Corso di dottorato di ricerca in:

"Alimenti e Salute Umana"

Ciclo 35°

Titolo della tesi

# "Fetal growth, maternal nutrition and placental oxidative stress"


Dottorando

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**Anno 2023**

UNIVERSITÀ DEGLI STUDI DI UDINE
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*To Cesare, Giulio and Vittoria with love*

*To my father and my mother with love and gratitude*

*"Education is the most powerful weapon which you can use to change the world"*

*Nelson Mandela*

**Publications during the PhD study period**

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## Abstract

### Background

An optimum nutrition during pregnancy will contribute to optimal fetal growth and to better long term health in offspring. However, most women of reproductive age worldwide do not adhere to the dietary guidelines for pregnancy, since food consumption pattern is strongly influenced by social aspects, cultural factors and consolidated behaviors. Fetal smallness is known to be influenced by maternal nutrition. Yet, to what extent placental oxidative stress, possibly related to maternal nutrition, is involved in pathophysiology of fetal smallness at term of gestation has to be still clarified.

### Objectives

The main objective of the first part of this study was to determine the prevalence of high-fat diet and, secondly, the potential association between maternal high fat and/or carbohydrates diet and fetal growth in a cohort of pregnant women referring to the university hospital of Udine for pregnancy follow up and delivery. For the second part of the study, the main purpose was to evaluate if oxidative stress is differently present within the placentae according to fetal growth pattern. The secondary intention was to assess if there is a correlation between placental histology and oxidative stress.

### Study design

This study was a prospective single-center cohort investigation conducted from May 2021 to August 2022. Pregnant women aged 18 years or older, with singleton pregnancy, alive fetus at the time of ultrasonography and fluency in Italian language were included, whereas women affected by fetal anomalies, diabetes, hypertensive disorders, major psychiatric disorders or with multiple gestation were excluded. Women enrolled were evaluated about their food consumption pattern through a food frequency questionnaire and fetal growth was regularly monitored through consecutive ultrasound scans. After delivery the placenta was sent to the Institute of Pathology for macroscopic and microscopic examination, tissue microarray preparation and for the immunohistochemical analysis.

**Results**

For the first part of the study a sample of 186 women was included in the statistical analysis. This study showed that most of the recruited women consumed a high-fat diet that exceeded the upper limit for fat intake by almost 10 percentage points and almost doubled the recommended upper limit for saturated fat. In addition, mothers of SGA newborns had significantly higher fat intake, especially saturated fat, and alcohol consumption compared to controls.

For the second part of the study a sample of 165 women was finally included. This study showed a sex specific pattern of 8OH-dG expression in placentae of single term pregnancies, with higher expression of 8OH-dG in the nuclei of syncytiotrophoblast cells and in stromal and endothelial cells among AGA males compared to AGA females. Secondly, we found a sex difference in the histological pattern among the late FGR placentae, with males exhibiting placental lesions from either maternal and fetal malperfusion, while females displaying only placental lesions from maternal malperfusion. Finally, male late FGR showed a significant correlation between high intensity 8OH-dG staining in cytoplasm of syncytiotrophoblast cells and thrombi in chorionic plate or villi, whereas female late FGR had a significant correlation between high intensity 8OH-dG staining within endothelial and stromal cells and high values of birthweight MoM.

**Conclusions**

To conclude, according to our data pregnant women in this region tend to have a worryingly high fat diet, especially high in saturated fat, which appears to be strongly related with fetal smallness at term birth. Fetal smallness was also strongly associated with a higher alcohol intake. Moreover, our findings suggest that oxidative stress may not be the only pathway involved in the pathophysiology of fetal smallness at term of gestation. Surprisingly, we observed a significant difference in the oxidative stress pattern between males and females, suggesting that fetal growth is differently regulated among the two sexes.

**Abstract**

**Introduzione**

Un apporto nutritivo ottimale in gravidanza è essenziale al fine di garantire un'adeguata crescita fetale e uno stato di salute migliore a lungo termine nel nascituro. Tuttavia, la maggior parte delle donne in età riproduttiva non aderisce alle linee guida sulla nutrizione per la gravidanza, poiché il consumo di cibo è fortemente influenzato da fattori culturali e da comportamenti consolidati. La ridotta crescita fetale è a sua volta influenzata dall'apporto nutritivo materno. Ciononostante non è ancora chiaro quanto lo stress ossidativo correlato con l'apporto nutritivo sia coinvolto nella patogenesi della ridotta crescita fetale.

**Obiettivi**

L'obiettivo primario della prima parte dello studio era quello di determinare la prevalenza di una dieta ricca di grassi e, secondariamente, di rilevare la potenziale associazione tra una dieta ricca di grassi e/o carboidrati e la crescita fetale in una coorte di donne afferenti all'ospedale universitario di Udine per il follow-up della gravidanza e il parto. Nella seconda parte dello studio l'obiettivo primario era quello di verificare se lo stress ossidativo risultava differentemente espresso in rapporto alla crescita fetale. L'obiettivo secondario consisteva nel valutare se esiste una correlazione tra istologia placentare e stress ossidativo.

**Disegno dello studio**

Questo studio prospettico monocentrico di coorte è stato condotto da maggio 2021 ad agosto 2022. Sono state incluse gravide di almeno 18 anni di età, con gravidanza singola, feto vivo al momento dell'ecografia e buona padronanza della lingua italiana; mentre sono state escluse le pazienti affette da anomalie fetali, diabete, disordini ipertensivi, disordini psichiatrici maggiori e gravidanza multipla. Le gravide arruolate sono state valutate in merito al loro consumo di cibo attraverso un questionario FFQ e la crescita fetale è stata regolarmente monitorata attraverso ecografie consecutive. Dopo il parto la placenta è stata inviata all'istituto di Anatomia Patologica per l'esame

macroscopico e microscopico, per l'allestimento del "tissue macroarray" e per l'analisi immunoistochimica.

**Risultati**

Per la prima parte dello studio è stato considerato per l'analisi statistica un campione di 186 donne. Lo studio ha dimostrato che la maggior parte delle pazienti arruolate aveva una dieta ricca di grassi, che superava il limite superiore raccomandato per i grassi di circa 10 punti percentuali e che quasi raddoppiava il limite superiore raccomandato per i grassi saturi. Inoltre, le madri di neonati piccoli per epoca gestazionale avevano una dieta significativamente più ricca di grassi, soprattutto di grassi saturi, e un consumo superiore di alcool rispetto ai controlli.

Nella seconda parte dello studio è stato considerato per l'analisi statistica un campione di 165 donne. Questo studio ha mostrato un pattern di espressione del biomarcatore 8OH-dG sesso specifico in placente di gravidanze singole a termine di gestazione, con una espressione significativamente più alta nei nuclei del sinciziotrofoblasto e nelle cellule endoteliali e stromali nei maschi con crescita appropriata per epoca gestazionale rispetto ai corrispettivi neonati femmina. In secondo luogo, abbiamo riscontrato una differenza sesso specifica nel pattern istologico delle placente dei neonati con tardiva ridotta crescita fetale. I maschi infatti manifestavano lesioni da malperfusione sia materna che fetale, mentre le femmine manifestavano lesioni attribuibili solo a malperfusione materna. Infine, abbiamo osservato una significativa correlazione tra elevata intensità della colorazione immunoistochimica nel citoplasma del sinciziotrofoblasto e la presenza di trombi a livello del piatto coriale o comunque nei villi tra i maschi con tardiva ridotta crescita fetale. Al contrario nelle femmine si è evidenziata una correlazione significativa tra elevata intensità della colorazione immunoistochimica delle cellule endoteliali e stromali ed elevati valori di peso alla nascita espresso in MoM.

**Conclusioni**

Per concludere, secondo i nostri dati le gravide in questa regione geografica tendono ad avere una dieta eccessivamente ricca in grassi, soprattutto in grassi saturi, che sembra essere fortemente correlata con una ridotta crescita fetale alla nascita a termine. Inoltre, la ridotta crescita fetale è stata fortemente associata a una elevata introduzione materna di alcool. I nostri dati suggeriscono anche che lo stress ossidativo non sembra essere l'unica via coinvolta nella patogenesi della ridotta crescita fetale a termine di gestazione. Infine, con sorpresa abbiamo osservato una differenza

significativa tra maschi e femmine per quanto riguarda l'espressione dello stresso ossidativo, suggerendo di fatto che la crescita fetale sia regolata in modo diverso tra i due sessi.

## Section I

# INTRODUCTION

## Fetal programming

After 4 years of German occupation, liberation of the Netherlands from German occupation seemed imminent. Allied forces advanced quickly across France, Luxembourg and Belgium. On 14th September, the Allied troops already entered in the Netherlands, approaching however formidable German defenses along the Siegfried line. Trying to bypass this famous German defense line, the Allied came up with a daring plan: crossing the lower part of the Rhine River, to drive into the industrial heartland of northern Germany. Operation “Market Garden” was set up. Plans were made to seize six bridges across Dutch rivers, nearly simultaneously. The sixth one was the bridge across the lower Rhine, near the city of Arnhem, giving a direct route to Germany for the Allies. The offensive called for Allied airborne divisions (the Market part of the operation) to drop by parachute, conquer bridges and seize key territory, so that ground forces (the Garden) could cross the Rhine. Unfortunately, operation “Market Garden”, a massive offensive into the Nazi-occupied Netherlands, failed dramatically. Most of the approximately 10.000 Allied forces who made it north of the Rhine were killed, wounded or taken prisoner.

If operation Market Garden had succeeded, the war might have ended before Christmas of 1944, in the Netherlands (but also in Europe). Instead, because of the failure of the plan, the conflict would drag on for five more months, leaving the northern part of the Netherlands under German occupation. The Nazis cut off all food supplies to the densely populated western provinces of the Netherland, as a countermeasure against the exiled Dutch government supporting the Allies. By the time, in early November 1944, an usually harsh winter made it very difficult to transport supplies via the country’s canal system. The famine had begun [1]. A severe food crisis started for the around 4,3 million people who lived in that region, including 2.6 million in urban areas (e.g., Rotterdam, Amsterdam and The Hague).

**Figure 1**. Paratroops land in Holland during the operation "Market Garden" (available from web).

It is known that the Dutch famine, also named the Dutch Hunger Winter - which took place from November 1944 until the beginning of May 1945 - has cost the lives of some 20.000 people, while 4.5 million were affected by the direct and indirect consequences of the famine.

The official daily rations for the general adult population, having decreased gradually from about 1800 calories in December 1943 to 1400 calories in October 1944, fell abruptly to below 1000 calories in late November 1944. At the height of the famine between December 1944 and April 1945 the official daily rations in Amsterdam were between 400 and 800 calories [2]. People had to eat grass and tulips to survive. Children under the age of one were relatively protected because their official daily rations never fell below 1000 calories, and the specific nutrient components were always above the standards adopted by the Oxford Nutritional Survey. Pregnant and lactating

women were entitled to supplementary rations. At the peak of the famine, these extra rations could however no longer be provided [1]. The total food intake may have been up to twice as high as the official rations indicate, thanks to the additional food provided by black market, church organizations and foraging trips around the countryside. Yet, the official rations do adequately reflect the variation over time of total food availability throughout the famine. After the liberation of the Netherlands on May 5th 1945, the food situation improved very rapidly. By June 1945, the rations had risen to over 2000 calories a day.

The famine had a profound effect on the general health of the population living in cities in the western part of the Netherlands. Most of the excess mortality is likely to have been due to starvation [1,3]. Despite the disastrous famine, women were still conceiving and giving birth to babies. It is in these babies that the effects of maternal malnutrition on health in adult life have been extensively studied. Data from a historical cohort of 300000 19-year old Dutch men exposed in utero to famine were first published in 1976 in the New England Journal of Medicine. This study showed that famine exposure during the last trimester of pregnancy and the first months of life produced significantly lower obesity rates, but famine exposure during the first half of pregnancy resulted in significantly higher obesity rates [2]. Later, studies in different cohorts and with different set-ups have provided support for the fetal origin of non-communicable diseases. Some Authors showed that people who had been exposed to the Dutch famine during any stage of gestation were found to have higher glucose levels in adult life [4-6]. Moreover, people exposed to famine in early gestation had more atherogenic lipid profiles [7-8] and higher odds for hypertension [9]. People conceived in famine not only had a higher cumulative incidence of cardiac heart disease [10], but the disease came up at an earlier age [11]. Interestingly, some studies investigated whether there was a relationship between in utero undernutrition and cognitive impairment in adult life, finding that male individuals were more vulnerable. In men, exposure to famine during gestation was not only associated with smaller intracranial volume [12], but also with an overall worse brain perfusion and an earlier brain senescence (as assessed by a machine-learning pattern recognition method capable to estimate individual brain ages based on MR images) [13-14]. Also, prenatal exposure to famine was associated with increased symptoms of depression and anxiety [15] and a lower physical performance score in men [16]. Thus, the so-

called Dutch Hunger Winter study provides an almost perfectly designed, although tragic, human experiment in the effects of intrauterine deprivation on subsequent adult health.

**The Barker Hypothesis: "The womb may be more important than the home"**

In the early 1980s, David Barker and others noted a paradox: although overall rates of cardiovascular disease increase with rising national prosperity, the least prosperous residents of a wealthy nation suffer the highest rates. As he stated in his correspondence on BMC in 1990: "studies in Norway, Finland, Britain, and the United States have shown that death rates from cardiovascular disease are inversely related to adult height, and geographical differences in cardiovascular mortality are related to past differences in infant mortality. These findings have been interpreted as evidence that adverse living conditions during childhood, such as poor housing and diet, increase the risk of ischemic heart disease" [17]. He then maintained that, thanks to the existence of accurate birth registries from 1911 onwards in Hertfordshire, detailed follow up studies could had been carried out. These studies conducted on men and women born 60 and more years earlier showed that those who weighed more at birth and, if they were breast fed, had lower death rates from ischemic heart disease and stroke. This was the background for his revolutionary hypothesis, according to which an adverse fetal environment followed by plentiful food in adulthood may be a recipe for adult chronic disease. This theory is known as the developmental origins of health and disease (DOHaD) [3]. One of the predictions made by the DOHaD is that fetal adaptations to scarcity become maladaptive only when affected individuals are later exposed to an environment of plenty [3]. This is dramatically shown by comparing individuals exposed to the Dutch Hunger Winter with those born after the siege of Leningrad. In both cases, pregnant women were exposed to severe hunger. However, in the Dutch case there was a sudden onset and a rapid relief from the famine, with the population returning quickly to a complete diet, whereas in the U.S.S.R. there were continuing shortages, so that those exposed to famine in utero did not exhibit higher rates of either obesity or cardiovascular disease as adults [18]. These large epidemiologic studies and cohorts that involve multiple generations provide the foundation for understanding fetal programming.

**Fetal growth restriction and fetal programming on heart and brain**

Fetal growth restriction (FGR) is defined as a failure to achieve the genetic growth potential. It affects 7-10% of pregnancies globally [19] and it is mostly the consequence of placental

insufficiency. According to the International Society of Ultrasound in Obstetrics and Gynecology (ISUOG) guidelines, FGR has two main clinical presentations depending on the gestational age of appearance: early and late onset [20]. Early FGR is essentially associated with maternal vascular malperfusion of the placenta, which is characterized by abnormal transformation of the spiral arteries, pathologic features of the placental villi and multifocal infarction. These disease components result in the so-called 'placental insufficiency' and form the most common basis for placenta-mediated FGR [21-22]. The typical pattern of decline progresses from escalating abnormalities in umbilical artery and venous Doppler parameters to an end-stage cardiovascular deterioration caused by severe hypoxemia followed by acidosis [23-25]. The rate and rapidity of alteration in umbilical artery Doppler, from increased blood-flow resistance to absent end-diastolic flow, determines the rate of fetal deterioration [26-28], often requiring a preterm delivery. Early FGR is strongly associated with preeclampsia and it is burdened by high perinatal mortality. Though challenging, the clinical management of this type of feto-maternal condition is based on the timely use of steroids, followed by magnesium sulfate, transfer to a tertiary care center and consideration of the safest mode of delivery [29]. Late FGR is characterized by milder and more aspecific placental lesions and/or alteration in oxygen and nutrient diffusion [20]. Consequently, the Doppler alterations typically found in early FGR are rare and fail to identify the vast majority of late FGR cases or to predict adverse outcome in these fetuses [30]. However, subtle changes between placental and cerebral blood flow perfusion may be appreciated using the ratios of middle cerebral artery pulsatility index (PI) and umbilical artery PI (cerebro-placental ratio, CPR and umbilical-cerebral ratio, UCR). The usefulness of these tools has been consistently demonstrated in the literature. For instance, several studies have found a link between middle cerebral artery vasodilatation (i.e. reduction in MCA-PI) or the alteration of its ratio with umbilical artery-PI and poorer perinatal outcome [31], including stillbirth [32], a higher risk of cesarean delivery [33-35], an increased risk of abnormal neurodevelopment at birth [36] and at 2 years of age [37]. Additionally, late FGR fetuses exhibit alterations in the biophysical profile only shortly before stillbirth. Therefore, the biophysical profile, which is a mainstay in the management of the early FGR, is not useful in determining the monitoring intervals in this case [32]. Interestingly, the TRUFFLE study (which has defined the background for the current management of fetal growth restriction) surprisingly showed that the risk of poor neurodevelopmental outcome in babies delivered after 32 weeks remained static until term [38]. This might be explained by a reduced tolerance to hypoxemia, possibly due to high metabolic requirements occurring near term of

gestation. Thus, frequent monitoring of pregnancies is equally warranted with late FGR and early FGR.

Fetal growth restriction represents a perfect model for fetal programming: structural, functional, and metabolic changes that occur in the fetus as an adaptive response to an adverse environment persist into postnatal life, leading to a higher risk of disease in adulthood. Metabolic programming was theorized by researchers to explain why cardiovascular diseases are strongly associated with low birthweight. The compromised nutrient and oxygen delivery to the fetus during fetal life would promote developmental pathways that best fit in this type of environment, through the selection of "thrifty genes" [39]. Since the nutrient and oxygen availability is normal after birth, this programming would facilitate a higher incidence of metabolic disease, including obesity, diabetes mellitus, and metabolic syndrome, which secondarily may lead to cardiovascular diseases. Yet, recent advances in fetal research have demonstrated that FGR fetuses may undergo direct changes in the cardiovascular system as well as in the central nervous system.

**Fetal programming on heart**

Placental insufficiency has two important consequences for the fetus. On one side it determines hypoxia to which cardiomyocytes respond by disrupting their structure, on the other side it causes villous hypoplasia and thrombosis thereby increasing the fetal cardiac afterload. The fetal heart turns in a more spherical shape, thus being able to better tolerate the pressure overload with a decreased energy expenditure. Indeed, this cardiac shape allows the fetus to maintain the stroke volume with less contraction force [40]. If this change occurs in one ventricle, an elongated phenotype could be appreciated by ultrasound; vice versa, if this change involves both ventricles, then a globular phenotype is obtained [41,42]. In more severe cases of fetal growth restriction, this sphericity might not be able to meet the physiologic requirements, and the fetal heart develops hypertrophy to increase contractility and decrease local wall stress. Early FGR is more associated with a hypertrophic response, whereas cardiac phenotypes late-onset FGR usually develops globular or elongated [41,42].

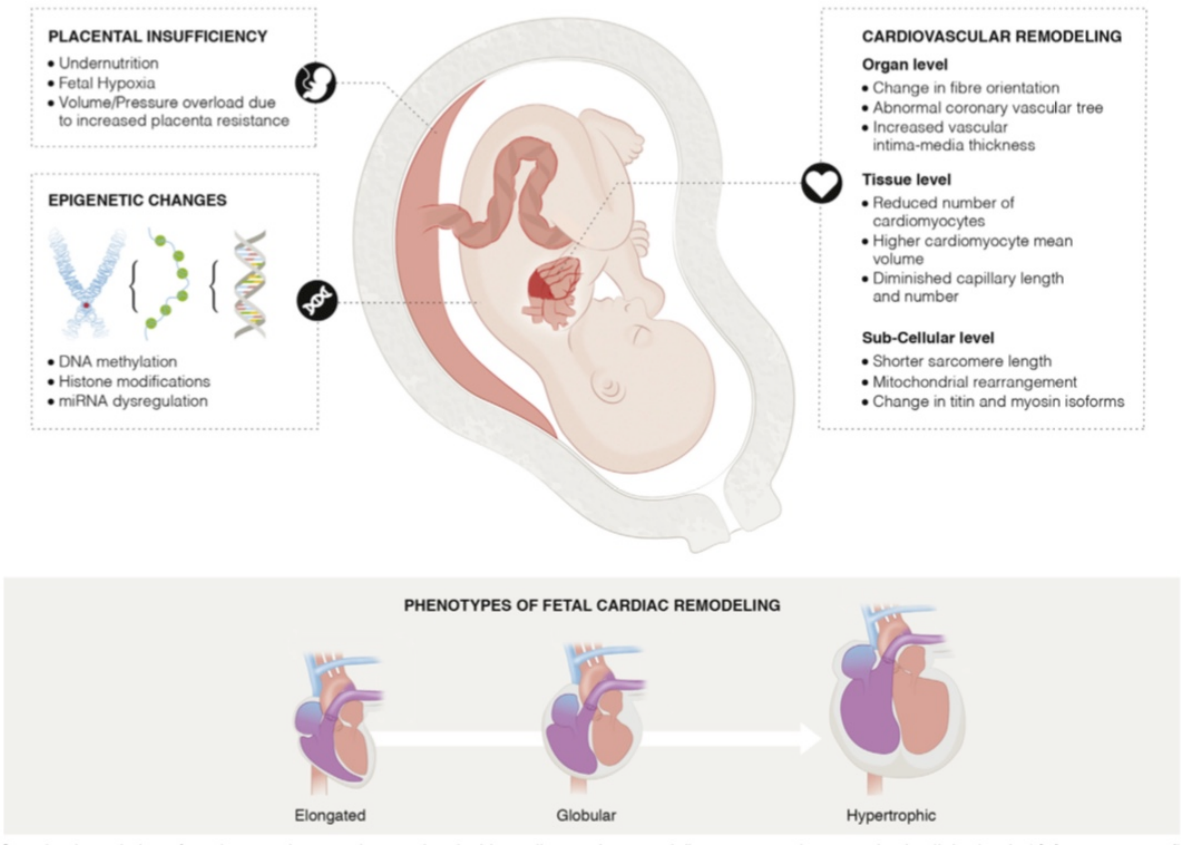



**Figure 2.** Sustained restriction of nutrients and oxygen is associated with cardiovascular remodeling at organ, tissue, and subcellular levels (right upper panel) and with epigenetic changes (mid left panel). Different fetal cardiac phenotypes elongated, globular and hypertrophic (lower panel) may be observed by cardiac imaging, depending on the severity/duration of the insult. Other abnormalities not included in this figure (such as hypertension, endothelial dysfunction, and insulin resistance) can operate simultaneously.

Source: Crispi F, Miranda J, Gratacós E. Long-term cardiovascular consequences of fetal growth restriction: biology, clinical implications, and opportunities for prevention of adult disease. Am Obstet Gynecol. 2018 Feb;218(2S):S869-S879. doi: 10.1016/j.ajog.2017.12.012. PMID: 29422215.

**Fetal programming on brain**

Brain sparing results from the chronically hypoxic fetal environment imposed by placental insufficiency [43]. This hemodynamic adaptation is build up to protect the brain with increased

oxygen supply, and is followed by decompensation. Brain sparing is therefore considered a useful indicator of the degree of compromise in growth-restricted fetuses [44]. However, brain sparing does not ensure normal brain development. Indeed it is clear that growth-restricted infants present a complex and distinct set of microstructural brain abnormalities not observed in their appropriately grown counterparts. In human FGR, decreased total brain volume is first apparent in utero, along with reduced total cell number, and a specific and independent vulnerability of the cortical grey matter to volume loss. Recent imaging advances have demonstrated the impairment of the connectivity networks, such as the long range cortico-basal ganglia thalamic tracts. Consequently, neurological impairments may be immediately evident in the newborn with altered attention and alertness and in school age children with gross and fine motor deficits, and specific learning disabilities encompassing cognitive, memory, and academic performance. Additionally, neurobehavioral dysfunctions have been also widely described in FGR children, including poor attention, hyperactivity and irritability. Intuitively, the more severe the fetal growth restriction, the most pronounced the neuropathology. Animal studies, where fetal growth restriction was artificially reproduced, showed a region specific loss of neuronal cells, deficits in white matter organization, axonal injury and a reduced structural integrity of the neurovascular unit, which may in turn cause an altered susceptibility to intraventricular hemorrhage [45].

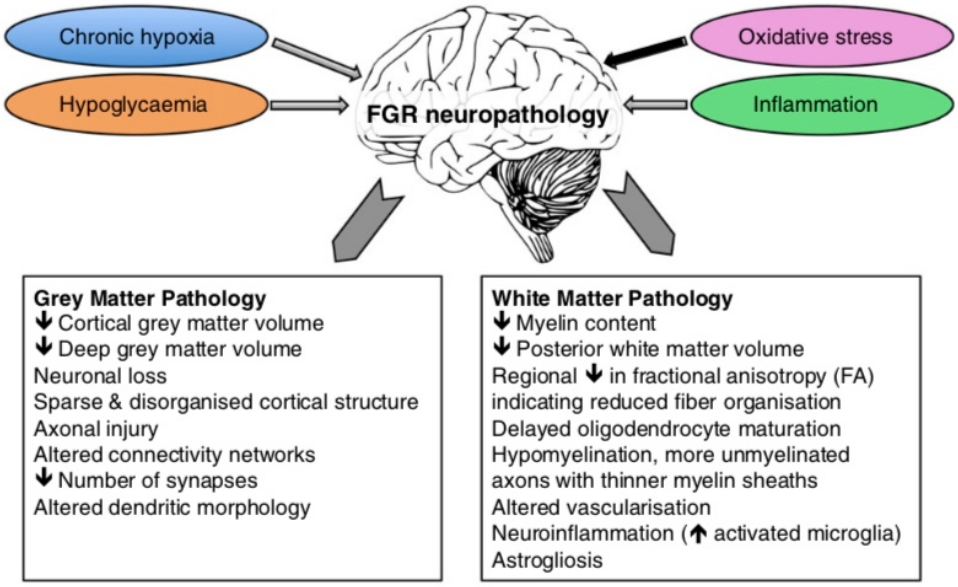



**Figure 3.** A summary of human and animal experimental results showing the principal adverse mechanisms contributing to grey matter and white matter pathology in FGR. Chronic fetal hypoxia, hypoglycemia, oxidative stress and inflammation are the likely causes of adverse neurodevelopment. Gross changes in brain volume are observed in both grey and white matter of the FGR brain, contributed by cell loss and sparsity of neuropil layers with altered axons, dendrites, synapses and myelination.

Source: Miller SL, Huppi PS, Mallard C. The consequences of fetal growth restriction on brain structure and neurodevelopmental outcome. J Physiol. 2016 Feb 15;594(4):807-23. doi: 10.1113/JP271402. Epub 2016 Jan 5. PMID: 26607046; PMCID: PMC4753264.

**Fast food, junk food, ultraprocessed foods.**

Although the main focus of the field of "developmental origins of adult health and disease" has been on the effects of poor fetal nutrition, the issue of maternal and hence fetal overnutrition is of growing importance in the context of the current global obesity epidemic (46). Over the last hundred years the human diet has shifted from a traditional pattern high in cereal and fiber towards a more western pattern high in sugars, fat, animal-source food. This transition is associated with two other remarkable epidemiological phenomena. The first of these is the demographic evolution from a condition of high fertility and high mortality to one of low fertility and low mortality, which

is typical of modern industrialized countries. The second historic course of change is conceptualized by the shift from a state of prevalent infectious diseases as a consequence of periodic famine and poor environmental sanitation to a context of prevalent chronic and degenerative diseases associated with urban-industrial lifestyles.

Throughout the 20th century, the development of technologies has been enhanced by the advances in science and engineering. Modern food processes vary greatly in the degree of complexity of the technologies employed. At the simplest level, food processing may involve no more than controlled storage such as refrigeration. At more complex levels, commodities may be processed to yield ingredients which are later combined to create foodstuffs such as frozen baked products, or chilled ready meals [47,48]. From a health perspective, more than 1/3 of the worldwide dietary guidelines advise to avoid fast foods [49], and herein fast food, industrialized food and junk food tend to be used interchangeably. Fast food is a type of mass-produced food designed for commercial resale, with a strong priority placed on speed of service. Over the last couple of generations, modern society has progressively incorporated fast food into people daily lives [50]. It is important to note that "fast" food does not always just refer to fast food restaurants. Convenience foods are available in corner stores, convenience markets, gas stations, and grocery stores just to name a few. Trends over time in food expenditures have demonstrated that expenditures "away from home" are rapidly rising to the detriment of food expenditures "at home". This means that people generally tend to spend more money outside of the home on the foods they eat than in the past. According to a definition recently provided by the World Health Organization (WHO), junk foods are high energy, low in nutrient content and/or high in fat snack foods that contain added sugar (i.e. sugary biscuits, cream-filled sponge cakes, candy, fizzy drinks) or have high salt content (i.e. fried potato crisps) [51]. This definition may fit well for a large number of foods, whose composition is often determined by industrial processing, such as packaged snack foods or many fast foods. However, it might also include those foods that are naturally rich in fats and can present a high salt content for their processing or storage (e.g. smoked salmon, caviar or anchovies). Ultra-processed foods (UPFs) are industrial formulations of processed food substances (oils, fats, sugars, starch, protein isolates) that contain little or no whole food and typically include flavorings, colorings, emulsifiers and other cosmetic additives. Processes and ingredients used for the manufacture of UPFs are designed to create low-cost, extremely palatable and convenient to use products [52]. Ultra-processed foods already make up more than half of the total dietary energy consumed in high-income countries such as the USA [53] and the UK [54] and between one-fifth

and one-third of total dietary energy in middle-income countries such as Brazil [55] and Mexico [56]. Of note, the average growth in sales of these products amounts to about 1 % per year in high-income countries and up to 10 % per year in middle-income countries [57]. Recently a narrative review showed that a high dietary intake of UPFs is associated with a range of adverse health outcomes and non-communicable diseases, thereby bearing the potential to significantly influence the global burden of disease. Moreover, there is evidence to support a strong association between high consumption of UPFs and a higher risk of all-cause mortality [58].

**Optimum maternal nutrition in pregnancy is of paramount importance**

From an obstetrical point of view, obesity and diabetes mellitus (DM) in the mother have been linked with large for gestational age (LGA) neonates. The pulsatile postprandial hyperglycemia, which typically occurs in these conditions, promotes fetal insulin secretion, that in turns leads to excess glycogen storage and fat accretion in the fetus, especially in pregnancies affected by diabetes and obesity concomitantly. Moreover, some researchers have demonstrated that the fetus increases fat mass when persistently exposed to high levels of glucose and lipids present in the maternal circulation [59,60]. Excess fat mass accreted in utero might contribute to later obesity, but postnatal fat mass accretion especially during the first 2 years of life can persist into later life leading to obesity in childhood. In a nonhuman primate model, a maternal Western-style diet resulting in intermittently higher postprandial glucose and lipid exposure to the fetus resulted in the 3-year-old offspring demonstrating higher glucose excursions. Furthermore, the child's pancreatic islets secreted more insulin, suggesting that these islets were primed before birth to hyper-secrete insulin [61]. In contrast, when the fetus is exposed to extremely high glucose concentrations such is in poorly controlled type 1 DM, insulin production may be suppressed, thus leading to fetal smallness. After birth this individual would also be at increased risk of later metabolic disease, especially when exposed to an obesogenic environment. In addition, maternal overnutrition plays an important role in the early origins of nonalcoholic fatty liver disease (NAFLD), the most common liver disease worldwide affecting 1 in 3 youth with obesity [62]. Finally, there is increasing evidence that persistent, very high fetal glucose concentrations can inhibit fetal neuronal development leading to reduced cognitive function in such offspring later in their lives [63].

**Section II**

**EXPERIMENTAL STUDIES**

**PART I**

# Nutrition in pregnancy: data from a prospective observational study in a pregnant population in the North-East Italy (epidemiological study)

**Part I at a Glance:**

1) Why was this study conducted? This study was conducted with the aim to evaluate the prevalent food consumption pattern and its relationship with the fetal growth at birth among a cohort of pregnant women referring to the university hospital of Udine for pregnancy follow up and delivery.
2) What are the key findings? This prospective observational study showed that most women had a high fat diet, exceeding the upper limit for the intake of fat of nearly 10 percentage points and almost doubling the upper limit recommended for saturated fat. Moreover, this study demonstrates that mothers of SGA neonates, when compared with controls, had a significantly increased fat intake, especially in saturated fat, and a significantly higher intake of alcohol.
3) What does this study add to what is already known? The high-fat diet during pregnancy among women in a geographical area in the North East Italy suggests that current nutritional guidelines lack efficiency. Social aspects, cultural factors and consolidated behaviors are important elements that strongly influence the population food consumption pattern. Therefore, adapting the guidelines to the social and cultural context should be encouraged to increase the people adherence to nutritional recommendations.

## Part I - Summary


### Background

An optimum nutrition during gestation will contribute to optimal fetal growth, favorable obstetrical outcomes and the potential for better long-term health in offspring. However, most women of reproductive age worldwide do not adhere to the dietary guidelines for pregnancy, since food consumption pattern is strongly influenced by social aspects, cultural factors and consolidate behaviors.

### Objectives

The main objective of this study was to determine the prevalence of high-fat diet in a cohort of pregnant women referring to the university hospital of Udine for pregnancy follow-up and delivery. Secondly, we aimed at evaluating the prevalence of maternal high carbohydrates diet, the association between a maternal diet high in fat and carbohydrates and fetal growth.

### Study design

This study was a prospective single-center cohort investigation conducted from May 2021 to August 2022. Pregnant women aged 18 years or older, with singleton pregnancy, alive fetus at the time of ultrasonography and fluency in Italian language were included, whereas women affected by fetal anomalies, diabetes, hypertensive disorders, major psychiatric disorders or with multiple gestation were excluded. Fetal growth was established according to Hadlock charts. The diet assessment was performed through a food frequency questionnaire, and the median energy intake of nutrients was calculated using CIQUAL database. A univariate and multivariate logistic regression analysis were performed in order to evaluate the relationship between the primary outcome and the fetal growth.

### Results

From a cohort of 242 eligible pregnancies, after exclusion of 49 women for gestational diabetes and 7 women for dropping out from the follow-up, we obtained a sample of 186 women included in the final analysis. We found that 95.7% of women enrolled had a high fat diet and no women had a high-carbohydrates diet. The univariate logistic regression analysis showed that compared to controls mothers of SGA neonates had a significantly higher fat diet (OR 1.09; IC 1.02 - 1.17), high saturated fat diet (OR 1.15; IC 1.03 - 1.29) and a significantly higher intake of alcohol (OR

2.52 gr; IC 1.33 - 4.76). These findings were confirmed by the multivariate logistic regression analysis, after adjusting for maternal age, parity and fetal gender.

**Conclusions**

This study shows that pregnant women in this region tend to have a worryingly high fat diet, especially high in saturated fat, which appears to be strongly related with fetal smallness at birth. Fetal smallness was also strongly associated with a higher alcohol intake. Further studies using more reliable methods to assess the population's diet are needed in order to guide people towards healthier food choices, that have long term consequences in offspring.

## Introduction

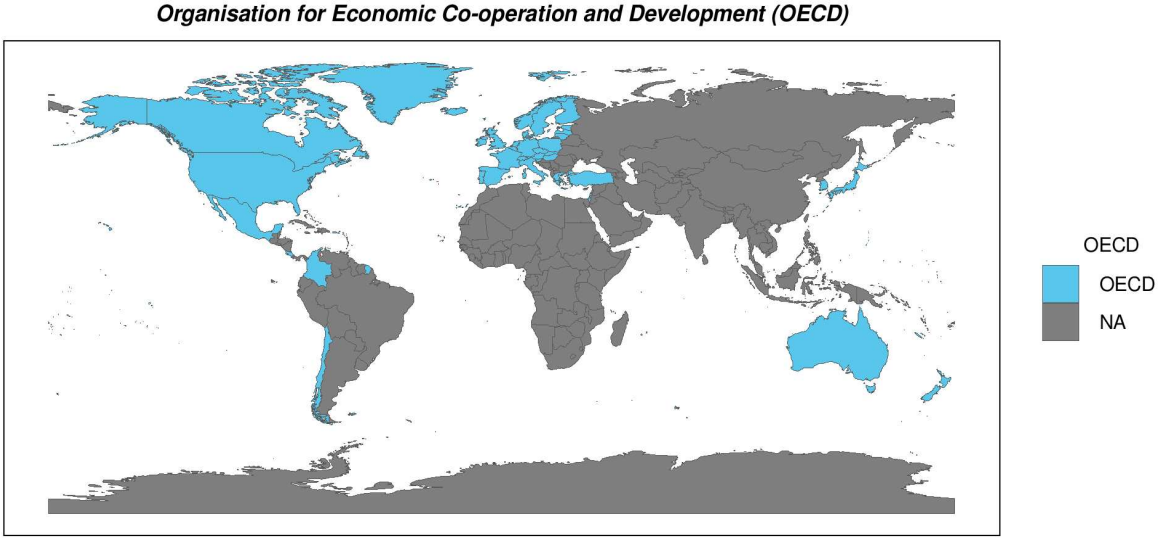
Italy ranks 25th (out of 37 OECD member countries in 2017) in the prevalence of overweight and obesity according to official statistics, with a worryingly high prevalence in childhood (Figure 1). Poor adherence to the Mediterranean diet by the Italian population, as well as a high proportion of sedentary lifestyles, may be among the main factors explaining the increase in obesity prevalence in Italy [64]. Recently, it has been widely demonstrated that obesity and many non-communicable diseases in adulthood have their origin already in periconception. The concept of the "first 1000 days" states that the period from conception to 2 years of age is considered the most critical for the induction of those pathophysiological disorders that eventually lead to obesity in childhood and later in adulthood [65]. Any intervention to reduce the risk of such imprinting should therefore focus on this specific period of early life. From this perspective, interventions to improve maternal, fetal and child growth are effective in improving adult outcomes. Therefore, the World Health Organization (WHO) states that nutrition, including maternal nutrition, is the highest priority for global public health [66]. It is estimated that optimal nutrition during pregnancy will lay the foundations for eliminating all forms of malnutrition, including premature deaths from non-communicable diseases, by 2030. However, most women of childbearing age do not adhere to the nutrition guidelines for pregnancy, according to a recent systematic review of international observational studies [67].

According to the FAO [68], the dietary guidelines are intended to provide a basis for public nutrition to promote healthy eating habits. However, it is now well documented that adherence to the Dietary Guidelines can be strongly influenced by social aspects, cultural factors and entrenched behaviors at the population level. The starting point for a shift to a health-promoting and sustainable diet should therefore be the assessment of dietary intake in a population-based mother-child cohort.

## Objective

The main objective of this study was to determine the prevalence of a high-fat diet in a cohort of pregnant women attending the University Hospital of Udine for antenatal care and delivery. Secondly, to investigate the prevalence of a high-carbohydrate maternal diet, the association between a high-fat and high-carbohydrate maternal diet and fetal growth.

*Figure 1- The OECD member countries.*




## Materials and methods

### Study design and setting

This study was a prospective cohort study conducted from May 2021 to August 2022 in the Department of Obstetrics and Gynecology of the University Hospital of Udine. This center is a tertiary level maternity hospital with advanced medical facilities for mother and newborn. The regional review board and the clinical research center of the hospital approved the present study (protocol number: asufc/2021/0044374), which complied with the requirements of the general authorization of the Italian Data Protection Authority for the processing of data for scientific research purposes. Furthermore, all ethical principles of the Declaration of Helsinki [69] were respected.

### Participants of the study

Women who attended routine ultrasound examinations at various times during pregnancy were recruited for this prospective observational study. Women were recruited in an outpatient facility during obstetrics visits, unaware of growth scan results but usually with anomaly scans available. Inclusion criteria were an age of at least 18 years, a singleton spontaneous pregnancy, a live fetus

at the time of the ultrasound scan and proficiency in Italian. Exclusion criteria were fetal abnormalities (including chromosomal abnormalities and structural malformations) detected antenatally, maternal diabetes, maternal hypertension, multiple pregnancies and severe maternal psychiatric illness.

**Variables considered**

Diet was assessed using a food frequency questionnaire (FFQ) previously validated for Italian adults through a three-day dietary protocol [70]. The medical staff, adequately trained on the study methodology, accurately explained to recruited patients how to fill out the questionnaire. Specifically, participants were asked about their consumption of 36 different foods based on frequently consumed foods. Participants were also asked to indicate their usual frequency of consumption, choosing from seven frequency categories ranging from "never" or "less than once a week" to "seven times a week". The foods were divided into the following categories: Drinks, Milk and Dairy Products, Meat, Fish and Eggs, Cereals, Vegetables, Pulses and Fruit, Fatty Sauces and Other (Sweets, Fried Foods and Fast Food). For coffee, alcoholic and soft drinks, the amount in cups or glasses was also reported. For each item of the questionnaire, a corresponding reference food was given, which was taken into account when calculating the energy and nutrient content using the CIQUAL database (https://ciqual.anses.fr/ accessed on February 2022).

Fetal growth was determined according to the Hadlock fetal weight growth charts [71]. Three different categories of fetal growth at birth were identified: (i) newborns of appropriate weight for gestational age (AGA); (ii) newborns who are small for gestational age (SGA, with a birth weight below the 10th centile); (iii) newborns who are large for gestational age (LGA, with a birth weight above the 90th centile) [72]. Recruitment and data collection took place only after women had been informed and had given written informed consent to participate. Residents and obstetricians involved in this study collected data on maternal characteristics, medical history, pregnancy complications, mode of delivery and neonatal health.

**Definition of outcomes**

A high-fat diet was defined when fat intake exceeded the 35% kcal limit of total energy, while a high carbohydrate intake was identified when carbohydrates accounted for more than 65% kcal of total energy [73].

**Sample size assessment and data analysis**

Assuming that the prevalence of a high-fat diet in this pregnant population is 14% (based on high BMI prevalence in the local population), the sample of pregnant women to be studied is 185 [74, 75]. With this sample size, the prevalence of high-fat diet could be estimated within five percentage points on either side of the estimated prevalence with a 95% confidence interval. However, given the likelihood of some women developing gestational diabetes or discontinuing pregnancy follow-up, a larger sample should be available to include 30 % more women, giving a total number of 241 women who could be included.

The statistical analyses were performed using R (version 4.2.1). The CIQUAL database was used to calculate the fat and carbohydrate content of the daily food intake. The normality of continuous variables was assessed employing the Kolmogorov-Smirnov test. Continuous parametric variables were presented using mean (± standard deviation) and non-parametric once using median and interquartile range (IQR). The logistic regression results are presented using odds ratio (OR) and 95% confidence interval (CI.95). The following tests were utilized for the analysis as appropriate: t-test, Wilcoxon test, chi-square test, or Fisher exact test. Univariate and, after adjusting for factors associated with the primary outcome, multivariate logistic regression analysis were performed to assess the relationship between the exposures and fetal growth. In the logistic regression models, the dependent variable was considered the fetal growth (i.e., SGA). The independent variables were the main exposure (a high-fat diet) and other diet-related variables. In the multivariate analysis also, possible confounders known from the literature were included.

**Results**

A total of 250 questionnaires were completed from May 2021 to August 2022. After excluding 2 multiple pregnancies and 6 cases of pre-gestational type 1 diabetes, 242 pregnancies were eligible (Figure 2). Subsequently, 49 women developed gestational diabetes and 7 women dropped out of the pregnancy follow-up, leaving the final cohort of 186 pregnant women for the final statistical analysis. The mean gestational age at the administration time of the food frequency questionnaire was 26.49 weeks of gestation (±5.04). The median maternal age was 33 years with an interquartile range (IQR) of 29 to 36 years. More than half of the women (56.25%) were nulliparous, and pre-pregnancy body mass index (BMI) was 22 (IQR 20-24). In addition, most of the women were from Italy (89.2%) (Table 1, Figure 3 and 4). Maternal complications during pregnancy were relatively rare in this cohort, with gestational hypertension and hyperthyroidism each accounting for 0.54%.

Gestational hypertension, pre-eclampsia and HELLP syndrome were not reported, while cholestasis and hypothyroidism were more common, with the prevalence of 4.84% and 7.53%, respectively.

The median gestational age at delivery was 39 weeks (IQR 38-40). In terms of fetal growth, 31 SGA with a fetal growth below 10th percentiles (16.67%), 147 AGA (79.03%), 8 LGA above the 90th percentile (4.30%). The median cord blood pH was 7.27 (IQR 7.22 - 7.32) and the median base excess was -3 (IQR -1 - -3), Apgar score at first and fifth minutes was 9 each (IQR 8-9 and 9-10 respectively). Neonates were admitted to the intensive care unit (ICU) in 3.76% of cases and neonatal resuscitation was performed in 16/176 cases (9.09%).

The median energy intake in our cohort was 14.42% of kcal from carbohydrates (IQR 11.42-18.13), 23.02 % from proteins (IQR 20.38 - 26.66), 44.88 % kcal from fat (IQR 41.49 - 47.6) and 18.87% (IQR 16.93-21.13) from saturated fats. Median water intake was 505.49 g (+ 154.05), median fiber intake was 13.51 (+ 5.79), while alcohol intake was minimal with a median of 0% and IQR of 0-0.01 (Table 2).

The primary endpoint of this study was to determine the prevalence of a high-fat diet in the cohort of pregnant women who participated, and we found that most women (95.7%) had a high-fat diet. In contrast, there were no women with a high-carbohydrates diet in this cohort.

Univariate logistic regression analysis showed that mothers of SGA newborns had a significantly higher fat diet (OR 1.09; IC.95 1.02 - 1.17), a diet high in saturated fat (OR 1.15; IC.95 1.03 - 1.29) and significantly higher alcohol consumption (OR 2.52 gr; IC.95 1.33 - 4.76) compared to controls. These results were confirmed by multivariate logistic regression analysis after accounting for maternal age, parity and fetal sex (Table 3).

*Figure 2- Population flowchart. Fetal growth was assessed according the antenatal standards (Hadlock growth charts).*

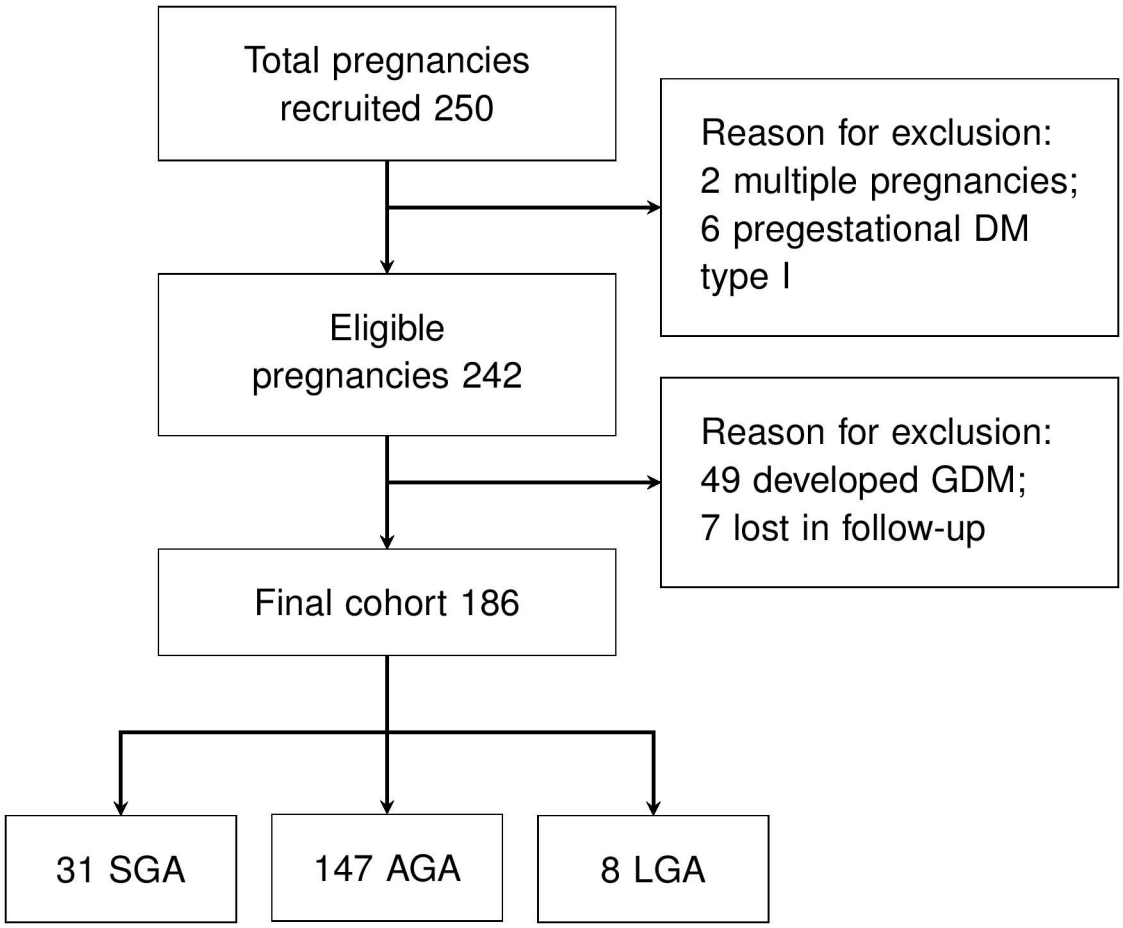


*Figure 3- Map of countries/regions from which women included in the study come.*

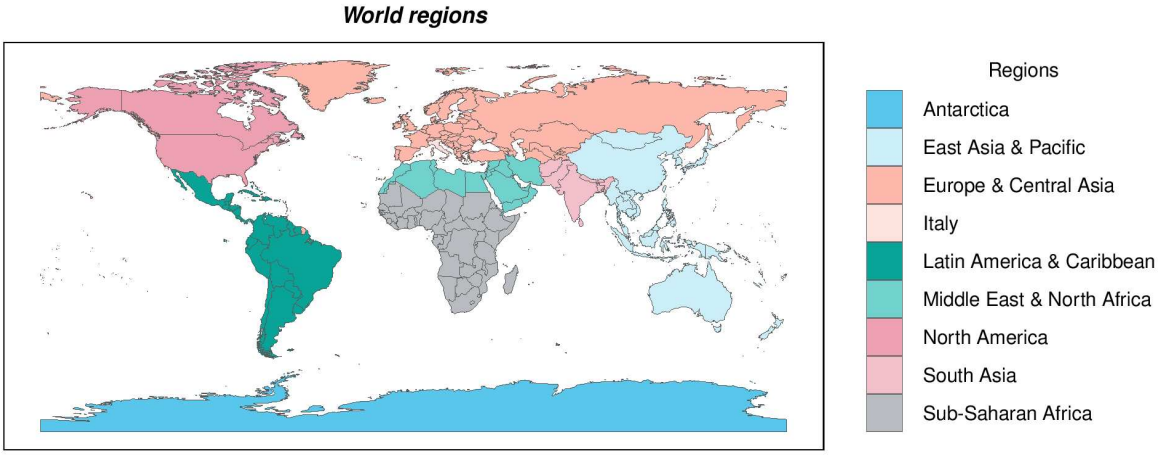



*Figure 4- Frequencies regarding the geographical region of origin of the included women*

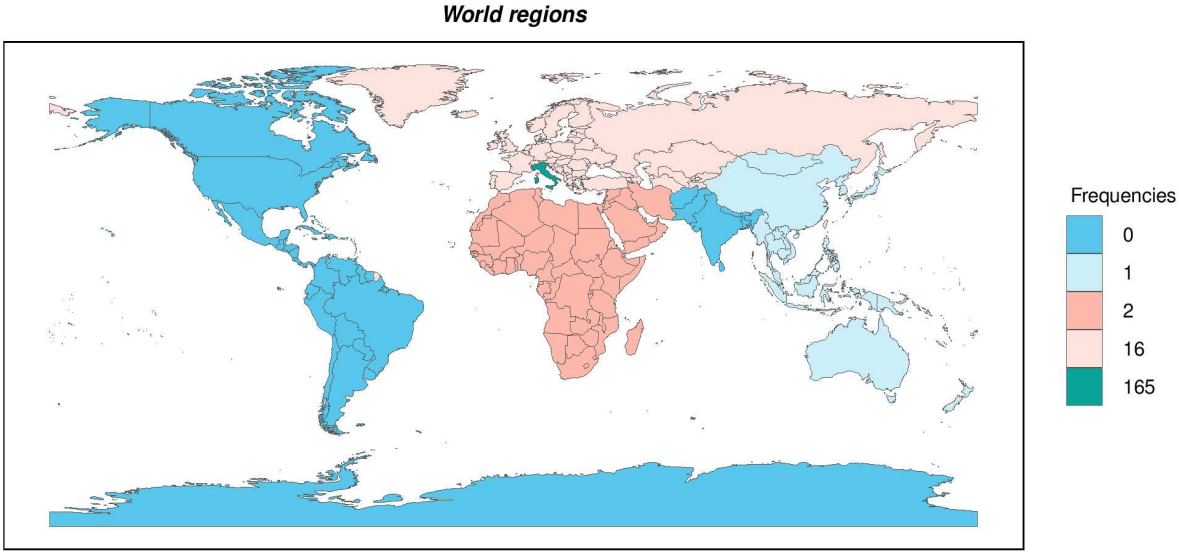


*Table 1- Population characteristics.*

| | |
|---|---|
| Maternal characteristics | |
| Maternal age (years) | 33 (29-36) |
| Nulliparity | 55.38% (103/186) |
| Pre-pregnancy BMI (kg/m²) | 22 (20-24) |
| Region of origin | |
| Italy | 88.71% (165/186) |
| Europe and Central Asia | 8.6% (16/186) |
| Middle East and North Africa | 1.08% (2/186) |
| Sub-Saharan Africa | 1.08% (2/186) |
| East Asia and Pacific | 0.54% (1/186) |
| Latin America and Caribbean | 0% (0/186) |
| South Asia | 0% (0/186) |
| Maternal outcomes | |
| Pregestational Hypertension | 0.54% (1/186) |
| Gestational hypertension | 0% (0/186) |
| Preeclampsia | 0% (0/186) |
| HELLP syndrome | 0% (0/186) |
| Cholestasis | 4.84% (9/186) |
| Hypothyroidism | 7.53% (14/186) |
| Hyperthyroidism | 0.54% (1/186) |
| Neonatal characteristics | |
| Fetal sex | |
| M | 46.77% (87/186) |
| F | 53.23% (99/186) |

| | |
|---|---|
| Gestational age (weeks) | 39 (38-40) |
| Neonatal weight (g) | 3323.89 (±454.91) |
| SGA <10th centile (*) | 16.67% (31/186) |
| LGA >90th centile (*) | 4.3% (8/186) |
| Apgar score 1st minute | 9 (8-9) |
| Apgar score 5th minute | 9 (9-9) |
| Cord blood pH | 7.27 (7.22-7.32) |
| Base excess | -3 (-1- -5) |
| NICU hospitalization | 4.3% (8/186) |
| Neonatal resucitation | 9.14% (17/186) |

(*) Based on Hadlock antenatal standards.

*Table 2- Maternal nutrient intake.*

| | |
|---|---|
| Sugar(% kcal) | 14.42 (11.42-18.13) |
| Protein (% kcal) | 23.02 (20.38-26.66) |
| Fat (% kcal) | 44.88 (41.49-47.6) |
| Fat saturated (% kcal) | 18.87 (16.93-21.13) |
| Water (g) | 505.49 (±154.05) |
| Fiber total (g) | 13.51 (±5.79) |
| Ash (g) | 7.56 (±2.31) |
| Alcohol (g) | 0 (0-0.01) |
| High fat diet (>35% kcal) | 95.7% (178/186) |
| High sugar diet (>65% kcal) | 0% (0/186) |
| Organic acids (g) | 0.05 (0-0.13) |
| Fatty acids total saturated (g) | 18.02 (13.93-22.98) |
| Fatty acids total monounsaturated (g) | 16.79 (12.85-20.88) |
| Fatty acids total polyunsaturated (g) | 3.88 (2.91-4.95) |
| FA4:0 (g) | 0.32 (0.21-0.45) |
| FA6:0 (g) | 0.23 (0.16-0.33) |
| FA8:0 (g) | 0.19 (0.13-0.27) |
| FA10:0 (g) | 0.42 (0.29-0.57) |
| FA12:0 (g) | 0.5 (0.33-0.71) |
| FA14:0 (g) | 1.38 (0.91-1.91) |
| FA16:0 (g) | 6.94 (5.14-8.84) |
| FA18:0 (g) | 2.93 (2.22-4.04) |
| FA18:1n-9cis (g) | 11.45 (8.6-15.27) |
| FA18:29c, 12c (n-6) (g) | 2.33 (1.77-3.09) |

| | |
|---|---|
| FA18:3c9, c12, c15 (n-3) (g) | 0.23 (0.19-0.29) |
| FA20:45c, 8c, 11c, 14c (n-6) (g) | 0.04 (0.03-0.05) |
| FA20:55c, 8c, 11c, 14c, 17c (n-3)EPA (g) | 0.06 (0.03-0.11) |
| FA22:64c, 7c, 10c, 13c, 16c, 19c (n-3)DHA (g) | 0.12 (0.07-0.23) |
| Cholesterol (mg) | 167.47 (123.29-209.63) |
| Salt (g) | 2.86 (2.26-3.48) |

*Table 3- Maternal diet and SGA <10th centile fetuses. Logistic regression analysis (dependent variable SGA <10th centile). The multivariate model was adjusted for neonatal sex, parity, maternal age, and pre-pregnancy BMI.*

| | OR (CI.95) | p | OR (CI.95)(*) | p(*) |
|---|---|---|---|---|
| Sugar(% kcal) | 0.99 (0.91 - 1.07) | 0.827 | 0.98 (0.9 - 1.07) | 0.677 |
| Protein (% kcal) | 0.95 (0.87 - 1.04) | 0.245 | 0.95 (0.87 - 1.04) | 0.307 |
| Fat (% kcal) | 1.09 (1.02 - 1.17) | <0.05 | 1.08 (1.01 - 1.17) | <0.05 |
| Fat saturated (% kcal) | 1.15 (1.03 - 1.29) | <0.05 | 1.13 (1 - 1.28) | <0.05 |
| Alcohol (g) | 2.52 (1.33 - 4.76) | <0.05 | 2.57 (1.32 - 5.03) | <0.05 |
| Fatty acids total saturated (g) | 1.03 (0.98 - 1.08) | 0.271 | 1.03 (0.97 - 1.09) | 0.356 |
| Fatty acids total monounsaturated (g) | 1.02 (0.96 - 1.08) | 0.485 | 1.02 (0.95 - 1.09) | 0.593 |
| Fatty acids total polyunsaturated (g) | 1.06 (0.87 - 1.29) | 0.557 | 1.04 (0.84 - 1.29) | 0.734 |
| FA4:0 (g) | 3.29 (0.55 - 19.69) | 0.193 | 3.3 (0.51 - 21.46) | 0.210 |
| FA6:0 (g) | 7.83 (0.66 - 92.7) | 0.103 | 9.09 (0.66 - 124.55) | 0.099 |
| FA8:0 (g) | 14.88 (0.49 - 447.37) | 0.120 | 18.6 (0.5 - 687.92) | 0.112 |
| FA10:0 (g) | 2.25 (0.58 - 8.66) | 0.238 | 2.39 (0.57 - 9.98) | 0.234 |
| FA12:0 (g) | 2.85 (0.89 - 9.16) | 0.079 | 2.95 (0.85 - 10.25) | 0.089 |
| FA14:0 (g) | 1.42 (0.88 - 2.29) | 0.155 | 1.42 (0.86 - 2.36) | 0.171 |
| FA16:0 (g) | 1.1 (0.97 - 1.25) | 0.133 | 1.1 (0.96 - 1.26) | 0.162 |
| FA18:0 (g) | 1.2 (0.94 - 1.55) | 0.145 | 1.23 (0.93 - 1.62) | 0.151 |
| FA18:1n-9cis (g) | 1.03 (0.96 - 1.11) | 0.438 | 1.03 (0.95 - 1.11) | 0.500 |
| FA18:29c, 12c (n-6) (g) | 1.1 (0.87 - 1.37) | 0.427 | 1.08 (0.84 - 1.38) | 0.562 |
| FA18:3c9, c12, c15 (n-3) (g) | 6.7 (0.14 - 326.3) | 0.338 | 2.92 (0.04 - 219.15) | 0.626 |

| | | | | |
|---|---|---|---|---|
| FA20:55c, 8c, 11c, 14c, 17c (n-3)EPA (g) | 0.01 (0 - 30.23) | 0.237 | 0.01 (0 - 38.6) | 0.248 |
| FA22:64c, 7c, 10c, 13c, 16c, 19c (n-3)DHA (g) | 0.07 (0 - 5.49) | 0.230 | 0.07 (0 - 6.46) | 0.248 |

## Comment

### Main results

This prospective observational study, conducted on a sample of pregnant women attending the University Hospital of Udine for antenatal care, showed that most of the recruited women consumed a high-fat diet that exceeded the upper limit for fat intake by almost 10 percentage points and almost doubled the recommended upper limit for saturated fat. In addition, this study shows that mothers of SGA newborns had significantly higher fat intake, especially saturated fat, and alcohol consumption compared to controls.

### Results in the context of what is known

As governments grapple with the increasing social and economic consequences of an alarming rise in non-communicable diseases, particularly obesity and its comorbidities, dietary recommendations in each country are evidence-based interventions to improve the quality, safety and sustainability of the population's diet [66]. Italian dietary recommendations, like those of other public health organizations, focus on reducing dietary fat intake, in particular reducing total fat consumption to 30% of total energy intake and, more importantly, reducing saturated fat consumption to 10% of total energy intake [76].

Saturated fatty acids are fully hydrogenated fatty acids that have a linear chain with no double bonds between the carbon atoms. The most abundant saturated fatty acids in the diet are lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0) and stearic acid (18:0), which are in a solid state at room temperature (76). In the complete list of food groups (meat, fish, eggs, dairy products, vegetables, fruits, nuts, seeds, legumes and cereals), dairy products are the only food group that contains more saturated than unsaturated fats. Meat, fish, eggs, nuts, seeds and even lard all contain more unsaturated than saturated fats. According to the literature, a high intake of saturated fat is positively associated with metabolic syndrome [77], and a reduction in dietary saturated fat lowers the number of cardiovascular events [78]. The role of saturated fat in pregnancy has been questioned, particularly in animal studies. Overall, these studies have demonstrated that the offspring of mothers fed a diet rich in saturated fat during pregnancy and/or lactation have alterations in the biological mechanisms that lead to the development of insulin resistance, obesity and cardiovascular disease [79-81]. The observation that the women in our study had significantly high fat intakes, especially of saturated fat, suggests that in this region there is little adherence to

dietary recommendations even during pregnancy, which usually makes women more likely to change their habits to healthier diets. Several explanations can be put forward to clarify the reasons for this observed trend. First of all, the food culture that is embedded in the core of a region's population has a great influence on food choices, and it is difficult to change. For example, some interesting surveys have been published showing that meat is a food with strong cultural resonance, and this cultural resonance is a barrier to reducing meat consumption [83,83]. We believe this may also be the case for the individuals recruited in this study, as saturated fats from animal sources are a typical feature of the food culture in this geographical region. Secondly, a shift towards a westernized dietary pattern is currently taking place all over the world, due to the increasing consumption of ultra-highly processed foods (UPFs) worldwide [84]. UPFs are industrially produced, processed foods that are usually inexpensive, highly palatable and convenient to use. Recently, it has been widely demonstrated that high intake of UPFs is closely associated with high levels of saturated fat. Thus, a strong cultural resonance influencing food choices and the increasing westernization of food consumption may be among the main factors explaining the high-fat diet in this cohort of pregnant women.

Fetal growth is largely dependent on the mother's ability to nourish her fetus. The provision of this nourishment depends on the ability of the placenta to provide nutrients and on maternal nutrition. Low birth weight is not only an indicator of fetal health, but also of adult health. Recent studies have demonstrated that fetal growth restriction and small for gestational age babies have both a suboptimal neurodevelopment and an increased cardiovascular risk, suggesting that SGA fetuses, rather than being a group of normal smaller fetuses, are a milder form of fetal growth restriction [85]. Our study found an association between fetal smallness and high fat and saturated fat diet in a cohort of women who delivered at term of gestation, were in good health and with a normal pre-pregnancy BMI. It could be speculated that the high levels of saturated fat in the diet of the pregnant women who participated in this study may have altered the uterine environment, leading to fetal programming. This phenomenon refers to the fact that stimuli applied during early development induce permanent changes that persist throughout life [86].

Interestingly, our study found that fetal smallness at birth was strongly correlated with alcohol consumption in the maternal diet. Ethanol crosses the placenta and enters the fetal circulation. Although ethanol administered to pregnant women in the second trimester has been shown to result in a 60% lower concentration in amniotic fluid than in maternal blood, the clearance of ethanol in amniotic fluid was slower than the clearance in maternal blood [87]. Therefore, fetal exposure to

ethanol can persist for long periods of time, long enough to cause damage to developing fetal structures. There is ample evidence that alcohol exposure during the prenatal period can lead to devastating consequences. Fetal alcohol syndrome (FAS), the most serious consequence of fetal alcohol exposure, consists of a series of abnormalities in children born to women who drank heavily during pregnancy, including characteristic facial dysmorphologies, growth restriction and central nervous system developmental abnormalities. However, since the initial recognition of FAS, a number of abnormalities attributable to prenatal alcohol exposure have been identified and are referred to as fetal alcohol spectrum disorders [88]. The association between fetal smallness and prenatal alcohol exposure is well established in the literature [88]. Our data appear to be consistent with a recent systematic review and meta-analysis of the effects of low to moderate maternal alcohol consumption (up to 32 g/week), which found that there is some increased risk of babies being born with SGA, but there is little direct evidence of other adverse effects [89-91].

**Clinical implications**

This study provides alarming results on food consumption patterns in a cohort of pregnant women from this region. Therefore, this analysis should be followed by further studies assessing diet with tools other than food frequency questionnaires, such as recall or 3-day dietary records. If these data are confirmed, policy makers and clinicians should cooperate in order to guide people towards healthier diets. This study also suggests that clinicians need to be properly trained in nutrition. Evidence suggests that women want more nutrition advice, consider nutrition in pregnancy important, and view clinicians as the most reliable source of this information [92].

**Implications for research**

Recent data suggest that processed foods account for the majority of saturated fat in the so-called "Western" diet [93]. By reducing the consumption of processed foods, saturated fat intake could easily fall into the range of what is tolerable. This could be possible if health policy organizations promoted the consumption of natural foods and discouraged the consumption of processed foods, biscuits, cakes, pizza, desserts and convenience foods. Further studies to examine food consumption at the national level, with a particular focus on processed foods, are warranted to understand what nutritional gaps exist in this population and to intervene appropriately.

## Strengths and limitations

A major strength of this study was the availability of data from a cohort of pregnant women living in a specific area in north-eastern Italy. This allowed a preliminary assessment of the dietary habits of the population, taking into account the social, economic and cultural context in which people live. However, the results on mean energy intake in our women cohort should be considered with caution. The main limitation of this study is reflected in the food frequency method, as it potentially contains a large number of measurement errors [94]. Quantification of nutrient intake may not be as accurate as with recollections or recordings, even though the FFQ used in this study was previously validated for a population of Italian adults using a three-day dietary protocol. In addition, the FFQ could contain an incomplete list of all possible foods or errors in frequency and usual portion sizes. There could also be a bias related to participants' social desirability, which could lead to overestimation of certain foods and underestimation of other items in certain cases. Furthermore, even though patients followed by a Dietologist during pregnancy were excluded from the study recruitment, enrolled patients might have received dietary indications during pregnancy follow up, thus hampering the study methodology. Last, the assessment of food consumption in our population was performed in the second trimester of pregnancy, but we lack data on the weight gain occurred in the first trimester of pregnancy, which could have had an impact on the study outcomes. The absence of this useful information could be considered another limitation of our study.

## Conclusions

In conclusion, according to this prospective observational study, pregnant women in this region tend to have a worryingly high-fat diet, especially high in saturated fat, which seems to be closely related to fetal smallness at birth. Women's limited knowledge of good nutritional practices, combined with inadequate support from health workers regarding nutritional management during pregnancy, may contribute to the overall prevalence of malnutrition during pregnancy and the potential long-term consequences for mother and child.

**PART II**

**Fetal growth at term and placental oxidative stress in a tissue micro-array model: a histological and immunohistochemistry study (tissue micro-array study)**

**Part II at a Glance:**

a) Why was this study conducted? This prospective study was conducted with the aim to clarify to what extent placental oxidative stress is involved in pathophysiology of fetal smallness at term of gestation.

b) What are the key findings? This study showed a sex specific pattern of 8OH-dG expression in placentae of single term pregnancies, with higher expression of this biomarker among AGA males compared to AGA females. Secondly, a sex difference in the histological pattern among the late FGR placentae, with males exhibiting placental lesions from either maternal and fetal malperfusion, while females displaying only placental lesions from maternal malperfusion was also found. Finally, male late FGR were found to have a significant correlation between oxidative damage in the syncytioblast and thrombi in chorionic plate or villi, whereas female late FGR had a significant correlation between oxidative damage within endothelial and stromal cells and growing birthweight.

c) What does this study add to what is already known? Our data suggest that oxidative stress may not be the only pathway involved in the pathophysiology of fetal smallness at term of gestation. Furthermore, our immunohistochemical and histological findings revealed an unexpected difference in the oxidative stress pattern between male and female placentae, suggesting that placental function and, as a result, fetal growth are differently regulated among the two sexes.

## Part II - Summary


### Background

To what extent placental oxidative stress is involved in pathophysiology of fetal smallness at term of gestation has to be still clarified.

### Objectives

The main objective of this study was to evaluate if there is a difference in 8OH-dG expression in placental tissue samples according to the different patterns of fetal growth at birth. The secondary objective was to evaluate the presence of a potential correlation between placental histology and the expression of 8OH-dG.

### Study design

This was a prospective cohort study including women of at least 18 years, with a singleton pregnancy, live fetus and fluency in Italian. Women enrolled were evaluated about their food consumption pattern and fetal growth was regularly monitored through consecutive ultrasound scans. After delivery, the placenta was sent to the Institute of Pathology for macroscopic and microscopic examination, tissue microarray preparation and for the immunohistochemical analysis.

### Results

A total of 165 women were included in this study: 131 AGA; ii) 10 LGA; iii) 9 SGA > 3° < 10° percentile and iv) 15 late FGR. When restricting the analysis only on AGA and comparing males versus females no significant histological differences were observed between the groups. Yet, compared to females, males had a significantly higher nuclear syncytiotrophoblast staining and a higher intensity score in stromal and endothelial cells staining. The female population consisted of 87 fetuses: 9 late FGR, 2 SGA, 72 AGA and 4 LGA. Smaller fetuses (late FGR and SGA) appeared to have more commonly placental hypoplasia (SGA 50.0% vs. AGA 25.0%) and accelerated villous maturation (FGR 11.11% vs AGA 0.00%). As for syncytiotrophoblast staining the nuclear intensity score was significantly higher in SGA than in AGA (2.75, IQR 2.62-2.88 vs. 2.00, IQR 2.00-2.25), while the percentage of positive nuclei was significantly higher in LGA compared to AGA (56.25, IQR 46.25-69.38 vs. 27.50 IQR 16.25-45.00). The male population accounted for 78 fetuses: 6 late FGR, 7 SGA, 59 AGA and 6 LGA. Compared to AGA, late FGR had more

frequently maternal vascular malperfusion (66.67% vs. 14.29%), accelerated villous maturation (33.3% vs. 0.00%), distal villous hypoplasia (33.3% vs. 0.00%) and avascular villi (33.3% vs. 14.29%). The intensity score of syncytiotrophoblast cytoplasm staining was significantly lower in SGA and LGA than in AGA (0.00, IQR 0.00-0.75 vs. 2.00, IQR 1.00-2.00; and 0.75, IQR 0.12-1.00 vs. 2.00, IQR 1.00-2.00). The intensity score staining in stromal and endothelial cells was higher in AGA than in SGA (3.00, IQR 2.00-3.00 vs. 2.00, IQR 1.00-2.00). Finally, two significant correlations were found: between high intensity 8OH-dG syncytioblast staining and thrombi in chorionic plate or villi among male late FGR, and between high intensity 8OH-dG staining within endothelial and stromal cells and high values of birthweight MoM among females.

**Conclusions**

To conclude, our data suggest that oxidative stress may not be the only pathway involved in the pathophysiology of fetal smallness at term of gestation. Furthermore, our immunohistochemical and histological findings revealed an unexpected difference in the oxidative stress pattern between male and female placentae, suggesting that fetal growth is differently regulated among the two sexes.

## Introduction

Fetal growth restriction (FGR) is one of the most studied topics in feto-maternal medicine since it is strongly burdened by adverse short and long term perinatal outcomes [95]. Fetal growth restriction is defined as the failure of the fetus to achieve its genetically determined growth potential, and it is primarily due to deficient remodeling of the uterine spiral arteries supplying the placenta during pregnancy. The resultant malperfusion induces cell stress within the placental tissues, ultimately leading to a compromised capacity to transfer nutrients to the fetus [96]. Recent studies have observed that not only FGR but also small for gestational age (SGA) fetuses have suboptimal neurodevelopment and an increased cardiovascular risk later in life, suggesting that SGA, rather than being a group of smaller normal fetuses, reflect a milder form of FGR [97,98]. In line with this evidence, some Authors suggested that less severe deficiencies in arterial remodeling result in ongoing pregnancies with differing degrees of fetal compromise, to which different degrees of fetal smallness correspond. At one extreme the FGR with early onset preeclampsia could be found, whereas at the opposite extreme there is SGA, passing through FGR alone and late FGR with late onset preeclampsia [99,96].

Malperfusion of any organ is a powerful inducer of oxidative stress, and the placenta is no exception [100]. Oxidative stress not only has a cytotoxic effect, but also plays an important role in the modulation of messengers that regulate the activity of redox-sensitive transcription factors to maintain metabolic homeostasis. However, when the generation of reactive oxygen species (ROS) overwhelms the cell's capacity to detoxify them, a widespread damage to any cell component occurs. Placental oxidative stress has been linked to complications of pregnancy, such as preeclampsia and FGR [101]. Yet, it is still to clarify to what extent placental oxidative stress is involved in pathophysiology of fetal smallness at term of gestation.

## Objective

The main objective of this study was to evaluate if there is a difference in the expression of 8 hydroxi-20deoxyguanosine (8OH-dG, a biomarker of oxidative stress) in placental tissue samples according to the different patterns of fetal growth at birth [102]. Secondly, we aimed at evaluating the presence of a potential correlation between placental histology and the expression of 8OH-dG.

## Materials and methods

### Study design and setting

This study was a prospective cohort study conducted from May 2021 to August 2022 at the University Hospital of Udine. Women who consented to participate in the study were enrolled at the Clinic of Obstetrics and Gynecology, where they were given a food frequency questionnaire (FFQ) aiming at evaluating their food consumption pattern [70]. Fetal growth was regularly monitored through consecutive ultrasound scans, including the first-trimester screening for chromosomal abnormalities and major defects, the second-trimester screening for anatomical defects, and two growth scans after 29 weeks of gestation at least two weeks apart. Moreover, further ultrasound evaluations were carried out according to women's clinical needs. Women were recruited in an outpatient facility during obstetrics visits, unaware of growth scan results but usually with anomaly scans available. After delivery, the placenta was sent to the Institute of Pathology for macroscopic and microscopic examination, tissue microarray (TMA) preparation, and for immunohistochemical analysis.

The regional review board and the clinical research center of the hospital approved the present study (protocol number: ASUFC/2021/0044374), which complied with the requirements of the general authorization of the Italian Data Protection Authority for the processing of data for scientific research purposes. Furthermore, all ethical principles of the Declaration of Helsinki [103] were respected.

### Participants of the study

Women who attended routine ultrasound examinations during pregnancy were recruited for this prospective observational study. Inclusion criteria were an age of at least 18 years, a singleton spontaneous pregnancy, a live fetus at the time of the ultrasound scan and proficiency in Italian. Exclusion criteria were fetal abnormalities detected antenatally, endocrine disorders (e.g., maternal pre-pregnancy diabetes, hypothyroidism, or hyperthyroidism), maternal hypertension, pregnancy-related diseases other than isolated fetal growth disorders (e.g. gestational diabetes or cholestasis), multiple pregnancies and severe maternal psychiatric illness.

**Variables considered**

Placental oxidative stress was evaluated through immunohistochemical analysis with Antibody anti 8OH-dG. Placental examination was performed according to the Amsterdam Placental Workshop Group Consensus Statement [104]. A food frequency questionnaire was used to assess the presence of a high-fat diet and diet with high levels of saturated fat.

Recruitment and data collection took place only after women had been informed and had given written informed consent to participate. Residents and obstetricians involved in this study collected data on maternal characteristics, medical history, pregnancy complications, mode of delivery and neonatal health.

**Definition of outcomes**

Fetal smallness was accurately defined according to the Delphi's consensus statement. The fetal ultrasound monitoring regularly performed at our Department allowed clinicians to discriminate between early and late fetal growth restrictions (FGR). Early FGR was defined when detected earlier than 32 weeks of gestation and presenting with three solitary parameters (abdominal circumference (AC) < 3rd centile, estimated fetal weight (EFW) < 3rd centile and absent end-diastolic flow in the umbilical artery (UA)) or four contributory parameters (AC or EFW < 10th centile combined with a pulsatility index (PI) > 95th centile in either the UA or uterine artery). Late FGR were identified after 32 weeks with two solitary parameters (AC or EFW < 3rd centile) or four contributory parameters (EFW or AC < 10th centile, AC or EFW crossing centiles by > two quartiles on growth charts and cerebroplacental ratio < 5th centile or UA-PI > 95th centile) [105]. Fetal growth was determined according to the fetal weight Hadlock charts [106]. Fetal abdominal circumference growth was assessed according to the IG-21 standard [107,108]. The estimated fetal growth at birth was then confirmed by newborn's birthweight for each patient included in the study. Fetal Dopplers were assessed according to previously published references charts [109,110]. Neonatal weights, in non FGR fetuses, were categorized according to the Italian growth post-natal standards in three further groups: (i) appropriate for gestational age (AGA); (ii) small for gestational age (SGA, birthweight between the 3rd and the 10th centile); and (iii) for gestational age (LGA, with a birthweight > 90th centile) [111,112]. The neonatal weight was also assessed as multiple of the median (MoM) as previously described [113]. The neonatal weight MoM is the ratio between the observed birthweight and the 50th percentile of birthweight (sex and parity specific) at the same gestational age [113,112].

**Placental examination**

The criteria contained in the Amsterdam Placental Workshop Group Consensus Statement were rigorously followed for placental tissue sampling [104]. The histologic sampling included five blocks: one block representing a roll of extraplacental membranes taken from the rupture edge to the placental margin; one block including three cross-sections of the umbilical cord (one from the portion next to the fetal insertion, one from the intermediate portion and one at approximately 3 cm from the placental insertion); three blocks each containing a full-thickness section of normal-appearing placental parenchyma.

**Tissue microarray (TMA) preparation**

The current standard of care for pathologic analysis of tissue is formalin fixation and embedding in paraffin, followed by histochemical staining and microscopic examination. However, a typical paraffin tissue block is exhausted after about 100 sections are cut (depending on the skill and care of the histotechnologist) [114]. TMA represents a mechanism for effective amplification of tissue. For the purpose of this study, sections of 4 μm thickness were obtained from samples fixed in formalin and included in paraffin. After section deparaffining and rehydration, glasses were stained in hematoxylin and eosin (EE), thus allowing pathologists to carefully examine the tissue morphology in order to identify the areas to subject to TMA preparation. The Beecher Instruments arraying device was used to prepare the TMA. The array construction is based on coring system. Two hollow needles (0.6 Gauge) were used, one in the donor block and the other in the receiver block, respectively. First of all, the receiver block was perforated, and then the donor block (an arrayed master block) was biopsied from specific areas previously labeled as worthy of interest. The tissue sampled was then cautiously released in the core of the receiver block. Two biopsies were obtained from each donor block. This procedure was repeated for each tissue sample. Once the array was constructed, the receiver block was placed facing downwards on a glass at 37°C for 15 minutes. In this way paraffin slightly melted and the cores adhered to paraffin. Following this step, the glass was gently pushed against the receiver block, in order to further improve the cores adherence to paraffin. Finally, the receiver block with the glass was placed in ice and only after cooling, the glass was separated from the block. Once the recipient block was completely filled (as determined by the design of the individual array), it was sectioned to reveal 0.6-mm-diameter circles of tissue from each case.

**Immunoistochemical analysis**

Four-micron thick sections were cut from the TMA blocks and then placed in a heater at 60°C for 20 minutes in order to increase their adherence to the glasses. Once deparaffinized and rehydrated, for Antigen retrieval the sections were incubated in Tris|/EDTA buffer (Dako Target Retrieval Solution pH 9, dilution 1:10) at 98°C for 5 minutes. Endogenous peroxidase activity was blocked by incubation in 0.1% hydrogen peroxide in absolute methanol for 10 minutes at room temperature. After a washing in PBS (Dulbecco's Phosphate Buffer Saline), sections were incubated in a humidity chamber at +4°C overnight with the Antibody anti-8-OHdG (JaICA, monoclonal antibody clone N45.1) diluted 1:10. The Dako REAL™ EnVision™ HRP Rabbit/Mouse was used as secondary Antibody, with incubation in whet chamber, at room temperature, 40 minutes long. After another washing in PBS, the peroxidase activity, which is used to mark the secondary Antibody, was retrieved through an incubation with the substrate Dako REAL™ DAB + Chromogen (diluted 1:50) in a humidity chamber at room temperature for 5 minutes. Finally, after a quick washing in distilled water, the slides were counterstained with Gill hematoxylin and mounted with Histomount (National Diagnostics, New Jersey, USA), being now ready for the evaluation. The stainings were reviewed and analyzed on a multi-head microscope by two observers blinded from the clinical data. The immunoreactivity was assessed separately in syncytiotrophoblastic, endothelial and stromal cells of the villi. The assessment was performed from one representative section by evaluating the whole section area. The immunohistochemical localization was scored in a semiquantitative fashion incorporating both the intensity and the distribution of specific staining [115]. Cytoplasm staining was evaluated by intensity score as strong 3, moderate 2, weak 1, and absent 0. Nuclear staining for 8-OHdG was evaluated by H-score (the product of actual percentage of positive-stained nuclei and intensity score—evaluated as strong 3, moderate 2 and weak 1—giving a possible range of 0–300). In case of discordance, a joint assessment was performed by the two pathologists [116,117].

**Data analysis**

With an estimated prevalence of late FGR at around 3%, we planned to collect samples from up to 500 pregnancies to achieve a case group of at least 14 cases. With a sample size of 14 cases, the target sample size for the control group was 130 pregnancies, which was large enough to detect differences in IHC scores, assuming a 80% power and a 5% (two-sided) significance level. A large Cohen effect size (d=0.8) was deemed relevant, and a nonparametric correction was used.

The data were analyzed using the R program (version 4.2.1) considering a p-value <0.05 as significant. The normality of the disruption was assessed by the Kolmogorov-Smirnov test. In case of non-parametric distribution, the continuous variables were described with median and interquartile range (IQR). In case of parametric continuous variables the mean (± standard deviation) was used. The categorical variables were described by percentages and absolute values. In addition, the following were performed where appropriate: T-test, one-way ANOVA, Wilcoxon test, Kruskal-Wallis test, Spearman test, chi-square test or Fisher's exact test.

**Results**

A total of 165 women were included in this study (Figure 5). Table 4 describes the general characteristics of the whole population. Of note, most women (78.18%) delivered vaginally, either spontaneously or with vacuum extraction, and labor induction occurred in 55.76% of cases. The median gestational age at birth was 39 weeks (IQR 38-40), the median birthweight was 3277.85 gr (±513.52). The median Apgar score at first and fifth minute was 8 and 9 respectively, the median umbilical artery pH at birth was 7.28 (IQR 7.23-7.32) and the median base excess at birth was 3 (IQR 2-6). Finally, 6.06% of neonates were admitted to NICU and 10.3% were resuscitated immediately after birth.

A total of 165 placentae were sent to the Institute of Pathology for macroscopic and microscopic examination, TMA preparation and the immunohistochemical analysis. The placentae were divided in 4 groups according to the fetal growth at birth: i) 131 were placentae of AGA neonates; ii) 10 were placentae of LGA neonates; iii) 9 were placentae of SGA > 3° < 10° percentile and iv) 15 were placentae of late FGR (Figure 5).

Table 5 illustrates the different histological findings overall retrieved in our sample. Maternal vascular malperfusion was found in 24.24% and included placental hypoplasia (9.09%,) placental infarction (10.91%), retroplacental hemorrhage (1.21%), accelerated villous maturation (2.42%) and distal villous hypoplasia (4.24%). Fetal vascular malperfusion was found in 12.12%, and incorporated avascular villi (5.45%) and thrombi (chorionic plate or major stem villi) 8.48%.

Table 6 shows the overall immunohistochemical findings. The nuclear staining of syncytiotrophoblast cells had a H-score of 65.00 (IQR 35.00-113.12), an intensity score of 2.00 (IQR 2.00-2.50) and a percentage of positive nuclei of 30.00 (IQR 17.50-50.62). The intensity score in the cytoplasm of syncytiotrophoblast cells resulted to be 1.50 (IQR 1.00-2.00) and the intensity score in stromal and endothelial cells was 2.50 (IQR 2.00-3.00).

The variables so far considered were then separately analyzed for each group and compared. Table 7 shows the general characteristics according to the 4 different fetal growth groups. There were no significant differences between the groups for maternal age, parity and principal pregnancy characteristics. However, pre-pregnancy BMI was significantly higher in AGA group compared to late FGR group (23.31 kg/m², IQR 20.31-27.06 vs. 19.10 kg/m² IQR 17.99-22.66) and, although not significant, the highest rate of labor induction (77.78%) and the highest mean gestational age at delivery, which was 40.00 weeks (IQR 39.00-40.00), were reported to be in the SGA group. Birthweight was significantly different among the 4 groups: 2496.00 gr (2252.50-2613.00) in late FGR group, 2900.00 gr (2700.00-3070.00) in SGA group, 3355.00 gr (3102.50-3637.50) in AGA group and 4096.00 gr (4007.50-4240.00) in LGA group. Also placental weight (expressed in MoM) was significantly different among the four groups, whereas the other outcomes at birth, such as Apgar score, umbilical cord gases and NICU admission or neonatal resuscitation, did not vary among neonates.

Table 8 shows the histological and immunohistochemical differences between the groups. Compared to AGA, late FGR had more frequently maternal vascular malperfusion (46.67% vs. 22.90%), placental hypoplasia (26.67% vs. 7.63%) and accelerated villous maturation (20% vs. 0.76%), while LGA had more commonly retroplacental hemorrhage (10.00% vs. 0.76%). The percentage of positive nuclei in nuclear staining of syncytiotrophoblast cells was significantly higher in LGA compared to late FGR and the intensity score in the syncytiotrophoblast cells cytoplasm staining was significantly lower in SGA and in LGA compared to AGA (0.50, IQR 0.00-1.00 vs. 1.50, IRQ 1.00-2.00 and 1.00, IQR 0.12-1.00 vs. 1.50, IQR 1.00-2.00).

Tables 9 and 10 display the histological and immunohistochemical differences between female and males among AGA neonates. This population accounted for 59 males and 72 females. No significant histological differences were observed between the two groups. On the contrary, compared to females, male counterparts had a significantly higher nuclear staining in syncytiotrophoblast cells and a higher intensity score staining in stromal and endothelial cells.

Table 11 shows the general characteristics according to the 4 different fetal growth groups in female fetuses. Table 12 shows the histological and immunohistochemical differences between female fetal groups. This population consisted of 87 fetuses: 9 late FGR, 2 SGA, 72 AGA and 4 LGA. Smaller fetuses (late FGR and SGA) appeared to have more commonly placental hypoplasia (SGA 50.0% vs. AGA 25.0%) and accelerated villous maturation (late FGR 11.11% vs. AGA 0.00%). The intensity score in nuclear staining of the syncytiotrophoblast cells was significantly

higher in SGA than in AGA (2.75, IQR 2.62-2.88 vs. 2.00, IQR 2.00-2.25), while the percentage of positive nuclei in nuclear staining of syncytiotrophoblast cells was significantly higher in LGA compared to AGA (56.25, IQR 46.25-69.38 vs. 27.50, IQR 16.25-45.00).

Table 13 shows the general characteristics according to the 4 different fetal growth groups in male fetuses. Table 14 shows the histological and immunohistochemical differences between male fetal groups. This population accounted for 78 fetuses: 6 late FGR, 7 SGA, 59 AGA and 6 LGA. Compared to AGA, late FGR had more frequently maternal vascular malperfusion (66.67% vs 14.29%), accelerated villous maturation (33.3% vs. 0.00%), distal villous hypoplasia (33.3% vs. 0.00%) and avascular villi (33.3% vs. 14.29%). The intensity score in cytoplasm staining of the syncytiotrophoblast cells was significantly lower in SGA and LGA than in AGA (0.00, IQR 0.00-0.75 vs. 2.00, IQR1.00-2.00 and 0.75, IQR 0.12-1.00 vs. 2.00, IQR 1.00-2.00). The staining intensity score in stromal and endothelial cells was higher in AGA than in SGA (3.00, IQR 2.00-3.00 vs. 2.00, IQR 1.00-2.00).

**Correlations**

We then evaluated the correlations between the neonatal weight expressed in MoM and the various parameters considered, stratifying by sex. Of note, in males there are statistically significant correlations between reduced fetal weight MoM and maternal vascular malperfusion ($p < 0.05$) (figure 6). Moreover, there are significant correlations between reduced neonatal weight MoM and placental hypoplasia or late FGR (Figure 2) ($p<0.05$).

Figure 7 shows the neonatal weigth MoM correlations in female population. A significant correlation emerges between an increase in the neonatal weight MoM and an increase in staining of syncytiotrophoblast nuclei (H-score) and stromal and endothelial cells (intensity score) ($p < 0.05$).

Figure 8 shows the correlations between different parameters and the immunostaining scores in males. There is a significant correlation between the cytoplasmic staining of syncytiotrophoblast and the thrombi formation in the chorionic plate or villi (Figure 8) ($p<0.05$).

Figure 9 shows that in females, there is a significant correlation between an increased MoM in fetal weight and the intensity of the immunostaining in stromal and endothelial cells. At the same time there is a correlation between an increased prevalence of positive-stained nuclei in the

syncytiotrophoblast and a reduced MoM of fetal weight. Finally, there is a significant correlation between a high-fat diet and increased immunoreactivity in stromal and endothelial cells ($p < 0.05$).

We then correlated the high-fat diet with different variables, starting from the female fetal population (Figure 10). Figure 10 shows a significant positive correlation between a high fat diet and a high intensity of stromal/endothelial staining with 8-OHdG ($p < 0.05$). A high intensity of stromal/endothelial staining with 8-OHdG is also directly related to the fetal weight MoM ($p < 0.05$). By contrast, there are indirectly proportional and insignificant correlations between a diet enriched in fat or in saturated fat and a reduction in neonatal weight MoM. In the case of the diet rich in saturated fat, the correlation reaches a level close to significance ($rho = -0.18$, $p = 0.092$). In Figure 7 we explored the same network of correlations in the male fetal population, where correlations with diet lose significance. Of note, a negative correlation persists between the MoM of fetal weight and the diet rich in saturated fat ($rho = -0.14$, $p = 0.224$). Furthermore, although not significant, there are positive correlations between the diet rich in saturated fat and distal villous hypoplasia ($rho = 0.22$, $p = 0.052$) and accelerated villous maturation ($rho = 0.19$, $p = 0.096$).

*Figure 5- Population flowchart. The late FGR category is based on the antenatal ultrasound standards, while SGA, AGA, and LGA are based on post-natal standards.*

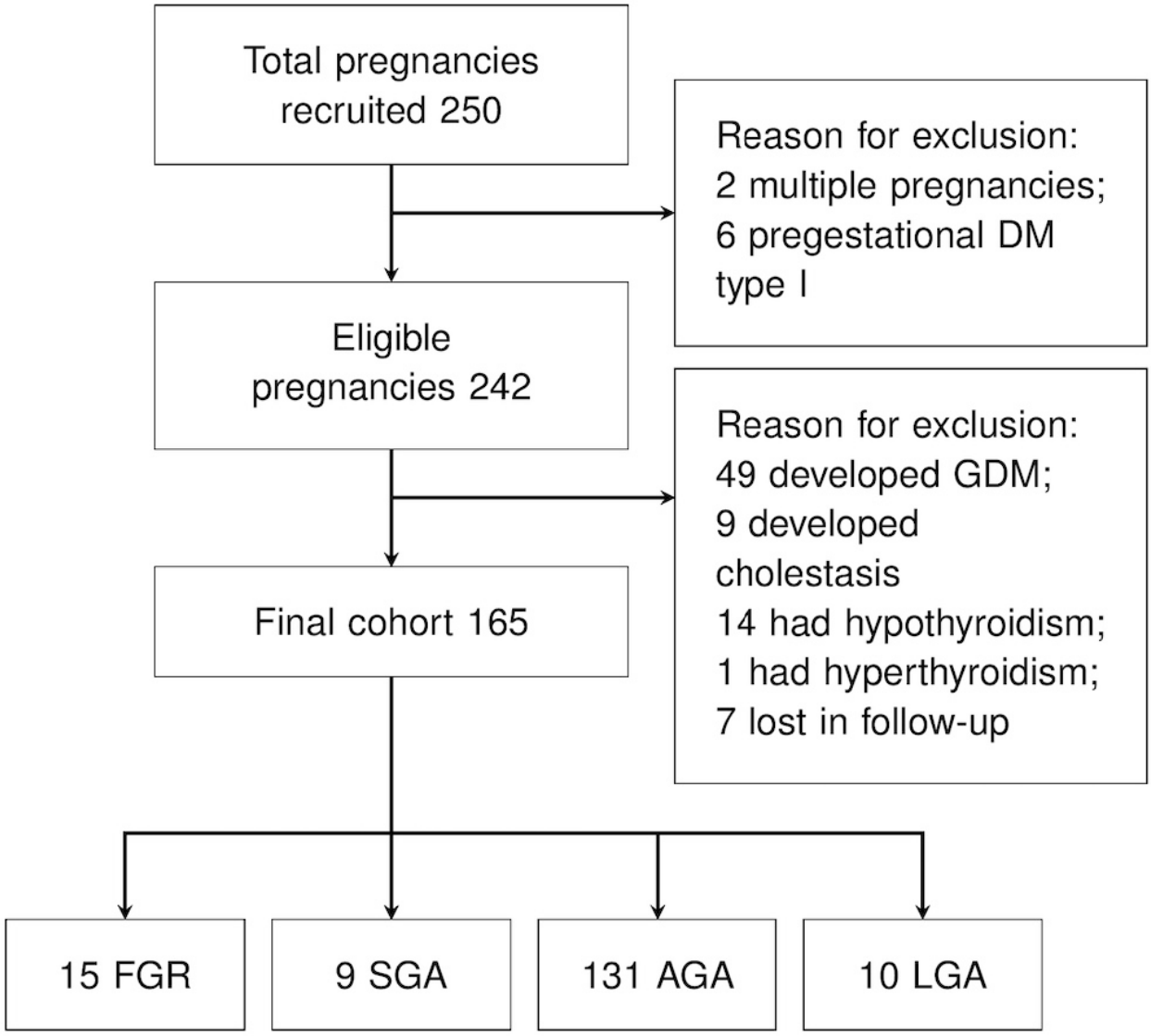


*Table 4- Population description.*

| | |
|---|---|
| Maternal characteristics | |
| Maternal age (years) | 33 (30-36) |
| Nulliparity | 53.33% (88/165) |
| Pre-pregnancy BMI (kg/m²) | 22.95 (19.92-26.45) |
| Region of origin | |
| Italy | 84.24% (139/165) |
| Europe and Central Asia | 9.7% (16/165) |
| Sub-Saharan Africa | 3.03% (5/165) |
| Middle East and North Africa | 1.82% (3/165) |
| South Asia | 1.21% (2/165) |
| East Asia and Pacific | 0% (0/165) |
| Latin America and Caribbean | 0% (0/165) |
| Pregnancy characteristics | |
| Tobacco smoke in pregnancy | 6.06% (10/165) |
| Labor mode | |
| Spontaneous | 31.52% (52/165) |
| Labor induction | 55.76% (92/165) |
| Without labor | 12.73% (21/165) |
| Delivery mode | |
| Spontaneous vaginal delivery | 61.82% (102/165) |
| Vacuum extraction | 16.36% (27/165) |
| Cesarean delivery | 21.82% (36/165) |
| Neonatal characteristics | |
| Male sex | 47.27% (78/165) |

| | |
|---|---|
| Gestational age (weeks) | 39 (38-40) |
| Neonatal weight (g) | 3277.85 (±513.52) |
| Neonatal weight (MoM) | 0.99 (±0.13) |
| Placental index | 0.13 (0.12-0.15) |
| Placental weight (MoM) | 1 (0.84-1.15) |
| Fetal growth | |
| FGR | 9.09% (15/165) |
| SGA | 5.45% (9/165) |
| AGA | 79.39% (131/165) |
| LGA | 6.06% (10/165) |
| Apgar score 1st minute | 8 (8-9) |
| Apgar score 5th minute | 9 (9-10) |
| Cord blood pH | 7.28 (7.23-7.32) |
| Base excess | 3 (2-6) |
| NICU hospitalization | 6.06% (10/165) |
| Neonatal resucitation | 10.3% (17/165) |

*Table 5- Placental histology assessed according to the Amsterdam criteria.*

| Histology | |
|---|---|
| **Maternal vascular malperfusion** | **24.24% (40/165)** |
| Placental hypoplasia | 9.09% (15/165) |
| Placental infarction | 10.91% (18/165) |
| Retroplacental hemorrhage | 1.21% (2/165) |
| Accelerated villous maturation | 2.42% (4/165) |
| Distal villous hypoplasia | 4.24% (7/165) |
| **Fetal vascular malperfusion** | **12.12% (20/165)** |
| Avascular villi | 5.45% (9/165) |
| Thrombi (chorionic plate or major stem villi) | 8.48% (14/165) |
| Delayed villous maturation | 60.61% (100/165) |

*Table 6- Placental immunohistochemical (IHC) 8-hydroxy-2'-deoxy-guanosine (8-OHdG) staining.*

| **IHC** | |
|---|---|
| Nuclear staining syncytiotrophoblast (H-score) | 65.00 (35.00-113.12) |
| Nuclear staining syncytiotrophoblast (intensity score) | 2.00 (2.00-2.50) |
| Nuclear staining syncytiotrophoblast (Percentage of positive nuclei) | 30.00 (17.50-50.62) |
| Cytoplasm staining syncytiotrophoblast (intensity score) | 1.50 (1.00-2.00) |
| Stromal and endothelial cells (intensity score) | 2.50 (2.00-3.00) |

*Table 7- Population characteristics differences between fetal growth groups.*

| | FGR (15) | SGA (9) | AGA (131) | LGA (10) | p |
|---|---|---|---|---|---|
| Maternal characteristics | | | | | |
| Maternal age (years) | 33.00 (30.00-34.50) | 37.00 (31.00-39.00) | 33.00 (30.00-36.00) | 31.50 (29.00-34.75) | NS |
| Nulliparity | 60.00% (9/15) | 66.67% (6/9) | 53.44% (70/131) | 30.00% (3/10) | NS |
| Pre-pregnancy BMI (kg/m²) | 19.10 (17.99-22.66) | 22.04 (18.78-24.39) | 23.31 (20.31-27.06) | 21.91 (19.53-24.96) | 2 |
| Region of origin | | | | | 0.880* |
| Italy | 73.33% (11/15) | 77.78% (7/9) | 85.50% (112/131) | 90.00% (9/10) | NS |
| Europe and Central Asia | 20.00% (3/15) | 11.11% (1/9) | 8.40% (11/131) | 10.00% (1/10) | NS |
| Sub-Saharan Africa | 6.67% (1/15) | 11.11% (1/9) | 2.29% (3/131) | 0.00% (0/10) | NS |
| Middle East and North Africa | 0.00% (0/15) | 0.00% (0/9) | 2.29% (3/131) | 0.00% (0/10) | NS |
| South Asia | 0.00% (0/15) | 0.00% (0/9) | 1.53% (2/131) | 0.00% (0/10) | NS |
| Pregnancy characteristics | | | | | |
| Tobacco smoke in pregnancy | 6.67% (1/15) | 11.11% (1/9) | 6.11% (8/131) | 0.00% (0/10) | NS |
| Labor mode | | | | | 0.558* |
| Spontaneous | 20.00% (3/15) | 22.22% (2/9) | 32.06% (42/131) | 50.00% (5/10) | NS |

| | | | | | |
|---|---|---|---|---|---|
| Labor induction | 66.67% (10/15) | 77.78% (7/9) | 54.20% (71/131) | 40.00% (4/10) | NS |
| Without labor | 13.33% (2/15) | 0.00% (0/9) | 13.74% (18/131) | 10.00% (1/10) | NS |
| Delivery mode | | | | | 0.366* |
| Spontaneous vaginal delivery | 60.00% (9/15) | 88.89% (8/9) | 58.78% (77/131) | 80.00% (8/10) | NS |
| Vacuum extraction | 26.67% (4/15) | 0.00% (0/9) | 16.79% (22/131) | 10.00% (1/10) | NS |
| Cesarean delivery | 13.33% (2/15) | 11.11% (1/9) | 24.43% (32/131) | 10.00% (1/10) | NS |
| Neonatal characteristics | | | | | |
| Male sex | 40.00% (6/15) | 77.78% (7/9) | 45.04% (59/131) | 60.00% (6/10) | NS |
| Gestational age (weeks) | 38.00 (37.00-39.50) | 40.00 (39.00-40.00) | 39.00 (38.00-40.00) | 39.00 (39.00-39.00) | NS |
| Neonatal weight (g) | 2496.00 (2252.50-2613.00) | 2900.00 (2700.00-3070.00) | 3355.00 (3102.50-3637.50) | 4096.00 (4007.50-4240.00) | 1,2,3,4,5,6 |
| Neonatal weight (MoM) | 0.78 (0.73-0.80) | 0.84 (0.81-0.86) | 1.01 (0.93-1.08) | 1.23 (1.22-1.28) | 1,2,3,4,5,6 |
| Placental index | 0.14 (0.12-0.16) | 0.13 (0.12-0.15) | 0.13 (0.12-0.15) | 0.13 (0.12-0.16) | NS |
| Placental weight (MoM) | 0.82 (0.72-0.89) | 0.88 (0.82-0.97) | 1.00 (0.88-1.13) | 1.19 (1.12-1.46) | 2,3,5,6 |
| Apgar score 1st minute | 9.00 (7.50-9.00) | 8.00 (8.00-9.00) | 8.00 (8.00-9.00) | 8.50 (8.00-9.00) | NS |

| | | | | | |
|---|---|---|---|---|---|
| Apgar score 5th minute | 9.00 (9.00-9.00) | 9.00 (9.00-9.00) | 9.00 (9.00-10.00) | 9.00 (9.00-9.75) | NS |
| Cord blood pH | 7.30 (7.26-7.31) | 7.25 (7.22-7.31) | 7.27 (7.23-7.32) | 7.29 (7.23-7.32) | NS |
| Base excess | 3.00 (2.00-6.50) | 5.00 (3.00-9.00) | 3.50 (2.00-6.00) | 2.00 (0.00-3.00) | NS |
| NICU hospitalization | 6.67% (1/15) | 0.00% (0/9) | 6.11% (8/131) | 10.00% (1/10) | NS |
| Neonatal resucitation | 6.67% (1/15) | 0.00% (0/9) | 11.45% (15/131) | 10.00% (1/10) | NS |

Differences statistically significant ($p<0.05$): 1) FGR v.s. SGA; 2) FGR v.s. AGA; 3) FGR v.s. LGA; 4) SGA v.s. AGA; 5) SGA v.s. LGA; 6) AGA v.s. LGA.

*Table 8- Histology and immunohistochemical 8-hydroxy-2'-deoxy-guanosine (8-OHdG) staining differences between fetal growth groups.*

| | FGR (15) | SGA (9) | AGA (131) | LGA (10) | p |
|---|---|---|---|---|---|
| Histology | | | | | |
| Maternal vascular malperfusion | 46.67% (7/15) | 22.22% (2/9) | 22.90% (30/131) | 10.00% (1/10) | NS |
| Placental hypoplasia | 26.67% (4/15) | 11.11% (1/9) | 7.63% (10/131) | 0.00% (0/10) | 2 |
| Placental infarction | 6.67% (1/15) | 22.22% (2/9) | 11.45% (15/131) | 0.00% (0/10) | NS |
| Retroplacental hemorrhage | 0.00% (0/15) | 0.00% (0/9) | 0.76% (1/131) | 10.00% (1/10) | NS |
| Accelerated villous maturation | 20.00% (3/15) | 0.00% (0/9) | 0.76% (1/131) | 0.00% (0/10) | 2 |
| Distal villous hypoplasia | 13.33% (2/15) | 0.00% (0/9) | 3.82% (5/131) | 0.00% (0/10) | NS |
| Fetal vascular malperfusion | 13.33% (2/15) | 22.22% (2/9) | 12.21% (16/131) | 0.00% (0/10) | NS |
| Avascular villi | 13.33% (2/15) | 11.11% (1/9) | 4.58% (6/131) | 0.00% (0/10) | NS |
| Thrombi (chorionic plate or major stem villi) | 6.67% (1/15) | 11.11% (1/9) | 9.16% (12/131) | 0.00% (0/10) | NS |
| Delayed villous maturation | 60.00% (9/15) | 88.89% (8/9) | 59.54% (78/131) | 50.00% (5/10) | NS |
| IHC | | | | | |
| Nuclear staining syncytiotrophoblast (H-score) | 45.00 (30.62-83.75) | 105.00 (12.50-125.00) | 65.00 (35.00-111.88) | 100.00 (36.25-120.31) | NS |

| | | | | | |
|---|---|---|---|---|---|
| Nuclear staining syncytiotrophoblast (intensity score) | 2.00 (2.00-2.00) | 2.00 (1.50-2.50) | 2.00 (2.00-2.50) | 2.00 (2.00-2.38) | NS |
| Nuclear staining syncytiotrophoblast (Percentage of positive nuclei) | 22.50 (17.50-35.00) | 52.50 (12.50-60.00) | 30.00 (17.50-50.00) | 46.25 (35.00-59.38) | 3 |
| Cytoplasm staining syncytiotrophoblast (intensity score) | 2.00 (1.00-2.00) | 0.50 (0.00-1.00) | 1.50 (1.00-2.00) | 1.00 (0.12-1.00) | 4,6 |
| Stromal and endothelial cells (intensity score) | 2.00 (2.00-2.75) | 2.00 (1.00-2.50) | 2.50 (2.00-3.00) | 2.50 (2.00-3.00) | NS |

Differences statistically significant ($p<0.05$): 1) FGR v.s. SGA; 2) FGR v.s. AGA; 3) FGR v.s. LGA; 4) SGA v.s. AGA; 5) SGA v.s. LGA; 6) AGA v.s. LGA.

*Table 9- Population characteristics differences between fetal males and females in AGA newborns.*

| | M (59) | F (72) | p |
|---|---|---|---|
| Maternal characteristics | | | |
| Maternal age (years) | 32.00 (29.50-35.50) | 34.00 (30.00-36.00) | 0.241 |
| Nulliparity | 45.76% (27/59) | 59.72% (43/72) | 0.111 |
| Pre-pregnancy BMI (kg/m²) | 23.18 (20.20-26.81) | 23.34 (20.40-27.09) | 0.982 |
| Region of origin | | | |
| Italy | 91.53% (54/59) | 80.56% (58/72) | 0.076 |
| Europe and Central Asia | 3.39% (2/59) | 12.50% (9/72) | 0.061 |
| Middle East and North Africa | 3.39% (2/59) | 1.39% (1/72) | 0.446 |
| Sub-Saharan Africa | 0.00% (0/59) | 4.17% (3/72) | 0.113 |
| South Asia | 1.69% (1/59) | 1.39% (1/72) | 0.887 |
| Pregnancy characteristics | | | |
| Tobacco smoke in pregnancy | 5.08% (3/59) | 6.94% (5/72) | 0.658 |
| Labor mode | | | 0.137 |
| Spontaneous | 28.81% (17/59) | 34.72% (25/72) | 0.471 |
| Labor induction | 50.85% (30/59) | 56.94% (41/72) | 0.486 |
| Without labor | 20.34% (12/59) | 8.33% (6/72) | <0.05 |
| Delivery mode | | | 0.341 |
| Spontaneous vaginal delivery | 54.24% (32/59) | 62.50% (45/72) | 0.339 |
| Vacuum extraction | 15.25% (9/59) | 18.06% (13/72) | 0.670 |
| Cesarean delivery | 30.51% (18/59) | 19.44% (14/72) | 0.143 |
| Neonatal characteristics | | | |

| | | | |
|---|---|---|---|
| Male sex | 100.00% (59/59) | 0.00% (0/72) | <0.05 |
| Gestational age (weeks) | 39.00 (39.00-40.00) | 39.00 (38.00-40.00) | 0.485 |
| Neonatal weight (g) | 3515.00 (3185.00-3685.00) | 3248.00 (3022.50-3462.50) | <0.05 |
| Neonatal weight (MoM) | 1.02 (0.97-1.09) | 1.00 (0.92-1.06) | 0.055 |
| Placental index | 0.12 (0.11-0.14) | 0.14 (0.13-0.16) | <0.05 |
| Placental weight (MoM) | 1.00 (0.88-1.10) | 1.02 (0.87-1.13) | 0.664 |
| Apgar score 1st minute | 8.00 (8.00-9.00) | 8.00 (7.00-9.00) | 0.671 |
| Apgar score 5th minute | 9.00 (9.00-9.50) | 9.00 (9.00-10.00) | 0.669 |
| Apgar score 1st minute | 8.00 (8.00-9.00) | 8.00 (7.00-9.00) | 0.671 |
| Apgar score 5th minute | 9.00 (9.00-9.50) | 9.00 (9.00-10.00) | 0.669 |
| NICU hospitalization | 1.69% (1/59) | 9.72% (7/72) | 0.056 |
| Neonatal resucitation | 8.47% (5/59) | 13.89% (10/72) | 0.333 |

*Table 10- Histology and immunohistochemical 8-hydroxy-2'-deoxy-guanosine (8-OHdG) staining differences between fetal males and females in AGA newborns.*

| | M (59) | F (72) | p |
|---|---|---|---|
| Histology | | | |
| Maternal vascular malperfusion | 20.34% (12/59) | 25.00% (18/72) | 0.528 |
| Placental hypoplasia | 6.78% (4/59) | 8.33% (6/72) | 0.739 |
| Placental infarction | 11.86% (7/59) | 11.11% (8/72) | 0.893 |
| Retroplacental hemorrhage | 0.00% (0/59) | 1.39% (1/72) | 0.364 |
| Accelerated villous maturation | 1.69% (1/59) | 0.00% (0/72) | 0.267 |
| Distal villous hypoplasia | 3.39% (2/59) | 4.17% (3/72) | 0.817 |
| Fetal vascular malperfusion | 8.47% (5/59) | 15.28% (11/72) | 0.237 |
| Avascular villi | 3.39% (2/59) | 5.56% (4/72) | 0.555 |
| Thrombi (chorionic plate or major stem villi) | 6.78% (4/59) | 11.11% (8/72) | 0.393 |
| Delayed villous maturation | 55.93% (33/59) | 62.50% (45/72) | 0.446 |
| IHC | | | |
| Nuclear staining syncytiotrophoblast (H-score) | 80.00 (35.00-163.75) | 60.00 (19.38-95.00) | <0.05 |
| Nuclear staining syncytiotrophoblast (intensity score) | 2.00 (2.00-3.00) | 2.00 (2.00-2.25) | <0.05 |
| Nuclear staining syncytiotrophoblast (Percentage of positive nuclei) | 35.00 (18.75-61.25) | 27.50 (16.25-45.00) | <0.05 |
| Cytoplasm staining syncytiotrophoblast (intensity score) | 2.00 (1.00-2.00) | 1.50 (1.00-2.00) | 0.063 |
| Stromal and endothelial cells (intensity score) | 3.00 (2.00-3.00) | 2.50 (2.00-3.00) | <0.05 |
| Blood nucleated cells (intensity score) | 1.00 (0.50-1.00) | 1.00 (0.00-1.00) | 0.866 |

*Table 11- Population characteristics differences between fetal growth groups. In this analysis, only female fetuses were included.*

| | FGR (9) | SGA (2) | AGA (72) | LGA (4) | p |
|---|---|---|---|---|---|
| Maternal characteristics | | | | | |
| Maternal age (years) | 31.00 (26.00-35.00) | 41.00 (39.00-43.00) | 34.00 (30.00-36.00) | 30.50 (29.00-32.50) | NS |
| Nulliparity | 66.67% (6/9) | 50.00% (1/2) | 59.72% (43/72) | 25.00% (1/4) | NS |
| Pre-pregnancy BMI (kg/m²) | 19.41 (18.23-21.66) | 20.41 (19.59-21.23) | 23.34 (20.40-27.09) | 23.65 (22.25-24.87) | 2 |
| Pregnancy characteristics | | | | | |
| Tobacco smoke in pregnancy | 0.00% (0/9) | 0.00% (0/2) | 6.94% (5/72) | 0.00% (0/4) | NS |
| Labor mode | | | | | |
| Spontaneous | 33.33% (3/9) | 50.00% (1/2) | 34.72% (25/72) | 50.00% (2/4) | NS |
| Labor induction | 66.67% (6/9) | 50.00% (1/2) | 56.94% (41/72) | 50.00% (2/4) | NS |
| Without labor | 0.00% (0/9) | 0.00% (0/2) | 8.33% (6/72) | 0.00% (0/4) | NS |
| Delivery mode | | | | | |
| Spontaneous vaginal delivery | 77.78% (7/9) | 100.00% (2/2) | 62.50% (45/72) | 100.00% (4/4) | NS |
| Vacuum extraction | 22.22% (2/9) | 0.00% (0/2) | 18.06% (13/72) | 0.00% (0/4) | NS |

| | | | | | |
|---|---|---|---|---|---|
| Cesarean delivery | 0.00% (0/9) | 0.00% (0/2) | 19.44% (14/72) | 0.00% (0/4) | NS |
| Neonatal characteristics | | | | | |
| Gestational age (weeks) | 39.00 (37.00-40.00) | 39.50 (39.25-39.75) | 39.00 (38.00-40.00) | 39.00 (39.00-39.25) | NS |
| Neonatal weight (g) | 2496.00 (2205.00-2646.00) | 2757.50 (2696.25-2818.75) | 3248.00 (3022.50-3462.50) | 4117.50 (4082.50-4172.25) | 2,3,4,6 |
| Neonatal weight (MoM) | 0.79 (0.74-0.80) | 0.83 (0.82-0.83) | 1.00 (0.92-1.06) | 1.26 (1.23-1.30) | 2,3,4,6 |
| Placental index | 0.14 (0.13-0.17) | 0.13 (0.12-0.14) | 0.14 (0.13-0.16) | 0.14 (0.13-0.15) | NS |
| Placental weight (MoM) | 0.81 (0.76-0.89) | 0.79 (0.75-0.84) | 1.02 (0.87-1.13) | 1.23 (1.21-1.32) | 2,3,6 |
| Apgar score 1st minute | 9.00 (8.00-9.00) | 9.00 (9.00-9.00) | 8.00 (7.00-9.00) | 9.00 (8.75-9.00) | NS |
| Apgar score 5th minute | 9.00 (9.00-9.00) | 9.00 (9.00-9.00) | 9.00 (9.00-10.00) | 9.50 (9.00-10.00) | NS |
| Apgar score 1st minute | 9.00 (8.00-9.00) | 9.00 (9.00-9.00) | 8.00 (7.00-9.00) | 9.00 (8.75-9.00) | NS |
| Apgar score 5th minute | 9.00 (9.00-9.00) | 9.00 (9.00-9.00) | 9.00 (9.00-10.00) | 9.50 (9.00-10.00) | NS |
| NICU hospitalization | 0.00% (0/9) | 0.00% (0/2) | 9.72% (7/72) | 0.00% (0/4) | NS |
| Neonatal resucitation | 0.00% (0/9) | 0.00% (0/2) | 13.89% (10/72) | 0.00% (0/4) | NS |

Differences statistically significant ($p<0.05$): 1) FGR v.s. SGA; 2) FGR v.s. AGA; 3) FGR v.s. LGA; 4) SGA v.s. AGA; 5) SGA v.s. LGA; 6) AGA v.s. LGA.

*Table 12- Histology and immunohistochemical 8-hydroxy-2'-deoxy-guanosine (8-OHdG) staining differences between fetal growth groups. In this analysis, only female fetuses were included.*

| | FGR (9) | SGA (2) | AGA (72) | LGA (4) | p |
|---|---|---|---|---|---|
| Histology | | | | | |
| Maternal vascular malperfusion | 33.33% (3/9) | 50.00% (1/2) | 25.00% (18/72) | 0.00% (0/4) | NS |
| Placental hypoplasia | 22.22% (2/9) | 50.00% (1/2) | 8.33% (6/72) | 0.00% (0/4) | 4 |
| Placental infarction | 0.00% (0/9) | 50.00% (1/2) | 11.11% (8/72) | 0.00% (0/4) | 1 |
| Retroplacental hemorrhage | 0.00% (0/9) | 0.00% (0/2) | 1.39% (1/72) | 0.00% (0/4) | NS |
| Accelerated villous maturation | 11.11% (1/9) | 0.00% (0/2) | 0.00% (0/72) | 0.00% (0/4) | 2 |
| Distal villous hypoplasia | 0.00% (0/9) | 0.00% (0/2) | 4.17% (3/72) | 0.00% (0/4) | NS |
| Fetal vascular malperfusion | 0.00% (0/9) | 50.00% (1/2) | 15.28% (11/72) | 0.00% (0/4) | 1 |
| Avascular villi | 0.00% (0/9) | 0.00% (0/2) | 5.56% (4/72) | 0.00% (0/4) | NS |
| Thrombi (chorionic plate or major stem villi) | 0.00% (0/9) | 50.00% (1/2) | 11.11% (8/72) | 0.00% (0/4) | 1 |
| Delayed villous maturation | 66.67% (6/9) | 100.00% (2/2) | 62.50% (45/72) | 25.00% (1/4) | NS |
| IHC | | | | | |
| Nuclear staining syncytiotrophoblast (H-score) | 40.00 (35.00-70.00) | 108.12 (75.94-140.31) | 60.00 (19.38-95.00) | 112.50 (83.75-161.25) | NS |

| | | | | | |
|---|---|---|---|---|---|
| Nuclear staining syncytiotrophoblast (intensity score) | 2.00 (2.00-2.00) | 2.75 (2.62-2.88) | 2.00 (2.00-2.25) | 2.00 (1.75-2.25) | 4 |
| Nuclear staining syncytiotrophoblast (Percentage of positive nuclei) | 20.00 (17.50-35.00) | 37.50 (27.50-47.50) | 27.50 (16.25-45.00) | 56.25 (46.25-69.38) | 6 |
| Cytoplasm staining syncytiotrophoblast (intensity score) | 2.00 (1.00-2.00) | 2.00 (1.50-2.50) | 1.50 (1.00-2.00) | 1.00 (0.75-1.12) | NS |
| Stromal and endothelial cells (intensity score) | 2.00 (2.00-2.00) | 2.75 (2.62-2.88) | 2.50 (2.00-3.00) | 3.00 (2.75-3.00) | NS |

Differences statistically significant ($p<0.05$): 1) FGR v.s. SGA; 2) FGR v.s. AGA; 3) FGR v.s. LGA; 4) SGA v.s. AGA; 5) SGA v.s. LGA; 6) AGA v.s. LGA.

*Table 13- Population characteristics differences between fetal growth groups. In this analysis, only male fetuses were included.*

| | FGR (6) | SGA (7) | AGA (59) | LGA (6) | p |
|---|---|---|---|---|---|
| Maternal characteristics | | | | | |
| Maternal age (years) | 33.50 (33.00-34.00) | 33.00 (30.50-38.50) | 32.00 (29.50-35.50) | 33.00 (27.25-35.75) | NS |
| Nulliparity | 50.00% (3/6) | 71.43% (5/7) | 45.76% (27/59) | 33.33% (2/6) | NS |
| Pre-pregnancy BMI (kg/m²) | 18.82 (17.63-22.66) | 24.11 (20.18-25.17) | 23.18 (20.20-26.81) | 20.09 (18.99-24.09) | NS |
| Pregnancy characteristics | | | | | |
| Tobacco smoke in pregnancy | 16.67% (1/6) | 14.29% (1/7) | 5.08% (3/59) | 0.00% (0/6) | NS |
| Labor mode | | | | | |
| Spontaneous | 0.00% (0/6) | 14.29% (1/7) | 28.81% (17/59) | 50.00% (3/6) | 3 |
| Labor induction/augmentation | 66.67% (4/6) | 85.71% (6/7) | 50.85% (30/59) | 33.33% (2/6) | NS |
| Without labor | 33.33% (2/6) | 0.00% (0/7) | 20.34% (12/59) | 16.67% (1/6) | NS |
| Deliery mode | | | | | |
| Spontaneous vaginal delivery | 33.33% (2/6) | 85.71% (6/7) | 54.24% (32/59) | 66.67% (4/6) | NS |
| Vacum extraction | 33.33% (2/6) | 0.00% (0/7) | 15.25% (9/59) | 16.67% (1/6) | NS |

| | | | | | |
|---|---|---|---|---|---|
| Cesarean delivery | 33.33% (2/6) | 14.29% (1/7) | 30.51% (18/59) | 16.67% (1/6) | NS |
| Neonatal characteristics | | | | | |
| Gestational age (weeks) | 37.50 (37.00-38.75) | 40.00 (39.00-40.00) | 39.00 (39.00-40.00) | 39.00 (39.00-39.00) | NS |
| Neonatal weight (g) | 2427.50 (2312.50-2557.50) | 3025.00 (2800.00-3072.50) | 3515.00 (3185.00-3685.00) | 4046.00 (3943.75-4229.25) | 1,2,3,4,5,6 |
| Neonatal weight (MoM) | 0.76 (0.73-0.78) | 0.85 (0.80-0.86) | 1.02 (0.97-1.09) | 1.22 (1.17-1.26) | 1,2,3,4,5,6 |
| Placental index | 0.14 (0.12-0.16) | 0.13 (0.12-0.16) | 0.12 (0.11-0.14) | 0.12 (0.11-0.15) | NS |
| Placental weight (MoM) | 0.83 (0.72-0.96) | 0.92 (0.83-1.04) | 1.00 (0.88-1.10) | 1.13 (1.09-1.43) | 3 |
| Apgar score 1st minute | 8.50 (7.25-9.00) | 8.00 (8.00-8.50) | 8.00 (8.00-9.00) | 8.00 (8.00-8.75) | NS |
| Apgar score 5th minute | 9.00 (8.25-9.00) | 9.00 (9.00-9.00) | 9.00 (9.00-9.50) | 9.00 (8.25-9.00) | NS |
| Apgar score 1st minute | 8.50 (7.25-9.00) | 8.00 (8.00-8.50) | 8.00 (8.00-9.00) | 8.00 (8.00-8.75) | NS |
| Apgar score 5th minute | 9.00 (8.25-9.00) | 9.00 (9.00-9.00) | 9.00 (9.00-9.50) | 9.00 (8.25-9.00) | NS |
| NICU hospitalization | 16.67% (1/6) | 0.00% (0/7) | 1.69% (1/59) | 16.67% (1/6) | 2,6 |
| Neonatal resucitation | 16.67% (1/6) | 0.00% (0/7) | 8.47% (5/59) | 16.67% (1/6) | NS |

Differences statistically significant ($p<0.05$): 1) FGR v.s. SGA; 2) FGR v.s. AGA; 3) FGR v.s. LGA; 4) SGA v.s. AGA; 5) SGA v.s. LGA; 6) AGA v.s. LGA.

*Table 14- Histology and immunohistochemical 8-hydroxy-2'-deoxy-guanosine (8-OHdG) staining differences between fetal growth groups. In this analysis, only male fetuses were included.*

| | FGR (6) | SGA (7) | AGA (59) | LGA (6) | p |
|---|---|---|---|---|---|
| Histology | | | | | |
| Maternal vascular malperfusion | 66.67% (4/6) | 14.29% (1/7) | 20.34% (12/59) | 16.67% (1/6) | 2 |
| Placental hypoplasia | 33.33% (2/6) | 0.00% (0/7) | 6.78% (4/59) | 0.00% (0/6) | 2 |
| Placental infarction | 16.67% (1/6) | 14.29% (1/7) | 11.86% (7/59) | 0.00% (0/6) | NS |
| Retroplacental hemorrhage | 0.00% (0/6) | 0.00% (0/7) | 0.00% (0/59) | 16.67% (1/6) | 6 |
| Accelerated villous maturation | 33.33% (2/6) | 0.00% (0/7) | 1.69% (1/59) | 0.00% (0/6) | 2 |
| Distal villous hypoplasia | 33.33% (2/6) | 0.00% (0/7) | 3.39% (2/59) | 0.00% (0/6) | 2 |
| Fetal vascular malperfusion | 33.33% (2/6) | 14.29% (1/7) | 8.47% (5/59) | 0.00% (0/6) | NS |
| Avascular villi | 33.33% (2/6) | 14.29% (1/7) | 3.39% (2/59) | 0.00% (0/6) | 2 |
| Thrombi (chorionic plate or major stem villi) | 16.67% (1/6) | 0.00% (0/7) | 6.78% (4/59) | 0.00% (0/6) | NS |
| Delayed villous maturation | 50.00% (3/6) | 85.71% (6/7) | 55.93% (33/59) | 66.67% (4/6) | NS |
| IHC | | | | | |
| Nuclear staining syncytiotrophoblast (H-score) | 63.12 (33.75-90.62) | 105.00 (11.88-122.50) | 80.00 (35.00-163.75) | 70.00 (36.25-104.69) | NS |

| | | | | | |
|---|---|---|---|---|---|
| Nuclear staining syncytiotrophoblast (intensity score) | 2.00 (1.62-2.38) | 2.00 (1.25-2.00) | 2.00 (2.00-3.00) | 2.00 (2.00-2.38) | NS |
| Nuclear staining syncytiotrophoblast (Percentage of positive nuclei) | 27.50 (18.75-34.38) | 52.50 (10.00-61.25) | 35.00 (18.75-61.25) | 38.75 (23.75-48.12) | NS |
| Cytoplasm staining syncytiotrophoblast (intensity score) | 1.00 (0.62-1.75) | 0.00 (0.00-0.75) | 2.00 (1.00-2.00) | 0.75 (0.12-1.00) | 4,6 |
| Stromal and endothelial cells (intensity score) | 2.25 (1.25-2.88) | 2.00 (1.00-2.00) | 3.00 (2.00-3.00) | 2.00 (2.00-2.75) | 4 |

Differences statistically significant ($p<0.05$): 1) FGR v.s. SGA; 2) FGR v.s. AGA; 3) FGR v.s. LGA; 4) SGA v.s. AGA; 5) SGA v.s. LGA; 6) AGA v.s. LGA.

*Figure 6- Correlations between the fetal weight expressed in MoM and different parameters analyzed in the male sex (in the figure only the correlations with rho greater than 0.2 using the Spearman test are shown). Acronyms: MVM = maternal vascular malperfusion, FGR = fetal growth restriction.*

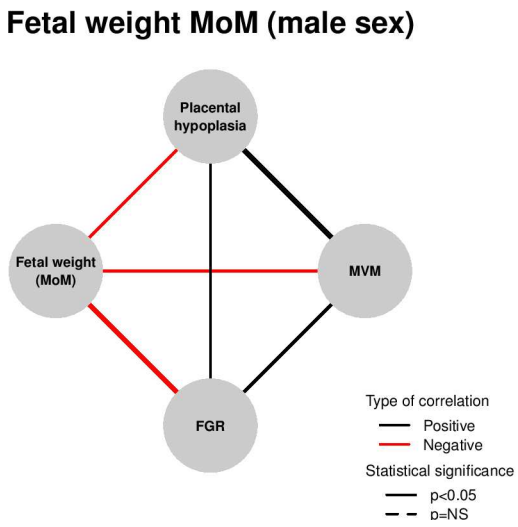



*Figure 7- Correlations between the fetal weight expressed in MoM and different parameters analyzed in the female sex (in the figure only the correlations with rho greater than 0.2 using the Spearman test are shown). Acronyms: FGR = fetal growth restriction.*

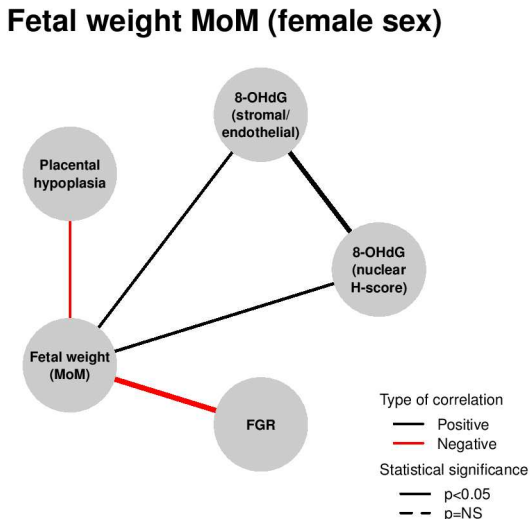


*Figure 8- Correlations between the 8-OHdG scores and different parameters analyzed in the male sex (in the figure only the correlations with rho greater than 0.2 using the Spearman test are shown).*

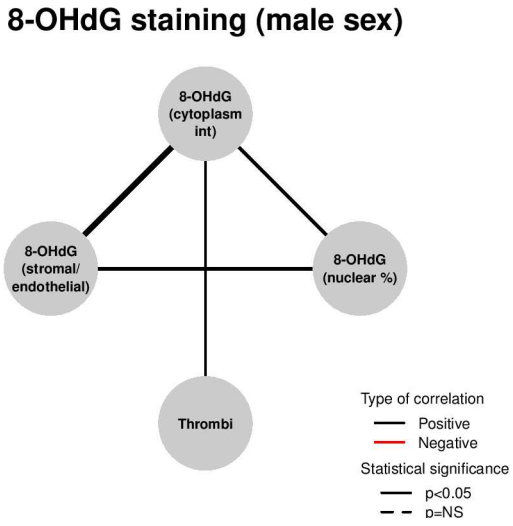



*Figure 9- Correlations between the 8-OHdG scores and different parameters analyzed in the female sex (in the figure only the correlations with rho greater than 0.2 using the Spearman test are shown). Acronyms: MVM = maternal vascular malperfusion.*

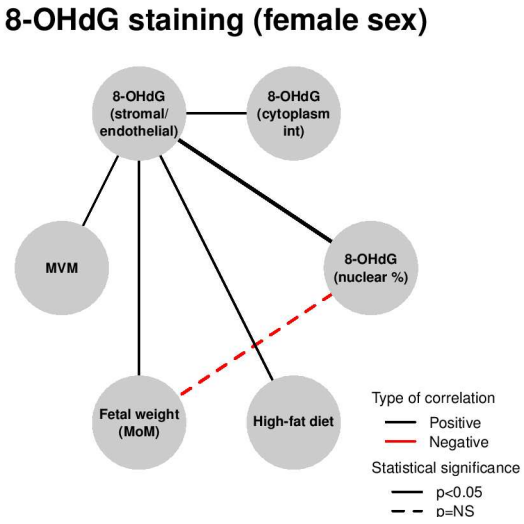


*Figure 10- Correlations between the high-fat diet and different parameters analyzed in the female sex (the figure shows all the correlations using the Spearman test).*

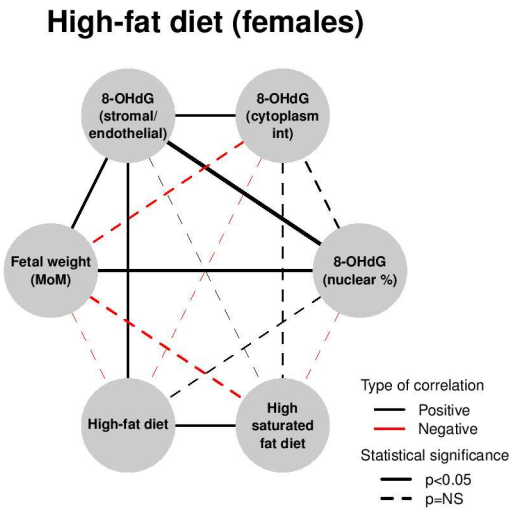
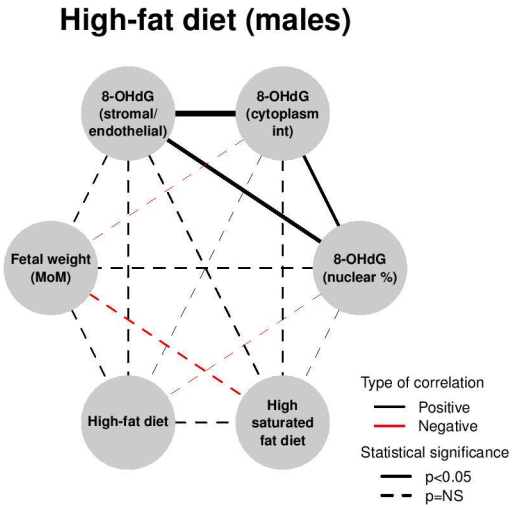



*Figure 11- Correlations between the high-fat diet and different parameters analyzed in the male sex (the figure shows all the correlations using the Spearman test).*



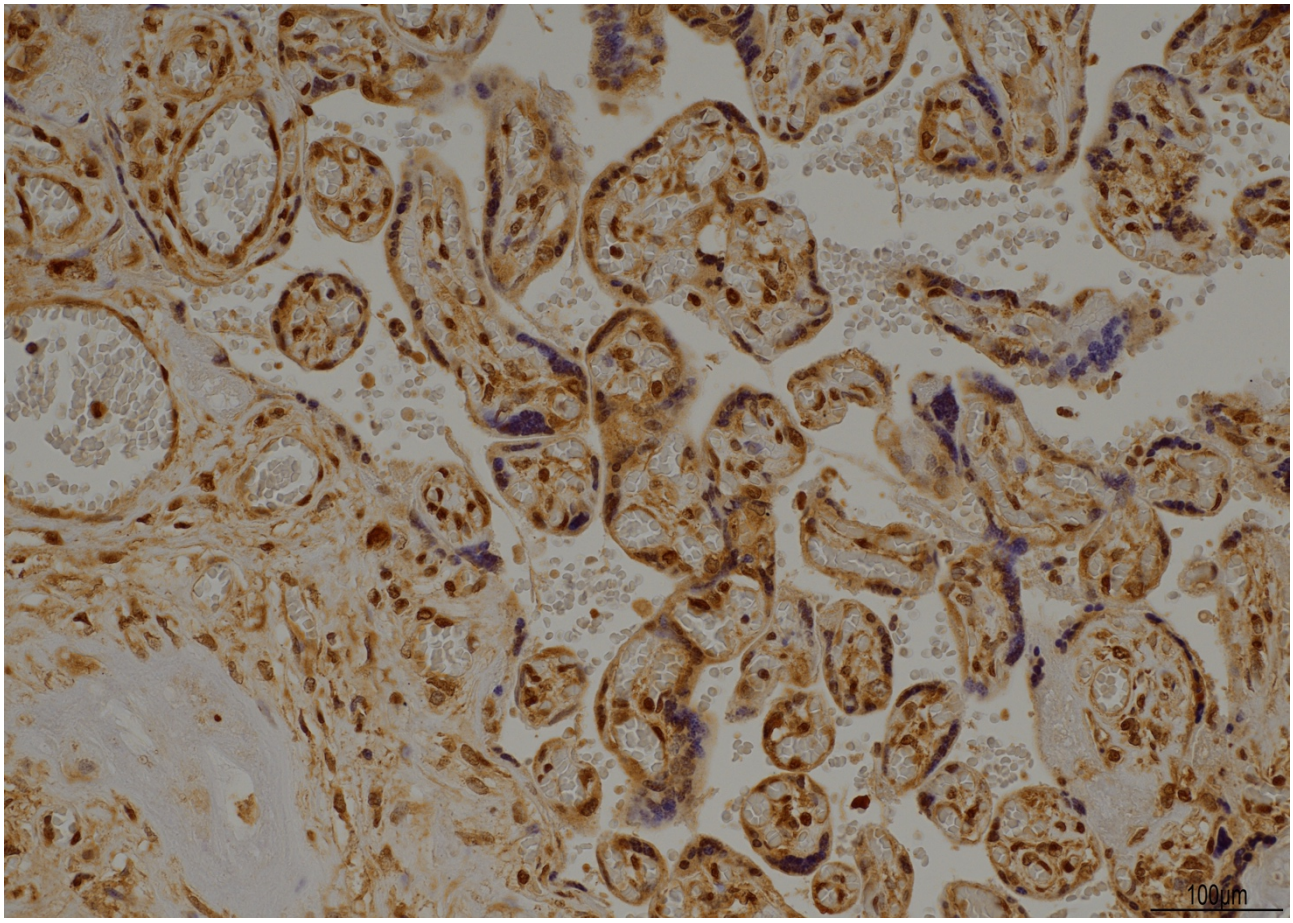



**Figure 12**: this section of placental parenchyma shows an overall low 8OH-dG immunoreactivity. Either the syncytiotrophoblast and the stromal and endothelial cells are poorly stained, thus reflecting a low grade of oxidative stress.

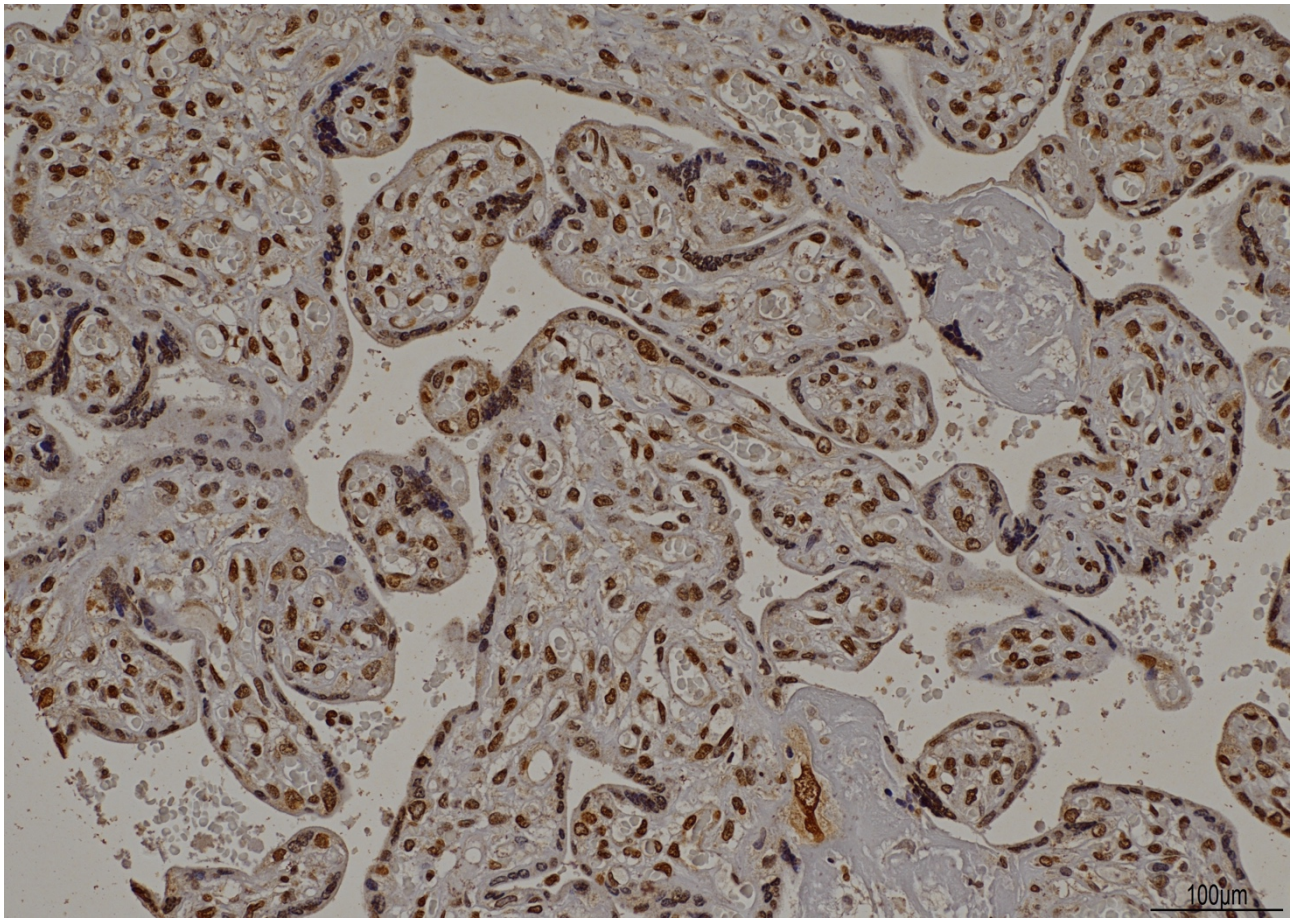



**Figure 13**: this section of placental parenchyma shows an overall intermediate 8OH-dG immunoreactivity.

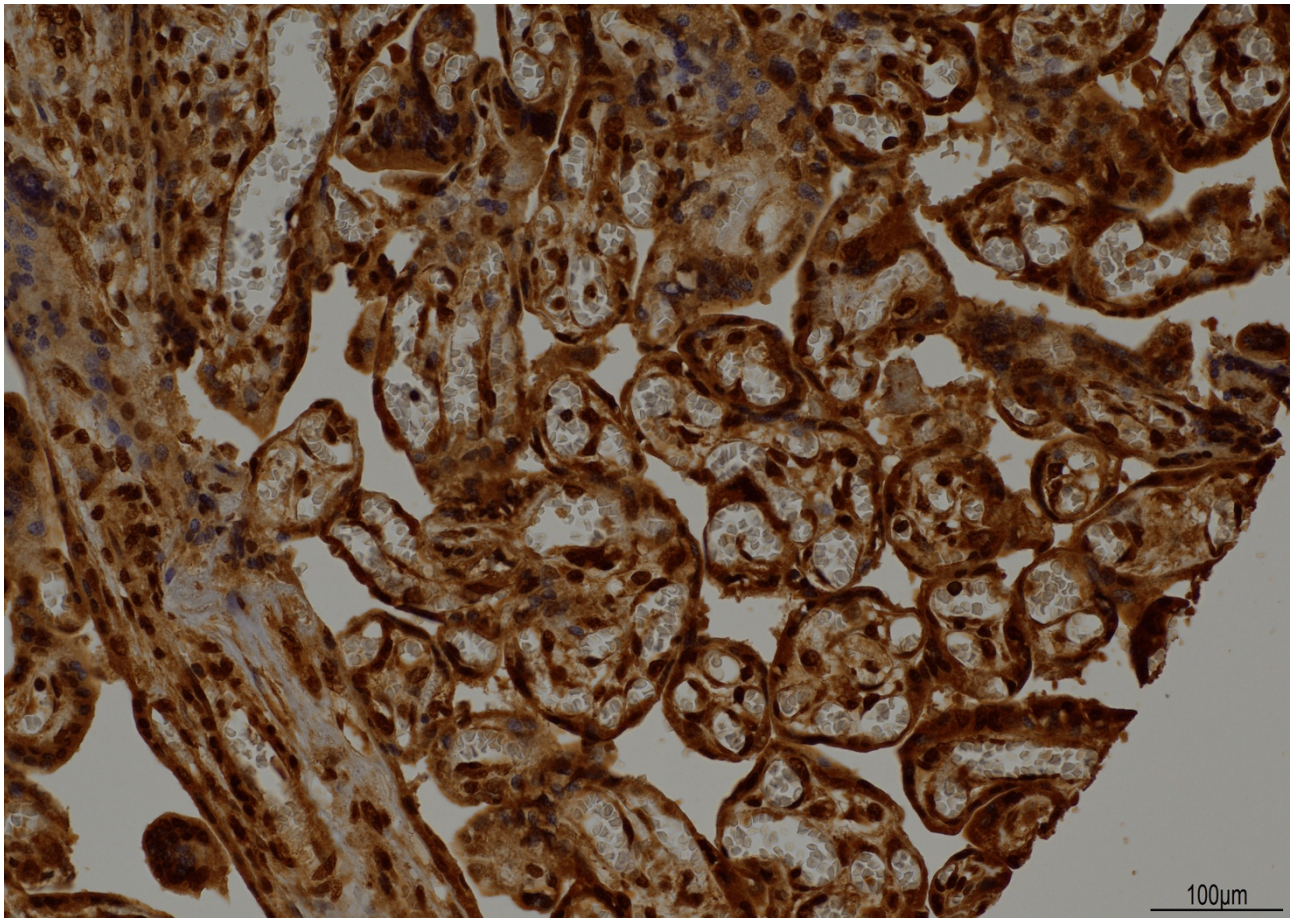



**Figure 14**: this section of placental parenchyma shows a high grade of 8OH-dG immunoreactvitiy. In this image there is strong evidence of oxidative damage in syncytiotrophoblast as well as in stromal and endothelial cells.

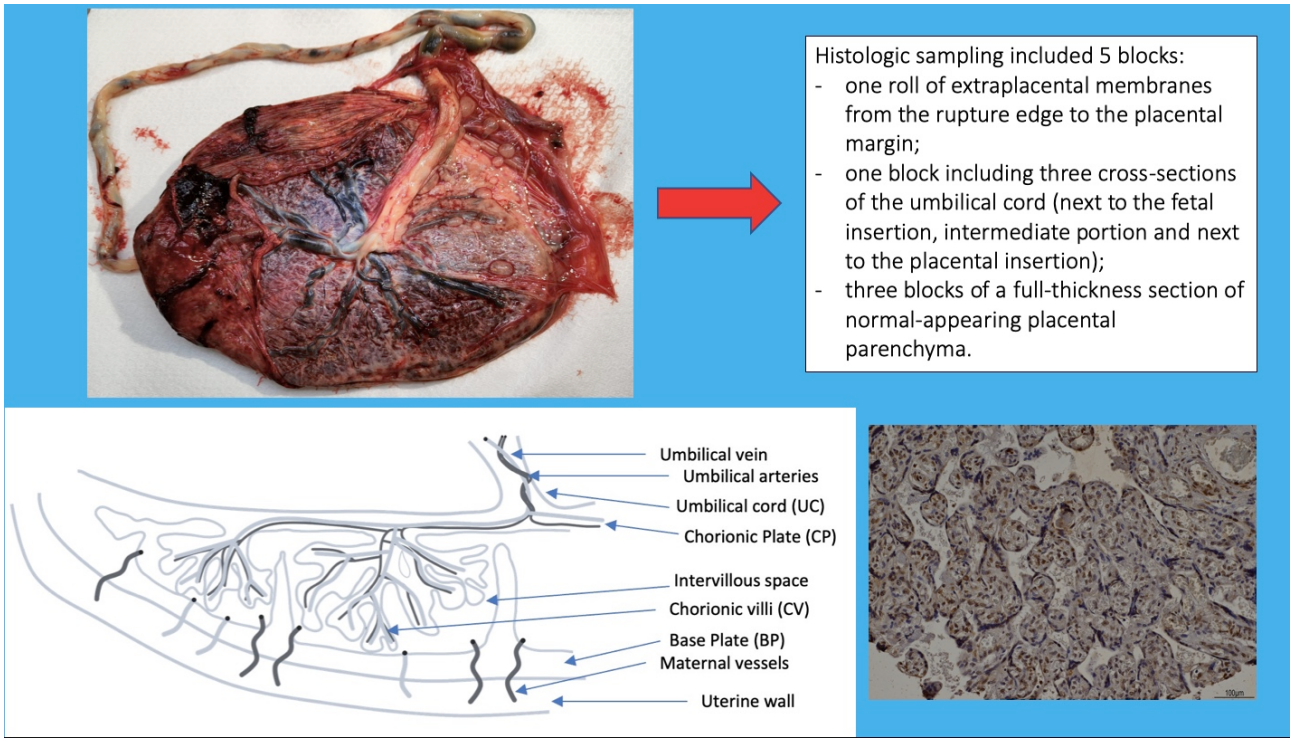



**Figure 15** shows how standardized was the sampling of placental tissue. The procedure followed the criteria included in the Amsterdam Placental Workshop Group Consensus Statement

## Comment

### Main results

This study showed a sex specific pattern of 8OH-dG expression in placentae of single term pregnancies, with higher expression of 8OH-dG in the nuclei of syncytiotrophoblast cells and in stromal and endothelial cells among AGA males compared to AGA females. Secondly, we found a sex difference in the histological pattern among the late FGR placentae, with males exhibiting placental lesions from either maternal and fetal malperfusion, while females displaying only placental lesions from maternal malperfusion. Finally, male late FGR showed a significant correlation between high intensity 8OH-dG staining in cytoplasm of syncytiotrophoblast cells and thrombi in chorionic plate or villi, whereas female late FGR had a significant correlation between high intensity 8OH-dG staining within endothelial and stromal cells and high values of birthweight MoM.

### Results in the context of what is known

#### Sex specific immunohistochemical pattern within placentae

The syncytiotrophoblast is the epithelial covering of the placental villous tree and is a multinucleated, terminally differentiated syncytium. It originates from the continuous fusion of underlying cytotrophoblast cells. It has been demonstrated that the number of syncytiotrophoblast nuclei increases ninefold from the end of first trimester to term of gestation [118] and consequently nuclei within the syncytium are of different ages, depending on their time of incorporation. Syncytiotrophoblast cells are particularly vulnerable to oxidative stress because of its intimate relationship with maternal blood and low amount of antioxidant activity [119].

Oxidative stress is defined as a condition in which the generation of highly reactive species of oxygen overwhelms a cell's capacity to detoxify them, leading to indiscriminate damage to any biological molecules in the immediate vicinity, including proteins, lipids, and DNA [102]. Consequently, cell function is impaired, and in the most severe cases cell death may be induced. ROS are generated physiologically as an inevitable by-product of both enzymatic and non-enzymatic activities [120, 96]. The principal source under normal conditions is the mitochondria, since electrons leakage occurs during their passage along the complexes of the electron transport chain [121, 96]. The acquisition of an unpaired electron generates the superoxide free radical, and 2% of oxygen consumed during quiet respiration is converted to superoxide. This acts as a

signaling intermediate, regulating the activity of redox-sensitive transcription factors to maintain metabolic homeostasis in accordance with the prevailing oxygen concentration. However, because of its potential harmful actions, excess superoxide is detoxified in the mitochondria by the enzyme superoxide dismutase through conversion to hydrogen peroxide, which can diffuse out from the mitochondria being further detoxified within the cytoplasm [96]. 8OH-dG is a biomarker for oxidative DNA damage that is formed by hydroxyl radicals on guanosine bases in DNA [122], thus facilitating the identification of damaged nuclei in syncytiotrophoblast cells.

Contrary to our expectations, we did not find a proportional relationship between higher degree of oxidative damage within placentae and decreased fetal growth. However, we surprisingly found a clear sexually dimorphic pattern of placental oxidative stress of term normally grown babies, with males exhibiting a significantly higher staining for 8OH-dG in syncytiotrophoblast nuclei as well as in stromal and endothelial cells than females. According to the literature, syncytiotrophoblast is a kind of steady state tissue with components existing in between cyto-syncytial fusion and syncytial shedding. More specifically, cytotrophoblast units proliferate, then fuse into the syncytiotrophoblast and ultimately its senescent nuclei and organelles are released into maternal circulation. It has been recently proposed that the rate of progression through the intrasyncytial phase is sex specific regulated. Barapatre et al reported a higher density of non-proliferating nuclei in villous trophoblast of females rather than males, thus advocating a slower rate of progression from the cyto-syncytial fusion to the syncytial shedding [123]. Due to longer permanence within the intrasyncytial phase, the organelles are exposed to increased oxidative damage in females. As opposed to these findings, we observed a higher density of damaged nuclei in syncytiotrophoblast cells in males, suggesting that oxidative stress labels male placentae preferentially. Additionally, male placentae in our study showed oxidative stress to be not only confined to the syncytiotrophoblast but to be also present in stromal and endothelial cells, thus advocating for a broader oxidative stress involvement. We believe that this difference between sexes could be attributed to the accelerated growth rates and increased growth outcomes of males. In fact, the current literature indicates a male-specific preference for growth signaling pathways throughout gestation, whereas females appear to prioritize pathways that increase feto- placental compliance and placental reserve capacity [124].

**Sex specific histological pattern within FGR placentae**

Our study reveals that the histological alterations more often reported in placentae of late FGR fetuses are due to maternal malperfusion, specifically placental hypoplasia and accelerated villous maturation. After having stratified the results according to sex, we observed lesions form maternal malperfusion (accelerated villous maturation) among females, and lesions from either maternal and fetal malperfusion (avascular villi) among males.

Maternal vascular malperfusion (MVM) is the histopathologic consequence of extravillous trophoblast's failure to implant deeply in the uterus and remodel the spiral arteries in early pregnancy [125]. Before the Amsterdam Placental Workshop Group Consensus MVM was termed as utero-placental insufficiency. Most investigators in the field agree that insufficient uterine vascular remodeling leads to abnormal placental perfusion, reducing placental growth, contributing to oxidative stress, and increasing the risk of premature uteroplacental separation [126]. The key features of MVM are decidual arteriopathy, accelerated villous maturation, villous infarction and abruptio placenta. Usually, the adverse outcomes associated with MVM include stillbirth, fetal death, FGR and preterm delivery [127, 125].

Fetal vascular malperfusion (FMV) FVM is a recognized cause of stillbirth and fetal death and central nervous system injury. Although having a multifactorial etiology, FMV is most commonly caused by obstructed umbilical blood flow [128]. Obstruction can lead to vascular stasis, which promotes fetal vascular thrombosis. Less commonly, fetal thrombi develop because of a prothrombotic state or damage to the endothelium or vessel wall, but even in these circumstances, stasis may be a contributing factor. Histopathologic findings in FVM include luminal thrombi, alterations to the wall of the large fetal vessels, and a significant number of downstream avascular villi. When these obstructions become critical, a generalized decrease in blood flow occurs leading to vascular degeneration in the most dependent portions of the villous tree [129].

A large number of studies have shown that male fetuses are more susceptible to adverse pregnancy conditions, showing a higher incidence of preterm birth, low birth weight, and adverse neonatal outcomes [130-133]. This suggests that male and female fetuses and neonates institute different strategies to cope with an adverse environment. For example in pregnant women with mild to moderate asthma, who did not use inhaled steroids, female fetuses were observed to have a significantly reduced birthweight, whereas male birthweights were unaffected [134]. The same trend was observed with preeclampsia, with normal growth trajectories in male fetuses, and

reduced growth patterns in female fetuses [135,136]. This evidence indicates that males and females respond to an adverse environment differently: while males continue to grow normally and are more vulnerable when a second stressful event occurs, females reduce their growth in order to diminish their energy expenditure. Female fetuses are thus more prone to survive any further compromises in the intrauterine environment to nutrition or oxygen supply as the pregnancy progresses.

Recently Chatterjie et al published the first study on the link between sex specific human placenta transcriptome and SGA outcomes. The Authors found that distinct molecular pathways are involved in the etiological mechanisms of SGA, with male-specific different expressed genes correlated with pathways of immune response and inflammation but female-specific different expressed genes correlated with pathways of cellular/organ growth and development [137].

In this scenario, it might be plausible to observe sex differences even in placental lesion patterns. In our study late FGR among males have more commonly maternal and fetal malperfusion lesions, whereas late FGR among females displayed only maternal malperfusion lesions. How can it occur? It might be speculated that, when the nutrient and oxygen supply is mild-moderately compromised such as in late fetal growth restriction, placentae of female fetuses are healthier than their male counterparts. Our data further support the concept of reduced placental reserve capacity in males that likely contribute to increased risks of intrauterine morbidities and mortality in the presence of an adverse maternal environment, when compared with females exposed to similar in utero conditions.

**Sex specific correlations between IHC and histology**

We observed a significant correlation between high intensity 8OH-dG syncytioplasm immunoreactivity and chorionic plate or villous thrombi among male late FGR, and between high intensity 8OH-dG immunoreactivity within endothelial and stromal cells and high values of birthweight MoM among females. Chorionic plate or villous thrombi originate from fetal malperfusion and might lead to increased oxidative stress. However, according to recent evidence it seems that the human placenta controls even the antioxidant capacity in a sexually dimorphic manner [138]. For example, it has been described that placentae of male fetuses in obese mothers have a significant decrease in enzymatic antioxidants [139]. If confirmed in non-obese mothers this mechanism together with the hypoxia generated by villous thrombi might explain the increased oxidative damage in the syncytioplasm. So far, a growing body of evidence indicates that females

preferentially express pathways, glucocorticoid mediated, that permit to increase their placental reserve capacity in order to better cope with insults, but at the expense of a reduced growth trajectory. Whereas in males the growth trajectory is sustained by pathways androgens mediated, that increase their intrauterine morbidity and mortality risk.

Recently, a research group analyzed the transcripts of cytotrophoblasts, syncytiotrophoblast, arterial and venous endothelial cells, which were isolated from male and female placentae. This research group found that sex differentially affected gene expression among all cell-phenotypes. More specifically, transcripts of male fetuses predominated in the epithelial compartment, represented by cytotrophoblasts and syncytiotrophoblasts, whereas the endothelial compartment, represented by arterial and venous endothelial cells, showed more female-biased genes. This might explain the higher oxidative damage retrieved among endothelial cells in female placentae as far as the female fetus is growing more [140].

**Clinical implications**

Our data provide evidence that sex is a biologic factor that influences fetal intrauterine growth and ultimately fetal birthweight. These data together with the observation that at term males weigh about 140 gr more than females [141] may suggest the usefulness of customized fetal growth charts in antenatal ultrasound monitoring. Of note, a vey recent French research aiming at evaluating the different performance between unisex and sex-specific estimated fetal weight charts during the third trimester ultrasound in detecting SGA newborns, found that the use of sex-specific charts would significantly reduce sex bias in intrauterine growth screening [142]. Prospective studies on the effect of specific charts rather than unisex charts are warranted in order to clarify this important issue, that have reverb on the daily clinical practice.

**Implications for research**

Future studies should assess the gene expression in placenta in order to understand what are the main cellular pathways involved in fetal growth under optimal and compromised condition in terms of nutrient and oxygen delivery to the fetus. Moreover, when these studies will be carried out, it should be emphasized the need to stratify according to fetal gender.

**Strengths and limitations**

Several strengths should be recognized in this study. First of all, women's enrollment occurred in a prospective way, which means that variables and outcomes were determined before the study beginning, and that patients were assessed at baseline and then followed in time to study the fetal growth pattern. Secondly gross examination and histologic sampling of the placenta was accurately performed in a standardized manner, according to the recommendations included within the Amsterdam Placental Workshop Group Consensus Statement [41]. Third, maximization of tissue resources has been reached through TMA. This method allowed to relocate tissue from conventional histologic paraffin blocks in a manner that tissue from multiple blocks could be seen on the same slide.

Nevertheless, our study is not exempt from limitations. The main limitation to be acknowledged is the low number of late FGR, SGA and LGA neonates, compared to AGA. As a result, the comparisons between the different fetal growth categories might have not been uniformly balanced, especially after stratification according to fetal sex.

**Conclusions**

To conclude, our data suggest that oxidative stress may not be the only pathway involved in the pathophysiology of fetal smallness at term of gestation. Furthermore, our immunohistochemical and histological findings revealed an unexpected difference in the oxidative stress pattern between male and female placentae, suggesting that fetal growth is differently regulated among the two sexes.

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**ADDENDUM: Preliminary data of RNA-seq analysis in placental sample tissues.**

## Introduction

Oxidative stress is known to be a key factor in the pathophysiology of fetal smallness. The starting point of this thesis was to explore whether the oxidative stress was increased in fetal smallness at term of gestation. Contrary to our expectations, we did not find a direct relationship between higher degree of oxidative damage within placental cells and decreased fetal growth. To investigate this unexpected result, we decided to analyze the placental samples of a predetermined number of pregnancies through the transcriptome analysis (RNA-seq).

In recent years, RNA sequencing (RNA-seq) has fundamentally changed the study of cell function. It offers researchers unprecedented insight into the entire transcriptional profile of cells. Using a high-throughput technique and next-generation sequencing, the expression levels of all transcripts in a cell are measured. This approach allows researchers to identify genome-wide differentially expressed genes (DEG) and their clustering into biological pathways and molecular mechanisms that provide key information about normal and disease status.

## Material and methods

This part of the study was carried out in collaboration with the Laboratory of Biochemistry of the DAME department. The placental tissue samples were divided into three categories according to the fetal growth at term. The first category included placentae of appropriate for gestational age (AGA) babies, i.e. our controls, the second category included placentae of small for gestational age neonates (SGA with birthweight under 10° percentile) and the third category encompassed late fetal growth restricted babies (also named SGA3, having a birthweight below the 3° percentile). Twenty-four placental tissue samples were subjected to the analysis: 10 AGA (5 females and 5 males), 10 SGA < 10 (5 females and 4 males) and 5 late FGR (2 females and 3 males) (Table 1).

| | AGA | SGA 10 | Late FGR (SGA3) |
|---|---|---|---|
| Number of cases | 10 | 9 | 5 |
| Female cases | 5 | 5 | 2 |
| Male cases | 5 | 4 | 3 |

**Table 1** shows the number of cases subjected to RNA seq analysis divided according to the fetal growth category (AGA, appropriate for gestational age, SGA 10, small for gestational age with a birthweight under 10° percentile and late FGR defined according to Delphi's Criteria). For each growth category the number of males and females are indicated.

Total RNA was extracted using Phenol/Chloroform standard extraction protocol. One ug of total RNA was DNAseI in-column digested following manufacturer's instructions (ZymoResearch, RNA Clean & Concentrator-5). rRNA was depleted by using Ribo-Zero Plus (Illumina). RNA fragmentation by RNase III (Ambion) was performed at 37 °C. First-strand cDNA was generated using random hexamer-primed reverse transcription, followed by a second-strand cDNA synthesis. The synthesized cDNA was end-repaired and 3' adenylated following BGI platform requirements. Adapters were ligated to the ends of these 3' adenylated cDNA fragments. PCR products were purified with Ampure XP Beads (AGENCOURT), and dissolved in EB solution. Library was validated on the Agilent Technologies 2100 bioanalyzer. The double stranded PCR products were heat denatured and circularized by the splint oligo sequence. The single strand circle DNA (ssCir DNA) were formatted as the final library. The library was amplified with phi29 to make DNA nanoball (DNB). The DNBs were load into the patterned nanoarray and paired end 100 bases reads were generated in the way of combinatorial Probe-Anchor Synthesis (cPAS).

**Results**

Out of 24 samples 3 samples were excluded from the RNA-seq analysis, because of their poor quality. The remaining 21 samples were considered to have an optimum quality. According to the threshold log2 FC $\geq$ 1and $P < 0.05$ for upregulated genes and log2 FC$\leq$ -1 and $P < 0.05$ for downregulated genes, our RNA-seq analysis revealed that:

| | **DEG** | **Upregulated** | **Downregulated** |
|---|---|---|---|
| **Late FGR vs AGA** | 662 | 190 | 472 |
| **SGA10 vs AGA** | 347 | 245 | 102 |
| **Late FGR vs SGA10** | 500 | 54 | 446 |

**Gene ontology enrichment analysis**

A preliminary gene ontology (GO) enrichment analysis showed that several metabolic pathways are downregulated in late FGR babies compared to the normal AGA newborns. In Figure 1 we report the downregulated metabolic pathways. The metabolic pathways upon which we will focus our attention, because they are likely to be linked with late FGR babies, are: i) Regulation of epidermal growth factor receptor signaling pathway; ii) mRNA export from nucleus; (iii) Protein metabolism; (iv) Control of redox homeostasis; (v) Cell death in response to oxidative stress.

**Nrf2 gene: the master regulator of redox homeostasis in eukaryotic cells**

An aspect of great interest revealed by the RNA-seq analysis is that the late FGR group had a significantly higher expression of the Nrf2 (or Nfe2l2) gene compared to the control group AGA (about 2-fold change, i.e. Nrf2 is 2-fold more expressed in late FGR than in AGA, $P<0.05$). The analysis shows that also NQO1, an antioxidant gene that is activated by Nrf2, is upregulated in the in late FGR babies, while HMOX is not. Other antioxidant genes have been found upregulated: HMOX2, CYP26A1 and ALDH16A1. In line with our results, we found in the *Human Protein Atlas* that Nrf2 is expressed, and probably exerts a key function, in the tissue group composed by Ovary, Fallopian tube, Endometrium, Cervix, Placenta and Breast. We therefore hypothesize that Nrf2 expression can be correlated with late FGR babies. A number of studies reported in the literature in the last decade support the notion that Nrf2 not only regulates the redox homeostasis (i.e. the level of oxidative stress) but also induces a metabolic reprogramming in tissues under stress conditions. Given the critical role of this gene also in the placenta, I briefly summarize here the mechanism through which it brings down the level of oxidative stress.

It is known that in eukaryotic cells oxidative stress is under the control of the nuclear factor Erytroid 2-Related Factor 2 (Nrf2) [1,2]. This protein is a redox-sensitive transcription factor that regulates the expression of antioxidant genes. These genes contain the antioxidant response element (ARE) in their promoter, which is recognized by Nrf2. Under increased oxidative stress, Nrf2 is upregulated and able to bind to ARE-related genes, promoting an antioxidant response that reduces the level of oxidative stress [3]. High levels of oxidative stress are harmful to cells and are reduced by Nrf2. Indeed, knocking down Nrf2 in mice increases susceptibility to a wide range of chemical toxicity, while overexpression of Nrf2 in cell lines dramatically drops the level of

ROS. The mechanism of activation of Nrf2 can be summarized as follows. Under normal redox conditions, cytoplasmic Nrf2 is bound to Keap1/Cul3 and the Nrf2:Keap1/Cul3 complex is ubiquinated and proteosomally degraded [4]. In this way Nrf2 does not have any redox function in the cell. However, when cellular ROS increases, ROS oxidize the cysteine residues of Keap1 inducing a conformational change in the protein that loses its affinity for Nrf2. The Nrf2:Keap1/Cul3 complex dissociates and free Nrf2 is able to translocate to the nucleus and activate the ARE-regulated genes (REF). The process is illustrated in figure 3. Other antioxidant genes have been found upregulated: HMOX2, CYP26A1 and ALDH16A1.

1. CYP26A1, which encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases that catalyze many detoxification reactions. It is also involved in the synthesis of cholesterol, steroids and other lipids. This enzyme also regulates the cellular content of retinoic acid, which is involved in the regulation of gene expression in both embryonic and adult tissues.
2. ALDH16A1 encodes a member of the aldehyde dehydrogenase superfamily. Members of the family act on aldehyde substrates and use nicotinamide adenine dinucleotide phosphate (NADP) as a cofactor.
3. HMOX2, heme-oxygenase, an essential enzyme in heme catabolism, cleaves heme to biliverdin, which is subsequently converted to bilirubin by biliverdin reductase, and to carbon monoxide, a putative neurotransmitter. The activity of heme oxygenase is induced by its substrate heme and by various non-hem substances.
4. NQO1 is a member of the NAD(P)H dehydrogenase (quinone) family and encodes a cytoplasmic 2-electron reductase. This protein's enzymatic activity prevents the one electron reduction of quinones that results in the production of radical species. Gene Ontology annotations related to this gene include *RNA binding* and *oxidoreductase activity*.

**Figure 1** shows the Gene Ontology (GO) enrichment analysis of genes downregulated in late FGR babies.

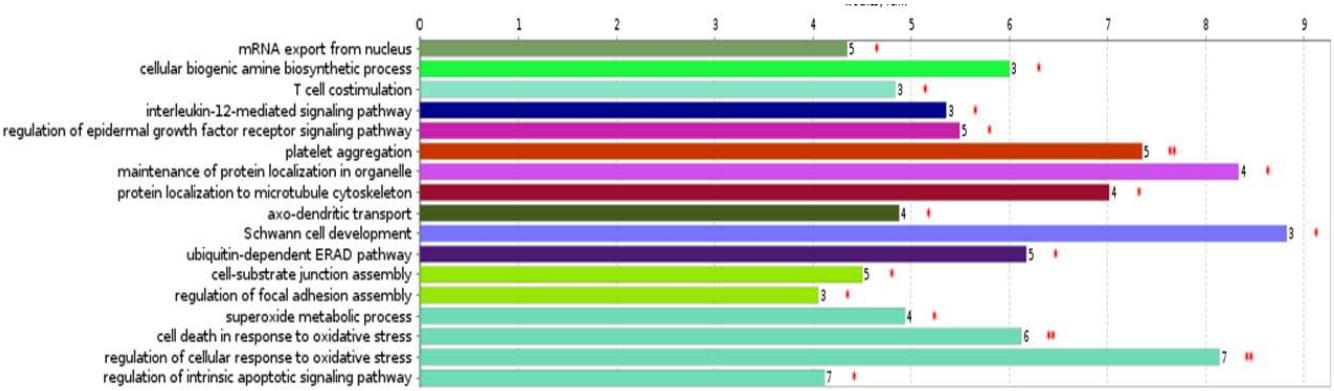
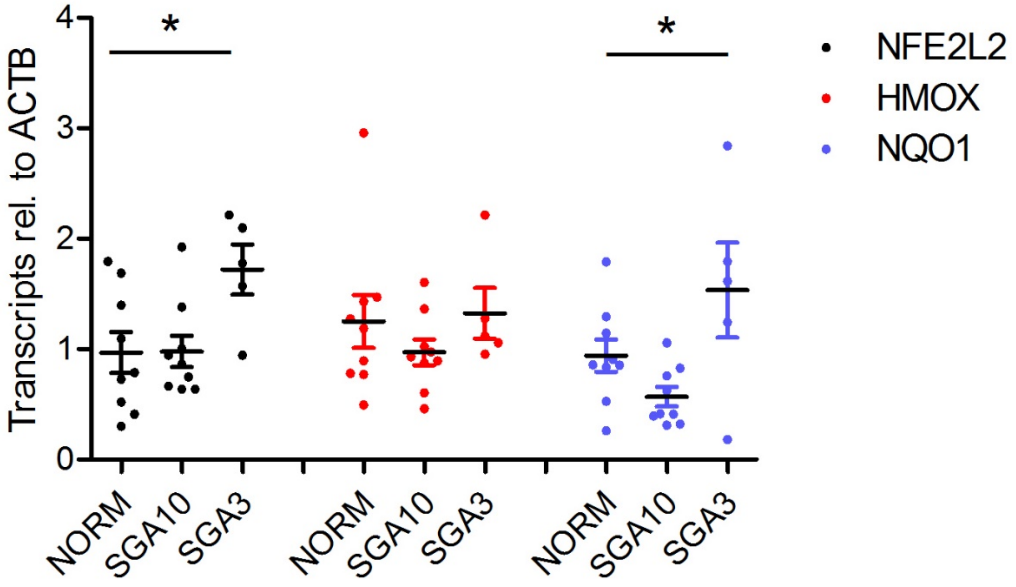



**Figure 2** shows the level of expression of Nfe2l2 (or Nrf2), HMOX and NQO1 within the placental cells of the three groups of fetal growth.



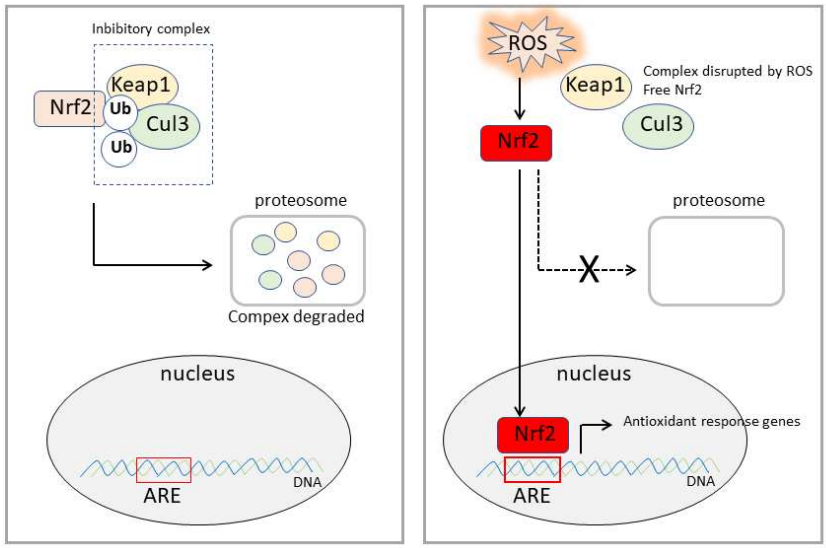



**Figure 3** shows the mechanism of activation of Nrf2. In basal conditions Keap1 binds to Nrf2 and Nrf2 is polyubiquitylated by Cul3 complex. This poly-ubiquitilation results in rapid Nrf2 degradation by the proteasome. A small proportion of Nrf2 escapes the inhibitory complex and accumulates in the nucleus to mediate basal ARE-dependent gene expression, thereby maintaining the cellular homeostasis. By contrast, under stress conditions, Keap1cysteines are oxidazied, thus leading to the inhibition of Nrf2 ubiquitylation via dissociation of the inhibitory complex. So, Nrf2 translocates into the nucleus, binds to the Antioxidant Response Element (ARE) and drives the expression of Nrf2 target genes.

**Concluding remarks**

At the moment, we are still elaborating our data. It is our intention to analyze all the dysregulated metabolic pathways characterizing late FGR babies, in order to define the "transcriptome profile" of babies affected by late growth restriction and how it is different from the transcriptome of larger babies. This is a unique opportunity to comprehensively explore the human placenta and to characterize the baseline placental transcriptional landscape that defines the patterns observed in different fetal growth trajectories. However, this process requires time and still a multidisciplinary work, with a pivotal role of biochemists and bioinformaticians. Therefore, we plan to complete the whole work within the end of the following year.

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*I remember that during the entrance exam for this PhD, one member of the committee asked me: why do you want to do this PhD? What is your goal, your objective? At that moment, I felt quite confused, I couldn't find the best reason to explain my ambition. Now, after three years, I can say that I'm grateful for this PhD. I learned so much in these three years. I improved my English, I read and wrote a lot, I published with Ambrogio and I felt progressively more comfortable standardizing my work method and elaborating what the clinical data suggest. On this occasion, I had the opportunity to realize a multidisciplinary project, thus facing some challenges and learning how to organize a job that involves other equips, other disciplines. In other words, thanks to this PhD I had the opportunity to learn. Learning for me is a mainstay of my life. As Albert Einstein stated once: "Intellectual growth should commence at birth and cease only at death".*