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First trimester plasma-derived exosomal proteins: Putative biomarker for early detection of pathological pregnancies

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Abstract

Background: Of the 130 million babies born each year, 8 million die before their first birthday. A contributing factor in many of these deaths is poor pregnancy outcome as a result of complications of pregnancy. Preeclampsia (PE) and Gestational Diabetes Mellitus (GDM) are the common pregnancy complications that have no effective antenatal treatment other than steroid administration and timely delivery. Each occurs with an incidence of 5-10% and is responsible for the majority of obstetric and paediatric morbidity and mortality. These can permanently impact on lifelong health. Early detection of disease risk and onset is the first step in implementing efficacious treatment and improving patient outcome. In the context of antenatal screening, the objective is to identify biomarkers (*e.g.* proteins) that are informative of the risk of asymptomatic pregnant women subsequently developing complications of pregnancy. Recent studies highlight the putative utility of tissue-specific nanovesicles (*i.e*. exosomes) in the diagnosis of disease onset and treatment monitoring. It was hypothesized that presymptomatic women who subsequently develop pregnancy complications display altered exosome profile (*i.e.* concentration and/or protein content) at first trimester of pregnancy (*i.e.* 6-12 weeks). The general aim of this thesis was to identify blood-borne biomarkers (*i.e.* exosomes) that may be used at the first antenatal visit to identify presymptomatic women who are at risk of developing complications of pregnancy.

Methods: A time-series experimental design was used to establish pregnancy-associated changes in maternal plasma exosome concentrations during first trimester. Serial samples of plasma were collected from normal healthy pregnant women (n=10) at 6, 7, 8, 9, 10, 11 and 12 weeks of gestation. Plasma samples from pregnant women at 11-14 weeks of gestation who developed preeclampsia (n=15) and gestational diabetes mellitus (n=7) were also collected. Exosomes were isolated by differential and buoyant density centrifugation and characterised by size distribution and morphology using nanoparticles tracking analysis (NTA; NanoSight™) and transmission electron microscopy (TEM) respectively. The total number of exosome vesicles and placenta-derived exosome vesicles were determined by quantifying immunoreactive exosomal CD63 and a placenta-specific marker (PLAP) by ELISA. Finally, the differentially expressed exosomal proteins and peptides were identified by Liquid Chromatography and Mass Spectrometry based approaches (LC-MS/MS). Statistical analysis was performed using the Graph Pad Prism software.

Results: EM and NTA identified the presence of 50 - 120 nm spherical vesicles in maternal plasma as early as 6 weeks of pregnancy. The number of exosomes in maternal circulation increased significantly (ANOVA, p=0.002) with the progression of pregnancy (from 6 to 12 weeks). The concentration of placenta-derived exosomes in maternal plasma (*i.e.* $PLAP^{+}$) increased progressively with gestational age, from 6 weeks 70.6 ± 5.7 pg/ml to 12 weeks 117.5 ± 13.4 pg/ml. Regression analysis shows that week is a factor that explains >70% of the observed variation in plasma exosomal PLAP concentration, total exosome number only explains 20%.

The total number of exosome was $1.31e^{12} \pm 1.60e^{12}$ (mean ± SD) during the first trimester in women who developed Gestational Diabetes Mellitus (GDM) in second trimester and $1.09e^{11} \pm 3.17e^{10}$ in women who developed Preeclampsia (PE). The statistical analysis showed that the total number of released exosomes in the first trimester of pregnancy complicated with GDM was three- fold higher than normal pregnancy and the concentration of the exosomes released from the placental cells was twofold compared to PLAP concentration in normal pregnancy. In addition, a significant ($p<0.001$) increase in the cumulative number of exosomes was observed during the first trimester of preeclamptic condition when compared with the first trimester normal pregnancy condition.

Conclusion: This study presents longitudinal data on placental-derived exosomes in the first trimester of pregnancy, starting from as early as 6 weeks. Early detection of women at risk of pregnancy complications would provide opportunity to evaluate appropriate intervention strategies to limit acute adverse sequelae. The rationale for developing early pregnancy screening tests is not only for the management of the contemporaneous pregnancy but also to optimise lifelong and intergenerational health. If this can be achieved, it will provide an opportunity for early assessment of risk and the implementation of an alternative clinical management strategy to improve outcome for both the mother and the baby.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Placenta-derived exosomes continuously increase in maternal circulation over the first trimester of pregnancy *(published in the Journal of Translational Medicine 2014)*

Suchismita Sarker, Katherin Scholz-Romero, Alejandra Perez, Sebastian E. Illanes, Murray D. Mitchell, Gregory E Rice, Carlos Salomon

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has been incorporated in chapter V and VI.

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Katherin Scholz-Romero	Assisted in conducting experiments
Alejandra Perez	Contributed in obtaining clinical samples
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Gregory E Rice	Assisted in study design Assisted in statistical analysis Edited the manuscript
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CHAPTER I

1. INTRODUCTION

1.1 Key concepts and variables

Pregnancy induces a number of alterations to maternal physiology for maintaining the correct course of pregnancy; however, when the maternal changes do not occur properly pathological complications appear such as gestational diabetes mellitus (GDM), preeclampsia (PE) and intrauterine growth restriction (IUGR) [1]. The in depth understanding of the aetiology of these complications is lacking that limits our ability to identify and implement efficacious management and intervention strategies to ameliorate adverse effects for both mother and baby arising from these complicated pregnancies [2]. The situation is further exacerbated by the lack of reliable screening test to identify presymptomatic women who are at risk of developing complications of pregnancy. Recent studies highlight the putative utility of tissue-specific nanovesicles (*e.g.* exosomes) in the diagnosis of disease onset and treatment monitoring [3-5]. To date, there is a paucity of data defining changes in the release, role and diagnostic utility of placenta-derived nanovesicles (*e.g.* exosomes) in pathological pregnancies. Exosomes are nanovesicles that are released from viable cells into the body fluids under certain pathological conditions [6]. Exosomes carry proteins, microRNA and bioactive lipids that are informative of disease risk [7]. Exosomes are expected to be released from different cells in particular from placental trophoblast cells carrying proteins (biomarkers) in abnormal concentration into the maternal circulation in pathological pregnancies [8, 9].

Keywords: pathological pregnancies, biomarker, exosome

1.2 Background and motivation to perform the study

Pathological pregnancies *i.e.* preeclampsia and gestational diabetes mellitus occur at a rate of 5-10% each in all pregnancies and the incidence of these events is increasing in Australia and other developed nations [2] . These common pregnancy pathologies are diagnosed at late second or third trimester when the underlying pathology is most likely established. The currently available management to combat these complications are not preventative. Once the condition is diagnosed, the management aims at limiting further maternal as well as perinatal complications to develop arising from these conditions. Pregnant women developing these complications remain asymptomatic in early gestational weeks (*i.e.* first trimester); however, when they develop symptoms generally in the late second or early third trimester, those conditions become clinically overt and is out of capability to reverse the conditions towards normal healthy pregnancy [2]. Therefore, developing an effective way to screen presymptomatic pregnant women in the first trimester to detect pathological pregnancies from early stage is a challenge and there are many opportunities and scopes to undertake research studies in this arena.

CHAPTER II

2. LITERATURE REVIEW

2.1 Human pregnancy

Pregnancy represents a significant homeostatic challenge to all body systems [10]. The progressive physiological changes that occur during pregnancy are essential to support and protect the developing fetus and also to prepare the mother for parturition [10] . This usually causes no major problems for healthy women. In the presence of clinical or subclinical pathology, the normal physiological changes of pregnancy can place significant strain on already compromised systems, threatening the lives of both mother and fetus [10]. Pregnancy is a time of great maternal metabolic and physiological challenges in order to adapt the mother to the needs of the developing fetus [11]. In pregnancy, the placenta plays the pivotal role in bringing out maternal physiological changes and fetal development. The placenta is a multifunctional organ as well as a rich source of several pregnancy associated hormones that influences fetal, placental and maternal metabolism [12]. Therefore, normal uncomplicated healthy pregnancy essentially relies on healthy placental development.

Successful pregnancy requires adequate implantation and invasion of the trophoblast [13, 14]. For the initial phase of trophoblast growth, the trophoblast must adopt an invasive phenotype and induce the formation of feto-maternal circulation via maternal vascular system remodelling [15]. This invasion process occurs during the early first trimester of pregnancy and does not go beyond the proximal third of the myometrium. This process is regulated by different soluble endocrine mediators *i.e.* autacoids secreted by the placental cells and is associated with an increased production of pro-inflammatory cytokines and chemokines [16]. Optimum and adequate proinflammatory, procoagulatory and immunosuppressive environment in the maternal circulatory system as well as vascular remodelling of the maternal uterine blood vessels to establish feto-maternal circulation essentially determine the outcome of pregnancy as normal to continue up to term delivery.

2.2 Aetiology of pregnancy pathologies

There are various pathologies that may occur during pregnancy such as gestational diabetes mellitus, preeclampsia and intrauterine growth restriction. Though the exact aetiology of these

conditions is not fully understood but it is considered that the placenta plays a vital role in developing these pathologies [17]; however, disordered placentation in terms of vascular connection establishment is considered responsible for evolving the complications [18].

During normal healthy pregnancy, the extravillous trophoblast cells invade into the inner third of the myometrium and the spiral arteries lose their endothelium and most of their muscle fibres [19]. These structural modifications are associated with functional alterations, such that spiral arteries become low-resistance and high-capacitance vessels, and thus become less sensitive or even insensitive to vasoconstrictive substances [20]. Therefore, there is effective first trimester invasion of trophoblast cells into the maternal uterine spiral arterioles. However, in most of the pregnancy complications this invasion process is anomalous leaving these vessels sensitive to vasoconstriction leading to placental ischemia [21].

Healthy pregnancy is associated with a state of relative systemic inflammation [22]. Notably, all the inflammatory changes of normal pregnancy are exaggerated in hypertensive disorders of pregnancy which manifest endothelial dysfunction and a wider stress response, including the acute phase response and metabolic effects of dyslipidaemia and increased insulin resistance [23].

During pregnancy, the placenta releases cortisol hormone (glucocorticoid) in higher concentration [22] which makes the pregnancy state more vulnerable to develop pregnancy-associated diabetes mellitus. Furthermore, the advanced maternal age, positive family history for diabetes and previous history of diabetes mellitus favour the condition (increased insulin resistance) to develop [22]. Pregnancy is a state of oxidative and nitrative stress resulting from generation of reactive oxygen and nitrogen species and this reactive oxygen species influence placental development [23]. However, in gestational diabetes mellitus (GDM) high glucose concentration yields excessive reactive oxygen species, especially superoxide free radicles that attributes to abnormal placentation [23].

2.3 Preeclampsia

Preeclampsia is a multisystem disorder of pregnancy which is life threatening to both mother and fetus. This maternal syndrome is characterised by new onset of hypertension and proteinuria in the second half of pregnancy. The criteria that define preeclampsia are: onset at >20 weeks' gestational age of 24-hour proteinuria ≥30 mg/day or, if not available, a protein concentration ≥30 mg (≥1+ on

dipstick) in a minimum of two random urine samples collected at least 4–6 hours but no more than 7 days apart, a systolic blood pressure >140 mmHg or diastolic blood pressure ≥90 mmHg as measured twice, using an appropriate cuff, 4–6 hours and less than 7 days apart, and disappearance of all these abnormalities before the end of the 6th week postpartum in both early and late onset preeclampsia [24]. Nonetheless, some presentations of pregnancy-related hypertension combined with clinical or biochemical abnormalities or intrauterine growth restriction should also be considered as potential preeclampsia [19].

2.3.1 Brief description of preeclampsia

Preeclampsia is a multisystem disorder that complicates 3%–8% of pregnancies in Western countries and constitutes a major source of morbidity and mortality worldwide [25]. The risk of pre-eclampsia is 2-fold to 5-fold higher in pregnant women with a maternal history of this disorder. Risk factors have been identified, including a medical history of chronic hypertension, kidney disease, diabetes, obesity, age ≥35 years, and some pregnancy characteristics, such as twin or molar pregnancy, previous preeclampsia, or fetal congenital abnormality [18]. Preeclampsia may be life threatening for both mother and child, increasing both fetal and maternal morbidity and mortality. In mother, preeclampsia may cause premature cardiovascular disease, such as chronic hypertension, ischemic heart disease and stroke. Later in life, while children born after preeclamptic pregnancies and who are relatively small for gestational age at birth, have an increased risk of stroke, coronary heart disease, and metabolic syndrome in adult life [26]. The current sole curative treatment is delivery. Screening pregnant women at high risk and preventing recurrences are becoming key issues in the management of preeclampsia [19].

2.3.2 Pathophysiology and immunology of preeclampsia

The placenta plays an important role in the pathophysiology of preeclampsia [27]. An inability to remodel maternal spiral arteries and subsequent suboptimal placental blood flow is one of the many underlying pathologies [27] (see figure 1). The placenta also produces factors that attribute to compromised blood flow and enhance placental damage [28].

The principal changes in the placenta with preeclampsia are decidual arteriolopathy, infarcts in the central area, abruption placentae and impaired growth and Tenney-Parker changes that refer to surface budding of the placental syncytium which is thought to be the adaptive changes to altered maternal blood flow [29]. There is also excessive systemic maternal inflammatory response in preeclampsia as placenta releases many cytokines and antiangiogenic factors when exposed to

preeclamptic environment [30]. These factors prevent proper placental development and establishment with uterine spiral arteries [29].

Preeclampsia manifests as a two stage disease. In first stage when the mother is asymptomatic, there is poor placentation due to inadequate uteroplacental circulation in hypoxic environment that leads to reperfusion injury, placental ischaemia and oxidative stress. This ischaemia leads to hypoxia that causes the release of proinflammatory, antiangiogenic and vasoconstrictive substances from the malfunctioning placenta that affect the maternal circulatory system and manifests as hypertension, proteinuria and glomerulopathies [31].

The current understanding of pathophysiology of preeclampsia emphasises on a wide variety of target organ involvement during the process of this pregnancy vascular complication. In preeclampsia, the underlying pathogenesis involves defective deep placentation, oxidative and endoplasmic reticulum stress, autoantibodies to type-1 angiotensin II receptor, platelet and thrombin activation, intravascular inflammation, endothelial dysfunction and the presence of an antiangiogenic state [32].

Furthermore, different immunological phenomena are implicated in preeclampsia pathogenesis, not merely defective placentation. In one study it was observed that during pregnancy natural killer cells and antigen presenting cells (including dendritic cells and macrophages) consisted a large proportion of the decidual leukocyte population, and were thought to modulate vascular remodelling and trophoblast invasion [33]. Additionally, T lymphocytes were involved in regulating maternal systemic immune tolerance that prevented the rejection of the fetal semiallograft and all these physiological processes attributed to normal healthy pregnancy [33].

Recently several studies reported the association of differentially expressed maternal circulatory immunological molecules and development of preeclampsia. The gene for CD4+ CD25 (bright) regulatory T cells displayed significantly decreased expression in preeclamptic condition [34]. Also, the defective expression of T helper 17 cells in maternal peripheral blood circulation was found to be responsible for developing preeclampsia [35]. CD4 (+) and HLA-G (+) T cells were observed to be present in significantly higher concentration in peripheral blood of preeclamptic mothers [36]. Higher plasma levels of Hsp60, Hsp70 [37, 38] , and their specific antibodies, tumour necrosis factor-alpha (TNF-alpha) [39, 40], interleukin (IL)-1, IL-12, and soluble TNF-alpha-receptor I (sTNFRI) [37] and lower level of IL-10 [37, 40, 41] were determined in preeclampsia compared to healthy uncomplicated pregnancy. Other studies also found the role of complement activation and

regulation in preeclampsia. Maternal C4 deficiencies were most common in the preeclamptic placentae whereas more C1q was measured in the same tissue. Activated C4 and membrane-bound regulators CD55, CD46, and CD59 were observed abundantly in the syncytiotrophoblast, syncytial knots of preeclamptic placentae [42].

2.4 Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is a syndrome characterised by "glucose intolerance with onset or first recognition during pregnancy" [43, 44].

2.4.1 Brief description of gestational diabetes mellitus

GDM affects ~5% of all pregnancies and parallels the global increase in obesity and type 2 diabetes. In USA alone, GDM affects more than 135,000 pregnancies per year. Lifestyle changes that impact adversely on caloric balance is thought to be a contributing factor in this emerging pandemic [45, 46]. The current 'gold standard' test for the diagnosis of GDM is the oral glucose tolerance test (OGTT) at 24–28 weeks of gestation [47]. Pathology is probably already established and reversal of the potential adverse perinatal outcomes may be restricted. The lack of a reliable early test for GDM has hampered the development of useful intervention therapies that may impact not only on the acute but also the long-term health outcomes. Thus, it is important to diagnose and predict GDM earlier so that appropriate management can be initiated and tailored to the needs of the patient in order to minimise perinatal complications and their sequelae.

According to HAPO study in 2008 [48], there is a strong correlation between maternal glucose levels below those diagnostic of diabetes in early pregnancy and adverse pregnancy outcomes (*i.e.* increased birth weight of babies) that similarly occurs in GDM.

2.4.2 Pathophysiology of GDM

During pregnancy, maternal metabolic status gets altered with changes in hormones, growth factors and nutrients [49]. Most of the pregnancy hormones *i.e.* Human Chorionic Gonadotropin (HCG), Human Placental Lactogen (HPL), placental glucocorticoids are gluconeogenic which leads to excess glucose production in the maternal circulation [48]. This hyperglycaemia affects and alters the level of insulin, insulin like growth factors 1 and 2 (IGF1 and 2), Fibroblast Growth Factor (FGF-2), Vascular Endothelial Growth Factor (VEGF), Tumour Necrosis Factor (TNF-α) and leptin which ultimately play role in abnormal placentation through inhibition of trophoblast invasion,

placental angiogenesis and placental vascularisation [23]. This change becomes evident in early pregnancy (at the end of first trimester) [23].

On the other hand, there is imbalance between ROS formation and the antioxidant defense mechanisms of a cell or tissue which may lead to damage to the placenta [23]. Other than abnormal placentation the changes in substrates, growth factors and hormones are expected to be observed in the maternal circulation in GDM during early pregnancy which may enhance exosome release from different tissues and those exosomal biomarkers can be identified.

2.5 Intrauterine growth restriction (IUGR)

IUGR is usually defined as fetal weight below the 10^{th} percentile for gestational age as determined by antenatal ultrasound [50]**.** Fetal growth restriction is the failure of the fetus to reach its genetically predetermined growth potential due to adverse genetic or environmental factors [51].

2.5.1 Brief description of IUGR

IUGR babies exhibit aberrant development and require higher neonatal intensive care [52]. In addition to the short-term risks, the long-term risk of developmental programming includes metabolic disorders later in life. Up to 63% of adult diabetes, hypertension and heart disease may be attributed to low-birth-weight conditions in conjunction with an accelerated newborn to adolescent weight gain and obesity [51].

2.5.2 Pathophysiology of IUGR and relationship with preeclampsia severity

A key aspect of successful placentation is the invasion of extravillous trophoblasts and transformation of the decidual and intramyometrial portions of uterine spiral arteries [53]. However, in IUGR, there are reductions in utero-placental blood flow as well as intervillous haemodynamic alterations resulting from retained spiral artery contractility as a result of inadequate remodelling by trophoblast cells [51]. The absence of optimal utero-placental blood flow through these unmodified vessels suggest that hypoxia, re-oxygenation, oxidative stress, or a combination of these, are the potential aetiologies for IUGR [51]. In fact, some genetic abnormalities and environmental cues like maternal under-nutrition during early pregnancy period are responsible for all these changes [51].

IUGR and preeclampsia display a common placental pathology, *i.e.* placental insufficiency [54] although some studies highlighted the separate entity of these two pregnancy complications. It was documented that IUGR can develop without the manifestation of preeclampsia [55]. The uterine artery pulsatility index shows a gradual increase with the gestational age in confirmed cases of IUGR without preeclampsia [56]. Also, different placental histopathological changes were observed in IUGR with preeclampsia and without preeclampsia [57]. Other studies found that IUGR was inconsistently related with preeclampsia and mostly as a result of severe preeclampsia [58, 59].

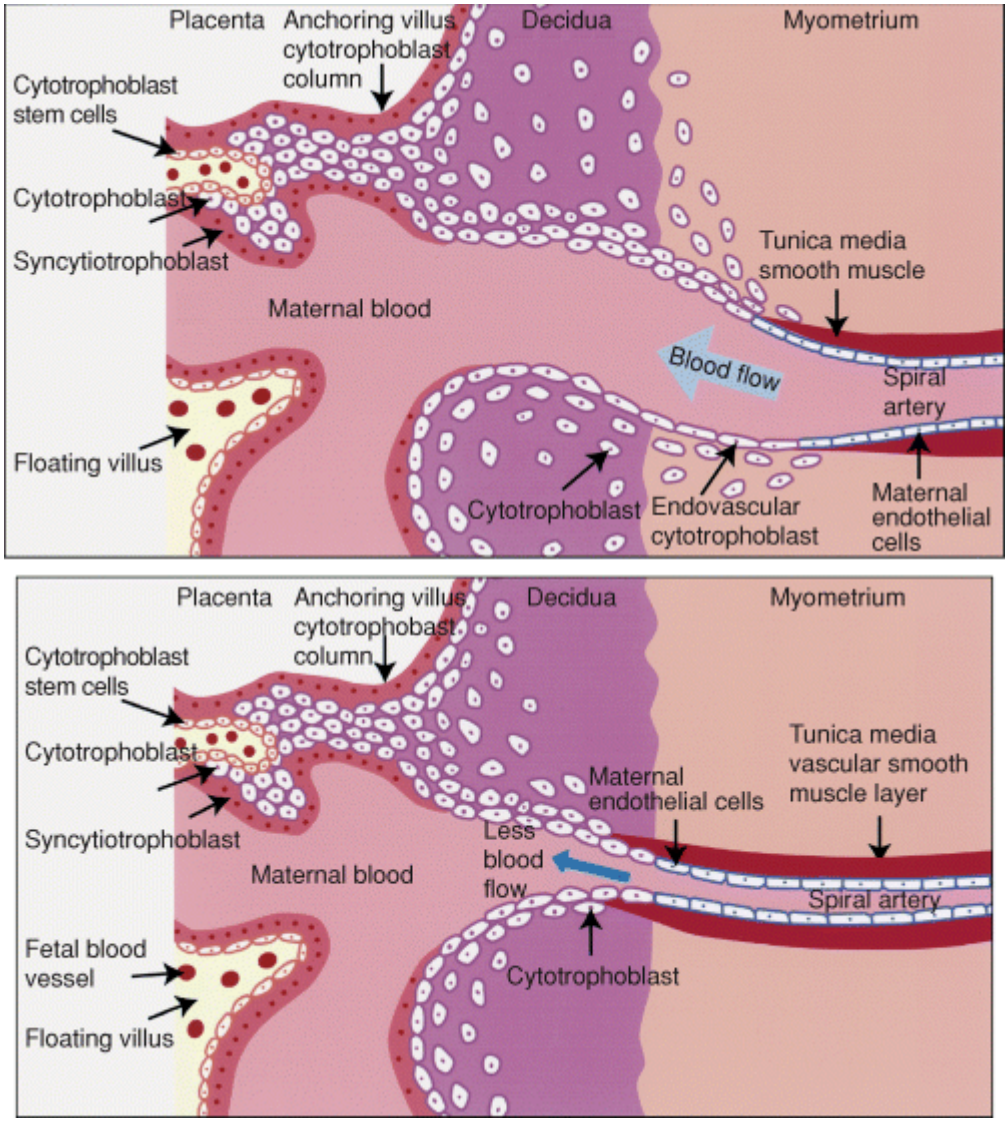


Figure 1. Placentation in normal healthy pregnancy and pregnancy complicated with preeclampsia. In normal placental development, invasive cytotrophoblasts of fetal origin invade the maternal spiral arteries, transforming them from small-calibre resistance vessels to high-calibre capacitance vessels capable of providing placental perfusion adequate to sustain the growing fetus (upper panel). In preeclampsia, cytotrophoblasts fail to adopt an invasive endothelial phenotype. Instead, invasion of the spiral arteries is shallow and they remain small calibre, resistance vessels (lower panel) which may result in placental ischemia. (*Ref: Karumanchi S. A. et al. 2005*)

2.6 Biomarkers

Biomarker is regarded as physiological or pathophysiologic biochemical or biomolecular alteration at the organ, tissue and cell or subcellular level in response to an exposure that is measureable in biological samples and became the signature of onset of disease, underlying disease process or phenotypic outcome [60]. Biomarkers are dynamic markers and their concentration can be influenced by a variety of confounders during pregnancy which include genetic, epigenetic, nutritional, behavioural, racial/ ethnic and many others [60].

2.7 Exosomes: tissue-specific nanovesicles in the diagnosis of disease onset and treatment monitoring

Exosomes are bioactive nanovesicles released from all types of viable cells into almost all biological fluids, including urine, blood, ascitic fluid, cerebrospinal fluid and even fractions of body fluids such as serum, plasma and cell conditioned media. Exosomes are synthesized by inward blebbing of the plasma membrane and are secreted by exocytosis process into the extracellular milieu [61-63]. Transmission Electron Microscopy (TEM) has described the morphology of exosomes as cup-shaped after fixation, adhesion, negative staining and visualization. Exosomes float on sucrose gradients and their buoyant density ranges from 1.13-1.19 g/ml [4]. Biochemically, the membrane of these vesicles is a lipid bilayer containing cholesterol, sphingomyelin, ceramide and detergent resistant membrane domains (lipid rafts) in very high concentrations that contribute to the stability of the exosomes in different types of environment [64]. These vesicles predominantly play role in intercellular signalling via sequestration of signalling molecules *i.e.* cytosolic proteins or proteins from the endocytic compartment or plasma membrane as well as mRNA and miRNA that act upon other distal target cells to modify their activity [65]. Exosomal proteins are from the protein families' tetraspannins, heat shock proteins and major histocompatibility complex (MHC) class I and II [66, 67].

Release of exosomes is dependent on the physiological state and the extracellular environment that also contribute to harbouring different proteins and mRNA content in exosomes [3]. The function of exosomes has been reviewed in several literatures. In particular, exosomes are capable of transferring the DNAs, microRNAs, non-coding RNAs and lipids with or without direct cell to cell contact and as such these nanosized vesicles display a key role as an intercellular messenger in cell-to-cell communication [68]. The major role of exosomes is to carry the information by delivering

various effectors or signalling molecules between specific cells contributing in immune suppression, antigen presentation, inflammation, exchanging genetic information, cell adhesion and waste management [68, 69]. This active interaction regulates various physiological and pathological conditions, including cancer, infectious diseases, and neurodegenerative disorders [70]. These varied functions enable exosomes to be utilised in different clinical implications to understand the physiology under normal condition and the pathophysiology of different disease conditions as well.

2.7.1 Biogenesis of exosomes

The suggested biogenesis of exosomes in Figure 2, separates them from all other extracellular vesicles (EVs) so far known [71]. They are formed in the late endosomal membrane compartment by inward budding of the limiting membrane of late multivesicular endosomes and multivesicular bodies (MVB) and contain cell surface–expressed proteins and cytosolic components [72]. They are actively secreted into the extracellular space by fusion of the MVB with the plasmatic membrane [72].

2.7.2 Exosomal proteins

While the composition of exosomes appears to be cell-specific, a subset of common proteins have been identified [72]. The lipid bilayer is composed of sphingomyelin [72]. Among the most commonly used markers for characterisation of exosomes are tetraspannin proteins, including: CD63, CD81; CD9; and CD82 [72]. Other families of common proteins in all exosomes include chaperone proteins such as: Hsc70 and Hsp90; cytoskeletal proteins including actin, tubulin and myosin; transport proteins; and annexins [72]. Exosomes derived from antigen-presenting cells (APC) express MHC-I and MHC-II on their surface [72].

2.7.3 Exosomes as biomarker

Cell derived vesicles *i.e.* exosomes may behold the unique source of clinically relevant and many non-invasive biomarkers as they are believed to play in various pathological conditions. Cell-lineage and state specific exosomes may harbour body fluid-based biomarkers of unparalleled accuracy [73]. Exosome specific membrane proteins provide markers enabling exosome identity and selection, while cell type and cell-condition specific protein, mRNA and miRNA provide a rich potential source of biomarkers [73]. Exosomes help the discovery of relatively less expressed biomarkers of different pathological conditions that normally remain undetected [73].

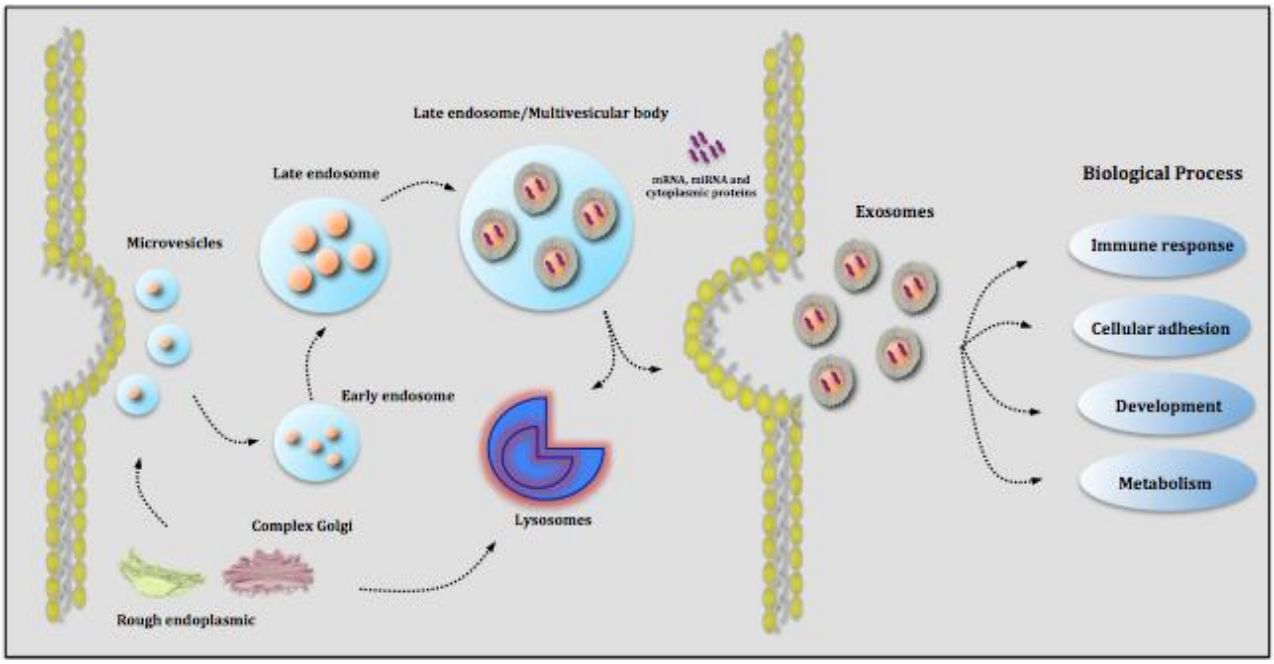


Figure 2. Biogenesis of exosomes – schematic presentation of the endosomal pathway by which exosomes are generated and secreted. CG, Complex Golgi; RER, rough endoplasmic reticulum; MV, microvesicle; MVB, multivesicular body. *(Ref: Salomon et al. 2013)*

2.8 Placental exosomal biomolecules: role in placental and maternal physiology

During pregnancy several kinds of microparticles including nanovesicles (*i.e.* exosomes) are secreted from the placenta into the maternal circulatory environment [74]. These vesicles containing various proteins, miRNA, mRNA and bioactive lipids play essential role in reproduction through adaptation of the maternal body system with the pregnancy related physiological changes [72]. Firstly, placental exosomes containing protein syncytin contributes to fusion of cytotrophoblast cells to form syncytiotrophoblast cells that are necessary for syncytiotrophoblast cells' invasion into the maternal uterine spiral arterioles [75, 76]. Another study reported that exosomes secreted from the extravillous trophoblast cells promote vascular smooth muscle cell migration which is particularly helpful for maternal uterine spiral arterial remodelling and properly controlled placentation [77].

Secondly, exosomes secreted from placenta consisting of specific proteins act upon target immune cells to provide immunosuppressive environment during pregnancy. Previously conducted several studies could isolate different proteins from trophoblast cells that exhibited their ability to suppress maternal immune system which is essential for fetal semiallograft survival. MHC class I chain related molecules [78], down regulated Natural Killer cell receptor (NKG2D) [78, 79], functional Fas ligand [80, 81] and TRAIL molecules [81], HLA-G and B7 family of immunomodulators [82] were isolated from first trimester placental tissues and these molecules suppressed T cell signalling components [83, 84]. Lastly, placenta specific miRNA molecules were sorted in placental exosomes [85, 86] that showed the property of resistance against viral infection to protect the fetus during pregnancy [87-89].

Exosomes are a specific subtype of extracellular vesicles (EVs) of endocytic origin with the capacity to incorporate specific molecules such as proteins and miRNAs in response to changes in the microenvironment milieu (*e.g.* hypoxia). The human placenta constitutively produces and secretes EVs that enter into the maternal circulation and have a participation in the maternal adaption to pregnancy related physiological changes. In early pregnancy, the concentration of placenta derived exosomes in maternal blood increases dramatically. These exosomes are biologically active and exert various functions to maintain the pregnancy successfully. Placental exosomal concentration gradually increases throughout pregnancy and decline in late pregnancy [90].

2.9 Placenta-derived exosomes: a potential biomarker of pregnancy complications

The human placenta is a fenestrated organ that produces and secretes exosomes which are released into the maternal peripheral blood circulation during pregnancy [1, 9, 91]. These exosomes are immunosuppressive as cause immunomodulatory decline in early pregnancy by suppressing the function of T cells and pluripotent carrying proteins, mRNA and miRNA which influence different biologic mechanisms throughout pregnancy [83]. There is evidence that exosomes are released in low concentration during normal pregnancy whereas this shedding from syncytiotrophoblast is enhanced in endothelial and immune-cell dysfunction which is associated with the placental pathophysiology in preeclampsia [91].

Exosomes have been identified in plasma under both normal and pathological conditions. There are a considerable number of proteins (biomarkers) commonly identified by proteomic analysis in plasma exosomes of pregnant women that are placenta-specific [92]. These placenta-specific exosome markers have been implicated to be involved in different pathological states of pregnancy. The concentration of exosomal proteins in plasma has been reported to be increased in association with disease severity and/or progression and in response to oxidative stress [5]. In preeclampsia, the impaired placental function with the placental apoptosis and necrosis causes increased release of microvesicles and nanovesicles (*i.e.* exosomes) [93, 94]. These exosomes contain different markers (proteins) that are actors or witnesses to the various stages of mechanisms of the disease [95]. In GDM pregnancies, oxidative stress and hyperglycaemia may be responsible for increasing the release of extracellular vesicles including exosomes into the maternal circulation that can be detected, characterised and may be of utility as early biomarkers of disease onset [71].

2.10 Biomarkers for the prediction of preeclampsia with their performance and limitation

Preeclampsia is a complex multisystem disorder of pregnancy that manifests in second trimester. The current preventive methods are based on obstetric history and clinical features which are not really helpful and the ultimate curative treatment is delivery which results in higher maternal as well as neonatal mortality and morbidity rate. Abnormal placentation with aberrant vasculogenesis is the basis of this pathology that begins in early first trimester [32]. All these findings necessitate the innovation of reliable non-invasive screening tests in first trimester that would allow for the early initiation of prophylactic therapies, institution of an appropriate model of care and recruitment of pregnant women at risk for interventions to ameliorate the condition.

Several research studies have been conducted to identify a set of parametres (*i.e.* biomarkers) in asymptomatic pregnant women's blood in first trimester that can indicate abnormal placental function (see Table I). Commonly detected blood-borne biomarkers are- Pregnancy Associated Plasma Protein-A (PAPP-A), Placental Protein 13 (PP13), soluble Fms-like tyrosine kinase -1(sFlt-1), pentraxin, inhibin-A, placental growth factor, vascular endothelial growth factor (VEGF) [96-98]. Some miRNAs, lipids and enzymes have been found to be deregulated in preeclamptic condition, *i.e.* miR455- 3P and miR455- 5P [99], miR-1233 [100], Glycerophosphoserines-GP01, GP02, GP03 and Flavanoids-PK12 [101], matrix metalloproteinase-2 and 9 [102]. Several biomarkers were found to be upregulated and some were downregulated with the pathological changes. However, most of these biomarkers cannot be detected at significant level in early first trimester. The relationship of these biomarkers with preeclampsia needs to be verified by performing large scale study on different ethnic population. Additionally, it is important to discover a panel of biomarkers with greater predictive value which necessitates further research. Finally, the available proteomic approaches and methods to identify the biomarkers need to be robust as well as cost-effective to introduce in clinical setting.

2.11 Final remarks

According to the World Health Organization, over 346 million people worldwide, roughly the combined populations of the United States and Canada have pregnancy complications (e.g. pre-eclampsia, GDM or IUGR) [100]. Therefore, it can be assumed that a large number (~15%) of the women who get pregnant will develop complications, which lead to maternal or fetal vital risk. Unfortunately, the incidence of disease alters physiological evolution of the fetal development as well as maternal health. In the context of antenatal screening, the objective of proteomic approaches is to identify proteins or peptides that are informative of the risk of pre-symptomatic early pregnant women who subsequently develop complications of pregnancy, meant a breakthrough for better management of the disease before symptoms begin. The exosomes research in human pregnancy is burgeoning and it is logical to expect that more exosomes-carrier molecules and mechanism of action will be revealed in the near future.

Table I. Commonly detected biomarkers in maternal blood to predict preeclampsia

Serum level (↑/↓)	Name of biomarkers
Upregulated in preeclampsia	Pregnancy associated plasma protein-A
	Placental protein-13/ Galectin family of proteins
	Inhibin-A
	P-selectin
	Pentraxin 3
	Fibronectin
	Heat-shock proteins (Hsp 70)
	Fms-like tyrosine kinase-1(SFlt1)
	Soluble Endoglin
	Cell free fetal DNA
	Adrenomedullin
	Calretinin
	Vimentin
Downregulated in preeclampsia	Annexin XI
	Placental growth factor (PlGF)
	Glypican 3
	insulin-like growth factor-2 mRNA-binding protein 3 (IGF2BP3)
	ICAM-1
	p-MAPK
	Bcl- 6

(Ref: Muller-Deile et al. 2014, Shu C et al. 2014)

CHAPTER III

3. HYPOTHESIS DEVELOPMENT

3.1. Rationale and research approach

Of the 130 million babies born each year, 8 million die before their first birthday [2]. A contributing factor in many of these deaths is poor pregnancy outcome as a result of pregnancy complications. Preeclampsia, gestational diabetes mellitus are important complications of pregnancy that have no effective antenatal management other than steroid administration and timely delivery [2]. Each of these complications occurs in 5 to 10% of pregnancies and are responsible for the majority of obstetric and paediatric morbidity and mortality [2]. When these pregnancy complications are diagnosed in the late second or early third trimester of pregnancy the 'pathology' is most likely well-established and the possibility to reverse or limit potential adverse effect on perinatal outcomes may be limited [2]. Early detection of predisposition to and/or onset of PE and GDM thus, are the first step in developing, evaluating and implementing efficacious treatment. Over the past 5 years, the role of exosomes in inter-cellular communication under both physiological and pathophysiological conditions has been recognised [10, 11]. The role of exosomal signalling in normal and pathological pregnancies, however, is only the beginning to be elucidated. Based on the available data, it was proposed that placental-derived exosomes might play a significant role in onset and development of pathologies in pregnancy (Figure 3).

3.2 Hypothesis

Presymptomatic women who subsequently develop pregnancy complications may display altered placental-derived exosome profile in the first trimester of pregnancy

3.3 General aim

The aim of this project is to identify blood-borne biomarkers (*i.e.* exosomes) that may be used at the first antenatal visit to identify presymptomatic women who are at risk of developing complications of pregnancy

3.4 Specific aims

1. To characterise gestational age related changes in the concentration of placenta-derived exosomes at first trimester of pregnancy (*i.e.* 6-12 weeks) in plasma from women with normal pregnancies.

2. To establish the placenta-derived exosomes profile (number of exosome release and their protein content) in early stages of pregnancy (first trimester) in plasma from pregnant women who subsequently develop GDM or PE

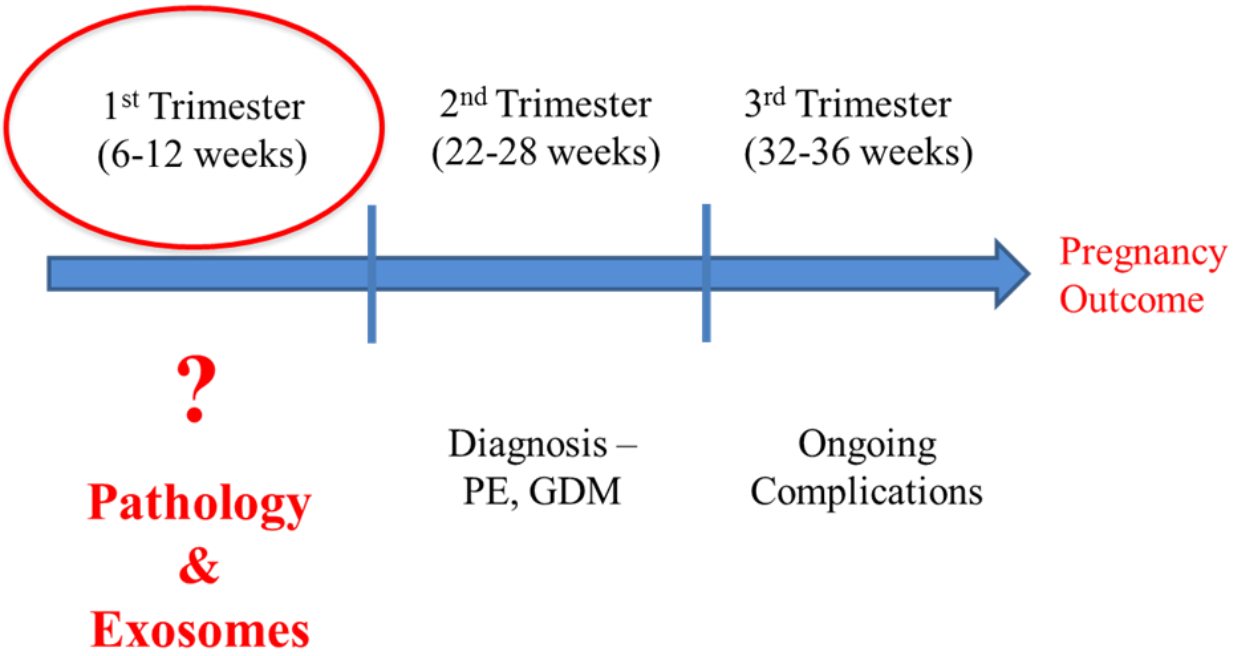


Figure 3. Schematic presentation of association between literature review and hypothesis. The arrow represents the total course of pregnancy. During the third trimester the pregnancy complications are established and affect the pregnancy outcome. The pregnancy complications are diagnosed currently at the second trimester. The hypothesis would test the relationship between pregnancy pathologies and placental exosome release including their protein content in the maternal circulation in the first trimester asymptomatic pregnant women predisposed to develop the pathologies in the second trimester.

CHAPTER IV

4. METHODOLOGY

4.1 Experimental design

1. Plasma sample collection (Figure 4)

- From women with normal healthy uncomplicated pregnancy (n=10, plasma was collected from 10 patients at 6-12 gestational weeks; total sample size was 70). These patients did not develop any complication during gestation.
- From women with preeclampsia (n=15, plasma was collected from 15 patients at 11-14 weeks; total sample size was 15). These patients developed preeclampsia (PE) in the late second or early third trimester of pregnancy.
- From women with gestational diabetes mellitus (n=7, plasma was collected from 7 patients at 11-14 weeks; total sample size was 7). These patients developed gestational diabetes mellitus (GDM) at 22-28 weeks of pregnancy.
- From non-pregnant women as control (n=5)

2. Extracellular vesicles including exosomes isolation from plasma (Figure 5)

- Differential ultracentrifugation (UT) and ultrafiltration

3. Exosome purification (Figure 6)

- Sucrose continuous gradient (0.25 M-2.5 M sucrose)
- Sucrose (30%) cushion
- ExoQuick kit

4. Characterization of exosomes

- Protein concentration determination by Bradford assay
- Size Distribution of exosomes using Nanoparticle Tracking Analysis (NTA) by NanoSight
- Transmission Electron Microscopic (TEM) analysis

5. Quantification of exosomes

- Quantification of number of exosome particle by ELISA for CD63 (specific exosomal endocytic marker)
- Placenta specific marker on exosome (PLAP) concentration measurement by ELISA for PLAP

6. Mass spectrometric protein (exosomal protein) analysis (Figure 7)

- Protein retrieval by Mascot^{TM} database searching against human species

Ethical clearance to perform the study:

THE UNIVERSITY OF QUEENSLAND

Institutional Human Research Ethics Approval

Project Title:	Mechanisms of Placental Growth and Function Related to Uncomplicated and Complicated Pregnancies
Chief Investigator:	Prof Murray D Mitchell, Prof Gregory E Rice, A/Prof Leonie Callaway
Supervisor:	Prof Murray D Mitchell
Co-Investigator(s):	Dr Jennifer M Ryan, Dr Hsiu-Wen Chan, Dr Marloes Dekker Nitert, Dr Carlos Salomon, Ms Kanchan Vaswani, Ms Hassendrini N Peiris, A/Prof Keith Ashman
School(s):	UQ Centre for Clinical Research
Approval Number:	2013000381
Granting Agency/Degree:	Murray D Mitchell Start Up Fund
Duration:	31st October 2015

Comments:

Expedited review on the basis of approval from the Royal Brisbane & Women's Hospital HREC, dated 21/12/2012.

Note: If this approval is for amendments to an already approved protocol for which a UQ Clinical Trials Protection/Insurance Form was originally submitted, then the researchers must directly notify the UQ Insurance Office of any changes to that Form and Participant Information Sheets & Consent Forms as a result of the amendments, before action.

Name of responsible Committee:
Medical Research Ethics Committee
This project complies with the provisions contained in the *National Statement on Ethical Conduct in Human Research* and complies with the regulations governing experimentation on humans.

Name of Ethics Committee representative:
Professor Bill Vicenzino
Chairperson
Medical Research Ethics Committee

Signature _____ Date 29.5.13

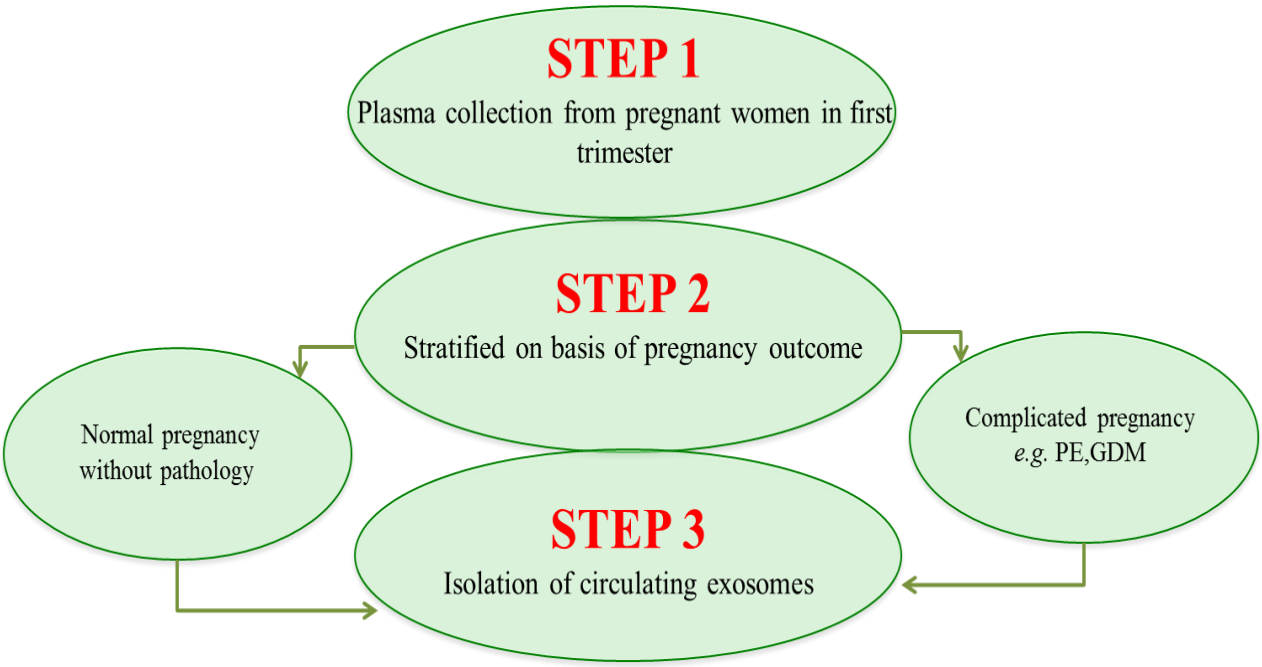


Figure 4. Plasma sample collection. Plasma was collected from healthy normal pregnant women at 6-12 gestational weeks and women with pregnancy complications *i.e.* preeclampsia and gestational diabetes mellitus in first trimester (11-14 weeks).

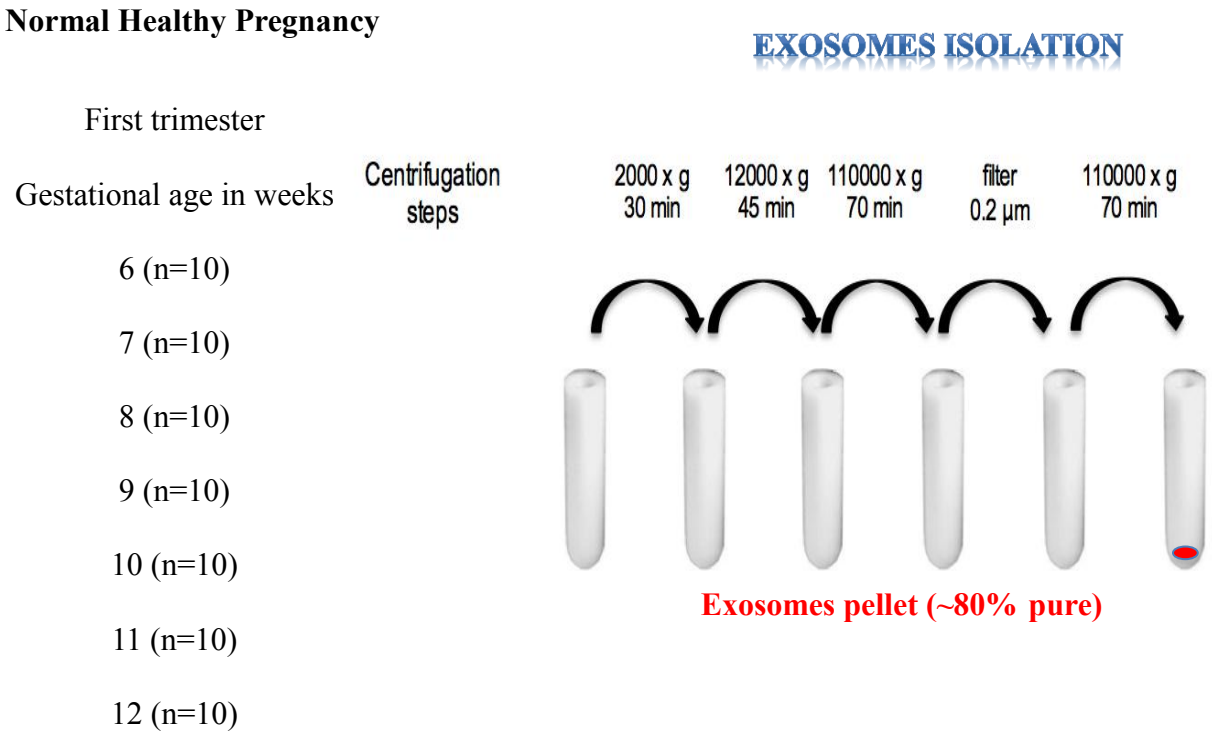


Figure 5. Steps of exosome isolation from plasma. Differential ultracentrifugation and ultrafiltration processes were involved to isolate exosome nanovesicles from plasma sample. n=10 (sample size) for each gestational age.

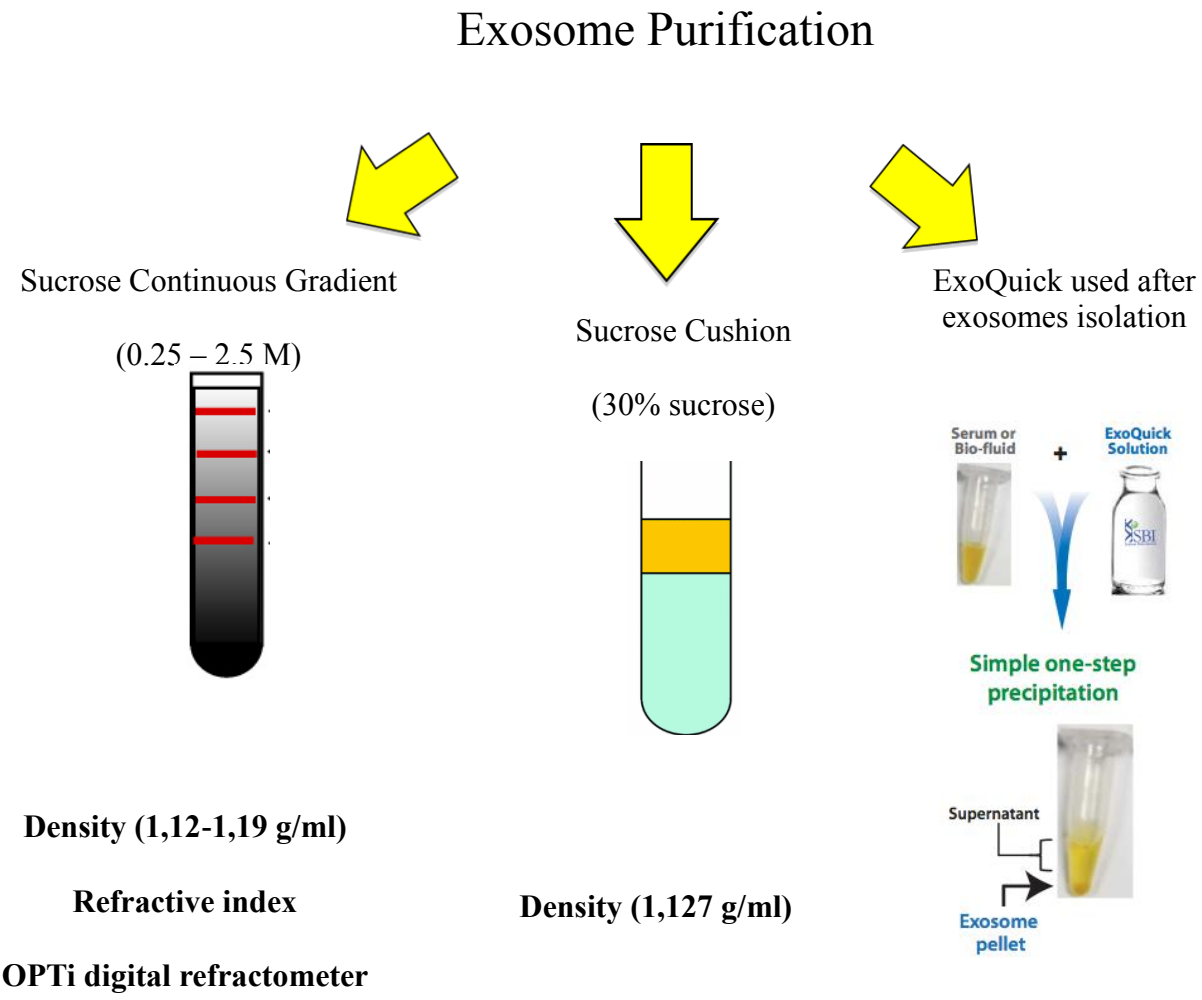


Figure 6. Different methods of exosome purification. Sucrose continuous gradient (0.25 M- 2.5 M), 30% sucrose cushion and ExoQuick kit were utilised to purify the isolated exosomes from plasma. Density of fractions was measured and compared with exosomal density.

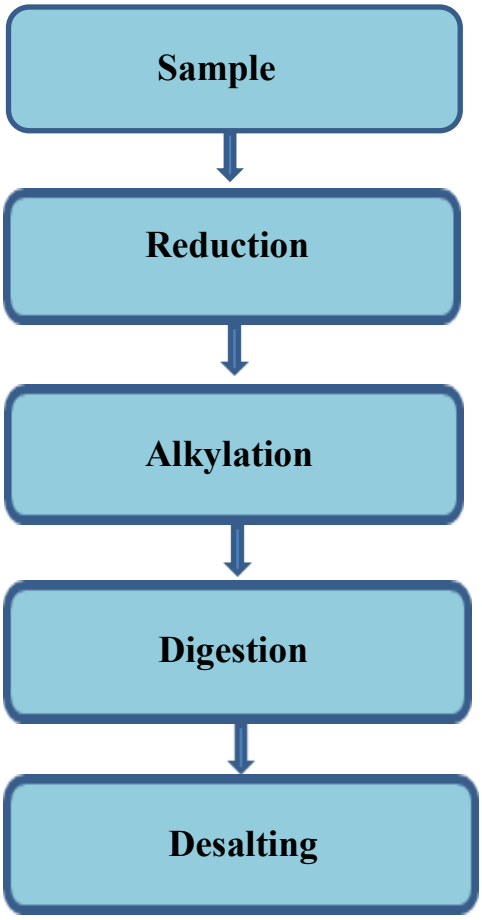


Figure 7. Workflow of exosomal sample processing for mass spectrometric protein analysis. 8 M urea was applied to exosome rich sample to breakdown the membrane of vesicles that releases the proteins (content of exosomes) in sample and afterwards reduction by using Dithiothreitol (DTT), alkylation by ammonium bicarbonate, digestion by trypsin enzyme and desalting to clean the peptides were performed.

4.2 Methods

Patient selection: Patients were selected based on certain demographic and clinical characteristics and plasma samples were collected in first, second and third trimester. In this study, only the first trimester samples were included. Ten healthy normal pregnant women at their 6-12 weeks (n=10 in each gestational week; total sample size is 70), 15 pregnant women at 11-14 weeks who developed preeclampsia later in their gestation (n=15 in first trimester) and 7 women at 11-14 weeks (n=7 in first trimester) who developed gestational diabetes mellitus in late second trimester were recruited. In addition, 5 non-pregnant women as control were recruited in this study with matching their demographic and clinical characteristics. Pregnant women underwent routine obstetrical care at Clinica Davilla (Santiago, Chile). All patients signed informed consent including the use of clinical data for research purposes and the protocol was approved by the Institutional Review Board of the University of Andes (Santiago, Chile). Obstetrical history and physical findings were recorded regarding previous spontaneous abortions, the course of previous pregnancies, hypertension, gestational diabetes and preeclampsia and different clinical and demographic characteristics *i.e.* maternal age, height, BMI, systolic blood pressure, HbA1c, basal glycaemic level. These characteristics were matched for each sample. Doppler ultrasound investigations of the umbilical arteries were also performed throughout the pregnancy period in all patients. The blood samples of those women were chosen who had none of these abnormal symptoms. Afterwards, all sample donors went through normal delivery at term.

Sample collection: Peripheral venous blood samples were collected from women with normal (n=10), preeclamptic (n=15) and gestational diabetic pregnancies (n=7; diagnosed in the second trimester between 22-28 gestational weeks) in EDTA treated tubes (BD Vacutainer® Plus plastic serum tube) from which plasma samples were separated by centrifugation at 4,200 x g at 4^{o}C for 10 minutes. The separated plasma samples were aliquot and stored at -80^{o}C until analysed (not more than three months). Care was taken to avoid repeated thaw and freeze cycles of the samples.

A time series experimental design was used to establish the variation in plasma exosome characteristics during normal pregnancy. All experimental procedures were conducted within an ISO17025 accredited (National Association of Testing Authorities, Australia) research facility. All data were recorded within a 21 CERF part 11 compliant electronic laboratory notebook (Iris note, Redwood city, CA, USA).

Isolation of extracellular vesicles (EVs): One (1) ml of plasma from each patient was utilized to isolate exosomes. The plasma was diluted with 1 ml of PBS (pH 7.4) and extracellular vesicles including exosomes were isolated through differential ultracentrifugation and ultrafiltration process (see Figure 8). Centrifugation was initially performed at 2,000 x g at 4^{o}C for 30 minutes (Thermo Fisher Scientific Ins., Asheville, NC, USA, Sorvall®, high speed microcentrifuge, fixed rotor angle: 90^{o}) followed by 12,000 x g at 4^{o}C for 45 minutes to eliminate cell debris from the supernatant fluid (~2 ml). Then the resultant supernatant fluid (~2 ml) was transferred to an ultracentrifuge tube (Sorvall® ultracentrifuge tubes) and was spun at 110,000 x g at 4^{o}C for 2 hours (Thermo Fisher Scientific Ins., Asheville, NC, USA, Sorvall®, T-8100, fixed angle ultracentrifuge rotor). The pellet was suspended in PBS and filtration was performed though 0.22 µm filter (Steritop™, Millipore, Billerica, MA, USA). The filtered solution was centrifuged finally at 110,000 x g at 4^{o}C for 70 minutes. The pellet was resuspended in 50 µl of PBS. Proteins were measured by using the DC™ Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) in each sample.

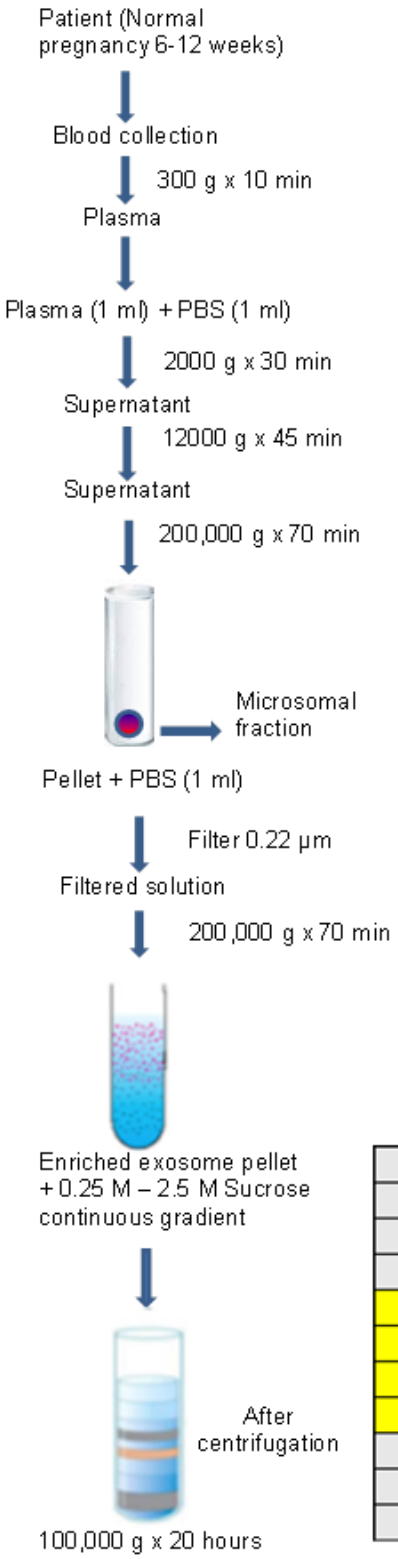
Purification of exosomes: EVs isolated from plasma were used to determine the more suitable method for purifying the exosome vesicles. The isolated exosomes were further enriched in samples through different purification procedures: (i) sucrose continuous gradient, (ii) 30% sucrose cushion and (iii) ExoQuick™ (System Bioscience).

(i) EVs were added on the top of the 0.25M-2.5M sucrose continuous gradient which was prepared through utilization of gradient maker (Hoefer SG30 gradient maker, GE Healthcare, NSW, Australia) and ultracentrifuged at 110,000 x g at 4^{o}C for 20 hours (Thermo Fisher Scientific Ins., Asheville, NC, USA, Sorvall®, SureSpinTM 630/360, Swinging-Bucket ultracentrifuge rotor). Ten fractions containing 3ml each were collected from bottom to top using highly reproducible automated fraction collector machine and the refractive indices were detected in those layers using the OPTi digital refractometre (Bellingham+Stanley Inc., Lawrenceville, GA, USA). The density of individual fractions was determined from the refractive index. Fractions that revealed the similar density to exosomes (1.11-1.19 g/ml) were again ultracentrifuged at 110,000 x g at 4^{o}C for 2 hours after adding PBS (60 ml). The pellet were dissolved in 50µl of PBS and preserved at -80^{o}C which seem to contain ~99% pure exosomes.

ii) In 30% sucrose cushion purification method, the EVs containing exosomes vesicles was added onto the top of 30% sucrose cushion and then ultracentrifuged at 110,000 x g at 4^{o}C for 70 minutes.

The comparatively pure exosomal solution was collected from the interface of the sample and the cushion by using 18G needle. This collected solution was further ultracentrifuged at 110,000 x g at 4^{o}C for 70 minutes. The pellets were dissolved in 50µl of PBS.

iii) EVs were diluted in 250 µl PBS and then 63 µl of ExoQuick solution was added. This solution was incubated for overnight. Sample was centrifuged at 1,500 x g for 30 minutes and the pellet was resuspended in 100 µl PBS.



Fractions	Density (g/ml)
1 st	1.069
2 nd	1.085
3 rd	1.101
4 th	1.122
5 th	1.137
6 th	1.171
7 th	1.197
8 th	1.226
9 th	1.247
10 th	1.258

Figure 8. Flow diagram for exosome isolation and purification procedure based on differential ultracentrifugation, ultrafiltration and sucrose density gradient centrifugation. Flow chart for exosome purification procedure based on differential ultracentrifugation to isolate extracellular vesicles removing contaminating cellular debris and dead cells. The flow chart for the exosome purification procedure based on sucrose continuous gradient centrifugation showing exosome enriched fractions in yellow 4-7.

Protein concentration determination: Total exosomal protein was estimated by utilizing the DC™ Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Sample was diluted with RIPA buffer (1:5) and then sonicated (Elmasonic S15H, iLABEQUIPMENT, New Jersey, USA) 3 times for 5 minutes to break down the membrane of the nanovesicles and release the protein content into the solution. In order to build the calibration curve; 0, 100, 200, 400, 800, 1000, 1500 times diluted solution of Bovine Serum Albumin (BSA, SIGMA-ALDRICH Life Science, Australia) were applied in triplicate manner into a 96 standard well plate. The samples were applied in duplicate manner. Two colorimetric reagents (Reagent A 25µl & Reagent B 200 µl, Bio Rad DC™ Protein Assay) were applied and the plate was covered with aluminium foil and then incubated for 15 minutes in dark for colour change. The absorbance was read at 750 nm wavelengths in a spectrophotometric plate reader (SPECTROstarNano, BMG Labtech, Australia). The device detects the absorbance of the colours that are produced after chemical reaction. From the absorbance protein concentration was determined. The limit of detection of the protein in BioRad DC™ protein assay is 0.1-2.0 mg/ml.

Identification of nanoparticles by NanoSight: NTA (Nanoparticle Tracking Analysis) measurements were performed using a NanoSight NS500 instrument (NanoSight NTA 2.3 Nanoparticle Tracking and Analysis Release Version Build 0033) following the manufacturer's instructions. The NanoSight NS500 instrument measured the rate of Brownian motion of nanoparticles. It contains a light scattering system that provides a reproducible platform for specific and general nanoparticle characterisation ((NanoSight Ltd., Amesbury, United Kingdom). Samples were processed in duplicate manner and diluted with PBS at concentration to obtain between 10 and 100 particles per image (optimal value is ~50 particles x image). The samples were mixed before introduction into the chamber (temperature: 25^{o}C and viscosity: 0.89 cP). The camera level was set to obtain an image that has sufficient contrast to clearly identify particles while minimising background noise during recording video (camera level: 10 and capture duration: 60 s). After capturing videos (2 videos per sample) these were processed and analysed. A combination of high shutter speed (450) and gain (250) followed by manual focusing enables optimum visualisation of maximum number of vesicles. A minimum of 200 tracks were completed per video. NTA post acquisition settings were optimised and kept constant between samples (Frames Processed: 1496 of 1496, Frames per Second: 30, camera shutter: 20ms; Calibration: 139 nm/pixel, Blur: 3x3; Detection Threshold: 10; Min Track Length: Auto; Min Expected Size: Auto). Each video was then

analysed to calculate the mean, mode, and median size of particles together with an estimate of the number of particles. An Excel spreadsheet (Microsoft Corp., Redmond, Washington) was also automatically generated, showing the concentration at each particle size.

Transmission electron microscopy (TEM): Exosome rich sample (30 µg protein) was fixed with an equal volume of buffer 3% (w/v) glutaraldehyde (pH 7.4) which is suitable for TEM. Exosome depleted sample was also prepared by performing ultrasonication (Elmasonic S15H iLABEQUIPMENT, New Jersey, USA) 3 times for 5 minutes and was fixed with glutaraldehyde (3% w/v). These two samples were then applied to a continuous carbon grid and negatively stained with uranyl acetate (2%). The samples were observed and examined in an FEI Tecnai 12 transmission electron microscope (FEITM, Hillsboro, Oregon, USA).

Quantification of placental-derived exosomes: (i) Total exosome vesicles: the exosome concentration in maternal circulation was determined using the total exosomal CD63 protein markers which was measured by ELISA (ExoELISA^{TM}, System Biosciences, Mountain View, CA) according to the manufacturer's instructions. The samples containing 10 µg of exosomal protein were bound and immobilised to a micro-titre ELISA plate wells using a binding buffer. The sample was then incubated by using monoclonal exosome specific primary antibody (Anti CD63) for an hour at room temperature with shaking. The plate was washed 3 times for 5 minutes with the wash buffer solution and then incubated with exosome validated secondary antibody (1:5000) for an hour at room temperature with shaking. Plate was washed again and incubated with super-sensitive TMB ELISA substrate at RT for 45 minutes with shaking in dark. Finally by using the stop buffer solution desired level of colour change was achieved by terminating the reaction. The number of exosome particles was determined in the samples from the absorbance identified at 450 nm spectrophotometric analysis and also by using an exosome protein standard curve calibrated by NanoSight instrument (provided by ExoELISA^{TM} kit). (ii) Placental-derived exosomes: PLAP (Placental Alkaline Phosphatase) is a specific protein marker of exosomes that is predominantly released from the placental trophoblast cells. To quantify the concentration of exosomal PLAP, a commercial ELISA kit (MBS701995, San Diego, CA, USA) was used and the protocol was followed according to the manufacturer's instructions. The samples containing 10 µg of exosomal protein (purified using sucrose continuous gradient) were added to each well of a micro-titre plate

coated with anti-PLAP antibody and incubated at 37^{o}C for 30 min, then was washed 3 times for 20 sec. 50 µl of HRP-conjugate (50 µl secondary antibody) was added to each well and incubated at 37^{o}C for 20 min. The plate was washed and incubated with 50 µl of substrate A and 50 µl of substrate B at 37^{o}C for 15 min. The incubation was terminated using 50 µl of stop solution at RT for 2 min with shaking. Absorbance was measured at 450 nm. The concentration of PLAP was determined from the absorbance.

Mass spectrometry for peptide profiling: 15 µl volumes containing 25 µg of protein from the exosomal fractions obtained in each gestational week were used for the LC-MS/MS sample processing. Reduction, alkylation, digestion and desalting were performed to remove all the contaminants from the sample and also to split the proteins into simple peptide form. Isolated exosome preparation was adjusted to 8 M urea in 50 mM ammonium bicarbonate (pH 8.5) solution. Reduction was performed with 20 mM Dithiothreitol (DTT) in 100 mM NH_4HCO_3 solution and was heated up at 60^{o}C for an hour. Alkylation was done with 100 mM Iodoacetamide (IAA) in 100 mM NH_4HCO_3 and was incubated at 37^{o}C for an hour. Trypsin (Promega) enzyme was used to digest the sample for overnight at 37^{o}C and salts were removed using stage micro column tips which were prepared in Eppendorf gel loader tips using the Empore C18 membrane. Digested peptides were further extracted with 60 µl 0.1% Formic acid in Acetonitrile (ACN) and 0.1% Trifluoroacetic acid (TFA). The sample was then dried by centrifugal evaporation and then redissolved in 50 µl of 1% Formic Acid (HCOOH). The sample was centrifuged at 15000 x g at RT for 2 min and 40 µl volumes from the top was transferred to the mass spectrometer glass tubes and analysed by LC-MS/MS. 5600 Triple TOF mass spectrometry-based profiling approach was utilised to identify peptides- which is a cutting edge Liquid Chromatography (LC) and Mass Spectrometry (MS) LC/MS/MS instrumentation.

Database searching and protein identification: Peak lists for each LC-MS/MS run were merged into a single MASCOT generic format file for MASCOT searches. LC-MS/MS spectra were searched against the human protein database using MASCOT search library. Peptide identifications were deemed significant if the ion score was greater than 30. The digested protein samples were analysed using a 5600 Triple TOF mass spectrometer (AB/Sciex) to obtain initial high mass accuracy survey MS/MS data to identify the peptides present in the samples. The in depth

proteomic analysis using Information Dependent Acquisition (IDA) experiments on the 5600 Triple TOF MS utilized an enhanced MS survey scan (m/z 350–1500) followed by 50 data-dependent product ion scans of the 50 most intense precursor ions.

Statistical analysis: Data were presented as mean ± SEM, for n=10 different patients with normal pregnancy, n=15 patients with preeclampsia, n=7 patients with gestational diabetes mellitus and n=5 non-pregnant women. The effect of gestational age on plasma exosome number, exosomal protein and PLAP concentrations were assessed using ANOVA, with variance partitioned between gestational age and subject. Statistical difference between group means was assessed by Dunnett's test to compare each treatment to the control group where the data distribution approximates normality and by Mann-Whitney U-test for distribution independent data analysis. Two group means were statistically assessed by Student's t-test. Statistical significance was defined as at least $p<0.05$.

CHAPTER V

5. RESULTS

5.1 Clinical data analysis

A set of demographic and clinical characteristics of pregnant women in the first trimester of pregnancy have been analysed and compared in three different groups: healthy uncomplicated pregnancies (n=10) and pregnancies complicated with preeclampsia (n=15) and gestational diabetes mellitus (n=7) that were diagnosed in second or third trimester of pregnancy. Maternal age, body weight, height, BMI, arterial blood pressure, echocardiographic findings, left and right uterine artery pulsatility index (diagnosed with transvaginal or transabdominal uterine artery doppler ultrasound) have been presented in Table II, III and IV. All pregnant women included in this study were normotensive (systolic / diastolic BP) (*i.e.* mean ± SD value for normal pregnancy 107.2 ± 10.94/64.62 ± 8.77; with GDM 111.4 ± 8.99/67.71 ± 9.12 and with PE 118.8 ± 13.71/71.93 ± 15.43 mmHg), had singleton pregnancy with no other medical or obstetrical complications. Mean maternal age with healthy pregnancy outcome was 24 ± 6.01 years; 29.14 ± 6.28 years with GDM and 29.93 ± 6.26 years with PE. Maternal body mass index (BMI) was calculated 25.91 ± 4.31 kg/m^2 in those women who had uncomplicated pregnancies. Fetal and placental weights were within normal range for both complicated (GDM) and uncomplicated pregnancies. The average values for right and left uterine artery pulsatility index were identified as within normal range according to gestational age for pregnancies complicated with preeclampsia and gestational diabetes mellitus. A comparative analysis of these characteristics in three different groups has been presented in Table V.

5.2 Exosome isolation and purification from plasma

Comparison of different methods of purification of exosomes

The total exosomal protein concentration was measured using DC™ Protein Assay in the initial plasma sample and in the samples following several steps of ultracentrifugation. There were 20-fold reductions in the amount of total proteins in the exosome enriched sample following differential ultracentrifugation compared to total protein concentration in plasma (Figure 9A). The

protein was also estimated and compared following ultracentrifugation and 3 different methods of exosome purification *i.e.* using 30% sucrose, sucrose continuous gradient and ExoQuick^{TM} kit. The total protein concentration reduced significantly ($p<0.05$) in the samples following these three purification steps compared to ultracentrifugation simply (Figure 9B). However, ExoQuick yielded more protein in the sample in comparison with 30% sucrose and sucrose gradient; though the difference in protein concentration was negligible using sucrose gradient and sucrose cushion (p-value was 0.115).

The microsomal (including exosomes) sample following purification steps was analysed by the NanoSight, which is a nanoparticle tracking device. The majority of the nanoparticles with size range of 30-150 nm were present in the sucrose purified samples. On the contrary, ExoQuick purified sample yielded less exosome like vesicles revealing size range 30-300 nm (Figure 10). It indicated that exosomal population was enriched in the sample after purification procedures.

The purity of these isolated vesicles was determined based on electron microscopic analysis, estimation of protein concentration, quantification of nanoparticles using NanoSight^{TM} technology and quantification of number of exosome vesicles using ELISA for exosome specific marker CD63. The analysis on the characterisation revealed that sucrose density gradient centrifugation procedure yielded pure exosome vesicles compared to other two methods. Therefore, sucrose density gradient centrifugation method was considered the best method of exosome purification as this method can yield higher protein amount that mostly originate from exosome nanovesicles showing size range of 30-150 nm which is similar to reported exosomal size range. The mean ± SD value of exosome size was 98 ± 39 nm after sucrose density gradient centrifugation purification procedure which was determined using NanoSight^{TM} device.

Exosome characterization

Maternal plasma exosomes isolated by differential and sucrose density gradient centrifugation were characterised by a buoyant density of 1.122 to 1.197 g/ml (fractions 4 to 7; see Figure 8). Nanoparticle tracking analysis showed a particle size distribution in 200,000 x g pellet (Figure 11A) ranging from 30 to 300 nm in diameter that corresponds to microsomal fraction (including exosomes particles) with an average size of 147 ± 71 nm (mean ± SD). After the sucrose continuous gradient centrifugation, the enriched exosomal fractions (1.122 to 1.197 g/ml) were mixed and a particle size distribution ranging from 50 to 140 nm in diameter, with an average of 98 ± 39 nm

(mean ± SD) (Figure 11B) was obtained. Electron microscopy revealed the presence of spherical vesicles, with a typical cup-shaped appearance and diameters ranging from 30 to 120 nm (Figure 11B, insert and Figure 12).

Table II. Clinical characteristics of patients in first trimester (6-12 weeks) of healthy normal pregnancy

Patient	**Maternal Data**				**Fetal and Placental Data**			**First Trimester**		
ID	**Age**	**Weight (Kg)**	**Height (metre)**	**BMI (Kg/m^2)**	**Fetal Sex**	**Placental weight (grams)**	**Fetal Weight (grams)**	**Left IP**	**Right IP**	**Blood Pressure (systolic/ diastolic)**
34	23	54.5	1.55	22.68	M	610	3365	2.51	2.43	90/60
39	21	54	1.62	20.57	NO DATA	NO DATA	NO DATA	1.8	1.77	100/60
48	22	108	1.73	36.08	F	712	3020	1.17	1.63	120/80
59	28	69	1.63	25.97	M	NO DATA	3975	1.13	1.5	100/60
61	19	57	1.49	25.67	F	702	3550	1.81	1.37	90/50
81	36	66	1.51	28.94	M	569	3005	2.76	2.79	120/80
85	31	54	1.52	23.37	NO DATA	NO DATA	NO DATA	0.94	0.71	110/60
104	20	64	1.51	28.06	M	731	3520	1.54	0.92	100/70
106	31	62	1.65	22.77	NO DATA	607	3885	3.4	1.49	110/60
108	22	57	1.51	24.99	F	543	4175	1.79	1.08	110/70
109	16	59	1.63	22.20	F	696	3095	1.92	1.13	120/60
113	17	57.5	1.55	23.93	M	501	2660	2.26	1.99	104/60
115	26	86	1.65	31.58	M	625	3690	1.64	0.69	120/70
Mean ± SD	24 ± 6.01	65.23 ± 15.52	1.58 ± 0.07	25.91 ± 4.31		629.6 ± 78.42	3449 ± 469.4	1.89 ± 0.69	1.5 ± 0.63	107.2 ± 10.94/64. 62 ± 8.77

Data were presented as mean ± SD (range). Fetal sex ‘F’ indicates female fetus and ‘M’ indicates male fetus. Left and right uterine arterial pressure was measured as pulsatility index (IP).

Table III. Clinical characteristics of patients in first trimester (11-14 weeks) diagnosed with gestational diabetes mellitus (GDM)

Patient	Maternal Data				Fetal and Placental Data			First Trimester		
ID	**Age**	**Weight (Kg)**	**Height (metre)**	**BMI (Kg/m^2)**	**Fetal Sex**	**Placental weight (grams)**	**Fetal Weight (grams)**	**Left IP**	**Right IP**	**Blood Pressure (systolic/ diastolic)**
56	25	69	1.62	26.29	F	697	3720	1.65	1.22	110/60
58	34	63	1.55	26.22	F	535	2340	1.2	0.88	120/80
143	32	75	1.65	27.54	F	590	3190	0.66	0.54	120/80
175	24	84	1.61	32.40	M	652	3530	1.25	1.2	100/60
183	24	73	1.54	30.78	M	730	3800	1.52	1.43	120/70
207	40	62	1.51	27.19	M	700	3635	0.91	1.21	100/60
353	25	40	1.58	16.02	NO DATA	NO DATA	NO DATA	1.87	1.77	110/64
Mean ± SD	29.14 ± 6.28	66.57 ± 13.91	1.58 ± 0.04	26.64 ± 5.23		650.7 ± 74.75	3369 ± 547.1	1.29 ± 0.42	1.17 ± 0.39	111.4 ± 8.99/67.71 ± 9.12

Data were presented as mean ± SD (range). Fetal sex ‘F’ indicates female fetus and ‘M’ indicates male fetus. Left and right uterine arterial pressure was measured as pulsatility index (IP).

Table IV. Clinical characteristics of patients in first trimester (11-14 weeks) diagnosed with preeclampsia (PE)

Patient	Maternal data			First Trimester of Pregnancy					
ID	**Age**	**Weight (kg)**	**Height (cm)**	**ECO**	**Art. Pressure**	**L Doppler**	**R Doppler**	**Gest. Age (weeks)**	**Gest. Age (days)**
7	25	80	154	29/04/2008		2.58	1.05	13	6
31	31	74	162	15/07/2008	120/70	1.4	2.03	12	4
40	32	65	153	24/07/2008	120/70	2.37	3.2	13	1
57	34	59	161	19/08/2008	110/70	2.53	4.4	13	4
104	20	64	151	30/10/2008	100/70	1.54	0.92	12	6
125	36	78.5	159	11/12/2008	120/80	2.17	2.03	12	3
137	33	84.9	149	20/01/2009	130/30	2.12	3.06	11	
156	36	69.5		10/03/2009	130/80	0.85	0.92	13	1
190	40	80	162	19/05/2009	110/80	1.07	0.94	12	5
192	18	65	160	26/05/2009	112/70	1.91	3.13	11	1
196	23	53	149	2/06/2009	100/60	2.79	5.21	12	
216	26	98	168	9/07/2009	140/80	1.46	2.71	12	6
319	30	86	151	21/01/2010	110/70	1.95	1.31	13	2
385	32	124	159	20/07/2010	146/102	1.17	2.01	13	
469	33	59	163	9/11/2010	115/75	1.28	1.06	12	2
Mean ± SD	29.93 ± 6.26	75.99 ± 18.02	157.2 ± 5.97		118.8 ± 13.71/71.93 ± 15.43	1.81 ± 0.60	2.26 ± 1.33	12.27 ± 0.70	3.41 ± 2.02

Data were presented as mean ± SD (range). Fetal sex ‘F’ indicates female fetus and ‘M’ indicates male fetus. Left and right uterine arterial pressure was mentioned as L and R Doppler. The date when echocardiography (ECO) was performed has been mentioned.

Table V. Comparative analysis of clinical characteristics of pregnant women in first trimester diagnosed with normal healthy pregnancy, gestational diabetes mellitus and preeclampsia

Condition	**Maternal Data**				**First Trimester of Pregnancy**		
	Age (years)	Weight (kg)	Height (metres)	BMI (kg/m^2)	Left IP	Right IP	Blood Pressure (systolic/diastolic)
Normal healthy pregnancy	24 ± 6.01	65.23 ± 15.52	1.58 ± 0.07	25.91 ± 4.31	1.89 ± 0.69	1.5 ± 0.63	107.2 ± 10.94/64.62 ± 8.77
Gestational diabetes mellitus	29.14 ± 6.28	66.57 ± 13.91	1.58 ± 0.04	26.64 ± 5.23	1.29 ± 0.42	1.17 ± 0.39	111.4 ± 8.99/67.71 ± 9.12
Preeclampsia	29.93 ± 6.26	75.99 ± 18.02	1.57 ± 0.05	NO DATA	1.81 ± 0.60	2.26 ± 1.33	118.8 ± 13.71/71.93 ± 15.43

Data were presented as mean ± SD (range). Left and right uterine arterial pressures were measured as pulsatility index (IP).

A B

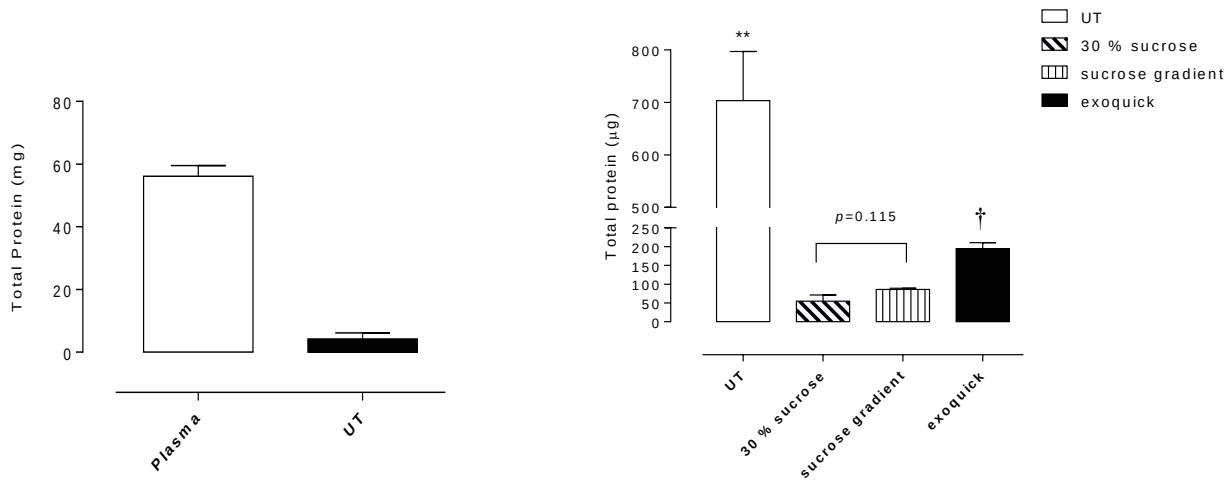


Figure 9. Comparison of different methods of purification of exosomes. The total protein concentration in plasma and following differential ultracentrifugation **(A)**. Comparison in protein concentration following ultracentrifugation (UT) and purification of exosomes using 30% sucrose, sucrose continuous gradient and ExoQuick **(B)**. **Significant ($p<0.001$) reduction in total protein concentration following purification. †Exoquick solution yielded significant ($p<0.05$) amount of total protein compared to 30% sucrose and sucrose continuous gradient centrifugation methods of exosome purification.

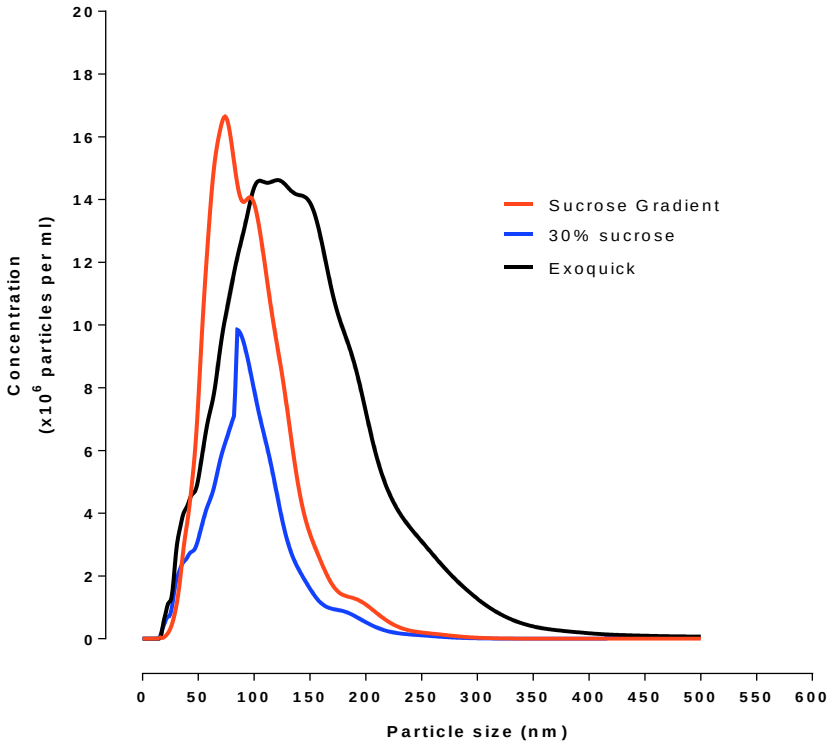


Figure 10. Size distribution of exosome vesicles using nanoparticle tracking analysis (NanoSight) following exosome purification by sucrose continuous gradient, 30% sucrose cushion and ExoQuick™. The nanoparticles with size range of 30-150 nm were present in higher population in exosomal sample purified by 30% sucrose and sucrose continuous gradient and of 30-300nm in ExoQuick purified sample. The mean ± SD value of the exosome size after sucrose gradient purification was 98 ± 39 nm and 147 ± 71 nm after purification using ExoQuick™ kit.

A **B**

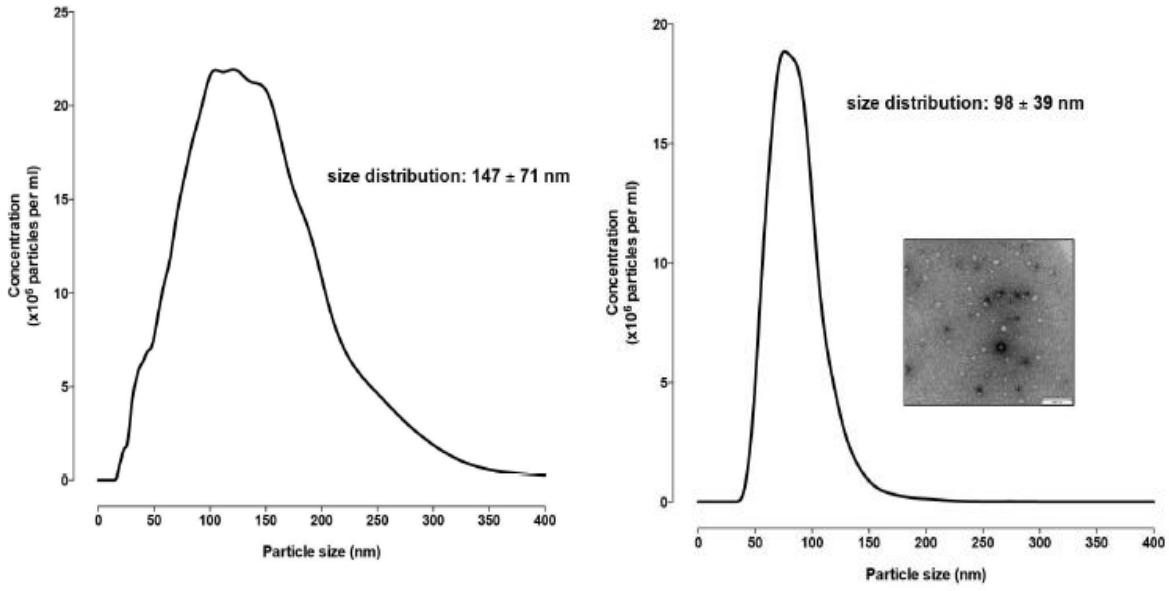


Figure 11. Characterisation of exosomes from maternal circulation. Exosomes were isolated from women with uncomplicated pregnancies during first trimester by differential and buoyant density centrifugation (see Methods). (A) Representative particles size distribution of microsomal fraction. Exosomes were purified based on sucrose continuous gradient centrifugation. (B) Representative particles size distribution of enriched exosomal fractions. Insert: Representative electron micrograph of pooled enriched exosome fractions, Scale bar 200 nm.

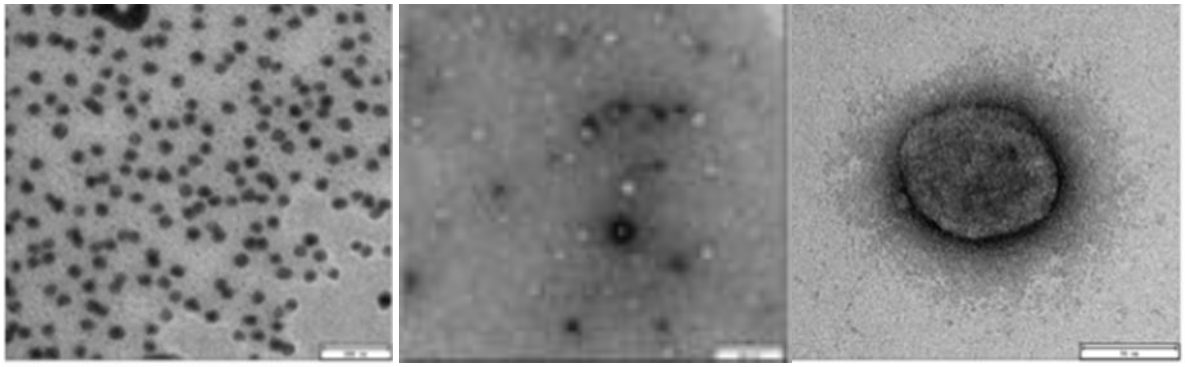


Figure 12. Transmission Electron Microscopic analysis of exosomes from plasma of normal healthy pregnant women following purification by sucrose continuous gradient. Numerous spherical cup shaped exosome like vesicles were present. The mean size of exosomes detected in the electron microscopic analysis was 34.16nm. The scale bar is 200 nm in the left two figures and 100 nm in the right figure.

5.3 Exosomal stability in frozen stored plasma

This study utilised exosomes from frozen stored (30 days) plasma sample; therefore, it was important to evaluate exosomes' stability in frozen condition. The stability of exosomes after a freeze and thaw cycle was assessed using fresh and frozen plasma. No significant difference was observed using fresh or frozen plasma in exosome quantification, exosomal marker expression, exosomal microRNA expression and exosomal protein content (Figure 13A-D and Table IX in Appendix A).

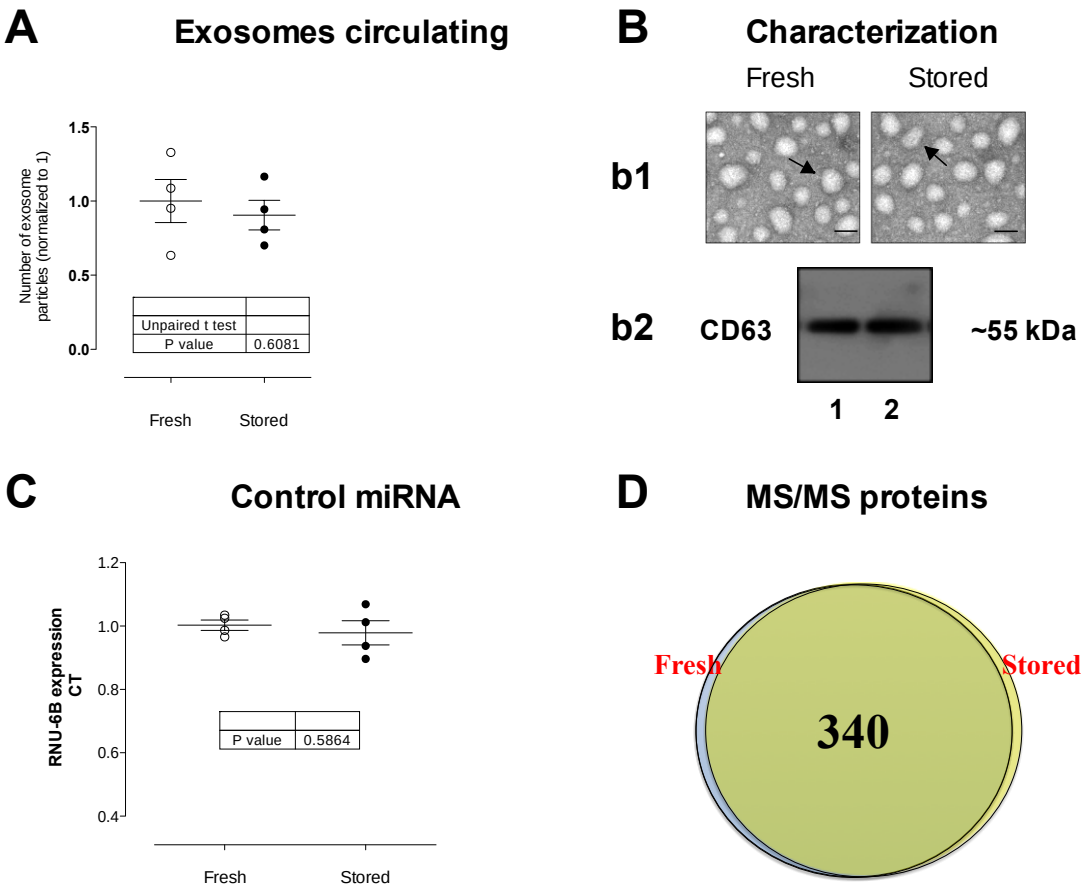


Figure 13. Characteristics of exosomes isolated from plasma immediately after phlebotomy and after 30 days stored at -80°C. (A) Number of exosome particles. (B) Exosomes characterization. b1: electron microscope (scale bar 100 nm) and b2: Western blot for CD63 (exosomal marker); lane 1: Fresh and lane 2: stored. (C) Expression of miRNA RNU6B in exosomes. (D) The Venn diagram of total number of proteins identified in fresh and stored exosomes isolated from plasma in normal healthy pregnant women (n=10) during the first trimester (6-12 weeks)

5.4 Plasma exosomal protein characterization in first trimester of normal healthy pregnancy

Difference of exosome release in pregnant and non-pregnant women

Exosomes were isolated from plasma during the first trimester in women with normal pregnancy (n=10) outcome without any complications and from women who were not pregnant (n=5). The number of exosomes was quantified in exosome rich samples using ELISA for CD63 (specific exosomal endocytic marker) where the total number of exosome particles in non-pregnant women was $0.6e^{11} \pm 0.2e^{11}$ (mean ± SD) and in pregnant women during first trimester was $1.33e^{11} \pm 1.27e^{11}$ (mean ± SD) and the statistical analysis (Student's t- test) was performed to compare between the samples. The number of exosomes displayed more than two- fold ($p<0.05$) increase in pregnant women's plasma exosomes compared to non-pregnant women's plasma exosomes (Figure 14).

Placenta-derived exosomes increased during first trimester of normal pregnancy

Pooled exosome-containing fractions (*i.e.* fractions 4 to 7) were further characterised by determining the number of exosome particle (NEP) and exosomal PLAP concentration in serial samples of maternal plasma obtained during the first trimester of pregnancy (*i.e.* 6-12 weeks).

The gestational age variation in plasma exosome number was analysed by two-way ANOVA with the variance partitioned between gestational age and subject. A significant effect of gestational age was identified (n=69, one missing value, $p<0.005$). A post-hoc multiple range test was used to identify statistically significant ($p <0.05$) differences between pairwise comparisons (Figure 15A). In addition, a significant effect of subject was identified (n=69, one missing value, $p<0.05$) (Figure 15B). In addition, NEP and gestational age (*i.e.* 6-12 weeks) displayed a significant positive linear relationship ($r^2 = 0.202$, $p < 0.001$, n=69, one missing value).

To assess gestational age associated variation in placenta-derived exosomes, exosomal immunoreactive (IR) PLAP was quantified using a commercial ELISA kit (see Methods). IR exosomal PLAP concentrations were analysed by two-way ANOVA with the variance partitioned between gestational age and subject. A significant effect of gestational age was identified ($p<0.0001$, n=69, one missing value) (Figure 15C). A post-hoc multiple range test was used to identify statistically significant ($p <0.05$) differences between pairwise comparisons (Figure 15D). No significant effect of patient on IR exosomal PLAP concentration was identified ($p=0.123$). Immunoreactive exosomal PLAP concentration and gestational age displayed a significant positive linear relationship ($r^2 = 0.711$, $p < 0.001$, n=69, one missing value).

Specific placental-derived exosomes

Exosomal PLAP concentration and exosome number were subjected to correlation analysis. The fitted linear model was described by the following equation: plasma exosomal PLAP pg/ml = 85.6 + 5.47 x 10^{-11} x exosome number/ml ($p < 0.05$, n= 69, one missing pair). The coefficient of determination (r^2) was 0.78 (Figure 16A) that indicated the simultaneous increase in the total exosome vesicles per ml of plasma and the concentration of placenta specific exosome marker (exosomal PLAP in pg/ml). However, no linear correlation was observed.

To estimate changes in the relative contribution of placental exosomes to total exosomes present in maternal plasma and to identify gestational age-related changes, the apparent PLAP content per 10^9 exosome (PLAP ratio) was determined. Overall PLAP ratio averaged 2.01 ± 0.33. The effects of gestational age on PLAP ratio were assessed by Kruskal-Wallis one-way ANOVA. No significant effect of gestational age on PLAP ratio was identified ($p = 0.06$) (Figure 16B).

The number of total exosome particle released into the peripheral circulation (CD63), amount of placenta specific exosomal marker (PLAP) and placental exosomal contribution to total exosomes in the circulation (PLAP/CD63) in each individual gestational week of mid-first trimester have been provided in Table VI.

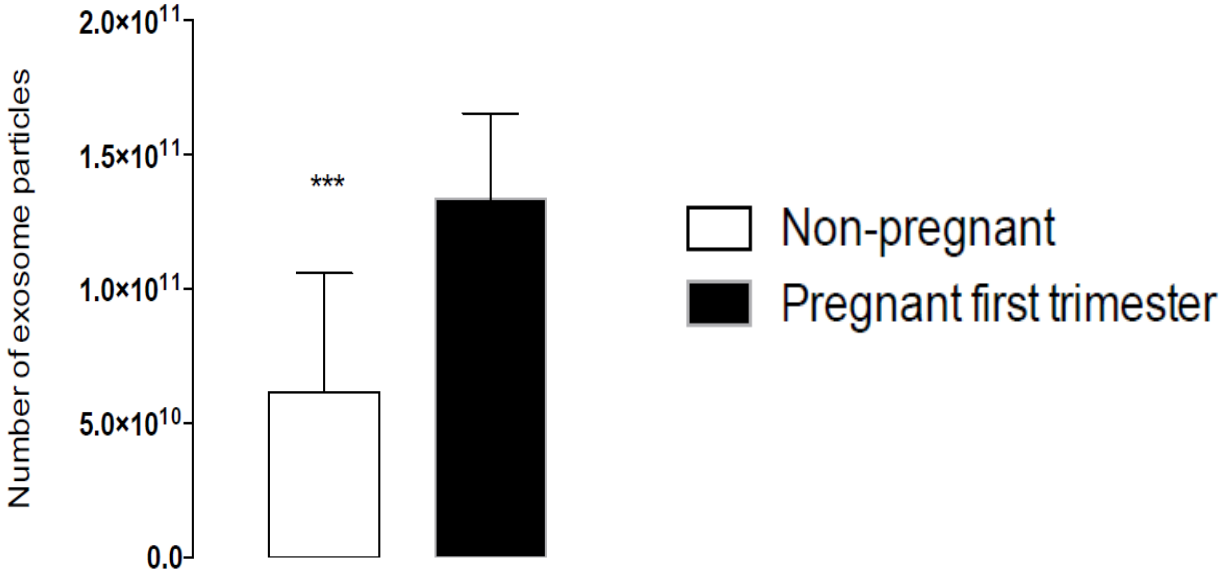


Figure 14. Exosome release in pregnant and non-pregnant condition. Exosomes were isolated from plasma during first trimester in women with normal healthy pregnancy and from non-pregnant women (control). The number of exosomes were quantified in the conditions and compared. The total number of exosome particles in non-pregnant women was $0.6e^{11} \pm 0.2e^{11}$ (mean ± SD) and in pregnant women during first trimester was $1.33e^{11} \pm 1.27e^{11}$ (mean ± SD). Exosome release increased by twofold ($p<0.05$) during first trimester of pregnancy than non-pregnancy condition.

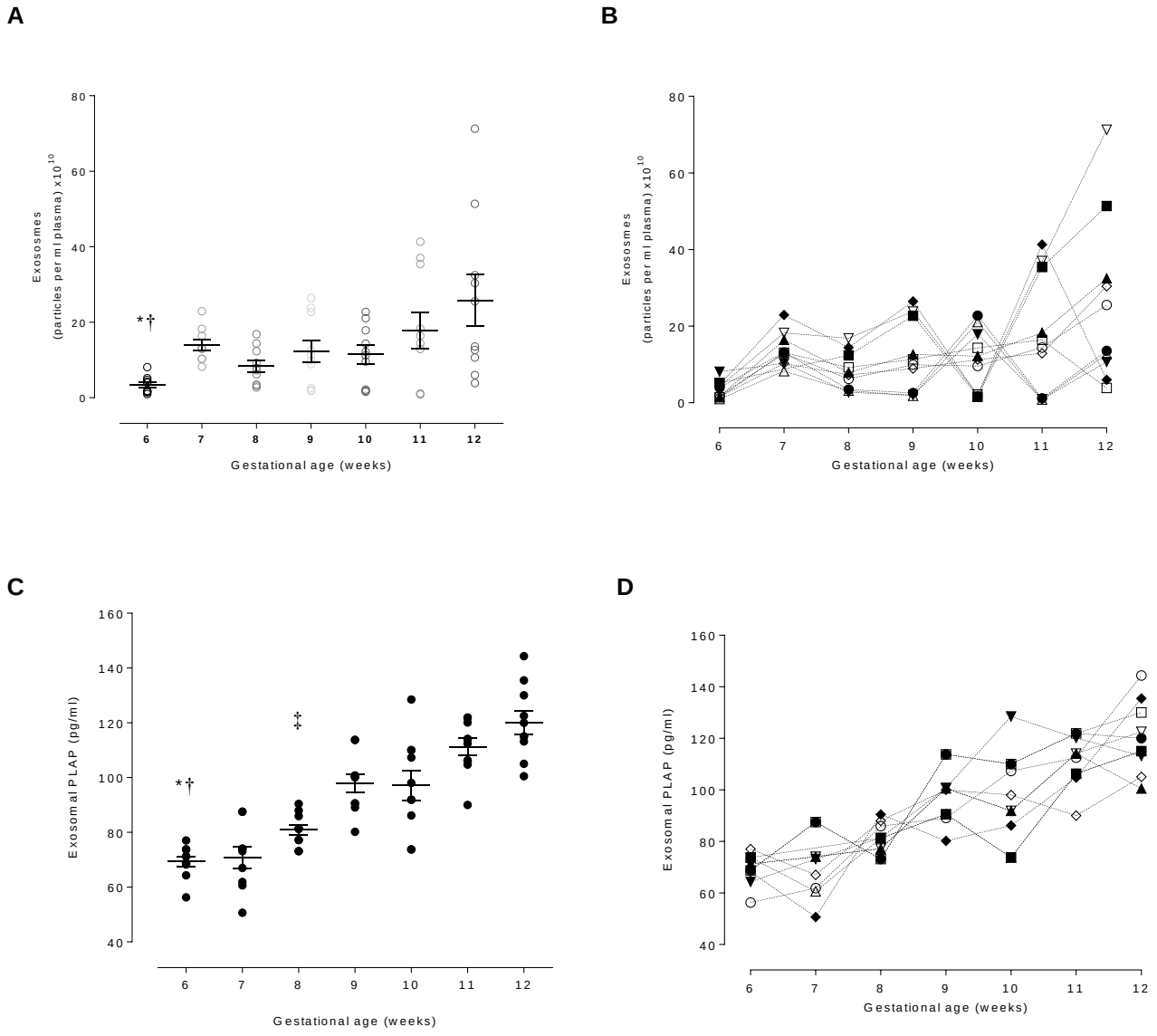


Figure 15. Exosome profiling across first trimester pregnancy. Enriched exosomal population (*i.e.* number of exosome particles) and placenta-derived exosomes (*i.e.* exosomal PLAP) were quantified in peripheral plasma of women in the first trimester of pregnancy by ELISA. (A) exosomes as particles per ml plasma. (B) individual variation in exosome number for each week (C) exosomal PLAP during first trimester of pregnancy (*i.e.* 6-12 weeks). (D) individual variation in exosomal PLAP for each week. Data are presented as aligned dot plot and values are mean ± SEM. In A, two-way ANOVA **p= 0.0048, Dunn's post-hoc test analysis = *p < 0.05 6 vs. 7 weeks and †*p*<0.005: 6 vs. 12 weeks. In C, two-way ANOVA ***p<0.0001, Dunn's post-hoc test analysis = *p < 0.05 6 vs. 9 and 10 weeks, †*p*<0.005: 6 vs. 11 and 12 weeks, and ‡*p*<0.005: 8 vs. 11 and 12 weeks.

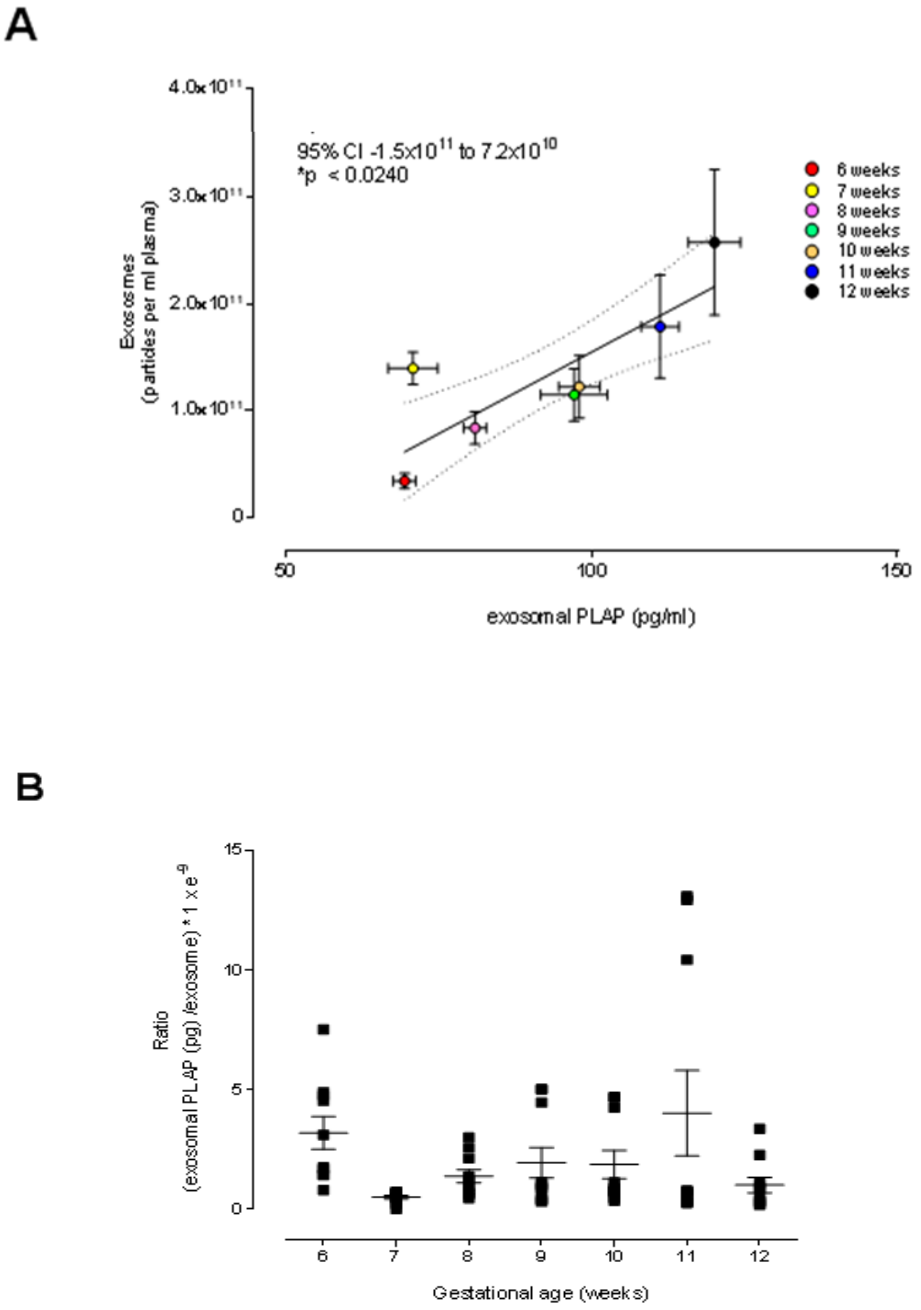


Figure 16. Contribution of placental-derived exosomes into maternal circulation. (A) Relationship between exosomal PLAP and total exosomes (particles per ml plasma) across first trimester of pregnancy (*i.e.* 6-12 weeks represented by colours). Coefficient of determination (r^2) was 0.78 and $p<0.05$. (B) Ratio of specific placental exosomes and total exosomes. In A, values were mean ± SEM, Linear correlation (-). In B, Data were presented as aligned dot plot and values were mean ± SEM, two-way ANOVA $p>0.05$.

Table VI. Number of Exosome Particle (CD63 and PLAP) in First Trimester of Pregnancy

Gestational age (week)	***CD63 (NEP)***	***PLAP (pg/ml)***	***PLAP/CD63***
6 (n=10)	$3.36e^{10} \pm 2.3e^{10}$	67.10 ± 6.16	$3.12e^{-9} \pm 2.12e^{-9}$
7 (n=10)	$1.45e^{11} \pm 4.66e^{10}$	67.98 ± 12.93	$5.09e^{-10} \pm 1.99e^{-10}$
8 (n=10)	$8.35e^{10} \pm 4.87e^{10}$	81.54 ± 6.12	$1.38e^{-9} \pm 8.56e^{-10}$
9 (n=10)	$1.22e^{11} \pm 9.24e^{10}$	95.81 ± 11.71	$1.95e^{-9} \pm 2.13e^{-9}$
10 (n=10)	$1.14e^{11} \pm 7.81e^{10}$	99.59 ± 19.55	$2.009e^{-9} \pm 2.16e^{-9}$
11 (n=10)	$1.78e^{11} \pm 1.53e^{11}$	113.2 ± 6.99	$4.11e^{-9} \pm 5.82e^{-9}$
12 (n=10)	$2.57e^{11} \pm 2.16e^{11}$	121.4 ± 15.94	$9.68e^{-10} \pm 9.32e^{-10}$

Data were presented as mean ± SD (range). CD63 was expressed in the number of exosome particle and PLAP was expressed in pg/ml. n=10 for each gestational week.

5.5 Plasma exosomal protein characterization in first trimester of pathological pregnancies

Placenta derived exosomes increased in first trimester of pregnancy with gestational diabetes mellitus (GDM) and preeclampsia (PE)

The cumulative number of combined exosomal population was quantified and placenta specific marker on exosomes (Placental Alkaline Phosphatase, PLAP) was measured in exosome rich sample from plasma collected during first trimester (11-14 weeks) complicated with gestational diabetes mellitus (n=7) using ELISA for specific exosomal endocytic marker CD63 and placenta specific marker PLAP respectively. The quantified total number of exosomes was $1.31e^{12} \pm 1.60e^{12}$ (mean ± SD) and the PLAP concentration was 171.7 ± 17.94 pg/ml. Also, the number of combined exosomal population counted in first trimester of preeclampsia (n=15) was $1.09e^{11} \pm 3.17e^{10}$ exosome vesicles/ ml of plasma and the PLAP concentration was 181.4 ± 28.37 pg/ml.

Statistical analysis showed that the total number of exosomes released in first trimester of pregnancy complicated with GDM was three- fold higher than normal pregnancy (Figure 17A) and the exosomes released specifically from the placental cells was two- fold compared to PLAP concentration in uncomplicated pregnancy (Figure 17B). Also, the number of exosomes that was detected in first trimester of preeclampsia significantly increased ($p<0.001$) compared to first trimester of healthy pregnancy (Figure 18). Student's t- test was performed to compare between normal healthy pregnancy (n =10) and preeclamptic (n =15) conditions. The total number of exosome particles in normal pregnancy was $1.33e^{11} \pm 1.27e^{11}$ (mean ± SD) and in preeclampsia was $1.09e^{11} \pm 3.17e^{10}$ (mean ± SD).

Placenta derived exosomal contribution to total exosomes in GDM

In order to know the contribution of placenta specific exosomes among combined exosome vesicles, ratio of PLAP and number of exosome particle (NEP) was calculated in GDM and was compared with uncomplicated pregnancies. The value of PLAP/NEP ratio was standardised as 1 in healthy pregnancy and approximately 64% reduction was observed in GDM (Figure 19).

This outcome may reflect two possibilities; (i) placenta-derived exosomes in GDM are more reactive to increase the non-placental exosome release in maternal circulation (ii) the PLAP content is less expressed in GDM than normal pregnancy.

The number of total exosome particle released into the peripheral circulation (CD63), amount of placenta specific exosomal marker (PLAP) and placental exosomal contribution to total exosomes

in the circulation (PLAP/CD63) in first trimester of normal pregnancy and gestational diabetes mellitus with ‘p’ values have been provided in Table VII. All data were presented as mean ± SD (range). CD63 was expressed in the number of exosome particle and PLAP was expressed in pg/ml. T test was performed to compare between normal pregnancy (n = 10) and gestational diabetes mellitus (n = 7) condition. The difference was statistically significant for CD63, PLAP and PLAP/CD63 in GDM compared to normal pregnancy with $p<0.05$, $p<0.001$ and $p<0.001$ respectively.

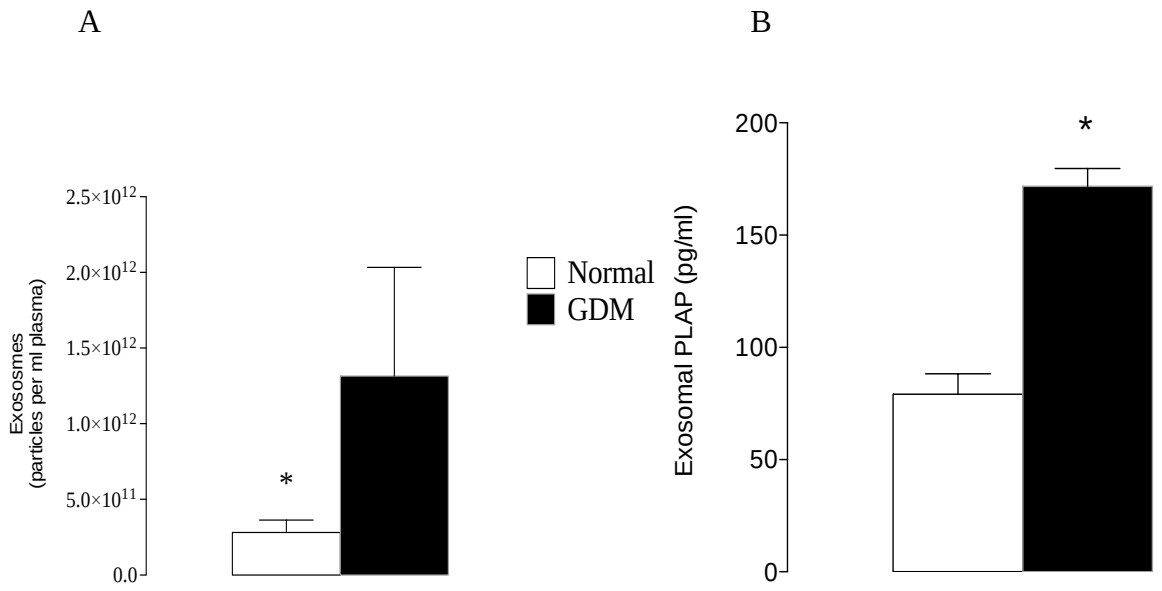


Figure 17. Placenta derived exosomes in maternal circulation during first trimester of normal uncomplicated pregnancy and gestational diabetes mellitus. Student's t- test was performed to compare between normal healthy pregnancy (n =10) and gestational diabetes mellitus (n =7) conditions. The total number of exosome particles in normal pregnancy was $1.33e^{11} \pm 1.27e^{11}$ (mean ± SD) and in GDM was $1.31e^{12} \pm 1.60e^{12}$ (mean ± SD). The measured exosomal PLAP concentration was 92.38 ± 22.89 pg/ml (mean ± SD) in normal pregnancy and 171.7 ± 17.94 pg/ml (mean ± SD) in GDM. The total number of exosomes in GDM was three- fold higher ($p<0.05$) than normal pregnancy (A) and the PLAP concentration (exosomes released from placental cells) was two- fold ($p<0.001$) compared to PLAP concentration in uncomplicated pregnancy (B).

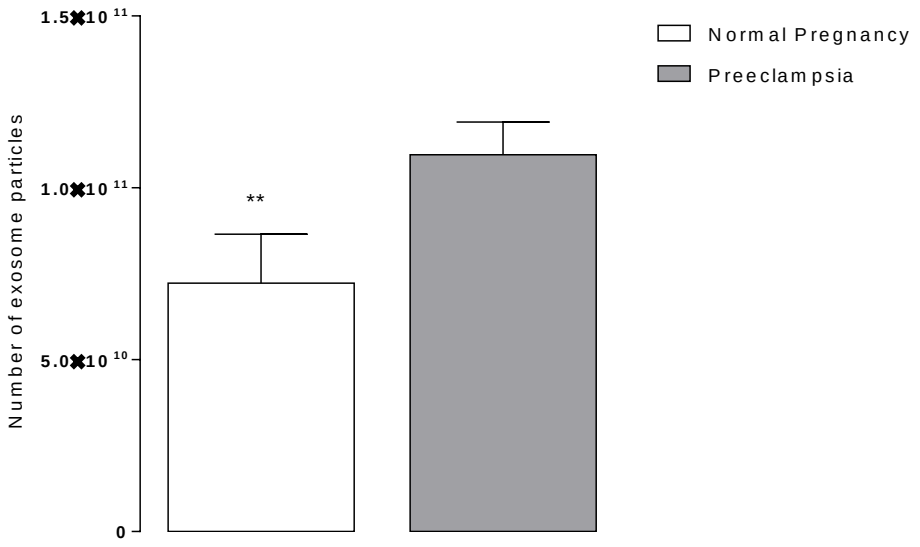


Figure 18. Exosomal vesicle release in maternal circulation during first trimester of normal healthy pregnancy and in preeclampsia. Student's t- test was performed to compare between normal healthy pregnancy (n =10) and preeclamptic (n =15) conditions. The total number of exosome particles in normal pregnancy was $1.33e^{11} \pm 1.27e^{11}$ (mean ± SD) and in preeclampsia was $1.09e^{11} \pm 3.17e^{10}$ (mean ± SD). The total number of exosomes in first trimester of pregnancy complicated with preeclampsia significantly increased ($p<0.001$) compared to healthy pregnancy.

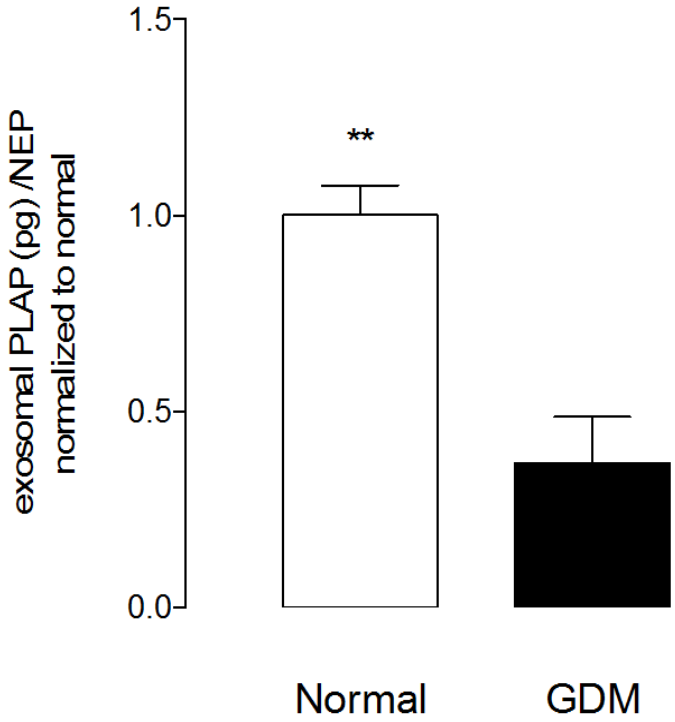


Figure 19. Placenta derived exosomal contribution to total exosomes in GDM. Student's t- test was performed to compare between normal healthy pregnancy (n =10) and gestational diabetes mellitus (n =7) conditions. PLAP/Number of Exosome Particle (NEP) in normal pregnancy was $6.91e^{-10}$ ± $5.11e^{-11}$ (mean ± SD) and in GDM was $2.53e^{-10}$ ± $3.3e^{-11}$ (mean ± SD). PLAP concentration/Number of Exosome Particle ratio was standardised as 1 in normal healthy pregnancy and compared with GDM. Approximately 64% reduction (significance level, $p<0.001$) was observed in GDM.

Table VII. Comparison of number of exosome particle (CD63 & PLAP) in normal pregnancy vs gestational diabetes mellitus

NEP	***Normal Pregnancy (n=10)***	***Gestational Diabetes Mellitus (n=7)*******	***P Value***
CD63	$1.33e^{11} \pm 1.27e^{11}$	$1.31e^{12} \pm 1.60e^{12}$	< 0.05
PLAP	92.38 ± 22.89	171.7 ± 17.94	< 0.001
PLAP/CD63	$6.91e^{-10} \pm 5.11e^{-11}$	$2.53e^{-10} \pm 3.3e^{-11}$	< 0.001

Data were presented as mean ± SD (range). CD63 was expressed in the number of exosome particle and PLAP was expressed in pg/ml. Student's t- test was performed to compare between normal pregnancy and gestational diabetes mellitus condition. The difference was statistically significant for CD63, PLAP and PLAP/CD63 in GDM compared to normal pregnancy with $p<0.05$, $p<0.001$ and $p<0.001$ respectively.

5.6 Mass spectrometry based proteomic profiling of plasma exosomes in first trimester of normal pregnancies

All mass spectra were analyzed using MASCOT™ and Protein Pilot™ search engines against the Swissprot database with the species set as human, specifying trypsin as the enzyme. The MS/MS analysis of exosomal proteins in each gestational week of normal first trimester pregnancy (healthy uncomplicated pregnancy, n=10) have been provided in Table IX as an appendix. Approximately 304 mass spectrometry identified exosomal proteins were detected in the plasma sample of women with normal pregnancy outcome (n=10) and 46 proteins in the plasma sample of control non-pregnant women (n=5). The functional analysis of these exosomal proteins was performed using a bioinformatics tool - Ingenuity Pathway Analysis (IPA) software (Figure 20). Collectively 204 canonical pathways regulated by these exosomal proteins (identified in first trimester of normal healthy pregnancy) were detected in Ingenuity Pathway Analysis. Of these 204 pathways, the activity of 113 canonical pathways was upregulated and downregulated for 91 pathways with the advancement of pregnancy from 6-12 weeks. A linear correlation was observed in between the pathway activation and the gestational age in all these pathways. Of these pathways, some pathways were associated with immune responses during pregnancy (assessed by functional analysis in IPA). The arbitrary scores in these pathways were estimated using IPA core, tox & comparison Analysis. The scores were calculated depending on the potential capability of the proteins to activate the specific immune response pathway in each gestational week. The identity of those pathways with their scores in each gestational week has been provided in Table VIII. The LC-MS/MS detected proteins were further analysed using another bioinformatics tool - PANTHER (www.pantherdb.org) software. The varied functions of the exosomal proteins in plasma of normal healthy pregnant women have been illustrated in the Venn diagrams (Figure 21). Comparative analysis of immune responses activated by the exosomal proteins identified in normal healthy pregnant women and in control non-pregnant women was performed using PANTHER gene analysis (Figure 22). It was observed that immune responses were more specific in pregnant women compared to non-pregnant women displaying additional immunological functions *i.e.* antigen processing and presentation, macrophage activation.

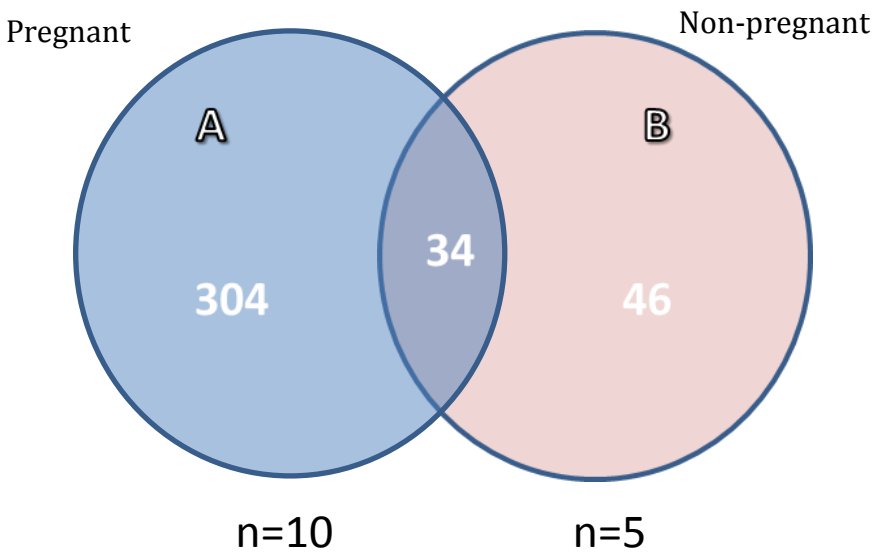


Figure 20. Comparative analysis of mass spectrometry detected exosomal proteins assessed in Ingenuity Pathway Analysis (IPA) bioinformatics tool. In Venn diagram, (A) 304 proteins were identified in the plasma sample of normal healthy pregnant women (n=10), (B) 46 proteins were identified in the plasma of control non-pregnant women (n=5).

Table VIII. Canonical pathways associated with immune responses with arbitrary scores in first trimester (6-12 weeks) of normal healthy pregnancy

Canonical Pathway	Scores in Gestational Weeks						
	6 week	**7 week**	**8 week**	**9 week**	**10 week**	**11 week**	**12 week**
Acute Phase Respose Signalling	5.93	6.64	6.92	6.94	7.30	7.84	9.17
Primary Immunodeficiency Signalling	2.06	5.87	6.04	5.32	4.40	5.95	5.84
B Cell Development	1.56	2.88	2.97	3.32	1.64	2.93	3.63
IL-12 Signalling and Production of Macrophages	1.02	1.88	0.68	1.57	1.11	1.93	1.85
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	0.75	2.12	0.504	1.26	0.83	1.52	1.53
IL-6 Signaling	0.62	0.26	1.43	1.74	0.29	0.75	1.15

The scores were arbitrary which were estimated using IPA (Ingenuity Pathway Analysis bioinformatics tool) Core, Tox & Comparison Analysis. The scores were based on the potential capability of the proteins to activate the specific immune response pathway.

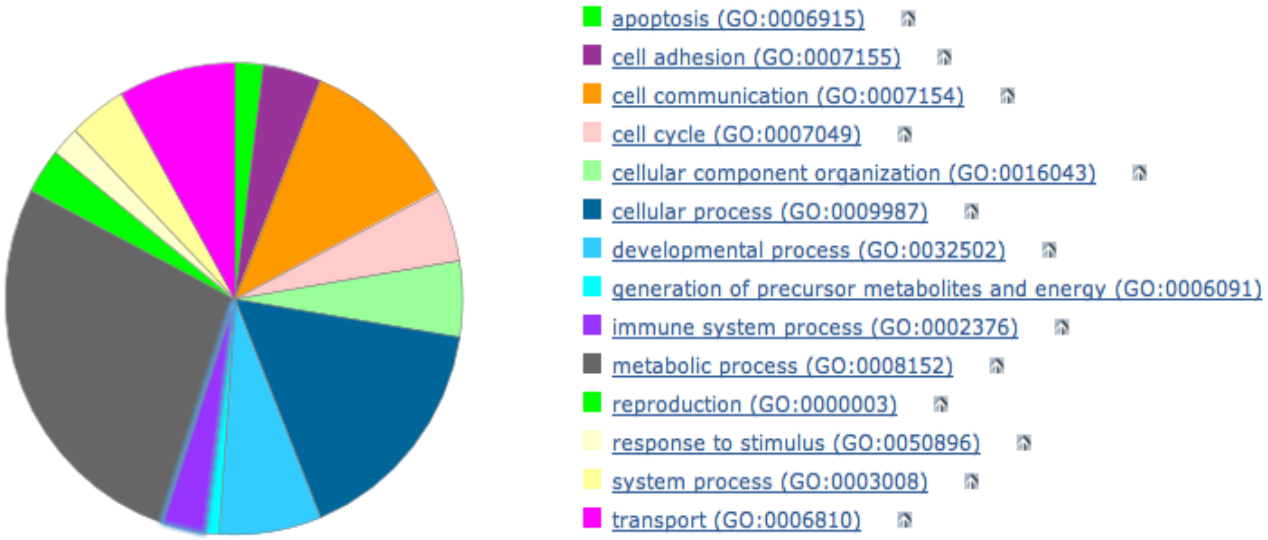


Figure 21. Different biological functions of LC-MS/MS detected exosomal proteins in maternal circulation using PANTHER bioinformatics tool. Different colours in the Venn diagram represent different biological functions of the proteins identified in plasma exosomes during first trimester (6-12 weeks) of normal healthy pregnant women (n=10).

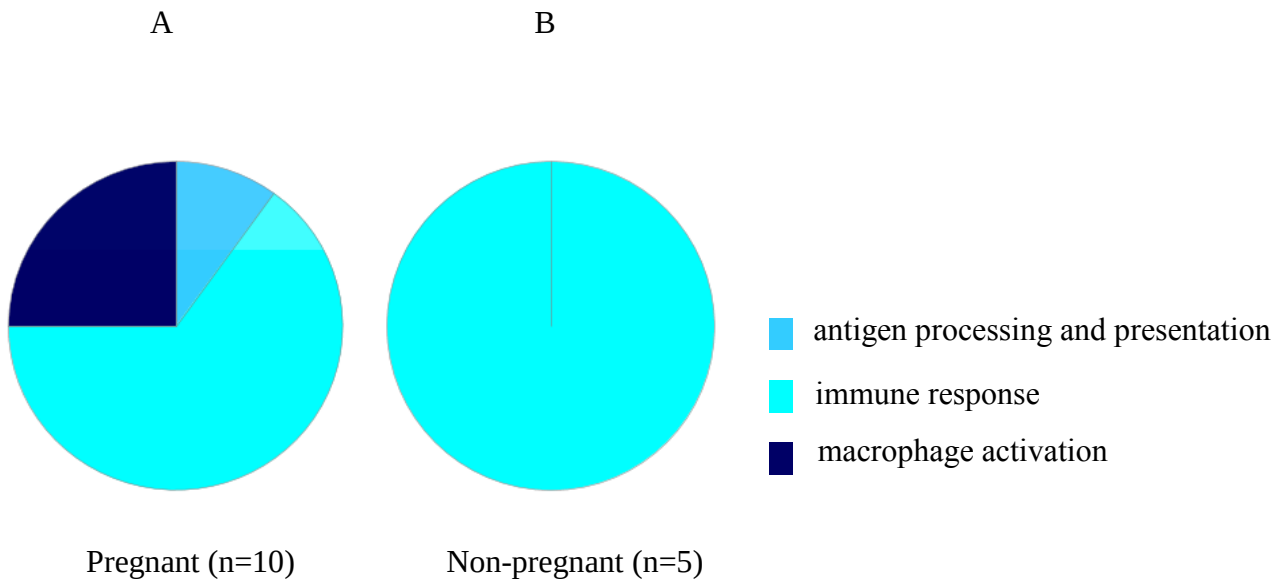


Figure 22. Comparative analysis of immune responses activated by the exosomal proteins identified in plasma of normal healthy pregnant women and control non-pregnant women using PANTHER gene analysis. Different colours in the Venn diagram represent different immunological functions. (A) Immunological functions of exosomal proteins in the plasma of normal healthy pregnant women (n=10) during first trimester (6-12 weeks) & (B) Immunological functions of exosomal proteins in the plasma of control non-pregnant women (n=5).

5.7 Mass spectrometry based proteomic profiling of plasma exosomes in first trimester of pathological pregnancies

10 µg of exosomal protein from plasma of preeclamptic condition (n=1) during first trimester (11-14 weeks) was processed for mass spectrometric proteomic analysis. The mass spectra were analysed using the protein pilot software specifying paragon method in swissprot database search engine setting the species as human and specifying the trypsin enzyme. 25 proteins were identified in preeclamptic condition (n=1). The proteins were considered significant with the presence of peptide >1%. All these mass spectrometry detected exosomal proteins were inserted into the IPA (Ingenuity Pathway Analysis) bioinformatics tool. 35 canonical pathways were detected in IPA core, tox and metabolomics analysis. Haematopoiesis, primary immunodeficiency signalling, acute phase response, B cell development, coagulation system signalling were the important pathways that were activated with these proteins (Figure 23). Some diseases and toxicity functions were identified that were associated with these proteins *i.e.* immunological diseases, cardiotoxicity, hepatotoxicity, nephrotoxicity (Figure 24).

Comparative analysis of plasma exosomal peptide profile in normal pregnancy and preeclampsia

10 µg of protein in exosome enriched sample from plasma of preeclamptic condition (n=1) and normal healthy pregnancy (n=1) during first trimester was processed for mass spectrometric proteomic analysis. The mass spectra were analysed using the protein pilot software specifying paragon method in swissprot database search engine setting the species as human and specifying the trypsin enzyme. 40 proteins were identified in first trimester of normal pregnancy condition (n=1) and 25 in preeclamptic condition (n=1). The proteins were considered significant with the presence of peptide >1%. 10 similar proteins were detected in both conditions (Figure 25). Among all these proteins, fibronectin or galectin-3 binding proteins were not observed in first trimester preeclamptic maternal plasma exosomal sample which were evident in first trimester normal healthy pregnant women's plasma exosomal sample. These proteins indicate normal placental function to maintain the pregnancy successfully. However, these discrepancies in exosomal proteins were observed in a single set of sample that needs to be verified for other samples and further study needs to be conducted where a large group of patients can be recruited.

These proteins were entered into the IPA (Ingenuity Pathway Analysis) bioinformatics tool. A comparison analysis between these two conditions was performed using the IPA core, tox and metabolomics analysis. The comparison was made on the basis of canonical pathways regulated by

the proteins in these conditions. Primary immunodeficiency signalling, haematopoiesis and B cell development were more activated with the proteins of preeclamptic condition compared to proteins of normal pregnancy condition. On the contrary, acute immune response pathway was potentiated with the proteins of normal pregnancy condition rather than preeclamptic condition (Figure 26).

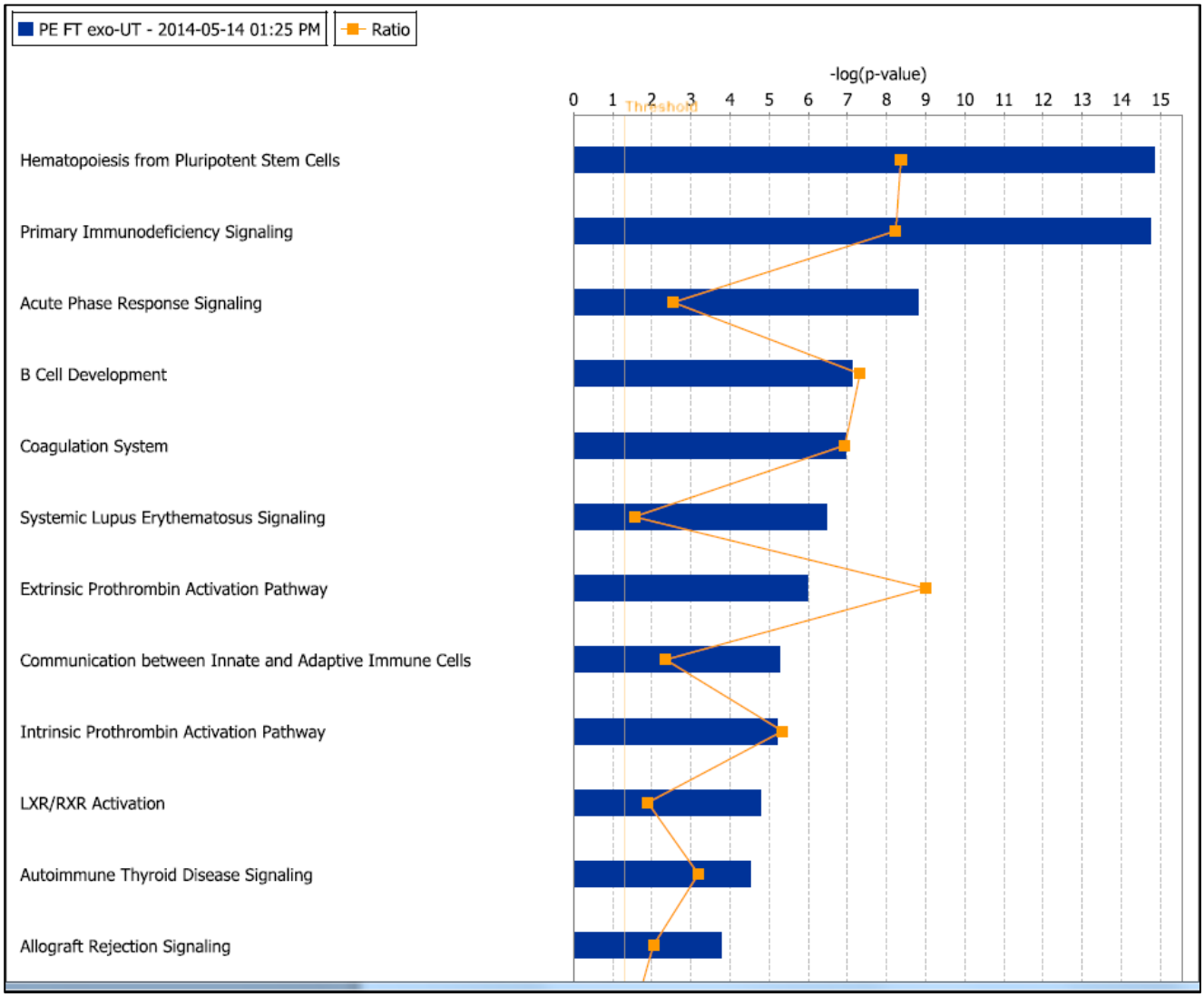
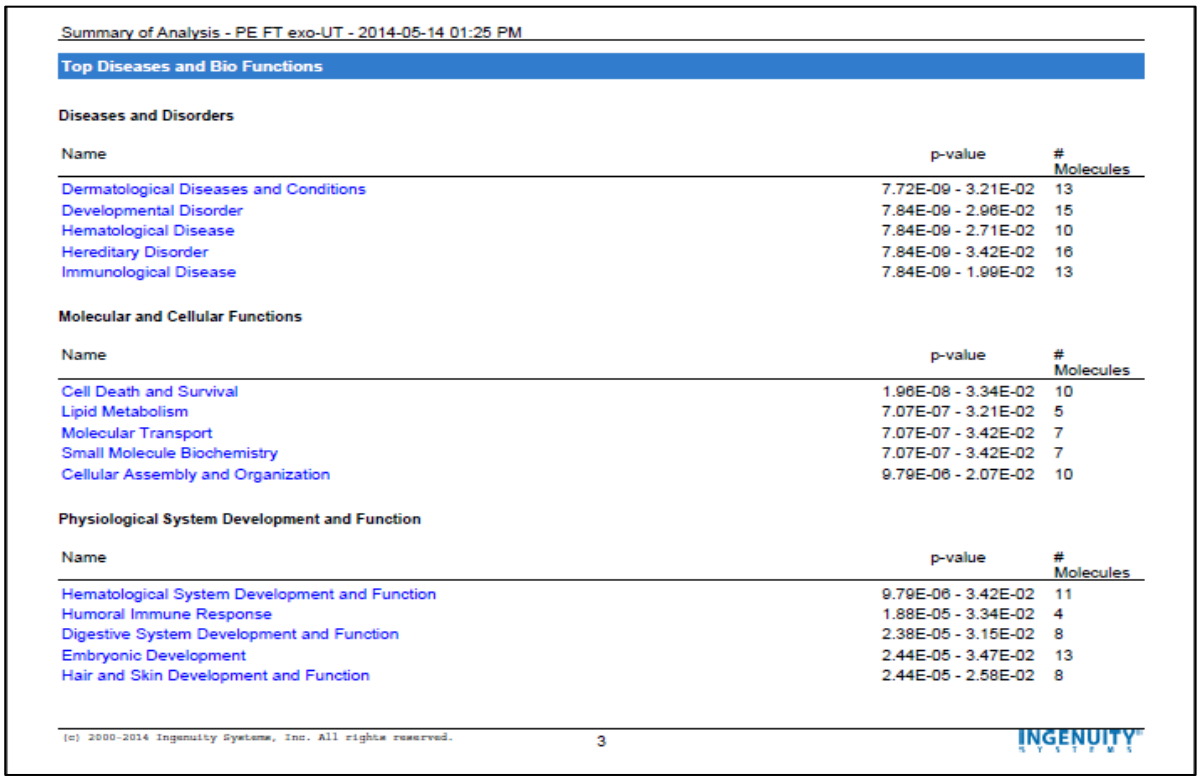


Figure 23. Canonical pathways using IPA bioinformatics tool for exosomal proteins identified in first trimester (11-14 weeks) preeclamptic women's (n=1) plasma sample. Haematopoiesis from pluripotent stem cells, primary immunodeficiency signalling, acute phase response signalling, B cell development, coagulation system were the top canonical pathways that were activated with the exosomal proteins identified in LC-MS/MS analysis.



Summary of Analysis - PE FT exo-UT - 2014-05-14 01:25 PM

Top Diseases and Bio Functions

Diseases and Disorders

Name	p-value	# Molecules
Dermatological Diseases and Conditions	7.72E-09 - 3.21E-02	13
Developmental Disorder	7.84E-09 - 2.96E-02	15
Hematological Disease	7.84E-09 - 2.71E-02	10
Hereditary Disorder	7.84E-09 - 3.42E-02	16
Immunological Disease	7.84E-09 - 1.99E-02	13

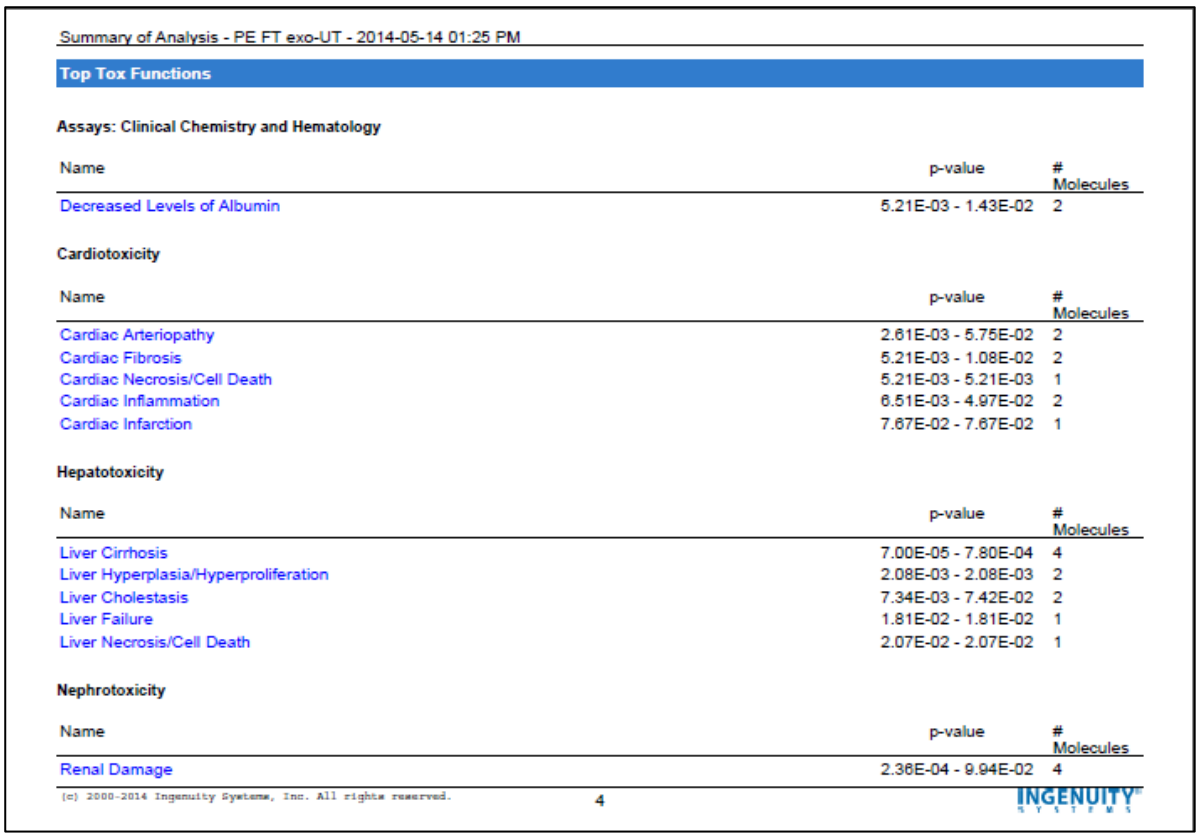
Molecular and Cellular Functions

Name	p-value	# Molecules
Cell Death and Survival	1.96E-08 - 3.34E-02	10
Lipid Metabolism	7.07E-07 - 3.21E-02	5
Molecular Transport	7.07E-07 - 3.42E-02	7
Small Molecule Biochemistry	7.07E-07 - 3.42E-02	7
Cellular Assembly and Organization	9.79E-06 - 2.07E-02	10

Physiological System Development and Function

Name	p-value	# Molecules
Hematological System Development and Function	9.79E-06 - 3.42E-02	11
Humoral Immune Response	1.88E-05 - 3.34E-02	4
Digestive System Development and Function	2.38E-05 - 3.15E-02	8
Embryonic Development	2.44E-05 - 3.47E-02	13
Hair and Skin Development and Function	2.44E-05 - 2.58E-02	8

3
INGENUITY SYSTEMS



Summary of Analysis - PE FT exo-UT - 2014-05-14 01:25 PM

Top Tox Functions

Assays: Clinical Chemistry and Hematology

Name	p-value	# Molecules
Decreased Levels of Albumin	5.21E-03 - 1.43E-02	2

Cardiotoxicity

Name	p-value	# Molecules
Cardiac Arteriopathy	2.61E-03 - 5.75E-02	2
Cardiac Fibrosis	5.21E-03 - 1.08E-02	2
Cardiac Necrosis/Cell Death	5.21E-03 - 5.21E-03	1
Cardiac Inflammation	6.51E-03 - 4.97E-02	2
Cardiac Infarction	7.67E-02 - 7.67E-02	1

Hepatotoxicity

Name	p-value	# Molecules
Liver Cirrhosis	7.00E-05 - 7.80E-04	4
Liver Hyperplasia/Hyperproliferation	2.08E-03 - 2.08E-03	2
Liver Cholestasis	7.34E-03 - 7.42E-02	2
Liver Failure	1.81E-02 - 1.81E-02	1
Liver Necrosis/Cell Death	2.07E-02 - 2.07E-02	1

Nephrotoxicity

Name	p-value	# Molecules
Renal Damage	2.36E-04 - 9.94E-02	4

4
INGENUITY SYSTEMS

Figure 24. Diseases and toxicity functions of exosomal proteins identified in first trimester of preeclamptic condition (n=1) using Ingenuity Pathway Analysis bioinformatics tool. The mass spectrometry identified exosomal proteins in plasma of first trimester preeclamptic condition that were associated with different diseases and disorders, molecular and cellular functions, physiological system development and several toxicity functions.

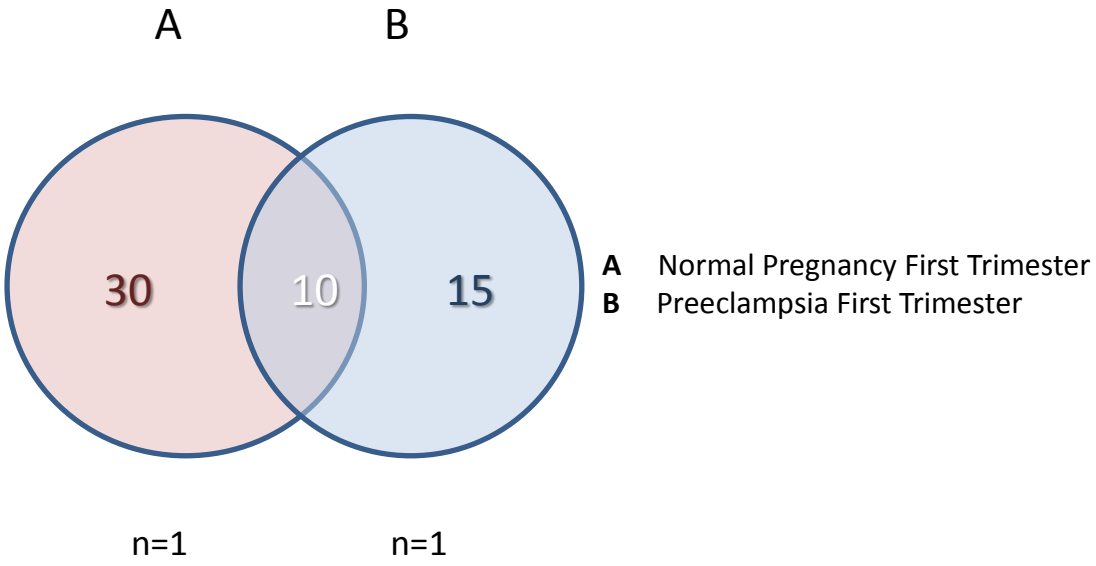


Figure 25. LC-MS/MS detected exosomal proteins from plasma in first trimester of normal pregnancy and preeclampsia. Comparison was made using Ingenuity Pathway Analysis (IPA) core, tox and metabolomics analysis. 30 proteins were identified in normal healthy pregnancy (n=1) and 15 proteins in preeclampsia (n=1). 10 similar proteins were detected in normal pregnancy and preeclampsia.

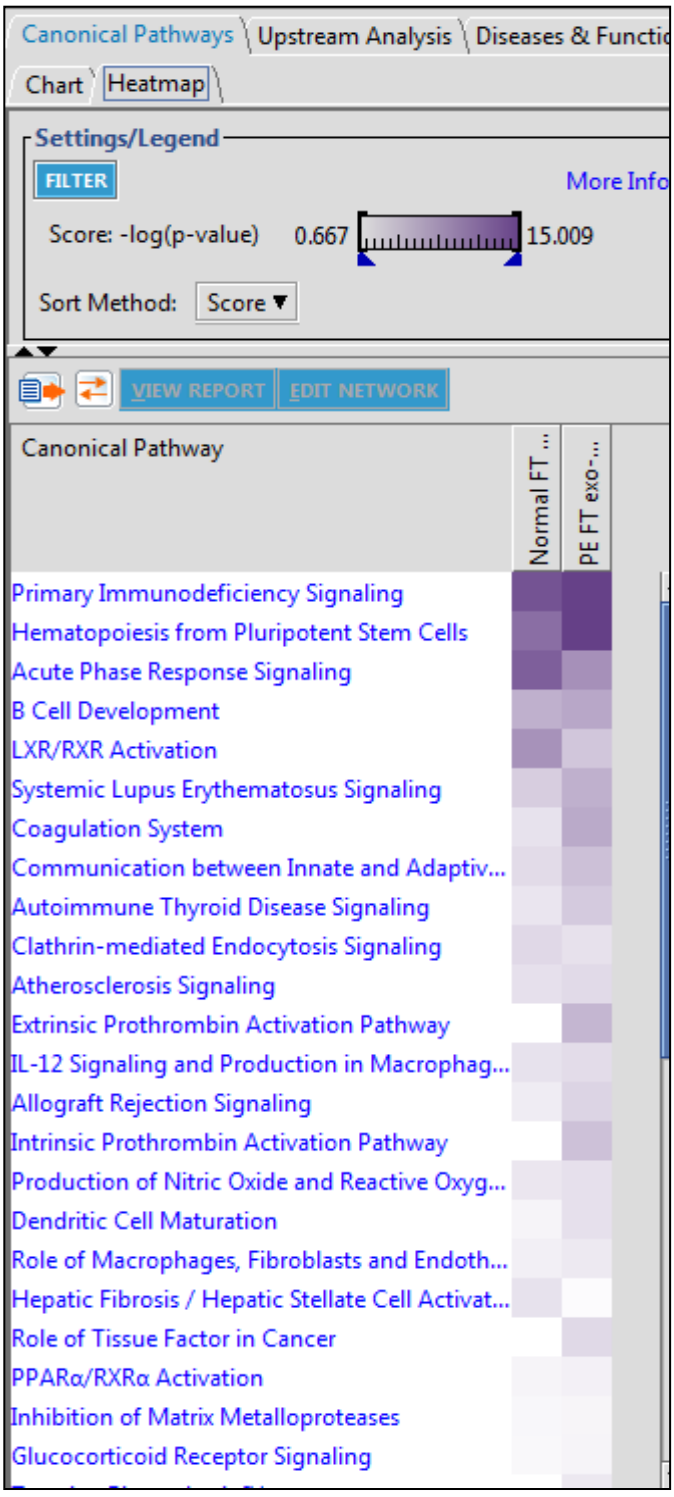


Figure 26. Activated canonical pathways in normal healthy pregnancy and preeclampsia using Ingenuity Pathway Analysis bioinformatics software. Primary immunodeficiency signalling, haematopoiesis and B cell development were significantly activated with the exosomal proteins identified in preeclamptic condition compared to normal pregnancy. Acute immune response pathway was potentiated with the exosomal proteins of normal pregnancy condition than preeclamptic condition.

CHAPTER VI

6. DISCUSSION

Currently, there are no proven means of identifying presymptomatic women who subsequently develop complications during early pregnancy. Most women who are triaged into high-risk clinical units on the basis of poor previous obstetric history ultimately have uncomplicated pregnancies. Early detection of women at risk of pregnancy complications would provide an opportunity to develop and evaluate timely and appropriate intervention strategies to limit acute adverse sequelae. The rationale for developing early pregnancy screening tests is not only for the management of the contemporaneous pregnancy but also to optimise lifelong and intergenerational health. Recent studies highlight the putative utility of tissue-specific nanovesicles (*i.e.* exosomes) in the diagnosis of disease onset and treatment monitoring; however, the exosome profile in early pregnancy (*i.e.* from 6 to 12 weeks) remains to be established. The overall aim of this study was to characterise gestational age associated variation in the concentration of placenta-derived exosomes in maternal plasma during first trimester of pregnancy from women with normal pregnancies (without any complication across pregnancy) and pre-symptomatic women who subsequently developed gestational diabetes mellitus (GDM) and preeclampsia (PE), pathologies that were diagnosed in the second trimester. Weekly serial blood samples (from 6 to 12 weeks) were collected and exosomes were isolated and characterised. The results showed that the number of exosomes vesicles present in maternal plasma increased progressively during first trimester, as did exosomal PLAP (placental marker) concentration in healthy women with normal pregnancy outcome. Patients who developed GDM and PE in the second trimester of pregnancy, exhibited higher amount in the number of exosomes and exosomal PLAP in the first trimester compared to healthy controls. Finally, using a mass spectrometry approach exosomal protein changes in patients with GDM and PE were attempted to identify. These results suggest that exosomes from placenta could potentially be used as surrogate diagnostic markers for placental function, extending its utility to screen asymptomatic population.

6.1 Exosomes isolation from maternal plasma

The purification of exosomes from plasma and other biological fluids is not trivial. Exosomes have been isolated from many different biological fluids with success and these procedures were largely developed using principally supernatants of cultured cells and plasma. In this study, exosomes were isolated from maternal plasma by differential and buoyant density centrifugation using a sucrose continuous gradient [103, 104]. In this regard, a straightforward method was utilised to estimate the number of exosomal vesicles that were obtained after the sucrose continuous gradient purification. An automatic system was used to collect the fractions after preparing a sucrose continuous gradient on the top of isolated exosomal sample dissolved in 2.5 M sucrose stock solution enables a highly reproducible density and decreasing the coefficient of variation between samples. In addition, using purification method based on the density of exosomes, vesicles were discarded with the same size of exosomes but not from endosomal origin (indicates high purity).

On the other hand, samples used in this study were kindly provided by Professor Sebastian Illanes (MD, Obstetrics and Gynaecology specialist) from *Clinica DAVILA* (Santiago, Chile). Serial blood samples were collected from pregnant women and plasma samples were separated from whole blood and stored at -80 ^{0}C until they were sent to UQCCR (Brisbane, Australia, within 4 weeks of collection). In order to discard any differences attributed to the freeze thaw cycle during exosomes isolation, their stability was determined using fresh and frozen plasma. In this study it was established that exosomes were very stable when stored at -80^{o}C. Similar number of exosomes yield from fresh and stored samples (*i.e.* plasma) were obtained and the gestational age differences in plasma exosomes number was identified in long-term stored samples. The isolation of exosomes from stored biofluids is quite normal rather than the exception. These results are consistent with those of other studies [105, 106] and suggest that the exosomal contents are protected inside these vesicles, highlighting the potential use of exosomes as biomarker based on their stability under different conditions.

To test biologic activities of plasma-derived exosomes, methods are necessary that ensure adequate recovery of exosome fractions free of contaminating larger vesicles, cell fragments and protein/nucleic acid aggregates. Exosome vesicles were isolated using the gold standard method of differential ultracentrifugation and filtration followed by sucrose gradient purification from maternal plasma sample [104]. The amount of protein was estimated in initial plasma sample, samples collected after different stages of differential centrifugation and also in purified samples. The measured protein concentration revealed gradual diminution over the isolation steps indicating

removal of cellular debris, apoptotic bodies, microparticles, highly abundant plasma proteins yielding exosome pure sample. There was ~10-fold reduction in the amount of total proteins compared to initial plasma sample following differential ultracentrifugation. The total protein concentration reduced significantly in all three purification (mentioned above) steps compared to ultracentrifugation simply. These isolated nanovesicles using these methods, displayed a diameter between 30-100 nm under transmission electron microscopic (TEM) analysis and their size distribution showed in the range of 30-150 nm with the majority (>95%) being 74 nm in the nanoparticle tracking analysis (NTA). Others studies have used flow cytometer to detect the size of the vesicles [31, 107]; however, this methodology cannot detect the smaller vesicles (*i.e.* exosomes). NTA can determine vesicle's size using the Brownian motion by tracking, visualizing and quantifying nanoparticles down to 50 nm. Exosomal population in the derived sample was further enriched by sucrose density gradient (1.11-1.19 g/ml) centrifugation. All these morphological characteristics are consistent with the exosomal characteristics that were evident in the previously published data [47, 108].

6.2 Placental-derived exosomes in maternal circulation

Exosomes are released from many different cell types, including the human placenta. Syncytiotrophoblast (more abundant cell type in the human placenta) constitutively release vesicles during normal pregnancy. Recently, it was established that normal pregnancy is associated with the increase in exosomes release into maternal plasma, and placenta-derived exosomes concentration increases a further 6-fold during uncomplicated healthy pregnancy from first to third trimester [103]. There is substantial evidence that these placental-derived exosomes are taken up by different cells in the maternal circulation and alter their phenotype [3, 109] In addition, it was found that the content of these released exosomes are placenta-specific [83], converting the study of these microvesicles in an excellent tool for the understanding of different physiological processes occurring during embryo/fetal development and the feto maternal interaction.

It has been established that the concentration of exosomes in maternal peripheral blood is greater than that observed in non-pregnant women [110]. In this study, exosomes of placental origin were specifically isolated from the maternal blood using anti-PLAP (anti-placental-type alkaline phosphatase) conjugated to agarose micro-beads. Studies completed to date provide persuasive evidence that placental cell-derived exosomes play a significant role in intercellular communication pathways that potentially contribute to placentation and development of maternal-fetal vascular exchange.

As exosomes carry different kinds of protein, mRNA and miRNA [69], engaging in cell-to-cell communication, it is likely that they play an important role in modifying the maternal physiological state to maintain a successful pregnancy [111]. Interestingly, in this study it was found that placental-derived exosomes increased systematically during the first trimester as early as sixth week of pregnancy when the intervillous circulation is not fully established. However, it has been observed that communication between placental and fetal circulation occurs at the beginning of the fourth week of post conception [112]. Moreover, the lacunar spaces are formed in the trophoblast from as early as nine days of post-ovulation and maternal blood flows into the trophoblast lacunae between ten and eleven days after fecundation. In addition, it has been reported that the intervillous blood flow is present in an early stage (*i.e.* < seventh week) [113] and increases gradually from fourth week during the first trimester of pregnancy [114].

Trophoblast plugs occlude the spiral arteries to prevent the contact of maternal blood flow into the intervillous space, however, at the same time trophoblast plug are in contact with the maternal blood, and could release soluble proteins (*e.g.* human chorionic gonadotropin, hCG) and vesicles (*e.g.* nanovesicles) into maternal circulation. It is interesting to highlight that hCG can be measured in maternal plasma as early as 4 weeks of gestation, confirming the presence of molecules released from the trophoblast in early pregnancy. Moreover, β-hCG and pregnancy-associated plasma protein A (PAPP-A) have been measured in maternal plasma as early as 6 weeks of gestation [115].

Recently, the exosome profile across pregnancy has been published. The concentration of total exosomes increased during normal gestation from first to third trimester. Moreover, the release of placenta-derived exosomes increased with the gestational age and displayed a positive correlation with the placental mass at third trimester of normal pregnancy [116]. However, the exosomal content with their potential different functions across gestation remains to be established. In terms of the function of these placental exosomes, Mincheva-Nilsson et al. [74, 117] have already published a series of manuscripts describing the role of exosomes as immunosuppressive agent regulating maternal immunity in several ways.

In regards to the clearance of placental exosomes from maternal circulation after delivery, Luo et al. [118] demonstrated by real-time PCR, the rapid clearance of the placenta-specific exosomal miRNAs after 3 days of delivery. The placental exosome profile during the first 24 hours of post-delivery has not been established yet.

Furthermore, there is robust evidence that these exosomes play significant role in pregnancy complications, especially in PE. Different studies demonstrated that extracellular vesicles (including exosomes) originating from placental explant and placental cells cause endothelial dysfunction [119, 120] and promote pro-inflammatory cytokines production [121]. Using a placental marker (*i.e.* Placental Alkaline Phosphatase, PLAP), placenta-derived vesicles was discovered in maternal plasma from first trimester of pregnancy [119, 121]. In this study, immunoreactive exosomal PLAP was not detectable in plasma of non-pregnant women, supporting previously conducted studies by other group [31, 90, 122]. In another study it was identified that placental cytotrophoblasts derived exosomes carry syncytin proteins that were responsible for increased cellular uptake and fusion to form the syncytiotrophoblast cells and contribute to proper establishment of uteroplacental circulation during pregnancy. However, this syncytin protein was less abundant in pregnant women's circulation who developed preeclampsia compared to those who went through uncomplicated pregnancies [76].

On the contrary, overall exosome number as well as placenta derived exosomes displayed a significant increase in maternal circulation during first trimester in both GDM and PE condition compared to normal pregnant state which is quite similar to results that were observed by other group [71, 120, 123, 124]. However, the contribution of specific placenta derived exosomes to cumulative exosomal population which was measured as PLAP/exosome number did not show significant variation in different gestational weeks in first trimester though this ratio showed significant reduction compared to that of normal healthy pregnancy condition. This result indicates that in pathological pregnancy, the specific placental-derived exosomes do not increase like total exosome population compared to healthy pregnancy. Therefore, it can be assumed that exosomes from placental origin are more reactive to other cells (*i.e.* endothelial cells, cells of immune system, platelets) which release exosomes and attribute to increase in overall exosome number. This noticeable finding is consistent to previously conducted studies [125-128].

6.3 Exosomal protein content and potential functions

Recently, several attempts and techniques were undertaken to determine and characterize the exosomal content in different biological fluids including normal human blood plasma As exosomes carry different kinds of protein molecules [69] and engage in cell to cell communication, it is likely that they play an important role in modifying the maternal physiological state to maintain the pregnancy successfully [111]. In this study, plasma exosomal proteomic profile was further elucidated by mass spectrometric analysis of protein biomarkers that were associated with the

physiological changes in pregnancy. Some exosomal complex proteins, pregnancy associated proteins, fibronectin, galectin-3 binding protein were identified in first trimester maternal plasma samples following numerous steps of isolation and purification of exosomes. All these proteins were evident in maternal circulation during first trimester in other placental exosome research studies [132] that indicate normal maternal physiological and placental function during healthy uncomplicated pregnancy. 304 different proteins were identified in 6-12 gestational weeks (n=10 in each gestational age) and in bioinformatics analysis, 6 canonical pathways were detected that were regulated by these proteins and displayed association with different immunological functions that may be of utility in providing a stable immune tolerance during pregnancy to prevent the fetal allograft rejection.

Exosome analysis provides diagnostic and therapeutic potential and biomarker opportunities for the early detection of diseases [91, 133, 134]. To date, several research studies have been performed to identify the morphologic and proteomic characteristics of exosomes [71] released from the placental extravillous trophoblastic cells and expression profile of these exosomal contents related to common pregnancy conditions [6, 73, 135].

Exosomal marker molecules such as MHC class I chain related antigens A and B were observed to be expressed in exosomes from syncytiotrophoblast during pregnancy [72]. Several studies have observed the effect of nanovesicles secreted from syncytiotrophoblast ($PLAP^+$) on endothelial cell, producing endothelial cell dysfunction through inhibition of their proliferation [119, 136, 137]. Moreover, many studies found different proteins (i.e. Flt-1, Endoglin, IL-1β, TFPI, CD81, Alix, cytoskeleton related proteins, Rab family of proteins) incorporated in nanovesicles shedding from placental syncytiotrophoblast cells in preeclampsia even in first trimester that can alter vascular endothelial cell function [6, 135, 138, 139].

In conclusion, placental microenvironment plays an important role during normal healthy pregnancy as well as in pathological pregnancy to determine the pattern of release and packaging of components (*i.e.* proteins) of the nanovesicles shedding from the placental cells that has been evidenced as early as in first trimester. Nevertheless, the sequelae of these pregnancy complications are the consequences of cellular interaction in maternal peripheral circulation by these placenta derived nanovesicles containing specific proteins. These ideas support this study to investigate the proteomic profile of nanovesicles in maternal plasma with manifestation of common pregnancy pathologies in the period of first trimester (early pregnancy).

6.4 Exosomes as potential biomarker of placental function

Previous studies have established that extracellular vesicles, including exosomes are released under normal physiological and pathophysiological conditions, and it has also been observed that exosome release is negligible in non-pregnant women's circulation compared to healthy normal pregnant women [92]. During gestation the release of these vesicles is increased and also in response to different pathological conditions [140]; presumably due to exosomal secretion from placental trophoblastic cells to the maternal peripheral circulation [71, 92]. However, all these studies considered the late second or third trimester of pregnancy plasma samples for analysis though provide valuable insights about the possibility of conducting this research in early pregnancy.

The total number of exosome vesicles is significantly increased in maternal circulation during pregnancy. The placenta derived exosomes are also increased in the circulation along with the advancing gestational age. In addition, exosomes isolated from maternal circulation during normal pregnancy exhibit changes in their bioactivity. Nanovesicles from first trimester of pregnancy were more bioactive in promoting endothelial cell migration than the vesicles isolated in second and third trimester. The effect of these nanovesicles could be associated with the cellular origin and/or exosomal content (*e.g.* proteins) which forecast the placental function in early normal pregnancy and could be potentially important to determine the disordered placentation in complicated pregnancies [90]. It has been demonstrated that exosomal protein content is different in women with preeclampsia [141]. Moreover, the specific syncytiotrophoblast protein, syncytin-2, is markedly down-regulated in exosomes derived from placenta of pregnant women with preeclampsia compared to healthy control (normal pregnancies) [94].

In this study, specific placental-derived exosomes were quantified in the maternal circulation using the immunoreactive placental protein PLAP. Recent studies have demonstrated the presence of exosomes- PLAP (+ve) only in peripheral circulation of pregnant women [90, 92]. PLAP is an integral membrane protein (enzyme) unique to the placenta (it has also been observed in some gynaecologic cancers), produced mainly by syncytiotrophoblast [142, 143]. Nevertheless, PLAP expression has been found in primary trophoblast cytotrophoblast cells [90] and ED27 trophoblast-like cells, both isolated from first trimester chorionic villi, and also in JEG-3 cells (a extravillous trophoblast model) [144]. In addition, using immunohistochemistry stain for PLAP, the majority of chorionic trophoblastic cells were positive for PLAP [145]. During the first trimester of pregnancy, the release of placental exosomes into the maternal blood may result from extravillous trophoblast

and/or syncytiotrophoblast cells; however, while a definitive answer awaits further investigation, it is of relevance to note that fetal cells are present in maternal blood from 4 weeks of pregnancy and that trophoblast cells invade the decidua and myometrium from the time of implantation. Thus, placental cellular and exosomal pathway exists for delivery into the maternal circulation.

Cell to cell communication via these exosomes in placental microenvironment is the platform for organisation of placental interface that provide fetal immune tolerance, protection against several viral infection and lastly potentiate cell fusion which is required for syncytiotrophoblast formation for the maternal-fetal communication during pregnancy [75]. In maternal peripheral circulation, exosomes derived from placental villous trophoblasts contain specific proteins such as syncytin-1 and syncytin-2. These protein act as mediators for the fusion, formation and maintenance of cytotrophoblast to syncytiotrophoblast in early pregnancy, which is essential for placentation to ensure optimum nutrient and oxygen levels as well as adequate immunosuppression for the developing fetus [94]. In addition, exosomal syncytin-1 derived from placental trophoblastic cells inhibited the production of cytokines (*i.e.* TNF-α and IFN-γ) by T lymphocyte. This inhibitory response of these exosomes during pregnancy could play imperative role in the maternal immune tolerance [146, 147].

On the other hand, placental exosomes carry specific miRNAs coming from placental cells that has been detected in maternal peripheral circulation [148]. During pregnancy placenta acts as a shield to protect the embryo from different pathogens. A distinctive type of miRNA has been identified and detected in higher concentration in maternal peripheral circulation during pregnancy that is packaged within the trophoblast-derived exosomes, which provide the resistance from many viral infections by local and/or systemic function [149, 150].

Complications of pregnancies are associated with hypoxia and poor placentation. Using primary trophoblastic cells, it has been demonstrated that exosome release and their protein content are very sensitive to the microenvironment milieu (*i.e.* oxygen tension, hyperglycaemia etc.) [3, 71, 109, 151, 152]. As such, the concentration, content and/or bioactivity of placenta-derived exosomes in maternal plasma may be informative of placental dysfunction. Altogether, the results of this study and previously published data show that in normal pregnancies, the concentration, protein content and bioactivity of placenta-derived exosomes in maternal plasma varied significantly with gestational age. These observations are consistent with the hypothesis that exosomes are released from the placenta during early pregnancy into maternal blood and may play a role in the maternal

adaptation to pregnancy. Gestational age related changes in the profile and characteristics of exosomes in maternal blood may be of clinical utility in the diagnosis of placental dysfunction.

6.5 Final remarks

The data obtained in this study establish that pregnancy is associated with a significant increase in the number of exosomes circulating in maternal blood due to the increased release from the placenta that varies with different gestational age in terms of their different characterisation and proteomic profiling. Available evidence supports the hypothesis that the aetiology of pregnancy complications begins during first trimester. If this is the case, then changes in the profile of placenta-derived biomarkers during early pregnancy may be common to and characteristic of, women at risk of developing complications of pregnancy. Identification of such molecules would provide an opportunity to develop clinically useful early pregnancy screening tests to identify those asymptomatic women who develop the common pathologies (GDM, PE) during pregnancy. If this can be achieved it will provide an opportunity for early assessment of risk and the implementation of an alternative clinical management strategy to improve outcome for both mother and baby.

Finally, in this study it was clearly established that overall exosome release is augmented during pregnancy that ensues as early as 6 weeks of pregnancy and exhibits increase in incremental manner with the progression of pregnancy. This increase was actually due to the boosted secretion of exosomes particularly from placental cells. However, in pregnancy pathologies (*i.e.* PE and GDM), both the total exosome number and placenta derived exosome showed significant increase compared to normal healthy pregnant state. In addition, the mass spectrometric proteomic study identified some plasma exosomal proteins in normal pregnant condition that are closely related with the pregnancy physiological processes whereas those were not evident in pathological pregnancy conditions.

6.6 Limitations

This study is based on utilising plasma sample from pregnant women. Plasma is a type of sample that contains a wide variety of plasma proteins, different hormones and tissue factors. Therefore, isolating exosomes with purity in higher concentration from plasma is a challenge. Additionally, exosomes in maternal circulation is mixed up of exosomes originating from different cellular sources. As this study considered pregnancy related conditions, it was important as well as challenging to separate the exosomes that are released specifically from placental trophoblast cells.

Another challenge was to remove contaminants from the exosomes to identify the protein contents of the exosomes in mass spectrometric proteomic study. However, all these difficulties were surpassed by using high- throughput technologies, *i.e.* mass spectrometry based proteomic studies.

6.7 Future directions

Persuasive evidence of specific exosomal protein in maternal peripheral circulation with pregnancy pathologies (*i.e.* GDM, PE) in first trimester of pregnancy would contribute to discover a panel of biomarkers associated with these complications. It is important to develop novel technique and approach that would monitor the fluctuation (*i.e.* increase, decrease) of these candidate protein biomarkers in maternal circulation. Subsequently there would be an avenue to develop screening tests utilising these potential biomarkers in early gestational period that can be further applied on pregnant women during antenatal visits as clinical trial to screen asymptomatic population who might develop pregnancy complications in their late second or third trimester of gestation. Therefore, conducting further exosomal proteomic studies using plasma sample of pathological pregnancies is of great value that would lead to proper direction of this pregnancy exosome research.

CHAPTER VII

7. CONCLUSIONS

7.1 Conclusions

The main purpose of this MPhil thesis was to identify blood-borne biomarkers (*i.e.* exosomes) that may be used in the first trimester of pregnancy to identify presymptomatic women who are at risk of developing complications of pregnancy at later stage of gestation. A time-series experimental design was used to establish pregnancy-associated changes in maternal plasma exosome concentrations during the first trimester. Four groups of women were involved in this study (normal healthy pregnant women, n=10; preeclamptic women, n=15; gestational diabetic women, n=7 and non-pregnant women, n=5). This study initially investigated the amount of circulating exosomes in healthy pregnant women during their first trimester. Further, the change in exosomal release with the progression of pregnancy was detected. The origin of these released exosomes was examined to see whether these were coming from the placenta. Afterwards, the ratio of the contribution of these placenta derived exosomes to the total exosomal population was measured. All these analyses were also conducted in pathological pregnancies (*i.e.* preeclampsia and gestational diabetes mellitus) in the first trimester samples. Preliminary studies were performed to identify exosomal protein contents from a small sample size in normal healthy pregnancies and complicated pregnancies.

The results were derived from overall and placenta specific exosomal release in maternal circulation as quantified by ELISA. For the first time, this study observed that a significant number of exosomes released in maternal blood circulation during pregnancy and the cumulative population of these exsosome vesicles increased from as early as 6 weeks of pregnancy. This exosomal release increased in incremental manner with the progression of pregnancy which was actually due to the enhanced secretion of exosomes particularly from the placental cells. In addition, it was noticed that the total and placenta derived exosomes varied with different gestational ages in terms of their different characterisation and proteomic profiling.

This study also found that women who developed pregnancy complications such as preeclampsia (PE) and gestational diabetes mellitus (GDM) displayed altered exosome concentration in the first trimester compared to healthy pregnant women. It is important to note that in pregnancy pathologies the total exosome number and placenta derived exosomes equally showed substantial increase compared to normal healthy pregnant state. This substantial increase is considered to be due to

abnormalities in the maternal circulatory environment (low oxygen tension in PE and high blood glucose level with insulin resistance in GDM) that act upon placental and other immune cells to favour the release of exosomes. Another noticeable finding was the ratio of placental derived exosomes to total exosomes in GDM which was lower compared to normal uncomplicated pregnancies. This outcome may reflect two possibilities; (i) placenta-derived exosomes in GDM are more reactive to increase the non-placental exosomal release in maternal circulation and/or (ii) the PLAP (placenta specific marker on exosomes) content is less expressed in GDM than normal pregnancy.

The preliminary mass spectrometry based proteomic studies identified some plasma exosomal proteins in normal pregnancies but not in pathological pregnancies. The identified proteins were fibronectin and galectin-3 binding protein. They are considered to be intimately related with the pregnancy associated maternal systemic changes (*i.e.* immunosuppression during pregnancy). These results are found to be consistent with other related studies. Their absence in preeclamptic plasma exosomal proteins may reveal aberrant maternal adaptation to changes in pregnancy complicated with preeclampsia. However, further studies are essential to validate these findings.

7.2 Significance of findings

To date, no study has been conducted in the arena of pregnancy exosome research in early pregnancy (*i.e.* first trimester). This area is still burgeoning. This study attempted to present the relationship between the release of exosomes in maternal circulatory system and the pregnancy related physiological changes in very early pregnancy. The significance of this study is to recognise the asymptomatic pregnant women in very early pregnancy (i.e. first trimester) that may develop pregnancy complications later.

The observation of altered concentration of exosome vesicles and/or the exosomal protein composition associated with pathological pregnancies can be utilised in the clinical implications *i.e.* in the diagnosis of placental dysfunction. Recently several research studies focussed the significance of utilising tissue-specific nanovesicles (*e.g.* exosomes) in the diagnosis of different disease diagnosis and prognosis after delivering treatment. Additionally, the exosomal release in plasma has been reported to be altered in relation to disease severity and/or progression.

This study establishes exosomal profile associated with the normal healthy pregnancy state as well as in some common pathological conditions of pregnancy *i.e.* preeclampsia and gestational diabetes mellitus. The currently available data from this study would contribute in the identification of reliable exosome based biomarkers in maternal blood. These exosomal biomarkers may be of utility in developing a classification model where different pregnancy conditions would be categorised depending on the presence or absence including fluctuation in concentration of specific exosomal biomarker. Eventually, the classification model can be utilised to generate antenatal screening tests of high sensitivity and specificity.

The ultimate goal is to screen pregnant mothers using the antenatal screening tests in their first trimester when they are asymptomatic but may develop preeclampsia or gestational diabetes in the late second or third trimester. This concept of screening is important for early therapeutic intervention and prevention of maternal and fetal complications arising from the common pregnancy complications before the manifestation of clinical signs.

7.3 Recommendations for future research

The presented work has identified many challenges regarding the methods of exosome vesicle isolation, characterisation, proteomic profiling and development of exosome based biomarkers. This requires further studies and specific suggestions regarding this are described below:

Firstly, this study was based on utilising plasma sample from pregnant women. Plasma is a type of sample that contains a wide variety of plasma proteins, different hormones and tissue factors. Therefore, isolating pure exosomes in higher concentration from plasma is a challenge. In order to yield pure exosome vesicles in increased concentration from plasma, the methods of exosome isolation need to be reviewed and further improved. This can be achieved by considering the application of optimum centrifugation force and time as well as selection of proper ultracentrifugation bucket and rotor. Secondly, exosomes in maternal circulation is mixed up of exosomes originating from different cellular sources. As this study considered pregnancy related conditions, it was important as well as challenging to separate the placenta specific exosomes. Novel techniques need to be developed to isolate the exosomes of specific interest that may be done by using magnetic beads or by immunoaffinity based isolation. Thirdly, exosomal characterisation methods need to be further advanced by using immunoblotting and nanoparticle tracking analysis.

Another challenge was to remove the contaminants and highly abundant plasma proteins from the exosomes to identify the specific exosomal protein contents in mass spectrometry based proteomic study. In order to surpass these difficulties, mass spectrometry based technologies have to be well developed which can be obtained by using some different methods of removal of highly abundant plasma proteins (*i.e.* albumin, fibrinogen, prothrombin etc.) as well as contaminants (*i.e.* keratin). Additionally, in order to retrieve specific exosomal proteins with accuracy offgel fractionation method can be followed. On the other hand, to discover the exosome based biomarkers related with different pregnancy conditions, a large group of patients with different ethnic background need to be recruited and further study need to be conducted. It is important to discover a panel of biomarkers with greater predictive value which also necessitates further research. This can be achieved by analysing various sets of samples to detect the individual variability between groups and also to identify commonly appearing proteins. Finally, the available proteomic approaches and methods to identify the biomarkers need to be robust as well as cost-effective to introduce in clinical setting.

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Appendix A: List of exosomal proteins in first trimester of pregnancy

Table IX. Mass spectrometry detected exosomal proteins in first trimester of normal healthy pregnancy

6 week (n=10)

ID	Symbol	Entrez Gene Name	Location	Type(s)
A2MG_HUMAN	A2M	alpha-2-macroglobulin	Extracellular Space	transporter
GABT_HUMAN	ABAT	4-aminobutyrate aminotransferase	Cytoplasm	enzyme
ACACA_HUMAN	ACACA	acetyl-CoA carboxylase alpha	Cytoplasm	enzyme
ACTA_HUMAN	ACTA2	actin, alpha 2, smooth muscle, aorta	Cytoplasm	other
ADAL_HUMAN	ADAL	adenosine deaminase-like	Cytoplasm	enzyme
ATS9_HUMAN	ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif, 9	Extracellular Space	peptidase
KFA_HUMAN	AFMID	arylformamidase	Nucleus	enzyme
ALBU_HUMAN	ALB	albumin	Extracellular Space	transporter
AMZ1_HUMAN	AMZ1	archaelysin family metallopeptidase 1	Other	peptidase
ANK2_HUMAN	ANK2	ankyrin 2, neuronal	Plasma Membrane	other
ANK3_HUMAN	ANK3	ankyrin 3, node of Ranvier (ankyrin G)	Plasma Membrane	other
ANR12_HUMAN	ANKRD12	ankyrin repeat domain 12	Nucleus	other
ANR23_HUMAN	ANKRD23	ankyrin repeat domain 23	Nucleus	other
ANKUB_HUMAN	ANKUB1	ankyrin repeat and ubiquitin domain containing 1	Other	other
ANXA1_HUMAN	ANXA1	annexin A1	Plasma Membrane	other
APOA1_HUMAN	APOA1	apolipoprotein A-I	Extracellular Space	transporter
APOB_HUMAN	APOB	apolipoprotein B	Extracellular Space	transporter
ARAP3_HUMAN	ARAP3	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 3	Cytoplasm	other
ASPM_HUMAN	ASPM	asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	Nucleus	other
ASXL2_HUMAN	ASXL2	additional sex combs like 2 (Drosophila)	Extracellular Space	other
RENR_HUMAN	ATP6AP2	ATPase, H+ transporting, lysosomal accessory protein 2	Cytoplasm	transporter
VPP1_HUMAN	ATP6V0A1	ATPase, H+ transporting, lysosomal V0 subunit a1	Cytoplasm	transporter
B4GT7_HUMAN	B4GALT7	xylosylprotein beta 1,4-galactosyltransferase, polypeptide 7	Cytoplasm	enzyme
BLM_HUMAN	BLM	Bloom syndrome, RecQ helicase-like	Nucleus	enzyme
BRPF1_HUMAN	BRPF1	bromodomain and PHD finger containing, 1	Nucleus	transporter
CS068_HUMAN	C19orf68	chromosome 19 open reading frame 68	Other	other

C1QC_HUMAN	C1QC	complement component 1, q subcomponent, C chain	Extracellular Space	other
CO3_HUMAN	C3	complement component 3	Extracellular Space	peptidase
CO4A_HUMAN	C4A/C4B	complement component 4B (Chido blood group)	Extracellular Space	other
C4BPA_HUMAN	C4BPA	complement component 4 binding protein, alpha	Extracellular Space	other
CF062_HUMAN	C6orf62	chromosome 6 open reading frame 62	Other	other
CAH3_HUMAN	CA3	carbonic anhydrase III, muscle specific	Cytoplasm	enzyme
CATIN_HUMAN	CACTIN	cactin, spliceosome C complex subunit	Nucleus	other
CACO1_HUMAN	CALCOCO1	calcium binding and coiled-coil domain 1	Nucleus	transcription regulator
CC175_HUMAN	CCDC175	coiled-coil domain containing 175	Other	other
CCD30_HUMAN	CCDC30	coiled-coil domain containing 30	Other	other
CENPH_HUMAN	CENPH	centromere protein H	Nucleus	other
CFAH_HUMAN	CFH	complement factor H	Extracellular Space	other
CHSS3_HUMAN	CHSY3	chondroitin sulfate synthase 3	Cytoplasm	enzyme
CMBL_HUMAN	CMBL	carboxymethylenebutenolidase homolog (Pseudomonas)	Other	enzyme
CO1A1_HUMAN	COL1A1	collagen, type I, alpha 1	Extracellular Space	other
CO1A2_HUMAN	COL1A2	collagen, type I, alpha 2	Extracellular Space	other
CROCC_HUMAN	CROCC	ciliary rootlet coiled-coil, rootletin	Plasma Membrane	other
DIAC_HUMAN	CTBS	chitobiase, di-N-acetyl-	Cytoplasm	enzyme
CTRL_HUMAN	CTRL	chymotrypsin-like	Extracellular Space	peptidase
CP1A2_HUMAN	CYP1A2	cytochrome P450, family 1, subfamily A, polypeptide 2	Cytoplasm	enzyme
DCD_HUMAN	DCD	dermcidin	Extracellular Space	other
DCX_HUMAN	DCX	doublecortin	Cytoplasm	other
DHX57_HUMAN	DHX57	DEAH (Asp-Glu-Ala-Asp/His) box polypeptide 57	Extracellular Space	other
DYH2_HUMAN	DNAH2	dynein, axonemal, heavy chain 2	Other	other
DSCAM_HUMAN	DSCAM	Down syndrome cell adhesion molecule	Plasma Membrane	other
DYHC2_HUMAN	DYNC2H1	dynein, cytoplasmic 2, heavy chain 1	Cytoplasm	other
COE2_HUMAN	EBF2	early B-cell factor 2	Nucleus	other
E2AK3_HUMAN	EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3	Cytoplasm	kinase
ELF2_HUMAN	ELF2	E74-like factor 2 (ets domain transcription factor)	Nucleus	transcription regulator
PERE_HUMAN	EPX	eosinophil peroxidase	Cytoplasm	enzyme
FABP5_HUMAN	FABP5	fatty acid binding protein 5 (psoriasis-associated)	Cytoplasm	transporter
F196B_HUMAN	FAM196B	family with sequence similarity 196, member B	Other	other

FIBA_HUMAN	FGA	fibrinogen alpha chain	Extracellular Space	other
FIBB_HUMAN	FGB	fibrinogen beta chain	Extracellular Space	other
FR1OP_HUMAN	FGFR1OP	FGFR1 oncogene partner	Cytoplasm	kinase
FIBG_HUMAN	FGG	fibrinogen gamma chain	Extracellular Space	other
FINC_HUMAN	FN1	fibronectin 1	Extracellular Space	enzyme
GSH0_HUMAN	GCLM	glutamate-cysteine ligase, modifier subunit	Cytoplasm	enzyme
TF3C4_HUMAN	GTF3C4	general transcription factor IIIC, polypeptide 4, 90kDa	Nucleus	transcription regulator
G6PE_HUMAN	H6PD	hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)	Cytoplasm	enzyme
HBB_HUMAN	HBB	hemoglobin, beta	Cytoplasm	transporter
HBG1_HUMAN	HBG1	hemoglobin, gamma A	Cytoplasm	other
HILS1_HUMAN	HILS1	histone linker H1 domain, spermatid-specific 1, pseudogene	Nucleus	other
H12_HUMAN	HIST1H1C	histone cluster 1, H1c	Nucleus	other
H2A1A_HUMAN	HIST1H2AA	histone cluster 1, H2aa	Nucleus	other
H2B1A_HUMAN	HIST1H2BA	histone cluster 1, H2ba	Nucleus	other
BPL1_HUMAN	HLCS	holocarboxylase synthetase (biotin-(proprionyl-CoA-carboxylase (ATP-hydrolysing)) ligase)	Cytoplasm	enzyme
HMCN2_HUMAN	HMCN2	hemicentin 2	Extracellular Space	other
GILT_HUMAN	IFI30	interferon, gamma-inducible protein 30	Cytoplasm	enzyme
IGHG1_HUMAN	IGHG1	immunoglobulin heavy constant gamma 1 (G1m marker)	Extracellular Space	other
IGHM_HUMAN	IGHM	immunoglobulin heavy constant mu	Plasma Membrane	transmembrane receptor
IGJ_HUMAN	IGJ	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	Extracellular Space	other
IGKC_HUMAN	IGKC	immunoglobulin kappa constant	Extracellular Space	other
KV401_HUMAN	IGKV4-1	immunoglobulin kappa variable 4-1	Extracellular Space	other
LAC2_HUMAN	IGLC2	immunoglobulin lambda constant 2 (Kern-Oz- marker)	Extracellular Space	other
INCE_HUMAN	INCENP	inner centromere protein antigens 135/155kDa	Nucleus	other
KDM5A_HUMAN	KDM5A	lysine (K)-specific demethylase 5A	Nucleus	transcription regulator
TALD3_HUMAN	KIAA0586	KIAA0586	Cytoplasm	other
KIRR1_HUMAN	KIRREL	kin of IRRE like (Drosophila)	Plasma Membrane	other
KLC2_HUMAN	KLC2	kinesin light chain 2	Cytoplasm	other
KLF1_HUMAN	KLF1	Kruppel-like factor 1 (erythroid)	Nucleus	transcription regulator
K1C10_HUMAN	KRT10	keratin 10	Cytoplasm	other
K1C18_HUMAN	KRT18	keratin 18	Cytoplasm	other
K1C9_HUMAN	KRT9	keratin 9	Cytoplasm	other

LDB1_HUMAN	LDB1	LIM domain binding 1	Nucleus	transcription regulator
LHPL3_HUMAN	LHFPL3	lipoma HMGIC fusion partner-like 3	Other	other
LMNA_HUMAN	LMNA	lamin A/C	Nucleus	other
MA2A2_HUMAN	MAN2A2	mannosidase, alpha, class 2A, member 2	Cytoplasm	enzyme
MAP4_HUMAN	MAP4	microtubule-associated protein 4	Cytoplasm	other
MCLN2_HUMAN	MCOLN2	mucolipin 2	Plasma Membrane	ion channel
MRE11_HUMAN	MRE11A	MRE11 meiotic recombination 11 homolog A (S. cerevisiae)	Nucleus	enzyme
COX2_HUMAN	MT-CO2	cytochrome c oxidase subunit II	Cytoplasm	enzyme
MYRIP_HUMAN	MYRIP	myosin VIIA and Rab interacting protein	Cytoplasm	other
ULA1_HUMAN	NAE1	NEDD8 activating enzyme E1 subunit 1	Cytoplasm	enzyme
NOTC3_HUMAN	NOTCH3	notch 3	Plasma Membrane	transcription regulator
NRX3A_HUMAN	NRXN3	neurexin 3	Other	transporter
NSN5C_HUMAN	NSUN5P2	NOP2/Sun domain family, member 5 pseudogene 2	Other	other
PALB2_HUMAN	PALB2	partner and localizer of BRCA2	Nucleus	other
MYOME_HUMAN	PDE4DIP	phosphodiesterase 4D interacting protein	Cytoplasm	enzyme
PDP2_HUMAN	PDP2	pyruvate dehyrogenase phosphatase catalytic subunit 2	Cytoplasm	phosphatase
PDYN_HUMAN	PDYN	prodynorphin	Extracellular Space	transporter
PI51B_HUMAN	PIP5K1B	phosphatidylinositol-4-phosphate 5-kinase, type I, beta	Cytoplasm	kinase
PKD2_HUMAN	PKD2	polycystic kidney disease 2 (autosomal dominant)	Plasma Membrane	ion channel
PMYT1_HUMAN	PKMYT1	protein kinase, membrane associated tyrosine/threonine 1	Cytoplasm	kinase
PLCL2_HUMAN	PLCL2	phospholipase C-like 2	Cytoplasm	enzyme
PNKD_HUMAN	PNKD	paroxysmal nonkinesigenic dyskinesia	Nucleus	other
RPC2_HUMAN	POLR3B	polymerase (RNA) III (DNA directed) polypeptide B	Nucleus	enzyme
PPIG_HUMAN	PPIG	peptidylprolyl isomerase G (cyclophilin G)	Nucleus	enzyme
PRDX2_HUMAN	PRDX2	peroxiredoxin 2	Cytoplasm	enzyme
PRDX6_HUMAN	PRDX6	peroxiredoxin 6	Cytoplasm	enzyme
PTGDS_HUMAN	PTGDS	prostaglandin D2 synthase 21kDa (brain)	Cytoplasm	enzyme
RAB10_HUMAN	RAB10	RAB10, member RAS oncogene family	Cytoplasm	enzyme
RAB6A_HUMAN	RAB6A	RAB6A, member RAS oncogene family	Cytoplasm	enzyme
RBM23_HUMAN	RBM23	RNA binding motif protein 23	Nucleus	other
REG1A_HUMAN	REG1A	regenerating islet-derived 1 alpha	Extracellular Space	growth factor
RS16_HU	RPS16	ribosomal protein S16	Cytoplasm	other

MAN				
RS27A_HUMAN	RPS27A	ribosomal protein S27a	Cytoplasm	other
RL1D1_HUMAN	RSL1D1	ribosomal L1 domain containing 1	Nucleus	other
RSPO1_HUMAN	RSPO1	R-spondin 1	Plasma Membrane	other
RXRB_HUMAN	RXRB	retinoid X receptor, beta	Nucleus	ligand-dependent nuclear receptor
DHSA_HUMAN	SDHA	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	Cytoplasm	enzyme
SEM5B_HUMAN	SEMA5B	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5B	Plasma Membrane	other
SPD2A_HUMAN	SH3PXD2A	SH3 and PX domains 2A	Cytoplasm	other
SHAN1_HUMAN	SHANK1	SH3 and multiple ankyrin repeat domains 1	Cytoplasm	other
CTL1_HUMAN	SLC44A1	solute carrier family 44 (choline transporter), member 1	Plasma Membrane	transporter
SMC5_HUMAN	SMC5	structural maintenance of chromosomes 5	Nucleus	other
SPAG7_HUMAN	SPAG7	sperm associated antigen 7	Nucleus	other
SPA2L_HUMAN	SPATA2L	spermatogenesis associated 2-like	Other	other
SRG2C_HUMAN	SRGAP2C	SLIT-ROBO Rho GTPase activating protein 2C	Other	other
SIA7B_HUMAN	ST6GALNAC2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2	Cytoplasm	enzyme
SYNE1_HUMAN	SYNE1	spectrin repeat containing, nuclear envelope 1	Nucleus	other
TBX20_HUMAN	TBX20	T-box 20	Nucleus	transcription regulator
TET1_HUMAN	TET1	tet methylcytosine dioxygenase 1	Nucleus	other
TLK2_HUMAN	TLK2	tousled-like kinase 2	Cytoplasm	kinase
T151A_HUMAN	TMEM151A	transmembrane protein 151A	Other	other
TM232_HUMAN	TMEM232	transmembrane protein 232	Other	other
TMM95_HUMAN	TMEM95	transmembrane protein 95	Other	other
TPD54_HUMAN	TPD52L2	tumor protein D52-like 2	Cytoplasm	other
TPRN_HUMAN	TPRN	taperin	Extracellular Space	other
TARA_HUMAN	TRIOBP	TRIO and F-actin binding protein	Nucleus	other
TTBK2_HUMAN	TTBK2	tau tubulin kinase 2	Other	kinase
TITIN_HUMAN	TTN	titin	Other	kinase
UBR2_HUMAN	UBR2	ubiquitin protein ligase E3 component n-recognin 2	Nucleus	enzyme
UN13A_HUMAN	UNC13A	unc-13 homolog A (C. elegans)	Plasma Membrane	other
UNC79_HUMAN	UNC79	unc-79 homolog (C. elegans)	Extracellular Space	other
MELT_HUMAN	VEPH1	ventricular zone expressed PH domain-containing 1	Nucleus	other
VIME_HU	VIM	vimentin	Cytoplasm	other

MAN				
WDR1_HUMAN	WDR1	WD repeat domain 1	Extracellular Space	other
WDR43_HUMAN	WDR43	WD repeat domain 43	Nucleus	other
1433T_HUMAN	YWHAQ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide	Cytoplasm	other
ZN345_HUMAN	ZNF345	zinc finger protein 345	Nucleus	transcription regulator
ZN516_HUMAN	ZNF516	zinc finger protein 516	Nucleus	other
ZN671_HUMAN	ZNF671	zinc finger protein 671	Nucleus	other

7 week (n=10).

ID	Symbol	Entrez Gene Name	Location	Type(s)
A2MG_HUMAN	A2M	alpha-2-macroglobulin	Extracellular Space	transporter
ATS16_HUMAN	ADAMTS16	ADAM metallopeptidase with thrombospondin type 1 motif, 16	Extracellular Space	other
DSRAD_HUMAN	ADAR	adenosine deaminase, RNA-specific	Nucleus	enzyme
ADCY7_HUMAN	ADCY7	adenylate cyclase 7	Plasma Membrane	enzyme
PLCA_HUMAN	AGPAT1	1-acylglycerol-3-phosphate O-acyltransferase 1	Cytoplasm	enzyme
AICDA_HUMAN	AICDA	activation-induced cytidine deaminase	Cytoplasm	enzyme
ALBU_HUMAN	ALB	albumin	Extracellular Space	transporter
AMZ1_HUMAN	AMZ1	archaelysin family metallopeptidase 1	Other	peptidase
ANR12_HUMAN	ANKRD12	ankyrin repeat domain 12	Nucleus	other
ANR26_HUMAN	ANKRD26	ankyrin repeat domain 26	Nucleus	transcription regulator
APOA1_HUMAN	APOA1	apolipoprotein A-I	Extracellular Space	transporter
APOB_HUMAN	APOB	apolipoprotein B	Extracellular Space	transporter
ASPH2_HUMAN	ASPHD2	aspartate beta-hydroxylase domain containing 2	Other	enzyme
BEND4_HUMAN	BEND4	BEN domain containing 4	Other	other
BLMH_HUMAN	BLMH	bleomycin hydrolase	Cytoplasm	peptidase
C1QC_HUMAN	C1QC	complement component 1, q subcomponent, C chain	Extracellular Space	other
CO3_HUMAN	C3	complement component 3	Extracellular Space	peptidase
CO4A_HUMAN	C4A/C4B	complement component 4B (Chido blood group)	Extracellular Space	other
C4BPA_HUMAN	C4BPA	complement component 4 binding protein, alpha	Extracellular Space	other
C8AP2_HUMAN	CASP8AP2	caspase 8 associated protein 2	Nucleus	transcription regulator
CC154_HUMAN	CCDC154	coiled-coil domain containing 154	Other	other
CCD70_HUMAN	CCDC70	coiled-coil domain containing 70	Plasma Membrane	other
CCD80_HUMAN	CCDC80	coiled-coil domain containing 80	Nucleus	other
CCHCR_HUMAN	CCHCR1	coiled-coil alpha-helical rod protein 1	Cytoplasm	other
CFAH_HUMAN	CFH	complement factor H	Extracellular Space	other
CHSTB_HUMAN	CHST11	carbohydrate (chondroitin 4) sulfotransferase 11	Cytoplasm	enzyme
CHSS3_HUMAN	CHSY3	chondroitin sulfate synthase 3	Cytoplasm	enzyme
CILP1_HUMAN	CILP	cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	Extracellular Space	phosphatase
CLUS_HUMAN	CLU	clusterin	Cytoplasm	other
CMBL_HUMAN	CMBL	carboxymethylenebutenolidase homolog (Pseudomonas)	Other	enzyme
CNO6L_HUMAN	CNOT6L	CCR4-NOT transcription complex, subunit 6-like	Cytoplasm	enzyme
CPXM2_HUMAN	CPXM2	carboxypeptidase X (M14 family), member 2	Extracellular Space	peptidase
DIAC_HUMAN	CTBS	chitobiase, di-N-acetyl-	Cytoplasm	enzyme
CTRL_HUMAN	CTRL	chymotrypsin-like	Extracellular Space	peptidase
C19L2_HUMAN	CWF19L2	CWF19-like 2, cell cycle control (S. pombe)	Other	other
DAPL1_HUMAN	DAPL1	death associated protein-like 1	Other	other
DCD_HUMAN	DCD	dermcidin	Extracellular Space	other

DMXL1_HUMAN	DMXL1	Dmx-like 1	Extracellular Space	other
DYH17_HUMAN	DNAH17	dynein, axonemal, heavy chain 17	Cytoplasm	other
EBP_HUMAN	EBP	emopamil binding protein (sterol isomerase)	Cytoplasm	enzyme
ENPP5_HUMAN	ENPP5	ectonucleotide pyrophosphatase/phosphodiesterase 5 (putative)	Extracellular Space	enzyme
EPHAA_HUMAN	EPHA10	EPH receptor A10	Plasma Membrane	transmembrane receptor
PERE_HUMAN	EPX	eosinophil peroxidase	Cytoplasm	enzyme
EXOS1_HUMAN	EXOSC1	exosome component 1	Nucleus	enzyme
FBF1_HUMAN	FBF1	Fas (TNFRSF6) binding factor 1	Nucleus	other
FIBA_HUMAN	FGA	fibrinogen alpha chain	Extracellular Space	other
FIBB_HUMAN	FGB	fibrinogen beta chain	Extracellular Space	other
FR1OP_HUMAN	FGFR1OP	FGFR1 oncogene partner	Cytoplasm	kinase
FGFR2_HUMAN	FGFR2	fibroblast growth factor receptor 2	Plasma Membrane	kinase
FIBG_HUMAN	FGG	fibrinogen gamma chain	Extracellular Space	other
FINC_HUMAN	FN1	fibronectin 1	Extracellular Space	enzyme
GLI2_HUMAN	GLI2	GLI family zinc finger 2	Nucleus	transcription regulator
AATC_HUMAN	GOT1	glutamic-oxaloacetic transaminase 1, soluble	Cytoplasm	enzyme
GP126_HUMAN	GPR126	G protein-coupled receptor 126	Plasma Membrane	G-protein coupled receptor
HERC1_HUMAN	HERC1	HECT and RLD domain containing E3 ubiquitin protein ligase family member 1	Cytoplasm	other
BPL1_HUMAN	HLCS	holocarboxylase synthetase (biotin-(proprionyl-CoA-carboxylase (ATP-hydrolysing)) ligase)	Cytoplasm	enzyme
HPTR_HUMAN	HPR	haptoglobin-related protein	Extracellular Space	peptidase
HS3S6_HUMAN	HS3ST6	heparan sulfate (glucosamine) 3-O-sulfotransferase 6	Cytoplasm	enzyme
5HT2A_HUMAN	HTR2A	5-hydroxytryptamine (serotonin) receptor 2A, G protein-coupled	Plasma Membrane	G-protein coupled receptor
IGHA1_HUMAN	IGHA1	immunoglobulin heavy constant alpha 1	Extracellular Space	other
IGHG1_HUMAN	IGHG1	immunoglobulin heavy constant gamma 1 (G1m marker)	Extracellular Space	other
IGHM_HUMAN	IGHM	immunoglobulin heavy constant mu	Plasma Membrane	transmembrane receptor
IGJ_HUMAN	IGJ	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	Extracellular Space	other
IGKC_HUMAN	IGKC	immunoglobulin kappa constant	Extracellular Space	other
KV401_HUMAN	IGKV4-1	immunoglobulin kappa variable 4-1	Extracellular Space	other
LAC1_HUMAN	IGLC1	immunoglobulin lambda constant 1 (Mcg marker)	Cytoplasm	other
IQCF6_HUMAN	IQCF6	IQ motif containing F6	Other	other
JARD2_HUMAN	JARID2	jumonji, AT rich interactive domain 2	Nucleus	transcription regulator
KTNB1_HUMAN	KATNB1	katanin p80 (WD repeat containing) subunit B 1	Cytoplasm	enzyme
KCND2_HUMAN	KCND2	potassium voltage-gated channel, Shal-related subfamily, member 2	Plasma Membrane	ion channel
KI13A_HUMAN	KIF13A	kinesin family member 13A	Cytoplasm	transporter
KIRR1_HUMAN	KIRREL	kin of IRRE like (Drosophila)	Plasma Membrane	other
KLC2_HUMAN	KLC2	kinesin light chain 2	Cytoplasm	other
K2C1_HUMAN	KRT1	keratin 1	Cytoplasm	other
K1C10_HUMAN	KRT10	keratin 10	Cytoplasm	other

K1C9_HUMAN	KRT9	keratin 9	Cytoplasm	other
LDB1_HUMAN	LDB1	LIM domain binding 1	Nucleus	transcription regulator
LIPC_HUMAN	LIPC	lipase, hepatic	Extracellular Space	enzyme
LZTR1_HUMAN	LZTR1	leucine-zipper-like transcription regulator 1	Cytoplasm	transcription regulator
MDN1_HUMAN	MDN1	MDN1, midasin homolog (yeast)	Nucleus	other
MKL1_HUMAN	MKL1	megakaryoblastic leukemia (translocation) 1	Nucleus	transcription regulator
ULA1_HUMAN	NAE1	NEDD8 activating enzyme E1 subunit 1	Cytoplasm	enzyme
NHS_HUMAN	NHS	Nance-Horan syndrome (congenital cataracts and dental anomalies)	Nucleus	other
NRX3A_HUMAN	NRXN3	neurexin 3	Other	transporter
NSD1_HUMAN	NSD1	nuclear receptor binding SET domain protein 1	Nucleus	transcription regulator
OR8K1_HUMAN	OR8K1	olfactory receptor, family 8, subfamily K, member 1	Plasma Membrane	G-protein coupled receptor
PCDBC_HUMAN	PCDHB12	protocadherin beta 12	Plasma Membrane	other
PEAK1_HUMAN	PEAK1	NKF3 kinase family member	Plasma Membrane	kinase
PFD6_HUMAN	PFDN6	prefoldin subunit 6	Cytoplasm	other
PGP_HUMAN	PGP	phosphoglycolate phosphatase	Other	enzyme
PIGS_HUMAN	PIGS	phosphatidylinositol glycan anchor biosynthesis, class S	Cytoplasm	enzyme
PNKD_HUMAN	PNKD	paroxysmal nonkinesigenic dyskinesia	Nucleus	other
PP1R7_HUMAN	PPP1R7	protein phosphatase 1, regulatory subunit 7	Nucleus	phosphatase
PREB_HUMAN	PREB	prolactin regulatory element binding	Nucleus	transcription regulator
RAB10_HUMAN	RAB10	RAB10, member RAS oncogene family	Cytoplasm	enzyme
RGPA2_HUMAN	RALGAPA2	Ral GTPase activating protein, alpha subunit 2 (catalytic)	Cytoplasm	other
RFX8_HUMAN	RFX8	RFX family member 8, lacking RFX DNA binding domain	Other	other
RN213_HUMAN	RNF213	ring finger protein 213	Other	enzyme
FTM_HUMAN	RPGRIP1L	RPGRIP1-like	Cytoplasm	other
RL37_HUMAN	RPL37	ribosomal protein L37	Cytoplasm	other
RL1D1_HUMAN	RSL1D1	ribosomal L1 domain containing 1	Nucleus	other
RSPO1_HUMAN	RSPO1	R-spondin 1	Plasma Membrane	other
RTN4_HUMAN	RTN4	reticulon 4	Cytoplasm	other
RYR2_HUMAN	RYR2	ryanodine receptor 2 (cardiac)	Plasma Membrane	ion channel
UTER_HUMAN	SCGB1A1	secretoglobin, family 1A, member 1 (uteroglobin)	Extracellular Space	cytokine
SHAN1_HUMAN	SHANK1	SH3 and multiple ankyrin repeat domains 1	Cytoplasm	other
S27A2_HUMAN	SLC27A2	solute carrier family 27 (fatty acid transporter), member 2	Cytoplasm	transporter
CTL1_HUMAN	SLC44A1	solute carrier family 44 (choline transporter), member 1	Plasma Membrane	transporter
SP100_HUMAN	SP100	SP100 nuclear antigen	Nucleus	transcription regulator
SPO11_HUMAN	SPO11	SPO11 meiotic protein covalently bound to DSB	Nucleus	enzyme
SRGP2_HUMAN	SRGAP2	SLIT-ROBO Rho GTPase activating protein 2	Cytoplasm	other
SP20H_HUMAN	SUPT20H	suppressor of Ty 20 homolog (S. cerevisiae)	Nucleus	other
SPT6H_HUMAN	SUPT6H	suppressor of Ty 6 homolog (S. cerevisiae)	Nucleus	transcription regulator
SVEP1_HUMAN	SVEP1	sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	Cytoplasm	other

BROMI_HUMAN	TBC1D32	TBC1 domain family, member 32	Other	other
TET1_HUMAN	TET1	tet methylcytosine dioxygenase 1	Nucleus	other
TLK2_HUMAN	TLK2	tousled-like kinase 2	Cytoplasm	kinase
TRA2B_HUMAN	TRA2B	transformer 2 beta homolog (Drosophila)	Nucleus	other
TRML4_HUMAN	TREML4	triggering receptor expressed on myeloid cells-like 4	Other	other
TARA_HUMAN	TRIOBP	TRIO and F-actin binding protein	Nucleus	other
TITIN_HUMAN	TTN	titin	Other	kinase
UBR2_HUMAN	UBR2	ubiquitin protein ligase E3 component n-recognin 2	Nucleus	enzyme
UGDH_HUMAN	UGDH	UDP-glucose 6-dehydrogenase	Nucleus	enzyme
VP13C_HUMAN	VPS13C	vacuolar protein sorting 13 homolog C (S. cerevisiae)	Other	other
WAC_HUMAN	WAC	WW domain containing adaptor with coiled-coil	Nucleus	other
WDR1_HUMAN	WDR1	WD repeat domain 1	Extracellular Space	other
WDR35_HUMAN	WDR35	WD repeat domain 35	Cytoplasm	other
ZFHX4_HUMAN	ZFHX4	zinc finger homeobox 4	Extracellular Space	other
ZMYM3_HUMAN	ZMYM3	zinc finger, MYM-type 3	Nucleus	other
ZN132_HUMAN	ZNF132	zinc finger protein 132	Nucleus	transcription regulator
ZN345_HUMAN	ZNF345	zinc finger protein 345	Nucleus	transcription regulator
ZN532_HUMAN	ZNF532	zinc finger protein 532	Other	other
ZN624_HUMAN	ZNF624	zinc finger protein 624	Nucleus	other

8 week (n=10).

ID	Symbol	Entrez Gene Name	Location	Type(s)
A2MG_HUMAN	A2M	alpha-2-macroglobulin	Extracellular Space	transporter
ACTN3_HUMAN	ACTN3	actinin, alpha 3	Other	other
ADA12_HUMAN	ADAM12	ADAM metallopeptidase domain 12	Plasma Membrane	peptidase
AICDA_HUMAN	AICDA	activation-induced cytidine deaminase	Cytoplasm	enzyme
AKAP9_HUMAN	AKAP9	A kinase (PRKA) anchor protein 9	Cytoplasm	other
ALBU_HUMAN	ALB	albumin	Extracellular Space	transporter
AMZ1_HUMAN	AMZ1	archaelysin family metallopeptidase 1	Other	peptidase
ANK2_HUMAN	ANK2	ankyrin 2, neuronal	Plasma Membrane	other
ANR12_HUMAN	ANKRD12	ankyrin repeat domain 12	Nucleus	other
APOA1_HUMAN	APOA1	apolipoprotein A-I	Extracellular Space	transporter
AT2A3_HUMAN	ATP2A3	ATPase, Ca++ transporting, ubiquitous	Cytoplasm	transporter
BAI2_HUMAN	BAI2	brain-specific angiogenesis inhibitor 2	Plasma Membrane	G-protein coupled receptor
BLMH_HUMAN	BLMH	bleomycin hydrolase	Cytoplasm	peptidase
BMP3_HUMAN	BMP3	bone morphogenetic protein 3	Extracellular Space	growth factor
CP013_HUMAN	C16orf13	chromosome 16 open reading frame 13	Other	other
CA174_HUMAN	C1orf174	chromosome 1 open reading frame 174	Other	other
C1QC_HUMAN	C1QC	complement component 1, q subcomponent, C chain	Extracellular Space	other
CO3_HUMAN	C3	complement component 3	Extracellular Space	peptidase
C4BPA_HUMAN	C4BPA	complement component 4 binding protein, alpha	Extracellular Space	other
CI078_HUMAN	C9orf78	chromosome 9 open reading frame 78	Other	other
CAND1_HUMAN	CAND1	cullin-associated and neddylation-dissociated 1	Cytoplasm	transcription regulator
CDRT1_HUMAN	CDRT1	CMT1A duplicated region transcript 1	Other	other
CHSS3_HUMAN	CHSY3	chondroitin sulfate synthase 3	Cytoplasm	enzyme
CLC4M_HUMAN	CLEC4M	C-type lectin domain family 4, member M	Plasma Membrane	other
CMBL_HUMAN	CMBL	carboxymethylenebutenolidase homolog (Pseudomonas)	Other	enzyme
DIAC_HUMAN	CTBS	chitobiase, di-N-acetyl-	Cytoplasm	enzyme
CTRL_HUMAN	CTRL	chymotrypsin-like	Extracellular Space	peptidase
DCD_HUMAN	DCD	dermcidin	Extracellular Space	other
DCR1B_HUMAN	DCLRE1B	DNA cross-link repair 1B	Nucleus	enzyme
DCSTP_HUMAN	DCSTAMP	dendrocyte expressed seven transmembrane protein	Plasma Membrane	other
DDX51_HUMAN	DDX51	DEAD (Asp-Glu-Ala-Asp) box polypeptide 51	Other	enzyme
DDX6L_HUMAN	DDX60L	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60-like	Other	other
DIP2B_HUMAN	DIP2B	DIP2 disco-interacting protein 2 homolog B (Drosophila)	Other	other
DYH11_HUMAN	DNAH11	dynein, axonemal, heavy chain 11	Cytoplasm	enzyme
DYH17_HUMAN	DNAH17	dynein, axonemal, heavy chain 17	Cytoplasm	other
DTNA_HUMAN	DTNA	dystrobrevin, alpha	Plasma Membrane	other
EXOC8_HUMAN	EXOC8	exocyst complex component 8	Plasma Membrane	other

NIBL2_HUMAN	FAM129C	family with sequence similarity 129, member C	Other	other
FIBA_HUMAN	FGA	fibrinogen alpha chain	Extracellular Space	other
FIBB_HUMAN	FGB	fibrinogen beta chain	Extracellular Space	other
FR1OP_HUMAN	FGFR1OP	FGFR1 oncogene partner	Cytoplasm	kinase
FIBG_HUMAN	FGG	fibrinogen gamma chain	Extracellular Space	other
FLNB_HUMAN	FLNB	filamin B, beta	Cytoplasm	other
FINC_HUMAN	FN1	fibronectin 1	Extracellular Space	enzyme
FSIP2_HUMAN	FSIP2	fibrous sheath interacting protein 2	Cytoplasm	other
GAK_HUMAN	GAK	cyclin G associated kinase	Nucleus	kinase
GDF6_HUMAN	GDF6	growth differentiation factor 6	Extracellular Space	growth factor
GIMA8_HUMAN	GIMAP8	GTPase, IMAP family member 8	Other	other
GMEB1_HUMAN	GMEB1	glucocorticoid modulatory element binding protein 1	Nucleus	transcription regulator
AATC_HUMAN	GOT1	glutamic-oxaloacetic transaminase 1, soluble	Cytoplasm	enzyme
GSHB_HUMAN	GSS	glutathione synthetase	Cytoplasm	enzyme
HAS2_HUMAN	HAS2	hyaluronan synthase 2	Plasma Membrane	enzyme
HBG1_HUMAN	HBG1	hemoglobin, gamma A	Cytoplasm	other
HERC1_HUMAN	HERC1	HECT and RLD domain containing E3 ubiquitin protein ligase family member 1	Cytoplasm	other
HILS1_HUMAN	HILS1	histone linker H1 domain, spermatid-specific 1, pseudogene	Nucleus	other
HIP1_HUMAN	HIP1	huntingtin interacting protein 1	Cytoplasm	other
BPL1_HUMAN	HLCS	holocarboxylase synthetase (biotin-(proprionyl-CoA-carboxylase (ATP-hydrolysing)) ligase)	Cytoplasm	enzyme
HS3S6_HUMAN	HS3ST6	heparan sulfate (glucosamine) 3-O-sulfotransferase 6	Cytoplasm	enzyme
5HT2A_HUMAN	HTR2A	5-hydroxytryptamine (serotonin) receptor 2A, G protein-coupled	Plasma Membrane	G-protein coupled receptor
IGHA1_HUMAN	IGHA1	immunoglobulin heavy constant alpha 1	Extracellular Space	other
IGHG1_HUMAN	IGHG1	immunoglobulin heavy constant gamma 1 (G1m marker)	Extracellular Space	other
IGHM_HUMAN	IGHM	immunoglobulin heavy constant mu	Plasma Membrane	transmembrane receptor
IGKC_HUMAN	IGKC	immunoglobulin kappa constant	Extracellular Space	other
KV401_HUMAN	IGKV4-1	immunoglobulin kappa variable 4-1	Extracellular Space	other
LAC1_HUMAN	IGLC1	immunoglobulin lambda constant 1 (Mcg marker)	Cytoplasm	other
IHH_HUMAN	IHH	indian hedgehog	Extracellular Space	enzyme
RED_HUMAN	IK	IK cytokine, down-regulator of HLA II	Extracellular Space	cytokine
IL1AP_HUMAN	IL1RAP	interleukin 1 receptor accessory protein	Plasma Membrane	transmembrane receptor
IRPL2_HUMAN	IL1RAPL2	interleukin 1 receptor accessory protein-like 2	Plasma Membrane	transmembrane receptor
KCNQ5_HUMAN	KCNQ5	potassium voltage-gated channel, KQT-like subfamily, member 5	Plasma Membrane	ion channel
KIF19_HUMAN	KIF19	kinesin family member 19	Extracellular Space	enzyme
K2C1_HUMAN	KRT1	keratin 1	Cytoplasm	other
K1C10_HUMAN	KRT10	keratin 10	Cytoplasm	other

K1C14_HUMAN	KRT14	keratin 14	Cytoplasm	other
K1C9_HUMAN	KRT9	keratin 9	Cytoplasm	other
LDB1_HUMAN	LDB1	LIM domain binding 1	Nucleus	transcription regulator
YP023_HUMAN	LOC100128265	uncharacterized LOC100128265	Other	other
LRC32_HUMAN	LRRC32	leucine rich repeat containing 32	Plasma Membrane	other
MBD5_HUMAN	MBD5	methyl-CpG binding domain protein 5	Other	other
MYBA_HUMAN	MYBL1	v-myb avian myeloblastosis viral oncogene homolog-like 1	Nucleus	transcription regulator
ULA1_HUMAN	NAE1	NEDD8 activating enzyme E1 subunit 1	Cytoplasm	enzyme
NUCL_HUMAN	NCL	nucleolin	Nucleus	other
NDUF4_HUMAN	NDUFAF4	NADH dehydrogenase (ubiquinone) complex I, assembly factor 4	Cytoplasm	other
NRX3A_HUMAN	NRXN3	neurexin 3	Other	transporter
ODFP2_HUMAN	ODF2	outer dense fiber of sperm tails 2	Cytoplasm	other
NOE2_HUMAN	OLFM2	olfactomedin 2	Cytoplasm	other
PARP4_HUMAN	PARP4	poly (ADP-ribose) polymerase family, member 4	Other	enzyme
PCDBC_HUMAN	PCDHB12	protocadherin beta 12	Plasma Membrane	other
PLCD3_HUMAN	PLCD3	phospholipase C, delta 3	Cytoplasm	enzyme
PNKD_HUMAN	PNKD	paroxysmal nonkinesigenic dyskinesia	Nucleus	other
PNKP_HUMAN	PNKP	polynucleotide kinase 3'-phosphatase	Nucleus	kinase
PPT2_HUMAN	PPT2	palmitoyl-protein thioesterase 2	Cytoplasm	enzyme
PRC2A_HUMAN	PRRC2A	proline-rich coiled-coil 2A	Cytoplasm	other
PTN13_HUMAN	PTPN13	protein tyrosine phosphatase, non-receptor type 13 (APO-1/CD95 (Fas)-associated phosphatase)	Cytoplasm	phosphatase
RAB10_HUMAN	RAB10	RAB10, member RAS oncogene family	Cytoplasm	enzyme
RBCC1_HUMAN	RB1CC1	RB1-inducible coiled-coil 1	Nucleus	other
REG1A_HUMAN	REG1A	regenerating islet-derived 1 alpha	Extracellular Space	growth factor
RFX8_HUMAN	RFX8	RFX family member 8, lacking RFX DNA binding domain	Other	other
RN180_HUMAN	RNF180	ring finger protein 180	Cytoplasm	enzyme
RL1D1_HUMAN	RSL1D1	ribosomal L1 domain containing 1	Nucleus	other
SARM1_HUMAN	SARM1	sterile alpha and TIR motif containing 1	Plasma Membrane	transmembrane receptor
SASH1_HUMAN	SASH1	SAM and SH3 domain containing 1	Extracellular Space	other
SPB9_HUMAN	SERPINB9	serpin peptidase inhibitor, clade B (ovalbumin), member 9	Cytoplasm	other
SHAN3_HUMAN	SHANK3	SH3 and multiple ankyrin repeat domains 3	Plasma Membrane	other
S27A2_HUMAN	SLC27A2	solute carrier family 27 (fatty acid transporter), member 2	Cytoplasm	transporter
SPAG7_HUMAN	SPAG7	sperm associated antigen 7	Nucleus	other
SPA2L_HUMAN	SPATA2L	spermatogenesis associated 2-like	Other	other
SRGP2_HUMAN	SRGAP2	SLIT-ROBO Rho GTPase activating protein 2	Cytoplasm	other
SRG2C_HUMAN	SRGAP2C	SLIT-ROBO Rho GTPase activating protein 2C	Other	other
SIA7B_HUMAN	ST6GALNAC2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2	Cytoplasm	enzyme
SVEP1_HUMAN	SVEP1	sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	Cytoplasm	other
TBX20_HUMAN	TBX20	T-box 20	Nucleus	transcription regulator

TFP11_HUMAN	TFIP11	tuftelin interacting protein 11	Extracellular Space	other
TMM95_HUMAN	TMEM95	transmembrane protein 95	Other	other
TARA_HUMAN	TRIOBP	TRIO and F-actin binding protein	Nucleus	other
TTBK2_HUMAN	TTBK2	tau tubulin kinase 2	Other	kinase
TITIN_HUMAN	TTN	titin	Other	kinase
TRXR3_HUMAN	TXNRD3	thioredoxin reductase 3	Cytoplasm	enzyme
UBR2_HUMAN	UBR2	ubiquitin protein ligase E3 component n-recognin 2	Nucleus	enzyme
VASH2_HUMAN	VASH2	vasohibin 2	Cytoplasm	other
WDR1_HUMAN	WDR1	WD repeat domain 1	Extracellular Space	other
YIPF1_HUMAN	YIPF1	Yip1 domain family, member 1	Cytoplasm	other
TUT4_HUMAN	ZCCHC11	zinc finger, CCHC domain containing 11	Nucleus	enzyme
ZF64B_HUMAN	ZFP64	ZFP64 zinc finger protein	Nucleus	other
ZNF14_HUMAN	ZNF14	zinc finger protein 14	Nucleus	transcription regulator
ZN285_HUMAN	ZNF285	zinc finger protein 285	Nucleus	other
ZN624_HUMAN	ZNF624	zinc finger protein 624	Nucleus	other

9 week (n=10).

ID	Symbol	Entrez Gene Name	Location	Type(s)
A2MG_HUMAN	A2M	alpha-2-macroglobulin	Extracellular Space	transporter
A2ML1_HUMAN	A2ML1	alpha-2-macroglobulin-like 1	Cytoplasm	other
ADAL_HUMAN	ADAL	adenosine deaminase-like	Cytoplasm	enzyme
KFA_HUMAN	AFMID	arylformamidase	Nucleus	enzyme
ALBU_HUMAN	ALB	albumin	Extracellular Space	transporter
AMZ1_HUMAN	AMZ1	archaelysin family metallopeptidase 1	Other	peptidase
ANKAR_HUMAN	ANKAR	ankyrin and armadillo repeat containing	Nucleus	transcription regulator
ANKL1_HUMAN	ANKLE1	ankyrin repeat and LEM domain containing 1	Other	other
ANR12_HUMAN	ANKRD12	ankyrin repeat domain 12	Nucleus	other
APOA1_HUMAN	APOA1	apolipoprotein A-I	Extracellular Space	transporter
RHG08_HUMAN	ARHGAP8/PRR5-ARHGAP8	Rho GTPase activating protein 8	Cytoplasm	other
ARHGB_HUMAN	ARHGEF11	Rho guanine nucleotide exchange factor (GEF) 11	Cytoplasm	other
B4GT7_HUMAN	B4GALT7	xylosylprotein beta 1,4-galactosyltransferase, polypeptide 7	Cytoplasm	enzyme
CA228_HUMAN	C1orf228	chromosome 1 open reading frame 228	Other	other
C1QC_HUMAN	C1QC	complement component 1, q subcomponent, C chain	Extracellular Space	other
CO3_HUMAN	C3	complement component 3	Extracellular Space	peptidase
C4BPA_HUMAN	C4BPA	complement component 4 binding protein, alpha	Extracellular Space	other
CAND1_HUMAN	CAND1	cullin-associated and neddylation-dissociated 1	Cytoplasm	transcription regulator
CAN1_HUMAN	CAPN1	calpain 1, (mu/I) large subunit	Cytoplasm	peptidase
CAN2_HUMAN	CAPN2	calpain 2, (m/II) large subunit	Cytoplasm	peptidase
CASC5_HUMAN	CASC5	cancer susceptibility candidate 5	Nucleus	other
ACHG_HUMAN	CHRNG	cholinergic receptor, nicotinic, gamma (muscle)	Plasma Membrane	transmembrane receptor
CHSS3_HUMAN	CHSY3	chondroitin sulfate synthase 3	Cytoplasm	enzyme
CLNK_HUMAN	CLNK	cytokine-dependent hematopoietic cell linker	Cytoplasm	other
CMBL_HUMAN	CMBL	carboxymethylenebutenolidase homolog (Pseudomonas)	Other	enzyme
DIAC_HUMAN	CTBS	chitobiase, di-N-acetyl-	Cytoplasm	enzyme
CP51A_HUMAN	CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1	Cytoplasm	enzyme
DDX51_HUMAN	DDX51	DEAD (Asp-Glu-Ala-Asp) box polypeptide 51	Other	enzyme
DUS3L_HUMAN	DUS3L	dihydrouridine synthase 3-like (S. cerevisiae)	Other	other
EIF3C_HUMAN	EIF3C/EIF3CL	eukaryotic translation initiation factor 3, subunit C	Cytoplasm	translation regulator
F208B_HUMAN	FAM208B	family with sequence similarity 208, member B	Other	other
FA78B_HUMAN	FAM78B	family with sequence similarity 78, member B	Other	other
FIBA_HUMAN	FGA	fibrinogen alpha chain	Extracellular Space	other
FR1OP_HUMAN	FGFR1OP	FGFR1 oncogene partner	Cytoplasm	kinase
FIBG_HUMAN	FGG	fibrinogen gamma chain	Extracellular Space	other
FHAD1_HUMAN	FHAD1	forkhead-associated (FHA) phosphopeptide binding domain 1	Other	other
FINC_HUMAN	FN1	fibronectin 1	Extracellular Space	enzyme
G6PC2_HUMAN	G6PC2	glucose-6-phosphatase, catalytic, 2	Cytoplasm	phosphatase
AATC_HUMAN	GOT1	glutamic-oxaloacetic transaminase 1, soluble	Cytoplasm	enzyme

HERC1_HUMAN	HERC1	HECT and RLD domain containing E3 ubiquitin protein ligase family member 1	Cytoplasm	other
HIP1_HUMAN	HIP1	huntingtin interacting protein 1	Cytoplasm	other
HJURP_HUMAN	HJURP	Holliday junction recognition protein	Nucleus	other
IGHA1_HUMAN	IGHA1	immunoglobulin heavy constant alpha 1	Extracellular Space	other
IGHG1_HUMAN	IGHG1	immunoglobulin heavy constant gamma 1 (G1m marker)	Extracellular Space	other
IGHM_HUMAN	IGHM	immunoglobulin heavy constant mu	Plasma Membrane	transmembrane receptor
IGKC_HUMAN	IGKC	immunoglobulin kappa constant	Extracellular Space	other
KV401_HUMAN	IGKV4-1	immunoglobulin kappa variable 4-1	Extracellular Space	other
LAC1_HUMAN	IGLC1	immunoglobulin lambda constant 1 (Mcg marker)	Cytoplasm	other
IL1AP_HUMAN	IL1RAP	interleukin 1 receptor accessory protein	Plasma Membrane	transmembrane receptor
K1161_HUMAN	KIAA1161	KIAA1161	Nucleus	other
KIRR1_HUMAN	KIRREL	kin of IRRE like (Drosophila)	Plasma Membrane	other
KLC2_HUMAN	KLC2	kinesin light chain 2	Cytoplasm	other
LIPC_HUMAN	LIPC	lipase, hepatic	Extracellular Space	enzyme
MANF_HUMAN	MANF	mesencephalic astrocyte-derived neurotrophic factor	Extracellular Space	other
MAP4_HUMAN	MAP4	microtubule-associated protein 4	Cytoplasm	other
RM32_HUMAN	MRPL32	mitochondrial ribosomal protein L32	Cytoplasm	translation regulator
ULA1_HUMAN	NAE1	NEDD8 activating enzyme E1 subunit 1	Cytoplasm	enzyme
NEBU_HUMAN	NEB	nebulin	Other	other
NEDD4_HUMAN	NEDD4	neural precursor cell expressed, developmentally down-regulated 4, E3 ubiquitin protein ligase	Cytoplasm	enzyme
NOA1_HUMAN	NOA1	nitric oxide associated 1	Cytoplasm	other
NUD15_HUMAN	NUDT15	nudix (nucleoside diphosphate linked moiety X)-type motif 15	Cytoplasm	phosphatase
OPN4_HUMAN	OPN4	opsin 4	Plasma Membrane	G-protein coupled receptor
OR4K1_HUMAN	OR4K1	olfactory receptor, family 4, subfamily K, member 1	Plasma Membrane	G-protein coupled receptor
PER3_HUMAN	PER3	period circadian clock 3	Nucleus	other
P3C2A_HUMAN	PIK3C2A	phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 alpha	Cytoplasm	kinase
DPOLQ_HUMAN	POLQ	polymerase (DNA directed), theta	Nucleus	enzyme
PMGT1_HUMAN	POMGNT1	protein O-linked mannose N-acetylglucosaminyltransferase 1 (beta 1,2-)	Cytoplasm	enzyme
PP12C_HUMAN	PPP1R12C	protein phosphatase 1, regulatory subunit 12C	Cytoplasm	phosphatase
PSB3_HUMAN	PSMB3	proteasome (prosome, macropain) subunit, beta type, 3	Cytoplasm	peptidase
PTPRM_HUMAN	PTPRM	protein tyrosine phosphatase, receptor type, M	Plasma Membrane	phosphatase
RAB10_HUMAN	RAB10	RAB10, member RAS oncogene family	Cytoplasm	enzyme
RB3GP_HUMAN	RAB3GAP1	RAB3 GTPase activating protein subunit 1 (catalytic)	Cytoplasm	other
RELN_HUMAN	RELN	reelin	Extracellular Space	peptidase
RFX8_HUMAN	RFX8	RFX family member 8, lacking RFX DNA binding domain	Other	other
RMND1_HUMAN	RMND1	required for meiotic nuclear division 1 homolog (S. cerevisiae)	Cytoplasm	other
SCUB3_HUMAN	SCUBE3	signal peptide, CUB domain, EGF-like 3	Plasma Membrane	other
SHAN1_HUMAN	SHANK1	SH3 and multiple ankyrin repeat domains 1	Cytoplasm	other
SPAG7_HUMAN	SPAG7	sperm associated antigen 7	Nucleus	other

SPA2L_HUMAN	SPATA2L	spermatogenesis associated 2-like	Other	other
SPO11_HUMAN	SPO11	SPO11 meiotic protein covalently bound to DSB	Nucleus	enzyme
SRGP2_HUMAN	SRGAP2	SLIT-ROBO Rho GTPase activating protein 2	Cytoplasm	other
SIA7B_HUMAN	ST6GALNAC2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2	Cytoplasm	enzyme
TBX20_HUMAN	TBX20	T-box 20	Nucleus	transcription regulator
TDRD1_HUMAN	TDRD1	tudor domain containing 1	Cytoplasm	other
TET1_HUMAN	TET1	tet methylcytosine dioxygenase 1	Nucleus	other
TM131_HUMAN	TMEM131	transmembrane protein 131	Extracellular Space	other
TRI65_HUMAN	TRIM65	tripartite motif containing 65	Other	other
TRPC5_HUMAN	TRPC5	transient receptor potential cation channel, subfamily C, member 5	Plasma Membrane	ion channel
GCP6_HUMAN	TUBGCP6	tubulin, gamma complex associated protein 6	Cytoplasm	other
USP9X_HUMAN	USP9X	ubiquitin specific peptidase 9, X-linked	Plasma Membrane	peptidase
VP13C_HUMAN	VPS13C	vacuolar protein sorting 13 homolog C (S. cerevisiae)	Other	other
WDR1_HUMAN	WDR1	WD repeat domain 1	Extracellular Space	other
WDR35_HUMAN	WDR35	WD repeat domain 35	Cytoplasm	other
ZFHX4_HUMAN	ZFHX4	zinc finger homeobox 4	Extracellular Space	other
Z286B_HUMAN	ZNF286B	zinc finger protein 286B	Other	other
ZN345_HUMAN	ZNF345	zinc finger protein 345	Nucleus	transcription regulator
ZN624_HUMAN	ZNF624	zinc finger protein 624	Nucleus	other

10 week (n=10).

ID	Symbol	Entrez Gene Name	Location	Type(s)
A2MG_HUMAN	A2M	alpha-2-macroglobulin	Extracellular Space	transporter
GABT_HUMAN	ABAT	4-aminobutyrate aminotransferase	Cytoplasm	enzyme
ABCF1_HUMAN	ABCF1	ATP-binding cassette, sub-family F (GCN20), member 1	Cytoplasm	transporter
ACTT1_HUMAN	ACTRT1	actin-related protein T1	Cytoplasm	other
ADA12_HUMAN	ADAM12	ADAM metallopeptidase domain 12	Plasma Membrane	peptidase
AICDA_HUMAN	AICDA	activation-induced cytidine deaminase	Cytoplasm	enzyme
ALBU_HUMAN	ALB	albumin	Extracellular Space	transporter
ANK2_HUMAN	ANK2	ankyrin 2, neuronal	Plasma Membrane	other
ANR12_HUMAN	ANKRD12	ankyrin repeat domain 12	Nucleus	other
ANR31_HUMAN	ANKRD31	ankyrin repeat domain 31	Extracellular Space	other
APOA1_HUMAN	APOA1	apolipoprotein A-I	Extracellular Space	transporter
APOL1_HUMAN	APOL1	apolipoprotein L, 1	Extracellular Space	transporter
APOP1_HUMAN	APOPT1	apoptogenic 1, mitochondrial	Cytoplasm	other
DP13B_HUMAN	APPL2	adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 2	Cytoplasm	other
ATOH8_HUMAN	ATOH8	atonal homolog 8 (Drosophila)	Other	other
OSTCN_HUMAN	BGLAP	bone gamma-carboxyglutamate (gla) protein	Extracellular Space	other
CO3_HUMAN	C3	complement component 3	Extracellular Space	peptidase
CO4A_HUMAN	C4A/C4B	complement component 4B (Chido blood group)	Extracellular Space	other
C4BPA_HUMAN	C4BPA	complement component 4 binding protein, alpha	Extracellular Space	other
CABIN_HUMAN	CABIN1	calcineurin binding protein 1	Nucleus	other
KKCC1_HUMAN	CAMKK1	calcium/calmodulin-dependent protein kinase kinase 1, alpha	Cytoplasm	kinase
CAND1_HUMAN	CAND1	cullin-associated and neddylation-dissociated 1	Cytoplasm	transcription regulator
CBX7_HUMAN	CBX7	chromobox homolog 7	Nucleus	other
CCD37_HUMAN	CCDC37	coiled-coil domain containing 37	Other	other
CP135_HUMAN	CEP135	centrosomal protein 135kDa	Cytoplasm	other
CHD9_HUMAN	CHD9	chromodomain helicase DNA binding protein 9	Cytoplasm	other
CHSS3_HUMAN	CHSY3	chondroitin sulfate synthase 3	Cytoplasm	enzyme
CPN2_HUMAN	CPN2	carboxypeptidase N, polypeptide 2	Extracellular Space	peptidase
DIAC_HUMAN	CTBS	chitobiase, di-N-acetyl-	Cytoplasm	enzyme
CUL9_HUMAN	CUL9	cullin 9	Cytoplasm	other
CWC25_HUMAN	CWC25	CWC25 spliceosome-associated protein homolog (S. cerevisiae)	Other	other
DCD_HUMAN	DCD	dermcidin	Extracellular Space	other
DCR1B_HUMAN	DCLRE1B	DNA cross-link repair 1B	Nucleus	enzyme
DDX51_HUMAN	DDX51	DEAD (Asp-Glu-Ala-Asp) box polypeptide 51	Other	enzyme
DGC14_HUMAN	DGCR14	DiGeorge syndrome critical region gene 14	Nucleus	other
DYH5_HUMAN	DNAH5	dynein, axonemal, heavy chain 5	Extracellular Space	enzyme
DNJC7_HUMAN	DNAJC7	DnaJ (Hsp40) homolog, subfamily C, member 7	Cytoplasm	other
EDN1_HUMAN	EDN1	endothelin 1	Extracellular Space	cytokine

EIF3C_HUMAN	EIF3C/EIF3CL	eukaryotic translation initiation factor 3, subunit C	Cytoplasm	translation regulator
ENPP1_HUMAN	ENPP1	ectonucleotide pyrophosphatase/phosphodiesterase 1	Plasma Membrane	enzyme
ESPL1_HUMAN	ESPL1	extra spindle pole bodies homolog 1 (S. cerevisiae)	Nucleus	peptidase
NIBL2_HUMAN	FAM129C	family with sequence similarity 129, member C	Other	other
FIBA_HUMAN	FGA	fibrinogen alpha chain	Extracellular Space	other
FIBB_HUMAN	FGB	fibrinogen beta chain	Extracellular Space	other
FR1OP_HUMAN	FGFR1OP	FGFR1 oncogene partner	Cytoplasm	kinase
FIBG_HUMAN	FGG	fibrinogen gamma chain	Extracellular Space	other
FINC_HUMAN	FN1	fibronectin 1	Extracellular Space	enzyme
GLTL5_HUMAN	GALNTL5	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 5	Cytoplasm	enzyme
AATC_HUMAN	GOT1	glutamic-oxaloacetic transaminase 1, soluble	Cytoplasm	enzyme
GSAP_HUMAN	GSAP	gamma-secretase activating protein	Cytoplasm	peptidase
GSHB_HUMAN	GSS	glutathione synthetase	Cytoplasm	enzyme
HBG1_HUMAN	HBG1	hemoglobin, gamma A	Cytoplasm	other
HIP1_HUMAN	HIP1	huntingtin interacting protein 1	Cytoplasm	other
BPL1_HUMAN	HLCS	holocarboxylase synthetase (biotin-(proprionyl-CoA-carboxylase (ATP-hydrolysing)) ligase)	Cytoplasm	enzyme
HNF4A_HUMAN	HNF4A	hepatocyte nuclear factor 4, alpha	Nucleus	transcription regulator
HPTR_HUMAN	HPR	haptoglobin-related protein	Extracellular Space	peptidase
HS3S6_HUMAN	HS3ST6	heparan sulfate (glucosamine) 3-O-sulfotransferase 6	Cytoplasm	enzyme
GILT_HUMAN	IFI30	interferon, gamma-inducible protein 30	Cytoplasm	enzyme
IBP2_HUMAN	IGFBP2	insulin-like growth factor binding protein 2, 36kDa	Extracellular Space	other
IGHA1_HUMAN	IGHA1	immunoglobulin heavy constant alpha 1	Extracellular Space	other
IGHG1_HUMAN	IGHG1	immunoglobulin heavy constant gamma 1 (G1m marker)	Extracellular Space	other
IGHM_HUMAN	IGHM	immunoglobulin heavy constant mu	Plasma Membrane	transmembrane receptor
IGJ_HUMAN	IGJ	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	Extracellular Space	other
IGKC_HUMAN	IGKC	immunoglobulin kappa constant	Extracellular Space	other
KV401_HUMAN	IGKV4-1	immunoglobulin kappa variable 4-1	Extracellular Space	other
IL25_HUMAN	IL25	interleukin 25	Extracellular Space	cytokine
IL26_HUMAN	IL26	interleukin 26	Extracellular Space	cytokine
INHBB_HUMAN	INHBB	inhibin, beta B	Extracellular Space	growth factor
JARD2_HUMAN	JARID2	jumonji, AT rich interactive domain 2	Nucleus	transcription regulator
KIF4B_HUMAN	KIF4B	kinesin family member 4B	Nucleus	other
KLC2_HUMAN	KLC2	kinesin light chain 2	Cytoplasm	other
K2C1_HUMAN	KRT1	keratin 1	Cytoplasm	other
K1C10_HUMAN	KRT10	keratin 10	Cytoplasm	other
K1C14_HUMAN	KRT14	keratin 14	Cytoplasm	other
K22E_HUMAN	KRT2	keratin 2	Cytoplasm	other
K2C4_HUMAN	KRT4	keratin 4	Cytoplasm	other
K1C9_HUMAN	KRT9	keratin 9	Cytoplasm	other

LDB1_HUMAN	LDB1	LIM domain binding 1	Nucleus	transcription regulator
LY75_HUMAN	LY75	lymphocyte antigen 75	Plasma Membrane	transmembrane receptor
MANF_HUMAN	MANF	mesencephalic astrocyte-derived neurotrophic factor	Extracellular Space	other
MA7D3_HUMAN	MAP7D3	MAP7 domain containing 3	Cytoplasm	other
MRE11_HUMAN	MRE11A	MRE11 meiotic recombination 11 homolog A (S. cerevisiae)	Nucleus	enzyme
RT18C_HUMAN	MRPS18C	mitochondrial ribosomal protein S18C	Cytoplasm	other
MTOR_HUMAN	MTOR	mechanistic target of rapamycin (serine/threonine kinase)	Nucleus	kinase
MYF6_HUMAN	MYF6	myogenic factor 6 (herculin)	Nucleus	transcription regulator
ULA1_HUMAN	NAE1	NEDD8 activating enzyme E1 subunit 1	Cytoplasm	enzyme
NCOA2_HUMAN	NCOA2	nuclear receptor coactivator 2	Nucleus	transcription regulator
NCOA5_HUMAN	NCOA5	nuclear receptor coactivator 5	Nucleus	other
NFL_HUMAN	NEFL	neurofilament, light polypeptide	Cytoplasm	other
PSA_HUMAN	NPEPPS	aminopeptidase puromycin sensitive	Cytoplasm	peptidase
NSD1_HUMAN	NSD1	nuclear receptor binding SET domain protein 1	Nucleus	transcription regulator
NET5_HUMAN	NTN5	netrin 5	Other	other
OCEL1_HUMAN	OCEL1	occludin/ELL domain containing 1	Other	other
PAR3L_HUMAN	PARD3B	par-3 family cell polarity regulator beta	Plasma Membrane	other
PCDH8_HUMAN	PCDH8	protocadherin 8	Plasma Membrane	other
SOX_HUMAN	PIPOX	pipecolic acid oxidase	Cytoplasm	enzyme
PK1L1_HUMAN	PKD1L1	polycystic kidney disease 1 like 1	Extracellular Space	other
PLEC_HUMAN	PLEC	plectin	Cytoplasm	other
PNKD_HUMAN	PNKD	paroxysmal nonkinesigenic dyskinesia	Nucleus	other
PNKP_HUMAN	PNKP	polynucleotide kinase 3'-phosphatase	Nucleus	kinase
PRP31_HUMAN	PRPF31	pre-mRNA processing factor 31	Nucleus	other
PRC2A_HUMAN	PRRC2A	proline-rich coiled-coil 2A	Cytoplasm	other
PRS7_HUMAN	PSMC2	proteasome (prosome, macropain) 26S subunit, ATPase, 2	Nucleus	peptidase
PTTG3_HUMAN	PTTG3P	pituitary tumor-transforming 3, pseudogene	Other	other
PZP_HUMAN	PZP	pregnancy-zone protein	Extracellular Space	other
REG1A_HUMAN	REG1A	regenerating islet-derived 1 alpha	Extracellular Space	growth factor
RFC4_HUMAN	RFC4	replication factor C (activator 1) 4, 37kDa	Nucleus	other
RFX8_HUMAN	RFX8	RFX family member 8, lacking RFX DNA binding domain	Other	other
RN219_HUMAN	RNF219	ring finger protein 219	Other	other
FTM_HUMAN	RPGRIP1L	RPGRIP1-like	Cytoplasm	other
RL29_HUMAN	RPL29	ribosomal protein L29	Cytoplasm	other
KS6A4_HUMAN	RPS6KA4	ribosomal protein S6 kinase, 90kDa, polypeptide 4	Cytoplasm	kinase
S27A2_HUMAN	SLC27A2	solute carrier family 27 (fatty acid transporter), member 2	Cytoplasm	transporter
GTR11_HUMAN	SLC2A11	solute carrier family 2 (facilitated glucose transporter), member 11	Plasma Membrane	other
SRGP2_HUMAN	SRGAP2	SLIT-ROBO Rho GTPase activating protein 2	Cytoplasm	other
SIA7B_HUMAN	ST6GALNAC2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2	Cytoplasm	enzyme
MENTO_HUMAN	STARD3NL	STARD3 N-terminal like	Cytoplasm	other
STXB1_HUMAN	STXBP1	syntaxin binding protein 1	Cytoplasm	transporter
SPT6H_HUMAN	SUPT6H	suppressor of Ty 6 homolog (S. cerevisiae)	Nucleus	transcription regulator

TADA3_HUMAN	TADA3	transcriptional adaptor 3	Nucleus	transcription regulator
TET1_HUMAN	TET1	tet methylcytosine dioxygenase 1	Nucleus	other
TLK2_HUMAN	TLK2	tousled-like kinase 2	Cytoplasm	kinase
TRIPB_HUMAN	TRIP11	thyroid hormone receptor interactor 11	Cytoplasm	transcription regulator
TRPC5_HUMAN	TRPC5	transient receptor potential cation channel, subfamily C, member 5	Plasma Membrane	ion channel
TSC2_HUMAN	TSC2	tuberous sclerosis 2	Cytoplasm	other
TTBK2_HUMAN	TTBK2	tau tubulin kinase 2	Other	kinase
TITIN_HUMAN	TTN	titin	Other	kinase
UBQLN_HUMAN	UBQLNL	ubiquilin-like	Other	other
UBR2_HUMAN	UBR2	ubiquitin protein ligase E3 component n-recognin 2	Nucleus	enzyme
UCKL1_HUMAN	UCKL1	uridine-cytidine kinase 1-like 1	Cytoplasm	kinase
UGDH_HUMAN	UGDH	UDP-glucose 6-dehydrogenase	Nucleus	enzyme
UTRO_HUMAN	UTRN	utrophin	Plasma Membrane	transmembrane receptor
WDR1_HUMAN	WDR1	WD repeat domain 1	Extracellular Space	other
WDR35_HUMAN	WDR35	WD repeat domain 35	Cytoplasm	other
WFDC3_HUMAN	WFDC3	WAP four-disulfide core domain 3	Extracellular Space	other
NIPA_HUMAN	ZC3HC1	zinc finger, C3HC-type containing 1	Nucleus	other
TUT4_HUMAN	ZCCHC11	zinc finger, CCHC domain containing 11	Nucleus	enzyme
ZF64A_HUMAN	ZFP64	ZFP64 zinc finger protein	Nucleus	other
ZNF14_HUMAN	ZNF14	zinc finger protein 14	Nucleus	transcription regulator
ZN215_HUMAN	ZNF215	zinc finger protein 215	Nucleus	transcription regulator
ZN345_HUMAN	ZNF345	zinc finger protein 345	Nucleus	transcription regulator
ZN624_HUMAN	ZNF624	zinc finger protein 624	Nucleus	other
ZN735_HUMAN	ZNF735	zinc finger protein 735	Other	other

11 week (n=10).

ID	Symbol	Entrez Gene Name	Location	Type(s)
A2MG_HUMAN	A2M	alpha-2-macroglobulin	Extracellular Space	transporter
ACTT1_HUMAN	ACTRT1	actin-related protein T1	Cytoplasm	other
ADAL_HUMAN	ADAL	adenosine deaminase-like	Cytoplasm	enzyme
ADA30_HUMAN	ADAM30	ADAM metallopeptidase domain 30	Plasma Membrane	peptidase
ATS16_HUMAN	ADAMTS16	ADAM metallopeptidase with thrombospondin type 1 motif, 16	Extracellular Space	other
ANGT_HUMAN	AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	Extracellular Space	growth factor
AICDA_HUMAN	AICDA	activation-induced cytidine deaminase	Cytoplasm	enzyme
ALBU_HUMAN	ALB	albumin	Extracellular Space	transporter
AMZ1_HUMAN	AMZ1	archaelysin family metallopeptidase 1	Other	peptidase
ANR12_HUMAN	ANKRD12	ankyrin repeat domain 12	Nucleus	other
APOA1_HUMAN	APOA1	apolipoprotein A-I	Extracellular Space	transporter
APOB_HUMAN	APOB	apolipoprotein B	Extracellular Space	transporter
RHG15_HUMAN	ARHGAP15	Rho GTPase activating protein 15	Cytoplasm	other
ASPM_HUMAN	ASPM	asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	Nucleus	other
ATG2B_HUMAN	ATG2B	autophagy related 2B	Other	other
AT2A3_HUMAN	ATP2A3	ATPase, Ca++ transporting, ubiquitous	Cytoplasm	transporter
ATR_HUMAN	ATR	ataxia telangiectasia and Rad3 related	Nucleus	kinase
BC11B_HUMAN	BCL11B	B-cell CLL/lymphoma 11B (zinc finger protein)	Nucleus	other
BRCA2_HUMAN	BRCA2	breast cancer 2, early onset	Nucleus	transcription regulator
CO3_HUMAN	C3	complement component 3	Extracellular Space	peptidase
CO4A_HUMAN	C4A/C4B	complement component 4B (Chido blood group)	Extracellular Space	other
C4BPA_HUMAN	C4BPA	complement component 4 binding protein, alpha	Extracellular Space	other
CC171_HUMAN	CCDC171	coiled-coil domain containing 171	Other	other
CCD30_HUMAN	CCDC30	coiled-coil domain containing 30	Other	other
CCD37_HUMAN	CCDC37	coiled-coil domain containing 37	Other	other
CAD26_HUMAN	CDH26	cadherin 26	Plasma Membrane	other
CDK10_HUMAN	CDK10	cyclin-dependent kinase 10	Nucleus	kinase
CHD4_HUMAN	CHD4	chromodomain helicase DNA binding protein 4	Nucleus	enzyme
CHSS3_HUMAN	CHSY3	chondroitin sulfate synthase 3	Cytoplasm	enzyme
CSRN1_HUMAN	CSRNP1	cysteine-serine-rich nuclear protein 1	Nucleus	transcription regulator
DIAC_HUMAN	CTBS	chitobiase, di-N-acetyl-	Cytoplasm	enzyme
CTRL_HUMAN	CTRL	chymotrypsin-like	Extracellular Space	peptidase
CX028_HUMAN	CXorf28	chromosome X open reading frame 28	Other	other
DCAF6_HUMAN	DCAF6	DDB1 and CUL4 associated factor 6	Nucleus	transcription regulator
DCD_HUMAN	DCD	dermcidin	Extracellular Space	other
DDX51_HUMAN	DDX51	DEAD (Asp-Glu-Ala-Asp) box polypeptide 51	Other	enzyme
DEN2D_HUMAN	DENND2D	DENN/MADD domain containing 2D	Cytoplasm	other
DESM_HUMAN	DES	desmin	Cytoplasm	other

DGAT1_HUMAN	DGAT1	diacylglycerol O-acyltransferase 1	Cytoplasm	enzyme
DHX30_HUMAN	DHX30	DEAH (Asp-Glu-Ala-His) box helicase 30	Nucleus	enzyme
DYH3_HUMAN	DNAH3	dynein, axonemal, heavy chain 3	Extracellular Space	enzyme
DOP1_HUMAN	DOPEY1	dopey family member 1	Cytoplasm	other
EVC_HUMAN	EVC	Ellis van Creveld syndrome	Cytoplasm	other
F150A_HUMAN	FAM150A	family with sequence similarity 150, member A	Other	other
FIBB_HUMAN	FGB	fibrinogen beta chain	Extracellular Space	other
FR1OP_HUMAN	FGFR1OP	FGFR1 oncogene partner	Cytoplasm	kinase
FGRL1_HUMAN	FGFRL1	fibroblast growth factor receptor-like 1	Plasma Membrane	transmembrane receptor
FIBG_HUMAN	FGG	fibrinogen gamma chain	Extracellular Space	other
FIGL2_HUMAN	FIGNL2	fidgetin-like 2	Other	other
FINC_HUMAN	FN1	fibronectin 1	Extracellular Space	enzyme
GCN1L_HUMAN	GCN1L1	GCN1 general control of amino-acid synthesis 1-like 1 (yeast)	Cytoplasm	translation regulator
CXB1_HUMAN	GJB1	gap junction protein, beta 1, 32kDa	Plasma Membrane	transporter
AATC_HUMAN	GOT1	glutamic-oxaloacetic transaminase 1, soluble	Cytoplasm	enzyme
GRID2_HUMAN	GRID2	glutamate receptor, ionotropic, delta 2	Plasma Membrane	ion channel
HCN2_HUMAN	HCN2	hyperpolarization activated cyclic nucleotide-gated potassium channel 2	Plasma Membrane	ion channel
HES1_HUMAN	HES1	hairy and enhancer of split 1, (Drosophila)	Nucleus	transcription regulator
HS3S6_HUMAN	HS3ST6	heparan sulfate (glucosamine) 3-O-sulfotransferase 6	Cytoplasm	enzyme
IGHA1_HUMAN	IGHA1	immunoglobulin heavy constant alpha 1	Extracellular Space	other
IGHG1_HUMAN	IGHG1	immunoglobulin heavy constant gamma 1 (G1m marker)	Extracellular Space	other
IGHM_HUMAN	IGHM	immunoglobulin heavy constant mu	Plasma Membrane	transmembrane receptor
IGJ_HUMAN	IGJ	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	Extracellular Space	other
IGKC_HUMAN	IGKC	immunoglobulin kappa constant	Extracellular Space	other
KV401_HUMAN	IGKV4-1	immunoglobulin kappa variable 4-1	Extracellular Space	other
LAC1_HUMAN	IGLC1	immunoglobulin lambda constant 1 (Mcg marker)	Cytoplasm	other
LAC2_HUMAN	IGLC2	immunoglobulin lambda constant 2 (Kern-Oz- marker)	Extracellular Space	other
IL25_HUMAN	IL25	interleukin 25	Extracellular Space	cytokine
IL26_HUMAN	IL26	interleukin 26	Extracellular Space	cytokine
ITK_HUMAN	ITK	IL2-inducible T-cell kinase	Cytoplasm	kinase
KDM2B_HUMAN	KDM2B	lysine (K)-specific demethylase 2B	Nucleus	other
KIRR1_HUMAN	KIRREL	kin of IRRE like (Drosophila)	Plasma Membrane	other
KLC2_HUMAN	KLC2	kinesin light chain 2	Cytoplasm	other
K2C1_HUMAN	KRT1	keratin 1	Cytoplasm	other
K1C10_HUMAN	KRT10	keratin 10	Cytoplasm	other
K1C14_HUMAN	KRT14	keratin 14	Cytoplasm	other
K22E_HUMAN	KRT2	keratin 2	Cytoplasm	other
K2C4_HUMAN	KRT4	keratin 4	Cytoplasm	other
K1C9_HUMAN	KRT9	keratin 9	Cytoplasm	other

LAMA5_HUMAN	LAMA5	laminin, alpha 5	Extracellular Space	other
LDB1_HUMAN	LDB1	LIM domain binding 1	Nucleus	transcription regulator
LIPC_HUMAN	LIPC	lipase, hepatic	Extracellular Space	enzyme
LRP1B_HUMAN	LRP1B	low density lipoprotein receptor-related protein 1B	Plasma Membrane	transmembrane receptor
LTBP2_HUMAN	LTBP2	latent transforming growth factor beta binding protein 2	Extracellular Space	other
MAP4_HUMAN	MAP4	microtubule-associated protein 4	Cytoplasm	other
MBIP1_HUMAN	MBIP	MAP3K12 binding inhibitory protein 1	Nucleus	other
MEX3B_HUMAN	MEX3B	mex-3 RNA binding family member B	Other	kinase
MFNG_HUMAN	MFNG	MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	Cytoplasm	enzyme
MORC1_HUMAN	MORC1	MORC family CW-type zinc finger 1	Nucleus	other
MYO15_HUMAN	MYO15A	myosin XVA	Cytoplasm	other
MYO6_HUMAN	MYO6	myosin VI	Cytoplasm	other
ULA1_HUMAN	NAE1	NEDD8 activating enzyme E1 subunit 1	Cytoplasm	enzyme
NCOA5_HUMAN	NCOA5	nuclear receptor coactivator 5	Nucleus	other
NFL_HUMAN	NEFL	neurofilament, light polypeptide	Cytoplasm	other
NEK10_HUMAN	NEK10	NIMA-related kinase 10	Other	kinase
NRX3A_HUMAN	NRXN3	neurexin 3	Other	transporter
NET5_HUMAN	NTN5	netrin 5	Other	other
OBSCN_HUMAN	OBSCN	obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	Cytoplasm	kinase
PCDBC_HUMAN	PCDHB12	protocadherin beta 12	Plasma Membrane	other
PNKD_HUMAN	PNKD	paroxysmal nonkinesigenic dyskinesia	Nucleus	other
PPCEL_HUMAN	PREPL	prolyl endopeptidase-like	Other	peptidase
PRG4_HUMAN	PRG4	proteoglycan 4	Extracellular Space	other
PZP_HUMAN	PZP	pregnancy-zone protein	Extracellular Space	other
RAB10_HUMAN	RAB10	RAB10, member RAS oncogene family	Cytoplasm	enzyme
RAB8B_HUMAN	RAB8B	RAB8B, member RAS oncogene family	Cytoplasm	enzyme
RFX8_HUMAN	RFX8	RFX family member 8, lacking RFX DNA binding domain	Other	other
R144B_HUMAN	RNF144B	ring finger protein 144B	Other	enzyme
RRNAD_HUMAN	RRNAD1	ribosomal RNA adenine dimethylase domain containing 1	Other	other
RTKN_HUMAN	RTKN	rhotekin	Cytoplasm	other
SACS_HUMAN	SACS	spastic ataxia of Charlevoix-Saguenay (sacsin)	Plasma Membrane	other
SAMD8_HUMAN	SAMD8	sterile alpha motif domain containing 8	Cytoplasm	other
SET1A_HUMAN	SETD1A	SET domain containing 1A	Nucleus	ion channel
SH24A_HUMAN	SH2D4A	SH2 domain containing 4A	Cytoplasm	other
SHAN1_HUMAN	SHANK1	SH3 and multiple ankyrin repeat domains 1	Cytoplasm	other
SLN14_HUMAN	SLFN14	schlafen family member 14	Other	other
SPAG7_HUMAN	SPAG7	sperm associated antigen 7	Nucleus	other
SPTN5_HUMAN	SPTBN5	spectrin, beta, non-erythrocytic 5	Plasma Membrane	other
SRGP2_HUMAN	SRGAP2	SLIT-ROBO Rho GTPase activating protein 2	Cytoplasm	other
SRG2C_HUMAN	SRGAP2C	SLIT-ROBO Rho GTPase activating protein 2C	Other	other
SIA7B_HUMAN	ST6GALNAC2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2	Cytoplasm	enzyme
SPT6H_HUMAN	SUPT6H	suppressor of Ty 6 homolog (S. cerevisiae)	Nucleus	transcription regulator
TBX20_HUMAN	TBX20	T-box 20	Nucleus	transcription regulator

TLK2_HUMAN	TLK2	tousled-like kinase 2	Cytoplasm	kinase
T132C_HUMAN	TMEM132C	transmembrane protein 132C	Other	other
TNFA_HUMAN	TNF	tumor necrosis factor	Extracellular Space	cytokine
TRI32_HUMAN	TRIM32	tripartite motif containing 32	Nucleus	transcription regulator
TROAP_HUMAN	TROAP	trophinin associated protein	Cytoplasm	peptidase
TRPC5_HUMAN	TRPC5	transient receptor potential cation channel, subfamily C, member 5	Plasma Membrane	ion channel
TSG13_HUMAN	TSGA13	testis specific, 13	Other	other
TTBK2_HUMAN	TTBK2	tau tubulin kinase 2	Other	kinase
TITIN_HUMAN	TTN	titin	Other	kinase
TRXR3_HUMAN	TXNRD3	thioredoxin reductase 3	Cytoplasm	enzyme
UBR2_HUMAN	UBR2	ubiquitin protein ligase E3 component n-recognin 2	Nucleus	enzyme
MELT_HUMAN	VEPH1	ventricular zone expressed PH domain-containing 1	Nucleus	other
WDR1_HUMAN	WDR1	WD repeat domain 1	Extracellular Space	other
WDR35_HUMAN	WDR35	WD repeat domain 35	Cytoplasm	other
ZNF14_HUMAN	ZNF14	zinc finger protein 14	Nucleus	transcription regulator
ZN561_HUMAN	ZNF561	zinc finger protein 561	Nucleus	other

12 week (n=10).

ID	Symbol	Entrez Gene Name	Location	Type(s)
A2MG_HUMAN	A2M	alpha-2-macroglobulin	Extracellular Space	transporter
ADAL_HUMAN	ADAL	adenosine deaminase-like	Cytoplasm	enzyme
ANGT_HUMAN	AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	Extracellular Space	growth factor
ALBU_HUMAN	ALB	albumin	Extracellular Space	transporter
AKD1B_HUMAN	ANKDD1B	ankyrin repeat and death domain containing 1B	Other	other
ANR12_HUMAN	ANKRD12	ankyrin repeat domain 12	Nucleus	other
APOA1_HUMAN	APOA1	apolipoprotein A-I	Extracellular Space	transporter
APOB_HUMAN	APOB	apolipoprotein B	Extracellular Space	transporter
BRCA2_HUMAN	BRCA2	breast cancer 2, early onset	Nucleus	transcription regulator
CO3_HUMAN	C3	complement component 3	Extracellular Space	peptidase
C4BPA_HUMAN	C4BPA	complement component 4 binding protein, alpha	Extracellular Space	other
CAND1_HUMAN	CAND1	cullin-associated and neddylation-dissociated 1	Cytoplasm	transcription regulator
CCD80_HUMAN	CCDC80	coiled-coil domain containing 80	Nucleus	other
CHSS3_HUMAN	CHSY3	chondroitin sulfate synthase 3	Cytoplasm	enzyme
CMBL_HUMAN	CMBL	carboxymethylenebutenolidase homolog (Pseudomonas)	Other	enzyme
COPA1_HUMAN	COL25A1	collagen, type XXV, alpha 1	Cytoplasm	other
DIAC_HUMAN	CTBS	chitobiase, di-N-acetyl-	Cytoplasm	enzyme
CP1A2_HUMAN	CYP1A2	cytochrome P450, family 1, subfamily A, polypeptide 2	Cytoplasm	enzyme
DCAF6_HUMAN	DCAF6	DDB1 and CUL4 associated factor 6	Nucleus	transcription regulator
EBP_HUMAN	EBP	emopamil binding protein (sterol isomerase)	Cytoplasm	enzyme
YV021_HUMAN	FAM230B	family with sequence similarity 230, member B	Other	other
FIBA_HUMAN	FGA	fibrinogen alpha chain	Extracellular Space	other
FIBB_HUMAN	FGB	fibrinogen beta chain	Extracellular Space	other
FR1OP_HUMAN	FGFR1OP	FGFR1 oncogene partner	Cytoplasm	kinase
FIBG_HUMAN	FGG	fibrinogen gamma chain	Extracellular Space	other
FIGL2_HUMAN	FIGNL2	fidgetin-like 2	Other	other
FINC_HUMAN	FN1	fibronectin 1	Extracellular Space	enzyme
FRMD3_HUMAN	FRMD3	FERM domain containing 3	Other	other
GLRA2_HUMAN	GLRA2	glycine receptor, alpha 2	Plasma Membrane	ion channel
GOGA3_HUMAN	GOLGA3	golgin A3	Cytoplasm	transporter
AATC_HUMAN	GOT1	glutamic-oxaloacetic transaminase 1, soluble	Cytoplasm	enzyme
GSAS1_HUMAN	GSN-AS1	GSN antisense RNA 1	Other	other
HERC1_HUMAN	HERC1	HECT and RLD domain containing E3 ubiquitin protein ligase family member 1	Cytoplasm	other
I23O2_HUMAN	IDO2	indoleamine 2,3-dioxygenase 2	Cytoplasm	enzyme
IGHA1_HUMAN	IGHA1	immunoglobulin heavy constant alpha 1	Extracellular Space	other
IGHG1_HUMAN	IGHG1	immunoglobulin heavy constant gamma 1 (G1m marker)	Extracellular Space	other
IGHM_HUMAN	IGHM	immunoglobulin heavy constant mu	Plasma Membrane	transmembrane receptor

IGKC_HUMAN	IGKC	immunoglobulin kappa constant	Extracellular Space	other
KV401_HUMAN	IGKV4-1	immunoglobulin kappa variable 4-1	Extracellular Space	other
LAC1_HUMAN	IGLC1	immunoglobulin lambda constant 1 (Mcg marker)	Cytoplasm	other
IRPL2_HUMAN	IL1RAPL2	interleukin 1 receptor accessory protein-like 2	Plasma Membrane	transmembrane receptor
KI13A_HUMAN	KIF13A	kinesin family member 13A	Cytoplasm	transporter
KLRF1_HUMAN	KLRF1	killer cell lectin-like receptor subfamily F, member 1	Plasma Membrane	transmembrane receptor
LDB1_HUMAN	LDB1	LIM domain binding 1	Nucleus	transcription regulator
KLP6_HUMAN	LOC100130097	kinesin-like protein family member 6-like	Cytoplasm	other
MACD1_HUMAN	MACROD1	MACRO domain containing 1	Cytoplasm	enzyme
MLP3A_HUMAN	MAP1LC3A	microtubule-associated protein 1 light chain 3 alpha	Cytoplasm	other
MYO3A_HUMAN	MYO3A	myosin IIIA	Cytoplasm	kinase
ULA1_HUMAN	NAE1	NEDD8 activating enzyme E1 subunit 1	Cytoplasm	enzyme
NRX3A_HUMAN	NRXN3	neurexin 3	Other	transporter
PARP4_HUMAN	PARP4	poly (ADP-ribose) polymerase family, member 4	Other	enzyme
PCLO_HUMAN	PCLO	piccolo presynaptic cytomatrix protein	Cytoplasm	transporter
PEG10_HUMAN	PEG10	paternally expressed 10	Nucleus	other
PLXA4_HUMAN	PLXNA4	plexin A4	Plasma Membrane	transmembrane receptor
PNKD_HUMAN	PNKD	paroxysmal nonkinesigenic dyskinesia	Nucleus	other
PRG4_HUMAN	PRG4	proteoglycan 4	Extracellular Space	other
REG1A_HUMAN	REG1A	regenerating islet-derived 1 alpha	Extracellular Space	growth factor
RFX8_HUMAN	RFX8	RFX family member 8, lacking RFX DNA binding domain	Other	other
RNF17_HUMAN	RNF17	ring finger protein 17	Cytoplasm	other
FTM_HUMAN	RPGRIP1L	RPGRIP1-like	Cytoplasm	other
KS6A4_HUMAN	RPS6KA4	ribosomal protein S6 kinase, 90kDa, polypeptide 4	Cytoplasm	kinase
SHAN1_HUMAN	SHANK1	SH3 and multiple ankyrin repeat domains 1	Cytoplasm	other
SNTAN_HUMAN	SNTN	sentan, cilia apical structure protein	Other	other
SOLH1_HUMAN	SOHLH1	spermatogenesis and oogenesis specific basic helix-loop-helix 1	Cytoplasm	transcription regulator
CYTSB_HUMAN	SPECC1	sperm antigen with calponin homology and coiled-coil domains 1	Nucleus	other
SRGP2_HUMAN	SRGAP2	SLIT-ROBO Rho GTPase activating protein 2	Cytoplasm	other
SYNJ1_HUMAN	SYNJ1	synaptojanin 1	Cytoplasm	phosphatase
TBX20_HUMAN	TBX20	T-box 20	Nucleus	transcription regulator
THMS1_HUMAN	THEMIS	thymocyte selection associated	Cytoplasm	other
T151A_HUMAN	TMEM151A	transmembrane protein 151A	Other	other
TTC12_HUMAN	TTC12	tetratricopeptide repeat domain 12	Other	other
GCP6_HUMAN	TUBGCP6	tubulin, gamma complex associated protein 6	Cytoplasm	other
UBQLN_HUMAN	UBQLNL	ubiquilin-like	Other	other
WDR1_HUMAN	WDR1	WD repeat domain 1	Extracellular Space	other
WDR35_HUMAN	WDR35	WD repeat domain 35	Cytoplasm	other
ZN532_HUMAN	ZNF532	zinc finger protein 532	Other	other
ZN624_HUMAN	ZNF624	zinc finger protein 624	Nucleus	other
ZNF74_HUMAN	ZNF74	zinc finger protein 74	Nucleus	other

Appendix B: Copy of ethics approval & funding

THE UNIVERSITY OF QUEENSLAND

Institutional Human Research Ethics Approval

Project Title:	Mechanisms of Placental Growth and Function Related to Uncomplicated and Complicated Pregnancies
Chief Investigator:	Prof Murray D Mitchell, Prof Gregory E Rice, A/Prof Leonie Callaway
Supervisor:	Prof Murray D Mitchell
Co-Investigator(s):	Dr Jennifer M Ryan, Dr Hsiu-Wen Chan, Dr Marloes Dekker Nitert, Dr Carlos Salomon, Ms Kanchan Vaswani, Ms Hassendrini N Peiris, A/Prof Keith Ashman
School(s):	UQ Centre for Clinical Research
Approval Number:	2013000381
Granting Agency/Degree:	Murray D Mitchell Start Up Fund
Duration:	31st October 2015

Comments:

Expedited review on the basis of approval from the Royal Brisbane & Women's Hospital HREC, dated 21/12/2012.

Note: if this approval is for amendments to an already approved protocol for which a UQ Clinical Trials Protection/Insurance Form was originally submitted, then the researchers must directly notify the UQ Insurance Office of any changes to that Form and Participant Information Sheets & Consent Forms as a result of the amendments, before action.

Name of responsible Committee:
Medical Research Ethics Committee
This project complies with the provisions contained in the *National Statement on Ethical Conduct in Human Research* and complies with the regulations governing experimentation on humans.

Name of Ethics Committee representative:
Professor Bill Vicenzino
Chairperson
Medical Research Ethics Committee

Signature _____ Date 29.5.13

Appendix C: List of Abbreviations

APC	Antigen Presenting Cell
ACN	Acetonitryl
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CERF	Collaborative Electronic Research Framework
cP	CentiPoise
DTT	Dithiothreitol
ELISA	Enzyme Linked Immunosorbent Assay
EDTA	Ethylenediaminetetraacetic acid
FGF	Fibroblast Growth Factor
GDM	Gestational Diabetes Mellitus
HAPO	Hyperglycaemia and Adverse Pregnancy Outcome
HCG	Human Chorionic Gonadotrophin
HPL	Human Placental Lactogen
Hsp	Heat Shock Protein
Hsc	Heat Shock Cognate
HbA1c	Glycated haemoglobin
HRP	Horse Radish Peroxidase
IPA	Ingenuity Pathway Analysis
IGF	Insulin like Growth Factor
IUGR	Intrauterine Growth Restriction
IAA	Iodoacetamide
IR	Immunoreactive
IL	Interleukin
IFN	Interferon

LC-MS	Liquid Chromatography - Mass Spectrometry
MVB	Multivesicular bodies
miRNA	micro RNA
mRNA	messenger RNA
MHC	Major Histocompatibility Complex
MV	Microvesicle
MD	Doctor of Medicine
NTA	Nanoparticle Tracking Analysis
NEP	Number of Exosome Particle
OGTT	Oral Glucose Tolerance Test
PE	Preeclampsia
PLAP	Placental Alkaline Phosphatase
PANTHER	Protein Analysis Through Evolutionary Relationships
PBS	Phosphate Buffered Saline
PAPP-A	Pregnancy Associated Plasma Protein –A
ROS	Reactive Oxygen Species
RIPA	Radioimmunoprecipitation assay
RT	Room Temperature
SD	Standard Deviation
SEM	Standard Error of Mean
TEM	Transmission Electron Microscopy
TNF	Tumour Necrosis Factor
TFA	Trifluoroacetic acid
TOF	Time of Flight
TFP	Tissue Factor Protein
UQCCR	University of Queensland Centre for Clinical Research
VEGF	Vascular Endothelial Growth Factor