Effect of maternal exposure to polystyrene micro- and nanoplastics on placental and fetal development in a mouse model of pregnancy

By

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Abstract

Plastics are ubiquitous and when released into the environment, they break down into smaller particles termed microplastics (MPs). These microparticles can be ingested by organisms and potentially accumulate in tissues and organs. Recently, MPs were found in the placentas of healthy women, raising the concern that plastic exposure may have an impact on pregnancy and fetal development. In this project, we studied the effect of maternal exposure to micro- and nanoplastics on placental and fetal growth and on placental metabolism using experimental mice. CD-1 pregnant mice were exposed to 5 μm polystyrene microplastics (PS-MPs) and, 50 nm polystyrene nanoplastics (PS-NPs) in filtered drinking water at one of four environmentally-relevant concentrations (0 ng/L (controls), 10^2 ng/L, 10^4 ng/L, 10^6 ng/L) from embryonic day 0.5 to embryonic day 17.5 (full term is 18.5 days). While the placental weights were constant in all groups at embryonic day 17.5, there was a significant effect on fetal weights, with a dose-dependent decrease in weight in the MP- and NP-exposed fetuses ($p<0.0001$). Maternal exposure to PS-MPs and PS-NPs also impacted the structure of the placenta and resulted in shorter umbilical cord lengths in all MP- and NP-exposed groups. Placental metabolite profiles were determined using ^{1}H high-resolution magic angle spinning magnetic resonance spectroscopy. The relative concentration of lysine ($p=0.003$) and glucose ($p<0.0001$) in the placenta were found to decrease with increasing MP concentration. This study highlights the impact of MP and NP exposure on pregnancy outcomes and that efforts should be made to minimize exposure to plastics, particularly during pregnancy.

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List of Abbreviations

Abbreviation	Description
ABS	Acrylonitrile butadiene styrene
ACOG	American College of Obstetrics and Gynecology
Ala	Alanine
ANOVA	Analysis of variance
Asn	Asparagine
ATR-FT-IR	Attenuated total reflectance-FTIR
ChoCCp	Choline-containing compounds
CIHI	Canadian Institute for Health Information
Cr	Creatine
DGE MRI	Dynamic glucose enhanced magnetic resonance imaging
E	Embryonic day
EDS	Energy-dispersive X-ray spectroscopy
FA	Fatty acid
FGR	Fetal Growth Restriction

FPA-FT-IR	Focal plane array-FTIR
FPWR	fetoplacental weight ratio
FTIR	Fourier-transform infrared spectroscopy
GC	Gas chromatography
GI	Gastrointestinal
Glc	Glucose
Gln	Glutamine
GSH	Glutathione
HRMAS MRS	High-Resolution Magic Angle Spinning Magnetic Resonance Spectroscopy
Ile	Isoleucine
Lac	Lactate
LC	Liquid chromatography
Leu	Leucine
Lys	Lysine
mAChRs	Muscarinic Acetylcholine Receptors
MAS	Magic angle spinning
MDA	Malondialdehyde

MPs	Microplastics
NMR	Nuclear Magnetic Resonance
NPs	Nanoplastics
PA	Polyamide
PCBs	Polychlorinated Biphenyls
PE	Polyethylene
PET	Polyethylene terephthalate
PFASs	Per- and polyfluoroalkyl substances
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctanesulfonic acid
PLS-DA	Partial least squares – discriminant analysis
PMMA	Poly methyl methacrylate
PND	Postnatal day
POPs	Persistent Organic Pollutants
PP	polypropylene
PPM	Part per Million
PS	Polystyrene

PVC	Polyvinyl chloride
Pyr-GC-MS	Pyrolysis gas chromatography-mass spectrometry
SEM	Scanning electron microscopy
SERS	Surface-enhanced Raman spectroscopy
SOD	Superoxide dismutase
TCE	Trichloroethylene
TCH	Total cholesterol
TED-GC-MS	Thermal extraction desorption-gas chromatography/mass spectrometry
TG	Hepatic triglyceride
TGA-MS	Thermo-gravimetric analysis-mass spectrometry
The	Threonine
Val	Valine
WHO	World Health Organization
WTP	Water Treatment Plants

List of Symbols

Symbol	Description
μm	micrometer
μs	microsecond
μM	micromolar
nM	nanomolar
°C	centigrade
mm	millimeter
GH	GigaHertz
kHz	KiloHertz
MHz	MegaHertz
nm	nanometer
ng	nanogram
mg	milligram
L	liter(s)
h	hour
s	second

θ	magic angle
B	magnetic field
D	dipole coupling constant
γ	gyromagnetic ratio
r	distance
$\hat{H}$	Hamiltonian
I	nuclear spin quantum number
E	energy level
N	number of nuclei
T	temperature
k_B	Boltzman constant
h	Planck constant
μ	magnetic moment
m	magnetic quantum number

Chapter 1

Micro- and Nanoplastics: A new environmental threat to human health

1.1. Definition of Microplastics and Nanoplastics

Plastics are synthetic organic polymers, derived from the polymerisation of monomers that are mainly produced from petroleum, coal, and natural gas (Thompson *et al*., 2009). Their durability, light weight, and reasonable price made them a suitable choice for manufacturing a wide range of industrial products (Andrady & Neal, 2009). It has been estimated that 33 billion tons of plastics will be produced by 2050 (Rochman *et al*., 2013). The widespread use of plastic has inevitably resulted in large amounts of plastic waste that can be found in a variety of environments (Gasperi *et al*., 2018; Rillig, 2012; Andrady, 2011; Mattsson *et al*., 2018), including the surface water of the open oceans where approximately 6600 to 35200 metric tons of plastics have been reported (Cózar *et al*., 2014). This plastic waste undergoes slow (physical and photo-oxidative) degradation into smaller pieces.

The term microplastics (MPs) was first used by Thompson *et al*. (Thompson *et al*., 2004) in 2004 when the group reported the presence of microscopic plastic fragments and fibers in marine sediments using Fourier Transform infrared spectroscopy. In 2009, Arthur *et al*. (Arthur *et al*., 2009) defined MPs as plastic particles smaller than 5 mm by proposing an upper size limit to the initial definition. Although there are still debates over the lower size limit, the definition of MPs as plastic particles with size ranging from 1 μm to 5 mm is most commonly adopted. MPs are

found in a variety of forms, mostly pellets, fragments and fibers that are insoluble in water (Frias & Nash, 2019). They are classified as primary when they are produced in the micron size range for commercial and healthcare purposes, such as their application in cosmetic products and toothpaste. Secondary MPs describe when natural environmental conditions such as abrasion, radiation, and photo-oxidation degrade plastic fragments into smaller pieces (Cole *et al.*, 2011). When plastic particles degrade into even smaller particles, they are termed nanoplastics (NPs). While the initial definition of microplastics (plastic particles <5 mm in diameter) includes particles in the nano-size range (Arthur *et al.*, 2009), they are recently defined as a separate group. Gigault *et al.* (Gigault *et al.*, 2018) proposed the definition of nanoplastics as plastic particles within a size ranging from 1 to 1000 nm that can show colloidal behavior.

1.2. Methods for the detection of MPs in the environment

Various analytical methods have been applied to study MPs in different samples such as the atmosphere, marine environment, soil, animal and human tissues.

Visual and manual screening is often the first step for detecting MPs (>500 µm) using a light microscope. Information regarding size, color, shape, and the number of particles can be obtained through observation, while polymer types are not distinguishable (Löder & Gerdts, 2015). Scanning electron microscopy (SEM) generates a high-intensity electron beam to scan the sample surface and can be applied to confirm MPs particle size. In addition, the elemental composition of MPs can be studied using a combination of SEM and energy-dispersive X-ray spectroscopy (SEM-EDS) (Crawford *et al.*, 2017).

Spectroscopic methods are widely used to determine the physical characteristics of isolated particles and are able to determine the polymer type (Löder & Gerdts, 2015). Vibrational spectroscopies, such as Raman spectroscopy and Fourier-transform infrared spectroscopy (FTIR), are the most commonly used techniques that can provide information related to MPs types, size, shape and the number of particles (Möller *et al*., 2020). In FTIR, changes in the permanent dipole moment of a chemical bond results in a signal, and this technique is able to detect MPs down to 20 μm in size (Nguyen *et al*., 2019). IR spectroscopy measures absolute frequency in which samples absorb the radiation while Raman spectroscopy works based on the polarizability of the bond and measures the relative frequency at which samples scatter the radiation. Compared with FTIR spectroscopy, Raman techniques have better spatial resolution (down to 1 μm), lower water interference, narrower spectral bands and higher sensitivity to MPs with non-polar functional groups such as polystyrene (Araujo *et al*., 2018). Polar molecules have a very weak Raman signal due to the presence of electronegative atoms which hold electrons closely, resulting in lower polarizability, while in non-polar groups the polarizability is larger resulting in intense Raman signals (Möller *et al*., 2020). Besides its application for identifying MPs in the marine environment, soil, and atmosphere, Raman spectroscopy is an appropriate vibrational technique widely used in biomedical studies. Ragusa *et al*., (Ragusa *et al*., 2021) applied Raman microspectroscopy to detect MPs in six healthy human placentas and showed that 12 different MPs fragments of 5 and 10 μm size were present in four placentas. While particles smaller than 1 μm show a very weak signal in Raman spectroscopy, Surface-enhanced Raman spectroscopy (SERS) is capable of detecting particles with sizes smaller than 1 μm (Xu *et al*., 2020). In addition, the development of Raman Tweezers has also been described as a tool for identifying NPs (<1 μm) (Gillibert *et al*., 2019).

It was recently demonstrated that quantitative determination of MP particles is possible using solution Nuclear Magnetic Resonance (NMR) spectroscopy (Peez *et al*., 2019; Peez & Imhof, 2020). Polyethylene (PE) granules (< 300 μm), Polyethylene terephthalate (PET) fibers (500 μm), Polystyrene (PS) beads (0.5-1 mm), Polyvinyl chloride (PVC) (<50 μm), Acrylonitrile butadiene styrene (ABS) (100–300 μm), and Polyamide (PA) (500 μm) were qualitatively and quantitatively analyzed at concentration ranges of 3.0×10^7- 9.3×10^8 ng/L and 5.0×10^8- 3.2×10^9 ng/L for PE, PET, PS and 2.8×10^8- 3.1×10^9 ng/L for PVC, ABS and PA in a model sample using a calibration curve method (Peez *et al*., 2019; Peez & Imhof, 2020). Quantitative NMR is used to determine the concentration of molecules using the integrated signal area of the resonances corresponding to each molecule. While this result is promising, the limit of detection for MPs using NMR (4.0×10^7-8.4×10^7 ng/L) is not sensitive enough to detect MPs at environmentally relevant concentrations. Moreover, while NMR acquisition is fast and non-destructive, to minimize overlapping resonances, the sample matrix must be removed. Thermoanalytical methods such as pyrolysis gas chromatography-mass spectrometry (Pyr-GC-MS), thermal extraction desorption-gas chromatography/mass spectrometry (TED-GC-MS), and thermo-gravimetric analysis-mass spectrometry (TGA-MS) are widely used for reliable identification and quantification of MPs in different environments (Prata *et al*., 2019). Unlike NMR, the detection limits are much lower (20 ng/L) (Ribeiro *et al*., 2020). Pyr-GC-MS starts with decomposition of samples in the heated pyrolysis unit at high temperatures (500-1400 °C) and transfer of products into the gas chromatograph for separation (Picó & Barceló, 2020). Sample preparation is required to dissolve biological matrices before performing Pyr-GC-MS, as the organic matter would interfere with particle extraction and quantification of particles in a biological tissue (Zhou *et al*., 2021). Pyr-GC-MS was recently applied for the identification and quantification of MPs in the

stomach and gastrointestinal (GI)-tract tissues of fish samples (Fischer & Scholz-Böttcher, 2017), five different seafood samples (Ribeiro *et al*., 2020), and for the first time, for quantifying polystyrene and polymethyl methacrylate (PMMA) NPs in freshwater animal tissue samples (Zhou *et al*., 2021).

Each one of the analytical techniques described above has some limitations in terms of qualitative or quantitative identification of MPs, sample preparation and detectable particle size, especially when it comes to NPs detection due to their small size range. As a result, the concentration of NPs in the environment remains unknown.

1.3. Routes of Exposure

Inhalation of indoor and outdoor air and ingestion of foods containing plastics are considered the main route of exposure to MPs and NPs for animals and humans (Prata *et al*., 2020). MPs in the air are an important source of MPs pollution with their ability to migrate from one environment to another one (Liu *et al*., 2019; Zhang, Zhao, *et al*., 2020). The main sources of MPs in the air are synthetic textile fibers and clothing, erosion of synthetic rubber tires, road dust and household furniture (Gasperi *et al*., 2018; Wang *et al*., 2021; Kole *et al*., 2017; O'Brien *et al*., 2020). The abundance of MPs in the air differs depending on the geographic region, weather conditions and human activities. For instance, more MPs can be found in urban areas compared to rural areas due to larger populations and higher atmospheric fallout, as a potential source of plastic pollution, and higher levels of MPs can be observed during times of increased precipitation (Dris *et al*., 2016; Zhang, Zhao, *et al*., 2020).

MPs are found in many food resources and ingredients such as seafood (Smith *et al*., 2018; Walkinshaw *et al*., 2020), table salt (Karami *et al*., 2017), honey, beer, milk (Diaz-Basantes *et al*., 2020), and drinking water (Koelmans *et al*., 2019). MPs have been reported in treated water from drinking water treatment plants (WTPs) (Pivokonsky *et al*., 2018), tap water (Eerkes-Medrano *et al*., 2019; Kosuth *et al*., 2018) and water bottles (Cox *et al*., 2019; Oßmann *et al*., 2018). Two important sources of drinking water, surface water and groundwater, are less polluted by MPs compared with tap water and bottled water (Koelmans *et al*., 2019). Koelman *et al.,* (Koelmans *et al*., 2019) summarized the MPs (> 1μm) concentration in different water sources and reported this concentration to be around 1.0×10^{-2} particles /L in groundwater, while it ranged from 0 to 10^4 particles/L in drinking water (Koelmans *et al*., 2019). Studies have shown that the main source of MPs in drinking water are water supply chain and product packages such as plastic bottles and caps (Zhang, Xu, *et al*., 2020; Oßmann *et al*., 2018; Schymanski *et al*., 2018), which is why people who drink bottled water could be exposed to a higher number of MPs compared to drinking tap water; 9.0×10^4 particles /year and 4.0×10^3 particles /year respectively (Cox *et al*., 2019).

The most common types of MPs in drinking water are PE, PP, PS, PVC and PET (PE ≈ PP > PS > PVC > PET) (Koelmans *et al*., 2019). This is in agreement with the high scale of their annual production and their wide range of industrial applications compared to other plastic types (Schymanski *et al*., 2018; Singh *et al*., 2021). Since MPs and NPs are present in different sources, the potential impacts of ingestion and inhalation by humans need to be investigated.

1.4. Bioaccumulation and Health Effects of Exposure to Microplastics

For a chemical to be considered of concern for human health, it must be persistent, bioaccumulative and toxic (termed PBT chemicals). To date, much of the research about MP exposure has been conducted in animals. MPs have been shown to accumulate and persist in organs of marine life (Worm *et al*., 2017). In mice, MPs and NPs have been shown to enter the body and accumulate in tissues (Deng *et al*., 2017); however, the toxicity of MPs and NPs is believed to be different due to differences in their bioavailability and the targeted organs. For instance, NPs are considered a greater threat to the nerves and reproductive system compared to larger size MPs, because studies show that organs with barriers such as the brain and gonads can block larger MPs, while NPs can pass and accumulate in those tissues (Prüst *et al*., 2020; Yin *et al*., 2021). A recent study using Pyr-GC-MS found plastic particles >700 nm in the blood of 18 of the 22 healthy participants. The plastics included PET, PE, and PS at the maximum concentration of 2.4 µg/ml, 7.1 µg/ml and 4.8 µg/ml respectively (Leslie *et al*., 2022).

Most common types of plastics are made from hazardous substances that can be released during their lifetime (Lithner *et al*., 2011). In addition, MPs and NPs have the ability to carry contaminants such as heavy metals, persistent organic pollutants (POPs), polychlorinated biphenyls (PCBs), and personal care products on their surface (Tang *et al*., 2020; Velzeboer *et al*., 2014; Wu *et al*., 2016). As such, the negative effects of MPs should be divided into the physical effects of the particles (e.g. causing gastrointestinal obstructions) and their chemical effects caused by the release of additives and adsorbed contaminants (Wang *et al*., 2021).

In vitro and animal studies have demonstrated that MPs and NPs relocate inside the body, causing metabolic disorder in adults and offspring (Lu *et al*., 2018; Luo *et al*., 2019) and have adverse effects on the brain (Prüst *et al*., 2020), behavior (da Costa Araujo & Malafaia, 2021), and the gastrointestinal (Tan *et al*., 2020), reproductive (D'Angelo & Meccariello, 2021), and immune systems (Canesi *et al*., 2015). Table 1 summarizes the experimental details of some of the latest studies on the health effects of MPs exposure in mice.

Suspended MPs and NPs in the air may enter the respiratory system and deposit in the upper airway or the smaller particles reach and deposit in the deep lung and may persist in the body for a long time (Gasperi *et al*., 2018). *In vitro* tests showed that polymeric organic fibers were present in synthetic extracellular lung fluid for at least 180 days (Law *et al*., 1990). Different toxic effects are associated with digestion of MPs when they enter the stomach and intestines through the mouth. MPs are considered as foreign bodies when they enter the tissues, so they activate the local immune system. Studies on aquatic organisms showed that particles may reach the gastrointestinal system possibly leading to an inflammatory response and to changes in the gut microbe composition and metabolism (Huang *et al*., 2020).

Table 1.1 Health effects of exposure to MPs

Ref	Mouse strain	Exposure time	Plastic type	Plastic size	Dosage (ng/L)	Results
(Lu *et al*., 2018)	CD-1 male mice	5 weeks	PS	0.5 and 50 μm	10^5 and 10^6	• PS-MPs exposure reduce hepatic triglyceride (TG) and total cholesterol (TCH) levels • PS-MPs exposure could modify the gut microbiota composition and induce hepatic lipid disorder
(Jin *et al*., 2019)	CD-1 male mice	42 days	PS	5 μm	10^5 and 10^6	• PS-MPs exposure causes metabolic disorders, induced gut microbiota dysbiosis and intestinal barrier dysfunction
(Luo *et al*., 2019)	CD-1 pregnant mice	During gestation (18 days)	PS	0.5 and 5 μm	10^5 and 10^6	• Maternal PS-MPs exposure altered the biochemical parameters of serum and liver in offspring • Maternal PS-MPs exposure increases the risk of fatty acid metabolism disorder in offspring
(Hou *et al*., 2021)	CD-1 male mice	35 days	PS	5 μm	10^5, 10^6 and 10^7	• PS-MPs exposure significantly reduced the number of viable epididymis sperm, and increased the rate of sperm deformity • PS-MPs exposure resulted in no significant change in body weight
(H. Jin *et al*., 2021)	Balb/c male mice	28 days	PS	0.5, 4 and 10 μm	10^{10}	• PS-MPs can accumulate in the testis of mice • PS-MPs exposure decrease sperm quality and testosterone • PS-MPs exposure induce testicular inflammation and the disruption of blood-testis barrier
(Choi *et al*., 2021)	CD-1 mice	2 weeks	PS	0.5 μm	10^4, $5*10^4$ and 10^5	• PS-MPs exposure results in significant changes in water consumption, stool weight, stool water contents, and stool morphology • PS-MPs exposure decreased gastrointestinal (GI) motility, intestinal length, GI hormone concentration, muscarinic acetylcholine receptors (mAChRs) expression and their downstream signaling pathway
(Zheng *et al*., 2021)	C57BL/6 male mice	28 days	PS	5 μm	$5*10^5$	• PS-MPs exposure induced inflammatory effects and exerted great disturbance on liver metabolites • PS-MP exposure increased intestinal permeability of mice •

(Y. Wang *et al*., 2022)	C57BL/6 male mice	30 days	PS	1–10 μm and 50–100 μm	10^7	• PS-MPs exposure of both sizes resulted in necroptosis and inflammation to bladder epithelium • PS-MPs with size of 1–10 μm resulted in more severe necroptosis, and 50–100 μm led to a higher degree of inflammatory injury
(S. Liu *et al*., 2022)	C57BL/6 male mice	7 days	PS	5 μm	$5*10^5$	• PS-MPs exposure significantly increased the expression of inflammation factors with intestinal immune imbalance • PS-MPs exposure aggravated the histopathological damage of colonic mucosa with intestinal immune imbalance, and exerted great disturbance on the colonic microbial community and metabolism
(Shi *et al*., 2022)	C57BL/6 male mice	180 days	PS	5 μm	10^6	• PS-MPs exposure aggravated vascular lesions and organ injuries, particularly liver, kidney, and heart injuries • PS-MPs exposure exacerbated oxidative injuries by inhibiting the activities of antioxidant enzymes and increasing the levels of the serum biochemistry indicator of organ damage
(Zhao *et al*., 2022)	C57BL/6 male mice	12 weeks	PS	0.5 and 5 μm	10^5 and 10^6	• PS-MPs exposure resulted in increased levels of fasting plasma glucose and fasting plasma insulin • PS-MPs exposure resulted in changes in the gut microbiome
(Mu *et al*., 2022)	CD-1 male mice	4 weeks	PP	5 μm	10^8, $5*10^8$ and 10^9	• PP-MPs exposure could damage liver structure and function with broken and reduced mitochondrial cristae • PP-MPs exposure induce oxidative damage through increase of malondialdehyde (MDA) and decrease of glutathione (GSH) and superoxide dismutase (SOD) in the liver

One significant knowledge gap is the impact of MPs and NPs on placental and fetal development during pregnancy. Ragusa *et al.,* (Ragusa *et al*., 2021) recently documented the presence of 12 MP fragments (three identified as polypropylene (PP) particles) with size range of 5-10 μm in the amniochorial, maternal and fetal membranes of human placenta, using Raman microspectroscopy. An *ex vivo* human placental perfusion model was studied previously to show PS NPs with diameter

up to 240 nm were able to cross the placental barrier (Wick *et al*., 2010). These studies raise concerns about the ability of MPs and NPs to penetrate in biological membranes and affect placental and fetal development.

1.5. The Placenta and Fetal Growth Restriction

The placenta is an essential organ for a successful mammalian pregnancy (Rossant & Cross, 2001). The placenta plays a vital role in fetal development as it acts like a lung, kidney and digestive and immune system. It functions as a lung to transfer oxygen, and act as a kidney in order to remove waste. As a digestive system, it absorbs all essential nutrients for fetal growth and its vital functions as the immune system protects the fetus from antigen attack (Yuping Wang, 2010). Normal placental function is required for a healthy pregnancy outcome allowing the placenta to act as the interface between the mother and the fetus, providing the hormones and growth factors necessary for fetal development and allowing the exchange of oxygen, nutrients, and waste products (Vrooman *et al*., 2016). Abnormal placental vascular development will have detrimental effects on pregnancy and will put the life of both mother and the fetus in danger (Yuping Wang, 2010). One of the most common complications of pregnancy is fetal growth restriction (FGR), defined as fetal weight below the third and 10th percentile by the World Health Organization (WHO) and American College of Obstetrics and Gynecology (ACOG) respectively (Nardozza *et al*., 2017; Chauhan *et al*., 2009). FGR is often caused by abnormal placental development (Woods *et al*., 2018). It affects 5–10% of pregnancies in the world and is the second most common cause of perinatal mortality (Nardozza *et al*., 2017). Canadian Institute for Health Information (CIHI) (Information, 2009) reported that the average rate of FGR is 8.3% in Canada which varied in

different provinces. It ranges from 5.9% in Newfoundland and Labrador and 6.8% in Prince Edward Island to 8.7% and 8.9% in Alberta and Ontario, respectively (Information, 2009).

1.6. Studying the Placenta Using Mouse Models of Pregnancy

The mouse is a well-established model to study pregnancy complications and placental dysfunction. Many of the vital genes for mouse placental development can also be found in the human placenta (Rossant & Cross, 2001). The mouse and human placenta have many similarities in vascular and cellular structure. They both have a villous hemochorial structure, meaning the villi are in direct contact with the maternal blood (Figure 1.1). The network of fetoplacental vessels in the mouse placenta are remarkably similar to the villous tree of a single cotyledon in the human placenta. This is unlike other animal models of pregnancy like sheep that have an epitheliochorial placenta where there is no trophoblast invasion of the uterine vessels (Hafez *et al*., 2010). Although the sheep is a well-established model to study fetal physiology, it is not a common model to study placental development. The rabbit has a discoid, hemochorial placenta with two cellular layers of chorion between the maternal and the fetal blood (Skoda *et al*., 2017). Rabbits used to be a common model to study placental circulation and placental transfer (Carter *et al*., 1971), but they are not very common these days due to strict rules and guidelines for housing of this animal (Carter, 2007). The guinea pig has a hemochorial placenta with a fetal/maternal transport barrier which is similar to that of the human placenta (Wilson *et al*., 2021) and is a well-established model for the study of placental transfer (Jansson & Persson, 1990) and fetal growth restriction (Anthony M Carter, 1993), but they require large housing spaces and have long generation time (gestation period is 59-72 days).

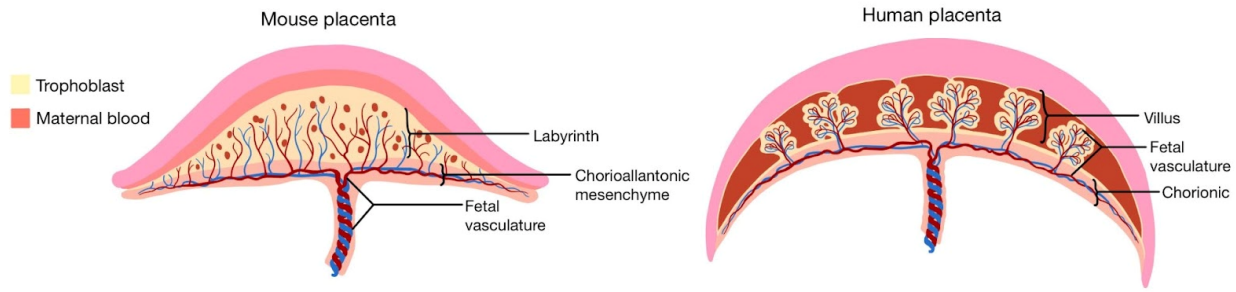

Figure 1.1: Comparing mouse and human placenta structure. Both have a discoid shape and have a hemochorial gas-nutrient exchange surface. They have similar cell types (e.g. trophoblast cells), regions (e.g. labyrinth) and both have highly branched, tree-like villi. While the mouse placenta has a single cotyledon, human placentas have multiple cotyledons and are much larger (0.12 g vs. 500 g at full-term).

Some of the advantages of working with mice compared to other animals is their small size, short gestation length, and the large litter size, which make them economically affordable. One of the most commonly used mouse strains for pregnancy research is CD-1, an outbred strain with average litter sizes of 13 (Rennie *et al*., 2012).

One difference between the mouse and human placenta is the presence of two umbilical arteries in the human placenta while the mouse has only one. There are other important differences between mouse and human pregnancy. For instance, gestational length is three weeks in mice and 40 weeks in humans. This relatively long gestation period in humans results in usually giving birth to one well-developed baby with open eyes and ears (Martin, 2003), while mice give birth to large litters of poorly developed offspring with no fur and eyes that only start to open around postnatal day (PND) 13 (Guan *et al*., 2017).

1.7. Effect of Environmental Exposure on Pregnancy

Many environmental pollutants can be transported across the placenta, potentially impacting pregnancy outcomes and resulting in reproductive toxicity (Wick *et al*., 2010; Myren *et al*., 2007). Human and animal studies have shown that environmental and genetic factors are able to affect placental development at any stage, resulting in placental insufficiency and FGR (Rossant & Cross, 2001). For instance, in studies of maternal exposure to per- and polyfluoroalkyl substances (PFASs) such as perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) in mouse models of pregnancy, the pollutants were detected in the placenta and found to be transferred across the placental barrier into the fetus. This resulted in abnormal fetal and placental weights and detrimental effects on postnatal development (e.g. neurological impairments and increased risk for chronic diseases in adulthood) (Aghaei *et al*., 2022). In a birth cohort study, it was shown that cadmium, a ubiquitous heavy metal, tends to accumulate in the placenta affecting neurodevelopment of children (Geng & Wang, 2019). Trichloroethylene (TCE) is a common environmental contaminant and an industrial solvent, that was reported to cross the placenta of pregnant women and result in FGR (Elkin *et al*., 2020). While the effects of many environmental

toxicants on placental development have been illustrated in available reports, how exposure to microplastics and nanoplastics impacts placental and fetal development is unknown.

1.8. Placental metabolism

To fully reach their growth potential, fetuses depend on a well-functioning placenta including appropriate placental metabolism. One way to study metabolism is by identifying and quantifying the metabolome, the complete set of low molecular-weight organic compounds in a biological sample, to understand metabolic networks. This approach is called metabolomics (Oldiges *et al*., 2007). It can play an important role in understanding the impact of environmental exposures on organisms, how contaminants lead to alteration in metabolites in the organism and providing insights into how biological pathways are altered following the exposure (Bundy *et al*., 2009). Metabolomics has been used widely in cancer studies including lung, bladder, breast and gastric cancer, with the goal of finding potential biomarkers and improving diagnosis of the diseases (Armitage & Ciborowski, 2017). Only a small number of studies have used metabolomics in human placental tissue to identify biomarkers of placental dysfunction and/or pregnancy complications (Dunn *et al*., 2009; Heazell *et al*., 2011; Horgan *et al*., 2010; Tissot van Patot *et al*., 2010; Kawasaki *et al*., 2019; Elshenawy *et al*., 2020; Austdal *et al*., 2015). While the sample sizes in these studies were small, the results suggested specific metabolites might be associated with FGR, preterm birth, preeclampsia and chronic maternal hypoxia.

One of the common analytical techniques to measure the metabolite concentration is NMR, which is capable of detecting numerous tissue metabolites and has been used frequently to show how exposure to environmental contaminants is changing the metabolome in the target tissue. Some of the benefits of this technique include high reproducibility, ability for quantitative analysis, simple

sample preparation, availability of online databases for confirming metabolites and also the acquired data can be used for pathway analysis using programs like Metaboanalyst (Labine & Simpson, 2020). On the downside, NMR spectra have lower sensitivity and spectral resolution compared to other methods like mass spectroscopy. High-resolution magic angle spinning (HRMAS) magnetic resonance spectroscopy at high magnetic field is capable of providing high-resolution spectra of intact biological samples without the need for complex sample preparation and metabolite extraction, so the sample can be preserved for further study using histological analysis to study the cellular structure (Kaebisch *et al*., 2017; Dietz *et al*., 2017). Dietz *et al*. (Dietz *et al*., 2017) summarized the application of HRMAS MRS to study metabolites in a wide range of human tissues and illustrated its application in the study of brain tumors and different kinds of cancers. Our group has recently used ^{1}H HRMAS MRS to study metabolites in mouse placental tissue throughout late gestation in healthy control CD-1 pregnancies (Schneider *et al*., 2022). We were able to unambiguously assign 14 different metabolites and found the relative concentration of 12 of the 14 metabolites remained constant over late gestation. This study in healthy tissue demonstrated that HRMAS MRS can be used to study placental tissue to determine the impact of environmental exposure on metabolite profiles and biochemical pathways.

1.9. Objectives and Outline of this Thesis

The goal of this thesis is to investigate the effects of gestational exposure to MPs and NPs on fetal and placental development using the mouse model of pregnancy, motivated by the urgent need for more investigation into the uptake, accumulation and the human health effect of this environmental exposure.

Chapter 2, describes the theoretical concepts of solid state NMR and how HRMAS MRS can be used for NMR-based metabolomics.

Chapter 3, presents the results of maternal exposure to Polystyrene MPs- and NPs on fetal and placental growth. Using a mouse model of pregnancy, it was shown that in late gestation, MP- and NP-exposed fetuses were significantly growth restricted.

Chapter 4, illustrates the effect of maternal exposure to Polystyrene MPs on placental metabolites using HRMAS MRS. It was shown that maternal MP exposure caused significant alterations in the relative concentration of lysine and glucose.

Finally, Chapter 5 provides a summary of the results of this thesis and discusses some interesting future research directions.

1.10. References

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Chapter 2

Nuclear Magnetic Resonance (NMR) Spectroscopy and its application in Metabolomics

2.1. Principles of NMR Spectroscopy

Nuclear Magnetic Resonance spectroscopy was first introduced by Bloch *et al,* and Purcell *et al,* in 1946, when they observed that some nuclei can absorb radio frequency radiation when they are placed in an external magnetic field (Cavanagh *et al*., 1996). Nuclei such as ^{1}H, ^{13}C and ^{31}P have properties defined as spin angular momentum which is characterized by the nuclear spin quantum number, *I*. The spin of nuclei with even numbers of protons and neutrons is zero, and the spin of atoms with odd numbers of protons and neutrons is non-zero. All molecules with a non-zero spin have a magnetic moment, μ, which is defined as:

$$\mu=\gamma\hbar m \qquad (2.1)$$

where γ is the gyromagnetic ratio, m is the magnetic quantum number:

$$m= (-I, -I+1, ..., I-1, I) \qquad (2.2)$$

and ħ is defined as:

$$\hbar=\frac{h}{2\pi} \qquad (2.3)$$

where h is the Planck constant.

In the absence of an external magnetic field (B_0) there will be 2I+1 degenerate energy states and the nucleus is at the ground state. When the sample is interacting with the external magnetic field, the energy levels will split and the energy of each state is defined as:

$$E=\mu.B_0 \qquad (2.4)$$

$$E=\gamma\hbar mB_0 \qquad (2.5)$$

The Boltzmann distribution, equation (2.6), describes the distribution of the population in each state:

$$\frac{N_m}{N} = \exp(\frac{-E_m}{k_B T}) \qquad (2.6)$$

Where N_m is the number of nuclei in state m and N is the total number of spins, T is the absolute temperature and $k_B$is Boltzman constant. The population in each state depends on the type of the nuclei and the strength of the magnetic field and is in the order of part per million (ppm), which is why NMR spectroscopy is considered a relatively low sensitive technique. Signal in NMR spectroscopy is generated when a spin flips between the energy levels after receiving radio frequency radiation (Figure 2.1) (Günther, 2013).

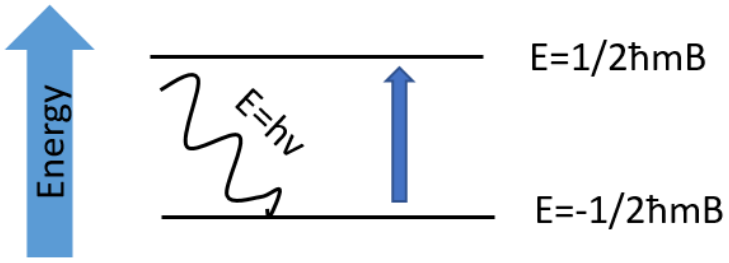

Figure 2.1: Transition of nuclear spin between energy levels after radiation of radio frequency pulse

In a molecule, all nuclei are surrounded by electrons and in the presence of an external magnetic field, the circulation of these electrons result in a local magnetic field (B_{local}). Consequently, the effective magnetic field (B_{eff}) experienced by each nuclei is different from the external magnetic field, and is defined as:

$$B_{eff}=B_0- B_{local} \qquad (2.7)$$

This local magnetic field will shield the nuclei and result in receiving a different magnetic field from the external applied magnetic field. As a result, the resonance frequency for each nucleus is different and depends on the amount of shielding and the electron environment of that nucleus.

In NMR spectroscopy, the signal comes from the sum of five Hamiltonians described in equation (2.8). $\hat{H}_z$ describe the interaction of spin with the external magnetic field which is the most dominant interaction, and other interactions which contribute to B_{local} are, dipole-dipole coupling ($\hat{H}_D$), chemical shielding ($\hat{H}_{CS}$), interaction of spin with the applied radio frequency field ($\hat{H}_{rf}$) and the quadrupolar coupling ($\hat{H}_Q$)

$$\hat{H}=\hat{H}_z+ \hat{H}_D+ \hat{H}_{rf}+ \hat{H}_{CS}+ \hat{H}_Q \qquad (2.8)$$

2.2. Solid State NMR Spectroscopy (SSNMR)

Solid-state NMR (SSNMR) spectroscopy is one of the valuable techniques for studying the molecular structure and dynamics in different systems. Besides the low natural abundances or the low gyromagnetic ratios of many NMR active nuclei, anisotropic effects are more dominant in SSNMR compared to liquid state NMR spectroscopy, resulting in the presence of broad peaks. Specifically, in solid, rigid samples the strong dipole interactions are not averaged to zero, leading to significant line broadening, while in solution, the rapid random tumbling of molecules averages the interaction to zero, and their direct effect is not observable in the NMR spectrum. Over the past 50 years the invention of techniques that enable scientists to acquire high resolution NMR spectra of a solid or semi-solid sample, increased the popularity of SSNMR spectroscopy (Duer, 2008). One of the progresses in the field of SSNMR spectroscopy is the concept of magic angle spinning (MAS) SSNMR (MRS) which allows for direct measurements of solid and semi-solid samples. HRMAS was first introduced by Andrew *et al.* (Andrew *et al*., 1958) in 1958, when the group reported that molecular rotation in solids samples decreased the width of the observed nuclear magnetic resonance spectra. MAS SSNMR significantly increases the spectral resolution of solid samples by averaging the anisotropic interactions, including dipole-dipole interactions, chemical shield anisotropy and quadrupole interactions as well as the isotropic bulk magnetic susceptibility.

The dipolar coupling (D), which is the interaction of the nuclear spin and the local magnetic field generated by other neighboring spins, is defined as:

$$D = \frac{\mu\hbar\gamma_I\gamma_S}{4\pi r^3} I_z S_z (3\cos^2\theta - 1) \qquad (2.9)$$

Equation 2.9 Shows that the dipolar constant, D, between two nuclei, I and S, is proportional to the gyromagnetic ratio, γ, the distance between the nuclei, r, and the angle (θ) between the external magnetic field (B_0) and the internuclear vector (Figure 2.2).

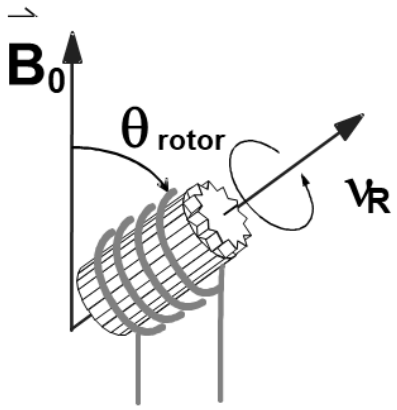

Figure 2.2: Illustration of the angle (θ) between the external magnetic field (B_0) and the sample inside the rotor.

In order to average the interactions, the term $(3\cos^2\theta - 1)$ must be equal to zero resulting in θ=54.74° , known as the magic angle. When the solid sample inside the rotor is inserted in the NMR spectrometer, the artificial movement at the magic angle results in higher resolution and sharper spectra, and increasing the spinning rate results in narrower peaks. The improved resolution of MAS SSNMR opened new avenues to study the structure and dynamics in solid samples such as polymers, nanoporous materials and solid-state conducting materials. The technology led to applications in the study of cellular metabolism in non-liquid biological tissue, termed high-resolution magic angle spinning magnetic resonance spectroscopy (HRMAS MRS) (Dietz *et al.*, 2017).

2.3. Application of ^{1}H NMR Spectroscopy in Metabolomics

Metabolomics is the identification and quantification of metabolites in a biological sample (Idle & Gonzalez, 2007). The focus of metabolomics is mostly the study of low molecular-weight metabolites including lipids, amino acids, peptides, and carbohydrates using different analytical methods. The two most common techniques for metabolomic studies are gas chromatography (GC) and liquid chromatography (LC) coupled to MS and NMR spectroscopy (Zhang *et al*., 2012). While more than 80% of publications in the metabolomic field have applied GC-MS or LC-MS, the application of NMR spectroscopy increased over the past fifteen years (Emwas *et al*., 2019). Unlike other available analytical methods, NMR is not limited by sample preparation and tissue extraction methods. Intact unprocessed tissue and organ samples, solid or semi-solid samples can be studied using SSNMR and HRMAS MRS (Blondel *et al*., 2016; Hong *et al*., 2009). HRMAS MRS has lots of applications in biomedical and biochemical fields and can be applied to study tissue metabolites, providing insight into how biological systems work. One of the advantages of NMR spectroscopy is its non-destructive nature compared to mass spectroscopic techniques. Consequently, NMR can be applied for real-time analysis of the metabolomic profile of living samples (Motta *et al.*, 2010). In addition, as HRMAS MRS will not change the structure of the tissue sample, the sample can be used for further analysis like histopathological analysis.

NMR spectroscopy also has lots of clinical applications providing metabolite imaging through MRS and magnetic resonance imaging (MRI) (A.-Q. Lin *et al*., 2014; G. Lin *et al*., 2017). Quantitative analysis of metabolites and the knowledge of metabolite profiles can help clinicians in early diagnosis and treatment of different diseases (Dietz *et al*., 2017; Kaebisch *et al*., 2017). This technique has been applied to study cancer and tumors to detect changes in metabolic pathways and also monitoring cancer drug therapy. For instance, metabolomic analysis using MRS

was shown to be able to provide useful biomarkers of pancreatic, gastric, breast and liver cancer (Beger, 2013).

Although NMR spectroscopy has a high level of reproducibility, one of the disadvantages of this technique is its low sensitivity compared to other methods. While mass spectroscopy is capable of detecting more than 1000 metabolites with concentrations of more than 10 to 100 nM, NMR spectroscopy can only detect about 50 to 200 metabolites with concentration of more than 1 μM (Emwas *et al.*, 2019). New improvements in NMR spectroscopy such as the application of HRMAS MRS is capable of improving signal sensitivity and spectral resolution. Other new improvements in NMR spectroscopy which results in having better signal sensitivity include access to more advanced pulse sequences, improvements in designing of probes and availability of higher external magnetic fields (1 GHz). While different reports are available showing the application of HRMAS MRS in studying tissue metabolites (Cheng *et al.*, 1998; Ratai *et al.*, 2005), the application of HRMAS MRS during pregnancy is very new. Austdal *et al.* (Austdal *et al.*, 2015) used ^{1}H HRMAS MRS to study placental tissue from 15 normotensive and 19 preeclamptic pregnancies. They identified 25 different metabolites in the tissue, several of which were correlated to disease severity. The long-term goal of HRMAS MRS studies in placental tissue is two-fold: first, to help identify causal metabolic pathways during post-mortem analysis and second, to be used *in utero* to improve diagnosis, prognosis and to optimize pregnancy management. Two small proof-of-concept studies during human pregnancy suggest specific metabolites may represent early biomarkers of placental dysfunction in women with growth-restricted fetuses (Denison *et al.*, 2012; Macnaught *et al.*, 2015). These studies suggest HRMAS MRS has tremendous potential for use during pregnancy.

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Chapter 3

Maternal exposure to micro- and nanoplastics causes fetal growth restriction in mice

3.1. Statement of Co-Authorship

This chapter was submitted under the above title and has been published at the journal of Environmental Science & Technology Letters, at April 2022.

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Author Contribution Statement: Z.A. performed the experiments. Z.A., L.S.C. analyzed the data. Z.A., L.S.C. interpreted results of the experiments. Z.A., L.S.C. prepared figures. J.G.S., A.A.B., J.C.K., P.A.H., K.J.J., L.S.C. conceived and designed the research and drafted the manuscript. Z.A., J.G.S., A.A.B., J.C.K., P.A.H., K.J.J., L.S.C. approved the final version of the manuscript.

3.2. Abstract

Plastics are ubiquitous and when released into the environment, they break down into smaller particles termed microplastics (MPs) and nanoplastics (NPs). These MPs and NPs can be ingested by organisms and potentially accumulate in tissues and organs. Recently, MPs were found in the placentas of healthy women, raising the concern that plastic exposure may have an impact on pregnancy and fetal development. In this study, we investigated the effect of maternal exposure to plastics on fetal and placental growth using experimental mice. In late gestation, MP- and NP-exposed fetuses were significantly growth restricted. This study represents a crucial first step towards evaluating risks of plastics exposure to human pregnancies.

3.3. Introduction

The worldwide use of plastics has resulted in a vast accumulation of microplastics (MPs) (plastic particles with a diameter of <5 mm) and nanoplastics (NPs) (diameter of <1 μm) in the air, soil, and water (Prata, 2018; Thompson *et al*., 2004; Yang *et al*., 2021). The detrimental effects of the accumulation of MPs in organs of marine life has been documented extensively (Worm *et al*., 2017); however, their effects on human health remain largely unknown. Inhalation and ingestion (via food and drinking water) are considered the main routes of exposure of humans to MPs (Zhang *et al*., 2020). MPs and NPs are known to enter the body and accumulate in tissues (Deng *et al*., 2017); however, the toxicities of MPs and NPs are believed to be different due to differences in bioavailability and the target organs (Yin *et al*., 2021). *In vitro* and animal studies have demonstrated that MPs and NPs relocate inside the body, causing metabolic disorder in adults and offspring (Lu *et al*., 2018; Luo *et al*., 2019), and have adverse effects on the brain (Prüst *et al*.,

2020), behavior (da Costa Araujo & Malafaia, 2021), and the gastrointestinal (Tan *et al*., 2020), reproductive (D'Angelo & Meccariello, 2021), and immune systems (Canesi *et al*., 2015).

One significant knowledge gap is the impact of MPs and NPs on placental and fetal development during pregnancy. Ragusa *et al.* (Ragusa *et al*., 2021), recently documented the presence of 5−10 μm MPs using Raman microspectroscopy in the human placenta. In four of six placentas from healthy pregnancies, 12 MP fragments were detected (four were identified as polypropylene and the remainder were from MPs used in paints, coatings, and dyes). This study raises concerns about the effect of MPs on placental and fetal development. The mouse is a well-established model of human pregnancy (Georgiades *et al*., 2002), providing the opportunity to establish causality between environmental exposure and adverse pregnancy outcomes such as fetal growth restriction. In the study presented here, we investigated the effect of maternal exposure to plastics on placental and fetal growth using experimental mice, specifically studying the impact of particle size and concentration on pregnancy outcomes.

3.4. Materials and Methods

3.4.1. Chemicals

The 5 μm polystyrene MPs (PS-MPs) and 50 nm polystyrene NPs (PS-NPs) were purchased from Microspheres-Nanospheres (Cold Spring, NY) (stock solution concentration of 25 mg/mL). The 5 μm particle size was selected to represent the MPs detected in the human placenta (Ragusa *et al*., 2021), and 50 nm was selected to represent exposure of NPs. All solutions for drinking water (10^2, 10^4, and 10^6 ng/L) were prepared using the stock solution and diluting with regular filtered water (three-phase micrometer filtration system; 5, 1, and 0.2 μm). The two lowest concentrations

were selected to span the range of exposure in human drinking water (Koelmans *et al*., 2019), and the third concentration was chosen to determine the effects of a high concentration. The control mice received the same filtered drinking water.

3.4.2. Animals

Healthy adult CD-1 female mice (7−16 weeks of age) were purchased from Charles River Laboratories (St. Constant, QC) and mated in house. The presence of a vaginal plug on the morning after mating was designated as embryonic day 0.5 (E0.5). The full term for CD-1 mice is 18.5 days. Each pregnant mouse was housed individually in a standard cage under a 12 h light:dark cycle and with ad libitum access to food and water (either regular filtered drinking water or filtered drinking water with PS-MPs or PS-NPs) during gestation. Pregnant mice were randomly assigned to one of seven groups: control (n = 14), 10^2 ng/L PS-MPs (n= 12), 10^4 ng/L PS-MPs (n= 11), 10^6 ng/L PS-MPs (n= 11), 10^2 ng/L PS-NPs (n= 12), 10^4 ng/L PS-NPs (n= 11), or 10^6 ng/L PS-NPs (n= 11). The sample size is unequal between groups because of high pregnancy success rates during the final timed matings. At E17.5, dams were euthanized by cervical dislocation and fetoplacental units were dissected from the uterus. All of the fetal weights, placental weights, and umbilical cord lengths were recorded. All animal experiments were approved by the Institutional Care Committee at Memorial University of Newfoundland and conducted in accordance with guidelines established by the Canadian Council on Animal Care.

3.4.3. Statistical Analysis

All statistical tests were performed using the R software package (www.r-project.org). All data are presented as means and 95% confidence intervals. The maternal weights, litter sizes, numbers of fetal resorptions (resorptions/total implantation sites per mouse), fetal weights, placental weights, fetoplacental weight ratios, and umbilical cord lengths were analyzed using a two-way analysis of variance (ANOVA) to evaluate the effects of particle size (5 μm and 50 nm) and concentration (0, 10^2,10^4, and 10^6 ng/L). Litter was a significant factor in the fetal and placental weights, fetoplacental weight ratios, and umbilical cord lengths; thus, litter means were used for statistical analysis. If the ANOVA is significant, Tukey post hoc tests were performed. A p value of <0.05 was taken to be significant.

3.5. Results and Discussion

At E17.5 (full term is 18.5 days), there was no effect of particle size or concentration on maternal weight, litter size, or fetal resorption (Figure 3.1).

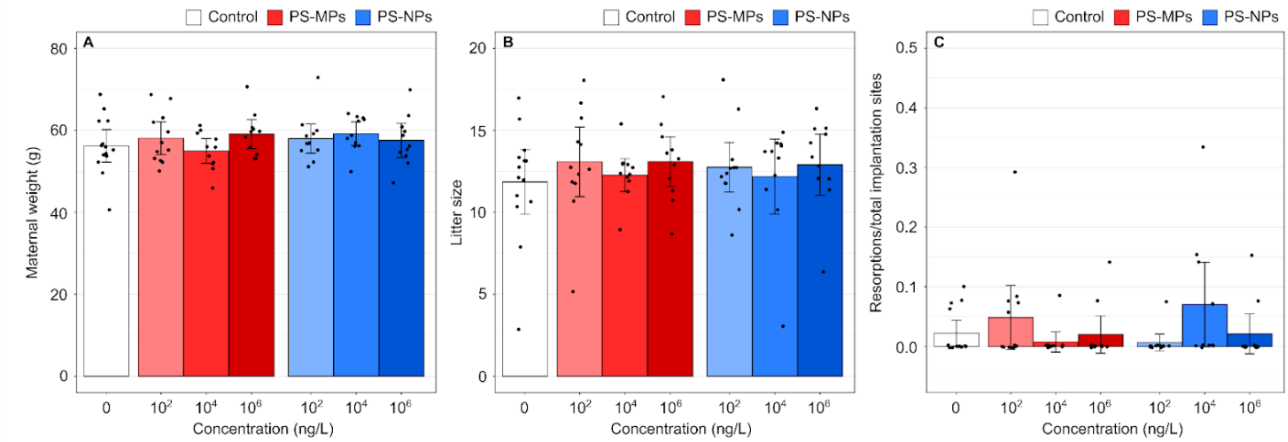

Figure 3.1. There was no effect of particle size or concentration on (A) maternal weight, (B) litter size, or (C) fetal resorptions in polystyrene microplastics (PS-MPs, red) or polystyrene nanoplastics (PS-NPs, blue) compared to controls (white). n = 11-14 dams/group. Data are shown as means and 95% confidence intervals.

After maternal exposure to PS-MPs and PS-NPs, the E17.5 fetuses weighed significantly less than controls at concentrations of 10^4 and 10^6 ng/L [$p < 0.0001$; two-way ANOVA (Figure 3.2A)], indicating that MP- and NP-exposed fetuses failed to reach their genetically predetermined growth potential. The difference in fetal weights was most pronounced in the group exposed to 10^6 ng/L PS-NPs, with a 12% decrease compared to the controls. In contrast, placental weights remained similar among all groups (Figure 3.2B). There Was a significant effect of concentration on the fetoplacental weight ratio (FPWR), a measure of fetal health and placental efficiency (Perry *et al.*, 1995) (Figure 3.2C). A reduced FPWR is consistent with fetal growth restriction and suggests the placenta has failed to maintain adequate nutrient transfer capacity to support fetal weight gain (Hayward *et al.*, 2016). Maternal exposure to PS-MPs and PS-NPs also impacted the structure of the placenta, with a significant effect of both concentration and particle size on the umbilical cord

length [$p < 0.0001$, and $p < 0.01$ (Figure 3.2D)]. The PS-NPs resulted in shorter cords at all concentrations and a decrease of ~30% at the highest concentration for both particle sizes. In humans, short umbilical cords have been associated with decreased fetal growth and fetal compromise (Krakowiak *et al*., 2004). Our group has previously found short umbilical cords in established mouse models of fetal growth restriction (Cahill *et al*., 2018; Cahill *et al*., 2019).

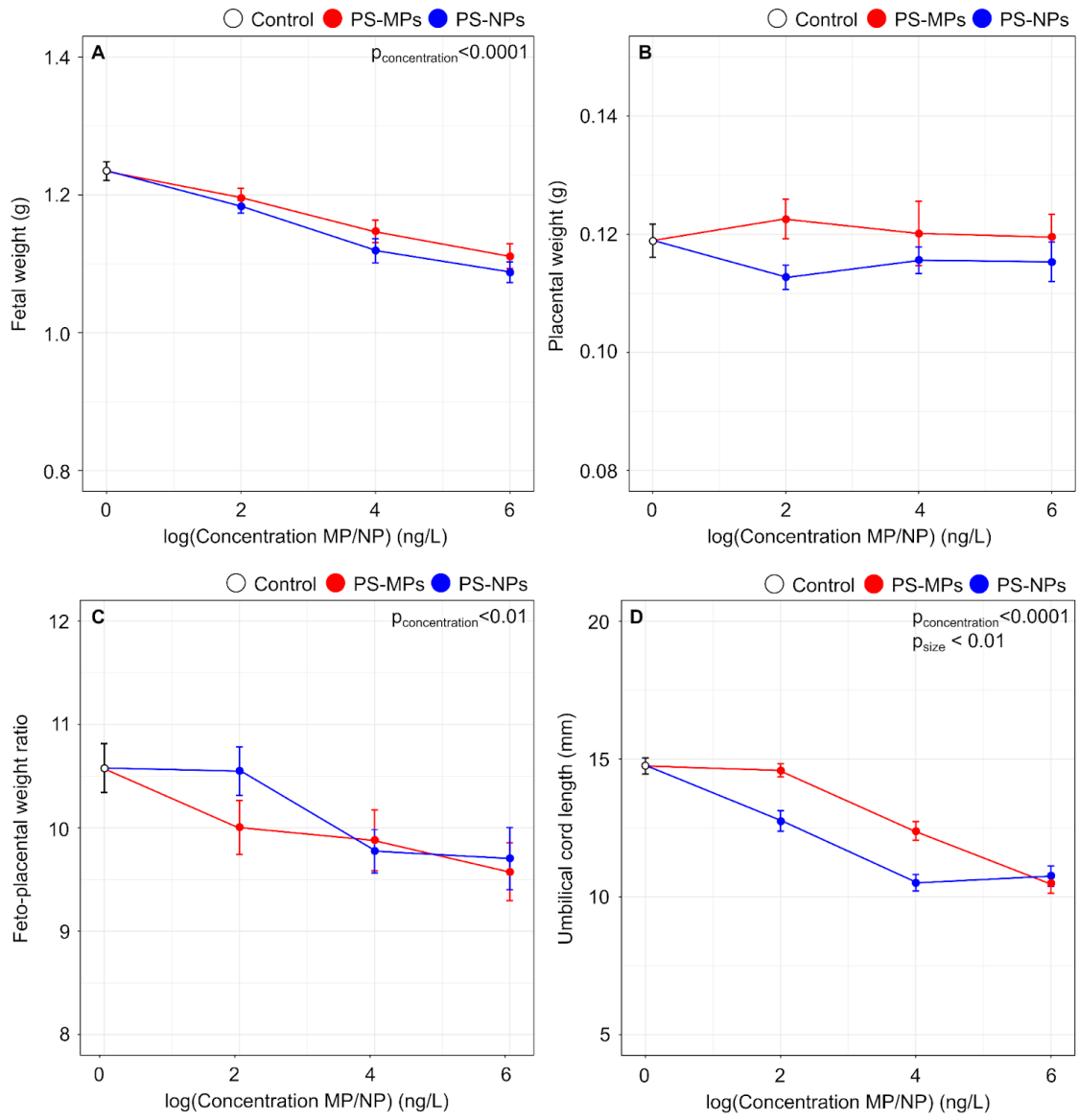

Figure 3.2: Developmental changes following maternal exposure to micro- and nanoplastics. (A) Fetal weights decreased significantly with an increase in the concentration of polystyrene microplastics (PS-MPs, red) and polystyrene nanoplastics (PS-NPs, blue) compared to controls (white) ($p < 0.0001$; two-way ANOVA). (B) There was no significant effect of particle size or concentration on placental weight. (C) The fetoplacental weight ratio decreased significantly with an increase in the concentration of MPs and NPs ($p < 0.01$; two-way ANOVA). (D) The umbilical

cord length decreased significantly with an increase in concentration ($p < 0.0001$; two-way ANOVA), and the cord length was significantly different for different particle sizes ($p < 0.01$; two-way ANOVA). The main effects of concentration and particle size are noted as Pconcentration and Psize, respectively. n=11−14 dams/group. Data are shown as means and 95% confidence intervals.

Maternal exposure to MPs and NPs resulted in fetal growth restriction that may have resulted from insufficient placental nutrient transfer to meet the demands of the growing fetus. Our study presents the first evidence of the detrimental effect of MPs and NPs on fetal growth. Previous work has demonstrated the negative impact on body weight of exposure to a high concentration of PS-MPs for at least 3 weeks in adult male CD-1 mice (Lu *et al*., 2018). A study exposing pregnant mice to PS-MPs throughout gestation did not find differences in offspring body weight at weaning (21 days of age) (Luo *et al*., 2019). In light of our results, this suggests rapid catch-up growth occurs during the first 3 weeks of life, consistent with humans born small for gestational age (Yanney & Marlow, 2004). The offspring prenatally exposed to MPs showed evidence of fatty acid metabolism disorder, a long-term health outcome that is associated with fetal growth restriction (Dessì *et al*., 2012). A striking feature of this study was the decrease in umbilical cord length, even at a low concentration of PS-NPs. It is believed that umbilical cord growth occurs in response to tensile forces resulting from fetal movement (Moessinger *et al*.,1982). Decreased fetal movement is a hallmark sign of central nervous system dysfunction and suggests abnormal brain development in the MP- and NP-exposed fetuses. It is well-established that growth-restricted fetuses exhibit abnormal neurodevelopment and decreased global activity prenatally as well as sustained developmental impacts in childhood (Leitner *et al*., 2007; Baschat, 2011). The impact of PS-NPs on postnatal brain development has recently been reported in mice, with evidence of

neuronal dysfunction and cognitive deficits after maternal exposure to very large doses (>500μg/day) (Jeong *et al.*, 2022). In 2019, the World Health Organization (WHO) published a report stating that MPs in drinking water pose a low concern for human health (Marsden *et al.*, 2019). However, they concluded that evidence is limited and further research is needed, particularly about the impact of NPs. As summarized in a recent systematic review (Koelmans *et al.*, 2019), the reported concentrations of MPs (diameter of >1μm) in drinking water ranged from 0 to 10^4 particles/L. In the study presented here, the concentrations ranged from 1.5×10^3 particles/L (10^2 ng/L MPs) to 1.5×10^{13} particles/L (10^6 ng/L NPs), with a significant impact on fetal growth starting at 1.5×10^5 particles/L (Figure 3.2 A,B).

In agreement with the WHO report, this suggests most human pregnancies will not be exposed to levels of plastics that cause significant growth restriction. However, there are areas with water sources that exceed this concentration, including the Los Angeles River, where MPs have been reported at levels of $>1.0 \times 10^6$ particles/L (Eerkes-Medrano *et al.*, 2015). Moreover, our data suggest NPs have an even greater impact on fetal outcomes and efforts should be focused on determining the levels of NPs in drinking water. It is clear from Figure 3.3 that it is not the number of particles alone but also particle size (MPs vs NPs) that needs to be considered when minimizing the impact of plastics on adverse fetal outcomes. This should be kept in mind when developing regulatory standards for ingested plastic, which may depend on particle size, akin to how ambient air quality criteria exist for particulate matter of different particle sizes (e.g., <2.5 μm vs<10 μm). It should be noted that we cannot extrapolate the results to conclude that even smaller plastics (<50 nm particles) will result in more severe fetal growth restriction. Without knowing the mechanism (e.g., blockage of capillaries in the placenta or accumulation in the fetal brain), the particle size-dependent effect needs to be explored further.

In summary, fetal growth restriction is one of the most common complications of pregnancy and exposure to plastics may be a risk factor. Similar to exposure to other environmental contaminants, exposure to MPs and NPs in utero could potentially impact neurodevelopment and chronic diseases later in life. While this study used pristine plastics, future work should focus on the impact of exposure to weathered plastic to more accurately represent environmental conditions. Moreover, there is a need for basic research into the mechanism of action of MPs and NPs that causes growth restriction. Our data support further studies of exposure in animal models and humans, particularly during pregnancy, that will inform regulations to minimize exposure to MPs and NPs.

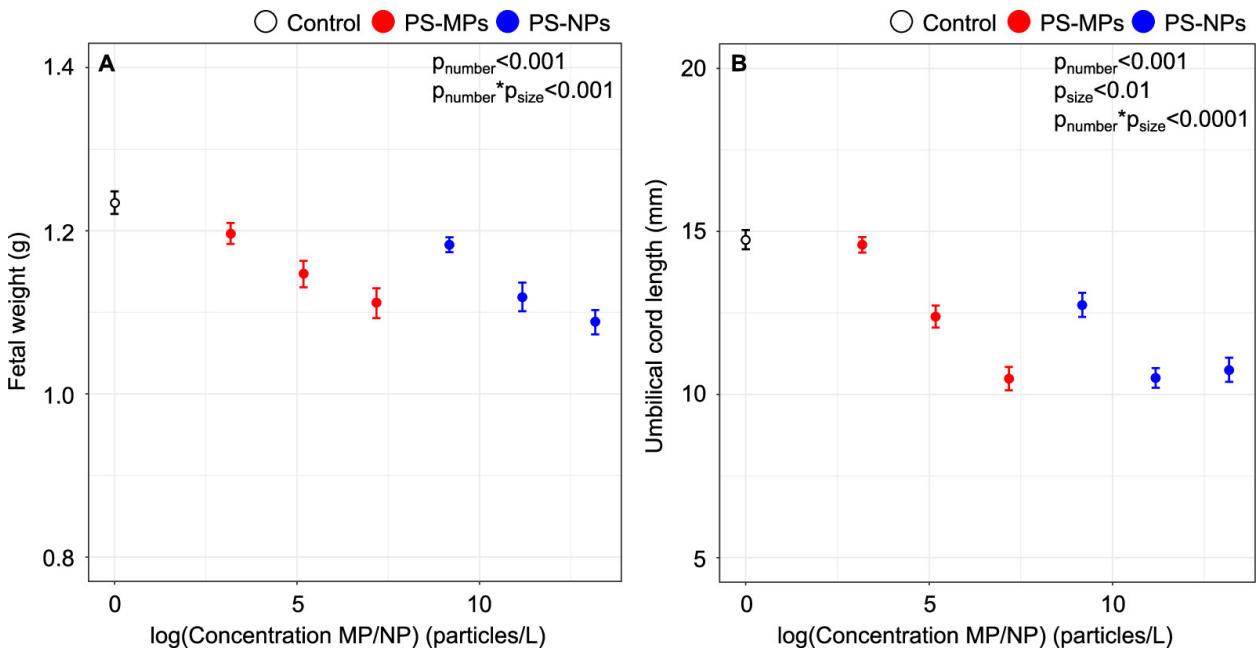

Figure 3.3: Impact of the number of particles on fetal and placental growth. (A) Fetal weights decreased significantly with an increase in the number of particles ($p < 0.001$; two-way ANOVA), and the rate of change depended on the particle size ($p < 0.001$; two-way ANOVA, interaction

term). (B) The umbilical cord length decreased significantly with an increase in the number of particles ($p < 0.001$; two-way ANOVA). The cord length was significantly different among different particle sizes ($p < 0.01$; two-way ANOVA). The rate of change with an increase in the number of particles depended on the particle size ($p < 0.0001$; two-way ANOVA, interaction term). The main effects of the particle number and particle size are denoted as p_{number} and p_{size}, respectively, and a size-by-number interaction is noted as $P_{number} * P_{size}$. n=11−14 dams/group. Data are shown as means and 95% confidence intervals.

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Chapter 4

Maternal exposure to polystyrene microplastics alters placental metabolism in mice

4.1. Statement of Co-Authorship

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Author Contribution Statement: Z.A., C.M.S. performed the experiments. Z.A., G.V.M., L.S.C. analyzed the data. Z.A., C.M.S., L.S.C. interpreted results of the experiments. Z.A., L.S.C. prepared figures. J.G.S., C.K.M., A.A.B., J.C.K., P.A.H., A.J.S., M.J.S., K.J.J., L.S.C. conceived and designed the research and drafted the manuscript. Z.A., G.V.M., C.M.S., J.G.S., C.K.M., A.A.B., J.C.K., P.A.H., A.J.S., M.J.S., K.J.J., L.S.C. approved the final version of the manuscript.

4.2. Introduction

Microplastics (MPs) are synthetic solid polymers (diameter < 5 mm) that humans are exposed to in everyday life, mostly as pellets, fragments and fibers that originated from sources including toys, food packaging, clothing, and construction materials (Frias & Nash, 2019). MPs are considered an important environmental concern (Wright & Kelly, 2017; Lambert & Wagner, 2018) with significant evidence of detrimental effects being observed upon marine life (Eerkes-Medrano *et al*., 2015; Botterell *et al*., 2019). Despite these concerns, the potential impact of MPs on human health remains largely unknown. The main route of exposure for humans to MPs are inhalation and ingestion in food and drinking water (Zhang *et al*., 2020). MPs can accumulate in the digestive system or relocate inside the body through the circulatory system (Campanale *et al*., 2020). Animal studies have shown the presence of MPs in many different organs including liver, kidney, gut, intestines, and muscle (Deng *et al*., 2017; Haave *et al*., 2021; Yin *et al*., 2021). A recent study reported 5-10 μm MPs in the placentas of healthy women (Ragusa *et al*., 2021), which raises concerns about the impact of MPs during pregnancy and their effects on placental structure and function.

Our group has recently demonstrated that maternal exposure to 5 μm and 50 nm polystyrene particles resulted in fetal growth restriction in late gestation in a mouse model of pregnancy (Aghaei *et al*., 2022). The fetoplacental weight ratio was also reduced, suggesting that following exposure to plastics, the placenta fails to maintain adequate nutrient capacity to support fetal growth. In addition to the need for adequate nutrient transfer from the placenta, fetuses depend on appropriate metabolic responses of the placenta (transplacental transport efficiency, intracellular

metabolism) to reach their growth potential. Our hypothesis is that the growth restriction associated with MP exposure is partially the result of alterations in placental metabolism.

Using ^{1}H high-resolution magic angle spinning magnetic resonance spectroscopy (HRMAS MRS) and unprocessed intact tissue samples, our group has studied metabolites in the mouse placenta throughout late gestation in healthy control pregnancies (Schneider *et al*., 2022). This work demonstrated the feasibility of HRMAS MRS to study placental metabolism and suggested NMR-based metabolomics may be a sensitive measure of fetal growth. In the present study, ^{1}H HRMAS MRS was used to study the effect of maternal exposure to MPs on placental metabolism.

4.3. Materials and Methods

4.3.1. Animals

Forty healthy adult CD-1 female mice (7-16 weeks of age) from Charles River Laboratories (St Constant, QC, Canada) were used and mated in-house. The presence of a vaginal plug on the morning after mating was designated as embryonic day 0.5 (E0.5). At E0.5, pregnant mice were randomly assigned to one of four groups: control (n=7), 10^2 ng/L polystyrene MPs (PS-MPs) (n=11), 10^4 ng/L PS-MPs (n=11) and 10^6 ng/L PS-MPs (n=11). These concentrations were chosen to span the range of exposure found in human drinking water (Koelmans *et al*., 2019). Each pregnant mouse was housed individually in a standard cage under a 12-hour light:dark cycle and with *ad libitum* access to food and water.

At E17.5, dams were euthanized by cervical dislocation and fetoplacental units were dissected from the uterus and weighed. Two placentas from the cervical end of the left uterine horn, and two

from the right, were snap-frozen in liquid nitrogen and then stored at -80 °C to prevent biochemical degradation (Beckonert *et al*., 2010; Waters *et al*., 2000). All animal experiments were approved by the Institutional Care Committee at Memorial University of Newfoundland and conducted in accordance with guidelines established by the Canadian Council on Animal Care.

4.3.2. Microplastics exposure

5 μm PS-MPs were purchased from Microspheres-Nanospheres (New York, USA). All solutions for drinking water (10^2 ng/L, 10^4 ng/L, 10^6 ng/L) were prepared using the stock solution (25 mg/mL) and diluting with regular filtered water (three-phase micron filtration system; 5 μm, 1 μm, and 0.2 μm). The control mice received the same filtered drinking water.

4.3.3. High-resolution magic-angle spinning magnetic resonance spectroscopy

The protocol for ^{1}H HRMAS MRS has been described in detail previously (Schneider *et al*., 2022). Briefly, the placental samples were allowed to thaw and then were placed in a 3.2 mm zirconium rotor (Cortecnet, Voisins-le-Bretonneux, France). ^{1}H HRMAS MRS was performed on a Bruker Avance II 600 MHz spectrometer with a Bruker H-C-P 3.2 mm MAS triple-tuned solid-state NMR probe. Spectra were acquired with a water presaturation pulse, a 90° pulse length of 3 μs, a recycle delay of 5 s, and 32 scans. The bearing gas was at physiological temperature (37 °C) and the spinning rate was 4 kHz. One placenta from the 10^6 ng/L group had to be excluded because of instrumentation failure during the acquisition.

4.3.4 Data processing

Spectra were processed using MestReNova (version 14.2.0, Mestrelab Research, S.L., Norwich, CT). The spectra were referenced to H_2O (l) at 4.8 ppm and were baseline corrected using a spline function. The ^1H chemical shifts were identified using literature values (Govindaraju *et al*., 2000; Schenetti *et al*., 2006) and 2D ^1H -^1H Correlation Spectroscopy as described previously (Schneider *et al*., 2022). To determine the relative concentration of each metabolite, automatic peak picking with global spectral deconvolution and peak integration was used to determine the relative concentration of each metabolite. The integration regions were kept constant across all datasets. The data were normalized to the total of all the resonance integrals from 0-6 ppm (excluding the water signal). In several of the spectra, peak picking failed because of low spectral resolution for four of the metabolites (leucine, valine, choline, threonine).

4.3.5 Statistical analysis

All statistical tests were performed using the R software package (www.r-project.org). All data are presented as means and 95% confidence intervals. The fetal and placental weights were analyzed using a linear mixed effects model with concentration (0 ng/L, 10^2 ng/L, 10^4 ng/L, 10^6 ng/L) as the fixed effect and litter as a random effect. To account for multiple comparisons, we first determined whether MP concentration influenced relative metabolite concentrations using a linear mixed effect model with MP concentration and metabolite as the fixed effects and allowing for interaction between the two. The relative metabolite concentrations were then analyzed post-hoc using a linear mixed effect model with MP concentration as the fixed effect and litter as a random

factor to account for similarity among placentas from littermates. A p-value of < 0.05 was taken to be significant.

Partial least squares – discriminant analysis (PLS-DA) and pathway analysis were performed using MetaboAnalyst (version 5.0) (Pang *et al*., 2021). From the PLS-DA matrix of scores, the separation between MP-exposed and the control group was determined using a two-way analysis of variance (ANOVA) followed by post-hoc analysis using Tukey tests. To validate the outcomes of the PLS-DA, permutation testing was used (using 1000 data resampling steps). For pathway analysis, a list of the metabolites that were statistically significant with increasing MP concentration was used. The KEGG pathway library for *Mus musculus* was selected as the reference organism. Pathways with a p-value of < 0.05 were taken to be significant.

4.4. Results

At E17.5, there was no effect of PS-MP concentration on maternal weight ($p=0.2$). Fetal weights decreased significantly with increasing exposure to PS-MPs in a dose-dependent manner ($p=0.005$), while the placental weights were unaffected by PS-MP exposure ($p=0.8$) (Figure 4.1).

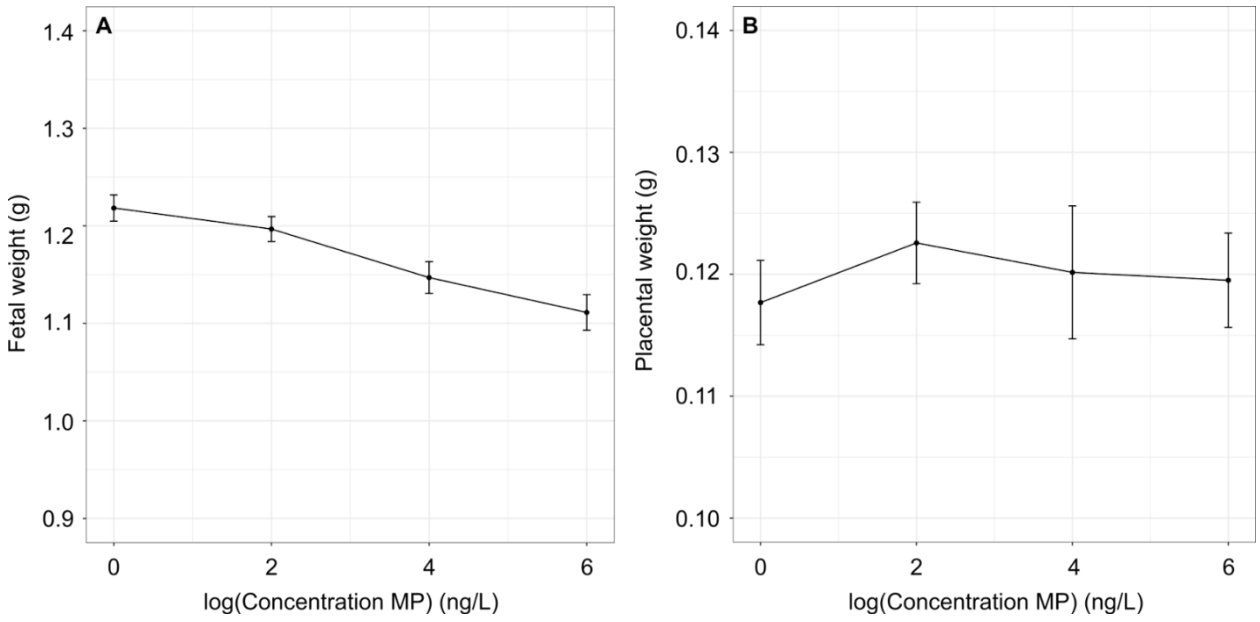

Figure 4.1: Effect of PS-MP concentration on fetal and placental weight. A Fetal weights decreased significantly with increasing concentration of 5 μm polystyrene microplastics (p=0.005). B There was no difference in placental weights with exposure to microplastics. Data are shown as means ± 95% confidence intervals. n = 86-148 fetuses/placentas per group.

We unambiguously identified a total of 14 metabolites in the placenta. Figure 4.2 shows a representative 1D ^{1}H NMR spectrum of a control placental tissue sample. Table 4.1 summarizes the relative metabolite concentrations in controls and MP-exposed placentas. While most of the metabolites remained constant with PS-MP exposure, lysine (p=0.003, Figure 4.3a) and glucose (p<0.0001, Figure 4.3b) varied significantly by concentration of PS-MPs. Post-hoc analysis using Tukey tests showed that only exposure to the highest concentration of PS-MPs (10^6 ng/L) resulted in a significant decrease in relative concentration of lysine and glucose compared to controls.

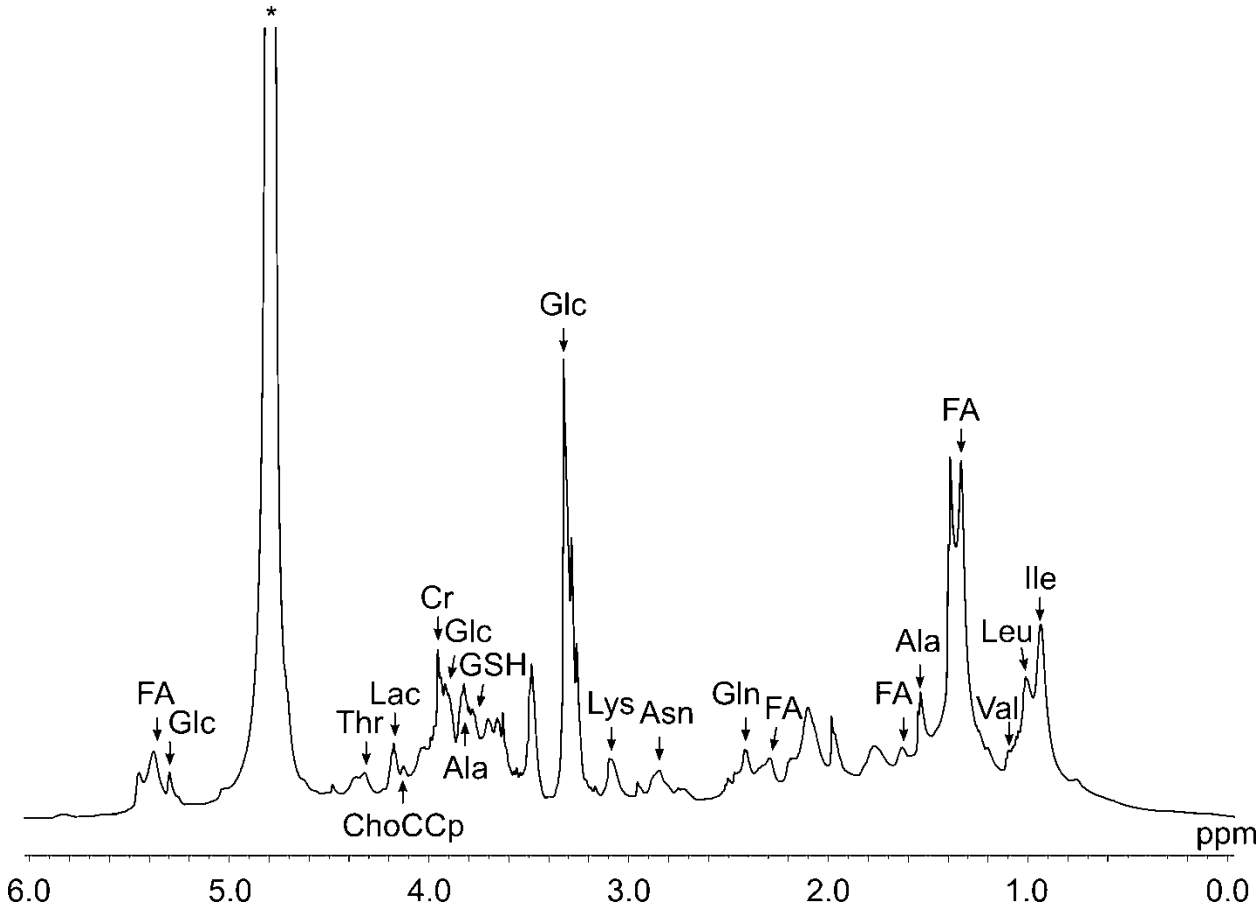

Figure 4.2: Representative ^{1}H HRMAS spectrum of a placental tissue sample from a control mouse at E17.5. Ala, alanine; Asn, asparagine; ChoCCp, choline-containing compounds; Cr, creatine; FA, fatty acid; Gln, glutamine; Glc, glucose; GSH, glutathione; Ile, isoleucine; Lac, lactate; Leu, leucine; Lys, lysine; Thr, threonine; Val, valine; *water.

Table 4.1: Relative metabolite concentrations

Metabolite	^{1}H chemical shift (ppm)	Relative concentration				$p_{concentration}$
		Control (0 ng/L) *n*=28	MP-exposed (10^2 ng/L) *n*=44	MP-exposed (10^4 ng/L) *n*=44	MP-exposed (10^6 ng/L) *n*=43	
Isoleucine	0.96	0.139±0.008	0.132±0.006	0.139±0.008	0.14±0.01	0.9
Leucine*	1.03	0.012±0.003	0.016±0.003	0.016±0.004	0.016±0.005	0.7
Valine**	1.09	0.0017±0.0007	0.0022±0.0009	0.0024±0.0006	0.0022±0.0008	0.5
Fatty Acids	1.36, 1.65, 2.31, 5.41	0.27±0.02	0.26±0.01	0.25±0.01	0.26±0.02	0.9
Alanine	1.56, 3.85	0.030±0.003	0.034±0.003	0.034±0.004	0.034±0.003	0.5
Glutamine	2.43	0.005±0.001	0.005±0.001	0.005±0.001	0.006±0.001	0.4
Asparagine	2.90	0.028±0.003	0.024±0.003	0.023±0.002	0.023±0.003	0.4

Lysine	3.12	0.017±0.002	0.016±0.002	0.016±0.002	0.012±0.002	0.003
Glucose	3.34, 3.94, 5.32	0.082±0.008	0.075±0.007	0.073±0.005	0.058±0.005	<0.0001
Glutathione	3.80	0.018±0.005	0.019±0.004	0.020±0.003	0.020±0.004	0.7
Creatine	3.97	0.030±0.003	0.034±0.002	0.032±0.002	0.031±0.002	0.6
Choline-Containing Compounds* **	4.15	0.003±0.001	0.003±0.001	0.004±0.001	0.004±0.001	0.4
Lactate	4.21	0.010±0.001	0.009±0.001	0.010±0.001	0.010±0.001	0.7
Threonine** **	4.35	0.010±0.002	0.090±0.001	0.010±0.002	0.010±0.001	0.9

Relative metabolite concentrations are shown as means ± 95% confidence intervals. $p_{concentration}$ as determined by a one-way ANOVA. *n* refers to the number of placentas. **n*=25 Controls, 39 MP-exposed (10^2 ng/L), 35 MP-exposed (10^4 ng/L), 37 MP-exposed (10^6 ng/L). ***n*=24 Controls, 40 MP-exposed (10^2 ng/L), 34 MP-exposed (10^4 ng/L), 23 MP-exposed (10^6 ng/L). ****n*=26 Controls, 29 MP-exposed (10^2 ng/L), 30 MP-exposed (10^4 ng/L), 29 MP-exposed (10^6 ng/L), *****n*=28 Controls, 38 MP-exposed (10^2 ng/L), 39 MP-exposed (10^4 ng/L), 42 MP-exposed (10^6 ng/L).

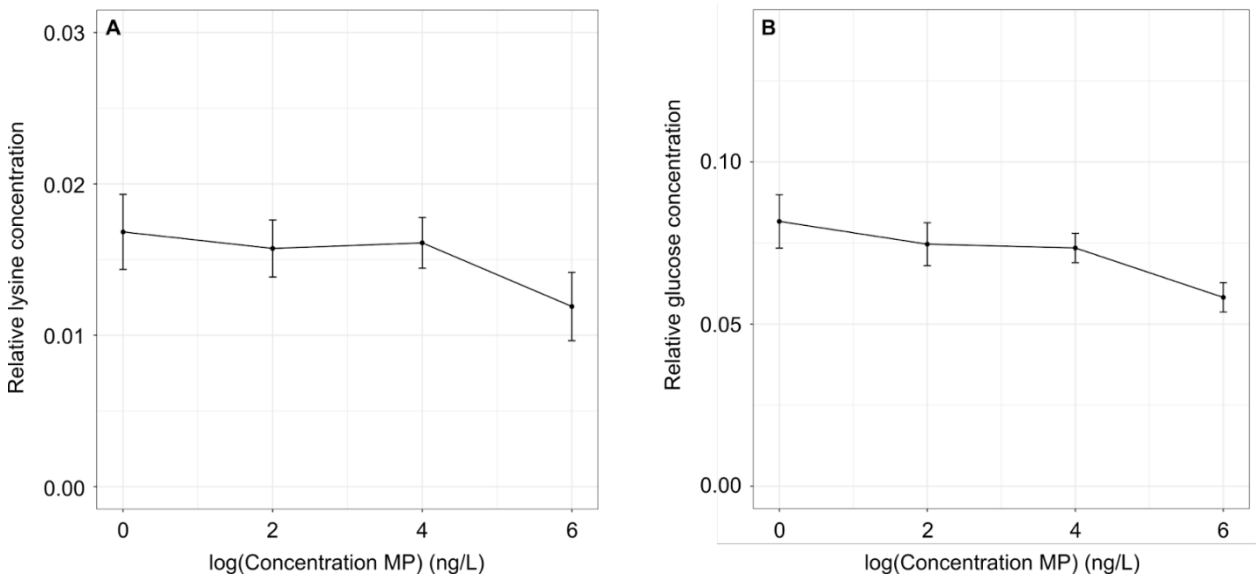

Figure 4.3: A Relative lysine concentration and B relative glucose concentration varied significantly with polystyrene microplastic concentration (p=0.003 and p<0.0001 respectively). Data are shown as means ± 95% confidence intervals. *n* = 28-44 placentas per group from 7-11 dams.

PLS-DA was performed to determine the ability of the metabolite patterns to discriminate between different exposure concentrations. Components 1 and 2 for the PLS-DA represented 77.1% of the total variation (Figure. 4.4). With increasing MP concentration, the PLS-DA exhibited a shift in the overall metabolic profile relative to the control, reaching statistical significance for the highest MP concentration (p=0.02). However, the separation between the four exposure concentrations was only modest, with an R^2 of 0.19 and Q^2 of 0.13. Pathway analysis revealed maternal MP exposure was associated with perturbations to three biochemical pathways (Table 4.2).

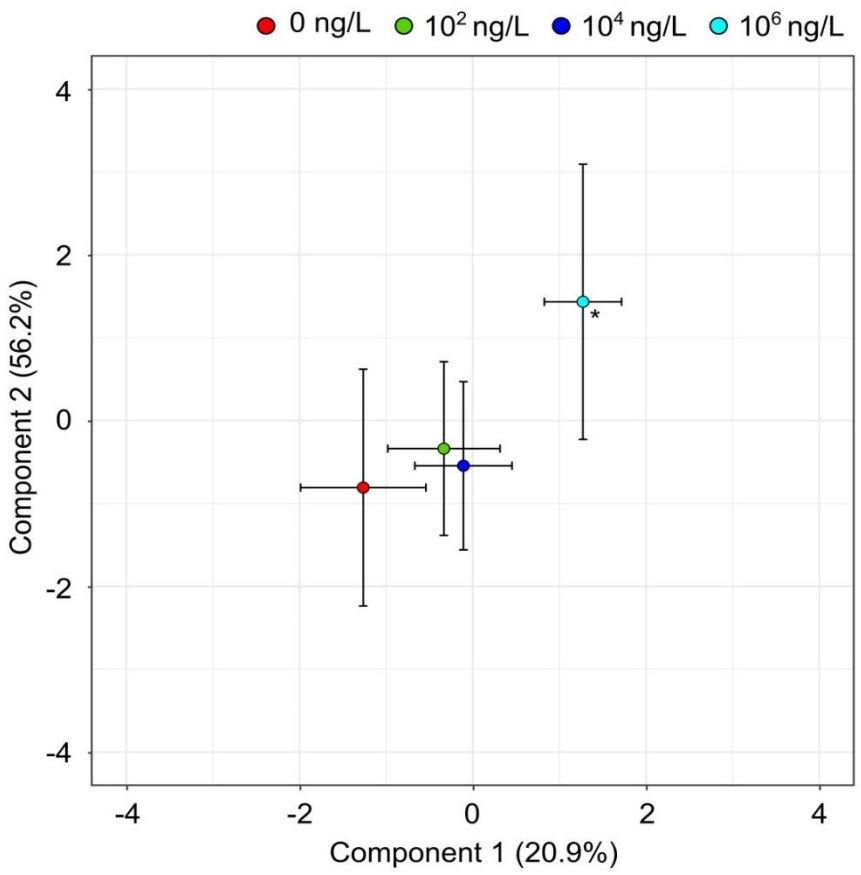

Figure 4.4: Averaged partial least squares – discriminant analysis (PLS-DA) scores plot showing differences in the murine placental metabolite patterns following maternal exposure to polystyrene microplastics. Statistically significant separation from the control group (p=0.02) is indicated with an asterisk (*). R^2=0.19, Q^2=0.13.

Table 4.2: MetaboAnalyst evaluation for major biochemical pathways associated with maternal polystyrene microplastics exposure ($p<0.05$)

Pathways	Metabolites Associated	p-value
Biotin metabolism	Lysine	0.01
Lysine degradation	Lysine	0.03
Glycolysis/Gluconeogenesis	Glucose	0.03

4.5. Discussion

Using experimental mice and HRMAS MRS, we found that maternal exposure to PS-MPs significantly altered fetal weights and two components of placental metabolism. Placental transport and metabolism determine the amount of nutrients available to the fetus and therefore play key roles in fetal growth. Studies of umbilical cord plasma have shown that concentrations of essential amino acids are reduced in growth restricted human fetuses (Cetin *et al*., 1988; Economides *et al*., 1989). In the present study, the relative concentration of lysine and glucose were significantly reduced in the placenta at a PS-MP concentration that resulted in a 9% reduction in fetal weight compared to healthy controls. Both lysine and glucose are essential nutrients for fetal development. In particular, glucose is the primary driver of fetal insulin-like growth factors and therefore is the main source of energy for fetal growth (Baschat, 2004). Reductions in glucose availability from the mother (hypoglycemia) (Caruso *et al*., 1998; Scholl *et al*., 2001) and in fetal blood glucose concentrations (Nicolini *et al*., 1989; Marconi *et al*., 1996) have been associated with fetal growth restriction in humans. Based on Hay's work on placental and fetal glucose

metabolism (Hay Jr, 2006), a recent study found fetal glucose consumption increased with birth weight and that fetal and placental glucose consumption are inversely correlated (Michelsen *et al*., 2019). A study using dynamic glucose enhanced magnetic resonance imaging (DGE MRI) to assess placental function reported that glucose uptake and transfer in the placenta was reduced in a mouse model with acute placental injury (Wu *et al*., 2018). This technique has also been used to measure placental utilization of glucose in a preliminary study of human pregnancy (Luo *et al*, 2019) but has not yet been used to study growth restriction. The importance of glucose metabolism on fetal growth was demonstrated in a study of high altitude human pregnancies where reduced fetal growth was found to result from decreased fetal glucose consumption caused by increased glucose consumption by the placenta (Zamudio *et al*., 2010). Similarly, our data suggest reduced fetal growth following maternal PS-MP exposure is associated with decreased delivery of lysine and glucose to the fetus. While MPs have been shown to enter directly into cells (von Moos *et al*, 2012), the fact that the placental weight does not change following exposure suggests the primary mechanism is not intracellular metabolism but alteration in transplacental transport efficiency. However, it is not clear from the present study whether the reduction in metabolites is due to decreased delivery from the dam or decreased transfer into the fetal compartment.

In addition to investigating the impact of maternal PS-MP exposure on the metabolite profiles, how the metabolites disrupt biochemical pathways provides additional information about the mechanism of action. Perturbations in lysine degradation and in glycolysis, the metabolic pathway that breaks down glucose, are consistent with abnormal levels of lysine and glucose in the placenta. The other biochemical pathway found to be perturbed with PS-MP exposure was biotin metabolism. Biotin is used in glucose metabolism and is essential for normal fetal development, with deficiencies in biotin leading to fetal growth restriction (Schenker *et al*, 1993).

Fetal growth restriction is associated with increased risk of metabolic diseases in adult life including diabetes, hypertension, obesity, and cardiovascular diseases (Barker, 2006; de Rooij *et al*., 2007; Dessì *et al*., 2012). The "fetal programming" hypothesis (Barker, 1995) suggests individuals are predisposed to these diseases in adulthood because of fetal metabolic adaptations that permanently change the function and structure of the adult body. How environmental toxicants cause metabolic adaptations during pregnancy is poorly understood at present. A recent study showed that exposure to fine particulate matter (< 2.5 μm) altered placental metabolic gene expression in humans, with implications for adverse effects later in life (Kaur *et al*., 2022). Our data support further studies of prenatal exposure to environmental contaminants such as MPs to determine the impact on chronic diseases later in life.

The present study has several limitations. One is that the use of a standard solid-state MAS NMR probe meant that several resonances could not be assigned because of spectral overlap. A dedicated high resolution MAS NMR probe with a magic angle gradient coil, lock, susceptibility matched coils/stators, and ^{1}H optimized tuned circuits that is specifically designed for biological tissue samples has the potential to significantly improve the sensitivity and spectral resolution of the data. Another limitation is that we did not determine the sex of the fetus and it is possible that some of the variability in our relative metabolite concentrations could be the result of sex differences in metabolism. The PS-MPs used in this study only represent one type of plastic to which humans are exposed. Future studies will investigate the impact of other plastics such as polypropylene and polyethylene to determine if the results are generalizable across all MPs. Finally, it is important to note that oxygen delivery to the fetus is known to impact fetal growth but was not determined in this study. The impact of maternal MP exposure on placental oxygen delivery and consumption should be the focus of future investigations.

In summary, the use of HRMAS MRS and experimental mice allowed us to gain a better understanding of how maternal exposure to PS-MP affects placental metabolism. Our data suggest PS-MP exposure results in a reduction in placental lysine and glucose that is associated with impaired fetal growth. This study adds to the growing literature that demonstrates the role of altered placental metabolism in fetal growth restriction.

4.6. References

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Chapter 5

5.1. Conclusion and Future Perspectives

MPs and NPs are emerging environmental concerns which are known to affect the health of animals and may have a negative impact on human health, including during pregnancy. Their toxic effects have been demonstrated in previous reports (de Souza Machado *et al*., 2018; Eerkes-Medrano *et al*., 2015; Ogonowski *et al*., 2018). In this thesis we studied the effects of exposure to PS-MPs and PS-NPs during pregnancy on fetal and placental development using experimental mice and our results showed that exposure to plastics could be a risk factor for FGR. Besides the genetic factors, normal fetal growth mostly depends on healthy placental structure (Miller *et al*., 2008). Reduction in the fetoplacental weight ratio is a sign of impaired placental function, increasing risk for neonatal morbidities (Lao & Wong, 1999). The results of this thesis demonstrated that insufficient placental transfer and abnormal placental metabolism (i.e. reduced placental lysine and glucose) may be the causes of FGR after maternal exposure to PS-MPs and NPs. Our study is the first to report the effects of gestational exposure to MPs and NPs on the umbilical cord length. The surprisingly large reduction of the umbilical cord length in both PS-MPs and NPs exposed groups suggests exposure to MPs and NPs could have adverse neurological consequences. Future studies will focus on the impact of maternal MPs and NPs exposure on fetal brain structure using high-resolution MRI and on postnatal brain development using well-established behavioural tests of learning and memory. We selected PS-MP and PS-NP to study the effect of exposure, as PS is one of the most common plastic types with many applications in different industries. It can be used to produce different consumer products and household appliances. The scale of PS production is in the range of several million tonnes per year (Ku,

1988). Consequently, the chance of being exposed to this type of plastic is relatively high for both humans and animals. As a recent published paper reported the presence of PET and PE as well as PS in human blood samples (Leslie *et al*., 2022), one of our future plans is to investigate the effect of different size and concentration of exposure to PET and PE during pregnancy to determine if our results are generalizable across all plastic types.

It was mentioned that some of the variability in our results could be the result of sex differences. Therefore, another important consideration is the importance of sex as a biological variable. Placental structure and function are known to be sexually dimorphic (Clifton, 2010). To better understand how exposure to contaminants impact birth outcomes and fetal development, we must include both sexes in the study design and include sex as a covariate in the analyses. The Cahill lab is currently setting up PCR protocols to determine the fetal sex (using the testis-determining Sry gene) from tail samples that were collected from all of the fetuses in this thesis. This will allow us to perform post-hoc analysis of the data in this thesis to determine whether maternal MPs and NPs exposure impacts males and females differently.

Another knowledge gap is the effects of MPs and NPs exposure on placental vascular morphology and blood flow. Two techniques to address this knowledge gap are *ex vivo* microcomputed tomography imaging of the fetoplacental arterial tree (Rennie *et al*., 2007) to study changes in vascular structure and the use of *in vivo* high-frequency ultrasound biomicroscopy (Zhou *et al*., 2014) to quantify changes in umbilical artery blood flow after exposure to MPs and NPs. Microcomputed tomography has shown the negative effects of maternal exposure to pollutants such as polycyclic aromatic hydrocarbons on placental vascular structure (Rennie *et al*., 2011), and high-frequency ultrasound has been used to investigate changes to placental blood flow in environmental exposure models of pregnancy complications such as in utero exposure to chronic

hypoxia (Cahill *et al*., 2019). The impact of maternal MPs and NPs exposure on placental vascular morphology and blood flow is the focus of some of our group's future studies.

Finally, the use of a standard solid-state MAS NMR probe prevented us from assigning more than 14 metabolites in the placenta because of spectral overlap. Future work, funded by the Natural Sciences and Engineering Research Council of Canada Research Tools and Instruments program, will allow us to use a high-resolution MAS NMR probe with a magic angle gradient coil, lock, susceptibility that matched coils/stators, and ^{1}H optimized tuned circuits that is specifically designed for biological tissue samples and has the potential to considerably improve the sensitivity and spectral resolution of the data.

In summary, this thesis presented the first evidence of the detrimental effect of MPs and NPs on fetal growth and placental structure and metabolism. This represents a crucial first step towards evaluating the risk of plastics exposure to human pregnancies, ultimately leading to practices and regulations to minimize these exposures.

5.2. References

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