# **Myo-inositol alters 13C-labeled fatty acid metabolism in human placental explants**

**Short title: Myo-inositol alters placental lipid metabolism**

### Oliver C. Watkins1, Mohammed Omedul Islam1, Preben Selvam1, Reshma Appukuttan Pillai1, [Amaury Cazenave-Gassiot](https://sg.linkedin.com/in/amaury-cazenave-gassiot-7585504)3,4, Anne K. Bendt4, Neerja Karnani2, Keith M. Godfrey5, Rohan M. Lewis5, Markus R. Wenk3,4, Shiao-Yng Chan 1, 2, †

1 Department of Obstetrics and Gynaecology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, 119228

2 Singapore Institute for Clinical Sciences, Agency for Science, Technology and Research, Singapore, 117609

3 Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, 117596

4 Singapore Lipidomics Incubator, Life Sciences Institute, National University of Singapore, Singapore, 117456

5MRC Lifecourse Epidemiology Unit and NIHR Southampton Biomedical Research Centre, University of Southampton and University Hospital Southampton NHS Foundation Trust, UK, SO16 6YD

† **Corresponding author: Shiao-Yng Chan**

E-mail: obgchan@nus.edu.sg, Phone: +65 67722672, Address: Department of Obstetrics and Gynaecology, Yong Loo Lin School of Medicine, National University of Singapore, National University Health System, 1E Kent Ridge Road, NUHS Tower Block, Level 12, Singapore 119228

Word count: 4980

**Abstract**

We postulate that myo-inositol, a proposed intervention for gestational-diabetes, affects transplacental lipid supply to the fetus. We investigated the effect of myo-inositol on fatty-acid processing in human placental-explants from uncomplicated pregnancies. Explants were incubated with 13C-labeled palmitic-acid, 13C-oleic-acid and 13C-docosahexaenoic-acid across a range of myo-inositol concentrations for 24 h and 48 h. The incorporation of labeled-fatty-acids into individual lipids was quantified by liquid-chromatography-mass-spectrometry. At 24 h, myo-inositol increased the amount of 13C-palmitic-acid and 13C-oleic-acid labeled lipids (median fold-change relative to control=1). Significant effects were seen with 30 µM myo-inositol (physiological) for 13C-palmitic-acid-lysophosphatidylcholines (1.26) and 13C-palmitic-acid-phosphatidylethanolamines (1.17). At 48 h, myo-inositol addition increased 13C-oleic-acid-lipids but decreased 13C-palmitic-acid and 13C-docosahexaenoic-acid lipids. Significant effects were seen with 30 µM myo-inositol for 13C-oleic-acid-phosphatidylcholines (1.25), 13C-oleic-acid-phosphatidylethanolamines (1.37) and 13C-oleic-acid-triacylglycerols (1.32) and with 100 µM myo-inositol for 13C-docosahexaenoic-acid-triacylglycerols (0.78). Lipids labeled with the same 13C-fatty-acid showed similar responses when tested at the same time-point, suggesting myo-inositol alters upstream processes such as fatty-acid uptake or activation. Myo-inositol supplementation may alter placental lipid physiology with unknown clinical consequences.

**Keywords:** Placenta, Pregnancy, Fatty acids, Phospholipids, Metabolism

**Introduction**

The naturally-occurring carbohydrate myo-inositol is the most abundant form of inositol and is present in all living cells (Noventa, et al. 2016). It is endogenously synthesized by the kidney, and is ingested in food (Noventa et al. 2016). Perturbations in myo-inositol synthesis, metabolism and excretion have been associated with the insulin-resistant conditions of polycystic ovary syndrome (PCOS), diabetes mellitus and metabolic syndrome (Croze and Soulage 2013), and with pregnancy disorders such as gestational diabetes (GDM) (Crawford, et al. 2015; D’Anna and Santamaria 2018), pre-eclampsia (D’Oria, et al. 2017), intrauterine growth restriction (Dessì and Fanos 2013), and fetal neural tube defects (Copp and Greene 2010). However, the extent and the focus of the myo-inositol-related pathophysiological events in these conditions are unclear (Xu and Ye 2018).

Inositols are the building blocks for phosphatidylinositol lipids, inositol phosphate derivatives and inositol-phospho-glycans (IPG). These compounds act as signaling molecules and participate in regulating membrane fluidity, trafficking and transport, organelle function, intracellular compartmentalization and enzyme activity (Balla 2013). Many inositol derivatives act as insulin-mimetics or interact with insulin second messenger pathways and therefore modulate glucose and lipid metabolism (Hansen 2015; Larner, et al. 2010). Any disruption in the bioavailability of inositols and inositol-derived signaling compounds could therefore have far reaching and pathological consequences.

In non-pregnant animal studies, myo-inositol deficiency increases lipid mobilization from adipose tissue and increases hepatic lipid accumulation (Hayashi, et al. 1978). Myo-inositol treatment during pregnancy reduced intra-abdominal adiposity in women (Croze and Soulage 2013), and reduced gonadal and perirenal adiposity in mice (Plows, et al. 2017).

The placenta plays a central role in the generation of GDM pathology, in part through the release of pro-diabetic biological signals (Jayabalan, et al. 2017). Furthermore, the placenta regulates transplacental lipid transfer from the mother to the fetus, and placental lipid uptake and metabolism are known to be disrupted in GDM (Gallo, et al. 2017; Uhl, et al. 2015). This has been postulated to contribute to dysregulated fetal growth and development (Herrera and Ortega-Senovilla 2018).

Myo-inositol supplementation for GDM prevention is being trialed (Godfrey, et al. 2017; Xu and Ye 2018) and, if demonstrated to be effective, widespread supplementation would invariably involve exposure of uncomplicated pregnancies to additional exogenous myo-inositol. Thus, its impact needs to be understood, not only in the diseased state, but also in normal pregnancies. Given the critical role of the placenta in regulating the physiology of both the mother and fetus, there is a need to understand the effects of myo-inositol on placental lipid biology and this, to our knowledge, has never been explored.

Our overarching hypothesis is that myo-inositol plays a physiological role in the regulation of placental FA uptake and lipid metabolism, and that both deficient and excessive myo-inositol states could impact placental lipid metabolism with consequences for fetal development. In this study we sought to address the specific hypothesis that myo-inositol treatment alters the processing of fatty acids in normal human term placenta.

We previously reported that human term placental explants incubated with stable-isotope labeled palmitic acid (PA; saturated FA), oleic acid (OA; monounsaturated FA), or docosahexaenoic acid (DHA; long-chain polyunsaturated FA (LC-PUFA)) incorporated the labeled FAs into phosphatidylcholines (PC), triacylglycerols (TAG), phosphatidylethanolamines (PE) and lysophosphatidylcholines (LPC) in a FA-specific manner (Watkins, et al. 2019). Here, we aimed to use this placental explant model to investigate changes in the amounts of stable-isotope labeled lipids with exposure to three myo-inositol concentrations consistent with deficient, physiologically-normal or supra-physiological states. For hypothesis generation, we also explored the potential persistent influence of maternal first trimester body mass index (BMI) and antenatal glucose regulation on the myo-inositol responsivity of placental explants, as well as the potential association between myo-inositol responsivity and fetal size.

## **Materials and Methods**

**Ethical Approval**

Women were recruited from the National University Hospital, Singapore with informed written consent. Ethical approval was obtained from the National Healthcare Group Domain Specific Review Board (2016/00183).

**Placental Tissue Collection**

Seven placentas from uncomplicated singleton pregnancies, delivered by elective cesarean section at term (mean ± SD: 38 weeks and 2 days’ gestation ± 5 days) were obtained. All mothers (aged 34 ± 6 years; maternal first trimester BMI 27 ± 6 kg/m2) were non-smokers with normal glucose tolerance at mid-gestation as assessed by a three time-point 75 g oral glucose tolerance test (OGTT) using WHO 2013 criteria (mean fasting plasma glucose: 4.3 ± 0.3 mmol/L, 1 h post-load plasma glucose: 7.3 ± 1.3 mmol/L, 2 h post-load plasma glucose: 6.2 ± 1.0 mmol/L). There were four male and three female babies who were all appropriately grown for gestational age based on customized birth weight centiles (for maternal age, ethnicity, BMI, parity and fetal sex) (Gardosi, et al. 2018), with a group mean of 55% (± 35).

**Placental tissue culture**

Placental explant culture was completed as previously described (Watkins et al. 2019) but with the addition of 0, 30 or 100 µM myo-inositol (Sigma, >99% pure, Saint Louis, MO) representing a myo-inositol deficient, physiological or supra-physiological environments (Brusati, et al. 2005). Myo-inositol was added to basal serum-free CMRL media (GIBCO 1066-L Glutamine, Thermofisher, New York, USA) containing 12 µM of myo-inositol and 5 mM glucose. Fresh placental explants (approximately 3 mm x 3 mm x 3 mm of villous placental tissue) were cultured for 24 h in CMRL media containing 1.5% BSA (HI Clone fraction V, Culture grade, pH 7.00 lyophilized powder, GE Life Sciences, South Logan, Utah), with the addition of 0, 30 or 100 µM myo-inositol. The normal physiological circulating concentration of maternal myo-inositol is 30-50 µM (Brusati et al. 2005). After 24 h the media was replaced with fresh media containing the same myo-inositol concentration and either no FA or 13C16-palmitic acid (13C-PA**;** 99 atom % 13C, 99% CP; Sigma-Aldrich, 300 µM), 13C18-oleic acid (13C-OA**;** 99 atom % 13C, 99% CP, Sigma-Aldrich, 300 µM) or 13C22-docosahexaenoic acid (13C-DHA; 99 atom % 13C, 99% CP, Cambridge isotope laboratories, 100 µM). Explants cultured in 12 well plates were incubated in 2 ml of media at 37 °C in a humidified atmosphere of 5% CO2/air.

Explants and the corresponding conditioned media were harvested at 24 h and 48 h after FA addition. DHA was only tested at 48 h. Harvested explants were washed with PBS and particulate matter removed from the supernatant by centrifugation before being stored at -80°C. Experiments were performed in triplicate for each placenta. To confirm the specificity of myo-inositol effects, explant culture with additional 30 µM myo-inositol was also performed (n=3) in the presence of 1 mM phlorizin (Cayman chemical company, MO, USA), a competitive inhibitor of myo-inositol binding sites in membrane transporters and enzymes (Van Steenbergen, et al. 2017). We found that lactate dehydrogenase (LDH) and human chorionic gonadotropin (HCG) levels were similar with 0, 30 and 100 µM myo-inositol at each time point suggesting that myo-inositol did not affect explant viability (Figure 1). Our previous work showed that explant viability was also not significantly affected by the addition of stable-isotope labeled FA (Watkins et al. 2019).

**Lipid quantification**

Lipid extraction and analysis was completed as previously described (Watkins et al. 2019). Briefly, placental explants were freeze dried and weighed, then lysed using an Omni bead Rupter homogenizer followed by lipid extraction using a modified Bligh and Dyer method (Bligh and Dyer 1959). Lysate (100 μl) or conditioned media (50 μl) were extracted with chloroform and methanol (1:2, 450 µl, containing internal standards (Watkins et al. 2019)). Water (300 μl) was added and the samples vortexed then centrifuged. The chloroform layer was collected and the aqueous layer re-extracted with chloroform (500 μl). Chloroform layers were combined, dried, dissolved in 200 μl 90% IPA, 5% chloroform, 5% methanol then stored at -80 °C until use. Triplicates were extracted as separate samples.

Analysis of lipids incorporating one or more 13C16-PA, 13C18-OA or 13C22-DHA was performed on the Agilent 6490 triple quadrupole liquid chromatography-mass spectrometry (LC-MS) instrument using reverse phase chromatographic separation and mass spectrometry with a targeted dynamic multiple reaction monitoring method (Watkins et al. 2019).

**Data processing and statistical analyses**

Data was processed using Mass Hunter quantitative analysis and Excel. Metabolites were quantified by integration, followed by normalization against internal standards (Watkins et al. 2019). Lipid amount is expressed as a function of dried placental weight (µmol/g). This data was used to calculate the equivalent lipid amount that would be expected in a normalized standard well system containing 30 mg dry weight explant and 2 ml media. Molar amount 13C-lipid by class is the sum of the molar amounts for all quantified individual 13C-lipids in class. Triplicate data was averaged to give a mean result for each placenta, which was then used for subsequent analysis. The effects of myo-inositol were expressed relative to controls (with no additional myo-inositol) whose mean lipid amount was assigned an arbitrary value of 1 for each lipid from each placenta. Myo-inositol response (i.e. fold change) was calculated as amount of labeled lipid in explants exposed to additional myo-inositol divided by amount of labeled lipid in control explants.

Non-parametric Wilcoxon signed rank tests were used to test whether (i) the addition of myo-inositol (30 or 100 µM) changed the amount of labeled lipid in placental explants at either 24 or 48 h, for any lipid class; i.e. whether the myo-inositol response was significantly different to 1; (ii) the myo-inositol response at 24 h was significantly different to the myo-inositol at 48 h, for any lipid class, with 30 or 100 µM myo-inositol. Multiple comparisons were accounted for using false discovery rate (FDR) adjusted P values (MetaboAnalyst 3.0 (Xia, et al. 2015))*.* The non-parametric Friedman test followed by the Dunn’s post-hoc test (Graph Pad Prism 7.00) were used to determine whether any lipid class showed a significantly different MI response to any other lipid class at each time and for each myo-inositol concentration. Correlations were measured using the non-parametric Spearman method (Graph Pad Prism 7.00). Statistical significance was taken as p<0.05.

# **Results**

Placental explants incubated with myo-inositol and stable-isotope labeled 13C-PA or 13C-OA demonstrated incorporation of labeled FAs into LPCs, PCs, PEs and TAGs at 24 h and 48 h. 13C-DHA incorporation was only reliably quantifiable in TAGs and was only tested at 48 h. Labeled LPCs were the only class of labeled lipids detectable by our LCMS method in the conditioned media after incubation with placental explants (Watkins et al. 2019).

**Myo-inositol responses in placental explants and conditioned media**

Figures 2 and 3 show the effects of 30 µM and 100 µM additional myo-inositol on the net incorporation of labeled FA into placental LPCs, PCs, PEs and TAGs in placental explants and conditioned media. This myo-inositol response is expressed as the relative amounts of labeled lipids in the treated experiment compared with controls (i.e. Molar Amount of 13C-lipid with 30 or 100 µM myo-inositol / Molar Amount of 13C-lipid with 0 µM myo-inositol).

Individual lipid species in the same class demonstrated similar myo-inositol responses (Figure 2), so the amounts for all quantified individual 13C-lipids in a given lipid class were summed in subsequent analysis (Figure 3). Lipid class data showed that the direction and magnitude of myo-inositol response was also similar across lipid classes (i.e. between TAGs, PCs, PEs and LPCs) for each labeled FA when tested at the same time point (Figures 3A, 3C).

In placental explants at 24 h, myo-inositol addition increased the amounts of labeled lipids compared to controls, with a similar effect seen for 13C-PA and 13C-OA labeled lipids. Myo-inositol response (median [inter-quartile range 25th - 75th percentile]) in explants treated with 30 µM myo-inositol was significantly different (p<0.05) to the 0 µM myo-inositol control (assigned value of 1) for 13C-PA-LPC (1.26 [1.07-1.38]) and 13C-PA-PE (1.17 [1.03-1.62]) (Figure 3A). DHA was not tested at 24 h.

At 48 h, myo-inositol addition increased the amount of 13C-OA labeled lipids compared to controls. Myo-inositol response in explants treated with 30 µM myo-inositol was significantly different to the 0 µM myo-inositol control (assigned value of 1) for 13C-OA-PC (1.25 [1.02-1.75]), 13C-OA-PE (1.37 [1.16-1.52]) and 13C-OA-TAG (1.32 [1.21-1.75]) (Figure 3A). Treatment with a higher dose of 100 µM myo-inositol showed a similar effect as treatment with 30 µM myo-inositol but the effect was not significantly different compared to the control (Figure 3C).

At 48 h, myo-inositol addition resulted in an overall decrease in the amount of 13C-PA-labeled lipids compared with controls but the effect was not significant. However, the difference between the myo-inositol response for 13C-PA compared with that for 13C-OA were statistically significant for every lipid class (Figure 3A). At 48 h, 100 µM myo-inositol also significantly decreased the amount of 13C-DHA-TAG (0.78 [0.72-0.86]) compared with 0 µM myo-inositol control (Figure 3C). The 13C-DHA response was also significantly different to that for 13C-OA (p<0.05). It is to be noted that these decreases represent decreases with respect to the control with no added MI and not changes in absolute amounts over the period in culture.

When comparing the difference in myo-inositol response between 24 h and 48 h, only 13C-PA showed divergent effects of an increase at 24 h but a decrease at 48 h for all four lipid classes. Differences in amount were significant for 13C-PA-PE (24 h: 1.17 [1.03-1.62] vs 48 h: 0.75 [0.57-0.99]) and 13C-PA-TAG (24 h: 1.05 [0.84-1.80] vs 48h: 0.73 [0.58-1.14]) with the addition of 30 µM myo-inositol (Figure 3A). For 13C-OA there was no further significant increase in labeled lipids from 24 h to 48 h compared to controls.

Labeled LPC levels were highly variable in conditioned media and no statistically significant effects in labeled LPC levels in conditioned media were observed between myo-inositol treatment groups or FA-type (Figures 3B and 3D).

To demonstrate that the changes seen in the amount of labeled lipid were a specific effect of myo-inositol, placental explants treated with the physiological dose of myo-inositol (additional 30 µM) were cultured in the presence and absence of the myo-inositol competitive inhibitor, phlorizin. The presence of phlorizin suppressed the 48 h myo-inositol-induced increases in 13C-OA labeled lipid classes relative to control (Figure 4).

**Maternal and fetal characteristics associated with amount of labeled lipid and MI responsivity**

For hypothesis generation purposes, we investigated if the preceding antenatal characteristics of first trimester maternal BMI and mid-gestation maternal glucose tolerance, reflected by the fasting glucose concentration and post-load glucose concentrations at 1 h and 2 h of an OGTT, could leave a placental “imprint” that persists into the time of *in-vitro* culture post-delivery to influence placental lipids. We also explored whether differences in placental MI responses could be associated with fetal size, by examining the relationship between placental labeled lipids and customized birthweight percentile.

We first examined the correlations between these characteristics and absolute molar amount of labeled lipid at the physiological myoinositol concentration (additional 30 µM). In general, across the BMI and glycemic ranges of our study population, LPC, PC and PEs labeled with 13C-PA and 13C-OA positively correlated with maternal first trimester BMI, but negatively correlated with antenatal fasting glucose concentrations as assessed at mid-gestation.

Meanwhile TAGs labeled with 13C-PA and 13C-OA positively correlated with antenatal 2 h glucose values and birthweight percentiles (Figure 5). Next we investigated the correlation between these factors and lipid class myo-inositol responsivity (ratio of amount with added myo-inositol / amount with no added myo-inositol). Correlations were derived only for conditions in which a significant myo-inositol response was observed (24 h, 30 µM myo-inositol for 13C-PA lipids and 48 h, 30 µM myo-inositol for 13C-OA lipids). Myo-inositol response for 13C-PA-LPC increased with fasting glucose (Rho 0.9, p<0.01, Figure 5A) in contrast to the negative correlation with the absolute amount of placental 13C-PA-LPC (Rho -0.9, p<0.01, Figure 6A). In comparison, the higher the maternal BMI, the lower the myo-inositol response for 13C-PA-PC (Rho -0.89, p=0.01, Figure 6B) even though the amount of 13C-PA-PC was positively associated with BMI. For 13C-OA at 48 h, the higher the post-load glycemia at 1 h and 2 h, the greater the 48 h myo-inositol response for 13C-OA-PE synthesis (Rho 0.77, p=0.05 and Rho 0.86, p=0.02 respectively, Figure 6D) and the higher the absolute amounts of placental 13C-OA-TAG (Rho 0.77, p=0.05 and Rho 0.93, p=0.007 respectively, Figure 6C). Birthweight percentile was positively correlated with both the absolute amount of 13C-PA-TAG with 30 µM myo-inositol at 24 h (Rho 0.79, p=0.048, Figure 6E) and myo-inositol response for 13C-OA-TAG (Rho 0.79, p=0.048, Figure 6F). These findings suggest that placental TAG metabolism could be associated with fetal growth. No significant correlations were observed for 13C-DHA labeled lipids with any of the maternal or fetal characteristics. Our study was underpowered to conclusively determine the effect of sex and ethnicity, and larger studies will be needed to investigate this variable.

# **Discussion**

This study has demonstrated that myo-inositol affects the metabolism of 13C-labeled PA, OA and DHA, in human term placental explants from uncomplicated pregnancies. At each time point, all measured lipids incorporating the same labeled FA showed a similar response to myo-inositol treatment; with myo-inositol initially increasing then decreasing the incorporation of the saturated palmitic acid into placental lipids over 48 hours, but continued increasing incorporation of the mono-unsaturated oleic acid and decreasing incorporation of the long-chained polyunsaturated docosahexaenoic acid compared to controls. This indicates that myo-inositol does not specifically act on biological pathways affecting the synthesis of any one lipid or lipid class. It instead suggests that myo-inositol affects common upstream processes such as FA transport into the placenta, or the activation of FAs that initiates the incorporation of FA into lipids.

The effect of myo-inositol did differ between the type of FA and over time. This suggests that several different FA-selective biological processes are affected by myo-inositol. The rate limiting step for each time and for each FA would depend on the placental capacity and speed of each process and whether these processes were close to equilibrium. We speculate that FA transport processes facilitating cellular entry should exert the greatest effect soon after FA addition to culture media and have less influence later. Synthetic and catabolic processes, in contrast, would exert a greater effect later. A balance of all these different processes would be reflected by the net incorporation represented by the absolute amount of labeled lipids quantified.

Myo-inositol most likely impacts lipid transport and metabolism though the effects of MI-derived signaling molecules such as phosphoinositides, phosphatidylinositol phosphates or inositol phosphoglycans, which are known to regulate lipid metabolism in various tissues (Hansen 2015; Larner et al. 2010).Myo-inositol may affect enzymes that catalyze the initial steps of lipid synthesis such as the Acyl-CoA long chain synthetazes (ACSL, ACSVL), which are important for facilitating placental FA uptake (Araújo, et al. 2013; Tobin, et al. 2009), or the glycerol phosphate acyl transferases (GPAT) (Wendel, et al. 2009). Alternatively myo-inositol could affect enzymes that regulate whether FA acyl-CoAs get directed into lipid synthesis or FA catabolism such as carnitine palmitoyltransferase-1 (CPT, increases catabolism) (Keung, et al. 2013; Wolf 1992) and acetyl-CoA carboxylase (ACC, decreases catabolism) (Wendel et al. 2009). Whether alterations in the activities of these enzymes occur in our explant model with myo-inositol treatment remains to be determined, but there is some literature evidence consistent with our hypothesis that these enzymes are likely candidates (Araújo et al. 2013; Hammond, et al. 2002; Hayashi, et al. 1976; Kunjara, et al. 1999).

The activities of GPAT and ACC are influenced by inositol-derived inositol phosphoglycans (Farese, et al. 1994; Huang, et al. 1993; Kunjara et al. 1999). Furthermore, myo-inositol deficiency in yeasts increased ACC activity resulting in an accumulation of lipids containing unsaturated FAs (Hayashi et al. 1976), an effect reversed by myo-inositol treatment. Intriguingly, phospholipids from Gpat1*−/−*mice contain less PA and DHA, and more OA, than lipids from control mice (Hammond et al. 2002); a pattern that matches our data at 48 h. Furthermore different isoforms of these enzymes are known to display different FA selectivity and could be differentially regulated by myo-inositol (Hammond et al. 2002; Watkins 2008; Wendel et al. 2009; Yan, et al. 2015).

ACSL in human trophoblasts and CPT in placental explants are decreased by GDM (Araújo et al. 2013; Visiedo, et al. 2013) and when exposed to high glucose *in-vitro* (Visiedo et al. 2013). Furthermore, ACC (Abu-Elheiga, et al. 2003; Harriman, et al. 2016; Kreuz, et al. 2009; Xu, et al. 2014), CPT (Keung et al. 2013; Wolf 1992) and GPAT (Bates and Saggerson 1977; Farese et al. 1994) are affected by glucose, insulin resistance and diabetes in other organs. This could explain why placental myo-inositol response was associated with maternal glycemia.

**Correlations of myo-inositol response with maternal and fetal characteristics**

Maternal obesity and hyperglycemia are associated with dysregulated placental lipid metabolism (Delhaes, et al. 2018; Gallo et al. 2017; Uhl et al. 2015). In our present study, the participants had a range of BMI but none were morbidly obese (BMI>40 kg/m2) and none had gestational diabetes. Even within the relatively narrow range of maternal glycemia and non-morbid BMI range, and the small sample size, correlations with placental explant incorporation of labeled FA and myo-inositol responsivity were already evident. It is intriguing that maternal BMI and glycemia is associated with divergent effects on labeled lipid incorporation in placenta with physiological myo-inositol exposure. This strongly suggests that these maternal characteristics antenatally leave a lasting influence on the placenta that persists into *in-vitro* culture, consistent with previous reports (Araújo et al. 2013; Visiedo, et al. 2015) and that this contributes to biological variability in myo-inositol response.

At 24 h higher maternal fasting glycemia was associated with a lower absolute amount of freshly synthesized placental 13C-PA-lipid; this contrasted with the increase in 13C-PA-lipid amount following placental myo-inositol treatment *in-vitro* (reflected by an increasing myo-inositol response). Furthermore, the magnitude of increase in 13C-PA incorporation induced by myo-inositol treatment was larger for placenta from pregnancies of higher fasting maternal glycemia. This suggests that increasing placental myo-inositol exposure could potentially promote compensatory mechanisms to combat the glycemia-induced disruptions in placental lipid processing. However, myo-inositol decreased 13C-PA lipid incorporation at 48 h suggesting that potential compensatory mechanisms may be transient, contrasting and complicated, involving multiple biological pathways.

With respect to 13C-OA incorporation at 48 h, myo-inositol response was also higher in those with higher levels of maternal glycemia, but only with higher 1 h and 2 h post-load glycemia rather than fasting glycemia. This further emphasizes the potential differences in the regulation of PA and OA metabolism in human placenta.

Interestingly, the maternal glycemia associated changes in the placental 13C-PA-lipid amount and myo-inositol response are opposite to those associated with maternal BMI, suggesting that maternal glycemia and BMI impact on placental lipid pathways differently, even though both characteristics are associated with increasing fetal adiposity and size. This may explain differences between clinical trials on the effects of myo-inositol supplementation on birthweight. Three small Italian trials had previously shown that myo-inositol supplementation reduced the GDM rate in at risk women; while a reduction in birthweight and fetal abdominal circumferences was found in the two studies that recruited women with clear dysglycemic risk (a borderline fasting glucose at booking or family history of diabetes) (D’Anna, et al. 2013; Matarrelli, et al. 2013), no birthweight reduction was found in the one conducted in women with a raised BMI (D’anna, et al. 2012).

The link between FA-incorporation and myo-inositol response with maternal glycemia and fetal size is intriguing, because placental lipid metabolism is thought to regulate fetal size (Haggarty 2002; Lager and Powell 2012). We previously suggested that placental TAGs act as an accessible FA reserve for transfer to the fetus (Watkins et al. 2019) to promote growth and hypothesized that fetal size may be partly regulated by how much FA is found in accessible placental lipid reserves such as TAGs compared with other less accessible reserves such as phospholipids. Our data of increasing absolute amounts of PA and OA labeled TAG with rising glycemia (2h) and birthweight percentile is consistent with this idea and consistent with other studies which have reported that placental TAGs are increased by hyperglycemia and associated with fetal macrosomia (Visiedo et al. 2015; Visiedo et al. 2013).

Conversely, sequestration of FA into inaccessible phospholipids could mean less FA available for transfer to the fetus, thus suppressing fetal growth. If high placental myo-inositol *in vivo* could increase FA incorporation into phospholipids, as we observed *in vitro* at 24h of culture, this would be consistent with the observation of intrauterine growth restriction in a sheep model of high placental inositol (Regnault, et al. 2010). However, in our small study, absolute amounts of labeled phospholipids were not significantly correlated with birthweight percentile, and myo-inositol’s effect on phospholipid synthesis was contrasting, depending on FA-type and time in culture. Further research is therefore needed to explore if myo-inositol could sufficiently influence the regulation of FA sequestration into phospholipids to have appreciable impact on birthweight. Nonetheless, our data overall suggests that myo-inositol has the potential to alter placental lipid synthesis, and this could affect fetal growth.

DHA is important for fetal development, particularly the brain (Yessoufou, et al. 2015). The reduction in DHA incorporation into TAGs induced by myo-inositol could result from decreased DHA uptake or from altered DHA metabolism. This could be problematic if myo-inositol is to be used in GDM management, since this could potentially exacerbate the already reduced placental DHA uptake in this condition (Gallo et al. 2017). On the other hand, it would be less concerning if our results reflect a myo-inositol-induced reduction in DHA incorporation into TAGs, implying that there could be more non-esterified DHA available for fetal transfer, rather than a reduction in overall DHA uptake. Unfortunately, non-esterified DHA was not quantified by our method to enable conclusions to be drawn.

This study is limited by its relatively small sample size (n=7) and only involved placentas from uncomplicated pregnancies without GDM. Thus, it is difficult to predict whether myo-inositol would be useful in preventing the macrosomia associated with GDM. Larger studies will be needed to confirm the effects of MI on the placenta and fetal growth with consideration of multiple maternal and fetal characteristics, and confounders. Even though our placental explant treatment with myo-inositol *in vitro* attempted to mimic the effects of maternal myo-inositol supplementation, this may not entirely recapitulate the *in vivo* situation, where the placenta synthesizes its own myo-inositol, whilst also receiving exogenous supply from both mother and fetus (Quirk and Bleasdale 1983). Since the relative contribution of each myo-inositol source is not yet known, it remains uncertain whether maternal myo-inositol supplementation will substantially increase placental myo-inositol and how placental lipid metabolism would be affected. Moreover, our placental explant method limits the interpretation of our findings since it cannot examine transplacental FA transfers between the maternal and fetal compartments. Placental explants also comprise many different cell types and can only indicate the composite response of the entire tissue. Other complementary methods such as placental perfusion or *in vivo* studies will therefore be necessary to further investigate the effects of myo-inositol.

**Conclusion**

This study shows that myo-inositol induces FA-specific effects on upstream placental lipid processes such as FA uptake or activation. Myo-inositol supplementation may have the potential to alter placental lipid physiology across a wide range of lipid classes even in uncomplicated pregnancies, with as yet unknown clinical consequences. The magnitude of the myo-inositol effect appears to be associated with maternal glycaemia, BMI and fetal birthweight indicating that the placental lipid processing effects of myo-inositol supplementation in pregnancy will likely depend on the population, consistent with the notion that placental metabolism is regulated by the maternal environment.

#### **Declaration of interest**

Chan S.Y., Karnani N. and Godfrey K.M. are part of an academic consortium that has received research funding from Abbott Nutrition, Nestec and Danone for work unrelated to this manuscript. Godfrey K.M. and Chan S.Y. are co-inventors on a pending patent, which covers the use of a novel inositol composition in human health applications. This invention is unrelated to the submitted work. The other authors have no financial or personal conflict of interest to declare.

#### **Funding**

This research is supported by a Clinician Scientist Award awarded to Chan S.Y from the Singapore National Medical Research Council (NMRC/CSA-INV/0010/2016), by the National University Health System Singapore and the Singapore Institute for Clinical Sciences A\*STAR. The Singapore Lipidomics Incubator receives funding from the Life Sciences Institute, the National University of Singapore Yong Loo Lin School of Medicine and the National Research Foundation (grant number NRFI2015-05). Godfrey K.M. is supported by the UK Medical Research Council (MC\_UU\_12011/4), the National Institute for Health Research (NIHR Senior Investigator (NF-SI-0515-10042) and the NIHR Southampton Biomedical Research Centre) and the European Union (Erasmus + Programme Early Nutrition eAcademy Southeast Asia-573651-EPP-1-2016-1-DE-EPPKA2-CBHE-JP).

**Abbreviations**

AA: Arachidonic acid, ACSL: Acyl-CoA long chain synthetazes, ACC: acetyl-CoA carboxylase  BMI: body mass index, BSA: bovine serum albumin, CPT: carnitine palmitoyltransferase-1, CMRL: Connaught Medical Research Laboratories, DHA: Docosahexaenoic acid, dMRM: dynamic Multiple Reaction Monitoring, FA: fatty acid, FDR: False Discovery Rate, GDM: gestational diabetes mellitus, GPAT: glycerol phosphate acyl transferases, HCG: human chorionic gonadotropin, IGF: Insulin-like growth factor, IPG: Inositol-phospho-glycans, IS: Internal standard, LC-MS/MS: tandem liquid chromatography mass spectrometry, LA: Linoleic acid, LC-PUFA: long-chain polyunsaturated fatty acids, LPC: lysophosphatidylcholine, LPE: lysophosphatidylethanolamine, LDH: lactate dehydrogenase, OA: Oleic acid, PA: Palmitic acid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PUFA: polyunsaturated fatty acids, TAG: triacylglycerol, QQQ: triple quadrupole.

#### **Acknowledgements**

We acknowledge Celes Maria Catherine Dado, Samantha Grace Loon Magadia and Chen Zhenzhi in coordinating the administration and recruitment of women, staff of the National University Hospital who kindly assisted with placental collection, and the women for generously donating their placenta for research.

# **References**

Abu-Elheiga L, Oh W, Kordari P & Wakil SJ 2003 Acetyl-CoA carboxylase 2 mutant mice are protected against obesity and diabetes induced by high-fat/high-carbohydrate diets. *Proceedings of the National Academy of Sciences* **100** 10207-10212.

Araújo JR, Correia-Branco A, Ramalho C, Keating E & Martel F 2013 Gestational diabetes mellitus decreases placental uptake of long-chain polyunsaturated fatty acids: involvement of long-chain acyl-CoA synthetase. *The Journal of nutritional biochemistry* **24** 1741-1750.

Balla T 2013 Phosphoinositides: tiny lipids with giant impact on cell regulation. *Physiol Rev* **93** 1019-1137.

Bates EJ & Saggerson D 1977 A selective decrease in mitochondrial glycerol phosphate acyltransferase activity in livers from streptozotocin-diabetic rats. *FEBS letters* **84** 229-232.

Bligh EG & Dyer WJ 1959 A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology* **37** 911-917.

Brusati V, Jóźwik M, Jóźwik M, Teng C, Paolini C, Marconi AM & Battaglia FC 2005 Fetal and maternal non-glucose carbohydrates and polyols concentrations in normal human pregnancies at term. *Pediatric research* **58** 700.

Copp AJ & Greene ND 2010 Genetics and development of neural tube defects. *The Journal of pathology* **220** 217-230.

Crawford TJ, Crowther CA, Alsweiler J & Brown J 2015 Antenatal dietary supplementation with myo-inositol in women during pregnancy for preventing gestational diabetes. *Cochrane Database of Systematic Reviews*.

Croze ML & Soulage CO 2013 Potential role and therapeutic interests of myo-inositol in metabolic diseases. *Biochimie* **95** 1811-1827.

D’anna R, Di Benedetto V, Rizzo P, Raffone E, Interdonato M, Corrado F & Di Benedetto A 2012 Myo-inositol may prevent gestational diabetes in PCOS women. *Gynecological Endocrinology* **28** 440-442.

D’Anna R & Santamaria A 2018 Myo-Inositol Supplementation in Gestational Diabetes. In *Nutrition and Diet in Maternal Diabetes*, pp 229-235: Springer.

D’Anna R, Scilipoti A, Giordano D, Caruso C, Cannata ML, Interdonato ML, Corrado F & Di Benedetto A 2013 myo-Inositol supplementation and onset of gestational diabetes mellitus in pregnant women with a family history of type 2 diabetes: a prospective, randomized, placebo-controlled study. *Diabetes care* **36** 854-857.

D’Oria R, Laviola L, Scioscia M, Fascilla F, Bettocchi S & Giorgino F 2017 PKB/Akt phosphorylation in human umbilical vein endothelial cells is highly induced by myo-inositol and D-chiro inositol: novel potential insights in the pathogenesis of preeclampsia. *Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health* **7** 56.

Delhaes F, Giza SA, Koreman T, Eastabrook G, McKenzie CA, Bedell S, Regnault TRH & de Vrijer B 2018 Altered maternal and placental lipid metabolism and fetal fat development in obesity: Current knowledge and advances in non-invasive assessment. *Placenta* **69** 118-124.

Dessì A & Fanos V 2013 Myoinositol: a new marker of intrauterine growth restriction? *Journal of Obstetrics and Gynaecology* **33** 776-780.

Farese RV, Standaert M, Yamada K, Huang L, Zhang C, Cooper D, Wang Z, Yang Y, Suzuki S & Toyota T 1994 Insulin-induced activation of glycerol-3-phosphate acyltransferase by a chiro-inositol-containing insulin mediator is defective in adipocytes of insulin-resistant, type II diabetic, Goto-Kakizaki rats. *Proceedings of the National Academy of Sciences* **91** 11040-11044.

Gallo LA, Barrett HL & Dekker Nitert M 2017 Review: Placental transport and metabolism of energy substrates in maternal obesity and diabetes. *Placenta* **54** 59-67.

Gardosi J, Francis A, Turner S & Williams M 2018 Customized growth charts: rationale, validation and clinical benefits. *American journal of obstetrics and gynecology* **218** 609-618.

Godfrey KM, Cutfield W, Chan S-Y, Baker PN & Chong Y-S 2017 Nutritional intervention preconception and during pregnancy to maintain healthy glucose metabolism and offspring health (“NiPPeR”): Study protocol for a randomised controlled trial. *Trials* **18** 131.

Haggarty P 2002 Placental regulation of fatty acid delivery and its effect on fetal growth—a review. *Placenta* **23** 28-38.

Hammond LE, Gallagher PA, Wang S, Hiller S, Kluckman KD, Posey-Marcos EL, Maeda N & Coleman RA 2002 Mitochondrial glycerol-3-phosphate acyltransferase-deficient mice have reduced weight and liver triacylglycerol content and altered glycerolipid fatty acid composition. *Molecular and cellular biology* **22** 8204-8214.

Hansen SB 2015 Lipid agonism: The PIP 2 paradigm of ligand-gated ion channels. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **1851** 620-628.

Harriman G, Greenwood J, Bhat S, Huang X, Wang R, Paul D, Tong L, Saha AK, Westlin WF & Kapeller R 2016 Acetyl-CoA carboxylase inhibition by ND-630 reduces hepatic steatosis, improves insulin sensitivity, and modulates dyslipidemia in rats. *Proceedings of the National Academy of Sciences* **113** 1796-1805.

Hayashi E, Hasegawa R & Tomita T 1976 Accumulation of neutral lipids in Saccharomyces carlsbergensis by myo-inositol deficiency and its mechanism. Reciprocal regulation of yeast acetyl-CoA carboxylase by fructose bisphosphate and citrate. *Journal of Biological Chemistry* **251** 5759-5769.

Hayashi E, Maeda T, Hasegawa R & Tomita T 1978 The effect of myo-inositol deficiency on lipid metabolism in rats: III. The mechanism of an enhancement in lipolysis due to myo-inositol deficiency in rats. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* **531** 197-205.

Herrera E & Ortega-Senovilla H 2018 Implications of Lipids in Neonatal Body Weight and Fat Mass in Gestational Diabetic Mothers and Non-Diabetic Controls. *Current diabetes reports* **18** 7.

Huang L, Fonteles M, Houston D, Zhang C & Larner J 1993 Chiroinositol deficiency and insulin resistance. III. Acute glycogenic and hypoglycemic effects of two inositol phosphoglycan insulin mediators in normal and streptozotocin-diabetic rats in vivo. *Endocrinology* **132** 652-657.

Jayabalan N, Nair S, Nuzhat Z, Rice GE, Zuñiga FA, Sobrevia L, Leiva A, Sanhueza C, Gutiérrez JA & Lappas M 2017 Cross talk between adipose tissue and placenta in obese and gestational diabetes mellitus pregnancies via exosomes. *Frontiers in endocrinology* **8** 239.

Keung W, Ussher JR, Jaswal JS, Raubenheimer M, Lam VHM, Wagg CS & Lopaschuk GD 2013 Inhibition of Carnitine Palmitoyltransferase-1 Activity Alleviates Insulin Resistance in Diet-Induced Obese Mice. *Diabetes* **62** 711-720.

Kreuz S, Schoelch C, Thomas L, Rist W, Rippmann JF & Neubauer H 2009 Acetyl‐CoA carboxylases 1 and 2 show distinct expression patterns in rats and humans and alterations in obesity and diabetes. *Diabetes/metabolism research and reviews* **25** 577-586.

Kunjara S, Wang DY, Greenbaum AL, McLean P, Kurtz A & Rademacher TW 1999 Inositol phosphoglycans in diabetes and obesity: urinary levels of IPG A-type and IPG P-type, and relationship to pathophysiological changes. *Molecular genetics and metabolism* **68** 488-502.

Lager S & Powell TL 2012 Regulation of nutrient transport across the placenta. *Journal of pregnancy* **2012** 1-14.

Larner J, Brautigan DL & Thorner MO 2010 D-chiro-inositol glycans in insulin signaling and insulin resistance. *Molecular Medicine* **16** 543.

Matarrelli B, Vitacolonna E, D’angelo M, Pavone G, Mattei PA, Liberati M & Celentano C 2013 Effect of dietary myo-inositol supplementation in pregnancy on the incidence of maternal gestational diabetes mellitus and fetal outcomes: a randomized controlled trial. *The Journal of Maternal-Fetal & Neonatal Medicine* **26** 967-972.

Noventa M, Vitagliano A, Quaranta M, Borgato S, Abdulrahim B & Gizzo S 2016 Preventive and therapeutic role of dietary inositol supplementation in periconceptional period and during pregnancy: a summary of evidences and future applications. *Reproductive Sciences* **23** 278-288.

Plows JF, Budin F, Andersson RA, Mills VJ, Mace K, Davidge ST, Vickers MH, Baker PN, Silva-Zolezzi I & Stanley JL 2017 The effects of myo-inositol and B and D vitamin supplementation in the db/+ mouse model of gestational diabetes mellitus. *Nutrients* **9** 141.

Quirk JG, Jr. & Bleasdale JE 1983 myo-Inositol homeostasis in the human fetus. *Obstet Gynecol* **62** 41-44.

Regnault TR, Teng C, de Vrijer B, Galan HL, Wilkening RB & Battaglia FC 2010 The tissue and plasma concentration of polyols and sugars in sheep intrauterine growth retardation. *Experimental Biology and Medicine* **235** 999-1006.

Tobin KAR, Johnsen GM, Staff AC & Duttaroy AK 2009 Long-chain Polyunsaturated Fatty Acid Transport across Human Placental Choriocarcinoma (BeWo) Cells. *Placenta* **30** 41-47.

Uhl O, Demmelmair H, Segura MT, Florido J, Rueda R, Campoy C & Koletzko B 2015 Effects of obesity and gestational diabetes mellitus on placental phospholipids. *Diabetes research and clinical practice* **109** 364-371.

Van Steenbergen A, Balteau M, Ginion A, Ferté L, Battault S, De Ravenstein CDM, Balligand J-L, Daskalopoulos E-P, Gilon P & Despa F 2017 Sodium-myoinositol cotransporter-1, SMIT1, mediates the production of reactive oxygen species induced by hyperglycemia in the heart. *Scientific Reports* **7** 41166.

Visiedo F, Bugatto F, Quintero-Prado R, Cózar-Castellano I, Bartha JL & Perdomo G 2015 Glucose and fatty acid metabolism in placental explants from pregnancies complicated with gestational diabetes mellitus. *Reproductive Sciences* **22** 798-801.

Visiedo F, Bugatto F, Sánchez V, Cózar-Castellano I, Bartha JL & Perdomo G 2013 High glucose levels reduce fatty acid oxidation and increase triglyceride accumulation in human placenta. *American Journal of Physiology-Endocrinology and Metabolism* **305** 205-212.

Watkins OC, Islam MO, Selvam P, Pillai RA, Karnani N, Cazenave-Gassiot A, Wenk MR, Bendt AK, Godfrey KM, Lewis RM, et al. 2019 Metabolism of 13C-labeled fatty acids in term human placental explants by liquid chromatography mass spectrometry.

Watkins PA 2008 Very-long-chain acyl-CoA synthetases. *Journal of Biological Chemistry* **283** 1773-1777.

Wendel AA, Lewin TM & Coleman RA 2009 Glycerol-3-phosphate acyltransferases: rate limiting enzymes of triacylglycerol biosynthesis. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **1791** 501-506.

Wolf H 1992 Possible new therapeutic approach in diabetes mellitus by inhibition of carnitine palmitoyltransferase 1 (CPT1). *Hormone and metabolic research. Supplement series* **26** 62.

Xia J, Sinelnikov IV, Han B & Wishart DS 2015 MetaboAnalyst 3.0—making metabolomics more meaningful. *Nucleic acids research* **43** 251-257.

Xu J & Ye S 2018 The efficacy of myo-inositol supplementation to prevent gestational diabetes onset: a meta-analysis of randomized controlled trials. *The Journal of Maternal-Fetal & Neonatal Medicine* 1-171.

Xu Y, Huang J, Xin W, Chen L, Zhao X, Lv Z, Liu Y & Wan Q 2014 Lipid accumulation is ahead of epithelial-to-mesenchymal transition and therapeutic intervention by acetyl-CoA carboxylase 2 silence in diabetic nephropathy. *Metabolism* **63** 716-726.

Yan S, Yang X-F, Liu H-L, Fu N, Ouyang Y & Qing K 2015 Long-chain acyl-CoA synthetase in fatty acid metabolism involved in liver and other diseases: an update. *World Journal of Gastroenterology: WJG* **21** 3492.

Yessoufou A, Nekoua MP, Gbankoto A, Mashalla Y & Moutairou K 2015 Beneficial effects of omega-3 polyunsaturated fatty acids in gestational diabetes: consequences in macrosomia and adulthood obesity. *Journal of diabetes research* 1-11.

# **Figure legends**

**Figure 1. Lactate dehydrogenase (LDH; A) and human chorionic gonadotropin (HCG; B) levels in conditioned media were not changed by the addition of 30 or 100 µM myo-inositol.** Levels of LDH and HCG in media were measured after initial 24 h of placental explant culture and at 24 and 48 h after media change. Boxes show median and the interquartile range, whiskers show 5th-95th percentile. LDH was measured using a Roche Cytoxicity detection kitPLUS (04744934001; Manheim, Germany) and HCG using a DRG HCG ELISA kit (EIA 1469; Marburg, Germany) following the manufacturers’ protocol (n=6 placenta). Media from well-triplicates were combined and measured in duplicate. Media + 1.5 % BSA was used to quantify background. Data was normalized such that each assay well contained the equivalent of 30 mg dry placental explant weight and 2 ml of media. Data was normally distributed (Shapiro-Wilk normality test) so data was analyzed by Repeated Measures two-way ANOVA with multiple comparisons tests using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (Graph Pad Prism 7). No significant differences were caused by the addition of myo-inositol.

**Figure 2. Heat map (median fold change) showing the effect of myo-inositol on net fatty acid incorporation into individual placental lipids, 24 h and 48 h after the addition of stable-isotope labeled fatty acids.** Effects were assessed by quantifying the amount of stable-isotope labeled lipids in placental explants with myo-inositol treatment (30 µM, n=7; 100 µM, n=6) relative to the respective controls (no added myo-inositol). Increased lipids are shown in red while decreased lipids are shown in blue. **Myo-inositol response (fold change)** = Amount of 13C-lipid with additional myo-inositol / Amount of 13C-lipid with 0 µM additional myo-inositol. MI: Myo-inositol, PA: Palmitic acid, OA: Oleic acid, DHA: Docosahexaenoic acid, PC: phosphatidylcholine, LPC: lyso-phosphatidylcholine, PE: phosphatidylethanolamine, TAG: triacylglycerol.

**Figure 3. Effect of myo-inositol on the net fatty acid incorporation into placental explant lipids by class, 24 h and 48 h after addition of stable-isotope labeled fatty acids.** Effects were assessed by quantifying the amount of stable-isotope labeled lipids in placental explants (**A, C**) with myo-inositol treatment (**A, B**: 30 µM, n=7 or **C, D**: 100 µM, n=6) and conditioned media (i.e. media incubated with placental explants, **B, D**), expressed relative to the respective controls (no added MI). **Myo-inositol response** (median fold change with 25th and 75th percentiles) = Amount of 13C-lipid in each lipid class with additional myo-inositol / Amount of 13C-lipid in each lipid class with 0 µM additional myo-inositol. Statistical significance \* by Wilcoxon Signed-Rank Test if the FDR adjusted P < 0.05 compared with control (dashed line = 1) or between different time points (indicated by \* above brackets). Letters indicate statistically significant differences in myo-inositol response between lipid classes at 48 h by Freidman test followed by Dunns Post-hoc tests with P < 0.05. Significantly different with 30 µM myo-inositol: **a vs b**. Significantly different with 100 µM myo-inositol: **c vs d** or **e vs f**: significantly different with 100 µM myo-inositol. MI: Myo-inositol, PA: Palmitic acid, OA: Oleic acid, DHA: Docosahexaenoic acid, PC: phosphatidylcholine, LPC: lyso-phosphatidylcholine, PE: phosphatidylethanolamine, TAG: triacylglycerol.

**Figure 4. Influence of the myo-inositol inhibitor, phlorizin (1 mM), on myo-inositol response of 13C-OA lipids with 30 µM myo-inositol at 48 h.**Effects assessed by quantifying the amount of stable-isotope labeled lipids in placental explants (n=3) with myo-inositol treatment (crosses) and myo-inositol with phlorizin (dots) relative to the respective controls (no added myo-inositol).  Lines show the decrease with myo-inositol inhibition for each placenta. Amount relative to control = Amount of 13C-lipid in each lipid class with additional MI / Amount of 13C-lipid in each lipid class with 0 µM additional myo-inositol. MI: Myo-inositol, OA: Oleic acid, PC: phosphatidylcholine, LPC: lyso-phosphatidylcholine, PE: phosphatidylethanolamine, TAG: triacylglycerol, Plz: Phlorizin.

**Figure 5. Heatmap showing correlations (Spearman Rho) between maternal glycemia, maternal BMI and birthweight percentile, with the amount of 13C-PA or 13C-OA labeled placental lipids when incubated with 30 µM MI (n=7).** Rho values are presented to show general trends. Positive correlations are shown in red while negative correlations are shown in blue. The sample size is too small to determine significance for all factors and lipids. Specific statistically significant correlations for lipids by class are highlighted in Figure 6. MI: Myo-inositol, PA: Palmitic acid, OA: Oleic acid, PC: phosphatidylcholine, LPC: lyso-phosphatidylcholine, PE: phosphatidylethanolamine, TAG: triacylglycerol.

**Figure 6. Illustrated correlations between 13C lipid amount, myo-inositol (MI) response and fetal and maternal characteristics with 30 µM MI (n=7).** Relative myo-inositol response (fold change, dots) = Amount of 13C-lipid in each lipid class with additional myo-inositol / Amount of 13C-lipid in each lipid class with 0 µM additional myo-inositol. Absolute molar amount of 13C-fatty-acid-lipid (crosses) expressed as the amount (nmol) in an average well system containing placental explants (30 mg dry) and media (2 ml). Overlapping points are shown by overlapped X and +. Correlations were measured using the non-parametric Spearman’s method.MI: Myo-inositol, PA: Palmitic acid, OA: Oleic acid, PC: phosphatidylcholine, LPC: lyso-phosphatidylcholine, PE: phosphatidylethanolamine, TAG: triacylglycerol.