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

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**Keywords:**

**Author contribution:** MCM, NEH, RJS, BEG, and DB conceptualized experiments. Data were curated by MCM, NEH, JRR, and HS. RJS provided the experimental animals. MCM completed the analysis and wrote the original draft of the manuscript. All authors contributed to the review and editing of the manuscript and approved its final format before submission.

**Funding:** MCM is supported by a Rackham Merit Fellowship. This study was supported by funds from the University of Michigan (MCubed to DB and RJS) and the National Institutes of Health (DK107535 and a Pilot and Feasibility Grant from P30DK020572 to DB).

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# Abstract

Growth differentiation factor-15 (GDF15) increases 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 consumed a similar amount of food and gained comparable 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 had similar survival rates in both genotypes. There were also no detectable differences in milk volume production, milk fat percentage, or 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, it was found that levels of circulating 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 increases in response to many stressors including 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 (Andersson-Hall et al., 2021; Chen et al., 2016; Marjono et al., 2003; Sugulle et al., 2009; Welsh et al., 2022).

Preclinical work with knockout or knockdown models have highlighted the role of GDF15in body weight regulation (Hsu et al., 2017, p. 15), appetite (Tsai et al., 2019), and emesis (Borner et al., 2020). In rodents, the effect of Gdf15 antagonism through antibodies or knockout on food intake depends on their diet. When consuming a high fat, high sucrose diet, food intake and body weight increases (Tran et al., 2018; Tsai et al., 2019); however, when consuming a chow diet, they remain similar to wildtype animals (Tran et al., 2018).These models show that *Gdf15* acts through the GFRAL receptor found in the area postrema of the brain. The role of GFRAL in body weight and food intake has been just as critical as *Gdf15*. One study showed a positive association between GFRAL positive neurons and fat mass/body weight gain (Tsai et al., 2019). Interrupting GFRAL receptors in preclinical models don’t produce consistent results on weight and feeding. One model showed ablating GFRAL in mice resulted in smaller mice at the beginning of the study that developed increased food intake and weight gain from eating a hyperpalatable diet (Mullican et al., 2017). Another study noted no differences in food intake, weight accretion, or in size at the onset of the experiment (Yang et al., 2017).

Pharmacologic administration or overexpression of Gdf15 induces weight loss through reductions in food intake (Hsu et al., 2017; Mullican et al., 2017; Yang et al., 2017). It also results in nausea-like behavior in mice and emesis in shrews (Borner et al., 2020), reduced changes in food preferences (Frikke-Schmidt et al., 2019), or a decrease in meal size (Emmerson et al., 2017). As such, evaluating *Gdf15* for its capacity to ameliorate metabolic illness is currently being explored.

During pregnancy, GDF15 increases across gestation and reaches its highest levels during the third trimester (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, secreted by the placenta? into parental circulation, and present in the amniotic fluid (Moore et al., 2000). In spite of these pregnancy-related increases, details on the functional role of GDF15 in pregnancy are just emerging. GDF15 has been linked to several 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 have also been linked to gestational weight gain, as elevations were negatively associated with cumulative gestational weight gain (P. Wang et al., 2020). Petry and colleagues found pre-pregnancy BMI was inversely related to GDF15 levels during pregnancy (Petry et al., 2018). 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 reductions (Chen et al., 2016), increases (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 gestational diabetes (GDM) (Yakut et al., 2021), or type 2 diabetes (T2DM) (Sugulle et al., 2009) while others find it is only significantly increased in pregnancies that are complicated by T1DM but not T2DM or GDM (Jacobsen et al., 2022). GWAS have indicated that *GDF15* variants in humans are associated with hyperemesis gravidarum, an extreme form of nausea and vomiting of pregnancy (Fejzo et al., 2018, 2019). Given the sometimes-conflicting human data, we sought to understand more about the effects of *Gdf15* loss of function during the course of murine pregnancy, including effects on weight gain, food intake, insulin sensitivity, and neonatal outcomes.

# Materials and Methods:

## Animal Husbandry

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. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

### Insulin resistance of pregnancy study

Virgin female C57BL/6J (RRID: IMSR\_JAX:000664) mice were ordered from The Jackson Laboratories. Mice were allowed to acclimatize for two weeks to the temperature and humidity-controlled facility with *ad lib?*free access to water and laboratory chow diet (CD, Picolab Laboratory Rodent diet 5L0D; 5% of Calories from fat, 24% from protein, 71% from carbohydrates). After acclimatizing, females were randomized into three groups, non-pregnant females (n=7), pregnant females (n=7), and pregnant females exposed to dexamethasone (1mg/kg/day Sigma-Aldrich catalog #D2915-100MG) in drinking water (n=7). One week after experimental treatment began, males were introduced to the dam’s cage and allowed to remain until gestational day 19. Body weight and food intake measurements occurred weekly from randomization until birth.

### Gdf15 study

Male and female *Gdf15* null animals are described by Frikke-Schmidt et al. (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. We chose to study *Gdf15+/+* mated pairs compared to *Gdf15-/-* pairs because comparing littermates of *Gdf15+/-* pairs would result in potential placental contributions to GDF1*5* in dam serum as the fetus provides a substantial amount of the placenta. To limit genetic drift, all homozygous parents were direct offspring of heterozygous crosses. We combined homozygous pairs, resulting in homozygous genotype progeny and placentae. Adult virgin female mice (*Gdf15*-/-n=8, *Gdf15*+/+n=6), between 45 and 119 days old (mean 82 days), were singly housed with *ad libitum* access to water and CD. 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 identified, 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).

## Genotyping

At 14 days of age, a small section of the tail of offspring 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 samples were amplified 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.

Insulin tolerance tests

On E16.5, dams underwent intraperitoneal insulin tolerance testing (ITT) (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 blood samples from non-fasted dams: 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 determinations were 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 Assessments

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 the 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 the amount of nutrition/milk produced by the dams and provided to each pup. Survival of pups to PND 3.5 was assessed by comparing the number of pups present at PND 3.5, prior to culling, 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 two 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; El 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 then reintroduced to the dam’s cage where they remained for 1 hour and were allowed to nurse/suckle/feed undisturbed. After one hour, the final weights were taken for both dams and pups in aggregate. The volume of milk produced is expressed by 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 (0.13g/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 promote milk 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 and 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 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.

Dexamethasone section in methods?

## 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 with 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 for offspring body weight were assessed for interaction of sex with time and genotype but neither were significant, so sex remained a fixed effect. 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 C57BL/6J? mice

Previous work has shown that pregnancy in mice results in maternal insulin resistance (Ladyman et al., 2018; Musial et al., 2016), so we sought to understand if GDF15 levels related to either pregnancy or a model of excess insulin resistance in pregnancy. We compared age-matched pregnant and non-pregnant C57BL/6J females using an intraperitoneal insulin tolerance test on day 16 of pregnancy (**Figure 2A**). Consistent with prior work, we found that pregnant dams responded less to insulin than non-pregnant females, though this did not reach statistical significance (**Figure 2A**, p=0.23 via mixed linear models). Inconsistent to Musial and colleagues, there were no significant differences in their fasting blood glucose (**Figure 2B**, p=0.020). We found that GDF15 is 49% (54 ±18.8 pg/dL) elevated in pregnant animals compared to non-pregnant mice (**Figure 2C,** p=0.007). As expected, body weights in pregnant females were 1.57± 0.55 grams heavier than non-pregnant females (**Supplemental** **Figure 2A**, p=0.0039).

To enhance insulin resistance in pregnancy, we leveraged prior work from our lab that has demonstrated that administering the glucocorticoid dexamethasone in their drinking water impairs insulin sensitivity in non-pregnant mice (Gunder et al., 2020; Harvey et al., 2018). We treated dams with 1 mg/kg dexamethasone one week before mating and throughout the length of the pregnancy. We compared dexamethasone-treated dams to age-matched pregnant dams who were provided normal drinking water. We found that dexamethasone dams did not respond to insulin compared to pregnant dams with plain drinking water (**Figure 2D**, pdex\*time=0.02 via linear mixed effect models). Dexamethasone-treated dams had 33% lower fasting blood glucose (**Figure 2E**, pdex=0.007) consistent with our findings in non-pregnant mice. GDF15 levels were not further increased by dexamethasone administration in pregnant dams (**Figure 2F**, p=0.11). Body weights in pregnant dams were 2.77±0.58 grams lighter in those treated with dexamethasone compared to water dams (**Supplementary Figure 2B**, p<0.0001). We were interested to see how pregnancy and dexamethasone administration in pregnancy related to GDF15 levels in these mice. Based on these data we conclude that while GDF15 is related to pregnancy, it is not elevated in insulin resistant dexamethasone treated dams.

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

To evaluate 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**). 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). Both genotypes consumed similar calories weekly (**Figure 3E**, pgenotype=0.23). Both genotypes had a rapid increase in food intake in the final trimester of pregnancy, with smaller increases in the *Gdf15-/-* dams. 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, 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 revealed no effect of the genotype (pgenotype= 0.71). This was confirmed by determining the area under the ITT curve, again showing similar responses (**Figure 4C**, p=0.74). Often an informative measure of the insulin response is the initial rate of drop of blood glucose. The initial rate of glucose decline 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 substantially affect 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 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/viable at birth?, that difference was only 7.8% larger (**Figure 5E**, p=0.70, or 0.46 pups/litter greater on average). The total pups who were born alive that lived to postnatal day 3 was variable within genotypes, resulting in 91.7% survival for *Gdf15+/+* dams and 90% for *Gdf15-/-* dams which was not statistically significant (**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 milk fat percentage

To determine the effect of *Gdf15* knockout during pregnancy on lactation in the postnatal period, we assessed milk volume 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) and weight gained by pups during nursing (**Figure 6B**, p=0.7) was similar between genotypes, though highly variable between dams. Next, we evaluated whether the major macronutrient in milk, fat, was changed by *Gdf15* knockout. To do this, we collected whole milk between PND 14-17 and evaluated milk fat percentage. We found that milk fat percentage was similar between genotypes (**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 after adjusting for sex differences). There was also no statistically significant modifying 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 complications of pregnancy in addition to its better understood role in signaling somatic stress throughout the body. In fact, pregnancy itself is an oft-underappreciated stressor on the body, an effect that is consistent with elevations in GDF15. The goal of this study was to understand the role of GDF15 in gestational health. To date, there are very few studies that evaluate GDF15 in human pregnancy. 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 prominent changes in gestational outcomes, although perhaps contrary to our prediction, is novel in the literature. Previous reports of *Gdf15* or *Gfral* null mouse models have generally not reported pregnancy or gestational outcomes during breeding or maintenance, but only describe differences as adults when used in experimental models. One study evaluating the transgenic expression of human *GDF15* in mice found that there was early mammary gland involution, reduced milk production, reduced survival in pups, and lower weight gain in the postnatal period in offspring born to transgenic dams (Binder et al., 2016). Previous work shows that external administration of GDF15, similar to the rising levels accompanying pregnancy, in mice results in reductions in food intake (Mullican et al., 2017; Patel et al., 2019). Our current study found that ablation of *Gdf15* and the resulting loss of 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, with only small reductions in pup birth weight. This suggests that GDF15 in pregnant mice is altered, but it is not necessary for changes in weight accretion during a normal pregnancy. It is possible that under conditions of elevated somatic stress, GDF15 may play a larger role.

Taken together, the lack of evidence of differences in 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 pregnancy-related inductions of GDF15are insufficient to meet the threshold to elicit an effect. *Gdf15* may act as a less acute stressor during pregnancy and more as a long-term indicator of feto-placental implantation. It could also imply that in the observational human studies, GDF15 is a biomarker of pregnancy related complications but not part of a causal pathway.

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 pairs. 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 could have provided more statistical power to detect differences in the outcomes evaluated. For example, via a reverse power analysis, we cannot rule out an effect size smaller than 15.3% difference in body weight gain during pregnancy between genotypes, but such a small effect would likely be physiologically insignificant. We also followed the pups for a relatively short period of time after birth (until PND14.5). So, any effect that would have manifested after the second week of life was not evaluated. Finally, we did not evaluate two other GDF15-associated complications, hypertension or nausea-related behavior in these mice.

In contrast to the human findings, this study had several strengths including strong environmental, genetic, and experimental consistency. Dams and sires were homozygous; they were derived from heterozygous crosses to limit genetic drift. In contrast to human observational studies demonstrating connections to pregnancy complications, we do not observe any detectable differences in litter sizes, glucose homeostasis, or gestational weight gain in the knockout mice. This study 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: Schematic of Experimental Manipulations

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 Co-occurs with Elevations in GDF15

A) Intraperitoneal insulin tolerance testing on E16.5 in pregnant C57BL/6J 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) GDF15 levels at ZT1 in pregnant and non-pregnant females, assessed as paired t tests. 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) GDF15 ELISA evaluating serum levels at ZT1 and ZT13 in pregnant dams given plain drinking water, pregnant dams given dexamethasone in drinking water, assessed as paired t tests.

## Figure 3:Gdf15 Knockout Does Not Impact Food Intake or Body Weight During Mouse 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: Gdf15 Knockout Has No Effect on Gestational 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:Birth Weight is Reduced in Gdf15 Knockout Pregnancies

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 Are not Changed in Gdf15 Knockout Dams

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: Offspring Postnatal Growth is Normal in Gdf15 Knockout Litters

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 and Body Weights in

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.

## Supplementary Figure 2: Pregnancy Increases Body Weight in Mice, but Weight Gain Is Impaired by Dexamethasone Treatment

A) Body weights of non-pregnant dams compared to pregnant dams, assessed via linear mixed effect modeling. B) Body weights of pregnant dams given plain drinking water and pregnant dams given dexamethasone in drinking water, assessed via linear mixed effects modeling.

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# References

Andersson-Hall, U., Joelsson, L., Svedin, P., Mallard, C., & Holmäng, A. (2021). Growth-differentiation-factor 15 levels in obese and healthy pregnancies: Relation to insulin resistance and insulin secretory function. *Clinical Endocrinology*, *95*(1), 92–100. https://doi.org/10.1111/cen.14433

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, *67*, 1–48. https://doi.org/10.18637/jss.v067.i01

Binder, A. K., Kosak, J. P., Janhardhan, K. S., Moser, G., Eling, T. E., & Korach, K. S. (2016). Expression of Human NSAID Activated Gene 1 in Mice Leads to Altered Mammary Gland Differentiation and Impaired Lactation. *PLoS ONE*, *11*(1), e0146518. https://doi.org/10.1371/journal.pone.0146518

Bootcov, M. R., Bauskin, A. R., Valenzuela, S. M., Moore, A. G., Bansal, M., He, X. Y., Zhang, H. P., Donnellan, M., Mahler, S., Pryor, K., Walsh, B. J., Nicholson, R. C., Fairlie, W. D., Por, S. B., Robbins, J. M., & Breit, S. N. (1997). MIC-1, a novel macrophage inhibitory cytokine, is a divergent member of the TGF-β superfamily. *Proceedings of the National Academy of Sciences*, *94*(21), 11514–11519. https://doi.org/10.1073/pnas.94.21.11514

Borner, T., Shaulson, E. D., Ghidewon, M. Y., Barnett, A. B., Horn, C. C., Doyle, R. P., Grill, H. J., Hayes, M. R., & De Jonghe, B. C. (2020). GDF15 Induces Anorexia through Nausea and Emesis. *Cell Metabolism*, *31*(2), 351-362.e5. https://doi.org/10.1016/j.cmet.2019.12.004

Boston, W. S., Bleck, G. T., Conroy, J. C., Wheeler, M. B., & Miller, D. J. (2001). Short Communication: Effects of Increased Expression of α-Lactalbumin In Transgenic Mice on Milk Yield and Pup Growth. *Journal of Dairy Science*, *84*(3), 620–622. https://doi.org/10.3168/jds.S0022-0302(01)74516-X

Böttner, M., Suter-Crazzolara, C., Schober, A., & Unsicker, K. (1999). Expression of a novel member of the TGF-beta superfamily, growth/differentiation factor-15/macrophage-inhibiting cytokine-1 (GDF-15/MIC-1) in adult rat tissues. *Cell and Tissue Research*, *297*(1), 103–110. https://doi.org/10.1007/s004410051337

Bridges, D., Mulcahy, M. C., & Redd, J. R. (2022, March 7). *Insulin Tolerance Test*. Protocols.Io. dx.doi.org/10.17504/protocols.io.b5zxq77n

Chen, Q., Wang, Y., Zhao, M., Hyett, J., da Silva Costa, F., & Nie, G. (2016). Serum levels of GDF15 are reduced in preeclampsia and the reduction is more profound in late-onset than early-onset cases. *Cytokine*, *83*, 226–230. https://doi.org/10.1016/j.cyto.2016.05.002

El Habbal, N., Meyer, A. C., Hafner, H., Redd, J. R., Carlson, Z., Mulcahy, M. C., Gregg, B., & Bridges, D. (2021). Activation of Adipocyte mTORC1 Increases Milk Lipids in a Mouse Model of Lactation. *BioRxiv*, 2021.07.01.450596. https://doi.org/10.1101/2021.07.01.450596

Emmerson, P. J., Wang, F., Du, Y., Liu, Q., Pickard, R. T., Gonciarz, M. D., Coskun, T., Hamang, M. J., Sindelar, D. K., Ballman, K. K., Foltz, L. A., Muppidi, A., Alsina-Fernandez, J., Barnard, G. C., Tang, J. X., Liu, X., Mao, X., Siegel, R., Sloan, J. H., … Wu, X. (2017). The metabolic effects of GDF15 are mediated by the orphan receptor GFRAL. *Nature Medicine*, *23*(10), Article 10. https://doi.org/10.1038/nm.4393

Fejzo, M. S., Fasching, P. A., Schneider, M. O., Schwitulla, J., Beckmann, M. W., Schwenke, E., MacGibbon, K. W., & Mullin, P. M. (2019). Analysis of GDF15 and IGFBP7 in Hyperemesis Gravidarum Support Causality. *Geburtshilfe Und Frauenheilkunde*, *79*(4), 382–388. https://doi.org/10.1055/a-0830-1346

Fejzo, M. S., Sazonova, O. V., Sathirapongsasuti, J. F., Hallgrímsdóttir, I. B., Vacic, V., MacGibbon, K. W., Schoenberg, F. P., Mancuso, N., Slamon, D. J., Mullin, P. M., Agee, M., Alipanahi, B., Auton, A., Bell, R. K., Bryc, K., Elson, S. L., Fontanillas, P., Furlotte, N. A., Hinds, D. A., … Wilson, C. H. (2018). Placenta and appetite genes GDF15 and IGFBP7 are associated with hyperemesis gravidarum. *Nature Communications; London*, *9*, 1–9. http://dx.doi.org.proxy.lib.umich.edu/10.1038/s41467-018-03258-0

Frikke-Schmidt, H., Hultman, K., Galaske, J. W., Jørgensen, S. B., Myers, M. G., & Seeley, R. J. (2019). GDF15 acts synergistically with liraglutide but is not necessary for the weight loss induced by bariatric surgery in mice. *Molecular Metabolism*, *21*, 13–21. https://doi.org/10.1016/j.molmet.2019.01.003

Gunder, L. C., Harvey, I., Redd, J. R., Davis, C. S., AL-Tamimi, A., Brooks, S. V., & Bridges, D. (2020). Obesity Augments Glucocorticoid-Dependent Muscle Atrophy in Male C57BL/6J Mice. *Biomedicines*, *8*(10), Article 10. https://doi.org/10.3390/biomedicines8100420

Harvey, I., Stephenson, E. J., Redd, J. R., Tran, Q. T., Hochberg, I., Qi, N., & Bridges, D. (2018). Glucocorticoid-Induced Metabolic Disturbances Are Exacerbated in Obese Male Mice. *Endocrinology*, *159*(6), 2275–2287. https://doi.org/10.1210/en.2018-00147

Hsiao, E. C., Koniaris, L. G., Zimmers-Koniaris, T., Sebald, S. M., Huynh, T. V., & Lee, S.-J. (2000). Characterization of Growth-Differentiation Factor 15, a Transforming Growth Factor ␤ Superfamily Member Induced following Liver Injury. *MOL. CELL. BIOL.*, *20*, 10.

Hsu, J.-Y., Crawley, S., Chen, M., Ayupova, D. A., Lindhout, D. A., Higbee, J., Kutach, A., Joo, W., Gao, Z., Fu, D., To, C., Mondal, K., Li, B., Kekatpure, A., Wang, M., Laird, T., Horner, G., Chan, J., McEntee, M., … Allan, B. B. (2017). Non-homeostatic body weight regulation through a brainstem-restricted receptor for GDF15. *Nature*, *550*(7675), 255–259. https://doi.org/10.1038/nature24042

Jacobsen, D. P., Røysland, R., Strand, H., Moe, K., Sugulle, M., Omland, T., & Staff, A. C. (2022). Cardiovascular biomarkers in pregnancy with diabetes and associations to glucose control. *Acta Diabetologica*, *59*(9), 1229–1236. https://doi.org/10.1007/s00592-022-01916-w

Kempf, T., Eden, M., Strelau, J., Naguib, M., Willenbockel, C., Tongers, J., Heineke, J., Kotlarz, D., Xu, J., Molkentin, J. D., Niessen, H. W., Drexler, H., & Wollert, K. C. (2006). The transforming growth factor-beta superfamily member growth-differentiation factor-15 protects the heart from ischemia/reperfusion injury. *Circulation Research*, *98*(3), 351–360. https://doi.org/10.1161/01.RES.0000202805.73038.48

Klein, A. B., Nicolaisen, T. S., Ørtenblad, N., Gejl, K. D., Jensen, R., Fritzen, A. M., Larsen, E. L., Karstoft, K., Poulsen, H. E., Morville, T., Sahl, R. E., Helge, J. W., Lund, J., Falk, S., Lyngbæk, M., Ellingsgaard, H., Pedersen, B. K., Lu, W., Finan, B., … Clemmensen, C. (2021). Pharmacological but not physiological GDF15 suppresses feeding and the motivation to exercise. *Nature Communications*, *12*, 1041. https://doi.org/10.1038/s41467-021-21309-x

Ladyman, S. R., Carter, K. M., & Grattan, D. R. (2018). Energy homeostasis and running wheel activity during pregnancy in the mouse. *Physiology & Behavior*, *194*, 83–94. https://doi.org/10.1016/j.physbeh.2018.05.002

Marjono, A. B., Brown, D. A., Horton, K. E., Wallace, E. M., Breit, S. N., & Manuelpillai, U. (2003). Macrophage Inhibitory Cytokine-1 in Gestational Tissues and Maternal Serum in Normal and Pre-eclamptic Pregnancy. *Placenta*, *24*(1), 100–106. https://doi.org/10.1053/plac.2002.0881

Moore, A. G., Brown, D. A., Fairlie, W. D., Bauskin, A. R., Brown, P. K., Munier, M. L., Russell, P. K., Salamonsen, L. A., Wallace, E. M., & Breit, S. N. (2000). The transforming growth factor-ss superfamily cytokine macrophage inhibitory cytokine-1 is present in high concentrations in the serum of pregnant women. *The Journal of Clinical Endocrinology and Metabolism*, *85*(12), 4781–4788. https://doi.org/10.1210/jcem.85.12.7007

Mullican, S. E., Lin-Schmidt, X., Chin, C.-N., Chavez, J. A., Furman, J. L., Armstrong, A. A., Beck, S. C., South, V. J., Dinh, T. Q., Cash-Mason, T. D., Cavanaugh, C. R., Nelson, S., Huang, C., Hunter, M. J., & Rangwala, S. M. (2017). GFRAL is the receptor for GDF15 and the ligand promotes weight loss in mice and nonhuman primates. *Nature Medicine*, *23*(10), 1150–1157. https://doi.org/10.1038/nm.4392

Musial, B., Fernandez-Twinn, D. S., Vaughan, O. R., Ozanne, S. E., Voshol, P., Sferruzzi-Perri, A. N., & Fowden, A. L. (2016). Proximity to Delivery Alters Insulin Sensitivity and Glucose Metabolism in Pregnant Mice. *Diabetes*, *65*(4), 851–860. https://doi.org/10.2337/db15-1531

Ost, M., Igual Gil, C., Coleman, V., Keipert, S., Efstathiou, S., Vidic, V., Weyers, M., & Klaus, S. (2020). Muscle-derived GDF15 drives diurnal anorexia and systemic metabolic remodeling during mitochondrial stress. *EMBO Reports*, *21*(3), e48804. https://doi.org/10.15252/embr.201948804

Patel, S., Alvarez-Guaita, A., Melvin, A., Rimmington, D., Dattilo, A., Miedzybrodzka, E. L., Cimino, I., Maurin, A.-C., Roberts, G. P., Meek, C. L., Virtue, S., Sparks, L. M., Parsons, S. A., Redman, L. M., Bray, G. A., Liou, A. P., Woods, R. M., Parry, S. A., Jeppesen, P. B., … O’Rahilly, S. (2019). GDF15 Provides an Endocrine Signal of Nutritional Stress in Mice and Humans. *Cell Metabolism*, *29*(3), 707-718.e8. https://doi.org/10.1016/j.cmet.2018.12.016

Petry, C. J., Ong, K. K., Burling, K. A., Barker, P., Goodburn, S. F., Perry, J. R. B., Acerini, C. L., Hughes, I. A., Painter, R. C., Afink, G. B., Dunger, D. B., & O’Rahilly, S. (2018). Associations of vomiting and antiemetic use in pregnancy with levels of circulating GDF15 early in the second trimester: A nested case-control study. *Wellcome Open Research*, *3*, 123. https://doi.org/10.12688/wellcomeopenres.14818.1

R Core Team. (2021). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Schmidt, A., Morales-Prieto, D. M., Pastuschek, J., Fröhlich, K., & Markert, U. R. (2015). Only humans have human placentas: Molecular differences between mice and humans. *Journal of Reproductive Immunology*, *108*, 65–71. https://doi.org/10.1016/j.jri.2015.03.001

Sugulle, M., Dechend, R., Herse, F., Weedon-Fekjaer, M. S., Johnsen, G. M., Brosnihan, K. B., Anton, L., Luft, F. C., Wollert, K. C., Kempf, T., & Staff, A. C. (2009). Circulating and Placental Growth-Differentiation Factor 15 in Preeclampsia and in Pregnancy Complicated by Diabetes Mellitus. *Hypertension*, *54*(1), 106–112. https://doi.org/10.1161/HYPERTENSIONAHA.109.130583

Suriben, R., Chen, M., Higbee, J., Oeffinger, J., Ventura, R., Li, B., Mondal, K., Gao, Z., Ayupova, D., Taskar, P., Li, D., Starck, S. R., Chen, H.-I. H., McEntee, M., Katewa, S. D., Phung, V., Wang, M., Kekatpure, A., Lakshminarasimhan, D., … Allan, B. B. (2020). Antibody-mediated inhibition of GDF15-GFRAL activity reverses cancer cachexia in mice. *Nature Medicine*, *26*(8), 1264–1270. https://doi.org/10.1038/s41591-020-0945-x

Tong, S., Marjono, B., Brown, D. A., Mulvey, S., Breit, S. N., Manuelpillai, U., & Wallace, E. M. (2004). Serum concentrations of macrophage inhibitory cytokine 1 (MIC 1) as a predictor of miscarriage. *The Lancet*, *363*(9403), 129–130. https://doi.org/10.1016/S0140-6736(03)15265-8

Tran, T., Yang, J., Gardner, J., & Xiong, Y. (2018). GDF15 deficiency promotes high fat diet-induced obesity in mice. *PloS One*, *13*(8), e0201584. https://doi.org/10.1371/journal.pone.0201584

Tsai, V. W.-W., Zhang, H. P., Manandhar, R., Schofield, P., Christ, D., Lee-Ng, K. K. M., Lebhar, H., Marquis, C. P., Husaini, Y., Brown, D. A., & Breit, S. N. (2019). GDF15 mediates adiposity resistance through actions on GFRAL neurons in the hindbrain AP/NTS. *International Journal of Obesity*, *43*(12), Article 12. https://doi.org/10.1038/s41366-019-0365-5

Wang, L., & Yang, Q. (2022). Circulating Growth Differentiation Factor 15 and Preeclampsia: A Meta-Analysis. *Hormone and Metabolic Research = Hormon- Und Stoffwechselforschung = Hormones Et Metabolisme*. https://doi.org/10.1055/a-1956-2961

Wang, P., Ma, W., Zhou, Y., Zhao, Y., Shi, H., Yang, Q., & Zhang, Y. (2020). Circulating metal concentrations, inflammatory cytokines and gestational weight gain: Shanghai MCPC cohort. *Ecotoxicology and Environmental Safety*, *199*, 110697. https://doi.org/10.1016/j.ecoenv.2020.110697

Welsh, P., Kimenai, D. M., Marioni, R. E., Hayward, C., Campbell, A., Porteous, D., Mills, N. L., O’Rahilly, S., & Sattar, N. (2022). Reference ranges for GDF-15, and risk factors associated with GDF-15, in a large general population cohort. *Clinical Chemistry and Laboratory Medicine (CCLM)*. https://doi.org/10.1515/cclm-2022-0135

Wischhusen, J., Melero, I., & Fridman, W. H. (2020). Growth/Differentiation Factor-15 (GDF-15): From Biomarker to Novel Targetable Immune Checkpoint. *Frontiers in Immunology*, *11*. https://www.frontiersin.org/articles/10.3389/fimmu.2020.00951

Yakut, K., Öcal, D. F., Öztürk, F. H., Öztürk, M., Oğuz, Y., Sınacı, S., & Çağlar, T. (2021). Is GDF-15 level associated with gestational diabetes mellitus and adverse perinatal outcomes? *Taiwanese Journal of Obstetrics and Gynecology*, *60*(2), 221–224. https://doi.org/10.1016/j.tjog.2020.12.004

Yang, L., Chang, C.-C., Sun, Z., Madsen, D., Zhu, H., Padkjær, S. B., Wu, X., Huang, T., Hultman, K., Paulsen, S. J., Wang, J., Bugge, A., Frantzen, J. B., Nørgaard, P., Jeppesen, J. F., Yang, Z., Secher, A., Chen, H., Li, X., … Jørgensen, S. B. (2017). GFRAL is the receptor for GDF15 and is required for the anti-obesity effects of the ligand. *Nature Medicine*, *23*(10), Article 10. https://doi.org/10.1038/nm.4394