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Jared Druss January 30, 2023

Grip Strength in GALT deficiency

by

Jared Druss

Dr. Judith Fridovich-Keil
Adviser

Biology

Dr. Judith Fridovich-Keil

Adviser

Dr. Leila Rieder

Committee Member

Dr. David Gorkin

Committee Member

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Jared Druss

Dr. Judith Fridovich-Keil

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

Grip Strength in GALT deficiency
By Jared Druss

Classic galactosemia (CG) is a rare autosomal recessive metabolic disorder resulting from deficiency of the enzyme galactose-1-phosphate uridylyltransferase (GALT), the second enzyme in the Leloir pathway of galactose metabolism. Despite early detection by newborn screening and lifelong dietary restriction of galactose, this deficiency results in potentially numerous complications, including cataracts, cognitive challenges, a growth delay, and a grip strength deficit, among others. Here we provide further evidence of a grip strength deficit, both in patients with CG and a GALT-null rat model of CG. Additionally, we demonstrate that, rather than representing an independent phenotype, the grip strength deficit that galactosemia patients and GALT-null rats experience can be accounted for by the growth delay observed in both cohorts.

Grip Strength in GALT deficiency

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Chapter 1: Introduction

Classic galactosemia is an autosomal recessive disorder caused by a deficiency of galactose-1-phosphate uridylyltransferase (GALT), the second enzyme in the Leloir pathway of galactose metabolism, pictured in Figure 1. This disorder affects approximately one in fifty thousand births and normally arises as a result of common hereditary variants in the *GALT* gene, such as Q188R, K285N, and S135L, as well as over three hundred other variants, ranging from missense mutations to indels and others[1, 2, 3]. Variants are found at different prevalence in different populations: for example, S135L typically affects Africans and those of African ancestry, whereas Q188R typically affects those of Northern European ancestry[2].

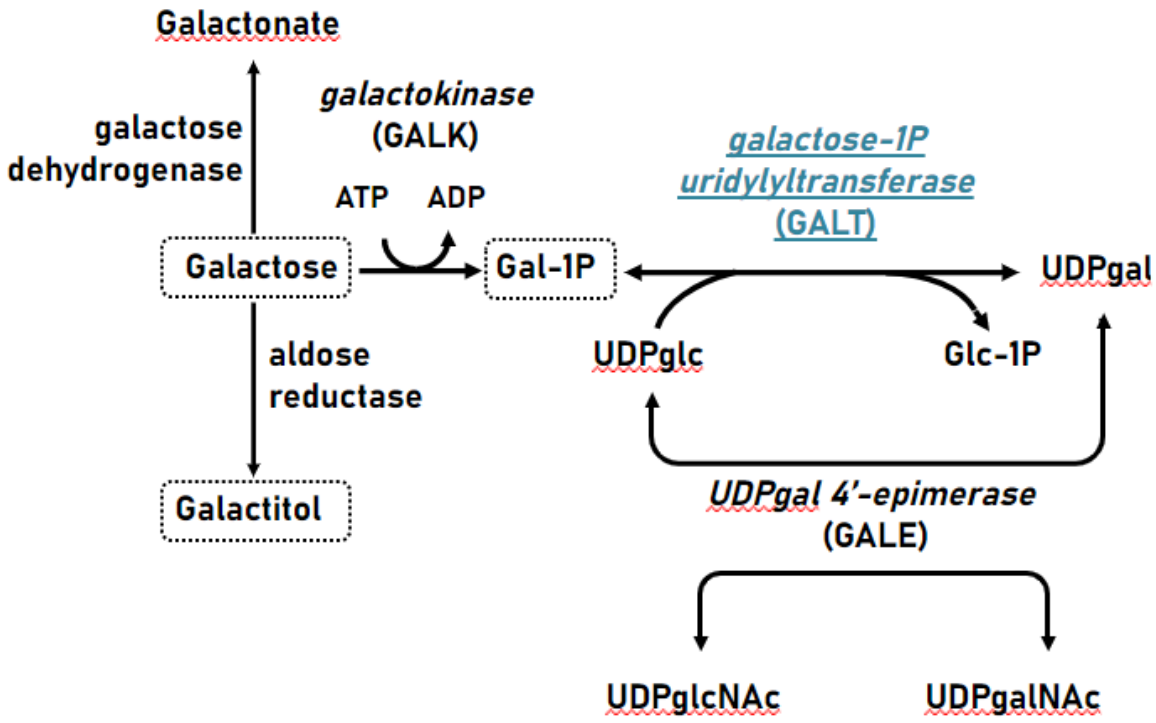

Figure 1: The Leloir pathway, adapted from Rasmussen et al. 2020.[8]

The catalytic mechanism of GALT involves transferring a Uridine Monophosphate (UMP) group from UDP-glucose to galactose-1-phosphate, forming UDP-galactose, which sets up for the final step of the Leloir pathway, the epimerization of UDP-Galactose to UDP-Glucose. The absence of GALT activity leads to an inability to efficiently metabolize galactose, which results in a buildup of metabolites including galactose, galactitol, galactose-1-phosphate, and in some tissues, galactonate[2].

For infants exposed to breast milk or a dairy milk-based formula, which contains high levels of galactose, classic galactosemia results in a sudden and severe onset of acute symptoms, starting with jaundice and feeding problems, and potentially leading to neonatal death[4]. For those who are diagnosed early with the condition and are changed to a diet restricted from high-galactose products such as milk, these severe symptoms may be avoided or mitigated, but there are numerous lifelong complications associated with CG that nonetheless occur, including cognitive challenges, a growth delay, and speech and motor difficulties, among others[4, 5, 6].

One less studied phenotype, diminished grip strength, was reported by Potter et al. in 2013. The study was conducted on a cohort of galactosemia patients with a history of treatment for speech difficulties. The study addressed the higher incidences of speech errors and coordination within this cohort through measures such as frequency of articulation errors, balance scores and manual dexterity scores. However, Dr. Potter and colleagues also noted that in both boys and men, as well as girls and women with CG, there was a noticeable deficit in grip strength for affected individuals. For boys and men, galactosemia patients appeared stronger at earlier ages tested, but over time unaffected (control) individuals' strength overtook that of cases.

Meanwhile, in girls and women, there was a relatively constant deficit in grip strength across ages for individuals with galactosemia relative to controls[7].

Another model of interest that may help characterize this phenotype is our GALT-null rat model. The M3 allele, a deficient form of the rat *Galt* gene, resulted from non-homologous end joining following a CRISPR-Cas9-mediated double stranded cut at the locus encoding the active site of the enzyme. This ultimately created a 2-base pair insertion in the sixth exon of the rat *Galt* gene that created a premature stop codon knocking out the gene, resulting in rats mimicking GALT deficiency in humans. Many of the phenotypes of classic galactosemia replicate in the Fridovich-Keil lab's GALT-null rat model of classic galactosemia[8]. First, there are heightened levels of galactose, galactitol and Gal-1P in these rats. They display striking bilateral cataracts from a young age that continue into adulthood. They additionally suffer deficits in motor and cognitive function, as characterized by the Rotarod test and the Morris Water Maze test. There is also an observed mild growth delay mirroring what has been seen in patients, which at least in female rats was shown to largely resolve itself by adulthood. Also, as in patients, a diet with a restriction of galactose lowers GALT-null rats' metabolite levels, although they remain elevated relative to wild-type rats[8]. This model has been used to assess the efficacy of novel gene-therapy-based treatments such as AAV9-h*GALT*[9] and scAAV9-h*GALT*[10], both of which have been shown to effectively mitigate at least some symptoms of GALT-deficiency in the rat model.

Beyond its original description, the grip strength phenotype has not since been addressed in other studies. Particularly, the mechanisms underlying this phenotype remain in question. A better understanding of how the grip strength deficit relates to other outcomes of galactosemia

could further a better understanding of the condition as a whole. Also, this phenotype has not previously been explored in any animal models of classic galactosemia. Characterizing the effects of GALT deficiency on grip strength in rats would potentially allow for another read out to test the efficacy of therapies in the rat model and would also provide a context to explore factors causing the grip strength deficit in this condition.

One particular phenotype of interest is the growth delay. As discussed prior, this is a manifestation of GALT deficiency both in rats and humans. Furthermore, weight is heavily associated with grip strength, with many studies of rat grip strength listing their results in terms of total grip strength, as well as a rat's grip strength on a per-gram of body weight basis[11, 12]. Similarly, the strong effects of weight on grip strength are well-documented in studies of grip strength in humans[13, 14].

Here, we characterize the grip strength phenotype in both humans (aged 4-16), and in rats. We measure grip strength for patients and controls using repeated measures of the Jamar Hand Dynamometer on each hand separately, while we determine rats' grip strength using repeated measures of the Columbus Grip Strength Meter with front paws only or repeated measurements using a custom device with all 4 paws. Furthermore, we take weight measurements of all study participants, human and rat, to determine whether size might be an explanatory, or at least associated, variable for any grip strength differences observed.

First, we address whether the patients and controls in our study replicate the grip strength phenotype first described by Potter and her colleagues[7]. Then, in our GALT-null rat model, we

analyze the growth delay: whether it is prevalent at early ages, as well as whether it continues on to later time points. Finally, we test for evidence of a grip strength deficit in our GALT-null rats, asking the same questions of whether there is a phenotype, the best way to characterize this phenotype, and whether rat weight might associate with this deficit.

Chapter 2: Grip Strength in Patients with Classic Galactosemia

Introduction:

We first address the grip strength phenotype in humans. To do so, we first determine whether a grip strength phenotype exists between individuals with galactosemia and unaffected people. We then explore whether galactosemia patients' lesser weight is associated with potential deficits in grip strength.

Methods:

Both cases and controls were recruited from among participants at the 2022 Galactosemia Foundation Conference in Orlando, Florida. Galactosemia patients and their families attended this conference, and unaffected family members of those with CG often acted as controls. We excluded any people above the age of 19 tested due to a paucity of data for that age group and additionally excluded people weighing above the 95th percentile of weight for age, as defined by the CDC clinical growth charts[15]. This is because when we consider weight as a modifier of grip strength, weight stored as fat does not contribute to a person's grip strength. This would mean that we would be accounting for weight excessively for larger individuals with larger body fat percentages, potentially skewing results. While no individuals were excluded from this dataset on the basis of weight, there were individuals excluded from Dr. Potter's dataset in Chapter 3 for that reason.

Individuals in this study are described as boys and men and girls and women. As discussed above, we are not including individuals above the age of 19. Regardless, we describe them as such given the genetics nomenclature of defining of defining those past the age of puberty as men and women.

We also do not distinguish between sibling controls who are carriers and non-carriers for galactosemia. We do not have this information since most healthcare providers do not support carrier testing prior to legal adulthood, given that children cannot consent to such a test.[16] Nonetheless, given that this is an autosomal recessive disorder, we are confident that distinguishing carriers from non-carriers among controls is unnecessary.

Table 1 describes summary statistics of the study sample after excluding individuals based on these criteria.

Table 1: Demographic characteristics of participants in this study.

Category	Cases (n=38)	Controls (n=19)
Sex Female Male	 22 (61.1%) 14 (38.9%)	 10 (52.6%) 9 (47.4%)
Mean age ± SD (age range in years)	10.63 ± 4.30 (4 to 18)	10.98 ± 4.25 (4 to 17)

Test Administration:

Participants were tested using the Jamar Hand Dynamometer, shown in Figure 2. All participants were given the same instructions prior to completing their grip strength testing. Specifically, they were instructed to place their feet flat on the ground, with their upper leg

(thigh) facing straight towards the wall in front of them, and their lower leg pointing straight towards the ground. Then, they needed to hold their arm in a perpendicular position, with their upper arm facing straight up to the ceiling and their forearm pointing straight toward the wall, as shown in Figure 3. While they positioned themselves, I adjusted the handle of the grip strength meter either outward or inward to accommodate the size of the individual's hand. I then handed them the grip strength meter in their right hand so that the display, as well as the protruding metal pieces, faced away from them, as shown in Figure 4.

The subjects were then instructed to squeeze the grip strength meter as hard as possible for 3 seconds and then release, with their maximum force achieved being measured in pounds of force. This same process was repeated for an individual's left hand. Then, after 15 seconds of rest, this process was repeated for the person's right and left hands again. Once both hands had been tested three times each, the test was completed for that participant. Of the six grip strength tests, only the peak grip strength value was used for graphing and statistics purposes. This better accords with multiple studies which, rather than averaging participants' grip strength values, takes the peak as their grip strength[7, 13].

In addition to grip strength testing, all subjects tested were weighed using a digital scale.

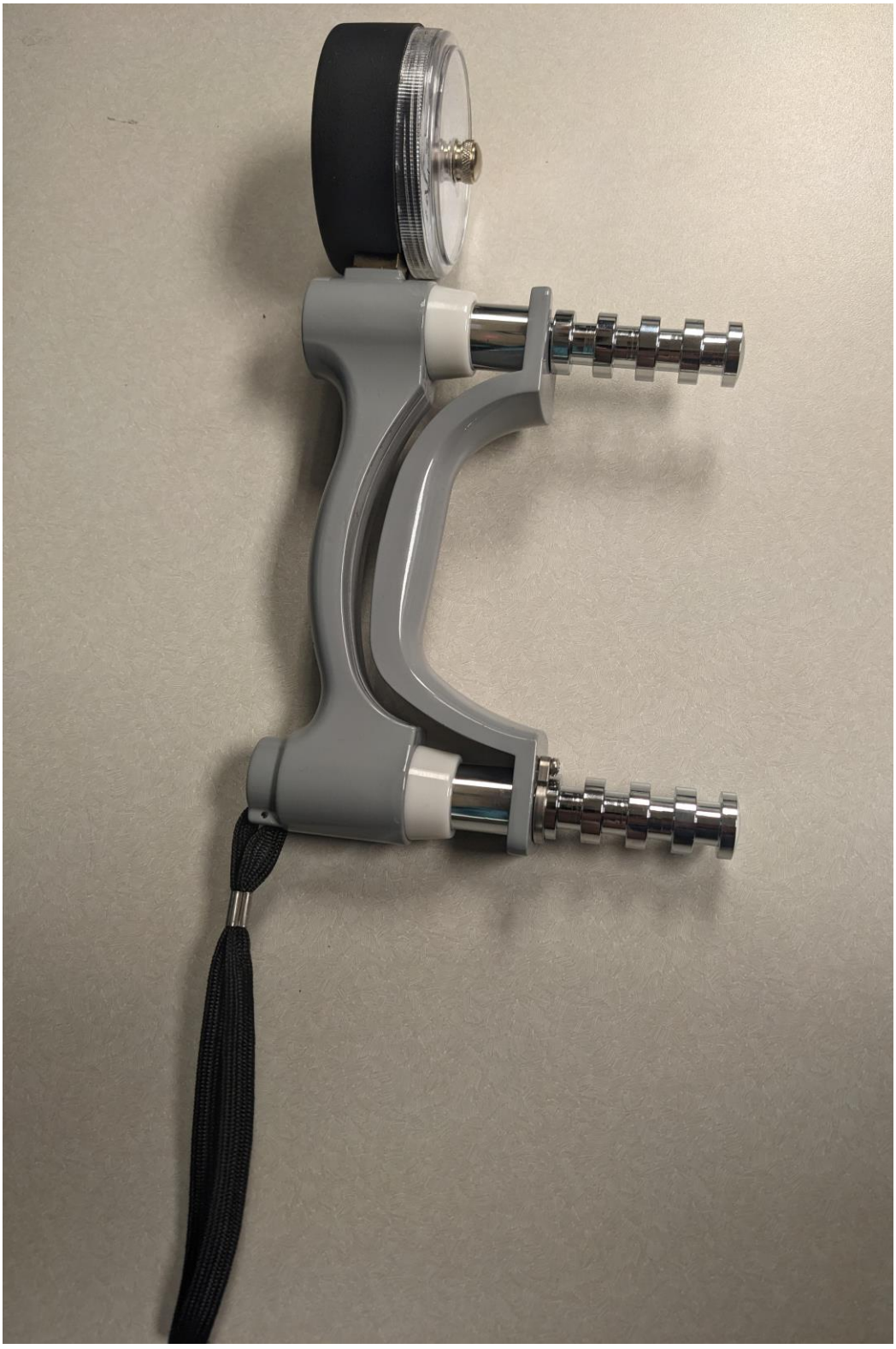

Figure 2: Jamar Hand Dynamometer.

Figure 3: Proper Positioning for Human Grip Strength Testing.

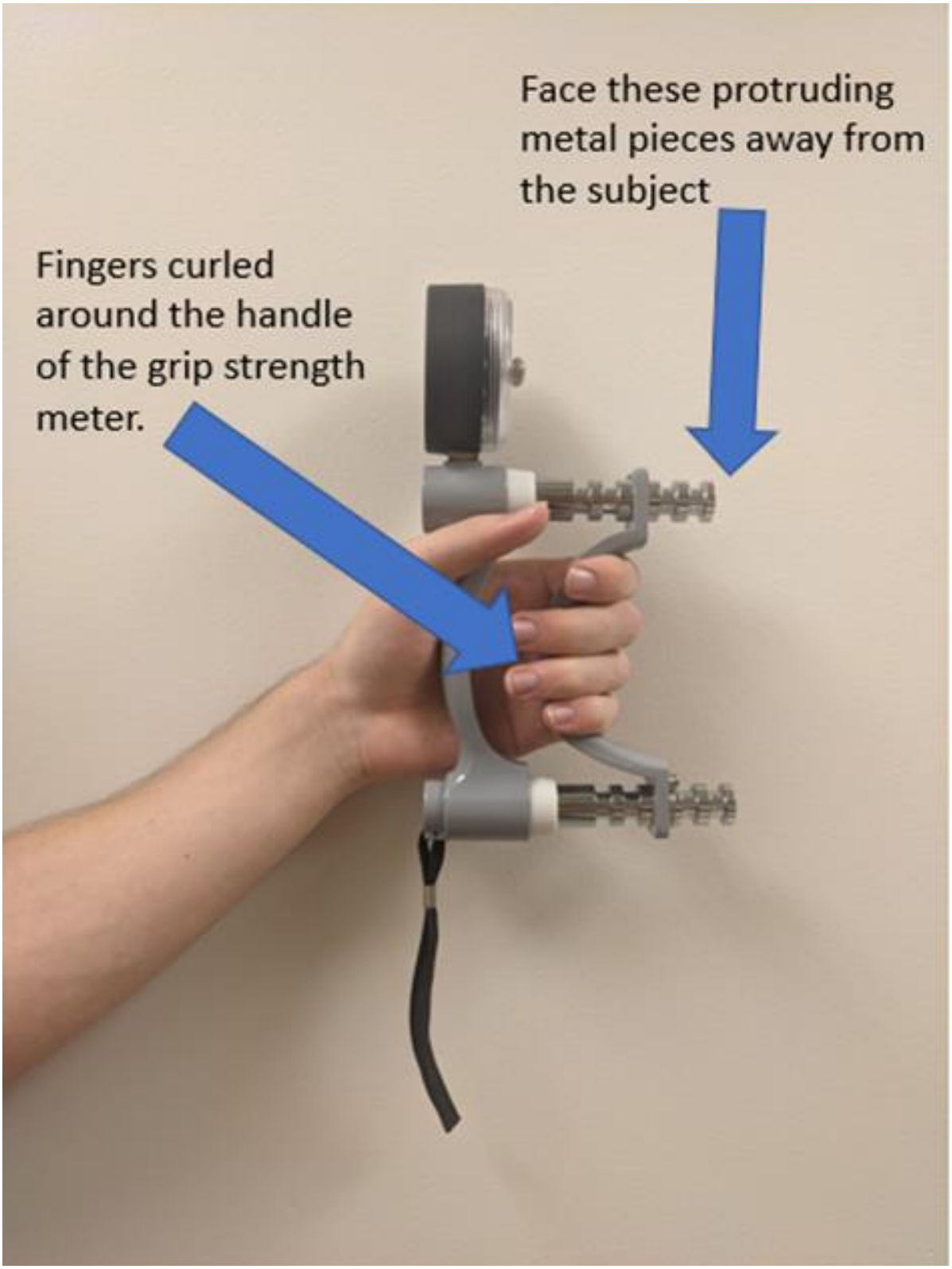

Figure 4: Hand Position for Grip Strength Testing

Graphing and Statistical Methods

For our graphs, we created a scatterplot with age on the x-axis and an individual's peak grip strength value on the y-axis. We added lines of best fit to assess how grip strength changes with age in our cross-sectional cohort of cases and controls. Additionally, we created shaded 95% confidence intervals surrounding the lines of best fit to better visualize whether the results may be statistically significant. When graphing the association between grip strength and weight, the same graph described above was created, but with an individual's weight, rather than age, graphed on the x-axis.

For all subjects, the relationship between grip strength and galactosemia was measured using a linear mixed effects model. A linear mixed effects model was used because of its ability to take into account covariates that could potentially affect the data. Since humans tested were widely distributed in age and weight, the model would be necessary to account for the effects of these variables and isolate the effects of whether someone was a case or control. Genotype and age were considered fixed variables, while a patient's study ID code was considered a random variable in this model.

When assessing whether differences in weight are associated with the grip strength differences seen in patients with Classic Galactosemia versus controls, we ran the same linear mixed effects model as before but replaced Age with Weight for our fixed variable.

Results:

Boys and men significantly differ in peak grip strength between cases and controls (Figure 5, linear mixed effects model, p=0.0071). As is visible in Figure 5 below, there appears to be a divergence in grip strength performance with age, where controls grow stronger more quickly than cases do.

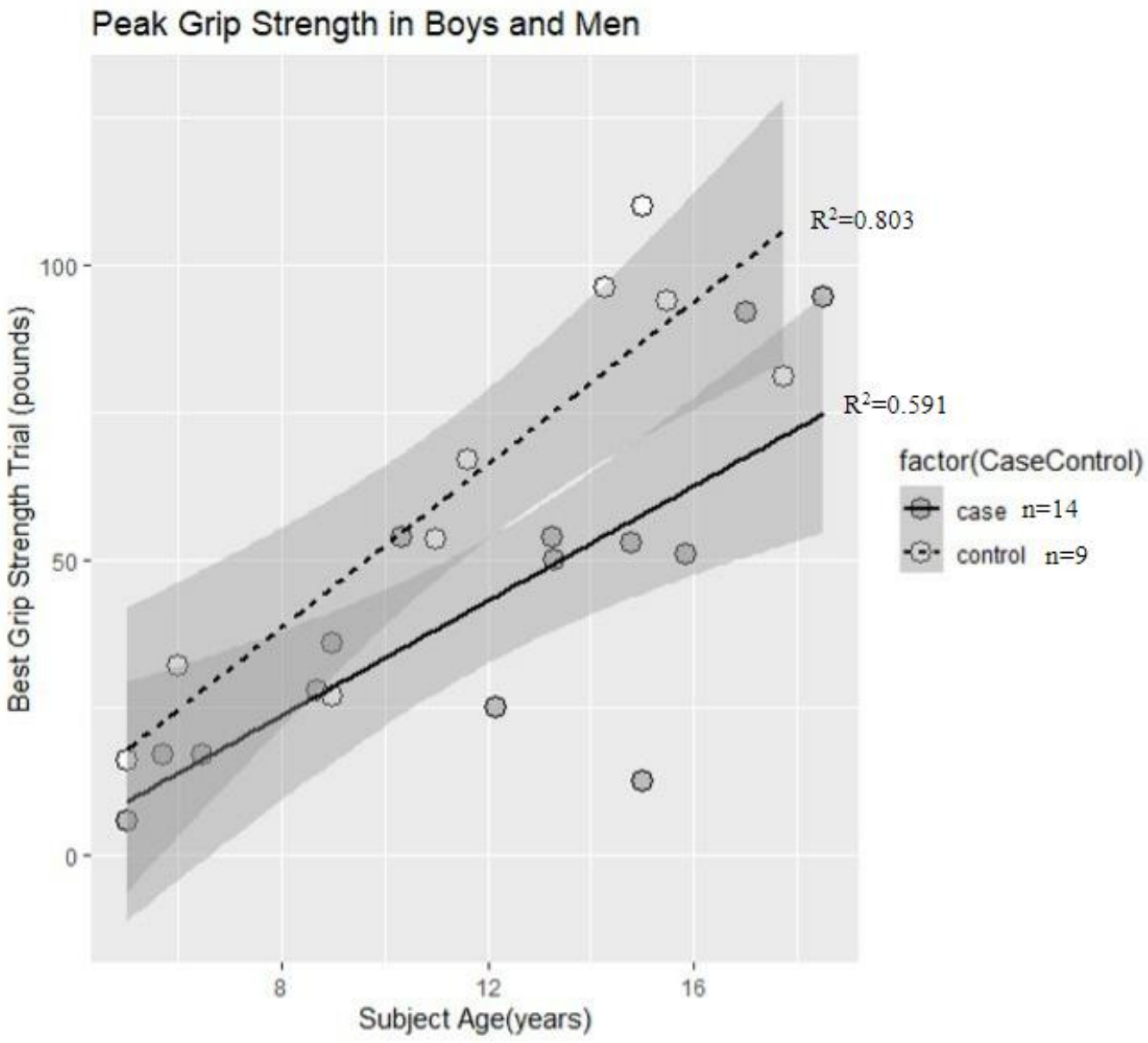

Figure 5: Grip Strength of Boys and Men. The dotted and solid lines represent the lines of best fit of the controls and cases respectively, while the shaded areas represent 95% confidence intervals for the grip strength of cases and controls. Each participant's peak grip strength (both hands) is graphed on the y-axis, while their age is on the x-axis.

Similarly, girls and women significantly differ in total grip strength as a function of case-control status (Figure 6, linear mixed effects model, p=0.0134). As with boys and men, genotypic differences appear to become more prominent over time. However, at no point do the 95% confidence intervals on the graph diverge. This indicates that there may be less of a difference in girls and women than in boys and men.

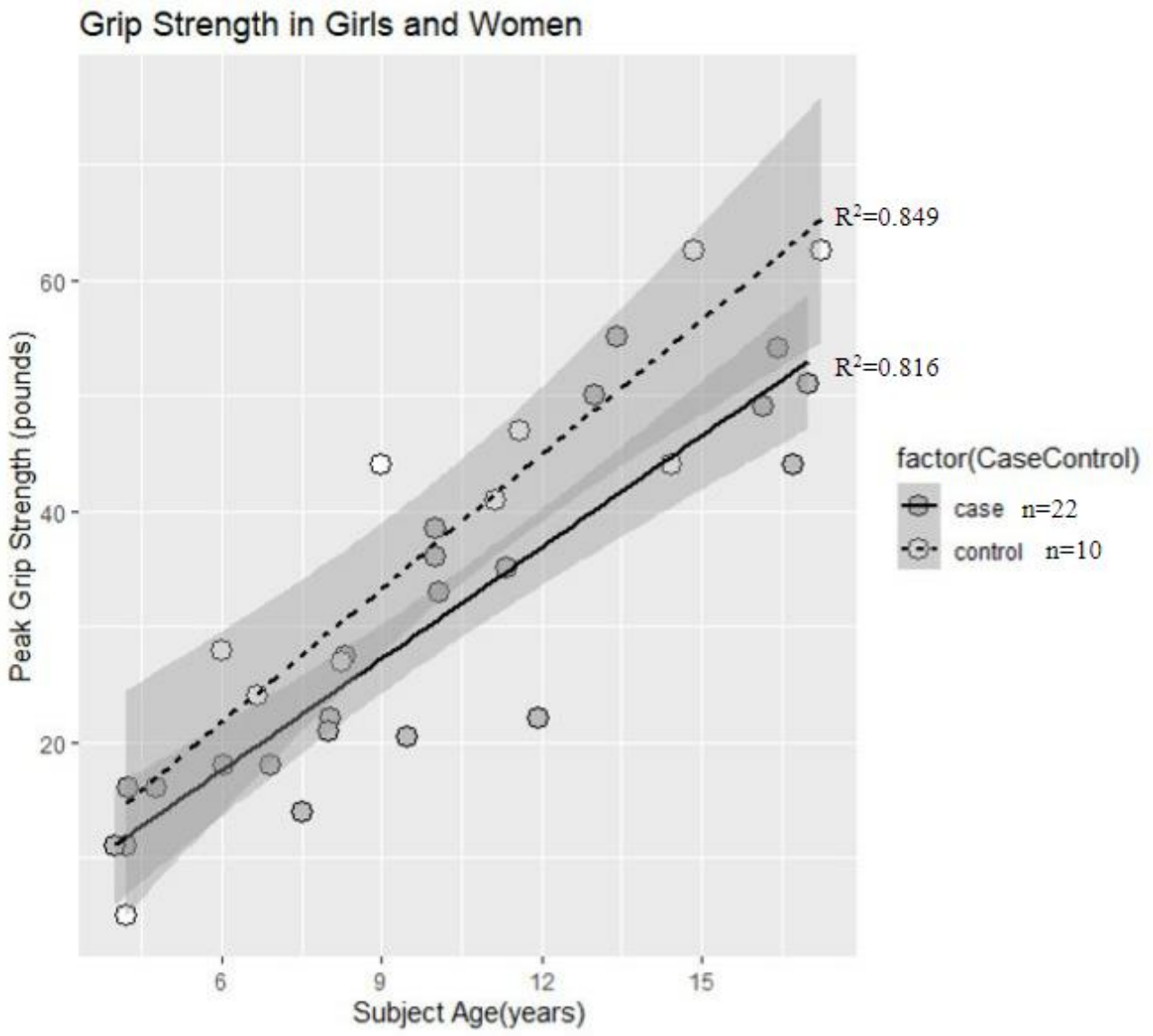

Figure 6: Grip Strength of Girls and Women. The graphing used is the same as described for Figure 5.

Differences in weight between cases and controls appear to associate heavily with differences in grip strength. When weight was considered by the linear mixed effects model, our p-value in boys and men increased to a statistically insignificant value (linear mixed effects model, p=0.1684). As can be seen by the graph below, though the controls participants' line of best fit is higher than the cases' at every point on the graph, the shaded 95% confidence intervals for cases and controls also overlap quite a bit along the entire length of the graph. This indicates that on a per-pound basis, controls do not differ significantly from cases in grip strength.

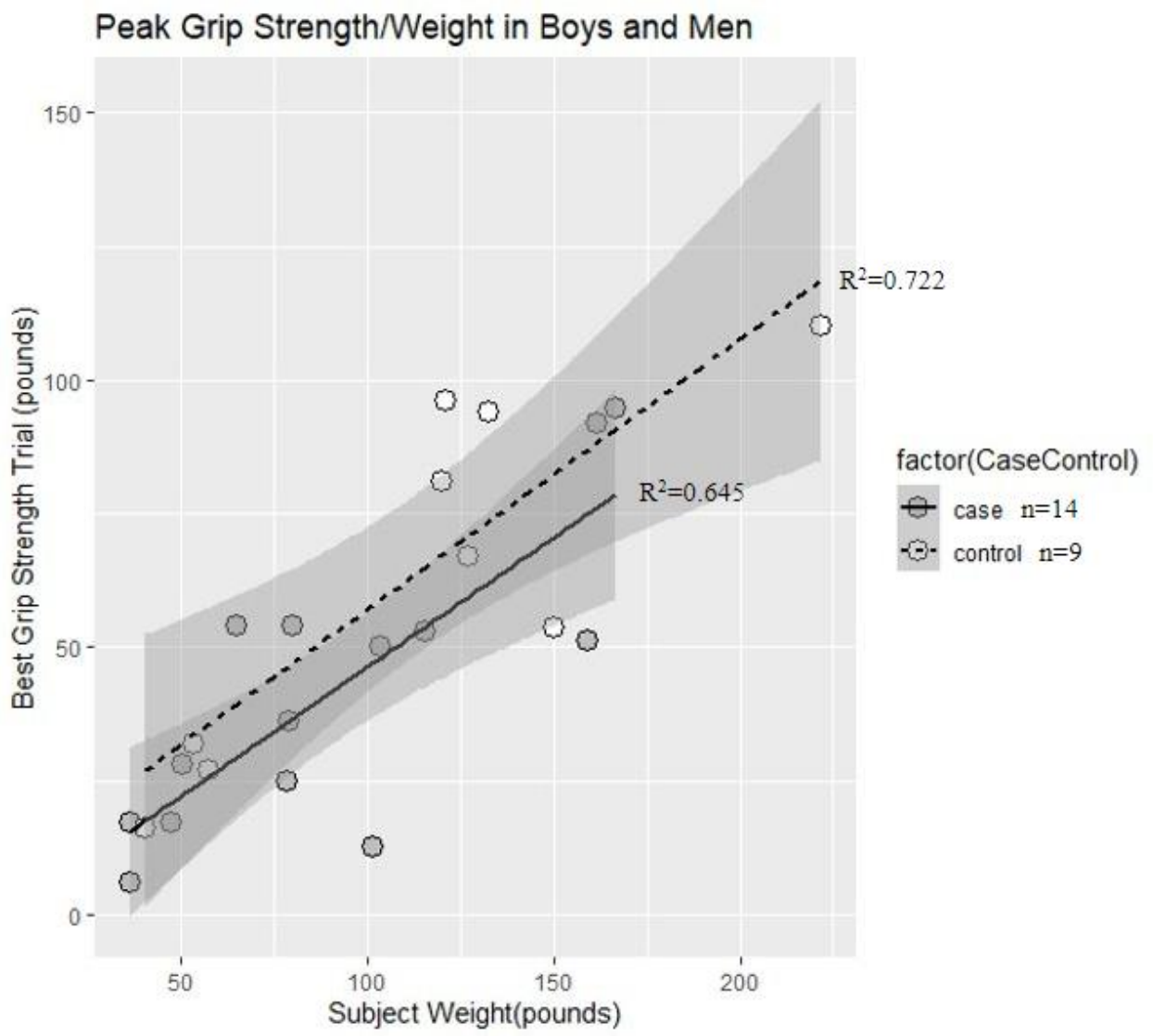

Figure 7: Grip Strength of Boys and Men, adjusted for participant weight. The graphing method used is the same as described in Figure 5, except that an individual's weight, not age, is graphed on the x-axis.

Similarly, accounting for weight in girls and women reduces genotypic differences in grip strength to a statistically insignificant level (linear mixed effects model, p=0.5197). In the graph below, not only do the confidence intervals surrounding the lines of best fit for cases and

controls overlap at every point on the graph, but the lines of best fit themselves overlap on the graph, with cases appearing to surpass controls in their per-pound grip strength.

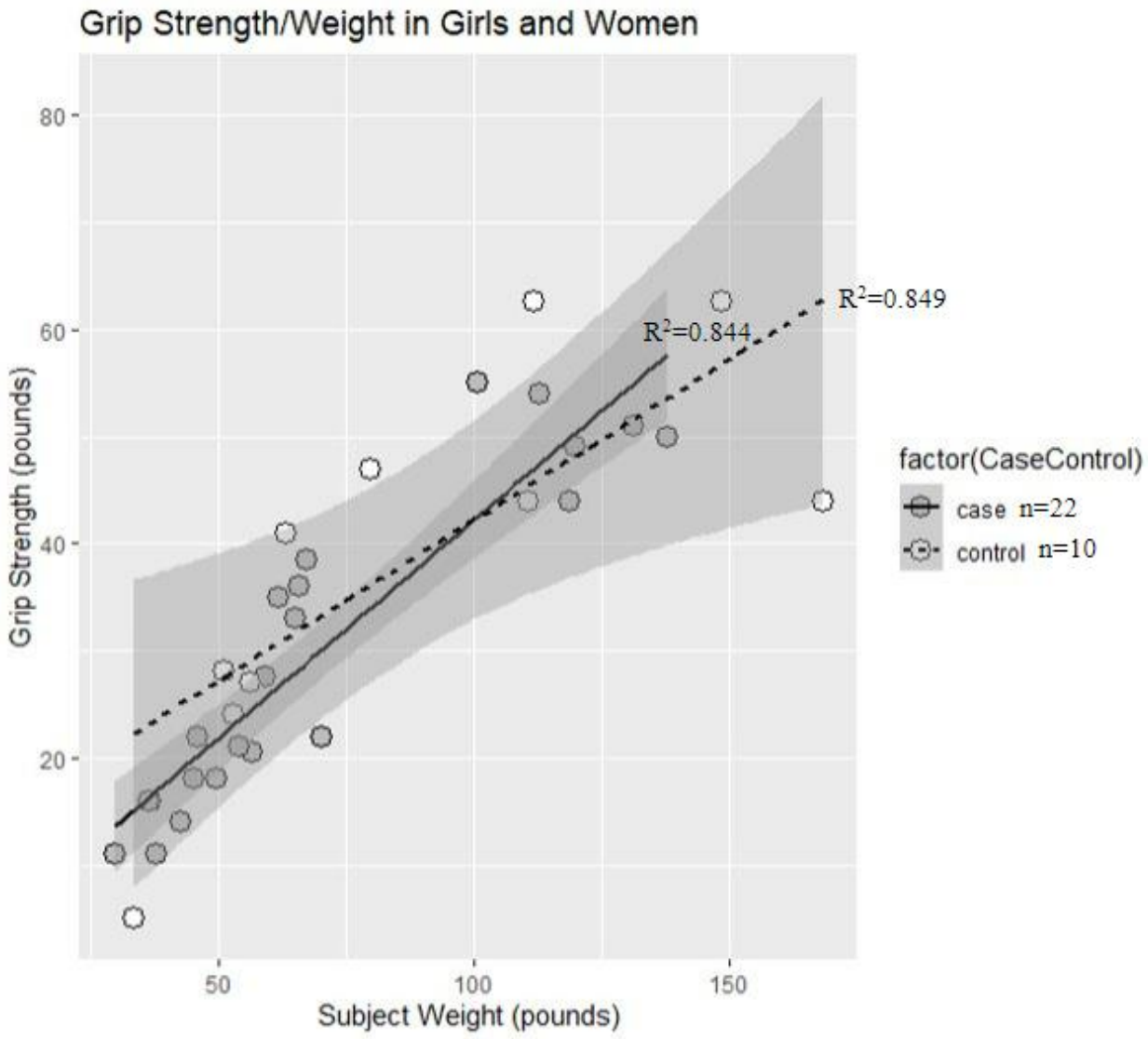

Figure 8: Grip Strength of Girls and Women, adjusted for participant weight. The graphing style here is the same as described in Figure 7.

Key findings:

The most important finding in this part of our study is first that we were able to recapitulate the grip strength phenotype shown by Dr. Nancy Potter, showing that a cohort of young people with galactosemia have significantly lesser grip strength than controls. Furthermore, it appears that when an individual's weight is taken into account, the grip strength deficit largely vanishes. The implications of these findings will be further discussed in Chapter 3, which compares the results of Potter et al. 2013 to our data collected in 2022. Additionally, it is shown in Appendix A, on page 60, that the results of our study do not differ in terms of significance when we take study participants' average grip strength versus their peak grip strength.

Limitations:

Our conclusions are limited first by the fact that this study is cross-sectional. Because data are being collected on individuals at a singular time point, our ability to make conclusions about how patients with CG develop over time versus controls is limited. Furthermore, our sample is of limited size, particularly among controls, limiting our ability to effectively define the extent and parameters of the grip strength phenotype. Our cohort is also limited by a lack of older participants. The Galactosemia Foundation Conference typically consists mostly of young children and their parents; few adult patients, particularly older adults, are present. This means that while we can discuss a grip strength phenotype among young people with galactosemia, we cannot extend this discussion into later adulthood.

Chapter 3: Results from Dr. Nancy Potter's 2013 Study:

Introduction:

In this chapter, we will compare our results collected in 2022 with human subjects to those collected by Dr. Potter more than 10 years ago. To do this, we must first acknowledge differences in our study cohorts and approach to data gathering. In Dr. Potter's study, data were collected using the Iowa Oral Performance instrument pictured in Figure 8, the same device used to collect tongue strength information. Data were collected by placing the air-and-silicone-filled hand bulb not shown in this figure at the center of a person's palm and asking them to put their fingers around the bulb and squeeze as hard as possible for a length between 2-3 seconds. Trial frequency and speed were almost the same as in our study, with 3 trials performed on both dominant and nondominant hands, with 30 seconds of rest instead of 15 between trials. Instead of patients' strength being measured in pounds, it was measured in units of pressure, specifically kilopascals.

Data Analysis and Graphing:

Potter et al. 2013 graphed males and females separately, and graphed age in months on the x-axis, and peak non-dominant hand strength on the y-axis. Similarly to our graphs, separate lines of best fit were made for cases and controls to indicate the increase in grip strength with age of young cases and controls.

Figure 9: Iowa Oral Performance Instrument used in data collection, courtesy of IOPI Medical

Methods:

To compare our data directly to Dr. Potter's data, we reached out to her and received a spreadsheet consisting of all the data collected during her original study. While her data have already been processed and analyzed in her paper, we want to analyze her data in a manner similar to our data. To make these data as comparable as possible to ours, we only used data from patients who had been both weighed and grip strength tested. Furthermore, for comparability of controls and cases, we only included ages where we had data for both groups. Also, we excluded 18 individuals above the 95th percentile of weight for the same reason as described in Chapter 2. Finally, we eliminated controls significantly lying outside the age range of cases. Table 2 describes a summary of the remaining data.

Table 2: Demographic characteristics of participants in Dr. Potter's study.

Category	Cases (n=18)	Controls (n=92)
Sex Female Male	 7 (38.9%) 11 (61.1%)	 36 (39.1%) 56 (60.9%)
Mean age ± SD (age range in years)	9.14 ± 3.31 (4 to 17)	10.14 ± 3.41 (4 to 17)

The main differences between our study cohort and Dr. Potter's are as follows: First, she has far more controls than we do, but fewer cases. Her controls consisted of any children unaffected by galactosemia, whereas our cohort of controls were siblings of patients with

galactosemia that we tested. So, despite our lower number of controls, our controls are better matched by many demographic markers to the cases in our study.

Beyond this fundamental difference in sampling, the mean ages of cases and controls in both our studies are relatively similar. Though there are some differences in sex distribution between our cohort and Dr. Potter's, both cohorts are relatively balanced overall.

Statistics and Graphing:

We graphed and re-analyzed Dr. Potter's data in the same manner as we did our own, showing individuals' peak overall grip strength value. This is in contrast to her original graphs, where she recorded individuals' maximum hand grip strength only for their non-dominant hand. We used a linear mixed effects model just as we did in our own data for the same reasons outlined in the chapter above.

Grip Strength Results:

While Dr. Potter previously reported that her data indicate a deficit in grip strength for cases relative to controls, we performed a more thorough comparison of the results. As described in Potter et al. 2013, there is a significant difference between cases and controls ($p=0.0002$, linear mixed effects model). In the grip strength of boys and men, Dr. Potter's data indicate that at an early age, there is virtually no difference between cases and controls, whereas, at later ages, the difference widens heavily, as shown in Figure 10. This appears to largely be a result of cases

of ages 5 to 13 giving relatively constant grip strength numbers, resulting in almost no increase in grip strength over time in this age window.

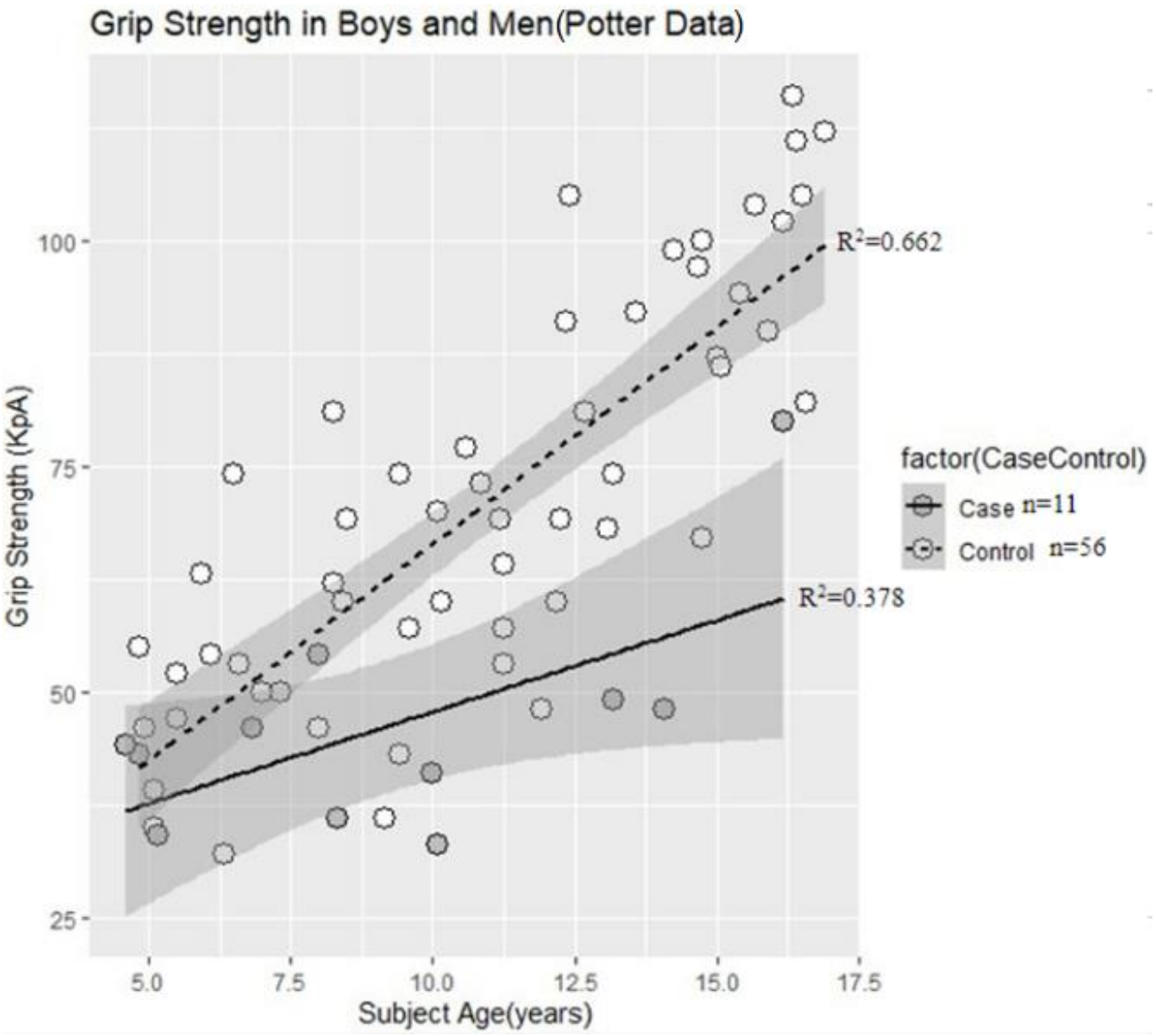

Figure 10: Grip Strength of Boys and Men, graphed from the data used in Dr. Nancy Potter's 2013 study of Classic Galactosemia patients. Graphing techniques are the same as described in Figure 5.

While our data indicate a divergence over time between cases and controls in grip strength, it is not nearly so stark; Cases in our cohort, but not Dr. Potter's, still appear to gain additional grip strength over time, just perhaps less than controls (although both studies are cross-sectional, so no definitive conclusions can be drawn about this).

For girls and women, Dr. Potter's data exhibit relatively similar trends to ours. There is a deficit in grip strength for cases ($p=0.0071$, linear mixed effects model) that is relatively constant over time, as both cases and controls increase in grip strength similarly over time, as seen in Figure 11. However, there is no clear divergence between the cases' and controls' confidence intervals at any time point. This is likely a result of the low number of cases, and the high level of variability.

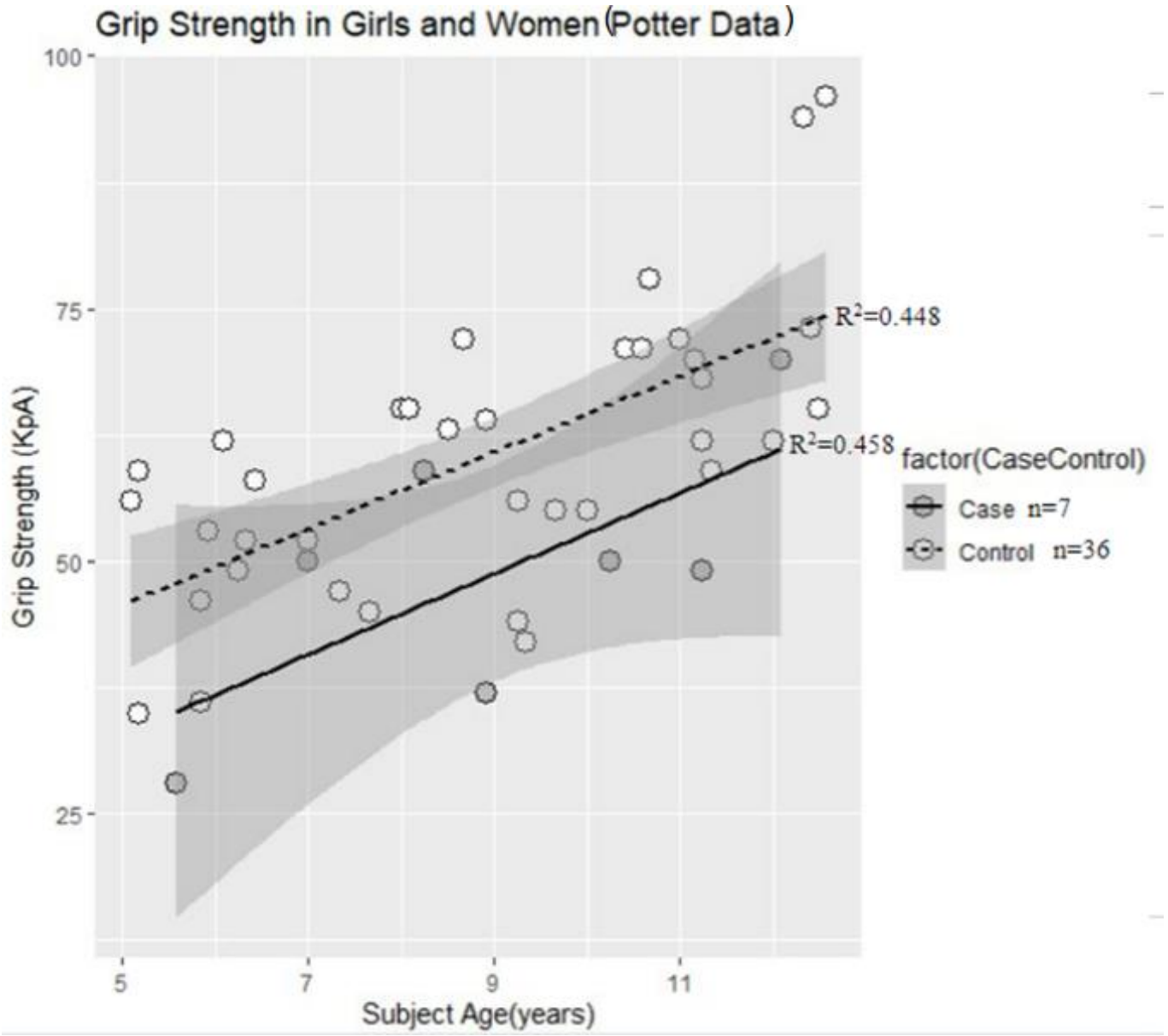

Figure 11: Grip Strength of Girls, graphed from the data used in Dr. Nancy Potter's 2013 study of Classic Galactosemia patients. Graphing techniques are the same as described in Figure 5.

Grip Strength vs Mass Results:

Unlike in our data, weight does not appear to account for much of the grip strength deficit in boys and men. While graphing weight rather than age on the x-axis indicates that cases in Dr. Potter's study increase in grip strength more so based on weight increases, rather than age, cases and controls are still clearly divergent from one another in terms of grip strength ($p=0.0031$, linear mixed effects model).

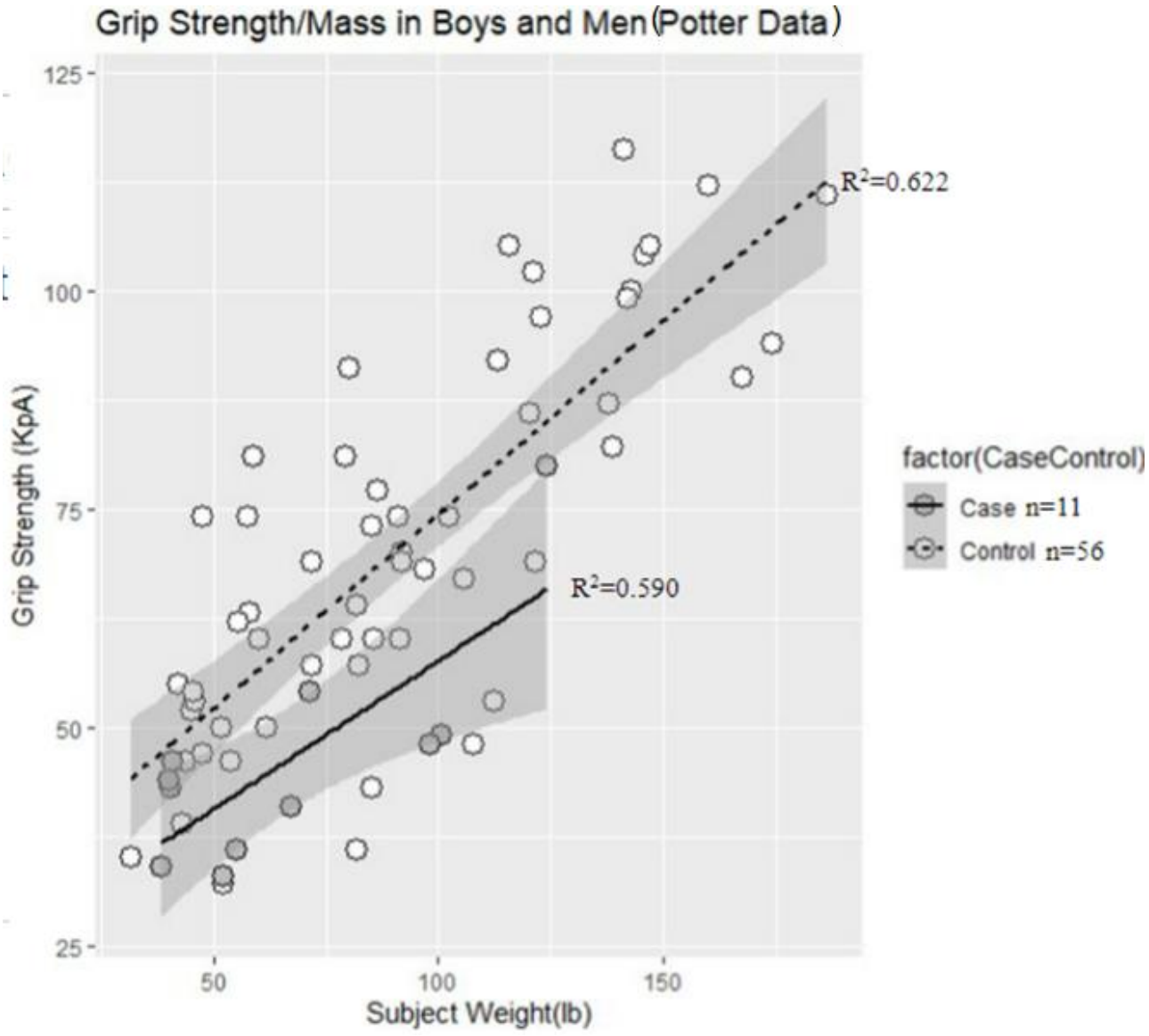

Figure 12: Grip Strength of Boys and Men, adjusted for weight. The graphing method used is the same as described in Figure 7.

However, for girls and women, weight appears to be more heavily associated with the grip strength deficit. As is visible, Dr. Potter's data show not only the 95% confidence intervals overlapping, but at one point the lines of best fit overlapping for cases and controls when weight

is graphed on the x-axis, something that we also see in our data. When we perform a statistical analysis, we see insufficient evidence of a genotypic difference in grip strength when mass is accounted for (p=0.1868, linear mixed effects model).

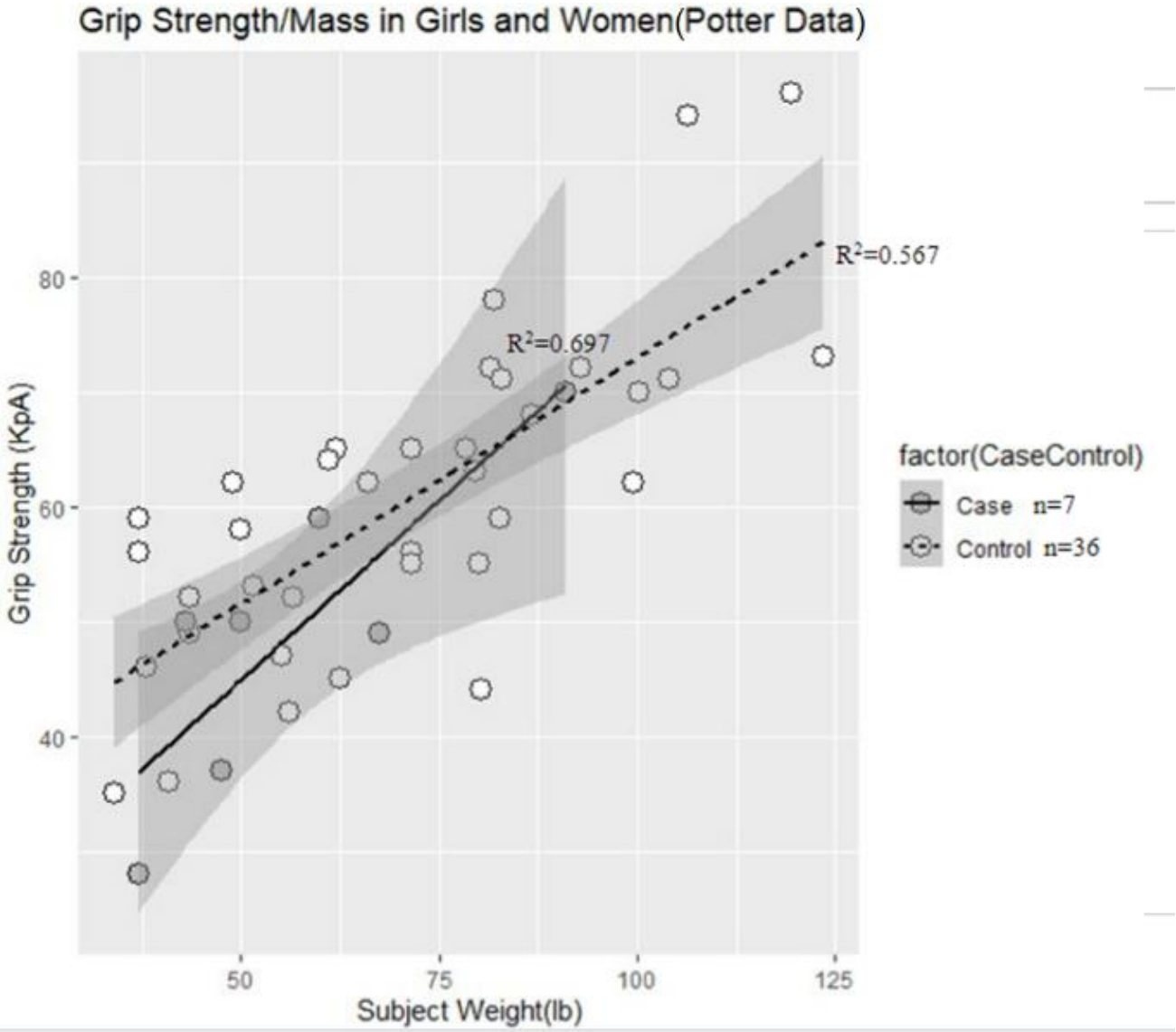

Figure 13: Grip Strength of Girls, adjusted for weight. The graphing method used is the same as described for Figure 7.

Main findings:

The grip strength findings we have are generally consistent with those previously reported by Potter et al. 2013, with a few differences. First, the slope for how grip strength develops over time in boys and men differs between our studies. Potter et al.'s data suggest only a small increase in grip strength over time for boys and men with CG. In contrast, our data suggest that while male cases develop in grip strength more slowly than controls, they still experience a relatively large increase in grip strength over time. This disparity may be due to three factors: First, for our comparison between our data and those of Dr. Potter, we only included participants for whom both weight and grip strength were known, leaving a very small number of cases in a less extensive age range than in our study. This may result in a few cases who perform less well at later ages skewing the data.

Second, our data include boys and men of a wider age range than Potter et al. While her number of study participants who are young men is far smaller than younger boys, our participants are spread more evenly across ages.

Third, because Dr. Potter's study focused on individuals with galactosemia who had motor and speech difficulties, her data included only galactosemia patients who had been treated in the past for speech disorders. While speech difficulties are more common among patients with galactosemia than in the general population, this outcome is not universal, and there are many cases with mild or no speech problems who do not require treatment. Dr. Potter additionally selected controls who had normal articulation levels and performed academically at their grade

level. Most controls fall within this range, but not everyone does, so a certain subset of the control population was excluded. Both these criteria potentially selected for cases with more severe phenotypic outcomes and controls who performed better relative to the cases.

Also, in the boys and men in Dr. Potter's study, accounting for weight appears to take away less of the grip strength deficit than in our study. There is still a clear difference between the cases and controls. This, too, may be a result of the low numbers of cases we are left with when we pare Dr. Potter's data down due to missing weights, which may result in a less representative sample.

Both studies, however, show an apparent trend of a lesser increase in grip strength over time in boys and men with CG than controls. An explanation for this is unclear, as there are no common male-specific symptoms of galactosemia known that would seem to cause or relate to this finding.

Limitations:

The comparison between our two studies is hindered first by sample sizes. As discussed above, Potter et al.'s data are hindered by a lack of cases, particularly after we excluded participants for whom weight data were not available. Meanwhile, both studies were hindered by issues with controls: our study by a lack of sufficient controls, and Dr. Potter's study by the controls' poor matching to cases.

Furthermore, there were differences in how the data were collected. The instrument used to collect grip strength data differed between the two studies, possibly creating differences in how grip strength was quantified.

A further hindrance to both datasets was the way data were pared down. As discussed in the methods section of Chapters 2 and 3, individuals were excluded from the study in our analysis on the basis of being outliers with respect to weight. Our rationale was that individuals with high weights could have large amounts of fat that would not contribute to grip strength. However, those of unusually high weight could also simply be very large, which should not necessarily warrant exclusion from the study. BMI would be a better measure of this, and therefore a better criterion to exclude people based on, but due to a lack of height data on Dr. Potter's data, we cannot include this measure here. Furthermore, in our study, no cases or controls needed to be excluded on the basis of weight, so a more precise measure for exclusion from our study would be unwarranted.

Chapter 4: Growth delay in GALT-null rats

Introduction:

Clearly, an association exists between grip strength and weight for patients with CG. To address whether the same is true in our GALT-null rats, we must first explore the growth pattern in GALT-null rats. Previous research has suggested that while there is a clear growth delay in young GALT-null rats, this largely resolves itself in adulthood. To assess whether this is true using a much larger data set, we analyzed rats' data at 1 and 4 months of age, because this was the same age they were grip strength tested, to determine whether growth delays exist at these time points.

Methods:

Rat Husbandry:

All rats in this study were fed and housed according to the conditions described in Rasmussen et al. 2019[8] in section Data S1. In short, rats are housed by Emory's Division of Animal Resources in a non-sterile environment, typically with two rats per cage. Rats are given ad libitum access to water and food (LabDiet 5053). Rats were regularly handled from shortly after birth until being euthanized.

Collection and analysis of mass data:

Mass data were collected on all rats that were also grip strength tested using a digital scale assessing rats' mass in grams. Data graphed represent an individual's mass at time points when they were grip strength tested. How rats housed/fed. Data were analyzed by using a Shapiro-Wilk test to assess normality, and then using either a t-test or Wilcoxon rank sum test as appropriate to analyze the data. We used these tests instead of a linear mixed effects model because rats were tested at the same time points. Therefore, it was unnecessary to account for the effects of covariates such as age.

Results:

At 1 month of age, rats are just starting puberty and there is a clear replication of the initial growth phenotype shown in Rasmussen et al. 2020[8]. The differences by genotype in both males ($p=0.0004$, Wilcoxon rank sum test) and females ($p=0.0000083$, Wilcoxon rank sum test) are both highly significant and clearly visible on the plot shown below. Also, it appears that males and females are relatively similar with respect to mass, with males that are both GALT-null and wild-type having slightly higher mass than their female counterparts. The genotypic differences in mass are also similar whether you look at males or females: while there appears to be a slightly larger gap between GALT-null and wild-type females, the difference is not much greater than the gap between GALT-null and wild-type males.

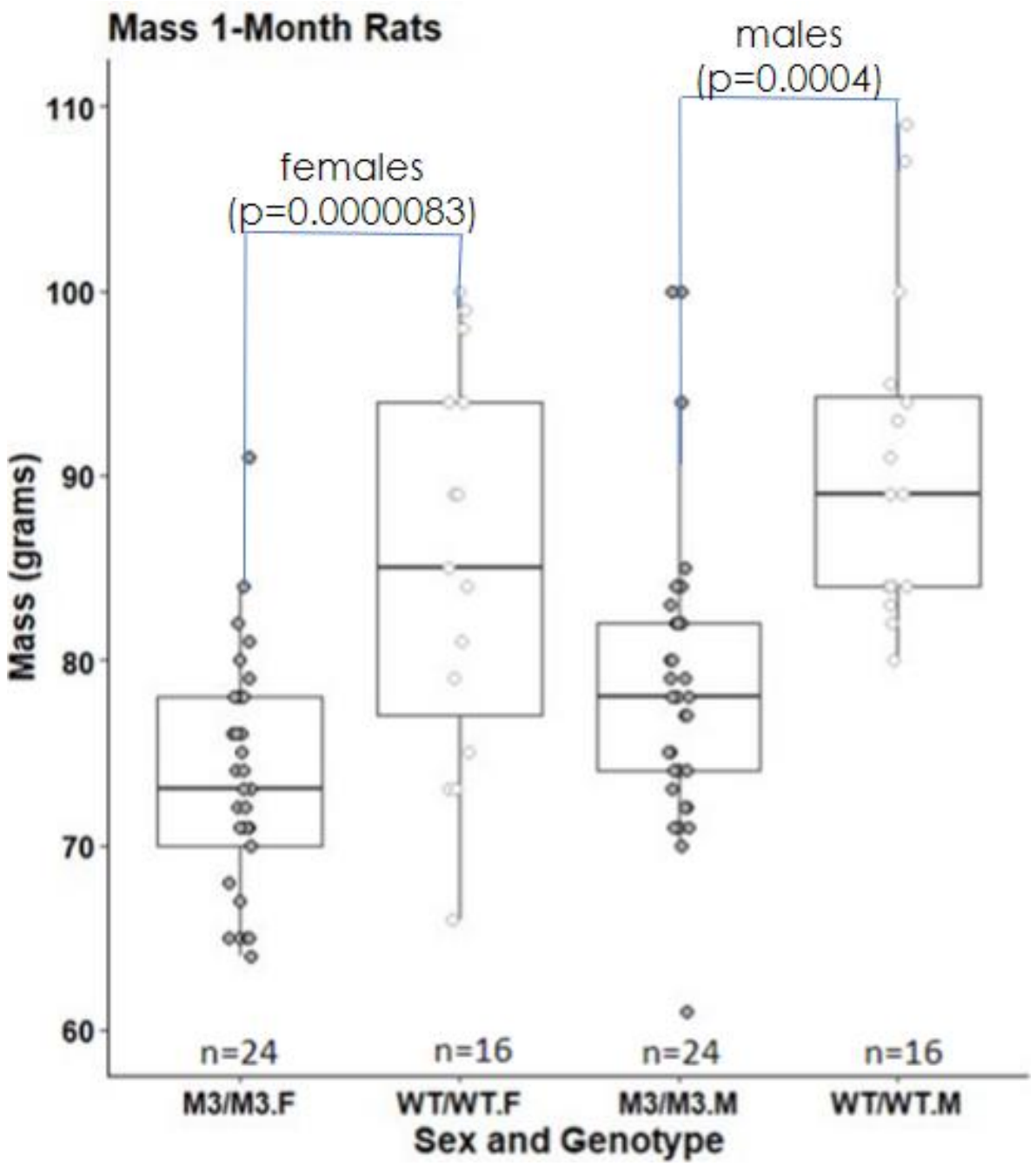

Figure 14: Rats' mass at 30 days of age graphed on the y-axis, with separate boxplots corresponding to rat genotype and sex. Rat genotype denoted by the label M3/M3 or WT/WT, and their sex denoted by M or F.

By 4 months, rats have long since completed puberty and there are larger differences between male and female rats, with males almost twice as heavy as females. Furthermore, the genotypic differences in mass are not as stark as they were at 1-month. However, they are also not fully resolved. In females, the difference between wild-type and GALT-null rats becomes insignificant ($p=0.1736$, t-test), despite the graph showing a higher median for wild type rats than for our GALT-null rats. In males, wild type animals remain significantly heavier than their GALT-null counterparts ($p=0.01497$, t-test), which is clearly visible on the graph. Therefore, though the genotypic differences we see lessen over time, in contrast to our lab's previous report[8] from a smaller cohort of rats we find that they do not fully disappear in adulthood.

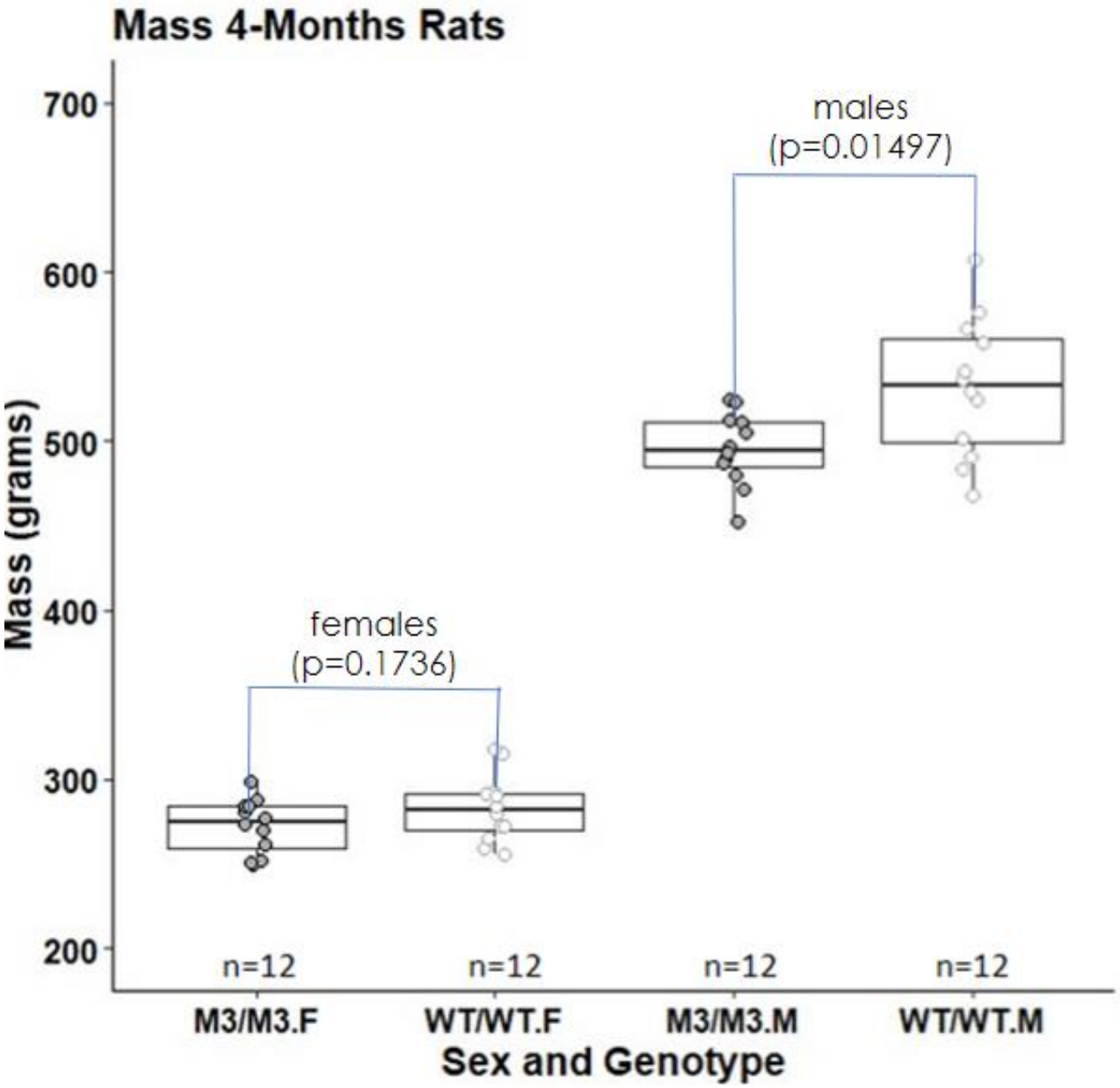

Figure 15: Rats' mass at four months of age graphed on the y-axis, with separate boxplots corresponding to rat genotype and sex.

Discussion:

Here, we find that genotypic differences by weight, at least in males, extend beyond the time points previously shown.[8] This may offer further insight into how the growth delay impacts grip strength in our GALT-null rats, which will be further discussed in Chapter 5.

Chapter 5: Grip Strength in GALT-null Rats

Introduction:

As aforementioned, our GALT-null rats mimic many of the adverse phenotypes seen in humans with CG. Testing whether these phenotypes are preserved or resolve during exposure to treatment allows us to assess the efficacy of different therapies. Therefore, finding additional measurable phenotypes would allow for further examination of how treatments can improve symptoms of GALT deficiency.

To find evidence of a grip strength phenotype, we test both wild-type and GALT-null rats at 1 month and 4 months of age and compare by genotype. We additionally weigh these rats at the times they are grip strength tested to evaluate associations between rat grip strength and weight.

Test Administration:

Testing was performed on our rats using two different grip strength meters. The first grip strength meter used, shown in Figure 16, is a homemade device. The centerpiece of the device is a pink plastic-coated metal grid with cross-sections between the bars of approximately 2.25 square inches. Under this mesh is a thin foil designed to prevent the rats' paws from slipping through the mesh bars and reaching the ground. The grid is supported on 4 roller ball bearings for minimal friction, and to hold the grid at a height above the smooth laminated base that puts it

parallel with an attached spring force measure. This grip strength device is hooked to a newton meter spring scale (which is the blue instrument shown in Figure 16) to measure a rat's grip strength when it is gripping the bars of the grid and pulled back from the base of the tail until its hold begins to slip.

Figure 16: Homemade (old) Grip Strength Meter.

To perform testing on this device, a rat is lifted from its cage by its tail. The rat is then lowered to the grip strength meter, where it is allowed to grip onto the device with all four of its paws in a particular fashion. Specifically, it must have its front paws on the frontmost bar of the pink mesh, with one front paw to the left of the hook that attaches the mesh to the newton meter, and the other front paw to the right. Once the rat has gripped onto the mesh bars, the rat is pulled

horizontally backward by the base of its tail. The rat shows no signs of pain or distress, but naturally resists the pull, thereby transmitting the force to the spring meter. The newton meter measures the force of the rat's pull based on how far back the rat pulls the grip strength meter before it lets go of the grid. This test is repeated over four trials, with one trial occurring on each of four days of testing.

The second grip strength meter, illustrated in Figure 17, is a device purchased from Columbus Instruments. It employs the same principle of allowing a rat to grip onto a grid platform, and then resist being pulled backward. However, instead of a rat gripping the grid with all four of its paws, it only holds onto the meter with its two front paws. Additionally, the meter is digital, meaning that it digitally reads a rat's force, rather than the manual grip strength read that the homemade grip strength meter requires. The grid of the device that the rat grips onto is inverted to ensure that when a rat is pulled back by the grip strength meter when it reaches its maximum force, it does not catch a nail on the remainder of the grid. This is because, as pictured in Figure 18, the outside edges of the bottom side of the mesh piece are raised above the inner bars, meaning that when the rat grips onto this raised mesh on the edge, and is then pulled horizontally off, its nails and paws remain elevated above the rest of the grid. While rats on the homemade meter are tested once a day for four days, on the Columbus grip strength meter, they are tested twice a day for two days. Sequential tests on the Columbus meter, however, must be separated by at least three hours.

Figure 17: Columbus Instruments (new) Grip Strength Meter

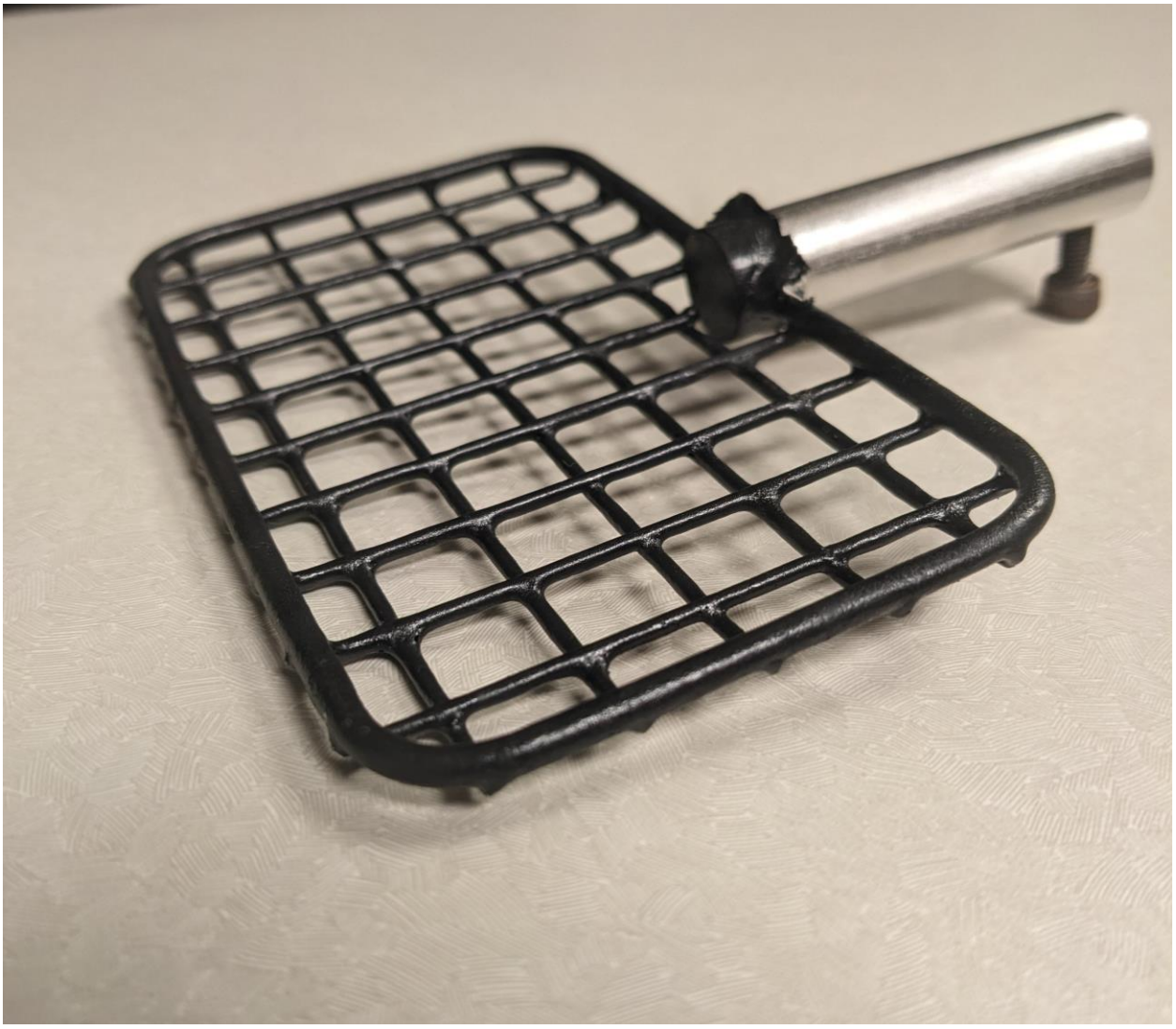

Figure 18: Columbus Grip Strength Meter Mesh Instrument

Graphing and Statistical Methods:

For our graphs, we created boxplots stratified by a rat's genotype and sex. On the y-axis, we included the average of a rat's four grip strength trials. This is because, while in the human studies peak grip strength is the standard, in most rat studies their grip strength is averaged[11, 17].

When graphing the association between grip strength and mass, the same graph was created, but with a rat's averaged grip strength divided by its weight on the y-axis.

For all rats, the significance of the relationship between grip strength as a response variable and genotype as an explanatory variable was measured using either Wilcoxon rank-sum tests or t-tests, depending on whether these data passed the Shapiro-Wilk test for normality. When assessing whether mass can account for the grip strength differences seen by genotype, a Shapiro-Wilk test followed by an appropriate t- or Wilcoxon rank-sum test was performed, but instead using the average of a rat's grip strength divided by its mass on the final day of testing as the response variable.

Testing the Efficacy of each grip strength meter:

To determine whether the Columbus device collected data similarly to the homemade device, we tested the performance of rats across these two devices. As a part of this study, rats between the ages of 2.5 and six months old were initially tested using the Columbus Instruments grip strength meter. Then, a week later, these same rats were tested using the homemade grip strength meter. To analyze the correlation between a rat's performance on each meter, rats' grip strength average on the old grip strength meter was graphed on the x-axis, while rats' grip strength average on the Columbus instrument was graphed on the y-axis. A line of best fit, along with an associated r^2 value, would indicate whether higher grip strength values on one meter associate with higher grip strength values on the other.

Additionally, to test whether the homemade grip strength meter was reproducible for individual rats from month to month of testing, rats were tested using the homemade grip strength meter at four months of age, and then the same rats were tested again using the homemade grip strength meter at six months of age. Like the method described in the previous paragraph, these rats' average grip strength at four months was graphed on the x-axis, and their average at six months was graphed on the y-axis. A line of best fit and r^2 value would indicate whether a rat's performance at four months was associated with its performance at six months.

Grip Strength Meter Correlation Results

We observed a strong association between rats' performance on the old and new grip strength meters, indicating that if a rat performed well on the homemade device (the old grip strength meter) it also likely performed well on the Columbus instrument (the new grip strength meter). There is not, however, a one-to-one correlation between increases in grip strength on the old grip strength meter and the new, resulting from the fact that the old grip strength meter uses all four of a rat's paws for testing, whereas the new grip strength meter only uses the front two.

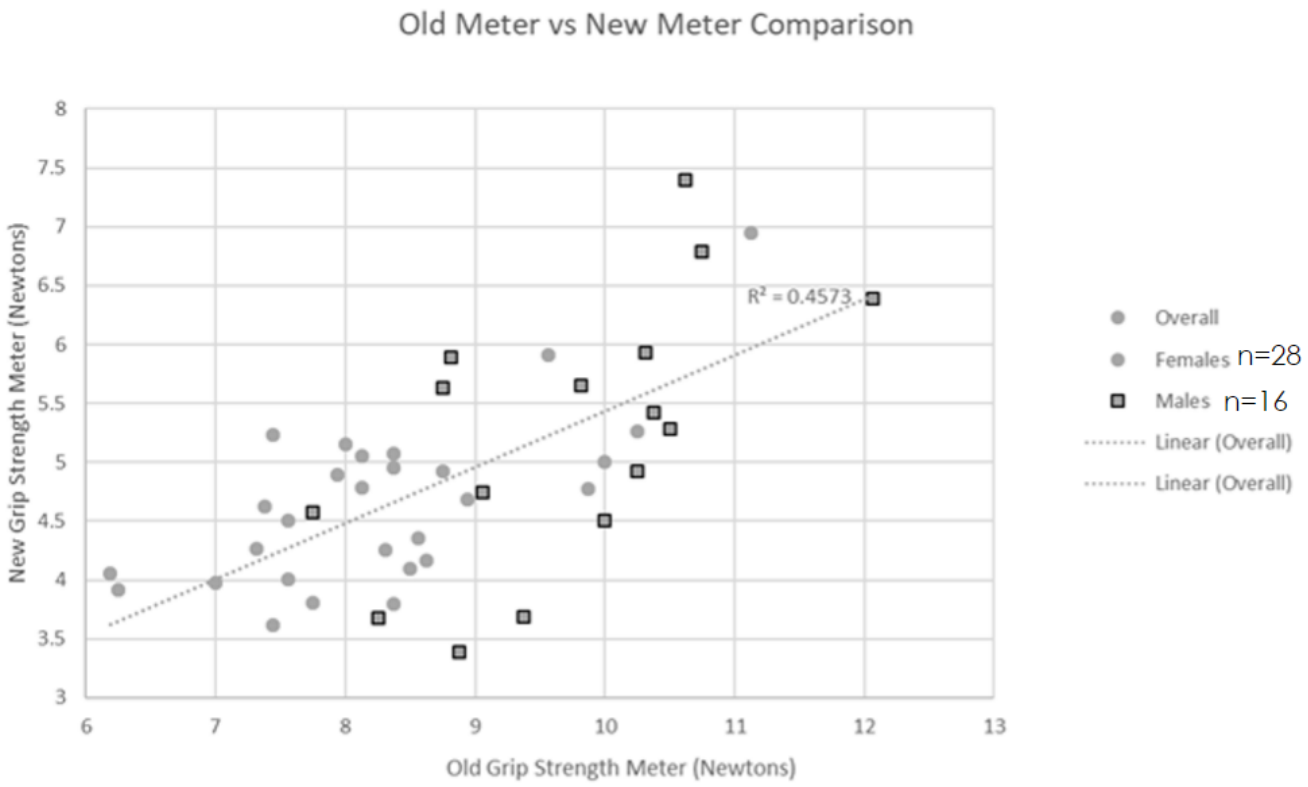

Figure 19: Correlation between rats' performance on the homemade (old) grip strength meter on the x-axis and the Columbus (new) grip strength meter on the y-axis. The dotted gray line indicates the line of best fit.

There is an even stronger correlation between a rat's longitudinal performance at different time points on the homemade meter, with an extremely high R^2 value.

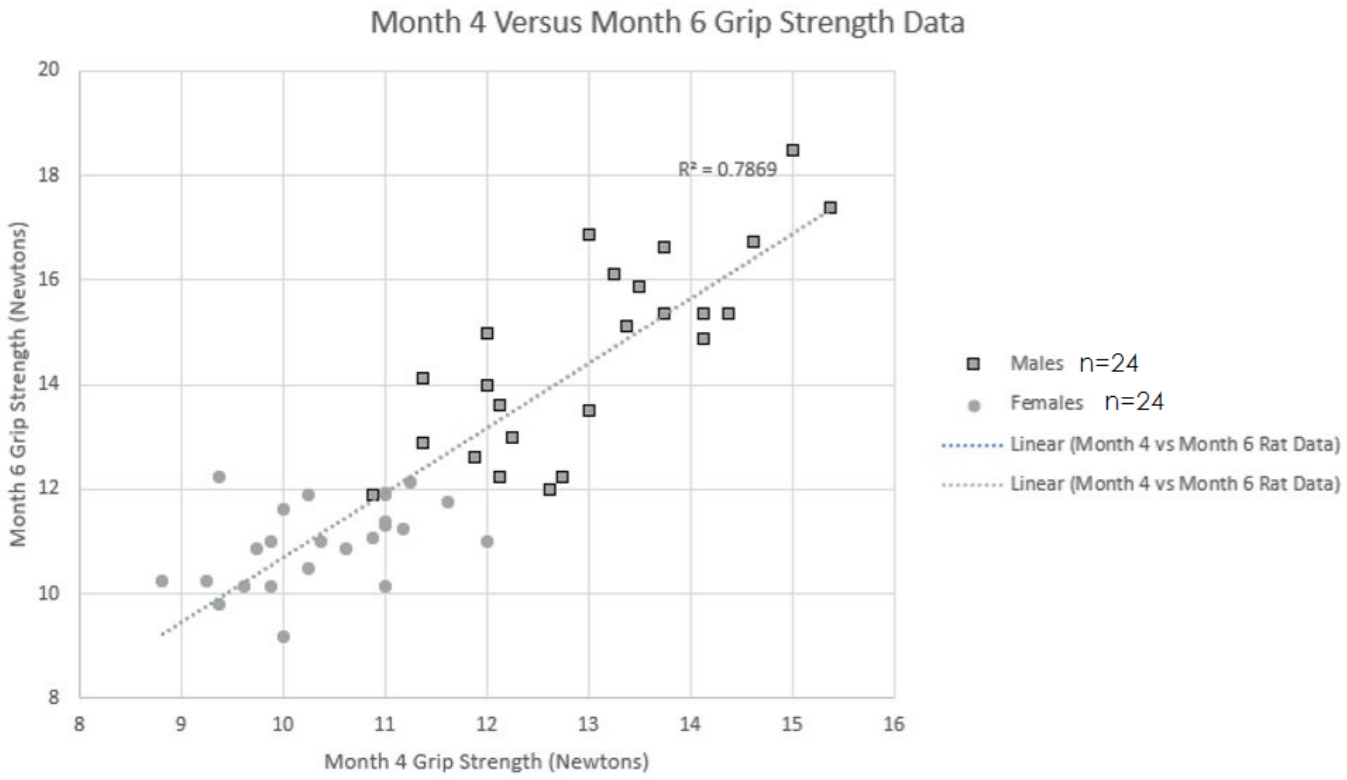

Figure 20: Rats' grip strength at 2 time points measured using the homemade grip strength meter (at 4 months on the x-axis and 6 months on the y-axis). The dotted gray line indicates the line of best fit.

Grip Strength Results:

In both male (p=0.00044, Wilcoxon rank sum test) and female rats (p=0.005, Wilcoxon rank sum test) at one month of age, wild-type rats outperformed their GALT-null counterparts in grip strength. Testing, in this case, was performed using the Columbus Instruments Grip Strength Meter.

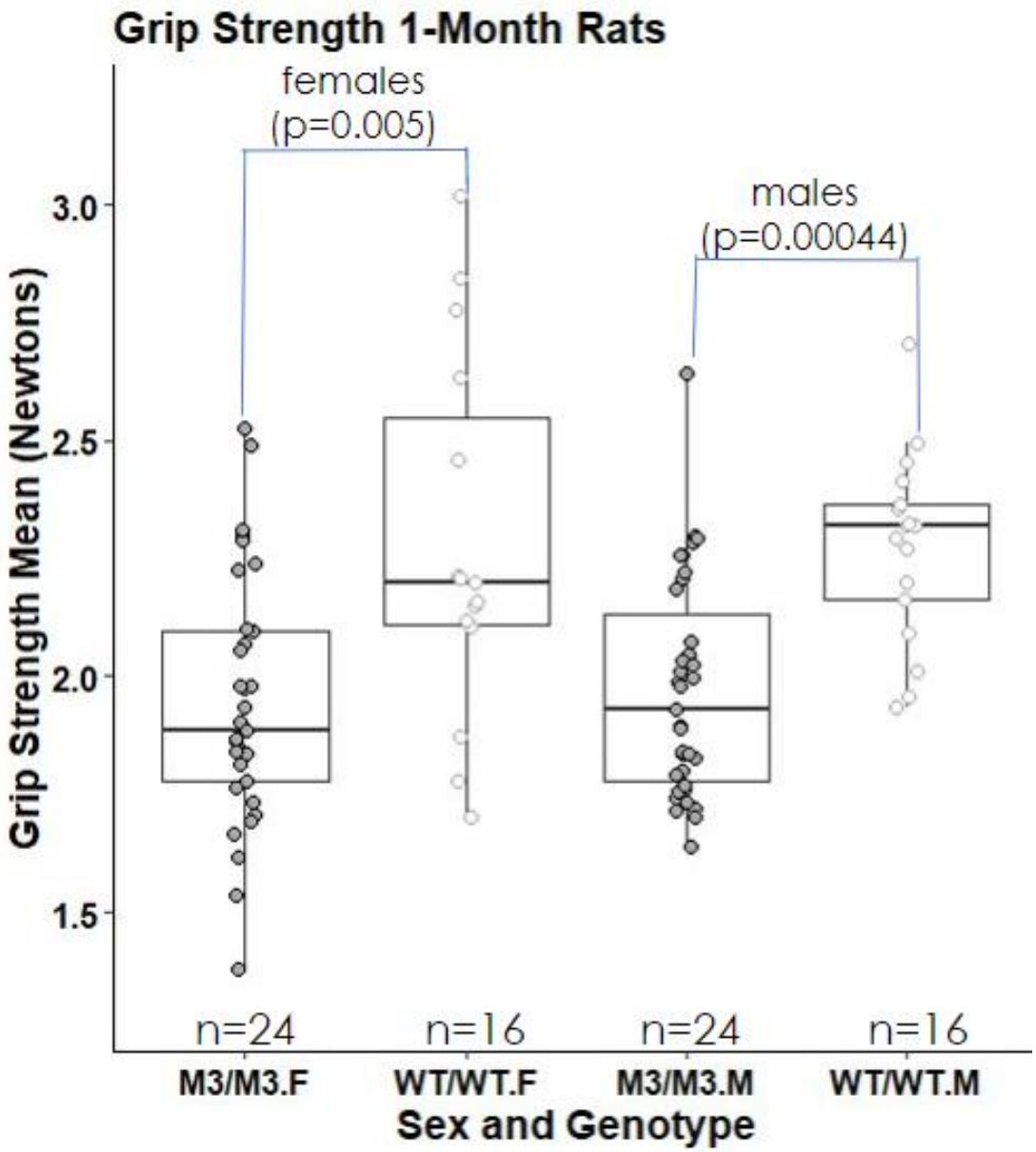

Figure 21: Rats' average grip strength measured at 1 month of age was graphed on the y-axis, with different boxplots corresponding to rats of different sexes and genotypes.

When a rat's mass is accounted for in one-month-olds, any differences in grip strength between GALT-null and wild-type rats largely disappears, both in males (p=0.954, Wilcoxon rank sum test) and females (p=0.83, Wilcoxon rank sum test).

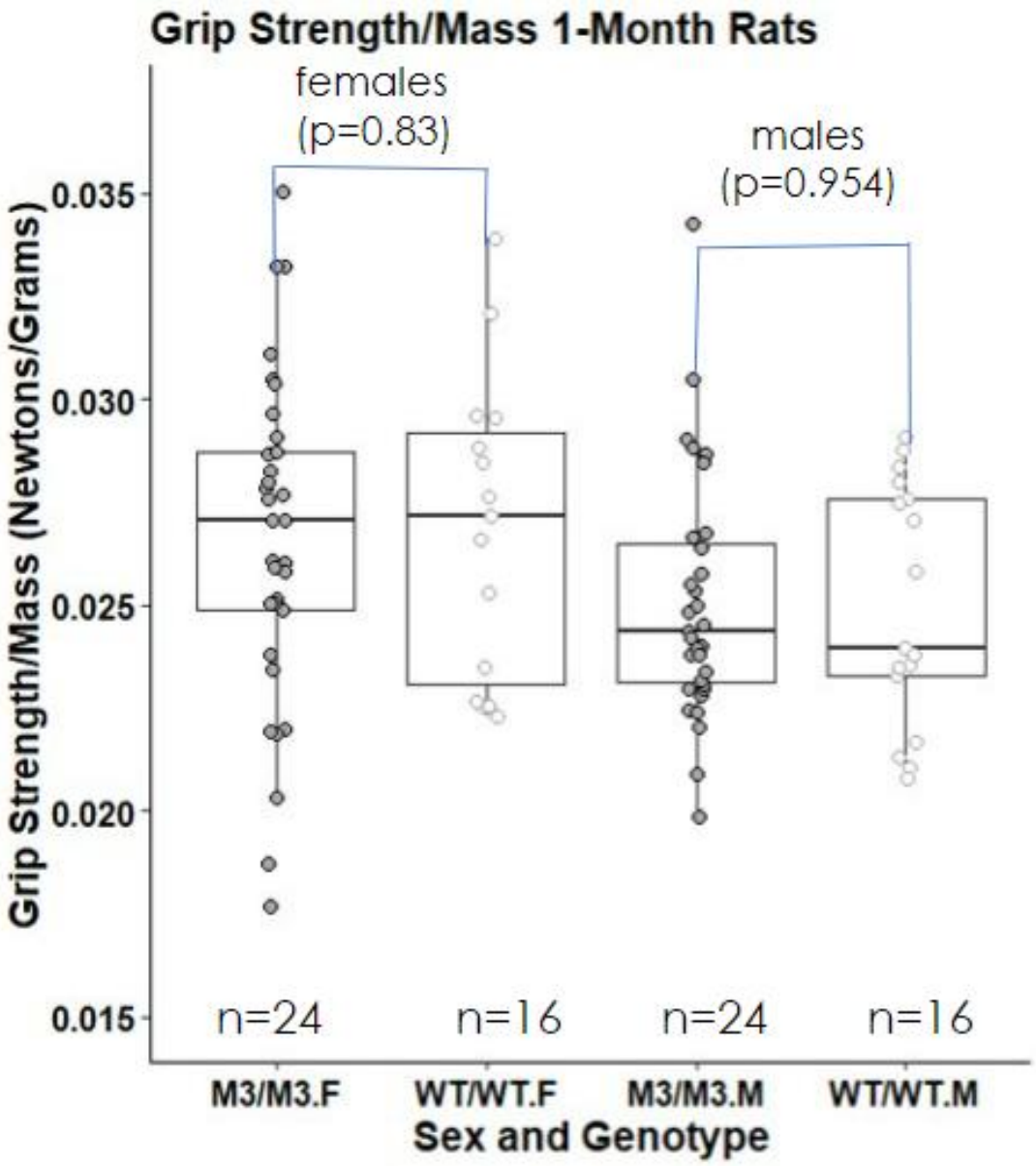

Figure 22: Rats' average grip strength divided by mass at 30 days of age was graphed on the y-axis, with different boxplots corresponding to rats of different sexes and genotypes.

At the age of four months, there is a clear difference between male rats based on genotype (p=0.001, t-test) but not such a clear difference between female rats (p=0.809, t-test). Testing here was performed using the homemade grip strength meter.

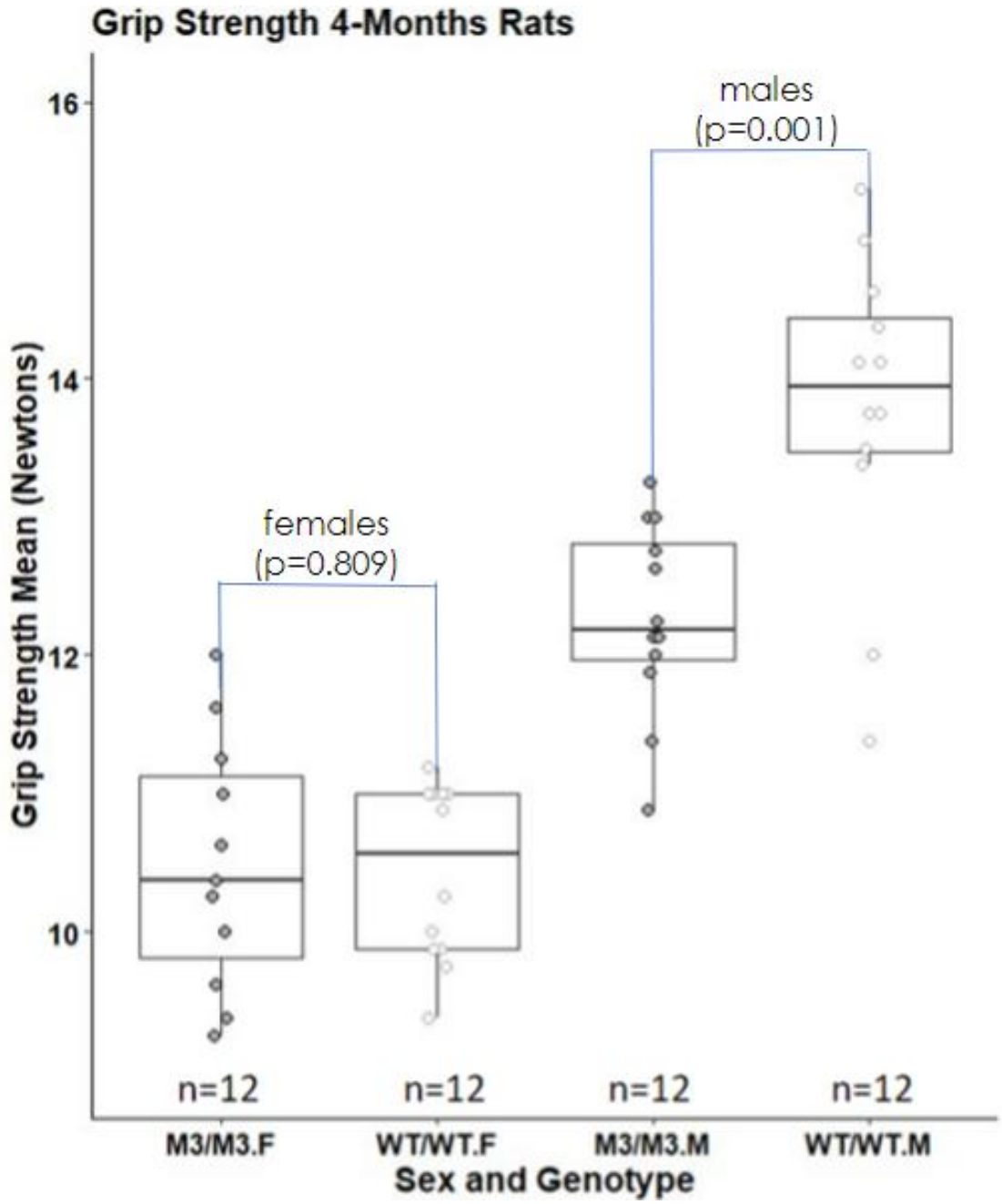

Figure 23: Rats' average grip strength measured at 4 months of age.

Accounting for mass somewhat resolves genotypic differences in grip strength at this age in males (p=0.088, t-test).

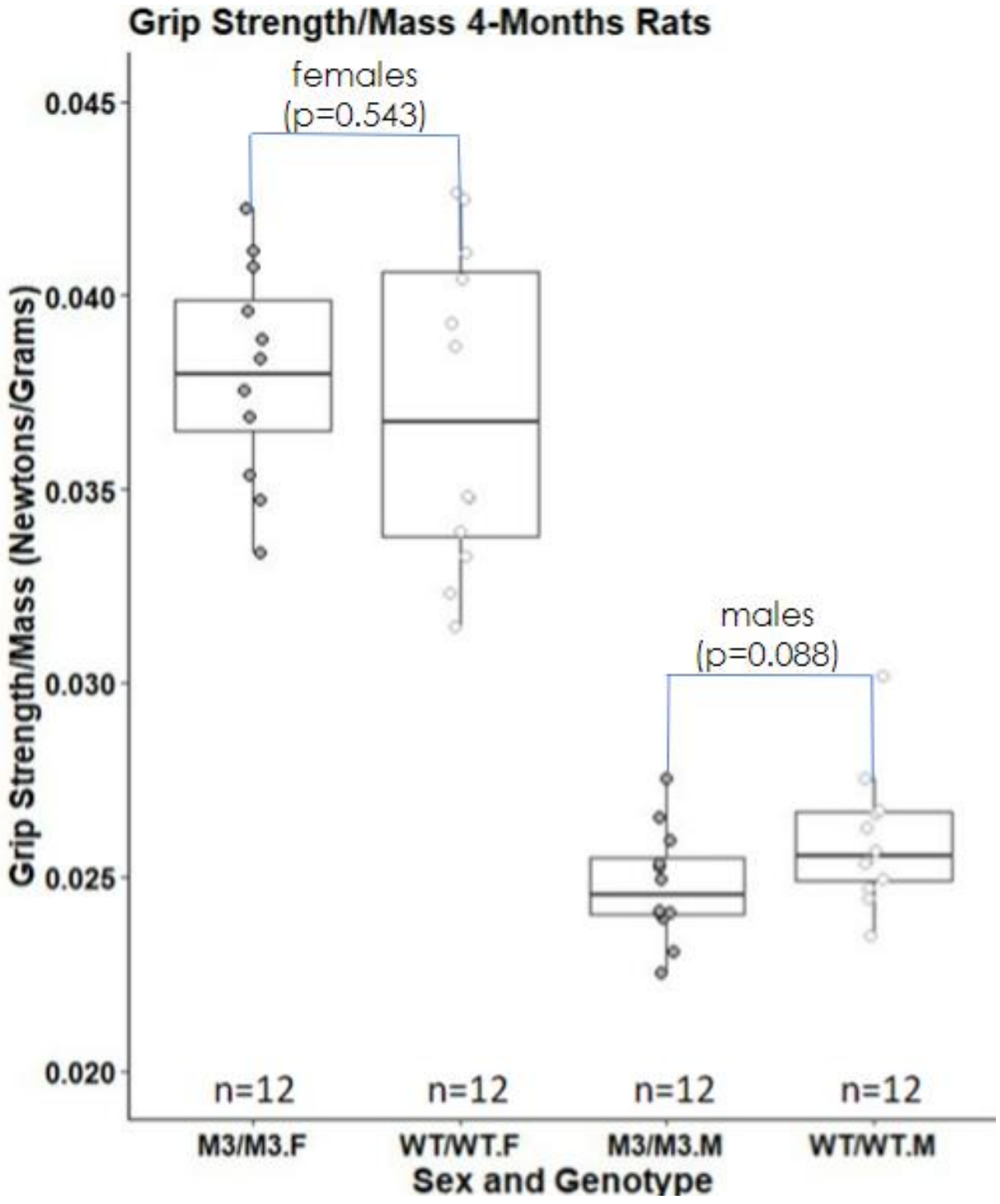

Figure 24: Rats' average grip strength divided by mass at 4 months of age.

Discussion:

Here, we demonstrate clear evidence of the reliability of our grip strength meters. First, we showed that our homemade grip strength meter produced reproducible grip strength values for the same rats over time. It is interesting to note that rats at 6 months showed slightly higher strength than at 4 months. Second, we present evidence that this grip strength meter produced comparable results to the Columbus Instruments Grip Strength Meter, a meter used in hundreds of previous studies[18, 19]. This evidence is especially striking given that we test rats on the two meters at different time intervals, and with different numbers of limbs. Clearly, despite these differences our numbers are reproducible, validating the use of both grip strength meters. It is interesting to note that in terms of Newtons, rats showed stronger grip strength on the old instrument than the new one, perhaps reflecting that rats held onto the grid with all 4 paws in the old instrument, and only with their 2 front paws in the new instrument.

Here we demonstrate the presence of a grip strength deficit in GALT-null rats parallel to that seen in patients. We observe that at early ages, wild-type rats outperform GALT-null rats in grip strength for both sexes. At later ages, it appears that while male wild-type rats significantly outperform their GALT-null counterparts, this difference is not evident among females.

Furthermore, we demonstrate that the grip strength differences between mutant and wild-type rats are largely accounted for by differences in mass at early ages, both in males and females. This is particularly true of our one-month-old rats but is also true of our four-month-old males. It is of note, however, that females no longer have a mass difference by 4 months of age,

whereas males still do, which may explain why there is also not a grip strength deficit at this age in females.

One potential explanation for why the grip strength phenotype is so much more prominent in males at later ages is their weight phenotype. As was shown above, the weight phenotype in males does not entirely disappear, and persists over time. Meanwhile, in females, there appears to be little if any evidence of this phenotype at later time points. While we cannot prove a causal relationship between weight and grip strength, it is interesting that these two phenotypes line up and associate with one another so strongly.

Limitations:

Some limitations include a lack of time points at which we have sufficient data to look at rats' grip strength. For example, while we have enough data for young (1 month old) and older (4-6 months old) rats, we do not have sufficient data for rats of intermediate ages (2-3 months old). This makes it more difficult to assess how the grip strength phenotype changes in our young rats. Furthermore, we are limited by potential behavioral difficulties among the rats. Though rats appeared to engage with the grip strength meter, it is possible that some rats did not try their hardest on the grip strength meter, skewing the results. Furthermore, these data are cross-sectional, not longitudinal. Just like in humans, this hampers our ability to make conclusions about how grip strength develops over time, and how differences in weight may or may not explain differences in grip strength.

Chapter 6: Discussion, Limitations, and Future Directions

Our results, both in rats and humans, deepen our understanding of how grip strength is affected in GALT deficiency. Though previous research has reported this phenotype in humans[7], our robust sample of relatively large numbers of individuals with CG spread across young ages, along with sibling controls, explores the grip strength deficit in a different way. In GALT-null rats, we provide strong evidence of a grip strength deficit. Unlike in humans, this was entirely unaddressed in previous studies. Seeing a cross-species decline in grip strength from GALT deficiency provides further evidence of this association.

The grip strength phenotype, which presents clearly at different ages and in both males and females, is a useful tool to assess the efficacy of therapies for CG. Furthermore, as aforementioned, grip strength in humans is often a proxy for determining morbidity, with high correlations between low grip strength values and incidence of prediabetes, diabetes type 2, and other conditions[20, 21]. In a similar manner, measuring an individual's grip strength in a condition like galactosemia that has such different severity for different people could offer an empirical measure to assess the severity of the condition, and whether certain therapies may be warranted. Of course, the utility of this approach would depend on whether the grip strength deficit in patients correlated with other adverse outcomes, which we did not assess here.

Limitations:

The primary limitation of this study is our inability to infer causality. Because the study is observational in nature, we can only identify potential associations between grip strength and mass. While this provides useful information, as discussed in the above section, an association cannot identify an underlying cause.

Another limitation is the time points at which we collected data. While we have sufficient data both on young rats and humans, we lack data for intermediate ages. In humans, we have sparse data past age 18, and in rats, we lack data for the ages of 2 months and 3 months, times when rats are still young and grip strength is still changing and developing. This further hampers our ability to make broader conclusions about grip strength, since we only have data from specific time points.

Future Directions:

It would be highly useful to collect longitudinal data on grip strength in humans to truly address how it develops over time for individuals with CG, as well as our GALT null rats. To do this in our rat model, we could sample rats throughout their lifetimes. For people, many individuals with CG attend the Galactosemia Foundation Conference every two years it is hosted. Continuing to grip strength test people at the Conference would not only eventually give us more data on grip strength in older individuals with CG but would allow us to see how grip strength develops over time in these individuals we were able to test multiple times.

Furthermore, it is important to focus on whether differences in grip strength cluster with other phenotypes of interest. Testing phenotypes such as motor difficulties, speech, and cognitive development and whether they associate with the grip strength deficit could provide further explanation for why the grip strength differences between cases and controls in Dr. Potter's cohort[7] were not as associated with differences in weight as in our cohort. Also, differences in weight appear to be the sole factor associated with the grip strength deficit, it may be unnecessary to test grip strength in gene therapy studies, as it would be redundant with simply testing changes in mass.

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Appendix A:

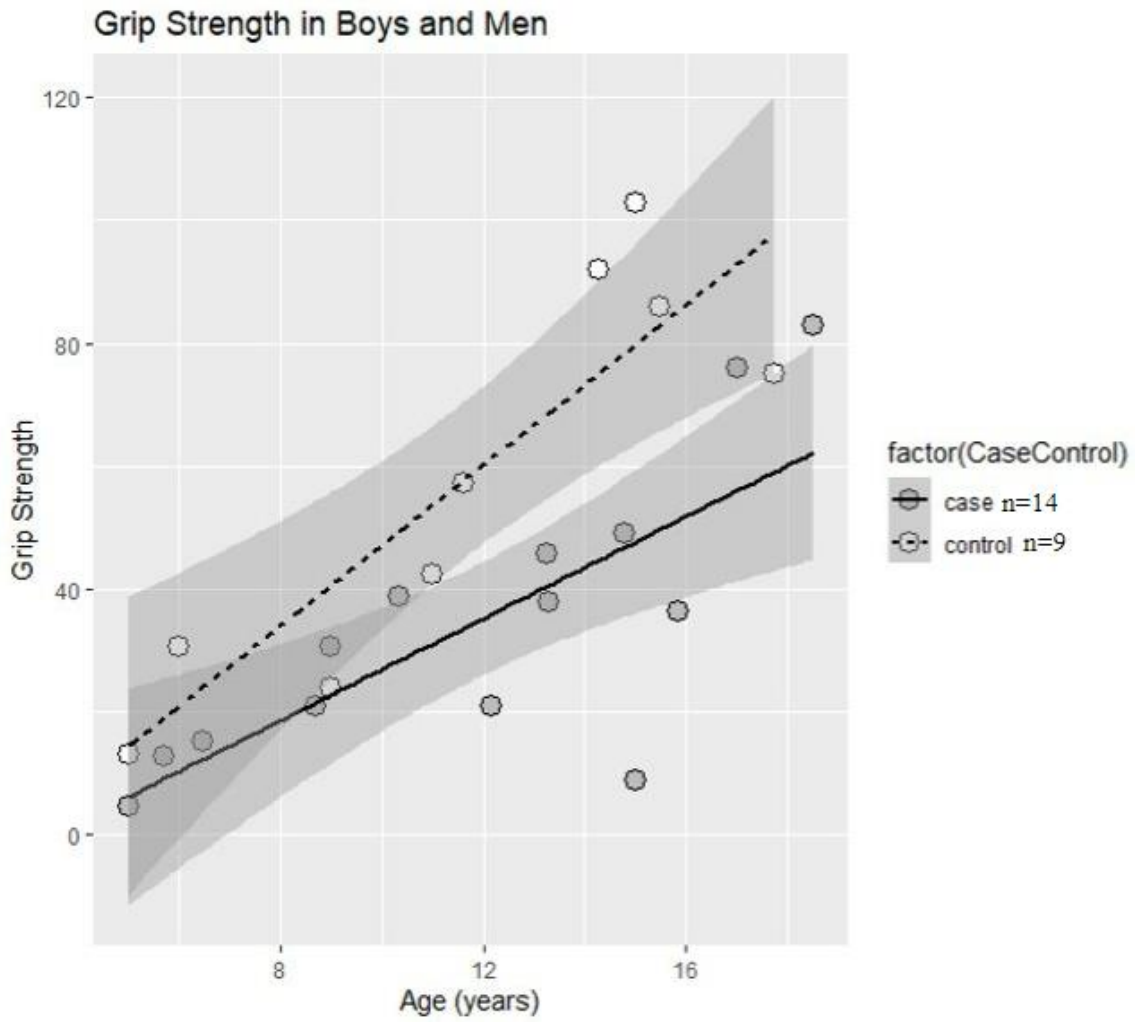

Figure 1: Boys' and men's average, rather than peak grip strength versus age. Separate lines of best fit for cases and controls, with 95% Confidence intervals.

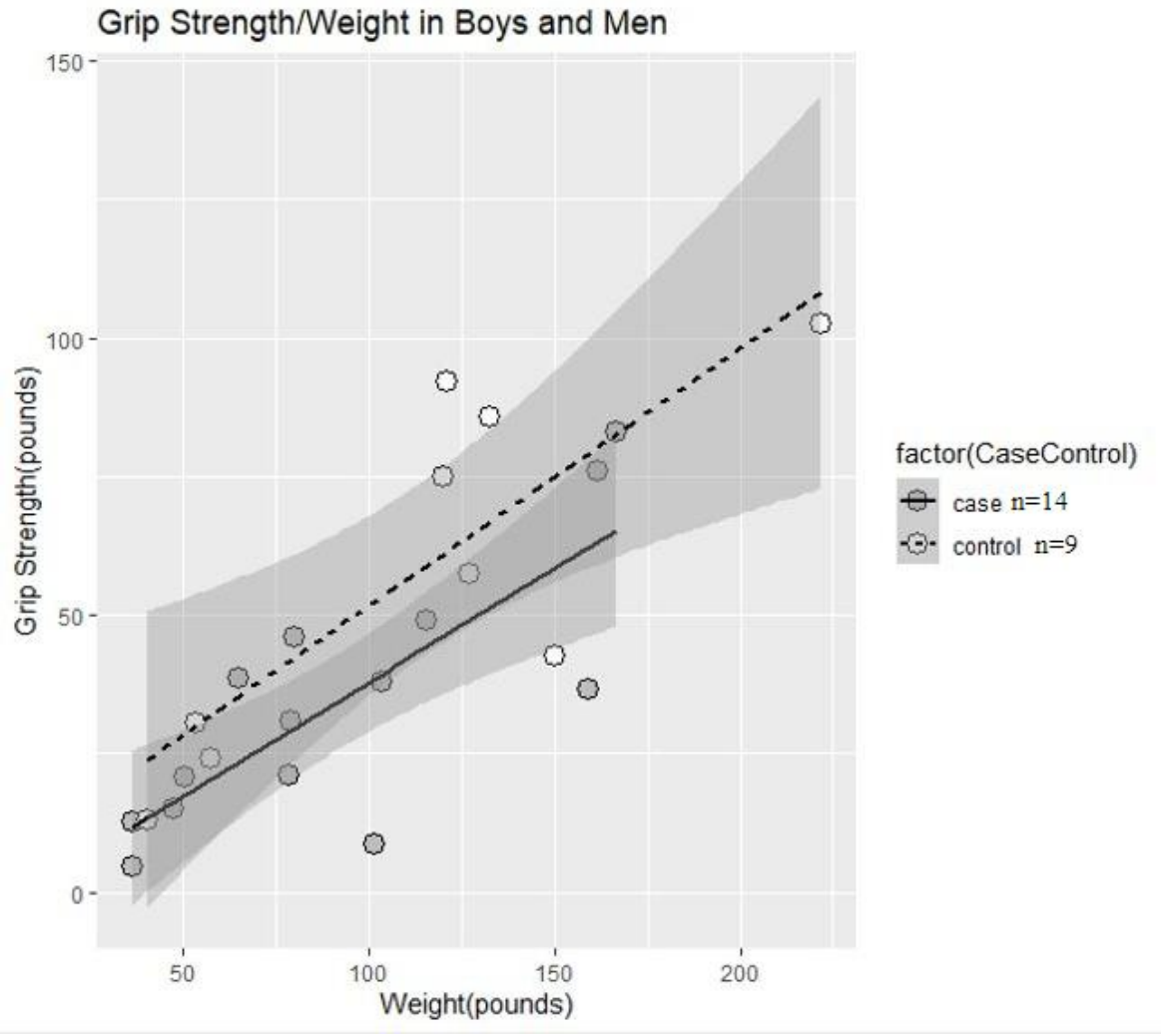

Figure 2: Same thing as Figure 1, but with grip strength divided by weight.

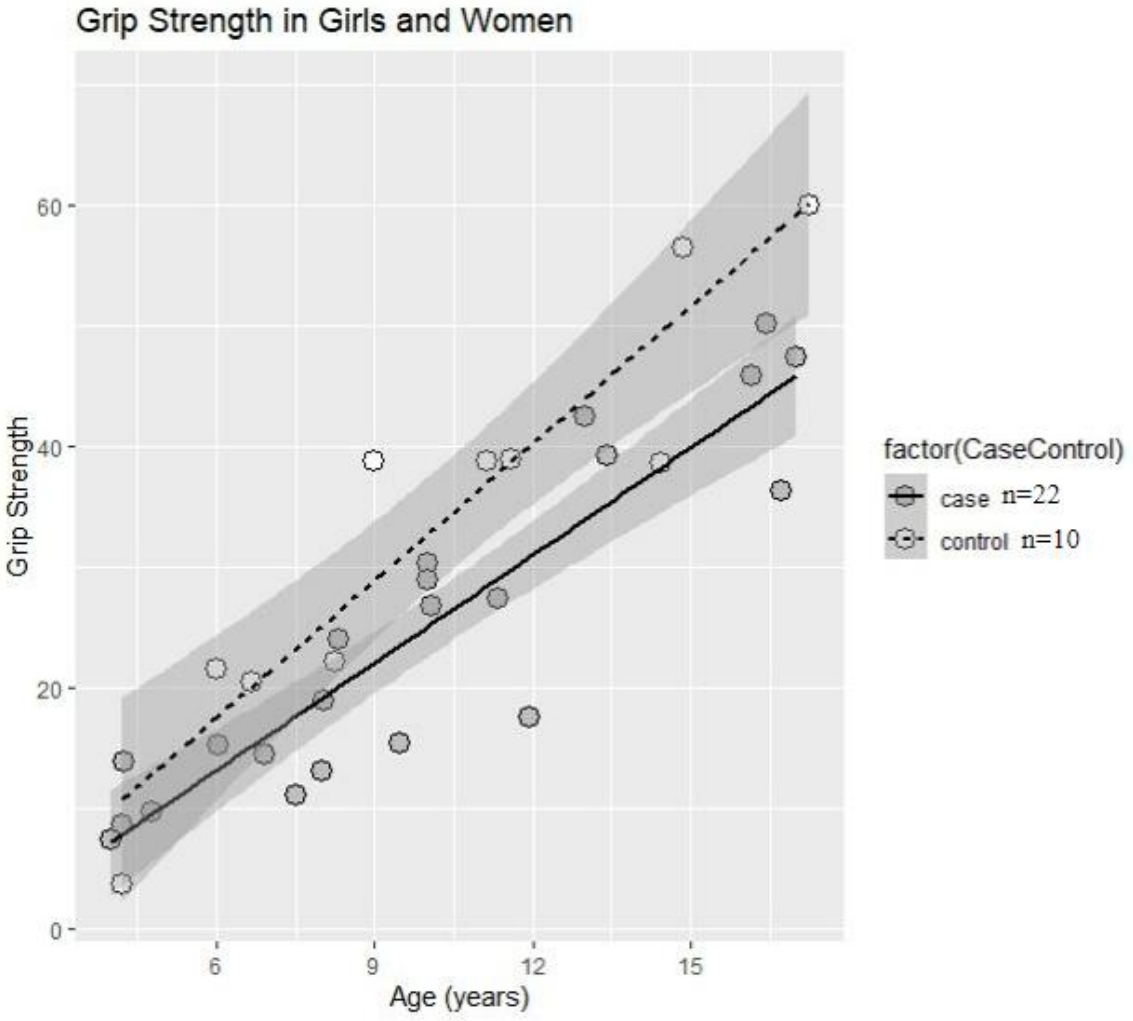

Figure 3: Girls' and women's average grip strength versus age. Separate lines of best fit for cases and controls, with 95% Confidence intervals.

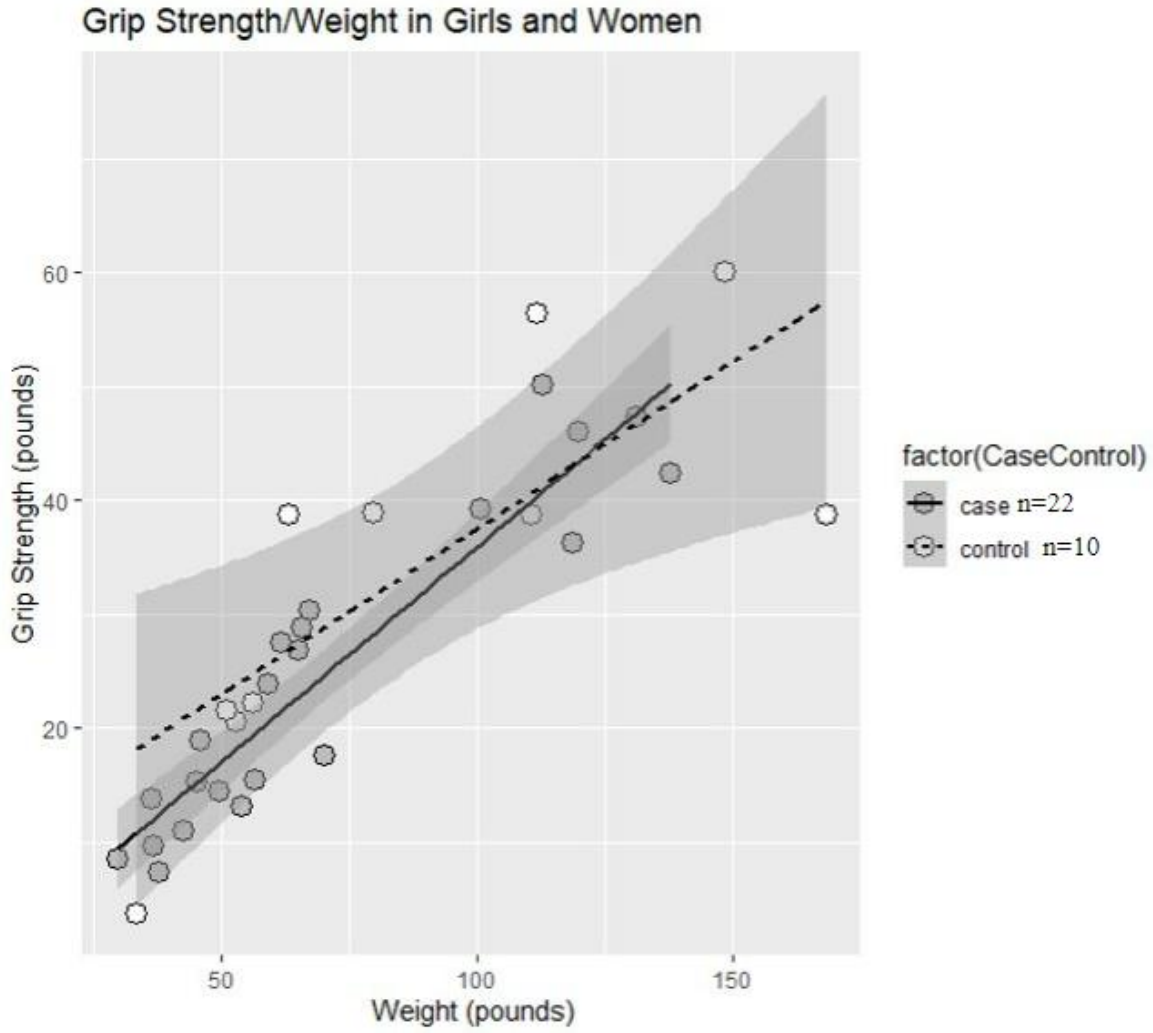

Figure 4: Same thing as Figure 2, but with weight on the x-axis.