# Myo-inositol alters $^{13}$C-labelled fatty acid metabolism in human term placental explants: Effects are fatty acid specific and quantifiable by LCMS

Oliver C. Watkins[1], Mohammad Omedul Islam[1], Preben Selvam[1], Reshma Appukuttan Pillai[1], Amaury Cazenave-Gassiot[3,4], Anne K. Bendt[4], Neerja Karnani[2], Keith M. Godfrey[5], Rohan M. Lewis[5], Markus R. Wenk[3,4], Shiao-Yng Chan*[1, 2]

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

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

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

[4] Singapore Lipidomics Incubator, Life Sciences Institute, National University of Singapore, Singapore

[5]MRC Lifecourse Epidemiology Unit and NIHR Southampton Biomedical Research Centre, University of Southampton and University Hospital Southampton NHS Foundation Trust, UK

***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

**Short title:** Myoinositol alters placental $^{13}$C-labelled fatty acid metabolism

## Key points summary

- The placenta regulates the supply of fatty acids and other nutrients from mother to fetus, a process that is disturbed in gestational diabetes.
- As myo-inositol is being investigated as an intervention to prevent gestational diabetes, we examined the effects of increasing myo-inositol concentrations on the processing of labelled fatty acids in small pieces of human placenta collected immediately after uneventful pregnancies.
- Myo-inositol's effect on fatty-acid incorporation into placental lipids depended on the type of fatty acid, with palmitic acid, oleic acid and docosahexaenoic acid (saturated, mono-unsaturated and polyunsaturated fatty acids, respectively) showing different changes.
- All lipids containing the same labelled-fatty-acid showed similar responses to myo-inositol, indicating that myo-inositol affects the early stages of fatty-acid processing.
- The degree of placental responsiveness to myo-inositol *in vitro* correlated with maternal BMI and glucose levels during pregnancy, suggesting that maternal metabolic characteristics may influence placental responses to myo-inositol.

## Abstract

Myo-inositol has been proposed for prevention of gestational diabetes, a condition where placental lipid-processing is disrupted. We investigated *in-vitro* the effect of myo-inositol on fatty-acid (FA) processing in human term placentas from seven uncomplicated singleton pregnancies, with normal mid-gestation oral glucose tolerance tests. Placental explants were incubated with $^{13}$C-labelled palmitic-acid (PA), $^{13}$C-oleic-acid (OA) and $^{13}$C-docosahexaenoic-acid (DHA) (saturated, mono-unsaturated and polyunsaturated respectively) across a range of myo-inositol concentrations for 24h and 48h. The incorporation of labelled-FA into phosphatidylcholines (PC), triacylglycerols (TAG), phosphatidylethanolamines (PE) and lysophosphatidylcholines (LPC) was quantified by liquid-chromatography tandem mass-spectrometry. At 24h, myo-inositol addition increased the amounts of $^{13}$C-PA and $^{13}$C-OA labelled lipids relative to controls. Effects ($p<0.05$, vs control=1) were seen with 30 µM myo-inositol (physiological dose) for $^{13}$C-PA-LPC (median fold-change: 1.26, IQR: 1.07-1.38) and $^{13}$C-PA-PE (1.17, 1.03-1.62). At 48h, myo-inositol addition increased the amount of $^{13}$C-OA labelled lipids but decreased $^{13}$C-PA and $^{13}$C-DHA labelled lipids. Effects were seen for $^{13}$C-OA-PC (1.25, 1.02-1.75), $^{13}$C-OA-PE (1.37, 1.16-1.52) and $^{13}$C-OA-TAG (1.32, 1.21-1.75) with 30 µM myo-inositol and $^{13}$C-DHA-TAG (0.78, 0.72-0.86) with 100 µM myo-inositol. All lipids labelled with the same $^{13}$C-FA showed similar responses to myo-inositol, suggesting that myo-inositol affects upstream processes such as FA uptake or activation. Lipid incorporation and myo-inositol responsivity of $^{13}$C-PA correlated with maternal fasting glycaemia and first trimester BMI but in opposite directions, whilst those of $^{13}$C-OA correlated with maternal post-load glycaemia. Our results suggest that myo-inositol effects on placental lipid processing is fatty-acid dependent and could be influenced by antenatal maternal factors.

## Introduction

The naturally-occurring carbohydrate myo-inositol is the most abundant isomeric form of inositol and is present in all living cells (Noventa *et al.*, 2016). It is endogenously synthesised by the kidney, and is also 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 & Soulage, 2013; Heimark *et al.*, 2014), and with pregnancy disorders such as gestational diabetes (GDM) (Crawford *et al.*, 2015; D'Anna & Santamaria, 2018), pre-eclampsia (D'Oria *et al.*, 2017), intrauterine growth restriction (Dessì & Fanos, 2013), and fetal neural tube defects (Groenen *et al.*, 2003; Copp & Greene, 2010). However, the extent and the focus of the myo-inositol-related pathophysiological events in these conditions are unclear (Tabrizi *et al.*, 2018; Xu & Ye, 2018).

Inositols are the building blocks for a wide range of phosphatidylinositol lipids, inositol phosphate derivatives and inositol-phosphate glycans (IPG). These compounds act as signalling molecules and participate in regulating membrane fluidity, trafficking and transport, organelle function, intracellular compartmentalisation and enzyme activity (Balla, 2013). Many inositol derivatives act as insulin-mimetics or interact with insulin and insulin-like growth factor (IGF) second messenger pathways and therefore modulate glucose and lipid metabolism (Müller *et al.*, 1998; Larner *et al.*, 2010; Hansen, 2015). It can be expected that any disruption in the bioavailability of inositols and inositol-derived signalling compounds could have far reaching and pathological consequences.

In non-pregnant animal studies, myo-inositol deficiency increases lipid mobilisation from adipose tissue and increase hepatic lipid accumulation (Hayashi *et al.*, 1978). Myo-inositol

87 treatment during pregnancy reduced intra-abdominal adiposity in women (Croze & Soulage,
88 2013), and reduced gonadal and perirenal adiposity in mice (Plows *et al.*, 2017).

89 The placenta plays a central role in the generation of GDM pathology, in part through the
90 release of pro-diabetic biological signals (Jayabalan *et al.*, 2017). Furthermore, the placenta
91 regulates transplacental lipid transfer from the mother to the fetus, and placental lipid uptake
92 and metabolism are known to be disrupted in GDM (Uhl *et al.*, 2015; Gallo *et al.*, 2017). This
93 has been postulated to contribute to dysregulated fetal growth and development (Philipps *et al.*,
94 2011; Larqué *et al.*, 2014; Herrera & Ortega-Senovilla, 2018).

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

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

107 We have previously reported that human term placental explants incubated with stable isotope
108 labelled palmitic acid (PA; saturated FA), oleic acid (OA; monounsaturated FA), or
109 docosahexaenoic acid (DHA; long-chain polyunsaturated FA (LC-PUFA)) took up and
110 incorporated the labelled 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 labelled 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.

## 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/m$^2$) 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 $^{13}C_{16}$-palmitic acid ($^{13}$C-PA**;** 99 atom % $^{13}$C, 99% CP; Sigma-Aldrich, 300 µM), $^{13}C_{18}$-oleic acid ($^{13}$C-OA**;** 99 atom % $^{13}$C, 99% CP, Sigma-Aldrich, 300 µM) or $^{13}C_{22}$-docosahexaenoic acid ($^{13}$C-DHA; 99 atom % $^{13}$C, 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% $CO_2$/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=2) 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 (Segal *et al.*,

1984; Novak *et al.*, 1999; 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 effect explant viability (Figure 1). Our previous work showed that explant viability was also not significantly affected by the addition of stable isotope labelled FA (Watkins *et al.*, 2019).

**Lipid quantification**

Lipid extraction and analysis was completed as we have previously described (Watkins *et al.*, 2019). Briefly, placental explants were freeze dried and weighed, then lysed using an Omni bead Rupter homogeniser followed by lipid extraction using a modified Bligh and Dyer method (Bligh & 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 $^{13}C_{16}$-PA, $^{13}C_{18}$-OA or $^{13}C_{22}$-DHA was performed on the Agilent 6490 triple quadrupole (QQQ)-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 normalisation 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 normalised

standard well system containing 30 mg dry weight explant and 2 ml media. Molar amount $^{13}$C-lipid by class is the sum of the molar amounts for all quantified individual $^{13}$C-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 labelled lipid in explants exposed to additional myo-inositol divided by amount of labelled 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 labelled 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 labelled $^{13}$C-PA or $^{13}$C-OA demonstrated incorporation of labelled FAs into LPCs, PCs, PEs and TAGs at 24 h and 48 h. $^{13}$C-DHA incorporation was only reliably quantifiable in TAGs and was only tested at 48 h. Labelled LPCs were the only class of labelled 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 labelled 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 labelled lipids in the treated experiment compared with controls (i.e. Molar Amount of $^{13}$C-lipid with 30 or 100 µM myo-inositol / Molar Amount of $^{13}$C-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 $^{13}$C-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 labelled FA when tested at the same time point (Figures 3A, 3C).

In placental explants at 24 h, myo-inositol addition increased the amounts of labelled lipids compared to controls, with a similar effect seen for $^{13}$C-PA and $^{13}$C-OA labelled lipids. Myo-inositol response (median [inter-quartile range 25$^{th}$ - 75$^{th}$ 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 $^{13}$C-PA-LPC (1.26 [1.07-1.38]) and $^{13}$C-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 $^{13}$C-OA labelled 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 $^{13}$C-OA-PC (1.25 [1.02-1.75]), $^{13}$C-OA-PE (1.37 [1.16-1.52]) and $^{13}$C-OA-TAG (1.32 [1.21-1.75]) (Figure 3A). Treatment with a higher dose of 100 µM myo-inositol showed the same effect as treatment with 30 µM myo-inositol but the effect was not significant compared to the control (Figure 3C).

231 At 48 h, myo-inositol addition resulted in an overall decrease in the amount of $^{13}$C-PA-labelled
232 lipids compared with controls but the effect was not significant. However, the difference
233 between the myo-inositol response for $^{13}$C-PA compared with that for $^{13}$C-OA were statistically
234 significant for every lipid class (Figure 3A). At 48 h, 100 µM myo-inositol also significantly
235 decreased the amount of $^{13}$C-DHA-TAG (0.78 [0.72-0.86]) compared with 0 µM myo-inositol
236 control (Figure 3C). The $^{13}$C-DHA response was also significantly different to that for $^{13}$C-OA
237 ($p<0.05$). It is to be noted that these decreases represent decreases with respect to the control
238 with no added MI. The absolute amount of labelled lipid is not lower at 48 h than 24 h (Watkins
239 *et al.*, 2019).

240 When comparing the difference in myo-inositol response between 24 h and 48 h, only $^{13}$C-PA
241 showed divergent effects of an increase at 24 h but a decrease at 48 h for all four lipid classes.
242 Differences in amount were significant for $^{13}$C-PA-PE (24 h: 1.17 [1.03-1.62] vs 48 h: 0.75
243 [0.57-0.99]) and $^{13}$C-PA-TAG (24 h: 1.05 [0.84-1.80] vs 48h: 0.73 [0.58-1.14]) with the
244 addition of 30 µM myo-inositol (Figure 3A). For $^{13}$C-OA there was no further significant
245 increase in labelled lipids from 24 h to 48 h compared to controls.

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

249 To demonstrate that the changes seen in the amount of labelled lipid were a specific effect of
250 myo-inositol, placental explants treated with the physiological dose of myo-inositol (additional
251 30 µM) were cultured in the presence and absence of the myo-inositol competitive inhibitor,
252 phlorizin. The presence of phlorizin suppressed the 48 h MI-induced increases in all three of
253 the significantly altered $^{13}$C-OA labelled lipid classes relative to control (Figure 4B).

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

Myo-inositol response data for $^{13}$C-OA was suggestive of a sex difference with placentas of female fetuses demonstrating a trend of greater myo-inositol responsivity for $^{13}$C-OA incorporation for all four lipid classes, than placentas from male fetuses, most prominently at 48 h (Figure 4A). This is consistent with general reports that the placenta is a sexually dimorphic organ (Bale, 2016; Auriccio *et al.*, 2017). Sex differences in myo-inositol responsivity for $^{13}$C-PA and $^{13}$C-DHA were not apparent with this small sample size.

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 labelled lipids and customized birthweight percentile.

We first examined the correlations between these characteristics and absolute molar amount of labelled lipid at the physiological myoinositol concentration (additional 30 μM). In general, LPC, PC and PEs labelled with $^{13}$C-PA and $^{13}$C-OA positively correlated with maternal first trimester BMI, but negatively correlated with antenatal fasting glucose concentrations as assessed at mid-gestation. Meanwhile TAGs labelled with $^{13}$C-PA and $^{13}$C-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 $^{13}$C-PA lipids and 48 h 30 μM myo-inositol for $^{13}$C-OA lipids). Myo-inositol response for $^{13}$C-PA-LPC increased with fasting glucose (Rho 0.9, p<0.01, Figure 5A) in contrast to the negative correlation with the amount of placental $^{13}$C-PA-LPC (Rho -0.9, p<0.01, Figure 6A). In comparison, the higher the maternal BMI, the lower the myo-inositol response for $^{13}$C-PA-PC (Rho -0.89, p=0.01, Figure 6B) even though the amount of $^{13}$C-PA-PC was positively associated with BMI. For $^{13}$C-OA at 48 h, the higher the post-load glycaemia at 1 h and 2 h, the greater the 48 h myo-inositol response for $^{13}$C-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 $^{13}$C-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 $^{13}$C-PA-TAG with 30 μM myo-inositol at 24 h (Rho 0.79, p=0.048, Figure 6E) and myo-inositol response for $^{13}$C-OA-TAG (Rho 0.79, p=0.048, Figure 6F). These findings suggest that placental TAG metabolism could be a factor associated with fetal growth. No significant correlations were observed for $^{13}$C-DHA labelled lipids with any of the maternal or fetal characteristics.

## Discussion

This study has demonstrated for the first time that myo-inositol affects the metabolism of $^{13}$C-labelled PA, OA and DHA, in human term placental explants from uncomplicated pregnancies. At each time point, all measured lipids incorporating the same labelled 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 labelled lipids quantified.

Myo-inositol most likely impacts lipid transport and metabolism though the effects of MI-derived signalling molecules such as phosphoinositides, phosphatidylinositol phosphates or inositol phosphoglycans, which are known to regulate lipid metabolism in various tissues (Varela-Nieto *et al.*, 1996; Larner *et al.*, 2010; Hansen, 2015). Myo-inositol may affect enzymes that catalyse the initial steps of lipid synthesis such as the Acyl-CoA long chain synthetases (ACSL, $ACS_VL$), which are important for facilitating placental FA uptake (Tobin *et al.*, 2009; Araújo *et al.*, 2013), or the glycerol phosphate acyl transferases (GPAT) (Kennedy, 1961; 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) (Wolf, 1992; Keung *et al.*, 2013) 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 (Hayashi *et al.*, 1976; Kunjara *et al.*, 1999; Hammond *et al.*, 2002; Araújo *et al.*, 2013).

The activities of GPAT and ACC are influenced by inositol-derived inositol phosphoglycans (Huang *et al.*, 1993; Farese *et al.*, 1994; 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).

The literature also suggests that these enzymes are effected by glycemia which may explain why placental myo-inositol response was associated with maternal glycemia. For example, 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; Kreuz *et al.*, 2009; Xu *et al.*, 2014; Harriman *et al.*, 2016), CPT (Wolf, 1992; Keung *et al.*, 2013) and GPAT (Bates & Saggerson, 1977; Bates *et al.*, 1977; Farese *et al.*, 1994) are affected by glucose, insulin resistance and diabetes in other organs.

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

Maternal obesity and hyperglycemia have been associated with dysregulated placental lipid metabolism (Uhl *et al.*, 2015; Gallo *et al.*, 2017; Delhaes *et al.*, 2018). In our present study, the participants had a range of BMI but none were morbidly obese (BMI>40 kg/m$^2$) and none had gestational diabetes. Even within the relatively narrow range of maternal glycaemia, and the

small sample size, correlations with placental explant incorporation of labelled FA and myo-inositol responsivity were already evident. 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).

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

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

Interestingly, the maternal glycaemia associated changes in the placental $^{13}$C-PA-lipid amount and myo-inositol response are opposite to those associated with maternal BMI, suggesting that maternal glycaemia and BMI impact on placental lipid pathways differently, even though both characteristics are associated with increasing fetal adiposity and size. This may in part 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 (D'Anna & Santamaria, 2018); 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 into TAGs and the myo-inositol effect on this process, with maternal glycaemia and fetal size is intriguing, because placental lipid metabolism is thought to regulate fetal size (Haggarty, 2002; Jansson *et al.*, 2006; Lager & Powell, 2012). Other studies have reported that placental TAGs are increased by hyperglycaemia which in turn promotes fetal macrosomia (Tewari *et al.*, 2011; Visiedo *et al.*, 2013; Visiedo *et al.*, 2015). We previously suggested that placental TAGs act as an accessible FA reserve for transfer to the fetus (Watkins *et al.*, 2019) and hypothesised 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 PCs. Our data therefore suggests that myo-inositol may have the potential to alter placental lipid metabolism to influence fetal growth. However, this study is relatively small and larger studies will be needed to confirm the implications of multiple maternal and fetal characteristics with adjustment for confounding factors.

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; Djelmis *et al.*, 2018). On the other hand, a reduction in DHA incorporation into TAGs may also leave more non-esterified DHA available for fetal transfer. Unfortunately, our placental explant method 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 to further investigate the effects of MI (Perazzolo *et al.*, 2016).

## 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 normal 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, since placental metabolism is regulated by the maternal metabolic environment.

## 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.

Auriccio G, Frank J, Baluta S, Bhaat H, Frank R, Dygulska S, Lederman S, Dygulska B & Salafia C. (2017). Gender and placental features at term. *Placenta* **57,** 283.

Bale TL. (2016). The placenta and neurodevelopment: sex differences in prenatal vulnerability. *Dialogues in clinical neuroscience* **18,** 459.

Balla T. (2013). Phosphoinositides: tiny lipids with giant impact on cell regulation. *Physiological reviews* **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.

Bates EJ, Topping DL, Sooranna SP, Saggerson D & Mayes PA. (1977). Acute effects of insulin on glycerol phosphate acyl transferase activity, ketogenesis and serum free fatty acid concentration in perfused rat liver. *FEBS letters* **84,** 225-228.

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.

Djelmis J, Ivanišević M, Desoye G, van Poppel M, Berberović E, Soldo D & Oreskovic S. (2018). Higher Cord Blood Levels of Fatty Acids in Pregnant Women With Type 1 Diabetes Mellitus. *The Journal of Clinical Endocrinology & Metabolism* **103,** 2620-2629.

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.

Groenen PM, Peer PG, Wevers RA, Swinkels DW, Franke B, Mariman EC & Steegers-Theunissen RP. (2003). Maternal myo-inositol, glucose, and zinc status is associated with the risk of offspring with spina bifida. *American Journal of Obstetrics & Gynecology* **189,** 1713-1719.

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.

Heimark D, McAllister J & Larner J. (2014). Decreased myo-inositol to chiro-inositol (M/C) ratios and increased M/C epimerase activity in PCOS theca cells demonstrate increased insulin sensitivity compared to controls. *Endocrine journal* **61,** 111-117.

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.

Jansson T, Cetin I, Powell T, Desoye G, Radaelli T, Ericsson A & Sibley C. (2006). Placental transport and metabolism in fetal overgrowth–a workshop report. *Placenta* **27,** 109-113.

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.

561 Kennedy EP. (1961). Biosynthesis of complex lipids. In *Fed Proc*, pp. 934-940.

562
563 Keung W, Ussher JR, Jaswal JS, Raubenheimer M, Lam VHM, Wagg CS & Lopaschuk GD.
564 (2013). Inhibition of Carnitine Palmitoyltransferase-1 Activity Alleviates Insulin
565 Resistance in Diet-Induced Obese Mice. *Diabetes* **62,** 711-720.

566
567 Kreuz S, Schoelch C, Thomas L, Rist W, Rippmann JF & Neubauer H. (2009). Acetyl-CoA
568 carboxylases 1 and 2 show distinct expression patterns in rats and humans and
569 alterations in obesity and diabetes. *Diabetes/metabolism research and reviews* **25,**
570 577-586.

571
572 Kunjara S, Wang DY, Greenbaum AL, McLean P, Kurtz A & Rademacher TW. (1999).
573 Inositol phosphoglycans in diabetes and obesity: urinary levels of IPG A-type and
574 IPG P-type, and relationship to pathophysiological changes. *Molecular genetics and*
575 *metabolism* **68,** 488-502.

576
577 Lager S & Powell TL. (2012). Regulation of nutrient transport across the placenta. *Journal of*
578 *pregnancy* **2012,** 1-14.

579
580 Larner J, Brautigan DL & Thorner MO. (2010). D-chiro-inositol glycans in insulin signaling
581 and insulin resistance. *Molecular Medicine* **16,** 543.

582
583 Larqué E, Pagán A, Prieto MT, Blanco JE, Gil-Sánchez A, Zornoza-Moreno M, Ruiz-
584 Palacios M, Gázquez A, Demmelmair H & Parrilla JJ. (2014). Placental fatty acid
585 transfer: a key factor in fetal growth. *Annals of Nutrition and Metabolism* **64,** 247-
586 253.

587
588 Matarrelli B, Vitacolonna E, D'angelo M, Pavone G, Mattei PA, Liberati M & Celentano C.
589 (2013). Effect of dietary myo-inositol supplementation in pregnancy on the incidence
590 of maternal gestational diabetes mellitus and fetal outcomes: a randomized controlled
591 trial. *The Journal of Maternal-Fetal & Neonatal Medicine* **26,** 967-972.

592
593 Müller G, Wied S, Piossek C, Bauer A, Bauer J & Frick W. (1998). Convergence and
594 divergence of the signaling pathways for insulin and phosphoinositolglycans.
595 *Molecular Medicine* **4,** 299.

596
597 Novak JE, Turner RS, Agranoff BW & Fisher SK. (1999). Differentiated Human NT2-N
598 Neurons Possess a High Intracellular Content of myo-Inositol. *Journal of*
599 *neurochemistry* **72,** 1431-1440.

600
601 Noventa M, Vitagliano A, Quaranta M, Borgato S, Abdulrahim B & Gizzo S. (2016).
602 Preventive and therapeutic role of dietary inositol supplementation in
603 periconceptional period and during pregnancy: a summary of evidences and future
604 applications. *Reproductive Sciences* **23,** 278-288.

Perazzolo S, Hirschmugl B, Wadsack C, Desoye G, Lewis RM & Sengers BG. (2016). The influence of placental metabolism on fatty acid transfer to the fetus. *Journal of lipid research***,** 072355.

Philipps L, Santhakumaran S, Gale C, Prior E, Logan K, Hyde M & Modi N. (2011). The diabetic pregnancy and offspring BMI in childhood: a systematic review and meta-analysis. *Diabetologia* **54,** 1957-1966.

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.

Santamaria A, Alibrandi A, Di Benedetto A, Pintaudi B, Corrado F, Facchinetti F & D'Anna R. (2018). Clinical and metabolic outcomes in pregnant women at risk for gestational diabetes mellitus supplemented with myo-inositol: a secondary analysis from 3 RCTs. *American Journal of Obstetrics and Gynecology* **219,** 300.

Segal S, Hwang SM, Stern J & Pleasure D. (1984). Inositol uptake by cultured isolated rat Schwann cells. *Biochemical and Biophysical Research Communications* **120,** 486-492.

Tabrizi R, Ostadmohammadi V, Lankarani KB, Peymani P, Akbari M, Kolahdooz F & Asemi Z. (2018). The effects of inositol supplementation on lipid profiles among patients with metabolic diseases: a systematic review and meta-analysis of randomized controlled trials. *Lipids in health and disease* **17,** 123.

Tewari V, Tewari A & Bhardwaj N. (2011). Histological and histochemical changes in placenta of diabetic pregnant females and its comparision with normal placenta. *Asian Pacific Journal of Tropical Disease* **1,** 1-4.

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.

Varela-Nieto I, León Y & Caro HN. (1996). Cell signalling by inositol phosphoglycans from different species. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **115,** 223-241.

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 & Chan S-Y. (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.

# Additional information

### Funding and Competing interests

This research is supported by a Clinician Scientist Award awarded to SYC 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. SYC, NK and KMG are part of an academic consortium that has received research funding from Abbott Nutrition, Nestec and Danone for work unrelated to this manuscript. 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). KMG 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). KMG and SYC 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.

### Author contributions and acknowledgements

Placental culture experiments were performed in the Department of Obstetrics and Gynaecology, Yong Loo Lin School of Medicine, National University of Singapore by Watkins O.C., Islam O.M., Selvam P. and Pillai R. A. LCMS data was acquired, analysed and interpreted at the Singapore Lipidomics Incubator, Life Sciences Institute, National University of Singapore by Watkins O.C. with the advice and assistance of Cazenave-Gassiot A., Bendt A. K., and Wenk M. R.. Experiment conception and design was completed by Watkins O.C.,

Chan S.Y., Lewis R.M., Cazenave-Gassiot A., Bendt A. K., and Wenk M. R with advice from Karnani N. and Godfrey K. M.. Manuscript was written by Watkins O.C., and Chan S.Y. with advice and editing provided by all other authors. We also 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.

## Abbreviations

AA: Arachidonic acid, BMI: body mass index, BSA: bovine serum albumin, CMRL: Connaught Medical Research Laboratories, DHA: Docosahexaenoic acid, dMRM: dynamic Multiple Reaction Monitoring, FA: fatty acid, GDM: gestational diabetes mellitus, IS: Internal standard, LC-MS/MS: tandem liquid chromatography mass spectrometry, LC-PUFA: long-chain polyunsaturated fatty acids, LA: Linoleic acid, LPC: lysophosphatidylcholine, LPE: lysophosphatidylethanolamine, OA: Oleic acid, PA: Palmitic acid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PUFA: polyunsaturated fatty acids, TAG: triacylglycerol.

## 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 kit$^{PLUS}$ (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 myoinositol.

**Figure 2. Heat map (median) showing the effect of myo-inositol on the net fatty acid incorporation into individual placental lipids, 24 h and 48 h after the addition of stable isotope labelled fatty acids.** Effects were assessed by quantifying the amount of stable isotope labelled 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** = Amount of $^{13}$C-lipid with additional myo-inositol / Amount of $^{13}$C-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 labelled fatty acids.** Effects were assessed by quantifying the amount of stable isotope labelled 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 with 25$^{th}$ and 75$^{th}$ percentiles) = Amount of $^{13}$C-lipid in each lipid class with additional myo-inositol / Amount of $^{13}$C-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 fetal sex (A) and myo-inositol inhibitor Phlorizin (B) on myo-inositol response.** Effects assessed by quantifying the amount of stable isotope labelled lipids in placental explants with myo-inositol treatment relative to the respective controls (no added myo-inositol). **Myo-inositol response** = Amount of $^{13}$C-lipid in each lipid class with additional MI / Amount of $^{13}$C-lipid in each lipid class with 0 µM additional myo-inositol. **(A)** The influence of fetal sex on the effect of 30 µM myo-inositol on labelled LPC, PC, PE and TAG in placental explants at 48 h. Males (dots), Females (crosses). **(B)** The effects of the myo-inositol inhibitor, Phlorizin (1 mM), on MI response of $^{13}$C-OA lipids with 30 µM MI at 48 h (n=2). Myo-inositol only (crosses), myo-inositol with Phlorizin (dots). MI: Myo-inositol, PA:

790 Palmitic acid, OA: Oleic acid, PC: phosphatidylcholine, LPC: lyso-phosphatidylcholine, PE:
791 phosphatidylethanolamine, TAG: triacylglycerol, Plz: Phlorizin.

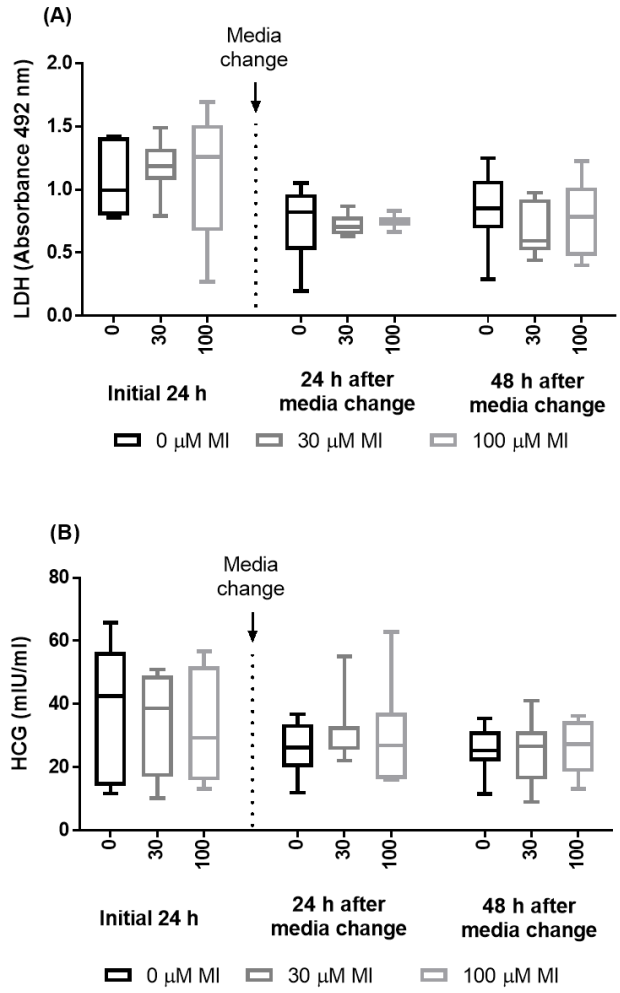
792 **Figure 5. Heatmap showing correlations (Spearman Rho) between maternal glycemia,**
793 **maternal BMI and birth weight percentile, with the amount of $^{13}$C-PA or $^{13}$C-OA labelled**
794 **placental lipids when incubated with 30 µM MI (n=7).** Positive correlations are shown in
795 red while negative correlations are shown in blue. MI: Myo-inositol, PA: Palmitic acid, OA:
796 Oleic acid, PC: phosphatidylcholine, LPC: lyso-phosphatidylcholine, PE:
797 phosphatidylethanolamine, TAG: triacylglycerol.

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

807

808

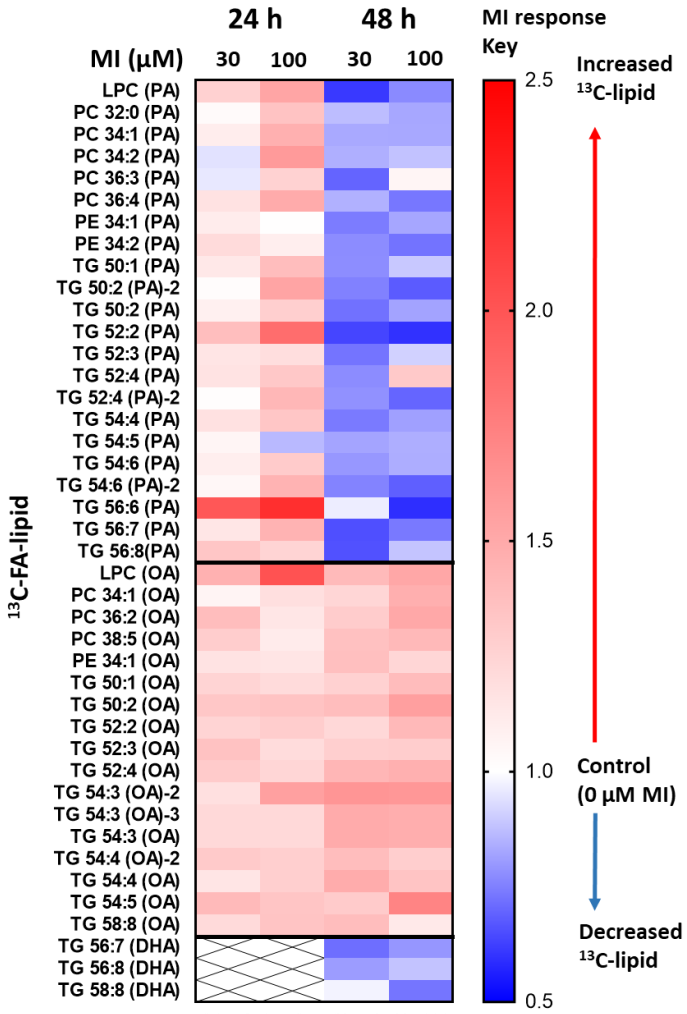
809 **Figure 1**



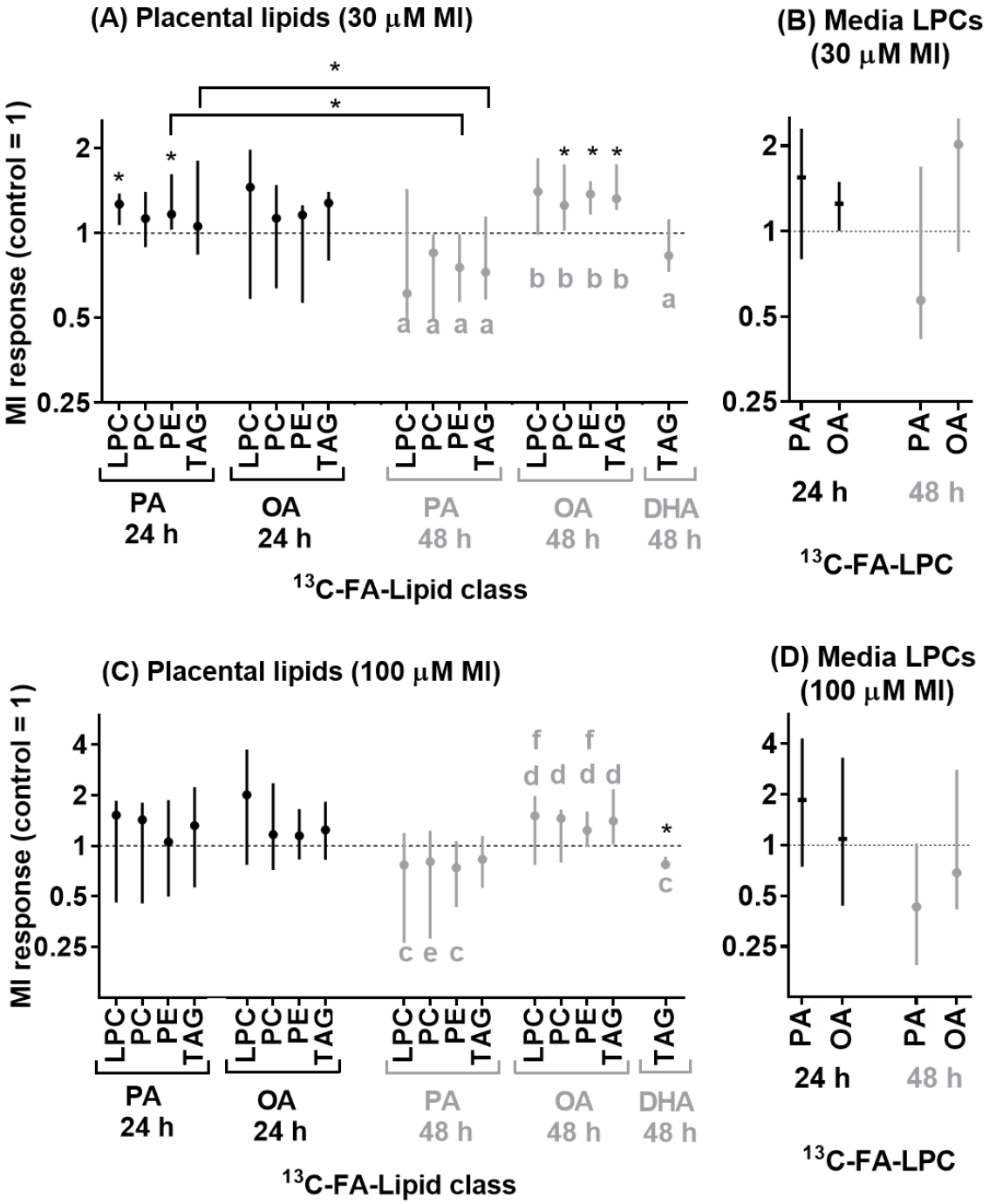

810

811

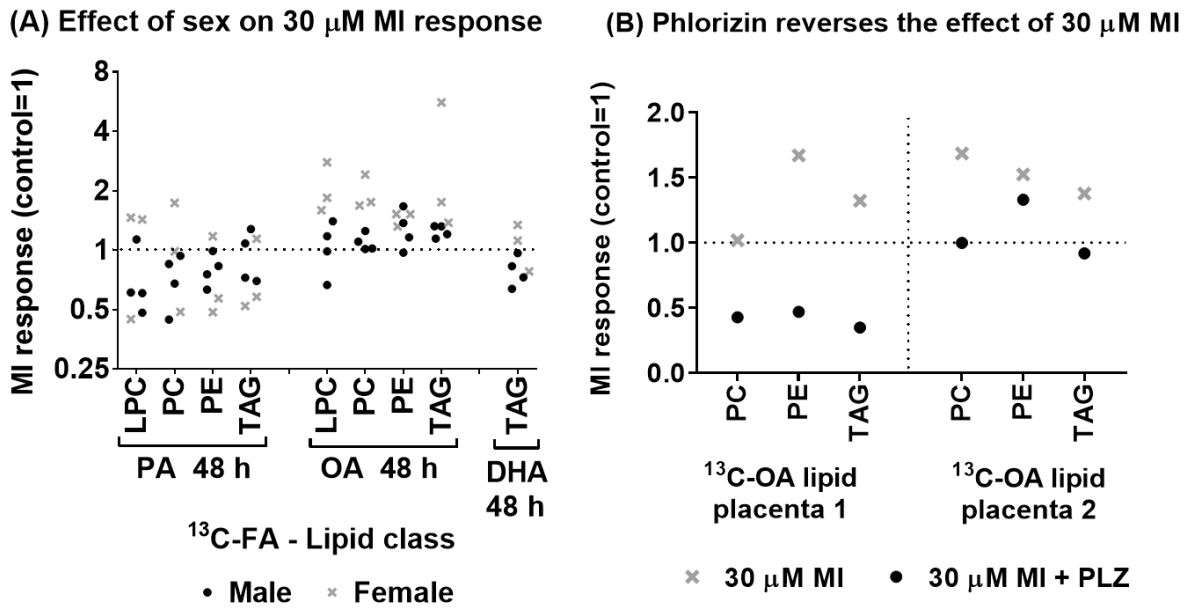
# Figure 2


24 h
48 h
MI response
Key
MI (μM)
30
100
30
100
Increased
13C-lipid
2.5
2.0
1.5
1.0
0.5
Control
(0 μM MI)
Decreased
13C-lipid
13C-FA-lipid
LPC (PA)
PC 32:0 (PA)
PC 34:1 (PA)
PC 34:2 (PA)
PC 36:3 (PA)
PC 36:4 (PA)
PE 34:1 (PA)
PE 34:2 (PA)
TG 50:1 (PA)
TG 50:2 (PA)-2
TG 50:2 (PA)
TG 52:2 (PA)
TG 52:3 (PA)
TG 52:4 (PA)
TG 52:4 (PA)-2
TG 54:4 (PA)
TG 54:5 (PA)
TG 54:6 (PA)
TG 54:6 (PA)-2
TG 56:6 (PA)
TG 56:7 (PA)
TG 56:8(PA)
LPC (OA)
PC 34:1 (OA)
PC 36:2 (OA)
PC 38:5 (OA)
PE 34:1 (OA)
TG 50:1 (OA)
TG 50:2 (OA)
TG 52:2 (OA)
TG 52:3 (OA)
TG 52:4 (OA)
TG 54:3 (OA)-2
TG 54:3 (OA)-3
TG 54:3 (OA)
TG 54:4 (OA)-2
TG 54:4 (OA)
TG 54:5 (OA)
TG 58:8 (OA)
TG 56:7 (DHA)
TG 56:8 (DHA)
TG 58:8 (DHA)

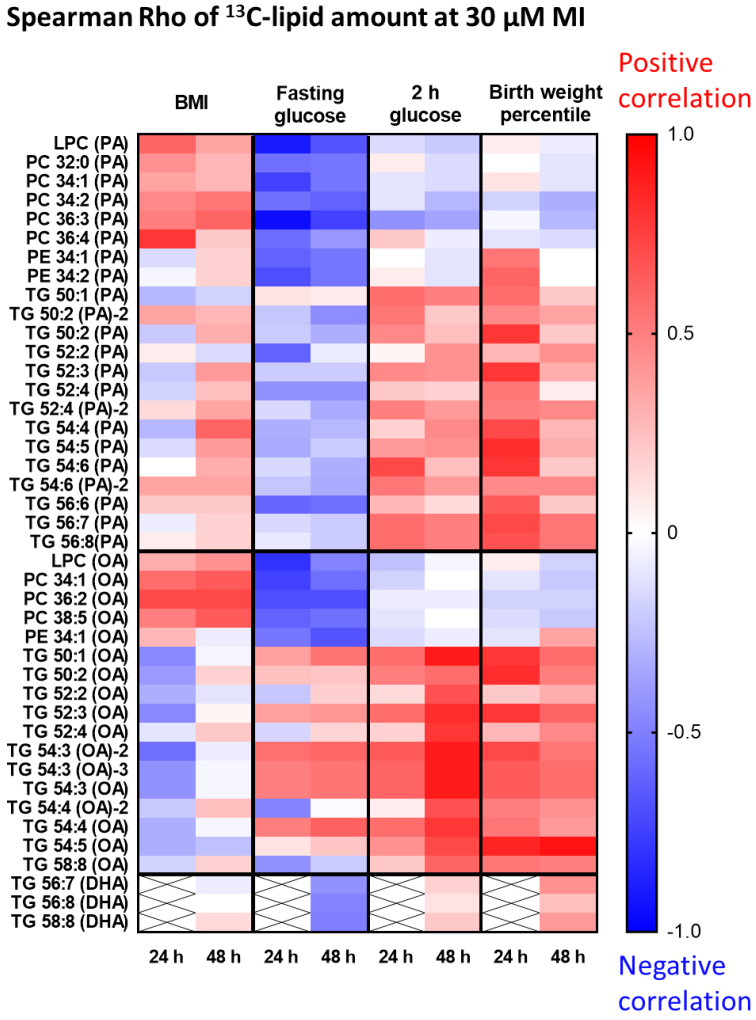
# Figure 3


(A) Placental lipids (30 μM MI)
MI response (control = 1)
2
1
0.5
0.25
LPC PC PE TAG
PA 24 h
OA 24 h
PA 48 h
OA 48 h
DHA 48 h
13C-FA-Lipid class
(B) Media LPCs (30 μM MI)
PA OA PA OA
24 h
48 h
13C-FA-LPC
(C) Placental lipids (100 μM MI)
4
2
1
0.5
0.25
(D) Media LPCs (100 μM MI)

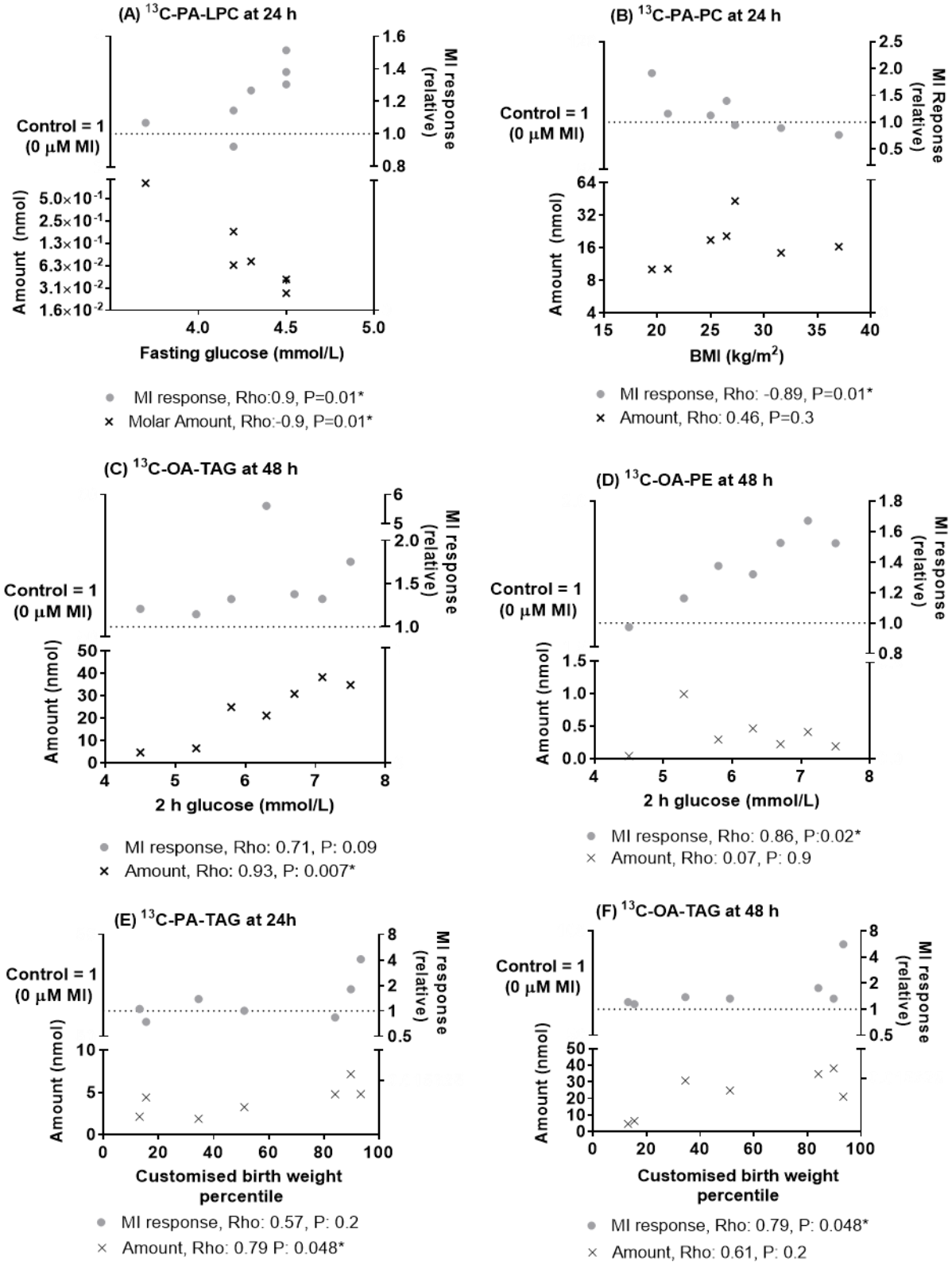
# Figure 4


(A) Effect of sex on 30 μM MI response
MI response (control=1)
8
4
2
1
0.5
0.25
LPC
PC
PE
TAG
LPC
PC
PE
TAG
TAG
PA 48 h
OA 48 h
DHA 48 h
13C-FA - Lipid class
Male
Female
(B) Phlorizin reverses the effect of 30 μM MI
MI response (control=1)
2.0
1.5
1.0
0.5
0.0
PC
PE
TAG
PC
PE
TAG
13C-OA lipid placenta 1
13C-OA lipid placenta 2
30 μM MI
30 μM MI + PLZ

# Figure 5


Spearman Rho of 13C-lipid amount at 30 µM MI
BMI
Fasting glucose
2 h glucose
Birth weight percentile
Positive correlation
1.0
0.5
0
-0.5
-1.0
Negative correlation
LPC (PA)
PC 32:0 (PA)
PC 34:1 (PA)
PC 34:2 (PA)
PC 36:3 (PA)
PC 36:4 (PA)
PE 34:1 (PA)
PE 34:2 (PA)
TG 50:1 (PA)
TG 50:2 (PA)-2
TG 50:2 (PA)
TG 52:2 (PA)
TG 52:3 (PA)
TG 52:4 (PA)
TG 52:4 (PA)-2
TG 54:4 (PA)
TG 54:5 (PA)
TG 54:6 (PA)
TG 54:6 (PA)-2
TG 56:6 (PA)
TG 56:7 (PA)
TG 56:8(PA)
LPC (OA)
PC 34:1 (OA)
PC 36:2 (OA)
PC 38:5 (OA)
PE 34:1 (OA)
TG 50:1 (OA)
TG 50:2 (OA)
TG 52:2 (OA)
TG 52:3 (OA)
TG 52:4 (OA)
TG 54:3 (OA)-2
TG 54:3 (OA)-3
TG 54:3 (OA)
TG 54:4 (OA)-2
TG 54:4 (OA)
TG 54:5 (OA)
TG 58:8 (OA)
TG 56:7 (DHA)
TG 56:8 (DHA)
TG 58:8 (DHA)
24 h
48 h
24 h
48 h
24 h
48 h
24 h
48 h

# Figure 6


(A) 13C-PA-LPC at 24 h
Control = 1
(0 μM MI)
MI response (relative)
Amount (nmol)
Fasting glucose (mmol/L)
MI response, Rho:0.9, P=0.01*
Molar Amount, Rho:-0.9, P=0.01*
(B) 13C-PA-PC at 24 h
Control = 1
(0 μM MI)
MI Reponse (relative)
Amount (nmol)
BMI (kg/m2)
MI response, Rho: -0.89, P=0.01*
Amount, Rho: 0.46, P=0.3
(C) 13C-OA-TAG at 48 h
Control = 1
(0 μM MI)
MI response (relative)
Amount (nmol)
2 h glucose (mmol/L)
MI response, Rho: 0.71, P: 0.09
Amount, Rho: 0.93, P: 0.007*
(D) 13C-OA-PE at 48 h
Control = 1
(0 μM MI)
MI response (relative)
Amount (nmol)
2 h glucose (mmol/L)
MI response, Rho: 0.86, P:0.02*
Amount, Rho: 0.07, P: 0.9
(E) 13C-PA-TAG at 24h
Control = 1
(0 μM MI)
MI response (relative)
Amount (nmol)
Customised birth weight percentile
MI response, Rho: 0.57, P: 0.2
Amount, Rho: 0.79 P: 0.048*
(F) 13C-OA-TAG at 48 h
Control = 1
(0 μM MI)
MI response (relative)
Amount (nmol)
Customised birth weight percentile
MI response, Rho: 0.79, P: 0.048*
Amount, Rho: 0.61, P: 0.2