Gastrointestinal Health in Classic Galactosemia

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Abstract Classic galactosemia (CG) is an autosomal recessive disorder of galactose metabolism that affects approximately 1/50,000 live births in the USA. Following exposure to milk, which contains large quantities of galactose, affected infants may become seriously ill. Early identification by newborn screening with immediate dietary galactose restriction minimizes or prevents the potentially lethal acute symptoms of CG. However, more than half of individuals with CG still experience long-term complications including cognitive disability, behavioral problems, and speech impairment. Anecdotal reports have also suggested frequent gastrointestinal (GI) problems, but this outcome has not been systematically addressed. In this study we explored the prevalence of GI symptoms among 183 children and adults with CG (cases) and 190 controls. Cases reported 4.5 times more frequent constipation (95% CI 1.8–11.5) and 4.2 times more frequent nausea (95% CI 1.2–15.5) than controls. Cases with genotypes predicting residual GALT activity reported less frequent constipation than cases without predicted GALT

activity but this difference was not statistically significant. Because the rigor of dietary galactose restriction varies among individuals with galactosemia, we further tested whether GI symptoms associated with diet in infancy. Though constipation was almost four times as common among cases reporting a more restrictive diet in infancy, this difference was not statistically significant. These data confirm that certain GI symptoms are more common in classic galactosemia compared to controls and suggest that future studies should investigate associations with residual GALT activity and dietary galactose restriction in early life.

Introduction

Classic galactosemia (CG) results from profound deficiency of galactose-1-phosphate uridylyltransferase (GALT) activity and affects approximately 1/50,000 live births in the USA (Pyhtila et al. 2015). Following exposure to milk, which contains large quantities of galactose, affected infants can become seriously ill and die if not immediately switched to a low-galactose formula (Berry 2014). Early identification by newborn screening and rapid dietary intervention generally prevents or resolves the potentially lethal acute symptoms of CG (Berry 2014).

Despite early diagnosis and intervention, most individuals with CG experience long-term complications that can include multiple developmental disabilities (Kaufman et al. 1995; Waggoner et al. 1990). The majority of girls and women with CG also experience primary or premature ovarian insufficiency (Fridovich-Keil et al. 2011; Kaufman et al. 1979; Spencer et al. 2013; Waggoner et al. 1990). For years, anecdotal reports of increased gastrointestinal (GI) health problems in CG have been shared by families but not

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investigated formally. To determine whether children and adults with CG indeed experience increased prevalence of GI symptoms, we performed a systematic survey of GI health among 183 individuals with CG (cases) and 190 controls. To address possible genetic and environmental modifiers of GI outcome in CG we also gathered *GALT* genotype and retrospective diet information for each case.

More than 300 different *GALT* variants have been reported (http://arup.utah.edu/database/GALT/GALT_display.php; Calderon et al. 2007) and this allelic heterogeneity has been a suspected modifier of outcomes (e.g., Tyfield et al. 1999). Recently, trace residual *GALT* activity predicted from a yeast model system for specific genotypes was associated with both improved scholastic (Ryan et al. 2013) and ovarian outcomes (Spencer et al. 2013), suggesting that residual *GALT* activity might also modify GI outcomes in CG.

Another potential modifier of GI outcomes in CG is diet. While the majority of healthcare providers recommend lifelong dietary restriction of milk and other dairy products for their patients with CG, some also recommend restriction of non-dairy foods that contain low levels of galactose (Gleason et al. 2010; van Calcar et al. 2014). As a result, rigor of dietary galactose restriction varies among individuals with CG.

Using GI health outcomes, *GALT* genotype, and retrospective diet information collected for volunteers in our study we sought to address: (1) whether cases reported more frequent GI problems than controls, (2) whether presence of predicted residual *GALT* activity associated with frequency of GI symptoms among cases, and (3) whether rigor of dietary galactose restriction in infancy associated with frequency of GI symptoms among cases.

Materials and Methods

Study Volunteers

Children and adults with classic galactosemia were ascertained by referral from healthcare professionals or self-referral, often following interactions facilitated by the Galactosemia Foundation (www.galactosemia.org). Controls were recruited in two ways. First, unaffected siblings of CG volunteers participating in the study were recruited as “related controls.” Second, “unrelated controls” were recruited by posting an IRB-approved flyer to the Centers for Disease Control (CDC) parents’ email listserv (a widely subscribed electronic mailing list). All study volunteers completed informed consent prior to joining this IRB-

approved study (Emory IRB00024933, PI: JL Fridovich-Keil).

Gastrointestinal Health Parent- or Self-Report Survey

We developed the gastrointestinal (GI) health survey used in this study to assess how frequently each study volunteer experienced different GI symptoms including abdominal pain, constipation, diarrhea, heartburn, nausea, and vomiting (see Supplemental data). The survey was administered online via Emory’s HIPAA-compliant Feedback Server in 2013 and 2014. Surveys were completed by parent/guardians for their children, or by adults for themselves. Symptoms of each GI outcome were rated by frequency: “never,” “less than once a month,” “at least once a month,” “weekly,” or “daily.” We classified problems that were experienced more than once a month as “frequent” and problems experienced less than or equal to once a month as “infrequent.”

In addition to measures of GI health, we also gathered data on potential covariates including probiotic/antibiotic usage within the prior 6 months, date of birth, gender, race, and ethnicity. Our study design did not allow calculation of an overall response rate because the survey distribution routes used prevented us from knowing how many eligible people received the invitation to participate.

Dietary Restriction Parent-Report Survey

Our diet survey was developed to assess historical dietary information retrospectively. For individuals with classic galactosemia, this included which food groups were restricted in infancy to avoid galactose exposure. Like the GI health survey, our diet survey was administered online via Emory’s Feedback Server. One hundred fourteen cases who responded to the diet survey also completed the GI health survey. We scored dietary restriction of milk/dairy only or milk/dairy plus legumes as “moderate” and restriction of milk/dairy, legumes, plus other food groups (e.g., some fruits or vegetables) as “strict.”

Predicted Residual *GALT* Activity

We collected all available *GALT* genotype information for cases and calculated predicted *GALT* enzyme activity using results from a previously described yeast expression system (Fridovich-Keil and Jinks-Robertson 1993; Riehman et al. 2001). Cases were classified as having either $\geq 0.4\%$ predicted residual *GALT* activity (approximately the limit of detection of the enzyme assay) or $< 0.4\%$ predicted

residual GALT activity based on the average of activities predicted for their two *GALT* alleles.

Statistical Analyses

We performed all statistical analyses in R (<https://www.r-project.org/>). Because there are no good estimates for the relevant population prevalence of the GI symptoms we report, we used the reported symptoms in our controls as a guide for calculating the statistical power of our study. Reported symptoms ranged from a prevalence of 1.6% (nausea) to 6.3% (heartburn) in our controls. With our sample size, we had 80% power to detect an increase in cases of 5.2–7.8%.

To determine if there were significant differences in population structure or outcomes between related and unrelated control groups, we used chi-square tests, *t*-tests, and Fisher's exact tests, as appropriate. For case/control comparisons we performed logistic regression using generalized estimating equations (GEE) (Liang and Zeger 1986) to account for within-family correlations. With "frequent" (symptom experienced more than once a month) or "infrequent" (symptom experienced once a month or less) GI symptom as the outcome, our full models included "case" or "control" diagnosis as the predictor of interest and age, gender, probiotic use, and antibiotic use as covariates. Covariates were tested individually for association with outcome and retained in our reduced model if their *p*-value was ≤ 0.1 . To adjust for multiple testing of various GI symptoms, we used permutation procedures that randomly shuffled each subject's set of GI symptoms within the study. To perform permutations while maintaining the existing familial structure in the dataset, we performed separate shuffling of unrelated subjects (unrelated cases and unrelated controls) and related subjects (related cases and controls). For related subjects, we assigned each individual's set of GI symptoms randomly among subjects from the same family. Symptoms significantly associated or close to associated with diagnosis ($p \leq 0.1$) were subjected to 10,000 such permutations of outcome to account for multiple testing.

For case-only diet and residual activity analyses we used Fisher's exact tests because all cases were unrelated (independent observations) and at least one cell in each comparison included fewer than five individuals.

Results

Study Population Characteristics

In total, 499 people responded to our GI health survey. However, we restricted analyses to respondents between

ages 1 and 55 because of differences in distribution of cases and controls outside this range. Additionally, because >90% of our cases self-reported as white and non-Hispanic, we restricted our analyses to this demographic. We ultimately analyzed GI health survey results from 183 children and adults with classic galactosemia (cases) and 190 children and adults without classic galactosemia (controls). These 190 controls included 75 volunteers who were related to cases in the study, and 115 unrelated volunteers. There were only 4 reports of frequent vomiting in our entire cohort (evenly split between cases and controls), so we excluded this outcome from our analysis.

Notably, GI outcomes were not significantly different between the related and unrelated control groups for abdominal pain, constipation, heartburn, or nausea (Fisher's exact test $p = 1$, $p = 1$, $p = 1$, and $p = 0.3$, respectively). This is important because it suggests there were no strong "household" effects impacting the outcomes studied here. However, 13 unrelated controls reported severe diarrhea, compared to 0 reports in the related control group (Fisher's exact test $p = 0.002$). Privacy issues prevented us from re-contacting these 13 individuals for clarification, and because they did not clearly differ from other controls with regard to other parameters assessed, we did not exclude them from the study but instead did not test diarrhea as an outcome in subsequent analyses.

Unrelated controls were significantly older than related controls (24 ± 14 years old compared to 18 ± 14 years old; *t*-test $p = 0.003$), and overall this combined control group was significantly older than the case group (22 ± 14 years old compared to 16 ± 12 years old; $p = 1\text{E-}05$ based on *t*-test). We therefore tested age as a potential covariate in all case/control analyses. We likewise tested gender as a potential covariate because of differences in gender distribution between related and unrelated controls (63 and 42% female, respectively; chi-square $p = 0.008$). However, the gender distributions of cases and combined controls were not significantly different (55 and 50% female, respectively). Table S1 shows a summary of the numbers of cases and controls used in all comparisons.

Individuals with Classic Galactosemia Experience Some GI Symptoms More Frequently Than Controls

Our final GEE models comparing frequency of GI symptoms between cases and controls included: probiotic usage for abdominal pain and constipation, age for heartburn, and antibiotic usage for nausea (Table S2 provides a summary of full and reduced models). Of note, both antibiotic and probiotic usage were similar between cases and controls, so this was not a confounding variable (Table S3). Gender did not approach significant association

Table 1 Odds ratios from logistic regression using generalized estimating equations (GEE) to calculate odds of cases experiencing frequent symptoms compared to controls, with 95% confidence intervals, *p*-values (^ indicates after 10,000 permutations), and the number of observations included in model

Symptom	Odds ratio for cases	95% CI	<i>p</i> -value	<i>N</i>
Abdominal pain	2.1	0.8, 5.4	0.1^	360
Constipation*	4.5	1.8, 11.5	0.0008^	362
Heartburn	1.2	0.5, 2.9	0.8	356
Nausea*	4.2	1.2, 15.5	0.03^	356

Asterisk (*) indicates outcome was significantly higher among cases

with any outcome ($p > 0.1$ for all analyses) and therefore was not included in any of our reduced models.

Using case/control status as a binary predictor in our GEE framework, we were able to calculate adjusted odds ratios for experience of frequent symptoms controlled for relevant covariates (Table 1). Comparing unadjusted prevalence numbers we found that a diagnosis of classic galactosemia was significantly associated with a 4.5-fold increase in frequent constipation (95% CI 1.8–11.5, permuted $p = 0.0008$) and a 4.2-fold increase in frequent nausea (95% CI 1.6–18.7, permuted $p = 0.03$) (Fig. S1). Differences in abdominal pain (2.1-fold, 95% CI 0.8–5.4) and heartburn (1.2-fold, 95% CI 0.5–2.9) were not significant.

Residual GALT Activity and GI Health

For the case-only residual GALT activity question, we had *GALT* genotype information for 153 of the 183 cases who completed our GI health survey, 29 of whom had *GALT* alleles either not yet tested or not appropriate for study in our yeast system (Fridovich-Keil and Jinks-Robertson 1993; Riehm et al. 2001). Of the 124 cases for whom we could predict GALT activity, 27 had $\geq 0.4\%$ predicted residual GALT activity and 97 had $< 0.4\%$. *GALT* genotypes and predicted activities for this study are summarized in Table S4.

While cases with predicted residual GALT activity $\geq 0.4\%$ reported one-fifth the frequent constipation reported by cases with lower predicted activity (Table 2, odds ratios, upper rows and Fig. S2A, unadjusted prevalence), this difference was not statistically significant (95% CI 0.005–1.6, $p = 0.2$). There was no evidence of a difference in frequency of nausea between the two groups ($p = 1$).

Dietary Restriction in Infancy and GI Health

We received completed parent-response diet surveys with historical galactose restriction data for 114 of the 183 cases

who also completed our GI health survey. The diet survey asked respondents to indicate categories of food restricted in infancy to avoid galactose. Options included: (1) milk and other high galactose dairy products, (2) legumes, (3) some fruits, (4) some vegetables, and (5) other. Milk and other dairy products were universally restricted among cases in infancy, and most families also restricted legumes which have long been considered a significant source of galactose (Acosta and Gross 1995). A smaller proportion of families also restricted some fruits/vegetables, or other foods believed to contain potentially concerning levels of galactose. Because of this distribution, we defined diets restricting only milk/dairy or milk/dairy plus legumes as “moderate” and diets restricting these plus any additional food groups (e.g., some fruits and vegetables) as “strict.”

Nausea was not significantly different between “moderate” and “strict” dietary groups (Table 2, odds ratios, lower rows and Fig. S2B, unadjusted prevalence). We noted a 3.9-fold increase in odds for frequent constipation in the “strict” group but this result was not significant (95% CI 0.8–38.3, $p = 0.1$). Importantly, our findings were not confounded by the effect of residual GALT activity, because similar proportions of cases in the “moderate” and “strict” dietary groups had $\geq 0.4\%$ predicted residual activity (19 and 22%, respectively, Table S5).

Discussion

The main goal of this study was to test whether there was a link between classic galactosemia and specific GI symptoms among a relatively large cohort of volunteers. Our results demonstrated that cases indeed reported significantly more frequent constipation and nausea than controls. Specifically, we found that individuals with classic galactosemia in our study were 4.5 times more likely to report frequent constipation and 4.2 times more likely to report frequent nausea compared to controls. It is important to note that while these increases were significant, the absolute prevalence of each symptom in our CG study group was fairly low at 11 and 5%, respectively. Therefore, while individuals with classic galactosemia do experience these GI problems more frequently than controls, these symptoms are not universal.

As a first step toward identifying possible genetic and environmental modifiers of GI health outcomes in classic galactosemia we addressed two obvious possibilities: predicted residual GALT activity and diet in infancy. We found suggestive trends for residual GALT activity: cases with $\geq 0.4\%$ predicted residual GALT activity reported less frequent constipation than individuals with $< 0.4\%$ predicted residual GALT activity (Table 2). We saw no evidence of a difference in frequency of nausea. However,

Table 2 Results of Fisher's exact tests for association of <0.4% predicted residual GALT activity (upper set of rows) or strict diet (lower set of rows) with frequent experience of GI symptoms

Symptom	Odds ratio for $\geq 0.4\%$	95% CI	<i>p</i> -value	<i>N</i>
Association of $\geq 0.4\%$ predicted residual GALT activity with frequent experience of GI symptoms				
Constipation	0.2	0.005, 1.6	0.2	122
Nausea	0.8	0.02, 7.4	1	118
Symptom	Odds ratio for strict diet	95% CI	<i>p</i> -value	<i>N</i>
Association of strict dietary galactose restriction in infancy with frequent experience of GI symptoms				
Constipation	3.9	0.8, 38.3	0.1	112
Nausea	1.2	0.2, 8.4	1	109

a larger study is needed to confirm or refute the significance of these results.

Considering dietary galactose restriction in infancy, we noted a nearly fourfold increase in reported frequent constipation among cases on strict compared to moderate galactose restriction in infancy. This difference was not statistically significant, but our sample size may not have been adequately powered to detect a difference. We saw no notable difference in the frequency of nausea between the two diet groups.

We did not have concurrent GI health and general nutritional information for our study cohort. It is therefore possible that cases on more restrictive diets in infancy also followed more restrictive diets later in life, potentially leading to lower fiber intake due to a reduction in fruit and/or vegetable consumption. A larger study, with data gathered concurrently for diet and GI symptoms, will be needed to test this possibility. We also did not have information concerning a number of other factors that might have potentially influenced the GI outcomes we measured here, including type of milk substitute consumed, if any, presence or absence of calcium supplementation, psychosocial distress or psychiatric comorbidity, alcohol ingestion, obesity, or use of medications not covered by our survey.

Because classic galactosemia is a rare disorder with limited treatment options, individuals experiencing complications may be more likely to participate in research than those not experiencing complications, resulting in ascertainment bias. However, our observation that less than 12% of cases reported "frequent" experience for each GI symptom helps counter the concern that only those with frequent GI problems were motivated to participate in this study.

One other study limitation is the retrospective nature of our diet survey. Because classic galactosemia is a rare condition (1/50,000 live births), it took many years to assemble our study cohort, at all times welcoming cases of any age to join. While recall bias is therefore potentially a concern, there was no practical way to conduct this study

otherwise. Of note, we have anecdotally found that parents of children with classic galactosemia tend to remember incredible detail of their child's early diet, perhaps because they worried about it so much.

Another potential limitation is our control group. We originally wanted to use siblings of cases to control for shared environment and genetics. However, we worried that as the parents raising a child with classic galactosemia might be so focused on the considerable health needs of their affected child they might under-report possible health concerns for their non-CG child. A comparison of related and unrelated control groups demonstrated no significant differences in reported frequency of GI symptoms between the two groups (with the exception of frequent vomiting in a small number of unrelated controls as a clear outlier). Additionally, performing GEE analysis of binary outcome data allowed us to account for within-family correlations that could have biased our results.

Importantly, our findings open up new avenues of investigation into pathophysiology of CG and possibilities for therapeutic intervention. One potential explanation for increased GI problems in CG is that defective glycosylation due to perturbation in UDP sugar substrate pools might impact the mucosal layer of the gut, compromising gut barrier function and potentially commensal bacterial population structure (reviewed in Bergstrom and Xia 2013). A "leaky" gut, microbiome dysbiosis, or both, could help explain increased GI problems as well as some of the other complications commonly seen in CG.

Importantly, diet also has a significant impact on establishment of the gut microbiome (Albenberg and Wu 2014; David et al. 2014), and the diet of infants and children with CG is fundamentally altered because of restriction of galactose-containing foods. Deficiency of the probiotic effect of milk and other dairy products alone could result in differences in the gut microbiome between cases and controls. Perhaps the most appealing aspect of testing this hypothesis is that it could offer opportunities for

therapeutic intervention such as dietary supplementation with appropriate probiotics.

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Compliance with Ethics Guidelines

Competing Interests

None of the authors have any competing interests to declare.

Human Subjects Ethics Approval

This study was conducted with approval of the Emory University Institutional Review Board (Emory IRB Protocol # 00024933, PI: JL Fridovich-Keil) and all study volunteers completed appropriate informed consent prior to participation.

Conflict of Interest

Kelly A. Shaw declares that she has no conflict of interest.

Jennifer G. Mulle declares that she has no conflict of interest.

Michael P. Epstein declares that he has no conflict of interest.

Judith L. Fridovich-Keil declares that she has no conflict of interest.

Author Contributions

Kelly A. Shaw helped to design the study, collected much of the data, conducted most of the statistical analyses, wrote the original draft of the manuscript, and contributed to editing the final manuscript.

Jennifer G. Mulle contributed to the study design, statistical analyses, and manuscript editing.

Michael P. Epstein contributed to the study design, statistical analyses, and manuscript editing.

Judith L. Fridovich-Keil oversaw the project and contributed to the study design and preparation and editing of the final manuscript.

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