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Characterization of Placental Autophagy and Bioenergetics, and Maternal MicroRNAs Profiling in Obese Pregnancies and Gestational Diabetes Mellitus

[BIO/12, MED/49, MED/40]

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Contents

1. ABSTRACT

BACKGROUND

Maternal obesity (MO) is expanding worldwide, contributing to Gestational Diabetes Mellitus (GDM) prevalence. The altered placental function and intrauterine environment of MO and GDM are associated with adverse maternal and fetal outcomes, with short- and long-term complications. Growing evidence suggests that both MO and GDM are characterized by placental autophagy disturbances, mitochondrial (mt) dysfunctions and epigenetic alterations, which could contribute to the pathogenesis of pregnancy-related metabolic diseases. For these reasons the aim of this work was to characterize placental autophagy and mitochondrial bioenergetics, and maternal plasmatic microRNAs profile in the context of MO and GDM.

METHODS

60 women with spontaneous singleton pregnancies delivering by elective Caesarean section were enrolled: 25 normal-weight (NW), 22 obese without comorbidities (OB/GDM(-)), 13 obese with GDM (OB/GDM(+)). The expression of antioxidant metabolism markers and autophagy-related genes expression was evaluated in placental villous tissue by Real-time PCR with SYBR green chemistry. In placenta, we also quantified ATP by CellTiter-Glo Luminescent Cell Viability Assay adapted to placental villous tissue, and mt Complexes I-V protein content by Western Blot experiments. Moreover, in a subgroup of 7 NW, 6 OB/GDM(-) and 6 OB/GDM(+), microRNA (miRNA) profiling in maternal plasma at delivery was performed with miRCURY LNA Serum/Plasma miRNA Focus PCR Panel (*Qiagen*).

Statistical analysis and bioinformatics predictive tools: SPSS v.27 (*IBM*); GeneGlobe (*Qiagen*); miTALOS v.2; miRPath v.3.

RESULTS AND CONCLUSIONS

Placental antioxidant genes expression (CAT, GPX1, GSS, GSR) tended to decrease, the pro-autophagic ULK1 increased and the chaperone-mediated autophagy (CMA) regulator PHLPP1 decreased in OB/GDM(-) vs NW. On the other hand, PHLPP1 expression increased in OB/GDM(+) vs OB/GDM(-). These preliminary findings showed placental alterations of autophagy in MO and GDM, paving the way for further analyses aimed at elucidating the role of placental autophagy in metabolic disorders during pregnancy.

Moreover, placentas of OB/GDM(-) and OB/GDM(+) women had reduced ATP level vs NW. All OB showed decreased Complex I vs NW. Furthermore, lower expression of Complexes III and V was found in OB/GDM(+) group (vs NW; vs NW and OB/GDM(-), respectively), along with a reduced fetal/placental weight ratio (i.e. placental efficiency) vs NW. These results might suggest impaired placental mt function in obese subjects, especially with GDM.

Sexual dimorphism in terms of the above-mentioned placental molecular markers was also investigated, with intriguing results suggesting that females and males might respond differently to insults and/or have differential, sex-dependent compensatory mechanisms in order to deal with the perturbed environment.

MiRNA profiling in maternal plasma highlighted patterns of significantly differentially expressed miRNAs between the three groups. In particular, OB/GDM(-) vs NW differed in the expression of 4 miRNAs, OB/GDM(+) vs NW of 1, and OB/GDM(+) vs OB/GDM(-) of 14. Pathway enrichment analysis by miRPath and miTALOS revealed many pathways associated with the set of miRNAs of each comparison, among which the most interesting and relevant in our context were related to nutrients and hormones metabolism, inflammation and oxidative stress. Indeed, miRNAs could be promising biomarkers of metabolic alterations in MO and GDM. Nevertheless, future investigations are needed to further deepen the pregnancy epigenetic landscape.

2. INTRODUCTION

2.1. The human pregnancy: the central role of the placenta

Pregnancy is a physiological event lasting about 40 weeks and including all the processes ranging from the conception of the embryo to delivery. This state is typically divided in three trimesters, during which it is possible to identify an embryonic period (the first 10 weeks), characterized by the organization of the body plan and the differentiation of the major organs, and a fetal period (from the 10th to the 40th week), in which the growth and maturation of the fetus prevail (Polin *et al.,* 2011). Pregnancy can be defined as a *three-compartment model* in which the mother, the fetus and the placenta have an active role, talking to each other in a continuous cross-talk. Each compartment has its own characteristics that can influence pregnancy outcome. By adapting and interacting, these cooperating compartments allow a healthy gestation, and, by keeping the mother and the fetus always in close contact, the placenta is fundamental for appropriate fetal growth and development (Mandò C., 2020).

The human placenta is a transient specialized organ of fetal origin representing the main interface between maternal and fetal blood circulations. It is formed in the uterus mainly during the first half of pregnancy, through a complex and synchronized process called placentation, which involves multiple interactions between maternal and embryonic cells (Gude *et al*., 2004). The human placenta is a discoid hemochorial organ, meaning that the fetal trophoblasts are in direct contact with maternal blood, while fetal and maternal blood never come in contact. The two sides of the placenta are, on one hand, the basal maternal plate that is connected to the endometrium, and on the other hand, the chorionic fetal plate to which the umbilical cord is attached (Malnou *et al*., 2019; Maltepe and Fisher, 2015) (**Fig.1**).

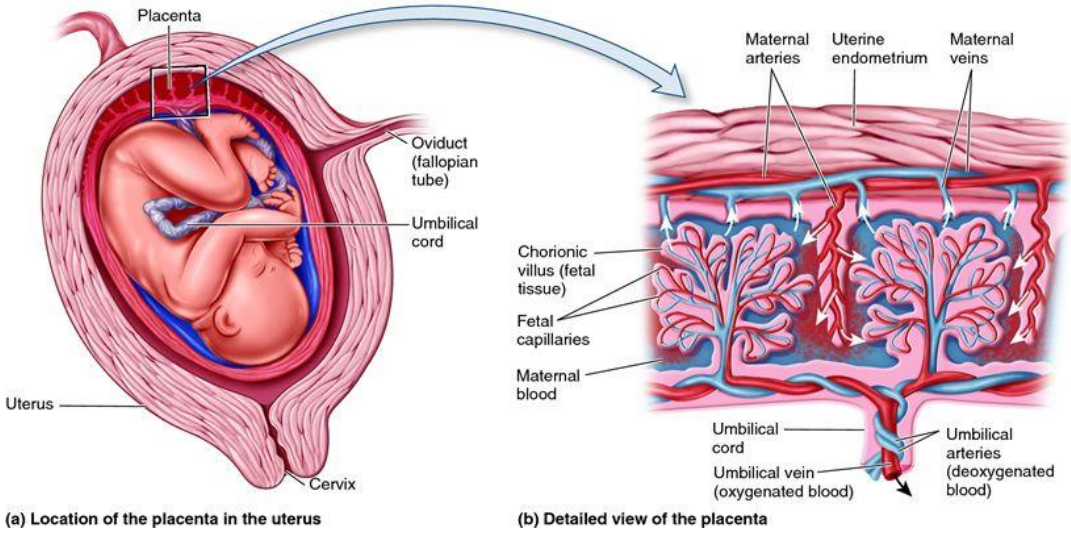


Figure 1 | *Anatomical location (a) and structural characteristics (b) of the human placenta* (https://biology-forums.com).

The structural and functional units of the human placenta are the chorionic villi, which consist of mononuclear cytotrophoblasts and multinucleated syncytiotrophoblasts; the syncytiotrophoblasts, located at the maternal-fetal interface, derive from the fusion of cytotrophoblasts and are involved in many placental functions (Guibourdenche *et al*., 2009; Sultana *et al*., 2017).

Notably, the placenta has multiple functions, including nutrients and O2 supply, elimination of waste products, selection and filtering of substances, hormones production (e.g. progesterone, estrogens, human chorionic gonadotropin, human placental lactogen, placental growth hormone) (Malnou *et al*., 2019). The placenta is far from being a passive barrier, in fact it is a metabolically active organ with its own energetic demands. Indeed, in the first half of pregnancy, the placenta uses the 50% of oxygen and glucose received from maternal circulation for its own growth and metabolic functions. Then, in the second half of pregnancy, when fetal growth is greater than placental, it transfers most of the nutrients to the fetus. The quality and quantity of these nutrients are conditioned by the functionality, the shape and the size of the placenta, which are in turn affected by the nutritional status, the lifestyle and the metabolism of the mother, influencing the entire fetoplacental compartment (Burton and Fowden, 2015).

Pregnancy is a crucial time-window during which perturbations to the intrauterine environment can have significant consequences (Carreras-Badosa *et al*., 2017; Myatt and Maloyan, 2016). The "First 1000 Days" of a new-born human are represented by the period ranging from the conception to the first two years of the child. This is a particularly sensitive time-frame characterized by a higher developmental plasticity, which is the capacity of a single genotype to generate different phenotypes (Barker *et al*., 2010). That is why adverse influences and placental dysfunctions during this critical phase can impact the fetal growth and the pregnancy outcome. Moreover, the quality of gestation not only influences pregnancy itself, but has also effects on the offspring's future health, according to the "fetal programming hypothesis" or "Developmental Origin of Health and Disease (DOHaD) hypothesis": exposure to an altered maternal-fetal milieu can result in permanent changes of structural and functional development of the fetus. This is due to adaptive responses that can modulate both physiological functions and susceptibility to diseases, increasing the risk of metabolic and cardiovascular pathologies (e.g. obesity, diabetes, hypertension and heart disease) in the child and in the adult, later in life (De Boo and Harding, 2016; Langley-Evans and McMullen, 2010).

2.2. Maternal obesity and Gestational Diabetes Mellitus

Maternal obesity is expanding worldwide representing a significant risk factor for adverse pregnancy outcomes, with short- and long-term consequences for both the mother and the offspring (Schaefer-Graf *et al*., 2018; Catalano *et al*., 2017). The short-term complications for obese mothers include Gestational Diabetes Mellitus (GDM), which is the most frequent complication of pregnancy presenting in 2–14% of all cases (Bordin *et al*., 2020), gestational hypertension, preeclampsia, pre-term delivery, respiratory complications and thromboembolic events. The offspring can be affected by congenital malformations, stillbirth, shoulder dystocia and, because of the altered nutritional status which finally results in malnutrition, either fetal-growth restriction or macrosomia (Parisi *et al.,* 2021). Both the mother and the baby, especially Large for Gestational Age (LGA) infants (i.e. > 4 kg), are also more susceptible to long-term complications later in life (e.g. obesity, type II diabetes, cardiovascular diseases, cognitive impairments, neuropsychiatric disorders).

The incidence of obesity among pregnant women is now estimated at 18.5-38.3% (Myatt and Maloyan, 2016). Moreover, the risk of developing GDM increases up to 50% in obese women. This, in turn, implies an increased risk for later development of type II diabetes in the mother, creating a vicious cycle of metabolic disorders (Álvarez *et al.,* 2017).

Maternal obesity and GDM are associated with low-grade chronic inflammation and excessive increase in oxidative stress, altered release of adipokines (e.g. leptin), cytokines (e.g. TNF-α, IL-6, IL-1β) and lipotoxicity (Parisi *et al.,* 2021; Howell and Powell, 2017). Altogether these alterations result in a perturbed intrauterine environment affecting placental structure and function. In particular, placentas from obese mothers are generally characterized by higher weight and thickness, lower placental efficiency, disturbed nutrient transport and altered energy modulation (Parisi *et al.,* 2021, **Fig.2**). Moreover, such a deranged intrauterine environment is among the main factors affecting the programming of fetal metabolism and health (Martino *et al.*, 2016), with possible transgenerational effects (Barker *et al.*, 2010; Saben *et al.*, 2014).

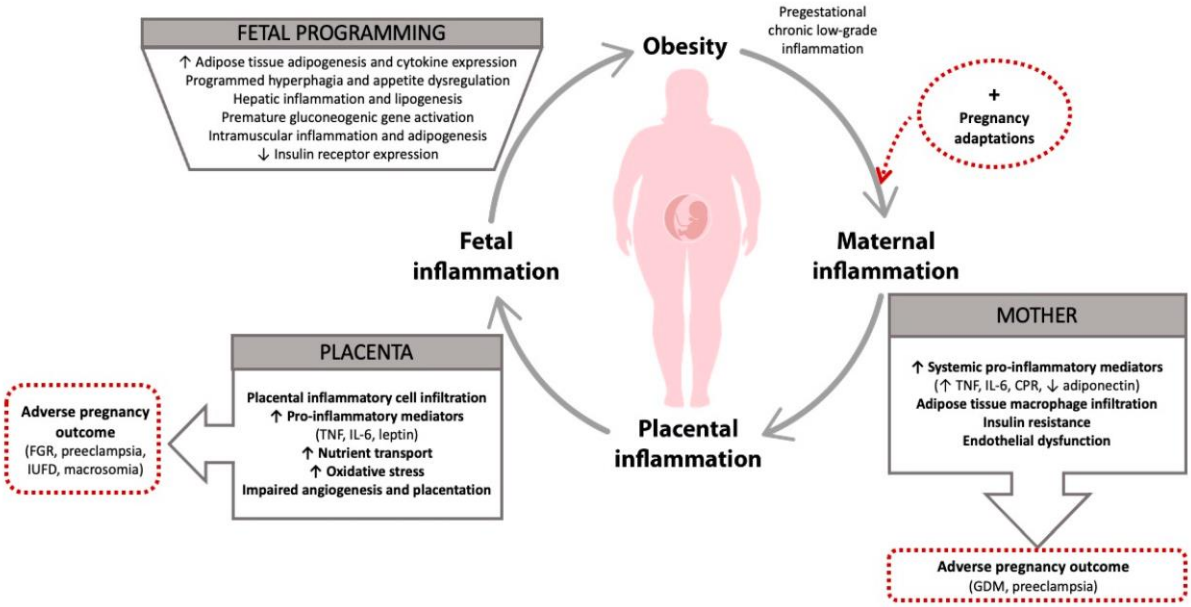


Figure 2 | *The vicious cycle linking maternal low-grade chronic inflammation, which characterize maternal obesity, to feto-placental derangements with multiple short- and long-term possible adverse outcomes* (Parisi *et al.*, 2021).

2.3. Autophagy in placenta, maternal obesity and GDM

Editor's note: the following paragraph and subparagraph are modified excerpts from the published review **"Serati A., Placental autophagy in maternal obesity and gestational diabetes mellitus"**, *Biochimica Clinica*, **2022**; Vol. 46 Supp. 1: S122-S128, DOI: 10.19186/BC_2022.029.

Autophagy, also known as autophagocytosis, is an evolutionarily conserved process and dynamic mechanism employed by eukaryotic cells to get rid of altered or defective proteins, macromolecules or organelles, in order to guarantee the cell homeostasis (Nakashima *et al.*, 2019). Importantly, autophagy is triggered by an initial detrimental stimulus such as starvation or over nutrition, hypoxia, oxidative damage and infections. Because of that, it is also considered a type of cellular death and, consequently, a pathway of cell survival and equilibrium maintenance (Gong and Kim, 2019).

The key actor in autophagy is the lysosome, first described by the Belgian biologist Christian de Duve in 1966 (De Duve and Wattiaux, 1966). Since then, the lysosome's multiple functions and complex pathways have continued to emerge. In the last 15-20 years several pathways of autophagic process have been partly dissected (Cuervo and Macian, 2012).

Autophagy includes macroautophagy and microautophagy, involved in the non-specific degradation of cellular components, and Chaperone-Mediated Autophagy (CMA), responsible for the degradation of specific cytosolic proteins inside lysosomes (Li *et al.*, 2012, Jacob *et al.*, 2017) (**Fig.3**).

In macroautophagy, the substrates (i.e. defective or damaged organelles and protein aggregates) are identified by soluble proteins and sequestered within cytosolic double-membrane vesicles termed autophagosomes, which are formed through a protein complex orchestrated by Beclin 1 (BECN1). Lysosomal delivery occurs through direct fusion of autophagosomes with lysosomes, then macroautophagy is activated by Unc-51 Like autophagy activating Kinase 1 (ULK1) (Feng *et al*., 2014). In contrast, in microautophagy the cytosolic cargos are sequestered by direct invagination of the lysosomal membrane; this creates small vesicles that are delivered directly to the lysosome lumen, where they undergo degradation along with their content (Li *et al*., 2012); however, in mammalian cells microautophagy has been poorly investigated and precise molecular mechanisms remain to be understood (Xu *et al*., 2021; Feng *et al*., 2014).

CMA is responsible for the degradation and recycling of single protein molecules and it specifically involves proteins presenting the penta-peptide consensus motif KFERQ (Kaushik and Cuervo, 2012). The waste proteins with accessible KFERQ tag are directly recognized by 70 kD Heat shock proteins (HSC70) and cross the lysosomal membrane through a protein-translocation complex formed by the multimerization of Lysosome-Associated Membrane Protein Type 2a (LAMP-2A). In the meantime, lysosomal degradation through CMA is specifically activated by PH-domain and

Leucine-rich-repeat Protein Phosphatase 1 (PHLPP1) (Gong and Kim, 2019, Tasset and Cuervo, 2016).

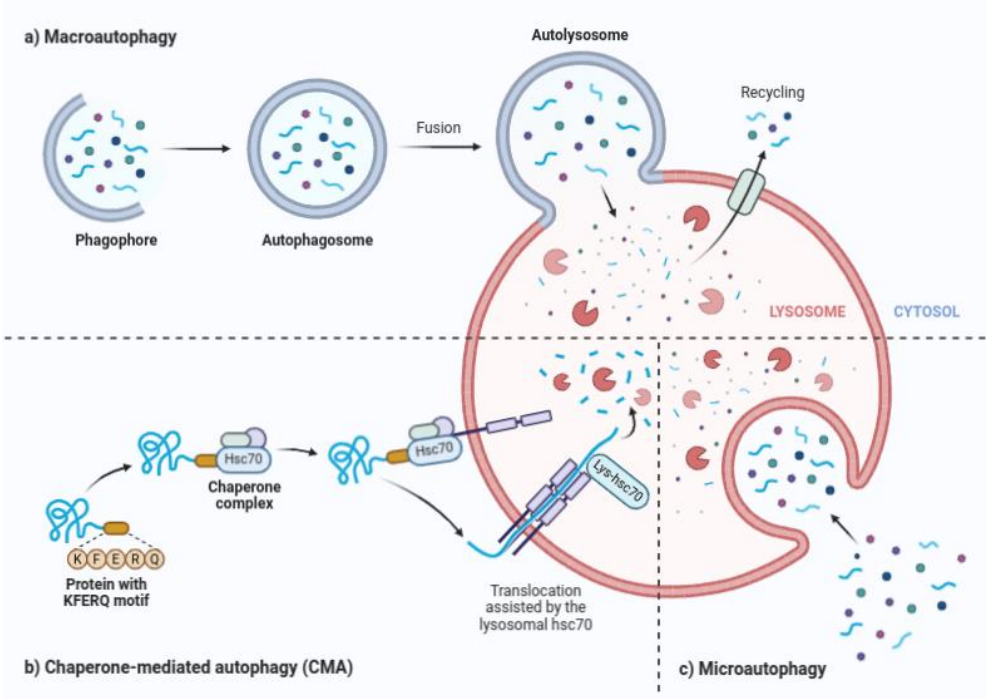


Figure 3 | *Schematic representation of the three main types of autophagy* (https://app.biorender.com).

In mammalian early pregnancy, autophagy appears to aid the development of zygotes, by clearing un-necessary maternal factors and eliminating some of the sperm mitochondria after fertilization. Moreover, further autophagy activation in the blastocyst, mediated by 17-β-Estradiol, contributes to its survival and implantation (Nakashima *et al*., 2019; Tsukamoto *et al*., 2008). During placentation, autophagy needs to be finely regulated, because both abnormal reduction and excess can lead to unfavourable outcomes. Physiologically, autophagy is activated in extravillous trophoblasts (EVTs) prior to implantation at 7 days of gestation, and this seems of critical importance for the proper invasion of maternal decidua. In both mouse and humans, autophagy is modulated by the physiological intrauterine hypoxia occurring during early pregnancy. In particular, low oxygen tension mimicking the early gestation intrauterine hypoxia was shown to induce autophagy in primary human trophoblasts. Moreover, *Hypoxia-Inducible Factor 1-alpha* (*HIF1-α*), a key regulator of hypoxia, is reported to be crucial during EVTs invasion. This factor is finely tuned by autophagic pathways, especially CMA, and when overexpressed it leads to invasion failure. Therefore, CMA is important for EVT invasion via modulating *HIF1-α* expression levels (Nakashima *et al*., 2019; Highet *et al*., 2015). Importantly, *HIF1-α* is both implicated in CMA and is

one of the main transcription factors driving *Vascular-Endothelial Growth Factor* (*VEGF*) expression (Diceglie-Anelli *et al.*, 2021), which stimulated angiogenesis and vascular network formation in the placenta. Alterations of VEGF pathway towards an increased angiogenesis and hyper-capillarization of villi have been found in the placenta of mild-hyperglycemic pregnant women, probably as a part of the compensatory mechanisms to maintain homeostasis at the maternal-fetal interface (Pietro *et al.*, 2010).

However, still little is known about placental autophagy in human physiological and pathological pregnancies during the third trimester and at term. Some evidence suggests an increase in autophagy flux in the whole placental tissue of intrauterine growth restriction (IUGR) pregnancies, although the mechanisms and the specific cells responsible for these results are still unclear (Hung *et al*., 2012). The metabolic impairment characterizing obesity and diabetes has been also linked to changes in autophagy. In particular, alterations of autophagy have been reported in the human adipose tissue of non-pregnant subjects with or without diabetes. Xu *et al*. reported upregulated mRNA levels of a few members of the *Atg (Autophagy-related genes)* family in the adipose tissue of both overweight and obese subjects, and this is suggestive of increased autophagy (Xu *et al*., 2018). Moreover, in visceral adipose tissue of obese patients with or without type II diabetes, several evidences of increased autophagic markers have been found. These demonstrate a significant activation of autophagy in the adipose tissue of obese patients, with or without diabetes (Kosacka *et al*., 2015).

Muralimanoharan and colleagues investigated autophagy dynamics in the human placental tissue of pregnancies characterized by maternal obesity and/or GDM and suggested a compensatory attempt of increased placental autophagy (Muralimanoharan *et al*., 2016). Interestingly, they also reported decreased gene expression of mitochondrial complexes of the respiratory chain and antioxidant defenses in the placental tissue of mice with a targeted autophagic gene deletion, discussing the similarities between these significant placental abnormalities in mice and those observed in human placentas with maternal obesity (Muralimanoharan *et al*., 2016). In addition, the offspring of these animals showed an increased sensitivity to obesity induced by high-fat diet, with excessive weight gain and adiposity, hyperglycemia, hyperinsulinemia and markers of cardiac remodelling, all of which resemble the phenotype of human babies from obese and diabetic pregnancies. Despite specific autophagic pathways were silenced in mouse placenta while it was overexpressed in human obese placenta, it is particularly interesting that both models are suggestive of placental abnormalities and that this placental dysfunction, possibly affected by altered autophagy, can impact the newborn's metabolic health (Muralimanoharan *et al*., 2016). Thus, placental autophagy disruption (i.e. both excess and deficiency) in this context supports the DOHaD hypothesis (Barker *et al*., 2010), correlating adverse conditions in maternal uterus with metabolic programming of chronic diseases in the offspring (Muralimanoharan *et al*., 2016). In

addition, Ji and colleagues reported the presence of autophagic vacuoles, autophagosomes and partially degraded organelles along with swollen and altered mitochondria in syncyziotrophoblasts of GDM placentas. Both placental mRNA and protein levels of autophagy markers were upregulated in these GDM patients compared to healthy women. Altogether, these evidences clearly indicate abnormal autophagy over-activation in diabetic mothers (Ji *et al*., 2017).

2.4. Mitochondrial bioenergetics, oxidative status and pregnancy

Mitochondria have a central role in the production of Adenosine Tri-Phosphate (ATP) through the Electron Transport Chain (ETC) and Oxidative Phosphorylation (OXPHOS), supporting the energy requirement of all tissues, including the placenta. Furthermore, mitochondria are important signalling organelles associated with metabolism of amino acids, lipids and nucleotides, calcium homeostasis, degradation processes (i.e. mitophagy and apoptosis), and production of steroids in the placenta and in the adrenal glands (Holland *et al.,* 2017; Pfanner *et al.,* 2019).

OXPHOS is characterized by the passage of electrons from the donors NADH and $FADH_2$ to the final acceptor, oxygen – producing H_2O – through ETC, which couples the electron transport with proton pumping out of the mitochondrial matrix across the inner mitochondrial membrane. In this way, an electrochemical gradient is created: its dissipation enables ADP phosphorylation and ATP production in the last step of OXPHOS (i.e. chemiosmosis). In particular, ETC consists of four multi-subunit enzymatic complexes, plus the ATP synthase, which is typically considered the fifth component: (1) Complex I (NADH dehydrogenase); (2) Complex II (Succinate dehydrogenase); (3) Complex III (Q-cytochrome c oxidoreductase); (4) Complex IV (Cytochrome c oxidase); (5) Complex V (ATP synthase) (Lu and Sferruzzi-Perri, 2021) (**Fig.4**).

Importantly, when mitochondria produce ATP through OXPHOS, reactive oxygen species (ROS) are also generated as a by-product due to the electron leakage occurring during the electron transfer steps (Holland *et al.,* 2017; Lu and Sferruzzi-Perri, 2021).

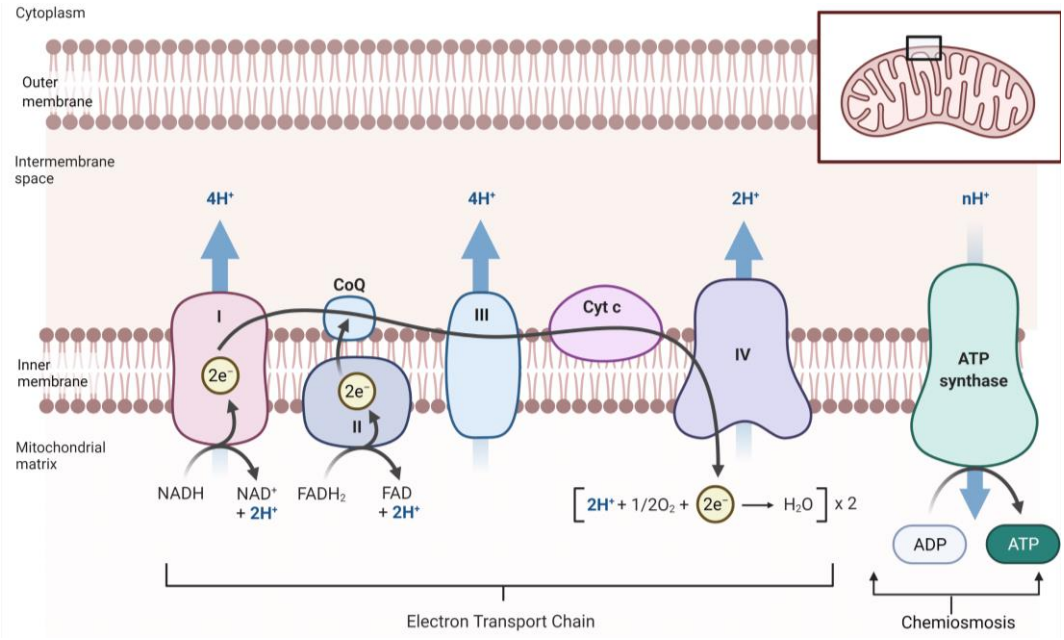


Figure 4 | *Schematic view of the Oxidative Phosphorylation (OXPHOS) process and of the Electron Transport Chain (ETC: CI, CII, CIII, CIV) + ATP synthase (CV)* (https://app.biorender.com).

Pregnancy is characterized by high energy demand and increased metabolic activity. Mitochondria are fundamental for maternal and placental metabolism and for supporting fetal growth and development. In the placenta, ATP is a fundamental energy molecule required for several biological processes, including macromolecules synthesis, active transport and hormones synthesis. Furthermore, ATP is also a signalling molecule released by placental cells throughout pregnancy, contributing to the maintenance of maternal-fetal microenvironment homeostasis (Sagrillo-Fagundes *et al.,* 2021). Indeed, structural and functional alterations in placental mitochondria could either result from or contribute to placental dysfunction and pregnancy disorders (Rodríguez-Cano *et al*., 2020, Sobrevia *et al*., 2020).

Both maternal obesity and GDM are characterized by low-grade inflammation and excessive ROS production from placental mitochondria, resulting in increased oxidative stress (OS) (Diceglie-Anelli *et al*., 2021; Kelly *et al*., 2020). These harms can lead to cell injury and chronic inflammation, inducing oxidative damage to DNA and RNA, proteins, lipids and entire organelles as mitochondria, resulting in mitochondrial dysfunctions. Mitochondria are able to tailor their functions and content based on different stimuli, allowing cellular adaption through changes in their dynamics (i.e. fission and fusion) and metabolism (e.g. OXPHOS, ATP and ROS production) (Pereira *et al*., 2017; Lu and Sferruzzi-Perri, 2021). In particular, altered mitochondrial content has been reported in placental cells of obese pregnant women; higher level of mitochondrial DNA (mtDNA), which is considered an indicator of the mitochondria number, has been described: this is probably an adaptive response to the intrauterine environment characterized by OS and lipotoxicity (Lu and Sferruzzi-Perri, 2021; Myatt and Maloyan, 2016). Our group previously reported that obese women with GDM had similar placental mtDNA level compared with normal-weight women, but an abnormal placental mitochondrial morphology (e.g. loss of matrix density, disorganization of inner membrane cristae and enlarged lipid droplets) (Mandò *et al*., 2018). Thus, this detrimental state of OS-induced damage could impair the main functions of mitochondria: cellular respiration through OXPHOS and ATP generation (Sobrevia *et al*., 2020) (**Fig.5**).

Oxidative status is determined by the balance between ROS formation and antioxidant defences. Normally, cells have learnt how to cope with OS to maintain the redox homeostasis by using enzymatic and non-enzymatic weapons. The first ones are Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPX), while the second ones are mainly represented by the tripeptide Glutathione (GSH), which is synthetized by Glutathione Syntethase (GSS) and restored to its reduced/active form by Glutathione Reductase (GSR) (Sultana *et al*., 2017; Al-Gubory *et al*., 2010, Peng *et al*., 2014).

ROS are not only associated with cell damage, they can also participate in various physiological processes as signalling molecules, including autophagy. In particular, mitochondrial ROS are

master inducers of autophagy: activation of autophagy is a key part of the cellular response to OS, because it allows scavenging of defective components before further damage, and regulates cellular homeostasis (Fang *et al*., 2017).

Molecular pathways involved in ROS-induced initiation of autophagy are sophisticated, but one of the main molecular protagonists is Nuclear Factor (Erythroid-derived-2)-like 2 (NRF2). When exposed to OS, NRF2 translocates into the nucleus, targeting Antioxidant Response Element (ARE) and leading to antioxidant enzymes expression. Moreover, NRF2 activates downstream target genes to promote autophagy in a positive feedback loop (Fang *et al*., 2017; Tang *et al*., 2019).

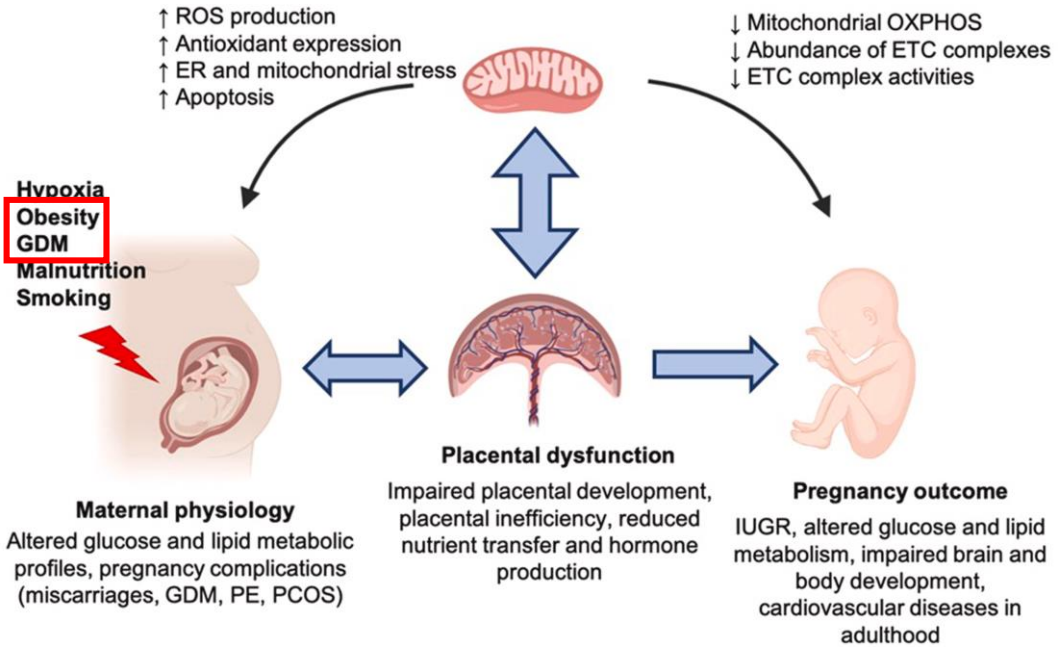


Figure 5 | *Detrimental triggers (e.g. obesity and GDM) altering maternal physiology and mechanisms linking placental mitochondria disturbance to placental dysfunction and pregnancy outcome* (Lu and Sferruzzi-Perri, 2021).

2.5. MicroRNAs as epigenetic regulators in pregnancy

During pregnancy, metabolic, physiological and immunological changes naturally occur to accommodate the development of the fetus and the placenta. These fluctuations are finely regulated, in particular through epigenetics (Sørensen *et al.*, 2022).

Epigenetics is defined as the complex molecular network regulating gene expression without changes in the DNA sequence. The main epigenetic modifications include DNA methylation, histone modifications and noncoding RNA-mediated control, especially through microRNAs (miRNAs) (Vaiman D., 2017). Epigenetic modifications are among the mechanisms through which placental dysfunction and perturbations of the intrauterine environment are able to influence pregnancy outcomes and fetal programming. Life-long consequences in offspring exposed to unhealthy maternal nutrition and lifestyle, which often occur in maternal obesity and GDM, can be conveyed by epigenetic changes (Franzago *et al*., 2019; Vaiman D., 2017). Increasing evidence points out that miRNA regulation is fundamental for the establishment and maintenance of pregnancy and is implicated in the pathogenesis of gestational disorders (Addo *et al*., 2020). Indeed, intrauterine exposures to obesogenic environment can influence birth and fetal outcomes *via* miRNA and alterations in miRNAs expression have been found in pregnancy-associated pathologies, including GDM (Poirier *et al*., 2017; Guarino *et al.,* 2018; Moean *et al*., 2017).

MiRNAs are endogenous, small non-coding, single-stranded RNAs (18-24 nucleotides) that modulate the gene expression of up to one-third of the human genome. They are implicated in many biological processes such as development, differentiation and cell-cycle regulation, acting mainly as transcriptional repressors by either degrading or inhibiting translation of messenger RNA (mRNA) targets (Franzago *et al*., 2019; Morales-Prieto *et al*., 2012). Recent evidences also suggest that miRNAs could be involved in alternative splicing events and in the regulation of other epigenetic machineries (Poirier *et al*., 2017).

The biogenesis of miRNAs (**Fig.6**) begins in the nucleus, where they are transcribed either from intragenic or intergenic regions, respectively by RNA Polymerase II or III. The transcription product, called pri-miRNA, is a long primary sequence with multiple hairpin loops. It is processed by the enzyme Ribonuclease III Drosha and the RNA binding protein DGCR8 (DiGeorge Syndrome Critical Region 8), generating a double-stranded hairpin-structured pre-miRNA of ~60-70 nucleotides. Then, Exportin-5 brings the pre-miRNA in the cytoplasm, where the RNase III Endonuclease Dicer further cleaves it, generating a 22 nucleotides-long miRNA duplex. One strand is degraded, while the most thermodynamically-stable one is loaded into Argonaute (AGO) proteins to form the RNA-Induced Silencing Complex (RISC). There are two possible isoforms of the mature microRNA, namely 5p and 3p: the 5p strand arises from the 5′ end of the pre-miRNA, while the 3p strand originates from the 3′ end. Both strands can be loaded into AGO proteins

depending on the specific cell type or cellular environment. The microRNA loaded in the RISC complex guides the recognition of complementary sequences on the target mRNA (i.e. RNA interference) (Cretoiu *et al.,* 2016; Guarino *et al.,* 2018; O'Brien *et al.,* 2018).

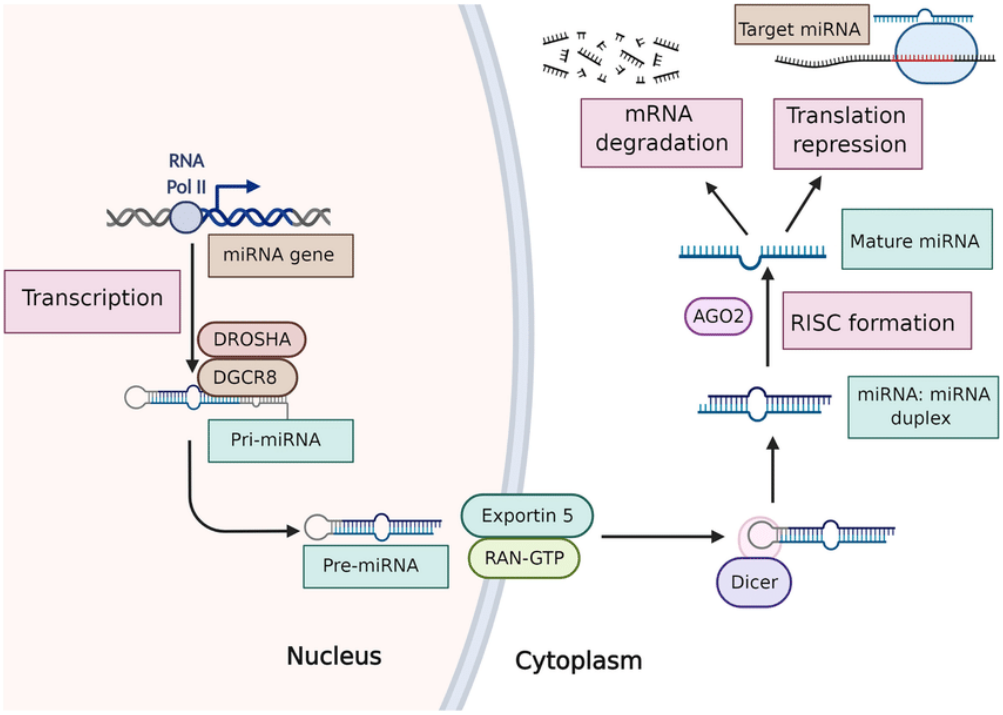


Figure 6 | *General overview of miRNA synthesis and mechanism of action* (Saleem *et al.*, 2022).

During pregnancy, miRNAs are produced by the mother, the fetus or the placenta. These pool of pregnancy-associated miRNAs can be tissue-resident and/or circulating. In fact, part of them is released into the blood flow, free or packaged into extracellular vesicles, which are effective means of communication between the three compartments of pregnancy. The miRNAs found into the maternal blood may reflect the status of the feto-maternal interface and of the intrauterine environment, thus representing potential non-invasive biomarkers for pregnancy disorders. Importantly, circulating miRNAs can be easily detected and quantitatively measured in the maternal blood. In-depth investigation on these miRNAs could provide valuable information to further understanding the physiological and pathological epigenetics status in pregnancy (James-Allan and Devaskar, 2021; Guarino *et al.*, 2018). Moreover, the placenta expresses more than 600 microRNAs, mainly encoded by three genic clusters: Chromosome 19 miRNA cluster (C19MC); Chromosome 14 miRNA cluster (C14MC); miR-371–3 cluster (Morales-Prieto *et al.*, 2013). The

expression of these miRNAs varies across gestation, with increasing C19MC and decreasing C14MC miRNAs expression from the first to the third trimester (Addo *et al*., 2020). MiRNAs from these clusters are mainly expressed by trophoblasts at the interface with maternal circulation, but they have also been reported in human embryonic stem cells. They seem to be implicated in the regulation of proliferation, invasion, differentiation, neurogenesis, embryonic development and in placental resistance to viral infections (Malnou *et al*., 2019; Mouillet *et al*., 2011; Mouillet *et al*., 2015; Paquette *et al*., 2018; Poirier *et al*., 2017).

Recently, a few studies analysed the expression of microRNAs associated with pregnancy complications, including maternal obesity and GDM. For instance, Zhao and colleagues analysed the serum miRNA profile of GDM patients at 16-19 weeks of gestation, reporting a set of differentially expressed miRNAs involved in glucose homeostasis, insulin sensitivity and β-cell function (Zhao *et al.*, 2011). Tsamou and colleagues investigated the association between maternal pre-pregnancy BMI and placental miRNA expression; in placental tissue of overweight and obese women, they found some altered miRNAs implicated in adipogenesis and obesity, but only in pregnancies with new-born girls, providing a sex-specific basis for epigenetic effects of pre-pregnancy BMI (Tsamou *et al.*, 2017).

3. AIMS OF THE STUDY

This study aimed at characterizing a cohort of Caucasian obese pregnant women without comorbidities (OB/GDM(-)) and with GDM (OB/GDM(+)) compared to normal-weight (NW), analysing both term placenta and maternal plasma. In particular, the main fields of investigation were:

1) **Placental autophagy, antioxidant metabolism and bioenergetics analyses**

We characterized the placental tissue of normal weight mothers and of women affected by maternal obesity with or without GDM under multiple points of view related to important cellular functions and mitochondrial bioenergetics. First, we studied the expression of autophagy-related genes (in particular Chaperone-Mediated Autophagy - CMA, and macroautophagy) and antioxidant metabolism markers in the three study groups. Then, we evaluated placental ATP levels and mitochondrial Complexes I-V protein content in the placental villous tissue, in relation to pre-pregnancy Body Mass Index (BMI) and to the presence of GDM.

2) **Maternal plasma miRNA profiling analyses**

We investigated the maternal epigenetic status by profiling maternal plasma miRNAs at delivery in a subgroup of our study population, to seek for possible associations with the dysregulated metabolic states of maternal obesity and GDM.

4. MATERIALS AND METHODS

4.1. Study population

The present study involved a cohort of pregnant women enrolled at the Obstetric Units of *L. Sacco* and *V. Buzzi Hospitals* (*ASST Fatebenefratelli-Sacco*) in Milan. The study protocol was approved by the University and Hospital Ethical Committees and all participants gave their informed consent for personal and clinical data treatment, and for biological specimens' collection and usage.

We recruited 60 Caucasian pregnant women aged 18-42, with single term pregnancies at elective (in absence of labor) Caesarean section (eCS). The study population was divided according to pre-pregnancy BMI (kg/m^2), following the *Institute of Medicine* (*IOM*) guidelines (Rasmussen *et al.*, IOM 2009):

- Normal-Weight (NW) women: 18.5 ≤ BMI ≤ 24.9 kg/m^2, n = 25;
- Obese (OB) women: BMI ≥ 30 kg/m^2, n = 35.

A subgroup of OB was affected by GDM (OB/GDM(+), n = 13), diagnosed accordingly to the *International Federation of Gynaecology and Obstetrics* (*FIGO*) guidelines: Oral Glucose Tolerance Test (OGTT)-75 g was performed at 24–28 weeks (Metzger *et al.*, 2010; Hod *et al.*, 2015).

OB women underwent regular glycemia checks and were specifically counselled for diet and lifestyle to avoid excessive Gestational Weight Gain (GWG), as recommended by *IOM* (Rasmussen *et al.*, IOM 2009):

- NW: 11.5 ≤ GWG ≤ 16 Kg;
- OB: 5 ≤ GWG ≤ 9 Kg.

We excluded mothers presenting comorbidities different from GDM or other pregnancy complications (e.g. genetic, oncologic, autoimmune disorders, or carrying fetuses with genetic/chromosomal syndromes, malformations etc.), as well as patients with GDM under pharmacological treatment (e.g. insulin therapy) and women with history of smoking, alcohol and/or drug abuse.

Maternal and fetal clinical and biochemical data were recorded at delivery.

4.2. Placental tissue and maternal plasma sampling

At eCS, placental weight and diameters were recorded after discarding umbilical cord and fetal membranes, and chorionic villi biopsies of almost 1 cm^3 were collected from the maternal side, after elimination of maternal decidua. Placental tissue samples were carefully washed in Phosphate Buffered Saline (PBS) to eliminate excessive blood and conserved in *RNA later* (for

gene expression analyses) or without any conservative solution (for ATP quantification and Western Blot analyses) at -80°C.

Placental area was calculated as the area of an ellipse from diameters (D x d x π/4). Assuming constant density, placental thickness was obtained as weight/area ratio. Placental efficiency was estimated by the fetal/placental weight ratio (Bianchi *et al.*, 2021).

Prior to eCS, maternal venous blood was collected from the cubital vein into EDTA tubes and centrifuged at 1500 rpm for 15 minutes at room temperature to obtain plasma (for microRNA profiling). Aliquots were stored at -80°C.

4.3. Experimental procedures

4.3.1. Gene expression of placental autophagy and antioxidant markers

Placental tissue (80-100 mg) homogenates were obtained by shredding in *Potter* homogenizer with *TRI Reagent.* RNA was extracted by *RiboPure Kit* (*Ambion-ThermoFisher Scientific*) following manufacturer's instruction. RNA concentration was determined by *NanoDrop ND-1000* (*NanoDrop Technologies*) and 2 µg of RNA retrotranscribed into cDNA using *GoScript Reverse Transcription Mix, Random Primers* (*Promega*), following manufacturer's instruction.
4 ng of cDNA were used for each sample amplification by Real-Time PCR. Gene expression of antioxidant metabolism (CAT, GSR, GSS, GPX1), chaperone-mediated autophagy (LAMP-2A, HSC70, PHLPP1, NRF2, VEGF), macroautophagy (ULK, BECN1) markers (**Tab.1**) was analysed (*7500 Fast Real-Time PCR System, Applied Biosystem*) with *SYBR Green* chemistry.

FUNCTION	GENE
Chaperone-Mediated Autophagy	Lysosome-associated membrane protein type 2A (**LAMP-2A**)
	Heat shock protein family A member 4 (**HSC70/HSPA4**)
	PH domain and leucine rich repeat protein phosphatase 1(**PHLPP1**)
	Nuclear factor erythroid 2-related factor 2 (**NRF2/NF2L2**)
	Vascular Endothelial Growth Factor (**VEGF**)
Macroautophagy	Unc-51 like autophagy activating kinase (**ULK1**)
	Beclin 1 (**BECN1**)
Antioxidant capacity	Catalase (**CAT**)
	Glutathione reductase (**GSR**)
	Glutathione Synthetase (**GSS**)
	Glutathione peroxidase 1 (**GPX1**)

Table 1 | *Function, complete name and acronym of the analysed genes.*

4.3.2. Placental ATP quantification

Placental ATP content was determined by *CellTiter-Glo® Luminescent Cell Viability Assay* (*Promega*), a luminescent assay based on a thermostable luciferase specifically adapted for placental tissue by Dr. Matteo Giovarelli (Dept. of Biomedical and Clinical Sciences, Unimi).
Briefly, frozen placental tissues were homogenized in 500 µl of cold lysis buffer (sucrose 0.25 M, HEPES-NaOH pH 7.4 10 mM) with *Ultraturrax*. The homogenate was centrifuged and 250 µl of the supernatant was added to an equal volume of ice-cold 10% trichloroacetic acid (TCA). The mixture was centrifuged and 200 µl of Tris-acetate buffer pH 8, 1 M was added to 400 µl of supernatant, to neutralize TCA. The obtained extracts were diluted 1:10, and also serial dilutions of standard ATP (10 nM, 20 nM, 50 nM, 100 nM, 1 µM) were prepared to generate the standard curve. 100 µl of samples and standards were plated in triplicate and 100 µl of *CellTiter-Glo Reagent* was added to each well. After mixing and incubating at RT, luminescence was read (*GloMax* luminometer, *Promega*). For each sample, final placental ATP content (nM/µg proteins) was obtained by normalizing raw ATP content (nM) on protein content (µg), which was quantified by *Lowry Protein Assay* (*Bio-Rad*).

4.3.3. Placental mitochondrial complexes quantification

Placental mitochondrial (mt) respiratory chain Complexes I-V protein expression was measured by Western Blotting in collaboration with Prof. Sara Castiglioni and Dr. Monica Zocchi (Dept. of Biomedical and Clinical Sciences, Unimi).
Frozen placental tissues (30-40 mg) were Potter-homogenized in 200 µl of lysis buffer (Tris HCl pH 7.4 20 mM, EGTA 10 mM, NaCl 150 mM, Triton 1%, Glycerol 10%) and homogenates were centrifuged to obtain proteins-enriched supernatants. Equal amounts of proteins (quantified by *Bradford Assay* (*Sigma-Aldrich*)) were separated by SDS-PAGE on 12.5% polyacrylamide gel and transferred to nitrocellulose membranes by using *Trans-Blot®* (*Bio-Rad*). After blocking with 5% powdered milk solution in TBS (Tris-Buffered Saline) for ~ 1 hour, Western blot analysis was performed using the *Total OXPHOS Rodent WB Antibody Cocktail* (*Abcam*) diluted 1:250 in PBS with 1% milk. This optimized premixed cocktail contains 5 mouse antibodies, against the 5 mt complexes (CI-CII-CIII-CIV-CV). After extensive washing, secondary antibody conjugated with horseradish peroxidase (*Abcam*) diluted 1:3000 in PBS was used. The emitted light from the chemiluminescent reaction was detected with *ChemiDoc MP Imaging System* (*Bio-Rad*).

4.3.4. MicroRNAs profiling in maternal plasma

For miRNA profiling, we employed ready-to-use Real-time PCR plates based on miRCURY LNA technology (*Qiagen*), followed by the analysis of significant results through the most updated targets/pathways prediction tools (*miRBase*, *miTALOS*) and their discussion according to literature data (*PubMed*), as described below.

In detail, non-hemolysed maternal plasma sample from 7 NW, 6 OB/GDM(-) and 6 OB/GDM(+) women were selected. Total RNA was extracted from plasma (200 µl) using *miRNeasy Serum/Plasma Advanced Kit*, which allows retrieval of low-abundance miRNA, in conjunction with the *miRNeasy Serum/Plasma Spike-In Control Cel-miR-39-3p*. 2 µl of the extracts were retrotranscribed into specific miRNA-based cDNA by *miRCURY LNA RT Kit*, using the *UniSp6* spike-in miRNA as retrotranscription control.

Real-time PCR experiments were performed on *Serum/Plasma-Focus PCR Panels* (YAHS-106YC-8), ready-to-use plates with specific miRNA primers for the 179 most expressed miRNAs in human serum/plasma, with reagents from the *miRCURY LNA SYBR Green PCR Kit.* Each plate contained a triplicate for the *UniSp3* IPC assay (complete of pre-spotted primers' pair + synthetic DNA), acting as Inter Plate Calibrator (IPC).

All experimental procedures were done according to manufacturer's instructions (*Qiagen*).

4.4. Statistical and bioinformatic analyses

4.4.1. Placental molecular data and clinical data analysis and statistics

For Real-time PCR data, normalization of gene expression data was performed according to Vasedompele *et al.*, using two different housekeeping genes, β-actin (Beta-actin) and YWHAZ (Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta), and considering the amplification efficiency of each assay (Vasedompele *et al.*, 2022). Only Ct values with standard deviation <0.25 across triplicates were included in the statistical analysis.

For Western Blotting, quantitative densitometry measurement of the bands was performed with the software *ImageLab* (*Bio-Rad*).

Normalized placental autophagy and antioxidant defense gene expression values, ATP content and mt complexes protein levels, as well as maternal, placental and fetal clinical data were analysed with *SPSS v.27* (*IBM*). Statistical significance was set for p-value <0.05.

According to data distribution (*Kolmogorov-Smirnov test*), *One-way ANOVA* (parametric statistics with *Tukey's HSD* as post-hoc analysis) or *Kruskal-Wallis test* (non-parametric statistics with

Pairwise comparison as post-hoc analysis) were performed to evaluate differences between NW, OB/GDM(-) and OB/GDM(+) groups, with *Bonferroni correction*. *Student's t-test* (parametric), corrected by *Levene's test* for equality of variances, or *Mann-Whitney U test* (non-parametric) were performed to evaluate differences between NW and all obese women together, with or without GDM (OB/GDM(±)).

Chi-Square Test for Independence was used to compare frequencies of fetal sex.

Correlations between variables were assessed by *Pearson Product-moment Correlation* (parametric) or *Spearman's Rank Order Correlation* (non-parametric alternative), depending on data distribution.

4.4.2. MicroRNAs profiling in maternal plasma: data analysis, statistics and bioinformatics pathway-enrichment analysis

MicroRNA data were analysed with the online statistical tool *GeneGlobe Data-Analysis Center* (*Qiagen*). Briefly, raw Ct were first obtained using *ThermoFisher Cloud*, and then they were uploaded on *GeneGlobe* software and project-specific settings were defined (i.e. experimental groups: OB/GDM(-) as Group 1, OB/GDM(+) as Group 2, NW as Control Group; internal controls: *Cel-miR-39-3p* as extraction control, *UniSp6* as retrotranscription control, *UniSp3* IPC as Inter Plate Calibrator). Lower limit of detection was set at 36, so that Ct values greater than 36 were not considered for further analysis. Data were normalized using *NormFinder* method; this algorithm attempts to find the optimum reference miRNAs out of a group of candidates, choosing the 10 most stably expressed miRNAs among all subjects (i.e. with standard deviation between samples' Ct <0.25) (Andersen *et al.*, 2004).

The software calculates: average ΔCt, $2^{(-\Delta Ct)}$, ΔΔCt, $2^{(-\Delta\Delta Ct)}$ (i.e. Fold Change) and Fold Regulation, referred to all the three possible comparisons: **A)** OB/GDM(-) (Case Group 1) vs NW (Control Group), **B)** OB/GDM(+) (Case Group 2) vs NW (Control Group), or **C)** OB/GDM(+) (Case Group 2) vs OB/GDM(-) (Case Group 1).

Fold Regulation conveys the same information as Fold Change, but with easier interpretation. If a Fold Change value is >1, the Fold Regulation and Fold Change values are identical. For Fold Change values (X) <1, the Fold Regulation is the negative inverse of the Fold Change (-1/x). Fold Regulation values >1 indicate increased gene expression, Fold Regulation values <0 indicate decreased gene expression, Fold Regulation =1 indicates no change. Fold Regulation cut-off was set for values >|2| (Fold Regulation >2 or <-2), meaning differential expression between groups of at least 2-times in positive (upregulation) or negative (downregulation).

Un-paired 2-tailed t-Test was used to compare the 2(-ΔCT) values for each miRNA in the two groups, for each of the three comparisons. Statistical significance was set for p-value <0.05.

For each of the three comparisons, miRNAs showing differential expression with a Fold Regulation >2 or <-2 and statistical significance (p-value <0.05) were further studied, trying to understand their biological role. Pathways prediction analyses were performed using the most updated and literature-accepted online bioinformatic tools: *miTALOS* and *mirPath*.

miTALOS v.2 integrates multiple databases collecting miRNA experimental data and bioinformatics prediction tools (i.e. *TargetScan*, *StarBase*, *European Bioinformatics Institute - EBI Expression Atlas*, Kyoto Encyclopedia of Genes and Genomes - *KEGG*, *Wiki Pathways* and *Reactome*) to identify specific associations between miRNAs and signalling pathways (Shaker *et al.*, 2020; Preusse *et al.*, 2016). *DIANA-miRPath v.3* uses data from the *KEGG*, *Gene Ontology - GO* and *TarBase* resources, to match each miRNA of interest to specific pathways (Vlachos *et al.*, 2015). For both *miTALOS v.2* and *DIANA-miRPath v.3*, the significance of the association (p-value) between miRNAs and the resulting pathways is calculated with *Fisher's Exact Test*; then, the results for multiple pathways are corrected using the *EASE Score* and *False Discovery Rate - FDR* (*Benjamini–Hochberg* procedure) (Benjamini *et al.*, 1995).

5. RESULTS

5.1. CLINICAL DATA

Tables 2A and **2B** summarize maternal, fetal and placental characteristics of the three study groups.

A	**NW** (***N*** **= 25)**	**OB/GDM(-)** (***N*** **= 22)**	**OB/GDM(+)** (***N*** **= 13)**
AGE [2] [years]	35.24 ± 3.53	30.82 ± 5.88 #/°	35.77 ± 4.29
PRE-PREGNANCY BMI [2] [Kg/m^2]	21.63 ± 2.11	33.92 ± 3.44 ###	34.89 ± 3.48 ###
GWG [2] [Kg]	11.17 ± 2.87	8.55 ± 5.74	6.63 ± 5.91 #
OGTT I [1] [mg/dL]	82.81 ± 5.21	80.67 ± 6.96	85.22 ± 12.51
OGTT II [1] [mg/dL]	125.69 ± 22.03	119.58 ± 30.10	164.67 ± 35.99 **/••
OGTT III [1] [mg/dL]	106.74 ± 31.02	100.50 ± 23.25	162.89 ± 32.28 ***/•••
GESTATIONAL AGE [2] [weeks]	39.21 ± 0.42	39.12 ± 0.32	39.09 ± 0.17
HEMOGLOBIN III T [2] [g/dL]	11.89 ± 1.10	11.38 ± 0.69	11.32 ± 1.07
HEMATOCRIT III T [2] [%]	35.42 ± 2.80	34.18 ± 2.56	34.31 ± 2.59

B	**NW** (***N*** **= 25)**	**OB/GDM(-)** (***N*** **= 22)**	**OB/GDM(+)** (***N*** **= 13)**
FETAL WEIGHT [1] [g]	3440.68 ± 365.10	3415.45 ± 352.41	3330.0 ± 336.55
	F: 3376.09 ± 259.43	F: 3346.25 ± 418.06	F: 3292.14 ± 283.58
	M: 3941.43 ± 433.61	M: 3455 ± 319.18	M: 3374.17 ± 413.50
FETAL WEIGHT CENTILE [1]	54.76 ± 25.38	52.77 ± 26.57	49.38 ± 26.99
FETAL SEX [%]	F: 48; M: 52	F: 36.4; M: 63.6	F: 53.8; M: 46.2
PLACENTAL WEIGHT [1][g]	442.38 ± 82.43	468.05 ± 85.10	497.46 ± 102.55
	F: 435.82 ± 78.61	F: 468.75 ± 71.80	F: 545.71 ± 76.35
	M:447.92 ± 88.32	M: 467.64 ± 94.45	M: 441.17 ± 105.75
PLACENTAL AREA [2] [cm^3]	238.72 ± 46.18	233.46 ± 75.44	245.57 ± 46.44
PLACENTAL THICKNESS [1] [cm]	1.90 ± 0.44	2.13 ± 0.59	2.09 ± 0.60
PLACENTAL EFFICIENCY [F/P] [2]	8.02 ± 1.58	7.50 ± 1.36	7.0 ± 1.84 #
	F: 7.93 ± 1.24	F: 7.26 ± 1.27	F: 6.09 ± 0.68°/##/##
	M: 8.09± 1.87	M: 7.63 ± 1.45	M: 8.05 ± 2.26

Table 2 | *Maternal (**A**), fetal and placental (**B**) characteristics of the population.*

Data were analysed according to their distribution with [1]*One-way ANOVA* (*Tukey's HSD* as post-hoc test with *Bonferroni Correction*) or [2]*Kruskal–Wallis* test (*Pairwise comparison* as post-hoc test with *Bonferroni Correction*). Data are presented as average ± standard deviation.

One-way ANOVA: *p<0.05, **p<0.01, ***p<0.001 vs NW; •p<0.05, ••p<0.01, •••p<0.001 vs OB/GDM(-) or vs OB/GDM(+). *Kruskal-Wallis*: #p<0.05, ##p<0.01, ###p<0.001 vs NW; °p<0.05, °°p<0.01, °°°p<0.001 vs OB/GDM(-) or vs OB/GDM(+).

Chi-Square Test for Independence: fetal sex (%) non-significant [χ^2 (2, *n* = 60) = 1.67, p = 0.56, Φ = 0.14].

[BMI: Body Mass Index; GWG: Gestational Weight Gain; Hemoglobin/Hematocrit III T: measured in the Third Trimester (29-40 weeks of gestation); OGTT: Oral Glucose Tolerance Test; smoking codes: N: never smoker; EX: ex-smoker; pluriparity codes: S: smoker; N: nulliparous; P: pluriparous; fetal sex codes: F: female (red); M: male (blue)].

Maternal age resulted to be significantly lower in the OB/GDM(-) compared to both NW (p = 0.036) and OB/GDM(+) (p = 0.026) groups (**Tab.2A**). However, this difference did not influence our molecular results (Two-way ANOVA analyses: non-significant; data not shown).

According to the inclusion criteria, pre-pregnancy BMI was significantly higher in both OB/GDM(-) and OB/GDM(+) vs NW (both p = 0.000), OGTT II was significantly higher in OB/GDM(+) vs both NW (p = 0.007) and OB/GDM(-) groups (p = 0.003), as well as OGTT III that was significantly higher in OB/GDM(+) vs both NW (p = 0.000) and OB/GDM(-) (p = 0.000). On average, all three groups did not exceed in GWG, according to IOM guidelines (Rasmussen *et al*., 2009). Interestingly, GWG was significantly lower in OB/GDM(+) vs NW (p = 0.033) (**Tab.2A**).

On average, all women delivered at full term (gestational age > 39 weeks), and hemoglobin and hematocrit, measured during the third trimester of gestation, were in line with the ranges referred to the third trimester of pregnancy (hemoglobin: 11-16 g/dL; hematocrit: 28-40%) (Khoigani *et al.,* 2012) (**Tab.2A**).

Placental efficiency, calculated as the ratio between fetal and placental weights [F/P], was significantly lower in OB/GDM(+) vs NW (p = 0.034) and tended to be lower also in OB/GDM(-), though not significantly; indeed, fetal weight tended to decrease in OB/GDM(-) and OB/GDM(+) and placental weight tended to increase in the same groups (**Tab.2B**).

Fetal sex frequencies did not differ between groups, meaning that the proportions of male and female fetuses were homogeneous (**Tab.2B**). Some additional analyses dividing the three groups according to fetal sex were also performed. Female placentas of OB/GDM(+) tended to be heavier compared to both sexes of other groups, though not reaching statistical significance. For fetal weight, no differences or particular trends between fetal sex-related groups emerged. As a consequence, female placentas of OB/GDM(+) displayed significantly lower placental efficiency compared to male placentas of the same group (p = 0.024) and to both sex of NW (vs male, p = 0.003¸vs female, p = 0.002) (**Tab.2B**).

5.2. MOLECULAR DATA

5.2.1. Autophagy and antioxidant metabolism in placenta

Editor's note: the following paragraph and subparagraph are modified excerpts from the original paper "Diceglie, C. and Anelli, G.M.; Martelli, C.; **Serati, A.** *et al.* **Placental Antioxidant Defenses and Autophagy-Related Genes in Maternal Obesity and Gestational Diabetes Mellitus**". *Nutrients* **2021**, 13, 1303. https://doi.org/10.3390/nu13041303.

5.2.1.1. Expression of antioxidant genes in placenta

To assess placental profile of redox homeostasis genes in NW, OB/GDM(-) and OB/GDM(+) women, we evaluated CAT, GSS, GSR, GPX1. Placental expression levels of all genes tended to be lower in OB/GDM(-) vs NW women, though not significantly (*data not shown*).

Interestingly, we found a strong positive correlation in the whole population between placental gene expression of CAT and placental efficiency (PEff) (r = +0.593, p = 0.000, n = 44) (**Fig.7**).

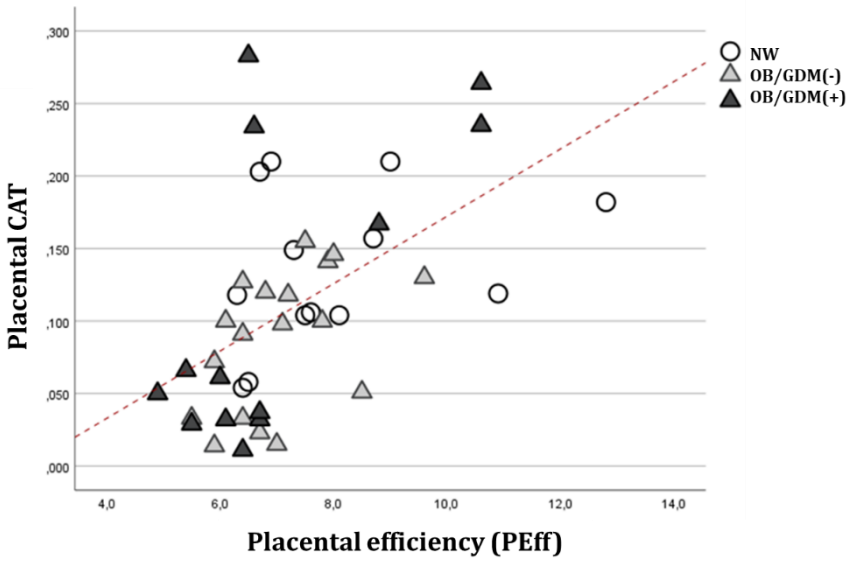


Figure 7 | *Significant positive correlation in the entire population* [NW (◯), OB/GDM(-) (▲), OB/GDM(+) (▲)] *between gene expression level of CAT in placenta and PEff.*
Spearman rank order correlation: r = +0.593, p = 0.000, n = 44.

5.2.1.2. Autophagy-related gene expression in placenta

To assess autophagy, the expression of the genes encoding for the main drivers of the autophagic pathways was analysed: ULK1, BECN1, HSC70, LAMP-2A, PHLPP1, NRF2 and VEGF genes.

Concerning macroautophagy, ULK1 significantly differed among groups (p = 0.011), being increased in OB groups vs NW (**Fig.8A**). BECN1 gene expression did not differ among the three groups, but BECN1 mRNA levels were significantly and strongly correlated with GSS levels (r = +0.758, p = 0.000, n = 42) in the whole population (**Fig.8B**), and in each subgroup.

We also measured the expression of genes involved in CMA activity or regulation. HSC70 expression was significantly different among groups (p = 0.019): both OB/GDM(-) and OB/GDM(+) presented lower levels vs NW (**Fig.8C**). LAMP-2A mRNA levels did not differ among groups; in the NW subgroup, LAMP-2A placental levels were significantly and positively correlated with those of GSS (r = +0.804, p = 0.002, n = 12; *data not shown*).

PHLPP1 expression was significantly different among groups (p = 0.001). *Post-hoc* analysis showed significantly increased levels in OB/GDM(+) vs OB/GDM(-) placentas (p < 0.001; **Fig.8D**).

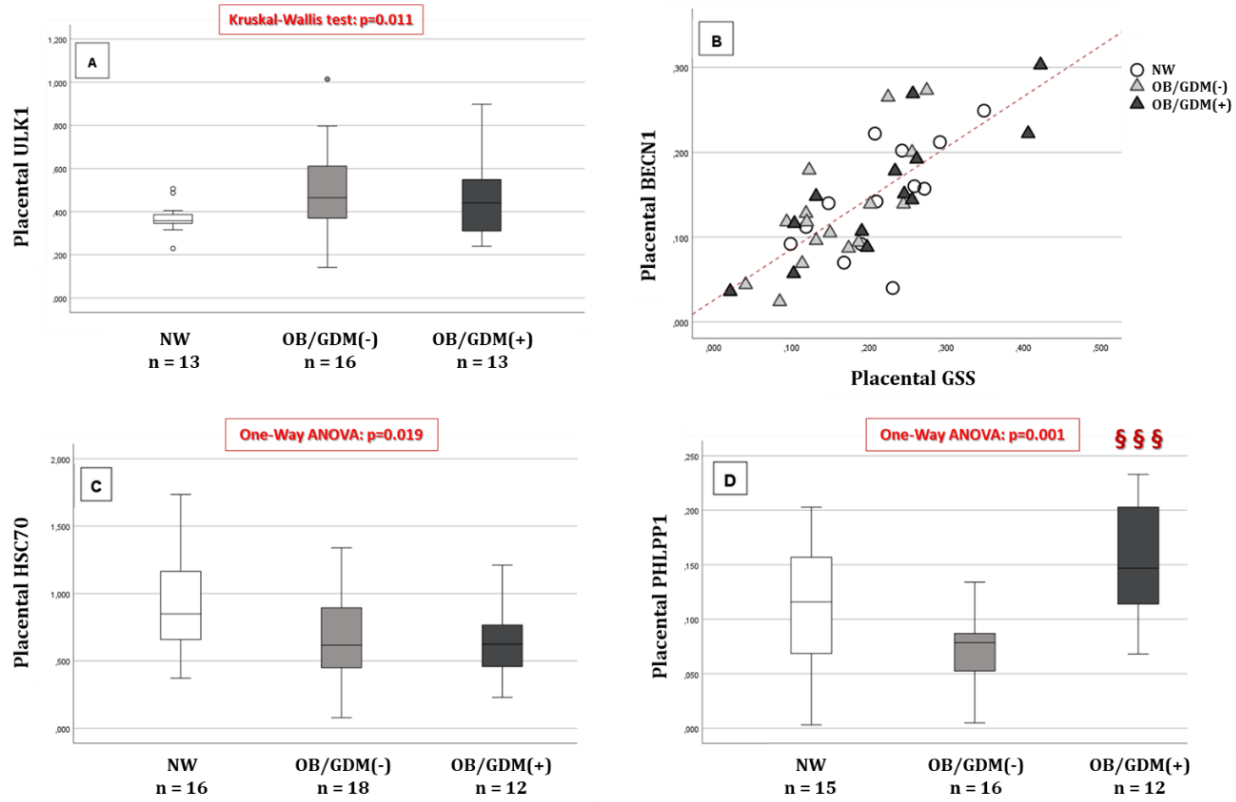


Figure 8 | *Expression of macroautophagy and CMA genes in placental tissue.*
(**A**) ULK gene expression levels in the three groups.
(**B**) Significant positive correlation in the entire population between GSS and BECN1 placental levels.
(**C**) HSC70 gene expression levels in the three groups.
(**D**) PHLPP1 gene expression levels among in the three groups.

(**A**, **C**, **D**) Gene expression levels were analysed with Kruskal–Wallis test or One-way ANOVA, depending on data distribution. Data are shown as box plots, indicating the median and the 25th and 75th percentiles; **§§§** $p < 0.001$ vs. OB/GDM(-) (all Tukey HSD post hoc tests).
(**B**) Spearman rank order correlation: r = +0.758, p = 0.000, n = 42, in NW (◯), OB/GDM(-) (▲), OB/GDM(+) (▲).

Finally, we evaluated NRF2, involved in the cell response to stressors and related to CMA activity. NRF2 placental levels did not differ among groups, as well as VEGF placental levels (*data not shown*), but NRF2 resulted significantly correlated with LAMP-2A in the whole population (r = +0.726, p = 0.000, n = 45) (**Fig.9**) and within each study group.

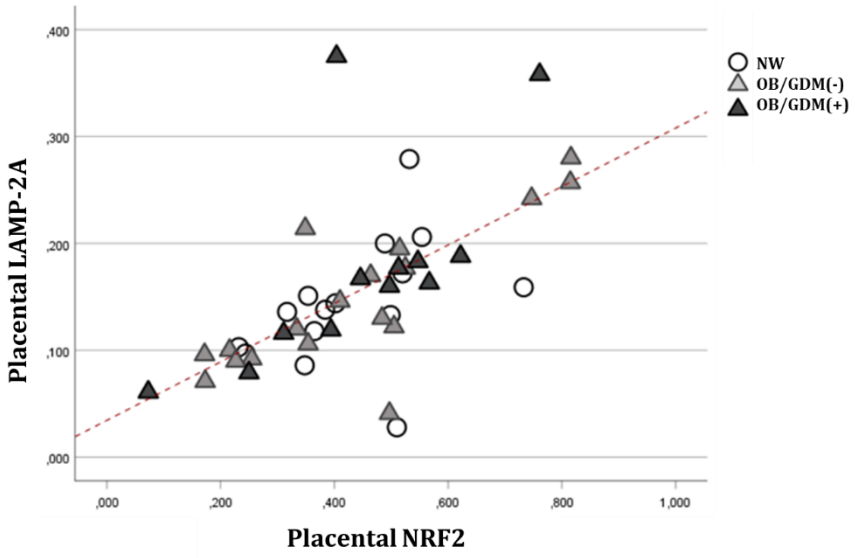


Figure 9 | *Significant and positive correlations in the entire study population* [NW (◯), OB/GDM(-) (▲), OB/GDM(+) (▲)] *between NRF2 and LAMP-2A placental levels.*
Spearman rank order correlation: r = +0.726, p = 0.000, n = 45.

5.2.1.3. Fetal sex-specific differences in autophagy-related genes

To understand if there is a possible interaction effect of fetal sex and maternal metabolic characteristics (BMI and absence/presence of GDM) on levels of the analysed genes, we performed additional analyses. A Two-way ANOVA explored the contribution of maternal BMI with absence/presence of GDM and fetal sex on our gene expression data, but no statistical significance was found. A comparison among the three BMI groups was then performed in female or male placentas, using non-parametric statistics due to the reduced sample sizes of the fetal sex subgroups.

In female, but not in male placentas, a generally lower expression was recorded for CAT, GSS and GPX1 in OB/GDM(-) vs NW, though not significantly. Macroautophagy genes did not show any difference when dividing by both BMI/GDM and fetal sex. Among CMA-related genes, similarly to antioxidant enzymes, a tendency for lower HSC70 expression was found in OB/GDM(-) compared to NW when analysing placentas from female fetuses.

Significantly lower values were observed for PHLPP1 in females' placentas in OB/GDM(-) vs. NW ($p = 0.009$; **Fig.10A**). Moreover, in placentas from female fetuses, PHLPP1 levels resulted significantly increased in OB/GDM(+) compared to OB/GDM(-). Interestingly, PHLPP1 levels were significantly increased also in males' placentas, in OB/GDM(+)compared to OB/GDM(-) ($p = 0.040$; **Fig.10A**).

An increased, though not significant, LAMP-2A expression was also observed in males' placentas, in OB/GDM(+) compared to NW.

Finally, a significant reduction in VEGF expression was observed in males' placentas in the OB/GDM(+) group when compared to both OB/GDM(-) and NW ($p = 0.005$). VEGF gene expression was also lower, though not significantly, in OB/GDM(-) male placentas vs NW (**Fig.10B**)

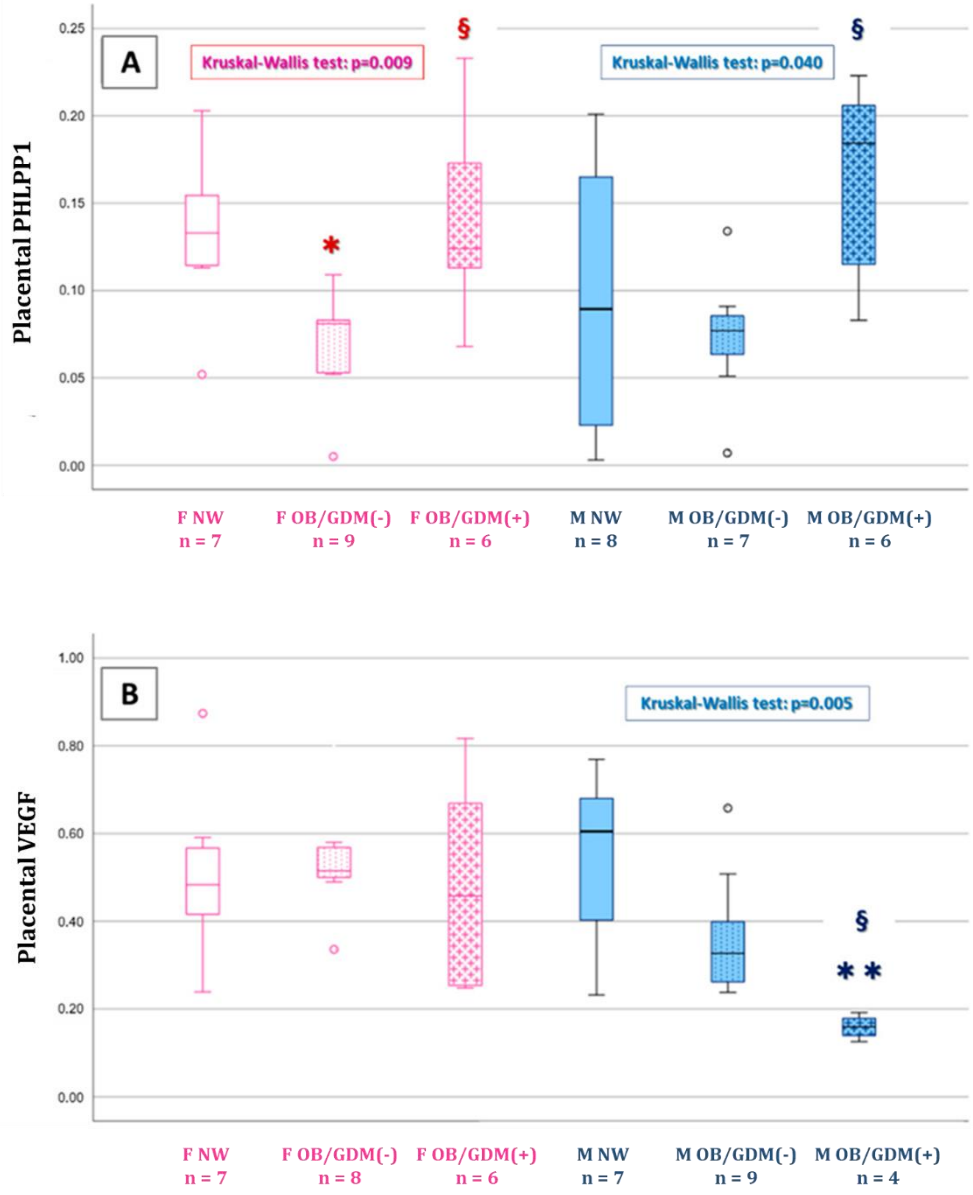


Figure 10 | *Sexually dimorphic expression of CMA or autophagy-related genes.*
(**A**) PHLPP1 and (**B**) VEGF levels among female or male placentas in NW, OB/GDM(−) and OB/GDM(+). Data were analysed with Kruskal–Wallis test (PHLPP1: p = 0.009 for FEMALE placentas, p = 0.040 for MALE placentas. VEGF: p = 0.005 for MALE placentas). Data are shown as box plots, indicating the median and the 25th and 75th percentiles. FEMALE Placentas: * $p < 0.05$ vs. NW or § $p < 0.05$ vs. OB/GDM(−). MALE Placentas: ** $p < 0.01$ vs. NW or § $p < 0.05$ vs. OB/GDM(−); post-hoc by Mann–Whitney U test with Bonferroni correction.

5.2.2. Mitochondrial bioenergetics analyses in placenta

5.2.2.1. Placental ATP

Significantly lower ATP levels were found in both OB/GDM(-) (p = 0.026) and OB/GDM(+) (p = 0.048) vs NW, while no significant difference was found among OB/GDM(-) and OB/GDM(+) (**Fig.11**).

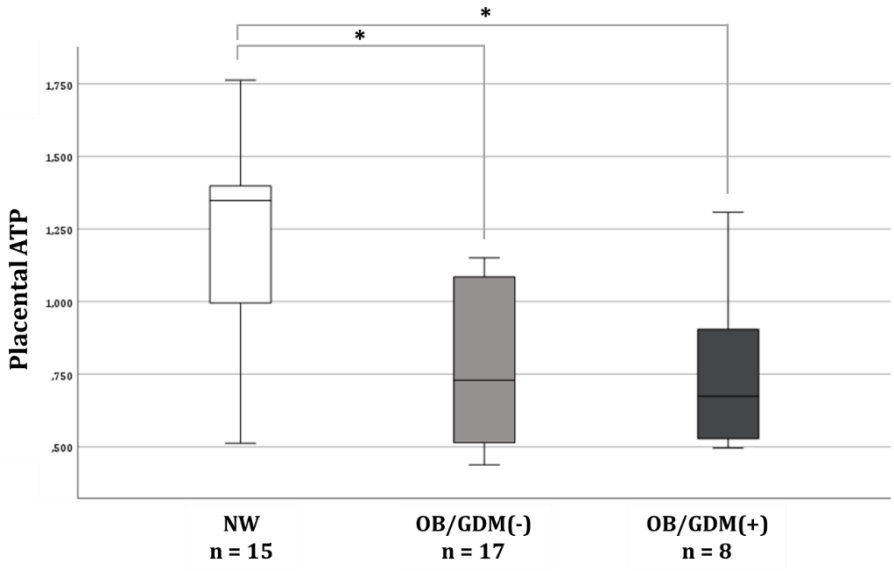


Figure 11 | *Placental ATP content in NW, OB/GDM(-) and OB/GDM(+) groups.*
Data are shown as box plots, indicating the median and the 25th and 75th percentiles.
Kruskal-Wallis test with *Pairwise Comparison* as post-hoc test (with *Bonferroni Correction*): *p<0.05 OB/GDM(-) and OB/GDM(+) vs NW.

5.2.2.2. Placental mitochondrial complexes

Since placental ATP was significantly different between the OB groups and NW controls, we deepened our research by investigating protein content of mitochondrial (mt) complexes in placenta. Because of the similar levels of placental ATP between OB/GDM(-) and OB/GDM(+), for each mt complex, we decided to perform both three-groups (NW vs OB/GDM(-) vs OB/GDM(+)) and two-groups (NW vs all obese women together = OB/GDM(±)) statistical comparisons. Parametric or non-parametric statistics was used according to data distribution.

CI.

Placental mt Complex I (CI) content did not significantly differ among the three study groups (**Fig.12A**). However, a significant difference was found when analysing all obese subjects together, with or without GDM (OB/GDM(±)) vs NW: Complex I was significantly lower in OB/GDM(±) vs NW (p = 0.02; **Fig.12B**).

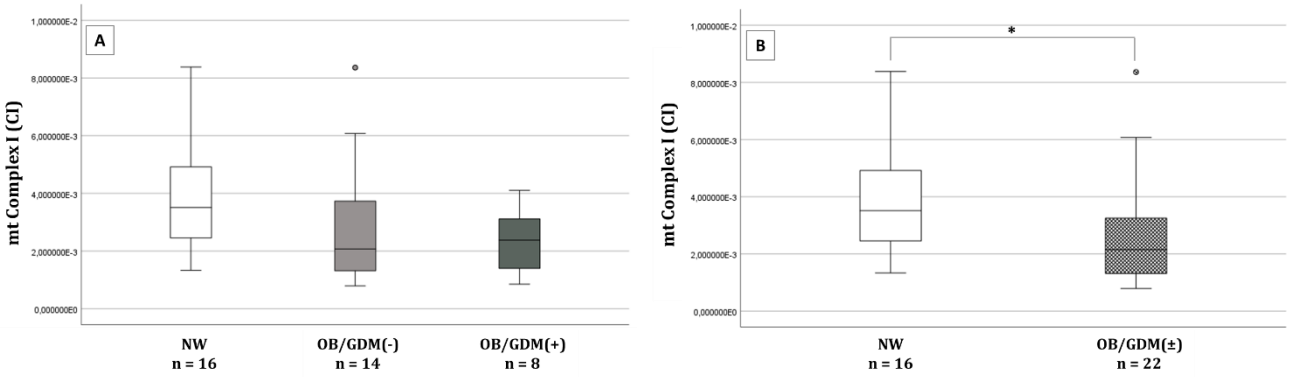


Figure 12 | *Placental mt Complex I in NW, OB/GDM(-), OB/GDM(+) (**A**) and in OB/GDM(±) vs NW (**B**).*
Data are shown as box plots, indicating the median and the 25th and 75th percentiles. Data were analysed using:
A) *Kruskal-Wallis* test with *Pairwise Comparison* as post-hoc test (with *Bonferroni Correction*): non-significant.
B) *Mann-Whitney U* test: *p<0.05, OB/GDM(±) vs NW.

CII.

Placental mt Complex II (CII) was analysed in NW, OB/GDM(-) and OB/GDM(+) groups (**Fig.13A)** and in all OB/GDM(±) vs NW (**Fig.13B**). In both cases, we did not found significant differences.

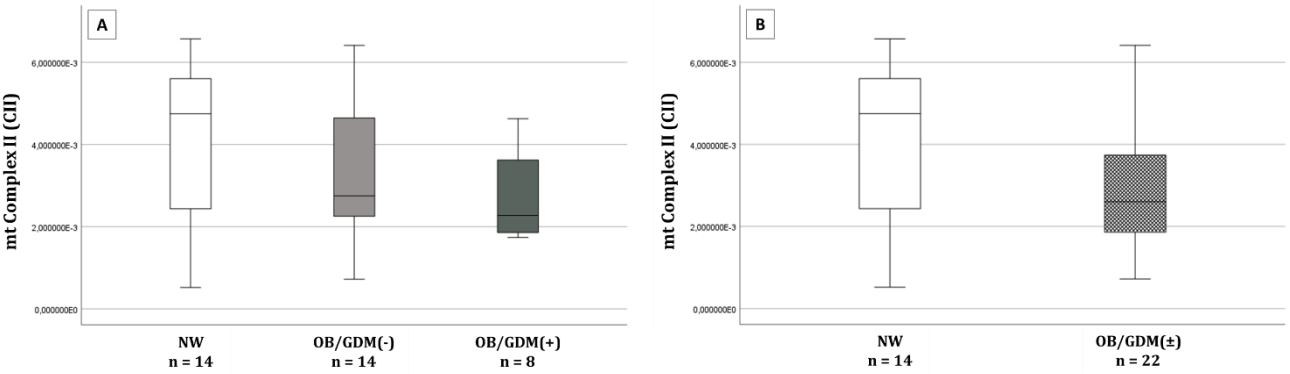


Figure 13 | *Placental mt Complex II in NW, OB/GDM(-), OB/GDM(+) (**A**) and in OB/GDM(±) vs NW (**B**).*
Data are shown as box plots, indicating the median and the 25th and 75th percentiles. Data were analysed using:
A) *One-way ANOVA* test with *Tukey's HSD* as post-hoc test (with *Bonferroni Correction*): non-significant.
B) *Student's t-test* corrected by *Levene's* test*:* non-significant.

CIII.

Placental mt Complex III (CIII), analysed in the three groups, was significantly lower in OB/GDM(+) vs NW (p = 0.032; **Fig.14A**). In two-groups comparison, it was significantly lower in OB/GDM(±) vs NW (p = 0.012; **Fig.14B**).

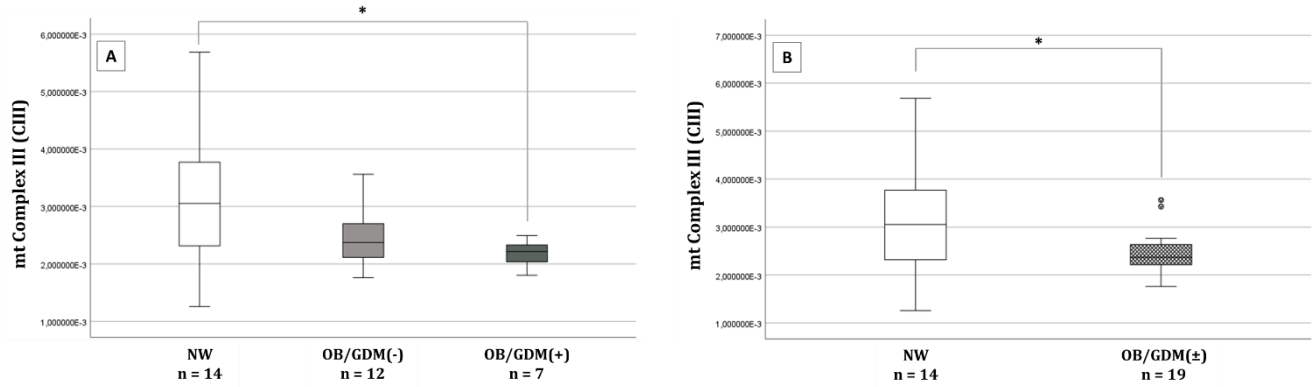


Figure 14 | *Placental mt Complex III in NW, OB/GDM(-), OB/GDM(+) (**A**) and in OB/GDM(±) vs NW (**B**).*
Data are shown as box plots, indicating the median and the 25th and 75th percentiles. Data were analysed using:
A) *Kruskal-Wallis* test with *Pairwise Comparison* as post-hoc test (with *Bonferroni Correction*): *p < 0.05, OB/GDM(+) vs NW.
B) *Mann-Whitney U* test: *p < 0.05, OB/GDM(±) vs NW.

CIV.

Placental mt Complex IV (CIV) was not different in NW, OB/GDM(-) and OB/GDM(+) (**Fig.15A**) nor in all OB/GDM(±) vs NW (**Fig.15B**) comparisons.

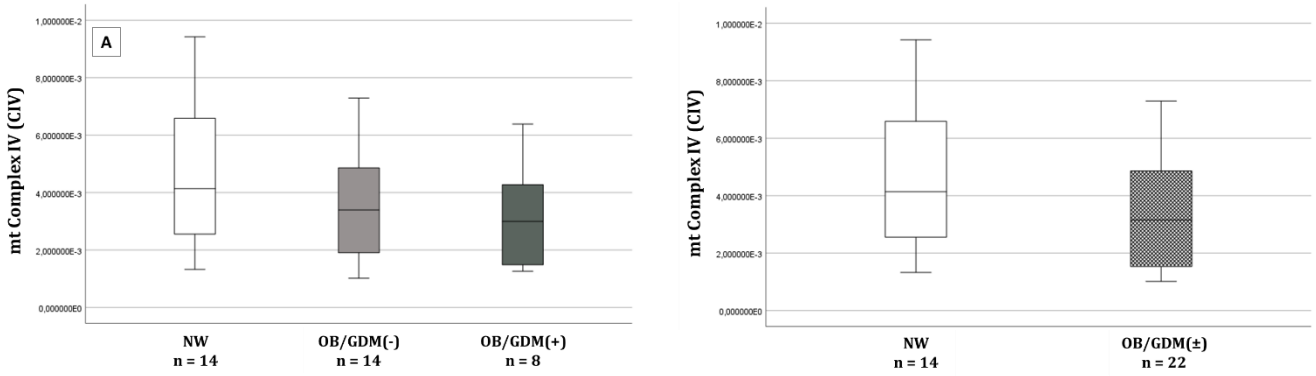


Figure 15| *Placental mt Complex IV in NW, OB/GDM(-), OB/GDM(+) (**A**) and in OB/GDM(±) vs NW (**B**).*
Data are shown as box plots, indicating the median and the 25th and 75th percentiles. Data were analysed using:
A) *One-way ANOVA* test with *Tukey's HSD* as post-hoc test (with *Bonferroni Correction*): non-significant.
B) *Student's t-test* corrected by *Levene's* test*:* non-significant.

CV.

Placental mt Complex V (CV) was analysed in NW, OB/GDM(-) and OB/GDM(+) groups. OB/GDM(+) had significantly lower content compared to both NW (p=0.013) and OB/GDM(-) women (p = 0.032; **Fig.16A**). Comparing NW and OB/GDM(±) women, no significant difference among groups was found (**Fig.16B**).

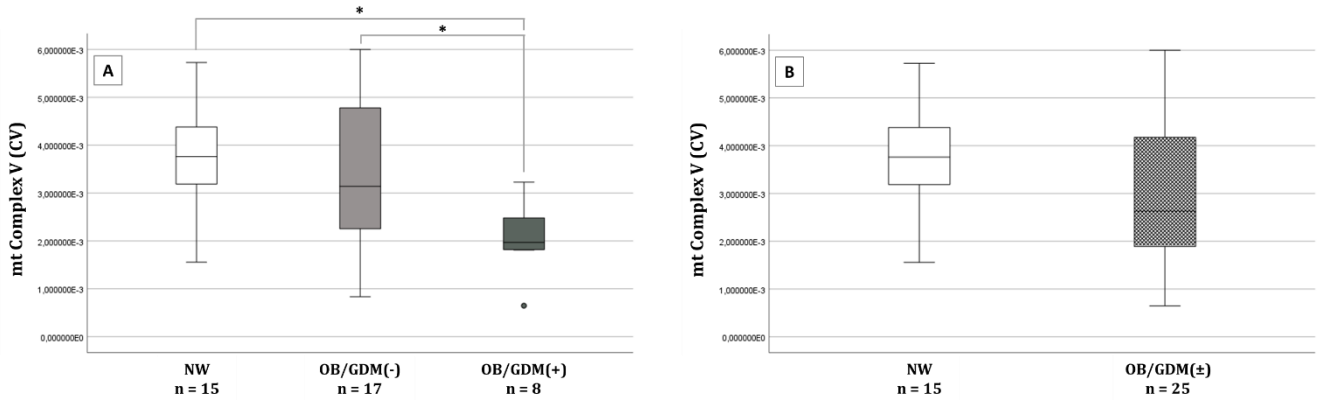


Figure 16 | *Placental mt Complex V in NW, OB/GDM(-), OB/GDM(+) (**A**) and in OB/GDM(±) vs NW (**B**).*
Data are shown as box plots, indicating the median and the 25th and 75th percentiles. Data were analysed using:
A) *One-way ANOVA* test with *Tukey's HSD* as post-hoc test (with *Bonferroni Correction*): *p<0.05, OB/GDM(+) vs NW and vs OB/GDM(-).
B) *Student's t-test* corrected by *Levene's* test*:* non-significant.

5.2.2.3. Correlations between mitochondrial complexes and placental efficiency

Placental efficiency (PEff) significantly and positively correlated with placental mt Complex I (CI) (*Pearson Correlation*, r = 0.431, p = 0.009, *n* = 36; **Fig.17A**) and Complex V (CV) (*Pearson Correlation*, r = 0.499, p = 0.001, *n* = 38; **Fig.17B**) contents in the entire study population.

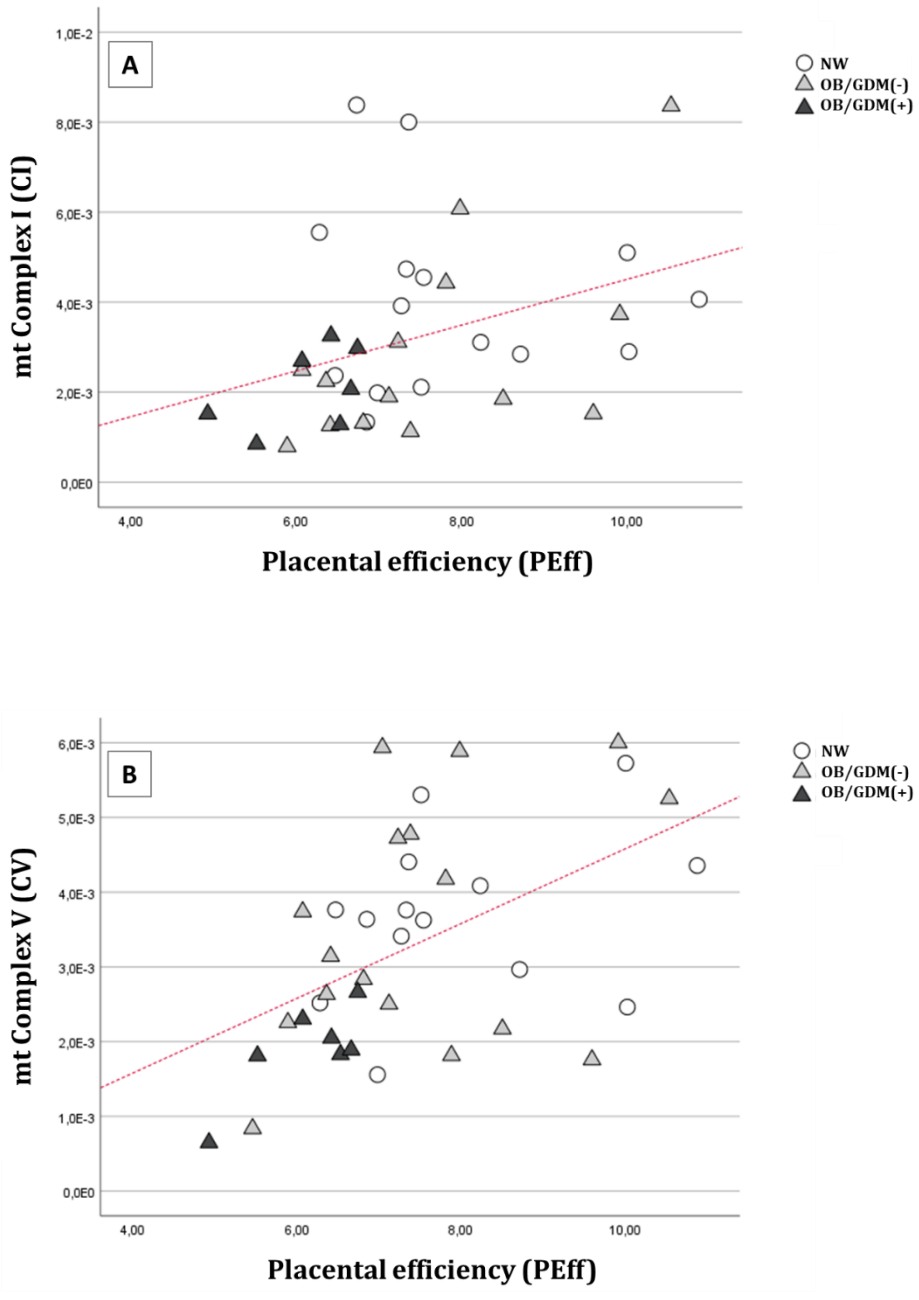


Figure 17 | *Significant positive correlations in the entire population between:*
A) Placental efficiency and placental mt Complex I (*Pearson Correlation*, r = 0.431, p = 0.009, *n* = 36).
B) Placental efficiency and placental mt Complex V (*Pearson Correlation*, r = 0.499, p = 0.001, *n* = 38).

5.2.2.4. Fetal sex-specific differences in placental mitochondrial complexes

Also in the case of ATP and mt complexes analyses, Two-way ANOVA excluded any contribution of maternal BMI with absence/presence of GDM and fetal sex on molecular data, since no statistical significance was found.

However, for these variables, it was not possible to perform a comparison among the three BMI groups further divided in women with female or male placentas/fetuses, because of extremely low size of these subgroups. Therefore, we considered comparisons between NW and all obese together (OB/GDM(±)), divided in pregnancies carrying females (F) or males (M).

Complexes II, III, IV and V did not significantly differ among subgroups, but we found a significant difference in placental mitochondrial Complex I between NW and all OB/GDM(±) according to fetal sex. In particular, considering only pregnancies carrying female fetuses, significantly lower mt Complex I content was present in OB/GDM(±) compared to NW women (p = 0.045; **Fig.18**).

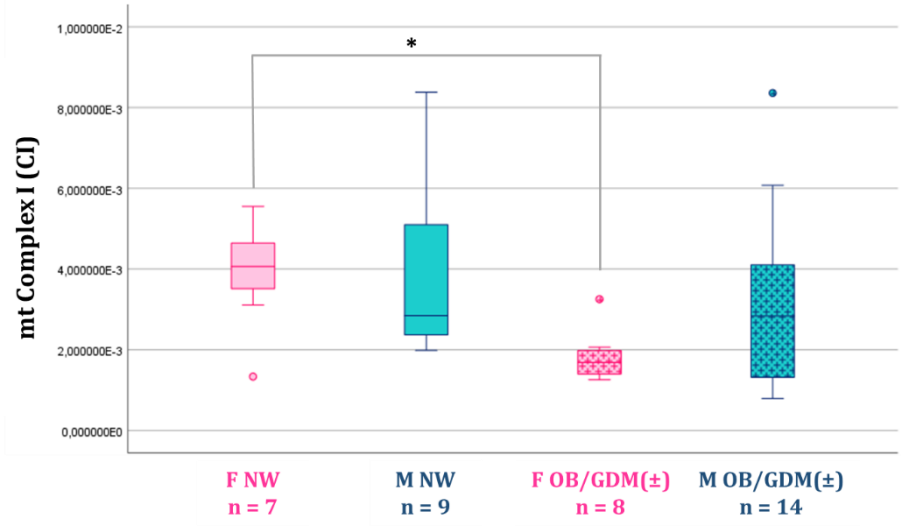


Fig. 18 | *Placental mitochondrial Complex I content in NW and OB/GDM(±) women according to foetal sex.* Data are shown as box plots, indicating the median and the 25th and 75th percentiles. Data were analysed using *Kruskal-Wallis* test with *Pairwise Comparison* as post-hoc test (with *Bonferroni Correction*): *p < 0.05, female OB/GDM(±) versus female NW.

5.2.3. MicroRNA profiling in maternal plasma

Editor's note: a new manuscript describing the following unpublished data about microRNA profiling in maternal plasma of obese women, with and without GDM, compared to normal-weight, is currently in draft.

The *miRCURY LNA miRNA PCR Panels & Assays* dedicated data analysis software *GeneGlobe* (*Qiagen*) selected data of 16 subjects (6 NW, 5 OB/GDM(-), 5 OB/GDM(+)) out of the 19 tested (7 NW, 6 OB/GDM(-), 6 OB/GDM(+)) for downstream analysis. 3 subjects (1 NW, 1 OB/GDM(-), 1 OB/GDM(+)) were excluded because they did not pass the quality control. The quality control standards are explained below:

- Extraction and retrotranscription controls: the amplification results of two synthetic spike-in miRNAs used as extraction and retrotranscription controls, respectively *Cel-miR-39-3p* and *UniSp6*, must be as similar as possible in all tested samples; data are considered acceptable if each spike-in's corrected Ct value falls in a range of 0-3 Cts.
  → The 3 excluded samples had *Cel-miR-39-3p* Ct exceeding the average range.
  → All samples had acceptable *UniSp6* Ct.

- Hemolysis assessment: ΔCt (hsa-miR-23a-3p — hsa-miR-450a) value allows calculation of hemolysis rate on a molecular level. Samples with a value >7 are considered contaminated with lysed erythrocytes.
  → All samples had Hemolysis Rate <7.

Table 3 recapitulates the lists of miRNAs that resulted significantly differentially expressed (p-value < 0.05), and with a fold regulation > |2|, in each of the three groups' comparison. Moreover, **Figure 19** contains Volcano Plots reporting the same results but graphically.

To resume, the three comparisons matched with the relative tables and figures are listed below:

A) OB/GDM(-) vs NW (significant miRNAs, n = 4; **Tab.3A** and **Fig.19A**);
B) OB/GDM(+) vs NW (significant miRNA, n = 1; **Tab.3B** and **Fig.19B**);
C) OB/GDM(+) vs OB/GDM(-) (significant miRNAs, n = 14; **Tab.3C** and **Fig.19C**).

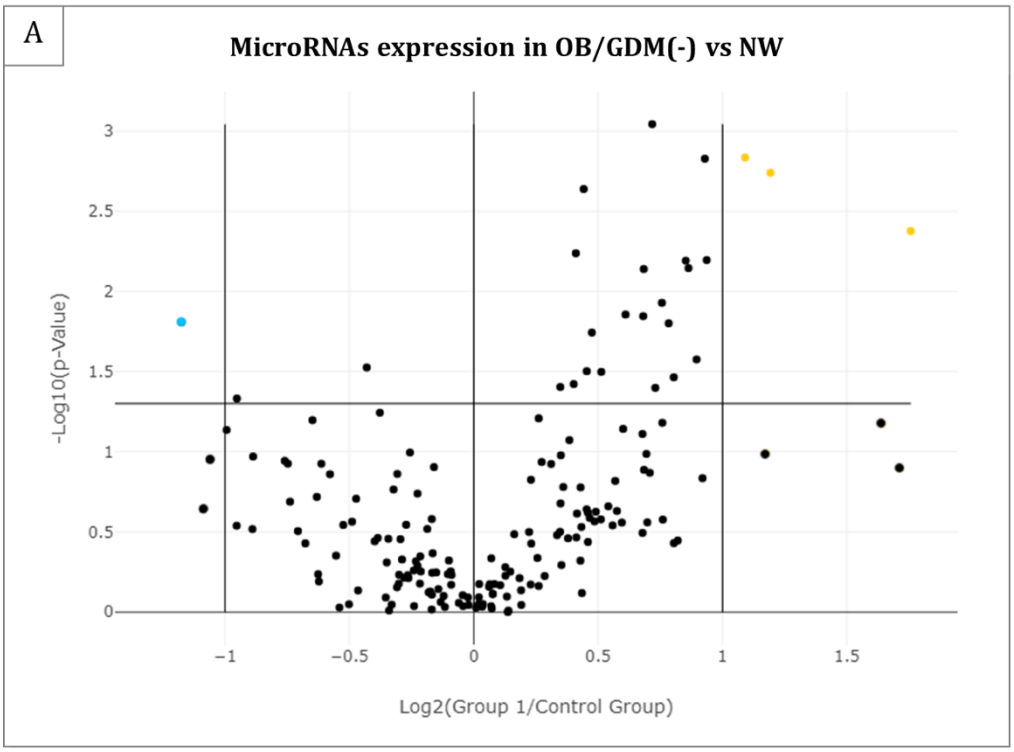
A	OB/GDM(-) vs NW		
	miRNA ID	**Fold Regulation**	**p-value**
	hsa-miR-27a-3p	2.13	0.0016
	hsa-miR-324-5p	2.29	0.0018
	hsa-miR-33a-5p	3.38	0.0042
	hsa-miR-186-5p	-2.26	0.0155

B	OB/GDM(+) vs NW		
	miRNA ID	**Fold Regulation**	**p-value**
	hsa-miR-454-3p	-2.32	0.0216

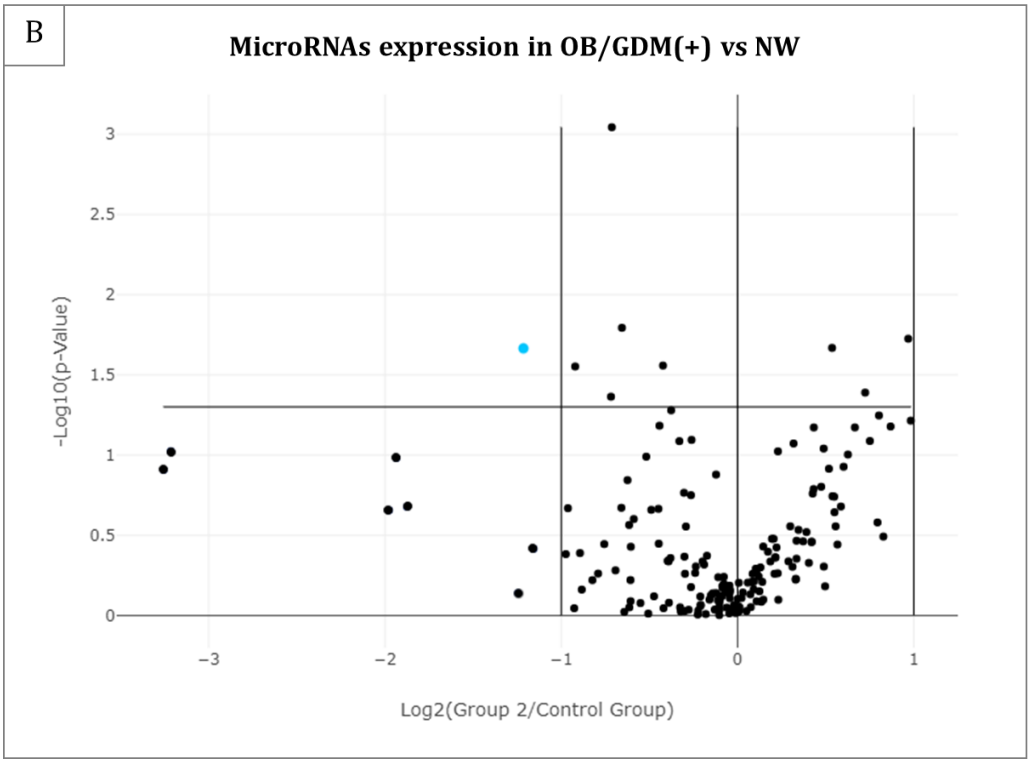
C	OB/GDM(+) vs OB/GDM(-)		
	miRNA ID	**Fold Regulation**	**p-value**
	hsa-miR-186-5p	2.13	0,0065
	hsa-miR-320d	2.18	0.0148
	hsa-miR-2110	2.04	0.0196
	hsa-let-7b-5p	2.09	0.0260
	hsa-miR-574-3p	2.45	0.0266
	hsa-miR-320c	2.26	0.0377
	hsa-miR-324-5p	-2.75	0.0023
	hsa-miR-142-3p	-3.03	0.0115
	hsa-miR-33a-5p	-4.09	0.0144
	hsa-miR-21-5p	-12.16	0.0162
	hsa-miR-27a-3p	-2.02	0.0186
	hsa-let-7f-5p	-3.45	0.0294
	hsa-miR-30e-3p	-4.57	0.0403
	hsa-miR-339-5p	-2.46	0.0467

Table 3 | *Statistically significant differentially expressed miRNAs in **A)** OB/GDM(-) vs NW, **B)** OB/GDM(+) vs NW, **C)** OB/GDM(+) vs OB/GDM(-).*
In yellow, upregulated miRNAs; in blue, downregulated miRNAs. For each table, upregulated miRNAs are listed first, downregulated miRNAs are listed latter; each section is ordered by increasing p-value (decreasing statistical significance).
Statistical significance: p-value <0.05; Fold Regulation > |2|, with positive values (>2) indicating upregulation and negative values (<-2) indicating downregulation in in each "case" group vs related "control". Data were analysed using *GeneGlobe* (*Qiagen*).

A


MicroRNAs expression in OB/GDM(-) vs NW
-Log10(p-Value)
3
2.5
2
1.5
1
0.5
0
−1
−0.5
0
0.5
1
1.5
Log2(Group 1/Control Group)

B


MicroRNAs expression in OB/GDM(+) vs NW
-Log10(p-Value)
3
2.5
2
1.5
1
0.5
0
−3
−2
−1
0
1
Log2(Group 2/Control Group)

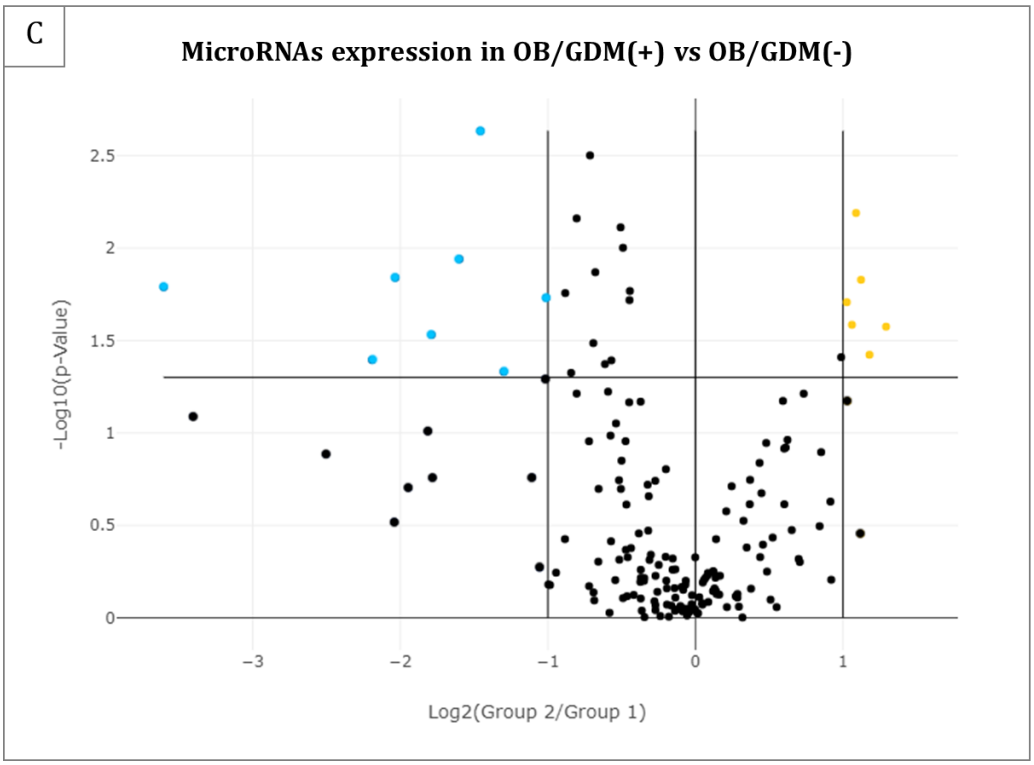


Figure 19 | *Volcano Plots representing the 179 microRNAs analysed in **A)** OB/GDM(-) vs NW, **B)** OB/GDM(+) vs NW, **C)** OB/GDM(+) vs OB/GDM(-).*
The Volcano Plots show significant miRNA expression changes, for each group comparison, by plotting the $\log_2$ of the Fold Changes in miRNA expression on the x-axis versus their statistical significance on the y-axis. The two vertical lines represent Fold Regulation cut-off of |2|, while the horizontal line indicates the statistical significance (p-value) of 0.05. Therefore, miRNAs with data points in the far upper left (downregulated: *blue*) and far upper right (upregulated: *yellow*) sections meet the selected fold regulation and p-value thresholds.
Data and figures obtained by *GeneGlobe* (*Qiagen*).

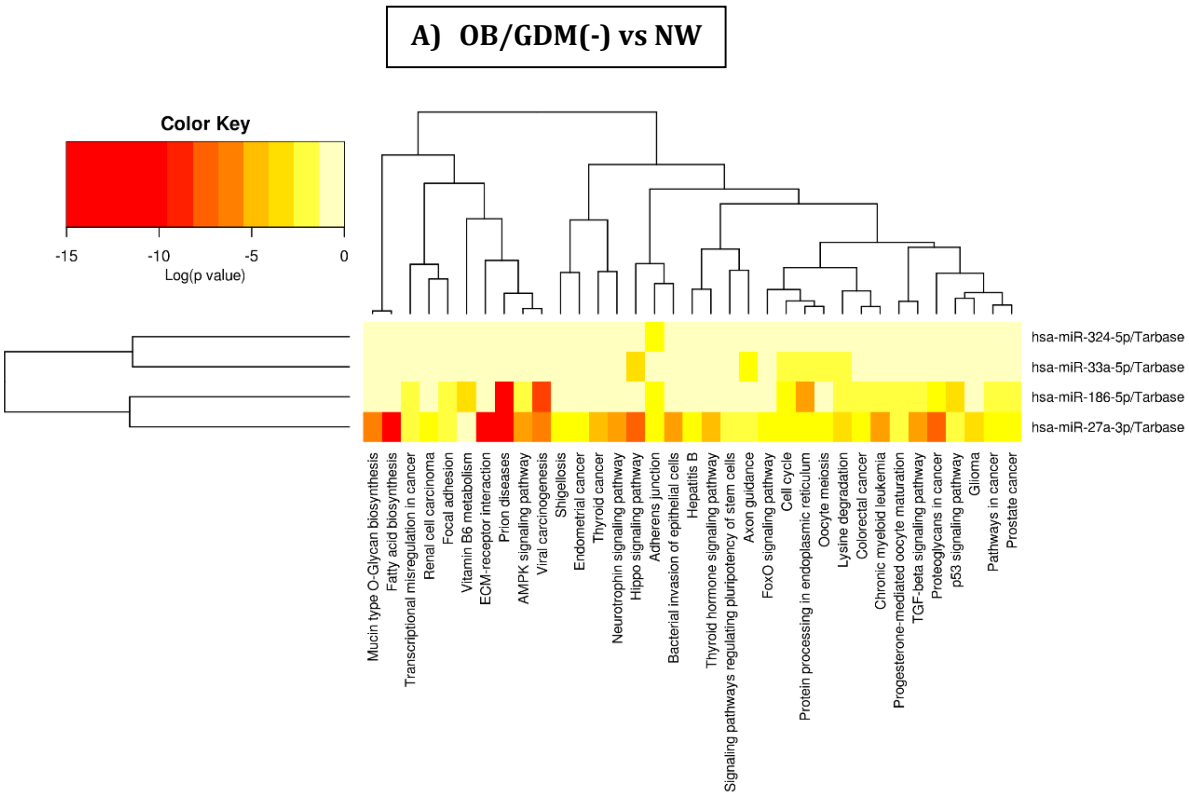
To gain insights into the miRNA profiling results, DIANA–miRPath and miTALOS online tools were used for miRNA pathway enrichment analysis.

First, each cluster of miRNAs that resulted significantly and differentially expressed between OB/GDM(-) vs NW, OB/GDM(+) vs NW, and OB/GDM(+) vs OB/GDM(-) was inserted in DIANA–miRPath v.3, and then *union analysis* (i.e. combinations of pathways related to the inserted miRNAs) was performed. **Table 4** shows the complete results for the three miRNAs' clusters related to each of the three comparisons (**A)** OB/GDM(-) vs NW, **B)** OB/GDM(+) vs NW, and **C)** OB/GDM(+) vs OB/GDM(-)).

Pathway Enrichment Analysis results (miRPath):		
A) OB/GDM(-) vs NW	**B) OB/GDM(+) vs NW**	**C) OB/GDM(+) vs OB/GDM(-)**
1. Prion diseases 2. Viral carcinogenesis 3. Hippo signalling pathway **4. Fatty acid biosynthesis** **5. ECM-receptor interaction** 6. Protein processing in endoplasmic reticulum 7. Proteoglycans in cancer 8. Adherens junction 9. Bacterial invasion of epithelial cells **10. AMPK signalling pathway** 11. Chronic myeloid leukemia **12. TGF-beta signalling pathway** **13. Lysine degradation** 14. Mucin type O-Glycan biosynthesis 15. Cell cycle 16. p53 signalling pathway **17. Oocyte meiosis** 18. Glioma 19. Neurotrophin signalling pathway 20. Thyroid cancer **21. Thyroid hormone signalling pathway** **22. FoxO signalling pathway** 23. Colorectal cancer 24. Pathways in cancer 25. Prostate cancer 26. Transcriptional misregulation in cancer 27. Axon guidance 28. Renal cell carcinoma 29. Shigellosis 30. Focal adhesion 31. Hepatitis B **32. Signalling pathways regulating pluripotency of stem cells** 33. Endometrial cancer **34. Progesterone-mediated oocyte maturation** **35. Vitamin B6 metabolism**	**1. Fatty acid elongation** **2. Fatty acid degradation** **3. Lysine degradation** **4. Fatty acid metabolism** **5. Signalling pathways regulating pluripotency of stem cells** **6. TGF-beta signalling pathway** **7. FoxO signalling pathway** **8. Valine, leucine and isoleucine degradation** 9. Bacterial invasion of epithelial cells **10. HIF-1 signalling pathway** 11. RNA transport 12. Ubiquitin mediated proteolysis 13. Glioma 14. Proteoglycans in cancer **15. Valine, leucine and isoleucine biosynthesis**	**1. Fatty acid biosynthesis** **2. ECM-receptor interaction** 3. Prion diseases 4. Viral carcinogenesis 5. Hippo signalling pathway **6. Lysine degradation** 7. Proteoglycans in cancer 8. Cell cycle 9. Adherens junction 10. Chronic myeloid leukemia **11. TGF-beta signalling pathway** 12. Hepatitis B 13. Glioma 14. p53 signalling pathway **15. FoxO signalling pathway** **16. Thyroid hormone signalling pathway** 17. Protein processing in endoplasmic reticulum 18. Bacterial invasion of epithelial cells **19. Fatty acid metabolism** 20. Pathways in cancer 21. Endocytosis 22. Colorectal cancer **23. Oocyte meiosis** 24. Thyroid cancer 25. Transcriptional misregulation in cancer 26. Prostate cancer 27. Epstein-Barr virus infection 28. Small cell lung cancer 29. Mucin type O-Glycan biosynthesis **30. AMPK signalling pathway** 31. MAPK signalling pathway 32. Pancreatic cancer 33. Neurotrophin signalling pathway 34. Melanoma 35. Renal cell carcinoma 36. Endometrial cancer 37. Steroid biosynthesis 38. Bladder cancer

Table 4 | *Results of the union analysis performed with DIANA–miRPath v.3, for each comparison:* ***A)*** *OB/GDM(-) vs NW,* ***B)*** *OB/GDM(+) vs NW,* ***C)*** *OB/GDM(+) vs OB/GDM(-).*
All listed pathways resulted significantly associated (force of association: p-value >0.05) with the related miRNAs' cluster, for each comparison (refer to Table 3 for the miRNA clusters). Pathways are ordered by descending statistical significance, p-values not showed. Pathways considered to be mostly interesting according to the context of maternal obesity, GDM, pregnancy, inflammation have been marked with bold character.

Heat maps depicting the force of association between specific miRNAs' cluster and related pathways was obtained for comparisons **A)** and **C)** (**Fig.20A** and **Fig.20C**). It was not possible to obtain the Heat map for comparison **B)** because it included only one miRNA (hsa-miR-454-3p).



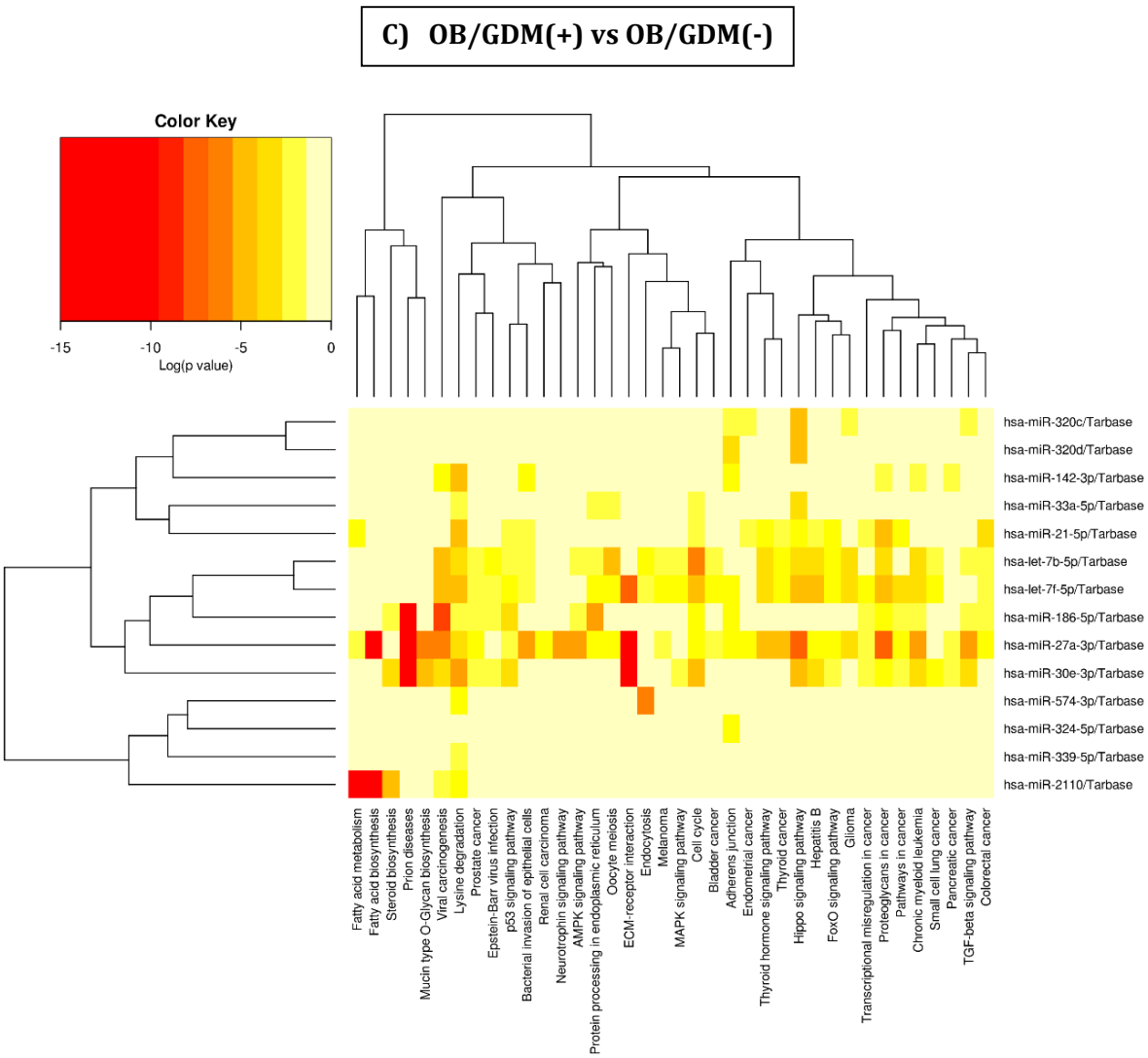


Figure 20 | *Heat maps by DIANA–miRPath v.3 showing miRNAs' clusters and associated pathways obtained by union analysis for the following comparisons:* ***A)*** *OB/GDM(-) vs NW, and* ***C)*** *OB/GDM(+) vs OB/GDM(-).* For each panel, the analysed miRNAs are listed on the right. The specific pathways, resulting in being significant from miRNA pathway enrichment analysis, are listed at the bottom. The force of association (Log(p-value)) between the analysed miRNAs and the pathways is indicated by the colour key reported at the upper left corner. Log: log10 p-value.

From the complete results obtained by DIANA-miRPath union analysis, resumed in **Table 4** and, when possible, in **Figure 20**, the most interesting pathways, i.e. the most coherent with the context of (maternal) obesity, diabetes/GDM, pregnancy, OS and inflammation, were selected for further discussion. Pathways related to cancer, infectious diseases, or to ubiquitous mechanisms present in every eukaryotic cell type have been omitted in the following sections because we considered that they were not inherent to the aims of this work. The selected pathways, with related force of association (i.e. p-value) and specifically-associated miRNAs, are reported in **Tables 5**.

A) OB/GDM(-) vs NW			
Pathway name	**Pathway information**	**p-value**	**miRNAs**
Fatty acid biosynthesis	Creation of fatty acids from acetyl-CoA and NADPH through fatty acid synthases.	3.2455 x e^{-12}	hsa-miR-27a-3p
ECM (ExtraCellular Matrix)-receptor interaction	Complex mixture of structural and functional macromolecules with important roles in cell, tissue and organ morphogenesis, structure and function.	1.6770 x e^{-8}	hsa-miR-27a-3p
AMPK (AMP-activated Protein Kinase) signalling pathway	Sensor of cellular energy status.	3.5242 x e^{-5}	hsa-miR-27a-3p hsa-miR-186-5p
TGF-beta (Transforming Growth Factor-beta) signalling pathway	Regulation of cellular functions such as proliferation, apoptosis, differentiation and migration	3.7782 x e^{-5}	hsa-miR-27a-3p hsa-miR-186-5p
Lysine degradation	Amino acid breakdown mainly taking place in hepatocytes' mitochondria.	6.9088 x e^{-5}	hsa-miR-27a-3p hsa-miR-33a-5p hsa-miR-186-5p
Oocyte meiosis	Maturation of female gametes.	0.000417	hsa-miR-27a-3p hsa-miR-33a-5p
Thyroid hormone signalling pathway	Thyroid hormones triiodothyronine (T3) and thyroxine (T4) are important regulators of growth, development and metabolism.	0.0016	hsa-miR-27a-3p
FoxO (Forkhead box O) signalling pathway	Transcription factors regulating apoptosis, cell-cycle control, glucose metabolism, oxidative stress resistance, and longevity.	0.003443	hsa-miR-27a-3p
Signalling pathways regulating pluripotency of stem cells	Pluripotent stem cells (PSCs) are self-renewal cells with the potential to generate all cell types of the three germinal layers.	0.030599	hsa-miR-27a-3p
Progesterone-mediated oocyte maturation	Insulin/IGF-1 or progesterone exposure induces maturation of the oocyte into a mature, fertilizable egg.	0.037975	hsa-miR-27a-3p hsa-miR-186-5p
Vitamin B6 metabolism	Coenzyme in amino acid, glucose, and lipid metabolism.	0.040007	hsa-miR-186-5p

B) OB/GDM(+) vs NW			
Pathway Name	**Pathway information**	**p-value**	**miRNA**
Fatty acid elongation	Part of the anabolic processes generating and modifying fatty acids.	1.3166 x e^{-11}	hsa-miR-454-3p
Fatty acid degradation	Fatty acids breakdown into their metabolites, and finally acetyl-CoA.	1.3726 x e^{-7}	hsa-miR-454-3p
Lysine degradation	Amino acid breakdown mainly taking place in hepatocytes' mitochondria.	8.4918 x e^{-5}	hsa-miR-454-3p
Fatty acid metabolism	Catabolic and anabolic processes involving fatty acids.	8.4918 x e^{-5}	hsa-miR-454-3p
Signalling pathways regulating pluripotency of stem cells	Pluripotent stem cells (PSCs) are self-renewal cells with the potential to generate all cell types of the three germinal layers.	0.009942	hsa-miR-454-3p
TGF-beta (Transforming Growth Factor-beta) signalling pathway	Regulation of cellular functions such as proliferation, apoptosis, differentiation and migration	0.010679	hsa-miR-454-3p
FoxO (Forkhead box O) signalling pathway	Transcription factors regulating apoptosis, cell-cycle control, glucose metabolism, oxidative stress resistance, and longevity.	0.037202	hsa-miR-454-3p
Valine, leucine and isoleucine (BCAA) degradation	Breakdown of the branched-chain amino acids (BCAA).	0.038549	hsa-miR-454-3p
HIF-1 (Hypoxia-inducible factor 1) signalling pathway	Transcription factor, master regulator of oxygen homeostasis. Involvement in autophagy, inflammation and oxidative stress.	0.043120	hsa-miR-454-3p
Valine, leucine and isoleucine biosynthesis	Enzymatic processed generating the branched-chain amino acids (BCAA).	0.049787	hsa-miR-454-3p

C) OB/GDM(+) vs OB/GDM(-)			
Pathway Name	**Pathway information**	**p-value**	**miRNAs**
Fatty acid biosynthesis	Creation of fatty acids from acetyl-CoA and NADPH through fatty acid synthases.	$<1 \times e^{-325}$	hsa-miR-2110 hsa-miR-27a-3p
ECM (ExtraCellular Matrix)-receptor interaction	Complex mixture of structural and functional macromolecules with important roles in cell, tissue and organ morphogenesis, structure and function.	$<1 \times e^{-325}$	hsa-miR-27a-3p hsa-let-7f-5p hsa-miR-30e-3p
Lysine degradation	Amino acid breakdown mainly taking place in hepatocytes' mitochondria.	$<1 \times e^{-325}$	hsa-miR-186-5p hsa-miR-2110 hsa-let-7b-5p hsa-miR-574-3p hsa-miR-142-3p hsa-miR-33a-5p hsa-miR-21-5p hsa-miR-27a-3p hsa-let-7f-5p hsa-miR-30e-3p hsa-miR-339-5p
TGF-beta (Transforming Growth Factor-beta) signalling pathway	Regulation of cellular functions such as proliferation, apoptosis, differentiation and migration	$9.0845 \times e^{-8}$	hsa-miR-186-5p hsa-let-7b-5p hsa-miR-320c hsa-miR-27a-3p hsa-miR-30e-3p
FoxO (Forkhead box O) signalling pathway	Transcription factors regulating apoptosis, cell-cycle control, glucose metabolism, oxidative stress resistance, and longevity.	$3.6540 \times e^{-6}$	hsa-let-7b-5p hsa-miR-21-5p hsa-miR-27a-3p hsa-let-7f-5p hsa-miR-30e-3p
Thyroid hormone signalling pathway	Thyroid hormones triiodothyronine (T3) and thyroxine (T4) are important regulators of growth, development and metabolism.	$3.9364 \times e^{-6}$	hsa-let-7b-5p hsa-miR-21-5p hsa-miR-27a-3p hsa-let-7f-5p
Fatty acid metabolism	Catabolic and anabolic processes involving fatty acids.	$1.9484 \times e^{-5}$	hsa-miR-2110 hsa-miR-21-5p hsa-miR-27a-3p
Oocyte meiosis	Maturation of female gametes.	$7.3982 \times e^{-5}$	hsa-let-7b-5p hsa-miR-33a-5p hsa-miR-27a-3p hsa-let-7f-5p
AMPK (AMP-activated Protein Kinase) signalling pathway	Sensor of cellular energy status.	0.004058	hsa-miR-186-5p hsa-let-7b-5p hsa-miR-27a-3p

Tables 5 | *Selected pathways, coherently with the context of this study, from the union analysis performed with DIANA–miRPath v.3, for each comparison: in **A)** OB/GDM(-) vs NW, **B)** OB/GDM(+) vs NW, **C)** OB/GDM(+) vs OB/GDM(-)*

Pathway information and description have been found on the online repository *https://www.genome.jp/*, in particular in the section *KEGG PATHWAY Database*; p-value indicates the force of association between the pathway and specific miRNAs (indicated in the last column); pathway are listed in order of increasing p-value (decreasing statistical significance).

To complete our bioinformatic search, an additional pathway enrichment union analysis was performed by miTALOS v.2, inserting the significantly differentially expressed miRNAs for each comparison (miRNAs' list at **Table 3**). For each comparison, the relevant pathways were selected and listed above:

A) OB/GDM(-) vs NW:

- *SREBF (Sterol Regulatory Element-Binding transcription Factor) and miR33 in cholesterol and lipid homeostasis* (p = 0.017981);
- *Insulin signalling* (p = 0.042847).

B) OB/GDM(+) vs NW:

- *TGF-beta (Transforming Growth Factor-beta) signalling pathway* (p = 0.005859).

C) OB/GDM(+) vs OB/GDM(-):

- *mTOR (mammalian Target Of Rapamycin) signalling pathway* (p = 0.044364);
- *Insulin signalling* (p = 0.013768).

6. DISCUSSION

6.1. Clinical characteristics of the study cohort

In this study, we characterized placental tissue by analysing some relevant markers of autophagy (in particular macroautophagy and CMA) and of antioxidant defense, as well as by exploring placental mitochondrial bioenergetics. We also focused on maternal plasmatic microRNAs profile, in relation to pre-pregnancy Body Mass Index (BMI) and to the presence of GDM.

Clinical characteristics were carefully considered. All pregnant women were enrolled at elective Caesarean section, in absence of labor, thus avoiding any bias related to labor-induced OS, inflammation and cell metabolism changes. Both NW and OB did not present any associated pathology, except for GDM in OB/GDM(+). All women were Caucasian, which avoided any genetic contribution to complex disorders (Kenney *et al.*, 2014; Gómez-Durán *et al.*, 2010). Possible confounding factors, such as maternal age differing among groups, were attentively checked during the statistical analysis and did not affect the molecular variables' results. Therefore, we may assume that the reported findings can be associated to metabolic dysfunctions related to increased BMI or hyperglycemia.

All mothers received nutritional and lifestyle counselling, especially on weight gain recommendations during pregnancy, which differ depending on pre-gestational BMI. This counselling was particularly important for obese subjects, and OB/GDM(+) women also followed a hypocaloric diet (1700 kcal/day), specifically set for diabetic patients, thus avoiding any pharmacologic therapy (e.g. insulin, metformin).

Indeed, all groups on average did not exceed in gestational weight gain (GWG) according to the IOM guidelines and obese women, accordingly, gained less weight. In particular, OB/GDM(+) had significantly lower GWG compared to NW (**Tab.2A**). This suggests that the nutritional counselling for all obese, and in particular the hypocaloric diet for diabetic women, were effective and allowed them to fit in the IOM guidelines.

Nevertheless, placental efficiency, estimated by calculating the ratio between fetal and placental weights, was significantly lower in OB/GDM(+) vs NW while it did not differ between OB/GDM(-) and NW (**Tab.2B**). These results are in line with previous data from our group, reporting decreased placental efficiency in OB/GDM(+) vs NW. Obese pregnancies affected by GDM, which is characterized by several systemic alterations such as inflammation, OS, insulin resistance and hyperglycemia, may present a particularly impaired intrauterine environment and deficient placental function. Normoglycemic obese women, instead, might exert some compensatory mechanisms contributing to maintain a fetal/placental weight ratio similar to NW (Mandò *et al.*, 2018; Bianchi *et al.*, 2021). However, other deficiencies/disturbances might be present in normoglycemic obese placentas, accounting for the molecular alterations that we found.

6.2. Evaluation of placental autophagy and antioxidant defense

Gene expression experiments showed a decreasing trend, though not significant, of placental antioxidant defense genes (CAT, GSS, GSR, GPX1) in OB/GDM(-), but not in OB/GDM(+), vs NW. In general, these results support the previously described alterations of placental antioxidant defenses in obese mothers (Saben *et al.*, 2014; Mandò *et al.*, 2018; Wallace *et al.*, 2012). CAT gene aroused particular attention because of its strong positive correlation with placental efficiency in the entire study population (**Fig.7**), suggesting the importance of the redox balance in the maintenance of organ functionality (Wallace *et al.*, 2012; Fattuoni *et al.*, 2018).

Interestingly, expression levels of these genes in GDM women were more similar to those of NW. This might be due to different responses in the obese and diabetic impaired environment (Al-Gubory *et al.*, 2010), which lead to alternative mechanisms in GDM, that of course are supposed to be deleterious as well, but resulting in unchanged expression of antioxidant genes. Indeed, previous studies showed that, while placentas from OB/GDM(-) women presented impaired mitochondrial biogenesis and mtDNA content (Scifres *et al.*, 2017; Hastie and Lappas, 2014), these were not altered in OB/GDM(+) placentas, although mitochondrial morphological abnormalities were shown, accounting for mitochondrial dysfunctionality (Mandò *et al.*, 2018).

Importantly, ROS are master inducers of autophagy. Thus, the activation of autophagy is a fundamental part of the OS-cellular response because it allows scavenging of defective components before further damage (De Andrade Ramos *et al.*, 2016). Autophagy activity has been documented in placentas (Oh and Roh, 2017), but only a few studies investigated it in obese and diabetic placentas (Hung *et al.*, 2020; Muralimanoharan *et al.*, 2016).

Macroautophagy acts by including cytoplasmic material and organelles within autophagosomes, whose fusion with lysosomes induces the degradation of the content (Feng *et al.*, 2014). On the other hand, CMA degrades proteins containing specific consensus sequences. These are usually bound by the chaperone HSC70, the complex is delivered to the lysosome, interacting with the LAMP-2A multimer, and the cargo is digested by proteases (Alfaro *et al*., 2019).

The ULK1 complex plays a central role in macroautophagy initiation and it's also involved in promoting autophagosome-lysosome fusion (Zachari and Ganley, 2017). Interestingly, our study showed an increase in placental ULK1 expression in both obese groups (**Fig.8A**), possibly due to the obesity-driven OS, activating macroautophagy.

Another principal driver of macroautophagy is BECN1 (Menon and Dhamija, 2018). No significant changes were observed in the three groups, but a strong and positive correlation with placental expression of GSS was noted (**Fig.8B**), as a clue of the relation between autophagy and the cell response to OS.

CMA is finely regulated by several proteins. Among them, PHLPP1 is a phosphatase inducing CMA by favouring the stabilization of the LAMP-2A multimers in the lysosomal membrane. Moreover, PHLPP1 supports insulin resistance by reducing insulin-dependent signal transduction, leading to a decrease in glucose transport and hyperglycemia (Leng *et al.*, 2010).

In our study, PHLLP1 expression was reduced in OB/GDM(-) vs NW placental tissues, while it resulted significantly increased in OB/GDM(+) vs OB/GDM(-) (**Fig.8D**), supporting a correlative hypothesis between PHLPP1 activity and GDM, as already hypothesized in type II diabetes (Andreozzi *et al.,* 2011). This could suggest a role for PHLPP1 in GDM placentas. However, whether it has a causative or a secondary role in relation to insulin resistance still needs to be investigated. In fact, it has been described that PHLPP1 overexpression induces insulin resistance. An increase in insulin levels also resulted in the overexpression of PHLPP1 itself, being probably involved in a feedback loop that is lost in diabetes (Mathur *et al.*, 2017).

Moreover, HSC70 expression decreased in both obese groups compared to NW (**Fig.8C**), indicating a potential impairment in CMA.

Finally, NRF2 is a transcription factor of different genes involved in cell detoxification and is also related to CMA activation because it triggers LAMP-2A expression (Pajares *et al.*, 2018). Our data confirm the strong positive correlation between NRF2 and LAMP-2A in the entire study population and in each group (**Fig.9**).

6.3. Evaluation of placental bioenergetics

In order to evaluate the placental mitochondrial bioenergetics status in our study population of pregnant women, we investigated ATP and mitochondrial (mt) Complexes I-V protein content in placental tissue at delivery.

Interestingly, placental ATP content was significantly lower in both OB/GDM(-) and OB/GDM(+), at a similar level, compared to NW women (**Fig.11**). This is in accordance with other studies reporting reduced ATP production in pregnancies affected by obesity and GDM, probably because of the enhanced OS and ROS production that impairs mitochondrial functions. For instance, Sobrevia and colleagues reported that placentas from GDM women showed higher ROS-induced mtDNA damage and lipid peroxidation, which limit mitochondrial respiration and ATP generation (Sobrevia *et al.,* 2020). Furthermore, the group of Rodríguez-Cano described a significantly higher generation of ROS with increasing maternal adiposity, along with lower mitochondrial respiration by OXPHOS and deficient ATP generation in the placenta (Rodríguez-Cano *et al*., 2020). Moreover, Lu and Sferruzzi-Perri reported that villous tissue from term placentas of obese mothers had decreased ATP levels, when compared to placentas from lean mothers (Lu and Sferruzzi-Perri, 2021).

In accordance with a reduced placental ATP production, we found decreased expression of several mt Complexes belonging to the respiratory chain. Concerning placental mt Complex I, we found a significant lower content in all OB/GDM(±) vs NW (**Fig.12B**). While placental mt Complexes II and IV did not show any significant differences among groups (**Fig.13A-B**, **Fig.15A-B**), placental mt Complex III was significantly lower in all OB/GDM(±) vs NW (**Fig.14B**) as well as in the group of OB/GDM(+) vs NW (**Fig.14A**) . Moreover, placental mt Complex V was significantly lower in OB/GDM(+) vs both NW and OB/GDM(-) (**Fig.16A**).

Interestingly, Sobrevia and colleagues also reported that placentas from GDM women under diet therapy showed lower expression of mt Complex III compared with placentas from healthy pregnancies (Sobrevia *et al.,* 2020). Importantly, the decreased expression of mt Complexes I-III-V proteins is coherent with the reduced placental ATP content. Taken together, these results suggest that less electrons may be delivered to Complex V, which is the ATP synthase catalysing ATP production, because some of the upstream complexes are reduced, together with a defective abundance of Complex V itself. As a final consequence, total placental ATP content could be reduced and mitochondrial respiration could be impaired. In addition, reduced ATP generation could also likely result from mitochondrial inner membrane cristae disorganization, which is a feature already reported in GDM pregnancies by our group (Mandò *et al.*, 2018).

We also found some interesting correlations. In particular, placental efficiency significantly and positively correlated with placental mt Complexes I and V content (**Fig.17A** and **Fig.17B**).

This suggests a possible association between placental mitochondrial bioenergetics and placental function, leading to the hypothesis that mitochondrial alterations found in obesity and GDM could contribute to placental dysfunction.

6.4. Sexual dimorphism in placental markers

Recently, the interest towards sex-based differences in response to adverse perinatal and intrauterine environments has grown increasingly (Osei-Kumah *et al.*, 2011; Yu *et al.*, 2021). Sexual dimorphism in terms of molecular differences (e.g. inflammatory/oxidative markers, autophagy pathways, mitochondria parameters) is still largely uncharted and represents a research field in flourishing progress, possibly including the study of sexually dimorphic placental epigenetic modifications (Nugent and Bale, 2015). In particular, "placental epigenome" might play a leading role in this dichotomy. For instance, emerging evidence suggests that more than 500 genes are differentially methylated in placentas of male and female fetuses, and these encode for proteins related to immune function, growth and transcription factor signalling and nutrients transport across cell membranes (Martin *et al.*, 2017).

Moreover, concerning mitochondria, a complex fetal sex-related placental dimorphism in mitochondrial biogenesis markers and mtDNA content have been reported (Jiang *et al.,* 2017). These molecular differences found in placenta could partially provide insight into different responses to the perinatal environment and consequent different infant outcomes.

Indeed, placentas of male and female fetuses seem to respond differently to obesity- and GDM-associated dysfunctions. Males and females appear to have differential placental profile of OS and antioxidant defense markers and, as a consequence, different signs of placental harm and related compensatory mechanisms (Hebert and Myatt, 2021). Importantly, Alexander and colleagues found sex-related differential genome-wide DNA methylation in placentas of diabetic mothers, in particular at loci related to mitochondrial function, DNA repair, inflammation and oxidative stress (Alexander *et al.*, 2018). Our group already reported heavier placentas in diabetic obese and overweight mothers carrying female newborns (Mandò *et al.*, 2016; Mandò *et al.*, 2018). Indeed, due to the intrinsic differences in gonadal steroids production in females (estrogens) and males (androgens), it is well-known that fetal sex impacts on the formation, biometrical and functional features of placenta (Myatt, 2018), but much remains to be clarified about the sex-specific placental responses to different adverse intrauterine stimuli (e.g. obesity, overweight, diabetes) and about the related sex-specific placental molecular patterns. In some cases, females seem to be more skilled to respond to insults and/or have compensatory mechanisms leading to a more rapid and efficient restoration of the perturbed environment, but of course this could not be assumed as a general rule (Mandò *et al.*, 2016; Anelli *et al.*, 2018; Serati, 2022).

In the current study, fetal sex was equally distributed in our population. Similarly to our previous results, female placentas of OB/GDM(+) tended to be heavier compared to male placentas of the same group and to both sexes of NW, though not reaching statistical significance. Fetal weight, however, did not differ among groups divided by fetal sexes. As a consequence, female placentas

of OB/GDM(+) displayed significantly lower placental efficiency (i.e. fetal/placental weights ratio) compared to male placentas of the same group and to both sexes of NW (**Tab. 2B**).

Concerning molecular variables, in the present study an interesting result was obtained for the expression of PHLPP1, which dropped significantly in females' placentas in the OB/GDM(-) group, while it increased in both male and female placentas in OB/GDM(+) (**Fig.10A**). However, PHLPP1 modulation seemed to have different consequences in the two sexes, with a significant decrease in VEGF expression only in males (**Fig.10B**), which might be due to CMA activation. In fact, HIF1-α is both a CMA target and one of the main transcription factors driving VEGF expression. Supporting the hypothesis of a CMA activation, an increase in the expression of its main player, LAMP-2A, has been detected in the same samples even if it did not reach statistical significance (*data not shown*). This mechanism was not observed in females' tissues, providing clues about a dimorphic regulation and possibly different GDM outcomes depending on fetal sex, accounting for the hypothesis that males could be more sensible and fragile to GDM insults (Diceglie-Anelli *et al.*, 2021; Serati, 2022).

Interestingly, we also found a significant sex-specific difference in placental mt Complex I content between NW and all obese women, with and without GDM. In particular, we reported a significantly lower content in OB/GDM(±) compared to NW women only in pregnancies with female fetus (**Fig.18**). However, we did not record any difference in placental ATP content depending on fetal sex. Because of that, we hypothesize that, despite the drop in Complex I in placentas of female fetuses in the obese groups, female placentas might be more skilled to respond to the adverse intrauterine environment, reacting more rapidly to the insults and restoring possibly compromised mitochondrial functions.

6.5. Evaluation of maternal plasmatic microRNAs profile

In this study, we profiled maternal plasma circulating miRNAs in a clinically well-selected cohort of pregnant obese women affected or not by GDM, compared to normal weight pregnant controls, at term, to characterize their specific miRNA patterns and to seek for possible associations with the dysregulated maternal metabolic state. In particular, we performed Real time-based miRNA profiling in OB/GDM(-), OB/GDM(+) and NW, analysing the expression of the 179 most expressed known microRNAs (miRNAs) in human plasma. Then, we analysed and compared the results among the three groups and finally we performed a pathway enrichment analysis using miRPath and miTALOS.

Considering the comparison **A) OB/GDM(-) vs NW**, we found 4 significantly differentially expressed miRNAs; in particular, 3 miRNAs were upregulated and 1 miRNA was downregulated in OB/GDM(-) compared to NW (**Tab.3A** and **Fig.19A**). The 4 miRNAs' entries inserted in miRPath gave back a set of 35 signalling pathways specifically associated with this miRNAs' pattern (**Tab.4**, **column A**, and **Fig.20A**).

For **B) OB/GDM(+) vs NW**, only 1 miRNA resulted differentially expressed, with statistical significance, between the two groups (**Tab.3B** and **Fig.19B**); miRPath analysis showed that hsa-miR-454-3p is associated with 15 molecular pathways (**Tab.4**, **column B**).

Finally, from comparison **C) OB/GDM(+) vs OB/GDM(-)**, 14 miRNAs resulted significantly differentially expressed in the two groups (**Tab.3C** and **Fig.19C**). According to miRPath pathway enrichment analysis, this miRNAs' pattern was associated with 38 pathways (**Tab.4**, **column C**, and **Fig.20C**).

For each comparison, among the A) 35, B) 15, C) 38 pathways, the most relevant ones in the context of pregnancy, maternal obesity, GDM and metabolic derangements were selected for further literature search and discussion (**Tab5A**, **5B**, **5C**). An additional pathway enrichment analysis with miTALOS was also performed, pointing out some supplementary potentially interesting pathways (namely, 2 pathways for comparison A, 1 for B, 2 for C). The main findings from these analyses are discussed below, divided in main topics (i.e. single pathways alone or pathways grouped together).

Germinal cells, gametes and pluripotency-related pathways

According to miRPath analysis, some pathways related to germinal cells maturation, oocyte meiosis and pluripotency of stem cells were common among all the three miRNA patterns. This could be quite surprising due to the fact that we analysed maternal plasma at term, while some miRNAs are known to finely regulate mechanisms going from gametogenesis to embryogenesis, implantation, and embryo development since the very first days after conception and in particular

during the first trimester of gestation (Salilew-Wondin *et al.*, 2020). Moreover, few mammalian studies revealed that generation of functionally competent gametes, oocytes and sperm cells, from primordial germ cells in the offspring depends to some extent on fundamental processes that occur early during fetal development, in utero (Czukiewska *et al.*, 2022). In females, indeed, germ cells enter meiosis during fetal ovaries development (Spiller *et al.,* 2012). According to the DOHaD hypothesis, it is well-known that environmental stressors, chemical compounds, such as endocrine disruptors, and poor intrauterine milieu are able to epigenetically influence not only maternal cells, but also fetal cells. When these harms alter the epigenetic programming of the fetal germ line, these changes can be transmitted across generations in the absence of direct exposure, an effect termed epigenetic transgenerational inheritance (Skinner, 2016). Therefore, it is tempting to speculate that these crucial cellular pathways related to germ cells homeostasis remain cautiously accessible and non-dormant across all the gestation, under the vigilant control of the epigenetic machinery, and of miRNAs, in particular. In the obese and/or diabetic pregnant mother, the unhealthy intrauterine and systemic environments, characterized by inflammation, lipotoxicity and oxidative stress, could alter some of these mechanisms, with negative impact on the development of fetal organs and cells, including gametes (Aiken *et al.*, 2016).

Insulin signalling pathway

As expected, also insulin signalling pathway was pointed out by miTALOS analysis, in the context of OB/GDM(-) vs NW and OB/GDM(+) vs OB/GDM(-) miRNA patterns. It is possible that this pathway did not emerge in OB/GDM(+) vs NW comparison because of the presence, in this case, of only one differentially expressed miRNA (i.e. hsa-miR-454-3p).

Of course, evidences from non-pregnant human adipose and skeletal muscle tissues showed that impaired insulin action in type II diabetes could also be due to direct defects of the insulin signalling pathway (Petersen and Shulman, 2018). Interestingly, in adipose and skeletal muscle tissues of pregnant women, Colomiere and colleagues demonstrated that the same pathway also plays a role in the pathophysiology of insulin resistance associated with GDM and maternal obesity. The same group also verified that endogenous expression levels of insulin signalling components in the human term placenta, in particular insulin receptors and their substrates but also glucose transporters, are altered in placental tissue from GDM women in comparison both with normal-weigh healthy controls and obese non-diabetic women (Colomiere *et al.,* 2009; Colomiere *et al.,* 2010).

Lipids, fatty acids and lysine metabolism pathways

Interestingly, plasmatic miRNA patterns from all the three comparisons resulted associated with fatty acid biosynthesis, elongation, metabolism and degradation, and in lysine degradation

pathways. This is in accordance with previous data recently reported by our group, showing a significant association between the same fatty acids-related and lysine degradation pathways, and maternal salivary miRNAs during the third trimester of pregnancy, in a population of obese pregnant women which have partially overlapping characteristics compared to the current study cohort (Mandò *et al.*, 2022). Together, these findings may be suggestive of a disarranged fatty acid metabolism, as well as an excessive lysine catabolism in obese pregnancies with or without GDM, being mediated by miRNA epigenetic alterations. Importantly, these two independent works lead us to hypothesize that such changes can be detected coherently in different non-invasive-collected body fluids, being conserved in both maternal saliva and plasma. In a previous metabolomics analysis of placentas from obese versus normal-weight women, we also showed amino acid and fatty acids changes in placental tissue of obese women, with marked lower amounts of lysine on one hand, and lower levels of Long Chain-Polyunsaturated Fatty Acid (LC-PUFA) derivatives, arachidonic acid, and Docosa-Hexaenoic Acid (DHA), plus significantly increased saturated palmitic acid levels on the other hand (Fattuoni *et al.*, 2018).

Moreover, miTALOS analysis also pointed out an association between OB/GDM(-) vs NW miRNA pattern and the pathway named "Sterol Regulatory Element-Binding transcription Factor (SREBF) and miR-33 in cholesterol and lipid homeostasis". This is very interesting because, among the miRNAs that resulted differentially expressed in the same comparison, we actually found hsa-miR-33a-5p. This is an intronic miRNA located within the gene encoding Sterol Regulatory Element–Binding Factor 2 (SREBF-2), a key transcriptional regulator of cholesterol synthesis, thus modulating the expression of genes involved in cholesterol metabolism. In mice, hepatic miR-33 levels were inversely correlated with cholesterol levels and positively correlated with SREBF2 transcript level, suggesting that miR-33 is regulated by dietary cholesterol in vivo and that miR-33 and SREBF2 are co-transcribed and co-regulated. MiR-33 seems to target and thus silence adenosine triphosphate–binding cassette (ABC) transporters ABC-A1 and ABC-G1, which normally promote cellular cholesterol efflux. Indeed, this was also verified in vitro: miR-33 up-regulation was inversely correlated with both ABC transcripts and cellular cholesterol levels in mouse macrophages (Rayner *et al.,* 2010).

TGF-beta signalling pathway

According to the miRPath analysis, miRNA patterns from all the three comparisons also resulted associated with the Transforming Growth Factor (TGF)-beta signalling pathway. MiTALOS analysis then confirmed significant association between TGF-beta signalling pathway and OB/GDM(+) vs NW miRNA pattern.

TGF-Beta1 is a widespread anti-inflammatory cytokine with multi-organ effects. It plays active roles in glucose, lipid, amino acid and redox metabolism, and represents a key cytokine in obesity

and insulin resistance. Indeed, elevated TGF-Beta1 levels were associated with glucose intolerance and increased adiposity, and have been reported in women with a prior history of GDM (Yadav *et al.*, 2017; Zhu *et al.*, 2015).

In the above-cited study from our group, analysing maternal salivary miRNAs during the third trimester of pregnancy in a population of obese pregnant women compared to normal-weight pregnant controls, the same pathway emerged from miTALOS analysis. Actually, a significantly decreased TGF-Beta1 gene methylation levels in the saliva of obese versus lean mothers was also reported, supporting TGF-Beta1 upregulation (Mandò *et al.*, 2022). Therefore, the current data reinforce our previous hypothesis of a complex and epigenetic-regulated inflammatory network in maternal obesity and GDM.

ECM-receptor interaction

The sets of differentially expressed miRNAs in OB/GDM(-) vs NW and OB/GDM(+) vs OB/GDM(-) comparisons resulted significantly associated with the pathway of ExtraCellular Matrix (ECM)-receptor interaction, according to miRPath. Interestingly, both associations of this pathway with the two miRNA panels, had hsa-miR-27a-3p as a common miRNA. Hsa-miR-27a-3p is involved in adipogenesis and adipocyte differentiation, suggesting a possible role of this miRNA in obesity (Shi *et al.*, 2016).

Physiologically, ECM remodelling consists of structural and compositional changes and, in fact, it is critical for the differentiation and expansion of adipocytes. In obesity-related adipose tissue hypertrophy, ECM synthesis and degradation can be altered, causing inflammation and mechanical stress (Pessentheiner *et al.*, 2020). Similarly, hyperglycemia induces excessive production of ECM collagens and altered proteoglycans by endothelial cells. Indeed, in patients with type II diabetes, the ECM of blood vessels present an altered ratio of proteoglycans and a thicker basement membrane compared to normoglycemic controls. These changes could represent pathogenic mechanisms for vascular diabetic complications, which have a strong inflammatory basis (Olsson *et al.*, 1999).

We already reported a significant association between maternal salivary miRNAs differentially expressed in obese pregnant women during the third trimester and the ECM-receptor interaction pathway, and therefore we speculated that the miRNA alterations found in these women could be part of the dysregulated network of ECM remodelling due to the obesogenic and inflamed environment (Mandò *et al.*, 2022). Moreover, in another recent study investigating miRNAs expression in plasma of overweight and obese women during the first trimester of pregnancy (14-16 weeks), a set of 38 miRNAs resulted associated with maternal BMI; according to our results, when these miRNAs were analysed in miRPath *union pathway analysis*, some of them appeared

enriched in the KEGG pathway "ExtraCellular Matrix (ECM)-receptor interaction" (Thibeault *et al.*, 2022).

Thyroid hormone signalling pathway

Thyroid hormones, namely triiodothyronine (T3) and thyroxine (T4), are vital for the proper functioning of the female reproductive system, since they modulate the development and metabolism of ovarian, uterine and placental tissues (Silva *et al.*, 2018). During pregnancy, maternal thyroxine is essential for fetal growth since it supplies thyroid hormone-dependent fetal tissues and organs (i.e. brain and skeleton) until the complete maturation of fetal thyroid, which takes place during the 12-14 weeks of gestation. In case of maternal hypothyroidism, hormonal substitution with levothyroxine must be started in early pregnancy. Indeed, after the 14th gestational week, fetal brain development may already be irreversibly affected by lack of thyroid hormones (Springer *et al.*, 2017).

Thyroid hormones have been also demonstrated to regulate many metabolic pathways potentially relevant for the basal metabolic rate, including lipid metabolism and the uncoupling of cellular metabolism from ATP synthesis, thus also modulating the efficiency of the mitochondrial transport chain. During obesity, the Thyroid Stimulating Hormone (TSH), which is produced by the pituitary gland and stimulates the thyroid to produce T4 and T3 with a negative feedback loop, tends to increase with consequent decrease of T3; this is associated with a reduction of the basal metabolic rate and, in turn, with weight gain. Therefore, it is not surprising that hypothyroidism prevalence is higher among obese subjects and thyroid dysfunction seems to correlate with increasing BMI. However, it is still not clear if hypothyroidism can be considered a causative event or a consequence of obesity, or whether hypothyroidism and obesity concur in a vicious cycle (Sanyal and Raychaudhuri, 2016; Reinehr, 2010).

In the current study, miRPath highlighted a significant association between the miRNA patterns of OB/GDM(-) vs NW and OB/GDM(+) vs OB/GDM(-) and Thyroid hormone signalling pathway. To date, solid evidences about a possible epigenetic regulation of thyroid function in physiological and pathological contexts, and during pregnancy, are still insufficient but preliminary in vitro studies suggested that some miRNAs could be implicated in TSH-related hormone axis and lipid metabolism (Dai *et al.,* 2019), as well as in thyroid cancer progression and aggressiveness (Fuziwara *et al.,* 2019).

FoxO signalling pathway

Another interesting pathway emerging in all the three comparisons was FoxO (Forkhead box O) signalling pathway. FOXO proteins represent a subfamily of transcription factors conserved from nematodes to mammals that act as key regulators of longevity downstream of insulin and insulin-

like growth factor signalling. In mammals, this subfamily is involved in a wide range of crucial cellular processes regulating stress resistance, OS, metabolism, cell cycle arrest and apoptosis (Martins *et al.,* 2016). In placenta from obese women, Saben and colleagues observed a significant decrease in total antioxidant capacity together with increased nuclear FOXO4 localization that was significantly associated with the stress-activated c-Jun amino-terminal kinase (JNK) activation. JNK, in turn, plays a pivotal role in regulating cellular OS in several metabolic conditions such as obesity, insulin resistance and type II diabetes, reinforcing the evidences that maternal obesity may also be associated with increased OS in placenta (Saben *et al.,* 2014; Vallerie *et al.,* 2010). Importantly, among the downstream effectors of both FoxO and JNK signalling there are HIF-1α and VEGF-A, all of which are relevant in the context of maternal obesity, placental hypoxia, inflammation and OS (Saben *et al.,* 2014).

HIF-1 signalling pathway

Hsa-miR-454-3p, the single differentially expressed miRNA between OB/GDM(+) and NW women, resulted significantly associated with HIF-1 signalling pathway. As already stated, HIF-1α is a key transcriptional factor activated upon hypoxia and playing crucial roles in autophagy, inflammation, OS and angiogenesis (Macklin *et al.*, 2017). It is particularly important during early pregnancy, especially in the first phases of placental and embryonic development, by regulating EVT invasion and driving the expression of the pro-angiogenesis factor VEGF. Moreover, it is a known target of CMA (Nakashima *et al*., 2019). In the context of diabetes, high glucose concentration has been shown to impair hypoxia-induced transcriptional activation of HIF-1α in human fibroblasts and endothelial cells, suggesting that diabetic hyperglycemia may result in the loss of cellular adaptation to low oxygen (Catrina *et al.,* 2004; Baryla *et al.,* 2021). Growing evidence is revealing that the transcriptional activity of HIF-1α and other HIF pathway members is regulated by epigenetic mechanisms at multiple levels, including DNA methylation, histone methylation/acetylation and non-coding RNAs, including miRNAs. Some examples are coming from the oncologic research, in particular showing that some specific miRNAs are able to contribute to HIF-1α stability, promoting VEGF expression and, consequently, angiogenesis and metastasis (Li *et al.,* 2020). A few studies also pointed out similarities between cancer and placenta, in terms of capacity to proliferate, to invade adjacent tissues, to generate a blood supply and avoid rejection by the immune system, and some of these functions are regulated by HIF pathway itself (Macklin *et al.*, 2017). To date, only a few specific studies about the regulation of HIF signalling during pregnancy by epigenetics and miRNAs have been conducted, mainly in the context of preeclampsia (Anton *et al.,* 2019).

Valine, leucine and isoleucine metabolism pathways

Hsa-miR-454-3p also resulted significantly associated with the valine, leucine and isoleucine degradation and biosynthesis pathways. The branched-chain amino acids (BCAAs), valine, leucine and isoleucine are essential amino acids, meaning that they must be primarily obtained from the diet, which have been studied in a number of disorders, notably obesity, metabolic syndrome, liver and kidney disease, muscle disorders, sepsis, cancer and diabetes mellitus. Unlike most amino acids, the initial step of BCAA catabolism does not take place in the liver due to low hepatic activity of branched-chain-amino-acid aminotransferase (BCAT), the first enzyme in the BCAA catabolism pathway. Therefore, BCAAs increase rapidly in systemic circulation after protein intake and are readily available to extrahepatic tissues. Among others, BCAAs also stimulate insulin production (Holeček, 2018; Zhao *et al.,* 2019). It was hypothesized that mammalian Target of Rapamycin Complex 1 (mTORC1) could be activated by BCAA, as well as by insulin, glucose and cellular ATP availability. This suggest that BCAA overload may cause insulin resistance, together with an increase in BCAA oxidation breakdown products, short-chain acylcarnitines (ACs) (Bloomgardern, 2018). Significant increase of plasma BCAAs and related metabolites was observed not only in type II diabetes, but also in GDM (Zhao *et al.,* 2019). Indeed, insulin resistance levels in late pregnancy have been shown to positively correlate to circulating concentrations of BCAAs and ACs. Moreover, also maternal BMI is positively related to circulating concentrations of BCAA and related short-chain ACs (Allman *et al.,* 2021). Because of that, several studies recently proposed plasma BCAAs as potential biomarkers of GDM (Zhao *et al.,* 2019).

mTOR signalling pathway

The above cited mammalian Target of Rapamycin (mTOR) signalling pathway also emerged from miTALOS analysis, as a significant pathway associated with the pattern of miRNA differentially expressed between OB/GDM(+) and OB/GDM(-). As anticipated, the mTOR pathway act as a sensor of nutrients (e.g. BCAA), oxygen and growth factors availability (Zhang *et al.,* 2017). In obese and diabetic pregnant women, high insulin and leptin activate mTOR signalling in the placenta, promoting protein synthesis, mitochondrial function and nutrient transport. Indeed, nutrients availability, as well as expression of mTOR, is generally increased in GDM, especially when associated to obesity. This pathway increases fetal nutrient supply and contributes to fetal overgrowth and excessive adiposity in obese and/or diabetic mothers' offspring (Kelly *et al.,* 2020; Tsai *et al.,* 2021). Moreover, mTOR activation is associated with an inhibition of autophagy through silencing of the macroautophagy activator ULK1. However, as discussed above, in our population, placental autophagy appears to be increased in obesity and GDM. These apparently incoherent evidences may be due to alternative mechanisms of autophagy activation/inhibition in placenta, probably different from mTOR signalling. Anyway, the precise relation between mTOR

and autophagy in human physiological and pathological placentas remains to be elucidated (Ji *et al.,* 2017; Zhang *et al.,* 2017; Serati, 2022).

AMPK signalling pathway

The miRNA patterns of OB/GDM(-) vs NW and OB/GDM(+) vs OB/GDM(-) resulted significantly associated with AMP-activated Protein Kinase (AMPK) signalling pathway. AMPK is a serine/threonine kinase that is conserved within eukaryotes. It regulates the cellular and whole-body energy homeostasis under stress condition by sensing the AMP/ATP ratio, in almost every cell type. Once activated by falling cellular energy levels (i.e. increased AMP/ATP ratio, meaning increasing AMP and decreasing ATP), it acts to restore energy homeostasis by switching on catabolic pathways that generate ATP, while switching off anabolic pathways and other processes consuming ATP (Kumagai *et al.,* 2018; Gowans and Hardie, 2014). AMPK orchestrates these changes by histone phosphorylation and regulation of proteins involved in nucleosome remodelling. At downstream levels, this process enhances mitochondrial biogenesis and function. Additionally, during acute metabolic stress, such as fasting or physical exercise, AMPK directly associates with chromatin at promoter regions of genes involved in lipid and glucose metabolism, and activates them to promote energetic molecules availability. These observations point to important roles of AMPK as epigenetic regulator (Gongol *et al.,* 2018).

AMPK signalling has very complex and broad functions, and for this reason the dysregulation of AMPK correlates with many different disorders such as cardiovascular disease, diabetes, inflammatory disease and cancer. During pregnancy, AMPK is necessary for the proper placental differentiation, nutrient transportation, glucose and lipids utilization, maternal and fetal energy homeostasis, and protection of the fetal membrane (Kumagai *et al.,* 2018; Gowans and Hardie, 2014). Insulin resistance, obesity-related inflammation and endothelial cell dysfunction present in placenta, maternal adipose and skeletal muscle tissues in maternal obesity and GDM are all possible stressors influencing or being influenced by AMPK activity. In non-pregnant obese and diabetic individuals, AMPK activity in skeletal muscle and adipose tissue is diminished or impaired compared to normal healthy individuals. Coherently, recent data also revealed that AMPK activity is significantly reduced in skeletal muscle and adipose tissue of women with GDM (Nguyen-Ngo *et al.,* 2019). Such disruption of AMPK activity could possibly have dramatic impact also because of concomitant loss of its epigenetic mechanisms associated with adaptation and survival, as already explained (Gongol *et al.*, 2018). Once again, we cannot exclude the contribution of miRNAs in this epigenetic mechanism. For example, the Let-7 family of miRNAs is able to target the gene encoding for AMPK2α protein, which is strongly implicated in hepatic lipid homeostasis. In obese mouse models' offspring, hepatic Let-7 was found increased while protein level of AMPK2 α was decreased, correlating with excessive fat accumulation in hepatocytes

(Simino *et al.,* 2021). Interestingly, in our study, hsa-let-7b-5p resulted differentially expressed in maternal plasma of OB/GDM(+) vs OB/GDM(-).

Vitamin B6 signalling pathway

Finally, vitamin B6 signalling pathway resulted associated with OB/GDM(-) vs NW miRNA pattern. Vitamin B6, or pyridoxine, is a water-soluble vitamin contained in many foods including meat, poultry, fish, vegetables and bananas. It acts as cofactor in several enzymatic reactions, including glycogen breakdown by glycogen phosphorylase, to obtain glucose for energy production (Thomas-Valdés *et al.,* 2017). It is also crucial for one-carbon metabolism related to homocysteine, for provision of methyl groups and sulphur-containing amino acids. Being involved in such enzymatic activities, similarly to vitamin B9 and folates, it is also epigenetically relevant, in particular for methylation of DNA and histones. These events influence chromatic remodelling, which in turn regulates genome accessibility, impacting on the expression of both gene-coding and non-coding regions. Therefore, alterations of these extremely complex epigenetic cross-talk, could also dysregulate the expression profiles non-coding RNAs, including miRNAs (Friso *et al.,* 2017).

Vitamin B6 deficiency has been associated with many inflammatory diseases, such as cardiovascular disease, rheumatoid arthritis, inflammatory bowel disease and diabetes. Patients with vitamin B6 deficiency showed higher plasmatic levels of systemic markers of inflammation and OS, including acute phase reactants such as C-reactive protein, cytokines and adhesion molecules. This suggests that inflammation and OS could be associated with a functional deficiency of vitamin B6 (Sakakeeny *et al.,* 2012). Vitamin B6 deficiency was observed in severe obese subjects, with a negative association between serum vitamin B6 level and BMI (Aasheim *et al.,* 2008). Moreover, lower levels of vitamin B6, together with deficiencies of other vitamins and micronutrients, have been reported also in obese pregnant women (Sen *et al.*, 2014), but, to our knowledge, data about vitamin B6 supplementation during obese pregnancy are still unavailable. Nevertheless, non-pregnant overweight or obese individuals with vitamin B6 long-term (i.e. on average 10 years) supplementation presented significantly lower weight gain compared to non-supplemented. Therefore, it is possible that the effect of vitamin B6 on energetic metabolism helps weight control (Nachtigal *et al.,* 2005).

During pregnancy, vitamin B6 is sometimes indicated to help reducing nausea and vomiting, especially during early pregnancy. However, to date there is not enough evidence to detect clinical benefits of vitamin B6 supplementation in pregnancy and there is some debate whether vitamin B6 could have protective roles in pregnancy outcomes including fetal orofacial clefts, cardiovascular malformations, neurological development, preterm birth and preeclampsia (Salam *et al.,* 2015).

6.6. Conclusions

In this study, we presented preliminary data on the expression of antioxidant and autophagy-related genes in placentas from OB/GDM(-) and OB/GDM(+), vs NW mothers. Our analysis showed intriguing results, reporting differential alterations depending on the presence/absence of GDM and on fetal sex. These results confirm how metabolism, nutrient availability and cellular mechanisms such as OS responses and autophagy are strictly connected. Further studies will be necessary to unravel precise molecular processes below this observation.

To our knowledge, this is the first report describing an increase in PHLPP1 expression in placentas in relation to GDM. Indeed, a role of PHLPP1 and CMA activation in GDM might be suggested, deserving further investigations. The elucidation of PHLPP1 involvement in maternal obesity and GDM will pave the way for further comprehension of its role in placental alterations in the context of metabolic disorders and its potential targetability for the treatment of negative consequences of diabetes (Diceglie-Anelli *et al.,* 2021).

Moreover, placentas of both OB/GDM(-) and OB/GDM(+) women were characterized by a reduced ATP production, and all OB/GDM(±) showed a decreased mt Complex I content. Furthermore, we found lower mt Complexes III and V in OB/GDM(+) group, along with a reduced fetal/placental weight ratio (i.e. placental efficiency) in the same group vs NW. These results might be suggestive of impaired mitochondrial respiration and placental function in our obese subjects, especially in those with GDM, compared to NW women. Concerning the next steps of the evaluation of placental mitochondrial bioenergetics, high-resolution respirometry experiments in whole placental tissue are ongoing to functionally evaluate placental mitochondrial respiration and oxygen consumption.

The evaluation of miRNA profile in maternal plasma reported sets of differentially expressed miRNAs between the three groups, some of which seem to be involved in different processes associated with obesity and GDM. Interestingly, Pathway enrichment analysis conducted with miRPath and miTALOS revealed many pathways associated with each set of miRNAs of each comparison, among which the most interesting and relevant in our context were related to nutrient (e.g. lipids, glucose, amino acids) metabolism, inflammation and OS. Indeed, in the next future miRNAs could become promising biomarkers of metabolic alterations in MO and GDM. These profiling results open up interesting perspectives to deepen the study of miRNAs as possible biomarkers of metabolic impairment in obese pregnant women affected or not by GDM.

7. REFERENCES

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