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Role of Inositols pathway and Nanoplastics in placenta. Effect on placental villous tissue and trophoblast cells

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ABSTRACT

Background: Maternal obesity in pregnancy set an adverse intrauterine environment, leading to altered fetal programming in utero and modification in placenta's cell functions, causing to the fetus later onset of adult disease. On this ground, Inositols (INO) are insulin-sensitizing agents that have been shown to improve insulin sensitivity in women with obesity, gestational diabetes, and metabolic syndrome. Moreover, nano plastics (NPs) contaminants in the environment can act as endocrine disruptors, altering normal functions of placental cells, and, possibly, causing damage to the fetus.

Hypothesis: We hypothesized that intracellular INO pathway is altered in obese placenta and INO supplementation modulate placental functions. NPs treatment in vitro might affect trophoblast cellular activity leading to placental dysfunctions during gestation.

Study design: In a non-randomized experimental study, thirty-four placental explants were collected from uncomplicated obese (OB) and normal weight (CTRL) women undergoing elective cesarean section, at term. Tissues were snap frozen in liquid nitrogen. Critical proteins involved in INO intake (SMIT1, SMIT2), synthesis (ISYNA) and calcium-release pathway (ITPR1 and CN) were analyzed. Proteins levels were analyzed by Western Blot (WB). Placental villous tissue was used for INO soluble quantification between the two groups by colorimetric assay. To investigate the effect of INO treatment on placental cells, a trophoblast human cell line (HTR8/SVneo) was used for in vitro experiments. Proliferation assay study was performed after 24h of INO stimulation. ITPR1, cleaved active CAS3 and VEGF protein level were quantified after INO stimulation, to investigate on its role in the modulation of placental functions, including cell proliferation, apoptosis and placental vascularization.
The effect of plastic contaminants on placental cells was analyzed via in vitro treatment on HTR8/SVneo cells with NP spheres to study cell morphological alterations and to perform cytotoxicity assay. Fluorescent NPs were used for immunofluorescent analysis for internalization of plastics in placenta.

Results:
Placenta and baby weight showed no differences between the two groups. Importantly, INO content was decreased in obese placentas compared to healthy lean controls. We did not observed modification in uptake, synthesis and catabolism of INO molecules. Intracellular INO pathway in obese placental villous tissue is altered as revealed from ITPR1 and cleaved CN upregulation. In vitro stimulation with INO for 24h showed a decreased in proliferation rate of HTR8/SVneo cell line in a dose dependent way. NPs treatment induced a morphological alteration of trophoblast cells. NPs in vitro stimulation increased cell death and showed a cytotoxic effect on placental cells. NPs were found in the cytoplasm modulating cellular functions.

Conclusions:
While no differences were observed at macroscopic level, we detected alterations in the inositol pathway at the molecular level in the placenta of obese women. This could represent an underlying mechanism in the disease development related to obesity. Inositol supplementation during pregnancy is a promising strategy to counteract placental damage acting on different pathways. Lifelong exposure to nano plastics cause trophoblast cell death and alteration on cellular morphology and might affect placental function and its physiology leading to abnormal maternal and neonatal health.

Key words: Obesity, Inositol, Placenta, Plastics, Endocrine disruption

Ruolo del pathway dell'inositolo e delle nanoplastiche nella placenta. Effetto sul tessuto villoso placentare e sulle cellule del trofoblasto

ITALIAN ABSTRACT

Background: L'obesità materna in gravidanza crea un ambiente intrauterino avverso, portando ad un'alterata programmazione fetale in utero e alla modificazione delle funzioni cellulari della placenta, causando nel feto una successiva insorgenza della malattia in età adulta. Su questa base, gli inositoli (INO) sono agenti insulino-sensibilizzanti che hanno dimostrato di migliorare la resistenza all'insulina nelle donne con obesità, diabete gestazionale e sindrome metabolica. Inoltre, le nanoplastiche (NP) presenti nell'ambiente possono agire come interferenti endocrini, alterando le normali funzioni delle cellule placentari e, di conseguenza, causare danni al feto.
Ipotesi: Abbiamo ipotizzato che il pathway intracellulare dell'INO sia alterato nelle cellule di placente obese e che il trattamento con INO moduli le funzioni delle cellule placentari. Il trattamento con NP in vitro potrebbe influenzare l'attività cellulare del trofoblasto portando a disfunzioni della placenta durante la gestazione.
Disegno dello studio: Nello studio sperimentale non randomizzato, sono stati raccolti trentaquattro espianti di placenta da donne obese senza complicazioni (OB) e di peso normale (CTRL) sottoposte a taglio cesareo elettivo, a termine. I tessuti sono stati congelati in azoto liquido e conservati a -80°C. Sono state analizzate proteine critiche coinvolte nel pathway di INO (SMIT1, SMIT2), nella sua sintesi (ISYNA) e nella via di rilascio del calcio (ITPR1 e CN). Le proteine totali sono state estratte con RIPA, le loro concentrazioni misurate con il test BCA e quantificate mediante Western Blot (WB). Il tessuto villoso placentare è stato utilizzato per la quantificazione dell'INO tra i due gruppi mediante saggio colorimetrico. Per studiare l'effetto del trattamento con INO sulle cellule placentari, è stata utilizzata una linea cellulare umana di trofoblasto (HTR8/SVneo) per gli esperimenti in vitro. Lo studio del test di proliferazione è stato eseguito dopo 24 ore di stimolazione con INO. ITPR1, CAS3 e il livello di proteina VEGF sono stati quantificati dopo la stimolazione con INO, per indagare sul ruolo di INO sulla modulazione delle funzioni placentari, tra cui proliferazione cellulare, apoptosi e vascolarizzazione. L'effetto di NP sulle cellule del trofoblasto è stato analizzato tramite trattamento in vitro su cellule HTR8/SVneo con sfere NP per studiare le alterazioni morfologiche cellulari e per eseguire test di citotossicità. NPs fluorescenti sono stati utilizzati per l'analisi immunofluorescenti per l'internalizzazione della plastica nella placenta.
Risultati: INO nel tessuto villoso placentare obeso risulta alterato come rivelato da over espressione di ITPR1 e dalla CN clivata. Non abbiamo osservato modifiche nell'assorbimento, nella sintesi e nel catabolismo delle molecole di INO. È importante sottolineare che il test di quantificazione dell'INO ha dimostrato una diminuzione del contenuto di INO nelle placente obese rispetto ai controlli magri sani. La stimolazione in vitro con INO per 24 ore ha mostrato una diminuzione del tasso di proliferazione della linea cellulare HTR8/SVneo in maniera dose-dipendente. Il trattamento con NP ha indotto un'alterazione morfologica delle cellule del trofoblasto e ha aumentato la morte cellulare mostrando un effetto citotossico.
Conclusioni:L'obesità materna in gravidanza ha gravi conseguenze sulle funzioni delle cellule e del tessuto villoso placentare. L'integrazione di inositolo durante la gravidanza è una strategia promettente per contrastare il danno placentare agendo su

diversi percorsi. L'esposizione permanente alle NPs causa la morte delle cellule del trofoblasto e l'alterazione della morfologia cellulare e potrebbe influenzare la funzione placentare e la sua fisiologia portando a condizioni di salute materna e neonatale anormali.

Key words: Obesità, Inositolo, Placenta, Nanoplastiche, Gravidanza

ABBREVIATIONS

OB: obesity

OW: overweight

INO: Inositol

GLUT4: glucose transporter type 4

IR-β: insulin receptor beta

Akt: Protein kinase B

GSK3: glycogen synthase kinase 3

ATP: adenosine triphosphate

GTT: glucose tolerance test

TGF-β: transforming growth factor beta

SBP: systolic blood pressure

BMI: body mass index

GDM: gestational diabetes mellitus

LGA: large for gestational age

SGA: small for gestational age

HTN: hypertension

NO: nitric oxide

VEGF: vascular endothelial growth factor

PCNA: proliferator cellular nuclear antigen

PE: preeclampsia

MI: myo-inositol

DCI: D-chiro inositol

ITPR1: inositol 3 phosphate receptor 1

IRS-1: insulin receptor substrate 1

PI: phosphatidylinositol

PI-3K: phosphatidylinositol 3-kinase

PIP: phosphatidylinositol phosphate

IPGs: inositol-phosphoglycans

IEL: Intraepithelial Lymphocyte

CAS3: caspase 3

CN: calcineurin

NPs: nanoplastics

ISYNA1: inositol-3-phosphate synthase 1

HTR8/SV40: human trophoblast- simian virus 40 large antigen T

DMEM: dulbecco's Modified Eagle's Medium

PBS: phosphate buffer saline

RPMI 1640: media known as Roswell park memorial institute

FBS: fetal bovine serum

P/S: penicillin-Streptomycin

CCK8: cell counting kit-8

DOHaD: Developmental origin of health and disease

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1. INTRODUCTION

1.1 Obesity and overweight in pregnancy

Obesity is becoming a pandemic problem, and it is defined as body mass index (BMI) ≥ 30 kg/m^2 according to the World Health Organization. It is considered as a complex disease and its etiology is a result between genetic, socioeconomic, cultural influences [1].

The estimates for global levels of overweight and obesity (BMI ≥25kg/m2), also referred to as high BMI throughout this Atlas, suggest that over 4 billion people may be affected by 2035, compared with over 2.6 billion in 2020. This reflects an increase from 38% of the world's population in 2020 to over 50% by 2035. Nowadays, half or more than half of the population in the Americas (61.1%), Europe (54.8%), and Eastern Mediterranean (46.0%) are overweight/obese, while a much lower prevalence is observed in Africa (26.9%), South-East Asia (13.7%), and the Western Pacific (25.4%) [2]. World Obesity Atlas 2023 showed the increase incidence of obesity in Italy in the next years as reported in fig 1.

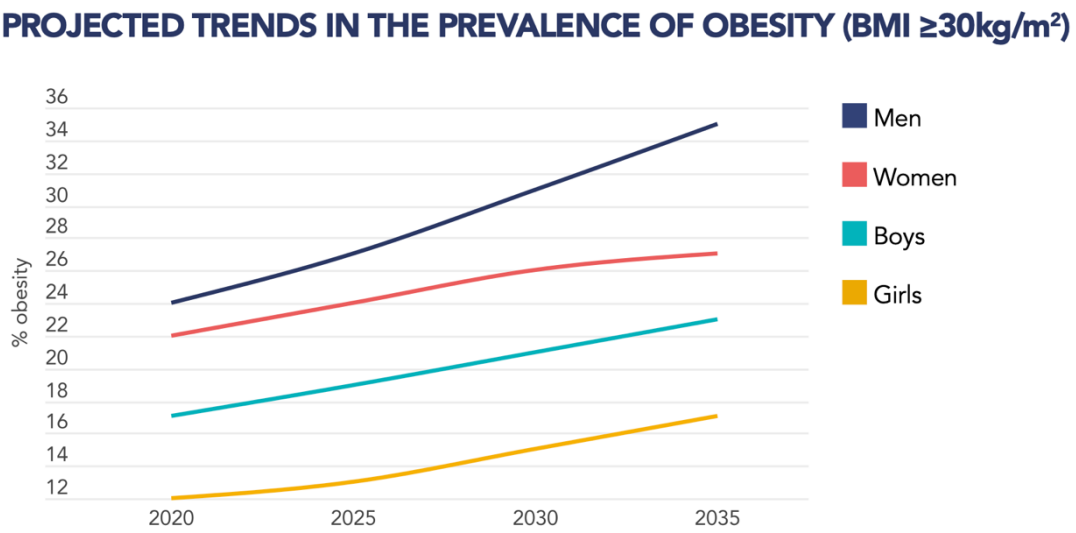


Fig 1. World Obesity Federation, World Obesity Atlas 2023. (https://data.worldobesity.org/publications/?cat=19)

Epidemiological studies performed in the USA, Europe, and Asia found that a high BMI was significantly associated with increased incidence of coronary artery disease (CAD) ischemic stroke and other relevel pathologies [3].

Obese women during pregnancy have an increased risk of maternal death and complications during pregnancy and labor than normal-weight women. In the United Kingdom, the latest Confidential Enquiry into Maternal and Child Health (CEMACH) reported that more than half of the deaths from direct or indirect causes during (late) pregnancy or labor were in overweight or obese women [4].

Obesity is associated with increased risk of almost all pregnancy complications such as gestational hypertension, preeclampsia, gestational diabetes mellitus (GDM), delivery of a large for gestational age (LGA) infant, and a higher incidence of congenital defects [5,6]. Cesarean section rates are also much higher, and anesthesia may be a serious problem in such cases. Notable exceptions are gastroschisis and spontaneous preterm labor, both of which occur less often. In addition, obese women often have difficulties in initiating and sustaining breastfeeding [7].

Given the negative consequences of excess weight in pregnant women, appropriate weight gain during pregnancy becomes crucial for maternal and fetal health. In 2009, in response to the ongoing increase in obesity in the United States, the Institute of Medicine (IOM) issued new recommendations on weight gain during pregnancy. The IOM's new guidelines are based on a reevaluation of the 1990 guidelines with some

exceptions: they consider the BMI categories of the WHO and recommend a restrictive range of weight gain for each category, emphasizing that weight loss during pregnancy is never advisable [8]. Table 1 shows the recommended weight gains by the IOM based on pre-pregnancy BMI.

Pre-pregnancy BMI	Total Weight Gain (kg)	Weight Gain 2nd-3rd Trimester (kg/week) (range)
Underweight (< 18.5 kg/m²)	12.5-18	0.51 (0.44-0.58)
Normal weight (18.5-24.9 kg/m²)	11.5-16	0.42 (0.35-0.50)
Overweight (25.0-29.9 kg/m²)	7-11.5	0.28 (0.23-0.33)
Obese (≥ 30 kg/m²)	5.9	0.22 (0.17-0.27)

Table 1. Recommendations for Gestational Weight Gain Based on Pre-pregnancy BMI

In this context, inositol diet supplementation represents a novel therapeutic strategy to overcome pregnancy obesity related conditions.

2 Placenta

The placenta is the main organ that interface between mother and fetus medium through which material passes from the maternal circulation to the fetal circulation by passive diffusion or active transport. It is one of the most important players, which determine pregnancy success. The core function of the placenta is to mediate the transport of nutrients between maternal and fetal circulation and it has critical endocrine functions that alter different maternal physiological systems to sustain pregnancy. Its development and functions can be active regulated by environmental factors, including nutrient status and tissue oxygenation [9]. Placenta development

begins between week 0 and 13, when the fertilized blastocyst roots in the uterine wall [10]. The placenta develops from the remaining cells surrounding the lacunae, called trabeculae, that penetrate the uterine wall and develop into the villous trees of the placenta. It derives from both maternal and fetal tissue [11].

Placental origin follows different stage of development during pregnancy. Vascular growth begins as early as 21 days and continues during gestation. When the primary villi are formed, the cytotrophoblast core is covered by a thick layer of syncytiotrophoblasts. With the development of secondary villi, connective tissue cells are homogeneous and can be recognized below the cytotrophoblast film [12]. At 6 weeks of gestation, there is a basal lamina around the villous vessels. Between weeks 10-12 of gestation, villous capillaries from hemangioblastic cells are developed. From 12 weeks, the capillaries coil, bulge, form sinusoids, and protrude towards the trophoblastic layers, forming a so-called syncytiocapillary membrane [13]. Around 32 days, villous endothelial tubes start to contact each other and the fetal allantoic vessels in the presumptive umbilical cord. A primitive fetoplacental circulation is established. From now until term, vasculogenic formation of capillaries is followed by angiogenesis of expansion of the villous vascular system. Placental vascular network development is a strictly controlled vasculogenic and angiogenic process across gestation [14]. Placenta morphology is summarized in fig 3.

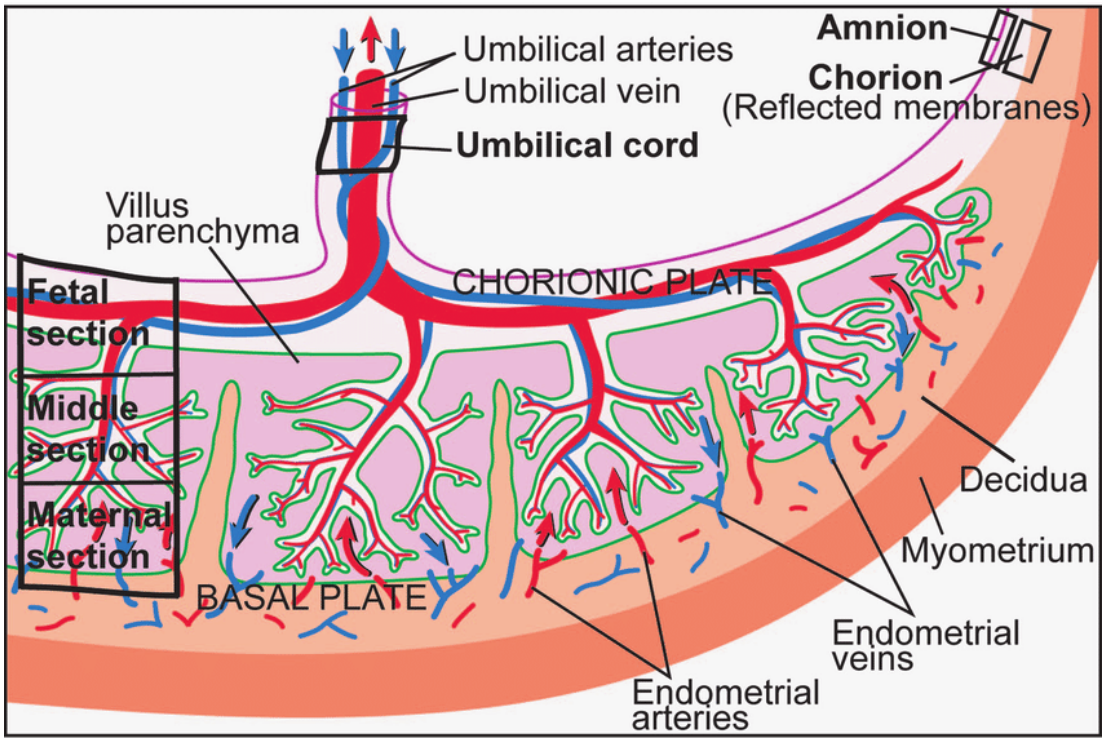


Figure 3: Placenta structure and vascularization. Image adapted from: [15]

Placenta villi are composed of three layers of components with different cell types in each: syncytiotrophoblasts/cytotrophoblasts that cover the entire surface of the villous tree; mesenchymal cells, mesenchymal derived macrophages (Hofbauer cells), and fibroblasts that are located within villous core stroma between trophoblasts and fetal vessels [16].

During vasculogenesis and angiogenesis, angiogenic factors produced by placental cells (trophoblasts, Hofbauer cells, pericytes, and endothelial cells) play a key role.

The trophoblast exerts a crucial role in implantation and placentation. Both processes properly occur because of an intimate dialogue between fetal and maternal tissues, fulfilled by membrane ligands and receptors, as well as by hormone and local factor release [17]. Vascularization of the trophoblast occurs to establish and maintain a feto-placental vasculature. Maternal vascular remodeling takes place to generate a utero-

placental circulation [16]. For successful placentation to occur, a highly orchestrated control of vasculogenesis, angiogenesis, and trophoblast functions is required [17]. This is operated by many heterogeneous factors which act by both autocrine and paracrine mechanisms. Perturbation of trophoblast functions may result in a range of adverse pregnancy outcomes such as malformation, fetal growth retardation, spontaneous abortion, and stillbirth [17]. A limited trophoblast invasion of maternal vessels has been correlated to both preeclampsia and fetal growth restriction, whereas an excessive trophoblast invasion is associated with invasive mole, placenta accreta and choriocarcinoma [17].

The placenta seems to function as a nutrient sensor, regulating nutrient transport according to the ability of the maternal supply line to deliver nutrients. By directly regulating fetal nutrient supply and fetal growth, the placenta plays a central role in fetal programming. Perturbations in the maternal compartment may affect the methylation status of placental genes and increase placental oxidative/nitrative stress, resulting in changes in placental function [18]. Placental nutrient transport capacity has been shown to be increased in animal models of maternal obesity and there is a strong association with birth weight in humans, providing mechanistic insight into the accelerated fetal growth associated with maternal obesity [19].

The maternal blood supply lies in the decidua basalis of the endometrium, which constitutes the maternal component of the placenta.

The placenta plays a vital role in maternal-fetal physiology. It has numerous functions during different phases of pregnancy: implantation, maternal recognition of pregnancy, nutrient and gas exchange, fetal protection from any immunologic attack, placental hormone syntesis [11]. Concerning the implant phase, the syncytiotrophoblast, which

later grows as part of the placenta, facilitates implantation by invading the wall of the endometrium in the uterus. Human chorionic gonadotropin (hCG) is synthesized and released from the syncytiotrophoblast to stimulate luteal progesterone production [20]. Without hCG production, the absence of progesterone would trigger menses and the flaking of the endometrium with the implanted zygote [21]. The exchange of nutrients and gases occur through terminal villi. Mother's blood provides oxygen, water with electrolytes, hormones and nutrients. The fetus excretes carbon dioxide, water, urea, hormones and other waste products. The placenta metabolizes numerous substances and protect against microbial infection [22]. Many hormones are released from the placenta to uphold a pregnancy. The placental growth factor is released from the placenta to prepare the mother's body for pregnancy in terms of cardiovascular adaption. Additionally, the placental growth factor promotes fetal development and maturity. Human chorionic somatomammotropin (HCS), also known as human placental lactogen (HPL) promotes breast development and alters the metabolism of the mother [9]. It decreases maternal insulin sensitivity so that more glucose is available for the fetus [11]. The placenta is a relatively low-oxygen environment at this point.

Placental villi are lined with cells known as cytotrophoblasts and syncytiotrophoblasts [10]. The cytotrophoblasts breach the uterine wall and begin reshaping blood vessels there. These remodeled vessels become a source of maternal blood for the placenta. In a normal pregnancy, cytotrophoblast cells from placental villi invade the space around the spiral artery, replacing cells that line the vessel [23]. This refinement makes the vessel larger and improves the blood flow to the placenta. In an abnormal pregnancy, cytotrophoblasts invade the space in and around the spiral artery [24]. The placenta

develops respiratory, nutritive and excretory functions while the fetal organs mature, and is also an important endocrine organ [25]. Placental hormones are secreted by the syncytiotrophoblast. These hormones are important for pregnancy establishment and maintenance. They exert autocrine and paracrine effects that regulate decidualization, placental development, angiogenesis, immunotolerance, endometrial receptivity, embryo implantation and fetal development. The hormonal profile levels in blood throughout pregnancy are reliable biomarkers to predict and diagnose pregnancy-associated complications, thus because they are released into maternal circulation. Altered levels of these hormones have been associated with some pathologies, such as chromosomal anomalies or pre-eclampsia [26].

2.1 Placenta and GDM

The placenta, being the interface between maternal and fetal circulation and overseeing multiple functions to ensure fetal well-being, plays an active role in the fetal healthy development. Because of this unique role, placenta is exposed to the regulatory of growth factors, cytokines, hormones and circulating plasma compounds. This leads high susceptibilities on development, morphology and cellular functions in GDM complicated gestations [27].

Many placentas from pregnancies affected by GDM exhibit characteristic histological features, including immature villi, villous fibrinoid necrosis, chorangiosis, and enhanced angiogenesis. The specific type of dysfunction is influenced by the timing of glycaemic disorders as gestation progresses. Typically, if impaired glucose metabolism is diagnoses early on, predominantly structural abnormalities are

observed. The nature and severity of these abnormalities are contingent upon various factors, including the degree of glycemic control attained during crucial stages of placental development [28]. At term, placentas of women affected by GDM appears larger than controls in volume [29,30] , thickness, diameter and weight. On the contrary, in some GDM cases, placenta weight results under the 10^{th} percentile [31].

A direct correlation between larger placental weight in GDM and larger infant birth weight has been demonstrated suggesting an increased placental vascularization and delivery of nutrients [32]. Collectively, the abnormalities in fetal growth point to placental dysfunction. Data from 2D and 3D ultrasonography shown no modification in placental size in GDM at 11-14 weeks compared to controls, but it increases significantly from the second trimester as pregnancy progresses [33,34]. Moreover, also abnormalities in villous structure and architecture are observed in GDM only since the second trimester, including reduced vascular flow.

In particular, insulin enhance fetal's utilization of glucose through aerobic metabolisms, consequently raising the oxygen requirements of the developing fetus. If there is an insufficient supply of oxygen due to decreased delivery to the intervillous space, caused by the increased oxygen affinity of glycated hemoglobin [35], the thickening of the placental basement membrane [36, 37], and as we described above, reduced blood flow [38] and fetal hypoxemia will occur, resulting in a state of oxygen deprivation for the fetus [39].

Placental changes in GDM are a result of cumulative dysregulations and aberrant signal molecules, including cytokines, adipokines which are responsible for regulation of inflammatory status and vascular development [40, 41].

However, the role of Ins in modulating GDM placental modifications still lacks comprehensive understanding. In the next paragraph, we aim to provide a concise overview of the current knowledge in literature in ex vivo and in vitro studies investigating Ins function in regulating insulin-glucose signaling and lipid biology in GDM women.

3 Inositols

Inositol (Ins) is a naturally occurring cyclic polyol. Scherer, during mid-1800s, isolated the compound from muscle tissue and named the substance “myo-inosite”, which in chemical language indicated a polyalcohol carbohydrate detectable in muscular fibers [42].

Years later, Maquenne extracted myo-inositol (MI) from leaves and determined its molecular weight and structure. Subsequently, he purified the compound from horse urine, demonstrating that Ins is present both in plants and in animals [43]. In 1919, Posternak isolated and characterized phytic acid from leaves. He found out that this compound is indeed the hexa-phosphate of MI, suggesting that Ins undergoes chemical reactions in the plants [44]. Later, he determined the structure of two different Ins isomers, myo- and scyllo-inositol, discovering that natural Ins makes up a family of isomers [45].

After optimizing the method for the purification, Needham demonstrated that animal do synthesize Ins, independently of dietary intake, also in a regimen of exogenous deprivation [46]. Twenty years later, Folch was studying cephalin, a membrane phospholipid of neurons and he found out that MI-based phosphates are constituents of structural lipids, providing the first insight into their crucial role in plasma

membranes, especially in the brain [47]. A few years later, the Hokins (husband and wife) discovered that Ins is not only a structural constituent of membranes, but also an active molecule in biological signaling. This finding led to the hypothesis that Ins undergoes release from the membranes and subsequently participates in intracellular signaling processes [48]. Irvine and Berridge identified inositol-trisphosphate, which is today considered as one of the most important second messengers in signaling processes [49].

After the discovery of inositol's signaling role, further evidence started emerging on this topic. The group of Larner isolated two different mediators of insulin signal, which they found to be characterized by diverse activities. The first mediator activates pyruvate dehydrogenase phosphatase, explaining some of the intracellular actions of insulin, while the second mediator had a pivotal role in the inhibition of protein kinase A. Later, Larner's group characterized these two mediators, revealing that they are indeed inositol-phosphoglycans [50]. The first one, inhibiting pyruvate dehydrogenase phosphatase, is composed by galactosamine and D-Chiro-Inositol (DCI) [51]. The second one, the inhibitor of protein kinase A, is made out of MI, glucosamine, galactose, and ethanolamine [52]. Thus, for the first time, Ins stereoisomers were noted to have different activities, evidence that will further open several debates.

On these bases, future investigations shed light on some of the biological features of inositols, including conversion between isomers and ratios in tissues and organs. Many of the roles of Ins in vivo are currently under investigation, and Ins physiology still represents a hot topic of research [53].

Now we know that Ins, belonging to the group of sugar alcohols (cyclic polyols or hexahydroxycyclohexanes), are white crystals, odorless, and with a slightly sweet

taste, soluble in water and insoluble in absolute alcohol and ether. Of note, they are stable to heat, strong acids, and alkalis.

Ins (formerly improperly called vitamin B_7) is involved in numerous biological processes including those of cellular signalling, and a structural element (in the form conjugated with lipids). They can occur in the form of different stereoisomers and present a more or less high degree of phosphorylation. In particular, when we talk about Ins, we commonly refer to the MI form, which does not have bound phosphoric groups. MI, which has deep structural similarities to glucose, is the most important and widespread, and it is detectable ubiquitously in almost all biological systems.

Overall, there are seven Ins stereoisomers that are naturally formed (scyllo-, muco-, epi-, neo-, allo-, D- and L-chiro-inositols), by means of different isomerases, and one, cis-inositol, whose existence in nature is unknown (Figure 2). Almost all Ins exert an insulin-mimetic activity and insulin-sensitizing effect, although with different effectiveness. Also, they have a huge number of other important physiological activities, such as promoting ovulation and fertility. Several Ins cross the cell membrane by two cation-coupled co-transporters (sodium or proton coupled) and become components of cellular metabolism. It is important to keep in mind that Ins can also act through a receptor pathway. This occurs only when these molecules are joined to one or more phosphoric groups, forming inositol phosphates, which have specific intracellular receptor to transmit the signal [54].

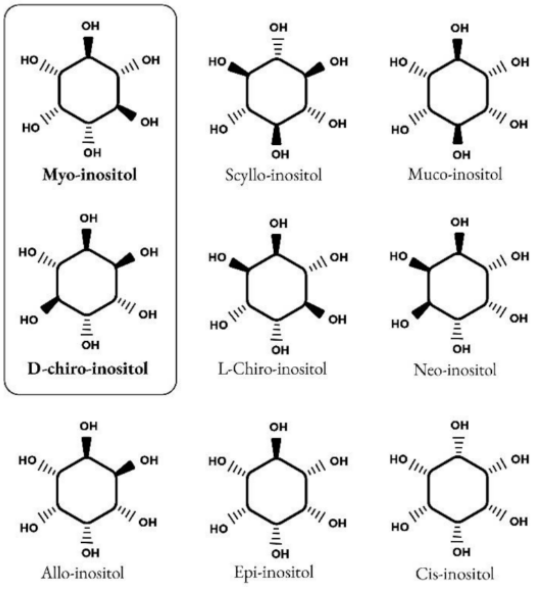


Figure 2 Chimical form of inositols

In addition diet, Ins are regularly synthesized in the liver and kidney from glucose 6-phosphate (the first product of glycolysis). In excess, they are catabolized and eliminated with urine.

MI stands out for its first-rate biological role abounding in animal tissues (meat and organs), where it is concentrated in phospholipids, and in plant tissues, where it occurs in the form of phytate - an antinutrient that binds calcium, iron and zinc. It forms insoluble complexes that are difficult to absorb. It is then possible to introduce MI both respecting an omnivorous and or vegan diet – the latter significantly reducing bioavailability. The most generous food sources of inositol are represented by bran and germ of cereals, whole grains, brewer's yeast, citrus fruits, meats in general and, of course, liver, kidneys, bone marrow and brain.

Once absorbed or synthesized, MI is introduced into the bloodstream, thanks to which - without the need for any transporter - it reaches peripheral tissues and cells. Here it is largely transformed into phosphatidylinositol, a substance endowed with numerous biological functions, some of which have yet to be clarified. We know that it is an active component of plasma membranes; as a precursor to second messengers, it participates in signal transmission systems that control cellular activity. It also stimulates the endogenous production of lecithin (phosphatidylcholine).

3.1 Activities of Inositols

The endogenous production of MI and DCI fluctuates depending on the specific tissue needs. For example, in healthy women, the plasma ratio is 40:1, whereas in ovarian follicular fluid, it is close to 100:1 [55,56]. Between 7% to 9% of MI is transformed into DCI as demonstrated using the radiolabeled [3H]-compound; instead, the other

stereoisomers resulted very low, not more than 0.06% of total radiolabeled MI [57]. Some body districts, such as brain, heart, and ovary, utilize high quantity of glucose; for this reason, they show significant amounts of MI in respect to other tissues [58].

One of the most significant activities exerted by MI and DCI concerns glycemic regulation. Both stereoisomers exert an insulin-mimetic activity and are also effective against insulin resistance. MI significantly inhibits the duodenal glucose absorption and reduces glucose rise in blood [59]. Moreover, it improves insulin sensitivity in adipocytes by increasing lipid storage capacity and glucose uptake, and by preventing lipolysis. It also downregulates the inflammatory response, mainly in macrophages, likely through the inhibition of proinflammatory transcription factors (e.g., Signal transducer and activator of transcription STAT, Nuclear factor kappa-light-chain-enhancer of activated B cells NF-κB, and Activator protein 1 AP-1). In addition, MI induces the translocation to the plasma membrane of GLUT4 - transporters, which are expressed in intracellular vesicles, leading to the increase in glucose uptake and the decrease in plasma glucose level, under glucose-loaded hyperglycemic condition. This effect mimics the insulin action, since insulin induces GLUT4 translocation from the endoplasmic reticulum to the plasma membrane to stimulate glucose uptake in skeletal muscle cells.

Since the discovery of their involvement in endocrine signal transduction, MI and DCI supplementation contributed to clinical approaches in many gynecological and endocrine disorders. Those compounds are useful in preventing and treating polycystic ovary syndrome (PCOS). In their meta-analysis, Zhao et al. reports that for women with PCOS, MI with DCI and metformin combined with thiazolidinediones appear superior to metformin alone in improving insulin resistance and decreasing total

testosterone. MI combined with DCI is particularly efficacious also in recovering menstrual cycle [60]. Ins showed non-inferiority in most outcomes compared to the gold standard treatment (metformin) decreasing the severity of hyperandrogenism, regulating menstrual function, and improving metabolic parameters such as decrease of insulin and Homeostasis Model Assessment index (HOMA index) [61]. Both MI and DCI are well-tolerated, effective alternative candidates to the classical insulin sensitizers, and can be administered for a long time [62]

Moreover, Ins play an essential function in the physiology of female and male reproduction. In women, MI (as InsP3) is one of the second messengers of FSH, and therefore, it is involved in regulating proliferation and maturation of granulosa cells. Due to this role, MI modulates the production of anti-Mullerian hormone (AMH), and consequently determines oocyte maturation and transport in the oviduct as well as guarantees the formation of good-quality embryos [63]. DCI promotes androgen synthesis, whereas MI reduction worsens the energy state of the oocytes, impairing FSH signaling and oocyte quality.

DCI has been extensively studied in its physiopathology and it is known to downregulate the gene expression of aromatase. In particular, a key solution to induce ovulation can be found in DCI since its role in helping the FSH axis restoration. In addition, due to its activity in aromatase downregulation and subsequently the estrogen level reduction, DCI supplementation could be useful in every condition where aromatase inhibitors are indicated. In particular, endometriosis, endometrial hyperplasia, endometrial and breast tumors, uterine myoma, and mood disorders can all benefit from DCI activity. This possible beneficial effect could therefore help in

reducing the amount of drug that needs to be administered, especially in chronic therapeutic protocols [62].

Inositols also influence male fertility and have been proven useful for treating sperm abnormalities and steroidogenesis, regulating the pools of androgens and estrogens, likely in opposite ways [64]. Since several steps have been done in ameliorating obesity and GDM related conditions in pregnancy, further studies are required to establish long-term maternal and foetal safety, involving a larger number of patients from different ethnicities and with different risk factors.

3.2 Safety of clinical use of inositols in pregnancy

Due to their metabolic activity, the interest toward Ins supplementation during pregnancy has risen greatly in recent years, and it is therefore appropriate to explore both their efficacy and safety. Both animal model studies and several clinical trials have been conducted in order to evaluate the safety of Ins supplementation [65].

Preclinical data indicate no toxic effects in terms of kidney and cognitive functions or carcinogenesis [66,67,68].

In mouse models, the preimplantation embryo exposure to MI (10mM) resulted in absence of early toxic effects, as suggested by normal prenatal and short-term postnatal development, and in a significant increase in the overall rate of the live births obtained, as compared with embryos cultured in absence of MI [69].

The safety of Ins supplementation was also demonstrated in humans. MI has been used in preterm infants (<29 weeks of gestational age) with respiratory distress syndrome in order to assess safety and pharmacokinetics of different daily Ins doses (10, 40, or

80 mg/kg/d). In this setting, treatment with MI 80 mg/Kg/die for more than 10 weeks did not result in increased incidence of any adverse event as compared with control babies [70].

According to the data, Ins are defined as generally recognized as safe by the Food and Drug Administration, which allows its use also in infants [71]. Regarding foetal safety, the transplacental passage of MI to the foetus seems not to be clinically relevant [72]. Furthermore, no side effects were observed in patients receiving intervention with DCI (27.5 up 500 mg/day) in the available clinical studies [73].

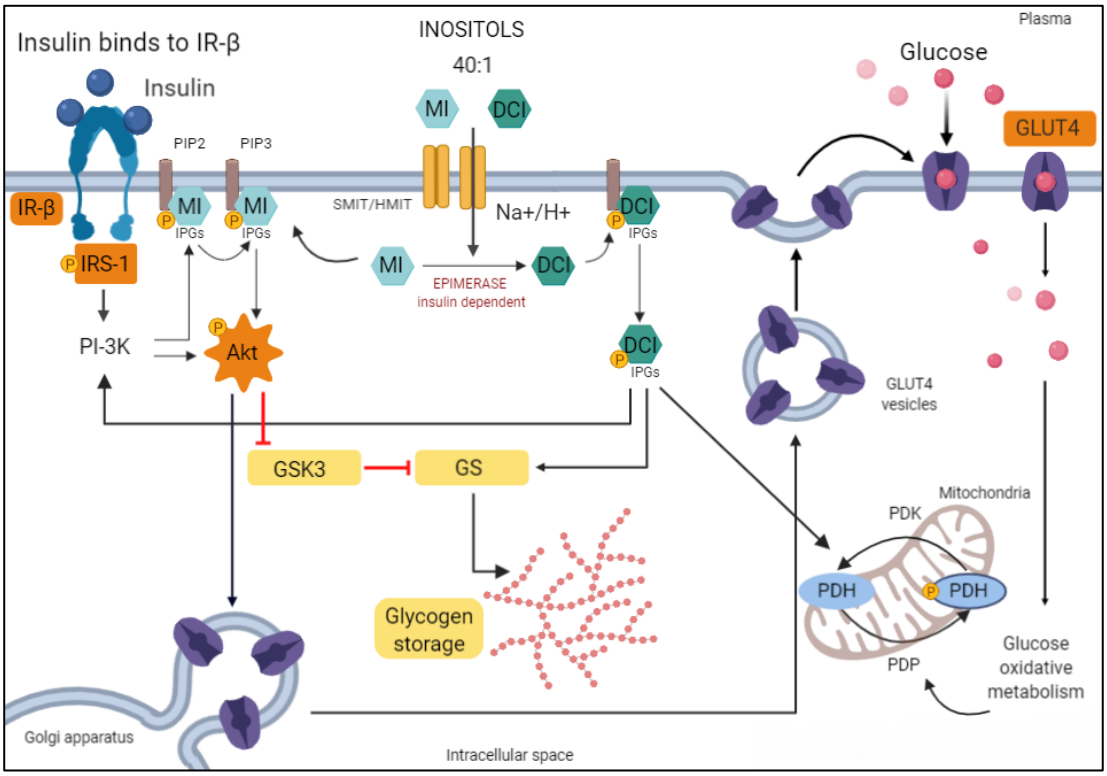
3.3 Inositols in placenta

Nowadays Ins placental biology is still poorly understood, but several studies are investigating on inositol metabolism and catabolism in placenta, as well, its effect as a second messenger in modulating cell processes.

A considerable portion of inositol is synthesized internally in the human body. As we already described, MI is the overwhelmingly prevalent isomer of inositol in mammals, and it can be generated from glucose molecules. Glucose-6-phosphate is isomerized by inositol-3-phosphate synthase 1 (ISYNA1) in inositol-3-phosphate (IP3). Subsequently, IP3 can be dephosphorylated by inositol monophosphates (IMPA1), leading to the formation of free MI. Here MI can be either become part of the cell membrane as phosphatidyl-inositol (PIPs) or acting as a second messenger in the cytoplasm, modulating glucose-insulin pathway. IP3 is, also, generated by phosphatidyl inositol 3 kinase IP3K.

The homeostasis of MI is critical, because it acts as a negative regulator (negative feedback effect), it can be converted to DCI via an insulin-depended epimerase. On the other hand, myo-inositol-oxygenase (MIOX) enzymatically degrades MI into D-glucuronate [74]. At the membrane, MI can enter in the cell mainly through three transporters/ion channels in placenta, i.e. the sodium/MI transporters 1 and 2 (SMIT 1/ SMIT2) and H^+-MI cotransporter 1 (HMIT). MI and DCI intracellular pathway in placenta can be summarized in the figure below (fig 3). The figure was created by Dr Daniela Menichini.

Figure 3. Roles of Myo-Inositol (MI) and D-Chiro-Inositol (DCI) in Cellular Insulin-Regulated Glucose Homeostasis Pathways.



Glucose uptake pathway
Glycogen synthesis pathway
Mitochondrial oxidative metabolism

Importantly, the fetal-placental unit contains a significant amount of Ins, and the level of MI in fetal blood is considerably higher compared to adults [75, 76]. Moreover, low Ins content in placenta is strongly linked with higher birthweight and maternal abdominal adipose tissue volume compared with those placentas with high Ins tertiles [77].

A recent study found that inositol content was 17% lower in GDM placental tissue compared with non-GDM samples. Ins content was associated with higher maternal mid-gestation glycemia [75]. This study suggests that elevated glycemia induced a dysregulation of placental inositol pathway in GDM gestations.

In a recent large study, Pillai et al., showed that elevated fasting glycemia was correlated with decreased protein levels of IMPA1, as well as the inositol transporters SMIT 2 and HMIT. The expression of these proteins was also found to be associated with the inositol level in the placenta. In addition, high concentration of glucose reduced the expression of IMPA1 and SMIT 2 mRNA. Furthermore, increasing fasting glycemia was positively associated with birthweight percentile, as expected in patients where placental inositol levels were low. They demonstrate that inositol pathway is impaired in GDM placentas via downregulation of Ins cell membrane transporters and monophosphates which generates free MI in the cytosol [77].

As we previously mentioned, GDM can causes an increase of lipid supply to the fetus, but MI supplementation might affect transplacental lipid supply to the fetus. In vitro MI stimulation showed to affect fatty acids amount, increasing C-palmitic acid-lysphosphatidylcholines and C-palmitic acid- phosphatidylethanolamines, but decreasing C-docosahexaenoic-acid lipids. These studies demonstrates that MI modifies upstream mechanisms like fatty acid uptake or activation [78]. GDM

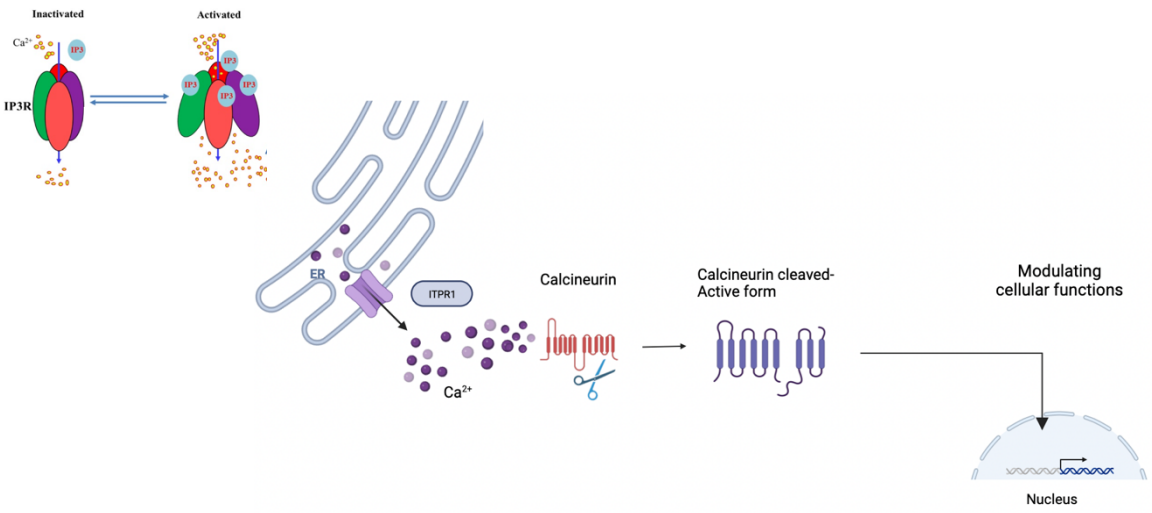
placental explants were susceptible to glucose stimulation in modulating fatty acid accumulation, but this effect was attenuated by concurrent MI treatment.

The supplementation of MI has the potential to alter placental lipid physiology in GDM, even if the clinical implications of this effect remain uncertain. This strongly indicates the physiological significance of inositol in placental function, fetal development, and potentially in maternal-placental-fetal communication as well. In addition, high placental inositol might protect the fetus from the pro-adipogenic effect of maternal glycaemia.

It is more evident that Ins regulates important cellular processes in placenta via its role as a second messenger.

PIP3 pathway

MI plays a role in regulation of calcium signal inside the cell. In fact, Ins-phosphate 3 (IP3) through its interaction with Inositol 3 phosphate receptor 1 (ITPR1), mediates the efflux of calcium ions from the endoplasmic reticulum into the cytoplasm, which modulate cellular functions and gene expression (Fig 4). Excess of calcium ions activates Calcineurin (CN) protein which undergoes to a protein cleavage and, subsequent, activation. Here CN modulates gene expression and cellular functions.



PI3K mRNA expression and protein level is downregulated in GDM and obese placentas, while a large recent study demonstrates that also ITPR1 gene is involved in fetal development [79].

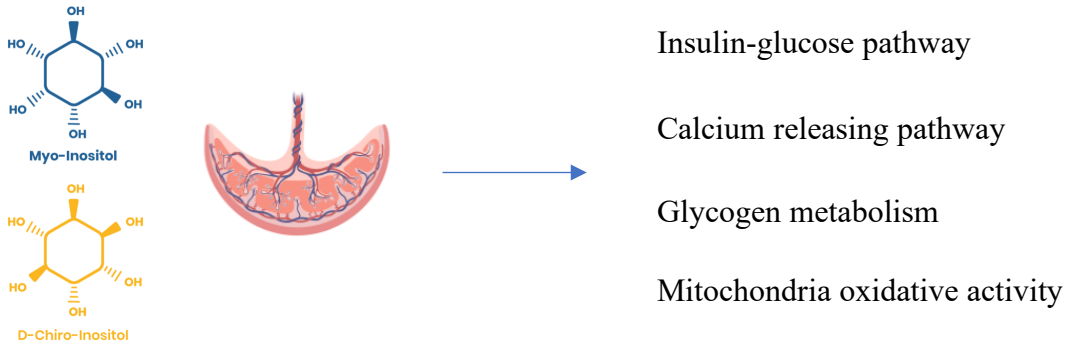
Tekola et al., identified a particular SNP (single nucleotide polymorphisms) in ITPR1 gene associated with reduced fetal weigh and head circumference [80]. Furthermore, ITPR1 placental expression was found downregulate in mRNA levels among male placentas, but not in female confirm that placentas metabolic profiles changed in a gender specific manner.

The involvement of PI3K-ITPR1 pathway and, consequently, its regulation of cytoplasmic calcium concentration in GDM placenta is still unknown. Changes in IP3 pathway might cause modifications of gene expression of specific targets via transcription factors resulting in a direct effect on cell processes.

It is becoming more and more evident that the placenta adapts differently in metabolic complicated pregnancies environment based on fetal gender. This has been observed also in pregnancies with a diet supplemented with MI-DCI in animal studies. Female placentas are more resourceful and able to respond to maternal insulin resistance, increasing insulin signaling pathway and glycogen storage. On the other hand, male placenta increased glucose uptake with high oxidative damage. Importantly, MI treatment enhanced placental glucose use for energy production in a gender-independent manner in offspring born to MS.

Moreover, adult offspring born to dams with MS benefit more from maternal MI treatment if exposed to environmental factors in utero. In conclusion, inositol supplementation improved glucose tolerance in placenta [81].

Biological effect of inositol in placenta can be summarized in the fig 5 below.



The current high prevalence of maternal obesity may lead to a perpetuating intergenerational cycle of increasing obesity and metabolic disorders as GDM.

Understanding the molecular mechanisms involved in placental inositol signaling in obese women will help in the development of new tools to be used in therapy to restore the metabolic profile and ameliorate fetus health. Indeed, the identification of inositol intracellular targets may pave the way for the development new approaches for diseases treatment or prevention and will help to stop the intergenerational transmission of obesity and GDM. Nowadays there are more evidence that a supplemented diet with inositol have a direct effect on placental biology and it might be a non-pharmacological therapy to ameliorate the adverse intrauterine environment

and, therefore, the altered fetal programming. The identification of biological alterations responsible for metabolic alterations in the placenta is fundamental to identify potential targets and develop novel therapeutic approaches and personalized therapies.

4. Nanoplastic as source of placental interfere molecules.

In the last century, the global production of plastics has grown exponentially to reach over 350 million tons per year produced in the world, part of which ends up polluting the environment [82]. The uncontrolled production of plastic led to excessive plastic waste in the environment. Consequently, once released into the environment, plastics are exposed to continuous processes like photo-oxidation, chemical weathering, mechanical forces, and biodegradation activities, which affect their structural integrity and result in the fragmentation of plastic components [83-85]. It is well known how commonly used plastics, such as polyethylene (PE), polypropylene (PP), polystyrene (PS), and polyethylene terephthalate (PET), undergo a very slow degradation process.

For instance, plastic bags, commonly used in everyday life, can last 500-1000 years in ecosystems. Plastic debris is now referred to as microplastics (MPs), defined by the European Food Safety Authority (EFSA), as plastic particles smaller than 5 mm. Particles smaller than 100 nanometers are defined as nanoplastics (NPs) [86,87]. Moreover, MPs are very heterogeneous in terms of diameter, chemical structure, shape, density, and color. For these features, they are so widely distributed in the environment [88]. MPs can be classified as primary and secondary based on their source of release into the environment. Primary MPs are intentionally produced with a size less than 5 mm and used for commercial purposes. Secondary MPs derive from environmental degradation processes [89-91]. MPs/NPs have been identified in packed food, especially seafood, and bottled water commercialized for human consumption [86,92,93]. Plastic debris can enter human bodies via three main routes: gastrointestinal, inhalation, and dermal contact. Among them, ingestion is considered the major source of contamination and recent studies estimated that a person intake from 39 to 52 thousand of MPs per year [94-96]. The potential health impairment caused by the internalization and accumulation of MPs is of prime concern. Although little is known on this topic, several recent studies reported evident toxic effects in animal models, marine organisms, and human cell lines [97-99]. Once internalized, MP/NPs can cross cell membranes [100-102], followed by accumulation or elimination by the onset of specific cellular mechanisms. In organic tissues, MPs/NPs are not inert as previously supposed, but they are considered foreign bodies by the cells and trigger local or systemic immunoreactions. Moreover, the particles can be the carrier for chemicals, including environmental pollutants and plastic additives, increasing the risk for human health (86, 103-107). Importantly, MPs/NPs can act as

endocrine disruptors, altering normal functions of the endocrine system, and causing damages to the entire organism, its progeny, or to a specific cell population of the organism itself (108). The scientific community is concerned about the effect of MP/NPs during pregnancy, a very delicate physiological process, susceptible to environmental factors, such as diet and lifestyle. Animal studies confirmed the toxic effects of plastic particles on offspring generation, which interfere with cellular energy production and lipid metabolisms, leading to oxidative stress and neurotoxic response, suggesting mitochondrial dysfunctions (109). MPs/NPs cause alterations at the phenotypic level in mice, modulating gene expression by epigenetic modifications, as demonstrated by brain abnormalities in mouse pups whose mothers were fed with plastic microparticles (110). Maternal exposure to PS during gestation and lactating periods affects offspring health.

In human studies, Ragusa et al. showed that human breastmilk contained plastic contaminants. The most abundant NPs were composed of PE, PVC, and PP, with sizes ranging from 2 to 12 μm. Interestingly, statistical analysis demonstrated that there was no significant relationship between MPs and patient characteristics (including lifestyle), suggesting that the ubiquitous MPs presence makes human exposure inevitable. Fig 6

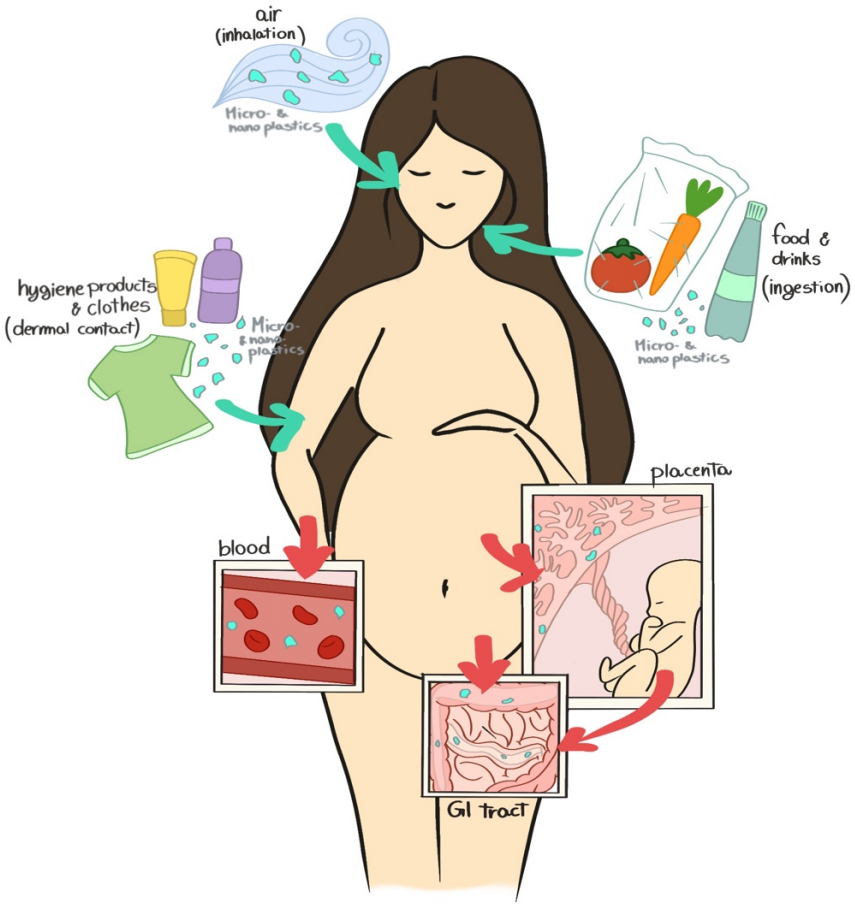


Fig. 6 **Nanoplastic contamination during pregnancy:** Schematic representation of nanoplastic contamination by inhalation from air pollution, ingestion from food and drinks and dermal contact from daily hygiene products and clothes. Consequent findings of NP in blood, in the gastrointestinal tract of the pregnant mother and fetus and in placenta are also illustrated. Image adapted from: Ragusa et al.2022

Furthermore, the placenta plays a main role in well-maintained gestation and fetus health. Importantly, ER stress causes autophagy in the human trophoblast cell line with cytoskeleton fragmentation and activates programmed cell death pathways. ER stress affects the pre-implantation process in pregnancy [111]. More recently Ragusa et al. correlated for the first time the presence of MPs to alterations of some organelles. The endoplasmic reticulum appeared dilatated, with many vesicles discreetly electrodense, with secretory material inside, covered by ribosomes and not (degranulation) communicating with each other [112]. Numerous electrodense swollen mitochondria

and whorled membranous bodies derived from involuting ER and other structures were observed. Results from this important study underline the role of MPs in human non-transmissible disease (NTD) progression.

2 STUDY DESIGN

In this study our first aim was to investigate the inositol pathway in the placenta from obese and overweight women and lean healthy controls. Since obesity alters placental biology and, as consequence, long term fetal health, we hypotized that inositol pathway and biology was altered in the placental villous tissue.

In addition, one of the main concerns of recent years is the effect of plastic pollution on human health. Nano plastics contaminants in the human body, as obesity, interferes

on the physiological cell biology, altering cell crucial functions, including cell proliferation, apoptosis, mitochondria, and it might also influence gene expression via epigenetic alterations. Investigating on these aspects will give an improvement in the wellbeing of mother and neonates.

In order to investigate on alterations of inositol pathway in obese/overweight women, we collected placentas from 34 women between 2021 and 2022 at the University Hospital of Modena (136/2015/SPER/AOUMO). Molecular analyses were performed in the Developmental origin of health and disease (DoHAD) laboratory headed by Doc Umberto Simeoni at the University of Lausanne, CHUV, under the supervision of PhD Benazir Siddeek. Inclusions and exclusion criteria of selection of the patients are summarized as follow:

The inclusion criteria for enrollment are:

- Age over 18 years
- Singleton pregnancy
- Gestational age less than 37 weeks
- Willingness to participate in the study
- Proficiency in the Italian language
- Signed informed consent for participation in the study
- Elective term cesarian sections

The exclusion criteria for enrollment are:

- Chronic diseases, in particular: Type I or II diabetes mellitus
- Vaginal delivery

- Covid positivity at the moment of partum
- Viral disease infections, in particular HIV

Patient's characteristics were collected the day of delivery including maternal clinical features and placenta and neonates data, including weight and gender.

To investigate on the Nanoplastic effect on placentas we used a human trophoblast cell line (HTR8/SV40) to perform cell in *vitro* experiments. Cells were treated with increasing concentration of plastic particles for 24 or 48 hours, in order to verify their toxic effect on human placental cells.

3 MATERIALS AND METHODS

3.1 Placental explants collection

Placentas were collected by Gynecology and Obstetrics Unit of University Hospital of Modena from elective cesarian section from women at term. Placentas were then weighted and placed in a sterilized box for delivery in the laboratory. Samples were processed within 1 hour from delivery in order to avoid oxidation stress and

contamination of the organ. The organ was carefully placed under flow cabin in sterilized sheets. Decidual and chorionic membranes were discarded in apposite boxes. Villous tissue was cut in 2x3 cm^2 fragments and placed in Nalgene™ General Long Term Storage Cryogenic tubes 2 ml (5000-0020, ThermoFisher) and immediately snap frozen in liquid nitrogen. Subsequently, cryo-preserved vials were placed in -80°C for further experiments. On the other hand, larger pieces of placental villous tissue with 5x6 cm^2 were included in formalin for morphological analysis.

3.2 Myo-inositol quantification

Myo-inositol quantification was performed using fluorometric myo-inositol assay kit (ab252896, Abcam) following protocol instructions. 10 mg of powder tissue was used for quantification and analyses. Sample were homogenized with 100ul ice-cold Inositol Assay Buffer. Centrifuge at 10000 g at 4°C for 5 minutes occurred to remove insoluble material. Samples required Sample clean-up mix step in order to clarify the samples, adding 2 ul of mix in 100 ul of clarified sample. Incubation of 1h at 37°C occurred. Samples were filtered with 10kD MWDCO spin column at 10000g for 10 minutes at 4°C. Assay buffers were added to reach final volume of 50ul. Standard were prepared in parallel. Inositol standards were composed by 50 ul of tubes with a myo-inositol concentration of 0,100,200,300,400 and 500pmol. Reaction mix was prepared with inositol assa buffer, inositol enzyme mix, inositol developer and prove. Well were mixed and incubated for 30 minutes at 37°C. Fluorescence was measured in end point mode at Ex/Em= 535/587 nm. Inositol concentration was measured with the following formula:

Inositol concentration = (B/V) x D = μM = (pmol/μL)

Where:

B = amount of myo-Inositol in the sample well from Standard Curve (nmol)

V = sample volume added into the reaction well (µl).

D = sample dilution factor (D = 1, if undiluted).

3.3 RNA extraction and analysis

Total mRNA is extracted with column-based methods and quantity and quality are evaluated by spectrophotometer measurements. RNA molecules were extracted following the instruction of QIAGEN KIT (RNeasy Kit 50).

3.4 Gene expression analysis by qRT-PCR

Expression of genes involved in inositol pathway including SMIT1, SMIT2, IMPA1, ISYNA1 and ITPR1 were evaluated in the placenta isolated trophoblasts through RT-qPCR. Total RNA have been isolated from placenta cells using column-based purification. The total RNAs quantities and quality were evaluated with a spectrophotometer (Nanodrop). 500ng RNAs were reverse transcribed into cDNA. cDNA was analysed through quantitative PCR using taqman reagent (Life Technologies) and specific primers. The relative expression levels of mRNAs were calculated using the comparative ΔΔCt method small by normalizing to Gapdh levels.

- Storage

Samples were stored into 1.5-mL Rnase-free Eppendorf DNA/RNA LowBind tubes and immediately freeze at −80 °C for RNA and -20°C for proteins.

3.5 Western Blot

Proteins were extracted from frozen placental villous tissue powder or from HTR8/SV40 cells with RIPA buffer. First, 20 to 40 µg of the samples were separated on SDS-PAGE. After the transfer, the membranes were blocked with PBST-BSA (3% Albumin). Primary antibodies for ITPR1, Calcineurin, cleaved Caspase 3 and VEGF were used (diluted at 1/1000 in PBST-BSA 1%). Secondary antibodies (anti-mouse or anti-rabbit-HRP, according to the primary antibodies, diluted 1/5000 in PBST-BSA 1%) were added, and the proteins were revealed with ECL. In order to check for the equal loading of the proteins, the membranes were re-incubated with anti-GAPDH (diluted 1/4000 in PBST-BSA 1%). A luminescent image analyzer camera G: Box (Syngene, Cambridge, UK) was used for luminescent signal scanning. The signals were quantified with Gene Tools software (Syngene, Cambridge, UK).

3.6 CCK8: proliferation assay

Trophoblast cells were plated in a 96 multi well plate for 24h to induce adhesion on the bottom plate at final concentration of 5000 cells/well. Nanoplastic were added to the cells in complete medium RPMI, 1xP/S and HEPES without serum (Fetal calf serum). Stimulation media was freshly prepared for the 24h stimulation to reach final concentration of 100, 50, 25, 1 uM. After 24h the cells were ready for further cytotoxicity/proliferation assay. For proliferation assay Cell counting Kit-8 (CCK8-

IK-11133-1000, Immunological science) has been used on trophoblast cell line. Cell suspension (100µL /well) was placed in a 96-well plate. The well plate has been pre-incubated in a humidified incubator (37 °C, 5% CO2). CCK-8 was thawed in a water bath at 37 °C. In order to stain the cells, 10µL of the CCK-8 solution was added to each well of the plate before the O.D. reading. The 96-well cell plate was incubated for 4 hours in the incubator. After the 4 the absorbance at 450 nm was assessed using a microplate reader (Thermoscientific Multiskan FC).

3.7 Immunofluorescence staining of trophoblast cells

Sterilized glass slides were placed on the bottom of a 6 well plate in order to let the cells grow on the support for further analysis. Cell media was composed by RPMI 1640 w/GLUTAMAX (61870044, Life technologies), Pen/strep and 5% of Fetal bovine serum Qualified hi (10500064, Life Technologies). HTR8/SV40 cells were plated on 6 well plate (140675, Life technologies) at final concentration of 30 000/cells per well. Cells were plated on glass support for 24h and then, treated with media supplemented with increasing concentration of Polybead® Microspheres 0.05 um conjugated with Fluoresbrite® YG (Tebubio, 17149-10). After 24h of incubation of 10, 25, 50, 100µg/ml of Polybeads, glass slides were washed 3 times with fresh Phosphate buffer saline solution (PBS without Ca^{2+}/Mg^{2+}) in order to remove the excess of culture media. Cells were fixed with 4% of paraformaldehyde and PBS for 10 mins at RT under chemical wood. Two washing steps with PBS of 5 minutes occurred. Slides were collected and placed on glass supports. Sliced of the different conditions were incubated with primary Ab- anti Phalloidin AF954 (A12381, Thermo

Fisher) diluted 1:200. Incubation time occurred for 2h at RT protected from the light. Cells were rinsed twice with PBS and them washed 3 times for 5 minutes. Secondary antibody anti-mouse was diluted in Bovine serum albumin 3% in PBS. Incubation time of 2 hours occurred at RT protected from the light. Cells were then washed 3 times with PBS and DAPI incubation occurred. DAPI was used at 1:1000 dilution for 7 minutes of incubation. Glass slides of the different conditions were then mounted with mounting solution in order to cover all the cells. Samples were either stored in the dark at 4°C until acquisition (withing 24 hours) or directly analyzed at the confocal microscopy.

3.8 Confocal microscopy

Stained cells on the glass supports were used for confocal microscopy analysis. Confocal microscopy LEICA TCS SP8 of the CIGS-Centro Interdipartimentale Grandi Strumenti of the Life Science Department, University of Modena and Reggio Emilia was used for the analysis.

3.9 Statistical analysis

The data from the different experiments were analyzed with GraphPad Prism software version 9.5.1 (GraphPad Software, LLC, San Diego, CA, USA.). The values were expressed as the mean ± SEM to account for sample variation within a dataset. Student's t-test was performed to determine whether there were differences between the two groups and an analysis of variance (ANOVA) with Fisher's LSD test when multiple groups were compared. $p < 0.05$ was considered statistically significant.

4 RESULTS

Inositol pathway alteration in obese placentas

4.1 Effects of Maternal obesity/ow on placenta and neonatal characteristics

Placentas collected from CS were weighted within 20 mins from the delivery. Data shown in fig 7 represents weight in grams of placentas and newborns. Data did no showed significant differences in weight between the two groups. Baby weight was

assessed and recorded after the delivery. No change was detected in placental weight and in baby weight between the two groups.

Study groups	BMI kg/m2	Placental weight (g)	Baby weight (g)
Controls (n= 15)	24,32 ± 1,37	594,7 ± 57,68	3407 ± 303,15
Obese (n= 16)	33,44 ± 4,24	660 ±100	3460 ± 222,18

A B

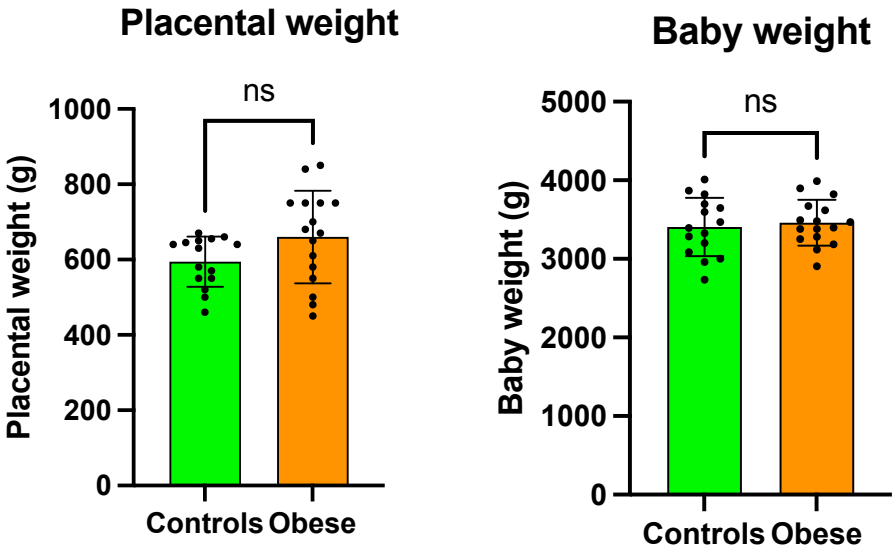


Figure 7. Effects of maternal obesity on neonatal and placental weight (A) Weight of whole placenta including umbilical cord, decidual and chorionic plate in grams (B) Baby weight comparison between the two groups. N=16-18 per group. The Student's t test was performed to compare control and obese women. Data were considered statistical significant if $p < 0.05$. ns: not statistically significant

4.2 Maternal obesity did not influence glycogen synthase kinase 3 beta and insulin-regulated glucose transporter 4 mRNA expression

To investigate whether the insulin-glucose pathway is altered in the obese placentas, we analyzed GSK3B and GLUT4 gene expression in placental tissue which encodes for glycogen synthase kinase 3 and insulin-regulated glucose transported 4. GSK3B is known to regulate glycogen synthesis, and, on the other hand, we aim to investigate on the intake of glucose mediated by insulin (GLUT4). Data from RT-qPCR analyses

are reported in fig 8. Our data indicates no change in GLUT4 and GSK3B mRNA levels between obese and controls.

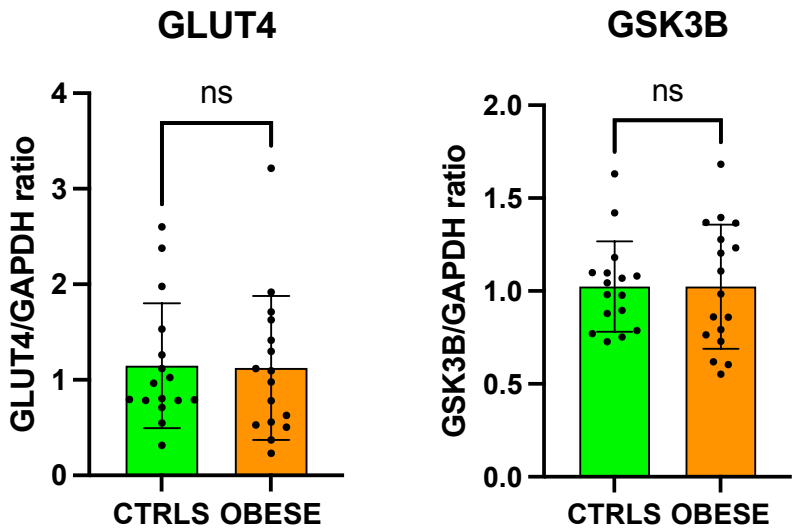


Figure 8. Maternal obesity did not influence Glucose transporter 4 (GLUT4) and Glycogen synthase kinase 3 beta (GSK3B) in placental villous tissue. GLUT4 and GSK3B mRNA levels are not altered in obese placenta compared to healthy controls. GAPDH is used as normalizer and data are expressed as SLC5A3/GAPDH ratio ± SD. N=16-18 per group. The Student's t test was performed to compare control and obese women. Data were considered statistical significance if $p < 0.05$. ns: not statistically significant

4.3 Effect of maternal obesity on MI amount in placental fetal side villous tissue

MI content was measured in placental villous tissue explants to investigate on the possible effect of obesity on inositol pathway in obese condition. As shown in fig 9, MI amount was decreased in obese placentas compared to controls. This data confirmed an alteration in MI content in placental villous tissue, suggesting that MI pathway is affected by maternal obesity in placenta.

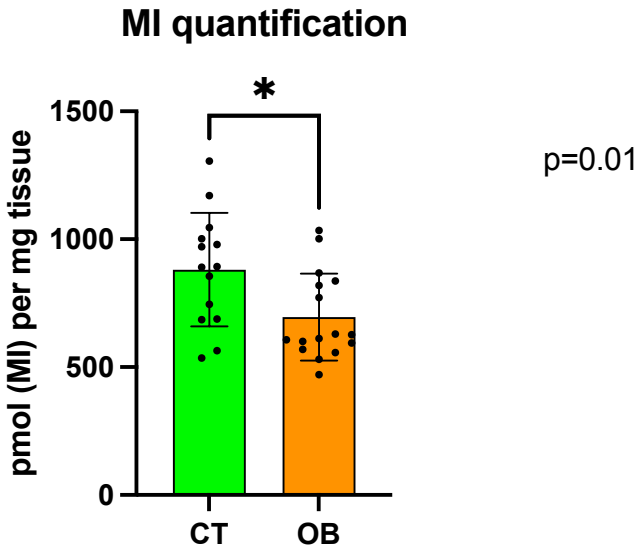


Figure 9. Maternal obesity influences MI amount in placental explants. MI amount is decreased in obese placenta compared to healthy controls. Data are expressed as pmol of MI per mg of placental tissue ± SD. N=16-18 per group. The Student's t test was performed to compare control and obese women. Data were considered statistical significant if $p < 0.05$.

4.4 SMITs transporters are not affected by maternal obesity.

To investigate whether the decrease in MI content in the obese placentas in related to MI cellular intake, we analyzed SLC5A3 gene expression in placental tissue which encodes for Sodium-myo-inositol co-transporter (SMITs). Data from RT-qPCR analyses are reported in fig 10. Our data indicates no differences in SLC5A 3 mRNA levels between the two groups, which seems to indicate that the decrease in MI in the placenta of obese women is not related to defects in MI uptake.

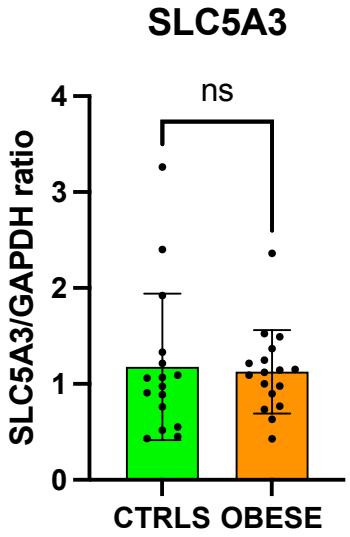


Figure 10. Maternal obesity did not influence Sodium-myo inositol transporter (SMITs) in placental villous tissue. SLC5A3 mRNA level is not altered in obese placenta compared to healthy controls. GAPDH is used as normalizer and data are expressed as SLC5A3/GAPDH ratio ± SD. N=16-18 per group. The Student's t test was performed to compare control and obese women. Data were considered statistical significance if $p < 0.05$. ns: not statistically significant

4.5 MI synthesis mRNA level is not modified by maternal obesity.

Then, we checked whether the decrease in MI placental content in the obese group was related to defects in MI synthesis. Analysis performed on MI biosynthesis target was assessed by RT-qPCR and normalized on GAPDH mRNA level. ISYNA 1 gene encodes for Inositol-3phospate synthase 1. No differences were detected in ISYNA1 mRNA levels between the two groups.

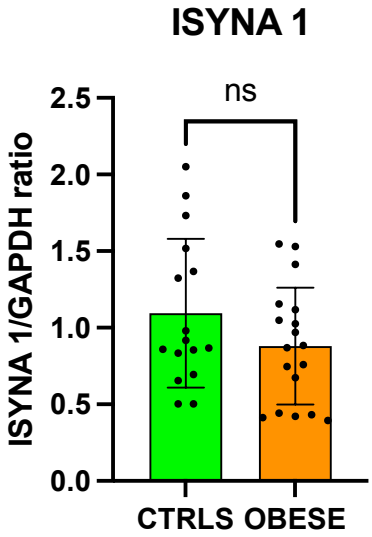


Figure 11. MI synthesis is not affected by maternal obesity in placental villus tissue. ISYNA 1 mRNA level is not altered in obese placenta compared to healthy controls. Target mRNA level was normalized on GAPDH mRNA and data are expressed as ISYNA1/GAPDH ratio ± SD. N=16-18 per group. The Student's t test was performed as statistical tool to compare differences in the groups Data were considered statistical significance if $p < 0.05$. ns: not statistically significant

4.6 Maternal obesity alters Phosphatidyl-inositol 3 phosphate pathway at protein level. ITPR1 is upregulated in obese placental tissue.

To test whether the decrease in MI content in the placenta of obese women has an impact on the inositol signaling pathway, we analyzed the expression level of the Inositol 3-Phosphate receptor 1 (ITPR1). Through western blot approaches we showed a significant increase in ITPR1 protein levels in the obese group compared to the lean healthy group (Fig 12).

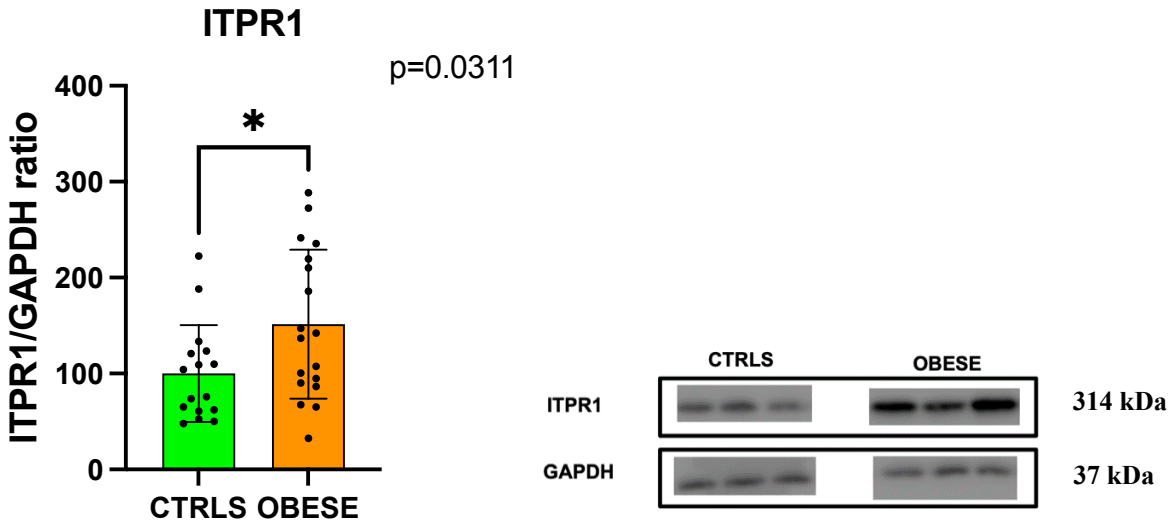


Figure 12. Maternal obesity impairs PIP3 pathway as revealed from the upregulation of ITPR1 protein level. Data are shown as percentage of protein expression difference compared to controls. Protein level was normalized on GAPDH protein and data are expressed as ITPR1/GAPDH ratio ± SD. N=16-18 per group. The Student's t test was performed as statistical analysis to compare differences in the groups. ∗ indicates statistical significance ($p < 0.05$).

4.7 Maternal obesity alters Calcineurin activation in obese placental tissue.

After the binding of inositol to ITPR1, the calcium release from the endoplasmic reticulum, has been described to activate calcineurin. Calcineurin is actively cleaved upon Ca^{2++} ion increasing concentration in cytoplasm after increasing ITPR1 activity. To verify if ITPR1 upregulation has an impact on calcineurin activation, we measured the protein levels of the full length Calcineurin (CN), and its active cleaved form (Cleaved CN). While we did not detect changes in the CN total form protein level

between the two groups (Fig 13, A), we observed a significant increase in the active cleaved CN protein levels with maternal obesity (Fig 13, B). These data suggest that maternal obesity influence PI3P pathway.

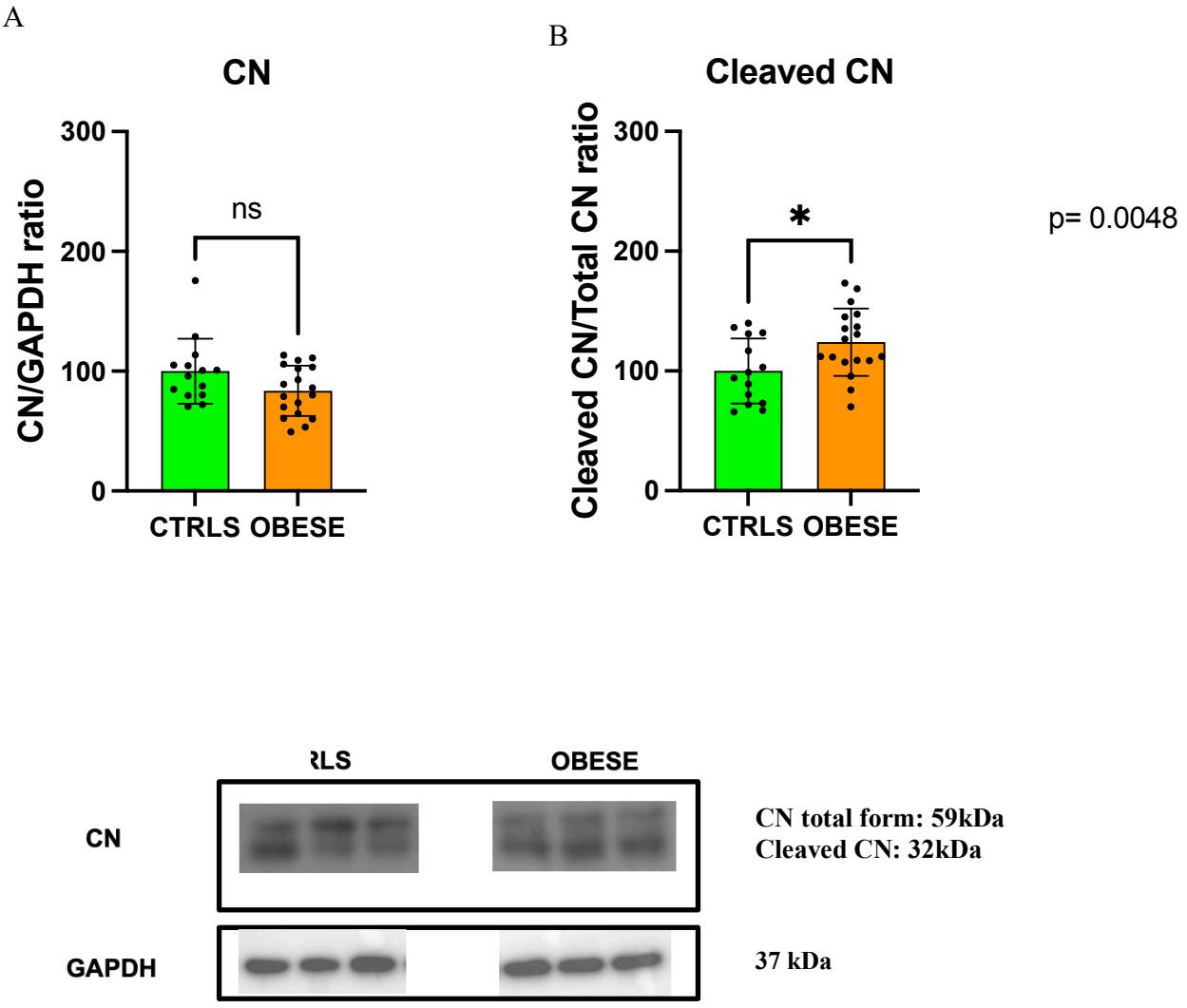
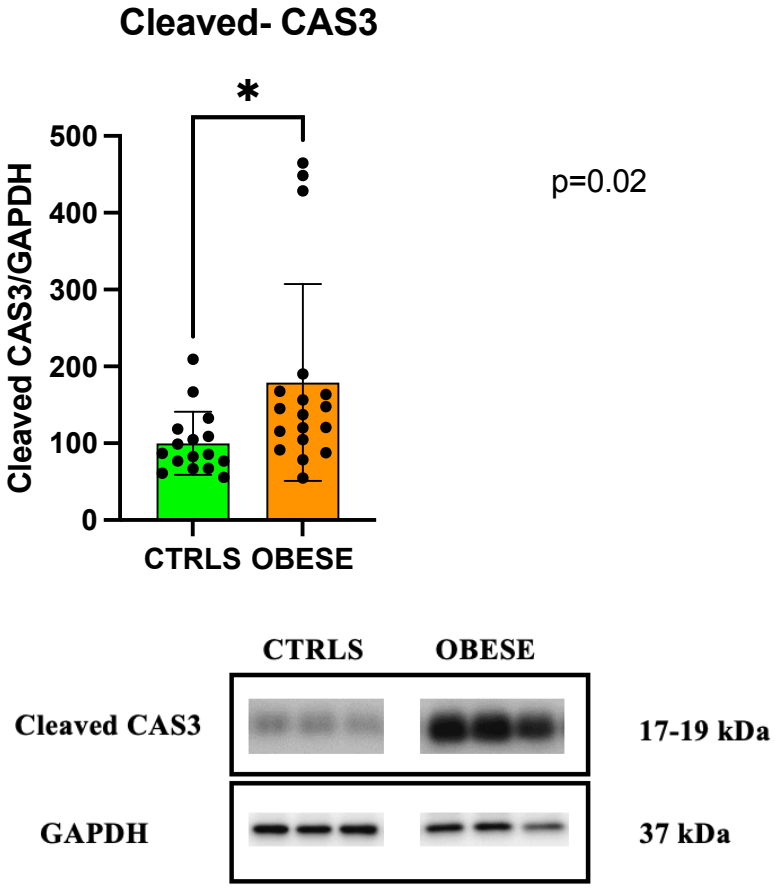


Figure 13. Maternal obesity increased active cleaved calcineurin in obese placenta. Data are shown as percentage of protein expression in study group compared to controls. Total form of CN was normalized on GAPDH protein (A) and cleaved active form was normalized on total CN ratio ± SD. N=16-18 per group. The Student's t test was performed as statistical analysis to compare differences in the groups. ∗ indicates statistical significance ($p < 0.05$). ns: not statistically significant

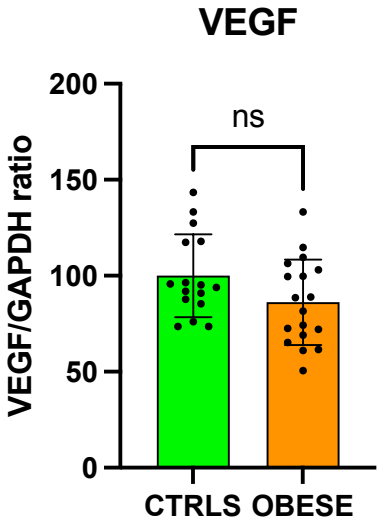
4.8 Maternal obesity is associated with altered cell death.

Once activated, Calcineurin regulates critical cellular pathways for the development and normal function of the placenta, such as cell proliferation, apoptosis, and vascularization. We, thus, measured the expression of key proteins involved in these processes: Proliferating cell nuclear antigen (PCNA), Cleaved Caspase 3 (CAS3) and Vascular endothelial growth factor-A (VEGF-A). Our analysis showed a significant increase of CAS3 protein level in the obese group, indicating an abnormal apoptosis level in the placenta from obese women. Maternal obesity rises placental apoptosis of villous tissue (Fig 14 A). VEGF-A data showed no significant differences in the two study groups, but we could observe a tendency of decreasing of VEGF protein expression in obese condition (Fig 14 B) ($p=0.07$). Proliferation seemed not to be affected by maternal obesity as showed in fig 14 C.

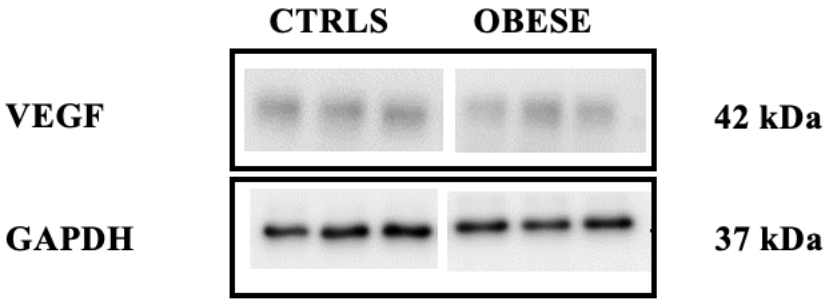
A


Cleaved- CAS3
*
500
400
300
200
100
0
Cleaved CAS3/GAPDH
CTRLS OBESE
p=0.02
CTRLS
OBESE
Cleaved CAS3
17-19 kDa
GAPDH
37 kDa

B


VEGF
200
150
100
50
0
VEGF/GAPDH ratio
ns
CTRLS
OBESE

p=0.07


CTRLS
OBESE
VEGF
42 kDa
GAPDH
37 kDa

C

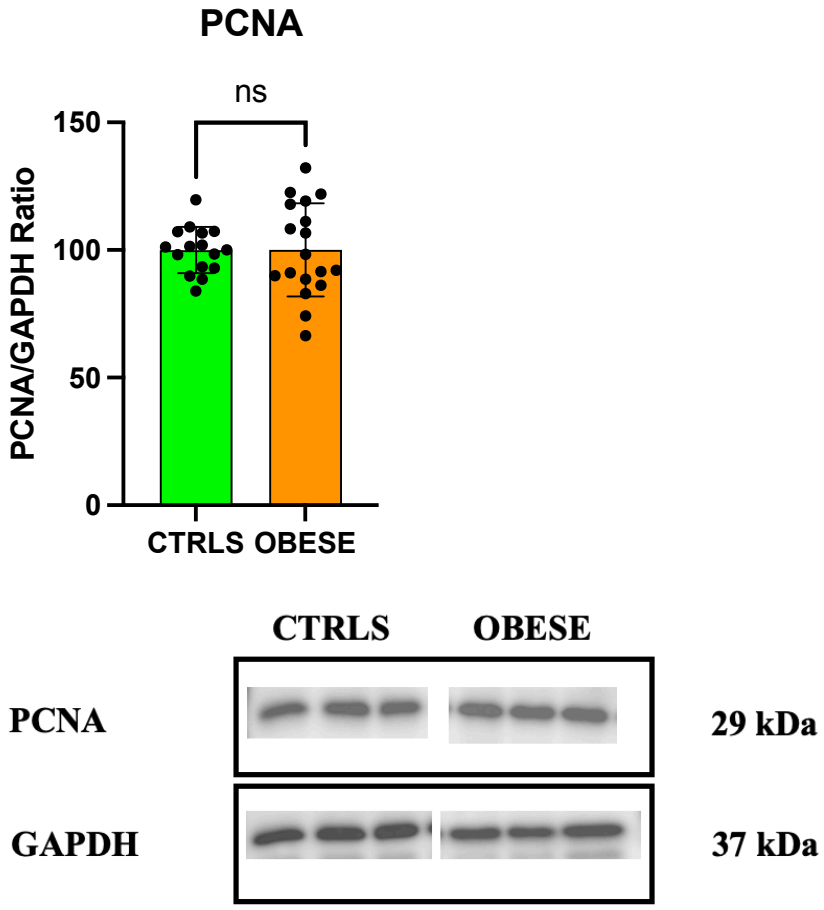
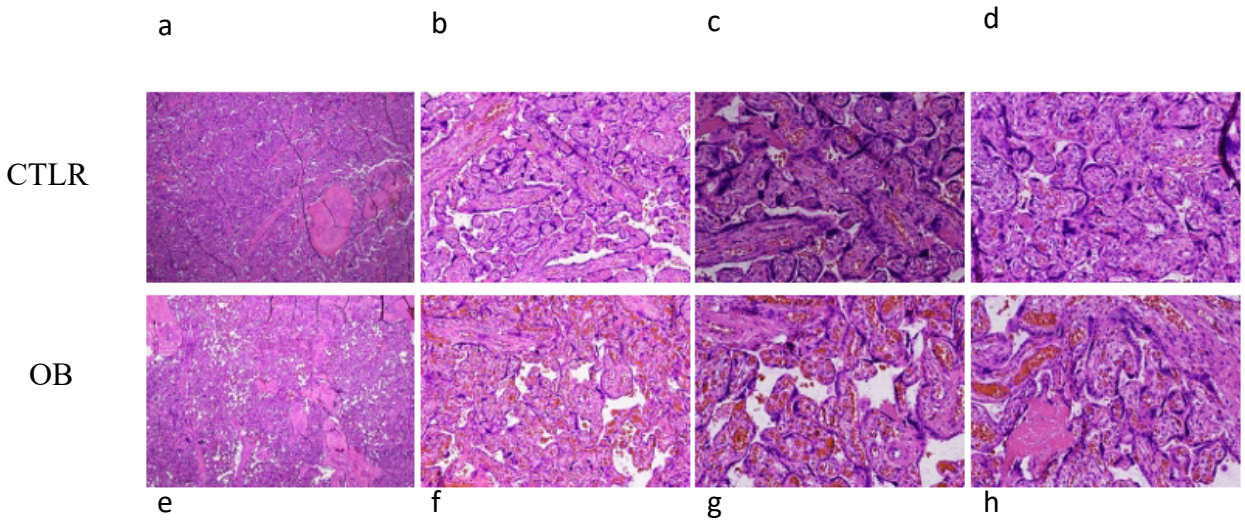


Figure 14. Maternal obesity increased active cleaved caspase 3 in obese placenta. Data are shown as percentage of protein expression in study group compared to controls ± SD. Target proteins were normalized on GAPDH level protein. N=16-18 per group. The Student's t test was performed as statistical analysis to compare differences in the groups. ∗ indicates statistical significance ($p < 0.05$). ns: not statistically significant.

4.9 Maternal Obesity influence placental villous tissue vascularization.

Placental villous sections were obtained from obese women and lean healthy controls to perform morphological and histological analysis. Histological evaluation of stained samples with eosin and hematoxylin underlined relevant impairments in obese placentas. In particular, obese villous tissues showed immature villi with a severe vascularization modification typical of obese placental tissue. Representative villous tissue images of two patients are shown in FIG 15 and histological analysis is summarized in the table below.



Healthy control	**Obese**
Small villi lined by monolayer of cells	Increase size of immature villi and with immature villi lined by two cell layers (*edematous*)
Small blood vessels	Increased number of blood vessels
Normal number of syncytial nodes (*Tenney Parker nodes*)	Hypoxia symptoms demonstrated by increased number of syncytial nodes
Calcifications typical of term placentas	*Corangious,* peri villous fibrin typical of obese placentas

Figure 15. Histological analysis of obese placenta: Placenta sections were fixed in 10% of formalin for 72 hours. The samples were dehydrated and embedded in paraffin and then sectioning at microtome occurred (thickness of 7um). Nuclei were stained by Hematoxylin 1:4 while eosin was used for cytoplasm visualization.

4.10 Myo-inositol stimulation did not alter Inositol 3-phosphate receptor 1

Myo-inositol treatment might have positive feedback in regulating PIP3 pathway and in our study human trophoblast cell line was used for *in vitro* myo-inositol treatment to evaluate modifications in ITPR1 protein level. Cells were treated for 24h with increasing concentration of myo-inositol in cell media (3 and 300 uM) while for negative control, complete cell media was used. Total protein were extracted and analyzed by and western blot. Data from our experiments showed no significant alteration in ITPR1 protein expression upon MI treatment in human trophoblast cell line (Fig 16). Myo-inositol did not impact Inositol-3phosphate receptor 1 protein level.

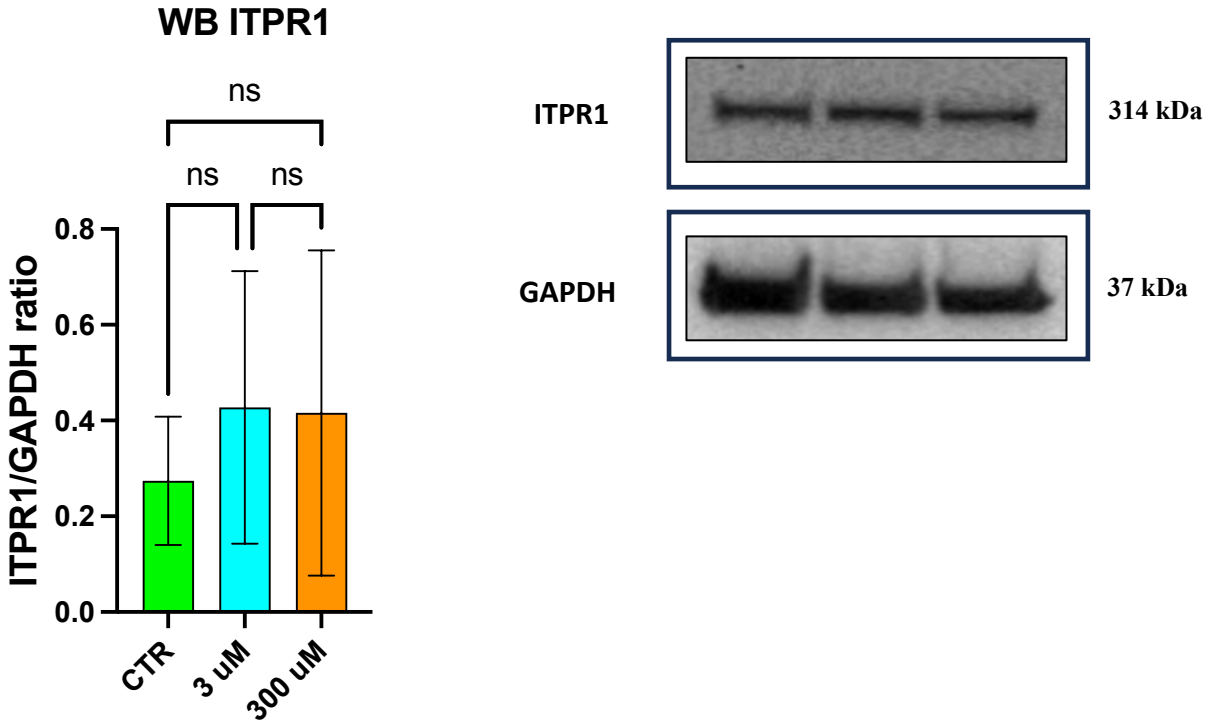
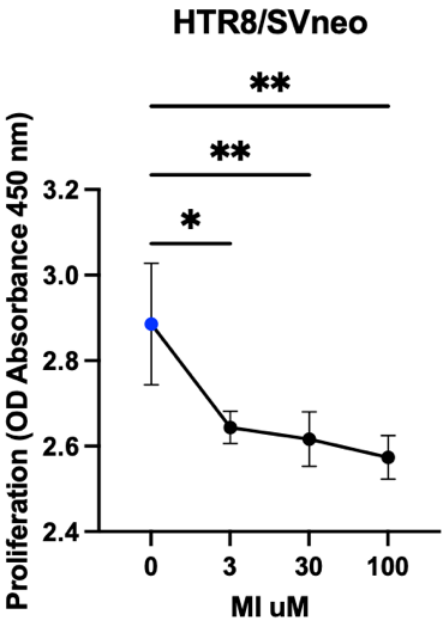


Figure 16. Myo-inositol did not influence ITPR1 expression in trophoblast cells. HTR8/SV40 cells plated in 6-well plates were treated for 24 hours with myo-inositol at 3 uM and 300 uM, or cell media (CTR). In these cells, ITPR1 protein levels were measured by western blot. GAPDH was used as loading control. Data are expressed as mean ± SD; ANOVA with a Fisher's LSD test was performed to compare control and MI treated cells. *$p < 0.05$. The results are representative of 3 independent experiments.

4.11 MI stimulation on human trophoblast cell line reduced cell proliferation

Human trophoblast cell line HTR8/SVneo was used for *invitro* experiments to investigate on MI treatment on trophoblast cell proliferation. We stimulated cells with 3, 30 and 100 µM of MI for 24h. After incubation time, CCK8 colorimetric proliferation assay was performed. Data in fig 17 showed a decrease in proliferation rate upon MI treatment in a dose dependent manner. Our results indicate that MI has an effect in reducing cell proliferation in human trophoblast cell line.



* p=0.012
** p=0.007; p=0.006

Figure 17. Myo-inositol treatment reduce proliferation rate in human trophoblast cell line. HTR8/SV cells plated in 96-well plates were treated for 24 hours with MI at 3µM, 30µM and 100µM or cell medium. Data are shown as mean of OD absorbance without blank. Values are expressed as treated/unstimulated sample ratio. The sum of three independent experiments is reported and error bars represent ± SEM. Experiments were performed in technical quintuplicates. The ANOVA test was performed as statistical analysis for multiple comparisons. P values were statistical significance when $p < 0.05$. p values were p=0.012 in the 3 µM condition; p=0.007 in 30 µM and p=0.006 in 100 µM.

4.12 Myo-inositol stimulation did not influence apoptosis in trophoblast cell line.

We aim to investigate on effects of MI treatment on cell apoptosis, regulated by upregulation of PIP3 pathway and after our previously described data in which MI decrease cell proliferation. Trophoblast cells were treated for 24h with increasing concentration of myo-inositol in cell media (3 and 300 uM) while for negative control, complete cell media was used. Active cleaved caspase 3 at Asp175 residue were analyzed as specific target of apoptosis pathways. When cells expressed cleaved CAS3 they undergo to irreversible apoptosis process, causing cell death. Data showed no

significant alteration in CAS3 protein level upon MI treatment (Fig 18). Myo-inositol did not influence cell apoptosis and did not cause cell death on placental trophoblast cells.

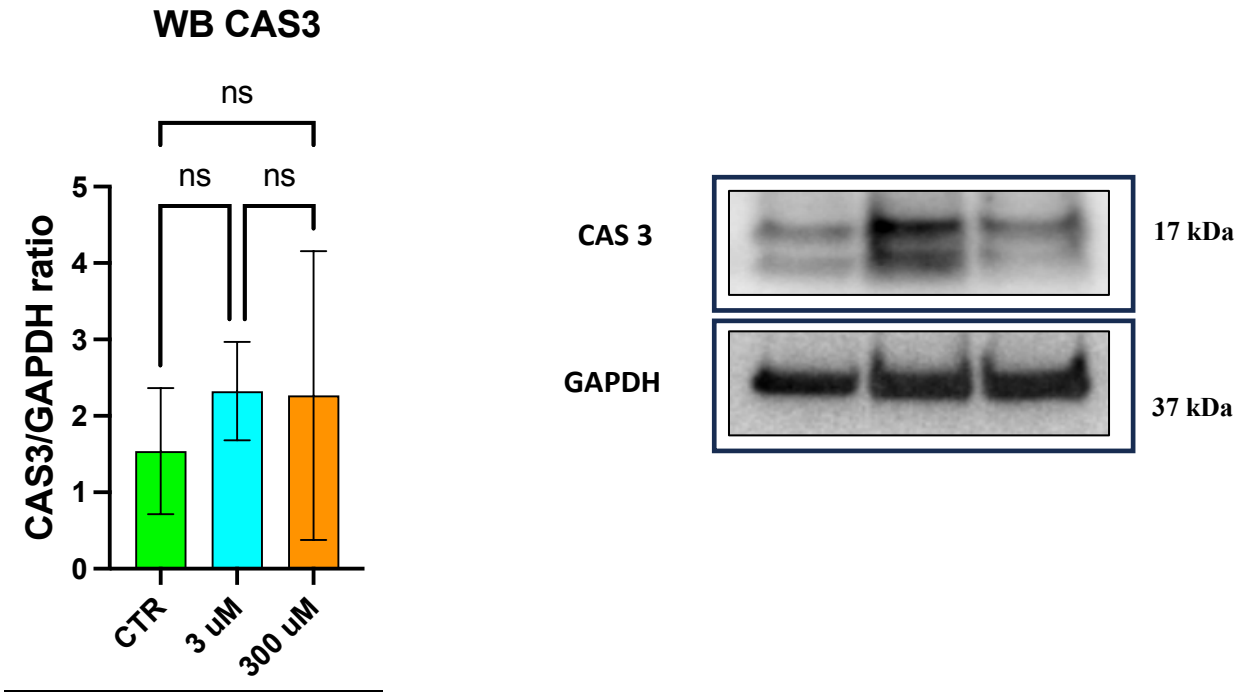


Figure 18. Myo-inositol did not influence apoptosis in trophoblast cells. HTR8/SV40 cells plated in 6-well plates were treated for 24 hours with myo-inositol at 3 uM and 300 uM, or cell media (CTR). In these cells, CAS3 protein levels were measured by western blot. GAPDH was used as loading control. Data are expressed as mean ± SD; ANOVA with a Fisher's LSD test was performed to compare control and MI treated cells. *$p < 0.05$. The results are representative of 3 independent experiments.

4.13 Myo-inositol treatment did not affect VEGF expression in trophoblast cell line

Vascularization in trophoblast cell line was evaluated after myo-inositol stimulation for 24 hours. In order to investigate on cellular vascular status, we analyzed vascular endothelial growth factor (VEGF) protein level by western blot. Cells were stimulated with different MI concentration 3 uM, 300uM and with complete cell media as control.

Data showed no significant alterations of VEGF between the three tested conditions (Fig 19). Myo-inositol did not affect vascularization via VEGF pathway in HTR8/SV40 cells.

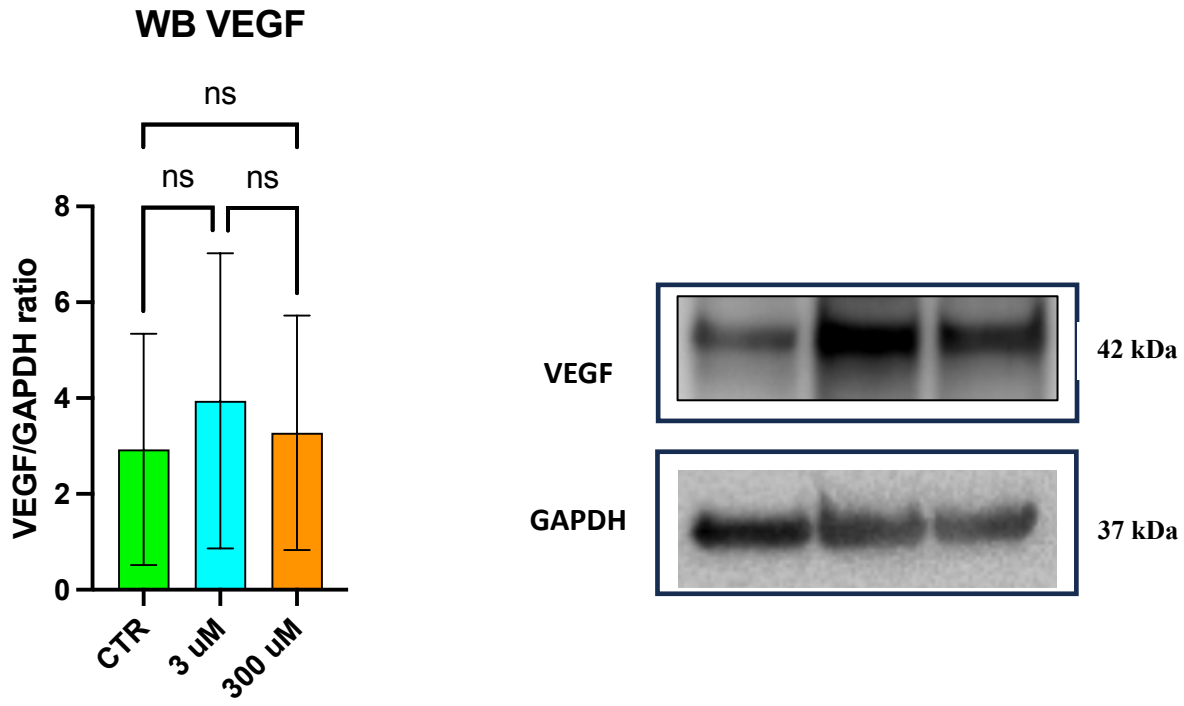


Figure 19. Myo-inositol did not influence vascularization via VEGF in trophoblast cells. HTR8/SV40 cells plated in 6-well plates were treated for 24 hours with myo-inositol at 3 uM and 300 uM, or cell media (CTR). In these cells, VEGF protein levels were measured via western blot. GAPDH was used as loading control. Data are expressed as mean ± SD; ANOVA with a Fisher's LSD test was performed to compare control and MI treated cells. *$p < 0.05$. The results are representative of 3 independent experiments.

Effects of nanoplastics treatment on human trophoblast cell line

4.14 Nanoplastics induce morphological alterations in trophoblast cells.

Environmental contaminants as nanoplastics have a concerning role in initiate a cell damage response (CDR) in human cells. To investigate on macroscopic alterations in placental trophoblast cell morphology, we treated human trophoblast cell line with increasing concentration of NPs for 24h (10, 50 and 100 ug/ml) in cell media without FBS. After 1 day of exposure time, microscopy observation occurred. We showed cell morphological alteration upon Polybeads nanoplastic treatment Fig 20 (A-D). Cells treated with microspheres 0.05um are morphological impaired with an elongated morphology that differ from CTRLs. We confirm that NPs exposure causes alteration in placental cell morphology and number after only 24 hours of supplementation. In addition, our data demonstrates that in higher concentrations, nano particles tend to aggregate forming bigger clusters.

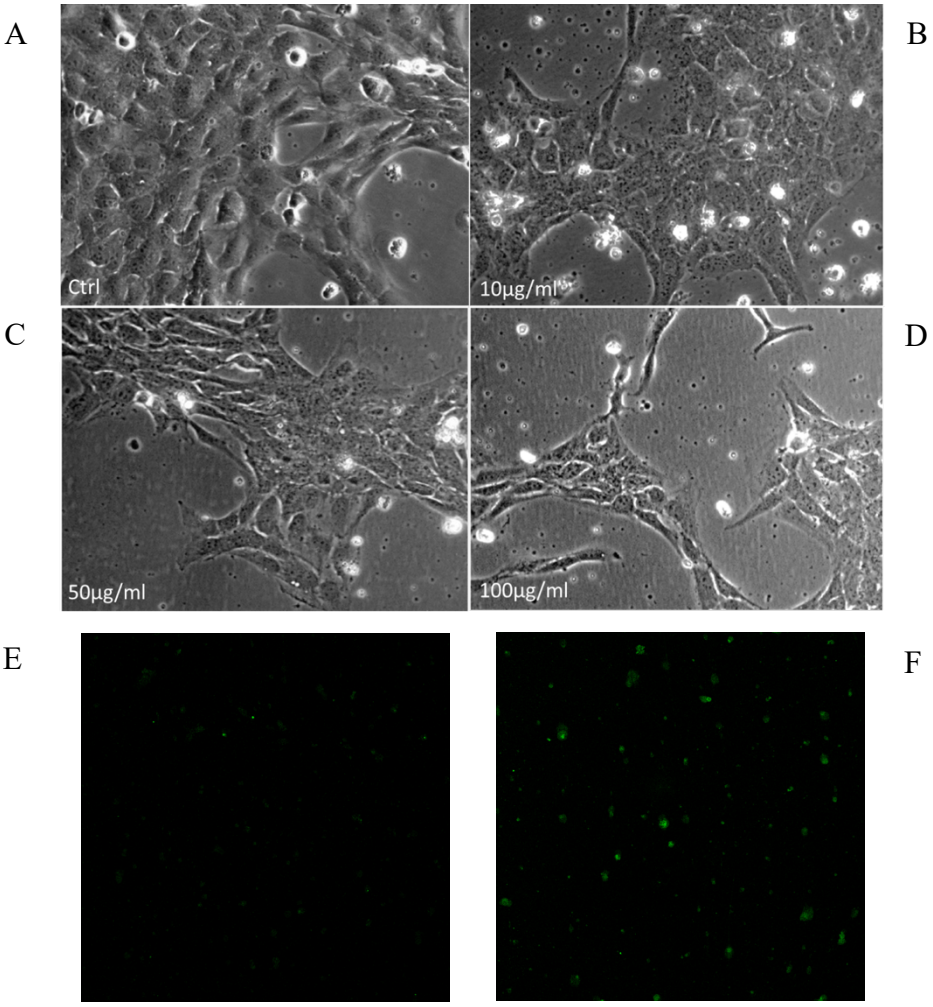


Figure 20. Effects of Polybeads treatment in placental trophoblast cells. HTR8/SV40 cells grown on coverslips were incubated with Polybeads microspheres 0.05um at 10 ug/ml 50 ug/ml, 100ug/ml or cell media (CTRL) for 24 hours. (A-D) (Scale bar = 20 µm). (E-F) In this figure we showed Fluorite Polybeads at 100ug/ml (E) or as stock concentration of 25mg/ml (F). Green signal represents conjugated Fluorite-Polybeads.

4.15 Microspheres are internalized in placental trophoblast cells

We demonstrate a morphological alterations of trophoblast cells treated with microspheres 0.05um of diameter. To investigate whether if NPs are able to enter in the cells we stimulate HTR8/SV40 with increasing concentrations of NPs 10ug/ml, 25ug/ml, 50 ug/ml, 75ug/ml and 100ug/ml or negative controls with complete cell media. Fluorescence staining was performed, and images were acquired at fluorescent microscope. Data showed that microspheres 0.05um enters in the cells at 10ug/ml conditions in the cell cytoplasm. We demonstrate internalization of nanoplastic in placental trophoblast cells (Fig 21). This accumulation of NPs might cause the morphologic alteration revealed in the previous data.

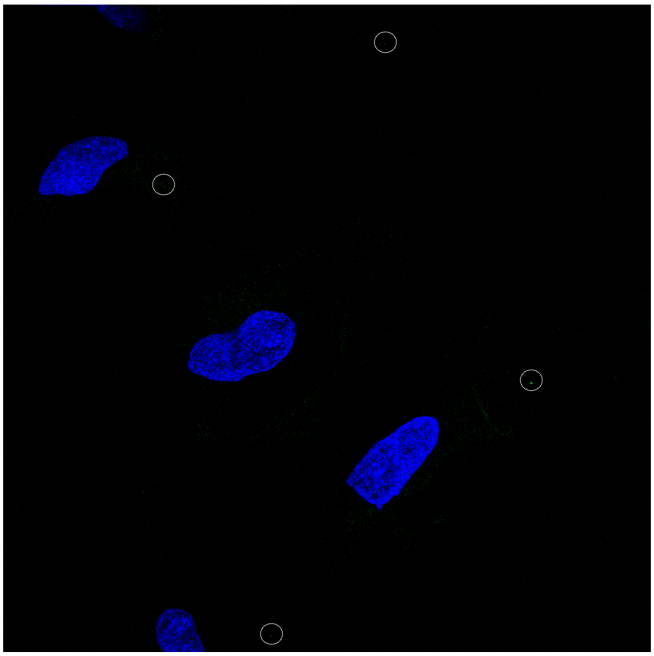


Figure 21. In vitro exposure of NPs on placental trophoblast cells cause internalization and accumulation of microspheres in the cell cytoplasm. HTR8/SV40 cells plated in 24-well plates were treated for 24 hours with Fluoresbrite YG microspheres 0.05um

(green, in white circles) in the cytoplasm at 10 ug/ml. Cells were stained with DAPI for nuclei detection (Blue). The image is representative of 2 independent experiments.

4.16 NPs exposure affect cell viability inducing cell cytotoxicity on trophoblast cells.

We aim to investigate on microspheres effect on placental trophoblast cell in proliferation and viability. The cell proliferation/cytotoxicity assay highlighted that a cytotoxic effect of microspheres is observed at 10 µg/ml concentration, determining the 60% of death. This effect, however, is not dose-dependent and results the same at the higher concentrations (25, 50, 75, 100 µg/ml), in contrast to observed morphological results. Data demonstrates that nanoplastic exposure causes cell cytotoxicity of placental trophoblast cells (Fig 22).

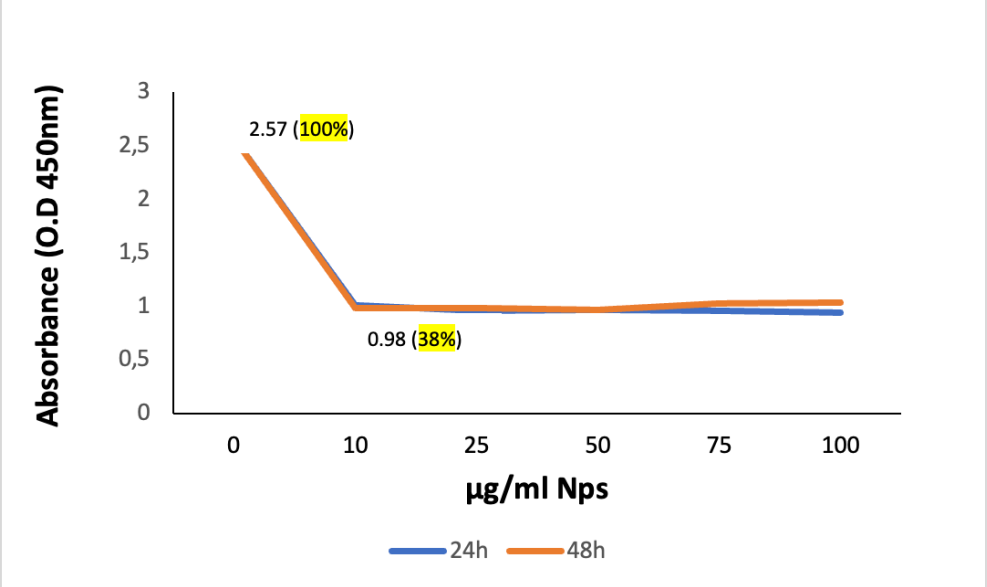


Figure 22. Nanoplastic microspheres influences HTR8/SV40 proliferation. HTR8/SV40 cells plated in 96-well plates were treated for 24 or 48chours with Polybeads microspheres 0.05 um at 10 ug/ml, 25 ug/ml, 50 ug/ml, 75ug/ml and 100 ug/ml or cell media (0). Cytotoxicity was measured by CCK8 assay following the protocol instructions. Absorbance at O.D 450 nm was used for cytotoxicity calculation. Data are expressed as percentage of cell vitality compared to untreated cells used as control. The results are representative of 5 independent experiments.

5 DISCUSSION AND CONCLUSIONS

Exposure to inference factors during pregnancy can influence disease risk lifelong and across generations for newborns. During adulthood, restoring nutritional imbalance or limit exposure to environmental pollution can reverse the health impact of a temporary challenge, however, the consequences are more detrimental when experienced early in life. Developmental origins of health and disease (DOHaD) describes that experiences during early life can alter developmental paths, leading to maladaptive responses to later-life environmental challenges [116]. Nowadays obesity and environmental pollutions play a fundamental role in influence maternal and baby health during pregnancy, in particular, placenta is the main organ in which such alterations take place.

Obesity is an excessive or abnormal accumulation of fat of adipose tissue in the body, leading to health impairments thought its association with an increased risk of developing conditions such as diabetes mellitus, cardiovascular disease, hypertension, and hyperlipidemia. This prevalent public health concern has steadily worsened over the last five decades. Obesity is characterized by a body mass index (BMI) greater then 30 kg/m^2 while overweight have a BMI between 26 kg/m^2 and 30 kg/m^2.

Placental metabolism perturbance leads to adverse intrauterine environment causing altered fetal programming. This is a strong predictor of long-term adverse outcome in offspring [117-118]. In the recent years it is becoming more evident that inositol diet supplementation has a beneficial role in modulating maternal and, consequently, fetal

health. Importantly, the intracellular pathway of myo-inositol in placenta is still largely unknown and knowing how it can restore healthy maternal and baby outcomes is fundamental to develop new therapies and to find predictive biomarkers in maternal obesity condition.

We aimed to investigate myo-inositol pathway in placenta complicated by maternal obesity, in order to find predictive biomarkers.

In our study we collected villous tissue from term placentas of obese/overweight and lean healthy control to investigate on the intracellular pathway of myo-inositol molecules in order to elucidate its possible alterations due to maternal obesity. In addition, we performed *in vitro* experiments on a human trophoblast cell line (HTR8/SV40) to analyze cellular processes, including cell proliferation, apoptosis, and vascularization upon myo-inositol treatment. We hypothesized that inositol intracellular pathway is modulated by maternal obesity in placental villous tissue.

Firstly, we collected maternal and baby characteristics to include in our analysis. Our data did not show differences in placental weight between the groups even if we can detect a tendency in increased weight in the obese one. In addition, baby weight showed no differences. These data are consistent with what observed in literature in which baby born from obese women have higher risk to have large for gestational age (LGA) or small for gestational age (SGA) but it is not always a direct consequence [119]. We did not observe macroscopic alterations in placental status and fetal weight, based on clinical data collection, but we detected differences at molecular level.

Data confirms that maternal obesity impairs myo-inositol placental biology, thus, we detected decreased amount of myo-inositol in the placenta of obese women confirming that its pathway is altered and modulated by maternal obesity. Importantly we found

decreased MI amount in the fetal part of placenta from obese women. This underlies an insufficient amount of MI supply to the fetus. In a recent large study, Pillai et al., described decreased myo-inositol amount in total placentas but only in gestational diabetes mellitus women [77]. Here, we showed a significant decrease in myo-inositol content in obese and overweight women. Moreover, our data demonstrated that not only GDM, but also maternal obesity affects myo-inositol amount and its biology in placenta villous tissue. In contrast with Pillai et al, we focused only on villous tissue which is the tissue responsible for the placenta oxygen and nutrient exchange function. It will be necessary to quantify MI amount in the maternal side of the placenta to verify its content and if this modification affect the total placenta of obese women. Furthermore, it may be necessary to find correlations with specific maternal characteristics and if they are involved in regulation of MI amount in placenta. In our study we showed no alterations in myo-inositol cellular intake via SMITs mRNA expression analysis. We focused on the RNA expression level, but it might be possible that a post transcriptional modification could change the protein level. Moreover, MI synthesis seems not to be affected by maternal obesity as revealed by ISYNA1 mRNA analysis. Importantly in the future, it is necessary to perform investigation on the protein level of such targets to really understand if the cellular intake and synthesis are affected in villous tissue from obese women. In addition, a recent study published in 2020 found a correlation with lower circulating myo-inositol amount in blood and 9 single nucleotide polymorphisms loci [120]. This might be a next step to include in our future projects, in fact, we may perform genetic associations studies in placenta tissue of obese women. It will allow us to verify if there is a correlation with those 9 SNPs and MI amount.

While our study could not permit the identification of the mechanisms involved in the decrease in MI amount, it highlighted alterations in the downstream target, phosphatidyl-inositol 3 phosphate pathway, as a potential key mechanism in the placenta dysfunctions in obesity. In particular the phosphoinositide-3-kinase (PI3K)-regulated pathway (PIP3 pathway) is involved in several cellular process. This pathway regulates proliferation and cell survival via PIK3 cascade [121]. We highlighted an upregulation of Inositol-3-phosphate receptor 1 (ITPR1) which is an intracellular receptor, expressed in endoplasmic reticulum. Upon stimulation by inositol 1,4,5-triphosphate, it mediates calcium ions release from the lumen of endoplasmic reticulum to the cytoplasm. Here, Ca^{2+} accumulation leads to activation of the serine/threonine phosphatase Calcineurin (CN). It is a crucial connection between calcium signaling and cellular functions including cell proliferation, apoptosis, and tissue vascularization process. We detected an upregulation of PIP3 as showed in ITPR1 and active calcineurin increased protein levels. Interesting the full length of CN is not altered indicating that it is the active pathway that is more active. This represents an original data and pave new horizons in find predictive biomarkers of placenta alterations due to maternal obesity.

In recent years it is well known that epigenetic represent a bridge between environmental factors, including nutrition, and baby health. We can hypothesize that ITPR1 is regulated via epigenetic modifications due to altered intrauterine environment caused by maternal obesity. It is known that ITPR1 epigenetic variations are crucial in several cellular processes and, therefore, altered in some diseases. For instance, ITPR1 protein is known to be fundamental in fertilization process and to be upregulated in non-obstructive azoospermia patients in testicular tissue [122]. Its

upregulation is found to be correlated with calcium-apoptosis pathway in such tissue. Importantly, a specific miRNA called has-miR-34b-5p/ITPR1 axis represent a valuable predictive biomarker for this disease [122]. It will be necessary investigate on this miRNA or others that are predicted to modulate ITPR1 expression. In another important large study, it was demonstrated that ITPR1 mRNA downregulation in placenta was correlated with reduced fetal growth in male babies and increased placental epigenetic age acceleration [80]. We observed an increase of ITPR1 protein level, but we did not analyze mRNA level, we can assume a modification in the mRNA expression, and it may be caused by epigenetic alterations. ITPR1 epigenetic altered regulation is involved in several disease such as thyroid cancer in mice, in which it has been demonstrated a down regulation of miRNAs able to silent ITPR1 expression [123]. Furthermore, also in human vascular dementia patients specific miRNAs as 10b-3p, miR-29a-3p and miR-130b-3p responsible for silence ITPR1 expression, where found differential expressed compared to healthy controls in plasma [124]. ITPR1 represent an important target to explore in obese placenta. In particular, we aim to investigate on previously mentioned miRNA targets, in particular those involved in vascularization impairments. In fact, histological analysis found significant alterations in the obese group, mainly in vascularization status as showed by less amount of blood vessels and, also, the presence of multilayer of endothelial cells around such vessels. This may be correlated by upregulation of ITPR1 protein level and, consequently, PIP3 pathway. Therefore, vascularization is slightly decreased in obese placental villous tissues samples as we can observed from VEGF protein level analysis ($p=0.07$). This data is in accordance with histological observations. In addition, ITPR1 plays a role in induce ER trigger apoptosis [125]. Importantly, we detected an increase in apoptosis

in the obese villous tissue explants and not differences in proliferation as revealed from proliferating cell nuclear antigen (PCNA) analysis. Indeed, we analyzed cleaved caspase 3 protein level that was significantly increased in the obese group. This indicates that PIP3 upregulation we observed by ITPR1 and active CN, is involved in increasing apoptosis in obese placentas compare to the controls. To verify if MI supplementation might restore healthy condition in trophoblast cells, we performed *in vitro* experiments of myo-inositol stimulation on HTR8 cell line.

ITPR1 protein level was not modified by free MI treatment after 24 hours, indicating that it is not altered by direct free MI amount in the cytoplasm. ITPR1 upregulation might be a result of a milieu of epigenetic factors typical of obese intrauterine environment.

In *in vitro* experiments MI supplementation did not modulate apoptosis, but it decreased cell proliferation rate in a dose dependent manner. Myo-inositol may play a role in protecting cells from excess cell proliferation typical of placenta of obese women. Future experiments will be focused on the investigation on the role of cytoplasmic free myo-inositol in modulating such processes. Importantly, it might be necessary to perform *in vitro* experiments on obese primary trophoblast cells that represent more valuable samples because its derivation from patients. It will allow, then, to investigate also on genetic variations between individuals ad find possible clinical co morbidities in addition to obesity. In order to better elucidate effect of ITPR1 upregulation we aim to include in the analysis also severe obese women (BMI greater than 35 or 40 kg/m^2) to verify the myo-inositol pathway alterations in such

women. Moreover, it might be necessary to perform our analysis on women who gave birth small for gestational age (SGA) or large for gestational age (LGA) babies.

One of the main limitations of our study is that we did not performed experiments on primary trophoblast cells. In fact, the data from tissue analysis are results of a pool of different cell types, including trophoblast cells, mesenchymal cell, epithelial cells, and blood cells. In future we aim to isolate primary trophoblast cells from obese and lean women and investigating on myo-inositol pathway and, in particular, on ITPR1. In vitro experiments on primary cells will allow us to stimulate them with increasing concentration of myo-inositol coupled with and without glucose and verify modulation in its pathway and in proliferation and apoptosis. In addition, we have already collected cord blood serum from vein and arteries of obese and controls. We aim to quantify MI amount in such samples to verify if there are modification sin the maternal MI supply to the fetus. It might also explain the decrease level of MI in obese fetal side villous tissue. Furthermore, we aim to include a third group in our study. We aim to collect placentas from women which undergoes to a diet supplemented with myo-inositol starting from the first trimester of pregnancy to verify the possible restoring of ITPR1 protein level due to a controlled diet. In conclusion ITPR1 represent a promising biomarker for health status of the placenta since it is involved in several cellular processes. Understanding the epigenetic mechanisms behind this upregulation in placenta from obese women will help in the development of new tools to be used in therapy to restore the metabolic profile and ameliorate fetus health condition.

Nanoplastic as interference particles on placental trophoblast cells

The global production of plastics has reached the impressive amount of more than 350 million tons per year. This extensive utilization of plastics has, in turn, resulted in their accumulation within landfills and the natural environment. Specifically, once introduced into the environment, plastic products undergo a degradation process initiated by atmospheric elements such as waves, abrasion, UV radiation, and photo-oxidation. This process, coupled with biological activities, contributes to the formation of nanoplalstics particles (NPs) [126-128]. The ubiquitous presence of NPs in the environment led to an inevitable exposure for the human body via dermal contact, inhalation, and ingestion [129]. In the recent study, Ragusa et al, demonstrated an abnormal accumulation of NPs occurred in human breastmilk. This is an important finding, point out that breastfeeding may cause a transfer of such particles to the offspring causing baby health issues [100]. Pregnancy is exposed to NPs contaminants and also placenta is known to be affected by plastic accumulation. It was showed the accumulation of plastic particles in both fetal side and maternal side, indicating that once NPs enter in the human body can reach placenta tissue in all layers. Moreover, using scan microscopy analysis it has been demonstrated the internalization of such particles in human placenta and their intracellular localization, in particular, polystyrene. In fact, some organelles were susceptible to NPs fragments including endoplasmic reticulum and mitochondria. These organelles in placentas were found morphological altered compatible with cell stress phenotype [130]. In particular,

abnormal amount of NPs in the cells might cause a cell damage response (CDR) due to the inability of degrades it and, subsequently, causing cell death. In the second part of the project included in the thesis, we aim to investigate on the effect of polystyrene NPs *in vitro* treatment on human trophoblast cell line. We demonstrated that trophoblast cells were susceptible to small concentration of NPs (10 ng/ml) for 24 hours, leading to 60% of cell death compared to untreated conditions. These data showed that even a small amount of NPs exposure for one day, strongly cause trophoblast cell death, blocking their proliferation. We also demonstrate that cytotoxicity was caused by an internalization of such particles as showed by our confocal microscopy data. Moreover, NPs not only cause cell death but also cell morphological alteration with altered cell shape compared to untreated condition. In future we aim to characterized and localized NPs fragments inside the cell, performing scanned electron microscopy investigations. Next, analysis on endoplasmic reticulum key targets will be done for better elucidate the pathway involved behind this increased trophoblast cell cytotoxicity. Moreover, mitochondria are thought to play a crucial role in cellular nanoplastic accumulation response, for this reason, future studies have to focus on mitochondria state, reactive species of oxygen damage and master regulators of mitochondrial functions and biogenesis. Importantly, the main limitation in our study is that we used plastic tools (pipets, tips, well-plates, media bottles) to perform *in vitro* studies and this represent a bias in the analysis. Moreover, we used only one type of plastic compound (polystyrene) while in the environment there are plenty of plastic fragments type. Our study is one of the first analysis on nanoplastic effects on trophoblast placental cells demonstrating their cytotoxic effects on placenta. In future is necessary to perform NPs treatment on primary trophoblast cells and do association

analysis with women habits in consuming plastic products, including food and cosmetics. Nano plastics will be a more serious concern about public health in future and knowing their effect on placental health will help us to understand how limit placental damage and improve maternal and newborn health.

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