Université de Montréal

# Effects of exercise training on intra-uterine growth restriction in an animal model

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# Résumé

Selon l'OMS, la retard de croissance intra-utérine (RCIU; 10% en dessous du poids normal pendant la grossesse) affecte 5-10% des grossesses et est une cause principale de la morbidité et de la mortalité périnatales. Dans notre étude précédente sur un modèle de souris transgénique de prééclampsie ($R^+A^+$), nous avons constaté que l'entraînement physique (ExT) avant et pendant la grossesse réduisait la pression artérielle maternelle et empêchait la RCIU en améliorant le développement placentaire. Dans le cadre de mon projet, nous avons confirmé les bénifices de l'ExT dans un modèle de RCIU (souris déficiente en $p57^{Kip2}$ ($p57^{-/+}$). Ainsi, nous avons observé la présence de RCIU, d'une masse placentaire réduite, d'une augmentation de la pathologie placentaire ainsi qu'une plus petite taille des portées chez les souris $p57^{-/+}$ sédentaire. L'ExT prévient la RCIU ainsi que tous les paramètres mentionnés ci-haut. Nous avons observé que l'expression du facteur de croissance de l'endothélium vasculaire, un régulateur clé de l'angiogenèse lors de la croissance placentaire, était réduite dans le placenta des souris $p57^{-/+}$ et normalisée par l'ExT. Nous avons également trouvé que l'expression en ARN dans le placenta de 2 facteurs inflammatoires (interleukine-1β et MCP-1) était augmenté chez les souris sédentaires $p57^{-/+}$ alors que ceci n'était pas présent chez les souris entraînées, ce qui suggère que l'inflammation placentaire peut contribuer à la pathologie placentaire. Toutefois, contrairement aux souris $R^+A^+$, le système rénine-angiotensine placentaire chez les souris $p57^{-/+}$ était normale et aucun effet de l'ExT a été observé. Ces résultats suggèrent que l'ExT prévient la RCIU en normalisant la pathologie placentaire, l'angiogenèse et l'inflammation placentaire.

Mots-clés: RCIU, entraînement physique, $p57^{Kip2}$, angiogenèse, inflammation, système rénine-angiotensine

# Abstract

Intrauterine growth restriction (IUGR) refers to the condition in which the baby's estimated weight is below the 10th percentile of babies of the same gestational age. It affects 5-10% of pregnancies and is a major cause of perinatal morbidity and mortality. In our previous studies in a transgenic preeclampsia mouse model ($R^+A^+$ mice), we found that exercise training (ExT) before and during pregnancy reduced maternal blood pressure (BP), and prevented IUGR by improving placental development. Here, we confirm the benefits of ExT in a mouse model of IUGR without preeclampsia ($p57^{kip2}$ KO ($p57^{-/+}$). We confirmed the presence of IUGR, reduced placental mass, increased placental pathology, smaller litter size and increased number of non-viable pups per litter in sedentary $p57^{-/+}$ mice. ExT prevented IUGR as well as normalized all the mentioned parameters. The expression of the vascular endothelial growth factor (VEGF), a key regulator of angiogenesis required for normal placental development, was reduced in pregnant $p57^{-/+}$ mice and normalized by ExT. The expression of 2 inflammatory factors (interleukin-1β and MCP-1 mRNA) in placenta was elevated in KO sedentary mice and MCP-1 was normalized by ExT, proposing that placental inflammation may contribute to placental pathology. However, in contrast to $R^+A^+$ mice, the placental RAS in $p57^{-/+}$ mice was found to be normal and there was no effect of ExT. Taken together, these results suggest that exercise training prevents intrauterine growth restriction by improving angiogenesis, placental alterations and placental inflammation.



Key words: IUGR, Exercise training, Angiogenesis, Placenta, Inflammation, $p57^{kip2}$

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# List of abbreviations

1. ACE2: Angiotensin-converting enzyme 2
2. AC: Abdominal circumference
3. Ach; Acetylcholine
4. ACOG: American college of obstetricians and gynecologists
5. AEDF: Absent end diastolic flow
6. AFP: Alpha-feto protein
7. AGA: Appropriate for gestational age
8. AGTR1: Angiotensin II receptor type 1
9. BMI: Body mass index
10. BP: Blood pressure
11. BPP: Biophysical profile
12. BWS: Beckwith-wiedemann syndrome
13. CHD: Coronary heart disease
14. CDK: cyclin dependent kinase
15. CDA: Canadian diabetes association
16. DV: Ductus venosus
17. CRH: corticotropin releasing hormone
18. EFW: Estimated fetal weight
19. ExT: Exercise training

20. FGR: Fetal growth restriction

21. GDM: Gestational diabetes mellitus

22. GPX: Glutathione peroxidase

23. hCG: Human chorionic gonadotropin

24. HIV: Human immune deficiency virus

25. HPA: Hypothalamic-pituitary-adrenal

26. IGF: Insulin growth factor

27. IL-1β: Interleukin 1β

28. IUGR: Intrauterine growth restriction

29. KO: Knockout

30. LTPA: Leisure time physical activity

31. Mas-R: Mas receptor

32. MCA: Median cerebral artery

33. METs: Metabolic equivalents

34. PAPA-A: Pregnancy associated protein-A

35. PCOS: Polycystic ovarian syndrome

36. PE: Preeclampsia

37. P-GR: Plasma glutathione reductase

38. PCR: Polymerase chain reaction

39. PIH: Pregnancy-induced hypertension

40. PLGF: Placental growth factor

41. RAS: Renin-angiotensin system

42. RI: Resistance index

43. ROS: Reactive oxygen species

44. SGA: Small for gestational age

45. SNP: Sodium nitroprusside

46. SOD: Superoxide dismutase

47. sFlt-1: soluble fms-like tyrosine kinase

48. UA: Uterine artery

49. UPD: uniparental disomy

50. VEGF: vascular endothelial growth factor

51. WHO: World health organization

52. WT: Wild-type

# *Dedication*

*To my mother and father for their*

*Encouragement and support to complete this work.*

*Thank you!*

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# *Chapter 1:*

# *INTRODUCTION*

# 1. Intrauterine growth restriction (IUGR)

## 1.1. Definition and epidemiology

Intrauterine growth restriction (IUGR)/Fetal growth restriction (FGR) refers to a condition in which a fetus is unable to reach its genetically determined potential size. In fact, it is a pathological reduction in an expected pattern of fetal growth that occurs *in utero*. IUGR is thus a major cause of perinatal morbidity and mortality[1]. Traditionally, in North America the standard definition of IUGR is a birth weight below the 10th percentile for gestational age. However, adverse consequences and mortality are also increased in infants with birth weights between 10th and 15th percentile. Conversely, many neonates whose weights are below the 10th percentile are healthy which are known as small for Gestational Age (SGA) babies [2]. Indeed, the SGA group includes fetuses that are constitutionally but not pathologically small and may reflect a normal pattern in a given population [1]. Several definitions of IUGR are accepted in different areas of the world. In Europe, for example, an abdominal circumference (AC) below the 10th or 5th percentile is the preferred diagnostic criteria. Published definitions include: weight at birth <2500 g, EFW (estimated fetal weight) <10th percentile, AC <10th percentile, EFW <10th percentile with abnormal Doppler indices in the umbilical artery or middle cerebral artery, and AC <10th percentile with abnormal umbilical artery or middle cerebral artery Doppler studies. Other diagnostic criteria utilize the fetus as a control for itself [3], or use customized fetal growth standards [4].

The prevalence of IUGR is about 5%-10% in the general obstetric population. Studies show that each year 18 million babies are born with low birth weight worldwide, half of which are born in

Asia [5]. Fetal Growth restriction is the second main cause of perinatal morbidity and mortality while prematurity is the leading cause [6].

## 1.2. IUGR risk factors:

Generally, the population at high risk for IUGR are women with low socioeconomic status, low weight before pregnancy, low weight gain during pregnancy, history of preterm delivery, stillbirth and previous pregnancies affected by IUGR [7-11]. Other risk factors are described as follow (**Table 1**).

### 1.2.1. Maternal nutrition

The specific responses of fetal growth to acute under nutrition at different points in pregnancy are still unclear. One study on the birth reports during the Dutch famine of 1944-1955 showed that only if the famine exposure happens late in pregnancy will result in low birth weight and declined crown-to-heel length [12]In contrast another revealed that, mothers of appropriate for gestational age (AGA) infants ate more servings of carbohydrate rich food and fruit, and were more likely to have taken folate and vitamin supplements than mothers of SGA infants at the time of conception. There was also an association between Iron supplementation when taken in the last month of pregnancy and a diminished risk of SGA [13]. These results suggest that malnutrition in early or late pregnancy may result in small for gestational age infants.

### 1.2.2. Multiple births

Newborns from multiple births are generally smaller than singletons. An analysis of birth weight and gestational age in twins and triplets in Norway showed that the intrauterine growth of both male and female twin diverged considerably from singletons starting at approximately 30 weeks of gestation [14]. Also monochorionic twins are twice more at the risk for IUGR compared to dichorionic

twins [15]. In a retrospective study of multi-fetal pregnancies it was revealed that birth weight of quadruplets and quintuplets was significantly lower than triplets [16].

### 1.2.3. Smoking

Smoking has been considered as one of the important risk factors for IUGR. One study on the impact of maternal exposure to environmental tobacco smoke on IUGR in a sample of 6866 singleton births represented a significant decrease in the mean birth weight of infants of active smoking mothers. This reduction was minimal but still present for mothers who stopped smoking after recognizing their pregnancy. Also, environmental tobacco smoke exposure in 1797 of 5507 non-smoking mothers decreased the mean birth weight of their infants by 53g[17]. Another study in Sweden demonstrated that babies of smoking mothers were at an increased risk for decreased head circumference, <32 cm [17, 18].

### 1.2.4. Adolescent pregnancies

It is reported that infants of adolescent mothers experienced almost twice the rates of preterm delivery (21.3%) and low birth weight (12.6%) compared to older mothers aged between 20 and 39 years [19]. Generally, adolescents most likely to become pregnant are those with insufficient nutritional status and unfavorable socio-economic status which may contribute to the higher rate of IUGR observed in this population [20].

### 1.2.5. Substance abuse

It is well known that alcohol and drug consumption are harmful to the developing embryo and fetus, and in the majority of cases cause IUGR. What we know as fetal alcohol syndrome includes pre- and postnatal growth deficiency, a “characteristic” facial appearance, microcephaly, mental retardation, and occasional major malformations [21]. In a 1-year study on all live singleton infants whose mothers were exposed to cocaine, it was observed that low birth weight (<2500 g) was more common among cocaine-exposed infants compared to non-exposed (31% versus 10%). In fact, cocaine use was

associated with a decrease in birth weight (154 g), length (1.02 cm), head circumference (0.69 cm), and duration of gestation (0.74 weeks). The birth-weight deficits were larger for infants born from mothers who used cocaine in combination with other drugs (195 g) and for infants born to mothers who specifically admitted using crack (200 g) [22]. Another study in china showed that mothers who had abused narcotics and heroin had SGA babies in 27.5% of pregnancies. The babies born to drug addicted mothers were on average 629 g lighter which was significantly different from the infants of the non-addicted mothers [23].

### 1.2.6. Inter-pregnancy interval

A shortened interval between pregnancies is associated with adverse perinatal consequences [24]. A research in Utah, USA showed that infants conceived 18–23 months after a previous live birth had the lowest risks of negative perinatal outcomes. However, in this study shorter intervals were related to higher risks for low birth weight [25].

### 1.2.7. High altitude

High altitude seems to reduce birth weight independently of other factors. It was found in a study in Colorado that birth weights at high altitude (2744–3100 m) were reduced due to IUGR [26]. The association between ethnicity and high altitude was also assessed in a study done in Tibet. Tibetans experience less altitude-associated IUGR than Chinese and have reduced levels of prenatal and postnatal mortality. When comparing the link between birth weight and altitude among these and other high-altitude populations, the results showed that those who have been living the longest at high altitude had the least altitude-associated IUGR. In general, the pregnant Tibetans had higher umbilical artery blood flow velocity and distributed a higher portion of common iliac blood flow to the umbilical artery compared to the Chinese women [27]. This might propose the occurrence of an evolutionary adaptation [28].

### 1.2.8. Congenital infections

Infections acquired *in utero* may often cause IUGR such as rubella [29], cytomegalovirus [30], herpes virus [31] and toxoplasma gondi which is less common than the others [32]. Moreover, HIV-Infected infants often suffer from IUGR [33].

### 1.2.9. Genetic and chromosomal factors

Chromosomal abnormalities are found in up to 7% of neonates with IUGR , which is over 10 times higher than in AGA (appropriate for gestational age) infants[34] . Moreover, the genomic imprinting, through which several genes in the human genome are differentially expressed based on whether they are located on the maternal or paternal chromosome, may play a role in embryonic and fetal growth. This has led to the theory that genomic imprinting regulates embryonic and fetal growth [35]. Silver-Russell syndrome which represents an extreme syndrome of IUGR and dysmorphic features, as well as maternal uniparental disomy (UPD: the inheritance of both chromosomes of a chromosome pair from only one parent) of human chromosome 7 has been observed in approximately 10% of these cases [36]. Other known imprinted genes where IUGR is the most common feature are maternal UDP14, maternal UDP20 and paternal UDP6q24 [37]. Also p57 $^{kip2}$ which is a paternally imprinted gene [38] has been related to severe growth restriction [39].

### 1.2.10. Preeclampsia and eclampsia

FGR can be related to preeclampsia (PE) as a result of impaired trophoblast invasion into the placental bed. In normal pregnancy, occlusion of the spiral arterioles by the endovascular trophoblast at the implantation site and the anatomical destruction of the distal spiral arteriole contribute to improved uterine blood flow. Failed interstitial invasion of spiral arterioles may lead to failure in local angiogenic and systemic cardiovascular adaptation signals that could be the main reason for early onset of IUGR and PE [40].

**Table 1:** Fetal growth restriction risk factors

| | |
|---|---|
| ***Fetal*** | ➢ Aneuploidy (trisomy 13, 18 and 21, triploidy, uniparental disomy)<br>➢ Fetal malformations (gastroschisis, omphalocele)<br>➢ Multiple gestation<br>➢ Infection (toxoplasmosis, rubella, cytomegalovirus, herpes) |
| ***Maternal*** | ➢ Hypertension<br>➢ Diabetes<br>➢ Renal disease<br>➢ Vascular disease<br>➢ Inflammatory bowel disease<br>➢ Hypoxia (pulmonary disease, cardiac disease)<br>➢ Systemic lupus erythematosus, antiphospholipid syndrome<br>➢ Thrombophilia (Factor V Leiden heterozygote, Prothrombin)<br>➢ Genetic (for instance, gene G20210A heterozygote, MTHFR heterozygote)<br>➢ Maternal uterine malformations (myomas, bicornuate, or septate uterus)<br>➢ Residing at high altitude |
| ***Placental*** | ➢ Placenta praevia<br>➢ Placental tumors<br>➢ Mosaicism |
| ***Environmental*** | ➢ Low socioeconomic status<br>➢ Malnutrition<br>➢ Smoking<br>➢ Alcohol<br>➢ Drugs (cocaine, heroin, methadone, cocaine therapeutic agents) |

## 1.3. Etiology

Growth and development of the fetus/embryo are complex biological procedures which are affected by different factors such as genetic, epigenetic, maternal age, environmental factors, etc. [41]. These factors can influence the size and efficiency of the placenta, uteroplacental transfer of nutrients and oxygen from mother to fetus, the endocrine environment of the fetus, and metabolic pathways [42-44]. Normal fetal growth consists of two phases. The first phase or embryonic life which starts from fertilization till the end of the 8$^{th}$ week includes proliferation, organization and differentiation of the embryo while the second phase, which we know as fetal life, starts at the end of week 8 and involves continuing growth and functional maturation of the different tissues and organs of the fetus [45]. The maternal-fetal-placental unit acts in harmony to fulfil the needs of the fetus, while supporting the physiological changes of the mother. The fetus has an inherent growth potential which results in a healthy newborn with appropriate size in normal situation. Studies showed that in IUGR, placentation is impaired [46, 47]. Dysregulation of endocrine-related factors such as, growth factor deficiencies, mainly insulin and the insulin-like growth factors (IGF) or their signaling pathway, often induce IUGR [45, 48]. Moreover, assessment of uterine, placental and umbilical blood flow shows that in growth restricted fetuses blood flows are decreased on both sides of the placenta. It is also reported that there is less placental exchange of essential nutrients such as amino acids in IUGR fetuses both *in vitro* and *in vivo* [49].

### 1.3.1. Normal placentation

Placentation starts with implantation of the blastocyst in the uterine epithelium and differentiation into embryonic and extra-embryonic tissues [50]. The trophectoderm of the blastocyst is the epithelium that is responsible for development of the human placenta. In other word, it is

necessary for successful embryonic development as it is integral in the transfer of nutrients from the mother to the child. [51]. Uteroplacental circulation is not fully established until the end of the first trimester. One proposed theory based on the observations of *ex vivo* histologic analysis of hysterectomy specimens of first-trimester placentas to explain the uteroplacental circulation states that trophoblasts invade decidual spiral arteries and form trophoblastic plugs. These trophoblastic plugs obstruct maternal blood flow into the intervillous space and prevent flow until the end of first trimester of pregnancy (10–12 weeks) [52]. Thus, human placental development during the first ten weeks of gestation occurs in a low oxygen environment with a $PO_2$ (oxygen pressure) measured at < 15 mmHg [53]. The plugs then loosen and permit continuous maternal blood flow into the intervillous space [52]. As a result, the low oxygen state changes and the pressure increases, so that the developing villous tree of the chorioallantoic placenta is then exposed to maternal blood with higher oxygen content.

### 1.3.2. Placental dysfunction in IUGR

Normal placental development is necessary for normal fetal growth. Failure of one or more of the components of the placentation process may result in pregnancy complications like preeclampsia, IUGR and placental abruption. There is considerable evidence showing that placentation is deficient in IUGR [46, 47]. Histologically, the features that can be observed in placenta from IUGR fetuses include: damage to branching angiogenesis with long unbranched intermediate and terminal villi, altered cytotrophoblast proliferation, trophoblast apoptosis, fibrin deposition, syncytial knotting and bridging, and enhanced villous maturation [54]. It is assumed that the reduced secretion of placental growth hormones (PGH) and IGF-1 which are some of the important determinants of fetal growth contribute to placental dysfunction [55] (**Figure 1**).

In IUGR, absence of endovascular trophoblast invasion of the myometrial segments of the spiral arterioles produces a high resistance vasculature in these arterioles. This lack of transformation leads to hypoperfusion, hypoxia, re-perfusion injury, oxidative stress and ultimately, to signs of villous tree maldevelopment in the second half of the pregnancy [56]. Villous cytotrophoblasts in IUGR have an augmented sensitivity to cell death in hypoxic situations when compared to normal pregnancies [57-59]. Although apoptosis is considered to be a normal part of villous trophoblast turnover and syncytiotrophoblast formation from cytotrophoblast [60, 61], in pregnancies complicated with IUGR this is augmented and produces an increase in syncytial knots [62]. This is also the case in the villi in IUGR and has been detected by the expression of cleavage products of caspase [62-64]. In IUGR, excess injury of the villous trophoblast layer decreases the functional mass of syncytiotrophoblast and restricts the capacity of the villi to transport nutrients. Furthermore, the microscopic injury has functional effects on placental permeability, as α-fetoprotein and small molecular weight compounds are able to pass between the maternal and fetal circulations [65, 66].

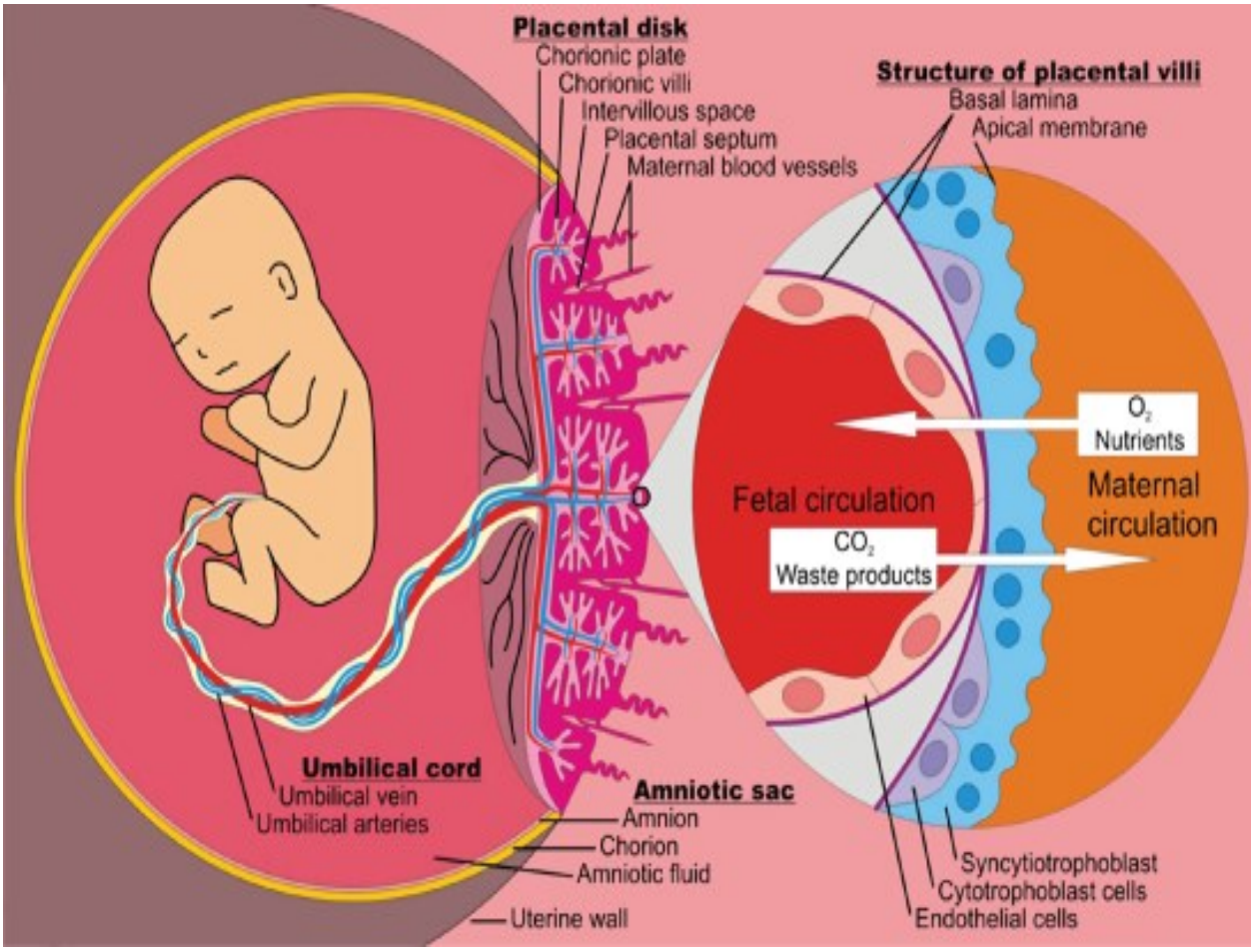


**Figure 1**: structure of placenta and placental villi [67].

### 1.3.3. Histopathology of placental injury

There are several stimuli and mediators that may contribute to the observed injury to the chorioallantoic villi but the most important one is oxidative stress [68]. Oxidative stress can be caused by ischemia in the placenta as a result of the insufficiently developed spiral arterioles. Indeed the production of reactive oxygen species (ROS) which promote oxidative stress contributes to tissue injury in many diseases including IUGR [69]. Studies show that the placentas of pregnancies complicated with IUGR demonstrate obvious signs of oxidative stress [70]. Moreover, hypoxia, ischemia, or both may contribute to placental injury via mechanisms other than ROS generation, as variable organ blood flow also activates the complement cascade [68, 71, 72]. Dysregulated complement activation in non-pregnant patients mediates immunological injury in the heart, lung and kidney, and recent data indicate that it also plays a role in abnormal human pregnancy [73]. For example, the kidneys of women with preeclampsia show deposition of complement split products in their glomeruli [74].

## 1.4. Classification

Growth during intrauterine life occurs during different stages. For example, growth in length happens in early prenatal life (during the 3$^{rd}$, 4$^{th}$ and 5$^{th}$ month) while weight gain develops later in prenatal life (during the last 2 months of gestation) [45]. Depending on the time of adverse intrauterine environment, IUGR fetuses are classified as symmetric or asymmetric. In symmetric growth restriction, the entire fetus body is proportionally small (small weight, length, and head circumference). In this situation, the adverse environment has happened early in pregnancy which can be as a result of genetic factors or congenital infections, syndromes, or toxic effects in early gestation [75]. On the other hand, in asymmetrical IUGR, adverse intrauterine environment happens

later during gestation. In this case, most of the energy is conducted for the maintenance of vital organs such as the brain and heart whereas liver, muscle and fat are less developed which results in smaller weight but normal length [76]. In asymmetrical IUGR , we observe normal head circumference but small abdominal circumference, skinny limbs, and thinned skin as a result of decreased liver size, muscle mass and subcutaneous fat[77].

## 1.5. IUGR and diseases later in life

Intrauterine life adapts the fetus to become mature and also overcome postnatal insults. During this period, the fetus goes through critical stages of tissue growth and elevated cell division. If the organism is affected in these critical periods it may have an important impact on organ development [78]. It has been reported in animals and also in some human cases that IUGR is associated with increased prevalence of many adult diseases [79]. In addition, results of a study by Park et al. demonstrated that low protein diet during pregnancy causes long-lasting changes in the liver and skeletal muscle mitochondria in the offspring [80].

### 1.5.1. IUGR and metabolic syndrome

According to the literature, there is an association between IUGR and the development of metabolic syndrome later in life, a condition associated with obesity, arterial hypertension, hypercholesterolemia, impaired glucose tolerance, and diabetes mellitus type 2 [81-90]. There is a hypothesis to explain this condition which is known as the *thrifty phenotype* or *Barker's hypothesis.* It proposes that in the case of an impaired intrauterine environment, such as nutrient restriction, the intrauterine milieu creates a "reprogramming" of the endocrine–metabolic status of the fetus in order to reach short-term survival benefits, although, it may be harmful in the long

term [81-84, 91, 92]. Barker was able to demonstrate that, the smaller the birth weight or the weight at 1 year of age, the greater was the prevalence of metabolic syndrome in adult life.

According to the thrifty phenotype, the connection of low birth weight and insulin resistance or diabetes in adulthood may be a result of fetal malnutrition due to poor nutritional reserves of the mother, not adequate flow of the blood in uterus, or destruction of nutrients in the placenta [93]. Hypothalamic-pituitary-adrenal (HPA) axis overacts by changing its set point in response to the adverse intrauterine environment, which can lead to increased cortisol levels [94-98]. This situation is similar to what can be seen with chronic stress [96, 99]. The HPA over-activation has been observed in both experimental animals and newborns with IUGR and both showed elevated cortisol levels in umbilical cord blood [100, 101]. This increase in cortisol during intrauterine life causes endothelial damage which contributes to the development of cardiovascular diseases. Furthermore, growth restricted babies have a reduced muscle mass and this deficiency will persist because the crucial period for muscle growth is at ~30 weeks *in utero*, and there is little cell replication after birth. As such, if they gain weight rapidly in childhood, they are more likely to increase their fat mass rather than their muscle mass, leading to a disproportionately high fat mass in later life resulting in an increase in the development of obesity and insulin resistance [85]. Ozanne et al. found decreased expression of specific insulin-signaling proteins, such as, protein kinase C (PKC) zeta, p85alpha, p110beta and GLUT4, in low birth weight subjects compared to controls [102] and it was also shown that, children born small for gestational age have reduced adiponectin levels, an adipokine with insulin-sensitizing and antiatherogenic properties [103], which may increase the risk for developing diabetes type 2.

Moreover, studies in Europe, North America, and India have shown association between coronary heart disease and small size at birth [104], as well as a study in Finland which reported that the

cumulative incidence of hypertension requiring medication was 20.2% in those weighing <3 kg at birth compared to 12.3% in those weighing >4 kg [105].

### 1.5.2. IUGR and reproduction problems

Some studies have demonstrated a link between IUGR and adrenarche (early sexual maturation), elevated prevalence of functional ovarian hyper-androgenism as well as the development of polycystic ovary syndrome [106-109]. Moreover, there has been reports of decreased uterine volume and smaller fraction of ovarian follicles in girls born with IUGR, [110, 111] which both may have a negative effect on fertility. Although it has not been clarified yet, there seems to be an association between IUGR and reproductive function

## 1.6. Clinical diagnosis of IUGR:

Abnormal fetal growth is suspected when there is a subnormal uterine size detected by abdominal palpation and direct measurement of the symphyseal-fundal distance [112]. Indeed, abdominal palpation has a sensitivity of 30% for detecting SGA fetuses, while the symphysis-fundal distance has a sensitivity of 27-86% and specificity of 80–93% [113]. Ultrasound has also been used for accurate pregnancy dating and for the diagnosis of IUGR, although it is reported that IUGR remains undetected in about 30% of routinely scanned cases and it is falsely detected in 50% of cases [114].

### 1.6.1. Serum biochemistry

In SGA and IUGR affected pregnancies, biochemical markers have been proposed. An increased maternal Serum alpha-fetoprotein (AFP) is correlated with an elevated risk of low birth weight [115]. Also low levels of maternal serum pregnancy-associated plasma protein A (PAPP-A) (at the lowest 5th percentile) are associated with higher risk of a SGA infant [116]. There are also other placental markers

like human chorionic gonadotropin (hCG), ADAM12 (A Disintegrin and Metalloprotease), placental protein 13 (PP13), serum soluble Fas (sFas) and placental growth factor (PlGF), amongst others. However, studies have shown that all of these markers are below the detection rate warranted for large population screening [117-122]. In low-risk populations, a combination which includes PP13, PAPP-A, ADAM12, activin A, or inhibin A, measured in the first or early second trimester and uterine artery Doppler in the second trimester, reveal sensitivities of 60%–80% and specificities >80%. Studies are still required to estimate the full potential of evaluating combining multiple markers and ultrasound in screening for IUGR.

### 1.6.2. Uterine artery doppler:

Since the 1980s, many progresses have been made in the utilisation of uterine artery Doppler in obstetrical practice, particularly, in the detection of maternal perfusion abnormalities in PE and IUGR [123-125]. Since trophoblastic invasion was thought to be completed by the second trimester of pregnancy, most of the studies were performed between 20 and 24 weeks of gestation. However, some believe that trophoblastic invasion peaks in the first trimester, so it would be more appropriate to screen for growth restriction in that period [126]. Although, the sensitivity of this test is not that high (24% and 16% according to some reports) [127, 128], women with a high uterine artery resistance index (RI), are 5.5 times more likely to have IUGR [128]. In more recent studies, they attempted to add serum biochemistry factors like PAPPA in order to increase detection rates. Unfortunately, sensitivity of these tests still remains low [117, 129].

## 1.7. Evaluating fetal wellbeing

### 1.7.1. Fetal heart rate monitoring

Fetal heart rate analysis is extensively used to detect pregnancies at high risk. It aims to determine fetal well-being by estimating the fetal heart rate baseline, variability, and periodic changes. A normal reactive test is likely to reflect adequate oxygenation of the fetal central nervous system. Since it is interpreted by visual inspection, it is prone to a significant intra-observer and inter-observer variation and therefore, there is a high rate of false positive. In premature fetuses, particularly those with IUGR, interpretation is challenging [130]. Computerized fetal heart rate analysis was introduced to decrease discrepancies in the interpretation. However, it was showed that the results from computerised cardiotocographic analysis agreed closely with visual assessment [113].

### 1.7.2. Biophysical profile

Ensuring fetal well-being and determining the optimal timing for delivery of an IUGR fetus is the primary goal of fetal specialists. However, the optimal method to use for fetal testing is also debatable; in the United States, the most frequently used test is the biophysical profile, whereas in Europe, cardiotocography (computerized fetal heart rate monitoring) is the preferred method [131].

The fetal biophysical profile (BPP) is a method for assessing fetal asphyxia and/or chronic hypoxia and is the most acceptable method of fetal well-being evaluation in the North America. It consists of a number of measurements including the amniotic fluid volume, fetal tone, fetal movements, fetal breathing movements, and fetal heart rate monitoring. In a normal situation, each parameter has a value of two out of total points of ten [132-134].

### 1.7.3. Doppler velocimetry of blood flow

Doppler blood flow has significantly changed the management of IUGR. We use it to assess vascular resistance and end organ function. There are three types: Doppler assessment of the umbilical artery (UA), middle cerebral artery (MCA) and ductus venous (DV). When the UA blood flow from the fetus to the placenta is determined, the placental vessel resistance can be evaluated. MCA detects fetal cerebral blood flow and DV reflects alterations in fetal cardiac function. In severe cases of IUGR the DV is abnormal [135, 136]. UA is the most frequently used Doppler test in women diagnosed with IUGR. It has the ability to distinguish IUGR caused by placental problems from SGA fetuses. Indeed, monitoring IUGR pregnancies with UA Doppler decreases the mortality rate and lowers the need for antepartum admissions, labor induction, and Caesarean deliveries [137, 138], as when IUGR is diagnosed, clinical management is performed by more frequent surveillance of fetal weight (every 2 weeks), along with UA and if available MCA and DV and in case of observation of adverse conditions like no change in fetal growth and decline in amniotic fluid index or fetal tone or gross movements more intensive surveillance (e.g., 2 to 3 times per week) or admission to hospital and delivery planning will be considered [139].

### 1.7.4. Histopathological and molecular diagnostics

Currently, sampling of amniotic fluid, fetal blood, maternal blood, and feto-placental or transabdominally obtained placental tissues is possible. Placental villi from human IUGR pregnancies show distinctive alterations in “hypoxic trophoblast signature transcripts”, for example, upregulation of transcripts for VEGF, connective tissue growth factor, follistatin-related protein, N-Myc downstream-regulated gene1, and adipophilin (ADRP), and downregulation of human placental lactogen and PHLDA2 [140] have been shown. For instance, dysregulation of transcripts like CRH, IGF1, IGF2,

AGTR1, leptin, and sFlt have also been described [141]. These techniques are novel; nevertheless, the potential combination of fetal biophysical testing and informatics-based molecular analysis may prove useful in the future management of IUGR [11].

## 1.8. Clinical management of IUGR

Currently, there are no standard prenatal therapies which are designed to specifically improve fetal growth or reverse the complications of IUGR. For management of IUGR, It is important to improve nutrition, stop smoking, avoid drug use, and control maternal disorders such as hypertension and renal dysfunction. If there is an infectious disease, it should be treated. Sonography is essential to identify fetal malformations particularly if lethal and offer fetal karyotyping. Previous studies demonstrated that administration of glucose or amino acids, and low-dose aspirin to the mother did not show a significant impact on perinatal outcomes [142-144]. It was also observed that smoking cessation and antimalarial therapy appeared to prevent IUGR, but they were not effective if IUGR was already established. Particularly, some studies propose that balanced energy/protein supplements may be beneficial in reducing the risk of IUGR [145, 146]. Experimental evidence from humans and animal models indicate that amino acid transport from mother to fetus and fetal amino acid metabolism are disturbed in IUGR [147]. As we know, accretion of amino acids into proteins is an essential component of fetal growth. Therefore, maternal protein supplementation to improve fetal growth is an attractive therapeutic option, especially when fetal growth is failing. Although this is supported by some studies [148-150], there have been some reports of adverse effects of protein supplementation on pregnancies with an increased risk of preterm and SGA delivery. Human trials generally show that increased maternal energy intake (in the context of malnutrition), without high amounts of dietary protein, improve fetal weight (though not necessarily lean mass) without significant adverse effects [151].

Timing the delivery of the growth restricted fetus is important. Currently there is no test which dictates the optimum time of delivery. When IUGR pregnancy is at full term (≥37 weeks), delivery is favored as there is no evidence that delaying delivery has benefits [152, 153]. At 34 to 37 weeks, the rate of significant neonatal morbidity is low, therefore, delivery is not a complex issue [154]. IUGR at 34 or more weeks gestation (late onset) is typically characterized by milder placental dysfunction and often may not produce an elevation in the umbilical artery Doppler resistance indexes [155]. When IUGR is detected before 34 weeks of gestation, decision to deliver is more difficult and is individualized [156]. Delivery of the IUGR fetus before 34 weeks gestation is associated with high rates of newborn morbidity and mortality. In the absence of clear indications for delivery, the emphasis should be on safely prolonging the pregnancy [156-158]. The decreased perinatal mortality that is found for each week that the IUGR fetuses remains *in utero* should be taken into account when a decision to deliver babies with less than 30 weeks age is made [157]. Factors like abnormal biophysical or modified biophysical profile score, oligohydramnios, repetitive FHR (fetal heart rate) decline are strong indicators that delivery is reasonable or warranted when IUGR is identified at or after 34 weeks of gestation. Also, a decrease in maternal perception of fetal movement indicates the need for further evaluation of the fetus [159].

After 34 weeks, the IUGR fetus in a singleton or twin pregnancy that develops either oligohydramnios or AEDF (Absent end diastolic flow) in the UA should be delivered proximate to the diagnosis of these complications. In singleton pregnancies in which the IUGR fetus has normal amniotic fluid volume, Doppler studies, and biophysical testing, the fetus is likely constitutionally small and may be managed expectantly until 38-39 weeks. If Doppler testing becomes abnormal indicating a placental etiology, delivery by 36-37 weeks is reasonable. In any of these scenarios, biophysical (weekly BPP or twice weekly modified BPP) and Doppler testing is warranted until delivery. When it comes to mode of delivery, there is contradictory evidence in the literature regarding the best mode of delivery of the

growth-restricted fetus. A vaginal delivery is rarely attempted when biophysical assessment of fetal status is not reassuring before labor because there is an increased risk of fetal hypoxia. Even when biophysical parameters are reassuring, clinicians vary in their decisions [156]. One study indicated that caesarean delivery for SGA fetuses was associated with a lower rate of respiratory distress syndrome, neonatal seizures, and death, but these trends were not statistically significant [160]. Certainly, other factors such as the gestational age, cervical status, fetal presentation, and maternal medical complications may influence the choice of delivery [50].

# 2. Exercise training

## 2.1. Exercise training and normal pregnancies

Regular exercise training for non-pregnant women has many benefits which are well recognized. Studies have shown no harm in doing exercise for the pregnant women and the fetus [161]. However, there are theoretic concerns regarding the effects of exercise during pregnancy, which are listed below.

### 2.1.1. Theoretic concerns regarding the effects of exercise on pregnancy

**2.1.1.1. Teratogenic effect:** One of the concerns about exercise during pregnancy is increasing the risk of teratogenic effect [162]. As far as we know, the metabolic rate increases during both exercise and pregnancy which results in higher heat production. Normally, fetal temperature is 0.5 to 1.0°C above maternal levels as a result of fetoplacental metabolism which generates additional heat. Theoretically, by doing exercise training during pregnancy, an increase in maternal core temperature may decrease fetal heat dissipation to the mother. Some data suggest a teratogenic potential when maternal temperatures rise above 39.2°C (102.6°F), especially in the first trimester [162]. According to these studies, pregnant women should perform exercise in thermoneutral conditions. However human studies are limited.

**2.1.1.2. Hemodynamic:** During exercise training, blood is diverted from abdominal viscera, including the uterus, to supply exercising muscle. The splanchnic blood flow can decrease to 50 percent and makes theoretic concerns about fetal hypoxemia [163]. However, measurements of the effect of exercise on fetal heart rate showed either no significant change or short-term increases of five to 15 beats per minute [164]. There is report of fetal bradycardia during vigorous exercise in untrained women performing near maximal

capacity which was resolved in less than two minutes. In the same women, submaximal exercise up to 70 percent of maximal aerobic capacity did not induce any fetal bradycardia [165, 166].

**2.1.1.3. Energy demand**: Both exercise and pregnancy are associated with high energy consumption. The competing energy demands of the exercising mother and the growing fetus raise the theoretic concern that excessive exercise might adversely affect fetal development. However, in clinical studies, there has been no significant difference in maternal weight during the first and second trimester of gestation among women who train during pregnancy compared to sedentary women. At the same time, some data propose that continuous exercise in the second and third trimesters is related with reduced maternal and fetal weight gain [167], However, the overall weight gain during pregnancy remains well within normal limits in exercising mothers [168]. Apparently, if pregnant women adjust their calorie intake to their energy demand, there should not be less fetal weight gain.

**2.1.1.4. Oxygen demand**: During pregnancy and exercise, adaptive changes happen in the pulmonary system. Pregnant and non-pregnant women have an equivalent respiratory frequency while resting. However, mild increases in tidal volume and oxygen consumption are noted in pregnant women probably as an adaptive mechanism to the increased oxygen requirement of the fetus [169]. With mild exercise, pregnant women have a greater increase in respiratory frequency and oxygen consumption to meet their greater oxygen demand. As exercise increases to moderate and maximal levels, they show a reduction in respiratory frequency, lower tidal volume and maximal oxygen consumption. The oxygen demand at high levels of activity seems to overwhelm the adaptive changes that occur at rest. This may be because of the obstructive effect of an enlarged uterus on diaphragmatic movement. However, several studies have shown a decreased maximal voluntary exercise performance in pregnant women [170, 171].

**2.1.1.5. Labor and outcomes**: There are some theoretic concerns about premature labor in women who exercise in late pregnancy. It is well known that exercise training increases circulating levels of norepinephrine and epinephrine [162]. Norepinephrine increases both the strength and the frequency of uterine contractions. Nevertheless, epinephrine has an inhibiting effect on uterine activity. Runners often have complaints of contractions during exercise, but actual measurements with external tokodynamometry have not indicated consistent changes in uterine contractility. Moreover, there is no evidence that supports an elevation in preterm labor related to exercise training [172], also, no significant difference in maternal weight gain, infant birth weight, length of gestation, length of labor or Apgar scores was found [173].

## 2.2. Positive effects of exercise training on normal pregnancies

### 2.2.1. Maternal wellbeing

Generally speaking, recent proofed guidelines indicate that regular maternal exercise is an important component of a healthy pregnancy [174]. Exercise training has been reported to have a positive effect on the experience of discomfort during pregnancy. In a study, women who exercised during three months before pregnancy felt during the first trimester than those who did not exercise (such as having less musculoskeletal discomfort, mood stability and decreased dyspnea, etc). Exercise in the first and second trimesters was associated with feeling better in the third trimester [175]. Another study on the effect of structured non-endurance antepartum exercise on pregnancy outcomes showed no adverse effect labor outcomes in the exercising group. They had significantly shorter first and second stages of labor compared to the sedentary group and they were less likely to need oxytocin augmentation and had spontaneous vaginal deliveries [176]. According to another study, continuing weight-bearing exercise during pregnancy helps to maintain the mother's fitness in the long-term and also reduces cardiovascular risks in

the premenopausal period [177]. It has also been proved that women with structured, supervised exercise training during gestation have 15% reduced risk for C-section compared to the non-trained group of study [178]. In the context of a normal and healthy pregnancy, the American College of Obstetrics and Gynecology (ACOG) guidelines encourages continuation of pre-pregnancy exercise activities and recommend that sedentary women start exercising during pregnancy. The intensity, duration and frequency of exercise should start at a level that does not result in pain, shortness of breath or excessive fatigue. Exercise may then progress at a rate that avoids significant discomfort. Patients should be counseled to perform frequent self-assessments of physical conditioning and well-being, including hydration, caloric intake, quality of rest and presence of muscle or joint pain. It should be emphasized that decreases in exercise performance are common, especially later in pregnancy. The goal is to obtain the maximal benefits of the mentioned benefits derived from exercise, while ensuring that there is no adverse effects on the mother or the fetus (**Table 2**) [179]. Also according to ACOG, regardless of physiological alterations during pregnancy which allow for the increased metabolic demands of the mother and fetus, women can benefit from regular exercise training during gestation as it has been demonstrated to result in marked maternal benefits including improved maternal cardiovascular and metabolic adaptations [180], limited pregnancy weight gain [181], decreased musculoskeletal discomfort [182], mood stability [183, 184] and decreased risk of dyspnea [185]. Many studies have reported elevated levels of stress and depressed mood during pregnancy. One study evaluated the outcomes of leisure time physical activity (LTPA) during pregnancy and its association to psychological well-being. When comparing exercisers to non-exercisers in each trimester, they found that exercisers had significantly less depressed mood, daily hassles, state-anxiety and pregnancy-specific stress in the first and second trimester. Women who exercised in the third trimester reported less anxiety in that trimester compared to non-exercisers. The results

showed that in healthy pregnant women, even low-intensity regular aerobic exercise may be potentially effective as a low-cost method of enhancing psychological well-being [186]. There is also data demonstrating improved placental development with exercise training. Although exercise during pregnancy can cause an intermittent reduction in oxygen and substrate delivery to the fetus while performing exercise, but it is probable that regular sessions of exercise training improves oxygen and substrate delivery at rest [187]. Women who start training in early pregnancy have elevated placental volumes and growth rates [188], as well as a decreased fraction of non-functional tissue and an increased volume of villous tissue [189].

### 2.2.2. Fetal benefits

Some reported fetal benefits include decreased fat mass, improved stress tolerance and advanced neurobehavioural maturation [190]. Barakat et al. reported that low intensity resistance training performed during the second and third trimester of pregnancy does not have a negative impact on the newborn's body size or overall health [191].

### 2.2.3. Preeclampsia and gestational diabetes

Several studies have found a reduced frequency of PE and pregnancy-induced hypertension (PIH) in women who participated in low- and moderate-intensity during physical activities [180, 192, 193]. In addition, epidemiological studies demonstrated that exercise training may be advantageous in prevention of gestational diabetes (GDM), specifically in obese women with BMIs that are more than 33 [194]. The prevalence of GDM in Canada may be higher than previously thought, ranging up to 4% in the general population [195] and as high as 18% in the Aboriginal population [196, 197]. Exercise is currently considered a complementary therapy for women with GDM. The Canadian Diabetes Association (CDA) recommends physical activity for women with GDM; however, the frequency, intensity, type, and duration of activity should

be based on each individual's condition [195]. The ACOG (2001) recommends that women with GDM who lead an active lifestyle be encouraged to continue an exercise program approved for pregnancy. These vague recommendations make it difficult for health professionals to give proper advice other than to increase physical activity. In one study where they examined the etiology of GDM in Saskatchewan, it was found that women who were the most physically active had the lowest prevalence of GDM [196]. It was also demonstrated that women who participated in any recreational physical activity within the first 20 weeks of gestation experienced a 48% reduction in the risk of GDM [198].

### 2.2.4. Obesity

Generally, exercise training can decrease the risk of obesity. Women who are overweight or obese have an increased risk of complications, including polycystic ovarian syndrome (PCOS) [199], menstrual irregularity, and infertility [200], that reduce the probability of conception. Clark et al. showed that regular exercise training is effective in restoring fertility in obese women [201]. In addition, overweight and obese women have an increased risk of maternal and fetal complications such as gestational diabetes, preeclampsia, increased risk for delivering at or before 32 weeks gestation, which contribute to longer hospitalization [202] and higher delivery costs [203]. In fact, the risk of maternal and fetal complications increases with the degree of obesity. The incidence of preeclampsia doubles with every 5–7 kg/m2 increase in pre-pregnancy BMI [204]. The risk of gestational diabetes also increases progressively in overweight, obese, and morbidly obese women [205, 206]. Overweight and obese women are more likely to deliver large for gestational age and macrosomic infants [207]. Infants of obese women are more likely to experience neonatal intensive care unit admission [208] and caesarean section [203]. In fact, infants from morbidly obese mothers (BMI ≥ 40 kg/m2) are twice as likely to demonstrate fetal distress and low APGAR (activity, pulse, grimace, appearance, and respiration) scores [206].

Regular exercise training, which includes exercise conducted before and during pregnancy, may act through several mechanisms to prevent obesity-related pregnancy complications. First, performing exercise before pregnancy may induce weight loss, resulting in a healthier BMI, which may prevent the risk of the obesity-related complications described above. Second, it has been suggested that regular aerobic exercise initiated during pregnancy may prevent gestational diabetes and preeclampsia [209]. Lowering the incidence of these 2 conditions among overweight and obese women may also prevent the resulting complications and adverse pregnancy outcomes associated with them. Third, exercise during pregnancy may assist women in preventing excessive weight gain [210]. Excessive gestational weight gain is associated with increased post-partum weight retention, and hence prenatal exercise may also be beneficial to facilitate return to pre-pregnancy weight after delivery [211].

## 2.3. General recommendations for exercise in pregnancy and post-partum period according to ACOG

- Recreational and competitive athletes with uncomplicated pregnancies can remain active during pregnancy and should modify their usual exercise routines as medically indicated. The information on strenuous exercise is scarce; however, women who engage in such activities require close medical supervision.
- Previously inactive women and those with medical or obstetric complications should be evaluated before recommendations for physical activity during pregnancy are made. Exercise during pregnancy may provide additional health benefits to women with gestational diabetes.
- A physically active woman with a history of or at risk for preterm labor or fetal growth restriction should be advised to reduce her activity in the second and third trimesters.

**Table 2:** Recommendations for sport activities during pregnancy

| | |
|---|---|
| Activities to encourage | ➢ Walking<br>➢ Stationary cycling<br>➢ Low-impact aerobics<br>➢ Swimming |
| Activities to discourage | ➢ Contact sports (increased risk of abdominal trauma)<br>➢ Hockey (field and ice)<br>➢ Boxing<br>➢ Wrestling<br>➢ Football<br>➢ Soccer |
| High risk sports(increased potential for falls/ trauma) | ➢ Gymnastics<br>➢ Horseback riding<br>➢ Skating<br>➢ Skiing (snow and water)<br>➢ Hang gliding<br>➢ Vigorous racquet sports<br>➢ Weight lifting<br>➢ Scuba diving |

## 2.4. Exercise training and IUGR:

Based on the reported data, the link between exercise training during pregnancy and birth weight is evident. Although there are studies stating that there is no relationship between maternal physical activity and fetal birth weight [161], others have suggested that babies from recreational athletes have lower body fat compared with offspring of sedentary mothers [161]. It has been shown that the rate of macrosomia and gestational diabetes in women who performed submaximal intensity exercise starting from week 6-8 of gestation is lower than the control group. The results also demonstrated that participation in moderate-intensity aerobic exercise did not increase the risk of IUGR [175]. Interestingly, Dr. Lavoie's group has demonstrated that IUGR in the context of preeclampsia can be prevented by exercise training when performed both before and during gestation in different mouse models of this disease. This was related to a reduction in blood pressure and placental development normalisation in the exercise group compared to sedentary counterparts [212, 213].

## 2.5. Hypothesized protective mechanisms implicated in the prevention of IUGR by exercise training

### 2.5.1. Enhanced placental development and vascularity

Abnormal placental development is a central cause of fetal growth-restriction. Insufficient trophoblastic invasion of the uterine spiral arteries in early pregnancy may contribute to an incomplete loss of sensitivity to vasoconstrictors in utero-placental vessels, causing intermittent hypoxia and reperfusion [68]. Conversely, regular physical activity in early pregnancy stimulates placental growth. Women who start training in early pregnancy have elevated placental volumes and growth rates [188], as well as a decreased fraction of non-functional tissue and an increased volume of villous tissue [189]. Interestingly, these adaptations are still noticeable at term even if the mother stopped training by 20 weeks gestation,

indicating that early pregnancy is a critical period for placental development. Additionally, if the mother continues to exercise until term a slightly additional increase in placental volume and surface area will be observed [189]. Improved placental growth and vascularity enhances its perfusion and transport capacity, and this may prevent reductions in fetal substrate and oxygen supplies during intermittent decreases in placental blood flow which may be associated with IUGR [188].

### 2.5.2. Prevention and/or reduction of oxidative stress

Regular physical training in non-pregnant rats, has been shown to augment antioxidant defense systems in heart, liver and muscle, which restricts cellular damage caused by oxidative stress related to acute bouts of exercise. Also, studies in animal models have shown that exercise training up-regulates antioxidants in skeletal muscles and growing evidence indicates that endurance exercise training promotes an elevation in both total SOD (superoxide dismutase) and GPX (glutathione peroxidase) activity in skeletal muscles. In this regard, it appears that high-intensity exercise training is generally more effective then low-intensity exercise in the up-regulation of muscle SOD and GPX activities [214, 215]. In a study consisting of a 16 week aerobic exercise training program with an individualized intensity in healthy men and women, the activity of superoxide dismutase in erythrocytes (E-SOD), glutathione peroxidase in whole blood (GSH-Px), and glutathione reductase in plasma (P-GR) were measured and GSH-Px and P-GR activity were found to be increased without any alteration in E-SOD activity [216]. In another study among a large group of Spanish women, with two categories of leisure time physical activity according to their intensity: low (<=6 METs) and high (>6 METs), a direct relationship between the amount and intensity of regular leisure physical activity and endogenous antioxidant enzymes was observed. Low intensity exercise training was associated with high SOD levels and high intensity exercise with high peroxidase levels. These results suggest a modulatory effect of leisure physical activity intensity on the anti-oxidative balance [217]. Although no differences could be observed in erythrocyte antioxidant enzyme activities (SOD, glutathione peroxidase, and

catalase) between active and sedentary pregnant women before delivery, SOD and catalase activities were dramatically elevated 1 h post-partum in trained women which seemed to inhibit labor-induced increases in malondialdehyde (an indicator of lipid peroxidation) [218]. These results propose that regular exercise training may enhance maternal antioxidant responses to augmented oxidative stress in normal pregnancies [218], which may prevent or improve endothelial dysfunction and thus IUGR. Indeed, in experimental animal models of atherosclerosis, hypercholesterolemia, hypertension, and diabetes, associations between oxidative stress and impaired endothelial function have been demonstrated [219-221].

### 2.5.3. Reduction of inflammation

There are many evidences supporting the anti-inflammatory effect of regular exercise training [222] in non-pregnant individuals and patients with heart failure [223] and coronary artery disease [224]. It has been shown in healthy men and women that plasma levels of sTNF-R1, sTNF-R2, (soluble tumor necrosis factor receptors 1, 2) interleukin-6, and C-reactive protein are decreased with physical training [225]. Exercise training can also attenuate interleukin-1, interleukin-6, and interferon-γ, while increasing the anti-inflammatory cytokine interleukin-10 [224]. Since there have been reports of increased inflammatory factors in IUGR, such as IL6 [226], if exercise training has similar anti-inflammatory effects in pregnant women, this could prevent or decrease the inflammatory response that may contribute to the development of IUGR.

### 2.5.4. Improving of endothelial dysfunction

It is proved that regular exercise training improves endothelial function in non-pregnant individuals with endothelial dysfunction [227], plus, it can favorably modify some risk factors of endothelium dysfunction like blood pressure [228]. Aerobic exercise has been demonstrated to raise local endothelium-dependent dilation in patients with endothelial dysfunction caused by aging [229] and type2 diabetes [230]. It has also been shown that in heart failure patients large muscle mass exercise improves systemic

endothelial function which was measured by assessing the response to acetylcholine (ACh) and sodium nitroprusside (SNP) [230]. If similar results are observed in women at risk for IUGR, training-induced correction of disease-related endothelial dysfunction may prevent the main pathological process leading to this disease.

# 3. Animal models of IUGR

Many of our knowledge regarding the short- and long-term effects of IUGR comes from animal studies. A number of animal models of maternal malnutrition and placental insufficiency have been developed over recent years to investigate the causes and consequences of IUGR. A variety of species have been studied, including: rodents, sheep and primates; and both, maternal dietary manipulations or surgical interventional techniques have been employed [231-237]. We use animal models as they better reproduce the human condition compared to *in vitro* studies. However, in spite of the advances made using "*in vitro*" models to study some aspects of pregnancy, the IUGR condition as a whole is more properly represented *in vivo*. Still other features of pregnancy, such as the development of the uteroplacental circulation, fetal growth velocity and fetal development have no *in vitro* counterpart. Moreover, when new therapies arrive, although they are first tested extensively *in vitro*, they must present a clean reproductive toxicology panel *in vivo* [238] before they can be considered for use in humans, hence animal experiments are necessary. The majority of experimental fetal growth restriction studies are performed in rats and mice [239] (**Table 3**). We chose to study a mouse model because in mice, environment and genetic background can be easily controlled. Furthermore, their gestation has many characteristics which are common to human pregnancy [240], which makes them a good model of the disease. Generally, animal models of IUGR fall into three categories when divided by method of intervention: fetal intervention, maternal intervention and genetic models.

## 3.1 Fetal intervention models:

The hypoxic chick is the primary model of fetal intervention [241-243]. Since many IUGR in human pregnancies are caused by placental insufficiency, there is no change in the health status of the mother. As such, the main advantage of this model is the ability to investigate the effects of hypoxia in the fetus in isolation, without any maternal effects. [244].

## 3.2. Maternal interventions

### 3.2.1. Surgical methods

There is a range of maternal interventions for inducing IUGR in animal models. The most frequent and oldest intervention is uterine artery ligation which was first introduced by Wigglesworth. In this method, the uterine arteries of the pregnant rat are permanently ligated near the cervical end of the arterial arcade at day 17 of pregnancy. It causes utero-placental insufficiency which involves altered intrauterine environment characterized by hypoxia, reduced growth factor and hypoglycemia [245]. Uterine artery ligation has been shown in other species to cause IUGR like guinea pigs and sheep [246-250]. Similarly, uterine artery embolization in sheep also results in IUGR [251-253]. Although these methods induce IUGR, the lack of an intact uteroplacental circulation in these models makes them less useful for testing maternal therapies that target uterine blood flow or the placental barrier directly.

Since bilateral uterine artery ligation obstructs blood supply, 30% of the fetuses die or go through partial resorption [254]. As a result, it causes severe maternal outcomes such as necrotic uterus, ectopic pregnancy, abortion, etc. Therefore unilateral ligation is preferred to provide chronic placental insufficiency. This procedure in guinea pigs is performed at mid-gestation. In about one-third of the cases, fetal death occurred, in another third, fetuses with less than 60% of normal weight were observed and in the remainder all fetuses were in the normal weight range. It produces fetuses that are growth

restricted and chronically hypoxic [248]. A related intervention is sheep, caruncleclectomy, in which the maternal portion of the placentome – the multiple contact points between maternal and fetal blood circulations in the placenta – are surgically removed from the uterus prior to pregnancy. This creates IUGR in about half of pregnancies [255].

### 3.2.2. Nutrient restrictions

Interventions such as calorie intake restriction can cause IUGR and the effect depends on which trimester of the pregnancy this occurs. A good example of this is the growth restrictions which occurred during World War II Dutch famine. This study was done to examine the effects of maternal intrauterine undernutrition on offspring birth weights in a cohort of women born between August 1944 and April 1946 in Amsterdam, The Netherlands [256]. The decrease in the offspring's birth weights was associated with famine exposure during the third trimester of pregnancy [257, 258]. This phenomenon has been reproduced in several models involving rat, guinea pig, rabbit, and sheep [259-264].

Interestingly, it was reported that overfeeding in an adolescent pregnant ewe also results in IUGR. In that study, the animals were split into 2 groups which were offered either a high or low quantity of a complete diet which consisted of 30% coarsely milled hay, 50% barley, 10% molasses, 9% fishmeal, 0.3% salt, 0.5% dicalcium phosphate and 0.2% of a vitamin-mineral supplement and had an average dry matter of 86%. Although not examined, it was suggested that when nutrient intakes are high, blood flow to support maternal tissue synthesis is maintained at the expense of utero-placental blood flow, resulting in reduced placental growth and functional development [265]. In fact, in human adolescents studies has been shown that continuing maternal growth at the time of conception is associated with a significant but modest reduction in birthweight (about 100 g) in both primiparous and multiparous mothers [266].

Moreover, nutrient restriction such as low protein or low sodium diets, have also been shown to affect the growth of the fetus using animal models [267-269]. Fernandez-Twinn et al. investigated the role of the endocrine system in IUGR induced by low protein (LP) diet in rat model by measuring circulating levels of several endocrine factors such as progesterone, insulin, prolactin in both maternal and fetal plasma. They found that the LP mothers were hyperglycaemic at day 14 of pregnancy and this was accompanied by an increase in their circulating insulin levels. Prolactin levels were also raised significantly in the LP dams on day 14 of gestation compared with the controls, whereas progesterone levels were reduced. Also, a significant decrease in maternal leptin levels was observed at gestation on day 21. It has been suggested that maternal low protein intake during pregnancy affects nutrient delivery to the fetus by downregulation of specific amino acid transport proteins [270]. In a study on male offspring of rat dams, it was shown that early growth restriction due to maternal protein restriction leads to the development of diabetes later in life [271].

## 3.3. Environmental restrictions

Environmental factors have also been shown to influence pregnancy outcome. For instance, low birth weight lambs born from sheep raised in hot conditions, a heat stress model of IUGR was developed. In this technique, animals are placed in a special chamber with daytime heat temperature of ~40 °C for 18 hours and ~35 °C for 6 hours each night with the humidity of 40-45%, from day 39 to 125 of gestation and studied in a normo-thermic environment at day 135. With this method, average fetal weight was decreased significantly to 53% of the control group [272]. Affected fetuses showed symptoms of brain sparing (which is a physiological mechanism used by the fetus to increase delivery of oxygenated blood to the brain at the expense of other organs), and umbilical and uterine blood flow were decreased [159, 272].

## 3.4. Genetic interventions

Generally, genetic models of IUGR have been created in mice [273], due to the richness of the molecular information available in this species and accessible embryonic stem cells. Early knockout models were overly severe, and resulted in embryonic lethal phenotypes. For instance, systemic disruption of Tissue Factor (also known as platelet tissue factor), factor III, thrombokinase, or CD142, resulted in fatal wasting of mouse offspring after embryonic day 9.5 [274]. Also, heterozygous knocking out the vascular endothelial growth factor (VEGF), a signal protein which is vital for angiogenesis, produces an embryonic lethal phenotype [275]. Alternative mouse models are now available, with conditional or tissue-restricted knockout of specific genes such as the placental specific insulin-like growth factor 2 (IGF2) knockout mouse model. In this model, a transcript of the gene which is expressed only in the placental labyrinthine trophoblast cells is deleted [276]. This causes impaired placental growth from embryonic day 12, and growth restriction in 96% of fetuses by embryonic day 16. Birth weight is approximately 69% of wild type, although the pups did exhibit postnatal catch up growth. In fact, impaired placental growth is seen earlier in gestation than reduced fetal growth, perhaps as a result of escalated placental System A activity which may contribute to maintaining fetal growth (system A transporter facilitates uptake of small non-essential neutral amino acids such as alanine, glycine, and serine [277]). When it becomes closer to term, the knockout placentas remain smaller, the System A activity is nearer to normal and there is less passive permeability as well, all of which contribute to the IUGR phenotype [276]. In contrast, in humans, the level of system A activity is associated with severity of IUGR [49].

Another mouse model is the systemic, knockout of endothelial nitric oxide synthase (eNOS) gene, an enzyme which converts arginine to nitric oxide (NO) which is responsible for vasodilation [278]. This results in impaired uterine artery function and reduced placental System A amino acid transporter

activity. An asymmetric growth and a reduction in extraction of oxygen by the fetus are observed in this model [278].

**Table 3:** Some IUGR animal model species

| Animal species | Advantage | Disadvantage |
|---|---|---|
| Mouse | • Small size and social nature → easy to maintain and relatively inexpensive<br>• Short gestation →less time and expense | • Small size → makes it hard to manipulate surgically<br>• Imaging the fetus and placenta is challenging<br>• Less common laboratory animal so specific reagents/equipment more expensive Differences between human and mouse physiology, though generally well characterized and understood |
| Rat | • Short gestation, large litters<br>• Large enough for complex surgical intervention<br>• Useful for intergeneration studies especially cognitive | • More expensive due to size increase over mice |
| Guinea pig | • Haemomonochorial placenta and Extensive trophoblast invasion<br>• Longer gestation → better for therapeutic evaluation<br>• brain development more like human than other rodents | • Longer gestation, larger animal, smaller litters thus more expensive<br>• Less common laboratory animal so specific reagents/equipment → more expensive |
| Sheep | • sampling from both sides of the placental barrier in un-anaesthetised and unstressed animal possible<br>• Sheep conceptus physiology relevant to human fetal physiology<br>• Consistent gestation with predominantly singleton pregnancies<br>• Good tolerance for in utero manipulation | • Placentation is not closely similar to human<br>• Large animal facility needed |
| Non-human primates | • Genetically, closest model to human<br>• Pregnancy characterised by trophoblast invasion of the spiral arteries | • Longer gestation, larger animal, smaller litters → more expensive<br>• Less common laboratory animal so specific reagents/equipment → more expensive |

## 3.5. P57 $^{kip2}$ knock-out mouse model:

P57 $^{kip2}$ is a paternally imprinted gene [38], meaning that only the maternal allele is expressed and it is present both in humans and mice. It can bind with different cyclin-cdk complexes and inhibit their kinase activity *in vitro*. P57 $^{kip2}$ belongs to the Cip/Kip family, and shares homology with p21$^{Cip1}$ and p27$^{Kip1}$ at the N-terminal domain (cdk-binding/inhibitory domain). Conversely, it distinguishes itself from p21$^{Cip1}$ and p27$^{Kip1}$ by its unique domains: a proline-rich domain and an acidic domain in mouse P57$^{kip2}$, and a PAPA domain in human P57 $^{kip2}$ [279, 280]. The p57$^{kip2}$ molecule is expressed in a tissue-specific manner in the placenta, skeletal muscle and heart. It can also be found in some other tissues like central nervous system and cartilage [279-282]. Although there is a wealth of information proposing that p57$^{kip2}$ may be associated with tumor suppression, the data provided by Takahashi et al. showed that p57$^{kip2}$ plays an essential role in mouse fetal development. Their results showed that p57$^{Kip2}$ may function in the proper development of labyrinthine and spongiotrophoblasts by pathways that are not involved with regulation of cdk activities. Moreover, there was no cancer predisposition in the mutant mice so far examined, suggesting that loss of p57$^{kip2}$ is not simply responsible for tumorigenesis [283]. On the other hand, there have been some reports of its role as a tumor suppressor. Mutations of this gene were observed among BWS (Beckwith–Wiedemann syndrome) patients. A reduction in its expression in most of the cases of Wilms' tumour tissues, adrenal tumour tissues, and cultured adrenocortical cells was also reported [284-286].

Generally, imprinted genes, including IGF-II, H19 and p57$^{Kip2}$, are related with trophoblastic disease [287-290]. Mice deficient in the p57$^{Kip2}$ gene (p57KIP2−/−) have shown altered cell proliferation and differentiation leading to abdominal muscle defects; cleft palate; endochondral bone ossification defects with incomplete differentiation of hypertrophic chondrocytes resulting in dyspnea, renal medullary dysplasia; adrenal cortical hyperplasia and cytomegaly; and lens cell hyperproliferation and

apoptosis. Most of the $p57^{Kip2}$-deficient mice died within 24 h after birth, while about 10% of them survived beyond the weaning period. Surviving mice exhibited severe growth restriction, immaturity of testes and uterus, and vaginal atresia [39, 281, 282]. Kanayama's group reported that mice that are heterozygote for a $p57^{kip2}$ gene deletion (p57−/+) presented with PE-like symptoms [291]. A clear disruption of normal architecture as well as an abnormal fibrin deposition in $p57^{kip2}$ mutant placentas was observed by Knox et al [292]. They also reported a significant decrease in the thickness of the labyrinth layer and evidence of extensive calcifications, indicative of decreased utero-placental blood flow [293]. In addition, there was evidence of infarction, fibrin extravasation and fibrinoid necrosis throughout the labyrinth of mutant placentas as well as increased numbers of nucleated RBCs are also identified [292]. Conversely, that group as well as ours have found that the $p57^{kip2}$ deficient mouse model does not develop preeclampsia features as it was observed by Kanayama group [291]. We did however find that there was an increased placental pathology, smaller litters [294] and incidence of IUGR. [39]. Furthermore, the $p57^{Kip2}$ mutant mouse has gained strong support from the high incidence of placental abnormalities which is considered to be one of the main etiologies of IUGR. As such, we chose to use this model to determine the impact of exercise training on the development of IUGR.

# *Chapter 2:*

# *Article*

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# Exercise training prevents intrauterine growth restriction in p57$^{kip2}$ knockout mice

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**Condensation:** Exercise training can prevent intrauterine growth restriction via improvement of placental pathology, angiogenesis and inflammation.

**Short title:** Exercise training prevents IUGR

# Abstract

**Background:** According to the WHO, intrauterine growth restriction (10% below normal fetal weight for gestational age) affects 5-10% of human pregnancies and is a major cause of perinatal morbidity and mortality worldwide. Preeclampsia, pregnancy induced hypertension and proteinuria, is one of the major risk factors for IUGR. In our previous study on preeclampsia mouse model (mice which overexpress human renin and human angiotensinogen), we found that exercise training before and during pregnancy reduced maternal blood pressure, and prevented IUGR by improving placental development.

**Objective**: The aim of this study was to investigate the beneficial effects of exercise training on intrauterine growth restriction in an animal model without preeclampsia.

**Study design:** In this study, we used heterozygous knockout mice of the $p57^{kip2}$ gene which is a cyclin-dependent kinase inhibitor that regulates the cell cycle of trophoblastic cells and as such, normal placental development. To investigate the role of exercise training we placed mice in cages with free access to an exercise wheel 4 weeks prior to and throughout pregnancy. At the end of gestation, mice were sacrificed to harvest and weigh fetus and placentas. All data are expressed as means ± SE. One-way ANOVA was used to determine the impact of exercise and genotype on most parameters, followed by Tukey's post hoc test when an interaction was detected. Placental pathology scores were analyzed by non-parametric Kruskal-Wallis-tests.

**Results:** We confirmed the presence of IUGR in sedentary $p57^{kip2}$ knockout mice, reduced placental mass, increased placental alteration as well as smaller litter size with increased numbers of necrotic pups. Exercise training prevented intrauterine growth restriction as well as normalized litter size and placental mass and alterations. The expression of the vascular endothelial growth factor, a key regulator of angiogenesis required for normal placental development, was reduced in pregnant knockout

sedentary mice and was normalized by exercise training. In contrast to data reported in our preeclampsia model, the placental renin-angiotensin system in the knockout mice was found to be normal and was unaffected by exercise training. Interestingly, we found that inflammatory markers in the placenta (interleukin-1β and monocyte chemoattractant protein-1 mRNA) were elevated, in sedentary knockout mice, which suggests that placental inflammation may contribute to the placental pathology in this model, whereas this was not present in the trained mice.

**Conclusion:** Taken together, these results suggest that exercise training prevents intrauterine growth restriction by improving angiogenesis, placental alterations and placental inflammation.

**Key words:** Exercise training, intrauterine growth restriction, p57 gene, knockout mice, placental pathology, angiogenesis, inflammatory factors

# INTRODUCTION

***Preeclampsia and intrauterine growth restriction (IUGR).*** Preeclampsia is characterized by the new occurrence of high blood pressure (BP) and proteinuria, placental pathology and inflammation, and IUGR (reviewed in [295, 296]). IUGR refers to a condition in which the estimated weight of the fetus is less than 10% of what is expected for its gestational age with abdominal circumference below the 2.5th percentile [297]. It affects 5-10% of human pregnancies and is a major cause of perinatal morbidity and mortality worldwide [298]. According to the WHO, each year approximately 18 million (14%) babies are born with IUGR and they account for 60-80% of neonatal deaths[5, 299]. IUGR places the fetus at risk of death and disease later in life such as, hypertension, cardiovascular disorders and renal disease [300-302]. The factors that increase the risk of IUGR in humans include poor maternal nutrition, preeclampsia, genetic and chromosomal factors (e.g. maternal and paternal imprinted genes, including $p57^{kip2}$) [34, 40], IUGR and still birth in previous pregnancies [7-10, 24]. Currently, there are no standard prenatal therapies which are designed to specifically improve fetal growth or reverse the complications of IUGR. The only therapeutic avenues to prevent or minimize IUGR is by improving maternal nutrition, smoking cessation, avoidance of drugs and control of maternal conditions such as hypertension and renal failure [142, 143]. Our research has focussed on the use of exercise training (ExT) to prevent and/or improve IUGR [212].

***ExT impact on placental development.*** Human placental cell proliferation is enhanced in placentas from active women during normal pregnancy compared to their sedentary counterparts [303]. Placentas from exercise trained mothers are larger and show less alteration [304]. Moreover, ExT during pregnancy was shown to decrease reactive oxygen species generation in human placenta which leads to reduced oxidative stress [305]. It was also reported that ExT increases circulating vascular endothelial growth factor (VEGF) in pregnant rats which is important in placental angiogenesis [306].

***Mouse models of preeclampsia and IUGR.***

*$R^+A^+$ transgenic mice.* The study of IUGR is challenging in humans as when it is detected, the pregnancy is likely to be interrupted if growth restriction is severe or if the babies are mature enough to have a better chance of developing *ex utero*. Our lab characterized a unique transgenic mouse model of preeclampsia (mice which overexpress human renin and human angiotensinogen – $R^+/A^+$ mice) in which placental pathology and IUGR were observed [307]. Local renin-angiotensin systems (RAS), in the placental and in the aorta, were found to be compromised in these mice, as well as placental angiogenesis. ExT improved both maternal and fetal outcomes mainly via normalization of placental and aortic RAS as well as angiogenic factors [212]. However, IUGR may have been improved indirectly in these mice, by decreasing BP as it correlates with adequate placental blood perfusion needed for normal placental development [213].

*$p57^{kip2}$knockout mouse model ($p57^{-/+}$).* $P57^{Kip2}$ (also called cyclin-dependent kinase inhibitor 1C (CDKN1C), p57 and Kip2) is a universal inhibitor of cyclin-dependent kinases. $P57^{Kip2}$ is a protein inhibitor that negatively regulates cell proliferation (arrests the cell cycle in G1 phase) and is encoded by an imprinted gene. Mutations in p57 are associated with loss of cell cycle control and increased risk of childhood cancers and congenital cellular overgrowth disorders, so it is a tumor suppressor candidate [280]. Takahashi et al. 2000 generated a mouse model ($p57^{Kip2-/+}$) with a heterozygous deletion of this gene [39]. It was reported by Kanayama's group that, when $p57^{-/+}$ females were bred with $p57^{-/+}$ males, they developed preeclampsia like symptoms including hypertension, proteinuria, thrombocytopenia and placental alterations [291]. Conversely, our research team and others have not been able to confirm preeclampsia like symptoms in this mouse model, however, we did confirm placental alterations, IUGR, reduced litter size and increased number of non-viable pups [292, 294]. The goal of the present study

was to investigate the beneficial effects of ExT on IUGR without the presence of preeclampsia.

# MATERIALS AND METHODS

*Animals.* Our experiments were performed on heterozygous $p57^{kip2}$ knockout (KO) mice which were provided by Dr. Keiichi I. Nakayama from Kyushu University, Fukuoka, Japan [308]. These animals were bred and maintained by backcrossing with C57BL/6 mice (strain code 027, Charles River, St.-Constant, Quebec, Canada) as described previously [294]. Mouse genotype was determined, as done previously by Takahashi et al. [39]. All animals, including the breeders, had access to water and standard Japanese laboratory chow [CA-1 Japanese (JPN); CLEA Japan, Tokyo, Japan *ad libitum.* Mice in these experiments were 12–15 weeks of age and their care met the standards set forth by the Canadian Council on Animal Care for the use of experimental animals. All procedures were approved by the Animal Care Committee of the CHUM Research Centre. To investigate IUGR, female $p57^{-/+}$ or wild-type ($p57^{+/+}$) mice were time-mated with identical genotype male.

*Exercise training (ExT).* To investigate the role of ExT in our animal model, we put female $p57^{-/+}$ mice in cages with free access to a running wheel starting at 4 weeks prior to pregnancy, as described previously [212, 213]. Each cage was connected to a computer and the number of wheel revolutions was counted to confirm running status (Compte-tour5, Aquila, Boucherville, Qc, Canada). The data were then compiled and analyzed as done previously in different mouse models of preeclampsia [212, 213].

*Tissue collection and histology.* On day 18 of gestation, mice were anesthetized with ketamine/xylazine. Placentas and fetus were collected and weighed individually. Non-viable fetuses did not have a detectable placenta. Placentas were then flash-frozen in liquid nitrogen or placed overnight

in 4% paraformaldehyde for fixation. The next day fixed placentas were rinsed with phosphate buffer and embedded in paraffin. Placentas were cut cross-sectionally using a microtome. Sections were stained with hematoxylin-phloxine-saffron to assess overall placental morphology. Embedding, sectioning, and staining were performed by the Histology Platform of the Research Institute in Immunology and Cancerology at the Université de Montréal.

Placental alterations were characterized by five criteria: necrosis, hyalinization, microcalcification, cytotrophoblastic island loss, and loss of labyrinthine trophoblast structure, as described previously [212, 213, 294]. For each criterion, changes were assigned a score from 0 to 3, where 0 was the absence of, 1 was mild, 2 was moderate, and 3 was severe alteration. All scores were summed up for a total evaluation of the placental pathology present. The pathologist scoring the placentas was blinded to the genotype and training status of the mothers.

To evaluate the impact of maternal genotype and exercise training on placental and fetal growth, we calculated total fetal and placental mass by adding the weights of all placentas and fetuses from each litter.

*Real-time PCR.* Total RNA was extracted from placentas with Trizol (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer's protocol. Removal of genomic DNA from total RNA and reverse transcription was carried out as described previously [212, 294]. Real-time PCR (qPCR; Rotor Gene RG-3000; Corbett Research) was performed using Faststart SYBR Green Master fluorescent dye (04 673 492 001; ROCHE) and specific primers for vascular endothelial growth factor (VEGF), Interleukin-1β (IL-1β) as well as Monocyte chemoattractant protein-1 (MCP-1). Gene levels were expressed as values relative to S16. PCR Primer sequences are presented in **Table 1**.

*Western Blot*. Proteins were extracted from homogenized tissue using RIPA lysis buffer containing 50 mM HEPES pH 7.5, 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 2 mM Na3VO4, 10 mM $Na_4P_2O_7$, 10 mM NaF, 2 mM EDTA, 1% NP-40, 34µg/mL PMSF, and added protease inhibitor cocktail tablets

(11 836 153001; ROCHE). Protein concentrations were assessed by the Bradford method (500-0006; Bio-Rad) Placental protein samples were separated by electrophoresis and transferred onto nitrocellulose membranes. RAS proteins were detected with Anti-Mas receptor (Alomone Labs, Jerusalem, Israel) and anti-human/mouse angiotensin-converting enzyme 2 (ACE2; R&D systems, MN, USA), using enhanced chemiluminescence West Pico kits (34080; Thermo-Scientific, MA, USA). Total protein content was calculated using the Image-J software (NIH) according to protein band intensity, and was normalized to α-tubulin protein expression (ab4074; Santa Cruz Biotechnology, Texas, USA).

*Drugs.* The following drugs were purchased for mouse anesthesia: ketamine (Bimeda-MTC, Cambridge, Ontario, Canada) and xylazine (Bayer, Toronto, Ontario, Canada).

*Statistical analysis.* All data were expressed as means ± SE. One-way ANOVA was used to determine the impact of ExT and genotype on most parameters, followed by Tukey's post hoc test when an interaction was detected. Placental pathology scores were analyzed by non-parametric Kruskal-Wallis-tests.

# RESULTS

***Maternal weight gain.*** In our previous study of p57 KO mice, we found that maternal weight at the end of pregnancy was not significantly different from WT mice [294]. The present study confirmed that maternal weight was not different between the three groups of mice (**Table 2**).

***IUGR is prevented by ExT.*** In previous studies in p57 KO mice, fetal mass was found to be significantly decreased and correlated with increased placental alterations [39, 294]. In the present study we confirmed a significant reduction in total fetal mass in $p57^{-/+}$ sedentary litters compared to their WT

counterparts ($p<0.01$) which was completely normalized by ExT (**Table 3**; $p<0.05$).

***Litter size and non-viable pups are normalized by ExT.*** We previously reported that litter size was reduced and the number of non-viable pups was significantly increased in p57 KO mice compared to WT mice [294]. The litter sizes in the present study varied from 8-12 pups in WT, 3-7 pups in KO sedentary and 6-11 pups in KO ExT (**Table 3**). Hence, ExT normalized litter size in p57 KO mice ($p<0.05$) whereas non-viable pup number was no longer significantly different from the WT.

***Placental pathology and mass is normalized by ExT.*** We previously reported that $p57^{-/+}$ KO mice exhibited placental pathology and, as a result, decreased placental mass [294]. In the present study, we confirmed that $p57^{-/+}$ KO mice had increased placental alterations which mainly resulted from cytotrophoblastic island loss (CIL) ($p<0.005$) and an increased total placental alteration in KO sedentary mice ($p<0.01$; **Table 4)**. We also found that, as previously reported, placental mass was decreased in the KO mice. Interestingly, ExT completely normalized CIL ($p<0.001$) and total placental alterations where no longer different from the WT animals (**Table 4)**. Consequently, placental mass was normalized (**Table 3;** $p<0.05$). The other placental alteration criteria (necrosis, hyalinization, microcalcification and loss of labyrinthine trophoblast structure) were not significantly altered by the genotype nor by ExT (**Table 4)**.

***Placental angiogenesis in p57 KO mice is normalized by Ext.*** We found that VEGF expression is reduced in p57 KO sedentary mice ($p<0.005$) compared to WT sedentary mice, and that ExT normalized VEGF mRNA expression (**Figure 1**; $p<0.005$).

***Placental inflammation in p57 KO mice is normalized by Ext.*** In the present study, we measured

mRNA expression of 2 inflammatory factors. We found that placental IL-1β and monocyte chemoattractant protein-1 (MCP-1) mRNA expression were significantly increased in the p57 KO sedentary vs. WT sedentary by 70% and 68% respectively (**Figure 2A and 2B**; $p<0.05$). ExT normalized the MCP-1 expression levels (**Figure 2B**; $p<0.02$), while IL-1ß was no longer different from the WT mice (**Figure 2A).**

***Local placental RAS and Ext.*** We previously found that in a preeclampsia mouse model, placental RAS, both MAS-receptor and ACE2 protein, was found to be decreased and ExT was able to partially normalize these effects as MAS-R was reduced by ACE2 was unchanged [212]. In contrast, in the present study, we found no difference in the above 2 components of the local placental RAS between p57 KO sedentary and WT mice (**Figure 3**). In addition, ExT had no effect on the expression of ACE2 and MAS-R (**Figure 3**).

# COMMENT

In this second study on the $p57^{Kip2-/+}$ mouse model, in agreement with our previous study [294], we confirmed the presence of IUGR, increased placental alterations as well as decreased placental mass and litter size and increased number of non-viable pups. We found that, similarly to what we previously found in preeclampsia animal models [212, 213], ExT in the $p57^{Kip2-/+}$ mice had significant beneficial effects by normalizing the above placental and fetal parameters.

In $R^+A^+$ mice, the beneficial effects of ExT on preeclampsia may have resulted from regulation of the local renin-angiotensin system (RAS) [212]. Indeed, a decrease in the protein expression of Mas-R and ACE2 in placenta of preeclamptic mice was reported [212] which would reduce the effects and production of Angiotensin-(1-7) whose effects oppose those of Angiotensin II which has been

implicated in the development of placental alterations through mediation of oxidative stress, inflammation and vasoconstriction [309-311]. In contrast, the local placental RAS was not found to be impaired in $p57^{Kip2-/+}$ mice, perhaps because preeclampsia was not present, nor was it affected by ExT. This suggests that the placental alterations observed in this model are not as a result of modulation in the RAS. Also, in $p57^{Kip2-/+}$ mice, the beneficial effects of ExT on pup and fetal parameters cannot be explained by improvements in maternal BP as it was the case in other preeclampsia mouse models as we had previously demonstrated [212, 213].

We suggest that the IUGR and reduced litter size observed in $p57^{Kip2-/+}$ sedentary mice, as well as the significant elevation in the number of non-viable fetuses, result from placental insufficiency, as the placental mass was clearly reduced and placental alterations were evident. We also noted significant improvement in placental mass, as well as in placental alterations in the trained mice, which is in line with our hypothesis that ExT improves placental function and development.

The noticeable decline in angiogenesis, as assessed by VEGF gene expression, in the placentas of the $p57^{Kip2-/+}$ sedentary mice, is in accordance with previous data from our laboratory and others which have shown a correlation between decreases in placental VEGF expression levels and IUGR [312-314]. The reduction in the placental VEGF level is typically associated with increased soluble fms-like tyrosine kinase (sFlt-1) expression and preeclamptic syndrome, and is considered a biomarker for predicting preeclampsia [315]. Therefore, we propose that the reduced expression of VEGF in the placenta of $p57^{Kip2-/+}$ mice could be responsible for the impaired development of vessels and villi of the placentas, as observed histologically by the increased CIL, which would lead to placental insufficiency. Interestingly, the VEGF expression was completely normalized by ExT in our study. It appears that regular maternal exercise is beneficial for placental and fetal growth as it diverts blood toward muscle and skin and thus creates a short-lived hypoxic environment which is needed for the secretion of VEGF

[316, 317]. Hence, the fact that ExT upregulated VEGF expression, increased placental mass and prevented placental pathology is in line with the literature regarding the benefits of ExT on placental perfusion and development in normal pregnancy [188, 209].

Along with other reports of increased inflammatory factors in IUGR [226], we found increased gene expression of IL-1β. Abnormally high placental and serum levels of IL-1β are associated with pregnancy complication, such as preeclampsia and IUGR [318]. This, along with the increase in MCP-1 observed in our study, may result in a proinflammatory environment. IL-1ß has been demonstrated to promote functional changes in endothelial cells which include oxidative stress, secretion of vasoconstrictors as well as microthrombis and infarction which may all contribute to a dysfunctional placenta [319]. In addition, as low grade inflammation has been related to insulin resistance in other tissues [320], it may also contribute to the development of IUGR in our model by reducing availability of nutrients to the placenta and as a result, the fetus. Interestingly, maternal ExT normalized MCP-1 and prevented the increase in IL-1ß. This is in line with previous studies which have shown that ExT can reduce pro-inflammatory factors in a non-pregnant state [321].

ExT before and throughout pregnancy has been shown to increase the villous area and vascular volume in the human placenta, suggesting improved placental perfusion and transport capacity [189]. In our study, we demonstrated that trained mice had significantly increased total placental and fetal mass by 55% and 46%, respectively, which may result from an increased trophoblast function stimulated by ExT (**Figure 4**).

Cardiovascular benefits associated with voluntary ExT in rodents have been demonstrated, such as increased VO2max and diminished adverse vessel remodeling [322-325], and similar improvements have been observed in humans with aerobic exercise programs [326]. This suggests that the beneficial effects of ExT on IUGR described in our mouse models may be translatable to the clinic to pregnant women, although studies will need to be conducted to confirm this finding. Furthermore, the molecular

mechanisms by which ExT can protect against IUGR and placental pathology require further investigation. IUGR can results from complications due to several diseases and syndromes, such as preeclampsia [40]. and infections like rubella [29], cytomegalovirus [30] and herpes virus [31]. As no specific treatment for IUGR and placental pathology are currently available in medical practices, our study, which shows the beneficial effects of exercise training on this condition, is an important advance.


# ACKNOWLEDGEMENTS

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**TABLES**

**Table 1:** Primer sequences

| Primers | Forward (5′-3′) | Reverse (5′-3′) |
|---|---|---|
| **S16** | *ATC TCA AAG GCC CTG GTA GC* | *ACA AAG GTA AAC CCC GAT CC* |
| **VEGF** | *CAG GCT GCT GTA ACG ATG AA* | *GCA TTC ACA TCT GCT GTG CT* |
| **IL-1β** | *CCT TCC AGG ATG AGG ACA TGA* | *AAC GTC ACA CAC CAG CAG GTT* |
| **MCP-1** | *ATT GGG ATC ATC TTG CTG GT* | *CCT GCT GTT CAC AGT TGC C* |

**Table 2:** Maternal weight is similar in sedentary and trained mice

| Training | Mother's genotype | Maternal weight(g) (baseline) | Maternal weight(g) (end of gestation) |
|---|---|---|---|
| **Sedentary** | $p57^{+/+}$ | 21.4±0.6 | 37.0±1.6 |
| | $p57^{+/-}$ | 19.8±0.7 | 34.0±1.3 |
| **ExT** | $p57^{+/-}$ | 21.0±0.9 | 36.3±1.3 |

N=6-7/group; values are expressed as mean ± SE.

**Table 3:** ExT normalizes IUGR, placental mass and litter size

| Training status | Mother's genotype | Fetal mass (mg) | Placental mass (mg) | Pups/litter | Non-viable fetuses |
|---|---|---|---|---|---|
| **Sedentary** | $p57^{+/+}$ | 6.7± 0.4 | 1.6 ± 0.1 | 9.6 ± 0.6 | 0 ± 0 |
| | $p57^{+/-}$ | 3.9 ± 0.5† | 0.6 ± 0.1† | 5.6 ± 0.6† | 0.8±0.2† |
| **ExT** | $p57^{+/-}$ | 5.8 ± 0.7* | 1.0 ± 0.1* | 8.2 ± 0.9* | 0.4±0.2 |

Values are expressed as mean ± SE. * $p<0.05$ significantly different from KO sedentary; † $p<0.05$ significantly different from $p57^{+/+}$ mice.

**Table 4**: Exercise training normalizes placental pathology observed in p57 -/+ (KO) mice

| | | | Necrosis | | Hyalinization | | Microcalcification | | CIL | | LLTS | | Total Pathology | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Training status** | **Mother's genotype** | **N** | Mdn | 75% | Mdn | 75% | Mdn | 75% | Mdn | 75% | Mdn | 75% | Mdn | 75% |
| **Sedentary** | $p57^{+/+}$ | 11 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 3 |
| | $p57^{+/-}$ | 10 | 0 | 1 | 1 | 2 | 2 | 2 | 1 † | 2 | 1 | 1 | 4 † | 7 |
| **ExT** | $p57^{+/-}$ | 10 | 1 | 1 | 0 | 2 | 1 | 1 | 0 * | 0 | 0 | 1 | 2 | 4 |

Placental scores for each parameter are expressed as median and 75th percentile. CIL, cytotrophoblastic island loss; LLTS, loss of labyrinthine trophoblast structure; Mdn, median; Total Pathology is the average value of all the different pathologies measured. * $p<0.05$ significantly different from KO sedentary; † $p<0.05$ significantly different from $p57^{+/+}$ mice

## FIGURE LEGENDS

**Figure 1. Placental VEGF mRNA expression is normalized in p57$^{+/-}$ mice by ExT.**

We found a significant decrease in VEGF in KO sedentary compared to WT sedentary group while ExT normalized this parameter (n= 10-14/ group). Values are expressed as mean ± SE. * $p<0.05$ significantly different from KO sedentary; † $p<0.05$ significantly different from p57 $^{+/+}$ mice. VEGF, vascular endothelial growth factor; KO, knockout; ExT, exercise training

**Figure 2. Inflammatory factors are increased with IUGR and normalized by ExT.** We found that both interleukin-1ß (IL-1ß; panel A) and the monocyte chemoattractant protein-1 (MCP-1; panel B) mRNA expression were significantly increased in the placenta of p57 $^{-/+}$ mice. Conversely, ExT normalized MCP-1 expression (B) and IL-1ß (A) was no longer significantly different from the WT mice. Values are expressed as mean ± SE, n=10-13/ group. * $p<0.05$ significantly different from KO sedentary; † $p<0.05$ significantly different from p57 $^{+/+}$ mice. ExT, exercise training; KO, knockout; WT, wild-type.

**Figure 3. Local placental RAS is similar in not modulated in our mouse model and by ExT.** Placental Mas receptor (Mas-R) and angiotensin-converting enzyme 2 (ACE2) protein were similar in all groups. Values are expressed as mean ± SE, n=6-8/ group. RAS, renin-angiotensin system; KO, knockout; ExT, exercise training; WT, wild-type.

**Figure 4. Mechanisms implicated in the effects of ExT on fetal outcome.**

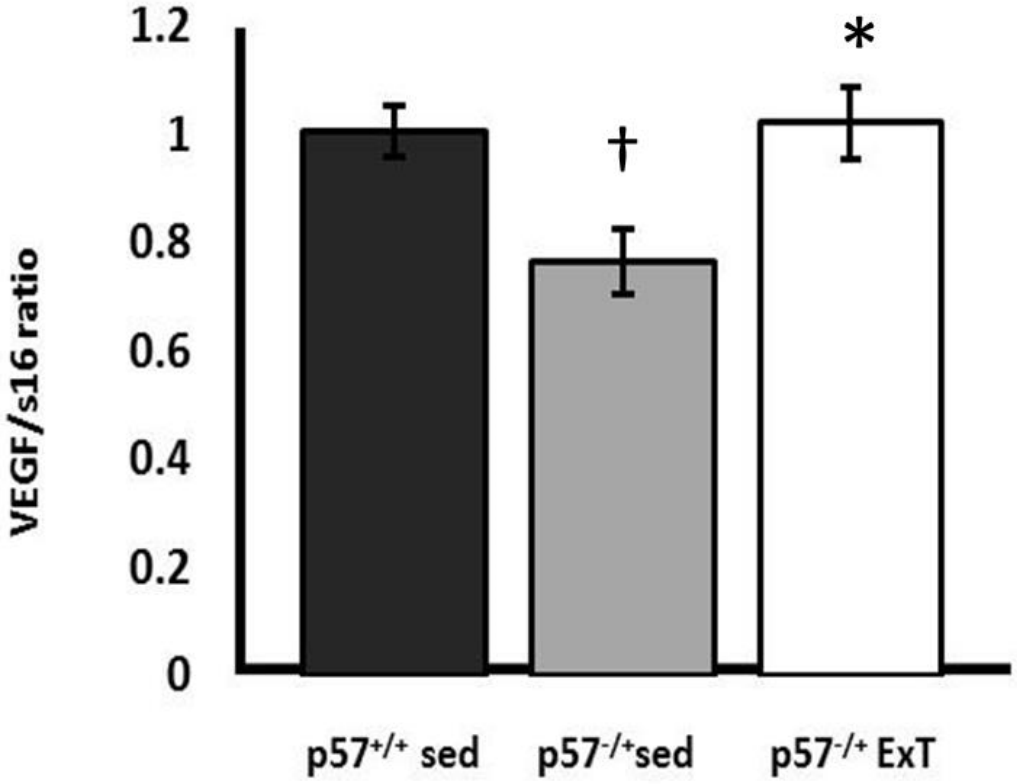



**Figure 1. Placental VEGF mRNA expression**

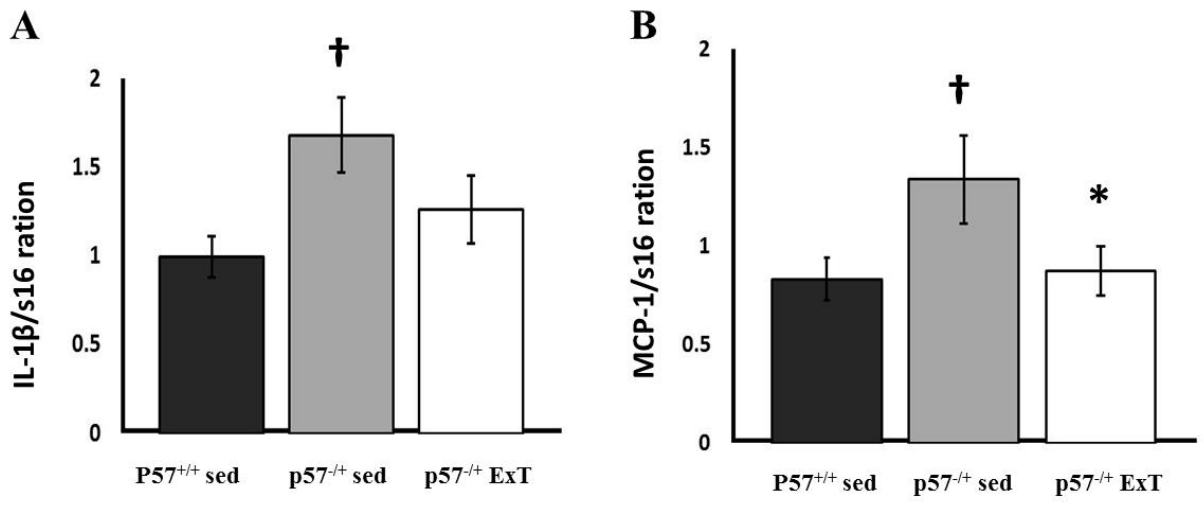



**Figure 2. Placental IL-1β (panel A) and MCP-1 (panel B) mRNA expression**

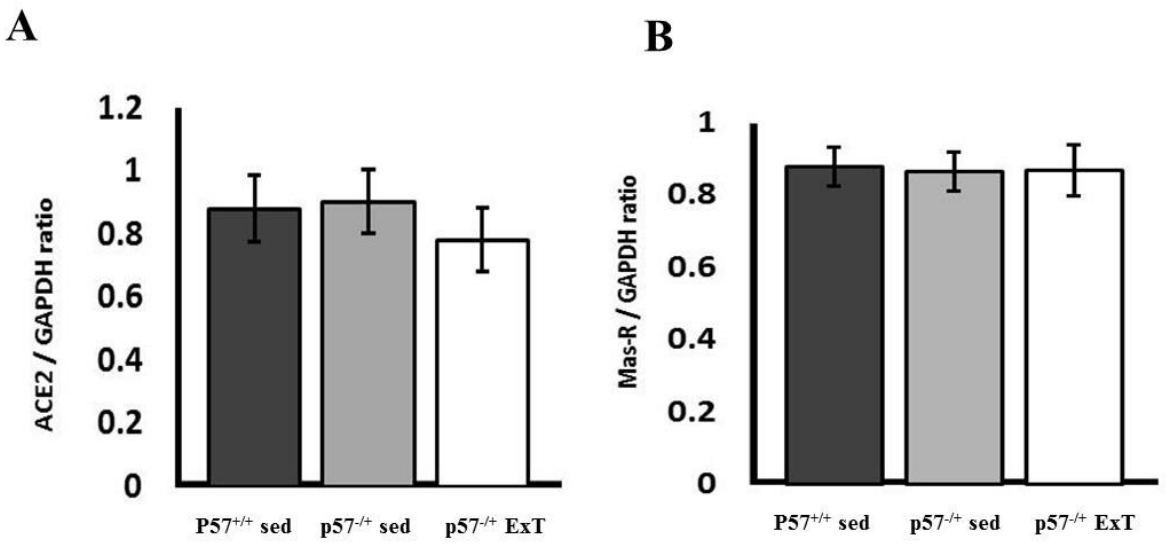
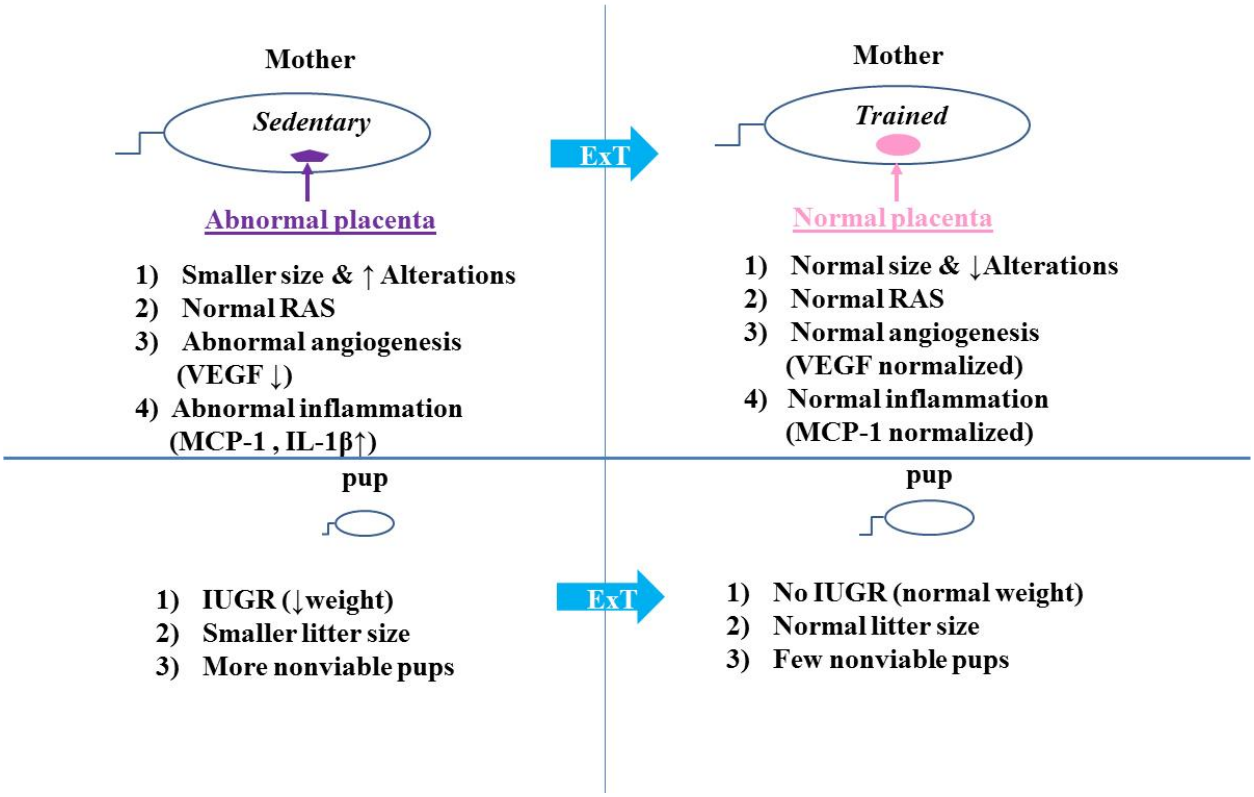



**Figure 3. placental ACE2 (panel A) and Mas-R (panel B)protein expression**

**Fig 4. Effect of Exercise Training (ExT) on Pup Outcomes**



# *Chapter 3:*

# *Discussion*

# Discussion

To our knowledge, we are the first group to study the effects of exercise training (ExT) on intrauterine growth restriction (IUGR). Our mouse model was heterozygous knockout (KO) of $p57^{kip2}$ gene ($p57^{-/+}$). Indeed, the homozygous deletion of this gene is lethal [281]. $P57^{kip2}$ which is a paternally imprinted gene [38] was shown to have an essential role in the proper development of labyrinthine and spongiotrophoblasts [283]. It was demonstrated by Kanayama et al that $p57^{-/+}$ mice show preeclampsia-like symptoms such as hypertension, proteinuria, thrombocytopenia, and excess trophoblast proliferation. [291]. Nonetheless, our research team and others have not found this syndrome in $p57^{-/+}$ mice [292, 294] and instead in our previous study on $p57^{-/+}$ mice we observed placental pathology and intrauterine growth restriction [294]. In fact, existence of placental pathology such as altered cytotrophoblast proliferation, trophoblast apoptosis, fibrin deposition, syncytial knotting and bridging, and enhanced villous maturation were reported by other groups in IUGR babies [54].

In the present study, we confirmed the presence of IUGR in this mouse model as the average fetal weight in the sedentary $p57^{-/+}$ litters was significantly decreased by 41% compared to the control group. We suggest that the remarkable growth restriction observed in this mouse model and the reduced litter size as well as the significant elevation in the number of nonviable fetus, are as a result of placental insufficiency, because similar changes to fetal weights were found in placenta as the average placental weight was decreased by 40%. Along with this observation in placenta, we noted an augmentation in cytotrophoblastic island loss in the placenta of sedentary KO mothers compared to their wild-type counterparts. Other placenta criteria, consisting microcalcification, necrosis, cytotrophoblastic island loss, hyalinization and loss of labyrinthine

trophoblastic structure, were not significantly changed, although tended to increase, suggesting the observed placental pathology in this mouse model is mainly due to the cytotrophoblastic loss. In addition, the total placental pathology was noticeably increased in the KO mice. Interestingly, all the mentioned fetal and placental parameters were significantly improved with ExT, except for the number of non-viable fetuses. These results propose that ExT can improve IUGR via increasing the placental mass, and reducing the placental pathology. However, ExT may not prevent fetal death, although it was tended to be decreased. These findings were in line with the literature stating Women who start training in early pregnancy have elevated placental volumes and growth rates [188], as well as a decreased fraction of non-functional tissue and an increased volume of villous tissue [189] and also with our previous study on the transgenic preeclampsia mouse model (R+A+) where a significant improvement of the placental pathology as well as an increase in placental mass in the trained mice was identified [212]. However, this might not be the direct effect of exercise training as we noticed a reduction in blood pressure as well as modulation of some of the renin-angiotensin system (RAS) components, like decreased Mas receptor (Mas-R) and angiotensin converting enzyme 2 (ACE2) in the placentas of pre-eclamptic mice and their normalization in the trained mice [212]. This would increase the sensitivity and production of Angiotensin-(1-7) whose effects oppose those of Angiotensin II (AngII), which is development of placental alterations through mediation of oxidative stress, inflammation and vasoconstriction [309-311].

In the present study, we also evaluated some components of the renin-angiotensin system (RAS) in the placenta. Interestingly, no difference in the placental Mas R and ACE2 protein expression between the KO and WT mice was observed, which proves that local placental RAS was not impaired in these mice, and it was not modulated by ExT which shows that the beneficial effects

of ExT on maternal and pup parameters cannot be explained by improvements in the local placental RAS.

Placentation includes extensive angiogenesis in maternal and fetal placental tissues, accompanied by a marked increase in uterine and umbilical blood flows [327-330]. Vascular growth, or angiogenesis, is indeed a major component of the increase in placental blood flow throughout gestation [327, 331, 332] and thus, reduced placental vascular development and increased vascular resistance have been associated with early embryonic mortality [333, 334].

Vascular endothelial growth factor (VEGF) is a homodimeric glycosylated heparin-binding glycoprotein [335, 336]. It is particularly known to promote angiogenesis and also can promote activation of eNOS (endothelial nitric oxide synthase) in uterine artery endothelium [337].

VEGF is expressed in several organs such as the heart, kidney, brain and lung. It has also been identified in endometrium [338] and in the placenta it has been identified in cytotrophoblast in first trimester and then in syncytiotrophoblast throughout the remainder of pregnancy [339].

It was shown by Lyall et al and that VEGF expression in placental villous tissue in pre-eclampsia and intrauterine growth restriction is suppressed [340]. In view of the placental pathological features of IUGR, especially impaired vascular development, and the known angiogenic effects of VEGF, we were interested to measure expression of this gene in placentas of our study groups. The noticeable decline that we observed in the level of VEGF gene expression in placentas of the $p57^{-/+}$ sedentary mice was in line with the other studies that found the correlation between decrease in the VEGF level and IUGR [312, 340]. The reduction in the VEGF level is typically associated with an increase in soluble fms-like tyrosine kinase (sFlt-1) expression and decline in the amount of VEGF may cause pre-eclamptic syndrome features. Also, VEGF is an angiogenic growth factor which is expressed in a temporal and spatial manner throughout

gestation [339] it is possible that the reduced VEGF expression observed in this study is linked to the abnormal vascular development in IUGR. Therefore, we propose that the reduced expression of VEGF in placenta may be responsible for the impaired development of vessels and villi of the placentas from IUGR pregnancies, which leads to placental insufficiency.

VEGF is known to be upregulated by hypoxia [341], hence, it would seem likely that if the placentas in the present study, were hypoxic then upregulation of VEGF would be anticipated.

Interestingly, the VEGF expression was completely normalized by exercise training in our study. It appears that exercise training may provide the hypoxic environment for the placenta which is needed for the secretion of angiogenic factors, like VEGF [317]. Thus, the fact that the up-regulation of VEGF and placental alteration were increased and reduced respectively by training is in line with the literature regarding the benefits of exercise training on placental perfusion and development in normal pregnancy.

Abnormally high placental and serum levels of IL-1β are associated with pregnancy complication, such as preeclampsia and IUGR [318]. Along with other reports of increased inflammatory factors in IUGR [226], we found increased gene expression of interleukin-1β (IL-1β). IL-1β is a member of the interleukin 1 family of cytokines. This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. There is data showing that IL-1β can destroy the cell-cell junction in placenta [342]. Moreover, increased level of IL-1β in the placenta of pre-term mice was found [343]. According to one study, increased systemic maternal or placental IL-1β level was shown to contribute to insulin resistance [344]. Thus, the significantly elevated levels of IL-1β observed in our study may attenuate the effects of maternal insulin on placental function and consequently reduces the fetal growth.

We also studied the mRNA gene expression of MCP-1 (monocyte chemoattractant protein-1) which is a known chemoattractant responsible for the migration of monocytes/macrophages and involved in the pathogenesis of chronic inflammation [345] [346]. IL-1β is known to induce MCP-1 synthesis and also it is upregulated by MCP-1[346]. Our data is in line with the literature because along with the increase in IL-1β, we also detected a rise in the placental MCP-1 mRNA expression, suggesting that increased inflammation is another possible mechanism for the decreased placental sufficiency observed in the $p57^{Kip2-/+}$ mouse model. MCP-1 expression was completely normalized by ExT. This data proves that ExT could partially control inflammation in the placenta of our mouse model.

ExT before and throughout pregnancy has been shown to increase the villous area and vascular volume in the human placenta, suggesting improved placental perfusion and transport capacity [189]. We have shown that all our trained mice had significantly increased total placental and fetal mass by 46% and 55%, respectively, which may result from an increased trophoblast function stimulated by ExT.

Cardiovascular benefits associated with voluntary ExT in rodents have been demonstrated, such as increased VO2max, diminished BP as well as diminished adverse vessel remodeling [322-325], and similar improvements have been observed in humans with aerobic exercise programs [326]. This suggests that the beneficial effects of ExT on IUGR described in our mouse models may be translatable to the clinic for pregnant women, although studies will need to be conducted to confirm this finding. Furthermore, the molecular mechanisms by which exercise training can protect against IUGR and placental pathology require further investigation. IUGR can results from complications due to several diseases and syndromes, such as preeclampsia [40]. and infections like rubella [29] cytomegalovirus [30] and herpes virus [31] No specific treatment for IUGR

and placental pathology are currently in medical practices. Our study, which shows the beneficial effects of exercise training on this situation, is an important advance.

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