Application of Circulating Large Extracellular Vesicles as Biomarkers in Type 1 Diabetes Mellitus and Pregnancy

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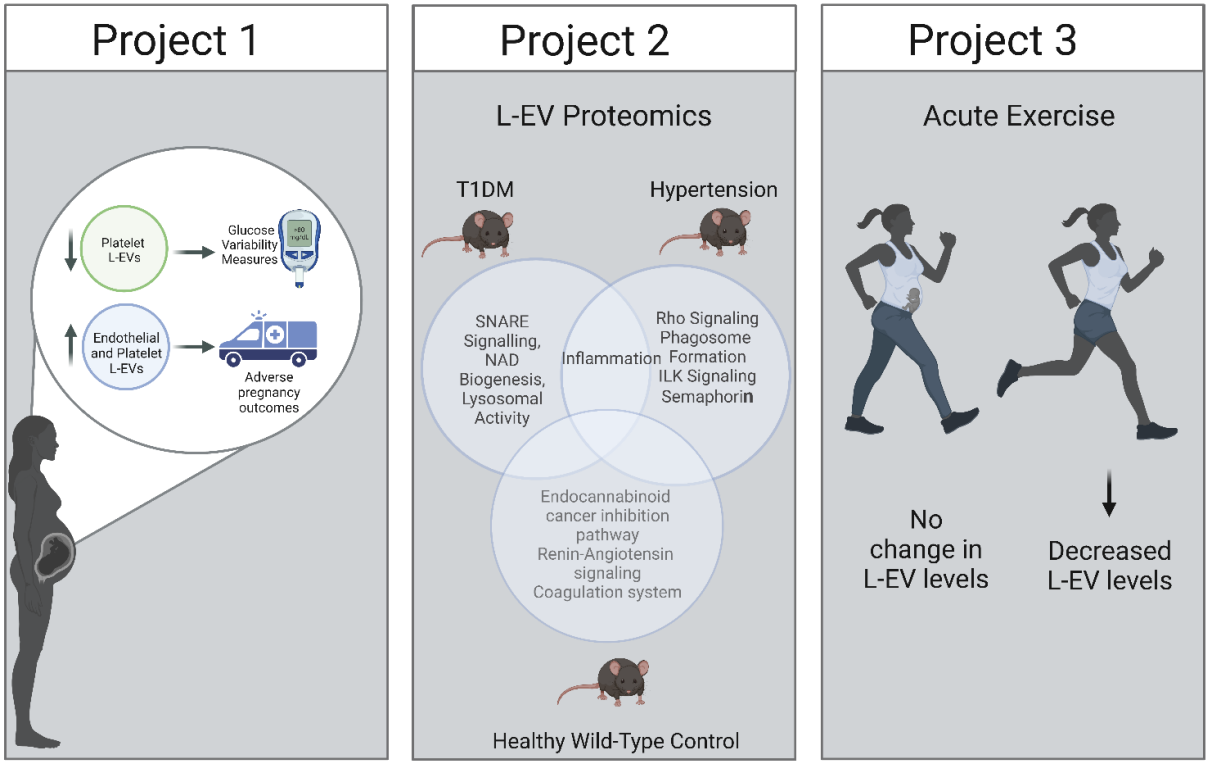
Abstract

Levels of circulating large extracellular vesicles (L-EVs) are increased in individuals with type 1 diabetes mellitus (T1DM) and associated with increased cardiovascular risk. T1DM in pregnancy induces vascular injury leading to adverse maternal and neonatal outcomes. Conversely**,** exercise has been shown to improve cardiovascular and metabolic health in pregnancy and may represent a non-pharmacological approach to improving pregnancy outcomes. Assessment of vascular health may aid in the identification of individuals at risk of complications and allow for intervention with strategies to improve the maternal vasculature. Unfortunately, there is a paucity of strategies for assessing vascular health in pregnant women. L-EVs are membrane-encapsulated particles released from stressed/injured cells. They are emerging biomarkers of vascular health. The purpose of this thesis was to assess the impact of T1DM and pregnancy on L-EV levels and protein composition, the relationship between L-EVs and pregnancy outcomes and the effect of exercise on L-EV levels. In aim #1, I observed that high levels of L-EVs are predictive of adverse pregnancy outcomes. In aim # 2, I examined the protein composition of circulating L-EVs in hypertensive, diabetic and healthy mice models. Diabetes-enriched proteins were involved in inflammation, SNARE signaling and NAD^+ biogenesis. The changes were found in L-EV protein content were consistent with proteins associated with inflammation, cytoskeletal organization, and angiogenesis. Finally, in aim #3, I examined the changes in plasma L-EVs after an acute bout of moderate-intensity aerobic exercise in healthy pregnant and non-pregnant women. I observed that circulating L-EVs significantly decreased after the acute exercise only in non-pregnant individuals. Taken together, my thesis work advances knowledge on L-EVs in T1DM, pregnancy, and

hypertension and sets the stage for future work on L-EVs as predictive biomarkers, for molecular profiling, and for monitoring of vascular health interventions in pregnancy.

Graphical Abstract



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Dedication

This PhD thesis and all my academic achievements are dedicated to the memory of my father, Ali Abolbaghaei, who passed away shortly after I started my PhD journey.

Statement of co-authorship

I performed all the studies outlined in Chapters 2-5 in the laboratory of Dr. Dylan Burger, with the assistance of co-authors as listed below.

Chapter 2 was a secondary analysis of CONCEPTT trial published previously [1]. I was responsible for all sample analysis, data analysis, drafting and preparation of manuscript. I also performed the flow cytometry with operational assistance from Vera Tang facility manager of the Flow Cytometry at the University of Ottawa or Fengxia Xiao a Research Associate in laboratory of Dr. Dylan Burger. The co-authors listed in this study, provided contributions to manuscript preparation and content.

For Chapter 3, the sample collection was done by Madisson Turner, and the post hoc analysis of all proteomics data, designing, drafting and preparation of the manuscript was performed by me. The co-authors listed in this study, provided contributions to manuscript preparation and content.

Chapter 4 was a secondary analysis of a prior study published by Hutchinson and Colleagues[2]. I did everything in this project including the study design, sample analysis, laboratory experiments, post hoc analysis, drafting and preparation of the manuscript. The data analysis was also done by me with the assistance of Danilo Fernandes da Silva and Shuhiba Mohammad The co-authors listed in this study, provided contributions to manuscript preparation and content.

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List of publications during Ph.D.

1. **Abolbaghaei A**, Langlois M-A, Murphy HR, Feig DS, Burger D, on behalf of the CONCEPTT Collaborative Group (2021) Circulating extracellular vesicles during pregnancy in women with type 1 diabetes: a secondary analysis of the CONCEPTT trial. Biomarker Research 9(1):67. https://doi.org/10.1186/s40364-021-00322-8
2. **Abolbaghaei A**, Mohammad S, da Silva DF, et al (2023) Impact of acute moderate-intensity aerobic exercise on circulating extracellular vesicles in pregnant and non-pregnant women. Appl Physiol Nutr Metab 48(2):198–208. https://doi.org/10.1139/apnm-2022-0288
3. **Abolbaghaei A**, Turner M, Thibodeau J-F, Holterman CE, Kennedy CRJ, Burger D (2023) The Proteome of Circulating Large Extracellular Vesicles in Diabetes and Hypertension. International Journal of Molecular Sciences 24(5). https://doi.org/10.3390/ijms24054930
4. **Abolbaghaei A** (2022) Proteome of large extracellular vesicle in type 1 diabetes. Hypertension News. https://doi.org/10.30824/2209-16
5. Bathla T, **Abolbaghaei A**, Reyes AB, Burger D (2022) Extracellular vesicles in gestational diabetes mellitus: A scoping review. Diabetes and Vascular Disease Research 19(2):14791641221093900. https://doi.org/10.1177/14791641221093901

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List Of Abbreviations

ANC	antenatal care
ANK1	ankyrin-1
ANOVA	analysis of variance
ARMMs	arrestin domain-containing protein 1 (arrdc1)-mediated microvesicles
ATP	adenosine triphosphate
BMI	body mass index
CEMACH	confidential enquiry into maternal and child health
CGM	continuous glucose monitoring
CLC1B	c-type lectin domain family 1 member b
CO3A1	collagen alpha-1 (iii) chain
CONCEPTT	continuous glucose monitoring in pregnant women with type 1 diabetes
CRP	c-reactive protein
CTRL	control
CV	coefficient of variation
CVD	cardiovascular disease
DAMP	damaged associated molecular pattern
DBP	diastolic blood pressure
DM	diabetes mellitus
DPP-IV	dipeptidyl peptidase IV
eEV	endothelial extracellular vesicle
EVs	extracellular vesicles
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

GDM	gestational diabetes mellitus
GFR	glomerular filtration rate
GWG	gestational weight gain
HDL	high-density lipoprotein
HPT	haptoglobin
HR	heart rate
HRR	heart rate reserved
HVM17	Ig heavy chain v region mopc 47a
IDDM	insulin-dependent diabetes mellitus
IGHA	Ig alpha chain c region
ILK	integrin-linked kinase
INS	human insulin gene
IPA	ingenuity pathway analysis
LC-MS/MS	liquid chromatography mass spectrometry
L-EV	large extracellular vesicle
lEV	leukocyte extracellular vesicle
LGA	large for gestational age
LPS	lipopolysaccharide
MAGE	mean amplitude of glycemia excursion
MCP	monocyte chemotactic protein
miRNA	Micro RNA
MPs	microparticles
MS	mass spectrometry

NGT	normal glucose tolerance
NICE	national institute for health and care excellence
NICU	neonatal intensive care unit
NPID	national pregnancy in diabetes
NTA	nanoparticle tracking analysis
Nu-P62	nucleoporin p62
OR	odds ratio
PA	physical activity
pEV	platelet extracellular vesicle
PGD	pre-gestational diabetes
PMGE	bisphosphoglycerate mutase
PPN	papilin
PS	phosphatidylserine
PSA3	proteasome subunit alpha type-3
PSA7	proteasome subunit alpha type-7
RHR	resting heart rate
rHRV	resting heart rate variability
RIPA	radioimmunoprecipitation assay
Rmcorr	repeated measures correlation
rMSSD	root mean square of successive r-r intervals differences
RPF	renal plasma flow
RT-CGM	real time continuous glucose monitoring
SAMP	serum amyloid p-component

SBP	systolic blood pressure
SD	standard deviation
sEV	small extracellular vesicle
SMBG	self-monitoring of blood glucose
SPSS	statistical package for the social sciences
SPTA1	spectrin alpha chain, erythrocytic 1
SPTB1	spectrin beta chain, erythrocytic
STB-EVs	syncytiotrophoblast-derived extracellular vesicles
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TAR	time spent above range
TBR	time spent below range
TEM	transition electron microscopy
tEVs	total extracellular vesicles
TIR	time in range
TNF	tumour necrosis factor
TRAP	thrombin-receptor-activating peptide
TSG101	tumor susceptibility gene 101
TSP4	thrombospondin-4
TtRhRen	Transthyretin Recombinant Human Renin
WHO	world health organization
WT	wild-type
ZPI	cluster of protein z-dependent protease inhibitor

Table of Contents

1 CHAPTER 1: INTRODUCTION

Portions of this chapter have been published in:

Bathla, T., **Abolbaghaei, A**., Reyes, A. B. & Burger, D. Extracellular vesicles in gestational diabetes mellitus: A scoping review. *Diab. Vasc. Dis. Res.* **19**, 14791641221093900 (2022). **Reproduced through a (CC BY-NC 4.0) license;**

as well as:

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1.1 DIABETES MELLITUS

Diabetes mellitus (DM) is a heterogeneous metabolic disease with complex pathogenesis [1]. It is characterized by chronic hyperglycaemia resulting from abnormalities in either insulin action, secretion, or both [2–5]. Further, it may appear in a heterogenous manner as fat, carbohydrate and protein metabolic dysfunctions [2–5]. According to World Health Organization (WHO) statistics, over 400 million people are living with DM today and by 2040 an estimated 642 million will live with DM [6,7]. The chronic hyperglycaemia of DM can cause numerous complications such as cardiovascular and reproductive dysfunction, neuropathy, retinopathy, and nephropathy [2,8]. High glucose conditions also promote proliferation of cancer cells and reinforce a suitable environment for infection [8]. Long-term poorly controlled glucose may cause various microvascular and macrovascular diabetic complications that are mainly responsible for diabetes-associated morbidity and mortality [8].

1.2 CLASSIFICATION AND PATHOGENESIS OF DIABETES MELLITUS

DM is a group of chronic disorders with varied presentation and any classification of this disorder is often influenced by the physiological conditions present at the time of assessment and diagnosis [4,8,9]. The current classification is according to both the pathogenesis and etiology of disease which may guide clinical assessment and therapeutic treatment. DM is classified into the following categories: type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM) and gestational diabetes mellitus (GDM).

Different types of DM may present pathophysiological differences. In T1DM, the destruction of beta cells causes failure to produce insulin or failure to produce adequate insulin to control glucose levels. Further, the insufficient insulin production which may lead to reduced glucose utilization by adipose tissues and muscle cells, increases glucose synthesis in liver and fatty acid production (Figure 1.1.A) [4,9–12]. This ultimately, results in an unbalanced glucagon response causing hyperglycaemia. T1DM arises mainly from autoimmune mediated injury to pancreatic islet cells. The treatment for T1DM is exogenous insulin which replaces the deficiency in insulin production. T2DM is quite different than T1DM; the body is producing insulin in the pancreatic beta cells (β- cells), but the cells don't respond to that insulin appropriately (insulin resistant) (Figure 1.1.B). The treatment of T2DM is not about giving insulin, however, giving insulin may overcome the failure to respond but the primary treatment is about increasing insulin sensitivity, or optimizing glucose utilization elsewhere as is the case with metformin.

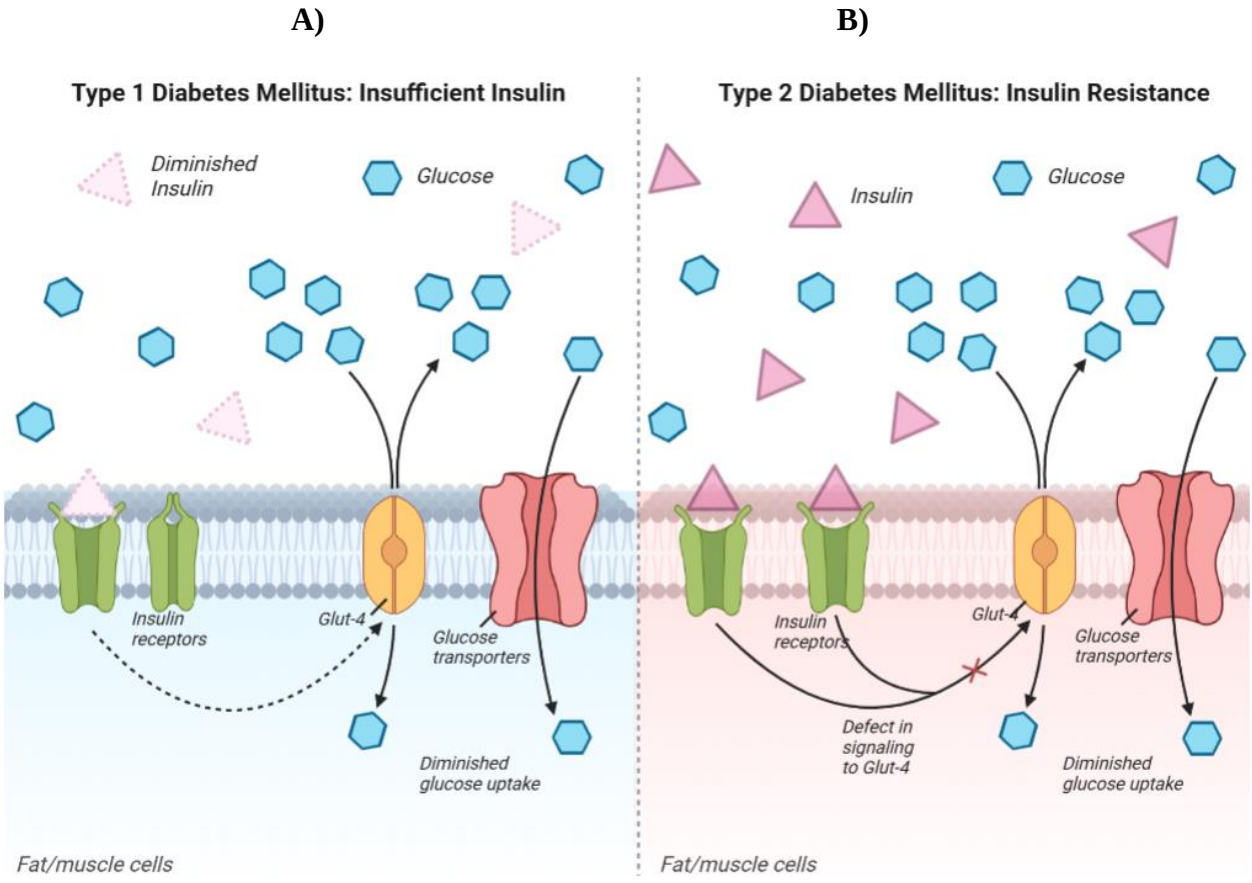

Figure 1.1. A. Pathophysiology of type 1 diabetes mellitus. B. Pathophysiology of type 2 diabetes mellitus. The figure is produced by BioRender.com.

1.2.1 Type 1 diabetes mellitus

T1DM, also known as insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes mellitus comprises nearly 5–10% of all diabetes worldwide [4]. It is an autoimmune disorder that results from the destruction of pancreatic β-cells in the pancreatic islets of Langerhans [13] which causes insulin deficiency and ultimately hyperglycaemia [4,10,14,15]. An important role of the pancreas in DM was first discovered in 1890 by removing the organ from dogs which resulted in permanent hyperglycaemia [16]. The pancreas is an organ of the digestive system responsible for both endocrine and exocrine functions in the body [17]. The exocrine pancreatic cells comprise around 98% of cells within the pancreas along with endocrine cells dispersed all over in small erratic clusters called the

islets of Langerhans [17]. The islets of Langerhans consist of four cell types including the insulin producing β-cells. These cells of the pancreatic islets release insulin, a peptide hormone encoded in humans by the INS gene [17]. Insulin is responsible for the uptake of glucose by cells in muscles, liver and adipose tissue. Glucose is stored as glycogen in the liver and muscle while stored as fat in adipose tissue. Autoantibodies targeting β-cell antigens including glutamic acid decarboxylase, insulin and a protein tyrosine phosphatase molecule named IA-2 are detected in most patients with T1DM. This is used to distinguish individuals from T1DM from other forms of diabetes [18,19]. Although T1DM usually appears in children and adolescents, it can occur at any age. Nevertheless, the incident rates are higher in younger individuals. The destruction of β-cells is highly variable but studies have shown that it tends to happen rapidly in infants and children and gradually in adults [2,20]. Numerous influences (genetic and environmental factors) such as the immune system, β-cells, and the environment in genetically susceptible individuals may also contribute to the development of T1DM [10].

1.2.2 Type 2 diabetes mellitus

T2DM is a chronic, multifactorial metabolic disease that has become a worldwide epidemic in recent decades [21,22]. Progression of T2DM is mostly dependent on a combination of two main factors; a) inability of insulin-sensitive tissues to respond appropriately to insulin (peripheral tissue insulin resistance) and b) β-cell dysfunction which results in defective insulin secretion [11,12,21,22]. These changes may cause (i) hyperglycaemia because of a reduction in glucose uptake, (ii) impaired ATP release and amino acid uptake into peripheral tissues including skeletal muscle because of reduced nutrient uptake, (iii) dyslipidaemia which is a hallmark of the metabolic syndrome (reduction of high density lipoprotein (HDL) cholesterol and hypertriglyceridemia) as a result of reduced fat uptake and (iv) increase in glucagon production signifying hyperlipidaemia

and hyperglycaemia [23–25]. T2DM is the most prevalent type of diabetes, accounting for approximately 90% of diabetes patients [25,26]. T1DM and GDM account for the remaining cases [25,27]. Individuals with T2DM are usually overweight or obese which aggravates insulin resistance [28,29]. The prognosis and treatment approach may vary between patients due to the heterogeneity of this disease as well as the severity of insulin deficiency [1,30]. According to previous epidemiological studies, approximately 90% of all cases associated to T2DM can be assigned to five major lifestyle factors: physical inactivity, smoking, diet, obesity and alcohol consumption [31,32]. There are several pharmacological agents available to treat T2DM such as metformin, sulfonylureas and meglitinides. Despite the improvements in treatment and prevention, T2DM remains a primary cause of end-stage renal disease, blindness, lower limb amputation and cardiovascular disease [25,33].

1.2.3 Gestational diabetes mellitus

GDM is defined as any degree of glucose intolerance that is diagnosed for the first-time during pregnancy [3,34]. GDM is recognized as one of the most common complications of pregnancy with a worldwide prevalence of ~6–13% depending on region and the availability of maternal care [34,35]. The timing of onset of GDM can vary during pregnancy but it is typically diagnosed at 24–28 weeks of gestation. Early diagnosis and management of GDM is a matter of great interest because of the associated adverse short and long-term maternal and fetal outcomes. During pregnancy, mothers with GDM have an increased risk of developing preeclampsia, post-partum hemorrhage, and a higher incidence of operative delivery [34,36]. There is also a high likelihood of recurrence of GDM in a subsequent pregnancy (up to 48%) [37].

Although glucose tolerance typically returns to normal in the postpartum period, women with GDM have a 20–70% risk of developing T2DM in the first decade after delivery [34,38]. Moreover, recent studies have demonstrated a relationship between GDM and later-life cardiovascular

disease. Women with GDM have a two-fold higher risk of developing cardiovascular disease independent of the intercurrent development of T2DM [34,39]. Post-delivery, mothers impacted by GDM are also at increased risk of developing renal disease [40]. GDM also has a significant impact on short and long-term outcomes in offspring. There is an increased risk of fetal macrosomia which in turn is associated with a higher incidence of shoulder dystocia and birth trauma [41]. Maternal GDM poses an increased risk of hypoglycaemia and respiratory distress in the newborn infant which in turn is associated with increased incidence of neonatal ICU admission [42]. Post-delivery, children born to mothers affected by GDM have increased lifetime risk of obesity, metabolic syndrome and cardiovascular disease [43]. It has been reported that the metabolic syndrome in pregnant women who eventually develop GDM is dysregulated before conception. [44]. The pathophysiology of GDM is usually linked to β-cell dysfunction and chronic insulin resistance during pregnancy [45].

1.3 PREGNANCY AND CARDIOVASCULAR PHYSIOLOGICAL CHANGES

During pregnancy, the woman undergoes a myriad of physiological, anatomical, biomechanical, and hemodynamic changes to meet the developmental needs of the fetus [46–48]. These changes occur after conception and affect all the organs in the body [48]. One of the maternal hemodynamic alterations in pregnancy is the vasodilation of the systemic vasculature, increased cardiac output and expanded blood volume [47]. Systemic vasodilation starts at 5 weeks gestation followed by full placentation and the complete development of the uteroplacental circulation [47]. Further, peripheral vascular resistance substantially decreases with a plateau or minor increase for the rest of the pregnancy [47,49]. In the first trimester, there is a substantial decrease in peripheral vascular resistance, which decreases to a nadir during the middle of the second trimester with a subsequent plateau or slight increase for the remainder of the pregnancy [48]. The decrease is ~ 35%

to 40% of baseline. Vasodilation of the kidneys results in a 50% increase in renal plasma flow and glomerular filtration rates by the end of the first trimester [48,50]. This may also decrease serum creatinine, urea, and uric acid values [51]. Another profound change occurring in pregnancy is hormonal, particularly involving the reproductive hormones (e.g., estrogen and progesterone) [52]. Studies have shown a relationship between increased levels of vasodilation, estrogen and progesterone, [47,50,51] and the levels of both increase noticeably throughout pregnancy [47]. Additionally, hormonal changes such as estrogen and progesterone can be the trigger for other important adaptations, such as cardiovascular alterations (e.g., higher heart rate and cardiac output) [50,52,53]. Vascular resistance increases during pregnancy, particularly when the third trimester approaches, causing an increase in both systolic and diastolic blood pressure.

1.4 WORLD HEALTH ORGANIZATION RECOMMENDATIONS ON ANTENATAL CARE FOR A POSITIVE PREGNANCY EXPERIENCE

Antenatal care (ANC) is defined as the routine health control of presumed healthy pregnant women without symptoms to diagnose diseases or obstetric complications and provide information about pregnancy, lifestyle modification and delivery. ANC decreases perinatal and maternal mortality and morbidity through treatment of complications of pregnancy and identifying individuals at greatest risk of developing complications during pregnancy, labour and delivery; hence, referring them to an appropriate level of care [54,55]. The WHO recommendations on ANC for a positive pregnancy experience developed 39 recommendations associated with five types of intervention: “1) Nutritional interventions, 2) Maternal and fetal assessment, 3) Preventive measures, 4) Interventions for common physiological symptoms and 5) Health system interventions to improve utilization and quality of ANC” [55]. These interventions were classified within three different categories: a) recommended upon specific conditions b) not recommended

and c) recommended [55]. Among the recommended interventions, counselling about healthy diet and keeping physically active during pregnancy have shown to reduce the risk of pregnancy complications [55].

1.4.1 Physical activity in pregnancy

Regular physical activity (PA) during pregnancy may improve metabolic, physiological and psychological parameters that reduce the risk of morbidity and mortality in both mother and fetus [56]. Of note are the maternal benefits including improved cardiovascular function, reduced incidence of muscle cramps, GDM, gestational hypertension, preeclampsia, depression symptoms during pregnancy and limited pregnancy weight gain [56–58]. Additionally, prenatal PA has been linked to the development of appropriate for gestational age-sized newborns [57,59,60] and reduction of fat mass at birth [61]. Studies have shown that PA has an impact on labour and delivery such that healthy women experience shorter labour and decreased incidence of operative delivery such as forceps assisted delivery [56]. Canadian guideline for PA throughout pregnancy (2019) recommends the engagement of pregnant women in a minimum of 150 minutes of moderate-intensity PA per week to reduce the risks associated pregnancy complications such as GDM, hypertension and preeclampsia [57,62]. These guidelines may also be applied to women who were previously diagnosed with GDM, inactive or classified as overweight or obese [body mass index (BMI) ≥25 kg/m2]. According to the guideline, greater engagement in PA including duration, volume, frequency etc. is associated with considerable benefits and PA below recommendations was still associated with benefits [62]. Figure 1.2 represents the health benefits of maternal PA for both offspring and mother.

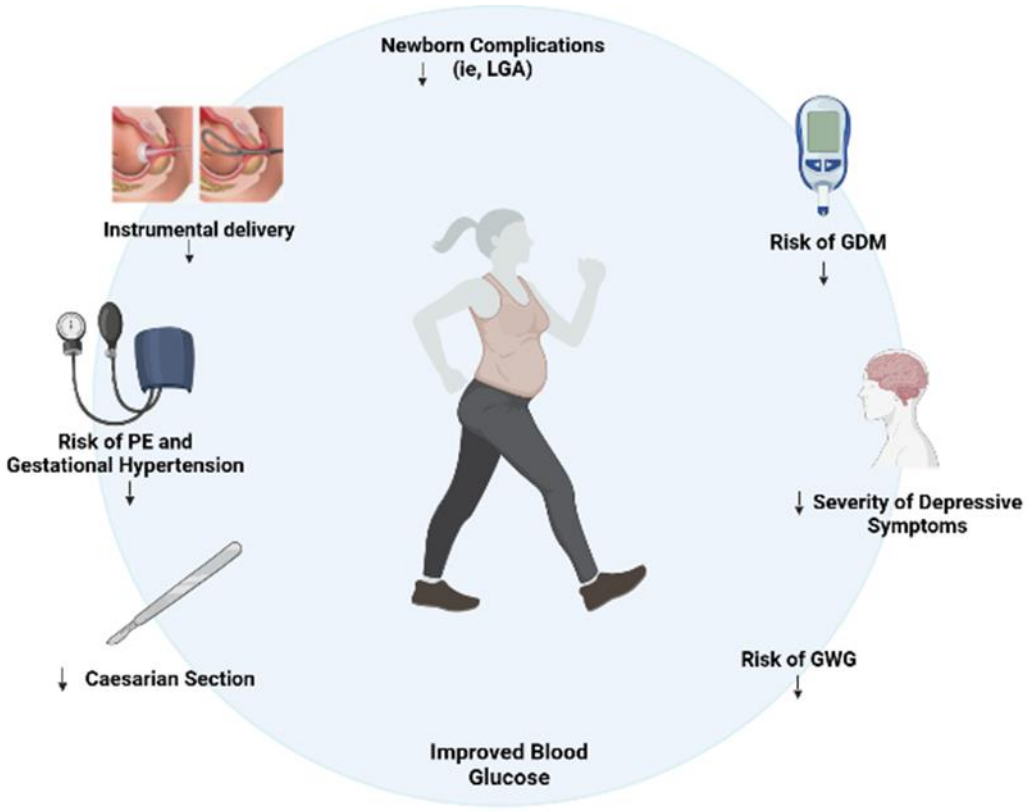

Figure 1.2. The health benefits of physical activity during pregnancy. LGA, large for gestational age; GDM, gestational diabetes mellitus; GWG, gestational weight gain; PE, preeclampsia. The figure is created using Biorender.com.

1.5 TYPE 1 DIABETES MELLITUS IN PREGNANCY

Women with T1DM have trouble achieving optimal glycemic control during pregnancy due to variability in insulin absorption, increased complexity of insulin dose adjustment and gestational changes in insulin sensitivity [63,64]. Poorly controlled T1DM is associated with a greater risk of adverse pregnancy outcomes [63,65,66]. In particular, pregnant women with T1DM have a 2-5-fold greater risk of stillbirth and congenital anomaly compared with women without T1DM [63,65,66]. T1DM in pregnancy is also associated with increased incidence of LGA infants and neonatal intensive care unit admission [63]. There is evidence that optimal glycaemic control early in pregnancy improves outcomes, reduces congenital abnormalities, preeclampsia and preterm delivery [63,65,66]. Nevertheless, despite

improved glucose control, maternal and neonatal risk remains higher with T1DM in pregnancy. Poorly controlled T1DM may also cause adverse obstetric outcomes [63]. Macrosomia which is associated with shoulder dystocia, birth injury, asphyxia and death, is ~2-fold higher than in women without T1DM [63,64]. There is also an increased risk of miscarriage and preterm delivery. Infants of mothers with T1DM may also face increased risk of operative vaginal delivery [63,64]. Murphy and colleagues conducted a cohort study in 2017 reporting that the stillbirth rates among women with T1DM and T2DM have decreased since 2002/2003 [63]. However, in 2015, the stillbirth rate was still high compared with the general maternity population [63]. There is also a reduction in rates of preterm delivery and LGA infants in women with T2DM compared with T1DM [63].

Maternal vascular health is one of the most important determinants of pregnancy outcomes and vascular dysfunction/injury may impact the maternal and fetal health across the lifespan. In addition to the short-term effects, a large body of evidence has found that it may exert a long-lasting effect on both mother and offspring [67-73]. Thus, assessment of maternal vascular health is a critical step in identifying individuals at greatest risk of complications and allow for intervention with strategies to improve maternal vascular health [73].

1.6 BIOMARKERS

Biomarkers have been defined as a wide array of analytical tools to measure biological parameters, assist in the evaluation and development of novel therapies and understanding of the differences in clinical response that may be influenced by uncontrolled variables such as drug metabolism [74]. Lately, biomarkers are defined as measurable, biological parameters that may provide important information to improve the prediction, diagnosis and prognosis of a disease, or

the effects of a given treatment [75]. In general, biomarkers are proteins or peptides, particles, cells, hormones, lipids, miRNA, metabolites, etc. that represent a sign of a normal or abnormal process of a condition or disease [76]. They can be isolated from urine, serum, plasma, and/or other body fluids that can be utilized as an indicator of the severity or existence of a disease state [77]. The critical role of biomarkers has also been reported in improving the drug development process [78] as it may be used to assess the effectiveness of a particular therapy. An ideal biomarker is one that is (1) sensitive and specific, (2) highly indicative of pathological or physiological effects, (3) easily obtained in a minimally invasive manner and (4) cost-effective [79]. In recent years, attention has been more towards biomarkers as a clinical tool for identifying pathology and evaluating risk of disease where the direct measurement of a biological state is very costly and invasive [80]. Furthermore, biomarkers may provide insight into early detection of pathology and facilitate earlier therapeutic intervention [78].

1.7 EXTRACELLULAR VESICLES

1.7.1 History of extracellular vesicles

Cell-derived vesicles were first evident in 1940s, by Chargaff and West when preliminary studies were conducted, addressing the "*Biological Significance Of The Thromboplastic Protein Of Blood*" [81,82]. Chargaff was a biochemist and West was a clinician interested in haemophilia and anaemia [81]. In the beginning, Chargaff had started his research in 1936 in the Journal of Biological Chemistry where he studied the chemistry of blood coagulation [83]. However, the study from Chargaff and colleagues (1945) highlighted the structure of cell and blood coagulation problems, can be interpreted as the beginning of the field of extracellular vesicles (EVs) biology [81]. In this study, Chargaff examined the effect of high-speed centrifugation on the clotting time of human

plasma. The main observation from this study was significantly shortened clotting time with the addition of high-speed sediment to the supernatant plasma [84]. Later, in a study which was performed by Randolph West in 1946 in New York, they discovered that centrifugation of plasma (31,000g for 150 mins) increased clotting time and they also re-introduced the pellet which reduced clotting times [82]. In fact, 17 years later Peter Wolf (1967) referred to EVs as "platelet dust" by publishing electron microscopy images of these particles [85]. In this study, Wolf identified a material sedimentable by high-speed centrifugation originating from platelets but distinguishable from intact platelets which are known as the EV fraction now [85]. A few years later, Neil Crawford revealed additional images of these vesicles which are now referred to as 'L-EVs' isolated from platelet-free plasma. Crawford also identified thrombosthenin, a contractile protein involved in clot formation, and ATPase activity in these particles [86]. The existence of other active enzymes in EVs was also revealed in 1995 [87]. A year later in 1996, it was discovered that EVs originated from immune cells are capable of presenting antigen (outside the field of platelet biology) [88]. This work showed that EVs had the potential to be used as anti-tumoural vaccines; indeed, led to clinical trials over the next decade and increased the interest in the field of EVs around the world [89]. Likewise, it was shown that EVs could play functional roles in biological processes. Importantly, the idea that EVs could have an important physiological role, led to the outbreak of interest in EVs used as a biomarker and therapeutic applications in the early 21st century. A decade later, it was demonstrated that EVs could functionally transfer RNA cargo to recipient cells [90–92]. The exponential increase in EVs field began in 2010. Since then, EVs have been shown to play an important role in several biological processes across many species; indeed, they contribute to a plethora of diseases when deregulated. The key discoveries are summarized in Figure 1.3.

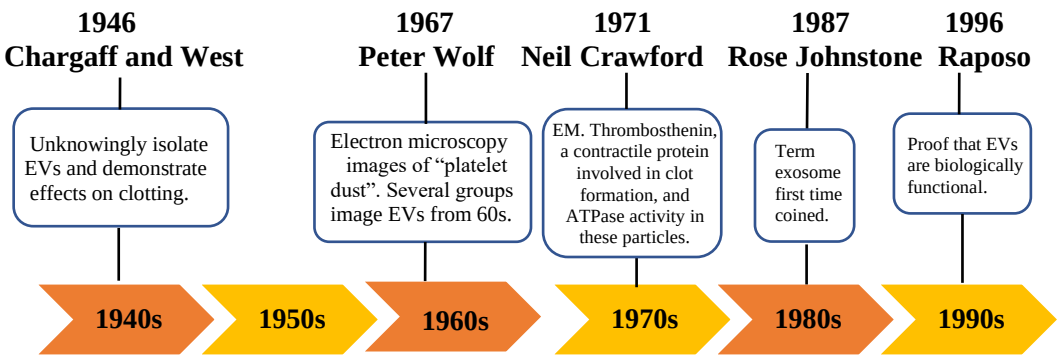

Figure 1.3. Timeline of key selected discoveries in the history of EVs field.

1.8 EXTRACELLULAR VESICLES CHARACHTERISTICS

EVs are membrane-encapsulated particles released by cells into their extracellular environment during physiological conditions as well as stress, injury or death [93,94]. Once released, EVs can accumulate in biological fluids including blood, cerebrospinal fluid, tears, urine and ascites fluid [94]. In the context of pregnancy, EVs from both mother and fetus can be found in maternal plasma. Indeed, EVs released from the placenta are seen in maternal circulation as early as 6 weeks and their concentration increase as gestation advances [95].

Historically, EVs have been subclassified based on cellular origin, function and size with the most prominent subclasses being exosomes, apoptotic bodies and microparticles (MPs) [93]. Exosomes, which range from 40 to 100 nm in size, exist possibly in all biological fluids [93,96]. They are characterized by the presence of tumor susceptibility gene 101 (TSG101), endosomal sorting proteins, membrane transport and fusion proteins and tetraspanins (CD63, CD81, and CD9) [97]. They do not appear to contain nuclear material and play a crucial role in intracellular communication [93,98]. By contrast, apoptotic bodies are the end product of cell shrinkage and fragmentation during apoptotic cell death [93,99]. Cell organelles, nuclear material and protein are all present in apoptotic

bodies which are rapidly removed by phagocytosis after their release in vivo [93,99]. They are greater than 1000 nm in size and appear to exert anti-inflammatory effects [93,99]. Microparticles/microvesicles are intermediate sized EVs (~100–1000 nm) that are released from stressed cells and contain miRNA, mRNA and membrane and cytosolic protein but appear to lack nuclear material [93,99]. In addition to large and small EVs, another functionally distinct EVs has recently been identified and referred to as exophers (>4 um in size) observed in model organisms ranging from *Caenorhabditis elegans*. They play an important role in mechanism for disposal of unwanted cellular material such as damaged organelles and protein aggregates [100]. Another subclass of EVs is referred to ARMMs (arrestin domain-containing protein 1 (ARRDC1)-mediated microvesicles) that bud directly at the plasma membrane (50-80 nm). However, the molecular composition and physiological function of these vesicles of ARMMs are not fully understood and they can overlap with small EVs isolation [101]. Figure 1.4 shows the biogenesis of major EV subtypes. While the various EV subpopulations differ in both size and biogenesis, there is considerable overlap and most isolates are heterogeneous in nature. Thus, the International Society of Extracellular Vesicles advocates for more inclusive nomenclature with ~100–1000 nm vesicles termed "medium/large EVs" (L-EVs) and ~40–100 nm vesicles termed "small EVs" (sEVs) [102]. For clarity, in this thesis the term L-EV will be referring to large extracellular vesicles and sEV will be referring to small extracellular vesicles.

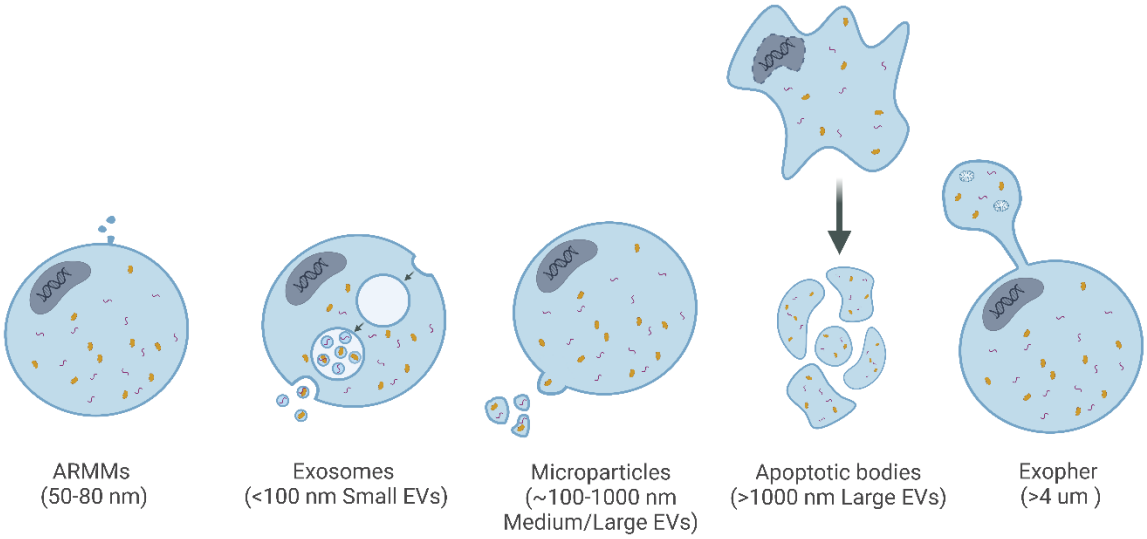

Figure 1.4. Biogenesis of extracellular vesicle (EV) subtypes. This figure is created using BioRender.com. ARMMs (arrestin domain-containing protein 1 (ARRDC1)-mediated microvesicles)

1.8.1 Biogenesis of L-EVs

EVs have traditionally been categorized according to differences in biogenesis; however, I will only focus on large EVs (microparticles/microvesicles) biogenesis. Formation of L-EVs involves external membrane budding (outward blebbing of the plasma membrane) and shedding in response to cell stress and activation during the early stages of apoptosis [80,99]. These small membrane blebs ubiquitously released and detected in all biological fluids in both disease and healthy conditions [103]. Many studies have revealed that plasma contains a variety of circulating L-EVs originating from different cell types including platelet, endothelial, erythrocyte and monocyte-derived L-EVs [80,104–106]. L-EV release is induced by various stimuli. In platelets, pro-inflammatory stimuli such as LPS (lipopolysaccharide), cytokines including IL (interleukin)-6 and erythropoietin and soluble CD40 ligand are known as potent stimuli for L-EVs formation. Furthermore, numerous stimulators of coagulation such as collagen, thrombin, proteinase-activated receptor agonists and TRAP (thrombin-receptor-activating peptide) are able to promote

platelet L-EVs production. Similarly, endothelial L-EVs production is stimulated by numerous stimuli such as lipopolysaccharide (LPS) (in the presence of fatty acids), TNF (tumour necrosis factor)-*α,* interleukin (IL-1*α)* and CRP (C-reactive protein), high glucose (HG) and shear stress. Although, the molecular mechanism induced via these stressors is not fully understood, they all stimulate an increase in intracellular Ca^{2+}. The influx of calcium ions activates calpain and scramblase which leads to a loss of membrane phospholipid asymmetry (scramblase action) and calcium dependent degradation of different proteins (calpain action) that cause outward budding of L-EVs from the plasma membrane [80,105]. The activation of these proteins including calpain, a proteolytic enzyme, protein kinases like Rho associated kinase 1 (ROCK-1) and cytoskeletal proteins, such as spectrin and actin are essential mediators in proteolysis and cytoskeletal reorganization [80,105]. Additionally, the influx of Ca^{2+} inhibits flippase enzymes while activating floppase which are responsible for inward (flippase) and outward (floppase) translocation [80]. PS is also a negatively charged phospholipid, particularly found on the inner portion of the plasma membrane of healthy cells, can be one of the phospholipids translocated in response to Ca^{2+} influx [104,107]. Further, PS gets externalized via activated floppases and there is no compensatory internalization with flippase inhibition; consequently, PS externalization is observed and begins to incorporate into membrane blebs [104,107].

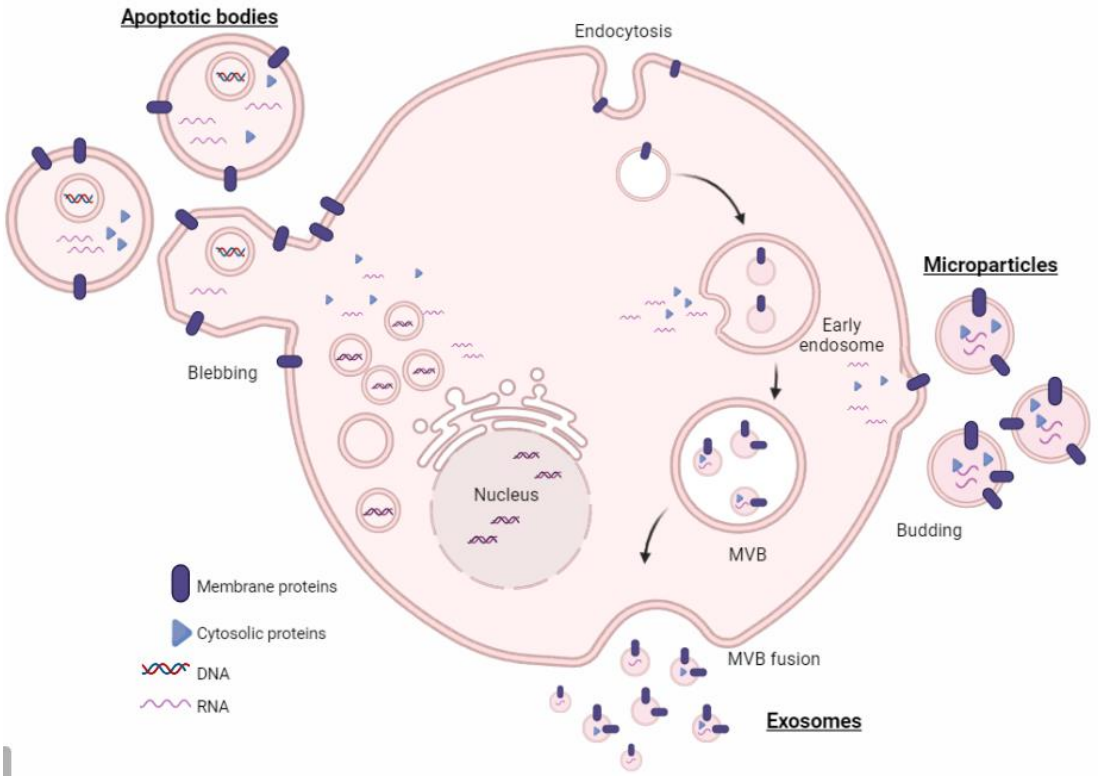

Figure 1.5. Biogenesis and release of extracellular vesicles populations.

As defined above, L-EVs retain many characteristics of the cells they originate from. Once released, they retain cell-surface proteins from their cell of origin as well as cytosolic proteins, RNA, miRNA (microRNA) and enzymes [80]. Antibodies specific to VE Cadherin (CD144), ICAM (CD54), E-selectin (CD62E) and Endoglin (CD105) are mostly used to identify endothelial L-EVs. Similarly, CD41, CD61 and P-selectin (CD62P), CD42a, CD42b, are known to identify platelet L-EVs and CD45 for leukocyte L-EVs [80,99,108].

Table 1.1. Summarizing the L-EVs origins and potentially associated biological processes along with antibodies for different types of cells.

L-EVs Origin	Relevant Biological Processes	Biological Fluid	Antibodies
Platelet	Coagulation	Plasma	**CD41**, CD61 and P-selectin (CD62P), CD42a and CD42b
Leukocyte	Inflammation	Plasma/Serum	**CD45**
Endothelial	Endothelial Dysfunction, Inflammation	Plasma/Serum	**VE Cadherin (CD144)**, ICAM (CD54), E-selectin (CD62E) and Endoglin (CD105)

1.8.2 Biological Role of L-EVs

Over the last decade, growing evidence suggests that L-EVs exert their biological effects as potent vehicles of intercellular communication by delivering proteins, nucleic acids and metabolites to their recipient cell [80,103]. Thus, they can influence numerous pathological and physiological functions of both parent and recipient cells and serve as putative biomarkers of disease [80,103]. Large EVs of different origins have been shown to play an important role in coagulation, inflammation, oxidative stress, angiogenesis, endothelial and vascular function and apoptosis [80,109].There are different types of extracellular vesicles; therefore this section will primarily focus on LE-EVs with the characteristics explained earlier.

One of the best recognized biological effects of L-EVs is possibly their capacity to enhance coagulation and participate in haemostasis [80,103]. It has been shown that L-EVs are increased in hypercoagulative disorders due to their active participation in the coagulation process [80,103].

Numerous observations indicate that L-EVs have the ability to represent both a consequence of and contributor to inflammation [80]. While numerous pro-inflammatory stimuli

induce the release of L-EVs, a few studies suggested that they may contribute to an inflammatory response [80,112].

Angiogenesis is involved in numerous pathological conditions such as diabetic retinopathy, tumor growth, inflammation and embryonic development and wound healing [120]. As mentioned earlier, the biological function of L-EV varies based on different types of cells which they originate and their composition. It was shown that L-EVs released from normal endothelial cells have been involved in angiogenesis whereas platelet L-EVs participate in blood coagulation [121,122].

Apoptosis is a potent stimulus for the release of L-EVs. Recent studies have highlighted their important role as a signalling element in cell-cell communication [80,123]. In this regard, Huang and colleagues observed that L-EVs released from hypertensive patients induce H_2O_2 production, apoptosis and cellular senescence [124]. Further, the number of endothelial L-EVs was greatly elevated in patients with systemic sclerosis and induced apoptosis in circulating angiogenic cells [125]. Numerous studies have reported that L-EVs may induce apoptosis in leukocytes and stimulate the secretion of cytokines and tissue factor expression in endothelial cells [112,123,126].

1.8.3 L-EVs clearance

At present, little is known about the mechanism of L-EVs elimination. A few studies observed that L-EVs are engulfed within phagocytic cells. Thus, phagocytosis is considered the primary mechanism by which L-EVs are eliminated/cleared in vivo [80]. Distler and colleagues have demonstrated that the co-culture of macrophages with L-EVs derived from T-cells *in vitro* results in phagocytosis of the L-EVs [127]. Internalization of L-EVs and phagocytosis result from the externalized phosphatidylserine, which may trigger endocytic/efferocytic pathways of L-EVs through scavenger receptors [128,129]. Efferocytosis is a process carried out by macrophages and other phagocytes such as dendritic cells, monocytes and epithelial cells [130]. This multi step process is

critical for tissue repair, tissue homeostasis and organismal health [130]. Interestingly, it is possible that macrophages prioritize the clearance of L-EVs and other types on the basis of glycosylation patterns [131]. It has also been shown that platelet L-EVs are endocytosed by brain endothelial cells and human umbilical vein endothelial (HUVECs) suggesting a role for endothelial cells in the phagocytosis of L-EVs cells [128,132].

1.8.4 L-EVs as biomarkers

In recent years, the interest in the use of L-EVs in diagnosis, prognosis and monitoring diseases such as DM, cancer, cardiovascular diseases etc. has substantially increased [133]. Since L-EVs are composed of cytosolic proteins, cell membrane and RNA, their molecular cargo is specific to their origins indicative of a unique molecular fingerprint [80]. The presence of L-EVs in circulating body fluids including urine, blood, cerebrospinal fluid, tears, amniotic fluid, breast milk, synovial fluid, ascites and saliva signify source of biomarkers that are easily accessible [80,134].

Much research has focused on the clinical implications of L-EVs as potential biomarkers and therapeutic approaches for DM complications. For example, L-EVs isolated from human plasma display upregulated levels of cytokines and angiogenic agents in diabetic patients [135]. Since L-EVs are formed early in the process of cell injury and the fact that they retain properties of the cell from which they originate, and can be identified by cell surface antigens, L-EVs are recognized as excellent biomarkers of cell/tissue injury with prognostic value [136]. Indeed, L-EVs of various origins may be altered under pathological condition. In particular, increases in circulating levels of endothelial L-EVs correlate with measures of vascular dysfunction and may predict risk of adverse cardiovascular events independent of traditional risk factors. Further, they have been known to play an important role as biomarker of endothelial dysfunction, inflammation, coagulation and other pathological processes [80,81].

1.8.5 L-EVs in health and disease

Previously, L-EVs were believed to be nothing more than cellular debris, however they are now recognized as a potent vehicle of intercellular communication that mediate pathological and physiological functions throughout the body fluids [80,137]. This is due to their capacity to interact with distant recipient cells transferring cargo into the target cells, thereby L-EVs influence various pathological and physiological functions of both parent and recipient cell [80,103]. To date, most observations have been made in the crucial roles of L-EVs in the pathogenesis of diseases, from cancer to autoimmune disease [103,138].

Since L-EVs are shed from the surface of cellular membranes under conditions of injury/stress, they have been examined as putative biomarkers of disease [80]. In general, plasma levels of L-EV is determined as a dynamic balance between their formation and elimination (both can affect the circulating levels) and may not be simply defined as an index of L-EVs formation [80]. Changes in L-EV formation are commonly seen in cardiovascular conditions including DM and this can be reflected in altered levels in various biofluids [103]. Depending on the source of the L-EVs studied (i.e., plasma vs urine or other biofluids), such changes may be reflective of diabetic complications including endothelial injury, kidney damage, or in the context of pregnancy, placental stress [139]. In particular, increases in circulating levels of endothelial L-EVs have been correlated with measures of vascular dysfunction and are independent predictors of cardiovascular risk [105]. In respect to their origin, the increases in endothelial L-EVs may indicate vascular injury, increases in platelet L-EVs may signify aberrant coagulation and increases in leukocytes L-EVs may suggest a pro-inflammatory state [80]. A significant overlap between these processes have been detected; hence, L-EVs of each origin may be increased in numerous pathologies. Several studies have reported increased levels of circulating L-EVs, specifically, endothelial L-EVs in patients

with T1DM and T2DM [105,140,141]. In one study, higher levels of platelet, endothelial and total annexin V positive L-EVs were observed in individuals with T1DM compared to age-matched control [141]. Further, increased L-EVs procoagulant activity was also detected in TIDM subjects as compared to control participants [141]. Similarly, monocyte and platelet L-EVs were elevated in individuals with T2DM [142]. A reduction in levels of circulating L-EVs was shown in individuals with T2DM with improved glycemic control after bariatric surgery [143]. These findings have also been replicated in both cellular and animal models that mimic high glucose or diabetic conditions [144]. Other studies have also investigated the association between circulating L-EVs level (originated from different cells) and other diseases. Amabile and colleagues have observed an increase in endothelial L-EVs in pulmonary hypertension [105,145] while Diehl has reported increases in platelet, endothelial and leukocyte L-EVs [146]. Further, alterations in the amount of endothelial L-EV formation have also been observed in association with physiological changes during pregnancy. In this regard, plasma levels of endothelial L-EVs were significantly higher in otherwise healthy pregnant women (non-complicated pregnancies) than in non-pregnant individuals [147]. However, elevation in endothelial L-EV levels has been detected during a normal pregnancy if preeclampsia is present [148–150].

1.8.6 Proteomics in L-EV Research

Proteomics is the large-scale study of protein expression, interaction, regulation, structure and physiological functions [151]. In recent years, proteomics has become a facet of L-EV exploratory research to uncover disease mechanisms with the goal of improving diagnosis, prognosis and monitoring in numerous diseases such as pulmonary, neurodegenerative, urological, cancers, cardiovascular diseases, preeclampsia and DM [136,152]. The two main goals of L-EV proteomic analysis are (1) the identification of disease-associated proteins and pathways that can be used as

diagnostic and prognostic biomarkers [153] and (2) the examination of the L-EVs role and their proteins in biological processes and phenomena such as disease progression [154].

Mass spectrometry (MS) with its sensitivity and specificity has been used as a foundational technique to identify and characterize L-EV protein cargo [155]. A workflow of a typical mass spectrometry-based approach includes (a) isolation of L-EVs from a biological fluid such as plasma; (b) extraction of L-EV proteins using a detergent or non-detergent lysis buffer; (c) separation of proteins and (d) digestion prior to loading in the mass spectrometer [155]. The concept that L-EVs are able to carry protein has long been known [86]. With the advancements in proteomics, multiple studies have completed large scale investigations to assess the contents of L-EVs.

Most diseases, including T1DM stem from abnormalities in the functioning of proteins and altered expression of many genes and their products [156,157]. In addition to the levels of L-EVs being altered in hyperglycemic conditions, both *in vivo* and *in vitro* studies have also suggested that the content of L-EVs can be affected under similar conditions. Extensive research has been conducted into the effect of high glucose and diabetic conditions on the molecular composition of L-EVs. Understanding how the content and function of L-EVs change in such environments may provide new insights into the etiology of diabetic complications.

Xu and colleagues examined the proteomic composition of plasma L-EVs isolated from newly diagnosed T2DM patients [158]. Their findings revealed that the proteins associated with inflammation, cell adhesion and platelet activation are well represented in L-EVs from the T2DM patients compared with healthy controls [158]. Thus, L-EVs appear to be pro-inflammatory and coagulant in T2DM and may ultimately lead to complications in diabetic patients [158]. In another study, Little and colleagues measured the plasma proteome of L-EVs in patients with cardiovascular diseases such as coronary artery disease, hypertension and DM. They identified a

total of 130 proteins involved in cytoskeletal regulation, integrin complexes, and hemostasis [159]. Similarly, Nunez Lopez and colleagues conducted a study to examine the proteomic of L-EVs in individuals during the development of T2DM (normal glucose tolerance (NGT), prediabetes (PDM), and diabetes (T2DM)) [160]. They identified upregulated L-EV proteins involved in coagulation, platelet activation, chemokine signaling and oxidative phosphorylation pathways early during the development of T2DM [160]. Proteomic analysis of L-EVs released from control and glucose-treated HUVECs identified approximately 1400 proteins in each group [161]. A subset of proteins (83) had transcriptional differences in the high glucose group, with 48 being upregulated and 35 being downregulated [161]. Burger and colleagues have conducted a study to examine the effect of high glucose on endothelial L-EVs content and formation [144]. The proteomic analysis of L-EVs derived from HUVECs exposed to high glucose conditions identified 68 unique protein molecules. The gene ontology analysis indicated that many of these proteins were involved in oxidation-reduction processes, hexose transport and protein complex assembly [144]. Further, the study showed that high glucose is a potent stimulus for endothelial L-EVs release which is functionally and compositionally different from the ones released under normal glucose conditions [144].

Changes in the composition of sEV populations in GDM have been reviewed elsewhere [162–165]. A list of altered sEV-associated proteins isolated from adipose tissues and plasma in GDM are summarized in Figure 1.6.

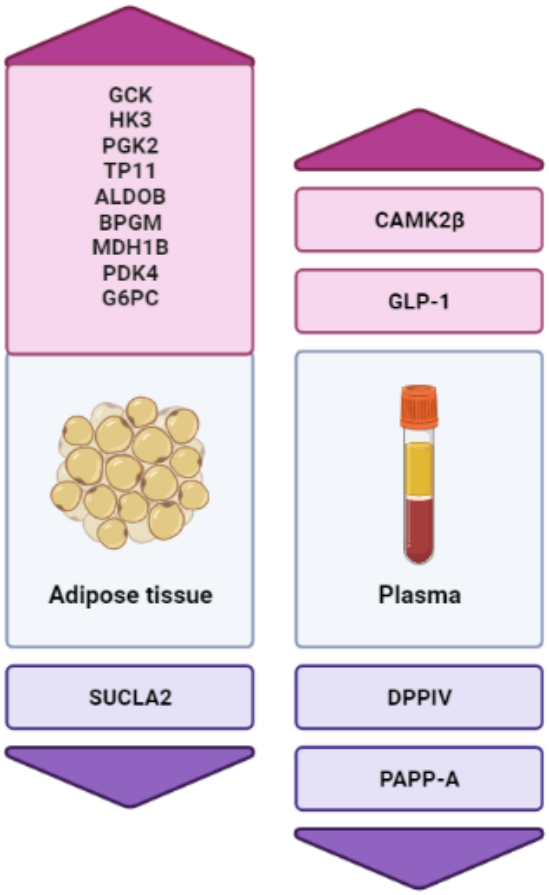

Figure 1.6. Altered small EV-associated proteins are represented in GDM. Up regulated proteins are displayed in fuchsia and down-regulated proteins in violet. This figure is created using BioRender.com. This figure originally was created from *Jayabalan et al., 2019* [162] ; *Shah et al., 2021* [166] and *Burger et al., 2015* [167].

Here, I focus on the most recent studies that have attempted to capture the proteomic of L-EVs in T1DM. The unbiased assessment of protein changes in T1DM could lead to the identification of dysregulated signaling responsible for diabetic complications. In this regard, Do Nascimento de Oliveira and colleagues previously conducted a proteomic study to identify candidate biomarkers associated with T1DM and investigate the differential expression of serum proteins in the plasma of patients with T1DM [156]. T1DM was associated with the upregulation of six proteins including prothrombin, alpha-2-macroglobulin, apolipoprotein A-II, β2 glycoprotein

I, Ig alpha-2 chain C region and alpha-1-microglobulin and down-regulation of two proteins (complement C4 and pregnancy zone protein) [156]. Such proteomic profiling of plasma is a powerful tool for the identification of altered biochemical pathways and biomarkers of disease states. However, a limitation is the complexity of the protein composition and uncertainty regarding the origin of differentially expressed proteins [168]. Assessment of L-EV protein composition may therefore provide greater insights given their ability to reflect the (patho) physiological status of their cell of origin.

Casu and colleagues conducted a study on patients with T1DM to uncover the correlation of circulating L-EVs cargo with key clinical features. They identified a total of 181 differentially expressed L-EV proteins and 15 differentially expressed L-EV phosphoproteins. Gene Ontology analysis revealed enrichment in proteins associated with platelets, neutrophils, immune response functions and neurodegenerative diseases [169]. While these observations require confirmation, analysis of proteomic signatures of L-EVs may aid in understanding of pathobiology of T1DM and its complications. Burger and colleagues have shown previously that high glucose is a potent stimulus for endothelial L-EVs formation [144]. They also reported that endothelial L-EVs released in high glucose are functionally and compositionally different from those formed under normal glucose conditions which may contribute to the progression and development of endothelial dysfunction in hyperglycaemia and diabetes [144].

1.9 OVERALL OBJECTIVES AND HYPOTHESIS

1.9.1 Overall objective

The overall objective of the proposed study is to investigate the impact of T1DM and pregnancy on L-EV levels and protein composition, the relationship between L-EVs and pregnancy outcomes and the effect of exercise on L-EVs in healthy pregnant and non pregnant women.

1.9.2 Overall hypothesis

Individuals with T1DM or poorly controlled glucose display increased L-EV levels with unique L-EV protein composition. Increased L-EV levels in pregnancy predicts adverse maternal and fetal outcomes while exercise reduces circulating L-EV levels.

1.9.3 Specific objectives and hypothesis

There are three specific objectives for this thesis:

1. Quantify L-EVs in a large cohort of pregnant women with T1DM and examine the association between circulating L-EVs and maternal and fetal outcomes in women with T1DM (Chapter 2).
2. Investigate the protein composition of circulating L-EVs in hypertensive, diabetic and healthy mice to identify common and disease-specific molecular changes (Chapter 3).
3. Investigate the changes in plasma endothelial L-EV levels after exercise in healthy pregnant and non-pregnant women (Chapter 4).

There are three specific hypotheses for this thesis:

1. Levels of L-EVs are increased in individuals who suffer complications during pregnancy (Chapter 2).

    - High baseline levels of L-EVs predict adverse maternal and fetal outcomes in pregnancy.
    - Levels of L-EVs are increased in individuals with poor glucose control.
2. The protein composition of circulating L-EVs in hypertensive, diabetic and healthy mice display common and disease-specific molecular changes (Chapter 3).
3. The levels of L-EVs decrease in response to an acute bout of moderate-intensity exercise in all women, regardless of pregnancy status (Chapter 4).

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2 CHAPTER 2- Circulating extracellular vesicles during pregnancy in women with type 1 diabetes: a secondary analysis of the CONCEPTT trial.

(Formatted as per Biomarker Research requirements)

Circulating large extracellular vesicles during pregnancy in women with type 1 diabetes mellitus: A secondary analysis of the CONCEPTT trial

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The authors declare no competing interests.

2.1 ABSTRACT

Background: Large extracellular vesicles (L-EVs) are membrane vesicles that are released into the extracellular environment and accumulate in the circulation in vascular disease. We aimed to quantify circulating L-EVs in pregnant women with type 1 diabetes mellitus (T1DM) and to examine associations between L-EV levels, continuous glucose measures, and pregnancy outcomes. **Methods:** We used plasma samples from the Continuous Glucose Monitoring (CGM) in Women with T1DM in Pregnancy Trial study and quantified circulating L-EVs by flow cytometry (n=163). Relationships with clinical variables were assessed by repeated measures correlation. Logistic regression was used to assess associations between elevated L-EV levels and pregnancy outcomes. **Results:** Platelet L-EV levels were inversely associated with glucose time above range and glycaemic variability measures ($P<0.05$). A weak positive association was observed between endothelial L-EVs and mean amplitude of glycemic excursion ($P<0.05$). In a univariate logistic regression model, high baseline endothelial L-EVs was associated with increased risk of neonatal intensive care unit (NICU) admission (OR: 2.06, 1.03-4.10), and respiratory distress requiring ventilation (OR: 4.98, 1.04-23.92). After adjusting for HbA1c and blood pressure the relationship for NICU admission persisted and an association with hyperbilirubinemia was seen (OR: 2.56, 1.10-5.94). Elevated platelet L-EVs were associated with an increased risk of NICU admission (OR: 2.18, 1.04-4.57), and hyperbilirubinemia (OR: 2.61, 1.11-6.12) after adjusting for HbA1c and blood pressure. **Conclusions:** High levels of L-EVs in early pregnancy were associated with adverse neonatal outcomes. Assessment of L-EVs may represent a novel approach to personalized care in T1DM pregnancy.

2.2 BACKGROUND

Maternal vascular health is a critical determinant of pregnancy outcomes and endothelial injury/dysfunction may play a causal role in acute and longer-term maternal fetal health outcomes [1,2]. Biomarkers of vascular injury, for example, soluble intercellular adhesion molecule-1 and soluble vascular cell adhesion molecule-1 elevations, are associated with preterm delivery [3]. Similarly, an increase in serum levels of the anti-angiogenic sFlt-1 is associated with a higher rates of early and late preterm births, low birth weight and preeclampsia [4]. Impairment in endothelial function (assessed by flow-mediated dilatation) is observed in women with preeclampsia as compared to women without complications which highlights the important role of the endothelium in disease pathogenesis [5,6]. Given the importance of the maternal vasculature to both maternal and fetal health, the assessment of maternal vascular health may aid in identification of individuals at greatest risk of complications and allow for intervention with strategies to improve maternal vascular health [7]. For example, the use of real time continuous glucose monitoring (RT-CGM) has been shown to improve maternal and neonatal outcomes, however its effect on maternal vascular health is not known.

One emerging approach for the assessment of vascular health is enumeration of circulating extracellular vesicles (EVs). EVs are membrane-enclosed vesicles lacking replicative capacity that are released into the extracellular environment and involved in cell signaling [8]. A subclass of EVs historically termed microparticles/microvesicles are ~100-1000nm in size and shed from the surface of cellular membranes under stress conditions [8,9]. L-EVs are comprised of miRNA, mRNA and membrane and cytosolic protein but generally lack nuclear material [8]. L-EVs exist in numerous biological fluids such as urine, blood plasma, ascites and sputum [8,9]. Circulating L-EVs have been extensively studied and primarily originate from platelets, endothelial cells, and leukocytes [10,11].

Notably, levels of circulating endothelial L-EVs demonstrate strong inverse correlations with arterial flow-mediated dilatation, and positive correlations with systolic blood pressure and pulse wave velocity [12,13]. Elevated circulating endothelial L-EVs, as assessed by flow cytometry, have been shown to predict risk of adverse cardiovascular events independent of traditional risk factors [14,15].

In diabetes, studies have consistently observed elevated levels of circulating L-EVs (particularly endothelial L-EVs) among individuals with T1DM and T2DM [16,17]. Sabatier and colleagues revealed that individuals with T1DM have higher numbers of circulating endothelial, platelet, and total annexin V–positive L-EVs and increased L-EV procoagulant activity in comparison with age-matched control subjects [17]. Similarly, Li, S. et al observed that platelet and monocyte L-EVs are elevated in individuals with T2DM [18]. Improved glycemia is associated with reduced levels of circulating L-EVs in individuals with T2DM after bariatric surgery [19]. Thus, assessment of L-EV levels may reflect dynamic changes to the vasculature in diabetes.

To date, the predictive value of circulating L-EVs in pregnancy is unclear. The aim of our study was to quantify circulating L-EVs in pregnant women with T1DM and to examine the associations between L-EV levels with glycaemia, blood pressure and pregnancy outcomes. We hypothesized that levels of endothelial L-EV would be positively correlated with glucose levels and that high levels would be associated with adverse pregnancy outcomes. A secondary goal of this work was to determine whether the use of RT-CGM alters levels of circulating L-EVs.

2.3 METHODS

2.3.1 Study participants and ethics

We examined plasma samples from the CGM in Pregnant Women with T1DM (CONCEPTT) trial biorepository [20]. Details of the clinical study protocol have been previously published [20,21]. In brief, women with T1DM who were pregnant or planning pregnancy were randomized to receive RT-CGM or standard care. Those receiving standard care wore a masked CGM at 4-12, 24 and 34 weeks gestation. We conducted a secondary analysis on biospecimens from all 163 pregnant participants with at least one plasma sample available for analysis. Samples from collections at 4-12 weeks (baseline), 24, and 34 weeks gestation were studied. A summary of baseline characteristics and pregnancy outcomes is shown in Table 2.1. The study was approved by the Mount Sinai Research Ethics Board (ID#: 17-0066-E) and the Ottawa Hospital Research Ethics Board (ID#: 20170658-01H). Samples were analyzed in a blinded fashion.

Table 2.1. Baseline characteristics and pregnancy outcomes.

Characteristic*	
Age- years	31.4 ± 4.8
BMI- kg/m²	25.7 ± 0.3
European/Mediterranean origin	141 (86.5%)
Duration of diabetes- years	16.7 ±7.9
Presence of diabetes complications	50 (30.7%)
Nulliparity	66 (40.4%)
Systolic blood pressure- mmHg	115.3 ± 16.1
Diastolic blood pressure- mmHg	69.8 ± 9.9
History of preeclampsia	10 (6%)
Maternal outcome	
Hypertension (Worsening Chronic, Gestational or Pre-eclampsia)	37 (22.7%)
Preeclampsia	20 (12.3%)
Gestational Hypertension	15 (9.2%)
New onset proteinuria	25 (15.3%)
Episodes of severe hypoglycemia	4 (2.5%)

Impaired liver function	15 (9.2%)
Vaginal labour	54 (33.1%)
Caesarean labour	104 (63.8%)
Maternal complications	22 (13.5%)
Maternal length of hospital stay- days	4.9 ± 3.5
Neonatal Outcome	
Early preterm (<34 weeks)	10 (6.3%)
Late preterm (34-37 weeks)	53 (32.5%)
Birthweight- g	3553 ± 737
Gestational age at delivery- weeks	37.0 ± 2.0
Neonatal length of hospital stay (days)	5.5 ± 5.8
Neonatal intensive care unit admission	52 (31.9%)
Neonatal hypoglycaemia	35 (21.5%)
Composite fetal outcome	70 (43%)
Respiratory distress	11 (6.8%)
Antenatal corticosteroids (y/n)	41 (25%)

*values are mean ± SD or n (%) as appropriate

2.3.2 Continuous glucose monitoring metrics

Summary metrics for CGM measures were obtained for baseline, 24, and 34 weeks gestation. Measures included the mean CGM glucose level, the percentage of time spent within the pregnancy glucose target range (TIR, 63–140 mg/dL), and time spent above (TAR, >140 mg/dL) and below (TBR, 63 mg/dL) the target range, mean amplitude of glycemia excursion (MAGE), and measures of glycemic variability (SD and coefficient of variation [CV]).

2.3.3 Isolation and immunolabeling of L-EVs from Archived Plasma

We isolated circulating L-EVs (often referred to as microparticles) from archived platelet-free plasma as described previously [22]. Plasma samples were thawed quickly in a water bath at 37 °C. 175 µl of samples were centrifuged at 12000 × g for 2 minutes at 4 °C. 150 µl of the supernatant was transferred to fresh tubes and centrifuged at 20000 x g for 20 min. The supernatant was discarded and L-EV pellets were re-suspended in 150 µl of Annexin V binding buffer (10mM

HEPES 140 mM NaCl, 2.5 mM CaCl2, pH 7.4). In fresh tubes, 30 µl of the samples were diluted in Annexin V binding buffer to a final volume of 200 µl for platelet labelling. The platelet samples labelling was performed using anti-CD41-APC (1:100) and Annexin V- FITC (1:50) as labels. Endothelial/leukocytes labelling was conducted by diluting 120 µl of resuspended samples in Annexin V binding buffer to a final volume of 200 µl. Anti-CD144-PE (1:100), anti-CD45-BV421 (1:25) and Annexin V-FITC (1:50) were used to label endothelial/leukocytes samples. After labeling, samples were re-centrifuged at 20,000g x 20 minutes after a two hours incubation in the dark. The supernatant was removed, and pellet was preserved and re-suspended in Annexin Binding Buffer (300 µL). The samples were transferred to flow cytometry tubes for analysis. All isotype controls, antibodies and Annexin V were purchased from BioLegend (San Diego, USA).

2.3.4 Quantitation of Circulating L-EVs by Nanoscale Flow Cytometry

L-EVs were quantified at the University of Ottawa Flow Cytometry and Virometry Facility using a Beckman Coulter CytoFLEX S with CyExpert Version 2.3.0.84. ApogeeMix beads (Apogee Flow Systems, Hertfordshire, UK) were used to establish a size gate of ~100-1000 nm. L-EVs were identified as ~100-1000 nm particles in size staining positive for the membrane marker Annexin V compared to negative controls. Results are expressed as the number of annexin V+ (Total), annexin V+ and CD41+ (platelet), annexin V+ and CD45+ (leukocyte) or annexin V+ and CD144+ (endothelial) L-EVs/ mL plasma. Samples labelled with isotype controls, antibodies alone in buffer, and unlabelled samples were analyzed as controls. FlowJo ver 7.6.5 was used for analysis.

2.3.5 Statistical analysis

Direct comparisons between L-EV levels were conducted by t-test or ANOVA (as appropriate) using GraphPad Prism version 5 (GraphPad Software).

Univariate and multivariate logistic regression models were tested using SPSS version 26 (IBM Corp, Armonk, NY). Logistic regression models with baseline L-EV levels as a dichotomous variable (above and below median value) were used to evaluate relationships with maternal and neonatal outcomes. Multivariate logistic regression models were designed *a priori* to adjust for known risk factors that have been shown to correlate with circulating L-EV levels in the general population (factors included were: systolic and diastolic blood pressure and HbA1c.) Relationships between L-EV levels and continuous variables throughout the study were assessed by repeated measures correlation in R 3.5.1 using the repeated measures correlation (rmcorr) package version 0.4.0 [23]. All graphs were constructed using GraphPad Prism. A *P* value <0.05 was considered significant.

2.4 RESULTS

2.4.1 L-EV levels throughout pregnancy

We first examined levels of circulating platelet, endothelial, leukocyte, and total L-EVs to determine if levels change throughout T1DM pregnancy. We did not observe significant differences in levels of endothelial (P=0.43), leukocyte (P=0.65), platelet (P=0.82), or total L-EVs (P= 0.58) between trimesters (Supplemental Figure 1).

2.4.2 Association between circulating L-EVs and glycemic variables

We next analyzed relationships of L-EVs with HbA1c and continuous glucose measures throughout pregnancy by repeated measures correlation (Table 2.2). We did not observe significant associations between L-EV populations and mean glucose (24h, obtained from CGM data) or HbA1c. A weak positive correlation between endothelial L-EVs and MAGE was observed but this was not accompanied by associations with other glycemic variability markers including CV and SD. Interestingly, we observed an inverse relationship between platelet L-EV levels and glucose SD, MAGE and TAR as well as a positive correlation with TIR suggesting an inverse relationship between platelet L-EVs and glucose levels. No relationships between leukocyte L-EVs and HbA1c or continuous glucose measures were observed**.**

We also examined the association between L-EV levels and blood pressure by repeated measures correlation. There was no correlation between endothelial or platelet L-EVs with either systolic or diastolic blood pressure. However, we did observe a positive correlation between leukocyte L-EVs and both systolic and diastolic blood pressure (Table 2.2).

Table 2.2. Association between circulating L-EVs populations and clinical variables by repeated measures correlation

L-EV population	Clinical Variable	r	95% CI	p
Endothelial	HbA1c	0.040	-0.072, 0.164	0.44
	Mean CGM Glucose	0.091	-0.006, 0.186	0.06
	% TIR (63-140mg/dl)	-0.005	-0.129, 0.118	0.94
	% TAR (>140mg/dl)	0.064	-0.060, 0.187	0.31
	% TBR (<63mg/dl)	-0.116	-0.236, 0.008	0.07
	Glucose CV	-0.012	-0.135, 0.112	0.85
	Glucose SD	0.054	-0.070, 0.177	0.39
	MAGE	**0.129**	**0.005, 0.248**	**0.04**
	SBP	0.082	-0.035, 0.196	0.17
	DBP	0.075	-0.043, 0.189	0.21
Platelet	HbA1c	0.009	-0.107, 0.126	0.88
	Mean CGM Glucose	-0.111	-0.231, 0.013	0.08
	% TIR (63-140mg/dl)	**0.159**	**0.035, 0.276**	**0.01**
	% TAR(>140mg/dl)	**-0.138**	**-0.257, -0.014**	**0.03**
	% TBR (<63mg/dl)	-0.055	-0.178, 0.068	0.38

	Glucose CV	-0.109	-0.229, 0.015	0.08
	Glucose SD	**-0.147**	**-0.267, -0.025**	**0.02**
	MAGE	**-0.152**	**-0.271, -0.029**	**0.01**
	SBP	0.051	-0.066, 0.167	0.39
	DBP	0.099	-0.017, 0.210	0.09
Leukocyte	HbA1c	0.001	-0.116, 0.116	0.99
	Mean CGM Glucose	0.003	-0.120, 0.127	0.95
	% TIR (63-140mg/dl)	0.007	-0.116, 0.131	0.91
	% TAR(>140mg/dl)	0.003	-0.121, 0.127	0.96
	% TBR (<63mg/dl)	-0.030	-0.154, 0.093	0.62
	Glucose CV	0.008	-0.115, 0.132	0.89
	Glucose SD	0.013	-0.110, 0.137	0.83
	MAGE	0.001	-0.124, 0.123	0.99
	SBP	**0.179**	**0.064, 0.289**	**0.002**
	DBP	**0.200**	**0.093, 0.315**	**0.001**

CGM (continuous glucose monitoring); TIR (time in range); TAR (time above range); TBR (time below range); CV (coefficient of variation); SD (standard deviation); MAGE (mean amplitude of glycemic excursion); SBP (systolic blood pressure); DBP (diastolic blood pressure). Significant relationships are bolded.

2.4.3 Effect of CGM on circulating L-EV

We next assessed whether using CGM compared with self-monitoring of blood glucose (SMBG) had an effect on circulating L-EVs. Figure 2.1 shows the effect of use of CGM on platelet, endothelial, leukocyte, and total L-EV levels at 34 weeks gestation. No differences between CGM and conventional self-monitoring of blood glucose (SMBG) were observed for circulating endothelial, platelet, leukocyte, or total L-EVs at 34 weeks gestation.

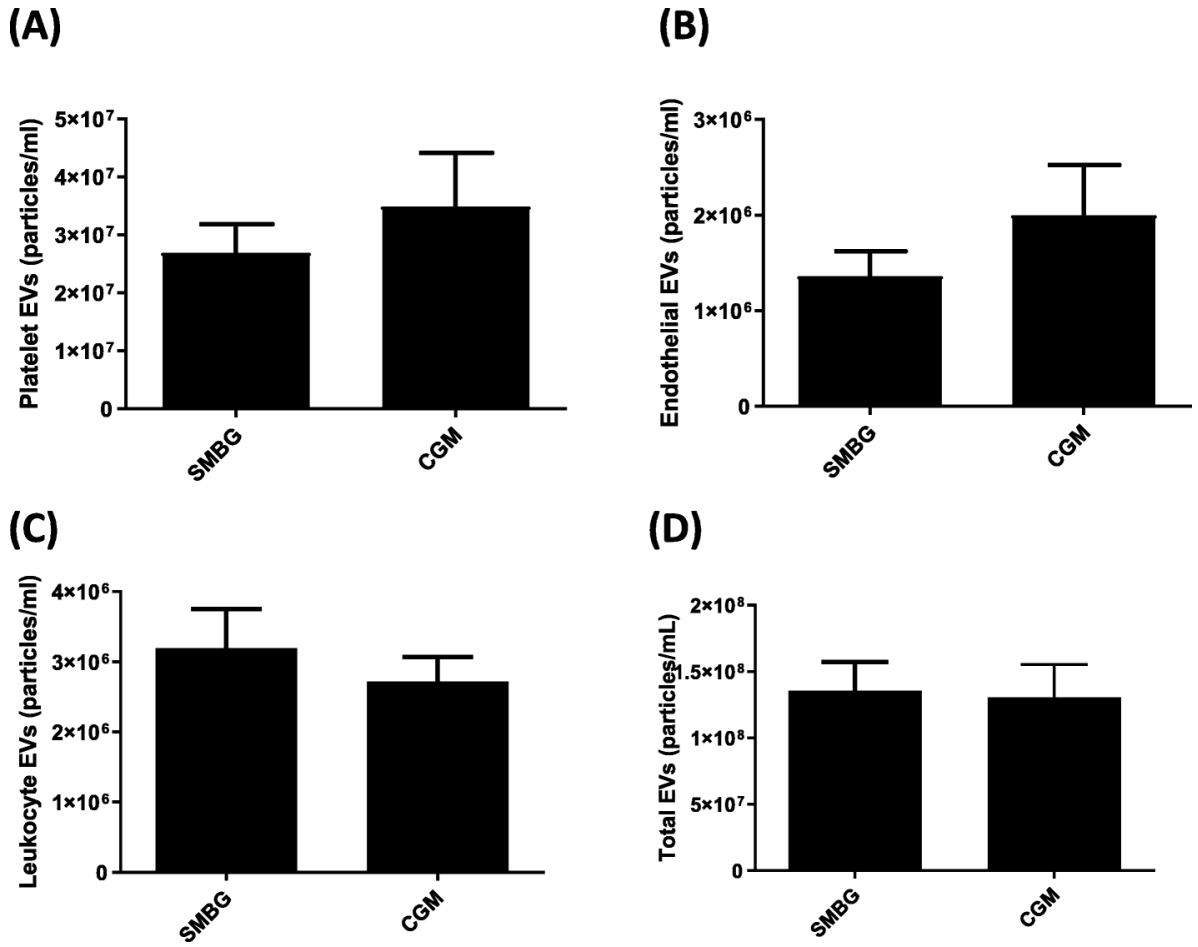


Figure 2.1. The effect of RT-CGM on levels of circulating L-EVs. Shown are values of circulating levels of L-EVs at 34 weeks gestation in individuals utilizing real time continuous glucose monitoring (CGM) compared with conventional self-monitoring of blood glucose (SMBG). A Platelet-derived L-EV; B. Endothelial-derived L-EV; C. Leukocyte-derived L-EV and D. Total annexin + L-EVs. The number of participants for CGM and SMBG are $n = 72$ and $n = 77$ respectively. No significant differences were observed for platelet-derived L-EVs (0.74), endothelial-derived L-EV (0.88), leukocyte-derived L-EVs (0.31), or total annexin V + L-EVs (0.17).

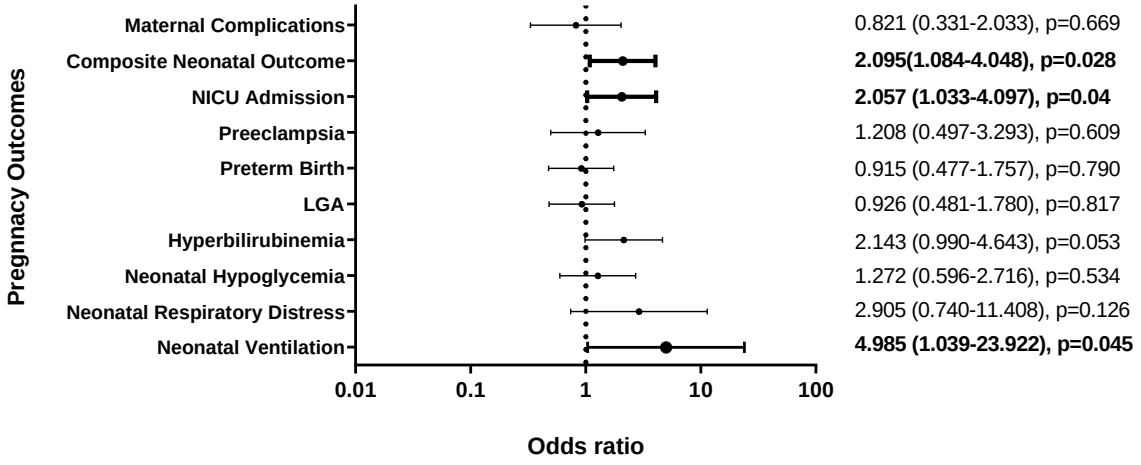
2.4.4 L-EVs and pregnancy outcomes

Finally, given that L-EV levels are reflective of vascular health, we assessed whether baseline levels of L-EVs were predictive of pregnancy outcomes. In a univariate logistic regression model, we observed that higher baseline levels of endothelial L-EVs (above median) were associated with increased risk of neonatal intensive care unit (NICU) admission, respiratory distress requiring positive pressure ventilation, and positivity for the composite fetal outcome (an

aggregate of pregnancy loss, birth injury, neonatal hypoglycaemia, and respiratory distress) (Figure 2.2A). After adjusting for HbA1c, systolic and diastolic blood pressure, we observed significant positive associations with NICU admission, composite neonatal outcome, and hyperbilirubinemia (Figure 2.2B). A weak positive association between baseline endothelial L-EV levels and maternal length of hospital stay was also observed ($p=0.03$, $r=0.17$).

With respect to platelet L-EVs, we observed that low levels of platelet L-EVs were associated with maternal complications in the unadjusted model (Figure 2.3A). After adjustment for baseline HbA1c and blood pressure, platelet L-EVs were inversely associated with maternal complications (Figure 2.3B). By contrast positively associations with neonatal intensive care unit admission, hyperbilirubinemia, and composite fetal outcome (Figure2.3B). We did not observe any relationships between leukocyte L-EVs and pregnancy outcomes (data not shown).

A



B

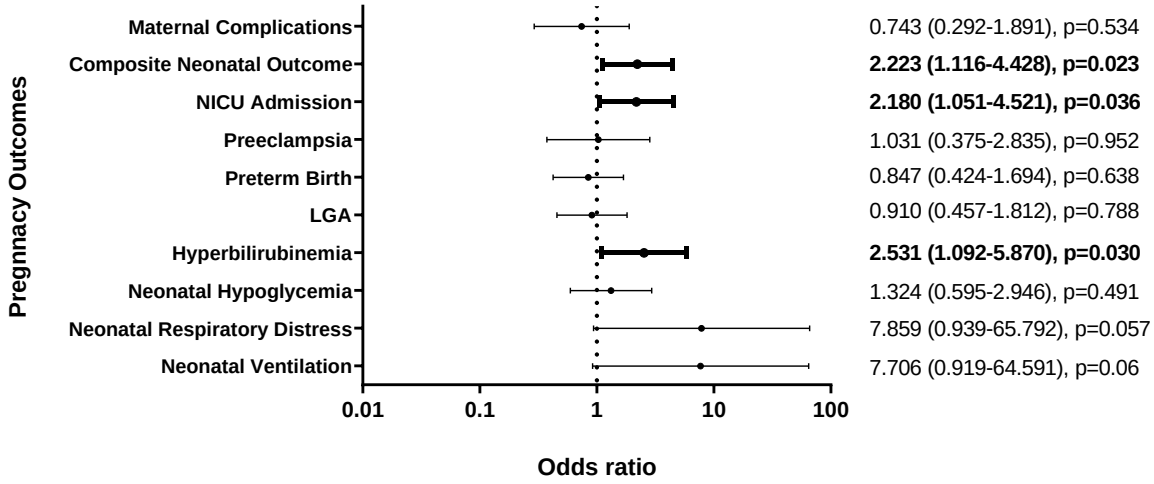
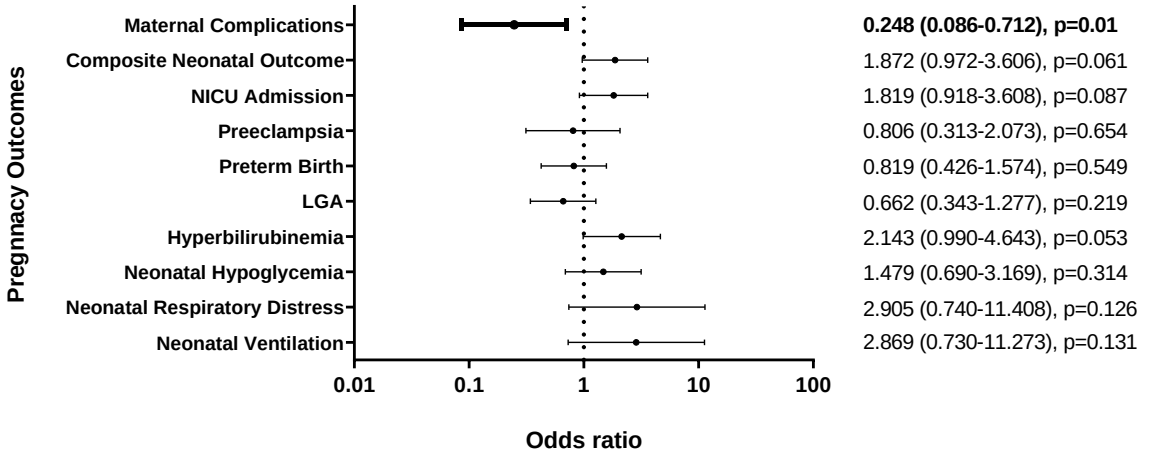


Figure 2.2. High baseline endothelial L-EV levels are associated with adverse pregnancy outcomes. A: Forest plot depicting the relationship between baseline endothelial L-EV levels and pregnancy outcomes. B: Forest plot depicting the relationship between baseline endothelial L-EV levels and pregnancy outcomes after adjusting for the HbA1c and systolic and diastolic blood pressure. Maternal complications indicate the presence of oliguria, cerebral of visual symptoms, impairment of liver function, pulmonary edema or cyanosis, epigastric or right upper quadrant pain, or thrombocytopenia. Composite Neonatal Outcome: aggregate measure of pregnancy loss, birth injury, neonatal hypoglycaemia, and respiratory distress; LGA: Large for gestational age (> 90 percentile); Ventilation: respiratory distress requiring positive pressure ventilation.

A



B

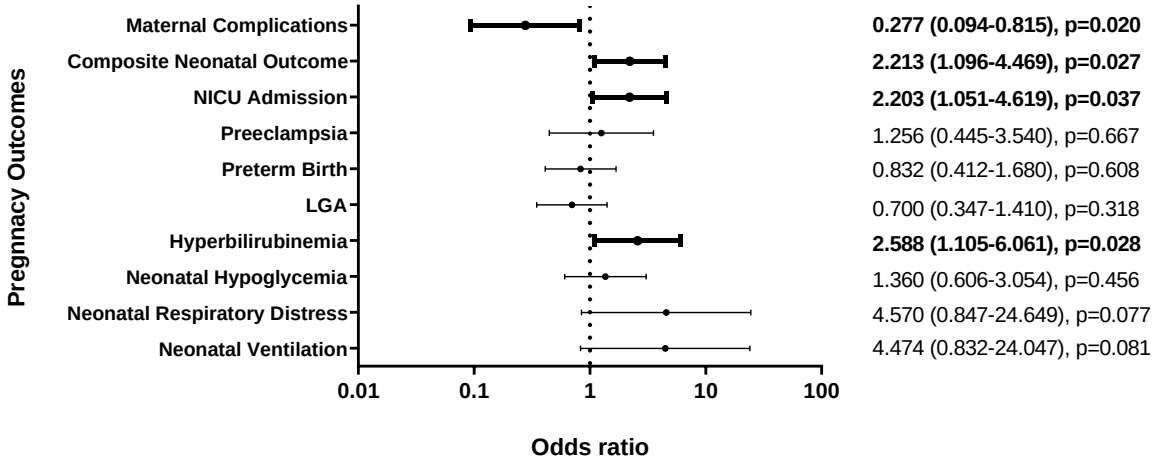


Figure 2.3. Relationship between platelet L-EV level and pregnancy outcomes. A: Forest plot depicting the relationship between baseline platelet L-EV levels and pregnancy outcomes. B: Forest plot depicting the relationship between baseline platelet L-EV levels and pregnancy outcomes after adjusting for the baseline HbA1c and systolic and diastolic blood pressure.

2.5 DISCUSSION

Our major finding is that baseline levels of circulating L-EVs are higher in women with neonatal complications. After adjustment for known risk factors (HbA1c and blood pressure), higher baseline endothelial L-EVs were associated with increased NICU admission and hyperbilirubinemia. Similarly, higher baseline platelet L-EVs were associated with increased risk of NICU and hyperbilirubinemia but decreased maternal complications after adjustment for HbA1c and blood pressure.

It is well established that glucose is a potent stimulus for L-EV formation. We have shown that high glucose induces L-EV release from cultured endothelial cells [24] and podocytes [25] and that acute hypoglycemia increases urinary podocyte L-EV levels [26]. Similarly, mesangial cells release more L-EVs in response to high glucose stimulation [27] and animal and clinical studies consistently show elevations in circulating L-EVs in DM [16–19]. Surprisingly, we did not observe any correlation between circulating L-EVs and HbA1c or mean glucose. We did observe a weak positive correlation between endothelial L-EVs and MAGE but other measures of glucose variability (SD, CV) did not show the same relationship. Similarly, we did not observe any associations between endothelial L-EVs and blood pressure values. Thus, while glucose and blood pressure may influence endothelial L-EV levels in T1DM in pregnancy, other factors are also contributing to L-EV dynamics. Perhaps more surprising is the observation that platelet L-EVs were inversely correlated with MAGE, SD, and time above range and positively correlated with time in range. These data suggest that platelet L-EV formation may be reduced with hyperglycaemia in pregnancy and are in contrast to studies outside of pregnancy including a systematic review which reported that platelet L-EVs are increased in hyperglycaemic conditions [17,18]. The reason for this discrepancy is unclear although ours is the first study to examine this relationship in T1DM in

pregnancy. Two previous studies, performed 15-20 years ago in normal, hypertensive and growth restricted pregnancies, reported reduced platelet L-EV levels in preeclampsia despite evidence of platelet activation [28,29] so it is possible that the molecular mechanisms underlying platelet L-EV formation are altered in pregnancy. Further study is needed to better understand the relationship between glucose levels and platelet L-EVs in pregnancies complicated by diabetes.

The use of CGM in individuals with T1DM and T2DM is well established to improve glycemic control and it has been speculated that the improved glycemia associated with CGM, may reduce microvascular complications [30–32]. Our laboratory and others have reported that endothelial L-EVs are biomarkers of glucose-induced endothelial injury [17,24]. Given this, we hypothesized that CGM, which was associated with improved glucose levels in the randomized controlled trial [20], would lead to reduced circulating endothelial L-EVs. However, we did not observe any differences in circulating L-EVs between the SMBG control and CGM intervention groups. Given the modest improvement in glucose levels, this secondary analysis was likely not powered to detect between-group differences in L-EVs. In addition, the short duration of the intervention (started in the late first/early second trimester) may not have been sufficient to impact cellular injury and resultant L-EV formation.

Our most striking observation is that high levels of endothelial and platelet L-EVs early in pregnancy were associated with adverse neonatal outcomes. Elevated levels of L-EVs have long been known to predict adverse cardiovascular outcomes in other populations. Sinning *et al* (2011) showed that high levels of endothelial L-EVs was associated with increased cardiovascular morbidity and mortality over a 6 year follow-up [15]. Similarly, Amabile *et al.* revealed that endothelial L-EVs are elevated in individuals with pulmonary hypertension and directly linked to hemodynamic severity of this condition [14]. In the present study we found that high levels of

endothelial L-EVs (above median value) were associated with increased NICU admission, neonatal respiratory distress requiring positive pressure ventilation, and hyperbilirubinemia. Similarly, high levels of platelet L-EVs, after adjustment for HbA1c and blood pressure, were associated with NICU admission, neonatal resuscitation, hyperbilirubinemia and positivity for the composite fetal outcome. Curiously, low levels of platelet L-EVs also seemed to predict maternal complications in this cohort. Of note is the observation that high levels of L-EVs were not predictive of preeclampsia in this population in contrast to other studies which found that L-EVs were increased in preeclampsia [33,34]. This may be related to the sample size, low number of cases in our study population and differences between normal, hypertensive and diabetes pregnancies.

Endothelial L-EVs are also engaged in pathological processes as they are able to induce vascular injury [24,35–37]. Previous studies have shown that L-EVs induce endothelial inflammation, haemostasis, angiogenesis, and endothelial dysfunction [35]. Thus, in addition to serving as biomarkers of vascular injury they may actually induce vascular dysfunction through direct actions on endothelial cells leading to increased oxidative stress, inflammation, and impaired endothelium-dependent vasorelaxation [24,36–40]. We and others have previously shown that endothelial L-EVs exert greater deleterious effects on the endothelium when formed under hyperglycaemic conditions [24,41]. Bidirectional L-EV-mediated cross-talk between the maternal vasculature and placenta may also play an important role in disease pathogenesis. In this regard, Kohli et al have shown that maternal L-EVs can promote inflammatory responses in trophoblasts [42] and Han et al. recently reported that placental-derived L-EVs can induce preeclamptic symptoms in mice [43]. Whether this is true in diabetes pregnancy and whether increased L-EVs played a causal role in the pregnancy-related complications observed in the present study is a subject for future investigation.

One of the strengths of this study is the use of human plasma samples from a multicenter, randomized controlled trial with extensive participant characterization. In addition, while many studies focus on a single L-EV population, our flow cytometry approach examined L-EVs from endothelial cells, leukocytes, and platelets. Indeed, we were able to identify predictive value for both endothelial and platelet L-EVs. In addition, the availability of CGM data allowed for assessment of relationships between L-EVs and glycaemia to an extent that has not been done previously. Nevertheless, our study also has limitations: First, while this represents a large cohort in the context of prior work, it is still a relatively small sample with limited neonatal complications. Second, relationships between L-EV levels were assessed at only three time points and this may have limited our ability to identify associations with clinical variables. Finally, our cohort was a secondary analysis of a clinical trial focused exclusively on pregnant women with T1DM. As such we were unable to compare values to those without DM or those with T2DM or GDM. Given differences in sample collection methods, interlaboratory differences in sample preparation, analysis, and instrument sensitivity numeric comparisons to previous data is challenging, however we note that the levels of endothelial and platelet L-EVs seen in our study are slightly higher than previous reports in non-pregnant healthy donors, individuals with T1DM or T2DM or in non-diabetic pregnant individuals [16,17,44]. However direct comparison may not be appropriate as these differences could be due to greater assay sensitivity.

2.6 CONCLUSIONS

In summary, our results suggest that elevated endothelial or platelet L-EVs are associated with perinatal complications in pregnant women with T1DM. Accordingly, assessment of L-EV levels early in pregnancy may aid in identifying women at high risk of neonatal complications.

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3 CHAPTER 3- The Proteome of Circulating Large Extracellular Vesicles in Diabetes and Hypertension

(Formatted as per International Journal of *Molecular Sciences* requirements)

The proteome of circulating large extracellular vesicles in type 1 diabetes mellitus and hypertension

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3.1 ABSTRACT

Hypertension and type 1 diabetes mellitus (T1DM) induce vascular injury through processes that are not fully understood. Changes in large extracellular vesicle (L-EV) composition could provide novel insights. Here we examined the protein composition of circulating L-EVs from hypertensive, diabetic and healthy mice. L-EVs were isolated from transgenic mice overexpressing human renin in the liver (TtRhRen, hypertensive), OVE26 type 1 diabetic, and wild-type (WT) mice. Protein content was analyzed using liquid chromatography/mass spectrometry. We identified 544 independent proteins of which 408 proteins were found in all groups, 34 were exclusive to WT, 16 were exclusive to OVE26 and 5 were exclusive to TtRhRen mice. Amongst the differentially expressed proteins, haptoglobin (HPT) was upregulated, and ankyrin-1 (ANK1) downregulated in OVE26 and TtRhRen mice compared with WT controls. Conversely, TSP4 and Co3A1 were upregulated and SAA4 was downregulated exclusively in T1DM while PPN was upregulated and SPTB1 and SPTA1 were downregulated in hypertensive compared to WT. Ingenuity pathway analysis identified enrichment in proteins associated with SNARE signaling, complement system, and NAD homeostasis in L-EVs from diabetic mice. Conversely, in L-EVs from hypertensive mice there was enrichment in semaphroin and Rho signaling. Further analysis of these changes may improve understanding of vascular injury in hypertension and T1DM.

3.2 INTRODUCTION

T1DM and hypertension are leading causes of cardiovascular disease (CVD) [1–4]. The two conditions may present independently or concomitantly where they synergistically increase cardiovascular risk. In this regard, the prevalence of hypertension is twice higher in individuals

with T1DM compared with those without T1DM [5] while cardiovascular risk in T1DM is exacerbated by coexistent hypertension [5]. A substantial overlap in etiology and disease mechanisms have been reported between the two conditions including involvement of oxidative stress, the renin–angiotensin–aldosterone system, sympathetic nervous system dysregulation, adipokines, and peroxisome proliferator-activated receptor signaling [6]. Nevertheless, there are also distinct pathways that are unique to T1DM or hypertension that may also cause vascular injury. Management of cardiovascular risk in these distinct but overlapping conditions requires a clear understanding of the molecular pathogenesis. However, despite significant progress in the understanding of the pathophysiology, the molecular alterations that mediate the initiation and progression of cardiovascular disease in T1DM and hypertension are not fully understood.

Circulating large extracellular vesicles (L-EVs) are novel biomarkers of cellular stress/injury [7,8]. L-EVs are 0.1-1.0 μm vesicles shed from the surface of cell membranes under conditions of stress [9,10]. Once formed, L-EVs contain membrane and cytosolic protein, mRNA and miRNA typical of their cell of origin, but lack nuclear material. They also play a crucial role in cell-to-cell communication as they may physically interact and transfer lipids, proteins and nucleic acids from a cell of origin to recipient cells [9]. Crucially, they are present in biological fluids such as urine, blood, saliva, and breast milk, and reflect a molecular fingerprint of the releasing cell type [9,11]. The protein composition of circulating L-EVs may therefore provide more insight into the molecular changes in the cell of origin, than analysis of whole plasma. This, in turn could identify key molecular changes that contribute to vascular injury in T1DM and hypertension. While all types of EVs may provide insight into the cell of origin, L-EVs are particularly suitable for identification of altered pathways in disease since the majority arise directly from stressed/injured cells [9,12].

The unbiased assessment of protein changes in T1DM has been employed in an effort to identify dysregulated signaling responsible for diabetic complications. Numerous proteomic studies on diabetic plasma have been conducted and candidate proteins such as TNFAIP6, CDNF, WIF1, TGFbR3 have been described as possibly involved in pathogenesis of vascular injury in DM [13–15]. Plasma protein profiling of newly diagnosed T2DM revealed proteins altered at the very early stage, reflecting key metabolic syndrome features such as insulin resistance, adiposity, fatty liver and hyperglycemia [15]. Similarly, the plasma proteome of patients with T1DM with diabetic nephropathy revealed new putative biomarkers kidney injury such as transthyretin, apolipoprotein A1, apolipoprotein C1 and cystatin C [16]. Another study observed that T1DM was associated with the upregulation of six proteins (prothrombin, alpha-2-macroglobulin, apolipoprotein A-II, β2 glycoprotein I, Ig alpha-2 chain C region and alpha-1-microglobulin) and down-regulation of two proteins (complement C4 and pregnancy zone protein) [17]. In contrast, the number of studies that define proteomic signatures of hypertension is comparatively small. A recent study employed proteomics on plasma from individuals that are hypertensive and matched healthy controls [18]. The study identified 27 molecular alterations such as osteocalcin, nexilin, and phosphoinositide 3-kinase regulator 1 and pathway alterations including atherogenesis, cellular calcium metabolism, cytoskeletal organization, angiogenesis [18–20]. Similarly, a plasma proteomics classifier based on a series of protein changes has been shown to improve risk prediction associated with renal disease in individuals with T2DM and hypertension [21].

Recently, several groups have examined the proteome of circulating EVs as a strategy to more specifically identify molecular alterations from stressed cells. For example, L-EVs from plasma of individuals diagnosed with T2DM are enriched in proteins involved in cell adhesion, inflammation and platelet activation such as S100A8, S100A9, and CD41 [22]. Interestingly,

assessment of circulating L-EVs in plasma samples from women with GDM showed altered protein expression as compared to healthy control with a shift towards proteins involved in metabolism, energy production and inflammation [23]. These studies suggest that there is alteration of the L-EV proteome in T1DM. However, further study and validation of differentially expressed proteins is necessary. Moreover, the L-EV proteome has not been examined in the context of hypertension. Thus, the aim of this study was to examine the effect of hypertension and T1DM on the molecular composition of circulating L-EVs.

3.3 RESULTS

3.3.1 Physiological and biochemical measures

Physiological parameters including blood pressure, blood glucose, heart weight, urinary albumin/creatinine, and body weight of healthy, OVE26 (diabetic) and TTRhRen (hypertensive) mice are presented in Table 3.1. As expected, blood pressure was elevated in TTRhRen mice while blood glucose was higher in OVE26 mice consistent with the expected phenotype of these two models. The urinary albumin/creatinine ratio was in-creased in OVE26 mice. A reduction was also observed in body weight in OVE26.

Table 3.1. Physiological parameters of the study population.

Background	Blood Pressure (mmHg)	Blood Glucose (mg/dL)	Heart Weight/Tibia Length (mg/mm)	Urinary Albumin/ creatinine (ug/mg)	Body Weight (g)
Wild-type	114.6±5.7	11.3±0.7	8.4±0.1	245±69	32.4±1.2
OVE26	123.5±4.9	29.9±0.8*	7.3±0.5	1026±204*	27.3±0.9*
TTRhRen	144.2±7.6*	12.3±1.0	9.3±0.6	504±166	32.3±1.1

*P<0.05 vs wild-type

3.3.2 Characterization of L-EV Isolates

Following differential centrifugation, L-EV isolates were assessed for size and morphology. Nanoparticle tracking analysis revealed a population of L-EVs with minimal presence of vesicles less than 100 nm in size (Figure 3.1A-D). We did not observe differences in L-EV size or concentration across treatment groups (Figure 3.1D, E). Transmission electron microscopy analysis showed vesicles approximately 150 nm in size with intact membranes (Figure 1F). Western blot analysis confirmed the presence of vesicle markers flotillin-1 and TSG-101 (Supplemental Figure 2).

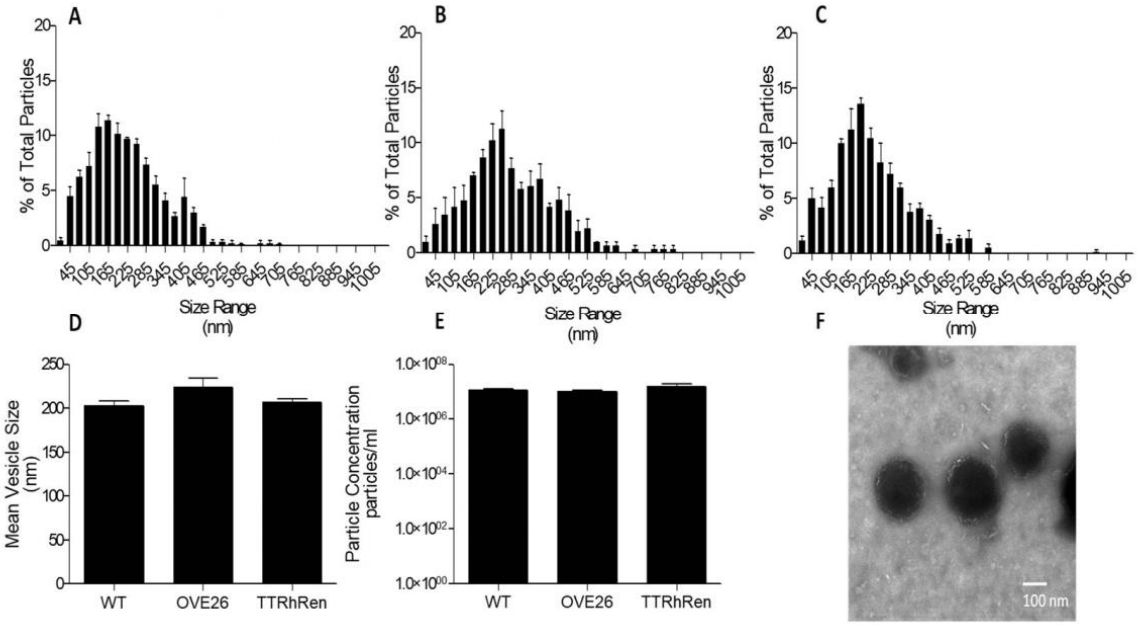


Figure 3.1. Nanoparticle tracking analysis and transmission electron microscopy of L-EV isolates. Shown are size distributions of L-EVs from WT (A), OVE26 (B), and TTRhRen (C) mouse plasma. (D) Comparison of mean particle size by NTA (P = NS, n = 3). (E) Comparison of particle concentration by NTA (P = NS, n = 3). (F) Representative transmission electron micrograph of pooled plasma showing distinct vesicle size and shape.

3.3.3 Proteomics analysis and associated signaling pathways

To gain insight of the molecular changes associated with hypertension and T1DM we next examined the protein composition of isolated circulating L-EVs. Across all samples LC–MS/MS analysis identified 544 proteins with a minimum of two spectral counts per sample with a 95% peptide threshold and 99% protein threshold. Of the 544 proteins identified, 408 were common to all groups, while 34 were exclusive to only healthy, 5 to hypertension and 16 to T1DM. Seven proteins were common in T1DM and hypertension groups, 34 were common between healthy and hypertension and 40 were common between healthy and T1DM (Figure 3.2A).

Notably, in hierarchical clustered heatmaps, we observed separation according to disease confirming that molecular profiles of L-EVs are most similar within disease conditions and suggesting that L-EVs may reveal disease-specific protein alterations (Figure 3.2B).

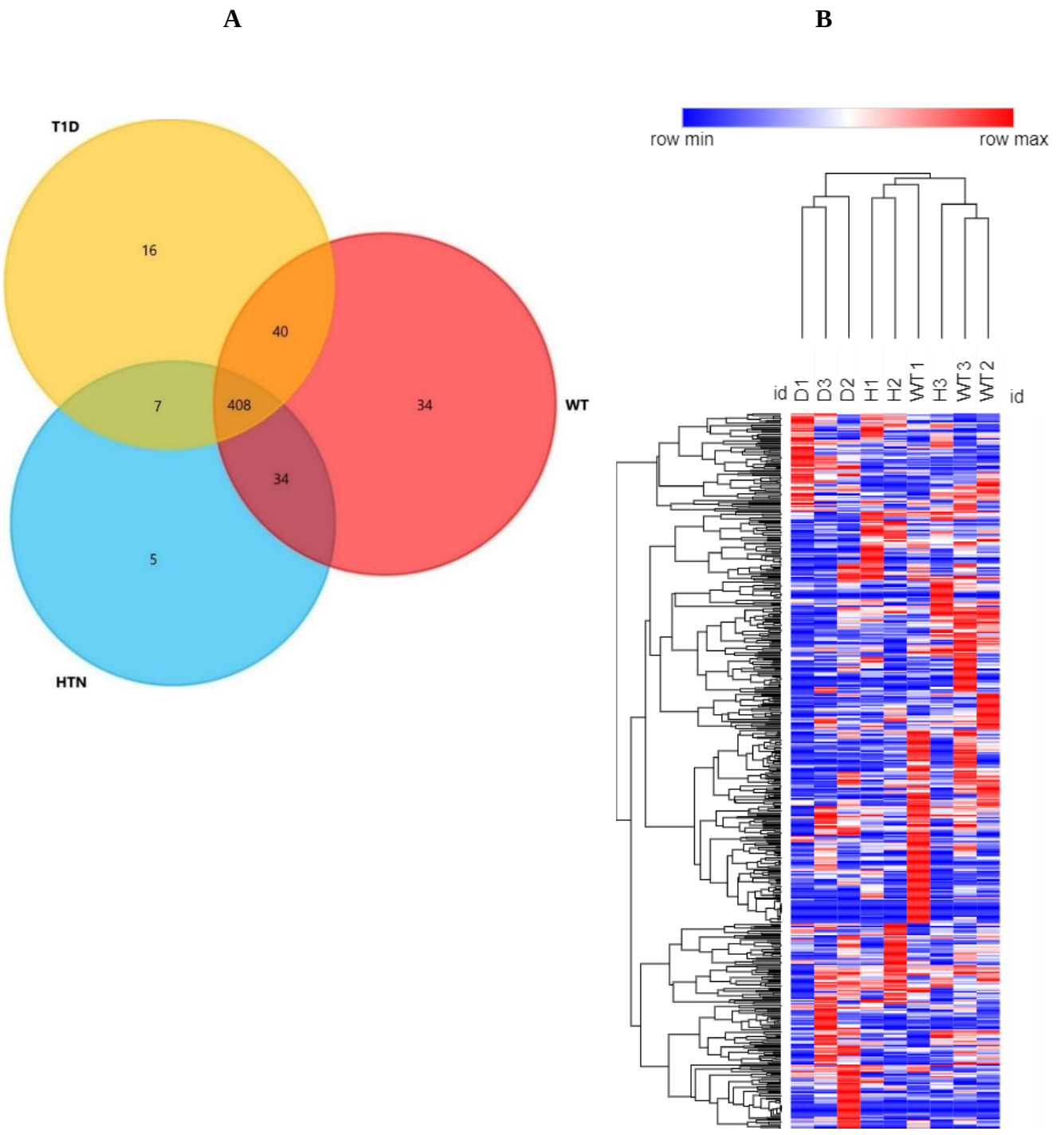


Figure 3.2. Venn diagram represents the differences in protein composition among T1DM, hypertension, and wild-type mice (A). Protein profile analysis of differentially expressed

proteins (Hierarchical clustering) across all three groups of mice using Morpheus (https://software.boardinstitute.org/morpheus/, (accessed on 1 June 2022)) (B).

The relative abundance of L-EV protein in diabetic mice in comparison to healthy mice is presented as a volcano plot in Figure 3.3A. A total of five differentially expressed proteins were identified (Table 3.2). Of these proteins, three were upregulated TSP4, HPT, CO3A1 and two were downregulated (ANK1, SAA4) (Figure 3.3A, Table 3.2).

With respect to hypertensive mice in comparison to healthy, a total of five differentially expressed proteins were identified (Table 3.3). Of these proteins, two proteins were upregulated HPT, PPN and three were downregulated (ANK1, SPTB1, SPTA1). (Figure 3.3B, Table 3.3).

Finally, for diabetic mice in comparison to hypertensive mice a total of 11 differentially expressed proteins were identified in L-EVs (Table 3.4). Eight proteins were upregulated (IGHA, TSP4, CLC1B, HVM17, CO3A1), and three proteins were downregulated (ZPI, SAMP, SAA4) (Figure 3.3C, Table 3.4).

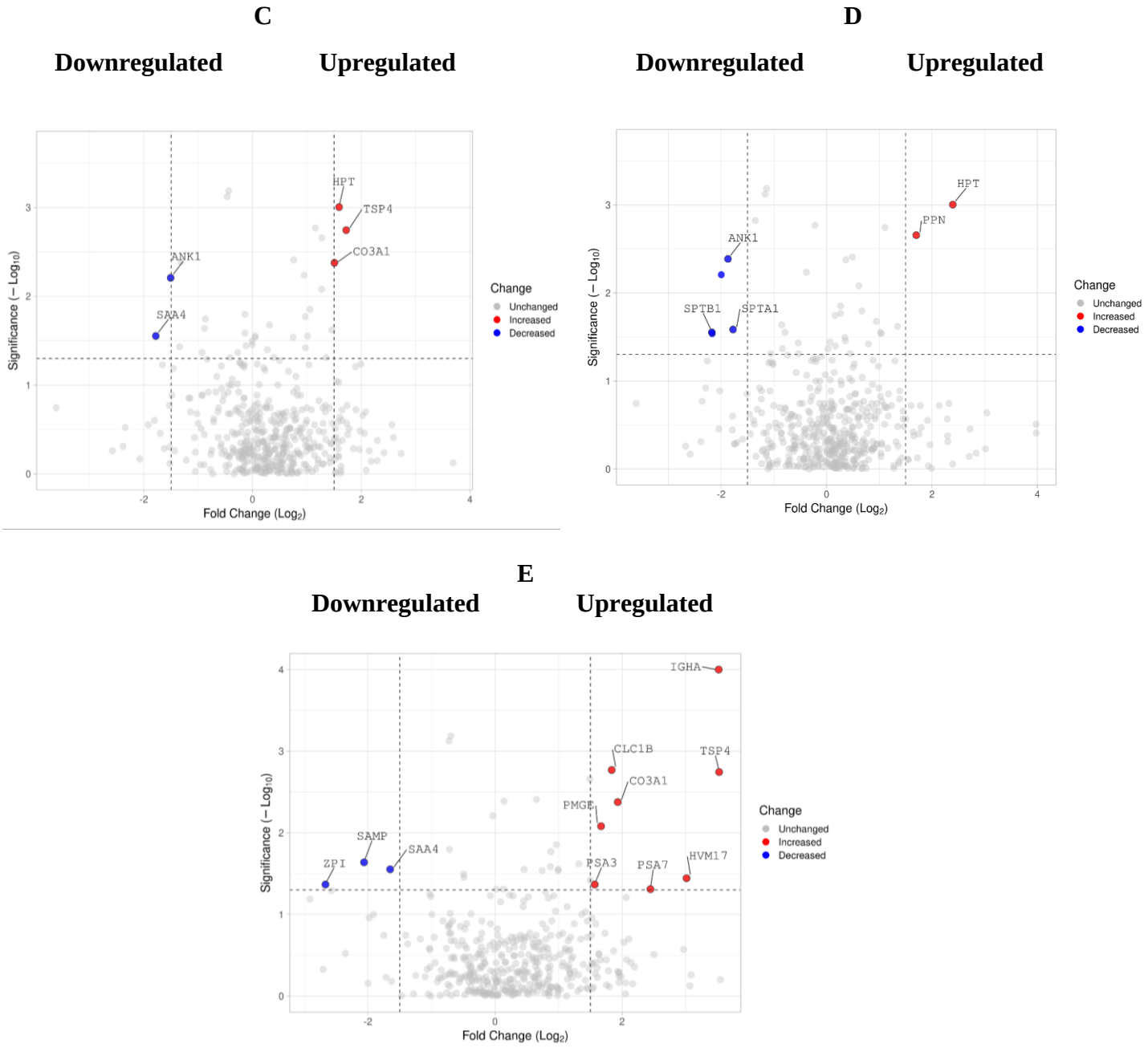


Figure 3.3. Volcano plot representing differentially expressed proteins comparison: A: Differentially expressed proteins in T1DM plasma L-EVs compared to wild-type plasma L-EVs. The horizontal axis represents the log2 of fold change and the vertical axis represent p-

value. Each gray dot represents a protein with red dots on the right above the dashed line are proteins upregulated while the blue dots on the left are downregulated. *(-log p value of 1.3010299957 is considered significant as it translates to a p value of 0.05*). VolcaNoseR. https://goedhart.shinyapps.io/VolcaNoseR/. B: Volcano plot representing differentially expressed protein in hypertensive plasma L-EVs compared to wild-type plasma L-EVs. C: Volcano plot representing differentially expressed protein in T1DM plasma L-EVs compared to hypertensive plasma L-EVs.

Table 3.2. Differentially expressed proteins in T1DM as compared to wild-type mice.

Protein	Change	Fold change (log2)	P value
TSP4	↑	1.721	0.0018
HPT	↑	1.591	0.00099
CO3A1	↑	1.505	0.0042
ANK1	↓	-1.507	0.0062
SAA4	↓	-1.780	0.028

↑ represents upregulated proteins. ↓ represents downregulated proteins.

*Proteins were selected using a cutoff point of $p < 0.05$.

Table 3.3. Differentially expressed proteins in hypertension as compared to wild-type mice.

Protein	Change	Fold change (log2)	P value
HPT	↑	2.395	0.00099
PPN	↑	1.701	0.0022
ANK1	↓	-1.871	0.0041
SPTB1	↓	-2.177	0.028

SPTA1	↓	-1.775	0.026

↑ represents upregulated proteins. ↓ represents downregulated proteins.

*Proteins were selected using a cutoff point of $p < 0.05$.

Table 3.4. Differentially expressed proteins in T1DM as compared to hypertensive mice.

Protein Name	Change	Fold change (log2)	P value
IGHA	↑	3.518	0.000302957
TSP4	↑	3.525	0.000298244
CLC1B	↑	1.835	0.014605208
HVM17	↑	3.013	0.000970275
CO3A1	↑	1.930	0.011735601
PSA3	↑	1.568	0.026992381
PSA7	↑	2.445	0.003588742
PMGE	↑	1.667	0.021479365
SAMP	↓	-2.060	115.0271922
SAA4	↓	-1.650	0.022372751
ZPI	↓	-2.668	0.002146066

↑ represents upregulated proteins. ↓ represents downregulated proteins.

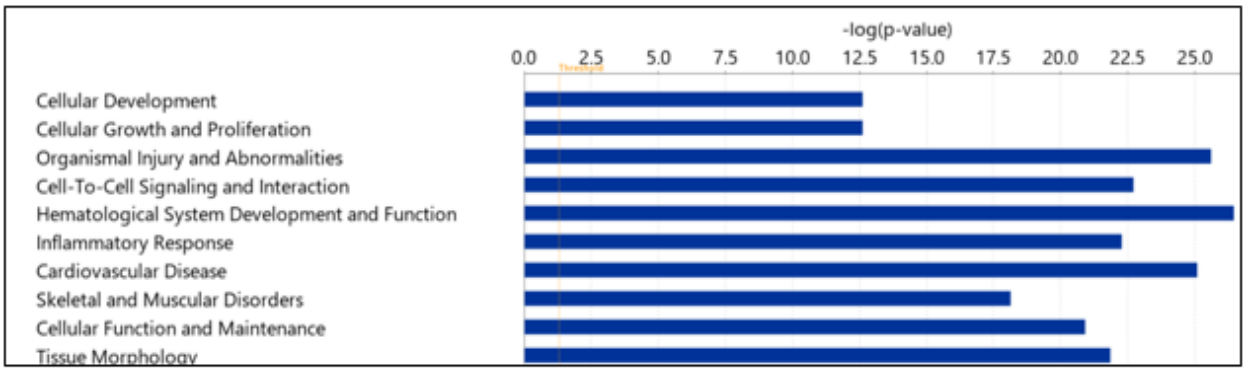
*Proteins were selected using a cutoff point of $p < 0.05$.

3.3.4 Protein ingenuity pathway analysis

To further understand the impact of T1DM and hypertension on the circulating L-EV proteome, Ingenuity Pathway Analysis (IPA) software was used to assess “disease and functions”, and “canonical pathway” of all identified proteins.

Using all proteins in L-EVs from T1DM compared to healthy mice, IPA noted enrichment in “disease and function” for cellular development, cellular growth and proliferation, organismal injury and abnormalities, cell-to-cell signaling and interaction, hematological system development and function, inflammatory response, cardiovascular diseases, skeletal and muscular disorders, cellular function and maintenance, and tissue morphology (Figure 3.4A). Similarly, IPA for “canonical pathways” identified pattern recognition, apelin cardiomyocyte signalling, white adipose tissue browning, SNARE signaling, complement system, PPARα, RxR α activation, IL-8 signalling, NAD homeostasis, and CLEAR signally as enriched in T1DM (Table 3.5). Among these pathways, apelin cardiomyocyte signally pathway, white adipose tissue browning pathway, apelin adipocyte signally pathway, PPARα, RxR α activation and IL-8 signal-ling pathway are enriched pathways associated with inflammation in this group. Other pathways such as SNARE signalling pathway is involved in extracellular vesicle formation or mediate vesicle fusion, NAD signalling pathway in mitochondrial biogenesis and CLEAR signalling pathway is responsible for lysosomal activity (lysosomal expression and regulation) (Figure 3.4B; Table 3.5). As shown in Table 3.5, significantly changed proteins participating in these pathways include complement (C1QA/C1QB), myosin (MYH10, MYH14, MYH9), and mitochondrial (ACADL, ACADM), etc.

A



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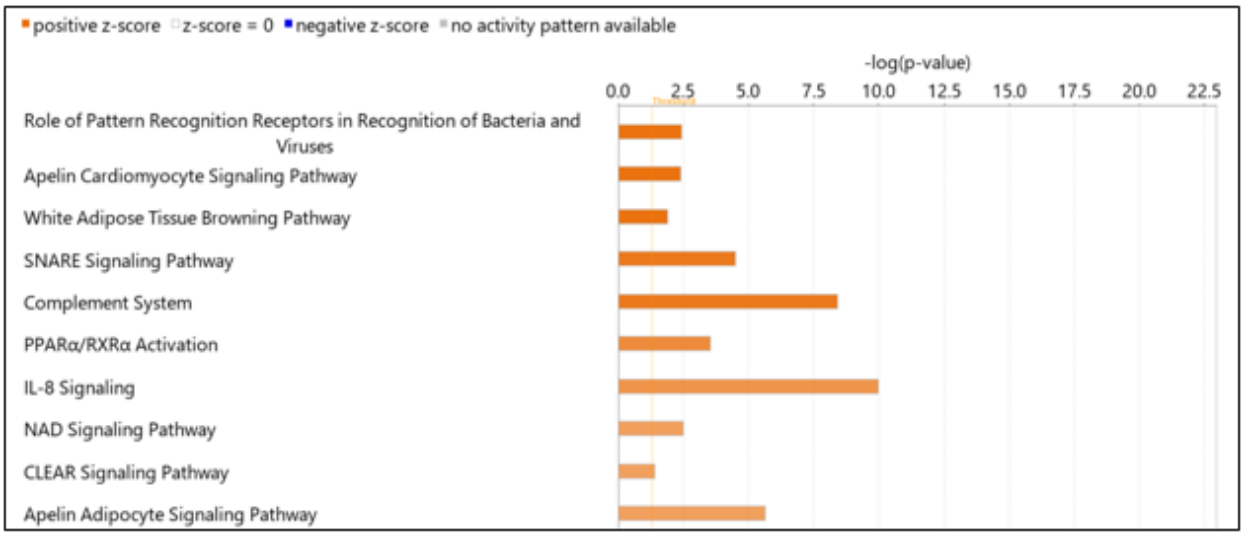


Figure 3.4. Summary of Ingenuity Pathway Analysis (IPA) for L-EV proteins in T1DM as compared to wild-type. Shown are disease and function (A) and Canonical pathways (B) The dotted orange line represents the threshold of significance (p = 0.05).

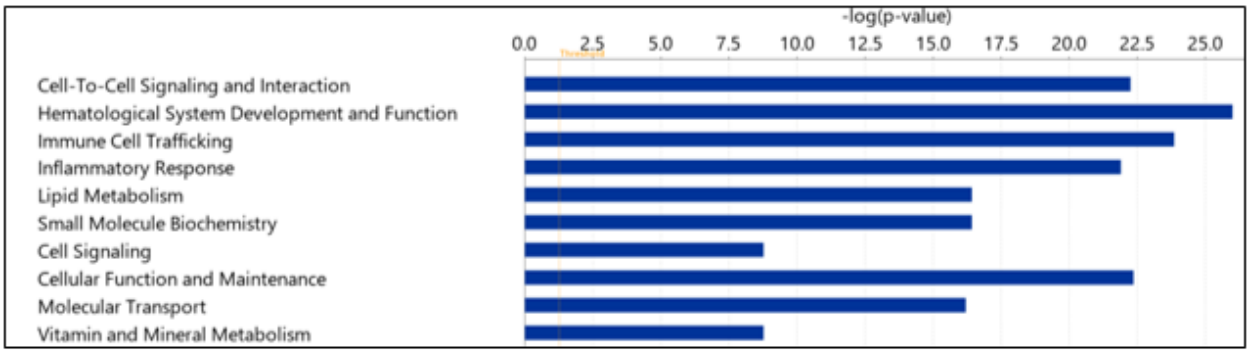
Table 3.5. Top 10 canonical pathways and related proteins in T1DM compared with wild-type mice.

Ingenuity canonical pathways	-log (pvalue)	Ratio	z-score	Proteins
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	2.45	0.0321	2	C1QA, C1QB, C1QC, MBL2, TGFB1
Apelin Cardiomyocyte Signaling Pathway	2.4	0.040	2	GNAI2, MYL6, MYL9, TGFB1
White Adipose Tissue Browning Pathway	1.9	0.029	2	CAMP, LDHA, LDHB, THRB
SNARE Signaling Pathway	4.51	0.051	1.89	MYH10, MYH14, MYH9, MYL6, MYL9, RAB6A, RAB7A
Complement System	8.43	0.189	1.89	C1QA, C1QB, C1QC, C4BPA, MASP1, MASP2, MBL2
PPARα/RXRα Activation	3.53	0.035	1.633	ACADL, APOA1, APOA2, GNAQ, RAP1A, RAP1B, TGFB1
I-8 Signaling	9.97	0.066	1.508	CDC42, EGFR, GNA13, GNAI2, GNAQ, GNAZ, RAC1, RAC2, RAP1A, RAP1B, RHOA, VCAM1
NAD Signaling Pathway	2.51	0.033	1.342	ACADL, ACADM, LDHA, LDHB, TGFB1
CLEAR Signaling Pathway	1.41	0.0175	1.342	EGFR, RAB7A, RAP1A, RAP1B, TGFB1
Apelin Adipocyte Signaling Pathway	5.66	0.0769	1.342	GNAI2, GPX1, GPX3, GSTM1, RAC1, RAC2, SOD1

*Ratio refers to the number of proteins from the dataset that map to the pathway listed divided by the total number of proteins that map to the canonical pathway from within the IPA knowledgebase.

Next, we examined the "disease and function" in hypertension compared to healthy. Cell-to-cell signaling and interaction, hematological system development and function, immune cell trafficking, inflammatory response, lipid metabolism, small molecule biochemistry, cell signaling, cellular function and maintenance, molecular transport, vitamin and mineral metabolism were noted as significantly enriched (Figure 3.5A). For "canonical pathways" the top pathways included signaling pathways such as pattern recognition receptor, white adipose tissue browning, semaphorin neuronal repulsive, RhoA signalling, regulation of Actin-based motility by Rho, phagosome formation, IL-8 signalling, ILK signalling, signaling by Rho family GTPases and actin cytoskeleton (Figure 3.5B, Table 3.6). The "canonical pathways" included as white adipose tissue browning pathway (CAMP, LDHA, LDHB, THRB) and IL-8 signalling pathway (CDC42, EGFR, GNA13, GNAI2, GNQ, GNAZ, MMP2, MYL9, RAC1, RAC2, RAP1A, RAP1B, RHOA, VCAM1).

A



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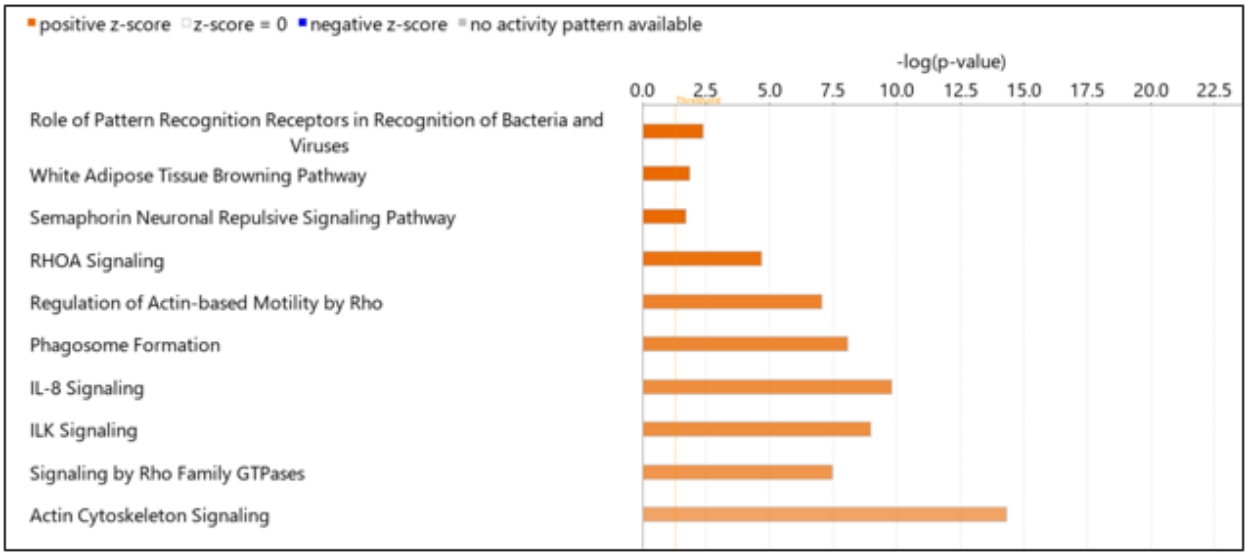

Figure 3.5. Summary of Ingenuity Pathway Analysis (IPA) for L-EV proteins in hypertension as compared to wild-type. Shown are disease and function (A) and Canonical pathways (B) The dotted orange line represents the threshold of significance (p = 0.05).

Table 3.6. Top 10 canonical pathways and related proteins in hypertensive compared with wild-type mice.

Ingenuity canonical pathways	-Log (pvalue)	Ratio	z-score	Proteins
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	2.39	0.032	2	C1QA, C1QB, C1QC, MBL2, TGFB1
White Adipose Tissue Browning Pathway	1.85	0.029	2	CAMP, LDHA, LDHB, THRB
Semaphorin Neuronal Repulsive Signaling Pathway	1.72	0.026	2	MYL6, MYL9, RAC1, RHOA
RHOA Signaling	4.68	0.056	1.89	ARPC2, ARPC4, ARPC5, GNA13, MYL6, MYL9, RHOA
Regulation of Actin-based Motility by Rho	7.04	0.077	1.66	ARPC2, ARPC4, ARPC5, CDC42, MYL6, MYL9, RAC1, RAC2, RHOA
Phagosome Formation	8.09	0.030	1.606	ARPC2, ARPC4, ARPC5, CDC42, IGHE, IGHG3, IGHM, IGKC, LCAT, MYH10, MYH11, MYH14, MYH9, MYL6, MYL9, RAC1, RAC2, RAP1A, RAP1B, RHOA, TLN11
IL-8 Signaling	9.79	0.066	1.508	CDC42, EGFR, GNA13, GNAI2, GNAQ, GNAZ, MMP2, MYL9, RAC1, RAC2, RAP1A, RAP1B, RHOA, VCAM1
ILK Signaling	8.99	0.065	1.508	CDC42, FLNA, ILK, MYH10, MYH11, MYH14, MYH9, MYL6, MYL9, PARVB, RAC1, RAC2, RHOA
Signaling by Rho Family GTPases	7.46	0.048	1.414	ARPC2, ARPC4, ARPC5, CDC42, GNA13, GNAI2, GNAQ,

				GNAZ, MYL6, MYL9, RAC1, RAC2, RHOA
Actin Cytoskeleton Signaling	14.3	0.077	1.213	RPC2, ARPC4, ARPC5, CDC42, FLNA, GNA13, KNG1, MYH10, MYH11, MYH14, MYH9, MYL6, MYL9, RAC1, RAC2, RAP1A, RAP1B, RHOA, TLN1

*Ratio refers to the number of proteins from the dataset that map to the pathway listed divided by the total number of proteins that map to the canonical pathway from within the IPA knowledgebase

3.4 DISCUSSION

Vascular injury and endothelial dysfunction are common features of both hypertension and T1DM. However, as pathogenic mechanisms driving such changes may differ between the two conditions, the approaches to therapeutic management of vascular injury may also differ. The present study examined the effect of hypertension and T1DM on the molecular composition of circulating L-EVs as an indirect measure of vascular alterations. Using well-established mouse models, we observed distinct protein signatures in L-EV populations with the greatest agreement within disease conditions. Further assessment with IPA identified enrichment in key signaling pathways including apelin and SNARE signaling (T1DM) and semaphorin and Rho signaling (hypertension). Our results suggest that L-EV protein composition is reflective of the underlying molecular changes driving disease pathogenesis.

In this study, we observed common/distinct changes in proteins in T1DM vs healthy. This study identified a total of five differentially expressed proteins in T1DM as compared with healthy. Of these proteins, three were upregulated (TSP4, HPT, CO3A1) and two proteins were downregulated (ANK1, SAA4). Some of these changes have been identified in other studies and are in accordance with our observations [24–28]. Thrombospondin-4 (TSP4) has been shown

previously to cause peripheral arterial disease in T1DM [24]. The fact that TSP4 is elevated in our vesicles suggest an activation of pathway that may contribute to this process. Increased amounts of type III collagen (CO3A1) have been noted in tubular epithelial cells in individuals with diabetic nephropathy, however to the best of our knowledge this has not been reported in the vasculature [25]. While previous reports have shown elevation in haptoglobin (HPT) in individuals with elevated glucose and metabolic syndrome [26], alterations in ANK-1 do not appear to have been reported previously [27]. Based on the protein composition of L-EVs, IPA identified the canonical pathways that are most enriched in diabetic vs control mice. These pathways included apelin signalling, white adipose tissue browning, SNARE signaling, complement activation, PPARα, and NAD biogenesis. Previous studies have reported that apelin (a peptide hormone linked with obesity and DM) and its receptor inhibit vascular injury in DM including endocrine response to stress, lipid metabolism, homeostasis and angiogenesis [28,29]. It is possible that enrichment in apelin signaling is a protective mechanism to limit vascular injury in diabetic mice. SNARE proteins are involved in insulin granule exocytosis but less is known about their relevance to vascular health [30–34] L-EV release and it is possible that their enrichment is simply a result of altered L-EV release under diabetic conditions [35]. PPARα signaling has been shown to lower blood pressure and reduce oxidative stress [36–38]. The enrichment in this signaling may therefore be evidence of a protective response. Finally, enrichment in proteins related to NAD+ biogenic pathways may be evidence of dysregulation of this pathway as has been reported in animal and human diabetes [39].

We also examined changes in proteins in L-EVs from hypertensive mice vs healthy. A total of five differentially expressed proteins were also identified in this group; two proteins were upregulated (PPN, HPT) and three proteins were downregulated (ANK1, SPTB1, SPTA1). Interestingly, the up-regulation of HPT and down-regulation of ANK1 were also seen in our

diabetic mice suggesting that these may be common pathways involved in vascular injury in both conditions. Conversely, the up-regulation of PPN and down-regulation of SPTB1 and SPTA1 were unique to hypertension. In addition to the previously described relationship with blood glucose, increases in HPT have been shown in individuals with elevated blood pressure and metabolic syndrome [26]. Mechanistically, HPT has been shown to lower blood pressure in a model of hemoglobin-induced hypertension [40]. Thus, increased HPT may be a common protective pathway activated in both hypertension and T1DM. When examining protein composition of L-EVs from hypertensive and healthy mice, IPA identified canonical pathways that are most enriched in hypertension. These pathways included semaphorin neuronal repulsive signaling, RhoA signaling, phagosome formation, ILK signaling, and actin cytoskeleton signaling. RhoA/Rho kinase signaling has long been implicated in hypertension due to its important role in smooth muscle contraction [41–43]. Thus, it is perhaps not surprising that this pathway is elevated in L-EVs from hypertensive mice in our study. As RhoA/Rho kinase also play important roles in cytoskeletal regulation [44] and phagosome formation [45], enrichment in these pathways may be related to convergent signaling. Interestingly, integrin-linked kinase (ILK) signaling has been implicated in hypertension-mediated organ damage [46,47]. However, to the best of our knowledge semaphoring signaling has not been implicated in blood pressure regulation and may represent a novel pathway for future study.

Our study identified over 500 proteins in circulating L-EVs with the vast majority of those proteins (>400) found in all groups. Interestingly, only a small number of proteins were found to be exclusive to a particular disease state. These proteins could represent those which are altered in response to the disease condition or they may be actively contributing to disease pathogenesis. Future research should seek to clarify the role of these proteins as biomarkers or pathogenic

mediators of hypertensive or diabetic vascular injury. Interestingly, our hierarchical clustering algorithm largely separated our L-EV isolates based on disease state. L-EVs from diabetic mice were distinctly categorized while those from hypertension and wild-type mice were more closely overlapping in protein signatures. While there were large variations within each group it is reassuring that the greatest similarities were seen within the same experimental group. Whether this will remain the case with larger and more heterogeneous populations (i.e. human cohorts) remains to be seen.

The present work represents one of the earliest to examine distinct proteomic changes in diabetic and hypertensive mice and the first to employ L-EVs as a tool to facilitate this analysis. One of the strengths of this study is the use of well-defined mouse models of T1DM and hypertension. In addition, the inclusion of both hypertension and diabetic mice allowed for the identification of both common and unique enriched pathways that may be contributing to disease pathogenesis and progression. We focused our efforts on the L-EV subpopulation due to the tight linkage between their formation and cellular stress [48]. Nevertheless, there is abundant evidence that other EV populations such as small EVs/exosomes play a role in cardiovascular physiologyy [49,50]**.** Future studies should strive to clarify the impacts of T1DM and hypertension on other EV populations. Our study also had some limitations to consider. First, relatively few male mice were studied, and although we did observe greatest similarity within disease, it is likely that the degree of heterogeneity was underestimated. Second, our observations require validation, and the potential for therapeutic targeting of dysregulated pathways is not known at this time. It is also worth noting that the approach to assessing protein signatures in circulating L-EVs does not provide a complete picture of molecular changes such as epigenetic alterations. Finally, there is also potential for differences in hypertension and diabetes-associated changes between mice and

humans. Thus, independent validation in humans is a logical next step. Nevertheless, our results suggest that circulating L-EVs may be used to assess protein changes to the vasculature in a minimally invasive fashion.

3.5 METHODS

3.5.1 Animals

Mouse models of hypertension, T1DM, and their wild-type (WT) littermates (healthy control) were employed on an FVB/N background and male mice were studied at 20 weeks of age. Hypertensive TTRhRen mice express a modified human pro-renin transgene under the control of the mouse transthyretin promoter [51–53]. These mice overexpress human renin and hemizygotes exhibit elevated systolic blood pressure and cardiac hypertrophy by 4 months of age. To model T1DM, we employed the transgenic OVE26 mice which have a pancreatic beta cell-specific overexpression of a calmodulin mini-gene and are insulinemic from birth [54]. Hypertensive and diabetic mice, as well as their healthy littermates were housed at the University of Ottawa Animal Care Facility with free access to food and water. Protocols were approved by the University of Ottawa Animal Care Committee and conducted in accordance with the guidelines of the Canadian Council on Animal Care.

3.5.2 Blood Pressure Measurement

Blood pressure was assessed by tail cuff plethysmography (BP 2000, Visitech) as described previously [51,52,55]. Following a five-day training period (10 BP readings/day), weekly BP measurements were obtained beginning at 10 weeks.

3.5.3 Physiological Parameters

Immediately prior to sacrifice, spot urine samples were collected and centrifuged at 2500 g for 10 minutes and stored at -80 ˚C. Urinary albumin was assessed with the Mouse Albumin Elisa Kit (Bethyl labs, Montgomery, TX.) following manufacturer's protocol. Albumin levels were normalized to creatinine concentration using the Creatinine Companion Kit (Exocell, Philadelphia, PA).

At sacrifice, blood samples were collected into heparinized syringes by cardiac puncture and immediately centrifuged at 2500 g for 10 minutes at 4˚C. Plasma glucose levels were determined by glucometry (Bayer Contour) and remaining plasma was used for L-EV isolation. Tibias, kidneys, and hearts were removed and weighed. Organ weights were normalized to tibia length**.**

3.5.4 L-EV isolation

Circulating L-EVs were isolated via differential centrifugation from plasma by centrifugation for 20 minutes at 20,000 x g to obtain a L-EV-rich pellet. The isolated vesicles were washed with 1xPBS and re-suspended in either PBS (nanoparticle tracking analysis), 2.5% glutaraldehyde in PBS (transmission electron microscopy), or RIPA buffer (proteomics) [10,56].

3.5.5 Nanoparticle Tracking Analysis

To confirm the presence of vesicles between 100-1000nm (L-EVs) in the vesicle population isolated with a 20,000 xg, Nanoparticle Tracking Analysis (NTA) was conducted to assess vesicle size. Briefly, samples were diluted in 1x PBS to the working range of the system and analyzed on a ZetaView PMX110 (Particle Metrix, Meerbusch, Germany) in size mode as we have done previously [57–60].

3.5.6 Electron Microscopy

L-EVs were examined by transmission electron microscopy (TEM) as described previously [59,61]. In brief, L-EVs were isolated from pooled plasma samples and fixed with 2.5% glutaraldehyde in PBS for four hours at room temperature. Next, the pellet was washed in 0.1 M Na cacodylate buffer, post-fixed in 2% OsO4, and dehydrated in a series of graded ethanols. Samples were embedded in Spurr Resin, and 60 nm sections were prepared on copper grids. Samples were visualized using a JEOL JEM-1400 Plus electron microscope.

3.5.7 Western blot analysis

L-EV isolates from pooled plasma samples were examined for the presence of vesicle protein markers by Western blot analysis as described previously [59,62]. Protein lysates were 10% polyacrylamide gels and levels of the vesicle-associated proteins flotillin-1 (1:2000, BD Biosciences, Franklin Lakes, NJ, USA) and TSG101 (1:2000, Abcam Inc, Toronto, Canada) were assessed.

3.5.8 Proteomic assessment of L-EVs

L-EV isolates were separated by gel electrophoresis on a 4-15% Mini PROTEAN TGX Gel. Separated proteins were excised by a gel excision tool (The Gel Company, San Francisco CA, USA) and placed in 1% acetic acid. In-gel proteins were digested with trypsin, purified by ZipTip, concentrated in an Eppendorf vacufuge (ThermoFisher Scientific) and re-suspended in 0.1% formic acid.

Digested peptides were then analyzed by label-free LC-MS/MS through the OHRI Proteomics Core Facility as described previously [63]. Briefly, the system consisted of an UltiMate 3000 RSLC nano HPLC, LTQ Orbitrap XL hybrid mass spectrometer, the XCalibur software

(version 2.0.7) and a nanospray ionization source. Peptides were eluted over a 60 minute gradient of 3% - 45% acetonitrile at a flow rate of 300nL/minute through a 10-cm long column with integrated emitter tip (Picofrit PF360-75-15-N-5 from New Objective packed with Zorbax SB-C18, 5 micron from Agilent, Santa Clara, CA). MS scans were acquired in FTMS mode at a resolution setting of 60,000. MS2 scans were acquired in ion trap CID mode using data-dependent acquisition of the top 5 ions from each MS scan. MASCOT software (Matrix Science, Boston MA, USA, version 2.5.1) was used to infer peptides and proteins from the observed MS/MS spectra and matched against mouse sequences from SwissProt. Mass tolerance parameters were MS ±10 ppm and MS/MS ±0.6 Da. Enzyme specificities were set to 'Trypsin' with <=2 miscuts, variable modifications were set to oxidation of methionine, protein N-terminal acetylation, pyrocarbamidomethlyation of N-terminal cysteine, and conversion of glutamine to pyroglutamate and fixed modifications was set to Carbamidomethylation of cysteine. "Identified MASCOT peptides and proteins were confirmed using Scaffold (Proteome Software Inc. Portland OR, USA version Scaffold_4.7.3, Proteome Software Inc., Portland OR, USA)" 79. The scaffold FDR algorithm accepted peptides with a greater than 95% probability and proteins were accepted if they contained at least 2 identified peptides and had a greater than 99% probability.

The differences in protein composition among T1DM, hypertension, and healthy mice was identified using Functional Enrichment analysis tool (FunRich), an open access standalone functional enrichment and interaction network analysis tool and presented as a Venn diagram [64].

3.5.9 Bioinformatics analysis

For hierarchical clustered heatmaps, Z-scores of log2 protein abundances (Normalized total spectra) were first calculated and column clustering was calculated using the linkage function (metric = 'Euclidean distance', Linkage method = 'average') with column clustering through

MORPHEUS by Broad Institute (RRID:SCR_017386), software tool for versatile matrix visualization. (https://software.broadinstitute.org/morpheus).

A volcano plot of log2 fold change versus −log10 (significance) of differentially expressed proteins comparing T1DM, hypertension and healthy mice was made using VolcaNoseR (**https://huygens.science.uva.nl/VolcaNoseR** (accessed on 1 June 2022)) [65] with a −log *p* value (a –log *p* value of <1.3010299957, corresponding to $p < 0.05$ was considered significant) and the fold change threshold of 1.5.

Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Mountain View, CA, USA; **www.ingenuity.com**, (accessed on 17 March 2022)) was used to identify "diseases and functions" and "canonical pathways" that are most significant to the dataset and to categorize differentially dysregulated proteins in specific diseases and functions for the proteins exclusive to three different types of mice. The pathways and diseases with $p < 0.05$ were listed and considered significantly different.

3.5.10 Statistical analysis

To analyze differences in physiological parameters between hypertensive, diabetic and healthy mice, a one way ANOVA was performed followed by Bonferroni correction test [66]. All statistical analyses were conducted using GraphPad Prism version 8.4.2 (GraphPad Software, La Jolla, CA). (GraphPad Software, La Jolla, CA). Statistical significance was considered when $p<0.05$.

3.6 CONCLUSIONS

In summary, circulating L-EVs have distinct molecular compositions that are dependent on pathogenic state. We also observed changes that were common to both hypertension and T1DM, and disease-specific changes. Further analysis of these changes may lead to the identification of novel pathways associated with the pathogenesis of vascular injury in hypertension and T1DM. Such knowledge is critical to optimizing and personalizing therapeutic management of vascular injury in these two conditions.

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Author Contributions

Conceptualization, A.A., M.T., C.R.J.K. and D.B.; methodology, A.A., M.T., J.-F.T. and C.E.H.; validation, A.A., C.R.J.K. and D.B.; formal analysis, A.A., M.T., J.-F.T. and D.B.; investigation, A.A. and M.T.; resources, C.R.J.K. and D.B.; data curation, A.A., M.T. and D.B.; writing—original draft preparation, A.A. and D.B.; writing—review and editing, A.A., M.T., J.-F.T., C.E.H., C.R.J.K. and D.B.; visualization, A.A.; supervision, D.B. and C.R.J.K.; project administration, D.B.; funding acquisition, D.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

All procedures were approved by the University of Ottawa Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

CHAPTER 4- Impact of acute moderate-intensity aerobic exercise on circulating extracellular vesicles in pregnant and non-pregnant women.

(Formatted as per Applied Physiology, Nutrition, Metabolism requirements)

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4 ABSTRACT

Introduction: Exercise has been shown to improve cardiovascular and metabolic health in pregnancy and may represent a non-pharmacological approach to improving pregnancy outcomes. Large Extracellular vesicles (L-EVs) are emerging biomarkers of endothelial dysfunction and offer the potential for evaluating vascular health non-invasively during pregnancy. The purpose of this study was to investigate changes in circulating L-EV levels after an acute bout of moderate-intensity aerobic exercise in healthy pregnant and non-pregnant women. **Methods:** We studied plasma samples from pregnant (N=13, 13-28 weeks) and non-pregnant (N=17) women. A pre-exercise blood sample was obtained followed by a 30 minute bout of moderate-intensity treadmill-based exercise. Immediately following the exercise, a post-exercise blood draw was collected. L-EVs were isolated from plasma by differential centrifugation and characterized by Western blot and electron microscopy. We quantified circulating L-EVs by nanoscale flow cytometry. Endothelial L-EVs were identified as VE-Cadherin+, platelet L-EVs as CD41+ and leukocyte L-L-EVs as CD45+ events. **Results:** Acute exercise was associated with a significant reduction in levels of circulating endothelial L-EVs in the non-pregnant group ($p=0.0232$) but not in the pregnant group ($p=0.2734$). A greater proportion of non-pregnant women (13/17, 76.47%) exhibited a reduction in endothelial L-EVs compared with their pregnant counterparts (4/13, 30.76%, $p<0.05$). We also observed a positive association between measures of fitness (average speed) and baseline levels of platelet ($r=0.5816$, $p=0.0159$,) and total L-EVs ($r=0.5325$, $p=0.0296$) in the non-pregnant group but not in pregnant individuals. **Conclusions:** Collectively, our study highlights that after a matched acute exercise, changes to circulating L-EV levels differ depending on pregnancy status.

4.1 INTRODUCTION

During pregnancy, the maternal cardiovascular system undergoes a series of changes, including an increased blood volume, higher heart rate and cardiac output, systemic vasodilation, and alterations in response to vasoactive factors [1–4]. These changes are necessary to provide sufficient uteroplacental circulation to support fetal growth. Optimization of maternal vascular health is therefore critical to achieving the best possible outcomes. Under certain conditions such as T1DM or gestational hypertension, the maternal vasculature may be perturbed, which can lead to short and long-term complications for mother and fetus. Conversely**,** exercise has been shown to improve cardiovascular and metabolic health in pregnancy and may represent a non-pharmacological approach to improving pregnancy outcomes. The *2019 Canadian Guideline for Physical Activity throughout Pregnancy* recommends that women without contraindications engage in a minimum of 150 minutes of moderate-intensity physical activity per week to improve health outcomes [5]. The benefits of such activity include a decrease in the risk of pregnancy complications such as pre-eclampsia, hypertension, and GDM [5,6]. Maternal exercise also reduces the odds of developing macrosomia [7]. It is therefore clear that exercise offers significant benefits in pregnancy. Mechanistically, benefits of exercise have been partly attributed to effects on endothelial function and associated benefits to both mother and fetus [8,9]. Identifying strategies for evaluating vascular health in pregnancy would be of significant value in evaluating the success of exercise interventions.

Circulating EVs offer potential as surrogate markers of vascular health. EVs are membrane-encapsulated packages released by cells that can be found in all biological fluids [10,11]. The main sources of EVs in plasma are platelets, with leukocytes, endothelial cells, and erythrocytes also notable contributors [10,12–15]. A subclass of large EVs (L-EVs), are typically defined as ~100-1000 nm vesicles shed from the surface of cellular membranes under conditions of stress [10,16,17] .

L-EVs have been extensively studied as biomarkers and effectors of tissue injury. In particular, we and others have shown that endothelial L-EVs induce oxidative stress, inflammation and impaired vasorelaxation in cultured endothelial cells and isolated vessels [18–20]. L-EVs also appear to have value as biomarkers of vascular disease. For example, high levels of endothelial L-EVs predict cardiovascular morbidity/mortality in patients with coronary artery disease [21], pulmonary hypertension, [22] and end-stage kidney disease [12]. Further, we recently showed that high levels of endothelial and platelet L-EVs in the first trimester were associated with adverse neonatal outcomes in women with type 1 diabetes mellitus (T1DM) [23].

Pregnancy-related physiological changes may affect levels of circulating endothelial L-EVs. Radu *et al*. (2015) reported that the plasma levels of endothelial L-EVs in non-complicated pregnancies were higher when compared to non-pregnant counterparts [24]. Moreover, endothelial L-EV levels may surpass those detected during an uncomplicated pregnancy if thrombotic disorders (e.g., thrombophilia) or pre-eclampsia are present [25–28]. In non-pregnant individuals, both acute and chronic exercise have been linked to alterations in endothelial L-EV levels, however, results have been inconsistent [29–34]. Lansford and colleagues have reported that the effects of acute exercise are not uniform among endothelial L-EV subtypes and that they differ between men and women [32]. For example, acute exercise increased CD62E+ endothelial L-EVs in men but increased CD34+ L-EVs in women with no effect on CD62E+ L-EVs. There are conflicting results on the impact of moderate-intensity vs high-intensity exercise on circulating L-EVs and the role of sex in exercise-associated changes [30–32]. Interestingly, little is known about the effect of exercise on L-EV levels in pregnant individuals.

Recently, our group showed that circulating small EVs increase after an acute bout of moderate-intensity exercise in pregnant compared to non-pregnant individuals [35]. However, the impact of acute exercise on circulating levels of L-EVs, which are more closely linked to vascular injury/dysfunction, has yet to be examined during pregnancy. Therefore, our goal was to examine the impact of exercise on L-EV levels after an acute bout of moderate-intensity aerobic exercise in healthy pregnant and non-pregnant individuals. An exploratory secondary aim was to determine whether circulating L-EV levels corresponded to cardiorespiratory fitness in these populations. We hypothesized that exercise would reduce levels of circulating L-EVs, reflecting beneficial effects on vascular health and that levels of L-EVs would be inversely associated with cardiorespiratory fitness in pregnant and non-pregnant individuals.

4.2 METHODS

4.2.1 Study participants

Pregnant (N=13) and non-pregnant (N=17) women were recruited from the Ottawa region (Ontario, Canada) using social media networks, and the Adamo Lab website advertisements for pregnancy studies. Study objectives and procedures were explained, and informed written consent was obtained from all study participants. Healthy women between the ages of 18-40 years with no contraindications to exercise and a self-reported body mass index (BMI) of 18.5-29.9 kg/m^2 were included. Participants were weight-stable (± 5 kg) for at least six months before the study. Additionally, pregnant participants were required to be carrying a singleton fetus between 13-28 weeks of gestation (2nd trimester). Those with thyroid disease, hypertension, DM, or frequent users of

drugs, tobacco, or alcohol were excluded. All experimental protocols were approved by the University of Ottawa Research Ethics Board (file number: H-06-18-634) and were conducted in accordance with the principles outlined in the Declaration of Helsinki.

4.2.2 Experimental protocol

The experimental protocol employed is as described by Hutchinson *et al*., (2019) and Mohammad *et al.*, (2021) [35,36]. Briefly, participants were asked to refrain from exercise for 12 hours and fast for 8 hours before study testing. Body weight was determined using a Tanita BWB-800 scale (accuracy: 0.1 kg; Lachine, QC), and height was assessed using a Tanita HR-200 wall-mounted stadiometer (accuracy: 0.1 cm). For the pregnant participants, gestational weight gain (GWG) was determined by subtracting the measured body weight from their self-reported pre-pregnancy weight. GWG was also expressed as the percent of the upper limit of weight gained according to the Institute of Medicine guidelines [37]. Participants were given a standardized snack before the exercise session of approximately 340 kCal. Then, resting heart rate (RHR) and resting heart rate variability (rHRV) were recorded using a Polar V800 heart rate (HR) monitor (Polar Electro, Lachine, QC) validated for this purpose [38]. Participants were seated comfortably in a quiet room at ambient temperature, with both feet flat on the ground and arms on armrests, while spontaneously breathing with eyes open during data acquisition [39]. rHRV data were analyzed using Kubios HRV analysis software (Department of Applied Physics, University of Eastern Finland, Kuopio, Finland). R-R intervals were recorded and analyzed according to the protocol outlined by da Silva *et al*., (2020) [40]. The R-R intervals were analyzed using the time-domain index root mean square of successive R-R intervals differences (rMSSD) [41]. As rMSSD was not normally distributed, data were transformed using the natural logarithm (i.e., Ln rMSSD), following recommendations from the literature [41,42]. This variable is expressed in milliseconds. Due to technical issues,

Ln rMSSD was captured for N=12 and N=15 participants in the pregnant and non-pregnant groups, respectively.

The acute bout of moderate-intensity exercise was consistent with current guidance for exercise in pregnancy [5]. A target of 40-59% of HR reserve (HRR, calculated by the Karvonen formula [43]) was used to stipulate moderate-intensity exercise [5,44]. Maximal HR was calculated using the formula: 220 - age. An incremental treadmill walking test was conducted with continuous measurement of HR to determine the speed necessary to maintain moderate-intensity exercise for the acute exercise session. A 3-min warm-up at 2.0 miles per hour (mph) at an incline of 2.0% was followed by an acclimation phase at 6.0% incline where the speed increased by 0.2 mph at 1-min intervals until the target moderate-intensity HR range was achieved. Once this range was reached, the participants exercised continuously for 30 min. The speed was adjusted accordingly to ensure the target HR range was maintained throughout the exercise session.

4.2.3 Blood sample collection

A pre-exercise blood sample was drawn from the median cubital vein and collected in potassium EDTA tubes. Immediately following the exercise session, a second, post-exercise blood sample was collected following the same protocol. Blood samples were immediately centrifuged following collection for 15 min at 4°C at a speed of 1700 × g. Plasma samples were stored at -80°C until further analysis.

4.2.4 Extracellular vesicle isolation and immunolabeling

Circulating L-EVs were quantified as described previously [20,23,45,46]. Plasma samples were thawed quickly in a 37°C water bath, transferred to fresh tubes, and centrifuged at 12,000 × g for 2 min at 4°C. The resulting supernatant was transferred to fresh tubes, and samples were further

centrifuged at 20,000 × g for 20 min. The supernatant was removed and L-EV pellets were re-suspended in Annexin V binding buffer (10mM HEPES 140 mM NaCl, 2.5 mM CaCl2, pH 7.4). For platelet labeling, resuspended samples were transferred to fresh tubes, diluted in Annexin V binding buffer, and labelled with Annexin V- FITC (1:50) and anti-CD41-APC (1:100). For endothelial/leukocyte labeling, resuspended samples were transferred to fresh tubes, diluted in Annexin V binding buffer and labelled with Annexin V-FITC (1:50), anti-CD144-PE (1:100) and anti-CD45-BV421 (1:25). The Annexin V, antibodies, and isotype controls were purchased from Bio-Legend (San Diego, CA). Samples were then incubated for two hours in the dark and re-centrifuged at 20,000 × g for 20 min. The labelled pellets were re-suspended in Annexin binding buffer and transferred to flow cytometry tubes for analysis. The following controls were used for analyses: (1) unlabeled samples, (2) samples labelled with isotype controls, and (3) antibodies in buffer alone.

4.2.5 Flow cytometry analysis

Isolated L-EVs were quantified at the University of Ottawa Flow Cytometry and Virometry Facility using a Beckman Coulter CytoFLEX S equipped with CyExpert version 2.3.0.84 (Beckman Coulter Inc., Indianapolis, IN). A size gate was prepared using ApogeeMix beads (Apogee Flow Systems, Hertfordshire, UK) as previously described [23,45] . Samples were analyzed using FlowJo™ version 7.6.5 (FlowJo, LLC, Ashland, OR). L-EVs were defined as approximately 100-1000 nm in size with greater Annexin V fluorescence than their respective negative controls. Results are shown as number of i) annexin V+ (total), ii) annexin V+, CD41+ (platelet), iii) annexin V+, CD45+ (leukocyte) or iv) annexin V+, CD144+ (endothelial) L-EVs/mL plasma [23,45,46].

4.2.6 Western blot analysis

Western blot analysis was performed as previously described, to assess for the presence or absence of L-EV markers [45,48]. As suggested in the Minimal Information for the Study of Extracellular Vesicles 2018 position statement [49] we examined cytosolic proteins recovered in L-EVs (class 2a positive markers, flotillin-1, TSG101), proteins associated to other intracellular compartments (class 4a negative marker, nucleoporin P62), and cytosolic proteins with promiscuous incorporation into L-EVs (class 2b, GAPDH). Immediately after isolation, L-EV pellets were resuspended in 120 ul of radioimmunoprecipitation assay (RIPA) buffer, with 1:100 protease cocktail (Sigma-Aldrich, St. Louis, MO, USA). As a positive control, a human podocyte cell line lysate was used [48,50]. For each sample, 5 ug was loaded onto a gel and subjected to sodium-dodecyl sulfate polyacrylamide gel electrophoresis. The gel was then transferred onto a PVDF membrane, washed thrice, and blocked in 5% non-fat milk in tris-buffered saline with Tween-20 (TBS-T). Membranes were incubated overnight in primary antibody (diluted in 5% milk, at 4°C as above). Blots were then washed in TBS-T and placed into secondary antibodies for 60 minutes at room temperature. Immunoreactivity was then assessed using chemiluminescence. Primary antibodies included, Flotillin (1:1000, Abcam, Cambridge, UK), tumor susceptibility gene 101 (TSG101, 1:500, System Biosciences, Palo Alto, CA, USA), Nucleoporin P62 (1:100, Santa Cruz, Dallas, TX, USA) and GAPDH (1:10000, Abcam). Secondary antibodies (1:2000, anti-rabbit or anti-mouse, Santa Cruz) were used as appropriate.

4.2.7 Electron microscopy

L-EVs were examined by transmission electron microscopy (TEM) as described in our previous publications [20,35]. Briefly, L-EVs were isolated by high-speed centrifugation (20,000 × *g*

for 20 min at 4°C). The supernatant was discarded, and the L-EV pellet was fixed with 2.5% glutaraldehyde in PBS for four hours at room temperature. The pellet was then washed in 0.1 M Na cacodylate buffer, post-fixed in 2% OsO_4, and dehydrated in a series of graded ethanols. Samples were then embedded in Spurr Resin, and 60 nm sections were prepared on copper grids. Samples were visualized using a JEOL JEM-1400 Plus electron microscope (JEOL Ltd, Tokyo Japan).

4.2.8 Statistical Analysis

Normality was assessed using the Shapiro Wilk test. Considering normality test results, characteristics from pregnant versus non-pregnant study participants and L-EV levels were compared using independent t-tests. A chi-squared test was used to explore whether there were differences in the percentage of participants that showed reductions in L-EV levels. Pearson's correlations were performed to assess associations between circulating L-EV levels and surrogate markers of cardiorespiratory fitness. All statistical analyses and graphs were conducted using GraphPad Prism version 8.4.2 (GraphPad Software, La Jolla, CA). (GraphPad Software, La Jolla, CA). Statistical significance was considered when $p<0.05$.

4.3 RESULTS

4.3.1 Participant characteristics

Participant characteristics are shown in Table 1. There was no difference between pregnant and non-pregnant women with respect to age, height, pre/non-pregnant weight/BMI, RHR or Ln rMSSD. Although participants in both groups completed the same moderate-intensity acute exercise session (40-59% HRR), non-pregnant women reached a significantly higher average speed during the exercise session compared to pregnant individuals ($p=0.009$). This higher speed resulted in a slightly shorter duration of total exercise in pregnant versus non-pregnant women

(p=0.006) however both groups engaged in 30 min of continuous moderate-intensity exercise (Table 1).

Table 4.1. Study participant characteristics.

	Pregnant (N=13)	Non-pregnant (N=17)	P-value
Age (years)	31.2 ± 3.5	30.2 ± 4.3	0.48
Gestational age (weeks)	20.1 ± 5.0	-	-
Height (cm)	166.7 ± 5.4	166.3 ± 6.3	0.87
Pre/non-pregnant body weight (kg)	63.7 ± 9.5	60.0 ± 8.4	0.27
Pre/non-pregnant BMI (kg/m^2)	23.7 ± 3.6	21.8 ± 2.3	0.09
GWG at time of session (kg)	5.8 ± 3.9	-	-
% upper limit of GWG	112.7 ± 46.9	-	-
Average speed during exercise session (mph)	3.2 ± 0.5	3.6 ± 0.3	0.009*
Total exercise duration (min)	40 ± 2.1	42 ± 1.6	0.006*
RHR (bpm)	81.4 ± 14.6	74.3 ± 9.3	0.11
Ln rMSSD (ms)[#]	28.1 ± 18.9	37.0 ± 23.7	0.46

Data are presented as mean ± SD. [#], N=12 and N=15 for pregnant and non-pregnant, respectively. *Indicates significance<0.05. BMI, body mass index; bpm, beats per minute; GWG, gestational weight gain; RHR, resting heart rate; Ln rMSSD, natural logarithm of root mean square of successive R-R interval differences; min, minutes; mph, miles per hour.

4.3.2 L-EV characterization

Prior to analysis, L-EV isolates were characterized for size, morphology and molecular composition. Electron microscopy revealed vesicles of ~100-400 nm in size with intact membranes (Figure 4.1A). Western blot analysis (Figure 4.1B) of L-EV isolates showed the presence of membrane (Flotillin-1) and cytosolic (TSG101, GAPDH) proteins but the absence of nuclear protein (nucleoporin 62).

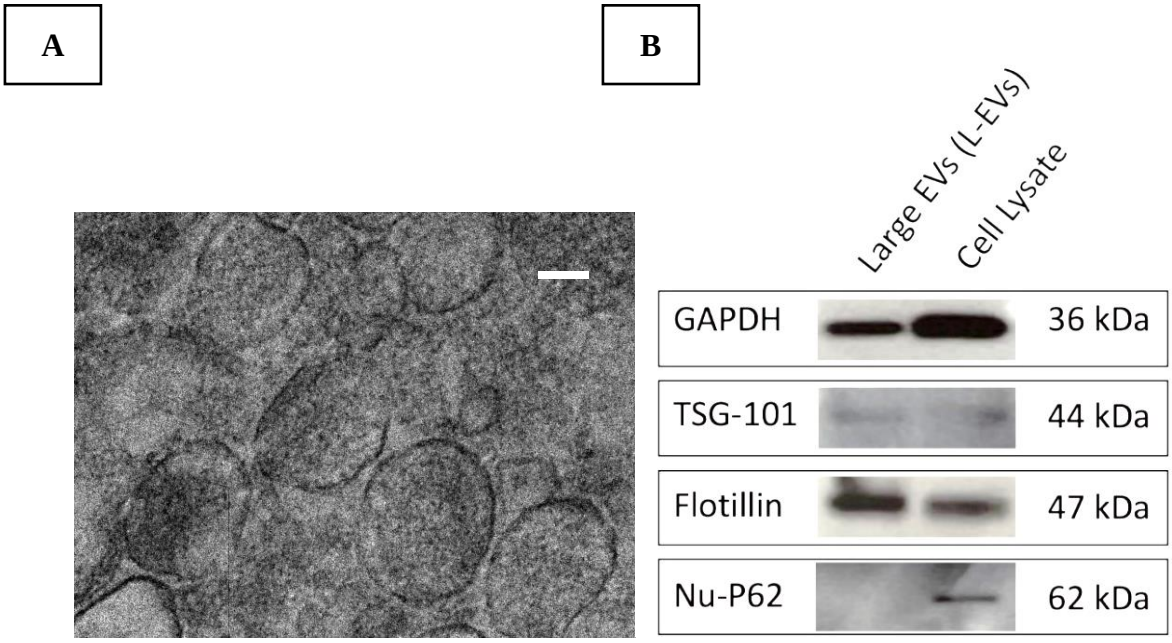

Figure 4.1. (A) Representative electron microscopy image of isolated L-EVs. Scale bar is 100 nm. (B) Representative western blot bands comparing a pooled L-EV preparation with a human podocyte cell line lysate (cell lysate). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was present in both the L-EVs and the cell lysate, which was expected. Tumor susceptibility gene 101 (TSG-101) was present in the L-EV isolate. Flotillin was enriched in our L-EV isolate, while Nucleoporin P62 (Nu-P62) was absent, as expected.

4.3.3 Changes in L-EVs after an acute bout of moderate-intensity exercise

To determine the impact of acute exercise on L-EVs we quantified circulating endothelial, leukocyte, and platelet L-EVs by nanoscale flow cytometry. We first determined if acute moderate-intensity exercise was associated with any changes in circulating L-EV levels. For total Annexin

V^{+ve} L-EVs, we did not observe any difference between pregnant vs. non-pregnant individuals. We detected a difference in pre vs. post-exercise levels for either the pregnant or non-pregnant groups (Figure 4.2A, B). No differences were observed in the proportion of individuals who exhibited reduction in total L-EVs between pregnant and non-pregnant individuals (data not shown).

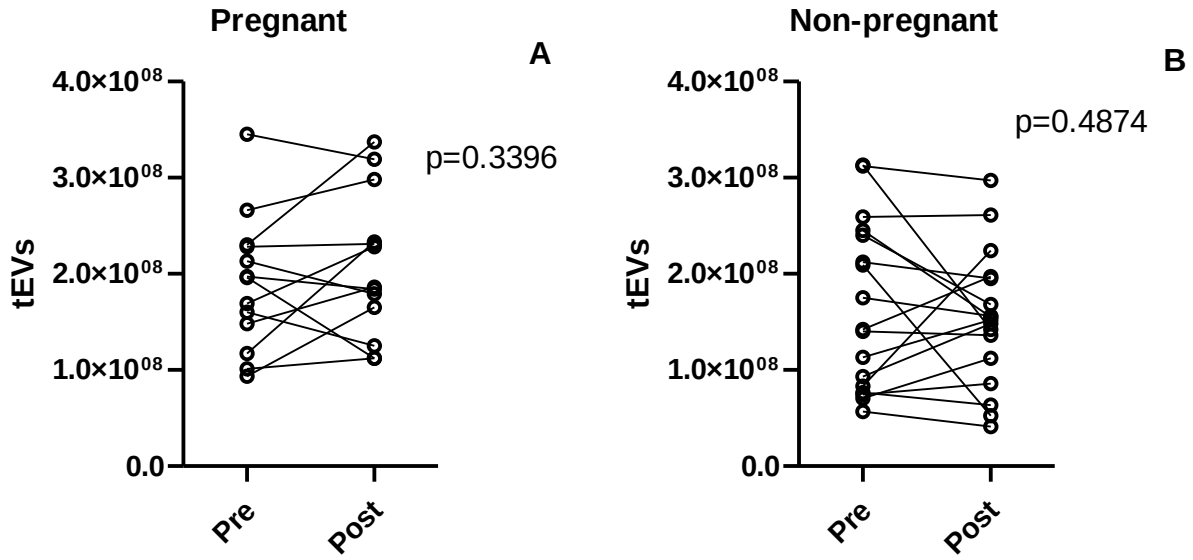

Figure 4.2. Total L-EV levels after an acute bout of moderate-intensity exercise in pregnant (A) and non-pregnant women (B).

As endothelial L-EVs are formed from stressed endothelial cells, reductions in their levels may reflect improved endothelial function. We observed a significant decrease in endothelial L-EVs post-exercise in the non-pregnant group (Figure 4.3B). However, this decrease was not observed in the pregnant individuals (Figure 4.3A). Consistent with these observations, a greater proportion of non-pregnant women (13/17, 76%) exhibited a reduction in endothelial L-EVs compared to pregnant women (4/13, 30.76%, $p<0.05$).

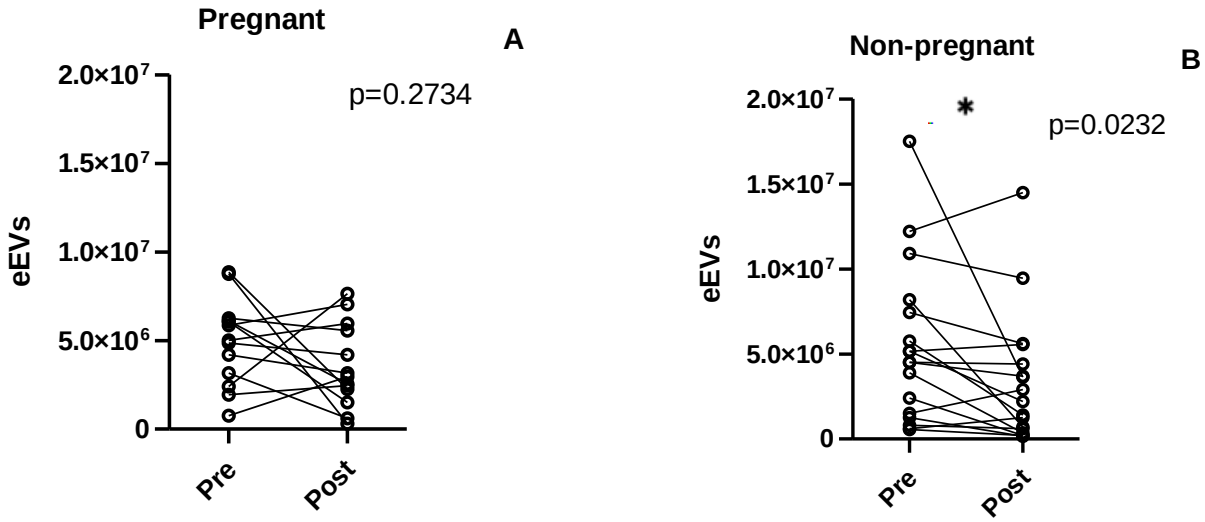

Figure 4.3. Endothelial L-EVs levels after an acute bout of moderate-intensity exercise in pregnant (A) and non-pregnant women (B).

We next assessed platelet L-EV levels, as a measure of platelet activation. We did not observe any difference between pregnant and non-pregnant individuals. There was no significant difference in pre vs. post-exercise levels in either the pregnant or non-pregnant groups (Figure 4.4).

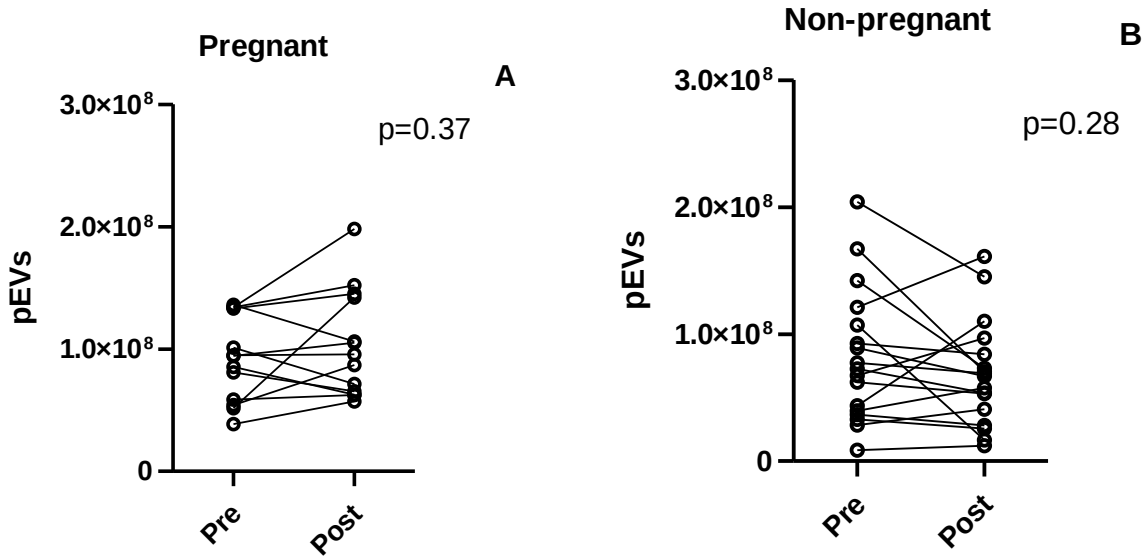

Figure 4.4. Platelet L-EVs levels after an acute bout of moderate-intensity exercise in pregnant (A) and non-pregnant women (B).

Finally, we assessed leukocyte L-EVs which may be altered in inflammatory conditions. There was no difference between pregnant and non-pregnant individuals. There was no significant difference in pre vs. post-exercise levels in either the pregnant or non-pregnant groups (Figure 4.5).

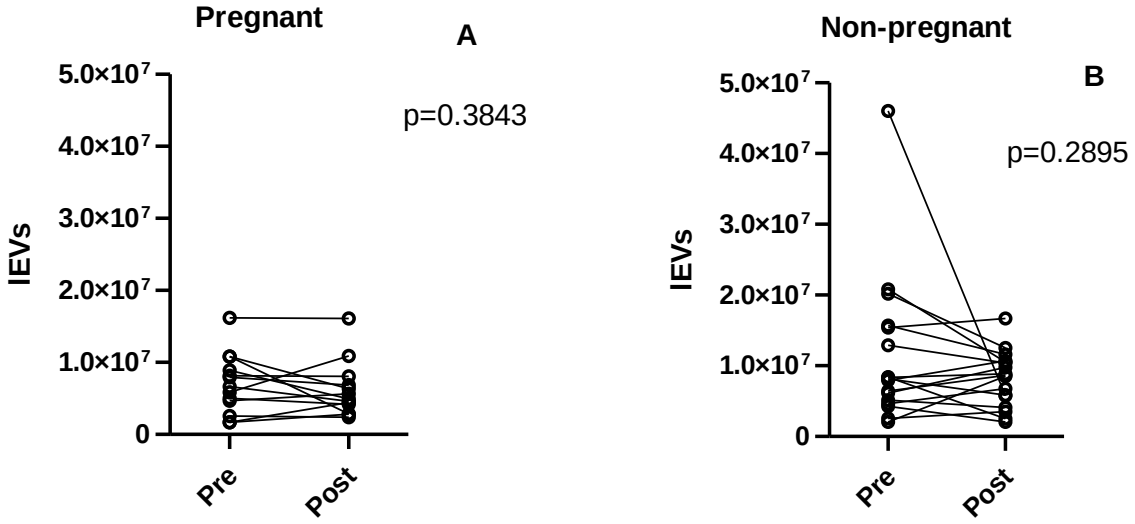

Figure 4.5. Leukocyte L-EVs levels after an acute bout of moderate-intensity exercise in pregnant (A) and non-pregnant women (B).

4.3.4 Correlation of fitness measures with L-EV levels

It has been suggested that L-EVs may be used as biomarkers of cardiorespiratory fitness, though this concept has not been examined in the context of pregnancy [51]. Therefore, we examined correlations between L-EV levels and surrogate measures of fitness including average speed, max speed, RHR, and Ln rMSSD.

When considering baseline (pre-exercise) L-EV levels, we observed a positive association between platelet L-EVs and maximum speed in non-pregnant individuals and the entire study population (Figure 4.6, Table 4.2). However, this association was not observed in the pregnant group alone. A similar trend was observed with total L-EVs and average speed in non-pregnant, but not pregnant women (Figure 4.6, Table 4.2). We did not observe any associations between endothelial or leukocyte L-EVs and surrogate measures of fitness.

We also examined associations between fitness-related variables and the change in L-EV levels after exercise. In pregnant women, there was non-significant trends towards negative

associations between change in endothelial L-EVs and rHRV (r=-0.641, p=0.063) and average speed (r=-0.550, p=0.125). However, no associations were found in non-pregnant women (not shown).

Table 4.2. Associations between baseline endothelial, platelet, leukocyte, and total L-EVs levels and fitness-related variables measured in pregnant and non-pregnant participants.

EV population	**Pregnant (N=13)**		**Non-pregnant (N=17)**		**Total study population (N=30)**	
Endothelial	***r***	***P***	***r***	***P***	***r***	***P***
Average speed achieved during exercise	0.4924	0.0896	0.1714	0.5072	0.1777	0.3565
Max speed achieved during exercise	0.3204	0.2837	0.1865	0.4699	0.1356	0.4750
RHR	0.07692	0.8064	-0.09147	0.7246	-0.0973	0.6087
Ln rMSSD (ms)	0.3257	0.2995	0.1486	0.5938	0.1624	0.4182
Platelet	***r***	***P***	***r***	***P***	***r***	***P***
Average speed achieved during exercise	0.3719	0.2094	**0.5816**	**0.0159***	0.2879	0.1228
Max speed achieved during exercise	0.5189	0.0713	**0.6229**	**0.0089***	**0.3675**	**0.0457***
RHR	0.1293	0.6724	0.1018	0.6960	0.0916	0.6304
Ln rMSSD (ms)	0.3018	0.337	-0.2359	0.3942	-0.1173	0.5602

Leukocyte	***r***	***P***	***r***	***P***	***r***	***P***
Average speed achieved during exercise	-0.1462	0.6297	-0.08589	0.7419	0.1812	0.3380
Max speed achieved during exercise	-0.05727	0.8518	-0.03020	0.9091	0.2527	0.1779
RHR	0.3967	0.1793	-0.05518	0.8333	-0.1457	0.4424
Ln rMSSD (ms)	-0.2070	0.5145	-0.1001	0.7213	0.0281	0.8895
Total L-EVs	***r***	***P***	***r***	***P***	***r***	***P***
Average speed achieved during exercise	0.2834	0.3455	**0.5325**	**0.0296***	0.2479	0.1866
Max speed achieved during exercise	0.3315	0.2665	**0.5662**	**0.0196***	0.2758	0.1401
RHR	-0.2527	0.4043	-0.2527	0.4043	-0.0169	0.9294
Ln rMSSD (ms)	0.4483	0.1446	-0.06613	0.8150	0.0218	0.9139

*P<0.05. L-EV, Large extracellular vesicle; Ln rMSSD, natural logarithm of root mean square of successive R-R interval differences; RHR, resting heart rate.

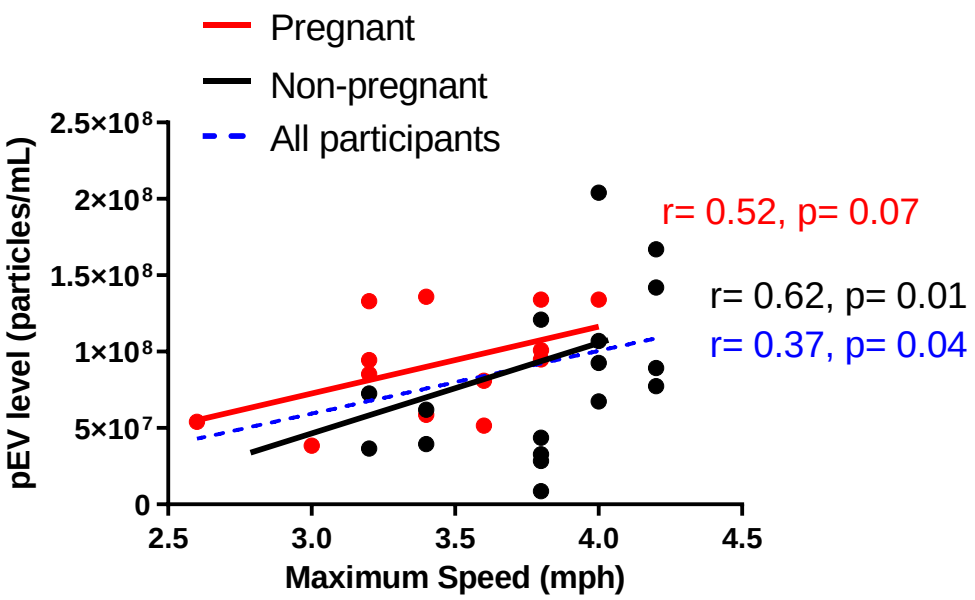

Figure 4.6. Relationship between platelet L-EV levels and maximum speed achieved during exercise in pregnant individuals (red), non-pregnant individuals (black), and the entire study population (blue line). Shown are linear regression lines with associated Pearson correlation coefficients and p values.

4.4 DISCUSSION

The present study aimed to assess circulating plasma L-EVs in healthy pregnant and non-pregnant women after an acute bout of moderate-intensity exercise. We also examined the association between L-EV levels and surrogate markers of cardiovascular health/fitness during an acute exercise session. The major finding is that acute exercise was associated with a significant reduction in levels of circulating endothelial L-EVs in the non-pregnant group, but not in the pregnant group. In addition, we observed associations between baseline levels of platelet L-EVs and surrogate markers of fitness in the non-pregnant individuals, yet this did not extend to those who were pregnant. Taken together these data suggest that circulating L-EVs may be differentially altered after acute exercise depending on pregnancy status, and that the relationship between L-EV levels and fitness is influenced by pregnancy status.

The link between physical activity and endothelial L-EVs in women is not well understood and to the best of our knowledge, the present study is the first to investigate this in pregnancy. Outside of pregnancy, the literature related to the acute effects of exercise on L-EVs has been equivocal with evidence of increases [29,31], decreases [30], or a lack of change [29,31,32]. Different factors may explain this discrepancy (e.g., age, physical activity levels, fitness, exercise type, and duration), but one key factor to consider is the intensity of physical activity. The literature suggests that exercise of moderate-intensity is less likely to induce an inflammatory response and shear stress compared to a high-intensity exercise session [52]. Indeed, a few studies reported a reduction in endothelial L-EVs two hours after moderate-intensity exercise in young and healthy individuals [30] and 30 minutes after exercise in pre-, peri-, and post-menopausal women [52].

Our study observed decreases in circulating endothelial L-EVs in non-pregnant women after moderate-intensity exercise. Albeit, this pattern did not extend to pregnant women. The reasons for this are not entirely clear. It is possible this is due to differences in hemodynamic effects of exercise on the vasculature and resultant L-EV formation dynamics [24]. Such differences could mean that the duration and/or intensity of exercise require personalized adjustment or repeated application in pregnancy to achieve benefits on endothelial health. Alternatively, the rate of L-EV removal may differ between pregnant and non-pregnant individuals. The primary mechanism by which L-EVs are removed from the blood is phagocytosis [53] and acute moderate intensity exercise has been shown to increase phagocytic activity [54]. However, phagocytic activity may be reduced in pregnant women [55–57] leading to impaired L-EV clearance in this population. Finally, we do note that there was a small but significant difference in total exercise duration between pregnant and non-pregnant individuals. However, as this difference is small (~ 2 mins), and both populations underwent a 30 min continuous exercise session at moderate-intensity, we

think that this difference is unlikely to result in altered L-EV dynamics. Nevertheless, further study is clearly needed to address this question.

A second novel observation from the present study was the association between baseline platelet L-EV concentrations and surrogate measures of fitness (average and maximum speed during exercise) in non-pregnant individuals. While we did not observe significant correlation between platelet L-EVs and these measures in pregnant individuals, it is possible that our study size was underpowered for this assessment. The correlation between platelet L-EVs and maximum speed approached significance (P=0.07) and the trend remained when the study population was examined as a whole. The positive correlations between platelet L-EVs and speed achieved during exercise are unexpected as platelet L-EVs are traditionally viewed as a measure of platelet activation. Thus one would expect that platelet activation would be lower in more "fit" individuals as men and women undergoing regular exercise training displayed reduced platelet activation [58]. Given this, one would expect to see negative associations between platelet L-EVs and cardiorespiratory fitness, yet our study suggests the opposite. The explanation for this is not presently clear.

Platelet L-EV levels may also be influenced by diet or medication [59] or blood collection, processing and storage conditions [60,61]. While our study protocol involved a standardized collection and storage protocol, diet and medication remain possible residual confounding variables. At present, it remains to be seen whether our observation is reproducible in other populations with more direct measures of fitness and the value of L-EVs as measures of cardiorespiratory fitness is uncertain at this time.

One of the strengths of this study is the use of human plasma samples from both healthy pregnant and non-pregnant individuals which have rarely been compared in previous studies. In

addition, our flow cytometry approach examined L-EVs from its main sources including endothelial cells, leukocytes, and platelets while many previous studies have focused on a single L-EV population [62,63] Our study also has some limitations. First, this is a comparatively small cohort and this may have reduced our ability to identify associations with clinical variables, including whether gestational age can influence the exercise-response of circulating L-EVs. Second, we assessed those from 13-28 weeks of pregnancy and, although this encompasses the 2nd trimester, interindividual variations stemming from physiological changes accompanying pregnancy could not be assessed. Future studies should consider how gestational age influences the effect of exercise on L-EV dynamics in pregnancy. In addition, relationships between L-EV levels were measured at two time points (pre- and post-exercise) and we focused on a single acute bout of moderate-intensity exercise on circulating L-EVs. Serial assessment of L-EV levels may provide greater insights into the impact of exercise on vascular health in pregnancy. Finally, the exercise duration chosen for this study was purposely based on current guidelines for physical activity during pregnancy[5] to represent an intensity and duration recommended for pregnant women. According to previous investigations, it seems reasonable that exercise mode, intensity, and duration all influence L-EVs concentrations. The L-EV response may differ with exposure to longer exercise durations or higher intensities. Therefore, future studies should examine the impact of differing exercise intensities or modalities such as cycling, swimming, or resistance training (which are all deemed safe during pregnancy), on L-EV release and kinetics.

4.5 CONCLUSION

In summary, our results suggest that an acute bout of moderate-intensity exercise reduced endothelial L-EVs in non-pregnant participants. By contrast, these changes were not seen in pregnancy. The difference in response to exercise could be indicative of differential benefit to vascular health of these individuals however further study is needed to clarify the value of circulating L-EVs as biomarkers of maternal vascular health in pregnancy.

4.6 REFERENCES

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5 CHAPTER 5- DISCUSSION

5.1 SUMMARY OF CHAPTERS

The overall goal of this thesis was to examine the role of L-EVs as a tool for the assessment of vascular health status in T1DM and pregnancy.

In Chapter 2, I quantified circulating L-EVs in women with T1DM during pregnancy at 4-12 weeks (baseline), 24 and 34 weeks gestation. I investigated the association between circulating L-EVs and glycemic control, maternal and fetal outcomes in this population. The major finding was that elevated endothelial and platelet L-EVs are associated with increased risk of adverse pregnancy outcomes in pregnant women with T1DM. In particular, higher baseline endothelial L-EVs were associated with increased risk of hyperbilirubinemia and NICU admission, even after adjusting for HbA1c and systolic and diastolic blood pressure. Likewise, higher baseline platelet L-EVs were associated with increased risk of hyperbilirubinemia and NICU but decreased maternal complications. Circulating L-EVs didn't show any significant correlations with mean glucose or HbA1c. I also explored the relationship between three measures of glycemic variability (MAGE, SD and CV) and circulating L-EVs within this study. I observed a weak positive correlation between MAGE and endothelial L-EVs, but not with SD and CV. Blood pressure values also didn't show any association with endothelial L-EVs. It is thus logical to conclude that other influences are also contributing to L-EV dynamics in pregnant women with T1DM. Remarkably, I observed an inverse correlation between platelet L-EVs and time above range, SD and MAGE and a positive correlation with time in range. Further study is needed to clarify whether glucose variability measures in pregnancy reduces platelet L-EV formation or platelet L-EV clearance.

In Chapter 3, using well-established mouse models, I employed L-EVs as an indirect measure of vascular alterations to distinguish the changes that appear in T1DM compared to another condition typified by vascular injury (hypertension). After assessment of protein composition of isolated circulating L-EVs, I observed common/distinct pathways enriched in T1DM vs wild-type. The resulting pathways that were enriched in T1DM include white adipose tissue browning, PPARα, RxR α activation, IL-8 signaling, apelin adipocyte and cardiomyocyte signaling pathways. I also observed that SNARE signaling which plays a vital role in extracellular vesicle formation is enriched in T1DM [1,2]. CLEAR signaling (responsible for lysosomal activity, lysosomal expression and regulation) and NAD signaling pathway (mitochondrial biogenesis) were also enriched in this group. Likewise, I observed changes in circulating L-EVs proteins in hypertensive mice vs wild-type. RhoA/Rho kinase signaling (smooth muscle contraction), integrin-linked kinase (ILK) signaling (hypertension-mediated organ damage and phagosome formation were unique pathways enriched in hypertension. Of note is the observation that semaphorin signaling was enriched as it has not been associated to hypertension previously. Finally, I observed pathways that were commonly enriched in both hypertension and T1DM including pattern recognition receptor signaling, white adipose tissue browning pathway and IL-8 signaling. Further assessment of these changes may provide important information regarding molecular underpinnings of vascular injury in hypertension and T1DM.

Finally, in Chapter 4, I assessed the impact of an acute bout of moderate-intensity aerobic exercise on circulating L-EVs in pregnant and non-pregnant healthy women. In this chapter, I applied exercise as an intervention to improve vascular health and reduce circulating L-EVs in this population. I demonstrated that levels of circulating endothelial L-EVs were significantly reduced after an acute bout of moderate intensity exercise in non-pregnant women. Notably, in non-

pregnant populations the vast majority of individuals had reduction in L-EVs while in pregnant individuals there was variability such that some individuals showed reductions while others did not. The greater variation in endothelial L-EV responses after exercise in the pregnant women may be due to pregnancy-related cardiovascular modifications [3]. Additionally, I observed a positive association between baseline levels of platelet and total L-EVs and measures of fitness in the non-pregnant group. Platelet L-EVs are traditionally known as a measure of platelet activation and platelet activation is expected to be lower in individuals with better physical fitness since regular exercise has also been shown to reduce platelet activation [4]. A relatively high individual variability was observed in this population regarding the L-EVs levels in response to exercise which may be linked to the exercise intensity spectrum. Exercise intensity seems to differentially affect L-EVs response to an acute bout of exercise. High-intensity exercise bouts appear to provoke higher shear stress and induce an elevated inflammatory response compared to moderate-intensity exercise. There is evidence supporting a decrease in endothelial L-EVs after a moderate-intensity exercise and maintenance/increase in endothelial L-EVs after a high-intensity effort [5,6].

5.2 INTEGRATION OF FINDINGS

In this thesis I employed L-EVs as a tool for assessing vascular health in pregnancy and T1DM with the goal of gaining insight into the health status and relating changes in levels or protein composition to physiological alterations and clinical outcomes. The specific objectives were to a) quantify L-EVs in a large cohort of pregnant women with T1DM and examine the association between circulating L-EVs and maternal and fetal outcomes in women with T1DM ; b) investigate the protein composition of circulating L-EVs in hypertensive, diabetic and healthy

mice to identify common and disease-specific molecular changes; and c) investigate the changes in plasma L-EV levels after exercise in healthy pregnant and non-pregnant women.

Since pregnancy complications continue to rise globally, significant efforts and interest have been made in investigating the first trimester (12 weeks gestation) as a window of opportunity for early identification of high-risk pregnancy. My first study exclusively focuses on L-EV levels in pregnant women with T1DM who have been diagnosed prior to pregnancy. This focus stemmed from the unique opportunity to study a population with extensive metabolic phenotyping including real time continuous glucose monitoring and relate these parameters to changes in L-EVs. I recognize that examining L-EVs in other types of DM including GDM and T2DM in pregnancy also merit future studies. For example, GDM represents the most common type of DM in pregnancy (prevalence of 3-20% according to Diabetes Canada). According to a cohort study that was conducted in 2016, the prevalence of T1DM between 1995 to 2015 in pregnancy increased from 1.56 to 4.09 per 1000 pregnancies. Likewise, a rapid increased was also observed in the prevalence of T2DM from 2.34 to 5.09 per 1000 pregnancies followed by even a more rapid increase to 10.62 per 1000 pregnancies by 2012 [7]. Thus GDM and T2DM represent additional populations where the relationship between L-EVs and pregnancy outcomes may merit further study. Novel findings from Chapter 2 include the observation that increased levels of L-EVs in pregnant women with T1DM are associated with increased risk of adverse outcomes. This finding highlights the prognostic value of L-EVs. If the results are confirmed, it may be possible to identify high-risk pregnancies by enumerating L-EV levels. It is crucial to note that the increased levels of L-EVs in my population may not simply be a result of increased formation. L-EV levels may be altered due to either increased formation or decreased removal. Thus, plasma levels represent a balance between elimination and formation of L-EVs. L-EVs clearance may occur via engulfment by macrophages

and phagocytosis is known as a primary mechanism by which L-EVs are eliminated [8,9]. Furthermore, several additional mechanisms for L-EV uptake have been proposed including micropinocytosis, clathrin-mediated endocytosis, macropincytosis and plasma or endosomal membrane fusion [10]. Thus populations of L-EVs may enter a cell through more than one route. Recently, the implications of L-EVs uptake have been demonstrated in the pathophysiology of various diseases such as inflammatory diseases, DM, cancer, autoimmune diseases, as well as neurodegenerative diseases [11]. Therefore, it is possible that the mechanism of L-EVs formation and clearance may be altered in diabetes. As such, a current limitation of this study is the inability to definitively determine if altered L-EV levels are related to increased formation or decreased removal.

I also examined circulating platelet, endothelial, leukocyte and total L-EVs in this cohort to determine if levels change throughout pregnancy. I didn't observe significant differences in levels of endothelial, leukocyte, platelet or total L-EVs between trimesters. Some other groups have previously suggested that levels of endothelial L-EVs are influenced by pregnancy-related physiological changes. Radu and colleagues observed that levels of circulating L-EVs are increased in pregnancy with the highest values reached in third trimesters [3]. However, this study examined the level of L-EVs in normal pregnancy and a group of age-matched healthy non-pregnant women acted as controls while my study exclusively focused on pregnant women with T1DM.

Taken together, the results from Chapter 2 suggested that L-EV enumeration may represent a novel test which could allow us to identify individuals at greatest risk of adverse outcomes in pregnancy. However, additional studies are required to assess circulating L-EV protein content which could provide more insight into molecular pathways driving disease pathogenesis.

Cardiovascular diseases including T1DM and hypertension are common comorbidities and the major cause of mortality and morbidity worldwide [12]. The incidence of hypertension is higher in individuals with T1DM compared to those without T1DM [13]. These two conditions share several similar pathological manifestations such as vascular inflammation, arterial remodeling and atherosclerosis and endothelial dysfunction [13]. Substantial pathophysiological overlap has been identified in the cardiovascular complications of T1DM and hypertension [13,14]. However, there are also distinct pathogenic pathways. Thus, the overlapping and distinct pathologies require a clear understanding of the molecular alterations to guide therapeutic management. On this point, it is very important to understand the molecular alterations involve in progression of cardiovascular diseases in T1DM and hypertension

In Chapter 3, I employed proteomic assessment of circulating L-EVs to address this knowledge gap. I observed pathways distinctly enriched in L-EVs of T1DM compared to wild-type (Figure 5.1). These pathways include SNARE signaling, NAD biogenesis and CLEAR signaling. Among the pathways enriched in T1DM, the most obvious targets of vascular injury are SNARE signaling and NAD biogenesis. SNARE proteins have shown to influence endothelial function and coagulation suggesting a link to vascular health. According to one study conducted in 2014, the SNARE protein syntaxin-binding protein STXBP5 is a novel regulator of endothelial exocytosis [15]. In addition to SNARE signaling, NAD biogenesis may also be a strong candidate for a role in vascular injury. Recent work has suggested a role of NAD signalling in mitochondrial health [16]. Further, replenishment of NAD^+ in vascular cells either by inhibition of its degradation or stimulation of NAD^+ synthesis has been shown to provide protection in endothelial dysfunction and age-related arterial stiffening [17]. The restoration of NAD^+ homeostasis may also diminish common vascular diseases such as coronary artery disease, hypertension and atherosclerosis [18].

Accordingly, further study of alterations to SNARE signaling and NAD biogenesis may provide more information on pathogenesis of vascular injury in T1DM.

Similarly, I observed pathways distinctly enriched in L-EVs of hypertension compared with wild-type such as semaphorin neuronal repulsive signaling, RhoA signaling, phagosome formation and ILK signaling (Figure 5.1). Among the pathways enriched in hypertension, the most obvious target of vascular injury is Rho signaling. Activation of RhoA/Rho kinase signaling pathway by agonists may induce vascular smooth muscle contraction: a critical determinant of blood pressure [19]. To the best of my knowledge no study has implicated the significance of semaphroin in hypertensive vascular injury. Thus, semaphroin signaling may constitute a novel pathway in hypertensive vascular injury.

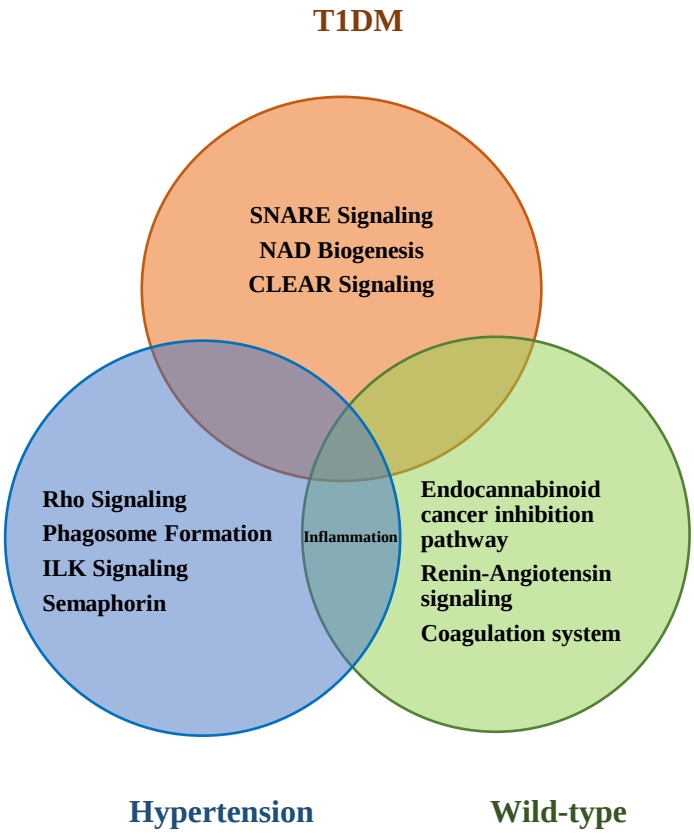

Figure 5.1. Venn diagram represents distinctly enriched and common pathways in T1DM, hypertension and wild-type.

The common and distinct proteins and pathways identified in this study could represent those which are altered in response to the disease condition. However, as yet, these changes have not been directly linked to clinical outcomes as I have shown with L-EV levels in Chapter 2. As such, a clear next step is to determine if protein signatures identified in Chapter 3 are associated with increased risk of T1DM or hypertension-related complications in pregnancy. Such studies would serve to clarify the utility of L-EV protein signatures and identify high risk of adverse pregnancy outcomes and intervene with strategies for improving vascular health. One such intervention is exercise. Exercise has been shown to improve the metabolic and cardiovascular health; specifically, during pregnancy it is well-known to bestow benefits on both mother and fetus by improving the vascular health and pregnancy outcomes in both generations [20]. As a first step in determining the value of exercise in mitigating vascular injury, I assessed the impact of a single acute bout of moderate-intensity aerobic exercise on circulating L-EVs in pregnant and non-pregnant healthy women in Chapter 4. In this chapter, I aimed to determine if exercise reduce L-EV levels, indicative of improved vascular health. Exercise is strongly advised in pregnancy guidelines and can be implicated without any regulations to reduce the vascular risk [21]. In fact, maternal physical activity is associated with decreased risks of pregnancy complications such as GDM, gestational hypertension and preeclampsia [22]. Further, improvement in prenatal depressive symptoms with a reduction in blood glucose levels has also been reported in pregnancy [23,24]. Engagement in regular exercise is also linked to significant health benefits to vasculature, reduced risk of chronic disease and all-cause mortality [25]. Mechanistically, exercise lowers blood pressure and blood glucose, increases shear stress and blood flow [26–29.] A major finding from this chapter is a significant reduction in circulating endothelial L-EVs after an acute bout of moderate intensity exercise only in non-pregnant women.

A two week exercise training program in pre-and post-menopausal women classified as obese or overweight resulted in a reduction in circulating endothelial L-EVs, coinciding with decreased glycemic variability [30]. Similarly, Kretzschmar et al. (2014) found that six months of regular aerobic exercise training was able to decrease endothelial L-EVs in women during pre- and post-menopausal periods [31]. An eight-week moderate intensity exercise intervention in women with the polycystic ovarian syndrome (PCOS) reduced circulating levels of endothelial L-EVs, but not in healthy control women [32]. Serviente et al. (2019) hypothesized that the chronic beneficial effect of exercise would be associated with the acute effect of one bout of exercise [5]. However, studies on the acute effects of exercise on endothelial L-EVs in women have shown that they can increase [33], reduce [6] or remain the same [33–35]. A study reported a reduction in circulating endothelial L-EVs after the acute effect of moderate-intensity exercise in women two hours after exercise in young healthy women [6]. Similarly, a reduction was also observed in endothelial L-EVs 30 minutes after exercise in pre-, peri-, and post-menopausal women [5]. Lansford and colleagues conducted a study to determine the impact of acute exercise on L-EVs subpopulations. Their results suggest acute exercise has different effects among endothelial L-EVs subtypes and they are not similar between women and men [35]. Recently, Rigamonti and colleagues conducted a study to measure the impact of an acute bout of exercise on circulating L-EVs of normal-weight and obese patients in both male and female. The results showed that acute exercise decreased the levels of total L-EVs in circulation in all participants [36]. Similarly, the levels of L-EVs were higher in normal-weight individuals compared to obese post exercise. Further, the levels of L-EVs were also higher in women after exercise compared to male participants [36]. Different factors may contribute to this discrepancy such as age, PA levels, exercise types, duration and fitness. However, the intensity of

PA appears to be the main factor with moderate-intensity exercise rarely associated with increased inflammatory response and shear stress compared to a high-intensity exercise session [5].

The variations in release of L-EVs in my populations may be triggered by an acute stress response in the body prompted by exercise. Therefore, it is possible that physiological stress response to exercise is elevated during pregnancy. It is also not clearly known whether the decrease in L-EV numbers observed post-exercise in non-pregnant women was due to secretion or decreased clearance/uptake when compared to pregnant individuals. While not the focus of this thesis, numerous groups have attempted to investigate the impact of acute and moderate exercise on circulating sEVs in male participants. However, the central focus of this chapter is to assess the impacts of exercise on healthy pregnant and non-pregnant participants. Upon review of the L-EV exercise literature, it was apparent that there were no studies on the circulating L-EV after acute exercise in the pregnant and non-pregnant populations. Additionally, all the literatures that currently examined the effects of acute exercise sessions to date were either endurance exercise until exhaustion or at a high intensity which do not deem safe according to recommendations for PA during pregnancy. Thus, this project was designed specifically to investigate the impact of an acute bout of moderate-intensity treadmill walking for 30 min in circulating L-EVs in pregnant and non-pregnant individuals. I designed the intervention model specifically in women (pregnant and non-pregnant) to model exercise intensity and modality recommended by the *2019 PA throughout pregnancy guidelines* [21]. A single bout of exercise model is just an initial step and I suggest looking at the repeated bout of exercise as a follow-up intervention. Only then, one can surmise whether L-EVs can be used as a non-invasive biomarker to improve vascular health in this study.

6 CHAPTER 6- FUTURE RESEARCH DIRECTIONS

My PhD studies highlight the important role of L-EVs as a tool for the assessment of vascular health status in pregnancy and T1DM. However, despite these advances (highlighted in Chapter 5), there is a significant gap between the current studies and translating them to strategies that identify individuals at high risk of neonatal and maternal complications and improve vascular health. Here, I discuss future work which could serve to bridge these gaps.

6.1 ADVANCES IN L-EVs RESEARCH BY EXTENDING THE POPULATION SAMPLE SIZE AND OTHER TYPES OF DIABETES IN PREGNANCY

While the study from Chapter 2 characterizes the largest cohort of pregnant women with T1DM ever to be screened for circulating L-EVs, it is relatively small cohort with selected participants. Therefore, it was very challenging to assess the relationship between L-EVs level and maternal and neonatal outcomes such as pregnancy loss, NICU admission etc. For example, level of L-EVs did not predict preeclampsia in this study. One possible explanation could be due to a very low incident of preeclampsia due to a small sample size (only 19 participants). Therefore, I would recommend extending the population sample size and investigating a larger study population to confirm strong, reproducible relationships between clinical outcomes and L-EV levels. Further, my study is a secondary analysis of CONCEPTT trial which focused only on T1DM in pregnancy and I was unable to compare values to those without diabetes. I think it is very important to look at other types of DM or different conditions and compare values to those without DM or those with GDM or T2DM. GDM is well-known as one of the most common complications of pregnancy with a prevalence of ~6–13% worldwide depending on region and the availability of maternal care [37,38] while T1DM and T2DM complicate up to 5-10% of all the pregnancies. Previous studies have

explored a relationship between L-EV changes in GDM and clinical variables, but they conducted these studies on small cohorts and selected populations. Therefore, the study of L-EVs in GDM on larger cohort may provide greater insight. Similarly, in Chapter 4, the true effects on L-EVs in healthy pregnant and non-pregnant individuals might have been masked or overestimated due to the small sample size. I would recommend further prospective studies with larger/extended sample size to clarify the impact of physical activity on L-EVs.

6.2 EXTEND THE APPROACHES TO IMPROVE VASCULAR HEALTH DURING PREGNANCY

In Chapter 4, I proposed to use physical activity as a possible intervention or therapeutic measure to improve the vascular health in my populations, reduce pregnancy complications and optimize maternal–fetal health. According to Canadian guidelines for Physical Activity throughout Pregnancy (2019), women without contraindications are recommended to engage in a minimum of 150 minutes of moderate-intensity physical activity per week to improve vascular health and reduce the risk of developing pregnancy complications such as GDM, gestational hypertension, and preeclampsia [21]. Similarly, the WHO recommendation is to engage in an aerobic training during pregnancy from 60 to 150 min/week [39,40]. Previous data suggest that physical activity during pregnancy has a lifelong protective effect which can result in reduction of cardiovascular risk. It is also beneficial for the fetus and improved cardiovascular health at a later age [41]. Therefore, I decided to apply exercise as an intervention because it is strongly advocated from guidelines and could be implemented immediately. In this study, I only focused on a single acute bout of moderate intensity exercise (treadmill walk) on circulating L-EVs. I would recommend examining the impact of differing exercise intensities or modalities such as cycling, swimming or resistance training (which are all deemed safe during pregnancy) on L-EV release and kinetics. Further, I just

examined the impact of an acute bout of moderate intensity exercise. I would also suggest assessing the repeated bout of exercise during pregnancy which may result in a different outcome. Longer exercise durations or bouts of higher intensity may also yield different responses.

In additional to physical activity, I would recommend using other approaches to improve the vascular health during pregnancy. One of the most important factors that could improve the vascular health during pregnancy is dietary changes. Studies have shown that maternal diet is the major intrauterine environment factor that is essential to fetal development [42,43]. Further, maternal diet can be easy intervened with low risk and low cost as one of the most important modifiable factors [43]. One of the most important factors in achieving healthy pregnancy according to WHO recommendations on antenatal care is having a healthy diet and eating food that provide the nutrients for support and energy during and prior to pregnancy [44]. Taking folic acid supplementation, daily oral iron and multivitamins are recommended during pregnancy to prevent puerperal sepsis, maternal anaemia, preterm birth and low birth weight. Dietary Approaches to Stop Hypertension (DASH) consumption for four weeks also shown to improved pregnancy outcomes in pregnant women with GDM [45]. Deficiency of specific antioxidants such as copper, zinc, micronutrients selenium and manganese may also cause poor pregnancy outcomes including preeclampsia, fetal growth restriction, and cardiometabolic diseases later in life [46]. Monitoring the healthy lifestyle parameters and gynecological such as preeclampsia, preterm delivery, recurrent pregnancy loss or intrauterine growth restriction and cardiovascular outcomes are also important factors to be controlled during pregnancy [43].

6.3 EXPLORE THE RELATIONSHIP BETWEEN GLUCOSE LEVELS AND PLATELET L-EVs IN PREGNANCIES COMPLICATED BY T1DM

The data from Chapter 2 revealed that platelet L-EV levels may be reduced with glucose

variability measures in pregnancy. Several lines of studies outside of pregnancy demonstrated that platelet L-EVs are increased in hyperglycaemic conditions [47,48]. The reason for this discrepancy is not well understood and this study is the first to assess this relationship in T1DM in pregnancy. Two previous studies in normal, hypertensive and growth restricted pregnancies, reported reduced platelet L-EV levels in preeclampsia despite evidence of platelet activation [49,50]. Hence, it is possible that the molecular mechanisms underlying platelet L-EV formation are altered in pregnancy. Evidently, there are certain determinants that require further investigation. Therefore, I would strongly recommend further study to better understand the relationship between platelet L-EVs and glucose levels and its mechanism in pregnancies complicated by T1DM.

6.4 INVESTIGATION OF MOLECULAR COMPOSITION/PROTEOMIC PROFILE OF L-EVs AFTER EXERCISE THERAPY

Recently, suggestions have been made towards considering preventive medicine strategies such as exercise within a broader scope of personalized care. However, biomarkers that guide disease therapy according to patients' individual molecular signature can provide more efficient information and treatment with lower incident of adverse events. One of the potential strategies to improve vascular health, increase individualization and allow for personalized medicine is to assess the protein composition of L-EVs. Physical activity can potentially be tailored based on the impact on the molecular composition of L-EVs, such that choice of exercise modality could be optimized. Other than nutritional and pharmacological approaches, exercise has also shown to restore NAD^+ homeostasis. It has been shown previously that exercise training increased NAD^+ levels and NAD^+ metabolism enzymes in numerous tissues. NAD^+ biogenesis has been shown an important role in skeletal muscle and promote mitochondrial adaptation to endurance exercise.

Given this, I recommend investigating proteins associated with NAD^+ biogenesis which may provide insight into dysregulation of NAD^+ and vascular injury. Similarly, based on results from Chapter 3, it may also be of interest to investigate SNARE signaling and semaphorin (novel pathway) in the pathogenesis of vascular injury in pregnant and non-pregnant individuals. In summary, I suggest investigating the proteomic profile of L-EVs in individuals with a single bout of moderate intensity exercise in healthy pregnant and non-pregnant individuals to identify dysregulated pathways that may benefit from exercise interventions. Extending from this I recommend examining the protein content of L-EVs in individuals with a repeated bout of exercise or different modalities in the same population.

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7 CHAPTER 7- SIGNIFICANCE OF STUDIES

My thesis highlights the use of L-EVs as a diagnostic tool to understand vascular health status in T1DM and pregnancy and offers promise for gaining a better understanding of disease pathogenesis and novel therapeutic approaches. To the best of my knowledge the results from Chapter 2 were the first to suggest that increased circulating L-EVs may be predictive of adverse outcomes in pregnancy. However, work in this area is at its infancy and significant challenges remain before L-EVs can graduate from research to clinical diagnostic tools. At this stage, external validation is lacking for the key observations and should be a priority going forward. Therefore, I suggest validating the results and expanding them to other populations such as other types of DM and healthy control groups.

These studies would be critical to identifying threshold levels above which there is a risk adverse pregnancy outcome. In my thesis, I identified the median value as a threshold above which risk is increased. However, it may be possible to identify different thresholds that provide better prognostic potential. Moreover, it is unclear whether the cut-off would be the same for the entire populations or if there might be a difference in diabetic and non-diabetic individuals. In the clinic, after a blood test with elevated L-EVs (indicative of risk of adverse outcomes such as NICU admission), individuals who are identified as high risk could be referred to a high-risk pregnancy clinic where a team of health care and specialists could provide more intensive care to ensure the best possible outcomes for these individuals.

If the above strategies are successful in identifying individuals at high risk, it will be important to identify therapeutic strategies to help reduce this risk. There are different strategies that can improve the health outcomes of both mother and fetus. Based on current available approaches,

to reduce the risk associated of adverse pregnancy outcomes (NICU admission, hyperbilirubinemia, etc.), I would recommend the use of (a) glucose reduction strategy such as CGM during pregnancy in these individuals to improve the glucose control and HbA1c; (b) dietary approaches such as folic acid and multivitamins supplementation as well as the Dietary Approaches to Stop Hypertension (DASH) which promotes the consumption of fruits, vegetables, low-fat dairy products, lean meat and carbohydrates[1] and (c) regular prenatal visits which can help the health care provider to coordinate and monitor the care plan and provide the interventions and support in a timely fashion. I would also recommend employing a vascular health intervention in pregnancy such as exercise which is recommended by pregnancy care guidelines to improve cardiovascular and metabolic health and reduce the risk for deleterious adverse outcomes in both fetus and mother during pregnancy and health across the lifespan. In Chapter 4, I conducted the first study to describe the impact of a single bout of acute exercise in healthy pregnant and non-pregnant group and to report a reduction in endothelial L-EV levels after exercise in only non-pregnant women. However, the benefits of exercise are fully achieved only with repeated administration. Therefore, as a next therapeutically relevant experimental approaches, I strongly recommend examining repeated bouts of exercise with different modalities and intensities. Activities such as swimming, stationary cycling, brisk walking or aquatic are associated with lower risk of physical contact or falling and moderate-intensity physical activity appear to be safe and does not significantly impact fetal or maternal well-being [2]. Ultimately, before this can be applied in high-risk pregnancy clinic, it is crucial to confirm whether exercise is beneficial from the vascular health standpoint in pregnancy.

From a more long-term perspective, I suggest extending the molecular profiling of L-EVs in these populations initially conducted in Chapter 4. In hapter 3, I identified several targets that

require further validation. One is semaphorin pathways which has not shown any significant association to blood pressure previously and needs to be further validated by western blot, ELISA or flow cytometry. I also recommend the proteomics profiling of L-EVs in larger and more heterogenous populations such as human cohorts. NAD biogenesis has been linked to diabetes previously; however, its impact in pregnancy has not been examined. I also recommend employing the L-EV molecular profiling approach in other types of DM, and expanding to human blood samples. With additional experimental molecular profiling, one might be able to identify pathways that are involved in the pathogenesis of injury in these individuals and ultimately, offer a long-term therapeutic treatment targeting these pathways. If validated by future research work, one can envision a world where assessment of L-EVs is used in early pregnancy to identify individuals at greatest risk. This could also benefit millions of women around the world who suffer from pregnancy complications.

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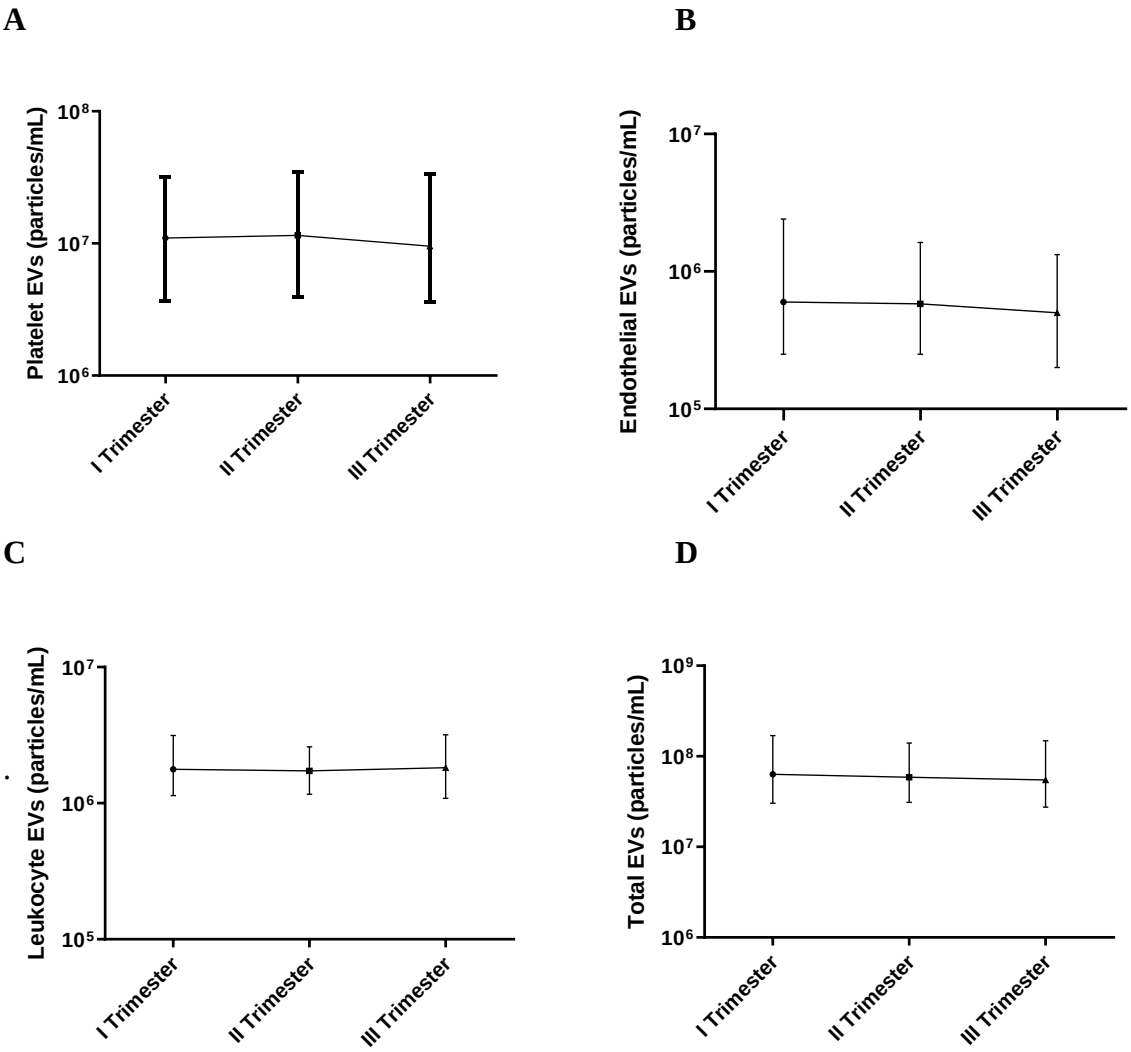
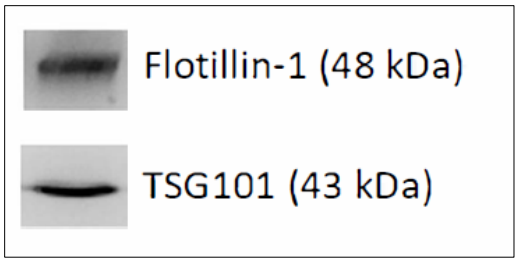
8 CHAPTER 8- CONCLUSION

This thesis delineates the role of L-EVs as a tool for the assessment of vascular health status in pregnancy and T1DM. In summary, my results showed that elevated endothelial and platelet L-EVs at baseline were associated with adverse pregnancy outcomes in women with T1DM. These observations were independent of HbA1c and blood pressure. Further, using a well-established mouse model, I examined the protein composition of circulating L-EVs in T1DM, hypertension and wild-type to identify distinct molecular compositions that are dependent on pathogenic state. Finally, I examined the changes in plasma L-EVs after an acute bout of moderate-intensity aerobic exercise in healthy pregnant and non-pregnant women to investigate the effect of exercise as an intervention on L-EVs level. I observed that circulating L-EVs significantly decreased after acute exercise in non-pregnant individuals but not in the pregnant women. Thus, this thesis advance knowledge into the value of L-EVs as tools for assessing vascular injury and pregnancy risk in health and T1DM.

APPENDIX 1: SUPPLEMENTARY FIGURES



Supplemental Figure 1: Circulating L-EV levels throughout pregnancy. Shown are L-EV levels from baseline (1st trimester), 24 weeks (2nd trimester), and 34 weeks (3rd trimester). A total of 134 individuals had samples available from all 3 time points. No significant differences between time points were seen for platelet-derived L-EVs (A, P=0.8237), endothelial-derived L-EV (B, P=0.4342), leukocyte-derived L-EVs (C, P=0.6491), or total annexin V+ L-EVs (D, P=0.5819).



Supplemental Figure 2. Representative Western blot images of L-EV isolates showing the presence of L-EV markers flotillin-2 and tumor suppressor gene-101 (TSG-101).