Maternal Exercise Improves the Metabolic Health of Adult Offspring Through Adaptations to Breastmilk

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Introduction

The prevalence of obesity and type 2 diabetes are increasing dramatically in the United States and worldwide, with recent projections indicating that the prevalence of type 2 diabetes is likely to increase to over 25% of the US population by 2050 (14). Type 2 diabetes is a complex disease that arises from a combination of environmental factors and genetic susceptibility. Increasing evidence has indicated that the *in utero* environment plays an important role in the development of diseases during adulthood, and numerous epidemiological and experimental studies have indicated a relationship between the maternal nutritional environment and obesity, type 2 diabetes, and cardiovascular disease in offspring (5-7, 31, 41, 52, 53, 58, 72, 73, 78, 84, 92, 102). The classic Dutch Famine studies demonstrated that maternal under-nutrition resulted in offspring with increased obesity later in life (78), while children with low birth weight had increased risk for cardiovascular disease (7), impaired glucose tolerance, and type 2 diabetes (31, 41, 73). Other studies have indicated that maternal over-nutrition is an important risk factor for childhood obesity (36, 54). In fact, the detrimental effects of maternal over- or under-nutrition on adiposity and metabolism in offspring have been well established in both human (7, 36, 41, 54, 78) and animal studies (44, 57, 65, 92, 101).

Regular physical exercise is an important preventive therapeutic for several diseases,

including type 2 diabetes. Physical exercise improves glucose homeostasis in people with type 2 diabetes due to enhanced glucose uptake and insulin sensitivity in the working skeletal muscles (24). In response to physical training, there are also molecular adaptations that enhance glucose homeostasis. The effects of exercise to improve glucose homeostasis are likely an important mechanism to explain the strong epidemiological evidence that regular exercise prevents or delays the onset of type 2 diabetes (49, 95).

Exercise during pregnancy has beneficial effects for the mother, including reduced rates of preeclampsia, gestational diabetes, heartburn, and the likelihood for cesarean section (51). The effects of exercise during pregnancy on fetal outcomes have been extensively investigated for many years. The majority of this work focused on fetal growth, as there is a strong association between offspring birth weight and postnatal health outcomes (5). However, until recently, much less was known about the effects of maternal exercise on the metabolic phenotype of offspring. This is an important issue, since insults to the intrauterine environment during pregnancy are a critical factor in the development of obesity and type 2 diabetes in offspring (38) . Here, we will discuss recent findings related to the effects of maternal exercise on offspring metabolic health, how maternal exercise effects an impaired maternal diet, and if maternal exercise influences male or female offspring differently.

Maternal Exercise Improves Offspring Health

In humans, maternal physical activity has been shown to influence perinatal outcomes. Studies investigating diet and physical exercise in humans during pregnancy have shown that exercise reduces gestational weight, decreases the risk for caesarean section, and regarding offspring, results in small but significant reductions in birth weight (1, 21, 66, 99). In one study, vigorous

weight bearing exercise throughout pregnancy in humans resulted in lower body weight of offspring at age 5, with no adverse postnatal health outcomes (21). Maternal exercise has also been associated with lower BMI in offspring at 8 years of age (66). Human studies, both retrospective to examine the effects of diet and exercise during pregnancy, or intervention studies introducing an exercise intervention, are incredibly important to determine the role of maternal exercise on offspring health. While these studies provide important data with regard to the health of the mother and the metabolic phenotype of the infant, it is difficult to follow the child throughout their lifespan and determine the effect of maternal exercise on offspring health. The majority of studies determining how the *in utero* environment affects offspring metabolic health in humans are the result of large epidemiological studies because the changes in health are not seen until adulthood (7, 31, 41, 73, 78). As a result, rodent models have been used to investigate the effects of maternal exercise on the metabolic health of adult offspring (17, 18, 50, 77, 83, 87, 88, 100). While there are discrepancies amongst these studies - different strains of mice and rats were used, different durations and modalities of maternal exercise were studied – the majority of studies resulted in the overall phenotype that maternal exercise before and during pregnancy improves glucose tolerance and insulin sensitivity in adult offspring (Table 1).

Optimal Timing of Maternal Exercise Intervention - Pre- or During Gestation?

An important question when investigating the beneficial effects of maternal exercise on offspring metabolic health is determining the optimal timing of the exercise intervention to confer maximal benefits to the offspring. The majority of studies investigating the effects of maternal exercise on offspring metabolic health subjected the dams to voluntary wheel cage running or swimming 7-21 days before gestation and during gestation (17, 18, 50, 100). While these studies established that exercise before and during pregnancy was important to observe improved

glucose tolerance and insulin sensitivity in adult offspring, it was not clear whether a specific time point of maternal exercise (pre-gestation, during gestation, during lactation, or all of the above) was required to determine the effects on the metabolic health of offspring.

Multiple studies have sought to address this question (17, 18, 83, 88). Recent work in our laboratory determined if the timing of maternal exercise pre-gestation, during gestation, or both, was important to confer the beneficial effects to the metabolic health of adult offspring (88). Female mice were divided into four subgroups: trained (mice housed with running wheels preconception and during gestation), pre-pregnancy trained (housed with wheels preconception), gestation trained (housed with wheels during gestation), or sedentary (housed in static cages). Maternal exercise was not performed during the lactation period. Male offspring of sedentary dams had a worsening of glucose tolerance as they aged, and this effect was negated in the offspring if maternal exercise was performed before and during gestation. Maternal exercise both before and during gestation improved glucose tolerance, lowered fasting insulin, and decreased % body fat in male offspring compared to all other groups. Maternal exercise only during gestation improved glucose tolerance at a young age (8 and 12 weeks), but not during adulthood. Maternal exercise only during pre-pregnancy did not alter glucose tolerance of offspring at any age (88). These data indicate that maternal exercise both before and during gestation is crucial to an improved glucose tolerance in the offspring.

Similar to these results, another study examined the effects of gestation-only exercise in rats on the metabolic health of adult male offspring (83). Female rats were given open access to a wheel cage only during the gestation period. They observed no effect on glucose tolerance in the adult male offspring, but determined decreased % body fat. The male offspring from

gestation-trained dams were also protected from high-fat diet induced hepatic steatosis and had increased expression of liver mitochondrial genes.

Another set of studies examined the effects of maternal exercise (voluntary wheel running) that was performed both pre- and during-gestation and throughout the lactation period (17, 18) using both a rat and mouse model. Adult male offspring (mouse) (17) and adult female offspring (rat) (18) had improved glucose tolerance and increased skeletal muscle insulin sensitivity. In these studies, each time point of maternal exercise (pre-gestation, during-gestation, and during lactation) was not independently investigated, but exercise continued throughout the pre-gestation, gestation, and lactation period.

Together these studies indicate that there are some beneficial effects in offspring if maternal exercise was performed only during gestation, but the maximal effects on offspring glucose tolerance and health are evident if maternal exercise is performed either before and during gestation, or before and during gestation and throughout lactation (17, 18, 83, 88). There was not an added improvement in glucose tolerance if maternal exercise was performed both before and during gestation and continued through lactation, however this has not been closely examined. Further investigation of maternal exercise only during the lactation period would allow insight into this, as well as a potential role for maternal exercise to alter the components of maternal milk that may improve glucose tolerance and metabolic health of offspring. Studies have shown that exercise does not affect the quality of breastmilk composition (25) but can alter the different components, including increasing insulin in the milk (79). From a therapeutic standpoint, the fact that exercise only during gestation can confer some beneficial effects to the offspring is exciting and important; if these results translate to humans it would indicate that a

previously sedentary woman could begin to exercise once she is pregnant and still provide some benefits to her offspring.

Influences of Maternal Exercise on Male vs. Female Offspring

There is an increasing amount of data indicating that metabolic insults and disease states differentially affect males and females. In fact, there are now strong efforts to understand the effects of interventions and treatments in both genders (22). With respect to maternal influences during pregnancy, studies investigating the effects of maternal over-nutrition on both male and female offspring have repeatedly shown that the male offspring have a more pronounced detrimental phenotype than the female offspring (9, 32, 48, 71, 73, 81). Here, we will discuss the effects of maternal exercise in chow-fed dams on the metabolic health of both male and female offspring; effects of maternal exercise in the presence of a maternal high-fat diet will be discussed at a later point.

The majority of studies investigating the effects of maternal exercise on offspring have primarily studied the male offspring (75-77, 83, 88, 100). Studies by our lab and others have shown that maternal exercise in chow-fed dams results in increased % lean mass (17) and decreased % fat mass (17, 88), and decreased body weight (83, 88) in adult male offspring compared to offspring from sedentary dams. Male offspring from exercise-trained dams also had significantly improved glucose tolerance (17, 50, 88), reduce fasting insulin (88), improved insulin tolerance (17), and increased energy expenditure (100).

Studies that have measured the effects of maternal exercise in chow-fed dams on metabolic health of female offspring have seen a somewhat tempered phenotype compared to male offspring. Carter et al. determined that glucose tolerance was improved in female offspring from exercise-trained dams (17, 18) and that female offspring had improved insulin tolerance

(17), and were more insulin sensitive when subjected to euglycemic-hyperinsulinemic clamps (18). Interestingly, work in our laboratory determined no difference in insulin sensitivity measured by euglycemic-hyperinsulinemic clamps in female offspring from chow-fed sedentary or exercise-trained dams (87). We also determined that female offspring from chow-fed, exercise-trained dams had reduced fasting insulin and % body fat compared to offspring from chow-fed sedentary dams (87).

Maternal Exercise Affects Function of Multiple Tissues in Offspring

Interestingly, while the improved glucose tolerance phenotype is more pronounced in male offspring than female offspring (17, 87, 88), the tissue responsible for the improved glucose tolerance has been more thoroughly investigated in female offspring. Work in our laboratory measured glucose clearance *in vivo* in skeletal muscle (tibialis anterior, soleus, gastrocnemius, and extensor digitorum longus). There was no difference in rates of basal or insulin-stimulated glucose clearance in skeletal muscles from offspring of sedentary or exercise-trained dams (88). Another study in male offspring revealed increased expression of hepatic *Pgc1α* and a reduction in the presence of hepatic steatosis, indicating that maternal exercise may exert beneficial effect on offspring through adaptations to the liver. It is important to note, however, that in this study the dams only exercised during gestation and there was no improvement in glucose tolerance observed in male offspring (83).

In female offspring, studies have indicated that maternal exercise causes adaptations to the skeletal muscle (17, 18), adipose tissue (17), and liver (18, 87). In vitro glucose uptake was measured in isolated soleus muscle and parametrial adipose tissue from female offspring. There was no difference in basal glucose uptake among groups, but insulin-stimulated glucose uptake

was significantly increased in both the soleus and parametrial adipose tissue in offspring from chow-fed exercise-trained dams compared to offspring from chow-fed sedentary dams (17). This indicates an important role for skeletal muscle and adipose tissue to mediate the improved glucose tolerance in female offspring from exercise-trained dams. Another study, this time in rats, determined an increase in skeletal muscle glucose uptake after euglycemic-hyperinsulinemic clamps in female offspring from chow-fed exercise-trained dams (18).

Other studies have examined the role of the liver in female offspring in response to maternal exercise. While both studies demonstrate an increase in hepatic insulin sensitivity and reduced hepatic glucose production, the data are slightly conflicting. In rats, the offspring from chow-fed sedentary and exercise-trained dams underwent euglycemic-hyperinsulinemic clamps. Female offspring from exercise-trained dams had increased glucose infusion rates, improved whole-body glucose turnover, and decreased hepatic glucose production (18). We performed a similar euglycemic-hyperinsulinemic clamps experiment in mice, and determined that there was no effect of maternal exercise on glucose infusion rates or whole-body glucose turnover in offspring from exercise-trained dams (87). While these data were perplexing in light of previous experiments, it is possible that the different species used (rats vs. mice) were partly responsible for the conflicting results. Further investigation on the effects of maternal exercise on the liver of female offspring revealed increased insulin sensitivity in isolated hepatocytes and expression of genes involved in hepatic metabolism. We measured glucose production in isolated hepatocytes and expression of hepatic genes involved in mitochondrial biogenesis, fatty acid metabolism, and Krebs cycle activity. Basal, insulin-suppressed, and glucagon-stimulated glucose production in isolated hepatocytes was significantly lower in female offspring from chow-fed, exercise-trained dams compared to offspring from chow-fed, sedentary dams. Several

hepatic genes involved in mitochondrial biogenesis, fatty acid metabolism, and Krebs cycle activity were also significantly higher in offspring from chow-fed exercise-trained dams compared to chow-fed sedentary dams (87). Together these data indicate that maternal exercise affects the skeletal muscle, adipose tissue, and liver in female offspring, and adaptations to one or all of these tissues likely contribute to an improved metabolic response.

The age of the offspring investigated and the intensity of the maternal exercise may also play an important role in which tissue is affected by maternal exercise. The studies described above measured skeletal muscle glucose uptake and methylation in adult offspring in response to voluntary maternal exercise (83, 88). Other studies have examined the effects of maternal treadmill exercise at various intensities before and during gestation in rats. When female rats were subjected to 4 wks of submaximal exercise (55% maximal aerobic speed), their male offspring had lower fasting glucose and pancreas weight, and a smaller islet cell size compared to offspring from sedentary dams at the time of weaning (3-4 wks of age) (76). However, at 7 months of age, male offspring from submaximally exercise-trained dams had a worsened glucose tolerance and impaired muscle insulin sensitivity compared to offspring from sedentary dams. This is in contrast to previous studies (83, 88) that determined improved glucose tolerance in adult male offspring after voluntary maternal exercise.

Controlled, low-intensity treadmill exercise for 4 wks prior to and during gestation improved skeletal muscle insulin sensitivity at 12 wks of age in the male offspring. At this time point there was no change in glucose tolerance or fasting glucose or insulin (75). It is not clear if this increased skeletal muscle insulin sensitivity would persist as the offspring age, but it is important to note that in this case, the skeletal muscle phenotype preceded the improvement in whole-body glucose tolerance. This study also used a controlled maternal exercise (treadmill)

(75) as opposed to voluntary wheel running (83, 88), which could potentially contribute to the different phenotypes observed in offspring. It is clear that the modality and intensity of the exercise are important factors in determining the offspring phenotype, but it has not been established why a more intense maternal exercise could have potentially detrimental effects in adult offspring. The effect of a more intense exercise training regiment on female offspring has also not yet been examined. More studies are needed to identify the optimal training paradigm to confer the beneficial effects of maternal exercise to offspring and to fully elucidate the mechanisms responsible for the improvement in metabolic health in response to maternal exercise, as well as to delineate the different responses in male and female offspring.

Maternal Exercise Increases Physical Activity of Offspring

An interesting question is whether maternal physical activity increases either activity or physical performance in offspring. This is a difficult question to address in humans because of the environmental factor; if the parents are physically active, it is likely that the offspring are in an active environment and continually exposed to activity. In the rodent studies discussed above, examining the effects of maternal exercise to alter offspring metabolic health, all offspring studied were maintained sedentary throughout their lifespan.

A recent study (27) used a rodent model to determine if maternal exercise increased voluntary physical activity in offspring. Female mice were given open access to a wheel cage one wk prior to gestation and throughout their gestation period. In contrast to previous studies, there were no effects determined on offspring body weight or composition during adulthood (17, 18, 87, 88). At 3, 10, and 23 wks of age, offspring from sedentary and trained dams were placed in metabolic cages and activity and energy expenditure. At 10 wks of age, female offspring from

exercise-trained dams had increased activity and energy expenditure compared to offspring from sedentary dams. At 43 wks of age, female offspring were given open access to a wheel cage to determine voluntary exercise over a 3 wk period of time; offspring from exercise-trained dams had a greater % body fat loss, likely due to an increased amount of exercise. This study raises several important points, particularly that minimal maternal exercise (1 wk prior to gestation and during gestation) can increase the volition of exercise on offspring. It is somewhat surprising that this effect was only observed in female offspring instead of male offspring, particularly because the metabolic phenotypes are more pronounced in the male offspring during adulthood. Determining if the amount or intensity of maternal exercise directly impacted the amount of voluntary exercise completed by the offspring will be of interest, and future studies will focus on investigating the mechanism for maternal exercise to increase voluntary exercise in adult offspring. Regardless, these data demonstrate the importance of the early environment to effect activity in offspring. If these data are translatable to humans, it will provide further support for maternal exercise to confer benefits to adult offspring and another potential mechanism for maternal exercise to protect against offspring obesity.

Maternal Exercise and Dietary Interventions

It is well established that maternal obesity and a maternal high-fat diet are major factors in the development of obesity and diabetes in offspring as they age, initiating a vicious cycle that likely contributes to the current rise in rates of obesity and diabetes (5, 9, 39, 41, 44, 45, 47, 57, 60, 73, 78, 101). Similarly, models of maternal under-nutrition result in offspring with increased obesity

during adulthood (78) and an increased risk for development of cardiovascular disease (7) and type 2 diabetes (31, 41, 73). Thus an essential question with regards to maternal exercise intervention is if maternal exercise can negate the detrimental effects of a maternal high-fat diet (Table 2).

Maternal Exercise Negates the Detrimental Effects of a High-Fat Diet

To determine the effects of maternal exercise on offspring metabolic health in the presence of a maternal high-fat diet, multiple studies investigated the effects of placing dams on a high-fat diet while simultaneously giving them open access to voluntary wheel running for 2-3 weeks prior to and during gestation (50, 87, 88). All offspring were sedentary and chow-fed and studied into adulthood.

Male offspring from high-fat fed sedentary dams had marked glucose intolerance as they aged and this was completely negated in offspring from high-fat fed exercise-trained dams (88). In fact, offspring from high-fat fed exercise trained dams had improved glucose tolerance compared to offspring from chow-fed sedentary dams at 52 weeks of age. Male offspring from high-fat fed exercise-trained dams also had lower fasting insulin, reduced body weight and % body fat, and improved insulin tolerance compared to offspring from high-fat fed sedentary dams. These data demonstrate that a maternal high-fat diet, even for a short period of time, has a detrimental effect on offspring metabolic health. Importantly, maternal exercise can prevent these deleterious effects in adult male offspring.

Studies investigating the effects of a maternal high-fat diet and exercise in female offspring also showed a striking effect for maternal exercise to counteract the effects of a maternal high-fat diet (50, 87). Similar to male offspring, a maternal high-fat diet impaired glucose metabolism in adult female offspring and maternal exercise reversed that effect (50, 87).

Female offspring from high-fat fed exercise-trained dams had lower fasting insulin, body weight, and % body fat compared to offspring of high-fat fed sedentary dams. Insulin tolerance was also improved in offspring from high-fat fed exercise-trained dams compared to offspring from high-fat fed sedentary dams (87).

The tissue likely contributing to the improved glucose tolerance in response to maternal exercise in the presence of a maternal high-fat diet was investigated in female offspring. Laker et al. (50) examined the role of *Pgc1α*-promoter methylation and saw that it was hypermethylated in skeletal muscle of female offspring from sedentary high-fat fed dams, but maternal exercise reduced the hypermethylation to that of offspring from chow-fed dams. Expression of genes involved in glucose metabolism were also significantly increased in skeletal muscle of offspring from high-fat fed exercise-trained dams. There was no effect of maternal exercise or high-fat diet to alter *Pgc1a* methylation in the liver or skeletal muscle of male offspring (50).

Our laboratory investigated the effects of maternal exercise in the presence of a maternal high-fat diet on liver function in female offspring. We measured glucose production in isolated hepatocytes and found that offspring from high-fat fed sedentary dams had impaired basal, insulin-suppressed and glucagon-stimulated glucose production, but offspring from high-fat fed exercise-trained dams had glucose production similar to that of offspring from chow-fed dams. Expression of hepatic genes involved in mitochondrial biogenesis, fatty acid metabolism, and Krebs cycle activity were significantly reduced in offspring from high-fat fed sedentary dams, but offspring from high-fat fed exercise-trained dams had gene expression similar to that of offspring from chow-fed sedentary dams (87). Together these data indicate that maternal exercise negates the detrimental effects of a maternal high-fat diet in both male and female

offspring. It is important to note that in the studies discussed, the maternal high-fat diet was over a relatively short time course (2-3 weeks prior to gestation). Even with this mild intervention, offspring from high-fat fed dams had significantly worsened glucose tolerance, and this effect was reversed in the presence of maternal exercise.

As stated above, these studies all examined the effects of maternal exercise that began simultaneously with the presence of a maternal high-fat diet on offspring health (50, 87, 88). While important, a more translational approach would be to examine the effects of maternal exercise in an already obese model and determine if this could negate or reverse the detrimental effects of maternal obesity on offspring metabolic health. A recent study investigated this using a rat model; female rats were placed on a high-fat diet for 6 wks and then given open access to a running wheel where they could complete voluntary exercise and maintained a high-fat diet for 4 wks prior to conception (97). Male offspring from high-fat fed dams who were exercise-trained had lower circulating leptin and triglycerides and decreased fat mass at 5 wks of age compared to male offspring from high-fat fed sedentary dams. Female offspring were not investigated, and offspring were not followed later than the 5 wk time point. These data are intriguing, indicating that maternal exercise could at least partially negate the detrimental effects of maternal obesity and could have a tremendous impact on the human population if translatable. More investigation is needed to determine if maternal exercise in an already obese mother can affect offspring metabolic health into adulthood.

Maternal Exercise Abolishes the Impaired Metabolic Response to Maternal Protein-Restriction

A maternal low-protein diet is one of the most well-studied models of early growth restriction. It is associated with elevated systolic blood pressure, increased fasting insulin and impaired glucose tolerance in offspring compared to offspring from dams fed a normal diet (70). Recent studies investigated the effects of maternal protein-restriction in the presence of exercise both before and during gestation (3, 30, 33) in rats on male offspring. Adult female Wistar rats were subjected to a controlled, moderate- to low-intensity exercise training regiment on a treadmill for 4 wks prior to gestation and maintained on the same program throughout the gestation period. After conception the dams were further subdivided into a group that received a normal protein (17% casein) diet or a low protein (8% casein) diet and remained on that diet through gestation and lactation.

Offspring from low protein fed, sedentary dams had decreased growth rates (3, 30), decreased reflex maturation (30), increased abdominal circumference, elevated fasting glucose and cholesterol, impaired glucose tolerance, and decreased plasma leptin (33) compared to offspring from normal protein fed, sedentary dams. Maternal exercise, however, attenuated the effects of a low protein diet. Offspring from low protein fed, exercise trained dams have increased growth rates (3, 30), improved reflex maturation (30), lower abdominal circumference, decreased fasting glucose and cholesterol, improved glucose tolerance (33) compared to offspring from low protein fed, sedentary dams. Maternal exercise did not completely normalize the effects of a maternal low-protein diet, but it did improve growth rates and most metabolic markers. These data indicate that maternal exercise could be a therapeutic option for disorders associated with perinatal under-nutrition (33)

Maternal Exercise vs. Maternal Under-Nutrition

To this point, there have been no studies investigating the effects of maternal exercise in an under-nutrition model or any type of dietary energy restriction. It is important to note, however, that exercise is a model of voluntary energy expenditure and results in an opposite phenotype to that of a dietary energy restriction, with improvements in glucose tolerance and adiposity. There has not been a mechanistic link established between these phenotypes, but future investigations focused on why energy restriction vs. energy expenditure result in contradicting metabolic phenotypes could be an important approach.

Maternal Exercise Prevents Obesity in Offspring Fed a High-Fat Diet

While numerous studies have examined the effects of maternal exercise to negate the detrimental effects of an impaired maternal diet on offspring metabolic health, a few recent studies have examined the effects of maternal exercise to protect or preserve the metabolic health of the offspring when the offspring are fed a high fat or high sucrose diet.

In one study, female rats were divided into a sedentary or exercise group 4 wks prior to gestation. The exercise group underwent 4 wks of low-intensity treadmill exercise and the exercise regimen continued during the gestation period (75). After weaning, offspring were either placed on a standard chow (5.1% fat, 4.4% other sugars) diet or fed a high-fat/high-sucrose (36% fat, 16.6% sucrose) diet for 10 weeks. There was no effect of maternal exercise to effect glucose tolerance, or fasting glucose or insulin in chow fed male offspring at 12 wks of age. Maternal exercise did improve muscle insulin sensitivity in chow fed male offspring.

Offspring fed a high-fat/high-sucrose diet had increased body weight, impaired glucose tolerance, increased fasting glucose and insulin, and reduced liver glycogen compared to offspring fed a standard chow diet. Maternal exercise, however, was protective against these

deleterious effects of a high-fat/high-sucrose diet. Offspring from exercise-trained dams fed a high-fat/high-sucrose diet had lower body weight, fasting glucose, and insulin, and increased liver glycogen compared to offspring from sedentary dams fed a high-fat/high-sucrose diet. Muscle insulin sensitivity was also increased in male offspring from exercise-trained dams fed a high-fat/high-sucrose diet compared to offspring from sedentary dams fed a high-fat/high-sucrose diet (75). It is important to note that the chow diet used in this study contained only 5% fat, while most chow diets contain ~20% fat. This does not take away from the effects of maternal exercise to protect against a high-fat diet, but should be considered a low-fat diet instead of a standard chow comparison.

Other studies have examined the effects of maternal exercise during gestation (83) or during gestation and lactation (79) to protect against the offspring being overfed or fed a high-fat diet. In both cases, offspring from exercise-trained dams had an improved metabolic phenotype compared to offspring from sedentary dams when overfed (79) or fed a high-fat diet (83). Maternal exercise improved glucose tolerance, insulin tolerance, and reduced the presence of hepatic steatosis in male offspring during adulthood when compared to offspring from sedentary dams (79, 83).

Together these data suggest that maternal exercise exerts a protective effect on offspring, even when the offspring are overfed or fed a high-fat or high-fat/high-sucrose diet. While the mechanism for this protective effect has not been established, increased muscle insulin sensitivity is likely important (75). The length of time the offspring were overfed or fed the high-fat or high-fat/high-sucrose diet also varied in each of these studies, and only male offspring were investigated (75, 79, 83). Future investigation will be imperative to determine how maternal exercise preserves metabolic parameters in offspring who are fed a high-fat diet, as well as to

establish when during gestation maternal exercise is required, and the intensity of the maternal exercise required to maximize these benefits. In terms of human physiology, if an ideal maximal time point and intensity of maternal exercise could be identified to confer beneficial effects to offspring to protect them from the detrimental effects of a high-fat diet, the implications on public health cannot be overstated.

Maternal Exercise and Gestational Diabetes

Gestational diabetes mellitus (GDM) is associated with both short- and long-term complications for the mother and her baby. It has been associated with disorders including hypertension, pre-eclampsia and early births, as well as an increased risk for perinatal morbidity, impaired glucose tolerance and type 2 diabetes following pregnancy (82). Recent studies have shown that exercise interventions may provide a protective effect on maternal glycemic control, thus improving maternal and infant outcomes (1, 15, 99).

The effect of maternal exercise in women with gestational diabetes on offspring metabolic health has not been extensively investigated, likely because epidemiological studies are difficult and there is not a clear animal model to use to study gestational diabetes. One study examined the effects of maternal exercise in diabetic rats and determined a beneficial effect on metabolic health of the offspring. Rats were given streptozotocin (STZ) to induce diabetes and 14 days later subjected to 14 days of pre-gestation exercise on a treadmill or kept sedentary. Offspring from exercise-trained diabetic dams had improved glucose tolerance at 4 weeks of age compared to offspring from sedentary diabetic dams (96). It is important to note that in this study, all offspring nursed with euglycemic foster moms and the offspring were only studied at 4 weeks of age. Regardless, maternal exercise in a diabetic dam improved glucose tolerance in

offspring. More studies are needed to fully understand the effects of maternal exercise in a diabetic mother on the metabolic health of the offspring.

Concluding Remarks and Future Perspectives

The intrauterine environment during pregnancy is a critical factor in the development of type 2 diabetes and obesity in offspring. Studies in both humans and rodents have shown that maternal over-nutrition and under-nutrition result in metabolic impairments during adulthood including increased rates of obesity and type 2 diabetes (5-7, 31, 36, 41, 44, 52-54, 57, 58, 65, 72, 73, 78, 84, 92, 101, 102). Regular exercise is an important therapeutic tool to combat obesity and improve metabolic health in the general population, but the role of maternal exercise during pregnancy on the metabolic health of the offspring has been poorly understood and human studies have been limited. Rodent models of exercise during pregnancy have been put forth to investigate these questions and have compellingly shown that maternal exercise improves the metabolic health of adult male and female offspring (3, 17, 18, 33, 50, 75, 83, 87, 88). Importantly, and with particular translatable ramifications, maternal exercise negates the detrimental effects of an impaired maternal diet on offspring metabolic health (3, 30, 33, 50, 87, 88).

These exciting studies stress the important of maternal exercise but leave us with several important questions. One such question to address is to determine the optimal timing of maternal exercise to confer metabolic benefits to the offspring. The studies discussed above examined offspring from maternal exercise that occurred: 1) pre-gestation; 2) during gestation; 3) both pre-gestation and during gestation; 4) pre-gestation, during gestation, and during lactation; and 5) during gestation and during lactation. Exercise only pre-gestation did not result in any beneficial

effects to the metabolic health of adult offspring. Investigating the timing of maternal exercise begins to address the question as to the mechanism of action through which maternal exercise improves offspring metabolic health. Because there is no effect of pre-gestation only exercise to affect offspring health (87, 88), it is unlikely that epigenetic changes to the oocyte are the mechanism responsible. Epigenetic changes to the placenta, however, have not been investigated. Changes in *Pgc1a* methylation in the liver (83) and skeletal muscle (50) of offspring were observed in previous studies, but other epigenetic changes in tissues have not been investigated. How these mechanistic changes occur to the offspring, and how they persist into adulthood is not clear and is an important topic of future investigation to maximize the potential therapeutic benefits of maternal exercise to improve the metabolic health of adult offspring.

In addition, another important question is to determine the intensity of maternal exercise required to confer metabolic benefits to offspring. The majority of the studies discussed above utilized either voluntary wheel cage running (17, 18, 50, 83, 87, 88) or low intensity treadmill exercise (3, 30, 33, 75, 79) and observed metabolic benefits in the adult offspring. However, a study that subjected the dams to a more intense exercise protocol (55% maximal aerobic speed) prior to and during gestation resulted in impaired glucose tolerance in adult offspring even though skeletal muscle insulin sensitivity was increased in offspring at 12 wks of age (76). The reasons for the discrepancies in these data are unclear. In all of these studies, the intensity of the exercise remained constant throughout the entire training period, regardless of whether it occurred pre-gestation, during gestation, or during lactation. In rodent studies using models of voluntary maternal exercise, the amount of exercise decreases throughout the gestation period. When using a submaximal treadmill exercise protocol, the pregnant mice were exercised at a

high intensity regardless of their point during gestation; it is possible that this caused a stress to the dam that resulted in a negative phenotype during adulthood in the offspring. The submaximal exercise regiment was chosen to correspond to the intensity guidelines for exercise in pregnant women around the world, and similar to the frequency recommended in the United States and Denmark (28). It is possible that the different exercise intensities affect placental blood flow differently thus resulting in varying offspring phenotypes. It is also possible that epigenetic changes induced by exercise differ with varying intensities of maternal exercise (8) thus affecting the phenotype of the offspring. More studies are needed to fully determine the optimal intensity of exercise, particularly in humans, to determine how maternal exercise can improve metabolic health of adult offspring.

Future studies investigating exercise interventions in pregnant women will provide insight into both the mechanism through which maternal exercise improves metabolic health in offspring, as well as to how translatable the rodent model is to humans. At least two such trials are underway, one investigating an exercise intervention in overweight and obese women prior to gestation (https://clinicaltrials.gov/ct2/show/NCT03146156) and one that will provide an exercise intervention in women who are ~12 wks pregnant (https://clinicaltrails.gov/ct2/NCT02125149). Both of these studies will investigate maternal health, body weight, and insulin sensitivity as a primary outcome, but will also include measures of the placenta (mitochondrial enzyme activity and lipid metabolism) (https://clinicaltrials.gov/ct2/show/NCT03146156) or will track the offspring for at least the first two years of life (https://clinicaltrails.gov/ct2/NCT02125149). These investigations, as well as others, will provide greater insight into the effects of maternal exercise on offspring metabolic health in humans.

In summary, maternal exercise before and during pregnancy significantly improves the metabolic health of adult male and female offspring in rodents and offsets the detrimental effects of an impaired maternal diet (high-fat or low protein). These findings, if translatable to humans, will have critical implications for the prevention of obesity and type 2 diabetes in future generations.

Gender	Species (strain)	Time of maternal exercise	Age of Offspring	Offspring Body Weight	Offspring Glucose Tolerance	Tissue Affected	Type and Intensity of exercise	Ref
Female	Mouse (C57BL/6)	Pre-Gestation	Adult	No change	Improves	_	VWR	22
Female	Mouse (C57BL/6)	During Gestation	Adult	No change	Improves	_	VWR	22
Female	Mouse (C57BL/6)	Pre & During Gestation	Adult	No change	Improves	Liver	VWR	22
Female	Mouse (ICR)	Pre & During Gestation & Lactation	Adult	No change	Improves	_	VWR	24
Female	Rat (Sprague Dawley)	Pre & During Gestation &Lactation	Adult	No change	Improves	Muscle	VWR	25
Male	Mouse (C57BL/6)	Pre-Gestation	Adult	No change	No effect	_	VWR	23
Male	Mouse (C57BL/6)	During Gestation	Adult	Reduced	No effect	Liver (27)	VWR	23,27
Male	Mouse (C57BL/6)	Pre & During Gestation	Adult	Reduced	Improved	Muscle (26)	VWR	23, 26
Male	Mouse (ICR)	Pre & During Gestation &Lactation	Adult	No change	Improved	Muscle	VWR	24
Male	Rat (Wistar)	Pre & During Gestation	Youth	No change	No effect	Muscle, Pancreas	Treadmill/Submaximal	39
Male	Rat (Wistar)	Pre & During Gestation	Adult	No change	Worsened	Muscle, Pancreas	Treadmill/Submaximal	39
Male	Rat (Wistar)	Pre & During Gestation	Adult	Reduced	No effect	Muscle	Treadmill/Moderate	38
Male	Rat (Wistar)	Pre & During Gestation	Adult	No change	No effect	_	Treadmill/Moderate	48
Male	Mouse (C57BL/6)	Pre & During Gestation	Adult	No change	No effect	Increased Activity	VWR	40
Female	Mouse (C57BL/6)	Pre & During Gestation	Adult	No change	No effect	Increased Activity	VWR	40

Table 1. Characteristics of male and female offspring in response to maternal exercise. Directionality (i.e. reduced, improved) is compared to offspring from sedentary dams. Age of offspring is defined as adult (>6 months of age), youth (<12 weeks of age), or young (<3 weeks of age). Voluntary wheel running (VWR).

Gender	Species (strain)	Time of maternal exercise	Maternal diet	Age of Offspring	Body Weight	Glucose Tolerance	Tissue Affected	Offspring diet	Type and Intensity of exercise	Ref

Female	Mouse (C57BL/6)	Pre-Gestation	High-Fat	Adult	No change	No change	_	Chow	VWR	22
Female	Mouse (C57BL/6)	During Gestation	High-Fat	Adult	No change	No change	_	Chow	VWR	22
Female	Mouse (C57BL/6)	Pre & During Gestation	High-Fat	Adult	Reduced	Improves	Muscle (26);Liver (22)	Chow	VWR	22,26
Male	Mouse (C57BL/6)	Pre-Gestation	High-Fat	Adult	No change	No change	_	Chow	VWR	23
Male	Mouse (C57BL/6)	During Gestation	High-Fat	Adult	No change	Improves	_	Chow	VWR	23
Male	Mouse (C57BL/6)	Pre & During Gestation	High-Fat	Adult	Reduced	Improves	Liver	Chow	VWR	23, 26
Male	Mouse (C57BL/6)	During Gestation	Chow	Adult	No change	Improves	Liver	High-Fat	VWR	27
Male	Rat (Wistar)	Pre & During Gestation	Chow	Adult	Reduced	No effect	Muscle	High-Fat/High-Sucrose	Treadmill/Moderate	38
Male	Rat (Wistar)	Pre & During Gestation	Low Protein	Adult	Reduced	Improves	_	Chow	Treadmill/Moderate	48
Male	Rats (Wistar)	Pre & During Gestation	High-Fat	Youth	No change	Improves	_	Chow	VWR	45
Female	Rats (Wistar)	Pre & During Gestation	High-Fat	Youth	No change	Improves	Muscle	Chow	VWR	45
Male	Rats (Wistar)	Gestation and Lactation	Chow	Young	Reduced	Improves	_	Over-fed	Treadmill/Moderate	38
Male	Rats (Wistar)	Gestation and Lactation	Chow	Youth	Reduced	Improves	_	Over-fed	Treadmill/Moderate	31

Table 2. Characteristics of male and female offspring in response to maternal exercise with a dietary intervention. Directionality (i.e. reduced, improved) is compared to offspring from sedentary dams. Age of offspring is defined as adult (>6 months of age), youth (<12 weeks of age), or young (<3 weeks of age). VWR=Voluntary wheel running.

Acknowledgements

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Maternal Exercise Improves Metabolic Health of Adult Offspring through Adaptations to Breastmilk

Introduction

A child's adult phenotype is influenced by many of the early life factors they encounter, including genetics, maternal factors, the intrauterine environment, and the early life environment. Exercise has been shown to be beneficial for many health conditions, including type 2 diabetes (49, 95), and maternal exercise has been shown to improve metabolic phenotypes of adult offspring (17, 18, 50, 77, 83, 87, 88, 100). In contrast, poor maternal diet (either overnutrition or undernutrition) and environment are linked to cardiac dysfunction (86), impaired leptin signaling, epigenetic markers, and endocrine function (26), as well as other metabolic health effects in adult offspring in humans and rodents (9, 30, 48, 60, 84). Exercise can be used as a treatment to affect these impacts on the offspring's adult outcomes. Poor outcomes for rodents when the mother consumes a high fat diet has been shown to be reversed when the mother exercises both before and during pregnancy (89). Other poor dietary conditions such as protein restriction have also been shown to be alleviated by maternal exercise (33). In a rodent model, exercise is a proven method for combatting potential negative effects on an offspring's health, and metabolic health specifically.

Studies so far have described the apparent physiological and phenotypical effects of maternal exercise on metabolic health, seen in the introductory chapter, but the mechanism through which maternal exercise exacts its effect on the offspring remains an unclear picture. There have been studies that show that exercise does not affect the oocyte (13), indicating that there is some other early-life factor that is being influenced by exercise. A potential factor that

could be influenced by maternal environment is the breastmilk. The importance of breastmilk has been shown in various studies that highlight its effect on reducing infection rates and supporting cognitive development (74, 98).

Some components of breastmilk are known to be susceptible to exercise and diet (23, 103), including human milk oligosaccharides (4). Human milk oligosaccharides (HMOs) have also been studied in humans to have an impact on early body composition (2). HMOs have been documented to have many roles in infant health (11, 12, 62). Thus, there is a need to evaluate how breastmilk, and milk oligosaccharides specifically, as a contributor to early life environment, is affected by exercise and how it could be affecting offspring metabolic health. Various experimental methods are used in order to determine the role breastmilk might play in the conference of exercise-induced metabolic health benefits to offspring. Here, we investigated the effects of maternal exercise and a maternal high-fat diet on the composition of breastmilk and determined if that could alter the metabolic health of adult offspring.

Methods

Mice and training paradigm. All studies were conducted in C57/BL6 mice from Charles River Laboratories. For cross-fostering experiments breeding mothers were split into two groups: Sedentary (housed in cages without additional exercise) and Trained (housed in cages with a running wheel from two weeks before conception). Both groups were fed a high fat diet (60% kcal from fat; Research Diets, Inc.) from two weeks before conception. After the initial two weeks of training, breeding was done with all sedentary (standard cage with no running wheel), chow fed (20% kcal from fat; PharmaServ 9F5020) males. Breeding was done in harems to control for differences in the fathers. Females were kept on high fat diet and in appropriate cages

through gestation until birth. Twenty-four hours after birth, litters were “cross-fostered” from their birth mother to a foster mother (59). Litters born to sedentary mothers were fostered with trained mothers, and vice versa; litters born to trained mothers were fostered with sedentary mothers. These litters were left nursing with their foster mother from the second day after birth until weaning, and after weaning were kept sedentary and on a chow diet. In addition to these two experimental groups were control groups, where litters born to sedentary mothers were fostered with a different sedentary mother, and the same for litters born to trained mothers. We grouped the arrangements under four headers: Sedentary raised by Trained, Trained raised by Sedentary, Sedentary raised by Sedentary, and Trained raised by Trained (“Birth mother condition” raised by "Foster mother condition”). Litters were culled in the cross-fostering process to ensure that the number of mice per mother was even across all four groups.

Milk Isolation

For isolation of milk, female mice were trained starting two weeks before pregnancy in running wheel cages, with four groups of sedentary chow fed, sedentary high fat fed, trained chow fed, and trained high fat fed. Pups were removed from the dams 24-h prior to milk isolation, and the milk was isolated 7 days after birth (56).

3’sialyllactose (3’SL) Feeding

Female mice were kept sedentary and placed on high fat diet starting two weeks prior to conception, then bred with chow fed sedentary male mice. The dams were split into two groups, PBS (phosphate-buffered saline) fed or 3’SL fed, and litters culled to the same size for each dam (64). Feeding was done by oral gavage. PBS fed received a phosphate-buffered saline (PBS)

vehicle and the other and fed an equal volume to the 3'SL fed pups each day. 3'SL fed pups were given 3'SL in PBS solution. Concentrations of 3'SL were increased as the mice grew, starting with 150 nmol of 3'SL on days one through three, then 300 nmol on days four through six, and 600 nmol on days seven to twenty-one. The PBS fed controls were fed the same volume solution as the 3'SL supplemented mice. These levels were determined based on the data from the 3'SL levels seen after milk analysis.

3'SL KO mice

3'SL knockout (KO) mothers were kept on chow diets (20% kcal from fat) as were wild-type (WT) females for control purposes. Both 3'SL KO and WT females were split into sedentary and trained groups, with training beginning two weeks before conception and lasting through pregnancy. The 3'SL KO mice were treadmill trained. The KO and WT mice for this experiment were run on the treadmill for 60 min/day, 5 days a week, at 21.0 meters/min and 10% incline for the duration of the training regimen (19).

High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) was performed on the milk samples (94) to determine the oligosaccharide composition in the milk in collaboration with Dr. Lars Bode at the University of California, San Diego. 3'-siallylactose (3'SL) and 6'-siallylactose (6'SL) were chosen for study because these two milk oligosaccharides are conserved between mice and humans.

Body Composition and Metabolic Testing.

Body weight was measured weekly through 52 weeks using an OHAUS NV212 scale. Body fat mass was measured using an EchoMRI instrument with canola oil calibration (55). Glucose Tolerance testing (GTT) was performed on a 12 hour fast (20:00h – 8:00h) with drinking water freely available. Blood glucose was assessed at baseline by a tail vein prick (87, 89, 90). Glucose was injected subcutaneously (2g glucose/kg body weight) at 0 min, and the tail vein prick was used to measure blood glucose levels at 0, 15, 30, 60, and 120 minutes post injection. Insulin Tolerance testing (ITT) was performed on a 4 hour fast (8:00h – 12:00h) with drinking water freely available. Baseline blood glucose levels were measured using a tail vein prick. Insulin was administered by subcutaneous injection (1 unit per kg body weight) at 0 minutes. Blood glucose levels were measured at 0, 10, 15, 30, 45, and 60 minutes post injection. If mice dropped below 40 mg/mL glucose they were given an injection of 200 μL of 10% glucose (0.1g/mL) to prevent seizures.

Biochemical Methods. Quantitative Polymerase Chain Reaction (qPCR) was performed on tissue after mice were sacrificed at 52 weeks. Tissue was frozen at -80°C between sacrifice and testing. mRNA levels in the tissue were measured by qRT-PCR using SYBR Green detection. The primers used are included in the following table (91).

Gene		Mouse (5’ to 3’)
G6pc	Forward	AGGTCGTGGCTGGAGTCTTGTC
	Reverse	GTAGCAGGTAGAATCCAAGCGC
Fbp1	Forward	TGCTGAAGTCGTCCTACGCTAC
	Reverse	TTCCGATGGACACAAGGCAGTC
Pgc130	Forward	GAATCAAGCCACTACAGACACCG
	Reverse	CATCCCTCTTGAGCCTTTCGTG
	Forward	GGCGATGACATTGCCTGGATGA

Pck1	Reverse	TGTCTTCACTGAGGTGCCAGGA
Pklr	Forward	TTCTGTCTCGCTACCGACCT
	Reverse	CCTGTCACCACAATCACCAG
Pcx	Forward	ATGTTGTGGACGTGGCAGTA
	Reverse	AATCGAAGGCTGCGTACAGT
Pfkl	Forward	CCATCAGCAACAATGTGCCTGG
	Reverse	TGAGGCTGACTGCTTGATGCGA
Pdha1	Forward	CTGTCAGAGTTTGTAGACACG
	Reverse	GACTACTGCTACCACATCACA
Pdk4	Forward	GTCGAGCATCAAGAAAACCGTCC
	Reverse	GCGGTCAGTAATCCTCAGAGGA
CS	Forward	ATAGTGAGGAGGTGGATTGG
	Reverse	GGGTGGTGTGAGCAGAAA
Idh3a	Forward	CGCGTGGGTGTCCAAGGTCTC
	Reverse	TGTGACATTGCGCTCCTCCAA
Mdh2	Forward	CAGAGCGTCCACTTTTCTAC
	Reverse	CTTGACCACTTCATCACCAC
Ogdh	Forward	GGTGTCGTCAATCAGCCTGAGT
	Reverse	ATCCAGCCAGTGCTTGATGTGC
Cd36	Forward	GGCACCACTGTGTACAGACAG
	Reverse	GGAAAGGAGGCTGCGTCTGTGC
Fatp4	Forward	GACTTCTCCAGCCGTTTCCACA
	Reverse	CAAAGGACAGGATGCGGCTATTG
Acox	Forward	GGGAGTGCTACGGGTTACATG
	Reverse	CCGATATCCCCAACAGTGATG
Cpt1	Forward	AAAGATCAATCGGACCCTAGACA
	Reverse	CAGCGAGTAGCGCATAGTCA
	Forward	TTTCCGGGAGAGTGTAAGGA

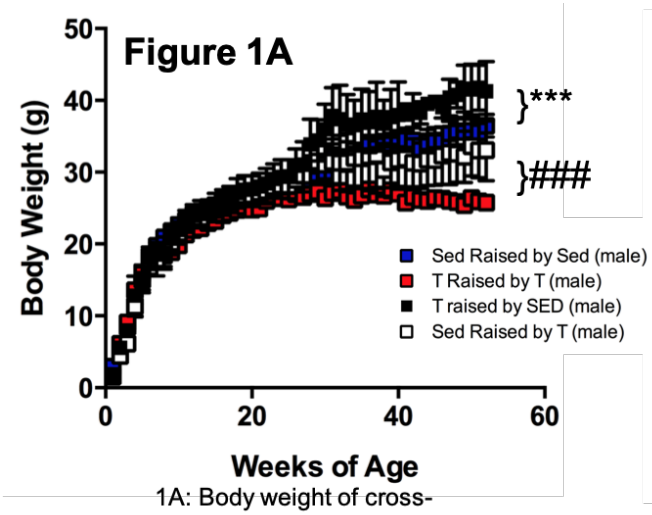
Lcad	Reverse	ACTTCTCCAGCTTTCTCCCA
Mcad	Forward	GATGCATCACCCTCGTGTAAC
	Reverse	AAGCCCTTTTCCCCTGAA

Results

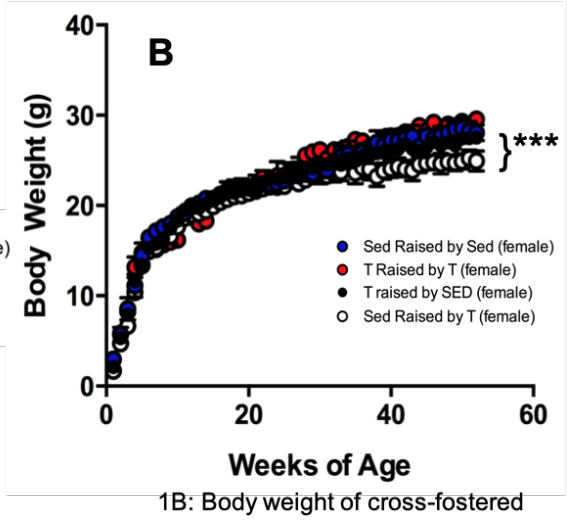
Cross-fostering Isolates Breastmilk as Factor in Exercise-Induced Changes.

Metabolic testing was performed at 8, 12, 24, 36, and 52 weeks of age in female and male offspring from cross-fostering dams, and for all following experiments. For cross-fostered offspring, body weight in male offspring was seen to differ significantly in adulthood between the groups raised by sedentary mothers, and the groups raised by trained mothers (Figure 1A). In fact, the group with the highest weight were male offspring from a trained mother raised by a sedentary mother. In female offspring, body weight was also found to be significantly lower in those offspring raised by trained mothers compared to those raised by sedentary mothers, when the offspring were born from a sedentary mother (Figure 1B). In both male and female groups, there was a significant improvement in the body fat mass (%) from the train raised by sedentary to the sedentary raised by trained groups. In the males, this difference held true for all groups raised by trained mothers, while in females, the sedentary raised by trained group was the only group with significant reduction in body fat mass compared to all groups (Figures 1C, D). Offspring that nursed with trained mothers (drank exercise-trained milk) had improved glucose tolerance. There is a significant difference between the male offspring groups raised by

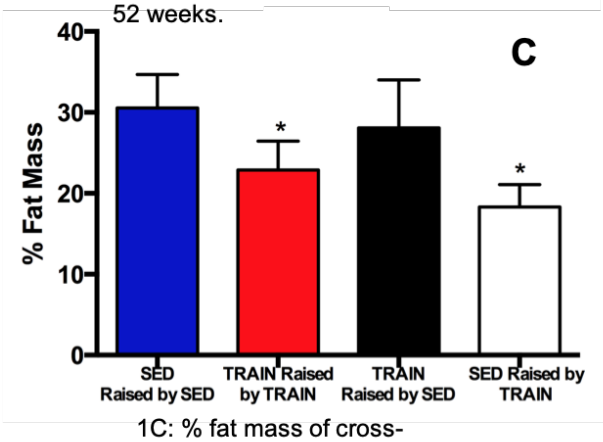
sedentary mothers and the groups raised by trained mothers (Figure 2A). In male offspring the same trend was found in insulin tolerance testing; the mice raised by trained mothers had significantly improved insulin tolerance compared to those raised by sedentary mothers (Figure 2C). Female offspring also showed that they had significantly improved glucose tolerance when they were raised by trained mothers, regardless of being born from a trained or sedentary mother (Figure 2B). Insulin tolerance tests showed no difference between the groups of female offspring (Figure 2D). In previous studies conducted in the Stanford lab, we have shown that maternal exercise increases expression of hepatic genes involved in glucose metabolism and mitochondrial activity. To see if the exercise-trained milk would have impacts on liver function, we measured liver gene expression in male and female mice at 52 weeks of age. In cross-fostered mice, there were some trends indicating that liver function could be impacted by the difference in trained or sedentary breastmilk. There were no significant differences, and only individual genes showed possible effects from trained milk (Figure 3A, B). The qPCR data is overall too variable to draw broader conclusions. The overall metabolic health improvements seen by cross-fostered offspring indicate that the milk from a trained mother is the variable that leads to metabolic health improvement in adulthood.



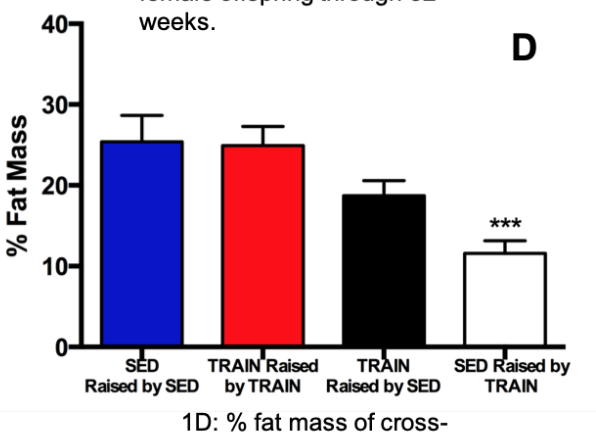
1A: Body weight of cross-fostered male offspring through 52 weeks.



1B: Body weight of cross-fostered female offspring through 52 weeks.

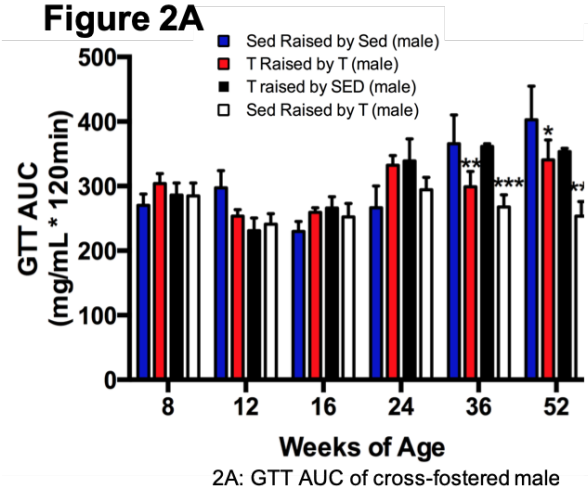


1C: % fat mass of cross-fostered male offspring at 52 weeks.

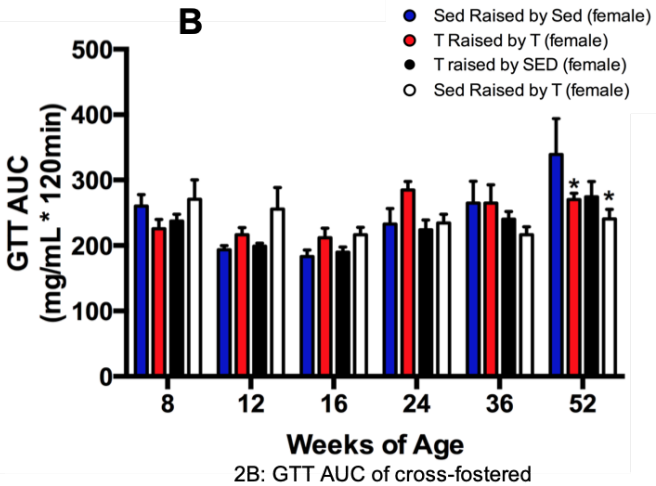


1D: % fat mass of cross-fostered female offspring at 52 weeks.

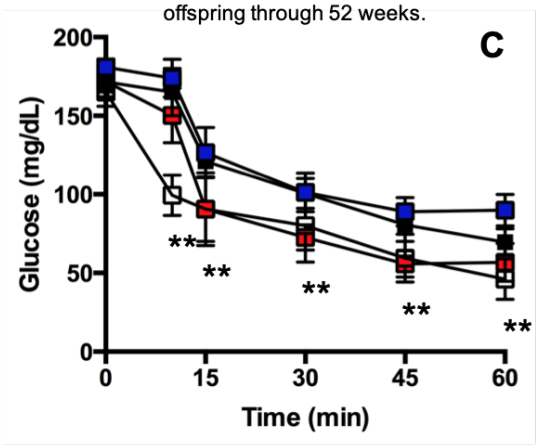
Figure 2A



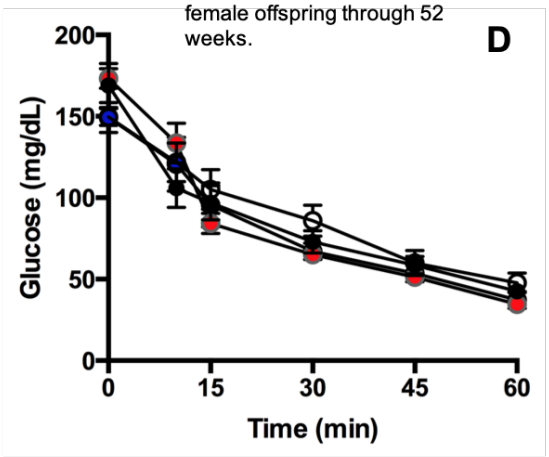
2A: GTT AUC of cross-fostered male offspring through 52 weeks.



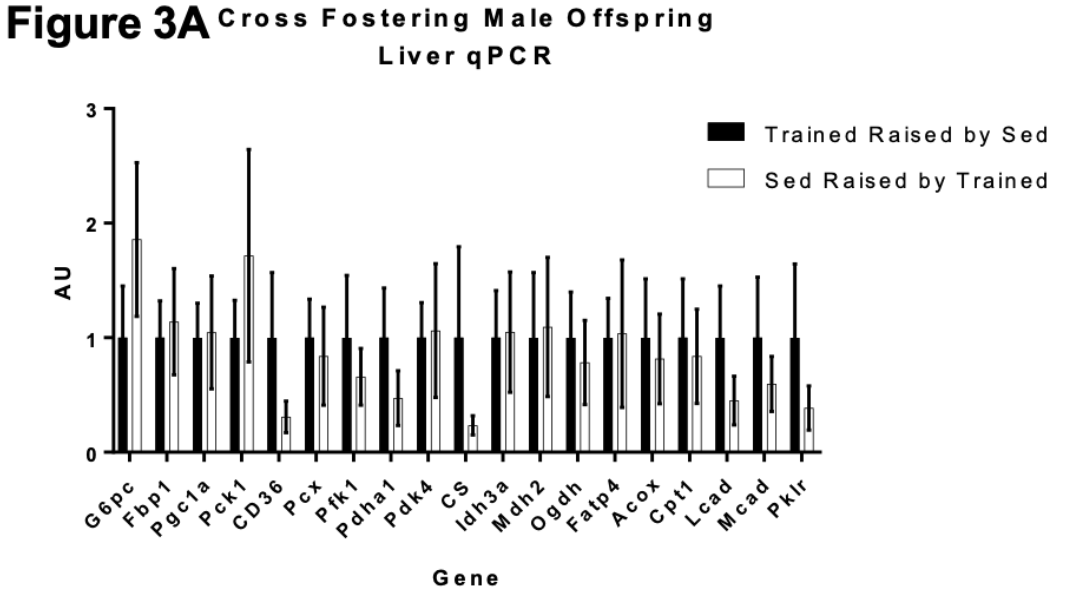
2B: GTT AUC of cross-fostered female offspring through 52 weeks.



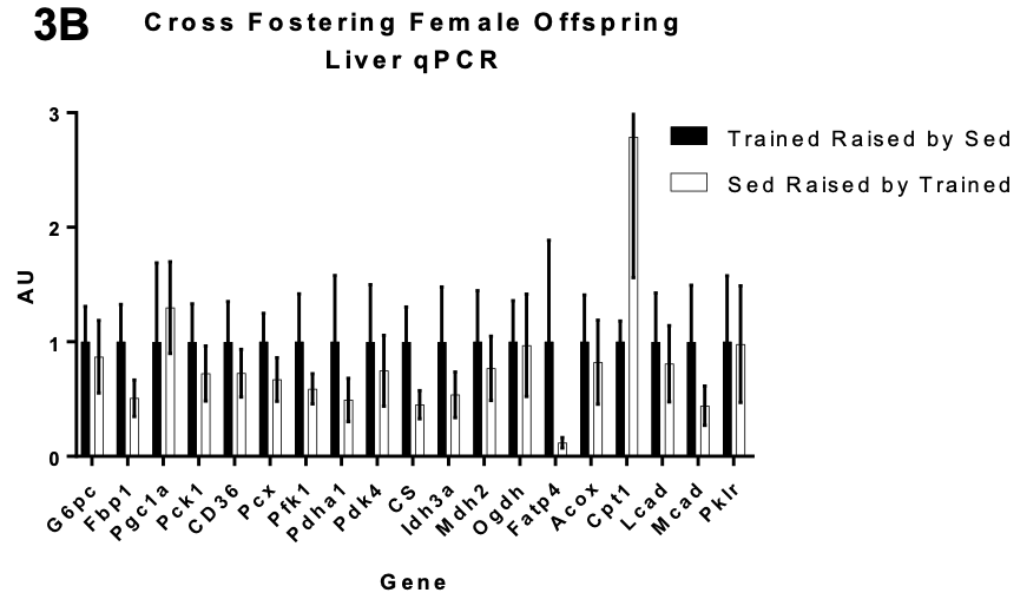
2C: Insulin Tolerance of cross-fostered male offspring through 52 weeks.



2D: Insulin Tolerance of cross-fostered female offspring through 52 weeks.



3A: Cross-fostering male offspring qPCR at 52 weeks.

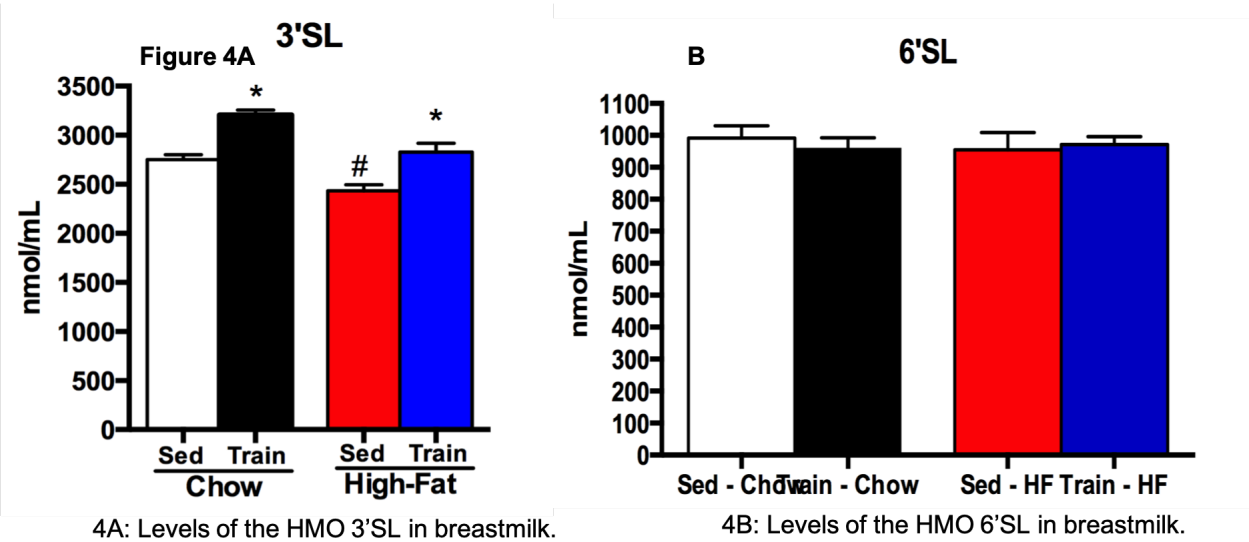


3B: Cross-fostering female offspring qPCR at 52 weeks.

Exercise Increases Levels of Milk Oligosaccharide 3'SL.

To investigate what component of breastmilk was affected by the maternal exercise, we isolated breastmilk and measured levels of human milk oligosaccharides present. Human milk oligosaccharides make up 5-15% of breastmilk and play important roles in infant development and other health benefits (2, 11, 12, 74). 3'SL and 6'siallylactose (6'SL) are human milk

oligosaccharides that are conserved between mice and humans. The milk oligosaccharide 6'SL was not changed in any of the four groups of mice (Figure 4B). However, 3'SL was significantly increased in the two trained groups and reduced in the sedentary high fat diet group. The trained chow fed group had higher levels of 3'SL compared to the sedentary chow fed group. The sedentary high fat diet group had significantly reduced concentrations of 3'SL than the sedentary chow fed group, and the trained high fat diet group had significantly increased levels of 3'SL compared to the sedentary high fat diet group (Figure 4A). These increases in 3'SL suggest the connection between maternal exercise and metabolic health could be mediated by 3'SL levels in the breastmilk.



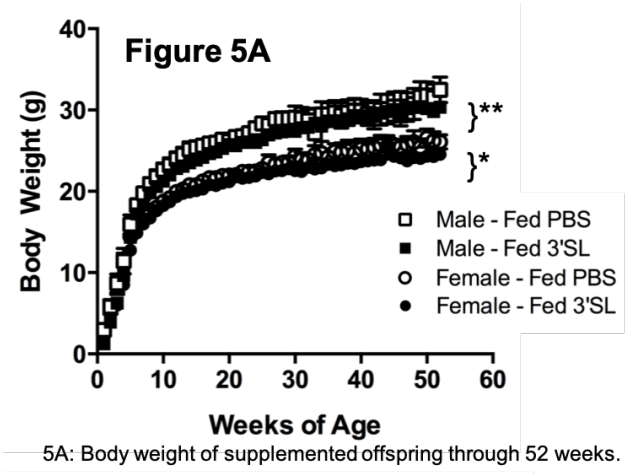
4A: Levels of the HMO 3'SL in breastmilk.

4B: Levels of the HMO 6'SL in breastmilk.

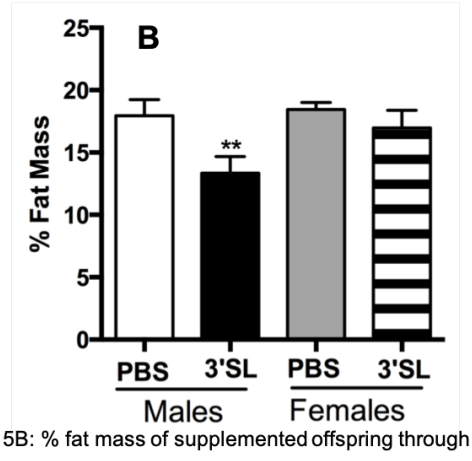
Postnatal Supplementation of 3'SL Leads to Metabolic Health Improvements.

To determine if 3'SL was the component in breastmilk inducing these metabolic changes in the offspring, we supplemented 3'SL to offspring postnatally for the duration of the nursing period, ceasing after the offspring were weaned. After 3'SL supplementation there was a significant reduction in body weight in 3'SL fed groups compared to PBS fed groups, in both the male and female offspring (Figure 5A). There was also a significant reduction in % fat mass in male

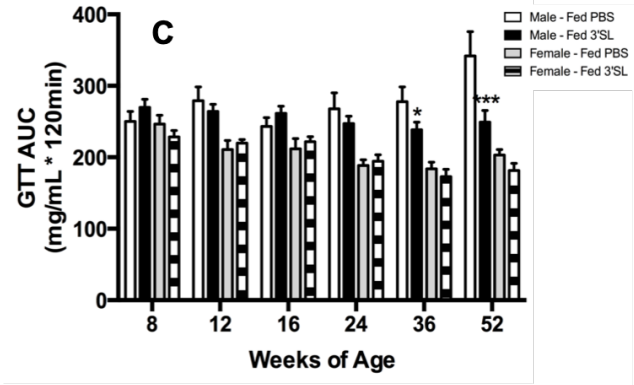
offspring who were supplemented with 3'SL (Figure 5B). Male 3'SL fed pups had significantly improved glucose tolerance in adulthood when compared to the PBS fed control (Figure 5C). This glucose tolerance improvement was not seen in the female offspring. For insulin tolerance, there was no difference in the tolerance between 3'SL fed, and PBS fed groups of either sex (Figure 5D). The improvements seen from 3'SL supplementation indicates that 3'SL itself has metabolic health benefits even out of breastmilk.



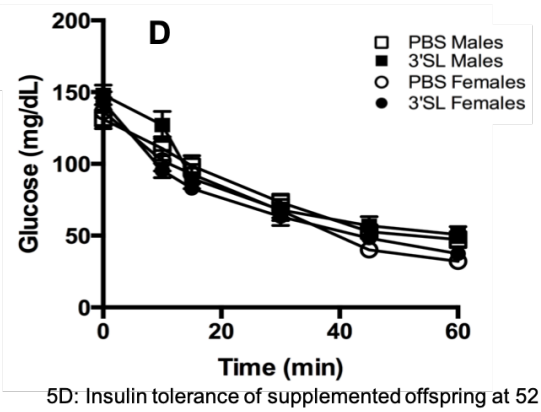
5A: Body weight of supplemented offspring through 52 weeks.



5B: % fat mass of supplemented offspring through 52 weeks.



5C: GTT AUC of supplemented offspring at 52 weeks.

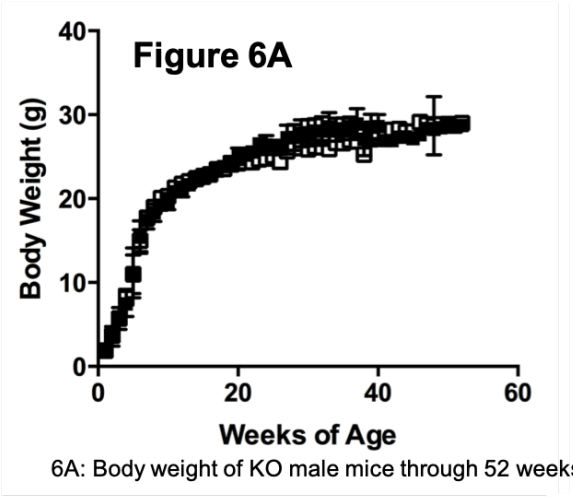


5D: Insulin tolerance of supplemented offspring at 52 weeks.

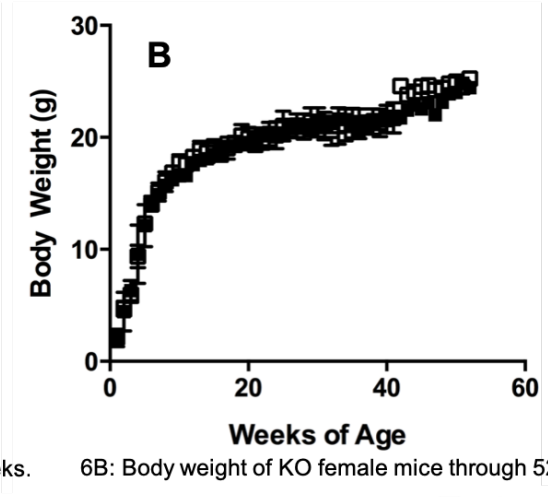
Knocking Out 3'SL Eliminates Exercise-Induced Metabolic Health Improvements.

The effects of 3'SL supplementation showed a clear benefit of 3'SL on the metabolic heatlh in the adult offspring. We then asked if removing the 3'SL would still result in exercise induced metabolic health benefits. The animals used for the knockout experiment were trained on a treadmill training regimen instead of a voluntary wheel cage. There was no difference in the

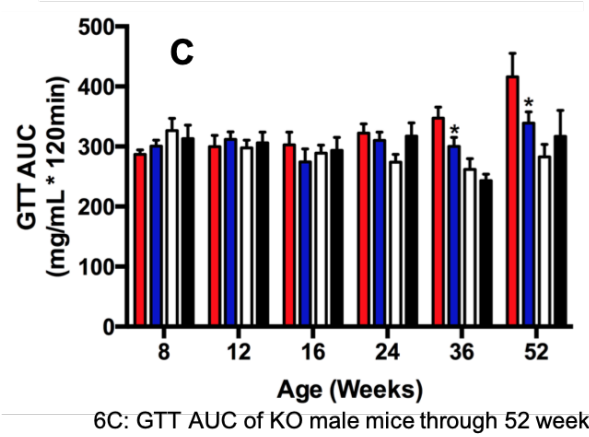
pups per litter, and animals completed this regimen successfully for all 5 weeks of training. In the knockout experiment, body weight was observed to be unchanged between KO groups (Figures 6A, B), both male and female. The KO male and female mice did not receive an exercise induced metabolic improvement (Figure 6C, D), however, in the WT mice improvement in glucose tolerance was observed in male offspring (6C). The female WT offspring did not have an improvement in glucose tolerance (6D). This indicates that 3'SL is key to the breastmilk related improvement of metabolic health in offspring from trained mothers.



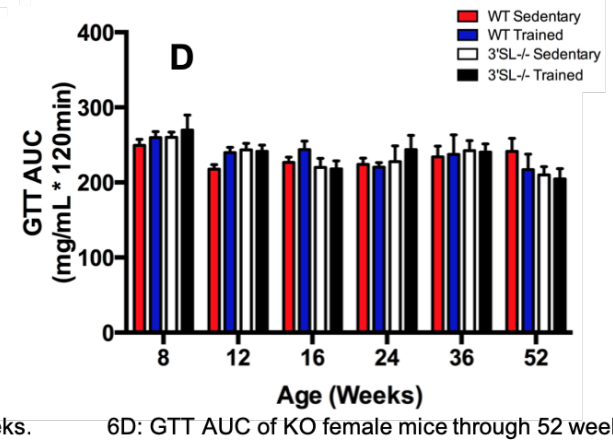
6A: Body weight of KO male mice through 52 weeks.



6B: Body weight of KO female mice through 52 weeks.



6C: GTT AUC of KO male mice through 52 weeks.



6D: GTT AUC of KO female mice through 52 weeks.

Conclusions

Demonstrated metabolic benefits to offspring from maternal exercise show the necessity in appropriate care and advice for mothers planning on having children. Importantly, with rates of diabetes and obesity increasing across the country (10), the prescribed exercise regimen of starting before pregnancy and maintaining it through gestation had a positive effect on mouse offspring of mothers who were on a high fat diet. How these benefits are conferred onto the offspring is the topic of this thesis, specifically how maternal exercise could be changing the composition of breastmilk. The experiments of this thesis give a clearer insight into how the maternal exercise might be working to improve the metabolic health of the offspring.

The results of cross-fostering indicate that there is a clear role being played by breastmilk in the metabolic improvements observed in adult offspring as a result of maternal exercise. The significant differences between the groups show that the difference made by breastmilk is more important than the difference made by the intrauterine environment. Regardless of whether the offspring were born to a sedentary or trained mother, they were more metabolically healthy when they received the milk from a trained mother opposed to the milk of a sedentary mother. The impact of the milk was the most holistic of all the experiments, showing improvements in all categories from body weight, fat mass (%), glucose tolerance, and insulin tolerance.

The increase in the 3'SL concentrations in the trained mouse breastmilk indicate that the 3'SL is an important component in the breastmilk that could confer the beneficial effects of exercise from the mother to the offspring. The lack of change in the 6'SL concentrations suggests that it is not involved. Additionally, the reduction in 3'SL levels in the sedentary high fat diet group and the subsequent increase in the trained high fat diet group shows promise for explaining the demonstrated effects of exercise on the metabolic health of offspring born from

mothers on a high fat diet (89). These data thus suggest that the effect seen from the cross-fostering could be explained through a 3'SL mechanism, prompting the need to conduct the 3'SL supplementation and knockout verification of the role of 3'SL.

Supplementation of 3'SL confirmed that 3'SL has an effect on the whole-body metabolism of the mouse offspring. While the metabolic improvements did not carry for the female offspring so much, there were still whole-body metabolic improvements in offspring as a result of 3'SL supplementation. This again indicates that the 3'SL can improve the metabolic health of offspring when given to them postnatally. The difference in the data between the supplementation experiments and the cross-fostering experiments suggests there could be some differences between the methods of delivery. It appears superficially that there could be a difference in whether the 3'SL is administered in something like a PBS solution, or if it is received through breastmilk.

The knockout results confirm that the breastmilk only confers the benefits of exercise with the presence of 3'SL. Breastmilk from an exercise-trained mother was demonstrated to not have the same effect without it, as offspring were no healthier than when they drank milk from a sedentary mother. Importantly, the WT mice in the knockout study did experience the metabolic improvements from the exercise even with the adjusted training regimen. This change was necessary as the 3'SL KO mice would not run on the running wheels in their cages even after prolonged exposure.

There were differences in the improvements of metabolic health between male and female offspring seen throughout the data. This difference between male and female offspring is consistent with past studies that show maternal exercise tends to affect male offspring more profoundly than female offspring (9, 32, 48, 71, 73, 81). It has been shown before that part of

this reason could be that female offspring do not see the same worsening in some metabolic characteristics with age that male offspring do (87), which is also seen in this study after cross-fostering. However, the improvements seen in the female offspring as a result of cross fostering with a trained mother are still remarkable. The differences between male and female offspring differed according to the experimental conditions, but this could be due to different delivery methods. As mentioned, there appears to be a possibility of 3'SL being more effective when delivered through breastmilk. The 3'SL supplementation studies found female offspring were not improving in % fat mass whereas they did after cross-fostering, as well as females improving in glucose tolerance after cross-fostering but not after supplementation.

The parameters used throughout the study of glucose and insulin tolerance, body weight and % fat mass are indicators of type 2 diabetes and obesity (55). There was no difference in weight between mothers fed a high fat diet and a chow diet, so the differences observed in offspring are likely not down to gestational obesity or diabetes. Seeing such effect as is shown at 52 weeks in mice corresponds to full adulthood in humans, crucially indicating that the benefit from this early life intervention manifests in later stages of life. While the benefit may not be present immediately in the offspring's life, the adult phenotype is greatly impacted which is of great importance to adult risk of type 2 diabetes and obesity.

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Future Directions of Research

These results could have enormous impact on maternal, prenatal, and postnatal care if the knowledge is able to be translated to clinical methods. With rising diabetes and obesity rates becoming an epidemic (10), the potential benefits of 3'SL and exercise should be a top priority for further research to make a difference in millions of future children's lives. The effects that exercise is shown to have in the offspring of mothers who exercise before and during pregnancy show great promise in being explained through the 3'SL mechanism. However, in order for that mechanism to be of use to actual people in the practice of obstetrics or nutritional and health guidance, more knowledge is needed of the 3'SL mechanism that will make it better understood and also, better utilized.

The observed physiological changes and positive health benefits in the mouse model are clear, so identifying the mechanisms through which this occurs will be imperative. There are some suspected pathways to follow for further study on this topic that are already of interest. Several options present themselves for how the 3'SL is affecting the human physiological state, that all carry different implications. Mainly, the questions that remain are, 1) How does 3'SL affect metabolic health; 2) How is 3'SL used by the animal; 3) How could we use 3'SL for therapeutic use?

How Does 3'SL Affect Metabolic Health?

Human milk oligosaccharides are most often associated with the colonization and development of the microbiome in the gut of the infant (11, 12, 46). There is a clear need to continue looking into what exact role 3'SL is playing in the gut of the organism. The knockout model offers the opposite side of a germ-free model, not removing the germs artificially, but

instead not allowing them to develop in the gut naturally. Additionally, each oligosaccharide has unique function of which many have not been studied to the full extent (11, 12). This suggests that 3'SL could have an effect specific to metabolically active microbiota or other function. Other milk oligosaccharides have been shown to cause changes in the composition of the gut microbiome when supplemented postnatally (11, 12, 20, 46, 64).

The data in this thesis indicate along with some previous research indicates that there is some effect specific to the liver as a result of maternal exercise (91). The role that microbiome plays in specific organ function has not been explored, so there is a need to explore that route. There could be other factors in liver or other tissue function that 3'SL or the microbiome affects that could be separate therapeutic targets for utilization. Hepatocyte study under exposure to 3'SL could be one appropriate experiment. The level of interaction that 3'SL could be having with organs is dependent upon the next question discussed as to how 3'SL is actually taken up by the microbiome or the host animal itself. After that question is answered, there will be a clearer path forward in elucidating the exact effect of 3'SL.

The KO mice had the unique problem that they would not run in the running wheels, which forced the switch to the treadmill training for that study. This unique behavioral trait also leads to some speculation about how the 3'SL could be performing other functions. There has been an increasing amount of research emerging looking into the gut brain axis, showing it could impact development of the central and enteric nervous systems, as well as a wide variety of other effects relating to behavior and physiological abnormalities (16, 35, 40). There is also increasing awareness around the relation between diabetes, obesity, and neural dysfunction, meaning there could be even more widespread therapeutic benefit to preventing the metabolic diseases (29). This secondary observation to the study leaves open the possibility that there are behavioral

aspects to the microbiome effect as well, and perhaps also that the nervous system could be involved in metabolic outcomes. 3'SL could be a larger player in offspring health in adulthood than previously imagined.

How is 3'SL Used by the Animal?

3'SL could be affecting metabolic health in several ways, and how it is taken up plays a large role in this. 3'SL contains a sialic acid group and so in this discussion is assumed it could be utilized by a sialic acid related protein. One possibility is that the 3'SL is exclusively used by the microbiome for its development and sustained vitality. Bacteria can use sialic acids, of which 3'SL is one, in a couple different ways, either for food or for help in immune evasion strategies (61, 93). This idea brings a couple of implications to the fore, mainly that the 3'SL is not acting directly on the human body. This would mean that in order to fully understand how the mechanism works, the bacteria of the microbiome would need to be isolated and examined for which use 3'SL the most, how they use 3'SL, and what effects it has on them. Additional studies would also need to be completed to examine how the presence of the specific 3'SL utilizing bacteria affects the metabolism of the organism in which it lives.

There are some suspected pathways through which 3'SL could be taken up by the bacteria in the microbiome. Sialic acids are taken up by a variety of proteins, such as the ATP-binding cassette, ATP-independent periplasmic (TRAP), major facilitator superfamily (MFS), and the sodium solute symporter (SSS) families of proteins. The first documented uptake of sialic acid in E.Coli was with the NanC porin (37), which is inducible by the NanCMS operon in *E. coli*. A more complex system was also found in *T. forsythia* and *B. fragilis* (80). This system featured the outer membrane protein complex NanO and NanU. NanU is a high affinity sialic

acid binding protein that sits on the surface of the outer membrane, and NanO completes the transport across the membrane (93).

The other possibility of uptake involves direct human uptake of the 3'SL, or at least some shared uptake between the microbiome and the human tissues themselves. There is evidence that the majority of the oligosaccharides taken in through breastmilk survive the gastrointestinal tract and make it into the feces and urine intact (20). The implications of this study suggest there is a protective effect of the oligosaccharides for the microbes in the microbiome. With the majority of the oligosaccharide surviving in the feces or urine intact, it also suggests that there may not be any significant absorption and metabolism. There have been more than a hundred human milk oligosaccharides discovered with potential for unique functionality for each (11), and more in-depth study is required of what happens in the gut with the oligosaccharides and the interactions they have with bacteria.

If there is no actual uptake of the 3'SL by the host, there could be some interaction with it on an extracellular level. Humans do have a gene called SLC35A1 that transports nucleotide sugars into the Golgi apparatus, including the nucleotide sugar CMP-sialic acid. Upon entry into the Golgi apparatus, the sugar is glycosylated (68, 85), which is another indication of the possible cellular fate of the 3'SL once it is taken up. Glycosylation of the 3'SL would indicate that the 3'SL is being exported for use in a specific area of the body, perhaps the liver as discussed already. If they are secreted intact in the feces and urine, then they could serve some endocrine-like function in the body.

Humans also have another group of proteins called sialoadhesins (42, 43). These are a group of immunoglobulins found on the surface of macrophages and other cell types. The tissues with the highest levels of sialoadhesins are the spleen, liver, lymph nodes, bone marrow,

colon, and lungs. This is not thought to be a phagocytic receptor, but its expression is thought to facilitate the activity of other phagocytic receptors. It is highly conserved between rodents and humans, which bodes well for the use of animal studies that could be directly translatable to humans. The gene for the protein is called Siglec1, which could be used to analyze levels of expression of the protein in the presence of the oligosaccharide.

How Could We Use 3'SL For Therapeutics?

A therapeutic area of interest that could be directly addressed by this study and by many others on human milk oligosaccharides is the lack of structurally complex milk oligosaccharides in infant formula milk. Formula milk is produced from bovine milk which has significantly lower levels of complex oligosaccharides (34). Human milk contains milk oligosaccharides at levels of 5 to 15 g/L (11), while sialic acid containing oligosaccharides comprise <20% of those (69). 3'SL specifically is found in human milk samples from a worldwide survey in concentrations of 0.26 to 0.39 g/L (62). In striking contrast to human milk where sialic acid containing milk oligosaccharides are found in 69-76 percent as oligosaccharides and 3% in their free form, in bovine milk they are mostly bound to proteins with only 27.8% present as oligosaccharides and <1% in their free form (98).

There is a clear difference between the two, and yet there has never been formula milk that has been supplemented with these sialic acid oligosaccharides. However, in studies where oligosaccharides were added to formula milk in pigs and in human infants, they led to microbiota changes (46, 63). There has already been demonstrated differences between breastfed and formula fed babies in gastrointestinal and respiratory tract infections in addition to demonstrated cognitive developmental differences (74, 98). The formula milk supplementation has also been shown to lead to changes in brain development in pigs with increased levels of sialic acids bound

to the hippocampus, prefrontal cortex, and corpus callosum (67). This adds another layer of possible future investigational routes to the results seen with the 3'SL KO mice.

It would seem adding a possibility of metabolic differences to the growing list of benefits seen from milk oligosaccharides could give even better reason for a push to be made by the FDA or formula milk producers to have these human milk oligosaccharides included in formula products. Most importantly, the formula supplementation strategy has been well tolerated in pig studies (64) indicating it could be a safe move to make, even though it would still require clinical trials in order to be approved for human use. Studies conducted in pigs are a great indicator as pigs have a very similar developmental trajectory to that of humans. Additionally, if these results can be taken one step further to determine exactly how the benefits seen are carried out in the body, it could lead to whole new ways of treating or preventing metabolic conditions such as diabetes or obesity.

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