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Inositols supplementation in pregnancies complicated by metabolic syndrome. Effects on metabolic and cardiovascular systems in the maternal/fetal unit

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List of Abbreviations

MS: Metabolic Syndrome

INO: Inositols

eNOS: endothelial nitric oxide synthase

HFD: high fat diet

WT: wild-type

GLUT4: glucose transporter type 4

IR-β: insulin receptor beta

Akt: Protein kinase B

pAktThr308: Protein kinase B threonine 308 phosphorylation

GSK3: glycogen synthase kinase 3

ATP: Adenosine triphosphate

GTT: glucose tolerance test

TGF-β: transforming growth factor beta

SBP: systolic blood pressure

BMI: body mass index

GDM: gestational diabetes mellitus

LGA: large for gestational age

IL6: interleukin 6

CRP: C-reactive protein

CVD: cardiovascular disease

PTB: preterm birth

HTN: hypertension

DBP: diastolic blood pressure

RAAS: renin-angiotensin-aldosterone system

NO: nitric oxide

VEGF: vascular endothelial growth factor

PIGF: placental growth factor

sFlt-1: placental soluble fms-like tyrosine kinase 1

T2DM: type 2 diabetes mellitus

PE: preeclampsia

MI: Myo-inositol

DCI: D-chiro inositol

TSH: thyroid-stimulating hormone

FSH: follicular stimulating hormone

LH: luteinizing hormone

IRS-1: insulin receptor substrate 1

PI: phosphatidylinositol

PI-3K: phosphatidylinositol 3-kinase

PIP: phosphatidylinositol phosphate

IPGs: inositol-phosphoglycans

PD: pyruvate dehydrogenase

PDP: pyruvate dehydrogenase phosphatase

PDK: pyruvate dehydrogenase kinase

PDH: pyruvate dehydrogenase

PCOS: polycystic ovary syndrome

PICP: Procollagen propeptides Type 1

PIIICP: Procollagen propeptides Type 3

CTX-I: Cross-linked C-Telopetides Type 1

CTX-III: Cross-linked C-Telopetides Type 3

ECM: extracellular matrix

GD: gestational day

L-NAME: N-nitro-L arginine methyl ester

Ach: acetylcholine

SNP: sodium nitroprusside

EDTA: ethylenediaminetetraacetic acid

% Max: percent maximal effect

ABSTRACT

Background: Metabolic syndrome (MS), obesity, and hypertension in pregnancy set an adverse intrauterine environment, leading to altered fetal programming in utero, predisposing the fetus to later onset of adult disease. On this ground, Inositols (INO) are insulin-sensitizing agents that have been shown to improve insulin resistance in women with obesity, gestational diabetes, and MS.

Hypothesis: We hypothesized that INO in pregnancy improves maternal and offspring metabolic and cardiovascular profile by reducing maternal end-organ damage and modulating placental glucose homeostasis pathways, therefore, improving maternal and fetal short and long-term health.

Study design: Female heterozygous for endothelial nitric oxide synthase (eNOS–/+) mice with moderate hypertension were either placed on a high-fat diet (HFD) for 4 weeks to induce a MS phenotype or fed with a regular diet to obtain the hypertensive phenotype. Similarly, wild-type (WT) mice were placed on an HFD for 4 weeks to induce a murine obesity model. Female mice were then bred with WT males. On gestational day 1, dams were randomly allocated to receive either INO or plain water as control. Dams were sacrificed, and maternal organs, blood, placentas, and pups were weighed and collected. Serum levels of biomarkers, relevant to the fibrosis pathway, were measured by a multiplex enzyme-linked immunosorbent assay. Cardiac, liver, and kidney tissues histology were performed for fibrosis deposition as organ damage.

To evaluate maternal organ damage, cardiac, renal, and liver tissues were stained with Masson's trichrome to assess connective tissue deposition. ELISA was used to measure the serum level of fibrogenic and collagen biomarkers.

Placentas per each group of dams were processed to obtain offspring genotyped for eNOS allele and gender to evaluate the level of proteins involved in glucose homeostasis. Specifically, glucose uptake (GLUT4 and IR-b), glycogen synthesis (Akt, pAktThr308 and GSK3), and ATP production (pPDH) were measured using Western blot, while glycogen storage was measured by ELISA assay.

A subgroup of the previously mentioned MS pregnant dams receiving INO or placebo were let deliver to evaluate vascular and metabolic profiles in their offspring. The offsprings developed in an abnormal uterine environment due to maternal MS underwent a GTT and SBP measurement at 9-10 weeks of age, then were sacrificed, and the carotid arteries were isolated for evaluation of vascular responses. Responses to phenylephrine, in the presence and absence of a nonspecific nitric oxide inhibitor, the vasodilator acetylcholine, and sodium nitroprusside were assessed.

Results: INO treatment during pregnancy significantly decreased maternal cardiac, renal, and liver fibrosis induced by the MS established before pregnancy by reducing serum levels of TGF-β and collagen-type 3 in MS dams. Moreover, it enhanced placental glucose use toward energy production in a gender-independent manner in offspring born to MS dams. Lastly, adult offspring born to dams with MS benefit more from maternal INO treatment if exposed to environmental factors in utero, and less if they inherited the altered genetic factors (eNOS). Indeed, inositol supplementation improved glucose tolerance, SBP, and vascular responses in those mice.

Conclusion: Metabolic and cardiovascular disease in pregnancy have serious consequences on maternal and fetal health. Inositol supplementation during pregnancy is a promising strategy to counteract the damages of dysmetabolism in both the mother and the fetus, acting on different pathways, showing improvement of short and long-term metabolic and cardiovascular outcomes.

Keywords: Inositol, Fetal programming, Metabolic Syndrome, Pregnancy, Placenta

Italian ABSTRACT

Contesto: La sindrome metabolica (SM), l'obesità e l'ipertensione in gravidanza creano un ambiente intrauterino avverso, che porta ad un'alterazione della programmazione fetale in utero, predisponendo il feto all'insorgenza della malattia in età adulta. Gli inositoli (INO) hanno dimostrato di migliorare la resistenza all'insulina nelle donne con obesità, diabete gestazionale e con SM.

Ipotesi: La somministrazione di INO in gravidanza migliora il profilo metabolico e cardiovascolare materno e della prole riducendo il danno agli organi materni e modulando le vie dell'omeostasi del glucosio placentare, migliorando quindi la salute materna e fetale a breve e lungo termine.

Disegno dello studio: Un modello murino di femmine eterozigoti eNOS–/+ con ipertensione moderata sono stati sottoposti ad una dieta ricca di grassi (HFD) per 4 settimane per indurre un fenotipo della SM o alimentati con una dieta regolare per ottenere il fenotipo ipertensivo. Allo stesso modo, i topi wild-type (WT) sono stati nutriti con HFD per 4 settimane per ottenere il modello di obesità murina. Dopo l'accoppiamento con maschi WT, le femmine gravide sono state assegnate casualmente a ricevere INO o acqua come controllo. Al termine della gravidanza (giorno di gestazione 18) le gravide sono state sacrificate, pesati e raccolti gli organi materni, il sangue, le placente e i feti. I livelli sierici dei biomarcatori, rilevanti per la fibrosi, sono stati misurati mediante un test multiplex di immunoassorbimento enzimatico. L'istologia dei tessuti cardiaci, epatici e renali è stata eseguita per la valutazione del danno d'organo. Per valutare il danno agli organi materni, i tessuti cardiaci, renali ed epatici sono stati colorati con la tricromia di

Masson valutando la deposizione di tessuto connettivo. Il livello sierico di biomarcatori fibrogenici e di collagene è stato misurato tramite ELISA.

Le placente di ciascun gruppo di madri sono state genotipizzate per l'allele eNOS ed il sesso per valutare il livello di proteine coinvolte nell'omeostasi del glucosio. In particolare, l'assorbimento del glucosio, la sintesi del glicogeno e la produzione di ATP sono stati misurati mediante Western blot, e l'immagazzinamento del glicogeno mediante test ELISA.

Un sottogruppo delle madri con SM che ricevevano INO o placebo, è stato lasciato partorire per valutare i profili vascolari e metabolici nella prole sviluppata in un ambiente uterino anormale a causa della SM materna. Nella prole sono stati valutati GTT e SBP a 9-10 settimane di età. Le arterie carotidi sono state isolate per la valutazione delle risposte vascolari alla fenilefrina, in presenza e assenza di un inibitore aspecifico dell'ossido nitrico, del vasodilatatore acetilcolina e del nitroprussiato di sodio.

Risultati: Il trattamento con INO durante la gravidanza ha ridotto significativamente la fibrosi cardiaca, renale ed epatica a livello materno indotta dalla SM stabilita prima della gravidanza, riducendo i livelli sierici di TGF-β e collagene di tipo 3 nelle madri con SM. Inoltre, ha migliorato l'uso del glucosio placentare favorendo la produzione di energia in modo indipendente dal genere nella prole nata da madri con SM.

Infine, l'INO ha migliorato la tolleranza al glucosio, la SBP e le risposte vascolari nei figli nati da madri con SM che non hanno ereditato i fattori genetici alterati (eNOS).

Conclusione: Le malattie metaboliche e cardiovascolari in gravidanza hanno gravi conseguenze sulla salute materna e fetale. L'integrazione di inositolo durante la gravidanza è una strategia promettente per contrastare i danni del dismetabolismo sia nella

madre che nel feto, mostrando un miglioramento degli esiti metabolici e cardiovascolari a breve e lungo termine.

Parole chiave: Inositolo, Fetal programming, Sindrome Metabolica, Gravidanza, Placenta

1. INTRODUCTION

1.1 Obesity in pregnancy

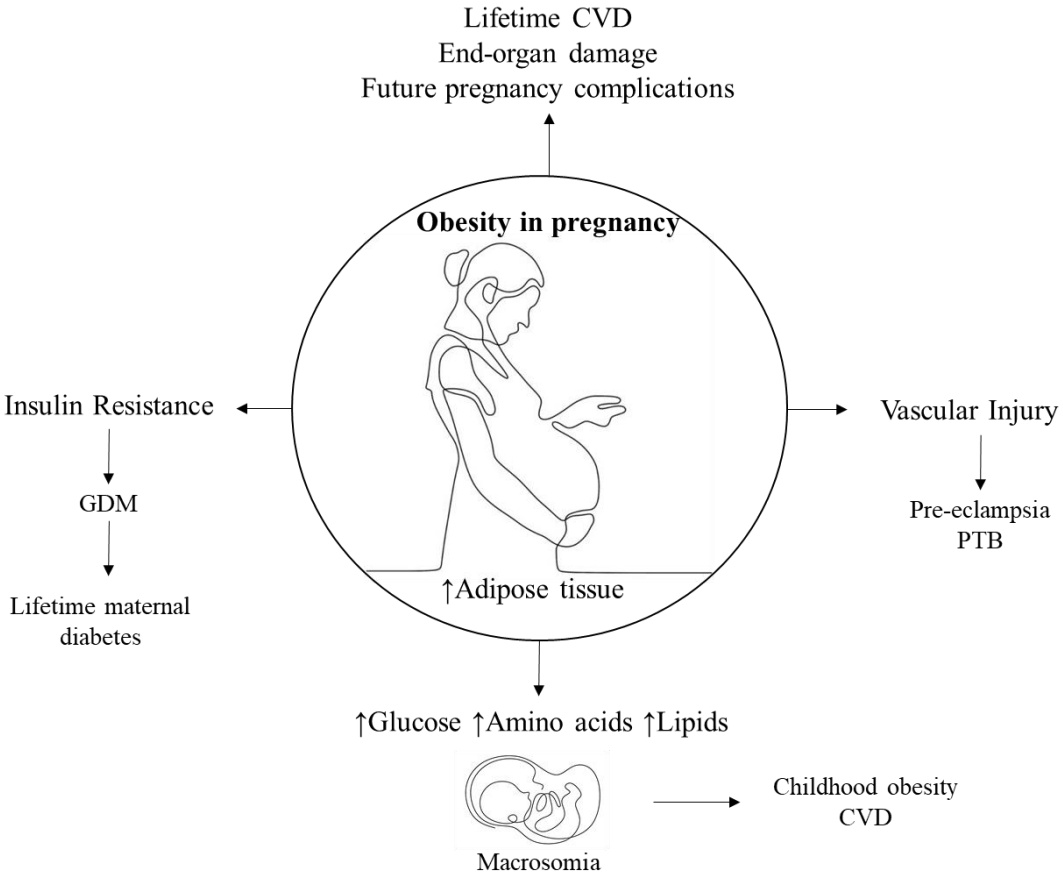
Obesity is becoming a pandemic phenomenon, and it is defined as body mass index (BMI) ≥ 30 kg/m^2 according to the World Health Organization. Approximately half or more than half of the population in the Americas (61.1%), Europe (54.8%), and Eastern Mediterranean (46.0%) are overweight/obese, while a much lower prevalence is observed in Africa (26.9%), South-East Asia (13.7%), and the Western Pacific (25.4%) (1). Epidemiological studies done in the USA, Europe, and Asia found that higher BMI was significantly associated with increased incidence of coronary artery disease (CAD) and ischemic stroke (1).

Obese women during pregnancy have an increased risk of maternal death and complications during pregnancy and labor than normal-weight women. In the United Kingdom, the latest Confidential Enquiry into Maternal and Child Health (CEMACH) reported that more than half of the deaths from direct or indirect causes during (late) pregnancy or labor were in overweight or obese women (2). Obesity is associated with increased risk of almost all pregnancy complications such as gestational hypertension, preeclampsia, gestational diabetes mellitus (GDM), delivery of a large for gestational age (LGA) infant, and a higher incidence of congenital defects (3,4). Cesarean section rates are also much higher, and anesthesia may be problematic. Notable exceptions are gastroschisis and spontaneous preterm labor, both of which occur less often. Obese women often face difficulties in initiating and sustaining breastfeeding (5) (Figure 1).

Several studies support a causal link between adiposity and adverse pregnancy outcome, that impact only maternal health but also the fetal one, representing a direct cause of the development of cardiovascular and metabolic disease later in life (6–8).

Obesity represents a state of altered hormonal and inflammatory activity, associated with the function of adipose or fatty tissue which has been demonstrated to be a source of production of peptides and nonpeptide compounds involved in cardiovascular homeostasis (9,10). These compounds in turn slow clot degradation increasing the prothrombotic state. The peptide, interleukin 6 (IL6) is secreted by adipose tissue and modulates the production of C-reactive protein (CRP). Elevated CRP is a known marker of chronic inflammation associated with an increased risk of cardiovascular disease (CVD) (10).

Figure 1. Pathological effects of obesity on pregnancy and fetal outcome.



CVD = cardiovascular disease; GDM = Gestational Diabetes Mellitus; PTB = preterm birth.

Obesity during pregnancy is characterized by an increase in adipose tissue that is linked to deposition of adipose cells in the vascular wall, causing vascular injuries that can result in pregnancy in the onset of hypertensive disorders and pre-eclampsia that can be itself the cause of preterm birth (iatrogenic of spontaneous). Moreover, obesity in pregnancy is characterized by the increase in glucose, amino acids and lipid levels that lead to macrosomia and lately to childhood obesity and cardiovascular disease. The obesity

condition during pregnancy leads also to an increased insulin resistance that cause the onset of GDM and its related long-term consequences for maternal life.

Adapted from Smith et al. (11).

1.2 Hypertension in pregnancy

Hypertension (HTN) is a worldwide health problem that affects about 25-40% of individuals. It is a major cardiovascular risk factor and is associated with many cardiovascular complications as stroke and heart failure.

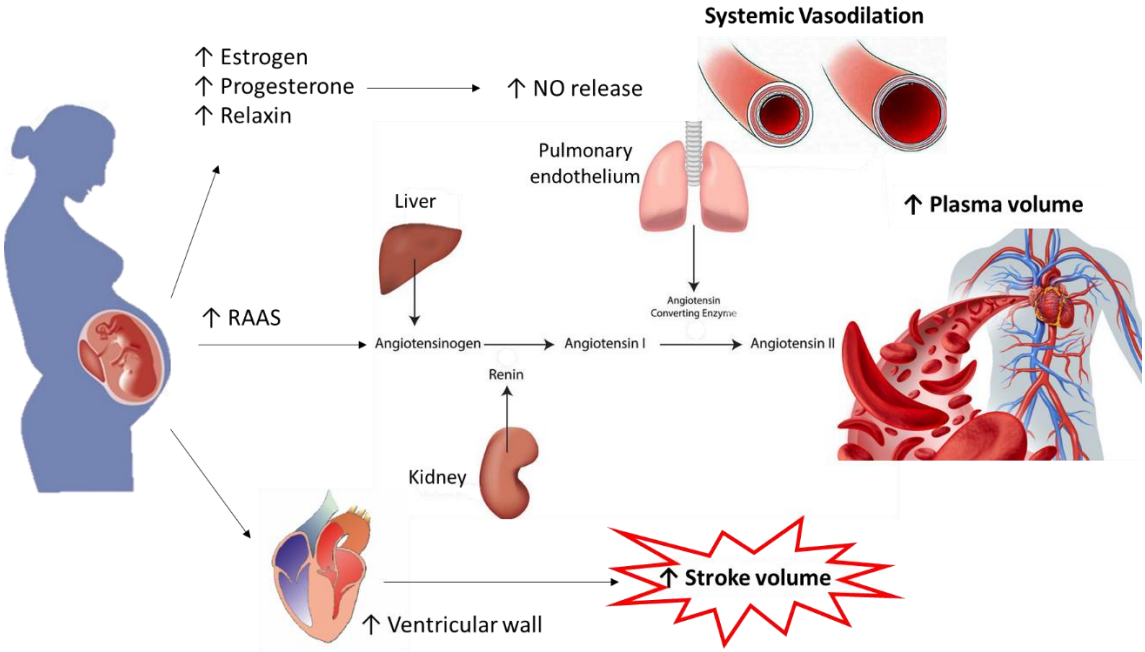
The prevalence of hypertension in reproductive-aged women is estimated to be 7.7% (12). Hypertensive disorders of pregnancy is an umbrella term that includes preexisting and gestational hypertension, preeclampsia, and eclampsia, complicate up to 10% of pregnancies and represent a significant cause of maternal and perinatal morbidity and mortality (13).

The definition of HTN in pregnancy has not always been standardized, but following the "National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy" recommendation is currently a systolic blood pressure (SBP) ≥ 140 mmHg and/or a diastolic blood pressure (DBP) ≥ 90 mmHg (14).

The hormonal changes of pregnancy induce significant adaptations in the cardiovascular physiology of the mother. Beginning early in the first trimester, there are surges of estrogen, progesterone, and relaxin (a hormone that, like progesterone, mediates nitric oxide release), leading to systemic vasodilation (15–17). Concurrently, the renin-angiotensin-aldosterone system (RAAS) is augmented to engender salt and water

retention, leading to an expansion in plasma volume (18). This, combined with an increased ventricular wall mass, leads to an increased stroke volume (Figure 2). The expansion in plasma blood volume also results in physiologic anemia, as the rate of increase is faster than that of the increase in red blood cell mass (19). To compensate for the aforementioned systemic vasodilation and physiologic anemia, heart rate raises (20). The combination of elevated stroke volume and tachycardia leads to an increase in cardiac output during pregnancy, which compensates for the decline in vascular resistance to maintain blood pressure at high enough levels for maternal and placental perfusion (20).

Figure 2. Cardiovascular physiology in pregnancy.



This figure represents the cardiovascular changes that occur during a physiologic pregnancy. These changes are mostly due to the increase in estrogen, progesterone and relaxin levels that lead to an increase in nitric oxide release which is responsible for the systemic vasodilation. Moreover, the renin-angiotensin-aldosterone system is solicited

increasing the plasma volume, and ultimately, the ventricular wall thickness is increased during pregnancy and this condition expose the woman to an increased stroke volume.

This figure was created by Dr Daniela Menichini.

The underlying pathophysiology that upholds the transition to hypertension or preeclampsia is not well understood; however, it is thought to be related to a mechanism of reduced placental perfusion inducing systemic vascular endothelial dysfunction (21). This arises due to a less effective invasion by the cytotrophoblast of the uterine spiral arteries (22). The resultant placental hypoxia induces a cascade of inflammatory events, disrupting the balance of angiogenic factors, and inducing platelet aggregation, all of which result in endothelial dysfunction manifested clinically as the preeclampsia syndrome (22).

Angiogenic imbalances associated with the development of preeclampsia include decreased concentrations of angiogenic factors such as the vascular endothelial growth factor (VEGF) and placental growth factor (PIGF) and increased concentration of their antagonist, the placental soluble fms-like tyrosine kinase 1 (sFlt-1) (23,24). Impeding the binding of VEGF and PIGF to their receptors is a factor in the reduction of nitric oxide synthesis, a crucial factor in vascular remodeling and vasodilation, which may otherwise be able to ameliorate placental ischemia (25).

1.3 Metabolic syndrome in pregnancy

Metabolic syndrome (MS) is a growing cause of morbidity and mortality worldwide. It is defined by a cluster of metabolic alterations: abdominal obesity, impaired glucose

tolerance, insulin resistance, hypertension, and dyslipidemia (26). It is one of the major health problems of the 21st century, and according to the World Health Organization (WHO), by 2020 will be the main risk factor linked to diabetes and cardiovascular diseases. Importantly, metabolic abnormalities as gestational diabetes, glucose intolerance, and obesity during pregnancy will affect not only the long-term maternal health but can expose infants to an increased risk of developing metabolic disorders later in life (27,28).

MS is a cluster of risk factors that encompass metabolic, vascular, and inflammatory indicators. The metabolic disturbances underpinning MS include atherogenic dyslipidemia, raised blood pressure (BP), insulin resistance, obesity, and pro-thrombotic and pro-inflammatory states.

While some expert definitions deem obesity an essential criterion (29), other definitions largely focus on insulin resistance (26,30). To date, there are no obligatory components to define MS but rather a constellation of risk factors that, irrespective of components and cut-offs, have consistently been demonstrated to increase the risk for CVD (31), some cancers (32), type 2 diabetes mellitus (T2DM) (33), and chronic kidney disease (34) in the adult population.

Normal pregnancy is a pro-inflammatory, pro-thrombotic, highly insulin resistant (35), and hyperlipidemic state (36). However, there are no recognized healthy metabolic variable cut points in pregnancy. Studies that have assessed metabolic components in pregnancy have generally used accepted definitions for the adult population (37). Appropriate definitions that associate metabolic health parameters and pregnancy

complications have yet to be defined, and the utility of using previously defined variables from the non-pregnant adult population is unclear.

The link between MS and chronic diseases in adulthood has been well established (31,33), as well as between pregnancy complications such as preeclampsia (PE) and GDM and later life T2DM and CVD (38,39). Therefore, pregnancy may offer a window of opportunity to identify women with MS and elevated risk of adverse pregnancy outcomes and reduce the onset of chronic diseases later in life.

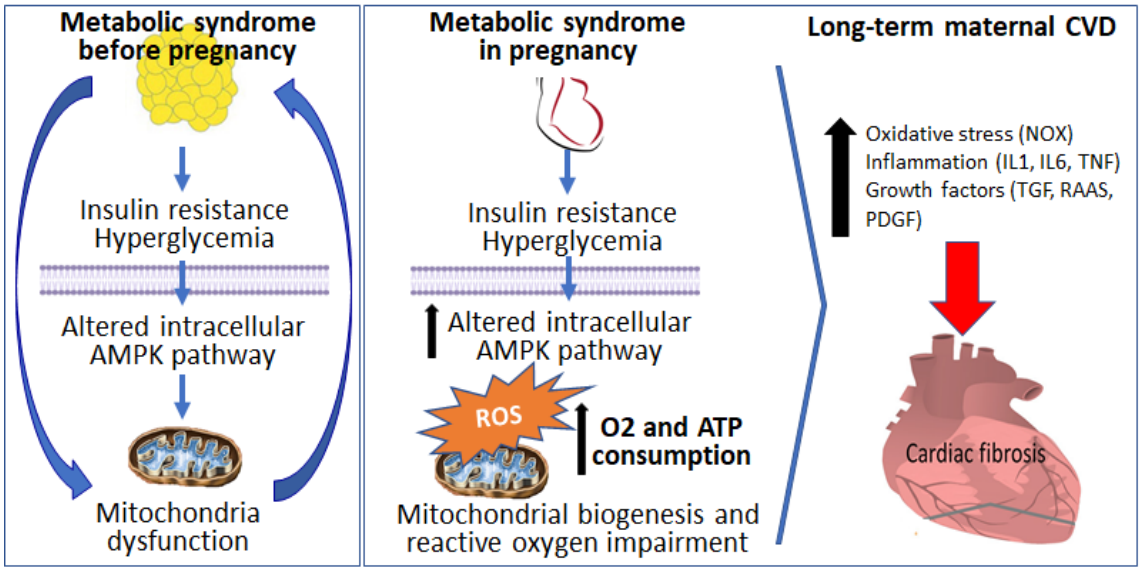
1.4 Metabolic disturbances and cardiovascular diseases

Hypertension, obesity, diabetes, and MS are the main risk factors for CVD in the general population as well as in pregnancy. Women developing those complications during pregnancy have a significantly increased risk of earlier onset in their lifetime of CVD (40,41). Maternal heart disease complicates up to 4% of pregnancies (42) and up to 16% of pregnancies in women with previous cardiac conditions (43), with risk depending on the underlying cardiac condition (43,44), becoming the single largest cause of indirect maternal mortality (45), accounting for over 33% of pregnancy-related maternal deaths (46–48).

Moreover, it is well established that there is a link between MS in pregnancy and chronic diseases in offspring adulthood (49), as well as between pregnancy complications such as preeclampsia and gestational diabetes and later onset of diabetes and CVD (50,51). Evidence has shown that excessive fatty acids, chronic inflammation, oxidative stress, and more recently an imbalance in mitochondrial dynamics have been linked to CVD due to

vascular damage (52,53). Subsequently, vascular injury contributes to fibrotic tissue remodeling through several mechanisms as the recruitment of fibrogenic macrophages and lymphocytes (54). Pre-pregnancy metabolic abnormalities (as hyperglycemia, hyperlipidemia, insulin resistance) can lead to impaired hemodynamic adaptations, placenta dysfunction, and inflammation culminating in pregnancy complicated by metabolic syndrome, diabetes, and hypertension that can prime to endothelial dysfunction, worsening metabolic adaptations leading to increased risk of maternal long-term chronic disease due to end-organ damage (55). Those chronic insults can cause alterations in oxidative stress, inflammation, and several growth factors that stimulate mesenchymal cells and fibroblasts leading to abnormalities in extracellular matrix turnover and ultimately fibrosis which can lead to heart, kidney, and liver damage (Figure 3). Those pregnancy complications could be either a marker uncovering underlying predisposition to CVD or risk factors. Hence intervention should target the pre-pregnancy and pregnancy period which may offer a window of opportunity to identify women with MS and elevated risk of adverse pregnancy outcomes, as well as later life chronic disease to improve long-term maternal health pregnancy.

Figure 3: Progression from pre-pregnancy metabolic syndrome to pregnancy and long-term maternal CVD.



This figure shows that pre-pregnancy metabolic syndrome (hyperglycemia, hyperlipidemia, insulin resistance) can impair the intracellular AMPK pathway with consequent mitochondria dysfunction. This alteration increases the oxygen and ATP consumptions with the production of reactive oxygen species. Those chronic insults can cause the increase of oxidative stress, inflammation and several growth factors that stimulate mesenchymal cells and fibroblasts leading to abnormalities in extracellular matrix turnover and ultimately fibrosis which can lead to heart, kidney, and liver damage. Those pregnancy complications could be either a marker uncovering underlying predisposition to CVD or risk factors.

This figure was created by Dr Daniela Menichini and Dr Monica Longo.

1.5 Metabolic disturbances and developmental origins of health and disease

In the 1980s, David Barker was the first to report on the theory of developmental origins of health and disease (7). Since then, several epidemiological and animal studies showed that fetal exposure to insults (i.e. obesity, diabetes, metabolic syndrome, hypertension, preeclampsia) during critical periods of fetal intrauterine development can induce fetal adaptations that can alter adult phenotype (56), highlighting the strong interactions between the genetic and uterine environment in determining the onset of adulthood disease (57).

These adaptations are called “Fetal programming” and take place when the optimal environment in which the fetus grows is disrupted by hostile factors, especially during critical periods of development of essential organs. It is an important mechanism that allows the new organism to maintain homeostasis in inadequate conditions. Once changes occur, the phenotype becomes permanent and may determine the outset of future health problems (58). The correlation between intrauterine stress and adverse effects in offspring has been confirmed for diseases such as atopic syndromes including dermatitis, asthma, and eczema, increased vulnerability to infections, metabolic dysfunction, cardiovascular disease, and cancer (especially lymphoma, hepatic cancer, and testicular cancer) (59).

Epigenetic researches investigate the hypothesis of developmental provenance of diseases, which presumes an association between intrauterine environment factors emerging from the availability of nutrients and the development of obesity and chronic diseases later in life (60). Metabolic disorders and deviations from appropriate nourishment during pregnancy can trigger fetal gene expression modifications, which lead to vulnerability to chronic diseases in the future. Various factors could trigger fetal programming, such as unhealthy habits including smoking, physical inactivity, psychosocial stress, mother’s neurological disorders, depression, anxiety, infections, endocrine diseases including

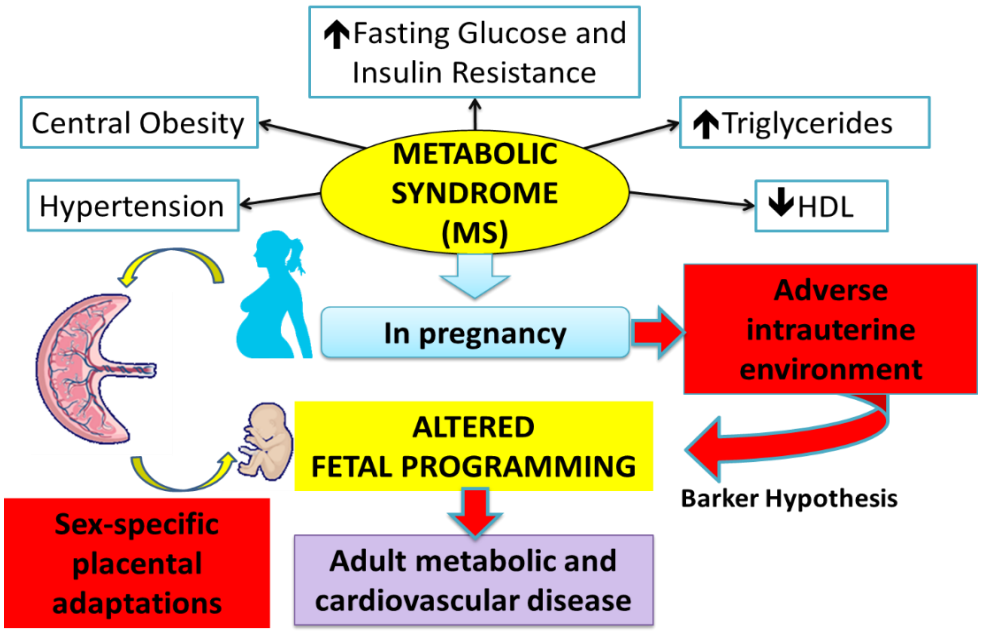
diabetes, complications such as preeclampsia, fetal hypoxia, and oxidative stress, or any deviations from the normal gestational environment (61). The placenta, being the interface between maternal and fetal circulation and overseeing multiple functions to maintain fetal well-being, plays an active role in fetal developmental programming (62–64).

Indeed, most of the above-mentioned conditions are determined by inadequate placental function, which allows us to ascertain that the placenta plays an immense role in fetal programming.

Mother-fetus transport is provided by the adequate activity of placental transporters, enzymes, vasculogenesis, and hormone secretion is disrupted when any pregnancy complications occur, which leads to a decrease of substrate delivered to the fetus, and eventually changes in its development and initiation of epigenetic alterations.

Interestingly, fetal adaptive responses can be gender-specific and can undergo gender-specific adaptive responses due to fetal developmental plasticity (Figure 4) (10,11). However, the mechanisms underlying fetal metabolic programming, as well as possible target therapeutic interventions, remain to be elucidated.

Figure 4: Metabolic Syndrome in Pregnancy.

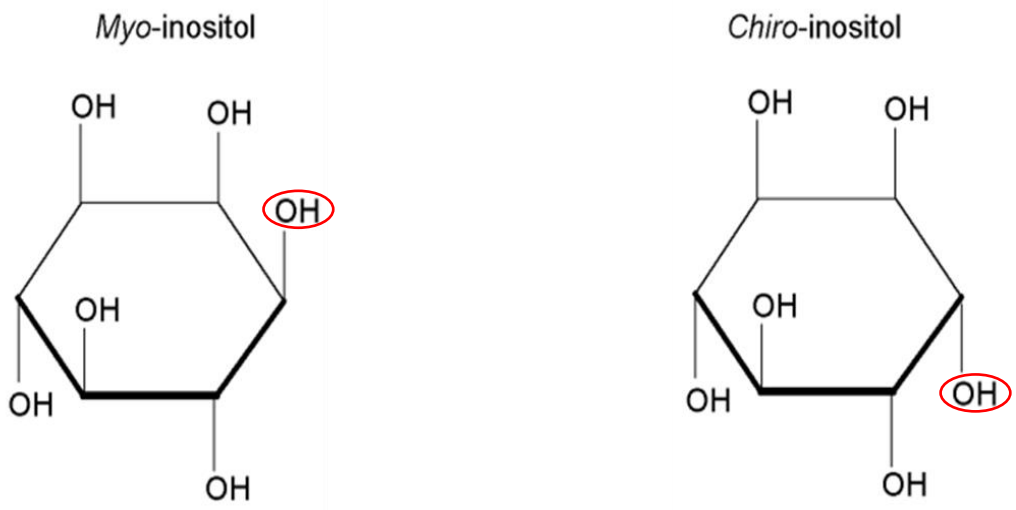


Correlation between metabolic syndrome features and their effects during pregnancy on fetal programming process and placental sex-specific alterations. This figure was created by Dr Daniela Menichini and Dr Monica Longo.

1.6 Inositols

Inositols are a family of 6-carbon, cyclic polyalcohols widely present in nature with nine stereoisomers, two of them predominantly found in eukaryotic cells: Myo-inositol (MI) and D-chiro inositol (DCI) (Figure 5).

Figure 5. Chemical structure of Myo-inositol and D-chiro inositol



The only difference between MI and DCI is a hydroxyl group, but their biological properties change significantly. MI and DCI are the main ones present in humans, with MI being the most abundant form in living cells and the most distributed in biological systems. In plants, it is generally represented in the form of hexaphosphate, and phytic acid or its salts (phytates). The greatest amounts of MI are found in fresh fruits and vegetables, and all foods containing seeds (beans, grains, and nuts). Especially high phytic acid contents are found in almonds, walnuts, and Brazil nuts; oats and bran contain more Myo-inositol than cereals derived from other grains. Among the vegetables, the highest contents are observed in the beans and peas, while leafy vegetables are the poorest vegetable sources (67). Inositols were once considered members of the vitamin B complex but they cannot

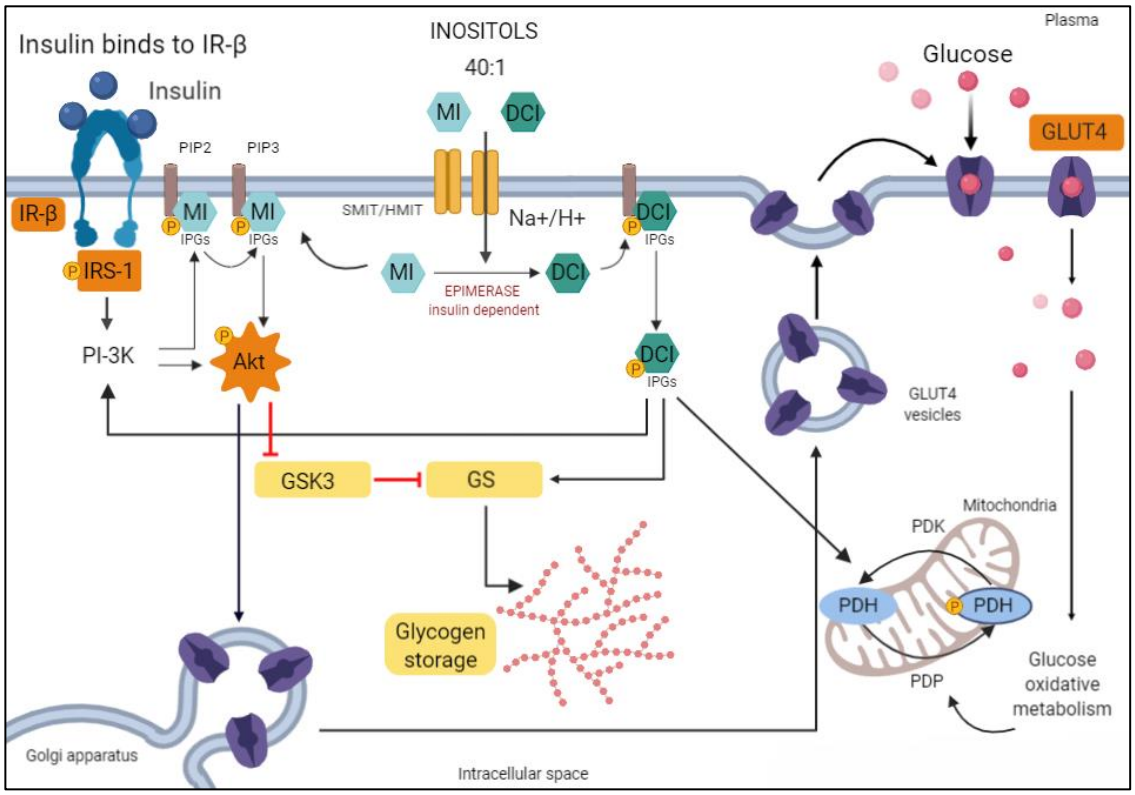
be deemed as a ‘true’ essential nutrient since they can be synthesized by the human body from glucose (mostly in the liver and kidney) (68). Indeed, in a two-step reaction, glucose-6-phosphate is first isomerized by inositol-3-phosphate synthase 1 into inositol-3-phosphate, and then inositol-3-phosphate is dephosphorylated by inositol-monophosphatase into MI (69). However, a major part of inositols comes from exogenous sources. In the cell, they are components of cell membrane phospholipids, with both structural and functional roles (70), and at the intracellular level, there is a conversion of MI to DCI regulated by an insulin-dependent epimerase which ensures the physiologic plasma ratio of MI/DCI at 40:1 (71).

Inositol pyrophosphates (PP-InsPs) acts as a protein pyrophosphorylation and have strong electrostatic interactions with target proteins. The cell membrane phospholipids are the source of inositol triphosphate, diacylglycerol, and inositol phosphoglycans that act as second messengers and regulate the metabolic signaling of many metabolic pathways, including those depending on thyroid-stimulating hormone (TSH), follicular stimulating hormone (FSH), luteinizing hormone (LH) and insulin.

1.6.1 Inositols intracellular mechanism of action

MI and DCI play a key role in intracellular insulin-regulated glucose homeostasis pathways. Their function is better elucidated in Figure 6.

Figure 6. Roles of Myo-Inositol (MI) and D-Chiro-Inositol (DCI) in Cellular Insulin-Regulated Glucose Homeostasis Pathways.



This figure represents a schematic glucose homeostasis pathway, with insulin on the top binding to its receptor IR-β activating the cascade downstream leading to the production of ATP used to store the glycogen and to activate the Golgi apparatus which produces GLUT4 vesicles that once reach the membranes favor the cellular glucose intake. These pathways involve MI and DCI and their products.

This figure was created by Dr Daniela Menichini and Dr Monica Longo.

As shown, insulin binds to its receptor (IR-β), which activates the insulin receptor tyrosine kinase (IR-β), which phosphorylates and activates the insulin receptor substrate 1 (IRS-1). The phosphatidylinositol (PI) 3-kinase (PI-3K) pathway is then activated and catalyzes the phosphorylation of PI acting as second messengers of other kinases PI dependent, resulting in the phosphorylation and activation of Akt cascade. This cascade induces glucose uptake into the cell via translocation of glucose transporter type 4 (GLUT4) vesicles from the Golgi apparatus to the plasma membrane. pAkT activation, also induces the inactivation of glycogen synthase kinase 3 (GSK3) by phosphorylation, leading to glycogen synthase (GS) activity and an increase in glycogen synthesis. MI and DCI are present in the cells in a free form or as part of the cell-membrane phospholipids as phosphatidylinositol phosphate (PIP). However, when they enter the cell from an exogenous source, intracellular uptake is regulated via Na+/MI cotransporters (SMIT) and H+/MI cotransporters (HMIT) on the cell membrane. The insulin-regulated epimerase enzyme establishes and maintains the physiologic tissue-specific MI: DCI ratio at 40:1.

To be functional, MI and DCI are converted to inositol-phosphoglycans (IPGs) and play a role as second messengers (MI-IPG and DCI-IPG) in the insulin-regulated glucose intracellular homeostasis. MI-IPG is involved primarily in cellular glucose uptake enhancing the PI-3K/Akt/GLUT4 pathway. DCI-IPG activates PI-3K enhancing glucose uptake and activating GS for glycogen synthesis. DCI-IPG improves glycolysis by acting on the pyruvate dehydrogenase (PD) phosphatase (PDP) and kinase (PDK) enzyme, setting a dynamic state of activation/inactivation of the pyruvate dehydrogenase (PDH) which regulates the oxidative metabolism of glucose via the Krebs cycle in the mitochondria.

In summary, MI acts by increasing the cell membrane permeability to glucose, which gets into the cell and is immediately available as substrate, while DCI determines the intracellular accumulation of glucose as glycogen improving the glycolysis process and regulating the glucose oxidative metabolism via the Krebs cycle.

1.6.2 Insulin Signaling role of Inositol

MI and DCI are insulin-mimetic when administered *in vivo* enhancing the physiologic insulin-receptor activity and reducing glucose levels in serum. Thus, they are regarded as insulin sensitizers compounds.

Indeed, for the past two decades, it has been acknowledged the fundamental role played by inositol in glucose homeostasis has. Several investigations have demonstrated that inositol and inositol-derived compounds can improve glucose metabolism (72,73). Indeed, insulin-resistant and diabetic patients present several alterations in inositol metabolism (74,75). Namely, animals and humans with insulin resistance show a decreased availability of inositol or inositol phosphoglycans (IPGs) that can be primarily ascribed to an increased urinary loss of MI. This effect is mostly due to the inhibition of MI reabsorption by the kidney which is glucose-mediated. The reduction in MI availability has a direct negative impact on the levels of its isomer, the DCI. Therefore, plasma and intracellular depletion of MI and its isomers or IPG metabolites worsen insulin resistance. Diabetes, insulin resistance, or metabolic syndrome are characterized by altered profiles of plasma/urinary levels of inositol and its metabolites/isomers, including DCI.

On these premises, inositols are increasingly used for the prevention and treatment of pathologies in which alterations in glucose metabolism and insulin resistance appear, such

as diabetes (76). Furthermore, since inositols are natural compounds, their applicability also extends to pregnancy, demonstrating safety and efficacy (77–79).

1.6.3 Studies on Inositols

Recently, several studies are supporting the beneficial role of dietary supplementation with inositols (INO), namely MI and DCI, in regulating glucose homeostasis during the periconceptional period throughout pregnancy (80).

Several RCTs in women with risk factors for metabolic diseases show that supplementation early in pregnancy with MI alone or in combination with DCI can positively affect maternal metabolic profile (decreasing GDM onset, fasting glucose levels, total cholesterol, low-density lipoprotein and triglycerides, and increasing high-density lipoprotein levels) and improve some neonatal outcomes (macrosomia, large for gestational age and preterm birth rate) (81–83). Animal studies using INO supplementation in murine models of diabetes, obesity, and metabolic syndrome have also shown similar metabolic improvement in both, mothers and offspring (84,85).

Moreover, myoinositol supplementation was proven to reduce insulin resistance in postmenopausal women with MS and women with polycystic ovary syndrome, a metabolic and endocrine disorder associated with insulin resistance (84).

However, to the best of our knowledge, the inositol supplementation effect has never been investigated in pregnancies complicated by MS.

Moreover, most of the studies in pregnant women focus on the effect of myoinositol supplementation alone, whereas mounting evidence in polycystic ovary syndrome studies suggest that the administration of combined myoinositol/D-chiro inositol at the physiologic plasma ratio (40:1) ensures better clinical results, such as the reduction of insulin resistance and cardiovascular risk parameters (86).

1.6.4 Inositol in pregnancies complicated by metabolic syndrome and obesity

Our research group, coordinated by Dr. Monica Longo, previously conducted a study in which they investigated the effect of a mixture of Myo-inositol/D-chiro inositol (MI/DCI) supplementation during pregnancy on the maternal metabolic profile in pregnancies complicated by metabolic syndrome and obesity using a pregnant mouse model (87).

For the MS group, they used female heterozygous endothelial nitric oxide synthase (eNOS +/-) mice with moderate hypertension, that once placed on a high-fat diet (HFD) for 4 weeks develop a MS phenotype. Similarly, for the obesity group, they used wild-type C57BL/6 mice placed on HFD for 4 weeks to induce a murine obesity model. Mice were then bred with wild-type males to obtain the pregnancy. On gestational day 1, dams were randomly allocated to receive either a mixture of MI/DCI in water (7.2/0.18 mg/mL, respectively) or water as a control (placebo). At term (gestational day 18), maternal weights, systolic blood pressure, and a glucose tolerance test were obtained. Dams were then killed; pups and placentas were weighed, and maternal blood was collected. Serum levels of metabolic biomarkers relevant to diabetes and obesity (ghrelin, gastric inhibitory peptide, glucagon-like peptide 1, glucagon, insulin, leptin, resistin) were measured by a multiplex enzyme-linked immunosorbent assay. Analysis was done comparing MS-MI/DCI-treated vs MS-nontreated mice and obese-MI/DCI-treated vs obese non-treated mice. The authors found that mean systolic blood pressure was lower in MS pregnant mice treated with MI/DCI compared with placebo ($p=0.04$), whereas there was no difference in systolic blood pressure between treated and placebo-treated obese pregnant mice. Pregnant MS mice treated with MI/DCI showed lower glucose values during the glucose tolerance

test and in the area under the curve, but no differences were seen in the obese pregnant mice.

Leptin serum levels were lower in the MS-MI/DCI-treated mice compared with the placebo group. No other differences were seen in any of the remaining serum metabolic biomarkers studied in MS and obese pregnant mice. Maternal weight gain was not different in the pregnant MS dams, whereas it was lower in the obese MI/DCI-treated dams compared with the placebo group. Fetal and placental weights did not differ between MI/DCI and nontreated pregnant dams with MS and obesity.

The authors concluded that combined inositol treatment during pregnancy improves blood pressure, glucose levels at the glucose tolerance test, and leptin levels in pregnant dams with MS but not in obese pregnant dams. In addition, inositol treatment was associated with lower gestational weight gain in the obese but not in the metabolic-like syndrome pregnant dams.

However, despite all the interesting data published so far, there is still a lack of knowledge on the impact that inositols supplementation in pregnancies complicated by obesity, hypertension, and metabolic syndrome can have on maternal organ fibrosis and subsequently on the placenta and ultimately on metabolic and vascular development of the offspring born to those mothers.

Moreover, the potential of a new nonpharmacological approach as a preventive and therapeutic agent is clinically relevant because appropriate preventive strategies for metabolic syndrome in pregnancy have not yet been identified.

1.7 Hypothesis

Based on the promising results that our group obtained with inositols supplementation in pregnancies complicated by MS and obesity (88), and considering that the more compromised the model was (MS), the more beneficial were the INO effects, we hypothesized that INOs supplementation in pregnancies complicated by MS will reduce the end-organ fibrosis in maternal heart, kidneys, and liver by activating the transforming growth factor-β (TGF-β), a central mediator of fibrogenesis upregulated and activated in fibrotic diseases and will stimulate specific glucose homeostasis pathways known to be modulated by INO such as glucose transport and uptake, oxidative mitochondrial metabolism, and utilization and storage as glycogen and ultimately will improve vascular and metabolic profiles in adult offspring born to pregnant mice with the MS phenotype.

The rationale behind our hypothesis is that improvement in the maternal metabolic and cardiovascular profile could translate into positive long-term maternal health effects by reducing the end-organ fibrosis and improving placental function, ameliorating the intrauterine environment, thus favoring a correct fetal development reducing the negative impact of maternal pre-pregnancy metabolic dysfunction on long-term fetal health.

To achieve our goal, we conducted the following studies evaluating metabolic and vascular parameters in the maternal/fetal unit:

1. <u>Maternal</u>
    - evaluating the effect of MS and HTN on maternal markers of fibrosis and assess if INOs supplementation in MS dams may prevent the end-organ

fibrosis in the heart, kidneys, and liver by activating the TGF-β, a central mediator of fibrogenesis upregulated and activated in fibrotic diseases.

2. Placental
    - evaluating the changes in protein level involved in specific glucose homeostasis pathways known to be modulated by INO such as glucose transport and uptake, oxidative mitochondrial metabolism, and utilization and storage as glycogen in placentas of MS dams.
3. Fetal and offspring
    - evaluating vascular and metabolic profiles in adult offspring born to pregnant mice with the MS to understand further the gene-environment relationship, and specifically the relative contribution of a hostile intrauterine environment (MS) and the fetal genotype on the fetal vascular and metabolic programming impact on adult long-term health.

2 STUDY DESIGN

2.1 Maternal Study

2.1.1 Organ fibrosis

We hypothesized that pre-pregnancy metabolic dysfunctions (HTN and MS) induce end-organ fibrosis, which is reduced by maternal INO supplementation, and this effect may be mediated by activating the TGF-β, a central mediator of fibrogenesis upregulated and involved in fibrotic diseases. It modulates fibroblast phenotype and function, inducing myofibroblast trans-differentiation while promoting matrix preservation (89). We aimed to evaluate the effect of MS and HTN on maternal markers of fibrosis and to assess INO supplementation effects in the MS model. To achieve our goal we collected heart, liver, and kidneys from heterozygous eNOS+/- female with a hypertensive and metabolic syndrome phenotype as described previously in Ferrari et al experiment (87), and females wild type as control (90).

2.2 Placental Study

We hypothesized that maternal INO supplementation in pregnancy complicated by MS improves placental metabolic pathways related to fetal metabolic development.

We aimed to evaluate changes in protein level involved in specific glucose homeostasis pathways known to be modulated by INO such as glucose transport and uptake, oxidative mitochondrial metabolism, and utilization and storage as glycogen.

To achieve our goal, we used placentas obtained from a well-characterized murine model of metabolic syndrome (91), heterozygous mice for endothelial nitric oxide (eNOS+/-)

gene (lacking the enzyme to produce nitric oxide and with a hypertensive phenotype) on high-fat (HF) diet, treated and non-treated with INO (92).

2.3 Fetal and Offspring Study

We hypothesized that INO supplementation during pregnancy will improve vascular and metabolic profiles in adult offspring born to pregnant mice with the MS phenotype. To test this hypothesis, we used a well-characterized murine model of MS, a heterozygous eNOS+/- mouse fed an HFD (previously mentioned). This model was used to generate the offspring in this study to understand further the gene-environment relationship, and specifically the relative contribution of a hostile intrauterine environment (MS) and the fetal genotype on the fetal vascular and metabolic programming impact on adult long-term health (93).

3 MATERIALS AND METHODS

This study has been carried out from 2016 to 2020, in the laboratory of Dr. Monica Longo in the Maternal-Fetal Division at The University of Texas Health Science Center at Houston (UTHealth), Texas, USA.

3.1 Animals

Two strains of female mice were used for the studies: wild-type (WT, strain C57BL/6J, stock number 000664) and homozygous for disruption of the eNOS gene (eNOS-knockout $^{-/-}$, strain B6.129P2, stock number 002684) both bred with male wild-type controls (WT, strain C57BL/6J, stock number 000664), purchased from Jackson Laboratory (Bar Harbor, ME) at 6 weeks of age and bred to obtain the heterozygous genotype.

The study was approved by the Animal Welfare Committee (AWC-19-0126) of the University of Texas Health Science Center at Houston. Mice were housed separately in temperature and humidity-controlled quarters with constant 12:12 hour light-dark cycles in the animal care facility at the University of Texas Health Science Center at Houston.

3.2 eNOS$^{+/-}$ a murine model of hypertension

Nitric oxide (NO) is synthesized from L-arginine by a family of enzymes, called NO synthases (NOSs). The constitutively expressed NOS isoforms, endothelial NOS (eNOS), and neuronal NOS (nNOS) are the major contributors to whole-body NO production. In particular, the eNOS gene, which maps on 7q35–36, is mainly expressed in endothelial cells and is associated with blood pressure regulation. Many studies strongly associated

polymorphisms of the eNOS gene with an increased risk of hypertension, cardiovascular disease, coronary spastic angina, myocardial infarction, and stroke (94).

Under physiologic conditions, insulin regulates many vascular functions (95,96) (besides metabolic functions), including the release of NO (97) and the regulation of mRNA expression of the endothelial nitric oxide synthase enzyme (98,99). Among the many activities of NO, it has been demonstrated its ability to modulate peripheral and hepatic glucose metabolism and insulin secretion (100). Indeed, endothelium-derived NO mediates insulin-induced stimulation of the perfusion of skeletal muscle (101), its main metabolic target tissue. In insulin-resistant individuals, insulin stimulation of endothelial NO synthesis is impaired and may contribute to defective skeletal muscle glucose uptake (102). Indeed, previous studies demonstrated that NOS inhibitors reduced insulin-stimulated muscle glucose uptake in rats in vivo (103) and NO donors stimulated glucose transport in isolated rat muscle preparations in vitro (104). Thus, NO alterations play an important role in the evolution of insulin resistance and type 2 diabetes (100). Consistent with those findings, eNOS null mice are insulin resistant and hypertensive (105–107). Extrapolation of these findings from mice to humans is problematic because eNOS gene total deficiency has not been reported in humans so far. There is evidence, however, that hypertension, coronary artery disease, and myocardial infarction are associated with eNOS gene polymorphism and impaired NO synthesis (108–111). Considering that complete gene deficiencies are seldom found in human disease, we decided to use a heterozygous murine model lacking only one gene for the endothelial nitric oxide synthase ($eNOS^{+/-}$).

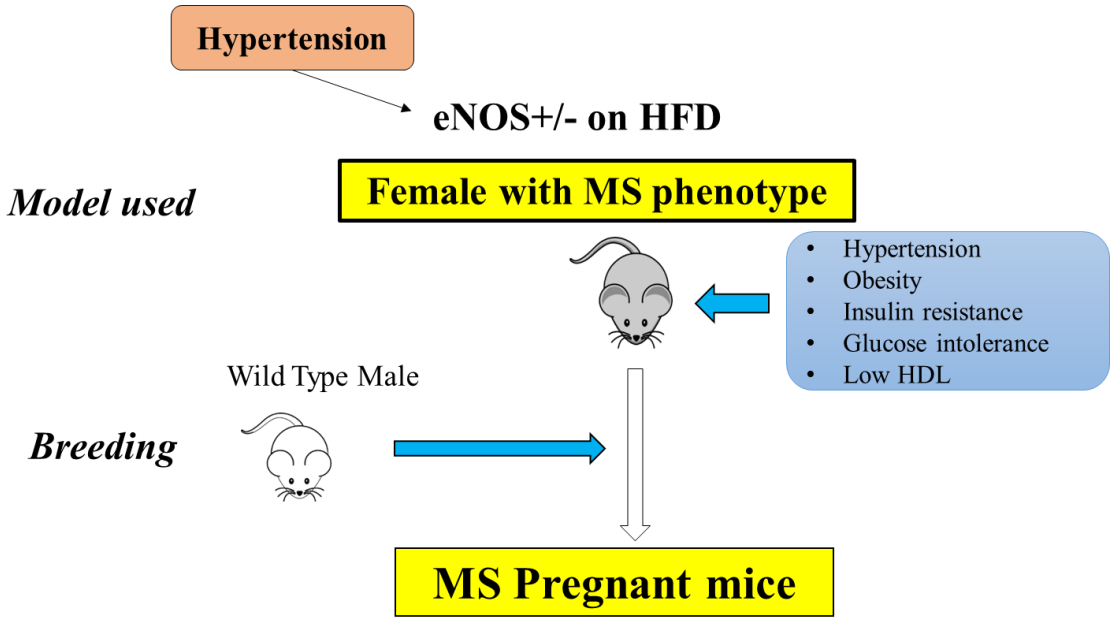
Indeed, as previously reported, when fed a high-fat (HF) diet for 8 weeks, $eNOS^{+/-}$ mice with a moderate hypertensive phenotype develop also insulin resistance, glucose intolerance, and further increase blood pressure (112).

3.3 Pregnant metabolic syndrome mouse model

Endothelial nitric oxide synthase knockout ($eNOS^{-/-}$) females, with a genetically induced hypertensive (HTN) phenotype, were bred with wild-type ($WT^{+/+}$) males to obtain endothelial nitric oxide synthase heterozygous ($eNOS^{+/-}$) females with a moderate HTN phenotype (91,113). As shown below in Figure 7, those $eNOS^{+/-}$ heterozygous females were placed either on a control diet to obtain an adult mild hypertensive phenotype, or on an obesogenic high-fat (HF) diet (D12492, 60% of fat) for 4 consecutive weeks after weaning to obtain an adult metabolic syndrome (MS) phenotype (113). Daily food consumption was estimated by subtracting the total amount of food left on the grid from the initial weight of food supplied.

At 7 - 8 weeks of age, $eNOS^{+/-}$ females were bred with wild-type ($WT^{+/+}$) males and gestational day (GD) 1 of pregnancy was determined by the presence of a vaginal plug after overnight exposure to male breeders.

Figure 7. Metabolic Syndrome pregnant mouse model



3.4 Inositol supplementation during pregnancy

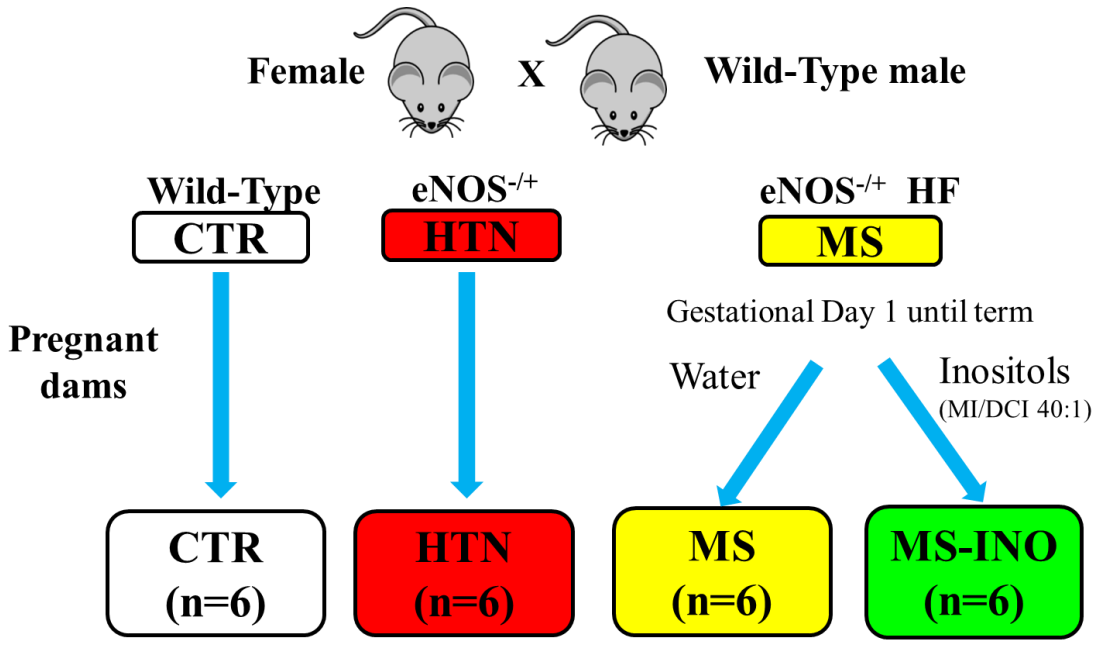
MI/DCI mixture corresponds to the physiologic plasma ratio of Myo-inositol and D-chiro-inositol equal to 40:1, which has been proven to be the most effective (71). On GD 1, dams with metabolic syndrome were randomly allocated to receive either plain water as control or INO [mixture of Myo-inositol/D-Chiro-inositol dissolved in water (7.2/0.18 mg/mL, respectively)] based on previous animals (74,114) and humans (81–83,115) studies. Blood volume during pregnancy increases physiologically by 20%, thus we adjusted the MI/DCI doses by a 20% increase to the previously established doses used in a non-pregnant obese mouse model. The treatment was maintained until term gestation (GD 18) when dams were euthanized with the use of CO_2 inhalation, in accordance with the 2000 Report of the AVMA Panel on Euthanasia, approved by the National Human Genome Research Institute

(NHGRI) (116). Pregnant mice were singly housed to be able to carefully evaluate water intake and consequently the daily dose of MI/DCI consumption. Daily water consumption was estimated by subtracting the total amount of water left in the bottle from the initial amount supplied. On average, pregnant mice drink 5 mL/day, so MI/DCI daily consumption was approximately 36/0.9 mg of, respectively, MI/DCI per day per mouse. At term gestation, dams were euthanized, and pups and placentas weights and numbers were collected.

3.5 Maternal organ fibrosis

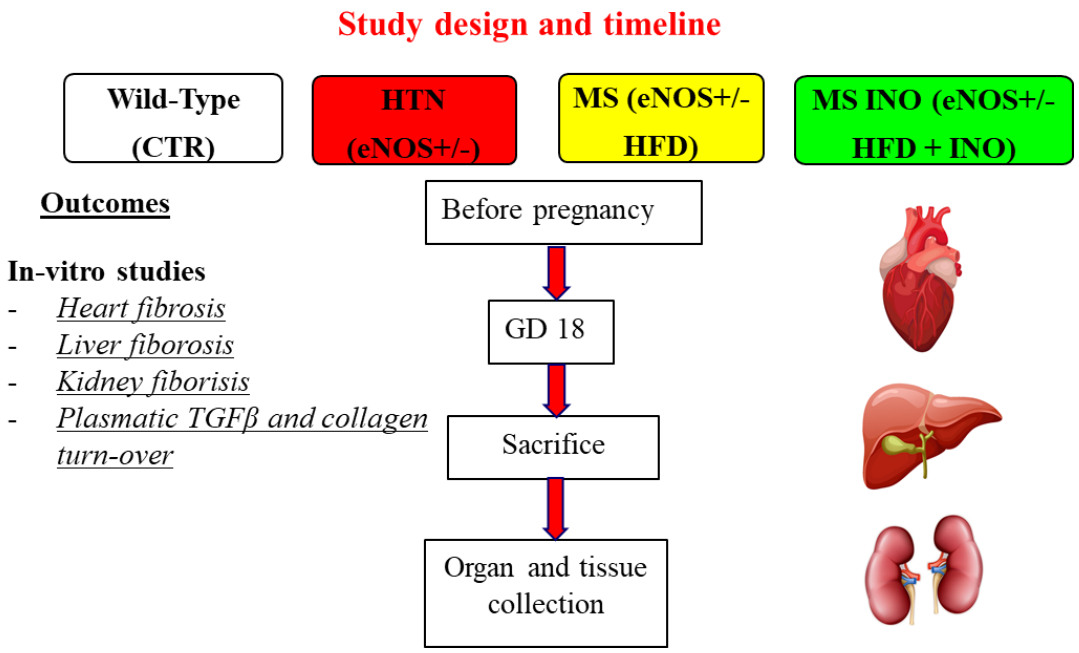
At 7-8 weeks of age, non-pregnant WT, eNOS+/- in control diet and eNOS+/- in high-fat diet females were mated with WT males to obtain 3 groups of pregnant dams: control (wild-type), hypertensive phenotype (eNOS+/-), and metabolic syndrome phenotype (eNOS+/- HFD) (Figure 8).

Figure 8: Experimental groups of maternal organ fibrosis study



For this study, we used females' wild type as baseline, eNOS+/- female with a hypertensive phenotype to evaluate the fibrotic alterations in pregnancy and metabolic syndrome phenotype treated or not with INO to evaluate the effects of the supplementation in the most compromised model. WT and eNOS+/- (hypertensive) females on control diet (CD) were mated with wild-type males to obtain pregnant dams (WT-CD, and eNOS CD) which were used as control, representing different stages of disease (i.e., normal and hypertension). Similarly, eNOS+/- female on high fat diet (metabolic syndrome) were mated with wild-type males to obtain pregnant dams (eNOS HFD). On the gestational day (GD) 1, MS dams (eNOS+/- on HFD) were randomly allocated to receive either a mixture of INO in water (MI/DCl) or water as a control for the duration of the pregnancy to obtain MS dams non treated and treated with INOs. Four groups of pregnant dams were obtained: CTR (WT-C), HTN (eNOS C), MS (eNOS HF), and MS-INO (eNOS HF on INO) (Figure 8). The study design and timelines are reported in figure 9.

Figure 9: Maternal organ fibrosis study design and timeline



3.5.1 Fibrosis evaluation

Dams were euthanized at term gestation (day 18) by CO_2 overdose, as previously described. The maternal cardiac, renal, and liver organs were collected for histological evaluation. The Masson's trichrome staining was used to assess for connective tissue deposition, and the percent of connective deposition was calculated by a J-image software, resulting in the ratio of the percentage of fibrosis present in the tissue (average percentage of fibrosis from 5 cardiac images per group).

3.5.2 Heart hypertrophy

The amount of cardiac hypertrophy was assessed by left ventricular wall thickness obtained by averaging 3 measurements of the left ventricular wall in the histological section at higher magnification (20×) from 5 heart images.

3.5.3 Renal damage

The extent of renal parenchyma damage was assessed by quantifying the glomeruli and tubules that showed characteristic features of damage as in chronic kidney disease: decreased Bowman's space, occlusion of capillary loop spaces, sclerosis, and necrosis in both glomeruli and tubules. To examine those features, the glomeruli and tubules were counted in 6 randomized fields of and blinded slides (×40 magnification), with each field counting at least 16-22 glomeruli and tubules, respectively.

The glomeruli and tubules in each field were given the highest score of 5 accorded to glomeruli and tubules with a normal amount of capillary space within Bowman's capsule and normal structures of tubules. A score of 1 was assigned to the glomeruli that showed complete loss of capillary space and sclerosis or necrosis. An intermediate score of 3 was assigned to the glomeruli that displayed reduced, but not completely obliterated, capillary space and partial necrosis. The lower the score the more renal parenchyma damage was seen (Table 1).

Table 1. Glomeruli and tubules score

Glomeruli and Tubules score	
5	Normal
4	Moderate
3	Mild
2	Severe
1	Abnormal

3.5.4 Quantification of the markers of fibrosis

ELISA was used to measure the serum level of TGF- β, a fibrogenic biomarker; PICP and PIIICP, biomarkers of collagen types 1 and 3 syntheses, respectively. 1-way or 2-way ANOVA was used for statistical analysis.

Then to confirm our data we evaluated circulating biomarkers of fibrosis ELISA assay was used to the measured serum level of Transforming growth factor-beta (TGF- β), a prosclerotic cytokine that is consistently implicated in organ fibrosis and hypertrophy; and markers of collagen Type 1 and Type 3 synthesis and degradation, respectively Procollagen propeptides Type 1 (PICP) and 3 (PIIICP), and the Cross-linked C-Telopetides Type 1 (CTX-I) and Type 3 (CTX-III).

Collagen, types I and III, is the principal structural protein found in the myocardium, and its pro- or telopeptides are released into the circulation in the course of cardiovascular diseases. Therefore, these peptides may reflect collagen synthesis and breakdown, representing a much more useful tool to address extracellular matrix (ECM) changes from a distance (117).

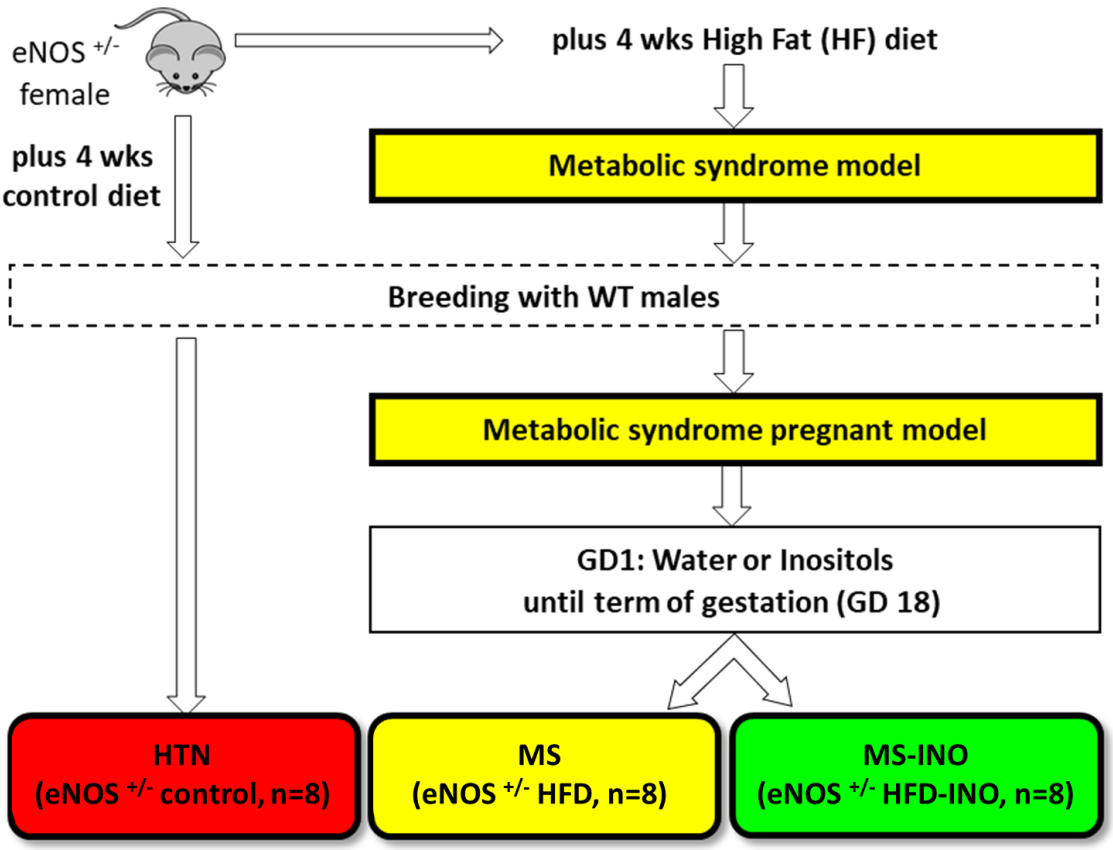
3.6 Placenta experiment

For the placenta experiment, only $eNOS^{+/-}$ were included, considering that the most relevant results in Ferrari et al. (87) experiment were obtained with the MS model.

Pregnant dams with a mild hypertensive phenotype ($eNOS^{+/-}$), fed with a control diet (HTN, n=8) were used as a control group. Pregnant metabolic syndrome dams were randomly allocated to receive either plain water as control (MS, non-treated, n=8) or INO

dissolved in water (MS-INO, treated with inositols, n=9) for the entire duration of pregnancy until term gestation (GD 18), as reported in Figure 10.

Figure 10: Experimental groups of placenta study: eNOS$^{+/-}$C, eNOS$^{+/-}$HF, and eNOS$^{+/-}$HF-INO.

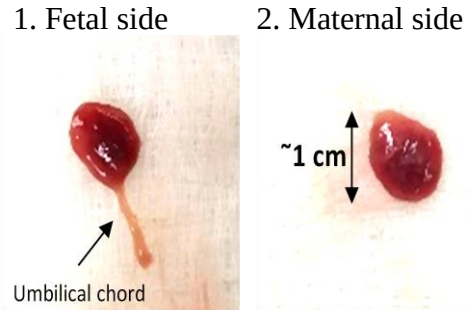


3.6.1 Placenta collection

At GD 18, pregnant HTN, MS, and MS-INO dams were fasted (6 h) and then euthanized by CO_2 inhalation. After laparotomy fetuses and placentas were collected. We obtained a total of n=175 placentas from the three experimental groups [HTN (n=8 dams, n=58 placentas), MS (n=8 dams, n=54 placentas) and MS-INO (n=9 dams, n=63 placentas)].

Placentas were frozen in liquid nitrogen before storage at -80°C. At the time of the experiments, placentas were thawed, and the maternal side was carefully separated from the fetal side through a longitudinal cut along the equatorial plane (Figure11). Considering that, due to Mendelian genetic inheritance, offspring placental genotype could be 50% heterozygous ($eNOS^{+/-}$) and 50% homozygous ($WT^{+/+}$), biopsies from the fetal placental side were obtained to determine the sex and genotype of each placenta. A double-check was done by genotyping each corresponding fetus for the same markers (eNOS allele and sex). Genotype was obtained by the Transnet YX Inc. system.

Figure 11: Murine placenta.



After genotyping for sex, we obtained:

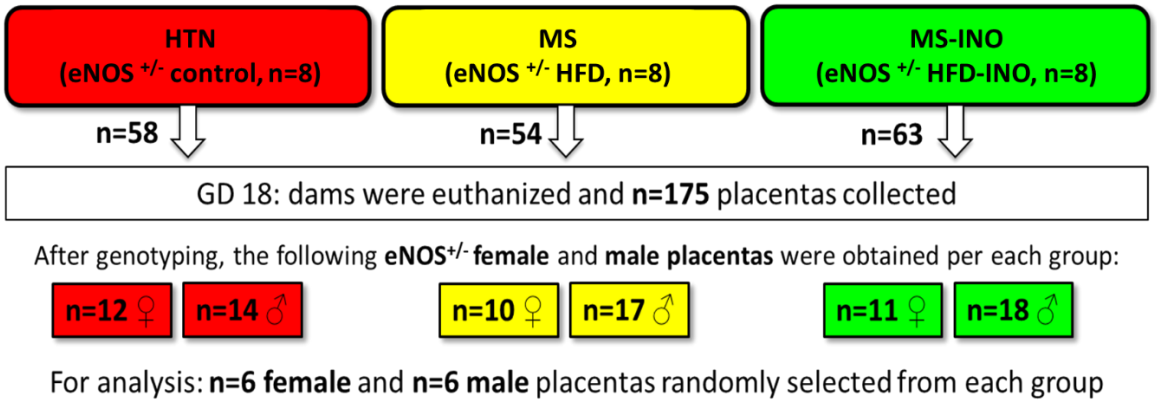
- HTN group: n=27 female and n=31 male placentas (tot. n=58).
- MS group: n=24 female and n=30 male placentas (tot. n=54).
- MS-INO group: n=25 female and n=38 male placentas (tot. n=63).

  After genotyping for the eNOS allele, we obtained the following eNOS heterozygous placentas:

- HTN group: n= 12 female and n=14 male placentas.
- MS group: n=10 female and n=17 male placentas.
- MS-INO group: n=11 female and n=18 male placentas.

In this study, only eNOS$^{+/-}$ offspring placentas were utilized, known to be genetically more vulnerable to the *in utero* influences compared to their WT counterpart (93). Thus, we randomly selected six females (F-PL) and six males (M-PL) eNOS$^{+/-}$ placenta for each group of pregnant dams, for a total of thirty-six placentas (Figure 12).

Figure 12: Placentas collection from each experimental group.



3.6.2 Primary antibodies

- Non-phosphorylated targets: Insulin receptor-β (IR-β, cat#3025S, Cell Signaling Technology, Danvers, MA); Glucose Transporter 4 (GLUT4, cat#ab35826, Abcam, Cambridge, MA); Glycogen Synthase kinase 3β (GSK3β, cat#SAB4300287, Millipore-Sigma, St. Lois, MO); Protein kinase B (Akt, cat#4961S, Cell Signaling Technology, Danvers, MA).

- Phosphorylated targets: Phosphorylated Insulin Receptor Substrate-1 at Tyr608 (pIRS-1^{Tyr608}, cat#09-432, Millipore-Sigma, St. Lois, MO); Phosphorylated protein kinase B at Thr308 (pAkt^{Thr308}, cat#13038, Cell Signaling Technology, Danvers, MA); Phosphorylated Pyruvate Dehydrogenase at Ser293 (pPDH^{Ser293}, cat#ab177461, abcam, Cambridge, MA)

- β-actin from Thermo Fisher Scientific (cat#MA5/15739, Rockford, IL).

3.6.3 Western blotting procedure

Frozen placenta tissue samples were homogenized using a hand-held motorized homogenizer (Huanyu, China) on ice in lysis buffer (RIPA buffer containing protease and phosphatase inhibitor cocktail, Thermo Fisher Scientific, Rockford, IL). Tissue homogenates were left on ice for 10 minutes and then centrifuged at 20000×g for 20 min/4°C. Supernatants (tissue lysates) were collected and protein concentrations were determined using a BCA kit (Thermo Fisher Scientific, Rockford, IL). Proteins in tissue lysate samples (30-50 µg/lane) were separated with electrophoresis on 10% precast TGX gels (BioRad, Hercules, CA). Resolved proteins were electro-transferred onto 0.45 µm nitrocellulose membranes (1.22 A for 35 min). Membranes were blocked for 1 h at room temperature (5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for non-phosphorylated proteins or 4% BLOCK ACE in Millipore water (BioRad, Hercules, CA). Membranes were incubated with primary antibodies for overnight transfer at 4°C. Membranes were then washed in TBST (3 × 5 min washes) and probed with appropriate HRP-labelled secondary antibodies from Abcam (Cambridge, MA) for 1 h at room temperature. β-actin was used as loading control either directly or after stripping and re-probing using a striping buffer according to manufacturer instructions (Thermo Fisher

Scientific, Rockford, IL). Target protein bands were visualized using Immobilon forte® ECL substrate (Millipore-Sigma, St. Lois, MO) and imaged using a BioRad Chemidoc MP imager (Hercules, CA). Densitometric analyses of obtained protein bands were performed using Viosionworks LS software version 8.20, Analytik Jena, Germany) and normalized to β-actin. Densitometric data were expressed as Relative Protein Level Normalized to β-actin for different experimental groups (Arbitrary Units, AU).

3.6.4 Glycogen assay

The total extracted placental proteins were quantified using a BCA assay. Glycogen levels were measured using a colorimetric Glycogen storage kit (cat#ab169558, Abcam, Cambridge, MA) according to the manufacturer's instructions. Results were expressed as µg glycogen/mg tissue protein.

3.7 Fetal and offspring study

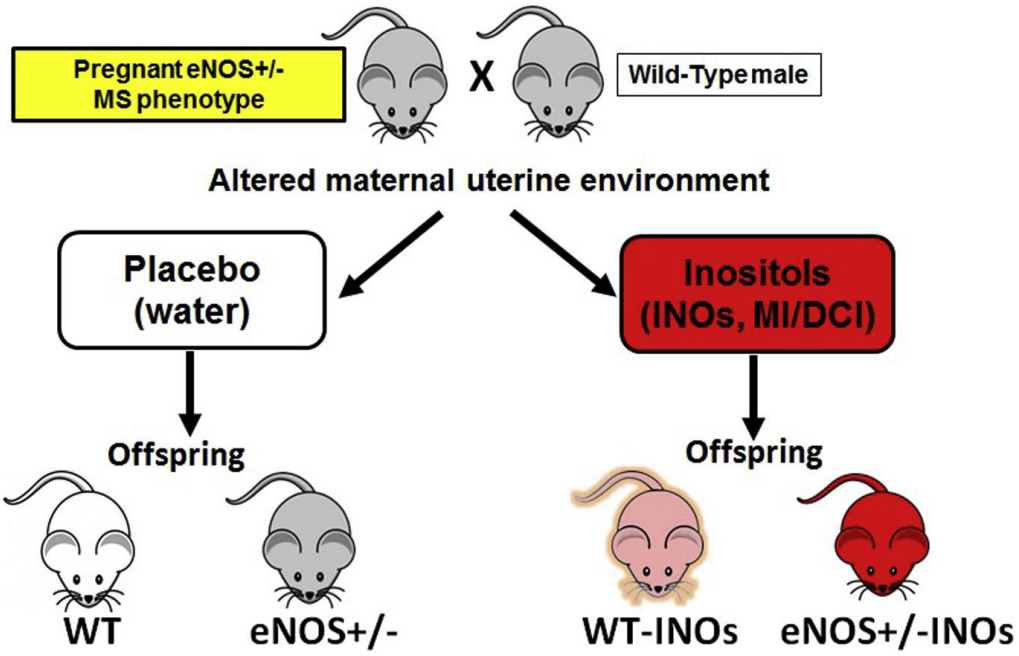
For the offspring study we used the murine model of MS to generate the offspring and understand further the gene-environment relationship, and specifically the relative contribution of a hostile intrauterine environment (MS) and the fetal genotype on the fetal vascular and metabolic programming impact on adult long-term health.

3.7.1 Female offspring genotype

Offspring obtained were either heterozygous eNOS+/- or WT. They were maintained on a regular diet until 9 -10 weeks of age, the time of sacrifice. Those offspring had a different fetal genotype but were born to the same dams, eNOS+/- heterozygous on HFD, with an

altered uterine environment due to MS and receiving either the INO mixture or placebo. Female offspring were genotyped at weaning (3 weeks of age) and divided into the following groups: nontreated WT offspring, with eNOS gene (WT, n = 8); eNOS+/- heterozygous, lacking one eNOS gene (eNOS+/-, n = 8); INO-treated WT offspring, WT INO (n = 10); and eNOS+/- INO offspring (n = 8) (Figure 13)(93).

Figure 13. Female WT and eNOS+/- offspring born to dams with MS treated with and without INO.



Female eNOS+/- heterozygous with MS phenotype were bred with WT males. MS dams were allocated to receive water as placebo (control) or Inositols (INO, red). Offspring were genotyped and divided: non-treated WT (n = 8) and eNOS+/- heterozygous (n = 8); INO treated, WT-INO (n = 10) and eNOS+/- INO offspring (n = 8).

At 9 - 10 weeks of age, mice were weighed, and after the glucose tolerance test (GTT) and systolic blood pressure (SBP) assessments, they were sacrificed for the vascular reactivity experiments.

3.7.2 In vivo experiments: Glucose tolerance test

After 6 hours of fasting, at 9 weeks of age, female offspring mice received 1.0 g/kg glucose intraperitoneally (I.P). for the glucose tolerance test (GTT). Plasma glucose levels were determined with the Accu-Chek Aviva Blood Glucose Meter System (Roche Diagnostics, Indianapolis, IN) via a tail nick at 0, 15, 30, 60, and 120 minutes after glucose administration.

3.7.3 In vivo experiments: Systolic blood pressure

At 10 weeks of age, systolic blood pressure (SBP) was measured using the noninvasive CODA tail-cuff system (Kent Scientific Corp., Torrington, CT). The animals were placed in a nose cone holder and on a warming plate (37°C). A tail cuff and a pneumatic pulse transducer were applied at the tail base. The tail cuff was programmed to insufflate to a maximal pressure of 250 mm Hg. A rest period of 15 seconds was allowed between cycles. The mice underwent 10 acclimation cycles, followed by 20 cycles for data collection (118,119).

3.7.4 In vitro experiments: Vascular reactivity

At 10 weeks of age, the offspring were sacrificed, and the carotid arteries were dissected. Two mm segments (2-4 segments per animal) were mounted on a wire-myograph system (model 410A, Danish Myo Technology, Aarhus, Denmark) using 25-mm tungsten wires.

Arteries were bathed in Krebs solution, maintained at 37°C with a pH of ≈ 7.4, and bubbled continuously with a mixture of 95% O2 and 5% CO2. The force was recorded by an isometric force transducer and analyzed with PowerLab data acquisition (ADInstruments, Castle Hill, NSW, Australia).

After stabilization of the vascular tone, the arteries were contracted twice with 60 mmol/L KCl for 30 minutes to stabilize vascular responsiveness. The second KCL contraction was used as a reference in the final calculations. After 1 hour of equilibration, contractile responses to cumulative concentrations of the a1-adrenergic agonist phenylephrine (PE, 10^{-10} – 10^{-5} mol/L) were assessed in the presence and absence of a nonselective nitric oxide synthase inhibitor (N-nitro-L arginine methyl ester [L-NAME], 10^{-4} mol/L). Then, relaxant responses to the endothelium-dependent acetylcholine (Ach; 10^{-10} – 10^{-5} mol/L) and the endothelium-independent sodium nitroprusside (SNP, 10^{-10} – 10^{-5} mol/L) (93).

3.7.5 Drugs and solutions

The composition of Krebs solution was as follows: NaCl, 119 mmol/L, KCl, 4.7 mmol/L, NaH2PO4 1.2 mmol/L, NaHCO3 25 mmol/L, MgCl2 1.2 mmol/L, CaCl2 2.5 mmol/L, ethylenediaminetetraacetic acid (EDTA) 0.026 mmol/L, and glucose 11.5 mmol/L. All the components were purchased from Sigma Chemical Company (St. Louis, MO) as well the Myo-inositol and D-chiro-inositol, PE, L-NAME, Ach, and SNP.

3.8 Statistical analysis

Stata 16.1 and Sigma Plot 12 were used for statistical analysis and to create the graphs. Results are expressed as mean ± SEM and a 2-tailed P value of < 0.05 was considered statistically significant.

3.8.1 Maternal Study

For the maternal organ fibrosis study, an average of 5 images per animal/group was used. One-way ANOVA followed by Newman-Keuls post hoc test was performed to compare the 4 study groups: CTR wild-type in control diet (WT-C) as baseline, HTN eNOS heterozygous +/- in control diet (eNOS C) as the first grade of cardiovascular alteration, MS eNOS heterozygous +/- in high-fat diet (eNOS HF), as the most compromised model and MS-INO MS eNOS heterozygous +/- in high-fat diet treated with Inositol (eNOS HF on INO).

3.8.2 Placental study

For the placental study, the statistical analysis was designed to evaluate the effects of maternal INO supplementation on placental protein levels involved in glucose homeostasis. The comparison was made between six females (F-PL) and six males (M-PL) $eNOS^{+/-}$ placenta obtained from the 3 groups of pregnant dams: $eNOS^{+/-}$C (HTN) as control, $eNOS^{+/-}$HF (MS), and $eNOS^{+/-}$HF-INO (MS-INO). Analysis was performed using 1-way ANOVA followed by Neuman-Keuls *post hoc* multiple comparisons test.

3.8.3 Fetal and Offspring study

Fetal and offspring weights, glucose levels at fasting, and at each time point of the GTT, SBP, and vascular reactivity were evaluated. Statistical tests were designed to compare the effects of genotype (WT vs eNOS+/-), the effects of INO supplementation (maternal

treatment vs no treatment), via a 2-way analysis of variance, followed by the Neuman-Keuls multiple comparisons test. In addition, for the vascular reactivity, the percent maximal effect (% Max) of the dose-response curve to each agent was calculated.

4 RESULTS

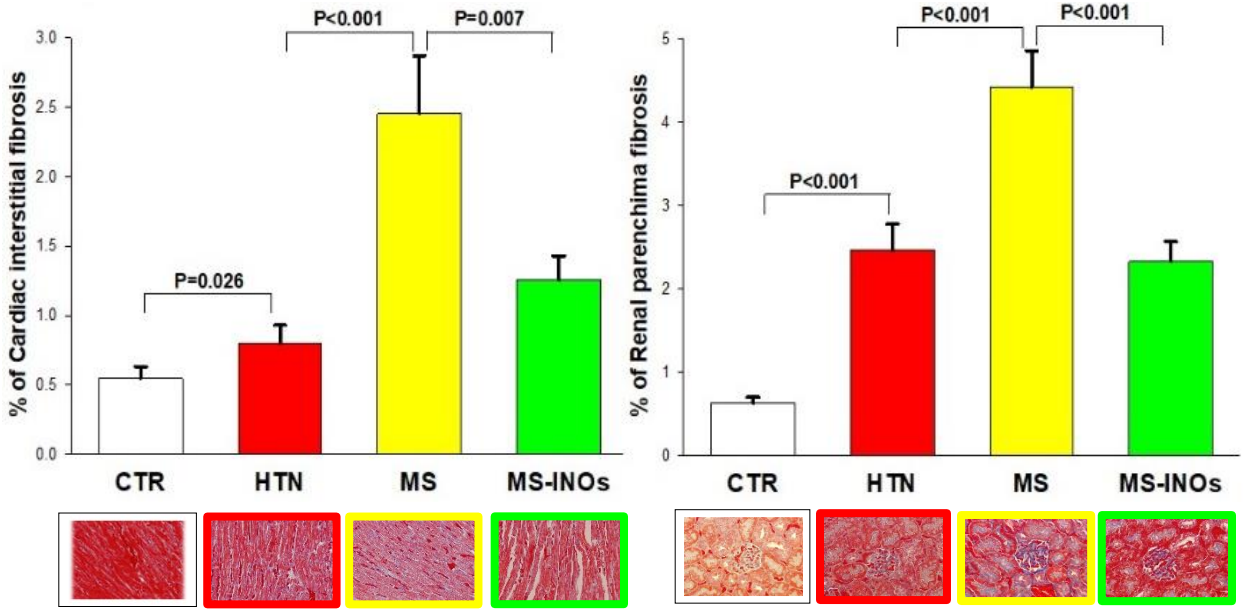
4.1 Maternal study: Organ Fibrosis

4.1.1 Histological End-organ damage

The percentage of cardiac fibrosis was higher in HTN and MS dams vs. CTR dams and reduced by INO treatment in MS dams (Figure 14 A). The level of fibrosis in the renal parenchyma, was higher in hypertensive pregnant dams and even more in metabolic syndrome dams compared with controls and was decreased by inositol supplementation (Figure 14 B).

Figure 14: Cardiac and renal parenchyma fibrosis.

A. Maternal Cardiac Parenchyma **B.** Maternal Renal Parenchyma

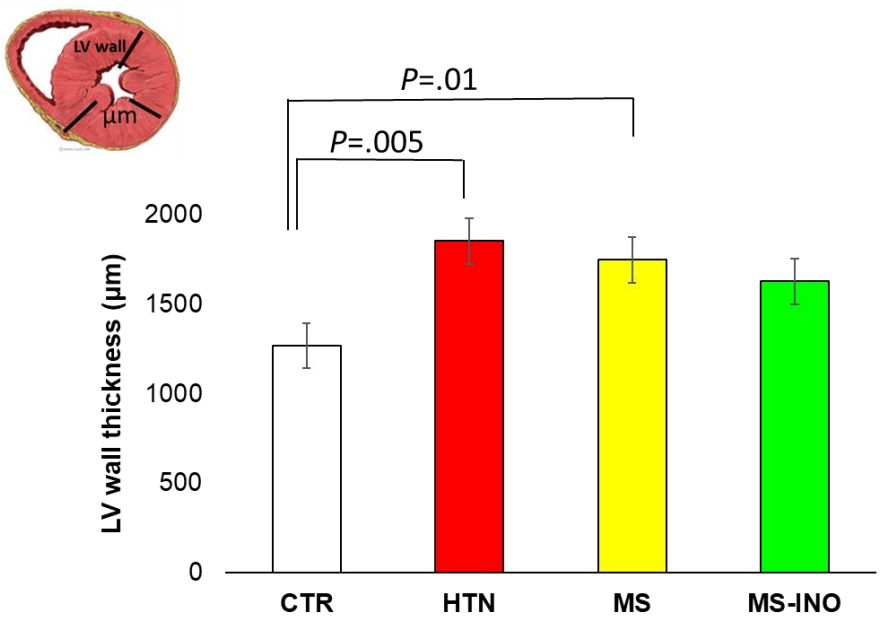


The Bar graphs represent the average of % area of fibrosis from 5 cardiac and renal images per group. Values are reported as means ± SEM

Hystologic images of Masson's trichrome staining at higher magnification (20×) of representative heart and kidney images are shown. One-way ANOVA followed by Newman-Keuls post hoc test was performed to compare the 4 study groups. The amount of fibrosis was higher in dams with HTN and MS groups compared to control dams and inositols treatment (in green) decreased the amount of fibrosis in the metabolic syndrome treated dams in both the cardiac and renal tissues.

The cardiac left ventricle wall thickness, which was higher in dams with pregnancy complicated by hypertension and metabolic syndrome compared to controls and was not affected by inositol supplementation (Figure 15).

Figure 15: Cardiac left ventricle wall thickness

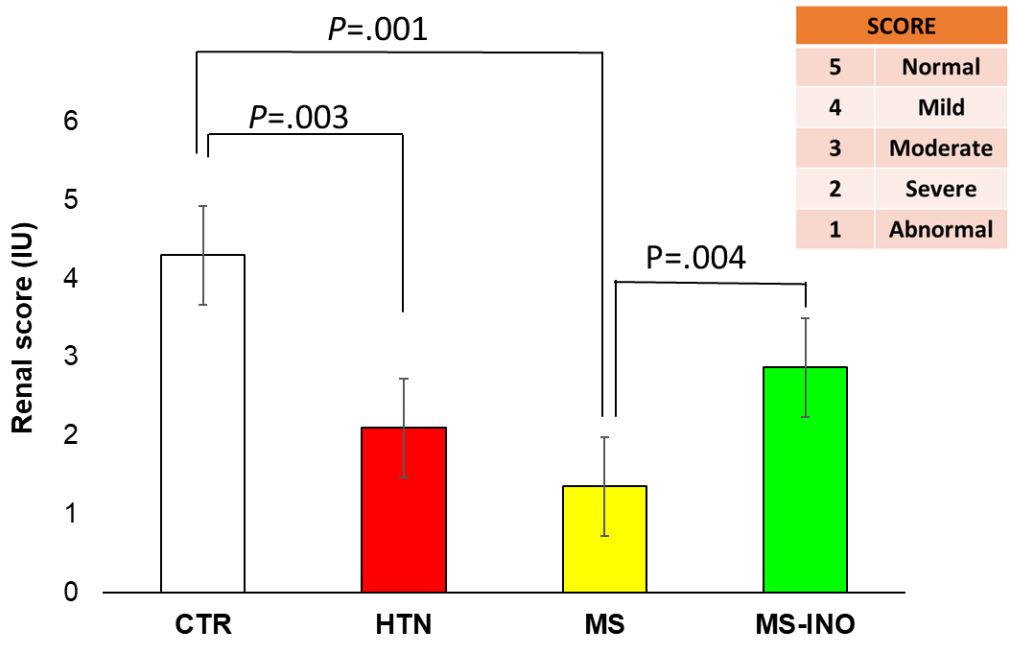


The Bar graph represents the average of the cardiac left ventricle wall thickness in µm, from 5 cardiac images per group. Values are reported as means ± SEM

The cardiac left ventricle wall thickness, which was higher in dams with pregnancy complicated by hypertension and metabolic syndrome compared to controls and was not affected by inositol supplementation. One-way ANOVA followed by Newman-Keuls post hoc test was performed to compare the 4 study groups.

As anticipated by the histological data, the healthy renal parenchyma (based on the renal score) was lower in the hypertensive and metabolic syndrome dams compared with controls and was improved in the MS dams treated with inositol (Figure 16).

Figure 16: Renal parenchyma score

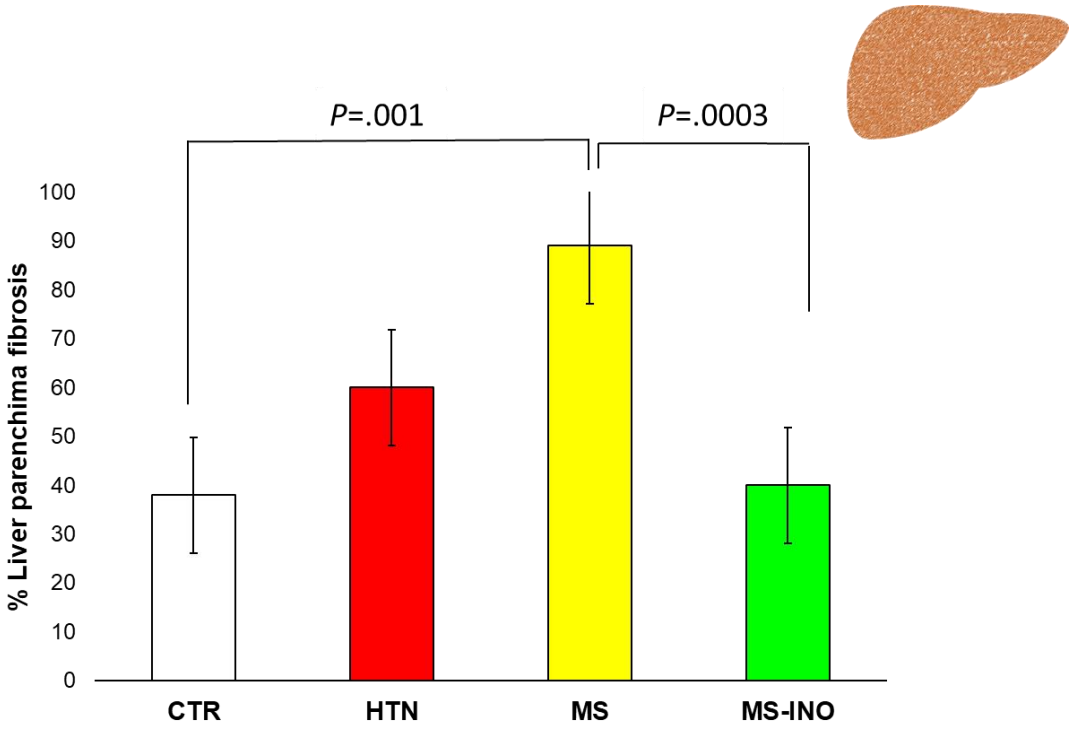


The Bar graph represents the average of the glomeruli and tubules score of the renal parenchyma. Values are reported as means ± SEM.

One-way ANOVA followed by Newman-Keuls post hoc test was performed to compare the 4 study groups. The score was lower in dams with HTN and MS groups compared to control dams and inositols treatment of the MS dams (in green) increased the score restoring the glomerular and tubular functionality.

In the liver parenchyma, the amount of fibrosis was increased only in dams with MS compared to HTN and CTR dams and was lowered to a level similar to controls after treatment with INO ($p < 0.03$) (Figure 17).

Figure 17: Liver parenchyma fibrosis



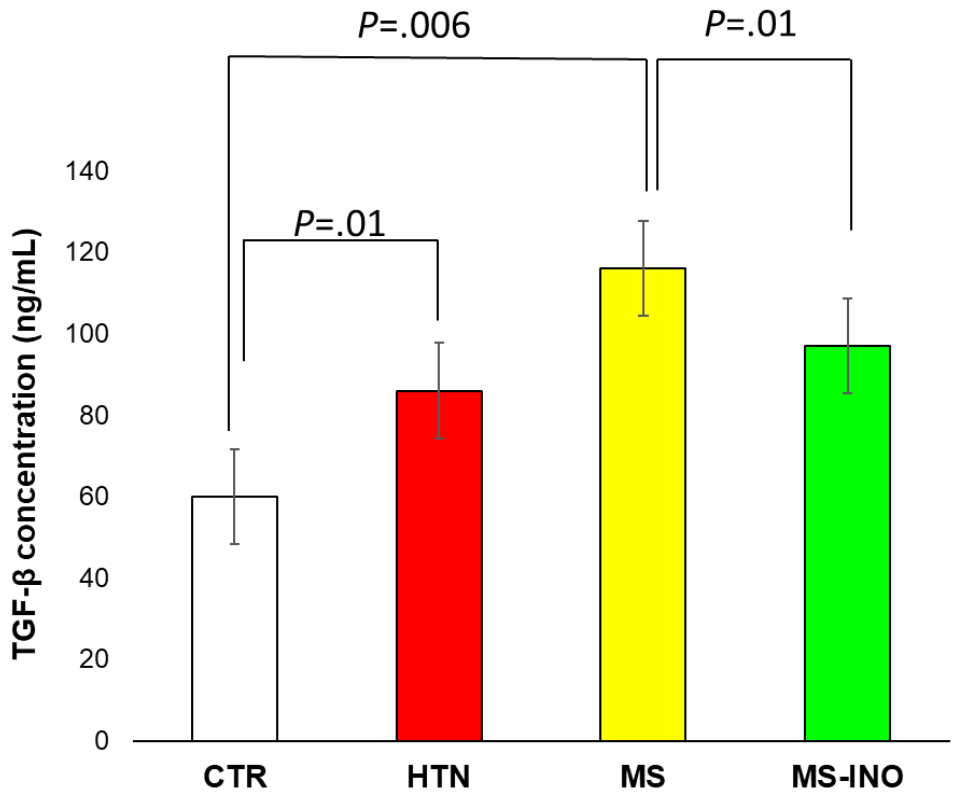
The Bar graph represents the average of % area of fibrosis from 5 liver parenchyma images per group. Values are reported as means ± SEM. One-way ANOVA followed by Newman-Keuls post hoc test was performed to compare the 4 study groups. The amount

of liver fibrosis was higher in dams with MS compared to control dams and inositols treatment (in green) decreased the amount of fibrosis in the metabolic syndrome treated dams.

4.1.2 Serum level of fibrosis biomarkers

The level of TGF-β was higher in MS as well as HTN dams compared with CTR which correlates with the presence of fibrosis seen in the organs. TGF-β level was lowered by INO treatment only in MS dams (Table 2, Figure 18).

Figure 18. Circulating serum level of TGF-β



The Bar graph represents the average of circulating serum level of TGF-β (ng/mL) in the four groups. Values are reported as means ± SEM. One-way ANOVA followed by Newman-Keuls post hoc test was performed to compare the 4 study groups. The level of TGF-β was higher in hypertensive as well as metabolic syndrome dams compared with controls which correlate with the presence of fibrosis seen in the organs. The TGF beta level was lowered by inositol treatment in metabolic syndrome dams.

The levels of collagen Type 1 (PICP) synthesis (Figure 19 A) and Type 1 (CTX-I) degradation (Figure 19 B) were higher in CTR vs. MS and HTN dams and were not affected by maternal INO supplementation. (Table 2). Meaning that in pregnancy

complicated by hypertension and metabolic syndrome there is a decreased capability of collagen degradation leading to a collagen type 1 buildup.

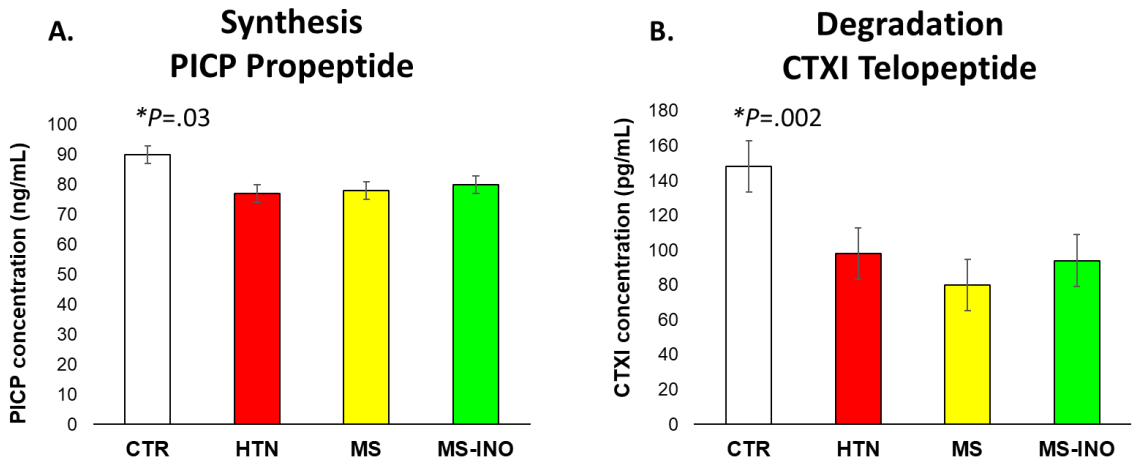
Table 2: Serum level of fibrosis biomarker (ng/mL)

	CTR	HTN	MS	MS-INOs	P-value
TGF-β	60.9 ± 3.3*	87.3 ± 2.4	117.1 ± 2.8*	101.7 ± 8.6	< **0.05**
PICP	90.9 ± 2.8*	76.3 ± 3.6	75.8 ± 2.8	80.9 ± 2.6	< **0.05**
PIIICP	1.1 ± 0.1	1.2 ± 0.1	1.4 ± 0.1*	0.7 ± 0.1	< **0.001**

Transforming growth factor-beta (TGF-β); Procollagen Type 1C-Terminal Propeptide (PICP); Procollagen type 3C-Terminal Propeptide (PIIICP)

** Significance: TGF-β: CTR vs Obese; MS vs HTN / PICP: CTR vs HTN, MS, and MS-INOs / PIICP: MS-INOs vs HTN and MS*

Figure 19. Serum Level of Collagen Type 1

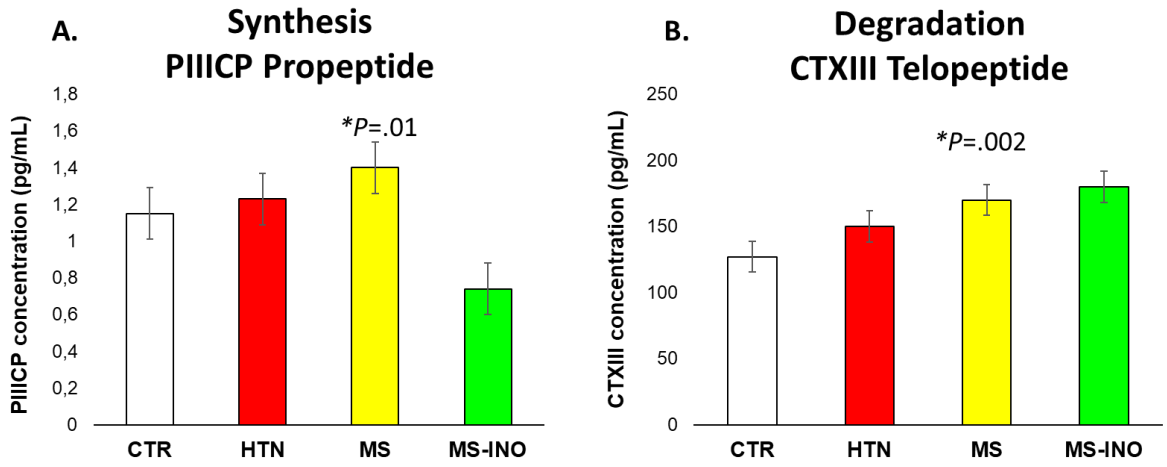


The Bar graphs represent the average of circulating serum level of collagen Type 1 (pg/mL) synthesis and degradation in the four study groups. Values are reported as means

± SEM. One-way ANOVA followed by Newman-Keuls post hoc test was performed to compare the 4 study groups. Collagen Type 1 synthesis, on the left, and degradation on the right, were lower in the hypertensive and metabolic syndrome dams compared with controls, and inositol treatment did not affect their levels.

The level of Collagen type 3 (PIIICP) synthesis (Figure 20 A), and type 3 (CTX-III) degradation (Figure 20 B), were higher only in MS dams compared to CTR and INO lower collagen type 3 synthesis in MS dams, without affecting its degradation (indicating that inositol might participate in collagen type 3 turnover).

Figure 20. Serum Level of Collagen Type 3



The Bar graphs represent the average of circulating serum level of collagen Type 3 (pg/mL) synthesis and degradation in the four study groups. Values are reported as means ± SEM. One-way ANOVA followed by Newman-Keuls post hoc test was performed to compare the 4 study groups. The levels of collagen type 3 synthesis, on the left, and degradation, on the right, were increased only in metabolic syndrome dams compared to controls. Inositol decreased collagen type 3 synthesis in MS dams, but did not affect its degradation.

4.2 Placental study

Glycogen assay results are reported as mean ± SEM in bar graphs. The *y* axis reports the concentration of glycogen expressed in μg/mg, while the *x-axis* reports the three studied groups: the eNOS$^{+/-}$C (HTN) in red, eNOS$^{+/-}$HF (MS) in yellow, and the eNOS$^{+/-}$HF-INO (MS-INO) in green.

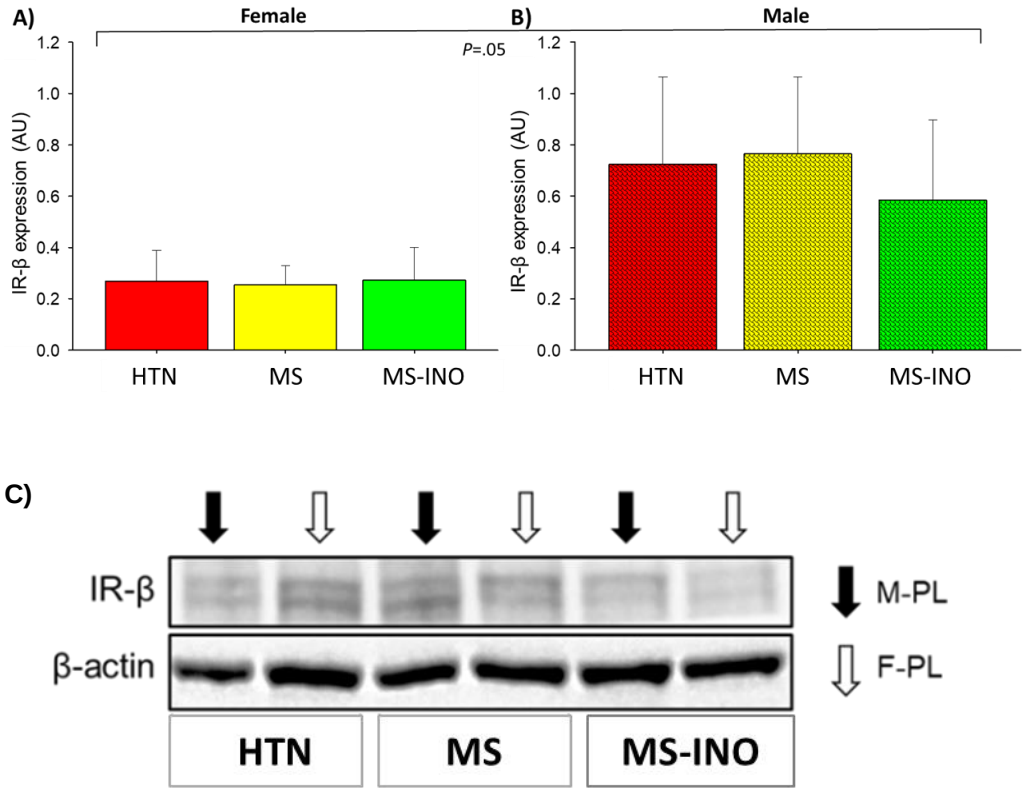
For each graph, *P* values are reported if significant ($P \leq .05$), and results are specified for both female and male placentas.

4.2.1 Glucose uptake pathway

- *Insulin receptor-β (IR-β)*

The IR-β level was not different in female and male placenta among the eNOS$^{+/-}$C (HTN), eNOS$^{+/-}$HF (MS), and eNOS$^{+/-}$HF-INO (MS-INO) group. There was a significant increase in IR-β levels in males compared with the female placenta (*P*=.05) (Figure 21 A, 21 B, 21 C).

Figure 21: Placental level of Insulin Receptor-β (IR-β). (A) Female placenta; (B) Male placenta; (C) Representative of western blot image.

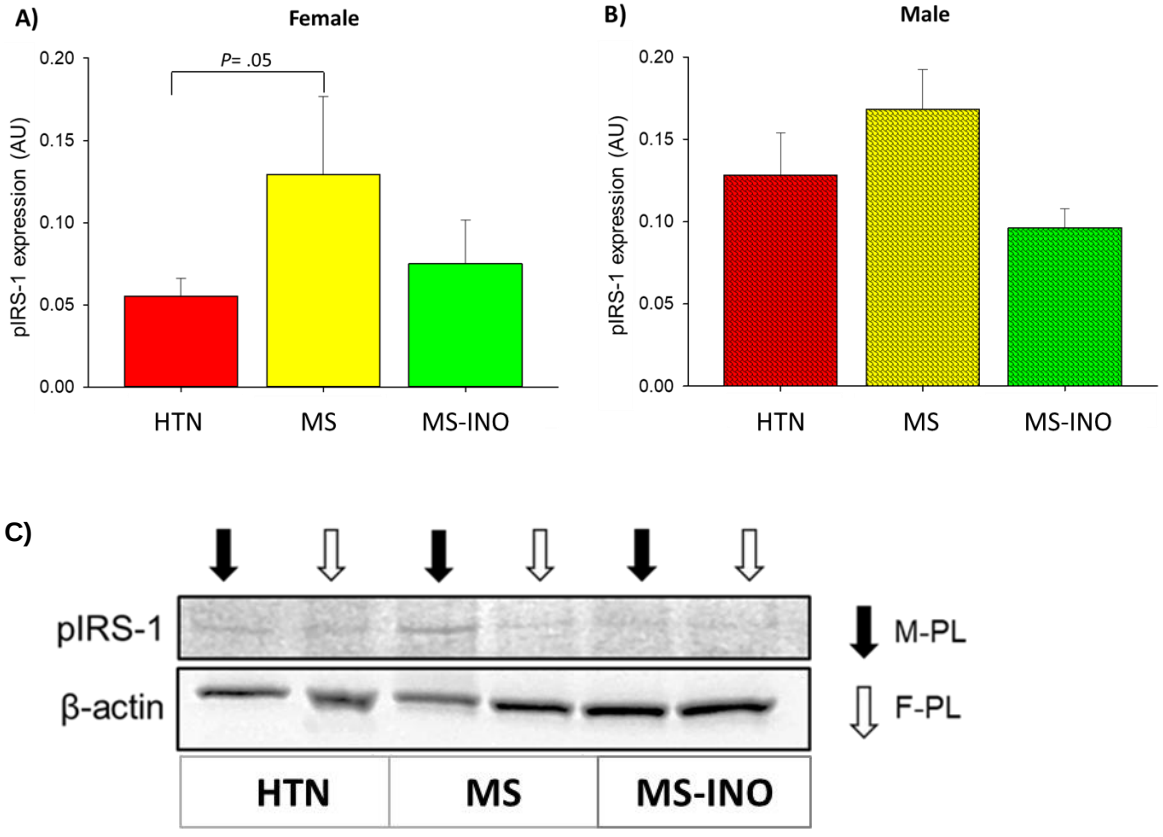


Data are reported as mean±SEM of protein expression normalized to β-actin in Arbitrary Unit (AU). The P-value significance is indicated in the figure

- *Phosphorylated insulin receptor substrate-1 (pIRS-1^{Tyr608})*

The level of phosphorylated insulin receptor substrate-1 at Tyr608 (pIRS-1^{Tyr608}, active form), was upregulated in the female MS placenta compared to the HTN group (*P*= .05) and was unchanged by maternal INO supplementation during pregnancy. A similar trend, although not significant, was observed in the male placenta (Figure 22 A, 22 B, 22 C).

Figure 22: Placental level of phosphor Insulin Receptor Substrate-1 (pIRS-1). (A) Female placenta; (B) Male placenta; (C) Representative of western blot image.

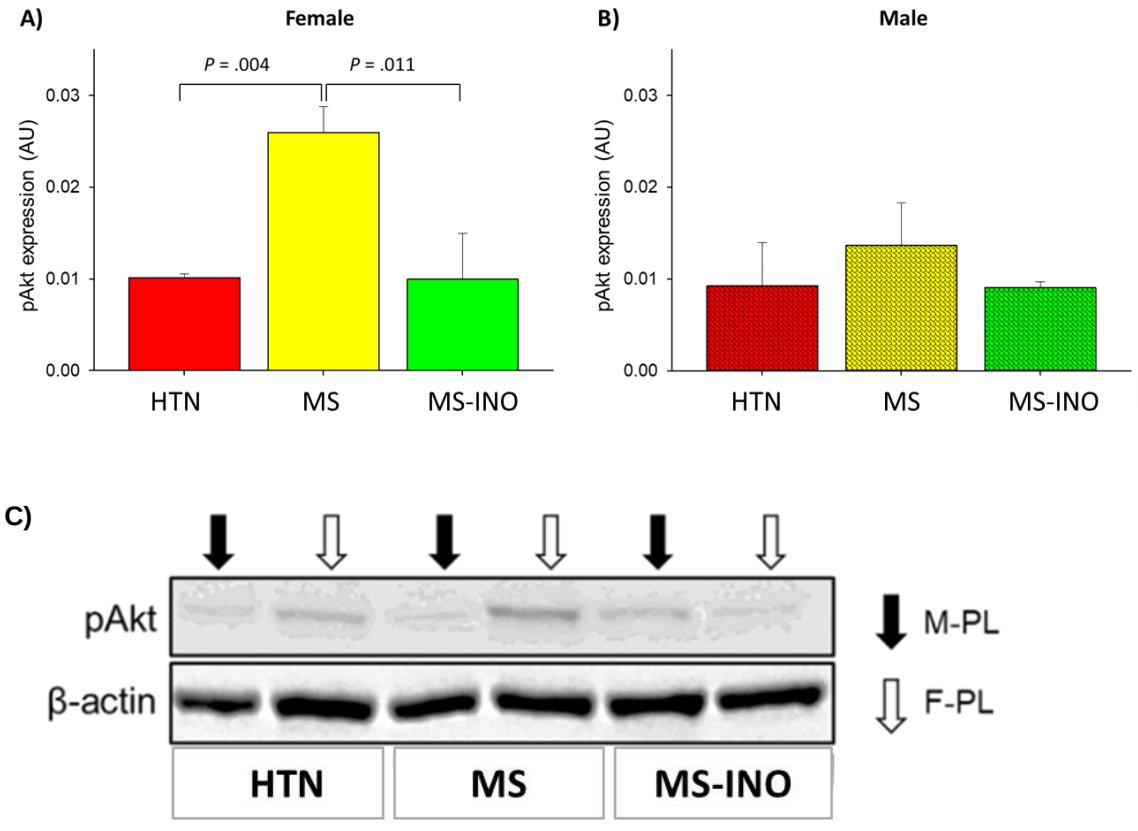


Data are reported as mean ± SEM of protein expression normalized to β-actin in Arbitrary Unit (AU). The P-value significance is indicated in the figure.

- *Phosphorylated protein kinase B (pAkt ^{Thr308})*

Similarly, phosphorylated protein kinase B at Thr308 (pAkt ^{Thr308}) level in its active form, was higher in placenta belonging to female MS compared with those obtained from HTN dams (*P*= .004) and was decreased by INO supplementation in the MS-INO placenta (*P* = .011). In males, this effect was not seen (Figure 23 A, 23 B, 23 C).

Figure 23: Placental level of phosphor Protein Kinase B (pAkt). (A) Female placenta; (B) Male placenta; (C) Relative western blot image.

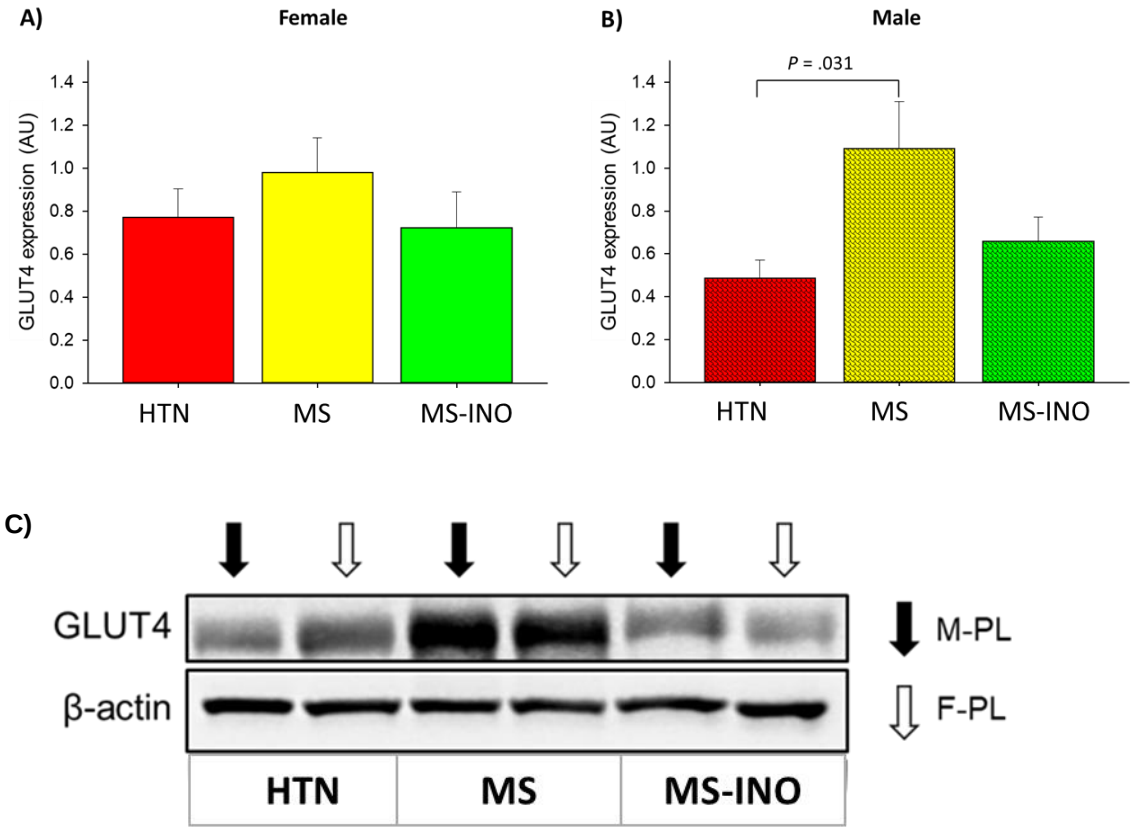


Data are reported as mean ± SEM of protein expression normalized to β-actin in Arbitrary Unit (AU). The P-value significance is indicated in the figure.

- *Glucose Transporter 4 (GLUT4)*

Glucose Transporter 4 (GLUT4) level was not different in female placenta across groups. Whereas in males, GLUT4 level was higher in the MS placenta compared to the HTN (*P*= .031) and maternal INO supplementation did not change GLUT4 level (Figure 24 A, 24 B, 24 C).

Figure 24: Placental level of GLUT4. (A) Female placenta; (B) Male placenta; (C) Relative western blot image.



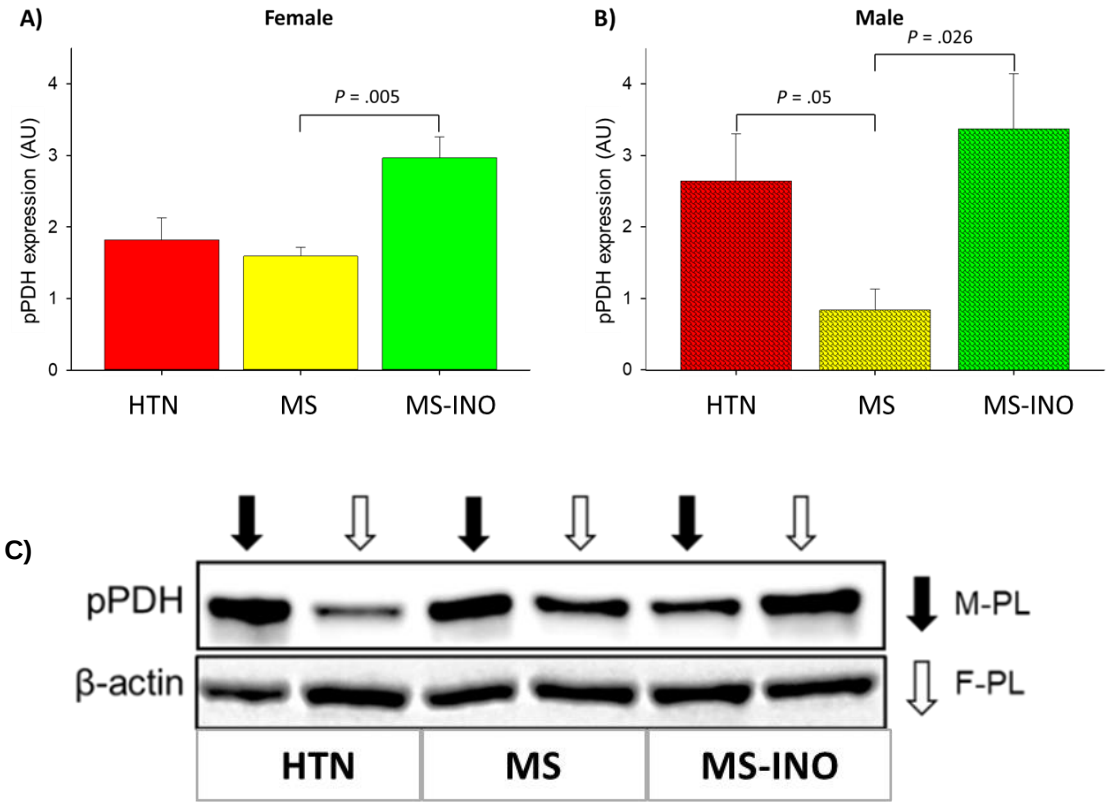
Data are reported as mean ± SEM of protein expression normalized to β-actin in Arbitrary Unit (AU). The P-value significance is indicated in the figure.

Mitochondrial Oxidative metabolism

- *Phosphorylated pyruvate dehydrogenase (pPDH^{Ser293})*

Phosphorylated pyruvate dehydrogenase at Ser293 (pPDH^{Ser293}) level in its inactive form, was similar between female HTN and MS placentas, while it was upregulated in MS-INO placenta (*P*= .005). In males, the pPDH^{Ser293} was significantly lower in the MS group compared to HTN (*P*= .05) and was upregulated in the MS-INO placenta group (*P*= .026) (Figure 25 A, 25 B, 25 C).

Figure 25: Placental level of phosphor Pyruvate Dehydrogenase (pPDH^{Se293}). (A) Female placenta; (B) Male placenta; (C) Relative western blot image.



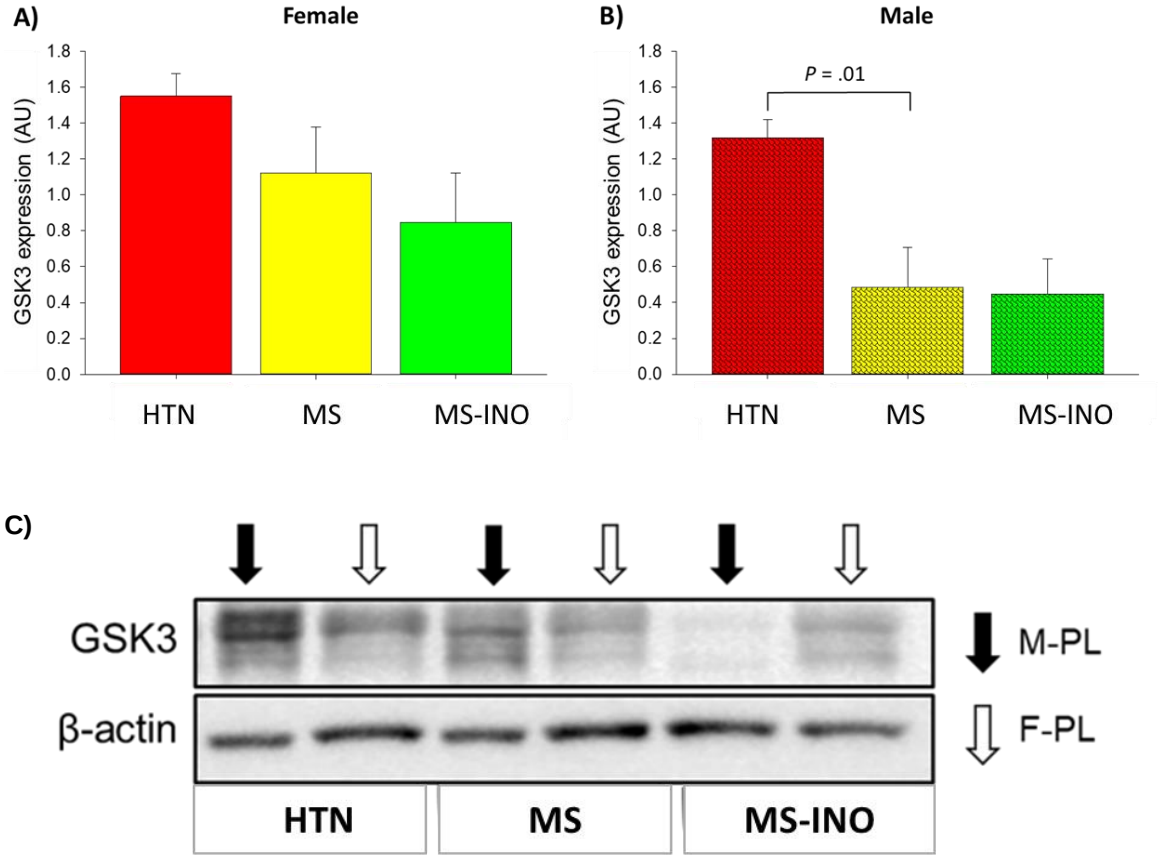
Data are reported as mean ± SEM of protein expression normalized to β-actin in Arbitrary Unit (AU). The P-value significance is indicated in the figure.

Glycogen synthesis pathway

- *Glycogen synthase kinase 3β (GSK3)*

Glycogen synthase kinase 3β (GSK3) level was not different in female placenta among groups. In males, GSK3 level was decreased in the MS compared to HTN (*P*= .01) and maternal INO supplementation did not change its level (Figure 26 A, 26 B, 26 C).

Figure 26: Placental level of GSK3. (A) Female placenta; (B) Male placenta; (C) Representative of western blot image.

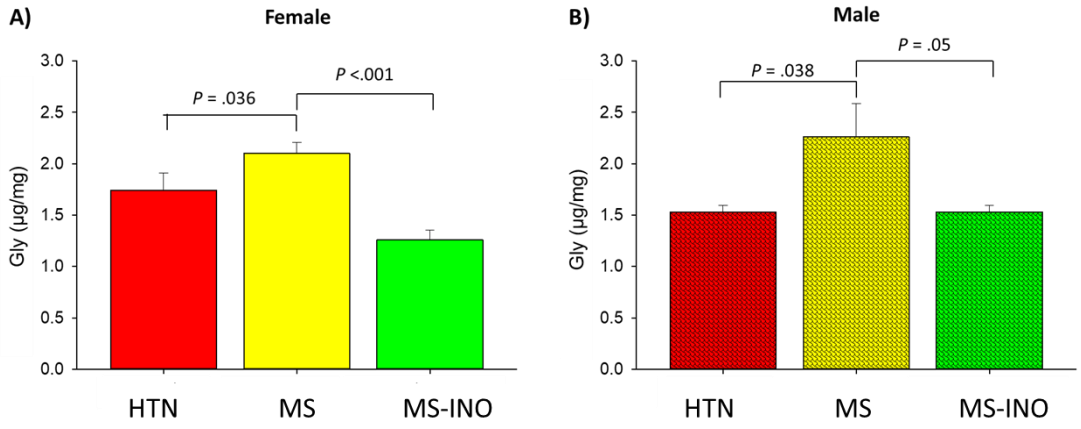


Data are reported as mean ± SEM of protein expression normalized to β-actin in Arbitrary Unit (AU). The P-value significance is indicated in the figure.

Glycogen storage

Placental glycogen storage (Gly) concentration was higher in both female and male placentas of the MS group compared to controls (F-PL, *P*= .036; M-PL, *P*= .038), and its level was lowered in the INO supplemented dams to a level similar to the HTN group (F-PL, *p*< .001; M-PL, *p*=0.05) (Figure 27 A, 27 B).

Figure 27: Placental Glycogen storage (Gly). (A) Female placenta; (B) Male placenta.



Data are reported as mean ± SEM of glycogen levels expressed in µg/mg. The P-value significance is indicated in the figure.

4.3 Fetal and offspring study

4.3.1 Food and water intake

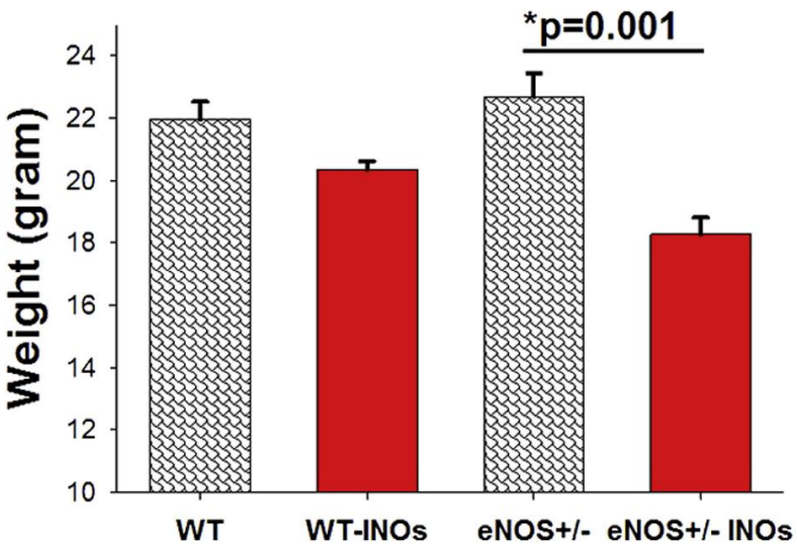
Daily food intake was similar between eNOS+/- and WT offspring born to MS dams treated with INOs mixture or placebo (eNOS+/-INOs, 8.1 ± 0.8 vs placebo, 8.6 ± 0.6 g)

and (WT-INOs, 8.2 ± 0.5 vs placebo, 8.1 ± 0.9 g). During pregnancy, daily water intake was not different between MS pregnant dams receiving INOs supplementation or placebo (INOs, 5.0 ± 0.8 vs placebo, 5.1 ± 0.9 mL).

4.3.2 Offspring weight

Maternal INOs treatment did not significantly decrease weight gain in WT offspring (WT, 21.9 ± 0.5 g vs WT-INOs, 20.3 ± 0.2 g, p=0.07). However, maternal INOs supplementation decreased the weight gain in eNOS+/- heterozygous offspring born to MS dams (eNOS+/-, 22.6 ± 0.8 g vs eNOS+/- INOs, 18.2 ± 0.5 g, p= 0.0001) (Figure 28).

Figure 28. Average weight in female offspring WT and eNOS+/- born to dams with MS with and without INOs mixture

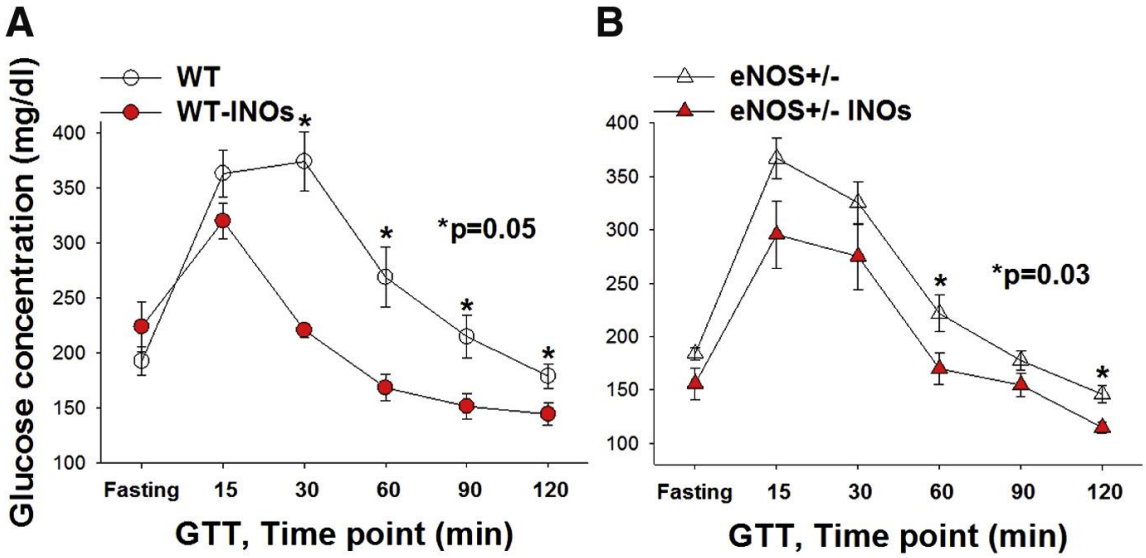


*At 10 weeks of age, weight gain was improved by INOs maternal supplementation in eNOS+/- INOs, but not WT-INOs offspring. Data are shown as mean ± standard error of the mean. Significance is indicated in the figure (*p=0.001).*

4.3.3 Offspring glucose tolerance test

The GTT showed that glucose levels at 60, 90, and 120 minutes were lower in the WT-INOs vs WT offspring born to untreated dams with MS (p=0.05) (Figure 29A). Similarly, lower glucose levels were noted at 60 and 120 minutes in the eNOS+/- INOs offspring vs eNOS+/- offspring born to untreated dams (p=0.03) (Figure 29 B).

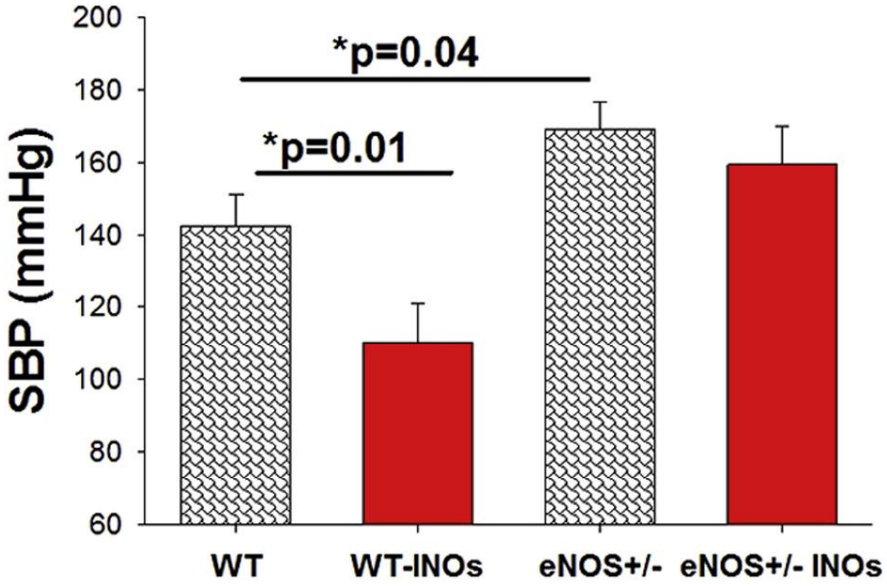
Figure 29. GTT in female offspring WT and eNOS+/- born to dams with MS treated with and without INOs mixture



4.3.4 Offspring systolic blood pressure

The average SBP was lower in WT versus eNOS+/- offspring born to dams with MS (WT, 142.34 8.79 mm Hg vs eNOS+/- 169.05 7.5 mm Hg, p =0.04). Maternal treatment of MS with INOs decreased SBP in WT-INOs offspring (110.15 10.8 mm Hg, p= 0.01) but not in eNOS+/- INOs offspring (159.24 10.7 mm Hg) (Figure 30).

Figure 30. Average SBP (mm Hg) in female offspring WT and eNOS+/- born to dams with MS treated with and without INOs mixture

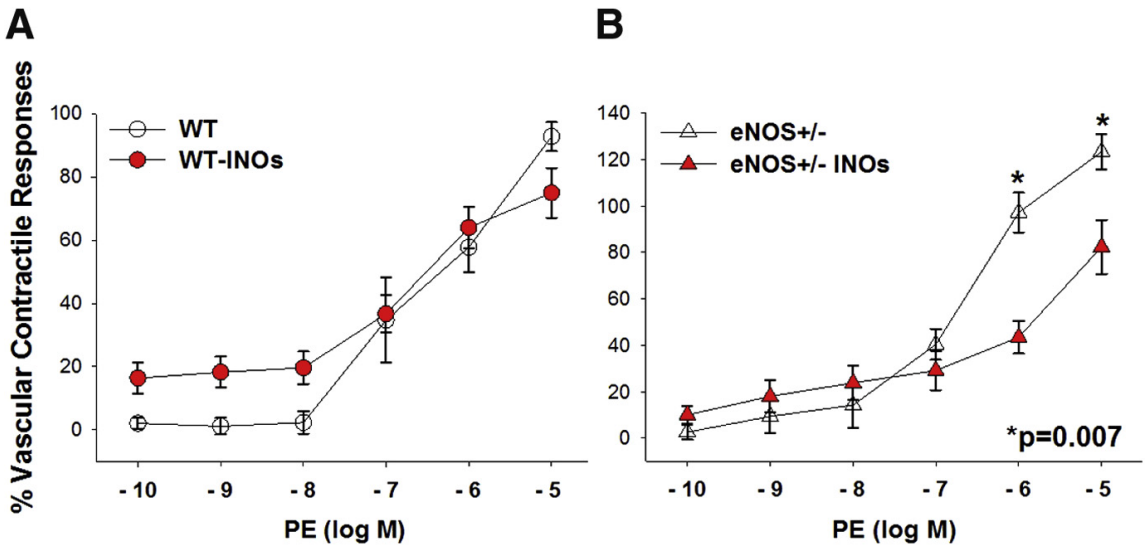


*SBP was higher in eNOS+/- offspring compared to WT born to MS dams (*p=0.04). Maternal treatment with INOs lowered SBP in WT-INOs offspring, but not in eNOS+/- INOs offspring (*p=0.01). Data are shown as mean ± standard error of the mean.*

4.3.5 Offspring vascular reactivity

The dose-response curve to PE was similar in WT offspring carotid arteries independent of INOs maternal supplementation (Figure 31A, Table 3). The vascular contractile responses to PE were decreased in eNOS+/- INOs compared to eNOS+/- offspring born to untreated MS dams (p=0.007) (Figure 31B, Table 3).

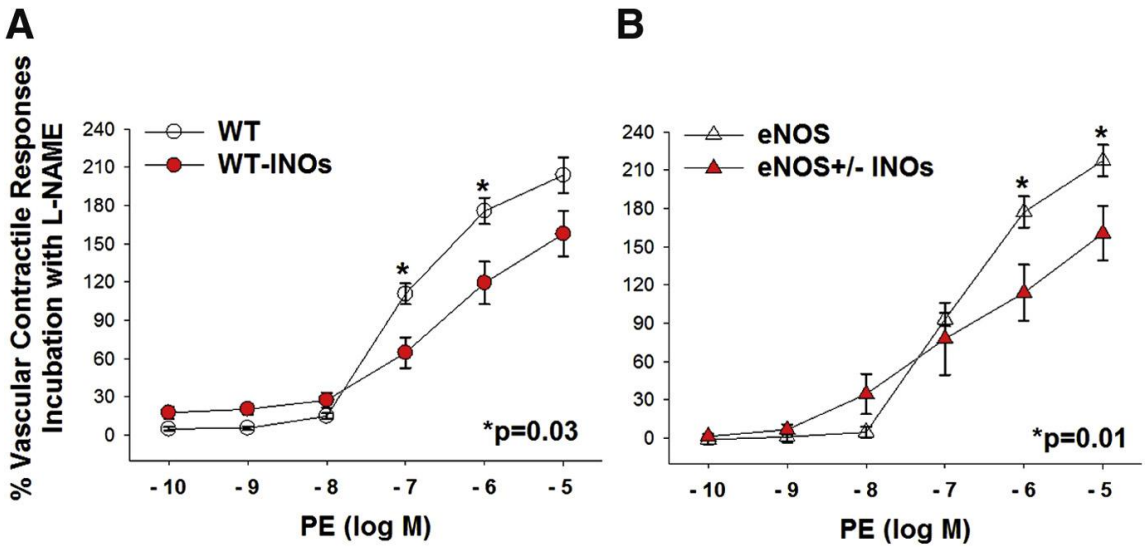
Figure 31. Effect of PE on the contractile responses in the carotid artery of female offspring WT and eNOS+/- born to dams with MS treated with and without INOs



A. *No contractile differences were seen between WT offspring born to either INOs-treated or untreated dams with MS (WT and WT-INO).* ***B.*** *PE contraction was lower in eNOS+/- INOs offspring born to INOs treated dams vs untreated ones (*p=0.007). Data are shown as mean ± standard error of the mean. Significance is indicated in the figure.*

After incubation of the carotid arteries with L-NAME, a nonspecific NO synthase inhibitor, the contractile responses to PE were decreased in WT-INOs and eNOS+/- INOs heterozygous offspring born to MS dams treated with INOs compared to offspring born to untreated dams (p=0.03, p=0.01, respectively) (Figure 32A and 32B, Table 3). This result suggested that the INOs mixture works on mechanisms different from the NO pathway in regulating vascular responses.

Figure 32. Effect of L-NAME on PE contraction in the carotid artery of female offspring WT and eNOS+/- born to dams with MS treated with and without INOs



PE response in the presence of the nonspecific NO synthase inhibitor L-NAME (10^{-4}). ***A.*** *WT-INOs had a lower contractile response to PE compared to WT (*p=0.03) offspring.* ***B.*** *Contractile PE response was lower in eNOS+/- INOs offspring compared to eNOS+/- offspring born to untreated dams (*p=0.01). Data are shown as mean ± standard error of the mean. Significance is indicated in the figure.*

The dose-response curve to the vasorelaxant ACh demonstrated that carotid artery vasorelaxation was altered in WT mice born to MS untreated dams and was improved in the WT-INOs offspring (p=0.03) (Figure 33A, Table 3). Similarly, ACh vasorelaxation, as expected, was abolished in eNOS+/- offspring and was improved in the eNOS+/- INOs offspring born to MS dams on INOs supplementation (p=0.01) (Figure 33B, Table 3). No changes were observed in any offspring group for any genotyped considered in response to SNP (Table 3).

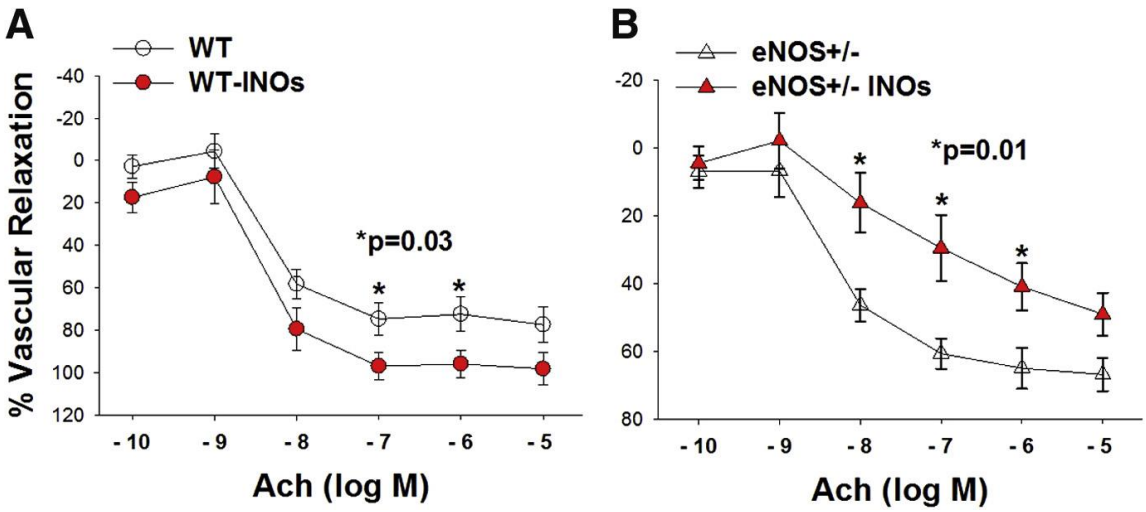
Table 3. Percent maximal effect in response to vascular contractile and relaxant agents

Drugs	**WT**	**WT-INOs**	**eNOS+/-**	**eNOS+/- INOs**
PE (10^{-10} – 10^{-5})	92.8 ± 4.6	75 ± 7.8	123.4 ± 7.5	82.4 ± 7.5*
L-NAME (10^{-4})	203.7 ± 13.7	157.8 ± 17.7#	217.4 ± 12.2	160.5 ± 21.4*
Ach (10^{-10} – 10^{-5})	77.4 ± 8.1	98.2 ± 7.6#	49 ± 6.3	66.8 ± 4.9*
SNP (10^{-10} – 10^{-5})	112.1 ± 4.7	103.5 ± 2.9	108.5 ± 7.8	112.9 ± 6.6

*Data are shown as mean ± SEM. % Max PE contraction was lower in eNOS+/- INOs compared to control eNOS+/- (*p=0.007); L-NAME increased % Max PE response, and INOs maternal treatment decrease it in eNOS+/- INOs (*p=0.03) and WT-INOs (p=0.01). ACh vasodilation was impaired in eNOS+/- offspring, and WT and was improved in WT-INOs (p=0.03) and eNOS+/- INOs (p=0.03) offspring born to dams with MS treated with INOs supplementation. No differences were seen in SNP responses. ACh, acetylcholine; eNOS, endothelial nitric oxide synthase; INOs, inositols; L-NAME, N-nitro-L-arginine methyl ester; PE, phenylephrine; SNP, sodium nitroprusside; WT, wild-type.*

** Refers to eNOS+/-; # Refers to WT*

Figure 33. Effect of ACh on the carotid artery of female offspring WT and eNOS+/- born to dams with MS treated with and without INOs



A. *ACh vasodilatory response was altered in WT offspring born to MS dams and was re-established in WT-INOs offspring born to treated dams (*p=0.03).* ***B.*** *ACh vasorelaxation was impaired in eNOS+/- offspring born to MS dams and was restored in eNOS+/- INOs offspring born to treated dams (*p=0.01). Data are shown as mean ± standard error of the mean. Significance is indicated in the figure.*

5 DISCUSSION

The murine model with partial lack of eNOS gene that we used in our experiments, combines genetic and environmental factors: the lack of maternal eNOS gene induced maternal moderate hypertension (due to decreased nitric oxide production) and *in utero* environmental abnormalities (due to HF diet-induced metabolic syndrome phenotype). The above genetic/environmental combination leads to insulin resistance and inflammation (120), participating in the dysregulation of many metabolic processes responsible for an altered fetal metabolic programming (121,122). Those features make this murine model suitable to study metabolic syndrome in pregnancy since polymorphism in the eNOS gene has also been associated with MS in humans (123).

5.1 Maternal organ damage

In the maternal study, we evaluated the end-organ damage in hypertensive and metabolic syndrome dams, compared to control wild-type pregnant mice, by looking at the fibrosis process in the main organs (heart, kidneys, and liver) and circulating biomarker of fibrosis. Furthermore, we assessed the effect of maternal inositol supplementation on maternal end-organ damage.

We found an increase in organ fibrosis, left ventricular wall thickness, and renal parenchyma damage in hypertensive and metabolic syndrome dams. Consistently we found an increase in circulating level of TGF-B and altered collagen Type 1 turnover more than Type 3, which are involved in the fibrosis process pathway.

Inositol supplementation in MS dams improved organs fibrosis, kidney parenchyma damage, and TGF-β level, and decreased collagen type 3 synthesis indicating that Inositols could influence extracellular matrix deposition.

5.1.1 Cardiac Fibrosis

Normal pregnancy is itself a pro-inflammatory, pro-thrombotic, highly insulin resistant, and hyperlipidemic state (124). The physiologic changes associated with pregnancy in the presence of an already underlying vascular disorder such as metabolic syndrome, diabetes, and hypertension can lead to further alteration in endothelial function, oxidative stress, inflammation, and growth factors that can impact an already compromised CV system, resulting in end-organ damage and increased risk of long-term maternal CVD.

The end-organ damage usually refers to damage occurring in major organs fed by the circulatory system (heart, kidneys, brain, eyes) which can sustain damage due to uncontrolled hypertension, hypotension, or hypovolemia.

A molecule, deeply involved in this process is the Transforming growth factor β1 (TGF-β1) is a pro-sclerotic cytokine that is consistently implicated in organ fibrosis and hypertrophy (125). TGF-β1 is overexpressed in hypertrophic myocardium during the transition from stable hypertrophy to heart failure (126), and up-regulation of TGF-β1 correlates with the degree of fibrosis in the pressure overloaded heart. It has been shown, in an animal model of diabetes that increased interstitial fibrosis and cellular hypertrophy are mediated by increased TGF-β1 activity and Smad2 Phosphorylation (127,128).

As far as cardiac fibrosis is concerned, collagen synthesis in the interstices of the myocardium represents its main feature. Collagens result from the synthesis of the

extracellular matrix (ECM) under physiologic and pathological conditions. Moderate amounts of collagens are beneficial for cardiac structure and function under physiologic conditions (129). On the other hand, diffuse fibrosis is associated with cardiac remodeling in conditions of pressure and/or volume overload, metabolic disorder, or ischemic insults and is characterized by unbalanced collagen turnover and excessive diffuse collagen deposition in the interstitial spaces (130). Some fibrotic factors, such as cytokines, chemokines, growth factors, hormones, and reactive oxygen species (ROS), are responsible for the activation of fibroblasts and the alteration of extracellular matrix (ECM) (131). Our results showed that the level of collagen was significantly increased in both cell lysates and supernatants. Generally, the homeostasis of the ECM is maintained by the balance between collagen synthesis and degradation.

However, when the balance breaks down due to high glucose the main characteristic of cardiac fibrosis induced by diabetes is collagen deposition in the interstices of the myocardium(132). It is reported that regulation of Smad 2/3 can adjust the levels of HG-induced collagen synthesis (133). The study of Zhang D. et al 2016 (134) confirmed that miR-155 regulates high glucose-induced cardiac fibrosis through direct targeting of TGFβ receptors, and it has an obvious impact on the TGF-β1–Smad 2 signaling pathway.

5.1.2 Renal fibrosis

Furthermore, regarding renal fibrosis, it might be the result of altered glucose metabolism, which plays a fundamental role in the inositol pathway. Indeed, the pathogenesis of diabetic nephropathy is multifactorial, and the advanced glycation end products have been postulated to play a vital role in the progression of this disease process (135). Alteration

in the inositol pathways phosphatidylinositol 3-kinase (PI3K) leads to increased cellular oxidative stress and activation of transcription factors triggering an increased synthesis of ECM proteins. As a consequence of these cellular perturbations, the tubular cells, besides the glomerulus, are likely to undergo apoptosis with worsening of tubular homeostasis, accentuation of renal injury, and acceleration of the progression of diabetic nephropathy (136).

Myo-inositol oxygenase is a cytosolic enzyme expressed predominantly in the renal proximal kidney tubules, where it catabolizes myo-inositol and channels it into the glucuronate-xylulose pathway (137,138). Myo-inositol oxygenase is the only enzyme that catabolizes Myo-inositol, and thus, plausibly acts as an important regulator of plasma inositol concentration in mammals (139). Its upregulation is associated with an intrarenal myo-inositol deficiency, which has been implicated in the acceleration of diabetic nephropathy with increased expression of fibronectin (140). Its upregulation was also noted to be associated with proximal tubular injury in vivo and that was accompanied by mitochondrial fragmentation, cytochrome-c release, and oxidative stress (141). Interestingly, overexpression of Myo-inositol oxygenase has been shown to accentuate the formation of ROS and exacerbation of injury under high glucose ambiance in renal tubular cells (142).

Moreover, mice overexpressing Myo-inositol oxygenase were noted to be susceptible to chemical injury that was confined to the proximal tubules and that seemed to be also mediated via excessive generation of ROS (143).

5.1.3 Liver Fibrosis

Moving to liver fibrosis, several studies have associated alterations in inositol metabolism with liver lipid accumulation, broadly associated with insulin resistance (144).

Other diseases involving insulin resistance such as diabetes and polycystic ovary syndrome are characterized by alterations in INOs metabolism. Therefore, given its role in other metabolic syndrome models, the hypothesis of an INS role as a supplement in liver lipid deposition and fibrosis is intriguing.

A recent systematic review on Inositol and Non-Alcoholic Fatty Liver Disease (144) reported that overall, INS deficiency was associated with increased fatty liver in animals while conversely, INS supplementation in animal models of fatty liver reduced hepatic triglycerides and cholesterol accumulation and maintained normal ultrastructural liver histopathology.

Another recent experimental study evaluated the effect of D-chiro-inositol in cholestatic liver diseases, which are important causes of liver cirrhosis and liver transplantation, and few drugs are available for treatment (145).

The authors used a cholestatic rat model, established via bile duct ligation (BDL), to whom orally administered DCI (150 $mg \cdot kg^{-1} \cdot d^{-1}$) in drinking water for 2 weeks.

They found that oral administration of DCI significantly decreased the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and attenuated bile duct proliferation, parenchymal necrosis, and fibrosis in BDL rats. Furthermore, DCI treatment significantly increased the serum and bile levels of total bile acid (TBA), and decreased TBA levels in the liver. Moreover, DCI treatment significantly increased the expression of the genes encoding bile acid transporters BSEP (Abcb11) and MRP2

(Abcc2) in liver tissues. DCI treatment also markedly decreased hepatic CD68 and NF-kappaB (NF-κB) levels, significantly decreased the serum and hepatic MDA levels markedly increased superoxide dismutase activity in both serum and liver tissues.

Ultimately, the authors used the whole-genome oligonucleotide microarray and revealed that DCI treatment altered the expression profiles of oxidation reduction-related genes in liver tissues. Collectively, DCI effectively attenuates BDL-induced hepatic bile acid accumulation and decreases the severity of injury and fibrosis by improving bile acid secretion, repressing inflammation, and decreasing oxidative stress (145).

All the above-mentioned studies can explain the promising results obtained in pregnant mouse models with metabolic syndrome and hypertension treated with Inositols in reducing the levels of cardiac, renal, and liver fibrosis by lowering circulating glucose level, indirectly modulating the extracellular matrix via the TGF-β pathway.

5.2 Placental study

In this animal model of hypertension and metabolic syndrome, no difference was found in IR-β levels among groups, but it was higher in males compared to the female placenta. Our findings support that the female placenta seems more resourceful and able to respond to the maternal insulin resistance showing increased intracellular insulin signaling pathway (↑pIRS-1 and ↑Akt); no change in glucose uptake (↔GLUT4); increased glycogen storage (↑Gly); no change in placental oxidative metabolism (↔pPDH). Males adapt differently, showing an increased placental glucose uptake (↑GLUT4) with higher oxidative damage

(↓pPDH) associated with an impairment in glycogen synthesis and storage (↓GSK3 and ↑Gly).

5.2.1 Glucose uptake pathway

Consistent with our data, other studies have found that IR-β level is higher in male placenta compared to females (146). This finding can be the result of a gender-specific adaptation to the maternal insulin resistance status. In our murine model, maternal INO supplementation did not affect IR-β placental level on either sex. Others have shown its upregulation but only in adipose tissue of diabetic rats after myo-inositol supplementation (147).

IR-β activation by insulin leads to phosphorylation of IRS-1 and Akt, which are key points in the insulin pathway. Their role in glucose homeostasis is well established in the liver, kidneys, or skeletal muscles (148,149). Studies with IRS-1 knockout mice reported that a decrease in pIRS-1 and pAkt activity in the liver and skeletal muscles is associated with insulin resistance onset (150,151). On the contrary, an increased IRS-1 and Akt phosphorylation has been demonstrated in the placenta of the murine model of maternal obesity (152).

Similarly, our data show that in response to maternal metabolic syndrome, there is an upregulation of pIRS-1 and pAkt level in female eNOS$^{+/-}$HF placenta but not in males, suggesting that the insulin signal may be tissues and gender-specific in response to maternal insulin resistance. INO supplementation did not affect the pIRS-1 level in both sexes, but it was able to restore pAkt level at a level similar to the control group in the female placenta, without affecting the GLUT4 level.

GLUT4 is the main insulin glucose transporter isoform involved in cellular glucose metabolism (152,153). Studies in adipose and liver tissue of diabetic rats have shown an increase in GLUT4 translocation in response to inositols supplementation (147). In our study, GLUT4 was upregulated in male eNOS$^{+/-}$HF placenta, unchanged in females, and was not affected by INO treatment in both sexes.

Our results highlight the diverse gender adaptations in the placental glucose uptake pathway in response to maternal metabolic status. The female placenta seems to ameliorate the intracellular insulin signaling downstream the receptor, without increasing placental glucose transport capacity. While male placenta seems to be more susceptible to perturbations in substrate availability and adjusts by increasing GLUT4 level and glucose uptake.

5.2.2 Mitochondrial oxidative metabolism pathway

PDH enzyme is part of the PD complex (PDC) which plays a dominant role in the aerobic mitochondrial metabolism of carbohydrates. Its activity is tightly regulated by the balance between the active (de-phospho-) and the inactive (phospho-) forms, modulated respectively by the pyruvate dehydrogenase kinases (PDK) and the pyruvate dehydrogenase phosphatases (PDP). PDH plays a vital role in cell survival by regulating oxygen consumption and radical oxygen species (ROS) production (154).

Our findings in this murine model reveal no changes in the female placenta, while pPDH level was decreased in male eNOS$^{+/-}$HF placenta compared to control. This supports that PDH activity increased in male offspring exposed to maternal metabolic syndrome, leading to an increase in ROS production. By our results, previous studies on animal models of

maternal obesity have shown that the male placenta is more susceptible to oxidative damage due to increased production of ROS (155,156).

Estrogens have also been implicated in modulating PDC activity (157), suggesting a gender-specific regulation of this enzyme in protecting the female placenta. Interestingly, maternal INO supplementation restored PDH activity in the placenta of both sexes by increasing its phosphorylation. Hence, INO seems to exert a gender-independent antioxidant activity in the placenta. A role of inositols in the regulation of PDC has already been reported, in balancing the dynamic state between PDH activation and inactivation by modulating PDK or PDP (158). The INO effect in the $eNOS^{+/-}$ placenta, might lead to a decreased glucose oxidation with a diversion of glucose metabolism away from glycogen storage and lactate production into oxidation.

5.2.3 Glycogen synthesis

GSK3 is a cellular regulator of metabolism, involved in cell proliferation, migration, and death (159,160). GSK3 overexpression in transgenic obese murine models was demonstrated to prevent insulin-resistance onset by increasing adiponectin production, which protects from metabolic syndrome (161,162).

Our findings show no differences in GSK3 level in the female placenta, while a decreased level was seen in male $eNOS^{+/-}$HF placenta compared to control. This suggests that male placenta exposed to maternal high fat diet might be more vulnerable as an adult to metabolic syndrome onset due to alteration in the GSK3 pathway.

Maternal INO supplementation did not affect GSK3 level in either sex. Studies in different animal models and tissues suggested that GSK3 is required for optimal myoinositol activity

and *de novo* inositol biosynthesis and that loss of GSK-3 activity causes inositol depletion (163). Furthermore, GSK3 plays a role in insulin-induced angiogenesis in fetoplacental endothelial cells, in parallel with IRS-1/Akt/eNOS pathway (164). In this animal model, the lack of eNOS production could further unbalance the GSK3 activity and disrupt the possible positive effect of myoinositol.

GSK3 inactivation leads to improvement of GS activity, thus increasing glycogen synthesis. We found an increased amount of glycogen in both female and male $eNOS^{+/-}$HF placenta compared to control.

Placental glycogen storage varies during pregnancy in a gender-dependent manner, with female fetuses showing higher levels of placental glycogen, peaking around mid-gestation (165). However, the significance of placental glycogen remains elusive: it could be a source of fuel for the placenta or a storage reservoir for later use by the fetus in times of need. In healthy pregnancies, stored glycogen is hypothesized to play a role when fetoplacental demand for glucose exceeds supply from the maternal circulation, such as in late gestation. Mounting evidence indicates that altered glycogen metabolism and/or deposition is linked to many pregnancy complications that adversely affect fetal development (166). In a poorly functioning placenta, as the one from our murine model, glycogen accumulation may reflect storage for its use, or the inability to utilize glucose from the glycogen stores when needed. In previous studies, DCI was proven to play a role in GSK3 activation, thus allowing intracellular glucose utilization when needed (167). In this model, maternal INO supplementation lowered the amount of stored glycogen in both sexes at similar levels of the control group, likely enabling the placenta to use its glycogen stores for energy production.

5.3 Fetal and Offspring Study

The fetal and offspring study demonstrated that at 10 weeks of age, eNOS+/- offspring compared to WT, both born to dams with MS, showed higher weight gain, higher glucose levels in response to GTT (lower glucose tolerance), higher SBP, and altered contractile as well as vasorelaxant responses. Maternal treatment of MS dams with the INOs mixture improved eNOS+/- INOs offspring weight, glucose tolerance, and vascular reactivity, and the WT-INOs offspring displayed better glucose tolerance, lower SBP, and improved responses to contractile and vasorelaxant agents.

INOs supplementation decreased the weight gain in the eNOS+/- INOs offspring but not in the WT-INOs mice (93).

The eNOS+/- dams, which exhibited MS during pregnancy, had an altered uterine environment due to genetically impaired NO production and an HFD.

In metabolic disorders, NO synthesis and stability are reduced (168,169).

Hence, NO is a key regulator of vascular and metabolic homeostasis. In the eNOS+/- heterozygous offspring compared to WT, NO production is lower, which can lead to further metabolic imbalance, resulting in a greater weight gain in adulthood without differences in their food consumption. The decrease in NO production leads to increased expression of proinflammatory cytokines and macrophages recruitment in adipose tissue (170,171), and INOs supplementation during pregnancy seems to prevent those derangements, avoiding the increase in weight gain in eNOS+/- heterozygous offspring.

5.3.1 Glucose metabolism

The glucose responses in the GTT were altered, being higher in WT offspring, similar to the eNOS+/- heterozygous offspring. This demonstrates that the insulin resistance was seen in the eNOS+/- offspring also is present in WT when developing in an abnormal uterine environment as in dams with MS. An HFD has been shown to impair NO production and induce insulin resistance by altering the glucose transport pathway (105,172,173). Thus, even the normal fetus with normal NO levels, developing in MS dams, might have altered NO production, leading to increased susceptibility to inflammation and altered insulin signaling in the adipose tissue and glucose homeostasis, which seems to be partially restored by INOs mixture supplementation during pregnancy (171,174). However, INOs treatment ameliorates glucose tolerance in the WT-INOs offspring, but not as much in the eNOS+/- INOs offspring. NO also is known to increase glucose transport, in part by increasing the cell membrane fraction of Glut 4, the active transporter of glucose (172,173). In eNOS knockout mice, studies have shown that lower NO levels lead to decreased insulin-stimulated glucose uptake (105). This observation theorized that the eNOS+/- offspring genotype contributes to the altered glucose responses, which cannot completely be re-established in the eNOS+/- INOs offspring in the presence of a lower level of NO production due to partial lack of the eNOS gene.

5.3.2 Vascular function

SBP was elevated in eNOS+/- and WT offspring born to untreated MS dams compared to those born to INOs-treated dams. INOs supplementation lowered SBP in WT-INOs, but not in eNOS+/- INOs offspring.

These data confirm that the hostile intrauterine environment (maternal MS) can alter fetal vascular programming regardless of offspring genotype (WT and eNOS+/-). Oxidative damage has been proved to have a pivotal role in the development of diabetic complications (175,176). INOs improved SBP in WT INOs, probably by decreasing radical oxidative species, enhancing endothelial NOS and NO bioactivity (177,178). The INOs effect became negligible in the eNOS+/- INOs offspring due to the combination of the following:

(1) eNOS deficiency, leading to lower basal levels of NO production compared to WT;

(2) an altered uterine environment due to maternal MS, causing damage in endothelial function, hyperinsulinemia, and further impairment in vascular NO synthesis (179).

Vascular contractile responses to PE were higher in eNOS+/- offspring and treatment with INOs decreased the contractile effect similar to that in WT offspring. It is known that a lack of eNOS can lead to increased vascular responsiveness to adrenergic agonists (180). The eNOS inhibitor L-NAME induced even higher PE contraction in both offspring, which was decreased by maternal treatment with INOs, and this effect was independent of the NO pathway. A reduction in eNOS activity is associated with increased susceptibility to fat-induced changes in gene expression that promote adipogenesis (180,181). Namely, adiponectin is an adipose tissue-specific protein that has been shown to improve insulin sensitivity and to exert anti-atherogenic effects by increasing NO production and preventing NO degradation by reducing superoxide anion production by endothelial cells (182,183). In support of adiponectin vaso-protective properties, studies have shown that adiponectin deficient mice display impaired endothelium-dependent vasodilation (181).

Thus, maternal MS seems to increase offspring susceptibility to changes in gene expression that alter adipogenesis and glucose homeostasis, which is worsened by the offspring genotype lacking in the eNOS gene.

Our findings suggested that the beneficial effects of INOs supplementation to dams with MS have been seen when testing the vascular function in response to ACh. Vascular relaxation in response to Ach was decreased in WT and eNOS+/- offspring to MS dams, and this effect was ameliorated by maternal INOs supplementation.

These data suggest that INOs enhance offspring tissue sensitivity through NO-independent pathways (184,185).

The relaxation to SNP, an endothelium-independent agonist, was not affected by maternal MS or by INOs treatment and offspring genotype. This was expected, as SNP is a NO donor, and the maximal relaxation of the arteries had already been achieved.

In MS, there is an increase in free radicals, which contributes to enhanced basal vascular responses, macrophage infiltration, and impaired endothelium-dependent relaxation (175,176,186,187).

Our findings suggested that maternal INOs treatment improved offspring endothelial function by reducing free radical levels in endothelial cells.

6 CONCLUSIONS

Cardiovascular and metabolic adaptations in pregnancies complicated by Obesity, HTN, and MS can provide a window of opportunity to understand the underlying pathways of early-onset CVD in women and to apply strategies that improve the intrauterine environment for optimal fetal development. In turn, this will counteract the end-organ damage which leads to long-term consequences on the mother's health.

Summarizing we found that inositols activities impacted:

1. the *maternal profile* by reducing the end-organ damage, lowering the circulating glucose level, and indirectly modulating the extracellular matrix via the TGF-β pathway;
2. the *placental metabolic pathways* in a gender-independent manner, playing a role in the placental and fetal metabolic programming since the placenta plays an essential role in nutrient transfer to the fetus and fetal metabolic programming;
3. the *fetal vascular-metabolic programming* in both normal WT and eNOS+/- offsprings by restoring the altered vascular-metabolic programming.

These data suggest a role of INOs as therapeutic agents for preventing and treating maternal obesity, hypertension, and metabolic syndrome, by their capability to regulate glucose homeostasis, improve insulin signals, modulate the extracellular matrix, reduce free radicals and enhance NO action.

Thus, it is imperative that more research is conducted to understand how introducing inositols supplementation in obstetric clinical practice could impact not solely maternal nutritional status and related fetal development, but also affect long-term health outcomes. So far, several clinical trials have shown the efficacy and safety of INOs periconceptional and gestational supplementation to counteract or prevent the onset of gestational diabetes mellitus (79,81,188,189). Meta-analyses seem to support INOs mixture as a promising intervention in pregnant women with MS to improve their vascular-metabolic profile (190,191).

Based on our findings, it is now the time to evaluate if INOs intervention would be able to improve long-term maternal and neonatal health, possibly breaking the vicious circle of non-communicable diseases.

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