

The Effect of Exercise-Induced Myokines on Placental Health and Function

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Abstract

Background: Exercise in pregnancy is associated with optimized fetal growth; however, the implicated mechanisms remain unknown. We hypothesize that exercise-induced myokines may be acting on the placenta to optimize fetal growth across gestation.

Methodology: 1) Circulating profiles of 11 myokines were analyzed in the 2nd trimester plasma samples from women characterized as active (N=14) or non-active (N=16) during pregnancy. 2) First trimester human placental explants (N=5) were treated with SPARC in a dose-dependent manner (0-150ng/ml). Metrics of placental health/function, including GLUT-4 expression/regulation, were assessed.

Results: 1) Active women demonstrated an elevation in circulating SPARC compared to non-active women (86 ± 19 pg/ml vs. 52 ± 18 pg/ml, $p=0.0001$). 2) Explants treated with SPARC at 100ng/ml demonstrated improved invasion, with improved maximum outgrowth distance (N=3; $p=0.0219$).

Conclusion: SPARC is a myokine that is elevated in the circulation of active pregnant women and is associated with improved placental invasion, suggesting a possible role of SPARC in placentation.

Résumé

Contexte: L'activité physique durant la grossesse est associée à une croissance optimale du fœtus; cependant, les mécanismes impliqués restent inconnus. Nous émettons l'hypothèse que les myokines agiraient sur le placenta afin d'optimiser la croissance fœtale pendant la grossesse.

Méthodologie: 1) Les profils circulants de 11 myokines ont été analysés par un test de multiplex dans des échantillons de plasma du 2^e trimestre des femmes enceintes caractérisées comme actives (N=14) ou non actives (N=16). 2) Des explants placentaires humains du 1^{er} trimestre (N=5) ont été traités par SPARC de manière dose-dépendante (0-150ng/ml). Les mesures de santé/fonction placentaire, y compris l'expression/la réglementation de GLUT-4, ont été évaluées.

Résultats: 1) Les femmes actives ont démontré une élévation de SPARC plasmatique par rapport aux femmes non actives (86 ± 19 ng/ml contre 52 ± 18 ng/ml, $p=0.0001$). 2) Un traitement des explants avec 100ng/ml de SPARC a amélioré la distance d'invasion maximale (N=3; $p=0.0219$).

Conclusion: SPARC est une myokine élevée dans la circulation des femmes enceintes actives et associée à une invasion placentaire améliorée, suggérant un rôle possible dans la placentation.

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Contribution of Authors

This thesis presents the work of Miss Chantal Dubé in collaboration with her thesis Supervisor, Dr. Shannon Bainbridge and co-supervisor, Dr. Kristi Adamo, and contributions from the following co-authors: Jeremiah Gaudet, Alysha Harvey, Céline Sayed, Audrey Caron, Dr. David Grynspan and Céline Aguer.

Manuscript 1: *A role for maternally derived myokines to optimize placental function and fetal growth across gestation.* Dr. Bainbridge, Dr. Adamo, and Dr. Aguer were responsible for the conceptualization of the content of this review article. The writing of the manuscript was performed by Chantal Dubé with the assistance of the aforementioned authors. All authors reviewed and agreed to the finalized content of this manuscript. This manuscript has been accepted for publication by the journal of *Applied Physiology, Nutrition and Metabolism*.

Manuscript 2: *SPARC is an exercise-induced myokine elevated in the plasma of active pregnant women.* Dr. Bainbridge, Dr. Adamo and Dr. Aguer were responsible for the initialization and conceptualization of this project. The retrieval of previously collected plasma samples and selection of study participants were performed by Chantal Dubé and Alysha Harvey. The multiplex and singleplex assays were performed by Chantal Dubé with the assistance of Jeremiah Gaudet. Statistical analysis, interpretation of results, and writing of the manuscript were performed by Chantal Dubé. All authors reviewed and agreed to the finalized content of this manuscript. This manuscript has been formatted and will be submitted to the *Journal of Applied Physiology*.

Manuscript 3: *The effect of SPARC on the health and functioning of first trimester human placentae.* Dr. Bainbridge, Dr. Adamo and Dr. Aguer were responsible for the initialization and

conceptualization of this project. Patient recruitment, placenta collection and dissection, experimental set-up, SPARC media preparation and treatments were performed by Chantal Dubé. The placental health and function assays were performed by Chantal Dubé with the assistance of Jeremiah Gaudet. Scoring of GLUT-4 syncytial staining of slides was performed by Chantal Dubé and Dr. David Grynspan. Statistical analysis, interpretation of results, and writing of the manuscript were performed by Chantal Dubé. All authors reviewed and agreed to the finalized content of this manuscript. This manuscript has been formatted and will be submitted to the journal *Placenta*.

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List of Abbreviations

AA	Amino acid
ACOG	American Congress of Obstetricians and Gynecologists
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANGPTL-4	Angiopoietin-like 4
AGA	At-gestational-age
BDNF	Brain-derived neurotrophic factor.
β-hCG	Beta-human chorionic gonadotropin
BMI	Body mass index
CCL2	C-C Motif Chemokine Ligand 2
CK18	Cytokeratin 18
CSEP	Canadian Society for Exercise Physiology
CT	Cytotrophoblast
CVD	Cardiovascular disease
CX3CL1	C-X3-C motif ligand 1
DMEM-F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
ELISA	Enzyme-linked immunosorbent assay
EPS	Electric pulse stimulation
EVT	Extravillous trophoblast
FA	Fatty acid
FATP4	Fatty acid transport protein 4

FFA	Free fatty acid
FKN	Fractalkine
GDM	Gestational diabetes mellitus
GLUT-1	Glucose transporter 1
GLUT-3	Glucose transporter 3
GLUT-4	Glucose transporter 4
GWG	Gestational weight gain
HBSS	Hank's Balanced Salt Solution
IFPA	International Federation of Placenta Associations
IGF-1	Insulin-like growth factor 1
IHC	Immunohistochemistry
IL-1ra	Interleukin-1 receptor agonist
IL-6	Interleukin-6
IL-7	Interleukin-7
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-13	Interleukin 13
IL-15	Interleukin-15
IOM	Institute of Medicine
IUGR	Intrauterine growth restriction
LDH	Lactate dehydrogenase
LGA	Large-for-gestational age

LIF	Leukemia inhibitory factor
LCPUFA	Long-chain polyunsaturated fatty acid
MCP-1	Monocyte chemoattractant protein-1
mTOR	Mammalian target of rapamycin
MOM	Maternal obesity management
MVM	Microvillous membrane
MVPA	Moderate-to-vigorous physical activity
NEFA	Non-esterified fatty acid
OW/OB	Overweight/obese
PA	Physical activity
PIH	Pregnancy-induced hypertension
PE	Preeclampsia
P-AMPK	Phosphorylated AMPK
PVDF	Polyvinylidene difluoride
P/S	Penicillin-Streptomycin
REB	Research Ethics Board
SD	Standard deviation
SGA	Small-for-gestational age
SNAT1	Sodium-coupled neutral amino acid transporter 1
SNAT2	Sodium-coupled neutral amino acid transporter 2
SNAT4	Sodium-coupled neutral amino acid transporter 4
SOGC	The Society of Obstetricians and Gynaecologists of Canada

SPARC	Secreted protein acidic and rich in cysteine
ST	Syncytiotrophoblast
TG	Triglyceride
TNF- α	Tumour necrosis factor alpha
TPA	Total physical activity
TSPC-2: CORE	Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans Course on Research Ethics
VEGF-IGF II	Vascular endothelial growth factor-insulin-like growth factor 2
VPA	Vigorous physical activity

Thesis Outline

- Chapter 1** Fundamental concepts are presented followed by a summary of the rationale, hypotheses, specific research aims and overview of the presentation of this thesis.
- Chapter 2** The first manuscript is presented, entitled “*A role for maternally derived myokines to optimize placental function and fetal growth across gestation*”, published in *Applied Physiology Nutrition and Metabolism*.
- Chapter 3** The second prepared manuscript is presented entitled “*SPARC is an exercise-induced myokine elevated in the plasma of active pregnant women.*”, prepared for submission to the *Journal of Applied Physiology*.
- Chapter 4** A preamble to manuscript three providing justification for the approach that was taken for the remainder of this research project followed by the third prepared manuscript entitled “*The effect of SPARC on the health and functioning of first trimester human placentae*”, prepared for submission to the journal *Placenta*.
- Chapter 5** A summary and discussion of the findings is presented along with limitations, future directions, clinical applications and final conclusions.

Chapter 1

Thesis Overview

1.1 The Threat of Maternal Obesity and Excessive Gestational Weight Gain

Maternal obesity is on the rise, as obesity continues to be a global epidemic. Today, more than two-thirds of North-American women of reproductive age are overweight/obese (OW/OB) (Yogev & Catalano, 2009). The many adverse maternal and fetal health outcomes associated with pregnancy complicated by obesity or excessive gestational weight gain (GWG) make this a matter of great concern (Adamo, Ferraro, & Brett, 2012). It has been found that 60% of pregnant women exceed the Institute of Medicine's (IOM) guidelines for gestational weight gain (GWG) during pregnancy (Ferraro, Gaudet, & Adamo, 2012). The evidence-based, GWG guidelines recommend a lower target weight gain range for women who are obese than non-obese (Kinnunen, Luoto, Gissler, & Hemminki, 2003). As a result, women who are obese often exceed GWG guidelines by 3-fold (Weisman, Hillemeier, Symons Downs, Chuang, & Dyer, 2010). Mean GWG for women, regardless of pre-pregnancy Body Mass Index (BMI) has risen, contributing to an overall increase in GWG by 10-15 kg over the last 4 decades (Kinnunen et al., 2003). As reviewed by Adamo et al. (2012), excessive GWG independent of pre-pregnancy BMI, is associated with many antenatal and postpartum risks including an increased risk of overweight for both mother and her child across the lifespan (Schack-Nielsen, Michaelsen, Gamborg, Mortensen, & Sørensen, 2010). These findings support the contention that controlling GWG is vital to mitigate the negative outcomes for the mother and fetus.

Obesity has a negative impact on maternal cardio-metabolic health. As the most common risk factor for insulin resistance (Adamo et al., 2012), there is a high prevalence of gestational diabetes mellitus (GDM) in pregnant women who are OW/OB (Kim et al., 2010). In fact, the risk of developing GDM increases exponentially with BMI (Torloni et al., 2009), and is higher in those

who experience high GWG. Women who develop GDM in one pregnancy are also at an increased risk to develop GDM in future pregnancies (Kim, Berger, & Chamany, 2007) and to later develop type 2 diabetes (Kim, Newton, & Knopp, 2002). Furthermore, women who are obese and/or have GDM during their pregnancies often experience dyslipidemia that can further promote insulin resistance and contribute to an increased risk of developing diabetes (Catalano et al., 2009; Huda, Brodie, & Sattar, 2010).

Sub-optimal weight management during pregnancy, independent of maternal obesity, is also associated with an increased risk of complications including pregnancy-induced hypertension (PIH) and preeclampsia (PE) (O'Brien, Ray, & Chan, 2003). Many studies have found a strong positive association between pre-pregnancy BMI and PE (O'Brien et al., 2003). PE is not only associated with an increased risk of maternal and fetal morbidity and mortality, but also with later development of cardiovascular disease (CVD) (Smith, Pell, & Walsh, 2001). These metabolic disturbances of excess GWG as well as the adverse pregnancy outcomes associated with maternal obesity, significantly increase the risk for future metabolic and cardiovascular diseases (Sattar & Greer, 2002).

1.1.1 Maternal Obesity and Subsequent Childhood Obesity

Maternal pre-pregnancy BMI is one of the greatest predictors of future obesity and metabolic disorders in children (Catalano et al., 2009). Being OW/OB and/or exceeding GWG guidelines significantly increases the risk of delivering a large-for-gestational age (LGA) baby (birth weight >90th percentile) (Ferraro et al., 2012; Mamun, Mannan, & Doi, 2014). Recent publications have confirmed the association between excess GWG and high birth weight or fetal macrosomia (birth weight >4000g), and an increased risk of obesity from childhood through to

early adulthood (Mamun et al., 2014; Yu et al., 2011). Fetal macrosomia is also associated with a predisposition to downstream chronic health conditions such as type 2 diabetes and CVD (Adamo et al., 2012; Yu et al., 2011). Infants born LGA as well as those that are exposed to GDM or type 2 diabetes during pregnancy are also at an increased risk of future obesity, type 2 diabetes and metabolic dysregulation (Dabelea et al., 2008; Hillier et al., 2007). These findings link maternal obesity and excessive GWG to later childhood obesity and an elevated risk of subsequent chronic health conditions.

1.2 Physical Activity during Pregnancy

Pregnancy has been identified as an important intervention period for making lifestyle changes, as pregnant women are thought to be motivated to adopt healthier behaviours to benefit the health of their unborn child (Adamo et al., 2013). Subsequently, such behaviours can impact multiple generations. Appropriate dietary habits and regular physical activity (PA) are important mediators of weight gain and weight maintenance, and have also been found to predict excessive GWG during pregnancy (Olson & Strawderman, 2003). Still, many women continue to feel that exercising during the gestational period may be unsafe, making them less likely to engage in regular PA (Duncombe, Wertheim, Skouteris, Paxton, & Kelly, 2009). Given the obvious benefits of exercise during pregnancy, national guidelines for PA during pregnancy and the postpartum period have been developed to encourage the participation of pregnant women in a healthy active lifestyle throughout gestation (Artal, O'Toole, & White, 2003; Davies, Wolfe, Mottola, & MacKinnon, 2003). Participation in regular PA not only regulates appropriate GWG, but also helps to reduce postpartum weight retention (O'Toole, Sawicki, & Artal, 2003). One of the major benefits of exercise during pregnancy is the reduction in fetal birth weight extremes,

including the risk of delivering both a small- or large-for-gestational age infant (SGA or LGA). Thus, exercise appears to have a role in the regulation of fetal growth (Clapp, 1996; Hopkins & Cutfield, 2011; Juhl, Olsen, Andersen, Nøhr, & Andersen, 2010; Owe, Nystad, & Bø, 2009). Overall, exercise has tremendous potential to minimize the negative effects of obesity and excessive GWG on maternal-fetal health.

An in-depth discussion of PA guidelines during pregnancy and the impact of exercise during pregnancy on maternal-fetal health can be found in Chapter 2 (published manuscript #1; *Appl Physiol Nutr Metab.* 2017; 42(5):459-469): “A role for maternally derived myokines to optimize placental function and fetal growth across gestation”. In this publication, a thorough review of the literature is provided, making a case for myokines as the critical mediators of optimized fetal growth in physically active pregnant women, emphasizing the possible beneficial effects of myokines on placental health and function.

1.3 Myokines

1.3.1 Skeletal Muscle as an Endocrine Organ

Skeletal muscle is a highly underappreciated endocrine organ. Upon contraction, hundreds of peptides and cytokines are produced and secreted by skeletal muscle – collectively known as myokines – which can participate in autocrine, paracrine and endocrine signaling throughout the body (Pedersen, Akerström, Nielsen, & Fischer, 2007). Research focused on identifying and characterizing myokine profiles in the context of human health and disease is still in its infancy; however, myokines are thought to mediate several protective effects (i.e. anti-inflammatory) of exercise in multiple organ systems and possibly provide protection from chronic diseases associated with low-grade inflammation and impaired metabolism (i.e. diabetes

and CVD) (Petersen & Pedersen, 2005). In the context of pregnancy, the effects of several of these peptides and cytokines on placental function have been studied in isolation, but never collectively as a secretome released by exercising skeletal muscle.

1.3.2 Physical Activity and Myokines

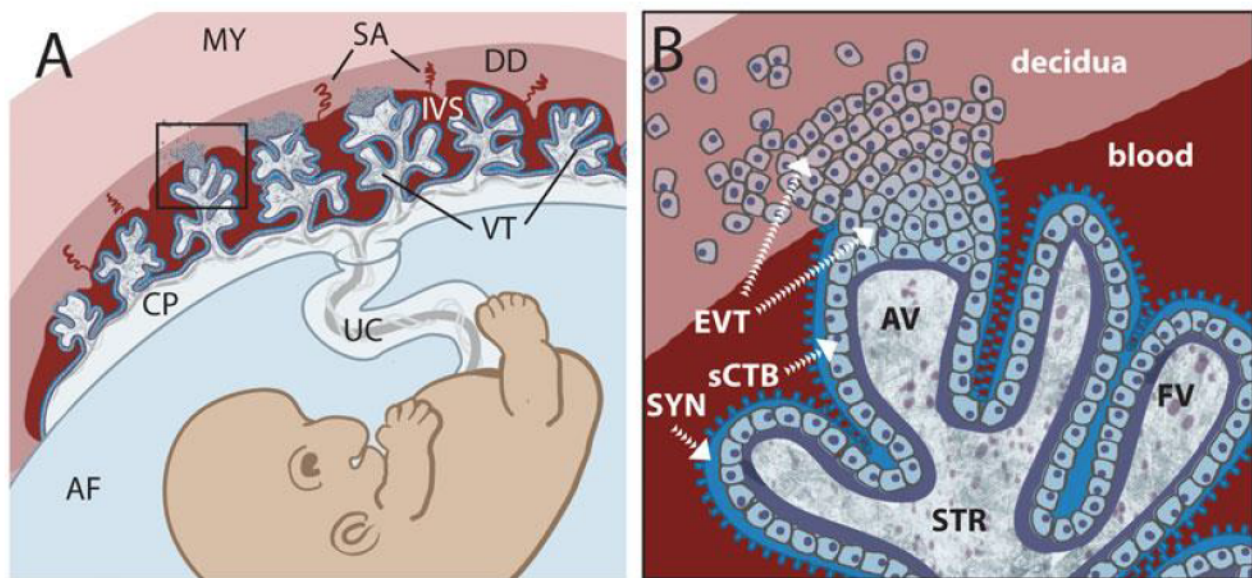
As detailed above, an in-depth discussion of the impact of physical activity on maternal myokine secretion, and how this may impact placental health and function, can be found in Chapter 2.

1.4 The Human Placenta

The placenta is the specialized organ of pregnancy that provides the vital connection between the mother and her developing fetus. It is the maternal-fetal interface where all exchange of nutrients, gases and waste products takes place. It functions as a protective barrier to the fetal-placental circulation and as well as an endocrine organ, synthesizing hormones that mediate maternal adaptation to pregnancy. Throughout gestation, the placenta supports the normal growth and development of the fetus.

The placenta is fetal in origin, composed of two functional compartments – the villous and extravillous compartments. On the fetal-facing surface of the placenta (the chorionic plate), a series of fetal vessels can be seen originating from the central umbilical cord (directly connected to the fetal vasculature). These chorionic vessels branch out towards the periphery of the placental disc, where they dive into the core of the placenta and further branch into the complex chorionic villous trees of the placenta. These tree-like structures of the placental villous compartment are bathed in maternal blood (found in the intervillous space), and mediate all maternal-fetal exchange during pregnancy. The fetal vasculature is encased in the core of these

trees, embedded within the stromal tissue. Overlaying the stromal core are 2 layers of placental trophoblast cells. The outermost layer – the syncytiotrophoblast (ST) - is comprised of a specialized multinucleated epithelial lining which is in direct contact with the maternal blood in the intervillous space. The inner layer consists of individual cytotrophoblast (CT) cells, acting as the precursor cells that support ST growth and regeneration (Benirschke, Burton, & Baergen, 2012).



Panels A and B of Figure 1 from Robbins JR, Skrzypczynska KM, Zeldovich VB, Kapidzic M, Bakardjiev AI (2010) Placental Syncytiotrophoblast Constitutes a Major Barrier to Vertical Transmission of *Listeria monocytogenes*. PLoS Pathog 6(1): e1000732. <https://doi.org/10.1371/journal.ppat.1000732>. Licensed under CC BY 4.0.

Figure 1. Early Placental Structure and Development. (A) The structure of the fetus and placenta in early pregnancy. Fetal structures are represented in blue and maternal structures represented in red. Maternal structures include the myometrium (MY), spiral arteries (SA), decidua (DD), and the intervillous space (IVS). Fetal structures include the villous tree (VT), chorionic plate (CP), umbilical cord (UC) and amniotic fluid (AF). (B) Early placental trophoblast development. A villous tree consists of both anchoring villi (AV) and floating villi (FV). Villi are enveloped by 2 layers of trophoblast cells, an outermost layer of syncytiotrophoblast (SYN) cells and an inner

layer of subsyncytial cytotrophoblast (sCTB) cells. The exchange of nutrients, gases and waste products occurs across the syncytiotrophoblast, where villi encounter maternal blood, to supply fetal capillaries in the stroma (STR). Extravillous cytotrophoblast (EVT) cells form cell columns that anchor the villous tree in the decidua. EVT cells invade into the decidua to remodel maternal spiral arteries to allow for maternal blood flow into the intervillous space.

The extravillous compartment of the placenta consists of a specialized group of extravillous cytotrophoblast cells (EVTs), which function to anchor the developing placenta to the uterine wall, and participate in the active process of uterine wall invasion and remodeling of the uterine vasculature (Benirschke et al., 2012). In the first trimester of pregnancy, the placenta develops under a hypoxic or low oxygen environment. The maternal spiral arteries are plugged by endovascular cytotrophoblast cells (Rodesch, Simon, Donner, & Jauniaux, 1992) until about 10-12 weeks' gestation when these plugs are removed, allowing for blood to be supplied to the intervillous space (Burton, Jauniaux, & Watson, 1999; Jaffe, Jauniaux, & Hustin, 1997). Appropriate EVT invasion and remodeling of the uterine spiral arteries into highly dilated vessels allows for optimized flow of maternal blood into the intervillous space of the placenta, supporting adequate fetal growth and development. The hypoxic environment in early pregnancy is an important regulator of EVT cell differentiation (Genbacev, Zhou, Ludlow, & Fisher, 1997). Approximately 3 weeks post-conception, villi undergo vasculogenesis and blood vessels are formed (Risau, 1997). As pregnancy progresses and the metabolic demands of the fetus increase, blood flow and placental growth increase, including increases in the branching of chorionic villi, to match the increasing needs of the developing fetus (Ahmed & Perkins, 2000).

1.5 Placental Macronutrient Transport

Specific transporters for all major classes of macronutrients (i.e. glucose, lipids, and amino acids) are present in abundance within the placenta, as these macronutrients serve as the building blocks for fetal growth. An adequate supply of nutrients to the fetus is essential to provide the energy necessary to support normal fetal growth. The availability of these macronutrients to the developing fetus depends both on maternal diet and metabolism, as well as placental expression of specific macronutrient transporters.

1.5.1 Placental Glucose Transport

Maternal intake of glucose is essential as it is the primary source of energy for fetal growth. Gluconeogenesis is not activated in the offspring until birth when the glucose supply from the mother is abolished (Kalhan & Parimi, 2000). Placental transport of glucose to the fetus across the microvillous membrane (MVM) of the ST occurs via facilitated-diffusion, powered by a variety of glucose transporters (Gude, Roberts, Kalionis, & King, 2004). The expression of one isoform, GLUT-1, increases in the latter half of pregnancy when increased placental glucose transport is essential to support the rapid growth of the developing fetus (Jansson, Wennergren, & Illsley, 1993), and is the primary glucose transporter present at the time of delivery (Illsley, 2000). However, early in pregnancy (1st-2nd trimester) the GLUT-3 and GLUT-4 isoforms are predominant (Ericsson, Hamark, Powell, & Jansson, 2005). For the purposes of this thesis, we will focus on GLUT-4, the major insulin-sensitive glucose transporter in the 1st trimester placenta. The abundance of placental insulin-sensitive receptors in early pregnancy is likely due to the high glucose requirements of feto-placental development in first trimester (Ericsson et al., 2005). It has been suggested that inappropriate expression and/or activity of these placental glucose

transporters may represent a mechanism by which fetal growth can become dysregulated (Ericsson et al., 2005).

1.5.2 Placental Lipid Transport

The transfer of maternal fatty acids (FAs) across the placenta is vital for fetal growth and development. FAs are taken up by the placenta as free FAs (FFAs) or as lipoproteins. Both FFAs and glycerol can cross the MVM and be transferred to the fetus via simple diffusion or FA transport proteins (Haggarty, 2010). Although, non-esterified fatty acids (NEFA) can be taken up directly by the placenta, triglycerides (TGs) must first be broken down into FFAs by placental lipases (Cetin, Alvino, & Cardellicchio, 2009). Long-chain polyunsaturated FAs (LCPUFAs) are primarily derived from maternal circulation and are critical for fetal development (Haggarty, 2010), and are therefore preferentially transferred from the mother to the fetus during pregnancy (Dutta-Roy, 2000).

1.5.3 Placental Amino Acid Transport

Specific transport proteins in the placenta actively transport amino acids (AAs) from the mother to the fetus. AA transport during pregnancy occurs across both the MVM or basal membranes of the ST and they are primarily used by the fetus for protein synthesis (Gude et al., 2004). There are two primary AA transporter systems in the placenta: System A and System L. As pregnancy progresses, the activity of System A increases in the MVM to meet the increasing energy demands of the fetus and to support optimal fetal growth (Mahendran et al., 1994).

1.5.4 Exercise and Placental Macronutrient Transport

Exercise during pregnancy has been found to alter the expression of certain genes involved in glucose, amino acid and fatty acid transport. Insulin-like growth factor 1 (IGF-1), found to be negatively correlated with moderate-to-vigorous PA levels (Brett, Ferraro, Holcik, & Adamo, 2015), is known to stimulate the expression of GLUT-1 in the placenta and up-regulates glucose transport (Baumann et al., 2014). Reduced IGF-1 levels, as a result of PA, are not associated with decreased birth weights indicating that PA may function to normalize rather than to reduce fetal growth (Brett et al., 2015). Previous studies have also identified a 2-fold decrease in the expression of FATP4, a transporter of FFAs across the placenta, when PA guidelines are followed during the 2nd trimester of pregnancy (Brett et al., 2015). This suggests that PA is associated with diminished potential for FA transport to the fetus that may explain the observed PA-induced reduction in neonatal fat mass (Harrod et al., 2014). Furthermore, following PA guidelines during pregnancy has also been associated with nearly a 2-fold increase in the expression of SNAT2 (Brett et al., 2015), a vital AA transporter in the placenta at term (Desforges et al., 2009; Desforges, Greenwood, Glazier, Westwood, & Sibley, 2010). Thus, women who are physically active during pregnancy may have increased potential for AA transport to the fetus via SNAT2, that along with the diminished potential for FA transport, could contribute to improved neonatal body composition.

The previously reported findings of the effect of maternal exercise on placental macronutrient transport are from studies with 2nd trimester placental tissues. From our knowledge, the effect of exercise on 1st trimester placental transporter activity has yet to be investigated. However, it is believed that maternal-fetal exchange via the placenta is initiated

within the 1st trimester of pregnancy. For example, blood flow into the intervillous space has been identified as early as the 1st trimester of pregnancy (Valentin, Sladkevicius, Laurini, Söderberg, & Marsal, 1996). It is near the end of the 1st trimester that hemotrophic nutritional pathways are thought to be established, when the maternal spiral arteries are unplugged (10-12 weeks' gestation), allowing for maternal blood flow in the intervillous space and for maternal-fetal exchange to become initiated (Burton, Hempstock, & Jauniaux, 2001; Jauniaux, Jurkovic, & Campbell, 1995). Thus, an understanding of the impact of exercise on maternal-fetal exchange in the 1st trimester placenta is warranted to gain insight into the impact of maternal PA on early placental macronutrient transport.

1.6 STUDY RATIONALE

The increasing population of individuals with obesity that are of reproductive age makes it a public health priority to find strategies to limit the adverse effects of obesity and excessive GWG on pregnancy. It is already known that exercise during pregnancy can both limit GWG and protect the fetus from the adverse effects of maternal obesity. During exercise, various myokines are secreted from skeletal muscle and exert beneficial endocrine effects on distant organs (Pedersen et al., 2007). The protective effects of exercise-induced myokine secretion have been examined at length in the non-pregnant population, and are believed to contribute to exercise-induced reductions in chronic disease onset and/or progression (Petersen & Pedersen, 2005). However, the impact of this skeletal muscle secretome has not been examined in the context of pregnancy, with little known about the impact of exercise-induced myokines on the vital organ of pregnancy – the placenta. It is extremely likely that, like in the non-pregnant population, maternal exercise would result in the production of myokines by the maternal

skeletal muscle, which could then act to improve maternal physiology as well as act distantly on the placenta to improve its development and function, and to ultimately optimize fetal growth. In several organ systems, myokines demonstrate potent effects on nutrient transport and metabolism. In isolation, not in the context of exercise, some of these same cytokines and peptides found in the muscle secretome have demonstrated similar effects on nutrient transport and metabolism within human placenta tissue, along with effects on placental development. However, to date, no group has collectively examined the myokine profiles of exercising pregnant women nor examined how this myokine secretome may affect placental development and function.

As we suggest in our published manuscript, to follow in chapter 2, exercise during pregnancy induces changes in maternal physiology, placental development and placental macronutrient transport that act in synchrony to optimize fetal growth. The exact mechanisms by which maternal exercise can alter placenta biology and function have yet to be fully understood. Although, it has been previously suggested that exercise regulates placental nutrient exchange to the fetus thereby optimizing fetal growth (Hopkins & Cutfield, 2011). There are various placental macronutrient transporters involved in glucose, lipid and amino acid transport that may be influenced by PA and improve nutrient supply to the fetus. However, the exact mechanisms by which maternal exercise can alter the expression and/or activity of these various nutrient transporters has not yet been explored. It is most likely a combination of exercise-induced changes in macronutrient transport and placental development/function that act in unison to promote optimal fetal growth. We propose that myokines secreted by exercising muscle may be the exercise-induced factors acting at the level of the placenta to enhance

placental development and function as well as the expression and/or activity of relevant placental macronutrient transporters and thus, have a role in optimizing fetal growth during pregnancy.

1.7 HYPOTHESES

The overarching hypothesis of this thesis is that in response to exercise, contracting maternal skeletal muscle cells secrete myokines which optimize fetal growth, at least in part through enhanced placental development/function and macronutrient transport to the developing fetus.

1.7.1 Specific hypotheses of this thesis

Hypothesis 1: Physically active pregnant women will demonstrate a distinct plasma myokine profile that differs from that of non-active pregnant women.

Hypothesis 2: Myokines secreted from maternal skeletal muscle during exercise will result in improved metrics of placental health and function as well as differences in expression and localization of GLUT-4, a key glucose transporter in the 1st trimester placenta.

1.8 RESEARCH AIMS

1.8.1 Specific research aims of this thesis

Aim 1: To compare the 2nd trimester circulating myokine profiles in the plasma of both physically active and non-active pregnant women.

Aim 2: To determine the impact of exercise-induced SPARC myokine exposure on placental health and function, and on placental GLUT-4 transport.

Sub Aim 2.1: Using an *ex vivo* human placenta explant model, determine the impact of exercise-induced SPARC myokine exposure on metrics of placental health and function, including proliferation, viability, apoptosis, cellular invasion and hormone production.

Sub Aim 2.2: Using an *ex vivo* human placenta explant model, determine the effects of exercise-induced SPARC myokine exposure on the expression and localization of placental GLUT-4.

1.9 PRESENTATION OF THESIS

The following Master of Science thesis conforms to the regulations provided by the University of Ottawa, Faculty of Health Sciences, School of Interdisciplinary Health Sciences and the University of Ottawa, Faculty of Graduate and Postdoctoral Studies. This thesis is presented in manuscript format. **Chapter 1** provides an overview of the current thesis as well as a review of the literature that is not covered in Chapter 2 (manuscript #1). **Chapter 2** provides a review of the literature for the remaining topic areas related to this thesis through the first published manuscript entitled “*A role for maternally derived myokines to optimize placental function and fetal growth across gestation*” (*Appl Physiol Nutr Metab.* 2017 May;42(5):459-469). **Chapter 3** presents the second prepared manuscript entitled “*SPARC is an exercise-induced myokine elevated in the plasma of active pregnant women*”. This manuscript has been prepared for submission to the *Journal of Applied Physiology*. **Chapter 4** begins with a preamble followed by the third prepared manuscript entitled “*The effect of SPARC on the health and functioning of first trimester human placentae*”. This manuscript has been prepared for submission to the journal *Placenta*. This thesis will conclude with **Chapter 5**, which provides the reader with a summary

and discussion of findings, limitations, future research directions, clinical applications and final conclusions.

Chapter 2

Manuscript 1: *A role for maternally derived myokines to optimize placental function and fetal growth across gestation*

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A role for maternally derived myokines to optimize placental function and fetal growth across gestation

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Abstract: Exercise during pregnancy is associated with improved health outcomes for both mother and baby, including a reduced risk of future obesity and susceptibility to chronic diseases. Overwhelming evidence demonstrates a protective effect of maternal exercise against fetal birth weight extremes, reducing the rates of both large- and small-for-gestational-age infants. It is speculated that this protective effect is mediated in part through exercise-induced regulation of maternal physiology and placental development and function. However, the specific mechanisms through which maternal exercise regulates these changes remain to be discovered. We hypothesize that myokines, a collection of peptides and cytokines secreted from contracting skeletal muscles during exercise, may be an important missing link in the story. Myokines are known to reduce inflammation, improve metabolism and enhance macronutrient transporter expression and activity in various tissues of nonpregnant individuals. Little research to date has focused on the specific roles of the myokine secretome in the context of pregnancy; however, it is likely that myokines secreted from exercising skeletal muscles may modulate the maternal milieu and directly impact the vital organ of pregnancy—the placenta. In the current review, data in strong support of this potential role of myokines will be presented, suggesting myokine secretion as a key mechanism through which maternal exercise optimizes fetal growth trajectories. It is clear that further research is warranted in this area, as knowledge of the biological roles of myokines in the context of pregnancy would better inform clinical recommendations for exercise during pregnancy and contribute to the development of important therapeutic interventions.

Key words: placenta, myokines, physical activity, exercise, pregnancy, skeletal muscle, physiology, development, transport, fetal growth.

Résumé : L'activité physique durant la grossesse est associée à une meilleure santé du bébé et de la mère et à une diminution du risque d'obésité ultérieure et de prédisposition aux maladies chroniques. Des preuves irréfutables indiquent l'effet protecteur de l'activité physique de la mère, contrant ainsi les poids extrêmes du fœtus, ce qui abaisse le taux de nourrissons gros et petits pour leur âge gestationnel. L'effet protecteur serait, selon toute spéculation, médié en partie par la régulation de la physiologie maternelle et le développement/fonction du placenta induits par l'exercice physique. Toutefois, on ne connaît pas encore les mécanismes spécifiques par lesquels l'exercice maternel régule ces modifications. Nous posons l'hypothèse selon laquelle les myokines, un ensemble de peptides et de cytokines sécrétés par le muscle squelettique en contraction au cours d'un exercice physique, seraient un important maillon manquant dans ce phénomène. Les myokines sont connues pour diminuer l'inflammation, améliorer le métabolisme et augmenter l'activité/expression des transporteurs des macronutriments dans divers tissus d'individus non gravides. Il y a peu d'études sur les rôles spécifiques des myokines dans le sécrétome en rapport avec la grossesse; toutefois, selon toute vraisemblance, les myokines sécrétés par le muscle squelettique à l'effort pourraient moduler le milieu maternel et avoir un impact direct sur le placenta, organe vital de la grossesse. Dans cette analyse documentaire, on présente des données solides à l'égard du rôle potentiel des myokines et de leurs sécrétions constituant un important mécanisme par lequel l'exercice maternel optimise les trajectoires de la croissance fœtale. De toute évidence, il faut réaliser d'autres études dans ce domaine ; les connaissances des rôles biologiques des myokines dans le contexte de la grossesse concourent à de meilleures recommandations cliniques en matière d'activité physique durant la grossesse en plus de contribuer au développement d'importantes interventions thérapeutiques. [Traduit par la Rédaction]

Mots-dés : placenta, myokines, activité physique, exercice physique, grossesse, muscle squelettique, physiologie, développement, transport, croissance fœtale.

Introduction

Gestation is the period between conception and birth, during which a one-cell zygote divides and develops into a fetus with fully formed and functional organ systems. This rapid period of fetal growth is accommodated by considerable adaptations to maternal physiology, including a 40%–50% increase in maternal blood

volume, increased cardiac output, systemic vasodilation, and maternal hyperinsulinemia to maintain normal glucose homeostasis (Hyttén and Paintin 1963; Sibai and Frangieh 1995; Baz et al. 2016). These maternal adaptations serve to increase placental blood flow, and maternal–fetal nutrient and gas exchange. Regular physical activity during this period can further modify maternal physiology

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and is associated with reductions in obstetrical complications, optimized fetal growth trajectories, and improved long-term health outcomes for mothers and their offspring (Ferraro et al. 2012).

The placenta is the vital organ of pregnancy, across which all maternal–fetal exchange takes place and, as such, plays a critical role in dictating fetal growth. Processes of early placentation, including uterine invasion by the placental trophoblast cells and establishment of a robust utero–placental circulation, are critical to ensuring optimized nutrient and gas delivery to the developing fetus. Across gestation the materno–fetal exchange surface area within the placenta expands from $\sim 1.5 \text{ m}^2$ in the first trimester up to 14 m^2 in the third trimester (Jackson et al. 1992), as the placental villous trees encasing the fetoplacental vasculature continue to branch and mature. Specific transporters for all major classes of macronutrients (i.e., glucose, lipids, and amino acids (AAs))—the building blocks for fetal growth—are present in abundance along the surface of the placental villous trees, and their expression and activity are directly associated with increased fetal growth (Jansson and Powell 2000). It has been postulated that physical activity during pregnancy not only improves maternal physiology to enhance utero–placental perfusion, but may play a more direct role in optimizing fetal growth by regulating critical processes involved in placental development and function. However, the specific mechanisms through which maternal exercise can directly modulate these processes within the placenta remain to be discovered.

In the current review, the potential role of maternally derived myokines as important regulators of placental development and function will be presented. Myokines, a collection of peptides and cytokines secreted from contracting skeletal muscles during exercise, which will henceforth be referred to as the “myokine secretome”, have beneficial effects on metabolism and macronutrient transport in nonpregnant tissues including the brain, liver, pancreas, skeletal muscle, and adipose tissue (Ellingsgaard et al. 2011; Pedersen 2011). While similar effects are likely present in the context of pregnancy, the exercising myokine secretome has not been adequately assessed in pregnant women nor have the effects of this secretome on placental function and (or) fetal growth been fully investigated. We hypothesize that myokines may be important signalling molecules acting at the level of the placenta linking maternal exercise to optimized fetal growth trajectories.

Exercise during pregnancy

Exercise is an important part of a healthy pregnancy. Studies have consistently demonstrated that regardless of prepregnancy activity levels, women with uncomplicated singleton pregnancies are at a low risk of adverse maternal and fetal exercise-related events (i.e., gestational hypertension and preterm birth) (Bredin et al. 2013; Cid and González 2016). With these findings, the previous contention that pregnancy is a medical condition requiring a sedentary lifestyle has been strongly disproven (Dempsey et al. 2005). Published literature now demonstrates numerous positive health benefits associated with maternal exercise, as detailed below, and has led to the encouragement of physical activity throughout gestation. Despite this evidence, most pregnant women still fail to meet the recommended levels of physical activity during pregnancy (Borodulin et al. 2008). Among the many self-reported reasons for the lack of adherence to exercise guidelines (i.e., nausea and fatigue), safety concerns of participating in physical activity during pregnancy predominate in women and these concerns have been found to predict the amount and (or) intensity of exercise that they participate in throughout their pregnancy (Duncombe et al. 2009). As such, an increased focus must be placed on highlighting the overwhelming evidence of the health benefits of exercise during pregnancy to reassure mothers that the many benefits largely outweigh the risks.

In 2002, the Canadian Society for Exercise Physiology developed the Physical Activity Readiness Medical Examination for Preg-

nancy, a screening tool for health care providers to use to clear women to safely participate in exercise during pregnancy (Wolfe and Mottola 2002). In 2003, the Society of Obstetricians and Gynecologists of Canada and the American Congress of Obstetrics and Gynecology (ACOG) developed recommendations for exercise during pregnancy and the postpartum period, recommending 30 min of aerobic exercise 4 times per week in uncomplicated pregnancies (i.e., in the absence of absolute contraindications like preeclampsia) (Artal et al. 2003; Davies et al. 2003). Currently, the ACOG guidelines recommend that women participate in physical activity on most, if not all, days of the week (The ACOG 2015). At present, an expert panel is working on updating the Canadian guidelines as well.

The benefits of exercise during pregnancy are extensive for the mother and her offspring, extending into the long term for both. Strong epidemiological evidence indicates that regular exercise during pregnancy prevents excessive maternal gestational weight gain and thus minimizes postpartum weight retention and the risk of developing health problems like diabetes, metabolic syndrome, and cardiovascular disease (Walker 2007; Ferraro et al. 2012; Nascimento et al. 2014). As reviewed, maternal exercise also minimizes the risk of developing pregnancy-specific conditions including gestational diabetes mellitus (GDM), and preeclampsia, a hypertensive disorder of pregnancy (Ferraro et al. 2012). The maternal health benefits achieved from engaging in exercise highlight the importance of promoting an active lifestyle before, during, and after pregnancy for all women. Further, the offspring of exercising mothers overwhelmingly benefit from the effects of physical activity during pregnancy. The offspring demonstrate optimized birth weights and body composition, with reduced birth weight extremes and reduced fat mass at birth (within the healthy range), and they maintain this morphometric profile into early childhood, with reduced rates of later childhood obesity (Clapp 1996). Evidence suggests that exercise later in pregnancy prevents excess fetal growth as women who maintain regular exercise during the second and third trimesters of pregnancy have a reduced risk of delivering a large-for-gestational-age (LGA) infant (Owe et al. 2009; Juhl et al. 2010; Hopkins and Cutfield 2011). Importantly, this decreased risk of LGA is not accompanied by an increased risk of delivering a small-for-gestational-age (SGA) infant (Hopkins and Cutfield 2011), but instead with a reduced risk of SGA (Juhl et al. 2010). Thus, exercise reduces the risk of birth-weight extremes while promoting appropriate-for-gestational-age offspring. This is important as children born at both birthweight extremes are at an increased risk of later obesity and subsequent comorbidities (i.e., diabetes and cardiovascular disease) (Pettitt and Jovanovic 2001; Oken and Gillman 2003; Whitaker 2004). The specific mechanisms underpinning the regulation of fetal growth by maternal exercise are still unclear but are likely the combined result of improved maternal physiology and optimized development and function of the placenta.

Potential mechanisms of optimized fetal growth mediated by maternal exercise

Exercise-induced adaptations to maternal physiology

Exercise during pregnancy results in several adaptations to maternal physiology. These changes are designed to protect the developing fetus and to ensure that both the needs of the mother and the fetus are met. For example, exercise during pregnancy is associated with changes in maternal blood glucose levels (Clapp and Capeless 1991; Clapp et al. 1995). In response to a bout of maternal exercise after 23 weeks of gestation, the increase in skeletal muscle glucose oxidation and decrease in hepatic glucose production results in a transient reduction in blood glucose levels (not associated with exercise intensity), likely due to increasing fetoplacental demands as pregnancy progresses (Clapp and Capeless 1991). This reduction in blood glucose levels in pregnancy

differs from the typical transient hyperglycemic response observed in nonpregnant individuals upon engaging in physical activity (Zinker et al. 1990). As reviewed by Ferraro et al. (2012), aerobic exercise has further been associated with improved insulin sensitivity in pregnant women, enhancing overall glycemic control and reducing the risk of developing GDM. Energy expenditure during exercise also increases during pregnancy. Total energy expenditure is represented by oxygen uptake ($\dot{V}O_2$), the sum of the calories used at rest and during participation in physical activities. Total energy expenditure increases by up to 19% in the third trimester of pregnancy (compared with nonpregnant individuals) due to gestational weight gain (Butte et al. 2004). As such, as pregnancy progresses the metabolic demands of exercise increase in proportion to the total amount of weight gained (O'Toole 2003). The maternal increase in oxygen consumption during pregnancy is essential to meet the energy demands associated with increased maternal body weight and increased work of breathing and to ensure that the fetus has a constant supply of energy for growth and development (O'Toole 2003). Further, maternal cardiovascular adaptations occur during pregnancy to ensure adequate delivery of oxygen and nutrient-filled blood to the developing fetus. As reviewed by Barakat et al. (2015), these adaptations consist of overall increases in maternal blood volume, heart rate, stroke volume, and cardiac output to enhance utero-placental perfusion, promoting fetal growth. During exercise, the chest wall of pregnant women also changes (i.e., increased elasticity and expansion) to enhance the capacity for ventilation as the fetus increasingly places pressure on the maternal diaphragm resulting in laboured breathing (Soultanakis et al. 1996). Although blood flow to skeletal muscle is enhanced during exercise, adequate delivery of oxygen and nutrients to the fetus is maintained, with no harms to the fetus (Clapp et al. 1995; Artal and Sherman 1999; Artal et al. 2003). These changes in maternal biology in response to exercise ultimately optimize fetal growth by providing the developing offspring with the appropriate substrates for growth.

Exercise-induced alterations in placental development

In addition to adaptations to maternal physiology, exercise during pregnancy can improve critical processes involved in placental development. Total placental volume, via ultrasound and gross measurements, is increased across gestation in exercising women. The rise in mid-pregnancy placental growth rates is estimated to be ~24% higher in exercising compared with sedentary pregnant women (Clapp et al. 2000a; Clapp 2003). Importantly, these changes in overall placental volume have been histologically attributed to changes in “functional” placental volume, with the largest increases seen in the terminal components of the placental villous trees (Clapp et al. 2000a). These terminal villi are active in maternal-fetal exchange as opposed to villi in the stem or intermediate components of the villous trees, which act as conduits for the larger fetal umbilical vessels (Clapp et al. 2000a). Detailed histomorphometric investigations of placental biopsies also demonstrate greater total vascular volume and total capillary volume in the placental villous trees of women regularly engaging in exercise (Jackson et al. 1995), coupled with an increased presence of proliferating trophoblast and endothelial cells (Bergmann et al. 2004). Taken together these findings are indicative of a pro-angiogenic effect of maternal exercise on the developing placenta. Based on previous findings regarding the effects of exercise on angiogenesis and placental perfusion (Feldser et al. 1999; Clapp et al. 2000b; Zygmunt et al. 2002; Herr et al. 2003), it has been speculated that this effect may be an adaptive response of the placenta to reduced tissue oxygenation and (or) glucose availability during bouts of vigorous exercise, resulting in activation of hypoxia-inducible transcription factors and stimulation of the Vascular endothelial growth factor-insulin-like growth factor 2 (VEGF-IGF II) pro-angiogenic pathways. However, the 10%–50% baseline augmentation in pregnancy-associated increases in

plasma volume, placental intervillous space blood volume, placental volume, and cardiac output observed in pregnant women who regularly participate in sustained weight-bearing exercise during pregnancy helps to buffer some of the acute reductions in placental and fetal oxygen content measured following bouts of acute maternal exercise typically observed in sedentary women (Clapp 2003). As reported by Davenport et al. (2016), current evidence suggests that blood flow delivery to the fetus is maintained during exercise, although the mechanisms underlying the protection of blood flow to the fetal unit are largely unknown. Current studies have found that the fetal heart adapts to exercise training, resulting in fetal cardiovascular adaptations to maternal exercise, including fetal heart rate modulation and improved autonomic control (May et al. 2010; Moyer et al. 2015). Studies have identified positive correlations between fetal heart adaptability and heart rate variability in response to maternal aerobic and resistance exercise training during pregnancy, with more fetal cardiovascular adaptations occurring in response to exercise of higher intensity and of longer duration (May et al. 2012). The occurrence of exercise-related events concerning fetal heart rate such as bradycardia (the slowing of fetal heart rate), a protective mechanism in response to maternal hypoxia, has been found to be low in relation to the number of exercise hours and in all instances, appears to be transient, with fetal heart rate always returning to normal in the postexercise period (Charlesworth et al. 2011).

Collectively, adaptations to placental structure observed in regularly active women are likely driven by additional exercise-induced mechanisms that warrant further investigation. What is clear, however, is that maternal exercise is capable of modulating placental development in such a way as to enhance the capacity for maternal-fetal exchange. It is therefore no surprise that these exercise-induced increases in functional placental volume and vascular development ensure adequate nutrient and oxygen delivery to the fetus to support optimal fetal growth trajectories (Clapp et al. 2000a). The proper early development and structure of the placenta is certainly paramount to successful fetal growth, but ultimately the optimized functioning of this organ across pregnancy will dictate fetal growth trajectories.

Exercise-induced alterations in placental macronutrient transport

There is a growing body of evidence suggesting a protective role of maternal exercise on several critical functions of the placenta across gestation that could serve to optimize fetal growth. With proper nutrition, the fetus is provided with adequate nutrient supply during bouts of maternal physical activity (Bessinger and McMurray 2003; Gavard and Artal 2008). The exact mechanisms by which maternal exercise mediates changes in substrate delivery across the placenta via alterations in the expression and (or) activity of placental macronutrient transporters are currently unclear. However, there is mounting evidence to support an “exercise-sensing role” for the mammalian target of rapamycin (mTOR) pathway, which is known to integrate maternal, fetal, and placental signals (i.e., growth factors, stress, energy status, oxygen, and AA) to regulate macronutrient transporter expression and activity (Roos et al. 2007, 2009; Laplante and Sabatini 2012). Jansson and Powell (2013) have suggested a “placenta nutrient sensing model” suggesting that mTOR integrates these signals from the mother and fetus to regulate fetal growth (Roos et al. 2009; Laplante and Sabatini 2012). Inhibition of the mTOR signalling pathway significantly reduces the activity of placental AA transporters (Roos et al. 2007, 2009). In the placentas of active pregnant women, the expression of members of the placental mTOR signalling pathway (i.e., positive regulators IGF1 and IRS1; negative regulators PRKAB1 and TSC2) are lowered (Brett et al. 2015). In fact, moderate-to-vigorous physical activity in pregnant women is inversely correlated with the expression of mTOR and IGF-1, a peptide that plays an important role in fetal growth

(Chiesa et al. 2008). Reduced expression of positive or negative regulators of mTOR, if accompanied by reduced protein levels, could result in either a reduction or increase in placental mTOR signalling, respectively (Brett et al. 2015). Because no differences in birthweights were observed between the offspring of active and inactive pregnant women, physical activity during pregnancy appears to optimize mTOR signalling and nutrient delivery to the fetus to optimize neonatal body composition and normalize fetal growth, protecting against fetal birth weight extremes (Brett et al. 2015). Thus, in response to exercise during pregnancy, mTOR is either upregulated in response to excess maternal nutrient availability to limit excess nutrient delivery to the fetus or downregulated when nutrient availability is limited, suggesting that the placenta matches fetal growth with the availability of maternal nutrients. The mechanisms through which the placenta may in fact 'sense' exercise during pregnancy to modulate the mTOR signalling pathway and subsequent placental functions is an area ripe for further investigation.

Exercise can both positively and negatively alter gene expression and (or) activity of many macronutrient transporters within the placenta, including transporters for lipids, AAs, and glucose. Pregnant women who meet exercise guidelines during the second trimester of pregnancy demonstrate a nearly 2-fold lowered mRNA expression of the free fatty acid (FA) transporter protein 4 (FATP4) compared with sedentary women (Brett et al. 2015), with no measurable changes in expression of any of the other FA transporters. This is particularly intriguing as FATP4 is considered the primary placental transporter for long-chained polyunsaturated FAs (Larqué et al. 2006), the FAs responsible for the rapid fetal fat accumulation in the third trimester (Haggarty 2010). Thus, lower FATP4 expression suggests a reduced potential for the transport of FAs to the fetus, a notion that is supported by the reduced neonatal fat mass observed in the offspring of exercising pregnant women (Clapp and Capeless 1990; Harrod et al. 2014). Placentas from these same physically active women also demonstrate an increase in mRNA expression of a key sodium-dependent neutral AA transporter, System A (SNAT2) (Brett et al. 2015). Placental SNAT2 expression is intimately linked to fetal growth profiles (Jansson and Powell 2006), with strong associations noted between insufficient placental SNAT2 expression and intrauterine fetal growth restriction (IUGR) (Mandò et al. 2013). It is postulated that the reduced potential for FA transport and increased potential for AA transport to the fetus in active pregnant women does not alter fetal birth weights, but rather improves neonatal body composition by increasing lean muscle mass and decreasing fat mass (Brett et al. 2015). Furthermore, when controlling for total sugar intake, moderate-to-vigorous physical activity has been inversely correlated with placental glucose transporter 1 (GLUT1) expression (Brett et al. 2015). This finding suggests a reduced potential for glucose transport to the fetus in active pregnant women. However, the strong positive correlation between maternal carbohydrate intake and the expression of GLUT1 also suggests increased potential for glucose transport to the fetus and provides evidence that the placenta acts as a nutrient sensor, altering glucose transport to match maternal carbohydrate availability (Jansson and Powell 2006; Brett et al. 2015). As demonstrated through these 3 examples, the effects of maternal exercise on macronutrient transporter expression and (or) activity appear to be transporter specific, with further evidence of differential responses to the maternal exercise in question (Brett et al. 2015). This complex regulatory dynamic may help explain the ability of maternal exercise to prevent fetal birth weight extremes. However, an important question still remains: how does maternal physical exercise—the process of repeat muscle contractions in the mother—directly signal to the feto-placental unit and result in optimized placental development and function? Myokines secreted from contracting skeletal muscle, may be a missing piece to this puzzle.

Myokines

During physical activity, skeletal muscle contractions stimulate the production and secretion of hundreds of peptides and cytokines, collectively known as myokines. Myokines participate in autocrine, paracrine, and endocrine signalling throughout the body (Pedersen et al. 2007). These exercise factors are known to mediate some of the beneficial health effects of exercise and protect against chronic diseases associated with low-grade inflammation and impaired metabolism (i.e., diabetes and cardiovascular disease) (Pedersen and Pedersen 2005). In nonpregnant individuals, myokines have been found to regulate cellular metabolism and macronutrient transport (i.e., promote lipid metabolism and glucose uptake in skeletal muscle and adipose tissue). In the context of pregnancy, there is a lack of work examining the effects of myokines, as a collective secretome, on maternal physiology and placental development and function. However, several individual cytokines, chemokines, and peptides identified within the myokine secretome have been found to alter placental development and function, including the expression and activity of placental macronutrient transport proteins. As such, we speculate that myokines may act as important signalling molecules between maternal skeletal muscle and the placenta, regulating maternal-fetal nutrient exchange and fetal growth. Although the exact mechanisms underlying the regulation of fetal growth in response to maternal exercise are still unclear, myokines may be one of the exercise-induced factors involved in promoting normal fetal growth trajectories, as they appear to have a potential role for improving maternal physiology and optimizing the development and function of the placenta. Evidence in support of this hypothesis for a few key constituents of the myokine secretome is presented below.

Interleukin-6

As the first myokine discovered (Pedersen and Febbraio 2008), the biological role of interleukin-6 (IL-6) has been extensively studied in the context of exercise physiology. Depending on the environment, IL-6 can act as an inflammatory cytokine produced by macrophages or as an anti-inflammatory myokine produced by exercising skeletal muscle (Pedersen and Febbraio 2008). IL-6 mRNA and protein expression as well as levels in circulation are highly elevated in response to acute exercise, increasing exponentially until reaching maximal plasma levels at the end of an exercise session (up to 100-fold) (Pedersen and Fischer 2007) and rapidly declining to basal levels in the postexercise period (Pedersen and Hoffman-Goetz 2000; Suzuki et al. 2002; Febbraio and Pedersen 2002). Several studies have provided evidence in support of the notion that skeletal muscle is responsible for the systemic increase in IL-6 in response to exercise. As reviewed by Pedersen and Febbraio (2012), this evidence is based on transcriptional analysis of IL-6, in situ hybridization, and immunohistochemistry of skeletal muscle biopsies, and microdialysis of exercising skeletal muscle. Further, arterial-femoral differences in IL-6 concentration across an exercising leg has shown that it is released in large amounts into circulation, accounting for the exercise-induced systemic increase in IL-6 (Steensberg et al. 2000). In contrast, chronic exercise training has been shown to decrease basal IL-6 levels and diminish the typical increase in IL-6 mRNA and plasma levels in response to endurance exercise in untrained men (Fischer et al. 2004). Endurance exercise has also been associated with enhanced gene and protein expression of the IL-6 receptor α , increasing the number of receptors and overall sensitivity to IL-6 (Keller et al. 2005).

IL-6 is believed to deliver the health benefits of regular exercise through its anti-inflammatory effects and ability to regulate lipid and glucose metabolism. IL-6 is thought to mediate the anti-inflammatory effects of exercise, in part through stimulating the release of anti-inflammatory cytokines IL-10 and IL-1 receptor an-

tagonist into circulation (Steensberg et al. 2003) and by inhibiting the production of tumour necrosis factor (TNF) α (Starkie et al. 2003). IL-6 is also thought to provide protection from TNF-induced insulin resistance (Petersen and Pedersen 2005). In skeletal muscle and adipose tissue, IL-6 stimulates glucose uptake and fat oxidation, partially via the activation of AMP-activated protein kinase (Kelly et al. 2004). IL-6 also increases whole-body lipolysis, fat oxidation, and FA re-esterification in the absence of hypertriglyceridemia (van Hall et al. 2003). Further, IL-6 exerts endocrine effects on the liver to induce hepatic glucose production (Febbraio et al. 2004; Pedersen 2009). These exercise-induced effects of IL-6 on glucose and fat metabolism and in reducing inflammation observed in nonpregnant individuals are likely to be mirrored in the pregnant population. The actions of IL-6 would provide an improved maternal milieu via enhanced glycemic control, use of nutrients and reduced inflammation to better support optimal fetal growth.

IL-6 has been shown to have important effects on glucose, AA, and FA transport in cultured skeletal muscle and in human placental trophoblast cells. In rat myotubes, IL-6 enhances glucose transport and translocation of GLUT-4 to the muscle plasma membrane, as well as insulin-stimulated glucose uptake (Carey et al. 2006). In another in vitro study, effects on glucose uptake via GLUT-4 were only observed at postexercise cytokine levels when IL-6 was combined with either IL-8 or IL-15, combinations likely present in circulation postexercise (Gray and Kamolrat 2011). GLUT-4 is also expressed in first trimester human placenta and appears to have a role in glucose uptake early on in pregnancy (as reviewed by Brett et al. 2014). Thus, IL-6 secretion in response to exercise early on in pregnancy may have an impact on placental glucose uptake via GLUT-4 and the subsequent transfer of glucose to the fetus, although this remains to be investigated. IL-6 has been shown to directly modulate placental FA transporters. In vitro studies have found that IL-6 stimulates FA accumulation by cultured primary human placental trophoblast cells via an unknown mechanism (Lager et al. 2011). FA accumulation by trophoblast cells in response to IL-6 could contribute to the adequate transfer of nutrients to the fetus during pregnancy (Lager et al. 2011). Interestingly, FATP4 mRNA expression is downregulated by long-term exposure to IL-6 (Lager et al. 2011), similar to the changes in FATP4 mRNA observed in the placentas of exercising women (Brett et al. 2015). With respect to AA transport, IL-6 at physiological concentrations has been found to increase the expression of SNAT2 in cultured human primary placental trophoblast cells, resulting in increased activity of system A (Jones et al. 2009). Interestingly, a similar 2-fold increase in SNAT2 expression is observed in the placentas of women adhering to the guidelines for exercise during pregnancy (Brett et al. 2015). This along with the known role of SNAT2 suggests that women who exercise during pregnancy may have increased potential for AA transport to the fetus via SNAT2 to meet the nutrient needs of the fetus and achieve optimal fetal body composition (Brett et al. 2015). It has previously been demonstrated that the activity of the insulin/IGF-1 and mTOR signalling pathways, system A activity, and protein expression of SNAT2 are increased in the placentas of obese women with LGA babies due to the overstimulation of AA transporters and excessive transfer of nutrients to the fetus (Jansson et al. 2013). In obese pregnant women, SNAT2 expression has also been positively correlated to maternal prepregnancy body mass index and birth weight, suggesting a mechanism linking maternal obesity to fetal overgrowth (Jansson et al. 2013). Thus, the decreased expression and activity of SNAT4 in obese compared with lean pregnant women (with no corresponding changes in infant birthweight) and unchanged expression of SNAT1 and SNAT2, suggests that obese pregnant women may have decreased transfer of amino acids and increased transport of free fatty acids (FFA) and (or) glucose across the placenta. We hypothesize that this could result in offspring of similar weight in obese and lean

women, with increased adiposity and lower lean mass in the offspring of obese mothers. Despite the contradictory results from the literature, which is likely the result of inadequately addressing other important contributors to energy balance (i.e., exercise and nutrition), some evidence suggests a possible link between maternal obesity, altered amino acid transport, and increased fetal growth. We propose that in response to exercise during pregnancy, the differential expression of macronutrient transporters in concert provides a specific compliment of nutrients to the fetus so that overall caloric exposure to the fetus and infant birthweight is maintained, but offspring body composition is largely improved (greater lean mass and decreased fat mass). We hypothesize that exercise-induced IL-6 myokine secretion (anti-inflammatory), shown to suppress TNF- α , could be regulating fetal growth by preventing the over-stimulation of these AA transporters and excessive AA uptake. In the context of pregnancy, increased maternal IL-6 levels in response to exercise may enhance glucose and AA transport and attenuate FA transport, to promote appropriate fetal growth trajectories and neonatal body composition (increased lean mass and decreased fat mass).

Interleukin-15

IL-15 is produced by skeletal muscle in response to exercise-induced contractions. Current published data are conflicting as to how IL-15 expression, protein level, and secretion from skeletal muscle are influenced by exercise type, duration, and intensity as well as the importance of individual activity level and health status (Görgens et al. 2015). Muscular IL-15 mRNA levels are upregulated 2-fold in skeletal muscle in response to a bout of resistance exercise whereas endurance exercise has been found to increase basal skeletal muscle IL-15 protein content by 40% (Nielsen et al. 2007; Rinnov et al. 2014). In these studies, neither resistance nor endurance exercise increased circulating plasma IL-15 (Nielsen et al. 2007; Rinnov et al. 2014). However, the subjects in these studies were previously active and it is possible that their basal IL-15 levels were already elevated. Other studies have found that acute endurance exercise increases systemic IL-15 levels (Riechman et al. 2004; Tamura et al. 2011), with the most significant effects resulting from acute, moderate intensity resistance exercise (Yeo et al. 2012). In both active and inactive individuals, no changes in resting circulating levels of IL-15 have been observed following any chronic exercise programs (Nieman et al. 2003; Riechman et al. 2004; Rinnov et al. 2014). Furthermore, although IL-15 is up-regulated in skeletal muscle in response to exercise, studies have yet to show that this directly contributes to the systemic increase in IL-15 concentration (Raschke and Eckel 2013). More work remains to be done to elucidate the effects of exercise on IL-15 expression, protein level, and secretion from skeletal muscle in humans.

IL-15 on its own is thought to play a role in modulating skeletal muscle and adipose tissue mass as well as in enhancing glucose metabolism and insulin sensitivity. It has been described as an endocrine modulator of visceral fat mass (Pedersen 2009) and likely has a role in modulating fat-to-lean body composition and insulin sensitivity (Pedersen et al. 2007). A study with transgenic mice exhibiting muscle-specific oversecretion of IL-15 found that IL-15 protects against diet-induced obesity, improves insulin sensitivity, and increases oxidative muscle metabolism (Quinn et al. 2011). IL-15 secreted in response to exercise during pregnancy may enhance the maternal milieu by regulating maternal body composition, increasing insulin sensitivity, and improving skeletal muscle oxygen metabolism.

The exact impact of IL-15 on placental development and function is unknown. However, IL-15 appears to have a beneficial effect on the process of uterine invasion and spiral artery remodelling by placental trophoblast cells, a critical process in the establishment of a robust utero-placental circulation. In vitro, IL-15 has

been found to directly increase human placental trophoblast cell migration and invasion in a dose-dependent manner (Zygmunt et al. 1998). This work has been complemented with work in an IL-15 knock-out murine model, in which IL-15 was shown to be essential for the recruitment of uterine natural killer cell to the uterine wall, a process required for the appropriate priming of uterine blood vessels for remodelling by the invading trophoblast cells (Barber and Pollard 2003). Pregnant IL-15 knock-out mice gave rise to growth restricted fetuses, secondary to insufficient remodelling of the uterine spiral arteries and reduced placental perfusion (Barber and Pollard 2003). Extrapolating these findings to the potential roles of exercise-secreted IL-15 in active pregnant women, we could speculate that IL-15 in the myokine secretome may enhance natural killer cell recruitment to the maternal decidua and increase early uterine trophoblast invasion, collectively augmenting the capacity of the utero-placental circulation and improving placental perfusion across gestation.

Similar to IL-6, IL-15 appears to have a role in skeletal muscle glucose metabolism and has been associated with increases in GLUT-4 mRNA expression and glucose uptake in C2C12 mouse myoblasts (Busquets et al. 2006; Gray and Kamolrat 2011). However, the effects on glucose uptake via GLUT-4 were only observed at postexercise cytokine levels when combined with IL-6, a combination likely to exist in circulation postexercise (Gray and Kamolrat 2011). Placental GLUT-4 has a role in glucose uptake early on in pregnancy, with GLUT-1 playing a greater role later in gestation. Thus, acute exercise-induced increases in IL-15 in the first trimester may have an impact on placental glucose uptake via GLUT-4 and the transfer of glucose to the developing fetus, although this warrants further investigation. IL-15 is a promising candidate for beneficial effects on placental development and function in response to its exercise-induced release during pregnancy.

Fractalkine/CX3CL1

Fractalkine (FKN/CX3CL1) is a newly described myokine whose expression and systemic concentration are elevated in humans in response to acute endurance exercise (Catoire et al. 2014). FKN/CX3CL1 is known to exert its effects locally to promote skeletal muscle regeneration, but it is unclear if the increase in circulating FKN/CX3CL1 levels postexercise results in other systemic effects (Catoire and Kersten 2015). One potential systemic effect of relevance may be the ability of FKN/CX3CL1 to stimulate cellular glucose uptake, as previously reported in pancreatic cancer cells (Ren et al. 2013). Deficiencies in FKN/CX3CL1 signalling have further been implicated in pancreatic islet β -cell dysfunction, insufficient insulin secretion, and decreased glucose uptake in cases of type 2 diabetes (Lee et al. 2013). While these findings have been reported outside the context of FKN/CX3CL1 as a myokine, they certainly provide an indication as to its potential role and securing its candidacy as a systemic regulator of glucose metabolism and insulin sensitivity in active individuals. This is particularly intriguing in the current discussion, considering the significance of appropriate maternal glycemic control during pregnancy to ensure optimal fetal growth.

Studied in isolation, FKN/CX3CL1 has emerged as a potent regulator of early placental development. Investigation of early implantation sites demonstrates abundant expression of FKN/CX3CL1 within the uterine decidual tissues and concurrent expression of the receptor for FKN/CX3CL1 on the surface of invading placental trophoblast cells, particularly those trophoblast cells responsible for remodelling the uterine spiral arteries into high conductance vessels (Hannan et al. 2006). In vitro data have demonstrated an increase in trophoblast adhesion molecules, an increase in secretion of extracellular matrix (ECM) proteases and a dose-dependent increase in trophoblast migration following FKN/CX3CL1 exposure (Hannan et al. 2006; Hannan and Salamonsen 2008). Taken together, these data indicate an important role for this chemo-

kine in the early invasion of the uterine wall by trophoblast cells and establishment of the utero-placental circulation. FKN/CX3CL1 and its receptor are also found in abundance within fetoplacental vascular endothelial cells (Szukiewicz et al. 2013). Given the known roles of FKN/CX3CL1 in the promotion of endothelial cell proliferation and capillary tube formation in vitro (Ryu et al. 2008), and the more recent findings of FKN/CX3CL1-mediated placental microvasculature remodelling in diabetic pregnancies (Szukiewicz et al. 2013), an important role for FKN/CX3CL1 in the development of the fetoplacental vasculature has additionally emerged. To date, no functional studies have been performed on placental tissues in response to FKN/CX3CL1. However, we would speculate that increases in systemic FKN/CX3CL1 levels from maternal exercise in pregnancy may serve to further promote placental development to optimize gas and nutrient supply to the developing fetus and thus aid in the optimization of fetal growth trajectories in exercising women.

Irisin

Irisin is a highly controversial myokine. Whether irisin is in fact secreted by human skeletal muscle has been called into question recently due to a reported lack of specificity in the enzyme-linked immunosorbent assay (ELISA)-based detection method used to measure irisin in several publications on this topic (Albrecht et al. 2015). This may serve to explain the inconsistencies reported in regards to circulating irisin concentrations following exercise (Catoire and Kersten 2015). However, in a recent study using tandem mass-spectrometry as a highly sensitive and more specific method of irisin detection, aerobic interval training was shown to increase circulating concentrations of irisin by ~15% (Jedrychowski et al. 2015). As reviewed by Chen et al. (2016), irisin could mediate some of the beneficial effects of exercise by regulating the browning of white adipose tissue (Stanford et al. 2015), improving lipid and glucose metabolism, and reducing insulin resistance as well as treating related metabolic diseases in humans and mice. Irisin has also been associated with more favourable lipid profiles (Oelmann et al. 2016). In mice, irisin secreted by contracting skeletal muscle induces the browning of white adipose tissue by up-regulating the expression of uncoupling protein 1 and by stimulating mitochondrial biogenesis in adipose tissue (Boström et al. 2012; Liu et al. 2013), increasing overall energy expenditure as well as reducing both weight and insulin resistance (Boström et al. 2012; Højlund and Boström 2013). Future studies are necessary to identify if these effects in mice can be translated to humans.

Very limited work has focused on the potential roles of irisin in the context of pregnancy. Placental expression of fibronectin type III domain-containing protein 5, the precursor of irisin, has been localized in uterine decidual tissues, in invading trophoblast cells and in the exchange region (villous trees) of the placenta across gestation (Garcés et al. 2014). Further, circulating concentrations of irisin have been measured in the serum of healthy pregnant women across pregnancy, with an increase of 16% and 21% in the second and third trimesters, respectively (Garcés et al. 2014). Of clinical importance, levels of circulating irisin measured in umbilical cord blood at the time of delivery are positively correlated with offspring birth weight (Joung et al. 2015), with the lowest irisin concentrations observed in infants demonstrating evidence of IUGR (Baka et al. 2015). Whether irisin confers any advantage to the processes involved in placental development and (or) function is currently unknown. However, the associations between circulating irisin concentrations and fetal growth profiles are certainly intriguing and demand further attention. These relationships also suggest a potential role for exercise-induced irisin secretion, as part of the myokine secretome, to further improve maternal and (or) fetoplacental physiology in such a fashion as to optimize fetal growth in utero.

Table 1. Other exercise-induced myokines that may play a role in optimizing fetal growth.

Myokine	Response to exercise	Potential effects on maternal physiology	Potential effects on placental development	Potential effects on placental function
IL-8	Skeletal muscle concentrations increase in response to strenuous exercise (Nieman et al. 2001, 2003, 2005; Marklund et al. 2013) Small and transient net release following exercise that does not contribute to an increase in circulating plasma levels (Åkerström et al. 2005)	Chemoattracts and degranulates neutrophils to mediate inflammation (Pedersen et al. 2007) Stimulates angiogenesis (Pedersen et al. 2007)	Promotes trophoblast cell migration (Hanna et al. 2006), viability, proliferation, and invasion (Jovanović et al. 2010)	Direct effects of IL-8 on placental function unknown Stimulates skeletal muscle glucose transport via AMPK phosphorylation during exercise (Gray and Kamolrat 2011)
LIF	Skeletal muscle mRNA expression is elevated following acute bouts of exercise (Broholm et al. 2008) and heavy resistance exercise (Broholm et al. 2011) Skeletal muscle protein levels and circulating levels are unchanged in response to acute exercise (Broholm et al. 2008)	Mediates inflammation (Chodorowska et al. 2004) Promotes the growth and differentiation of various types of target cells (Chodorowska et al. 2004)	Facilitates trophoblast adhesion to uterine endometrial cells and regulates invasion of the uterine wall to allow for spiral artery remodelling (Tapia et al. 2008, Winship et al. 2015)	Direct effects of LIF on placental function unknown
BDNF	Skeletal muscle mRNA and protein expression are increased by acute endurance exercise (Matthews et al. 2009) Exercise-induced increases in circulating levels of BDNF attributed to production by the brain (Gold et al. 2003; Ferris et al. 2007; Matthews et al. 2009)	Enhances lipid oxidation in via the activation of AMPK (Matthews et al. 2009)	Both in vitro model in vivo paracrine signalling by the BDNF/TrkB system is important for placenta trophoblast growth and survival (Kawamura et al. 2009, 2011)	Direct effects of BDNF on placental function unknown

Note: IL-8, interleukin-8; AMPK, activated protein kinase; LIF, leukemia inhibitory factor; BDNF, brain-derived neurotrophic factor.

Other myokines

There are many other myokines that have been shown to respond to skeletal muscle contractions but for which evidence is either nonexistent or lacking to demonstrate a systemic increase. Therefore, it is unclear whether these myokines act locally in skeletal muscle alone, or whether they have the ability to act distally on distinct tissue beds to alter cellular function (i.e., the placenta). These myokines include: IL-7, IL-8, leukemia inhibitory factor, brain-derived neurotrophic factor, myostatin, decorin, myonectin, fibroblast growth factor 2, monocyte chemoattractant protein-1, angiopoietin-like 4, and secreted protein acidic and rich in cysteine. Of these “other myokines”, IL-8, leukemia inhibitory factor, and brain-derived neurotrophic factor when studied in isolation outside of the context of exercise physiology, have been found to have positive influences on processes of early placental development and (or) function across pregnancy (Tapia et al. 2008; Kawamura et al. 2009, 2011; Jovanović et al. 2010; Krishnan et al. 2013; Winship et al. 2015) (Table 1). As such, further investigation into the potential roles of these myokines as mediators of optimized fetal growth observed in exercising women is strongly encouraged. Specifically, determination of whether these myokines have the ability to exert endocrine function is paramount. If these myokines are found to act in an endocrine fashion, their ability to improve maternal physiology and the development and function of the placenta suggests that they may be a set of exercise-induced factors involved in the optimization of fetal growth across gestation.

Clinical application

Hundreds of myokines are secreted in response to exercise-induced skeletal muscle contraction. Many of these myokines

have important anti-inflammatory and metabolic effects, accounting for some of the protective benefits of engaging in regular exercise. For the vast majority of myokines, their effects in the context of pregnancy, and more specifically on the development and function of the placenta—the vital organ of pregnancy—remain unknown. For the handful of myokines that have been examined in isolation for their impact on the placenta, observed effects include enhanced aspects of early placental development, including increased trophoblast migration and uterine wall invasion, and establishment of robust utero-placental and fetoplacental circulations. Moreover, some of the individual chemokines and cytokines found in the myokine secretome are known to directly influence placental function across pregnancy, capable of regulating nutrient sensing and macronutrient delivery to the fetus. The current published literature suggests that IL-6, IL-15, FKN/CX3CL1, and irisin are elevated systemically in response to acute bouts of exercise in nonpregnant individuals. The specific intensities of exercise required to stimulate the production and secretion of these myokines in the nonpregnant population are not fully understood and for some vigorous and (or) endurance-type physical activity levels may be required. For the other myokines discussed in this review, the influence of the type and intensity of exercise on the levels of these myokines in circulation in nonpregnant individuals have yet to be fully characterized. As this is a newer area of research, the effects of exercise on the production secretion of myokines in the pregnant population remains to be investigated. At this time, we do not if the current recommendations of regular bouts of 20–30 min of moderate physical activity during pregnancy may influence the production and secretion of myokines. Without further investigations of myokines in the context of pregnancy, the impact of maternal exercise on the myokine

secretome, and the subsequent effects of myokines on the placenta and on the developing fetus cannot be discounted.

Current evidence overwhelmingly suggests that maternal exercise during pregnancy serves to optimize the uterine environment to allow for appropriate fetal growth, protecting against fetal growth extremes (SGA and LGA). We must now focus our attention on understanding the nuanced mechanisms underlying the beneficial effects of exercise on the placenta, and we speculate that the myokine secretome may hold many of the answers to this question. Through our discussion on the effects of myokines in the nonpregnant population and the effects of certain myokines in isolation on placental tissues, it is clear that myokines could be signalling molecules involved in regulating fetal growth via our proposed mechanisms of improved maternal physiology, enhanced placental development, and improved placental macronutrient transport. Awareness of the exact mechanisms through which myokines exert their effects will provide clinicians with important mechanistic knowledge to support public health guidelines and recommendations as well as appropriately educate pregnant women about the benefits of exercise during pregnancy. Knowledge about the specific mechanisms whereby exercise alters organ function and metabolism is also required to prescribe the appropriate form of exercise as therapy (Pedersen and Saltin 2006). Although we acknowledge that historically implementing lifestyle interventions during pregnancy has been very challenging and most often women are noncompliant, there is currently a lack of guidance with respect to exercise during pregnancy and a lack of promotion of exercise in the pregnant population as a whole. A better understanding of myokine signalling in the context of pregnancy may be helpful in public health messaging by offering an additional benefit of engaging in physical activity. This knowledge would be an important motivational for physicians to promote this type of lifestyle intervention during pregnancy and to encourage women to engage in these activities during their pregnancy. Further, understanding how myokine signalling modulates placental development and function may lead to the discovery of new therapeutic targets for pregnancy-specific diseases such as IUGR, GDM, and preeclampsia and thus improve maternal and fetal health outcomes. Additionally, this knowledge may contribute to the future development of other therapeutic interventions, which while recognizing the ongoing challenges with compliance to lifestyle interventions during pregnancy, may allow all pregnant women to benefit from the advantageous effects of myokine secretion during pregnancy on placental development and function and on the regulation of fetal growth.

Conclusion

It is well known that regular exercise is an important part of a healthy lifestyle. Most importantly, exercise during pregnancy confers health benefits for 2 individuals, both the mother and her developing fetus. We propose that myokines are important exercise factors produced and secreted by skeletal muscles during pregnancy and act as signalling molecules systemically, including at the level of the placenta, to optimize fetal growth. Specifically, we have proposed 3 potential mechanisms through which myokines may act to optimize fetal growth trajectories: namely, improvements in maternal physiology, placental development, and placenta macronutrient transport to the fetus. As maternal physical inactivity continues to prevail, understanding the mechanisms underlying the benefits of exercise during pregnancy are of utmost importance. This understanding would allow for the capitalization of the “teachable moment of pregnancy”, motivating women to engage in regular physical activity to benefit themselves as well as their baby.

Conflicts of interest statement

There are no conflicts of interest to declare.

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Chapter 3

Manuscript 2: *SPARC is an exercise-induced myokine elevated in the plasma of active pregnant women*

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Title: *SPARC is an exercise-induced myokine elevated in the plasma of active pregnant women.*

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Abbreviated Title: Elevated circulating SPARC concentrations in active pregnant women.

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SPARC is an exercise-induced myokine elevated in the plasma of active pregnant women.

Abstract

Introduction: Lifestyle interventions in pregnancy, such as exercise, have been associated with improved maternal-fetal health outcomes, including reduced fetal birthweight extremes.

Currently, the mechanisms whereby maternal exercise optimizes fetal growth remain unknown.

Secreted myokines have been associated with some of the major health benefits of regular

exercise; however, they have yet to be investigated in pregnancy. We hypothesize that

circulating exercise-induced myokines in active pregnant women may act on the placenta, the

site of maternal-fetal exchange, to optimize fetal growth. As a first step in confirming this

hypothesis, the aim of the current study is to compare the circulating myokine profile in active

vs. non-active pregnant women. **Methods:** Circulating profiles of 11 different myokines were

analyzed via multiplex assay in 2nd trimester maternal plasma samples, collected from women

characterized as active (N=14) or non-active (N=16) during pregnancy. Using the multiplex assay

format, IL-6 levels were undetectable in most samples, therefore a high sensitivity IL-6 single-

plex assay was also performed. **Results:** Active women demonstrated an elevation in circulating

SPARC concentration compared to non-active women ($86 \pm 19 \text{ ng/ml}$ vs. $52 \pm 18 \text{ ng/ml}$, $p=0.0001$).

No significant differences were observed for the other myokines analyzed. **Conclusion:** SPARC is

an exercise-induced myokine that is elevated in the circulation of active pregnant women. The

possibility that increased SPARC exposure in active pregnant women may improve placental

development and function, and ultimately fetal growth, is intriguing and requires further

investigation.

Key Words: SPARC, myokines, pregnancy, exercise, physical activity.

Introduction

Maternal obesity and excessive gestational weight gain (GWG), regardless of pre-pregnancy body mass index (BMI), are well-known risk factors for a myriad of adverse pregnancy outcomes (1, 26). Specifically, maternal obesity is associated with an increased risk of pregnancy-specific conditions like gestational diabetes mellitus (GDM) (33), pregnancy-induced hypertension (PIH) and preeclampsia (PE) (42). Further, maternal obesity and/or excessive GWG significantly increase the risk of delivering either a large-for-gestational-age or small-for-gestational age (SGA or LGA) infant (19, 37, 45, 57, 63, 64). Many national guidelines endorse regular physical activity (PA) as it mediates weight gain/maintenance at all ages, as well as predicts maternal obesity, excessive GWG (44), and GDM (25). These national guidelines, including those from the Society of Obstetricians and Gynecologists of Canada/Canadian Society for Exercise Physiology (SOGC/CSEP), and the American Congress of Obstetrics (ACOG), recommend that pregnant women participate in regular PA, as a safe preventative intervention (15, 58).

Exercise during pregnancy is associated with many improved maternal-fetal health outcomes. PA has been found to significantly reduce the risk of pregnancy-related complications, including GDM (16, 17) and PE (4, 16, 51, 54, 61), as well as help to manage GWG and reduce post-partum weight retention (43, 55). These effects associated with exercise work to promote maternal health and subsequently the health of the developing fetus. Exercise during pregnancy has also been found to directly benefit the fetus through improved growth and development. Maternal PA has been found to optimize fetal growth trajectories by promoting appropriate-for-gestational-age (AGA) birth weights (5, 25, 46). As such, fetal

birthweight extremes, both SGA and LGA infants, are reduced in exercising mothers (13, 25, 31, 46). These beneficial effects of PA on pregnancy outcomes have, in part, been attributed to adaptations of maternal physiology (i.e. optimized glucose metabolism, reduced inflammation, increased oxygen consumption, and increases in maternal blood volume, heart rate, stroke volume and cardiac output). However, as we have previously suggested (18), it is likely that these adaptations to the maternal *milieu* are complimented by adaptations to the feto-placental unit, including improvements in placental development and function. The mechanisms through which maternal exercise may directly impact the feto-placental unit to optimize fetal growth currently remain unclear, leaving this area ripe for further investigation.

During exercise, contracting skeletal muscles secrete hundreds of cytokines and peptides, collectively known as myokines (47). In the non-pregnant population, myokines are thought to mediate several protective effects of exercise. Participation in regular exercise has been associated with anti-inflammatory effects, with PA reducing both visceral fat mass and accompanying systemic low-grade inflammation (48), as well as improved metabolism, by decreasing the risk of insulin resistance and improving lipid profiles (14). These beneficial effects of exercise are thought to protect against the development of chronic diseases, including type 2 diabetes and cardiovascular disease (48).

To-date, the myokine secretome has only been studied in the non-pregnant population and has yet to be investigated in the context of pregnancy. Moreover, investigations into the potential protective effects of this exercise-induced secretome on the vital organ of pregnancy – the placenta – are limited. In isolation, individual myokines/cytokines have been found to have important effects on placental health and function, including the promotion of placental

cellular invasion (22, 23, 27, 30, 56, 60, 65), and the expression/activity of placental macronutrient transport proteins (8, 9, 21, 28, 34, 49). Therefore, it is hypothesized that the circulating myokine secretome of active pregnant women may act directly on the developing placenta, improving its health and function and ultimately optimizing fetal growth. As a first step towards confirming this hypothesis, the aim of the current study is to determine the unique myokine secretome profile of women characterized as active during pregnancy (as per Canadian Physical Activity Guidelines). The identification of specific myokines which become altered in the maternal circulation in response to exercise will help to inform future mechanistic studies aimed at determining the effects of exercise-induced myokines on placental health and function.

Materials and Methods

Study Participants

Maternal plasma samples were previously collected through the Maternal Obesity Management (MOM) and ActiveMOM trials (2). The primary research aim of the MOM trial was to determine whether a structured exercise and nutrition intervention program could optimize GWG during pregnancy (2). The aim of the ActiveMOM trial was to identify any associations between physical activity and the quality of maternal diet on the expression of macronutrient transporters (7). Women recruited into these trials were from the Ottawa area (Ontario, Canada), aged 18-40 with a pre-pregnancy BMI $\geq 18.5 \text{ kg/m}^2$ for the MOM trial and between 18.5 and 24.9 kg/m^2 for the ActiveMOM trial. The exclusion criteria included smokers, diabetics, or those with evidence of fetal growth restriction or pregnancy-induced hypertension. For both trials, women were categorized as either active or non-active as per the CSEP Canadian Physical

Activity Guidelines (59). Based on these guidelines, pregnant women were characterized as active if they participated in at least 150 minutes of aerobic MVPA per week in bouts of 10 minutes or more (59). Directly measured accelerometry data was used for categorization of women into active and non-active groups. The current study utilized plasma samples that were obtained during the 2nd trimester visits (25-28 weeks' gestation) from these two original trials. The study participants had not engaged in PA for at least 12 hours prior to sample collection. Included plasma samples were specifically selected from women with the highest and lowest PA extremes, for whom we had a 2nd trimester sample available (active, N=14; non-active, N=16).

Human Myokine Multiplex Assay

A custom Human Magnetic Luminex Screening Assay (R&D Systems, USA) was used to assess the circulating levels of a panel of known myokines/cytokines and peptides previously reported to be present in the exercise-induced myokine secretome (11, 12, 47, 52). The customized panel tested for the following analytes: IL-1ra, IL-6, IL-7, IL-8, IL-10, IL-13, IL-15, ANGPTL-4, SPARC, MCP-1/CCL2 and Fractalkine/CX3CL1. The maternal plasma samples were prepared and analyzed using a Bioplex 200 Suspension Array System (Bio-Rad, USA) as per the manufacturer's instructions. Analyte concentrations were calculated from the appropriate optimized standard curves using Bio-Plex Manager software version 6.0 (Bio-Rad, USA).

Ultrasensitive IL-6 Singleplex Assay

A Human IL-6 Ultrasensitive Magnetic Bead Kit and corresponding Human Ultrasensitive Buffer Reagent Kit (Thermo Fisher Scientific, USA) were used to assess the circulating levels of IL-6 in maternal plasma samples. As 80% of the plasma samples had IL-6 levels below the

threshold for detection in the original multiplex panel, a high sensitivity IL-6 kit was subsequently used. The plasma samples were prepared as per manufacturer's instructions and analyzed using a Bioplex 200 Suspension Array System (Bio-Rad, USA). IL-6 concentrations were calculated from the optimized standard curve using Bio-Plex Manager software version 6.0 (Bio-Rad, USA).

Statistical Analysis

Demographic, anthropometric, pregnancy outcome and PA continuous variables are presented as means $\pm$ SD. For continuous variables, normally distributed data was analyzed using a student's t-test and non-normally distributed data using a Mann-Whitney U test. Categorical variables were analyzed using chi-square analysis with Fisher's exact test. Concentrations (pg/ml) of individual myokines measured in the multiplex assay were compared in active vs. non-active samples using a student's t-test. Concentrations (pg/ml) of IL-6 measured in the singleplex assay were compared in active vs. non-active samples using a Mann-Whitney U test. Pearson's correlational analyses were performed to identify any linear relationships between physical activity levels and myokine concentrations for all study participants. For all statistical analyses, *p<0.05 was considered statistically significant.

Results

Maternal-fetal characteristics

No differences were observed in maternal-fetal characteristics (demographic, anthropometric and pregnancy outcome data) between the active vs. non-active study participants (**Table 1**). By virtue of the study design, differences in physical activity levels were

observed in the active vs. non-active pregnant women (**Table 1**) for MVPA ($p<0.0001$; **Figure 1A**) and TPA levels ($p<0.0001$; **Figure 1B**).

Myokine profiles in active vs. non-active pregnant women

Using the multiplex panel assay, SPARC was the only myokine found to be significantly elevated in the plasma of active compared to non-active pregnant women ($86\pm 19\text{ng/ml}$ vs. $52\pm 18\text{ng/ml}$, $p=0.0001$) (**Figure 2**). No significant differences were observed for any of the other myokines analyzed. As previously identified, most samples fell below the threshold of detection for the measurement of IL-6 using the multiplex panel. However, the ultrasensitive IL-6 singleplex assay also identified no differences in plasma IL-6 levels in active vs. non-active pregnant women ($p=0.8573$).

Correlation between circulating myokine concentrations and physical activity levels

Correlational analysis was performed for all myokine concentrations measured, with PA level assessed as a continuous variable (instead of the binary separation of active vs. non-active). Correlations with $*p<0.05$, were considered statistically significant. A positive correlation was observed with circulating SPARC concentrations and both MVPA ($r^2=0.5520$; **Figure 3A**) and TPA levels ($r^2=0.3727$; **Figure 3B**). A similar positive correlation was observed between circulating MCP-1/CCL2 concentrations and TPA ($r^2=0.1350$; **Figure 3C**). A negative correlation was observed with circulating ANGPTL-4 and MVPA levels ($r^2=0.1399$; **Figure 3D**). No correlation between circulating myokine concentrations and maternal physical activity levels was observed for the other myokines measured.

Discussion

This study aimed to compare the circulating myokine profiles of active vs. non-active pregnant women. We anticipated that we would identify a set of plasma myokines that differed between active and non-active pregnant women. However, only one of the 11 myokines measured was found to be significantly elevated in the circulation of active pregnant women. Specifically, SPARC was found to be elevated in 2nd trimester plasma samples of active vs. non-active pregnant women, identifying SPARC as a potentially important exercise-induced myokine in the context of pregnancy. While the make-up and protective effects of the myokine secretome has been detailed in the non-pregnant population, to the best of our knowledge, this is the first report of myokine profiling in the pregnant population. As such, the results of this study are an important first step towards identifying specific myokines (i.e. SPARC) which serve to improve maternal and fetal health outcomes in physically active women.

The presented finding of elevated SPARC concentrations in the plasma of active pregnant women is consistent with the published literature in physically active non-pregnant individuals. In this population, both acute exercise and exercise training results in measured increases in skeletal muscle SPARC expression (3, 12), with a corresponding ~1.5-fold increase in serum SPARC levels measured immediately following a bout of acute exercise (3). In the current study, active women exhibited a ~1.7-fold higher level of SPARC in their plasma compared to non-active pregnant women. Although, due to the original study designs of the MOM and ActiveMOM trials, these levels were not measured following an acute exercise intervention and we were therefore unable to obtain baseline and post-exercise plasma myokine profiles for direct comparison to the previously published literature. Because of this, it is likely that if we would have been able to measure SPARC in plasma collected immediately

following a bout of acute exercise, that we may have detected much higher levels of a variety of myokines.

SPARC, also known as osteonectin or BM-40, is a matricellular glycoprotein expressed in a variety of tissues during remodeling and repair (6, 20). Reported roles of SPARC include local effects on skeletal muscle development and muscle regeneration (29, 40), glucose metabolism (increased GLUT-4 expression in L6 myocytes) (53), insulin secretion (24), erythropoiesis (36), as well as an inhibitory effect on colon tumorigenesis by increasing apoptosis in colon cells (3). SPARC has only recently been investigated in the context of pregnancy. An *in vitro* study found that SPARC may play an important role in the regulation of normal placentation by promoting the invasion of extravillous trophoblast cells (27). Other studies have also identified a potential role for SPARC in regulating glucose metabolism in rat skeletal muscle cells (53) and thus, it is possible that SPARC could influence placental glucose transport to the fetus during pregnancy. Overall, these studies identify a potentially important role for SPARC in the optimization of placental development (i.e. cytotrophoblast cell invasion into the maternal decidua) and placental glucose transport. As it is clear that SPARC is elevated in the circulation of active pregnant women, it would be important for future investigations to examine the potential effects of SPARC in the context of pregnancy. Specifically, determining the impact of SPARC, if any, on the health and functioning of the placenta, the vital organ of pregnancy.

While the circulating concentrations of most myokines tested were similar across both the active and non-active pregnant groups and we had anticipated a more decisive difference, it must be considered that the myokine levels were measured using the plasma of women that were generally more or less active than one another and not following an exercise stimulus.

Thus, although this finding is intriguing as it is not perfectly in line with previous findings, it likely reflects our study design. Of all the myokines measured, this finding was particularly unexpected in the case of IL-6 - a widely reported exercise-induced myokine. As extensively reviewed by Catoire and Kersten, an abundance of literature has identified IL-6 as an important exercise-induced myokine that is elevated in plasma in response to exercise in the non-pregnant population (11). It is important to consider that in most of these studies blood samples were obtained from extremely active individuals and often following intense exercise interventions. The current study design categorized women to an “active” or “non-active” group, according to accelerometry data. These samples were not obtained immediately following a bout of exercise and certainly not high intensity exercise. Although the women categorized as “active” were significantly more active than their “non-active” counterparts, there were only a few women who fit the traditional model of highly active. Also, although we had hoped to be able to clearly differentiate a group of highly active vs. non-active pregnant women, it is important to consider that, in North America, few non-pregnant individuals are highly active and ever fewer whom are pregnant. Ideally we would have preferred access to a population of women training during their pregnancy vs. non-active or sedentary women, but we were limited by the women who participated in the original set of studies, which may contribute to the lack of difference in concentrations of circulating myokines.

In order to address this limitation in the sample set, circulating myokine concentrations were additionally examined in relation to levels of PA as a continuous variable. When this analysis was performed, concentrations of SPARC along with two additional myokines – MCP-1/CCL2 and ANGPTL4 – were found to correlate with maternal PA levels, measured either as daily minutes of MVPA or daily minutes of TPA. As SPARC was found to be significantly elevated

in the active group, it is not surprising that SPARC levels were positively correlated with all PA levels. This means that similar to the non-pregnant population, SPARC tends to increase in circulation in response to increases in all forms of PA (3). If a pregnant woman were to engage in exercise in early pregnancy, elevated SPARC levels in circulation could contribute to improved placentation and later on, potentially to improved glucose transport to the developing fetus.

Although the correlation was not as strong, MCP-1/CCL2 levels also tend to increase with TPA levels of pregnant women. It is difficult to directly compare our finding of a positive correlation between MCP-1/CCL2 and habitual PA to studies in the non-pregnant population because these studies have largely focused on exercise at higher intensities. Long-term moderate to high intensity exercise has been associated with decreased plasma levels of MCP-1/CCL2 in non-pregnant individuals (35, 38). In another study, an acute endurance exercise intervention also increased MCP-1/CCL2 levels (12). MCP-1/CCL2 is a known marker of inflammation and has a role in recruiting macrophages and other immune cells in response to skeletal muscle damage to initiate muscle repair (41, 62). Although currently unclear, the increase in MCP-1/CCL2 in response to acute exercise in non-pregnant individuals and small increase in response to habitual exercise in pregnant women suggests that it may have some type of systemic role (11). The significant p-value associated with this positive correlation suggests that there is a very small probability that this observation of a small positive correlation would have arisen if maternal MVPA and concentration of MCP-1/CCL2 were truly unrelated.

Our finding that ANGPTL-4 is negatively correlated with MVPA in pregnant women suggests that habitual engagement in higher intensity PA is associated with moderate

decreases in systemic ANGPTL-4. Our finding that ANGPTL-4 is not elevated in active pregnant women is consistent with the published literature (10). ANGPTL-4 is a protein that inhibits lipoprotein lipase and prevents the uptake of LPL-dependent free fatty acids (FFAs) into tissues (50). Exercise-induced increases in systemic ANGPTL-4 are known to be mediated by elevated levels of FFAs (32). As non-exercising muscle has been found to have significantly higher levels of ANGPTL-4 compared to exercising muscle (32), our observed negative correlation between ANGPTL-4 in plasma and PA levels likely reflects a systemic reduction in ANGPTL-4 in order to increase FFA availability, as a result of exercising skeletal muscle utilizing FFAs as fuel. This is suggestive of a potential role of ANGPTL-4 as a regulator of lipid homeostasis during exercise (11). The significant p-value associated with this small negative correlation suggests that there is a very small probability that this observation would have arisen if maternal TPA and concentration of ANGPTL-4 were truly unrelated.

Prior to our study, little was known about myokines in the context of pregnancy. This is the first study to examine the myokine profiles in active vs. non-active pregnant women as well as to investigate any associations between maternal PA and systemic myokine concentrations. Now that we have established a circulating plasma myokine profile for active and non-active pregnant women in the 2nd trimester and have identified SPARC as a potentially important exercise-induced myokine during pregnancy. Further investigations are warranted to identify the role of exercise-induced SPARC secretion in pregnancy as well as to determine the exact effects of SPARC on the placenta. We also recommend that future studies investigate myokine profiles in pregnant women after acute exercise for comparison to our findings that reflect chronic exercise involvement. An understanding of the mechanism(s) underlying the benefits of exercise during pregnancy will allow us to better capitalize on the “teachable moment” of

pregnancy and encourage women to engage in regular exercise across gestation. Furthermore, identifying which types of exercise promote systemic increases in specific myokines, along with an understanding of the effects of these myokines in the context of pregnancy, may contribute to the development of important therapeutic interventions that can deliver the health benefits of regular exercise to all women and their developing offspring.

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Disclosures

There are no conflicts of interest to declare

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Figure Captions

Figure 1. Physical activity levels of active vs. non-active pregnant women. The active pregnant women included in this study demonstrated significantly higher levels of both moderate-to-vigorous physical activity (MVPA; **panel A**; * $p < 0.0001$, Mann-Whitney U test) and total physical activity (TPA; **panel B**; * $p < 0.0001$, student t-test) compared to non-active pregnant women. Data are presented as means $\pm$ SD.

Figure 2. SPARC concentrations in the plasma of active vs. non-active pregnant women. Of all the myokines tested, SPARC was the only myokine found to be significantly elevated in the plasma of active (N=11) compared to non-active (N=14) pregnant women. Of the samples measured, 2 active and 2 non-active women had SPARC concentrations that were out of the detectable range (above the standard curve). One outlier in the active group was excluded (>2 SDs above mean). Data are presented as mean $\pm$ SD. * $p = 0.0001$, student t-test.

Figure 3. Correlation between circulating myokine concentrations and maternal physical activity levels. When physical activity levels were assessed as a continuous variable across all patients, significant correlations were observed between circulating SPARC and moderate-to-vigorous physical activity (MVPA) (**panel A**; * $p = 0.0009$), SPARC and total physical activity (TPA) (**panel B**; * $p < 0.0001$), MCP-1/CCL2 and TPA (**panel C**; * $p = 0.0458$), and ANGPTL-4 and MVPA (**panel D**; * $p = 0.0417$). Of the samples measured, 2 were excluded in the SPARC analysis as the SPARC levels were above the detectable range. * $p < 0.05$ considered statistically significant.

Figures

Figure 1

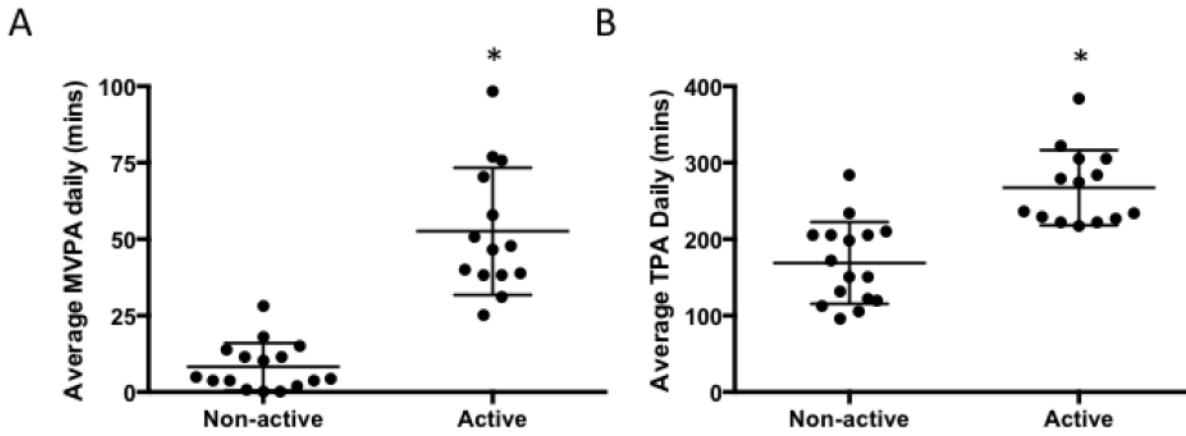


Figure 2

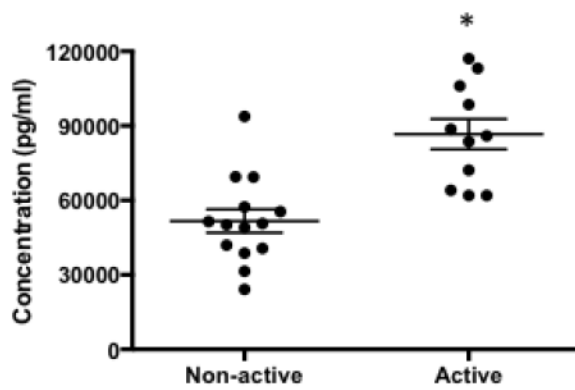
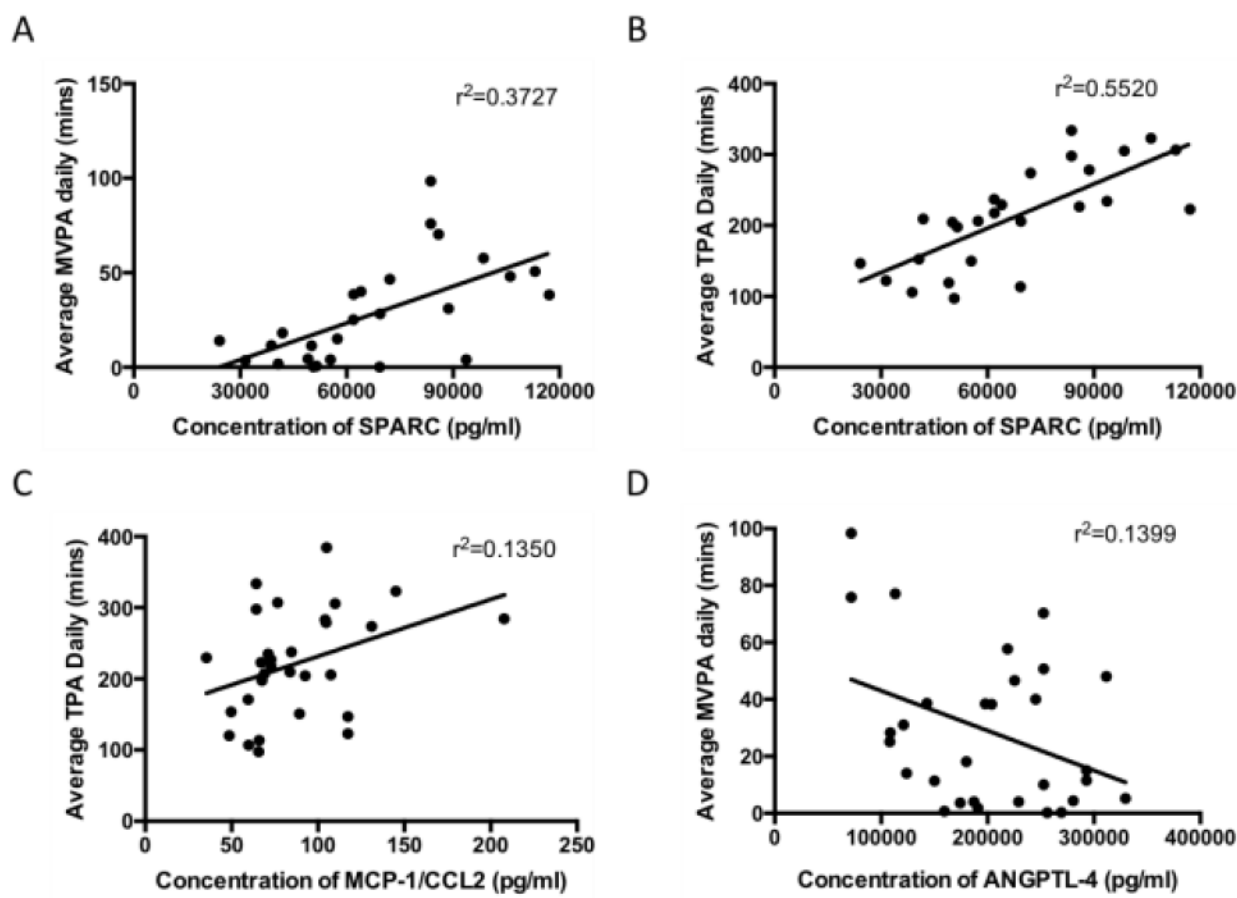


Figure 3



Tables

Table 1. Maternal and fetal characteristics.

Characteristic	Non-active (N=16)	Active (N=14)	p-value
Age at Delivery (years)	32.88±4.06	32.29±3.36	0.6713 ^a
Pre-pregnancy BMI (kg/m ²)	27.81±6.16	25.06±5.76	0.1757 ^b
GWG (kg) ^d	13.67±4.46	13.38±4.50	0.8750 ^a
Women who exceeded GWG ^e	66.67%	25.00%	0.0542 ^c
Birth Type (vaginal or C-section)	Vaginal=66.67% C-section=33.33%	Vaginal=78.57% C-section=21.43%	0.6817 ^c
Sex of Infant (male or female)	Male=68.75% Female=31.25%	Male=50.00% Female=50.00%	0.4572 ^c
Birth Weight (g) ^f	3384.20±449.67	3544.00±363.62	0.3041 ^a
Placenta Weight (g) ^g	588.61±113.01	607.47±150.79	0.7296 ^a
Maternal MVPA daily (mins)	8.28±7.79	52.58±20.79 [*]	p<0.0001 ^b
Maternal TPA daily (mins)	168.93±53.53	267.34±49.26 [*]	p<0.0001 ^a

Abbreviations: BMI=body mass index; GWG=gestational weight gain; MVPA=moderate-to-vigorous physical activity; TPA=total physical activity.

Statistical test used to derive p-value: ^aStudent's t-test; ^bMann-Whitney U test; ^cChi-square analysis and Fisher's exact test. ^{*}Indicates a significant difference at p<0.05.

Some characteristic values were missing for some participants: ^dN=15 non-active women, N=11 active women; ^eN=15 non-active women, N=13 active women; ^fN=15 non-active women, N=14 active women; ^gN=13 non-active women, N=11 active women.

Chapter 4

Preamble & Manuscript 3: *The effect of SPARC on the health and functioning of first trimester
human placentae*

Prepared for submission to the journal *Placenta*.

Preamble to manuscript 3

From our knowledge, we are the first group to investigate the myokine secretome in the context of pregnancy. For our first aim (manuscript #2), we analyzed the myokines profiles of women characterized as active or non-active during pregnancy to identify any differences in circulating myokine levels. Initially, our second aim was to examine the effects of myokine media on placental health and function. In the Aguer lab, myokine media was prepared using an *in vitro* exercise model involving electric pulse stimulation (EPS) of differentiated human myotubes and by collecting the myokine secretions in the surrounding media. Placental explants were dissected and treated with either control, skeletal muscle-conditioned non-stimulated media (media surrounding skeletal muscle cells collected prior to EPS) or stimulated skeletal muscle media (media surrounding skeletal muscle cells after EPS). However, after analyzing the myokine profiles of the media produced using this exercise model, we found that the measured myokine levels didn't correspond to the measured profiles reported in the published literature from which our protocol was obtained (Scheler et al., 2013). We treated a handful of first trimester human placentae (N=4) with this myokine media before discovering that the EPS protocol wasn't working as expected. We proceeded to analyze some of the metrics of placental health and function (viability, proliferation, apoptosis and β hCG secretion) using these explants as preliminary data for future work (See Appendix A for all methods and results).

For this thesis project, the purpose of aim 1 was to compare the myokine profiles in active and non-active pregnant women to determine which individual myokines may be important to study in isolation for their individual effects on placental health and function. As

outlined in manuscript 2, of all the myokines analyzed, SPARC was the only myokine found to be significantly elevated in the plasma of active compared to non-active pregnant women.

Based on our findings, we shifted the focus of aim 2 to specifically examine the impact of SPARC treatment on placental health and function. Thus, we treated first trimester human placental explants with exogenous SPARC in a dose-dependent manner. The findings from the treatment of first trimester human placentae with increasing levels of SPARC are outlined in Manuscript 3:

“The effect of SPARC on the health and functioning of first trimester human placentae”.

Manuscript 3

Title: *The effect of SPARC on the health and functioning of first trimester human placentae*

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The effect of SPARC on the health and functioning of first trimester human placentae

Abstract

Exercise in pregnancy is associated with improved maternal-fetal health outcomes, including a reduction in fetal birthweight extremes. We previously hypothesized that myokines, secreted from contracting skeletal muscle, may be impacting placenta functionality and thus contributing to the optimization of fetal growth. The aim of the current study is to determine the effects of SPARC, a myokine that we previously identified to be elevated in the plasma of active pregnant women, on the placenta. **Objectives:** To use an *ex vivo* 1st trimester human placental explant model to determine the impact of SPARC treatment on 1) placental health/function and 2) placental GLUT-4 expression and localization, as a surrogate of glucose transport. **Methods:** 1) First trimester human placental explants (N=5) were treated with SPARC in a dose-dependent manner (0-150ng/ml) for 72-hours. Placental trophoblast proliferation, viability, apoptosis, invasion and β -hCG production were assessed as metrics of placental health/function. 2) Expression of GLUT-4 along with the activation status of the regulatory signalling pathway for glucose uptake (AMPK/P-AMPK) were assessed through western blot analysis. GLUT-4 localization was further assessed using immunohistochemistry. **Results:** Explants treated with SPARC at 100ng/ml demonstrated improved invasion, with a significantly greater change in maximum outgrowth distance (N=3; p=0.0219). No significant differences were observed for the other metrics of placental health/function. No significant differences were observed in GLUT-4 expression nor in the activation status of AMPK. **Conclusion:** SPARC is an exercise-induced myokine that is associated with improved placental invasion *in vitro*, and may improve the process of placentation in early pregnancy.

Highlights:

- SPARC improves human trophoblast invasion *in vitro*
- SPARC demonstrates no additional effects on metrics of placental health or function
- Exogenous SPARC exposure results in no change in GLUT-4 expression

Key Words: Placenta, pregnancy, exercise, myokines, SPARC.

Introduction

Physical activity (PA) is an important part of a healthy lifestyle. In the context of pregnancy, the added benefit of regular participation in PA is that it provides benefits to both the mother and her developing fetus. Exercise during pregnancy not only reduces the risk of future obesity and chronic health conditions for both mom and baby [1–8], but also regulates fetal growth by protecting against fetal birth weight extremes (small- and large-for-gestational age infants) [9–12]. During PA, contracting skeletal muscles produce and secrete hundreds of peptides and cytokines known as myokines [13]. In the non-pregnant population, myokines have been identified as mediators of many important health benefits associated with regular PA, providing protection against the development of chronic health conditions like type 2 diabetes and cardiovascular disease [14]. In non-pregnant tissues, these exercise factors have also been found to have important effects on metabolism and macronutrient transport [13,15,16]. However, myokines have yet to be investigated in pregnancy and thus their potential effects on the vital organ of pregnancy - the placenta - remain unknown. In isolation, some of these myokines have been found to have important effects on placental health and function, including the promotion of placental cellular invasion [17–23], and the expression/activity of certain placental macronutrient transport proteins [24–29]. It is therefore likely that the collective myokine secretome may serve to optimize placentation and the delivery of macronutrients to the developing fetus.

Previously, we analyzed the circulating myokine profiles in active vs. non-active pregnant women in the 2nd trimester. SPARC was the only myokine found to be significantly elevated in the plasma of active pregnant women, with active women exhibiting a ~1.7-fold increase in

SPARC compared to their non-active counterparts [30]. SPARC is a matricellular glycoprotein that is expressed in tissues during remodeling/repair [31,32]. In response to both acute and chronic PA, the expression of SPARC within contracting skeletal muscle cells is elevated in humans [33,34], and is accompanied by a measurable increase in circulating levels of SPARC [33]. A recent study has found that PA duration appears to be more important than intensity in promoting SPARC secretion [35].

SPARC has been reported to have a variety of roles including in skeletal muscle development/muscle regeneration [36,37], glucose metabolism (increased GLUT-4 expression in L6 myocytes) [38], insulin secretion [39], erythropoiesis [40], and an inhibitory effect on colon tumorigenesis [33]. Only recently has SPARC been investigated in the context of pregnancy. Thus far, Jiang et al. have investigated the role of SPARC in the process of trophoblast invasion, a process that is similar to tumor cell invasion [22]. Downregulation of SPARC expression in HTR8/SVneo cells, an extravillous placental trophoblast cell line, was found to inhibit cell invasion through matrigel *in vitro* [22]. The process of trophoblast cell invasion into the uterine decidua is critical to early placentation, as this process is essential to the establishment of a robust utero-placental circulation required to sustain fetal growth across pregnancy [41]. Exercise-induced increases in SPARC in maternal circulation could play an important role in the regulation of normal placentation by promoting the invasion of EVT cells.

Another role for SPARC has been suggested in placental glucose transport and metabolism during pregnancy. GLUT-4 is the major insulin-sensitive glucose transporter in early pregnancy and is expressed in the cytosol of 1st trimester placental syncytiotrophoblast [42]. SPARC has been shown to affect AMPK-mediated glucose metabolism through the regulation of

GLUT-4 expression in L6 myocytes [38]. The knockdown of SPARC in these skeletal muscle cells reduced AMPK-stimulated GLUT-4 expression and likewise, the overexpression of SPARC enhanced AICAR-stimulated AMPK α phosphorylation as well as GLUT-4 expression [38]. The mechanism of insulin-stimulated GLUT-4-mediated glucose uptake involves the translocation of GLUT-4 transporters to the plasma membrane and an increase in the overall activity of GLUT-4 transporters (reviewed in [43]). The AMPK signaling pathway has the same effect on GLUT-4 expression and translocation during exercise, however this effect is independent of the insulin signaling pathway (reviewed in [38]). Thus, SPARC may have an impact on placental glucose uptake via GLUT-4 in early pregnancy. Further investigation into the effects of SPARC on placental biology and function are necessary to understand the potential benefits of exercise-induced SPARC secretion from skeletal muscle during pregnancy.

As we have previously demonstrated an elevation in SPARC in the circulation of active pregnant women, we hypothesized that this exercise-induced myokine may play an important role in optimizing fetal growth in active women, through direct beneficial effects on critical aspects of placental development and function. As such, the primary objective of the current study is to investigate the effects of increasing SPARC treatment on metrics of placental health and function using an *ex vivo* human placental explant model. As previous studies have identified a role for SPARC in the regulation of GLUT-4 expression in skeletal muscle [38], we similarly examined the effects of SPARC exposure on placental GLUT-4 expression and localization. In other tissue types, SPARC has been shown to regulate GLUT-4 expression and localization through the activation of AMPK signalling pathway [38], thus we further examined the activation (phosphorylation) status of AMPK in the same SPARC-treated human placental

explants. An understanding of the potential effects of SPARC on the placenta may uncover one of the mechanisms whereby PA during pregnancy works to optimize fetal growth across gestation. Such a discovery may further contribute to the development of innovative therapeutic interventions that could deliver some of the major benefits of exercise-induced SPARC secretion during pregnancy to all women and their developing offspring.

Materials and Methods

Placenta Tissue Collection and Preparation

Women undergoing surgical elective terminations from healthy 1st trimester pregnancies (6-12 weeks' gestation) were recruited from The Ottawa Hospital (TOH). Ethics approval was obtained from both TOH and the University of Ottawa, and informed consent was obtained from all study participants. Placenta tissues were collected in ice-cold HBSS supplemented with calcium and magnesium (HBSS++) immediately following the termination procedure and transported to the laboratory on ice. Depending on the gestational age of tissue collected, they were either prepared for health metric assays (floating explants) or *in vitro* functional invasion assays (extravillous trophoblast (EVT) Explants).

Floating Explants

Placental specimens approximately 8-11 weeks' gestation were dissected in ice-cold HBSS++ with 1% 100x Penicillin-Streptomycin (P/S) solution in a petri dish under a Leica® dissecting microscope. Large tissue explants (1-3mg) of floating villi were dissected using microsurgical scissors and fine tweezers. Approximately 4 dissected explants were placed onto each Corning® Netwell® insert (membrane diameter 15mm, membrane size 74µm; Sigma-Aldrich, USA) in a 12-well cell culture plate and 2ml of warmed experimental media (DMEM/F12,

2% FBS, 1% 100x P/S) was added to each well. The explants were sustained under normoxic culture conditions of 8% O₂/5% CO₂/balance N₂ (physiologically relevant oxygen tension [44]) at 37°C for 48 hours. A series of experimental SPARC treatments (50, 100 and 150ng/ml) were prepared by diluting Recombinant Human SPARC Protein (abcam, USA) in experimental media (DMEM/F12, 2% FBS, 1% 100x P/S). For each floating explant experiment (N=5), SPARC treatments were performed over a 72-hour period. At the end of the 72-hour experimental treatment period, weights of explants from each netwell were recorded and tissue was either flash-frozen for subsequent analysis of GLUT-4 expression, or fixed in 10% buffered formalin phosphate for 24 hours at room temperature (RT) and subsequently transferred to 70% ethanol for storage at 4°C for later immunohistochemical analysis of Ki-67, M30 and GLUT-4. Overlying culture media from each was also collected and stored at -80°C for future analysis of lactate dehydrogenase (LDH) release and beta-human chorionic gonadotropin (β -hCG) production.

Extravillous Trophoblast Explants

Placental specimens approximately 6-7 weeks' gestation were dissected in ice-cold HBSS++ with 1% 100x P/S solution in a petri dish under a Leica® dissecting microscope. Smaller tissue explants (~1mg) of terminal EVT villi were dissected using microsurgical scissors and fine tweezers. Individual dissected explants were carefully plated on a matrigel-coated Millicell® cell culture insert (0.4µM, 12mm diameter; EMD Millipore, USA) in a 24-well cell culture plate using fine tweezers and a small-gauge needle. Once all the explants were plated, 400µl of warmed experimental media (DMEM/F12, 2% FBS, 1% 100x P/S) was added to the exterior of each insert and the explants were sustained under normoxic culture conditions at 3% O₂/5% CO₂/balance N₂ (physiologically relevant oxygen tension [44]) at 37°C. The following day, 200µl of warmed

experimental media was carefully added to the interior of each insert to hydrate the explants along with another 200µl to the exterior. The explants were then sustained under normoxic culture conditions of 3% O₂, 5% CO₂/balance N₂ at 37°C for an additional 24 hours prior to experimentation. Prior to SPARC treatment, time zero pictures were taken of each explant. A series of experimental SPARC treatments (50, 100 and 150 ng/ml) were prepared by diluting Recombinant Human SPARC Protein (abcam, USA) in experimental media (DMEM/F12, 2% FBS, 1% 100x P/S) and the culture media in both the interior and exterior chambers of the culture insert was replaced. For each EVT explant experiment (N=3), placental explants were treated with their respective SPARC treatment for 72-hours, followed by the capture of final images of each explant for the assessment of their degree of invasion through the matrigel (as detailed below).

Assessment of Placental Health

Cellular Proliferation

The effect of increasing concentrations of SPARC on cytotrophoblast cell proliferation was examined through immunohistochemical analysis of Ki-67, an established marker of cellular proliferation [45,46]. Following SPARC treatments, explants from each experimental SPARC treatment (N=5 experiments) were fixed, paraffin embedded and processed for immunohistochemistry (IHC) according to standard protocols. Slides were incubated with primary monoclonal mouse anti-human Ki-67 antibody (1:100; Dako, USA) overnight at 4°C, followed by incubation with a biotinylated goat-anti mouse IgG secondary antibody for 1 hour at RT. Immunoreactivity was visualized through incubation with peroxidase-conjugated streptavidin (DAKO LSAB2 System-HRP (Dako, USA) for 30 minutes at RT followed by incubation with 3,3'-

Diaminobenzidine (DAB) tetrahydrochloride (Sigma-Aldrich, USA). Slides were counterstained with hematoxylin and mounted. A negative control was run in parallel with the omission of the primary antibody. High-quality images of each slide were captured using the Leica Aperio ScanScope® and Ki-67-positive cells were counted from four fields of view per placenta (10x magnification) from each treatment group via the thresholding and particle analysis tools in ImageJ Software v1.50i. Tissue area was measured using thresholding and particle analysis or by freehand selections and individual measurements of tissue areas in ImageJ. Data was expressed as the mean number of proliferative cells per mean area of tissue imaged.

LDH Cytotoxicity

The release of LDH, a cytoplasmic protein, into cell culture media is a marker of non-viable cells with compromised plasma membranes [47]. For each experiment (N=5), the media overlying the placental explants was collected following a 72-hour experimental SPARC treatment and stored at -80°C. Viability of placental tissue following SPARC treatment was assessed by measuring LDH content in the collected explant supernatant following 72-hr of experimental treatment, using a colorimetric Pierce LDH Cytotoxicity Kit (Thermo Fisher Scientific, USA) per manufacturer's instructions. Colorimetric detection of LDH activity was measured at an absorbance of 490nm and 690nm using POLARstar® Omega microplate reader and MARS Data Analysis Software (BMG Labtech, GER). For each experiment, a set of 4 placental explants (similar to what was placed in each experimental netwell) were weighed and flash frozen at the time of dissection. These experimental control explants were thawed and homogenized in 1ml 1X DMEM to release all cytoplasmic LDH. Cell debris was removed from the tissue homogenate using a centrifuge for 5 minutes at 14 000 RPM and supernatant was

collected. This sample was considered the 100% LDH control for each respective experiment. Measured LDH levels were normalized to the mass (mg) of each sample of placental explants (performed in duplicate), and expressed relative to the 100% LDH control for each placenta. Additionally, explants treated with staurosporine (1 μ M) were included as a positive control for cellular stress.

Apoptosis

The monoclonal antibody M30 has been established as an effective antibody to evaluate placental trophoblast apoptosis as it is directed against the epitope of cytokeratin 18 (CK18), that is cleaved by activated caspases during the apoptotic cascade [48]. The effect of increasing concentrations of SPARC on placental explant apoptosis was determined through M30 immunoreactivity. Explants from each experimental SPARC treatment (N=5 experiments) were fixed and processed as detailed above, using the monoclonal mouse anti-human M30 antibody (1:100; CytoDEATH, Roche Diagnostics, USA) under the same conditions. A positive stress control treatment of 1 μ M staurosporine was included. A negative control was run in parallel with the omission of the primary antibody. High-quality images of each slide were captured using the Leica Aperio ScanScope® and M30 positive cells were counted manually from four fields of view per placenta (10x magnification) from each treatment group using the cell counter tool in ImageJ Software v1.50i. Tissue area was measured using thresholding and particle analysis or by freehand selections and individual measurements of tissue areas in ImageJ. Data was expressed as the mean number of apoptotic cells per mean area of tissue imaged.

Assessment of Placental Function

Invasion

The ability of EVT cells to actively migrate into the maternal decidua and remodel uterine spiral arteries is vital in the establishment of a healthy pregnancy [41], thus the effects of increasing concentrations of SPARC on EVT explant invasion through an extracellular matrix were examined. As indicated above, EVT explants were plated on matrigel-coated Millicell® cell culture inserts (0.4µM, 12 mm diameter; EMD Millipore, USA). Images were taken of each explant using a Leica® dissecting microscope before and after a 72-hr treatment period. Explant outgrowth area was analyzed by using the freehand selection tool and measuring outgrowth area in ImageJ. The change in maximum outgrowth distance was determined using measurements from images taken before and after the 72-hour treatment period using a ruler in ImageJ. For each placenta, the mean change in outgrowth area and mean change in maximum outgrowth distance were calculated for each treatment and expressed relative to untreated controls.

β-hCG Secretion

Human chorionic gonadotropin hormone production and secretion is an essential function of the placenta during pregnancy [41,49]. The effects of increasing concentrations of SPARC on β-hCG hormone secretion by placental explants was examined. For each experiment, the media overlying the floating placental explants was collected following a 72-hour experimental SPARC treatment and stored at -80°C. β-hCG hormone secretion into the overlying culture media was analyzed using an hCG + beta enzyme-linked immunosorbent assay (ELISA) Kit (IBL International, GER) per manufacturer's instructions. The absorbance of each well was measured at an absorbance of 450nm using a POLARstar® Omega microplate reader and MARS Data Analysis Software (BMG Labtech, GER). Using the standard curve, the concentration

of β -hCG secreted was calculated using a 4 Parameter Logistics (4PL) curve fit, normalized to the mass (mg) of each sample of placental explants (performed in duplicate), and expressed as mIU/mL/mg relative to untreated controls.

GLUT-4 Measurement

Protein extraction from human placental explants was performed using the SurePrep™ RNA/Protein Purification Kit (Fisher Scientific, USA) per manufacturer's instructions. Flash-frozen SPARC-treated floating placental explants were homogenized using a mechanical homogenizer in the supplied lysis buffer. The DC Protein Assay Kit (Bio-Rad, USA) was used to quantify the protein in each sample. Multiple aliquots containing 10 μ g total protein were prepared and stored at -20°C for future western blot analysis.

Western Blot Analysis

Western Blot Analysis was performed to examine the expression of GLUT-4, AMPK and Phospho-AMPK (P-AMPK) in SPARC-treated human floating placental explants. Acrylamide gels were prepared using TGX Stain-Free™ FastCast™ Acrylamide Starter Kit (10%; Bio-Rad, USA). Aliquots of 10 μ g of explant protein for each SPARC treatment were prepared as previously described, with the exception of the boiling process (5 mins at 95°C for the AMPK and P-AMPK gels only). Electrophoretic separation was performed on 28 μ l samples using 1x running buffer at ~200V for ~1 hour. Afterwards, stain-free images were taken of each gel for later normalization. Electrophoretic transfer onto activated Immun-blot® PVDF membranes (Bio-Rad, USA) were performed at 30V overnight at 4°C. After the transfer, the membranes were washed in TBST twice for 5 mins each, in water twice for 5 mins each and in 100% ethanol once for 5 mins, then allowed to dry. For the primary antibody, Anti-Glucose Transporter GLUT4 antibody (abcam,

USA) was diluted to 1:2000 in 3% milk blocking solution and the membrane was incubated at 4°C overnight. The primary antibodies (Rabbit anti-human AMPK α mAb and rabbit anti-human Phospho-AMPK α mAb; Cell Signaling Technology, USA) were both diluted to 1:1000 in 5% BSA and used for AMPK and AMPK-P, respectively. After incubation with the primary antibody, the membranes were washed 5 times in 1X TBST for 5 minutes each. The secondary antibody, goat anti-rabbit IgG (H+L)-HRP conjugate (Bio-Rad, USA) was used for all membranes and was diluted to 1:10 000 in 5% milk and the membranes were incubated for 1 hour at RT on a rotating mixer. After incubation with the secondary antibody, the membranes were washed 5 times in 1X TBST for 5 minutes each. Visualization was conducted using the Clarity™ Western ECL Substrate kit (Bio-Rad, USA) and the membrane was exposed to chemiluminescence using a ChemiDoc™ Touch Gel Imaging System (Bio-Rad, USA). A Western blot band intensity analysis was conducted using Image Lab version 5.2 software (Bio-Rad, USA). The lanes and individual bands for each gel were selected, lane profiling was performed and each lane was normalized to the corresponding lane in the stain-free gel. Results are presented as band intensity volumes relative to controls per placenta.

Immunohistochemical Analysis

GLUT-4 is expressed in the syncytiotrophoblast of 1st trimester human placenta and has a role in placental glucose transport in early pregnancy [42]. In order to assess the effect of increasing concentrations of SPARC on GLUT-4 localization, GLUT-4 immunoreactivity was examined. Floating explants from each experimental SPARC treatment (N=5 experiments) were fixed and processed as detailed above, using the rabbit polyclonal anti-glucose transporter GLUT-4 antibody (1:1000; abcam, USA) overnight at 4°C, under the same conditions. A negative control

was run in parallel with the omission of the primary antibody. High-quality images were taken as previously described. A scoring rubric was prepared for the assessment of syncytial GLUT-4 staining intensity, using a scoring system (0-2) based on DAB staining intensity (**Figure 1**). When establishing the scoring rubric, the intent was to score based on localization. However, the staining intensity in all samples was much stronger than anticipated, making the scoring of changes in localization not possible. As such, we were only able to score the staining intensity in the syncytium. Thus, the assigned scores reflect the level of GLUT-4 expression in the syncytium rather than changes in localization. A pathologist (D.G.) and researcher (C.D.) were fully blinded to the treatments and asked to use the scoring rubric to assign a global score to each slide for syncytial staining intensity (0=no staining, 1=weak staining, 2=strong staining). Once all of the slides were scored, the pathologist and researcher compared scores and were required to reach consensus for all slides for which contrasting scores were assigned.

Statistical Data Analysis

All results were presented as means $\pm$ SD from independent experiments. Unless otherwise indicated, all experimental SPARC dose-response data was analyzed by a one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-hoc analysis using Prism software, version 6.0 (GraphPad, USA). The semi-quantitative assessment of GLUT-4 staining intensity was performed by chi-square analysis using Prism software, version 6.0 (GraphPad, USA). Differences between experimental treatments were considered statistically significant if * $p < 0.05$.

Results

Effect of SPARC on Placental Health

Cellular Proliferation

Following SPARC exposure, no significant changes were observed in the number of Ki-67 positive cells (**Figure 2**). For all experimental treatments (0-150ng/ml SPARC), 140 ± 60 cytotrophoblast cells were actively undergoing proliferation as indicated by Ki-67 immunoreactivity across 4 random fields of view at 10x magnification.

LDH Release

LDH release by placental explants remained unchanged across all SPARC treatments (50-150ng/ml) (**Figure 3**). For all experimental treatments, $4.1 \pm 1.9\%$ of intracellular LDH was released into cell culture media per milligram of placental explants. Staurosporine ($1\mu\text{m}$) treated explants were included as a positive stress control, with significantly reduced placental cell viability, as indicated by increased LDH release (10.75% LDH/mg).

Apoptosis

In all samples, apoptosis was primarily restricted to the syncytiotrophoblast layer (**Figure 4A-D**). No significant changes in apoptosis were observed in response to increasing SPARC exposure (50-150ng/ml) (**Figure 4E**). For all experimental treatments, 139 ± 60 cytotrophoblast cells were actively undergoing apoptosis across 4 random fields of view at 10x magnification. Staurosporine ($1\mu\text{m}$) treated explants were included as a positive control for stress, resulting in a 158% increase in apoptosis compared to SPARC-treated explants.

Effect of SPARC on Placental Function

Cellular Invasion

The effects of increasing concentrations of SPARC treatment on placental EVT invasion was examined through the measurement of outgrowth area and maximum outgrowth distance.

Representative images of all SPARC treatments (50-150ng/ml) pre- and post-treatment have been included for reference (**Figure 5A-D**). Percent change (pre- and post- treatment) in outgrowth area remained unchanged across all SPARC treatments ($650\pm327\%$) (**Figure 5E**). However, the change in maximum outgrowth distance, expressed as percent change, was significantly increased following treatment with 100ng/ml compared to 150ng/ml of SPARC and controls ($0.39\pm0.34\text{mm}$ vs $0.37\pm0.20\text{mm}$ and $0.26\pm0.19\text{mm}$, respectively; $p=0.0219$) (**Figure 5F**).

β -hCG Secretion

β -hCG secretion by floating placental explants remained unchanged across all SPARC treatments (50-150ng/ml) (**Figure 6**). For all experimental treatments, the mean concentration of β -hCG secreted into the cell culture media, normalized to placental explant weight, was $9.20\pm13\text{mIU/ml/mg}$.

Effect of SPARC on GLUT-4 Protein Expression

Western blot analysis detected a band for GLUT-4 at $\sim 45\text{kDa}$, corresponding to the expected size of the transport protein (**Figure 7A**). No significant differences were observed in the band intensities of SPARC treated explants compared to untreated controls (**Figure 7B**). The effects of increasing concentrations of SPARC treatment on GLUT-4 was also examined using IHC. Using semi-quantitative analysis, GLUT-4 staining intensity was found to be similar across all SPARC treatments and was no different from untreated controls (**Table 1 and Figure 7C**). Chi-square analysis for trend identified no significant differences in the syncytial expression of GLUT-4 between SPARC treatments.

Effect of SPARC on AMPK and P-AMPK Protein Expression

Western blot analysis detected a band for AMPK and P-AMPK at ~ 62kDa, corresponding to the expected size of the proteins (**Figure 8A-B**). A band intensity analysis identified no increases in AMPK protein content in SPARC treated explants relative to controls (**Figure 8C**), nor in the ratio of activated P-AMPK protein content to total AMPK content in the SPARC treated explants compared to untreated controls (**Figure 8D**).

Discussion

In the current study, we have characterized for the first time, the impact of the exercise-induced myokine SPARC on placental health/function, and on placental GLUT-4 transporter expression. We focused on the myokine SPARC as our team previously identified SPARC to be the only myokine that is significantly elevated in the circulation of physically active pregnant women [30]. As the mechanisms associated with the exercise-induced optimization of fetal growth are currently unknown, we explored the potential effects of SPARC on the placenta to identify if it is a potential candidate. The literature supports a role for SPARC in the regulation of important processes of early placentation and glucose transport/metabolism. As such, an *ex vivo* 1st trimester human placental explant model was used to determine the impact of exogenous SPARC treatment on trophoblast cell viability, proliferation, apoptosis, hormone secretion and invasive capabilities. Further, the impact of SPARC treatment on placental GLUT-4 transporter expression was assessed. The results of the current study demonstrate that exogenous SPARC exposure results in an increase in 1st trimester human trophoblast invasion *in vitro*, with no measurable impact on other metrics of placental health and function.

To determine the impact of SPARC treatment on the placenta, various metrics of placental health and function were examined. Through the analysis of placental function, which

included the assessment of cellular invasion and β -hCG production, at 100ng/ml SPARC caused a significant increase in the maximum invasive outgrowth distance. However, due to high variability between samples, a similar significant effect of SPARC wasn't observed on total area of placental invasion. Improved placental invasion distance in response to SPARC treatment supports our hypothesis that SPARC improves placental function. However, SPARC treatment wasn't found to improve placental endocrine function, as assessed via the measurement of β -hCG production. Thus, SPARC treatment promotes one vital function of the human placenta by contributing to improvements in placental invasion.

The finding that SPARC improves placental invasion supports previously published literature that has identified a role of SPARC in the regulation of cellular invasion. An *in vitro* study identified that the downregulation of SPARC expression in a placental trophoblast cell line (HTR8/SVneo) inhibits invasion through matrigel [22]. Together, the findings of the current study and those of Jiang et al., complement each other in illustrating a key role of SPARC in the process of placental invasion. As the invasion of EVT cells through the maternal decidua is essential to form a functional placenta and to establish a successful pregnancy [41], exercise-induced increases in systemic SPARC during pregnancy could promote the invasion of EVT cells. Trophoblast invasion through the maternal decidua is essential for the remodeling of uterine spiral arteries, a process that establishes the blood supply to the developing fetoplacental unit and allows for the transfer of nutrients, gases and waste products [41]. As SPARC appears to be elevated in the plasma of active pregnant women, exercise-induced increases in SPARC in circulation could act on the placenta to enhance the process of placental invasion through the decidua and subsequent remodeling of the maternal spiral arteries. The establishment of a

robust feto-placental circulation is essential to ensure that the developing fetus is supplied with an adequate supply of nutrients to support optimal fetal growth and development.

Other than clear improvements in placental invasion, no other aspects of placental health were significantly altered in response to increasing SPARC treatment. This includes proliferation, viability and apoptosis. While we anticipated that we would observe increases in trophoblast proliferation (as the placenta is a growing organ undergoing morphogenesis), we also speculated that SPARC would have no harmful effects on the placenta, and therefore the lack of decreased viability or increased apoptosis is in line with what we anticipated.

Among the many functions of SPARC, it has been shown to have proliferative and anti-apoptotic effects (cell survival) [39]. From the observations in our study, we can say with certainty that SPARC does not reduce proliferation, apoptosis or viability at physiologically relevant levels representative of the levels in the circulation of active pregnant women. However, it is still unknown what role, if any, SPARC plays in regulating the processes of proliferation and apoptosis in placental trophoblast cells. As the concentrations of SPARC in circulation following an acute bout of exercise are currently unknown, although likely higher than the measured levels in our subset of active pregnant women, it would be interesting to expand the dose-response curve in future studies (beyond 150ng/ml SPARC) and to observe if SPARC at higher concentrations has other effects on placental health/function (i.e. improved proliferation and reduced apoptosis).

The placenta plays a vital role in facilitating nutrient transport imperative for fetal growth and development. In this study, the impact of SPARC treatment on placental GLUT-4 was investigated because SPARC has been shown to increase glucose uptake/metabolism in other

tissues. Also, we hypothesized that in exercising women, SPARC may play a similar role to optimize placenta glucose uptake and ultimately to optimize fetal growth trajectories. GLUT-4 is the major insulin-sensitive glucose transporter expressed in the syncytium of 1st trimester placenta and plays a key role in glucose metabolism in early pregnancy [42]. Song et al. previously identified that SPARC is involved in the regulation of glucose metabolism by enhancing the expression of GLUT-4 in rat skeletal muscle cells [38]. Based on these findings, we anticipated that SPARC treatment would upregulate GLUT-4 expression and contribute to increased glucose uptake/metabolism. We also examined the activation of AMPK, an energy-sensing enzyme that is known to be a critical regulator of glucose metabolism [50]. Under certain conditions (i.e. physical activity), AMPK is activated by a rise in cellular AMP levels, and phosphorylation by one or more AMPK kinases [51]. Although we hypothesized that SPARC would increase GLUT-4 expression, suggesting that it may enhance glucose uptake and transfer to the developing fetus during pregnancy, no change in the expression of GLUT-4 in response to increasing SPARC treatment was identified. Also, no significant effect of SPARC treatment on the proportion of P-AMPK to AMPK was observed. Thus, SPARC treatment also appears to have no effect on the regulatory signalling pathway for glucose uptake.

No observed effect of increasing SPARC treatment on GLUT-4 in 1st trimester human placental explants suggests that SPARC has a different impact on GLUT-4 in skeletal muscle compared to in the placenta. There are many possible explanations for this observation. SPARC is primarily expressed and secreted by contracting skeletal muscle cells, with important local effects in muscular tissues (i.e. skeletal muscle development [36,37] and glucose metabolism [38]). However, SPARC also has numerous other roles in processes such as wound healing/tissue

regeneration, inflammation, extracellular protease activity, angiogenesis, regulation of growth factor signalling [31], insulin secretion [39], erythropoiesis [40], as well as inhibitory effects on carcinogenesis [52]. Considering the multi-functional role of SPARC, it is possible that SPARC in circulation results in some direct effects on the placenta (i.e. enhanced cellular invasion), but that this myokine primarily exerts systemic effects, resulting in overall improvements in maternal physiology. As we have previously hypothesized [53], such improvements in maternal physiology (i.e. improved insulin uptake and glucose metabolism), could contribute to an improved maternal *milieu* to better support optimal fetal growth.

Although it is known that SPARC is expressed by both the placenta and skeletal muscle [54], the relative production of SPARC by both tissue types is currently unclear. It has been established that exercise contributes to significant elevations in SPARC in circulation [30,33]. In our previous study, we identified that SPARC was significantly elevated in women characterized as active vs. non-active during pregnancy ($86 \pm 19 \text{ ng/ml}$ vs. $52 \pm 18 \text{ ng/ml}$) [30]. It is important to consider that SPARC concentrations likely peak in maternal circulation immediately following a bout of PA, as observed in the non-pregnant population [22]. These levels likely also decline rapidly in circulation, similar to non-pregnant individuals [22]. In this case, immediately following a bout of exercise, there may be significantly higher concentrations of SPARC in circulation compared to those that we measured in our subset of pregnant women and compared to the physiologically relevant concentrations that we used in the treatment of placental explants. It is possible that these higher concentrations of SPARC are required to observe an impact of SPARC on GLUT-4 expression in placental tissue. Measuring SPARC levels in the plasma of pregnant women immediately following an acute bout of PA would identify if SPARC is further elevated in

circulation, and identify higher concentrations of SPARC that should be used in the treatment of human placental explants.

As the maternal spiral arteries remain plugged until closer to the end of the 1st trimester (10-12 weeks' gestation), the feto-placental unit primarily depends on glucose as an energy substrate [42]. Accordingly, GLUT-4 is highly expressed in 1st trimester placenta to accommodate the glucose demands of early pregnancy. As GLUT-4 is already highly expressed in placental tissue, it is also possible that SPARC treatment at levels similar to those measured in our subset of active pregnant women, is not a strong enough stimulus to further promote an elevation in the expression of GLUT-4 in 1st trimester placental explants. Furthermore, it also cannot be discounted that SPARC may be influencing some of the many other macronutrient transporters expressed in the 1st trimester human placenta. In the current study, our group focused on examining GLUT-4 because SPARC was specifically identified to have an impact on this transporter in a different tissue type.

The stimulation of GLUT-4-mediated glucose uptake involves the translocation of GLUT-4 transporters to the plasma membrane and an increase in the overall activity of GLUT-4 transporters in individual cells [43]. Since no changes were observed in the overall protein expression of GLUT-4 within the placenta tissue homogenate in response to SPARC treatment, we attempted to look more specifically at GLUT-4 localization following SPARC treatment using IHC protocols. Unfortunately, in all tissues samples, the syncytial staining intensity of GLUT-4 was very strong, indicative of high amounts of GLUT-4 protein. This high degree of immunoreactivity made evaluating localization patterns quite challenging, and unfortunately we were unable to draw any conclusions on the impact of SPARC on GLUT-4 localization within the syncytium. We

therefore limited our assessment to overall staining intensity within the exchange surface of the placenta, and in line with our western blot results, found no change in GLUT-4 immunoreactivity across all SPARC treatments. Since the mechanism of insulin-stimulated GLUT-4-mediated glucose uptake involves the translocation of GLUT-4 transporters to the plasma membrane and an increase in the overall activity of these transporters [43], it is possible that SPARC may impact GLUT-4 localization in individual placental cytotrophoblast cells. Future investigations should utilize more sensitive measurements including immunofluorescence and confocal microscopy and/or real-time analysis to identify GLUT-4 trafficking and localization in response SPARC treatment.

As PA in pregnancy has been associated with tremendous health benefits for both the mother and her developing fetus, knowledge of the effects of these myokines on the placenta along with furthering our understanding of some of the potential mechanisms whereby PA works to improve maternal-fetal health outcomes is essential. As it has now been identified that the *in vitro* treatment of human placental explants with exogenous SPARC improves a critical function of the placenta, cellular invasion, it is possible that other exercise-induced myokines may also be acting on the placenta during pregnancy to contribute to optimized fetal growth during gestation. Improved placentation and placental development could promote improved fetal growth during pregnancy. Our findings suggest that maternal PA (pre-conception and in early pregnancy) contributes to elevated SPARC levels in circulation that could improve placental invasion through the maternal decidua. Improved invasion would promote the appropriate remodelling of maternal arteries in order to establish a robust feto-placental circulation to support optimal fetal growth across gestation. Due to limitations in access to 1st trimester

placental tissue, it is recommended that further replicates be performed to confirm these findings in a larger sample size. For now, it is clear that SPARC is an exercise-induced myokine that contributes to improved placental invasion *in vitro* and may have an important role in the promoting the early establishment of pregnancy.

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Disclosures

There are no conflicts of interest to declare.

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Figure Legends

Figure 1. Scoring Rubric for Semi-Quantitative Analysis of GLUT-4 Expression in human

Placental Explants. GLUT-4 immunohistochemistry staining intensity was assessed through a semi-quantitative analysis performed by two blinded reviewers using an in-house developed scoring rubric. The negative control represents a score of 0. The rubric contains representative images of both strong (score of 2) and weak (score of 1) syncytial staining intensities.

Figure 2. Effect of SPARC on cytotrophoblast proliferation in human placental explants.

Representative images showing proliferative cells (black arrow) after a 72-hour treatment with **(A)** control and **(B)** 100 ng/mL of SPARC, as indicated by Ki-67 immunoreactivity. Level of proliferation represented as mean number of proliferating cytotrophoblast cells (Ki-67 positive) per total area of tissue in 4 random fields of view at 10x magnification **(Panel C)**. Data is presented as means $\pm$ SD (N=5) relative to untreated controls. Statistical significance was assessed using a one-way ANOVA with Tukey's multiple comparison post hoc analysis (*p<0.05).

Figure 3. Effect of SPARC on lactate dehydrogenase (LDH) release by human placental explants.

No difference in placental LDH release following a 72-hour treatment with increasing concentrations of SPARC (50-150 ng/ml). Measured LDH release for each treatment group was normalized to the mass of the corresponding placental explants, expressed as a percentage of a 100% LDH positive control. The 100% LDH control represents the amount of total cytoplasmic LDH present in an equivalent mass of placental explants. As a positive stress control explants (N=1) were treated with staurosporine (ST; 1 μ M) for 72 hours. Data is presented as means $\pm$ SD (N=5). Statistical significance was assessed using a one-way ANOVA with Tukey's multiple comparison post hoc analysis (*p<0.05).

Figure 4. Effect of SPARC on apoptosis in human placental explants. Representative images showing apoptotic cells (black arrow) after a 72-hour treatment with **(A)** Control, **(B)** 100ng/ml SPARC and **(C)** 1 μ M staurosporine (ST). Level of apoptosis represented as mean number of apoptotic cells per total area of tissue in 4 random fields of view at 10x magnification **(Panel D)**. As a positive stress treatment control (N=1), one set of explants were treated with 1 μ M staurosporine (ST) for 72 hours. Data is presented as means $\pm$ SD (N=5) relative to controls per placenta. Statistical significance was assessed using a one-way ANOVA with Tukey's multiple comparison post hoc analysis (*p<0.05).

Figure 5. Effect of SPARC on invasion through an extracellular matrix by human placental explants. Representative images showing invading extravillous trophoblast cells from the tips of 1st trimester human placenta explants after a 72-hour treatment with **(A)** 0ng/ml (control), **(B)** 50ng/ml, **(C)** 100ng/ml and **(D)** 150ng/ml SPARC treatment. Quantification of the percent change in area of invasion following treatment with increasing concentrations of SPARC (50-150ng/ml) **(Panel E)**. Quantification of the change in maximum distance of invasion following treatment with increasing concentrations of SPARC (50- 150ng/ml). Data is represented as mean % change $\pm$ SD (N=3) relative to untreated controls **(Panel F)**. Statistical significance was assessed using a one-way ANOVA with Tukey's multiple comparison post hoc analysis (*p<0.05). Columns labelled with different letters are significantly different from each other.

Figure 6. Effect of SPARC on β -hCG secretion by human placental explants. The concentration of β -hCG released following a 72-hour treatment with increasing concentrations of SPARC (50- 150 ng/ml) was determined using an enzyme-linked immunosorbent assay (ELISA), and was normalized to tissue mass (mg). Data is presented as the mean concentration (mIU/mL/mg) of β -

hCG secretion $\pm$ SD (N=4) relative to untreated controls. Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison post-hoc analysis (* p <0.05).

Figure 7. Effect of SPARC on GLUT-4 protein expression in human placental explants. Western blot for GLUT-4 protein expression in human placental explants following treatment with increasing concentrations of SPARC (0-150ng/ml). A band was detected at ~45kDa (indicated by arrows) for GLUT-4 (**Panel A**). Data is presented as band intensity volumes normalized to the corresponding lane in the stain-free gel and relative to untreated controls (N=5) (**Panel B**). Statistical significance was assessed using a one-way ANOVA with Tukey's multiple comparison post-hoc analysis (* p <0.05). Using a semi-quantitative scoring rubric, no changes in GLUT-4 staining intensity was observed in SPARC treated explants (0-150ng/ml) (**Panel C**). Statistical significance was assessed using chi-square analysis for trend (* p <0.05).

Figure 8. Effect of SPARC on AMPK and P-AMPK protein expression by human placental explants. Western blot of human placental explants treated with increasing concentrations of SPARC (0-150ng/ml) looking at the expression of (**A**) AMPK and (**B**) P-AMPK. A band was detected ~62kDa for both AMPK and P-AMPK (indicated by arrows). Data is presented as band intensity volumes normalized to the corresponding lane in the stain-free gel and relative to untreated controls (N=5) (**Panel C**). The intensity volumes of AMPK and P-AMPK are expressed as a ratio of P-AMPK/AMPK relative to controls per placenta (**Panel D**). Statistical significance was assessed using a one-way ANOVA with Tukey's multiple comparison post-hoc analysis (* p <0.05).

Tables

Table 1. Semi-quantitative analysis of GLUT-4 staining intensity in the syncytium of human placental explants.

SPARC Treatment (ng/ml)	Weak Staining	Strong Staining	Total (N)
Control	20%	80%	5
50	60%	40%	5
100	40%	60%	5
150	40%	60%	5
Total	40%	60%	20

Figures

Figure 1

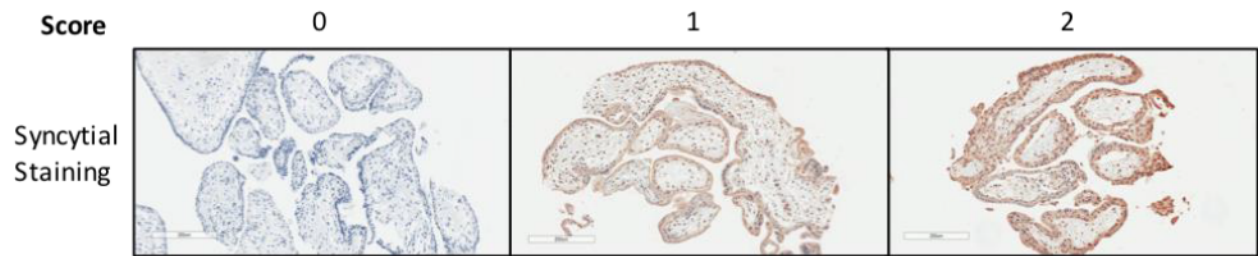


Figure 2

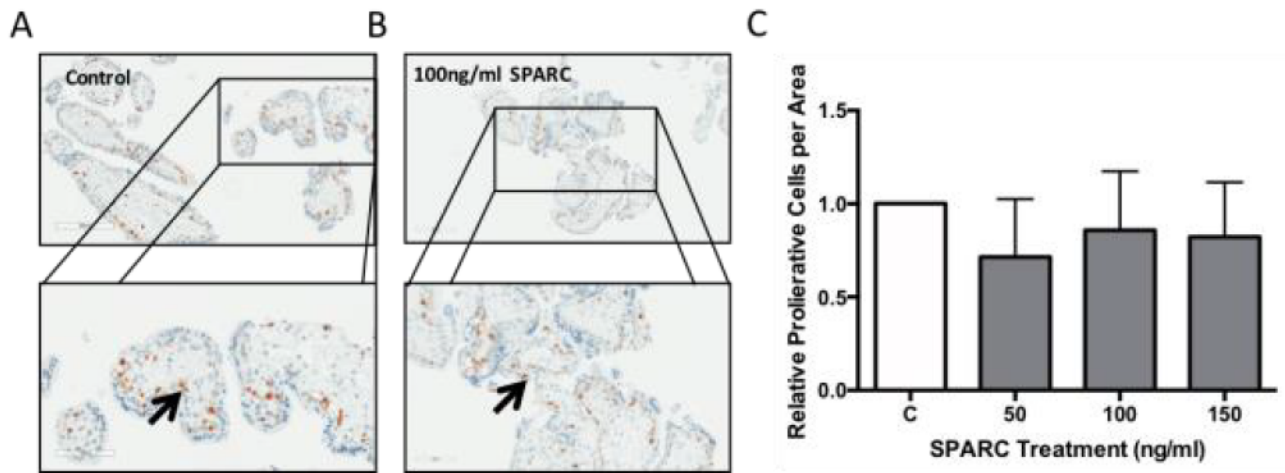


Figure 3

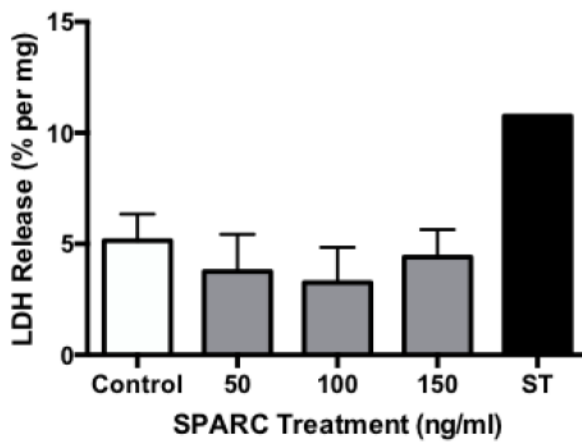


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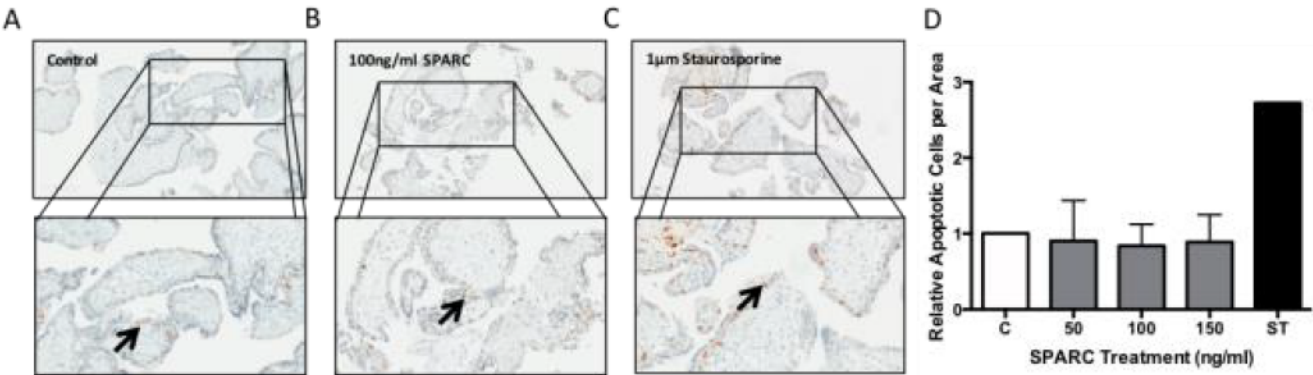


Figure 5

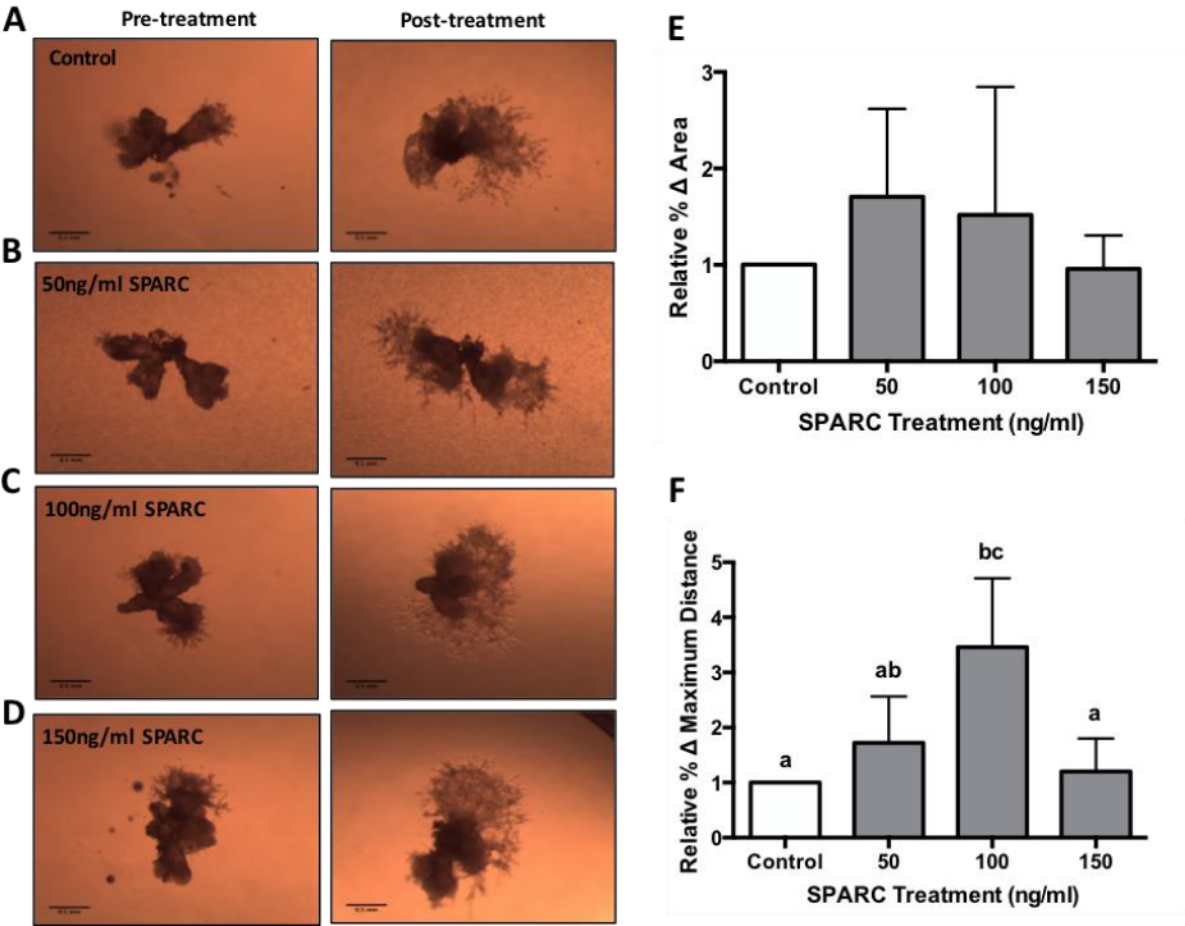


Figure 6

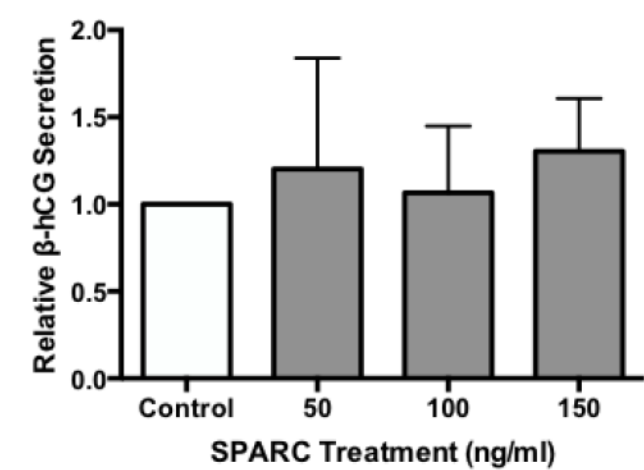


Figure 7

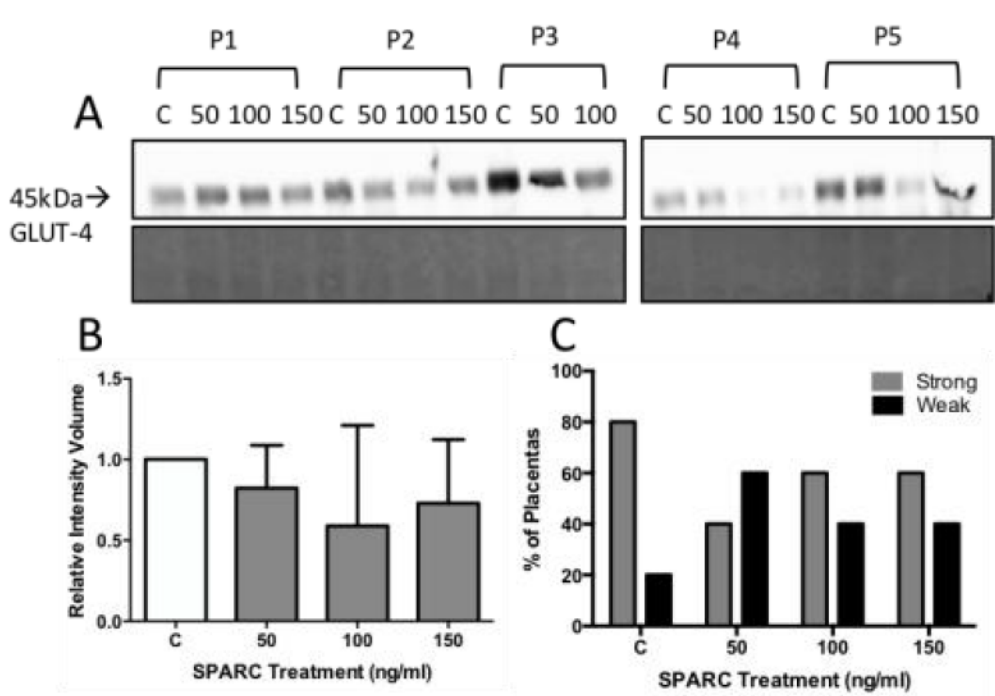
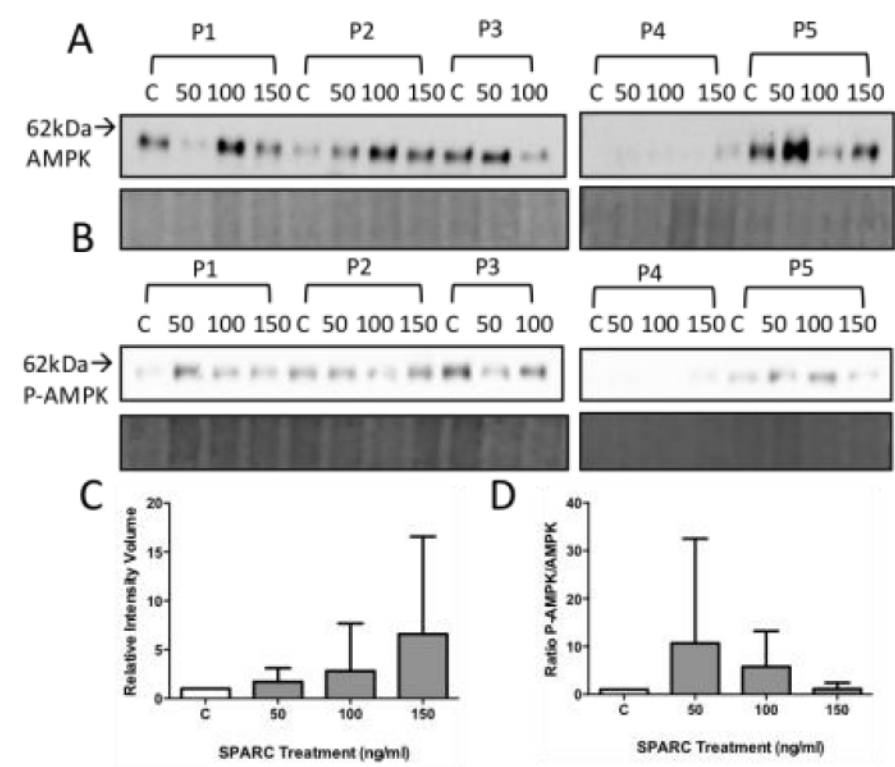


Figure 8



Chapter 5

Final Discussion and Conclusion

5.1 Summary of Findings

In this set of studies the primary research aims were 1) to characterize the circulating myokine profiles of active vs. non-active pregnant women and 2) to investigate the direct effects of any myokines found to be elevated in the circulation of active pregnant women on standard metrics of placental health/function. We hypothesized that we would observe an elevation in some commonly recognized exercise-induced myokines, and that these identified myokines may act as the missing link in trying to understand the mechanism through which maternal exercise may improve fetoplacental health. The major findings of the current studies are as follows:

- 1) SPARC is elevated in the circulation of active pregnant women. No other myokines were found to be elevated in the subset of active pregnant women included in our study.
- 2) SPARC, at concentrations similar to those of active pregnant women, resulted in an increase in 1st trimester human trophoblast invasiveness. None of the other metrics of placental health and function, including the analysis of GLUT-4 transport, were found to be impacted by SPARC treatment.

Overall, this project has illustrated that SPARC, an exercise-induced myokine, is elevated in the plasma of active pregnant women and exogenous SPARC promotes the cellular invasion of 1st trimester human placental explants *in vitro*. Thus, exercise-induced increases in SPARC in the circulation of active pregnant women could potentially contribute to improvements in early placentation and promote the establishment of a successful pregnancy.

5.2 Discussion

5.2.1 Myokine Profiles of Active and Non-Active Pregnant Women

Myokine profiles have been extensively studied in the non-pregnant population following both acute and chronic bouts of physical activity (PA). However, prior to this research project, exercise-induced myokines had yet to be examined in the context of pregnancy. Our group measured the 2nd trimester myokine profiles of women that were categorized as active vs. non-active in pregnancy. SPARC was the only myokine, out of a panel of 11 well-described exercise-induced myokines, with measurable elevations in active compared to non-active pregnant women. One possible explanation for this unexpected finding may be the study design from which the plasma samples were originally collected. In these primary studies the subset of active pregnant women recruited were engaging in habitual moderate-to-vigorous PA, as measured by accelerometry, however their plasma samples were not collected immediately following a bout of PA. It is possible that additional myokines may have been elevated in the circulation of active pregnant women, had the sampling taken place immediately following an acute bout of PA. Consistent with our findings, SPARC has been previously demonstrated to be elevated in non-pregnant individuals in response to chronic PA (Aoi et al., 2013). Furthermore, we recognize the possibility that there are other myokines that are elevated in active pregnant women that were not measured for the purposes of our study.

5.2.2 The Effect of SPARC Treatment on 1st Trimester Human Placental Explants

As we are the first group to measure maternal myokine profiles and identify SPARC as a myokine that is elevated in the plasma of active pregnant women, we are also the first to treat human placental explants with exogenous SPARC. Based on the results of Aim 1, we treated 1st trimester human placental explants *in vitro* with a range of physiologically relevant SPARC concentrations (0-150 ng/ml), including concentrations equivalent to those measured in our

subset of active and non-active pregnant women. Our finding that SPARC treatment at 100ng/ml promotes the maximum outward distance of invasion of 1st trimester placental explants compliments the previous findings by Jiang *et al.* who identified that the downregulation of SPARC in a placental trophoblast cell line (HTR8/SVneo) inhibited cellular invasion through matrigel *in vitro* (Jiang et al., 2013). Put together, our findings and those of Jiang *et al.*, suggest that SPARC has a potentially important role in regulating placental invasion. In early pregnancy, extravillous trophoblast (EVT) cells must actively invade the maternal decidua and remodel the uterine spiral arteries into highly dilated vessels. The appropriate remodeling of spiral arteries by EVT cells allows for optimized flow of maternal blood into the intervillous space of the placenta, supplying the developing fetus with essential nutrients required to support fetal growth. It is possible that elevated systemic SPARC levels in physically active pregnant women could promote the invasion of EVT cells into the maternal uterus and contribute to the establishment of a successful pregnancy (Jiang et al., 2013). In doing so, exercise-induced SPARC may link maternal exercise to direct improvements in placental health/function, which may promote optimized fetal growth.

Previous studies have also identified a role of SPARC in AMPK-regulated glucose metabolism through the regulation of GLUT-4 expression in L6 myocytes (Song, Guan, Zhang, Li, & Dong, 2010). Our group aimed to determine if the treatment of placental explants with SPARC would have a similar impact on the expression of placental GLUT-4. This is important as GLUT-4 is one of the primary glucose transporters expressed in the 1st trimester of pregnancy (Ericsson et al., 2005). Our examination of the effect of increasing SPARC treatment on GLUT-4 in 1st trimester human placental explants identified no impact of SPARC on the expression of this

transporter. This suggests that SPARC may have a different impact on GLUT-4 in skeletal muscle compared to placental tissue. Some possible explanations for this finding may relate to the consideration that SPARC is largely expressed and secreted by contracting skeletal muscle cells, with high concentrations of SPARC exhibiting local effects on skeletal muscle development (Jørgensen et al., 2009; Nakamura, Nakano, Miyoshi, Yamanouchi, & Nishihara, 2013) and glucose metabolism (GLUT-4 expression) (Song et al., 2010). SPARC also has many other functions in the human body including roles in wound healing/tissue regeneration, inflammation, extracellular protease activity, angiogenesis, regulation of growth factor signalling (Bradshaw, 2012), insulin secretion (Harries et al., 2013), erythropoiesis (Luo et al., 2012), and an inhibitory effect on carcinogenesis (Nagaraju, Dontula, El-Rayes, & Lakka, 2014). Although SPARC is expressed by the placenta (Wang et al., 2014), exercise has been shown to contribute to significant elevations in SPARC in circulation (Aoi et al., 2013; Dubé, Gaudet, Aguer, Adamo, & Bainbridge, 2017), likely larger than those produced by the placenta, although this is currently unknown. In our population of pregnant women, SPARC myokine levels were 86 ± 19 ng/ml for active vs. 52 ± 18 ng/ml for non-active pregnant women (Dubé et al., 2017). Our study didn't examine maternal myokine profiles in plasma following a bout of acute exercise, and studies in the non-pregnant population have only measured this in serum (Aoi et al., 2013). Aoi et al. identified that serum SPARC levels are transiently increased in young healthy men immediately after a bout of cycling (70% VO_2 max for 30 min) and gradually declined to baseline levels 6 hours post-exercise (Aoi et al., 2013). Thus, the extent to which SPARC is elevated in plasma in response to acute PA has yet to be investigated. With the multi-faceted role of SPARC, it is possible that SPARC in circulation results in certain direct effects on the placenta (i.e. improved

invasion), but that the true impact of this myokine is to induce systemic physiological changes in the maternal *milieu* via some of its many previously described functions.

Placental GLUT-4 is highly expressed in the syncytiotrophoblast of 1st trimester placenta (Ericsson et al., 2005). As the maternal spiral arteries remain plugged until nearly the end of the 1st trimester (10-12 weeks' gestation), it has been suggested that feto-placental tissue depends on anaerobic metabolism, relying largely on glucose as an energy substrate (Ericsson et al., 2005). GLUT-4 is highly expressed in 1st trimester placenta to meet these high glucose requirements of early pregnancy. Therefore, it is possible that SPARC treatment at concentrations similar to the levels detected in our subset of active pregnant women is not enough of a stimulus to promote the increased expression of GLUT-4 in 1st trimester placental tissue. The possibility also remains that SPARC concentrations are highest in circulation immediately following a bout of PA. If that is the case, concentrations greater than those detected in the circulation of our subset of active pregnant women may be necessary to influence the expression of GLUT-4 in placental tissue. The identification of SPARC levels in active pregnant women following an acute bout of PA is therefore highly important to determine if higher concentrations of SPARC are present in maternal circulation that could potentially exert effects on placental glucose metabolism.

In our study, the expression of both AMPK and P-AMPK in the same SPARC-treated explants was also examined, as SPARC has been found to increase AMPK phosphorylation (activate AMPK) in rat skeletal muscle cells (Song et al., 2010). However, we didn't observe any significant changes in the ratio of P-AMPK to AMPK in these SPARC-treated explants. Considering that activated AMPK is linked to GLUT-4 expression and we detected no changes in the

expression of placental GLUT-4 in response to SPARC treatment, it is not surprising that no difference was observed in the signaling pathway we are proposing would be responsible. Thus, unlike in rat skeletal muscle cells, the AMPK regulatory pathway was not upregulated in 1st trimester placental tissue in response to SPARC treatment and thus, didn't contribute to the increased expression of placental GLUT-4.

5.3 Limitations

In our studies, we were limited to previously collected plasma samples that reflected habitual exercise engagement. Unfortunately, we didn't have access to plasma samples representative of the effects of acute exercise on myokine profiles for comparison. This limitation makes it difficult to make comparisons with the published literature for the non-pregnant population. However, it is also important to consider that the active pregnant women in our study had PA levels that are, in general, lower than those observed in the active non-pregnant population, even though these women were significantly more active than their non-active counterparts. As a result, the myokine profiles of active pregnant and non-pregnant individuals in response to both acute and chronic PA may be different as a reflection of differing levels of engagement in an active lifestyle. Another limitation of this research project is that immunohistochemistry (IHC), the methodology that we used to examine GLUT-4 localization, was unable to identify intracellular localization of placental GLUT-4 within the syncytium. Future investigations should utilize methods of immunofluorescence and confocal microscopy to identify any differences in placental GLUT-4 localization in response to SPARC treatment. Another important consideration is that the PA levels of the women who donated their 1st trimester placentae for the purposes of this research project are unknown. It is possible that

some of these collected placental tissues came from active women and had been exposed to a more “active” maternal intrauterine environment. Furthermore, it is important to consider that there is a high degree of variability observed when working with primary tissues such as the human placenta. It is suggested that future studies use an increased sample size when examining the effects of exercise-induced myokines on placental health and function to account for this high degree of inter-placental variability.

5.4 Future Research Directions

Future experiments are necessary to compare the impact of acute vs. chronic PA on the myokine profiles of active and non-active pregnant women. As previously established in the non-pregnant population (Catoire & Kersten, 2015), it is likely that acute and chronic PA differentially stimulate the secretion of myokines from skeletal muscles in pregnant women. In non-pregnant individuals, participation in chronic PA results in changes to the myokine profile; namely elevated apelin, BDNF, IL-7, IL-15, SPARC, or reduced IL-6, MCP-1/CCL2 whereas there are inconsistencies in others (irisin, myostatin) (reviewed in (Catoire & Kersten, 2015)). Many of the myokines that we measured, including ANGPTL-4, MCP-1/CCL2, CX3CL1, IL-6, IL-8, IL-15 and SPARC, have been shown to be elevated in plasma in response to acute PA in the non-pregnant population (reviewed in 3). These myokines would likely also be elevated in the plasma of active pregnant women following a bout of acute PA. We only identified one of these myokines, SPARC, to be elevated in our subset of pregnant women who, by definition, were habitually active during pregnancy but had not engaged in PA for at least 12 hours (not acutely active). Despite our findings, it is likely that many other myokines are elevated in maternal circulation following an acute bout of exercise and thus could be acting on the placenta during pregnancy, warranting

an investigation of their effects on placental health/function. The establishment of maternal myokine profiles in response to PA along with the precise effects of any elevated myokines on placental health/function (including macronutrient transport) is vital. This knowledge will bring us one step closer to uncovering some of the mechanisms whereby maternal exercise during pregnancy improves maternal-fetal health outcomes, including optimized fetal growth.

Future investigations should further examine the potential effect of increasing SPARC treatment on placental GLUT-4 localization using more sophisticated techniques. SPARC has been found to activate AMPK to promote the translocation of GLUT-4 to the plasma membrane in mouse skeletal muscle cells (Song et al., 2010), suggesting that it is possible that increasing SPARC treatment could have the same effect in individual placental trophoblast cells. Due to time constraints for this thesis, it was not feasible to perform immunofluorescence protocols following the IHC experiments. Future experiments are warranted to determine if increasing SPARC treatment enhances GLUT-4 translocation in individual placental trophoblast cells using immunofluorescence and confocal microscopy. Since no effect of SPARC on GLUT-4 was observed, it is also possible that SPARC may influence other macronutrient transporters or other macronutrients (i.e. amino acids or fatty acids), contributing to the mechanistic link between exercise-induced SPARC and optimization of fetal growth in early gestation. The effects of other exercise-induced myokines on placental macronutrient transporters should also be examined. If other myokines are found to improve the expression/activity of macronutrient transporters in placental trophoblast cells, this could identify important roles of myokines in regulating the transport of nutrients to the developing fetus, and in the regulation of fetal growth across gestation.

5.5 Clinical Application

Prior to this thesis project, little was known about exercise-induced myokines in relation to pregnancy. An investigation of the effects of myokines that are elevated in maternal circulation in response to PA is important as it may uncover some of the potential mechanisms whereby maternal exercise during pregnancy optimizes fetal growth. It is well-established that exercise during pregnancy has a role in the reduction of fetal birth weight extremes (Clapp, 1996; Hopkins & Cutfield, 2011; Juhl et al., 2010; Owe et al., 2009). Knowledge regarding potential mechanisms through which maternal exercise promotes such improved maternal-fetal health outcomes will provide further evidence to support exercise as a preventative therapy or as treatment for pregnancy-related conditions. The discovery of new pathways and the dissemination of this knowledge will help to inform clinicians as to which type of exercises would be most beneficial as therapy based on the associated myokine profiles, along with an understanding of how myokines may improve placental health/function or placental macronutrient transport to the developing fetus. It is believed that this type of knowledge will further contribute to the development of important and innovative therapeutic interventions. Specifically, it may inform the development of therapeutics that could deliver myokines or other systemic signaling molecules into maternal circulation that reflect the myokine profiles of active pregnant women. As many women are unable to exercise during pregnancy due to health contraindications or other factors (i.e. feeling unwell, pain and discomfort), therapeutics could deliver the benefits of exercise during pregnancy to all women and their developing offspring regardless of having the ability/inability to engage in routine PA. This type of innovation could

contribute to improved maternal-fetal health outcomes on a global scale, such as promote AGA fetal growth and reduce the number of offspring born at both fetal birth weight extremes.

5.6 Conclusion

Through the analysis of the circulating myokine profiles in our subset of pregnant women characterized as active or non-active, we identified one myokine, SPARC, to be significantly elevated in the plasma of active pregnant women. Subsequently, human placental explants were treated with SPARC in a dose-dependent manner, identifying a significant effect of SPARC on maximizing the mean distance of outward invasion by human placental explants. Identifying SPARC as an important exercise-induced myokine in active pregnant women with effects on placental invasion is an important first step in progressing towards an understanding of the benefits of exercise during pregnancy. Once the effects of exercise during pregnancy are better understood, including the specific impacts on the placenta, the mechanisms whereby maternal exercise may optimize fetal growth may become clear and contribute to the development of interventions, including exercise therapy and therapeutic agents that can prevent fetal birth weight extremes and promote optimal fetal growth.

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APPENDIX A: PRELIMINARY DATA FROM THE TREATMENT OF HUMAN PLACENTAL EXPLANTS

WITH MYOKINE MEDIA

Methods

Refer to detailed methods as outlined in Chapter 4 (manuscript #3) for placental collection and dissection of floating human placental explants.

Placental explants were cultured in experimental media supplemented with 2% FBS, and 1% 100X P/S. Myokine treatment media was prepared in the Aguer lab at the Montfort Hospital. Previously obtained skeletal muscle cell biopsies were thawed and differentiated into human primary myotubes. Human primary myotubes were stimulated using electric-pulse stimulation (EPS) for 24 hours as per the protocol established by Scheler et al. (89). Control media, skeletal muscle conditioned non-stimulated media (media surrounding skeletal muscle cells prior to stimulation) and skeletal muscle stimulated media (media collected after 24-hr EPS of skeletal muscle cells) were transported on ice from Montfort to the laboratory at the University of Ottawa. For each floating explant experiment (N=4), approximately 3-4 individual explants per treatment group were placed on a Netwell® support and incubated in the corresponding experimental treatment media (control, non-stimulated and stimulated) for 72 hrs. Afterwards, all floating explants from each treatment group were collected, weighed and either flash-frozen or processed for immunohistochemistry. All treatment media was also collected immediately and stored at -80°C. For all floating explant experiments (N=4), a control was included and all experimental treatments were performed in duplicates per placenta.

Refer to detailed methods for assessment of placental health (cellular proliferation, LDH cytotoxicity and apoptosis), assessment of placental function (β -hCG Secretion) and statistical

data analysis as outlined in Chapter 4 (Manuscript 3). Assessment of the effect of myokine media on cellular invasion has yet to be investigated.

Results

Effect of Exposure to Myokine Media on Placental Health

Cellular Proliferation

Following exposure to myokine treatment media, no significant changes were observed in the number of Ki-67 positive cells (**Figure 1**). For all experimental treatments (control, non-stimulated and stimulated), the mean number of cytotrophoblast cells undergoing proliferation was 152 ± 61 across all treatments, as indicated by Ki-67 immunoreactivity across 4 random fields of view at 10x magnification.

LDH Release

LDH release by placental explants remained unchanged across all media treatments (control, non-stimulated and stimulated) (**Figure 2**). For all experimental treatments, $9.1 \pm 5.6\%$ of intracellular LDH was released into cell culture media per milligram of placental explants. Staurosporine ($1\mu\text{m}$) treated explants were included as a positive stress control, with significantly reduced placental cell viability, as indicated by increased LDH release (10.75% LDH/mg).

Apoptosis

Following exposure to treatment media, apoptosis was primarily restricted to the syncytiotrophoblast layer in all samples (**Figure 3A-D**). No significant changes in apoptosis were observed across all treatments (control, non-stimulated and stimulated). For all experimental treatments, 90 ± 48 cytotrophoblast cells were actively undergoing apoptosis as indicated by M30

immunoreactivity across 4 random fields of view at 10x magnification (**Figure 3E**). Staurosporine (1 μ m) treated explants were included as a positive control for stress, resulting in a 243% increase in apoptosis compared to the other experimental treatments.

Effect of Exposure to Myokine Media on Placental Function

β -hCG Secretion

β -hCG release by placental explants remained unchanged across all experimental treatments (control, non-stimulated and stimulated) (**Figure 4**). For all experimental treatments, the mean concentration of β -hCG normalized to placental explant weight was 11.7 $\pm$ 16.4 mIU/ml/mg secreted into cell culture media

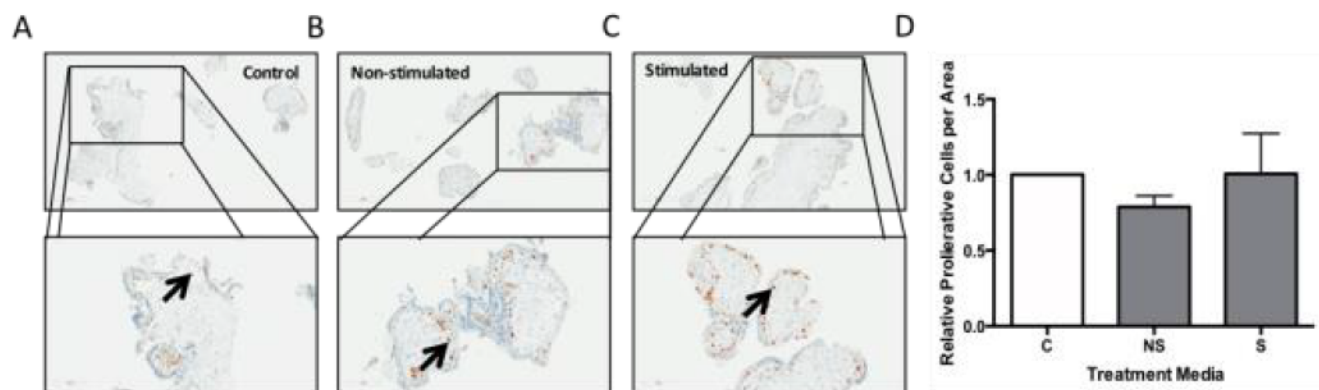


Figure 1. Effect of myokine treatment on cytotrophoblast proliferation in human

placental explants. (A-C) Representative images showing cytotrophoblast proliferation after

exposure to **(A)** control (C), **(B)** non-stimulated (NS) and **(C)** stimulated (S) media, as indicated by

Ki-67 immunoreactivity (black arrow). No difference in cytotrophoblast proliferation observed

following a 72-hour treatment with experimental media **(Panel D)**. Level of proliferation

represented as mean number of proliferating cytotrophoblast cells (ki-67 positive) per total area

of tissue in 4 random fields of view at 10x magnification. Data is presented as means $\pm$ SD (N=4).

Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison

post hoc analysis (*p<0.05).

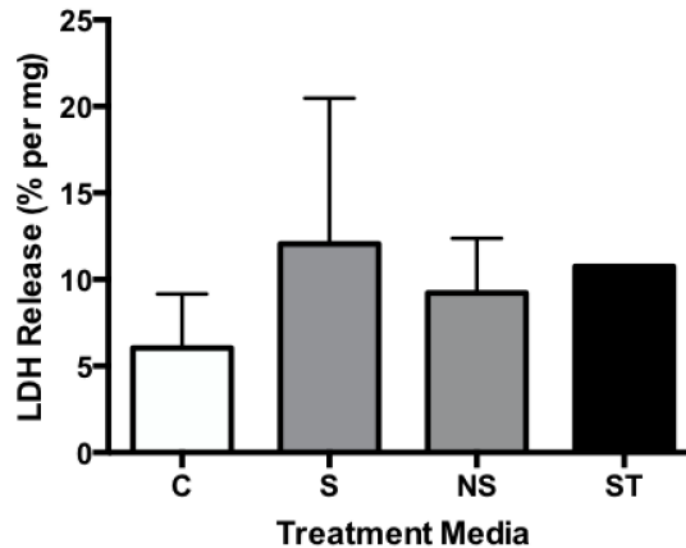


Figure 2. Effect of myokine treatment on lactate dehydrogenase (LDH) release by human placental explants. No difference in placental LDH release by placental explants following 72-hour treatments with experimental treatment media (control (C), non-stimulated (NS) and stimulated (S)). Measured LDH release for each treatment group was normalized to the mass of the corresponding placental explants and expressed as a percentage of a 100% LDH positive control. The 100% LDH control represents the amount of total cytoplasmic LDH present in an equivalent mass of placental explants. As a positive stress control explants (N=1) were treated with staurosporine (ST; 1 μ M) for 72 hours. Data is presented as means $\pm$ SD (N=4). Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison post hoc analysis (*p<0.05).

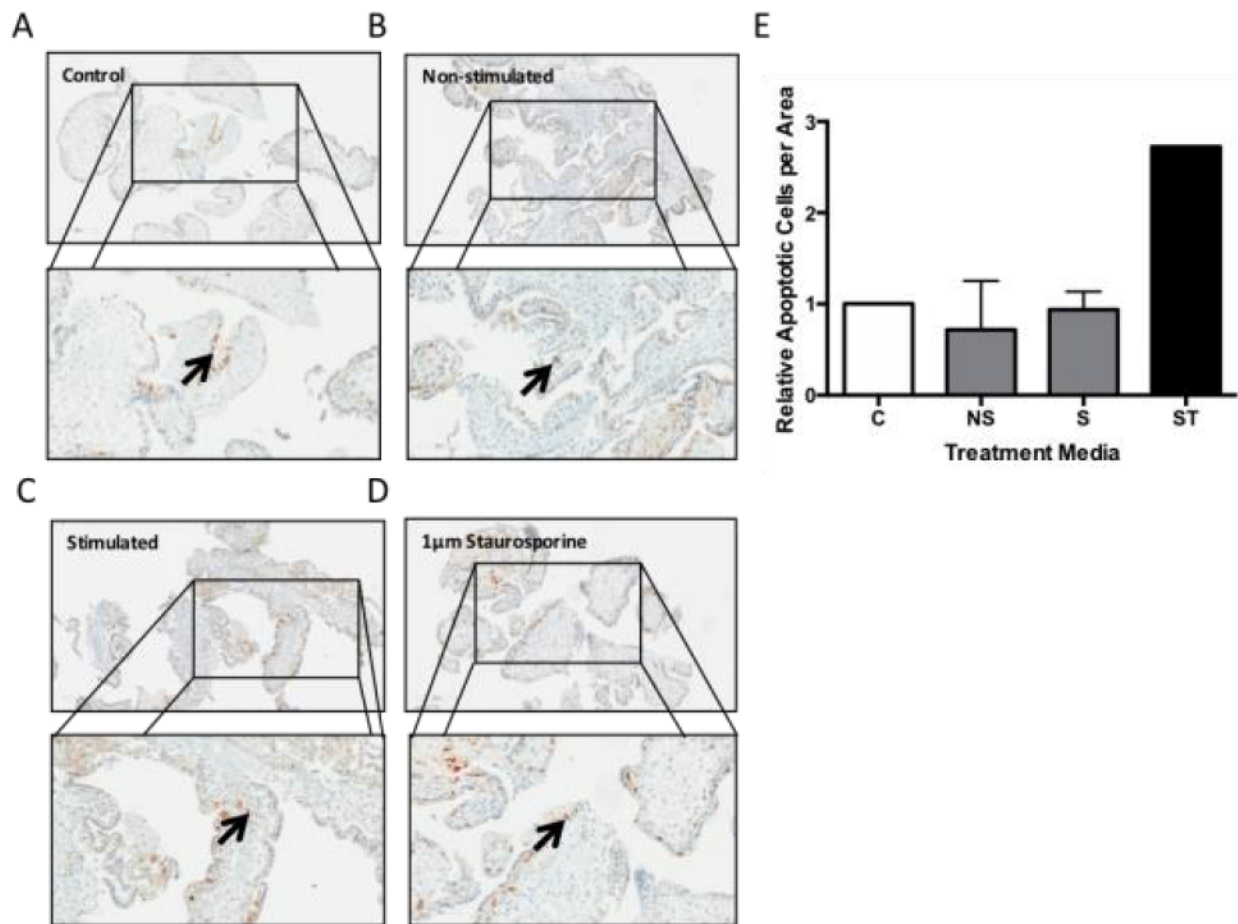


Figure 3. Effect of myokine treatment on apoptosis in human placental explants. (A-D)

Representative images showing apoptotic cells (black arrow) after 72-hour exposure to **(A)** control (C), **(B)** non-stimulated media (NS), **(C)** stimulated (S) media and **(D)** 1µM staurosporine (ST). Level of apoptosis represented as mean number of apoptotic cells per total area of tissue in 4 random fields of view at 10x magnification **(Panel E)**. As a positive stress treatment control (N=1), one set of explants were treated with staurosporine for 72 hours. Data is presented as means±SD (N=4). Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison post hoc analysis (*p<0.05).

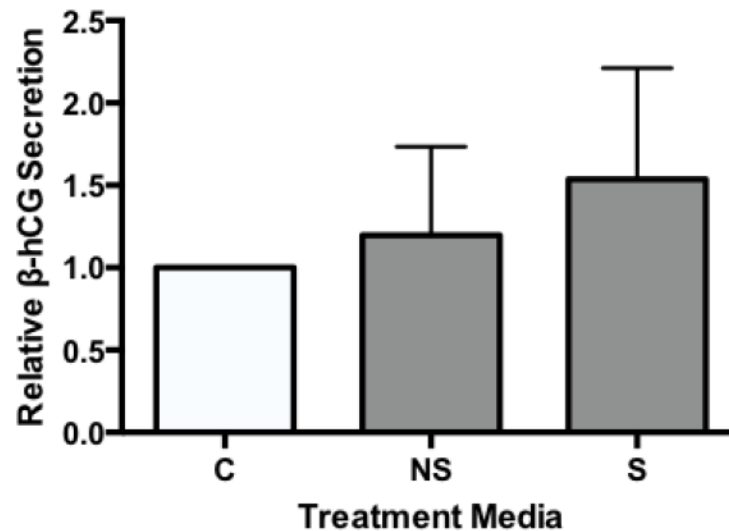


Figure 4. Effect of myokine treatment on β -hCG hormone secretion by human placental explants. The concentration of β -hCG released following 72-hour exposure to experimental treatment media (control (C), non-stimulated (NS) and stimulated (S)) was determined using an enzyme-linked immunosorbent assay (ELISA), and was normalized to tissue mass (mg). Data is presented as the mean concentration (mIU/mL/mg) of β -hCG hormone secretion $\pm$ SD (N=3) related to untreated controls. Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison post-hoc analysis (* p <0.05).

APPENDIX B: RESEARCH ETHICS BOARD (REB) APPROVALS AND PERMISSIONS

B.1 Ottawa Health Science Network (OHSN) REB Renewal



Ottawa Health Science Network Research Ethics Board/ Conseil d'éthique de la recherche du Réseau de science de la santé d'Ottawa

Civic Box 411 725 Parkdale Avenue, Ottawa, Ontario K1Y 4E9 613-738-5555 ext. 14902 Fax : 613-761-4311
<http://www.ohsn.ca/ohsn-reb>

October 21, 2016

Dr. Laura Gaudet
Ottawa Hospital - General Campus
Division of Maternal Fetal Medicine
501 Smyth Road
Ottawa, ON
K1H 8L6

Dear Dr. Gaudet:

RE: Protocol# - 20130677- Understanding the molecular basis of placental mediated diseases
01H

Renewal Expiry Date - October 27, 2017

I am pleased to inform you that your Annual Renewal Request 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.

The Protocol Amendment Report dated September 24, 2016 extending the projected date of study completion to October 27, 2017 is approved.

Renewal is valid for a period of one year. Approximately one month prior to that time, a single renewal form should be sent to the REB office.

The REB no longer requires a 'valid until' date at the bottom of all approved informed consent forms. The consent forms currently approved for use by the REB are:

- English Information Sheet and Consent Form- First Trimester (version 1) dated August 13, 2014
- English Information Sheet and Consent Form- Second and Third Trimester (version 1) dated August 13, 2014
- French Information Sheet and Consent Form- First Trimester (version 1) dated August 13, 2014
- French Information Sheet and Consent Form- Second and Third Trimester (version 1) dated August 13, 2014

The OHSN-REB complies with the membership requirements and operates in compliance with the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans; the International Conference on Harmonization - Good Clinical Practice: Consolidated Guideline and the provisions of the Personal Health Information Protection Act 2004.

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

B.2 OHSN REB Protocol Amendment-Addition of Chantal Dubé as a Co-investigator



**Ottawa Health Science Network Research Ethics Board/ Conseil d'éthique de la recherche du
Réseau de science de la santé d'Ottawa**

Civic Box 411 725 Parkdale Avenue, Ottawa, Ontario K1Y 4E9 613-708-5555 ext. 14902 Fax : 613-761-4311
<http://www.ohsn.ca/ohsn-reb>

01 April, 2016

Dr. Laura Gaudet
Ottawa Hospital - General Campus
Division of Maternal Fetal Medicine
501 Smyth Road
Ottawa, ON
K1H 8L6

Dear Dr. Gaudet:

Re: Protocol # 20130677-01H Understanding the molecular basis of placental mediated diseases

Thank you for the Protocol Amendment Report of January 26, 2016 for the purpose of adding Chantal Dubé as a co-investigator to the study. The amendment is approved.

OHSN-REB complies with the membership requirements and operates in compliance with the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans; the International Conference on Harmonization - Good Clinical Practice: Consolidated Guideline; and the provisions of the Personal Health Information Protection Act 2004.

Ethical approval remains in effect until October 27, 2016.

Yours sincerely,

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

/km

Appendix C: ETHICS AND LABORATORY TRAINING

C.1. Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans Course on Research Ethics (TCPS 2: CORE)



C.2. Lab Safety



C.3. Principles of Biosafety



Appendix D: Scholarly Achievements

D.1. Scholarships

2015-2017 University of Ottawa Graduate Admission Scholarship

D.2. Abstracts

2017 Dubé, C., Aguer, C., Adamo, K. and Bainbridge, S. (2017). The Effect of Exercise-induced Myokines on Placental Health & Function. Abstract accepted for International Federation of Placenta Associations (IFPA) Meeting 2017.

D.3 Academic Conferences and Workshops

2016 10th Annual Human Placenta Summer Workshop. Queen's University, Kingston, Ontario, Canada. July 24-29, 2016 (See certificate section appendix D.5).

2015 Strengthening Maternal-Child health in the Community: A Research Think Tank. University of Ottawa, Ottawa, Ontario, Canada. November 30, 2015.

D.4 Publications

2017 Dubé, C., Aguer, C., Adamo, K., & Bainbridge, S. (2017). A role for maternally derived myokines to optimize placental function and fetal growth across gestation. *Applied Physiology, Nutrition, and Metabolism*, 42(5), 459–469. <https://doi.org/10.1139/apnm-2016-0446>.

D.5. 10th Annual Human Placenta Summer Workshop Certificate

