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

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**Keywords:**

**Author contribution:** MM, NEH, and DB conceived of experiments. Data were collected by MM, NEH, JRR, and HS. RS provided the experimental animals. MM carried out analysis and wrote the initial draft of the manuscript. All authors contributed to the editing of the manuscript and approved its final format before publication.

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**Journals:**

# Abstract

# Introduction

Growth-like differentiation factor-15 (Gdf15), a Transforming Growth Factor-ß superfamily member and cytokine, was discovered in 1997 and dubbed macrophage-inhibiting cytokine-1 (MIC-1) (Bootcov et al., 1997). Since this time, Gdf15 has been known by many names and is associated with various physiological states, pathologies, and stressors. However, the full extent of the role in GDF15 in health and disease remains to be characterized. Recent work has focused on identifying its usefulness as a biomarker of illness, or as a target for pharmacotherapy.

Average 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 physiological states; such 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).

Much 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 (Patel et al., 2019). Genetic ablation of Gdf15 in mice results in reduction of body weight, fat mass, food intake, and improvement in glucose tolerance (Macia et al., 2012). Furthermore, 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.

What is less explored is the role of Gdf15 in a normal, human or mouse pregnancy. Although Gdf15 is well known to be associated with pregnancy and its related comorbidities, very little has been done to mechanistically understand the role of GDF15 in carrying an infant to term. GDF15 increases across gestation and reaches its highest levels during the third trimester of pregnancy (Moore et al., 2000). It is heavily expressed in the placenta, and secreted into parental circulation, and is also present in the amniotic fluid (Moore et al., 2000). Trophoblasts are thought to be the cells that produce GDF15 in the placenta (Moore et al., 2000).

The role of *Gdf15* in pregnancy is currently unknown. Although previously work has hypothesized that GDF15 and other TGF-ß family members may play a role in trophoblast invasion and implantation of the pregnancy early after conception (Moore et al., 2000). This is supported by evidence showing that 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 (Wang et al., 2020).

Different levels of GDF15 are secreted in concert with complications of pregnancy. Most interestingly, we see the directionality of the associations in pregnancy are not always similar among studies. 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), and no changes (Marjono et al., 2003) in Gdf15 in serum compared to non-preeclamptic, normotensive parents. Diabetes is a common complication in pregnancy and may be pre-existing (type 1 or type 2 diabetes / T1DM or T2DM) or develop during the course of pregnancy (Gestational Diabetes/ GDM). Some studies find that GDF15 is higher in pregnancies complicated by GDM, 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 were related to greater reports of pregnancy related vomiting and diagnosis of hyperemesis gravidarum(Fejzo et al., 2019; Petry et al., 2018). Furthermore, Petry and colleagues found pre-pregnancy BMI was inversely related to GDF15 levels during pregnancy (Petry et al., 2018).

Since the majority of the literature about Gdf15 during pregnancy comes from human trials comparing GDF15 levels between parents with or without certain complications or biological factors, we sought to understand more implicitly what role Gdf15 during the course of pregnancy might play on energy metabolism, insulin sensitivity, and neonatal outcomes. We compared Gdf15 null dams to wildtype dams while evaulatinge weight, food intake, insulin sensitivity, and pup neonatal health and growth to 2 weeks of age. We anticipated that Gdf15 null dams would have high body weights, would consume more food, and would have XXX insulin sensitivity compared to their wildtype counterparts.

# Materials and Methods:

## Animal Husbandry and Protocol

Animals were housed in a temperature and humidity-controlled facility with a 12-hour light:dark cycle. Male and female *Gdf15* null animals were generated by the Seeley lab as detailed 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 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 (pH8.0), 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 utilized 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 was put through gel electrophoresis on a 2% agarose gel at 130V and imaged on a gel documentation system using UV light. 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 confirmed via maternal serum ELISA.

## Mating

*Gdf15* is highly expressed in placental tissue during mouse pregnancy (REF). We chose to study *Gdf15+/+* mated pairs compared to *Gdf15-/-* pairs because comparing littermates of *Gdf15+/-* pairs would result in placental contribution of *Gdf15* to dam serum (**Figure 1A**). Our primary outcome of concern was maternal food intake, for dam serum levels of *Gdf15* to reflect the genetic knockout, we combined homozygous genotype mating pairs, resulting in homogenous genotype progeny and placenta (**Figure 1B**). Adult female mice (GDF15-/-n=8, GDF15+/+n=6), at least 70 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). 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. 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 decanted 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 ITT. 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 (ref). Dams were weighed using an analytical scale to the nearest 0.01 gram 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. Mammary glands were dissected.

## 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 version4.2.0 (R Core Team, 2021) and are represented 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 indentifiers with respect for 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. Effect sizes are reported as the effect of that variable on body weight in grams. 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. P-values <0.05 were considered statistically significant.

# Results

## 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 1A&B**). 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).

As measured by ELISA, Gdf15 levels in serum was reduced by XX% in *Gdf15-/-*dams (FIGURE 2)

*Gdf15-/-*dams consumed 2.18 kcals fewer per day than*Gdf15+/+* counterparts (**Figure 3A**, pgenotype=0.23). This resulted in a 75.52 kcal lower cumulative food intake in *Gdf15-/-*dams during the course of pregnancy (pgenotype=0.031). Despite the difference in cumulative food intake, Gdf15-/- dams had body weights only 0.96 grams greater than Gdf15+/+ counterparts (**Figure 3B**, pgenotype=0.47)

*Gdf15-/-* dams averaged 1.62 grams higher body weights than *Gdf15+/+* dams in the postnatal period, but this failed to reach statistical significance (pgenotype=0.20). During the postnatal period, despite having slightly higher body weights, *Gdf15-/-* dams consumed 1.13 kcal per day fewer than *Gdf15+/+* dams (pgenotype=0.79). This resulted in *Gdf15-/-* dams having cumulative postnatal food intake that was 101.12 kcals lower than *Gdf15+/+*dams (pgenotype=0.011).

## 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 13.46% lower in *Gdf15-/-* dams compared to *Gdf15+/+* dams (**Figure 4B**, pgenotype = 0.20). Linear mixed effect modeling of the experiment revealed a significant and expected effect of time, and an average of 6.37 mg/dL lower glucose values in -/- dams (pgenotype = 0.71). This was confirmed by -/- having comparable areas under the curve, only 5.22% lower than *Gdf15+/+* counterparts (**Figure 4C**, p=0.74). The initial rate of drop of blood glucose was 9.34% lower in *Gdf15-/-* dams compared to *Gdf15+/+* dams, but did not reach statistical significance (**Figure 4D**, p=0.082)

## Gdf15-/- dams have normal rates of pregnancy, gestational age, post-natal survival, and birth weight

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. 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). Pups from *Gdf15+/+* dams were 3.4% larger than those from *Gdf15-/-* dams (**Figure 5C**, p=0.050). 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).

## 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 weigh-suckle-weight test at postnatal day 10. We found no appreciable 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 milkfat using the milk creamatocrit method, expressing the total milk fat as a percentage. We found that milk fat percentage was only 3.1% greater in *Gdf15+/+*milk (**Figure 6C**, p=0.93). Despite reductions in maternal levels of *Gdf15* during pregnancy, mammary gland development, and lactation there is no apparent impact lactational volume or number of calories provided in milk.

## 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 with random intercept effects of unique dam and pup identifiers, a random slope effect of day for each unique pup, and fixed effects of sex, age in days, and genotype. Our model detected no differences (a 16 mg smaller weight in *Gdf15-/-* pups) in body weight between birth and 14 days of age in *Gdf15+/+* and *Gdf15-/-*pups (**Figure 7A**, pgenotype=0.81). The only significant effect in the model was that of age in days, where each postnatal day averaged an additional 0.42-gram increase from birthweight (page<0.0001). There was no statistically significant effect of sex on body weight from birth to PND 14.5 (psex=0.26).

# Conclusion

Despite the well-known, multi-fold rise in *Gdf15* during mouse and human pregnancy, we found no evidence that *Gdf15* knockout 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 and were therefore indistinguishable from age-matched *Gdf15+/+* pups.

# Discussion

* GDF15 is known to be elevated/reduced in complications of pregnancy.
* Similar to KO models in mice, we find X
* Dissimilar to other KO models in mice, we find Y
  + No increases in bw, food intake, or alterations in glucose/insulin tolerance (
* Because we see GDF15 high in pregnancy and in DM pregnancy, it makes sense that we don’t see statistically sig differences in glycemia makes sense.
* Limitations
  + Std err from MLM, plug that back into to power = did not detect and effect but cannot rule out an effect of X size. (FI or BW in pregnancy)

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