, Vogel HJ, **Mohammad S**, Bainbridge S, Adamo KB. Maternal and cord blood metabolite associations with gestational weight gain and pregnancy outcomes. *J Proteome Res*. 2021 Mar 5;20(3):1630-1638. doi: 10.1021/acs.jproteome.0c00854.
5. Bhattacharjee J, **Mohammad S**, Goudreau AD, Adamo KB. Physical activity differentially regulates VEGF, PlGF, and their receptors in the human placenta. *Physiol Rep.* 2021 Jan;9(2):e14710. doi: 10.14814/phy2.14710.
6. Everest C, Nagpal TS, Souza SCS, da Silva DF, Gaudet L, **Mohammad S**, Bhattacharjee J, Adamo KB. The Effect of Maternal Physical Activity and Gestational Weight Gain on Placental Efficiency. *Med Sci Sport Exerc*. 2021 Apr 1;53(4):756-762. doi: 10.1249/MSS.0000000000002524.
7. Nagpal TS, Bhattacharjee J, da Silva DF, Souza SCS, **Mohammad S**, Puranda JL, Abu-Dieh A, Cook J, Adamo KB. Physical activity may be an adjuvant treatment option for substance use disorders during pregnancy: A scoping review. *Birth Defects Res*. 2021 Feb 1;113(3):265-275. doi: 10.1002/bdr2.1803.
8. Dobson KL, da Silva DF, Dervis S, **Mohammad S**, Nagpal TS, Adamo KB. Physical activity and gestational weight gain predict physiological and perceptual responses to exercise during pregnancy. *Birth Defects Res*. 2021 Feb 1;113(3):276-286. doi: 10.1002/bdr2.1808.
9. Nagpal TS, Everest C, Souza SCS, da Silva DF, **Mohammad S**, Bhattacharjee J, Adamo KB. Does ‘sitting’ stand alone? A brief report evaluating the health effects of prenatal sedentary time on maternal and newborn outcomes related to the future risk of obesity. *J Phys Act Health*. 2020 Aug 17;17(9):915-919. doi: 10.1123/jpah.2020-0175.

10. da Silva DF, **Mohammad S**, Hutchinson KA, Adamo KB. Cross-Validation of Ratings of Perceived Exertion Derived from Heart Rate Target Ranges Recommended for Pregnant Women. *Int J Exerc Sci*. 2020 Sep 1;13(3):1340-1351. PMCID: PMC7523906.
11. Hutchinson KA, Vuong NH, **Mohammad S**, Everest C, Leung ML, Bhattacharjee J, Adamo KB. Physical Activity During Pregnancy Is Associated with Increased Placental FATP4 Protein Expression. *Reprod. Sci.* 2020 Oct;27(10):1909-1919. doi: 10.1007/s43032-020-00210-w.
12. da Silva DF, **Mohammad S**, Hutchinson KA, Adamo KB. Determination of minimal recording period to assess resting heart rate variability during pregnancy. *Appl Physiol Nutr Metab.* 2020 Apr;45(4):431-436. doi: 10.1139/apnm-2019-0351.
13. Hutchinson KA, **Mohammad S**, Garneau L, McInnis K, Aguer C, Adamo KB. Examination of the Myokine Response in Pregnant and Non-pregnant Women Following an Acute Bout of Moderate-Intensity Walking. *Front Physiol*. 2019 Oct 10;10:1188. doi: 10.3389/fphys.2019.01188.
14. Dutton H, Doyle M, Buchan CA, **Mohammad S**, Adamo KB, Shorr R, Fergusson D. Antibiotic exposure and risk of weight gain and obesity: Protocol for a systematic scoping review. *Syst Rev.* 2017 Aug 24;6(1):169. doi: 10.1186/s13643-017-0565-9.

Published Abstracts

1. Klein M, Shearer JE, **Mohammad S,** Ferraro ZM, Holcik M, Bainbridge S, Adamo KB. Second trimester serum metabolomics profiles can predict excessive gestational weight gain. *J Dev Orig Health Dis.* (2017), 8 (Suppl I), p.S319 (PA3.06.06).

APPENDIX M: List of presentations during Ph.D.

* denotes as presenting author

Oral Presentations

1. **Mohammad S*,** Hutchinson KA, da Silva DF, Bhattacharjee J, McInnis K, Burger D, Adamo KB. Pregnant women release greater levels of small extracellular vesicles after an acute bout of moderate-intensity physical activity compared to non-pregnant women. **2020 Ottawa Extracellular Vesicle e-Symposium**. University of Ottawa, Ottawa, ON, 2020.
2. **Mohammad S***, Hutchinson KA, da Silva DF, Bhattacharjee J, McInnis K, Burger D, Adamo KB. Extracellular vesicles and exercise in pregnancy. **Placenta Interface Virtual Seminar Series 2020**.
3. **Mohammad S***, Hutchinson KA, Burger D, Adamo KB. Deducing the role of exercise-induced extracellular vesicles in placenta vascularity. **Annual Human Kinetics Graduate Students Conference 2019**, University of Ottawa, Ottawa, ON, 2017. *(2nd place winner)*
4. Rankin J, **Mohammad S***, Cuillerier A, Menzies KJ, Adamo KB. Exploring the relationship between maternal physical activity and mitochondrial function in the placenta. **Canadian Society for Exercise Physiology Annual Conference 2018**. Niagara Falls, ON, 2018. *(On behalf of J Rankin)*
5. **Mohammad S***, Bitar D, Harvey AJ, Brett KB, Holcik M, Adamo KB. The effects of physical activity on PAPPA expression during pregnancy. **Annual Human Kinetics Graduate Students Conference 2017**, University of Ottawa, Ottawa, ON, 2017.

Poster Presentations

1. **Mohammad S***, Hutchinson KA, da Silva DF, Bhattacharjee J, McInnis K, Burger D, Adamo KB. Pregnant Women Release Greater Levels of Small Extracellular Vesicles After an Acute Bout of Moderate-Intensity Physical Activity Compared to Non-Pregnant Women. **Society for Reproductive Investigation – 68th Annual Scientific Meeting**. Boston, MA, USA, 2021. *(Virtual)*
2. **Mohammad S***, Hutchinson KA, Burger D, Adamo KB. Characterization of the maternal circulating extracellular vesicle profile after an acute bout of moderate-intensity physical activity. **7th Canadian National Perinatal Research Meeting**, Banff, AB, 2020.
3. Hutchinson KA, Vuong N, **Mohammad S**, Bhattacharjee J, Everest C, Leung M, Adamo KB. The role of acute and chronic exercise on placental nutrient transporter expression and localization. **7th Canadian National Perinatal Research Meeting**, Banff, AB, 2020.
4. Bhattacharjee J, **Mohammad S**, Adamo KB. Maternal physical activity is associated with differential expression of VEGF, PlGF and their receptors in human term placenta. **7th Canadian National Perinatal Research Meeting**, Banff, AB, 2020.
5. da Silva DF, Colley RC, Souza A, **Mohammad S**, Nagpal T, Adamo KB. Three valid days are enough to measure physical activity by accelerometers in pregnant women. **7th Canadian National Perinatal Research Meeting**, Banff, AB, 2020.

Forthcoming Presentations

1. **Mohammad S***, Bhattacharjee J, Tzaneva V, Adamo KB. Assessment of fetoplacental vascular alterations associated with prospective objectively measured physical activity in pregnancy. **9th Annual Canadian Perinatal Research Meeting (CNPRM)**. Virtual, 2022.
2. **Mohammad S***, Bhattacharjee J, Tzaneva T, Hutchinson KA, Shaikh M, Burger D, Adamo KB. The influence of exercise-associated small extracellular vesicles on trophoblasts *in vitro*. **DOHaD World Congress 2022**. Vancouver, BC, 2022.