Maternal metabolic health drives mesenchymal stem cell metabolism and infant fat mass at birth

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Graphical abstract

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(A) TITLE: Maternal Metabolic Health Drives Mesenchymal Stem Cell Metabolism and Infant Fat Mass at Birth.

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(E) KEY WORDS: mesenchymal stem cells, fatty acid metabolism, pediatric obesity, maternal obesity

(F) CONFLICT OF INTEREST: The authors have declared that no conflict of interest.

(G) FUNDING: MLE supported by NIH T32 DK064584. This work and KEB were supported in part by NIH R01DK117168 and the American Diabetes Association (1-18-ITCS-016).
The Healthy Start BabyBUMP Project was supported by the American Heart Association (14PRE18230008), the Colorado Nutrition and Obesity Research Center (NORC; NIH P30DK048520), and by the parent Healthy Start study: (NIH R01DK076648, UH3OD023248, DD) and NIH/NCATS Colorado CTSA (UL1TR001082). LR is supported in part by NIH R01DK124806 and R01NR017644.
Exposure to maternal obesity may promote metabolic dysfunction in offspring. We use infant mesenchymal stem cells (MSC) to experimentally examine cellular mechanisms of intergenerational health transmission. Our earlier reports show MSCs collected from infants of mothers with obesity had a dichotomous distribution in metabolic efficiency; they were either efficient (Ef-Ob) or inefficient (In-Ob) with respect to fatty acid oxidation (FAO). Here, we sought to determine if this was due to a primary defect in FAO. Accordingly, we measured FAO in myogenic differentiating MSCs under three conditions: 1) myogenesis alone, 2) excess fatty acid exposure, and 3) excess fatty acid exposure plus a chemical uncoupler to increase metabolic rate. Compared to NW and Ef-Ob MSCs, In-Ob displayed lower FAO in myogenesis alone and after fatty acid plus uncoupler, indicating In-Ob were less metabolically flexible after increasing lipid availability and metabolic rate, demonstrating a primary deficit in FAO. MSC FAO was negatively associated with fasting maternal glucose and insulin, and positively associated with fasting HDL-cholesterol. MSC FAO was negatively associated with infant fat mass. These data indicate a less favorable maternal metabolic milieu, independent of maternal BMI, reduces intrinsic MSC FAO and is linked to higher infant adiposity as early as birth.

**Brief Summary:** Independent of maternal BMI, worse maternal metabolic milieu is linked to reduced fatty acid oxidation in mesenchymal stem cells from offspring, and adiposity at birth.
INTRODUCTION

Childhood obesity is a global public health crisis in need of efficacious prevention strategies (1). Several observational studies in humans show that exposure to maternal obesity in utero is associated with obesity and metabolic disease risk later in life (2-10). This is in alignment with the Developmental Origins of Health and Disease (DOHAD) hypothesis, which posits that the propensity for obesity may be impacted during fetal development. The DOHAD hypothesis provokes the notion that intervening to improve maternal health will in turn improve offspring health, which illuminates maternal metabolic health endpoints as novel clinical treatment targets. Efforts to empirically test the DOHAD hypothesis at the cellular and molecular levels are however limited due to ethical issues associated with accessing fetal tissue. Previous research by ourselves and others shows the innovative Wharton’s jelly-derived mesenchymal stem cells (MSC) model may overcome this limitation.

MSCs are derived from umbilical cords collected at the time of birth and can be experimentally differentiated along mesodermal lineages. Mesodermal tissues (e.g., adipose, skeletal muscle) are important for the regulation of whole-body metabolism; thus, the MSC model affords an opportunity to directly interrogate human neonatal samples. These in vitro experiments can be used to probe MSC metabolic function, which may reveal a molecular source of metabolic deficiencies potentially promotive of offspring metabolic disease later in life. We have previously employed this model to examine the effects of maternal obesity on MSC metabolism in cells undergoing myogenesis. In support of DOHAD, our work has shown that maternal obesity alters MSC metabolism by promoting excessive fat storage, dysregulated AMPK, and reduced total mitochondrial fatty acid oxidation (FAO) (11). Moreover, we uncovered two distinct phenotypes
among MSCs from infants of mothers with obesity; metabolically efficient and metabolically inefficient with respect to FAO (12).

In our previous study (11), metabolic efficiency was assessed as a ratio of acid-soluble metabolites (i.e., partially oxidized fats) to fatty acids completely oxidized to CO$_2$ (ASM/CO$_2$). A lower ASM/CO$_2$ ratio indicates increased metabolic efficiency, whereas a higher ratio indicates lower metabolic efficiency. The previously observed dichotomy in MSC efficiency may be clinically meaningful, as comparisons of maternal metabolic circulating factors revealed corresponding differences in insulin, HOMA-IR, and free fatty acids. These data suggest that maternal metabolic health may be paramount to transmitting poor metabolic phenotype to their offspring, rather than maternal weight status per se. This unanticipated finding supports the importance of deepening our understanding of the MSC metabolic phenotypes. Here, we tested the hypothesis that differences in metabolism among the Ob-MSCs were due to intrinsic deficits in mitochondrial fatty acid oxidation.

Using metabolic flexibility assessments in vitro, we can more clearly define the origin of metabolic inefficiency. Metabolic flexibility is broadly defined as the ability to respond or adapt to conditional changes in metabolic demand (13). Changes in substrate availability or energetic demand are two common ways to assess such metabolic flexibility in cells, as we and others have considered previously in myotubes from patients with insulin resistance or type 2 diabetes (12, 14-17). For example, it is possible that inefficient MSCs oxidize fewer fatty acids because there is less fatty acid available for oxidation in the mitochondria. Or perhaps basal metabolic rate is lower leading to a backup of TCA cycle products causing fewer fatty acids to be completely oxidized to CO$_2$. If neither of these challenges ameliorate differences in metabolism, then lower FAO represents a true deficit in lipid flux within the mitochondria. Therefore, the primary objective here
was to compare MSC FAO in response to multiple metabolic challenges including excess fatty
acid exposure and chemical uncoupling, which increases cellular metabolic rate, across three MSC
metabolic phenotypes (Normal Weight, Efficient-Ob, and Inefficient-Ob). Given that *in utero*
exposures may be driving MSC metabolism, secondary objectives were to examine relationships
between MSC fat metabolism and maternal cardiometabolic risk factors during pregnancy, as well
as body composition during childhood.
RESULTS

Mesenchymal stem cell donor characteristics

Experimental Groups. Examination of MSCs collected from infants of mothers with obesity revealed two distinct phenotypes, characterized as either high or low metabolic efficiency (11). The ratio of incomplete to complete fat metabolism (ASM/CO2) was used to experimentally characterize MSC metabolic efficiency, as shown in Supplementary Fig. 1, (11). Thus, we defined three phenotypically distinct groups: MSCs from infants of mothers with normal weight (NW-MSCs), metabolically efficient MSCs from infants of mothers with obesity (Ef-Ob), and metabolically inefficient MSCs from infants of mothers with obesity (In-Ob). Importantly, these three groups exhibited a stepwise increase in maternal insulin (11).

Maternal. Participant characteristics for the three groups of MSCs including NW-MSCs (n=15), Ef-Ob-MSCs (n=5), and In-Ob-MSCs (n=9) are shown in Table 1. In earlier reports, we observed significant linear associations between groups using a trend analysis thus, results in Table 1 consistently report trend analysis results (12). Pre-pregnancy weight (P=8.4E-9) and BMI (P=2.3E-12) showed a linear increase across the three groups (NW, Ob-Ef, and Ob-In, respectively). Maternal metabolic panel assessed at late gestation also revealed group differences. This includes a linear decrease in fasting total cholesterol (P=0.027) and HDL cholesterol (P=0.013) across the three groups (NW, Ob-Ef, and Ob-In, respectively). In addition, HOMA-IR (P=0.007) increased linearly across the three groups (NW, Ef-Ob, and In-Ob, respectively).

Infant. Participant characteristics of offspring assessed approximately at birth are shown in Table 1. Cord blood insulin increased (P=0.001) across NW, Ob-Ef, and Ob-In groups. Body composition was assessed 24-48 hours after delivery. Percent fat free mass decreased (P=0.02)
across NW, Ob-Ef, and Ob-In groups. Fat mass, expressed as absolute kg ($P=0.02$) and percent ($P=0.02$), increased across NW, Ob-Ef, and Ob-In groups.

**Experimental design**

Given our previous observation of divergence among MSCs from infants born to mothers with obesity, suggesting of metabolically efficient and inefficient subgroups (11), the primary objective of this study was to determine whether deficits in In-Ob were due to limitations in mitochondrial fat availability or lower cellular metabolic rate that may display as reduced fat metabolism. We measured fat metabolism in myogenic differentiating MSCs under three metabolic conditions: 1) myogenesis alone (control, CT), 2) following 24h of excess fatty acid exposure (oleate/palmitate mix, 24hFA), and 3) following 24hFA, with fat metabolism measures in the presence of FCCP, a chemical uncoupler (24hFA+FCCP). If the Inefficient-Ob MSCs are less metabolically flexible with respect to complete FAO under the metabolic challenge conditions, this would support our hypothesis that they are truly metabolically inefficient with respect to fat oxidation rather than simply oxidizing available substrate at a lower metabolic rate, as would be evident if the metabolic challenges eliminated differences in fat oxidation.

**Cellular fat content is higher in both Ob-MSCs**

We have previously reported that cellular lipid content was higher in Ob-MSCs (18). Here, we measured cellular fat content by Oil Red-O (ORO) staining in CT and 24hFA conditions. As previously shown, ORO was higher in both Ef-Ob and In-Ob, compared with NW-MSCs (Figure 1A; t=2.24, $P=0.04$) during myogenesis (CT), but this effect was diminished with 24hFA. To explore this further, we next measured stored triglyceride (TAG) content and phosphorylation of
proteins involved in lipogenesis. We observed Ef-Ob MSCs stored more TAG than In-Ob MSCs (Figure 1B; t=-2.60, P=0.02). However, we observed no differences in protein content of lipogenesis regulators peroxisome proliferator-activated receptor (PPAR)-\(\gamma\) or sterol regulatory element binding protein (SREBP)1 (Figure 1C), or in lipogenesis proteins fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD)1, diacylglycerol acyltransferase (DGAT)1, and acetyl CoA synthetase (ACS) (Figure 1D, chemiluminescent plots are in Supplementary Fig. 2). Based on these results, it does not appear that enhanced lipogenesis is accounting for differences in cellular fat content observed with ORO staining or TAG measures.

**Fat uptake and esterification is not different between MSC Metabolic Phenotype**

We first examined whether fatty acid uptake or esterification rates were different between groups to account for potential differences in cellular fatty acids available for oxidation. We measured total cellular uptake of C\(^{14}\)-labelled fatty acids and esterification rates in CT, 24hFA, and 24hFA+FCCP conditions. We observed a decrease in total fatty acid uptake during 24hFA+FCCP relative to CT (Figure 2A; F=7.49, P=0.002), which was observed in all three groups (Figure 2A; F=0.121, P=0.97). This decline in fatty acid uptake with uncoupling plus fatty acid excess may be evidence of a preferential substrate shift from fatty acids to carbohydrate, even in the presence of adequate fatty acid availability. Similarly, fatty acid esterification rates in response to 24hFA or 24hFA+FCCP were not different between NW, Ef-Ob, and In-Ob phenotypes (Figure 2B; F=1.15, P=0.36). These results confirm that cellular fatty acid availability is similar among the groups.

**Mitochondrial lipid availability is not different between MSC Metabolic Phenotype**
Total fat oxidation is the sum of two component measures: complete oxidation of fatty acids to CO$_2$ and incomplete oxidation (ASMs), which represents fatty acids that entered the mitochondria but were not completely metabolized. Thus, total fatty acid oxidation represents all fatty acids taken up into the mitochondria during the measurement period. As expected, total fat oxidation increased from CT with 24hFA and returned to CT levels with 24hFA+FCCP (Figure 3A and B; $F=9.78$, $P=0.0004$); however, these changes were not different between the NW, Ef-Ob, and In-Ob groups. Lactate accumulation rates in response to 24hFA did not change (Supplementary Fig 4, $F=0.51$, $P=0.48$) in either MSC group (Supplementary Fig 4, $F=0.24$, $P=0.79$), indicating no change in glycolysis. Taken together, these results indicate that, by design, the 24hFA condition increased mitochondrial lipid availability, and this was not different between groups.

Inefficient-Ob MSCs have intrinsic deficits in FAO

The ASM/CO$_2$ ratio represents mitochondrial efficiency for FAO, where higher values reflect lower mitochondrial fatty acid flux. This could either occur in $\beta$-oxidation or the tricarboxylic acid (TCA) cycle, given that the complete product is derived from the TCA cycle. ASM/CO$_2$ is often higher in skeletal muscle tissue or progenitor cells from animals and humans with established obesity (16, 19-21) and mitochondrial inefficiency for fat oxidation induces insulin resistance in animal models (19).

Mixed model analysis revealed differences in complete oxidation of fatty acids to CO$_2$ among the MSC groups, with a significant Condition by Phenotype interaction (Figure 4A; $F=3.29$, $P=0.02$). Post-hoc analysis showed complete fat oxidation was lower in In-Ob compared to both NW (Figure 4B, $P=0.002$;) and Ef-Ob (Figure 4B, $P=0.006$) in the CT condition. This persisted
during co-incubation with 24hFA+FCCP, where complete oxidation was lower in In-Ob than NW
(P=0.02; Figure 4B) and tended to be lower than Ef-Ob (P=0.11; Figure 4B). Though complete
oxidation tended to be lower in In-Ob compared with NW in the 24hFA condition, this did not
reach statistical significance (P=0.13). We also examined complete oxidation during FCCP
incubation alone, which produced similar results as FA+FCCP (Supplementary Fig 2). These
results are consistent with increased metabolic rate in the FCCP conditions, where increased
metabolism should increase reliance on carbohydrate metabolism. As electron transport and TCA
cycle flux increases, acetyl CoA products from β-oxidation will also be metabolized at a faster
rate. Thus, we can consider this as a maximal flux of fatty acids through β-oxidation and the TCA
cycle.

The ASM/CO₂ ratio tracked with differences in complete oxidation with a significant
Condition by Phenotype interaction (Figure 4C; F=5.32, P=0.002) and higher ratio in In-Ob
compared to both NW (Figure 4C and D; P=0.02;) and Ef-Ob (Figure 4C and D; P=0.0007) in the
CT condition. Likewise, in the 24hFA+FCCP condition, ASM/CO₂ was tended to be higher in In-
Ob compared to Ef-Ob (Figure 4C and D; P=0.09). In general, ASM measures tracked closely
with total fat oxidation measures, which is expected given that ASMs represent the bulk of
mitochondrially oxidized fat in a resting cell. ASMs increased from CT with 24hFA and returned
to CT levels with 24hFA+FCCP (Figure 4E and F; F=12.60, P=6.15E-5). These changes were not
different between groups.

Together, these metabolic measures highlight several important concepts. First, the
experimental conditions achieved changes in fat availability and energetic demand as
demonstrated by increased total FAO with 24hFA and increased complete FAO with
24hFA+FCCP, respectively. Second, there were no differences between groups for fat
esterification or for cellular uptake or mitochondrial availability of fatty acids. Thus, fat availability does not account for observed differences in complete oxidation. Third, group differences in ASM/CO$_2$ ratio were largely driven by differences in complete oxidation of fatty acids to CO$_2$, given that ASM measures were not different between groups. Lastly, increasing mitochondrial lipid availability with 24hFA normalized differences in complete FAO between groups, but the added effect of FCCP demonstrates that, at higher metabolic rates, lipid oxidation through β-oxidation and/or the TCA cycle is impaired in the In-Ob group. Moreover, robust ability to increase FAO in the NW-MSCs indicates that the wide variation in initial metabolic efficiency measures do not necessarily reflect intrinsic capacity for mitochondrial lipid oxidation. Overall, these metabolic flexibility assessments have demonstrated reduced ability to respond to metabolic demand with respect to fat oxidation in the In-Ob group.

MSC FAO correlates with maternal metabolism

Despite striking group differences in complete FAO in the 24hFA+FCCP condition, reflecting differences in intrinsic capacity for fat metabolism, we still note a large degree of variation and overlap among individual values across all three groups. This overlap among the groups indicates they are not phenotypically distinct, and suggests that MSC metabolism is likely influenced along a continuum of various in utero exposures, rather than maternal pre-gravid obesity per se. In our previous work, we observed step-wise changes in maternal metabolic factors for fasting insulin, HOMA-IR, and free fatty acids across MSC groups (12). For the current investigation, we tested correlations among these variables, and also maternal fasting total cholesterol, triglycerides, and glucose (totaling 6 variables) versus MSC FAO after exposure to excess fatty acids and a chemical uncoupler to increase metabolic rate (CO$_2$ 24hFA+FCCP). We
chose to use this MSC experimental condition as it represents maximal FAO during both metabolic challenges, rather than FAO during basal conditions. Of these tested clinical variables, we observed that fasting maternal glucose ($r=-0.41$, $P=0.04$; Figure 5A) and fasting insulin ($r=-0.46$, $P=0.02$; Figure 5B) were negatively correlated with MSC complete FAO, adjusted for maternal BMI and ethnicity. NW-MSCs are interspersed across fasting glucose and insulin correlations highlighting the potentially influential role of maternal metabolic milieu in MSC metabolism, rather than BMI. In addition, fasting HDL cholesterol was positively correlated with MSC complete FAO ($r=0.45$, $P=0.02$; Figure 5C). Furthermore, infant fat mass assessed at birth was negatively correlated with MSC complete FAO ($r=-0.41$, $P=0.046$; Figure 5D), adjusted for gestational age, infant sex, and ethnicity.
DISCUSSION

Maternal obesity alters fetal tissue development in animal models (22-24) providing mechanistic links for intergenerational obesity transmission. In our previous work, we observed that MSCs differentiating into myotubes collected from infants of mothers with obesity were phenotypically different than MSCs from infants of normal weight mothers, and further examination revealed a bimodal distribution of metabolic efficient and inefficient subtypes among MSCs from infants of mothers with obesity (11). Here, we sought to uncover whether differences in MSC metabolic efficiency reflected a primary defect in FAO by assessing cellular metabolic flexibility. We experimentally challenged cells by altering substrate supply and metabolic rate, which are central components of metabolic flexibility. We found that MSC inefficiency was not due to deficits in substrate supply, as fatty acid uptake and esterification were not different between groups. Furthermore, 24hFA incubation increased lipid entry into the mitochondria (assessed as total FAO), as expected, and this was consistent across all groups further underscoring that substrate supply does not contribute to the observed metabolic deficits. Rather, the In-Ob MSCs had lower complete fat oxidation compared to both NW and Ef-Ob MSCs (assessed as complete CO$_2$) that was not normalized when challenged with a chemical uncoupler or uncoupler plus FA. This indicates an inherent, primary deficit in mitochondrial fat oxidation. Taken together, our series of in vitro experiments revealed that the source of MSC metabolic inefficiency was due an inability of the cell to meet the prevailing metabolic demand and are thus metabolically inflexible.

Skeletal muscle plays a primary role in maintaining whole-body metabolic health by regulating substrate metabolism (25). The ability of an organism to modify substrate oxidation during a metabolic challenge, known as metabolic flexibility, is a hallmark of health and can be specific to skeletal muscle (13). We, and others, have previously shown that primary human
myotubes cultured from adults with established obesity are unable to robustly increase FAO rates when challenged with excess fatty acids (14-17, 26). To our knowledge, we are the first to report similar findings in MSCs from infants exposed to maternal obesity in utero as early as fetal development. In the current study, a subset of MSCs collected from infants of mothers with obesity were also unable to increase FAO to the same extent as MSCs from NW or Ob-Ef in response to increasing metabolic demand. This limitation in complete fat oxidation is likely due to a primary deficit in the ability to flux lipids through β-oxidation or the TCA cycle. It is possible there may be a secondary deficit in overall cellular respiration or electron transport flux, but this is not likely to be rate-limiting for FAO. We also observed reduced triglyceride storage in the In-Ob MSCs compared to Ef-Ob MSCs. While speculative, this may be indicative of more favorable lipid storage in Ef-Ob MSCs, as opposed to bioactive lipids such as diacylglycerols or ceramides, perhaps contributing to better metabolic health.

Our findings are consistent with earlier work in which maternal obesity was associated with alterations in fetal skeletal muscle development. In sheep models, fetuses of mothers with obesity have been shown to exhibit defects in skeletal muscle insulin signaling as well as increased fat accumulation and fibrosis (22). Skeletal muscle fiber size may also be detrimentally influenced; maternal obesity has been associated with the downregulation of myogenesis, β-catenin signaling (27), and AMPK signaling (24). In humans, umbilical vein endothelial cells from offspring of mothers with obesity have been shown to exhibit downregulated lipid metabolism and mitochondrial function genes (28). We also observed a downregulation in PI3K and AMPK energy-sensing pathways in MSCs undergoing adipogenesis, (29), as well as in our previous report of myogenic differentiating MSCs AMPK activation was reduced in In-Ob MSCs compared with NW and Ef-Ob MSCs (11). These earlier reports suggest that maternal obesity may predispose
offspring to metabolic disease by altering energy-sensing pathways in myocytes and adipocytes.

Here, we are the first to show that maternal obesity is linked to offspring metabolic inflexibility using human cells.

Chronic exposure to excessive maternal substrates in utero increases fetal growth and adiposity (30). For example, elevated glucose that occurs during gestational diabetes is associated with the delivery of infants large for gestational age (31). Similarly, elevated fasting and postprandial triglycerides in maternal obesity predicts newborn adiposity (% fat) (32). Accordingly, we tested associations between circulating metabolic markers assessed under fasted conditions in late gestation and MSC FAO after excess fatty acid exposure and increased metabolic rate. We observe that FAO (24hFA+FCCP) was inversely correlated with fasting plasma glucose and insulin, and positively correlated with maternal HDL-cholesterol, independent of maternal BMI. The overlap of MSC phenotypes within these correlations strengthens the notion that maternal BMI is not the primary determinant of MSC metabolism. The metabolic milieu of the maternal donors of the metabolically inflexible MSCs also tended to have decreased total cholesterol, HDL cholesterol, and increased HOMA-IR (Table 1). Importantly, these effects were independent of differences in gestational weight gain between groups and FAO (24hFA+FCCP) was not related to gestational weight gain (data not shown). It may be that chronic maternal substrate exposures are recognized by fetal tissues, including MSCs, as nutrient overload that induces metabolic dysregulation. This may partially explain why lifestyle interventions aimed at reducing gestational weight gain during pregnancy have failed to attenuate infant fat accretion (33-35). Rather, excessive substrate availability in the in utero metabolic milieu may contribute to altered MSC metabolic flexibility. Epigenetic mechanisms by which intrauterine exposures alter fetal tissue development are well-established in animal models (36, 37), though these mechanisms
are difficult to define in humans. We previously reported that MSCs from infants of mothers with obesity have hypermethylation and lower mRNA content of genes regulating FAO and AMPK activity (11). We postulate that maternal substrates, that are typically elevated with maternal obesity, may alter MSC epigenetic signatures and subsequent metabolic function. These associations provoke the idea that normalization of the metabolic milieu in women with pre-gestational obesity may re-program MSC metabolism, including metabolic flexibility, and improve offspring outcomes.

Consistent with maternal metabolic milieu, we observed differences in infant body composition between MSC metabolic phenotypes; and also a linear decrease in fat-free mass, and linear increase in fat mass and cord blood insulin across the MSC metabolic phenotypes (NW, Ef-Ob, and In-Ob, respectively). We observed that MSC FAO (24hFA+FCCP) was inversely correlated with neonatal fat mass after adjusting for gestational age, infant sex, and ethnicity. This correlation suggests that greater infant fat mass is related to reduced metabolic health of myogenic differentiating MSCs, which are the precursors to skeletal muscle tissue. These results are similar to findings from animal models, which show that maternal obesity is linked to greater lipid deposition in skeletal muscle, smaller muscle fibers (27) and reduced oxidative metabolism in muscle-derived progenitor cells from offspring skeletal muscle tissue (38). Overall, these novel observations support the notion that maternal metabolic health contributes to altered MSC oxidative metabolism, which may play a role in fetal fat accrual, as suggested by the correlation with neonatal fat mass. However, it must be noted that none of the mothers had developed gestational diabetes, or even clinically elevated fasting glucose or insulin levels. Neither were any of the infants born large for gestational age. Our observations are distinguishing metabolic and phenotypic differences among a relatively healthy group of mothers and infants. Therefore,
whether this infant phenotype drives body composition and skeletal muscle metabolism during childhood is an ongoing investigation in The Healthy Start Study cohort.

Here, we have demonstrated that a subset of MSCs from infants born to mothers with obesity had lower complete fat oxidation compared to both NW and Ob-Ef MSCs that did not normalize when metabolically challenged with a chemical uncoupler or uncoupler plus excess fatty acids. While the causes of intrinsic deficits in FAO are not fully understood, this work shows that it may be evident as early as fetal development and our previous report indicates it may be epigenetically programmed in the MSCs (11). Associations between maternal metabolic health markers and MSC FAO suggest that MSC metabolic programming is driven in part by the maternal metabolic milieu during gestation. The observed associations between infant fat mass and MSC FAO indicates that a less favorable maternal metabolic milieu may induce a primary deficit in skeletal muscle FAO during fetal development, and this is related to greater adiposity as early as birth. Whether clinical intervention restores maternal metabolism and improves fetal tissue development may be of paramount importance for improving body composition and slowing the development of metabolic syndrome in adolescents. Future studies should address this link, as these findings have the potential to impact clinical practice by providing identifying modifiable targets that promote metabolic health at birth and during childhood.
METHODS

Participants

As described previously, MSCs were collected from 165 infants born to mothers participating in the Healthy Start Study (18). Briefly, women were eligible for enrollment if they were 16 years of age or older, currently pregnant with a singleton carry, and ≤ 23 weeks of gestation. Women were excluded for prior diabetes, premature birth, serious psychiatric illness, or multiple pregnancies.

Maternal Phenotyping: Maternal metabolic phenotyping from the Healthy Start Study have been published elsewhere (7). As described, women were characterized at mid-gestation (median of 17 weeks). Assessments include demographics, tobacco use, height and weight. Pre-pregnancy BMI was obtained through medical records (84%) or self-report (16%). Fasting blood samples collected at mid-gestation were analyzed for glucose, insulin, triglycerides, and free fatty acids (FFA).

Infant Phenotyping: Infant weight at time of birth was obtained through medical records. Additionally, infant weight, length, and body composition was assessed within 24-48 h after birth using whole body air plethysmography (PEA POD, COSMED, Inc). Body composition consists of estimates fat mass and fat-free mass.

Mesenchymal Stem Cell Procedures

The MSC culture and isolation procedures have be described previously (18). In brief, MSCs were cultured from umbilical cord explants and all experiments were conducted on cells within passages 4-6. Myotubes were differentiated for 21 days using myogenic induction media.
and then collected for protein or fat content measures or exposed to experimental conditions for
FAO assays.

Measures of stored fats

Following 21 days of myogenesis, cells were fixed with 4% formaldehyde and stained for
oil-red-O lipid content, as described (18). For triglyceride measures, following 21 days of
myogenesis, cells were harvested in ice cold PBS, then pelleted by centrifugation and flash frozen
in liquid nitrogen. Cells were thawed and resuspended and lysed in PBS. An aliquot was removed
for measures of total protein content by bicinechonic acid (BCA) assay. Then, triglyceride content
was measured as described by us (39) using a modified Bligh and Dyer method (40). Resultant
triglyceride concentrations were normalized to starting protein concentration.

Protein content

Cells were harvested in lysis buffer (CelLytic™ MT, Sigma–Aldrich, St. Louis, MO)
supplemented with protease and phosphatase inhibitor cocktails (Sigma–Aldrich). Total protein
was determined by BCA assay. Protein content of peroxisome proliferator-activated receptor
(PPAR)-γ, sterol regulatory element binding protein (SREBP)1, fatty acid
synthase (FAS), stearoyl-CoA desaturase (SCD)1, diacylglycerol acyltransferase (DGAT)1,
and acetyl CoA synthetase (ACS), with β-actin as reference control by Simple Western size-based
protein assay (WES, ProteinSimple, Santa Clara, CA) following manufacturer's protocol, and as
described (11). Results from WES were analyzed using ProteinSimple Compass software. All
antibodies were optimized in-house for this system and antibody specifics and assay conditions
are listed in Supplemental Table 1. Antibodies and conditions are detailed in Supplemental Table 1.

**14C-Fatty Acid Oxidation (FAO) Metabolism Assays**

14C-labeled FAO was assessed as previously described (12). Briefly, 21-day myogenic differentiating MSCs were incubated with containing 200 μM oleate and palmitate (2:1 ratio) with 1 mM carnitine, spiked with 0.25 μCi/mL [14C]-oleate and 0.25 μCi/mL [14C]-palmitate (PerkinElmer Life Sciences, Waltham, MA), as described(11) After a two-hr incubation period, the rate of FAO was determined by measuring 14CO2 released from the media after acidification with perchloric acid. All measurements were performed in triplicate. This method allows for assessment of both complete FAO to CO2 as well as incomplete FAO, quantified as acid soluble metabolites (ASM). The sum of CO2 and ASM measures represent total FAO, and the amount of 14C fatty acids that entered the mitochondria. In addition, fatty acid esterification was measured using 14C-esterified fatty acids extracted with chloroform/methanol. Total cellular fatty acid uptake is calculated as the sum of 14C measured in the cell lysate plus total FAO. These measures were made in three conditions following 21 days of myogenesis: 1) myogenesis alone (control, CT), 2) following 24h of excess fatty acid exposure (24hFA; 200 μM oleate and palmitate [2:1 ratio] plus 1 mM carnitine,), and 3) following 24hFA, with FAO measures in the presence of 8 μM FCCP, a chemical uncoupler (24hFA+FCCP).

**L-lactate index of glycolysis**

Following 21 d of myogenic induction, spent media was replaced with fresh media. Cells were incubated 2 h at 37 °C, then l-lactate accumulation was measured in the media using
the Glycolysis Cell-Based Assay Kit (Cayman Chemical, Ann Arbor, MI). Data were corrected for values in a no cell control well and for total protein content, as measured by BCA method.

Statistics

Data are presented as mean ± SE unless otherwise noted. Homogeneity of variances was tested using Levene’s test. For comparison of participant characteristics listed in Table 1, linear analysis of variance (ANOVA) was used. For comparisons of three groups for lipids and protein measures, data were analyzed using ANOVA with planned contrasts to evaluate the data as three independent groups, as well as NW versus both Ef-Ob and In-Ob groups. This was to account for the possibility that some variables would be similar between Ef-Ob and In-Ob groups, while also testing for individual group differences. For experimental conditions, fixed effects linear mixed models were computed in SPSS to test for an interaction effect of group by condition (repeated measure). If no interaction was observed, the main effects of group and condition were interpreted. Post hoc pairwise testing is adjusted for multiple comparisons using Sidak adjustments.

Correlation analyses were used to test associations between FAO assays and maternal metabolism during mid-gestation, computed in GraphPad Prism. Data were tested for normality using D’Agostino & Pearson tests. Partial correlations were used to determine relationships between MSC metabolic phenotype and maternal or infant variables. Maternal correlations were adjusted for maternal BMI and self-reported ethnicity (41). Infant correlations were adjusted for gestational age, infant sex, and maternal ethnicity. Based on a relatively small number of tested variables, we did not adjust for multiple comparisons.

Study Approval
Umbilical cord tissue samples used in this study were originally collected from participants volunteering in the Healthy Start Study (Clinical Trials.gov, NCT02273297). This study was approved by the Colorado Multiple Institutional Review Board. Written informed consent was obtained from all study participants prior to study inclusion.
AUTHOR CONTRIBUTIONS: M.L.E. analyzed and interpreted the data and drafted the manuscript. Z.W.P. conceptualized study, conducted experiments, acquired data, and edited and approved the manuscript. A.D. conducted experiments, acquired data, and edited and approved the manuscript. D.D. conceptualized and implemented the parent Healthy Start study and edited and approved the manuscript. L.M.R. analyzed and interpreted data and edited and approved the manuscript. K.E.B. conceptualized study, conducted experiments, acquired data, analyzed and interpreted data, as well as edited and approved manuscript.

ACKNOWLEDGEMENTS: Authors would like to thank research participants for their dedication to The Healthy Start BabyBUMP Project. This work was supported by grants the University of Colorado Center for Women's Health Research (KEB), the National Institutes of Health (NIH, R01DK117168, KEB) and the American Diabetes Association (1-18-ITCS-016l, KEB). The Healthy Start BabyBUMP Project was supported by grants from the American Heart Association (14PRE18230008), the Colorado Nutrition and Obesity Research Center (NORC; NIH P30DK048520), and by the parent Healthy Start study (NIH R01DK076648 and 1UG3OD023248, DD) and NIH/NCATS Colorado CTSA (UL1TR001082). Contents are the authors' sole responsibility and do not necessarily represent official views of the funding agencies.


## Table 1

### Characteristics of Mother-Infant Dyads

<table>
<thead>
<tr>
<th>Maternal Characteristics</th>
<th>Normal Weight (N=15)</th>
<th>Obese Efficient (N=5)</th>
<th>Obese Inefficient (N=9)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>28.0 ± 1.5</td>
<td>32.6 ± 4.0</td>
<td>23.4 ± 1.1</td>
<td>0.119</td>
</tr>
<tr>
<td>Race/Ethnicity</td>
<td></td>
<td></td>
<td></td>
<td>0.174</td>
</tr>
<tr>
<td>Hispanic</td>
<td>3 (20%)</td>
<td>2 (40%)</td>
<td>5 (55%)</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>8 (53%)</td>
<td>1 (20%)</td>
<td>3 (33%)</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>1 (6%)</td>
<td>2 (40%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>3 (20%)</td>
<td>0 (0%)</td>
<td>1 (11%)</td>
<td></td>
</tr>
<tr>
<td>Pre-pregnancy Weight (kg)</td>
<td>56.3 ± 1.2</td>
<td>92.8 ± 6.1</td>
<td>88.6 ± 4.5</td>
<td>8.4E-9*</td>
</tr>
<tr>
<td>Pre-pregnancy BMI (kg/m²)</td>
<td>21.1 ± 0.3</td>
<td>34.5 ± 2.0</td>
<td>34.6 ± 1.2</td>
<td>2.3E-12*</td>
</tr>
<tr>
<td>Gestational Weight Gain (kg)</td>
<td>14.2 ± 0.9</td>
<td>7.7 ± 2.6</td>
<td>11.6 ± 3.1</td>
<td>0.231</td>
</tr>
<tr>
<td>Fasting Plasma Glucose (mg/dL)</td>
<td>77.5 ± 2.9</td>
<td>76.4 ± 1.5</td>
<td>80.8 ± 3.0</td>
<td>0.458</td>
</tr>
<tr>
<td>Fasting Plasma Insulin (mU/dL)</td>
<td>12.9 ± 2.3</td>
<td>12.6 ± 1.3</td>
<td>13.4 ± 2.6</td>
<td>0.213</td>
</tr>
<tr>
<td>Free Fatty Acids (mmol/L)</td>
<td>374.1 ± 29.6</td>
<td>476.2 ± 89.3</td>
<td>478 ± 59.7</td>
<td>0.098</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dL)</td>
<td>225.1 ± 9.9</td>
<td>186.3 ± 6.8</td>
<td>192.4 ± 11.4</td>
<td>0.027*</td>
</tr>
<tr>
<td>HDL Cholesterol (mg/dL)</td>
<td>70.7 ± 4.7</td>
<td>59.8 ± 3.1</td>
<td>54.7 ± 4.0</td>
<td>0.013*</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>146.8 ± 12.9</td>
<td>141.8 ± 26.5</td>
<td>179.0 ± 13.5</td>
<td>0.122</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.4 ± 0.1</td>
<td>2.0 ± 0.2</td>
<td>2.6 ± 0.6</td>
<td>0.007*</td>
</tr>
</tbody>
</table>

### Infant Characteristics

<table>
<thead>
<tr>
<th>Sex (M/F)</th>
<th>8/7</th>
<th>4/1</th>
<th>5/4</th>
<th>--</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>3.2 ± 0.08</td>
<td>3.0 ± 0.14</td>
<td>3.3 ± 0.14</td>
<td>0.428</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>12.7 ± 0.23</td>
<td>12.6 ± 0.24</td>
<td>13.3 ± 0.27</td>
<td>0.107</td>
</tr>
<tr>
<td>Fat Free Mass (g)</td>
<td>2,877.8 ± 318.1</td>
<td>2,734.7 ± 170.8</td>
<td>2,881.5 ± 113.9</td>
<td>0.953</td>
</tr>
<tr>
<td>Fat Free Mass (%)</td>
<td>91.6 ± 0.93</td>
<td>91.0 ± 1.69</td>
<td>88.0 ± 0.97</td>
<td>0.020*</td>
</tr>
<tr>
<td>Fat Mass (g)</td>
<td>270.0 ± 31.8</td>
<td>268.9 ± 50.8</td>
<td>398.0 ± 39.2</td>
<td>0.021*</td>
</tr>
<tr>
<td>Fat Mass (%)</td>
<td>8.4 ± 0.93</td>
<td>9.0 ± 1.69</td>
<td>12.0 ± 0.97</td>
<td>0.020*</td>
</tr>
<tr>
<td>Cord Blood Glucose (mg/dL)</td>
<td>75.6 ± 5.94</td>
<td>67.5 ± 7.35</td>
<td>74.0 ± 5.16</td>
<td>0.822</td>
</tr>
<tr>
<td>Cord Blood Insulin (uU/mL)</td>
<td>5.8 ± 0.76</td>
<td>5.5 ± 1.04</td>
<td>11.8 ± 1.39</td>
<td>0.001*</td>
</tr>
<tr>
<td>Cord Blood Free Fatty Acids (uEq/L)</td>
<td>277.6 ± 51.7</td>
<td>201.3 ± 17.3</td>
<td>303.2 ± 51.3</td>
<td>0.685</td>
</tr>
<tr>
<td>Cord Blood Total Cholesterol (mg/dL)</td>
<td>57.2 ± 3.67</td>
<td>46.5 ± 5.68</td>
<td>52.1 ± 6.04</td>
<td>0.425</td>
</tr>
<tr>
<td>Cord Blood HDL Cholesterol (mg/dL)</td>
<td>23.8 ± 1.78</td>
<td>22.0 ± 3.61</td>
<td>24.4 ± 3.01</td>
<td>0.865</td>
</tr>
<tr>
<td>Cord Blood Triglycerides (mg/dL)</td>
<td>42.6 ± 7.4</td>
<td>44.0 ± 7.7</td>
<td>47.6 ± 9.06</td>
<td>0.657</td>
</tr>
</tbody>
</table>

Maternal fasting plasma assessments occurred during late gestation. Values are presented as average ± SE. P statistic represents linear ANOVA tests among normal weight, efficient-obese efficient, versus inefficient-obese. * indicates P ≤ 0.05.
Figure 1
FIGURE 1. Panel A: Cellular lipid content assessed by Oil Red-O (ORO) staining in CT and 24hFA conditions. ORO was higher in both Ef-Ob and In-Ob, compared with NW-MSCs (t=2.25, P=0.04) during myogenesis although this did not persist during 24hFA assessed by ANOVA. Panel B: Stored triglyceride (TAG) content and phosphorylation of protein involved in lipogenesis, in which Ef-Ob MSCs stored more TAG than In-Ob MSC (t=-t.260, P=0.02) assessed by ANOVA. Panel C: Protein content of lipogenesis regulators PPAR γ or SREBP1 not different among MSC metabolic phenotypes assessed by ANOVA. Panel D: Protein content of lipogenesis protein FAS, SCD1, DGAT, and ACS not different among MSC metabolic phenotypes assessed by ANOVA. WES chemiluminescent plots for all proteins are shown in Supplementary Figure 2.
Figure 2

A. Fat Uptake
Condition P=0.002

B. Fat Esterification

not significant
FIGURE 2: Fat uptake and esterification is not different between MSC metabolic phenotypes. Differences in ASM/CO2 among NW, Ef-Ob, and In-Ob MSC metabolism is not explained by differences in fatty acid uptake or esterification. Panel A. Linear fixed effects mixed model ANOVA of fatty acid uptake among MSC metabolic phenotypes (Interaction: F=0.121; P=0.974; Condition: F=7.49, P=0.002; Phenotype: F=0.121; P=0.974). Post-hoc analysis for Condition revealed that 24hFA+FCCP was lower compared with CT for all groups (P=0.002). Panel B. Linear fixed effects mixed model ANOVA of fat esterification among MSC metabolic phenotypes (Condition: F=0.506, P=0.609; Phenotype: F=2.319; P=0.125; Interaction: F=1.145; P=0.358). Data are presented as means ± SE. *indicates significant difference from CT condition.
Figure 3

A. Total Fat Oxidation

Condition P=0.0004

B. NW
Efficient-Ob
Inefficient-Ob
FIGURE 3: Mitochondrial lipid availability is not different between MSC metabolic phenotypes. Differences in ASM/CO$_2$ among NW, Ef-Ob, and In-Ob MSC metabolism is not explained by differences total lipid oxidation. **Panel A.** Linear fixed effects mixed model ANOVA of total lipid oxidation among MSC metabolic phenotypes (Interaction: $F=1.394$; $P=0.255$; Condition: $F=9.783$, $P=0.0004$; Phenotype: $F=2.060$, $P=0.149$). Post-hoc analysis for Condition revealed that the 24hFA condition was elevated compared with both CT and 24hFA+FCCP conditions ($P=0.0002$ and $P=0.02$, respectively). **Panel B.** Individual complete fat oxidation values shown. Data are presented as means ± SE. *indicates significant difference from CT and 24hFA+FCCP conditions.
Figure 4

A. Complete Fat Oxidation
   Interaction P=0.023
   \( \text{CO}_2 \) (pmol/mg protein/hr)
   CT  24hFA  24hFA + FCCP

B. Metabolic Efficiency
   Interaction P=0.002
   \( \text{ASM/CO}_2 \) (pmol/mg protein/hr)
   CT  24hFA  24hFA + FCCP

C. Incomplete Fat Oxidation
   Condition P=6.2E-5
   ASM (pmol/mg protein/hr)
   CT  24hFA  24hFA + FCCP
FIGURE 4: Inefficient-Ob MSCs have intrinsic deficits in FAO. Panel A. Linear fixed effects mixed model ANOVA reveals that complete FAO differs among MSC metabolic phenotypes at different experimental conditions, including control and 24hFA+FCCP (Interaction: $F=3.29$; $P=0.02$ indicated by *). Panel B. Individual complete fat oxidation values shown. Sidak-adjusted pairwise comparisons reveal increased lipid oxidation in NW versus Inefficient-Ob ($P=0.002$), as well as increased fat oxidation in Efficient-Ob vs. Inefficient-Ob ($P=0.006$) at control condition indicated by a and b symbols. Similarly, NW displays increased complete fat oxidation versus Inefficient-Ob at 24hFA+FCCP condition ($P=0.017$) indicated by a and b symbols. Panel C. Linear fixed effects mixed model ANOVA reveals three distinct MSC metabolic phenotypes assessed by ASM/CO$_2$ ratio, an index of metabolic efficiency (Interaction: $F=5.319$; $P=0.002$ indicated by *; Condition: $F=58.97$ $P=8.9E-13$; Phenotype: $F=4.16$; $P=0.027$). Panel D. Individual ASM/CO$_2$ values shown. Sidak-adjusted pairwise comparisons reveal increased ASM/CO$_2$ in NW versus Inefficient-Ob ($P=0.024$), as well as increased FAO in Efficient-Ob vs. Inefficient-Ob ($P=0.001$) at control condition indicated by a and b symbols. Panel E. Linear fixed effects mixed model ANOVA reveals that distinct MSC metabolic phenotypes are not due to differences in incomplete fat oxidation assessed by ASMs (Interaction: $F=1.61$; $P=0.192$ indicated by *; Condition: $F=12.61$; $P=6.2E-5$; Phenotype: $F=1.96$; $P=0.161$). Post-hoc analysis for Condition revealed that the 24hFA condition was elevated compared with both CT and 24hFA+FCCP conditions ($P=0.0002$ and $P=0.0005$, respectively). Panel F. Individual ASM values shown. Data are presented as means ± SE.
Figure 5

A. Maternal HDL-Cholesterol (mg/dL) vs. CO$_2$ 24hFA+FCCP (pmol/mg protein/hr) with correlation coefficient $r = 0.44$, $P = 0.024$

B. Maternal Fasting Insulin (mU/dL) vs. CO$_2$ 24hFA+FCCP (pmol/mg protein/hr) with correlation coefficient $r = -0.48$, $P = 0.013$

C. Maternal HDL-Cholesterol (mg/dL) vs. Infant Fat Mass at Birth (g) with correlation coefficient $r = 0.41$, $P = 0.046$

D. CO$_2$ 24hFA+FCCP (pmol/mg protein/hr) vs. CO$_2$ 24hFA+FCCP (pmol/mg protein/hr) with correlation coefficient $r = -0.46$, $P = 0.017$
FIGURE 5: MSC FAO correlates with maternal metabolism and infant fat mass at birth. Panel A: Partial correlation between CO$_2$ 24hrFA+FCCP and maternal fasting glucose assessed at late gestation (r=−0.46, $P=0.017$, n=28). Panel B: Partial correlation between CO$_2$ 24hrFA+FCCP and maternal fasting insulin assessed at late gestation (r=−0.48, $P=0.013$, n=27). Panel C: Partial correlation between CO$_2$ 24hrFA+FCCP and maternal HDL-cholesterol assessed at late gestation (r=0.44, $P=0.024$, n=23). All maternal correlations were adjusted for maternal BMI and ethnicity. Panel D: Partial correlations between infant fat mass assessed at birth and CO$_2$ 24hrFA+FCCP (r=−0.41, $P=0.046$, n=27), adjusted for gestational age, infant sex, and ethnicity. 95% confidence intervals shown on all panels.
Graphical Abstract

<table>
<thead>
<tr>
<th>MOTHER</th>
<th>INFANT MSCs</th>
<th>NEONATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORMAL WEIGHT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>more favorable metabolic milieu</td>
<td>robust response to metabolic challenge</td>
<td>fat mass</td>
</tr>
<tr>
<td>favorable metabolic milieu</td>
<td></td>
<td>fat mass</td>
</tr>
<tr>
<td>less favorable metabolic milieu</td>
<td>intrinsic deficit in fat oxidation</td>
<td>fat mass</td>
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MYOGENESIS