**Title:** GDF15 knockout does not impact perinatal body weight or neonatal outcomes in mice

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# Abstract

Growth differentiation factor-15 (Gdf15) is known to increase in circulation during pregnancy and has been implicated in food intake, and weight loss, complications of pregnancy, and dysmetabolism. We used a *Gdf15* knockout mouse model to assess the role of *Gdf15*  in body weight regulation and food intake during pregnancy. We found that *Gdf15-/-*dams ate a similar amount of food and gained similar amounts of weight during the course of pregnancy as their *Gdf15+/+* counterparts. Insulin sensitivity on gestational day 16.5 was also comparable between dams. In the postnatal period, pups were of similar birthweight, litter size, and has similar survival rates in both genotypes. There were also no detectable differences in milk volume production, milk fat percentage, or in offspring postnatal body weights until day 14.5 of life. These data suggest that elimination of Gdf15 is inessential for differences in food intake, weight gain, and dysmetabolism during pregnancy in a mouse model. Further research is warranted to evaluate the role of Gdf15 in pregnancy, outside of its role in body weight and food intake regulation.

# Introduction

Growth-like differentiation factor-15 (Gdf15), a Transforming Growth Factor-ß superfamily member, placental derived growth factor, and cytokine, was discovered in 1997 and dubbed macrophage-inhibiting cytokine-1 (MIC-1) (Bootcov et al., 1997). Circulating levels of Gdf15 in adults vary based on sex, age, disease status, and physiological state. In a large sample from Scotland, they found that levels of Gdf15 increase with age in both men and women and tended to be higher in those who had cardiovascular disease, cancer, or diabetes (Welsh et al., 2022). GDF15 is known to increase in response to many stressors including as cardiac injury (Kempf et al., 2006), cachexia of cancer (Suriben et al., 2020), mitochondrial stress (Ost et al., 2020), intense exercise (Klein et al., 2021), and most relevant to this work, during pregnancy (Welsh et al., 2022).

Preclinical work with knockout models have highlighted the role of *Gdf15* in body weight regulation (Hsu et al., 2017, p. 15), appetite, and emesis (Borner et al., 2020). These models show that physiological and pharmaceutical *Gdf15* levels acts through the GFRAL receptor found in the area postrema of the brain. Antagonism of Gdf15 by blocking GFRAL has been shown to increase fat mass and inflammation in mice (Tsai et al., 2019). As such, evaluating *Gdf15* for its capacity to weight ameliorate metabolic illness is currently being explored.

During pregnancy GDF15 increases across gestation and reaches its highest levels during the third trimester of pregnancy (Andersson-Hall et al., 2021; Chen et al., 2016; Moore et al., 2000; Sugulle et al., 2009). It is heavily expressed in the placental trophoblasts, and secreted into parental circulation, and is also present in the amniotic fluid (Moore et al., 2000). In spite of these pregnancy-related increases, the functional role of *Gdf15* in pregnancy is largely unknown. Studies of clinical outcomes connect GDF15 to many complications and conditions that can arise in pregnancy. Lower levels of GDF15 during early pregnancy were present in patients who later suffered a miscarriage (Tong et al., 2004). GDF15 levels may also be play a role in gestational weight gain, as greater levels were negatively associated with cumulative gestational weight gain (P. Wang et al., 2020), however, this metric is not often evaluated in clinical work. Different levels of GDF15 are secreted in concert with complications of pregnancy. In several cases, the epidemiological data is in conflict. For example, pre-eclampsia, a life-threatening complication involving critically high blood pressure and protein loss in urine, has been found to be associated with a reduction (Chen et al., 2016), an increase (Sugulle et al., 2009; L. Wang & Yang, 2022), and no changes (Marjono et al., 2003) in GDF15 in serum compared to non-preeclamptic, normotensive parents. Similarly, some studies find that GDF15 is higher in pregnancies complicated by GDM (Yakut et al., 2021), while others find it is only significantly increased in pregnancies that are complicated by T1DM (Jacobsen et al., 2022). Other studies find that GDF15 is higher in those who have T2DM and are pregnant (Sugulle et al., 2009). Recent GWAS studies have indicated that GDF15 function in pregnant humans is causally related to risk of developing hyperemesis gravidarum, an extreme form of nausea and vomiting of pregnancy (Fejzo et al., 2018, 2019). This is supported by other studies that those with higher levels of GDF15 during pregnancy and variants in GDF15 were associated with greater reports of pregnancy related vomiting and increased risk of diagnosis of hyperemesis gravidarum (Fejzo et al., 2019; Petry et al., 2018). Finally, Petry and colleagues found pre-pregnancy BMI was inversely related to GDF15 levels during pregnancy (Petry et al., 2018). Given the sometimes-conflicting human data, we sought to understand more about the effects of *Gdf15* loss of function during the course of pregnancy, including energy metabolism, insulin sensitivity, and neonatal outcomes.

# Materials and Methods:

## Animal Husbandry and Protocol

Animals from both studies described below were housed in a temperature and humidity-controlled facility with a 12-hour light: dark cycle, with lights on being zeitgeber time (ZT) 0 and lights off being ZT 12.

### Insulin resistance of pregnancy study

Virgin female C57BL/6J (RRID: IMSR\_JAX:000664) mice were ordered from Jackson Laboratory. They were allowed to acclimatize for two weeks to the temperature and humidity-controlled facility with free access to water and laboratory chow. After acclimatizing, females were randomized into 3 groups, non-pregnant females (n=7), pregnant females (n=7), and pregnant females exposed to dexamethasone in drinking water (n=7).

### Gdf15 study

Male and female *Gdf15* null animals were described in (Frikke-Schmidt et al., 2019). Null animals were generated using CRISPR Cas-9 deletion of Exon 2 of *Gdf15*. Exon 2 (translational start site), which we ablated, is present in every known *Gdf15* transcript.

## Genotyping

At 14 days of age, a small section of the tail was collected and digested in 100uL of lysis buffer (10 mM Tris pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.1% SDS and 1 mg/ml proteinase K) at 55°C for 4 hours. Digested DNA sample was processed with DreamTaq Green to generate PCR product (ThermoFisher Scientific, Catalog #K1081). Genotyping by PCR was conducted with 2 forward and one reverse primer sets (forward 1: 5' GAT TCC CGC CCG AAT TAG C 3', forward 2: 5' CCG AAT TAG CCT GGT CAC CC 3', Reverse: 5’ ATC CGT CCT ACT CTG GCT AAG 3'). Initiation of PCR was at 95 °C for 3 minutes, followed by 38 cycles of denaturation (95°C for 30 seconds), annealing (60°C for 40 seconds), and elongation (72°C for 1 minute), and a final amplification step at 72°C for 5 minutes. PCR product resulted in 2 visible bands, one at 200bp *Gdf15-/-* and another at 600bp *Gdf15+/+.* Mice with both bands were considered *Gdf15+/-*. Dam genotype was secondarily confirmed via maternal serum ELISA.

## Mating

*Gdf15* is highly expressed in placental tissue during mouse pregnancy (GEO dataset GDS3052). We chose to study *Gdf15+/+* mated pairs compared to *Gdf15-/-* pairs because comparing littermates of *Gdf15+/-* pairs would result in placental contributions to GDF1*5* in dam serum. Therefore, we combined homozygous genotype mating pairs, resulting in homogenous genotype progeny and placentae. Adult virgin female mice (GDF15-/-n=8, GDF15+/+n=6), between 45 and 119 (mean=82.4) days old, were singly housed with *ad libitum* access to water and a standard chow diet (CD, Picolab Laboratory Rodent diet 5L0D; 5% of Calories from fat, 24% from protein, 71% from carbohydrates). Once single-housed, weekly food intake and body weight measurements began and continued throughout the experiment. After one week of food and body weight monitoring, males of like-genotype for *Gdf15* were introduced into the dam’s cage. Males were allowed to remain in the breeding cage until a copulatory plug was discovered, indicating pregnancy (E0.5). Body weight and food intake measurements continued weekly through gestation and postnatal day 14.5. Their resultant offspring and their placentae were homozygous *Gdf15+/+ Gdf15-/-* and were studied until postnatal day 14 (PND14).

Mating for the comparator c57BL/6J dams were also occurred by adding the male to the dam’s cage and allowing them to remain until gestational day 19. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

## Insulin tolerance test

On E16.5, dams underwent insulin tolerance testing (Bridges et al., 2022). Dams were placed in clean cage without access to food but with ad libitum access to water at ZT 2. Dams were fasted for 6 hours (ZT2-ZT8). Baseline blood glucose was assessed using a tail clip and a handheld glucometer (OneTouch Ultra). After initial blood glucose measurement, an intraperitoneal injection of insulin was administered (Humulin, u-100; 0.75U/kg lean mass). Blood glucose was measured in 15-minute intervals for 2 hours. Area under the curve was calculated by taking the sum of all glucose values for each animal and averaging by genotype. We then calculated the rate of initial drop in blood glucose after insulin administration. We limited data to the first 45 minutes after injection and modeling the exponential rate of decay in glucose for each animal as a slope. This rate was then averaged by genotype.

24 hours after ITT, we collected two fed blood samples: at ZT1 and ZT13. Dams were lightly anesthetized via inhaled isoflurane and whole blood was collected by retroorbital bleed in a heparinized capillary tube. Blood was allowed to clot on ice for 20 minutes then was spun down in a cold centrifuge (4°C, Eppendorf microcentrifuge, model 5415R) for 20 minutes at 2000 g. Serum was pipetted off after centrifugation and stored at -80°C until used for analysis.

*Serum GDF15 Quantification*

Serum Gdf15 analysis was completed using maternal serum collected 24 hours after insulin tolerance tests on E16.5 in the Gdf15 and maternal comparator C57BL/6J studies. Gdf15 levels were determined via ELISA according to manufacturer guidelines (R&D system, catalog # MGD150).

## Offspring

Pups were counted and body weights were recorded within 24 hours of birth, postnatal day (PND 0.5). Latency to copulatory plug was defined as the number of days between the introduction of the male and appearance of a copulatory plug. Gestational age was determined as difference between birth dates and dates of appearance of copulatory plug. At PND 3.5, litter sizes were culled to 2 male and 2 female pups, to standardize amount of nutrition provided to each pup. Survival of pups to PND 3.5 was assessed by comparing the number of pups present at PND 3.5 to the number present on PND 0.5 and is expressed as a percentage. Body weight was assessed for each pup on PND 0.5, 3.5, 7.5, 10.5, and 14.5. Pups were euthanized by decapitation on the 2 hours before milk collection began (PND 14.5-17.5).

## Weigh-suckle-weigh, milk volume production

On postnatal day 10.5, we assessed milk volume production by the weigh-suckle-weigh method (Boston et al., 2001; Habbal et al., 2021). Dams were weighed using an analytical scale to the nearest 10 mg and placed in a clean cage with free access to food and water. Pups were then weighed in aggregate and placed in a clean cage on top of a heating pad without access to food or water. Dam and pups remained separated for 2 hours. After 2 hours, weight measurements were repeated, and pups were reintroduced to the dam’s cage where they remained for 1 hour. After one hour, the final weights were taken for both dams and pups in aggregate. Volume of milk produced is expressed the average weight lost by each dam after 1 hour of nursing divided by the number of pups in the litter.

## Milk collection

Milk collection took place on PND 14.5-17.5. Pups were separated from dams and sacrificed 2 hours before milk collection began. Dams were allowed to *ad libitum* access to food and water in a clean cage during that time. Dams were anesthetized with intramuscular injection of Ketamine/Xylazine (0.1275g/kg body weight) into forelimb muscle. Once the dam was fully anesthetized, an oxytocin injection (2U per dam) was given in the forelimb muscle to begin let-down. Milk was collected with a pipette after manually expressing milk from nipples and stored in a 1.5 mL Eppendorf tube. Following milk collection, dams were immediately euthanized via isoflurane inhalation and cervical dislocation.

## Milk fat percentage determination

Whole milk was collected from dams at Postnatal day 14.5-17.5 and was stored at -80° C until analyzed. Whole milk was thawed on wet ice then homogenized by pipetting up and down. Milk was then diluted in PBS+EDTA in a 1:3 ratio and mixed thoroughly by pipetting up and down.

Capillary tubes were filled with the diluted milk solution and one end was double-sealed with crit-o-seal. Sample tubes were spun in 8 consecutive 120-second cycles in a mini hematocrit spinner (Iris Sample Processing, StatSpin CritSpin M961-122). In the capillary after 16 total minutes of spinning, total fat and aqueous layers were visible. These layers were measured using a 150mm dial caliper (General Tools, 6” Dial Caliper). Percentage of milk fat was determined with based on total volume of diluted milk sample. Milk samples were analyzed in duplicate, or triplicate if milk fat percentage differed by more than 25% in the first two samples.

## Statistical Analyses

Data were analyzed in R Studio version 4.2.0 (R Core Team, 2021) and are presented as mean ± standard error. Longitudinal analyses, such as food intake, body composition, and insulin tolerance testing were assessed using linear mixed effects modeling using R package lme4 (Bates et al., 2015) with random slopes and intercepts for the dam and pup with respect to time and fixed effects of genotype, age, and sex. Models of offspring body weight were assessed using ANOVA where sex was used as a covariate in a non-interacting model. For offspring body weight analyses we evaluated sex for an interaction effect with both time and with genotype and found it was not significant, so sex remained a fixed effect without interaction. Pairwise values were assessed for normality by the Shapiro-Wilk test and equivalence of variance by Levene’s test. Variables that were not normally distributed or of equivalent variance underwent non-parametric testing via Mann-Whitney U test. Those that were normally distributed and of equivalent variance were assessed via Student’s *t*-test as noted in the figure legends. For this study, p-values <0.05 were considered statistically significant.

# Results

GDF15 is elevated during pregnancy in mice

Previous work has shown that pregnancy in mice results in maternal insulin resistance (Ladyman et al., 2018; Musial et al., 2016). We wanted to recapitulate this phenotype, so we tested compared age-matched pregnant and non-pregnant females using an intraperitoneal insulin tolerance test on day 16 of pregnancy (**Figure 1A**). We found that non-pregnant mice had greater reductions in blood glucose when we administered an insulin compared to pregnant females(**Figure 2A**, p=0.23). There were no differences in their fasting blood glucose (**Figure 2B**, p=0.020), or area under the curve (**Figure 2C**, p=0.70). Body weights in pregnant females tended to be 1.57 grams heavier than non-pregnant females (**Figure 2H**, p0.0039). Previous work from our lab has demonstrated that administering the glucocorticoid dexamethasone (dex) in drinking water impairs insulin sensitivity (Gunder et al., 2020; Harvey et al., 2018). We wanted to test whether administering dexamethasone to pregnant dams in their drinking water worsened pre-existing insulin resistance of pregnancy. We began dex one week before mating and it continued for the length of the pregnancy. We compared dex-treated dams to age-matched pregnant dams who were given plain drinking water. We found that dex dams did not respond to insulin compared to pregnant dams with plain drinking water (**Figure 2D**, pdex\*time=0.0204). Dex-treated dams also had 33% lower fasting blood glucose (**Figure 2E**, pdex=0.007), but similar areas under the curve (**Figure 2F**, p=0.80). Body weights in pregnant dams were 2.77 grams lighter in those treated with dex compared to water dams (**Figure 2H**, p<0.0001).We were interested to see how pregnancy and dex administration in pregnancy related to GDF15 levels in these mice. We found that GDF15 is 49% (54 pg/dL) elevated in pregnant animals compared to non-pregnant mice (**Figure 2G,** p=0.007), but was not increased by dexamethasone administration(**Figure 2G**, p=0.11). Since GDF15 is known to increase during pregnancy, supported by our own results, we sought to understand the role *Gdf15* plays in on insulin sensitivity and body weight regulation during pregnancy in mice.

## Gdf15-/- dams have normal weight gain and modestly reduced food intake during pregnancy and lactation

To assess the role of *Gdf15* ablation in maternal food intake and body weight accretion during mouse pregnancy, we mated *Gdf15+/+* dams with *Gdf15+/+* males and compared them to *Gdf15-/-* mated pairs (**Figure 1B**; **Supplementary Figure 2**). Dam body weight and food intake were measured weekly, beginning one week before mating and continued until pups reached 14 days of age (PND14.5).

*Gdf15-/-*dams consumed similar cumulative kilocalories during the prenatal period (**Figure 3A**, p=0.52). They also had a similar weight change when compared to *Gdf15+/+* dams during the course of pregnancy (**Figure 3B**, p=0.99).

consumed similar calories weekly compared to *Gdf15+/+* counterparts (**Figure 3E**, pgenotype=0.23). Both genotypes had a rapid increase in food intake in the final trimester of pregnancy, with a smaller increase in the *Gdf15-/-* dams. Using linear mixed effect modeling, we found that *Gdf15-/-* dams consumed 1.13 kcal per day fewer than *Gdf15+/+* dams (pgenotype=0.79).

In the postnatal period, cumulative food intake was similar between genotypes (**Figure 3C**, p=0.94). *Gdf15-/-* dams had 54% lower postnatal weight loss than *Gdf15+/+* dams did but this failed to reach statistical significance (**Figure 3D**, p=0.20; **Figure 3F**). This suggests that *Gdf15* is not a major determinant of either body weight or food intake during first pregnancy in the mouse.

## Gdf15-/- dams have normal insulin tolerance during pregnancy

On Gestational day 16.5, we conducted an intraperitoneal insulin tolerance test to assess the effect of *Gdf15* ablation on maternal insulin sensitivity during pregnancy (**Figure 4A**). Fasting blood glucose was slightly but insignificantly lower in *Gdf15-/-* dams compared to *Gdf15+/+* dams (**Figure 4B**, p= 0.20). Overall, linear mixed effect modeling of the experiment revealed a significant and expected effect of time on blood glucose but no effect of the genotype (pgenotype = 0.71). This was confirmed by evaluating total areas under the ITT curve, again showing similar responses (**Figure 4C**, p=0.74). Often the most informative insulin response is the initial rate of drop of blood glucose. The initial rate of glucose declines was 9.3% less in *Gdf15-/-* dams compared to *Gdf15+/+* dams but again, did not reach statistical significance (**Figure 4D**, p=0.082). These data suggest that ablation of *Gdf15* is not sufficient to effect insulin sensitivity in the pregnant mouse.

## Gdf15-/- dams have normal fertility, gestational age, post-natal survival, and pup birth weights

To understand the role of *Gdf15* knockout on pregnancy and early post-partum outcomes in the pups, we calculated latency to plug, gestational age and measured litter size, birth weight, and 3-day survival in all mated dams. Pups from *Gdf15-/-* dams were 3.4% smaller than those from *Gdf15-/-* dams (**Figure 5C**, p=0.05). The latency to copulatory plug a measure of xxx was similar between genotypes, averaging 3 days (**Figure 5A**, p=0.74). Gestational age was similar between genotypes, averaging 20 days (**Figure 5B**, p=0.76). The total number of pups born in a litter was 27% greater in *Gdf15-/-* dams (1.6 pups greater on average) compared to *Gdf1+/+* dams (**Figure 5D**, p=0.15). When comparing litter size, counting only pups who were born alive, that difference was only 7.8% larger (**Figure 5E**, p=0.70, 0.46 pups greater on average). The total pups who were born alive that lived to postnatal day 3 was highly variable between genotypes, resulting in 8.3% dead for *Gdf15+/+* dams and 10% for *Gdf15-/-* dams which did not reach statistical significance (**Figure 5F**, p=0.99). Together these data show that aside from modest decreases in birthweights, *Gdf15-/-* mice are similarly fertile, and carry pregnancies to a similar effectiveness as their wild-type counterparts.

## Gdf15-/- dams have no differences in milk production or milkfat percentage

To determine the effect of *Gdf15* knockout during in pregnancy on lactation in the postnatal period, we conducted a milk volume assessment at postnatal day 10. We found no differences between *Gdf15+/+and Gdf15-/-* dams in the volume of milk produced at peak lactation. The amount of weight lost by dams after nursing (**Figure 6A**, p=0.7) or and weight gained by pups after nursing (**Figure 6B**, p=0.7) were similar between genotypes. Next, we evaluated whether the major macronutrient in milk, fat, was changed by *Gdf15* knockout. To do this, we took whole milk collected between PND 14-17 and evaluated milk fat percentage. We found that milk fat percentage was similar to *Gdf15+/+*milk (**Figure 6C**, p=0.93). Despite reductions in maternal levels of *Gdf15* in the *Gdf15-/-* dams during pregnancy, mammary gland development, and lactation there is no apparent impact lactational volume milk fat content.

## Gdf15-/- pups accrete body mass at similar rates compared to Gdf15+/+ pups

To assess the effect of Gdf15 knockout during pregnancy and lactation on early pup postnatal growth, we weighed male and female offspring of *Gdf15+/+* and *Gdf15-/-*dams on PND 0.5, 3.5, 7.5, and 14.5. We used linear mixed effect modeling which detected no differences in body weight between birth and 14 days of age in *Gdf15+/+* and *Gdf15-/-*pups (**Figure 7A**, pgenotype=0.81). There was also no statistically significant effect of sex on body weight from birth to PND 14.5 (psex=0.16). Therefore consistent with similar milk production and composition, we did not detect any effects of GDF15 ablation on perinatal growth.

# Discussion

GDF15 has recently been tied to several compilations of pregnancy in addition to its better understood role in signaling stress throughout the body. In fact, pregnancy itself is an oft-underappreciated stressor on the human body, an effect that is consistent with elevations in GDF15. The goal of this study was to understand the role of *Gdf15* on gestational health. To date there are very few studies that evaluate GDF15 in human pregnancy and its effect on weight and none that evaluate it as a primary outcome. One study found no differences in circulating GDF15 between mothers with obesity and mothers of normal weight status (Andersson-Hall et al., 2021). Another that found that GDF15 was negatively associated with total gestational weight gain (P. Wang et al., 2020). The lack of gestational outcome differences, although perhaps contrary to our prediction, is novel in the literature. Previous reports of *Gdf15* null models do not formally evaluate pregnancy or gestational outcomes in null mice during breeding or maintenance, but only describes differences as adults when used in experimental models (Frikke-Schmidt et al., 2019; Mullican et al., 2017). One study has evaluated transgenic expression of human *GDF15* in mice found that there was reduced survival in pups and lower weight gain in the postnatal period born to transgenic dams (Binder et al., 2016).The current study found that ablation of *Gdf15* and the resulting dramatic reduction in GDF15 in maternal circulation (**Supplementary Figure 1A**) does not result in any differences in body weight accretion during the prenatal period and resulted in non-statistically significant higher body weights during the postnatal period in mice. This suggests that normal physiological levels of GDF15 in pregnant mice does not impact weight accretion or loss during a normal pregnancy or at least *Gdf15* in mice plays less of a role in body weight regulation than it does in humans.

Previous work shows that external administration of GDF15, similar to levels that are seen in the rise that accompanies pregnancy, in mice results in reductions in food intake (Mullican et al., 2017; Patel et al., 2019). Furthermore, Day and colleagues found that eliminating *Gdf15* in mice results in ablation of the reduction of food intake with metformin treatment (Day et al., 2019). Therefore, we hypothesized that observing pregnancy in the mouse that lacked GDF15 at both the maternal and feto-placental level would result in unrestrained food intake. Contrary to our expectations, we saw no significant effect on food intake during the course of pregnancy or lactation. This may mean that reduction of GDF15 levels is in the wrong direction to impact where *Gdf15* signaling most contributes to body weight regulation. More significant findings with GDF15 and body weight come from studies that deliver exogenous GDF15 (Mullican et al., 2017; Patel et al., 2019) or induce a Gdf15 stress response (such as the use of metformin (Day et al., 2019)). In fact, *Gdf15* null mice have previously been found to have no differences in food intake, only in macronutrient preferences to wild type animals(Frikke-Schmidt et al., 2019). Most similar to our own findings, GFRAL null animals displayed no differences in food intake, body weight, glucose tolerance, or fasting blood glucose during young adulthood while fed chow (Mullican et al., 2017).

The most compelling evidence for the role of GDF15 during the perinatal period is that when human studies evaluate complications of pregnancy. The most consistent are those that study excessive vomiting and emesis in pregnancy, or hyperemesis gravidarum (HG). Those patients who had HG or had higher than expected levels of vomiting during their pregnancies were more likely to have elevated GDF15 levels (Fejzo et al., 2019; Petry et al., 2018). A later study found that SNPs near GDF15 that were associated with loss of function were protective for HG (Fejzo et al., 2019). It should be noted that as mice do not exhibit emesis, any HG related phenotypes would not be expected. Because of these relationships, we expected *Gdf15-/-*dams to have greater food intake related to reduced aversion from circulating GDF15. However, this was not apparent in these data, which is more in line with the mouse literature.

Diabetes and glucose tolerance during pregnancy and the impact of GDF15 levels in humans is less clear. Some groups find that GDF15 levels rise in mothers whose pregnancies are complicated by type 2 diabetes (Sugulle et al., 2009). Others find elevated GDF15 in association with gestational diabetes, especially in the third trimester (Tong et al., 2004; Yakut et al., 2021). Still others looking at all types of diabetes during pregnancy found that GDF15 levels were only associated with type 1 diabetes (Andersson-Hall et al., 2021). Our findings must be interpreted carefully, as we did not evaluate diabetes in our model, but we found no detectable differences in insulin tolerance between *Gdf15-/-* and *Gdf15+/+* dams. This again may be because the strongest evidence of effect with *Gdf15* appear to be in relation to overexpression or external administration, meaning ablation may be insufficient to affect maternal insulin sensitivity.

Lactation is another period of life that is critically understudied in the context of GDF15 production. To our knowledge, no work has been done on the effect of *Gdf15* ablation on lactation before our study commenced. In fact, only two studies that mention lactation to date in relation to *Gdf15*. One study found that overexpression of *Gdf15* in mice resulted in impaired lactation and poor pup survival in (Binder et al., 2016). The other found simply found that the mammary gland was a prominent place of GDF15 production in their model (Böttner et al., 1999). We found that there was no difference in survival, milk volume production, or milk fat percentage when we modulated *Gdf15* in pregnant dams.

Taken together, the lack of evidence of change for food intake, body weight, insulin sensitivity, and lactation in our *Gdf15* null model suggests that there may be a threshold effect for GDF15 during pregnancy. Only those studies that overexpress, deliver exogenous, or induce long-term highly disruptive stressors to their model show differences in GDF15 in relation to food intake and body weight. Therefore, it might be that ablation of *Gdf15* fails to reach the threshold of stressor to elicit an effect. *Gdf15* may act as a less acute stressor during pregnancy and more as a long-term indicator or feto-placental implantation. This also could mean that *Gdf15* imparts effects on other systems that have been reported, but that were not evaluated in our study, such as miscarriage and maternal blood pressure (Chen et al., 2016), immunity and inflammation (Wischhusen et al., 2020), tissue injury (Hsiao et al., 2000), or macronutrient preference (Frikke-Schmidt et al., 2019).

Although the model of this work was intentional, there are several limitations to our study. Murine pregnancy is not entirely comparable to human pregnancy. The majority of human pregnancies are singleton and mice are multi-parous, the placental structure is also different when compared with human pregnancy in the level of invasion of the tissue into the maternal uterus and the structure of the zones of the placenta itself (Schmidt et al., 2015). The approach we took eliminated feto-placental contribution of GDF15 to maternal serum during pregnancy by the use of homozygous breeding. As a result, all knockout pups had knockout dams and sires, and all wild-type pups had wild-type dams and sires. Even though we did not detect any differences in offspring growth, the genotypes of these mice are not the same. A larger sample size would have conceivably facilitated more statistical power to detect differences in the outcomes evaluated. For example, via a reverse power analysis, we cannot rule out an effect size of <0.132g on maternal body weight gain during pregnancy but such a small effect would likely be physiologically insignificant. We also followed the pups for a relatively short period of time after birth. So, any effect that would have manifested after the second week of life could not be observed.

In contrast to the mixed human findings, this study had several strengths including strong environmental, genetic and experimental consistency. While dams and sires were homozygous, they were derived from heterozygous crosses to limit genetic drift. This is the first report of the loss of GDF15 in pregnancy and provides strong evidence for a lack of effect on body weight, food intake, or offspring health.

# Conclusion

Despite the well-known, multi-fold rise in GDF15during mouse and human pregnancy, we found no evidence that *Gdf15* ablation during mouse pregnancy and lactation causes metabolic, body weight, appetite, or lactational differences compared to *Gdf15+/+*counterpart dams. In the neonatal period, we did not observe any differences in survival, gestational age, litter size or birth weight between genotypes. Despite monitoring growth for 14 days after birth, there were no differences in body weight accretion in *Gdf15-/-* pups of either sex; indistinguishable from age-matched *Gdf15+/+* pups. More studies with larger sample sizes are needed to confirm these findings.

# Figure Legends

## Figure 1: Experimental Schema

A) Insulin resistance of pregnancy study, comparing age-matched females in 3 groups; non-pregnant females (n=7), pregnant females given plain drinking water (n=7), pregnant females given 1.0 mg/kg dexamethasone in drinking water (n=7). B) *Gdf15* Knockout study in pregnancy. *Gdf15+/+* females (n=6) were mated with *Gdf15+/+* males. *Gdf15-/-* females (n=7) were mated with *Gdf15-/-* males. Food intake and body weight was measured weekly from one week before mating until 14-16 days after pups were born.

## Figure 2: Insulin Resistance of Pregnancy in Mice

A) Intraperitoneal insulin tolerance testing on E16.5 in pregnant mice given plain water and age-matched non-pregnant females. Values are relative to fasting blood glucose and were assessed using a linear mixed effect model. B) Fasting blood glucose values in pregnant dams given water and non-pregnant females, assessed using student’s T test. C) Area under the curve during insulin tolerance test, defined as sum of glucose values for each animal in pregnant dams given plain water and age-matched non-pregnant females, assessed using students t test. D) Intraperitoneal insulin tolerance testing on E16.5 in pregnant dams given water or 1mg/kg dexamethasone in drinking water, assessed via linear mixed effect modeling. Values are relative to fasting blood glucose levels. E) Fasting blood glucose values in pregnant dams given plain drinking water or dexamethasone in drinking water, assessed via student’s t test. F) Area under the curve during insulin tolerance test, defined as sum of glucose values for each animal in pregnant dams given plain water and pregnant dams given dexamethasone in drinking water, assessed using students t test. G) GDF15 ELISA evaluating serum levels at ZT1 and ZT13 in pregnant dams given plain drinking water, pregnant dams given dexamethasone in drinking water, and age-matched non-pregnant females. Assessed as paired t tests between females given water, and pregnant animals. H) Body weights of pregnant dams given plain drinking water, pregnant dams given dexamethasone in drinking water, and age-matched non-pregnant females. Assessed via linear mixed effects modeling.

## Figure 3:Maternal Response to Gdf15 knockout during pregnancy

A) Cumulative food intake during the prenatal period (pre-mating through final measurement before birth), assessed via student’s t test. B) Weight gained during prenatal period, assessed via student’s t test. C)Postnatal cumulative food intake (after birth of pups-end of experiment), assessed via student’s t test. D) Weight lost in the postnatal period, assessed via students’ t test. E) Plot of the weekly food intake in both genotypes from 1 week before mating until end of the experiment. F) Plot of maternal body weight throughout the experimental period.

## Figure 4: Maternal Insulin Tolerance

A) Intraperitoneal insulin tolerance test in *Gdf15+/+* and *Gdf15-/-* dams at E16.5. Values are relative to fasting blood glucose levels. Assessed via linear mixed effects modeling. B) Fasting Blood glucose levels in dams, assessed by students t test. C) Area under the curve defined as sum of all glucose values for each animal, assessed by student’s t test. D) Rate of drop in blood glucose in the first hour of the insulin tolerance test, assessed by student’s t test.

## Figure 5:Neonatal Health Outcomes

A) Latency to copulatory plug (time from introduction of male into cage until copulatory plug is discovered), assessed via student’s t test. B)Gestational age in days, calculated as the number of days from appearance of copulatory plug until birth of the litter. Assessed via Mann-Whitney test. C)Average birth weight of pups, calculated as the average birth weight for each dam, then averaged by genotype. Assessed by student’s t test. D)Total litter size (including those who were dead), assessed via student’s t test. E)Number of pups born per litter that were alive, assessed via student’s t test. F) Percentage of pups in each litter who were dead by postnatal day 3.5, assessed by Mann Whitney test.

## Figure 6: Milk volume and milkfat percentage

A) Total mass (in grams) lost by dam during the suckling period of the weigh-suckle-weigh test on PND10.5, assessed by student’s t test. B)Total mass (in grams) gained cumulatively between all pups in the litter during suckling period during weigh-suckle-weigh test, assessed by Mann Whitney test. C)Percentage of fat found in mouse milk collected PND 14-16.5, assessed by student’s t test.

## Figure 7: Pup Postnatal Growth

A) Postnatal bodyweight measurements from birth through PND14.5 in male and female pups, assessed via linear mixed effect models.

## Supplementary Figure 1: Gdf15 levels in Knockout animals

A) GDF15 levels in mouse serum (pg/mL) collected E16.5 at ZT1 and ZT13 in *Gdf15-/-* and *Gdf15+/+* dams. Assessed via students t test.

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