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APPROVAL SHEET

Gamma-glutamyltransferase levels at age 2 stratifies progression of portal hypertension in biliary atresia

By

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ABSTRACT COVER PAGE

Gamma-glutamyltransferase levels at age 2 stratifies progression of portal hypertension in biliary atresia

By

A. Jay Freeman
M.D., American University of the Caribbean, 2008

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An abstract of
A thesis submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of
Master of Science in Clinical Research
In Clinical Research
2016

ABSTRACT

Gamma-glutamyltransferase levels at age 2 stratifies progression of portal hypertension in biliary atresia

By A. Jay Freeman, MD

Background & Aims: Approximately 50% of patients with biliary atresia (BA) will survive with native liver (SNL) past 2 years of age. Most, but not all, BA SNL patients will develop sequelae of portal hypertension during childhood, yet there are no means to accurately predict those who will develop portal hypertension. We sought to determine if serum GGT levels could predict subsequent thrombocytopenia, as a marker of portal hypertension, in BA SNL patients after age 2.

Methods: Retrospective chart review was completed for 46 children diagnosed or cared for with BA SNL and born between November 2010 and January 2013 at three large pediatric liver centers. An association between elevated GGT level (≥ 100 U/L) at age 2 with platelet counts at 4-6 years of age was evaluated using a generalized linear mixed model.

Results: GGT ≥ 100 U/L at 2 years of age has a predictive negative relationship with thrombocytopenia at 4, 5 and 6 years of age ($p < 0.01$ at each age). BA subjects with a GGT ≥ 100U/L at age 2 have a statistically significant lower median platelet level compared to those patients with a GGT < 100 U/L at 2 years of age (160 vs. 211 x 10^3/μL, $p=0.04$). Additionally, patients with a GGT ≥ 100 U/L at 2 years of age exhibited significant yearly worsening of their thrombocytopenia until 6 years of age (median platelet level 109 x 10^3/μl by age 6, $p < 0.01$ for median platelet level at each yearly interval from 2 to 6 years of age).

Conclusion: A serum GGT ≥ 100U/L at 2 years of age predicts worsening thrombocytopenia through age 6 in patients with BA. Thus, serum GGT levels may serve as a serum biomarker for the development of portal hypertension in patients with BA SNL patients.

COVER PAGE

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ACKNOWLEDGMENTS

Saul J Karpen, MD, PhD for his exceptional mentorship and guidance both through the MSCR program and for my career as a whole.

Vicky Lee Ng, MD, FRCP for her assistance in study design and data collection at The Hospital for Sick Children in Toronto, Canada.

Sanjiv Harpavat, MD, PhD for his assistance in study design and data collection at Texas Children's Hospital in Houston, Texas.

Alexander Hrycko, MD for his assistance in data collection at The Hospital for Sick Children in Toronto, Canada.

Pinar Bulut, D for her assistance in data collection at Children's Healthcare of Atlanta in Atlanta, Georgia.

Traci Leung, PhD for her assistance with the more rigorous statistical portions of the project, specifically her aid in SAS programing for CART analysis.

To the parents and patients who have to live with biliary atresia every day of their lives, they are the inspiration that pushed our work forward.

TABLE OF CONTENTS

INTRODUCTION

Biliary atresia (BA) is an obstructive cholangiopathy of unknown etiology with an estimated incidence between 1/5,000 and 1/20,000 (1). The diagnosis of BA is suspected based on clinical indicators, but is associated with high levels of the cholangiocyte-specific serum marker gamma-glutamyl transferase (GGT). Although no definitive therapy exists for patients with BA, the standard of care is to perform a palliative procedure known as a Kasai hepatoportoenterostomy (HPE) as a way to extend the life of the native liver and serve as a bridge to ultimate therapy, which is liver transplantation (LT). Typical progression of the disease from early infancy includes common bile duct obstruction and rapid progression to biliary cirrhosis, requiring LT in approximately 50% of patients by 18-24 months of age (2,3). Several variables have been identified that predict outcomes post-HPE and clinical course through the first few months of life, but no marker has been identified to predict the clinical course of the ~50% of patients with BA who maintain their native liver past 2 years of age.

The primary indication for liver transplant in patients with BA is portal hypertension as result of biliary cirrhosis (1,4). Direct measurement of portal pressures in patients is clinically unrealistic. Thrombocytopenia (defined as serum platelet levels < 150 x $10^3/\mu L$) has been shown to be a reliable predictor of portal hypertension in cirrhotic liver diseases, including BA (5-8). The purpose of the study is to determine if GGT may accurately predict the development of portal

hypertension, as measured by serum platelet levels, among those BA patients who have maintained their native liver past 2 years of age (Figure 1).

This question was addressed by conducting a retrospective chart review of all patients with BA born between 1987 and 2010 who were cared for at 1 of 3 large pediatric liver transplant programs in North America. GGT at 2 years of age and platelet levels at 2, 4, 5 and 6 years of age were examined. Classification and Regression Tree (CART) analysis was utilized to determine an optimal cut point for GGT. Generalized linear mixed models were then employed to evaluate the association between GGT and platelets. Finally logistic regression models were used to determine the sensitivity and specificity of GGT at age 2 as a predictor of thrombocytopenia at 4, 5, and 6 years of age.

BACKGROUND

BA occurs when the biliary system, which is responsible for moving bile form the liver into the small intestine, fails to form properly (Figure 2) and is currently the principal indication for pediatric LT, accounting for nearly 40% of all pediatric liver transplants (2,3). While initial damage to the biliary tree from BA likely occurs in utero (9) or soon thereafter (10,11), clinical presentation often occurs in the first few weeks of life with the classic triad of (i) jaundice with persistent cholestasis (yellow-appearing baby), (ii) acholic/pale colored stools, and (iii) an enlarged liver (hepatomegaly) (1). For those patients diagnosed early in the first few months of life and without features of end-stage cirrhosis, the current standard therapy is a Kasai HPE (Figure 3), ideally performed before 45 days of age (3,12). Unfortunately, for ~40-50% of patients with BA, the Kasai HPE is ineffective, as evidenced by persistent cholestasis and progressive complications of biliary cirrhosis, usually by three months after HPE (1,12-15). Patients who present after 120 days and/or those with early cirrhotic changes on liver biopsy are often not surgical candidates and typically develop end-stage liver disease within months. Thus, there is clinically relevant evidence soon after presentation and by three months post-Kasai HPE indicating those who would benefit from an evaluation for transplantation (15). However, there is incomplete information regarding the clinical course of the ~50% of children with BA who do not require early LT by age 2 and survive with their native liver (SNL).

Avoidance of early transplantation in BA does not usually lead to an absence of liver-related sequelae in those who achieve SNL status at age 2. One of the major complications of BA is the development of portal hypertension, which is the most common indication for liver transplant among patients with BA. Shneider, et al. previously reported that clinically definable portal hypertension was present in two-thirds of a North American cohort of long-term BA survivors with their native livers, using thrombocytopenia and/or splenomegaly as a priori research criteria to indicate portal hypertension (8). Following a cohort of 219 BA SNL patients over 5 years of age, Ng et al., from the NIH-supported ChiLDReN consortium found that > 98% have clinical or biochemical evidence of chronic liver disease, with indicators of portal hypertension such as splenomegaly, thrombocytopenia, ascites, and variceal hemorrhage seen in 56%, 43%, 17%, and 9% of the cohort, respectively (16). A long-term large French study revealed that over 80% of BA SNL subjects who reached 20 years of age had evidence of cirrhosis (17). Moreover, those that reached 2 years of age with native liver often developed serious complications of portal hypertension or cholestasis necessitating liver transplantation at various time points during childhood, evidenced by the sequential reductions in SNL in older patients: 5 y (40%), 10 y (36%), 15 y (32%) and 20 y (30%) (1,4). Thus, the majority of BA subjects will ultimately develop progression of complications of fibrosis, cirrhosis and portal hypertension leading to death or LT during childhood. However, not all BA SNL patients will experience progression, or even evidence of, portal hypertension

with up to 10% showing no evidence of chronic liver disease in some studies and SNL status at 20 years of age reported as high as 20% (17-19).

A major impediment to stratifying the progression of portal hypertension in those BA SNL subjects who avoid the need for LT before age 2 involves a lack of knowledge of markers that correlate with disease progression. While early intervention with Kasai HPE predicts a greater chance of jaundice clearance and avoidance of LT (with total serum bilirubin at 3 months post-HPE as a validated marker), biochemical or clinical data that model the course for BA SNL subjects are not readily available (3,4,12,15,20-24). Portal hypertension at time of the Kasai procedure has been associated with higher failure rates, and is a strong indicator of hepatic dysfunction post surgically, but the association is only applicable to those patients undergoing early LT prior to 2 years of age (25). Following 163 older BA subjects from the ChiLDReN study, Shneider et al identified patients as having “definite” (49%) or “possible” (17%) evidence of portal hypertension using a platelet count < 150,000/μL, splenomegaly, or complications of portal hypertension (e.g. ascites, variceal bleeding) as defining criteria (8). Thus, there is a potential to use a common laboratory test (i.e. platelet count) or physical examination (spleen size by palpation) to define portal hypertension in these subjects, but the means to predict or stratify any progression of the clinical course of BA SNL subjects remains elusive.

Gamma-glutamyl transferase (GGT) is a microsomal enzyme constituent of the epithelial cells that line the biliary tract (cholangiocytes) that is highly associated with cholestatic liver disease processes and becomes elevated in serum when biliary tract damage occurs as in cholangiopathies such as BA (26). Although an elevated GGT may be seen in a number of neonatal liver diseases, mean GGT levels are 3-fold higher on average in BA subjects than in other neonatal liver diseases, with a GGT > 300 U/L in the first 8 weeks (normal range 12-132 U/L) of life strongly suggesting the diagnosis of BA (27,28). Moreover, although other serum markers such as alkaline phosphatase has been validated in adult biliary tract diseases as a biochemical marker for testing efficacy of novel treatments (29,30), use of this enzyme as a liver marker in BA is precluded due to non-hepatic sources (i.e. bone), especially in childhood where bone turnover is high during periods of growth. Finally, the majority of BA SNL subjects have elevated GGT levels that can vary over time, yet whether the degree of elevation correlates with outcomes are unknown (16). Intriguingly, in adults, GGT can serve as a biochemical marker of efficacy in the treatment of biliary diseases such as primary biliary cholangitis, as well as alkaline phosphatase (31), which strongly suggests that were it utilized and validated, GGT levels in pediatric biliary tract diseases like BA would make an ideal, readily available marker to explore in developing a method to predict the development of portal hypertension and long-term disease course. If shown to be predictive, GGT levels could ultimately serve as a potential marker to monitor the efficacy of therapeutic interventions.

Thus the aim of this study was to determine if serum GGT levels were able to accurately predict the level and degree of portal hypertension (as measured by serum platelet count) in children with BA whom have maintained their native livers past 2 years of age.

METHODS

Hypothesis: We hypothesized that the degree of GGT elevation at 2 years of age would serve as a marker for risk of progression of biliary tract fibrosis during the early childhood years through 6 years of age, allowing stratification of BA SNL subjects by subsequent risk of progression of features of portal hypertension. The hypothesis was tested through the following three specific aims:

1. Determine a clinically relevant cut point for GGT at 2 years of age that correlates with thrombocytopenia (defined as serum platelet level < 150 x 10^3/μL), an accepted surrogate biomarker for portal hypertension in BA
2. Evaluate the association between an elevated GGT at 2 years of age with platelet levels at 4, 5, and 6 years of age utilizing generalized linear mixed models
3. Calculate the sensitivity and specificity of subsequent regression models to identify risk of progression.

Study design: A multicenter, retrospective cohort study was performed in which the underlying population was all children with BA diagnosed and/or cared for between November 1, 2010, and January 31, 2013 at one of three large pediatric liver transplant centers in North America (Children's Healthcare of Atlanta/Emory University School of Medicine in Atlanta, GA, Texas Children's Hospital/Baylor College of Medicine in Houston TX and The Hospital for Sick Kids/University of Toronto in Toronto, Canada). Time zero for the study was 2 years of age among BA SNL patients. A medical chart review was completed for all children

diagnosed with BA. Diagnosis of BA was established by accepted guidelines including surgical intraoperative cholangiogram and/or pathological examination of the bile duct remnant recovered at time of Kasai HPE in all cases. A small subset of subjects was diagnosed with BA at outside centers, but all liver biopsies and surgical specimens were examined and diagnoses confirmed by senior pathologists at one of the three participating study centers.

The variables collected for analyses are considered part of routine medical care for patients with BA and where readily obtained form the patients' medical chart. These included: serum GGT at 2 years of age (exposure), and serum platelet levels at 4, 5 and 6 years of age (outcome), age, gender, race and institution (covariates). In instances where multiple labs were obtained within one calendar year, the value closest to the patient's most recent birthday was selected.

Characteristics of the study population: Eligible subjects were born between 1987 and 2010, diagnosed with BA (diagnosed or confirmed at one of the participating study institutions), and survived with their native liver past two years of life. Patients were excluded if they died (n=1) or received a liver transplant before two years of age (n=38), were not yet four years of age at time of review (n=5) or had not undergone yearly laboratory testing for GGT and/or platelet level (n=2) (Table 1). In instances where laboratory data were missing for a patient, the patients were excluded from the analysis for that year and no imputations were performed

(complete case analysis). A total of 46 children met criteria and all were included in the analyses.

Analytical Plan: Baseline descriptive statistical analysis for GGT and platelets were performed (both as continuous variables), as well as for the covariates as categorical variables. Additionally all variables were assessed for missingness. Initial review showed the distribution of platelets to be slightly skewed, with the distribution of GGT being very skewed. Given these finding we decided to report median values with 95% confidence intervals for all subsequent analyses as this was felt to be a better measure of central tendency. Scatter plots were created to determine visually whether there appeared to be an inverse relationship between GGT at 2 years of age and platelet levels at 2, 4, 5 and 6 years of age.

To address Aim #1 (determine a clinically relevant cut point for GGT at 2 years of age that correlates with thrombocytopenia), Classification and Regression Trees (CART) analysis was utilized to calculate an optimal cut point of GGT that correlated with thrombocytopenia (platelet count $< 150 \times 10^3/\mu L$) at age 6. GGT as a continuous variable and platelets at 6 years of age (as a dichotomous variable: thrombocytopenia vs. no thrombocytopenia) were included in the analysis, as well as the covariates gender, race, and institution (categorical variables). CART analysis produced a GGT level cut point of 109 U/L at age 2 that correlated with thrombocytopenia at age 6. However, since the goal was to determine a clinically significant cut point, we utilized a practical cut point GGT

level of 100 U/L for subsequent analyses, since the findings did not significantly differ from those utilizing the calculated value of 109 U/L.

With a cut point determined, descriptive statistics were once again performed to compare covariates (gender, race and institution) between the "high" GGT group (GGT $\geq$ 100 U/L) and the "normal" GGT group (<100 U/L). For this set of descriptive analysis GGT was considered a dichotomous variable (high or normal) while platelets were utilized as a continuous variable. Student's t tests were used to determine if statistical significant differences existed between the two groups. P-values < 0.05 were considered significant.

Addressing Aim #2 (evaluate the association between a high GGT level ($\geq$ 100 U/L) at age 2 with platelet counts at 4, 5 and 6 years of age controlling for race, gender and study location) we conducted a univariate analysis utilizing generalized linear mixed models. Mixed modeling was used to account for the lack of independence of platelet levels within patients at each age. For the analysis platelets were utilized as a continuous variable while all other variables were categorical. The variables for the model included included:

1) GGT ≥ 100 (yes vs. no)
2) Gender (male vs. female)
3) Race (Caucasian vs. African American vs. Asian vs. Hispanic)
4) Institution (Texas Children's Hospital vs. Children's Healthcare of Atlanta vs. Hospital for Sick Children)

To ensure that GGT was a better predictor of thrombocytopenia at ages 4, 5 and 6 years, independent of the baseline platelet level, platelet level at age 2 was added to the model and recalculated as a continuous variable. Variables with a p value < 0.05 were considered statistically significant and remained in the subsequent models.

Given the covariates gender, race and institution were not considered influential to the model; we were able to proceed to Aim #3, calculating the sensitivity and specificity of subsequent regression models. Univariate logistic regression models were used with GGT as a categorical predictor variable (high vs. normal) to determine how well the models performed at predicting thrombocytopenia at 4, 5 and 6 years of age (dichotomous outcome variable). Given the nature of the predictive model, likelihood estimates were not of importance and were not reported, but p-values were accessed to confirm that GGT remained a significant predictor in the model (p value < 0.05 considered significant). Since sensitivity and specificity of the model was of primary importance, receiver operating characteristic (ROC) curves were constructed for each model along with subsequent calculation of the curve's area under the curve (AUC). Sensitivity and specificity was calculated for each of the 3 models. The model used was: Logit (platelets < 150 x $10^3/\mu L$) = $\beta_o + \beta_1 GGT + \varepsilon$ (a separate model was created at 4, 5 and 6 years of age).

Based on the results of our models, we were confident that GGT was a significant predictor of thrombocytopenia at 4, 5, and 6 years of age. Comparisons of median platelet levels between GGT < 100 U/L and GGT $\geq$ 100 U/L were conducting utilizing Student's t tests and a figure was created to show the difference in platelet levels between the two groups.

An overview of the study design is shown in Figure 4. SAS version 9.4 was utilized for all statistical analysis. Microsoft PowerPoint and Excel were utilized for constructing tables and figures.

RESULTS

Descriptive analysis of variables began simply by assessing missingness of the data. There was no missing data for GGT at 2 years of age, platelets at 2 years of age, or for any of the covariates (gender, race and institution). At 4, 5, and 6 years of age there were 2, 5 and 3 patients missing platelet levels respectively. It was decided that for the year that the patient was missing a platelet level that they would be excluded from that year's final model.

Among the categorical variables, of the 46 children who met inclusion criteria, there were slightly more females (n=25, 54%) and the majority were Caucasian (n=19, 43%), similar to other BA studies conducted in North America (3,14,17,21). As expected by United Network for Organ Sharing (UNOS) reported transplant volumes, Texas Children's Hospital contributed the most patients to the study (n=21, 46%), followed by The Hospital for Sick Children (n=14, 30%), then Children's Healthcare of Atlanta (n=11, 24%). Baseline demographics are shown on the left side of Table 2. Skewness was then assessed for the categorical variables, GGT and platelet levels. As shown in Figure 5, GGT at age 2 and platelet level at age 6 show considerable right-sided skewedness (GGT at 2 years of age: Skewedness 2.29, Kurtosis 7.05, Platelet level at 6 years of age: Skewedness 0.62, Kurtosis 0.68). Given the degree of skewness it was decided that medians with 95th percentile confidence intervals would be used when reporting appropriate findings. Scatter plots were then

created showing a visual representation of the inverse relationship between GGT at 2 years of age and platelet level at 2, 4, 5 and 6 years of age (Figure 6).

We sought to determine if there was a statistically significant relationship between initial GGT levels at age 2 (as a measure of ongoing peri-biliary damage/fibrosis) with platelet counts (as a measure of portal hypertension) through 6 years of age. CART analysis was used to determine if there was a clinically relevant cut point for GGT levels at 2 years of age that stratified BA patients by those with thrombocytopenia or without using the preselected platelet value <150 x 10^3/µl (the laboratory definition of thrombocytopenia) at age 6. Results of CART indicated a cutoff GGT level of 109 U/L at age 2 as discriminatory in separating the two groups. In particular, the analysis showed that those patients with a lower GGT (< 109 U/L) were highly likely to be categorized as having a normal platelet level (≥150 x 10^3/µL) at 6 years of age (i.e., an essentially normal GGT level at 2 years of age predicted a normal platelet level at 6 years of age). CART analysis also suggested that those patients with more advanced peri-biliary damage/fibrosis evident by a high GGT (≥ 109U/L) were more likely to be categorized as being thrombocytopenic (Figure 7). Although gender, race and institution were included in the CART analysis, they were not effective in further discriminating amongst patients. Given the aim was to determine a clinically effective cut point, the analysis was repeated with the practical GGT value of 100 U/L. The findings did not significantly differ using the new cut point and upheld the stratification observed utilizing the calculated

value of 109 U/L, and thus the cutoff off 100 U/L was used for all subsequent analyses and models.

With a cut point determined, baseline demographics were once again evaluated. There were no significant differences between the two groups among the covariates when stratified by GGT level at 2 years of age (Table 2). Median platelet levels along with interquartile range, maximum, minimum and 95% confidence intervals at 2, 4, 5, and 6 years of age were then calculated and stratified by GGT at age 2 (Table 3). It was observed that, among the high-GGT patients (>100 U/L), platelet levels appeared to be lower at 2 years of age and continued to decrease over the next 4 years, while those patients with BA and a lower GGT (<100 U/L) had platelet levels that appear to stay relatively unchanged (Table 3).

Given the apparent associations of low serum GGT values with higher platelet levels and vice versa, we sought to determine if those subjects with higher GGT levels at 2 years of age demonstrated greater degrees of portal hypertension, as indicated by a serum platelet < 150 x 10^3/μL, at subsequent ages. A generalized linear mixed model was first employed to determine if GGT at 2 years of age or any of the covariates were significant in predicting platelet level at 4, 5 and/or 6 years of age. Results of the model showed only GGT to be statistically significant, $p < 0.01$. Subsequently, platelets at 2 year of age were added to the model to determine if it might be a better predictor of future platelet trend but was

not found to be significant, p = 0.32. Thus, controlling for gender, race and institution of care, only GGT at 2 years of age was statistically significant in predicting platelet level at 4, 5 and 6 years of age.

Knowing that only GGT stratification was significant in predicting platelet level, logistic regression was employed to determine the relationship between GGT and yearly time points for platelets and to calculate the sensitivity and specificity of a GGT level ≥ 100 U/L at 2 years of age at predicting thrombocytopenia at 4, 5, and 6 years of age. The models performed well as evident by the calculated AUC (0.98, 0.91 and 0.95 at 4, 5, and 6 years respectively) from the produced ROC curves (Figure 8). The models showed that GGT $\geq$ 100 U/L at 2 years of age was discriminatory in predicting thrombocytopenia at 4 years of age (sensitivity=0.88, specificity=0.57), at 5 years of age (sensitivity=0.89, specificity =0.64) and at 6 years of age (sensitivity=0.94, specificity of 0.61).

Knowing that our cut point for GGT was significant in predicting thrombocytopenia, median platelet levels stratified by GGT (Table 3) were further evaluated. BA subjects with a high GGT (≥ 100 U/L) at age 2 had a statistically significant lower median platelet level compared to those patients with a GGT < 100 U/L (160 x 10^3/μL vs. 211 x 10^3/μL), and this discrepancy widened with each subsequent platelet measurement at ages 4, 5 and 6 years of age. It is relevant that those subjects with an initial elevation of GGT ≥ 100 U/L at age 2 showed a significant decline in median platelet level at each subsequent

measurement, while those BA subjects with lower GGT values (< 100 U/L) had essentially stable platelet counts over the next 4 years (Table 3). Platelet counts in subjects with high GGT values declined from a median (interquartile range) of 160 (127-231) to 109 (72-166) x 10^3/uL from ages 2 to 6. In comparison, those with low initial GGT levels were essentially unchanged during these same ages from 211 (182-300) x 10^3/uL at age 2 to 239 (159-320) x 10^3/uL at age 6 (Figure 9).

DISCUSSION/CONCLUSIONS

The first Kasai HPE was performed in 1959 (32). In the over 50 years since, it has become evident that the child's age at time of Kasai, classification type of BA, presence of other congenital defects, experience of the surgeon and episodes of cholangitis can all contribute to short-term success after the operation (4,19,20,24). However, predictive models in BA have focused largely on forecasting immediate post-Kasai success and have yet to provide a reliable marker or clinical means to identify those patients likely to obtain long-term survival of their native liver. Moreover, there are no current indices or biomarkers that accurately predict the clinical course of those who have not needed an early LT (before age 2). This study aimed to utilize a group of well-characterized subjects with BA SNL at age 2 from three large North American liver centers to determine if the readily available serum GGT level can be utilized as a highly sensitive marker to predict subsequent changes in serum platelet values over the next 4 years. When stratified by a GGT < or ≥ 100 U/L at age 2, there was a clear distinction not only in initial platelet counts (211 vs. 160 x 10^3/μL), but also in the progression of thrombocytopenia seen exclusively in those with a high GGT at age 2. Among those BA patients with a GGT ≥ 100 U/L, median thrombocytopenia developed by 4 years of age (median value of 143 x 10^3/μL), and worsened each subsequent year to median values of 106 x 10^3/μL at age 5 and 109 x 10^3/μL at age 6. This progression was not seen in those with a GGT < 100 U/L at age 2, whose platelet counts were, on average, essentially unchanged from the value at 2 years to a median 239 x 10^3/μL at age 6. Thus,

already at 2 years of age, those patients with BA who are likely to develop and worsen their thrombocytopenia can be predicted by their serum GGT levels at age 2.

Also of interest, among the 46 patients who met inclusion criteria, 6 are known to have undergone liver transplant since the conclusion of our review. Of those 6 patients, 5 had GGT levels > 100 U/L at 2 years of age. Given the small sample size and lack of longitudinal data on the majority of the remainder of the cohort, no statistical analysis or interpretation could be explored at this time.

Thrombocytopenia is a well-established indirect marker of portal hypertension and hepatic fibrosis and has been shown to be an independent risk factor for the development of esophageal varices, a life-threatening sequelae of portal hypertension (5-7) with progressively decreasing levels imparting increased risk of variceal formation and bleeding (33-35). Moreover, thrombocytopenia is a defining measure of portal hypertension in BA (8). This study links serum GGT levels to future platelet levels that are likely to be a viable surrogate for the development of portal hypertension and its sequelae. Since the sequelae of portal hypertension in BA SNL occurs at a low rate, larger long-term studies will be needed to identify if GGT levels at age 2 predict these clinical events.

The ability to predict the progression of thrombocytopenia in the approximately 50% of BA patients who have maintained their native livers at 2 years of age will

be useful in a number of clinical applications. This study suggests that serum GGT at age 2 can stratify those subjects who may or may not develop progressive thrombocytopenia over the next few years, and thus can serve as a new biomarker. To date, there are no biomarkers that can predict progression of BA SNL patients. This is of particular importance since the United Network for Organ Sharing (UNOS), which manages LT allocation, utilizes the Pediatric End-Stage Liver Disease (PELD) score to determine organ allotment to patients on the transplant list, which imperfectly corresponds with disease severity (36,37). Perhaps GGT or platelet count progression may serve such a role after careful evaluation and currently may help guide the clinician in asking for an exception score above a patient's natural PELD score which is done to show a patient is "more ill" - and thus can get higher priority for a LT - than their score represents.

Another pertinent decision point in the care of patients with BA is when and how frequently to perform surveillance endoscopy to detect and address esophageal varices. In order to prevent bleeding, the hepatologist may perform endoscopy and place "bands" around the varices in order to prevent bleeding. Current clinical practice is caregiver- and patient-specific, often relying upon following platelet levels at unspecified time intervals to identify at risk patients, or simply waiting for a bleeding event to occur. By knowing those patients at highest risk to develop thrombocytopenia and at what rate, meaningful clinical practice guidelines may be able to be developed to determine which patients would benefit from surveillance endoscopy.

Beyond the clinical implications, these findings may provide a new, easily obtained biomarker for future drug and/or intervention studies in BA SNL subjects. Note that the majority (28/46, 60%) of BA SNL subjects were in the GGT ≥ 100 U/L group, suggesting a prime group of patients to undergo testing of newly FDA approved antifibrotic or anticholestatic agents since existing studies have not identified effective agents. Ursodeoxycholic acid (UDCA), a common drug used in cholestasis, has been studied sparingly in BA with some promising results, although in cohorts of 16 patients or less (38,39). These studies have followed routine hepatic markers such as alanine and aspartate aminotransferases (AST and ALT) and GGT, but without any clear endpoint of how a change in these markers represents a physiologic change or measure of function in the liver of BA patients. These studies have also focused on short-term changes over approximately 6 months and only in younger patients immediately post Kasai. Large studies of UDCA or other agents have not been pursued in part due to the lack of a clinically meaningful marker that measures outcomes. These findings show that GGT, a readily available serum marker that can be performed in any essentially inpatient or outpatient laboratory, may be a meaningful target for studying various medical therapies and/or surgical procedures in BA patients who retain their native liver past 2 years of age.

The main strengths of this study are that is a “large” study in terms of BA research utilizing a well-described cohort from three of the largest pediatric liver

centers in North America. Additionally, the findings have direct clinical implications as outlined above and utilize a serum biomarker that can processed in essentially any laboratory worldwide. While the results of this study are promising, there are limitations as well. Despite utilizing the databases of three large pediatric liver transplant centers, we were still only able to identify 46 patients that met inclusion criteria, which is still a relatively small sample size. Additionally, due to the sample size we are limited by how many variables we can evaluate and there may be additional confounders (both measured and unmeasured) we are not accounting for or other variables that may contribute more to the model (i.e. socioeconomic status, spleen size on physical exam, etc.). The AUC calculations from our models must be interpreted with some caution, especially at age 6, as platelet level at age 6 was utilized to determine GGT discriminatory cut points and therefore likely over estimate the predictive ability by some factor. Larger sample sizes in future studies will allow for crosss-validation and solve this problem. Finally, given the exclusion of patients that died prior to 2 years of age (n=1) and the tendency of these three centers to care for patients with more advanced disease within their respected regions/country, a selection bias may be present, although unlikely to be of a large magnitude.

Future studies that examine larger cohorts prospectively are needed to determine what effect, if any, other covariates such as socioeconomic status, physical exam findings, including splenomegaly, or other variables might have on the rate of platelet decline in patients with BA. Ideally patients will be followed for

a longer period of time and outcomes such as the timing of those receiving transplant (as in the 6 patients from our cohort) can be further explored. Although still retrospective, the Childhood Liver Disease Research Network (ChiLDReN), which currently contains information on over 2,000 patients with BA, could be accessed and allow exploration over a longer duration of disease course as well as evaluation of additional covariates and multivariate analysis of multiple disease outcomes.

Conclusion: To our knowledge, this study is first to show that an elevated GGT $\geq$ 100U/L at 2 years of age predicts early and progressive thrombocytopenia in patients with BA. This is of high clinical importance as platelet counts < 150 x 10^3/μL are associated with portal hypertension and hepatic fibrosis and is independently known to significantly increase a patient's risk of esophageal varices and bleeding. Patients with GGT levels ≥ 100 U/L at 2 years of age had lower platelet values over the next 4 years (median 160 to 109 x 10^3/μL), while those with GGT < 100 U/L had stable, normal platelet values over this same time period. These studies may have a profound impact on determining which patients require earlier screening for significant sequelae of portal hypertension (e.g., esophageal varices). Finally, these findings provide support for GGT as a new biomarker for clinical progression as well as any proposed therapeutic interventions for patients with BA.

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FIGURE 1:
Disease progression in biliary atresia

Figure 1:

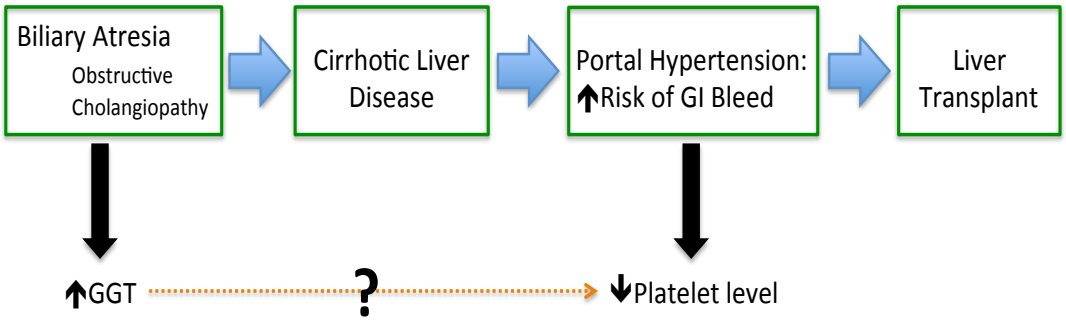

Figure 1: Disease progression in biliary atresia. This diagram shows the natural disease progression in patients with biliary atresia. The purpose of the study is to determine if GGT (as a marker of peri-biliary inflammation/fibrosis) accurately predicts the development of portal hypertension as measured by serum platelet level in patients with biliary atresia who have maintained their native liver past 2 years of age.

FIGURE 2:
Illustration of biliary atresia

Figure 2:

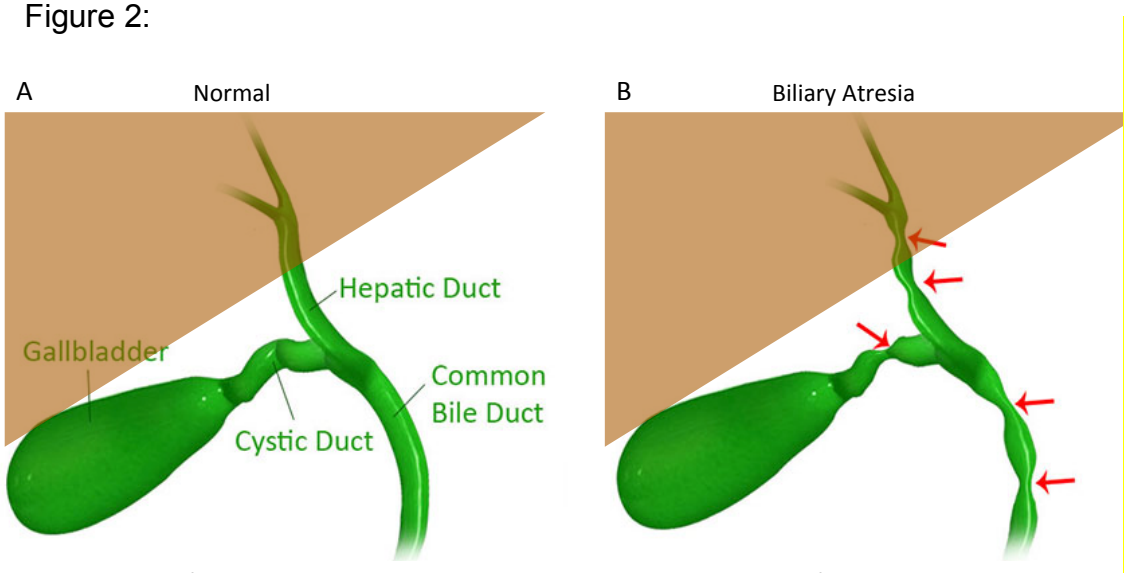

Figure 2: **Illustration of biliary atresia**. The biliary system when normally formed drains bile form the liver and either stores it in the gallbladder or empties it into the intestine (A). Biliary atresia (B) occurs when the biliary ducts that transport bile form the liver into the small intestine fail to form fully resulting in a fibrous cord rather than a hollow tubular structure (red arrows). This result in bile accumulation within the liver that is toxic to the liver cells, which leads to fibrosis, and ultimately to cirrhosis.

FIGURE 3:
Patient anatomy status post Kasai hepatoportoenterostomy

Figure 3:

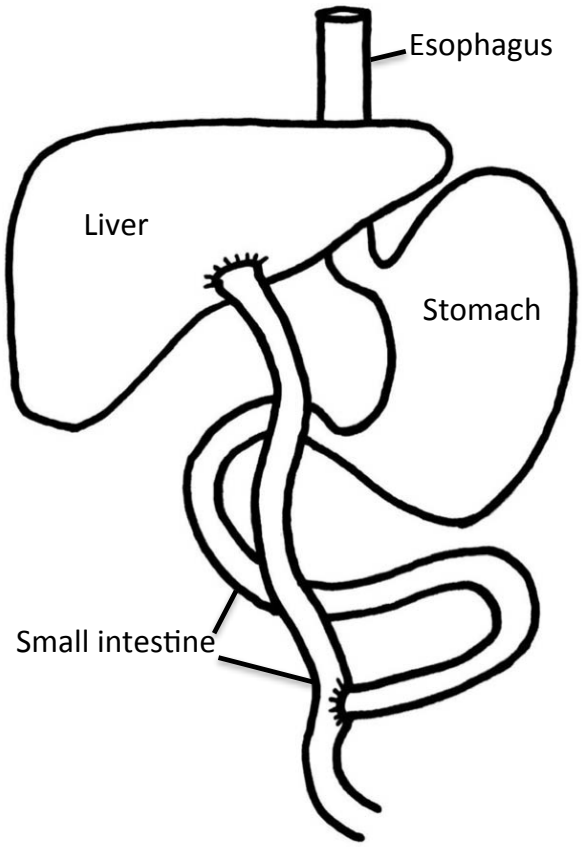

Figure 3: **Patient anatomy status post Kasai HPE**. During the Kasai HPE, a portion of the small intestine will be cut. The distal portion will be connected to the liver at the point where the bile ducts leave the liver in hopes to restore bile flow. The proximal portion will then be reconnected back to the small intestine further down.
(Imaging used and modified with permission, courtesy of Simon Ling, MD, The Hospital for Sick Children, Toronto, Canada)

TABLE 1:
Inclusion and Exclusion Criteria

Table 1:

Inclusion Criteria	Exclusion Criteria
Diagnosed with BA between November 2010 and January 2013	Unconfirmed diagnosis of BA by participating study center
Diagnosed made or confirmed at 1 of the 3 participating study institutions	Deceased prior to 2 years of age
Survived with native liver past 2 years of age	Received liver transplant prior to 2 years of age
At least 4 years of age at time of review	Not yet 4 years of age at time of review
Undergone routine laboratory testing yearly	Missed more than 1 year of routine laboratory testing

Table 1: Inclusion and exclusion criteria.

FIGURE 4:
Study overview

Figure 4:

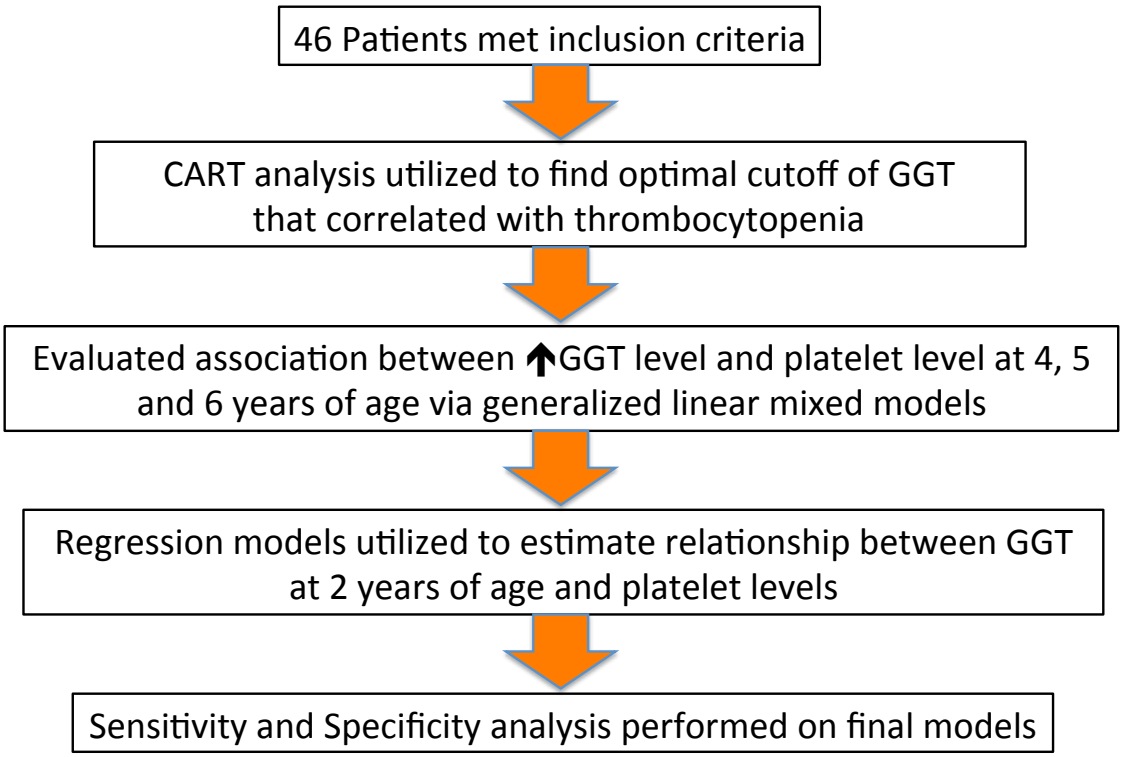

Figure 4: Study overview. Diagram outlines the study overview for the 46 patients who met inclusion criteria and highlights key statistical analyses conducted including Classification and Regression Tree (CART) analysis, general linear mixed models and logistic regression models to estimate sensitivity and specificity of the final predictive models.

TABLE 2:
Baseline demographics

Table 2:

Characteristics	GGT<100 U/L at 2 years	GGT≥100 U/L at 2 years	p-value
All (n=46)	18 (39%)	28 (61%)	0.39
Gender			0.37
Male (46%)	10 (48%)	9 (47%)	
Female (54%)	8 (32%	17 (68%)	
Race			0.26
Caucasian (43%)	10 (53%)	9 (47%)	
African American (14%)	2 (33%)	4 (67%)	
Asian (25%)	4 (36%)	7 (64%)	
Hispanic (18%)	1 (12%)	7 (88%)	
Institution			0.59
Texas Children's Hospital (46%)	10 (48%)	11 (52%)	
Children's Healthcare of Atlanta (24%)	3 (27%)	8 (73%)	
The Hospital for Sick Children (30%)	5 (36%)	9 (64%)	

Table 2: **Baseline Demographics.** Baseline demographics of study subjects stratified by GGT < or ≥ 100 U/L. p-values calculated by χ^2 or Fisher's exact test as appropriate.

FIGURE 5:
Histograms of GGT and platelets

Figure 5:

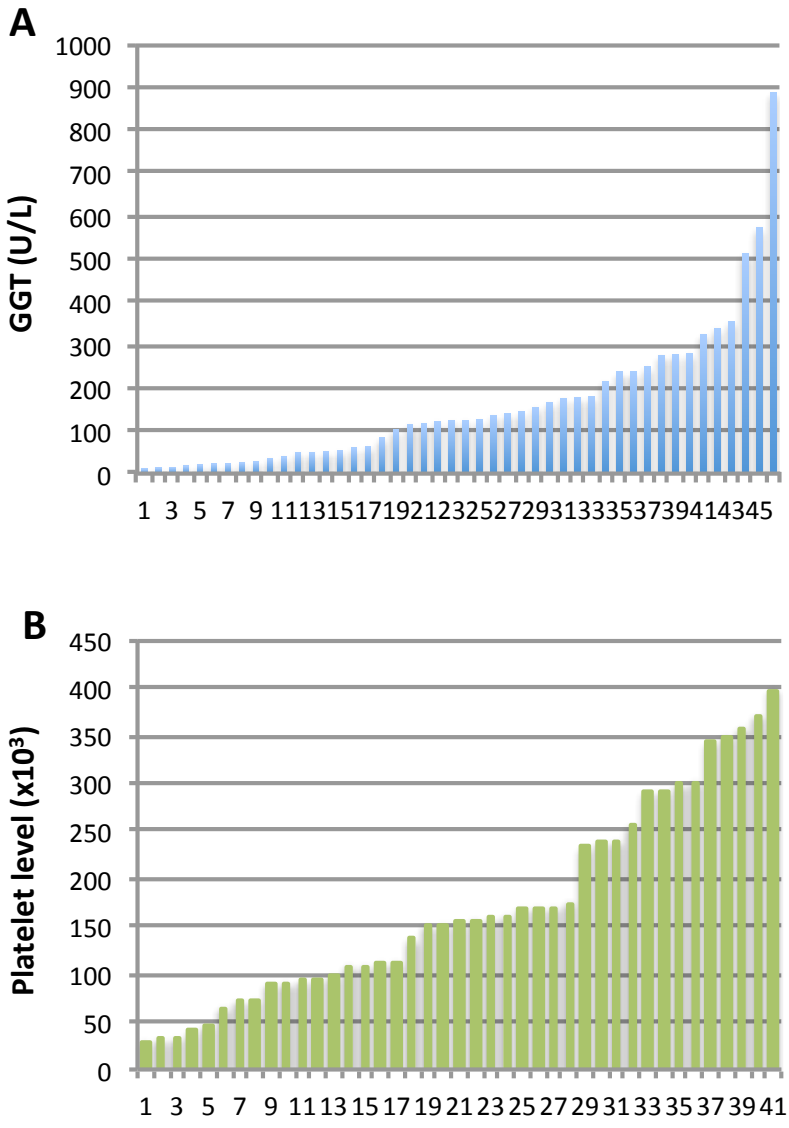

Figure 5: Histograms of GGT and platelets. Histogram A shows the distribution of serum GGT at 2 years of age (y-axis) with an obvious right-sided skewedness (Skewedness 2.29, Kurtosis 7.05). Similarly, histogram B shows a less pronounced right-sided skewedness (Skewedness 0.62, Kurtosis 0.68) of platelet levels at 6 years of age. Non-specific identifiers of patients are shown on the y-axis.

FIGURE 6:
GGT at age 2 inversely correlates with platelet counts at each age

Figure 6:

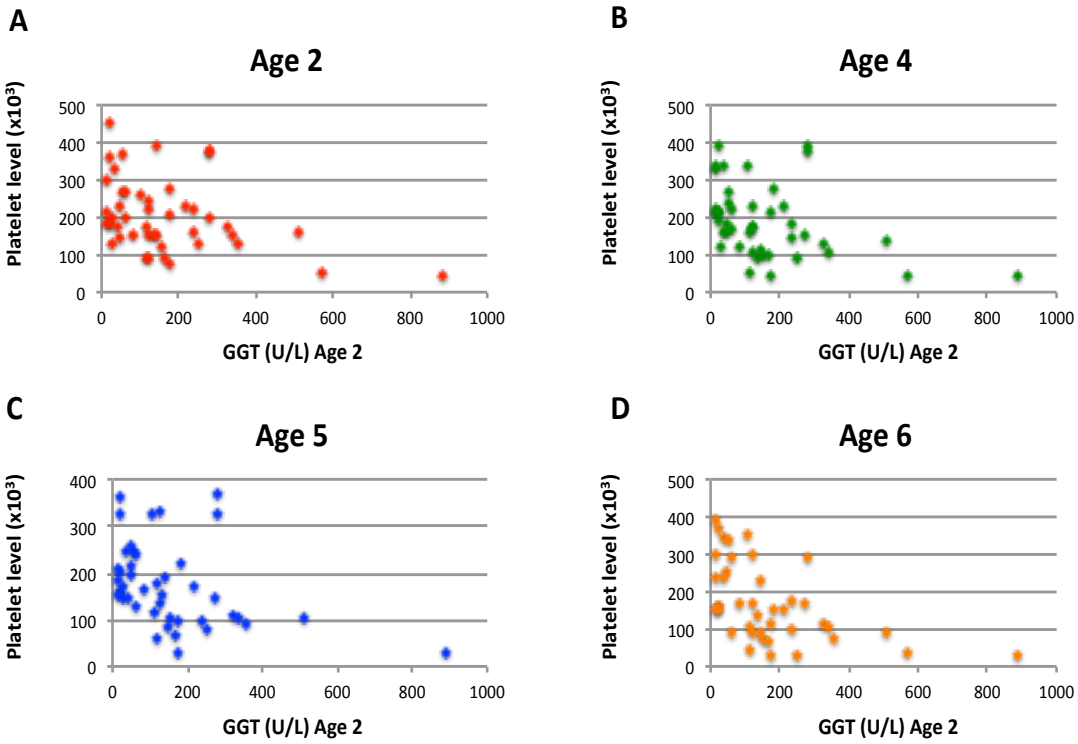

Figure 6: **GGT at age 2 inversely correlates with platelet counts at each age.** Scatter plots showing the inverse relationship between GGT at 2 years of age (x-axis) and platelet level (y-axis) at 2 years of age (A), 4 years of age (B), 5 years of age (C), and 6 years of age (D) among patients with BA, SNL past 2 years of age.

FIGURE 7:
CART Analysis

Figure 7:

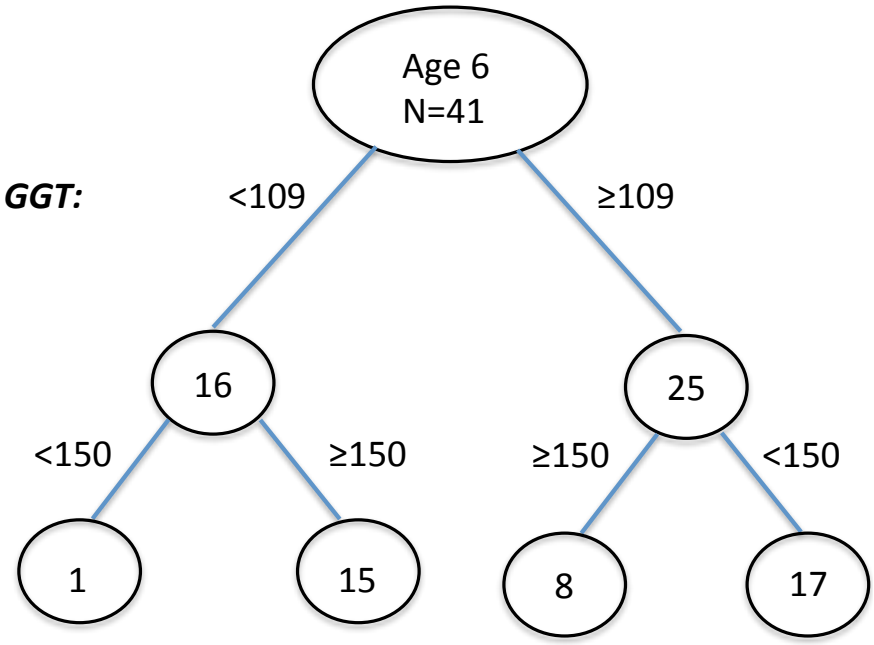

Figure 7: CART analysis. Results of CART analysis indicated a cutoff GGT level of 109 U/L at age 2 as discriminatory at predicting the development of thrombocytopenia 6 years of age. A lower GGT was particularly effective in discriminating between those patients who would go on to have a normal platelet level, compared to those that would develop thrombocytopenia at 6 years of age.

TABLE 3:
Median platelet levels with IQR, maximum, minimum and 95% confidence intervals stratified by GGT

Table 3:

Platelet Counts related to GGT level at Age 2								
	GGT < 100				GGT > 100			
Age	2	4	5	6	2	4	5	6
Minimum	131	122	130	90	46	45	31	30
25th percentile	182	189	158	159.5	127	100	89	72.3
50th percentile (median)	211	221	195	239	160	143	106	109
75th percentile	300	302	244	320	231	212	163	166
Maximum	459	391	368	397	381	375	221	357
95% CI (mean)	203--286	203—277	176--239	201--288	149--218	125--200	106--187	97--161
n=	18	16	17	18	28	26	23	28

Table 3: Median platelet levels with IQR, maximum, minimum and 95% confidence intervals stratified by GGT. Among the high GGT group (>100 U/L) platelet levels appear to be lower at 2 years of age and continue to decrease over the next 4 years while those patients with BA and a lower GGT (<100 U/L) appear to stay relatively unchanged.

FIGURE 8:
ROC curves with AUC for logistic regression models at 4, 5 and 6 years of age

Figure 8:

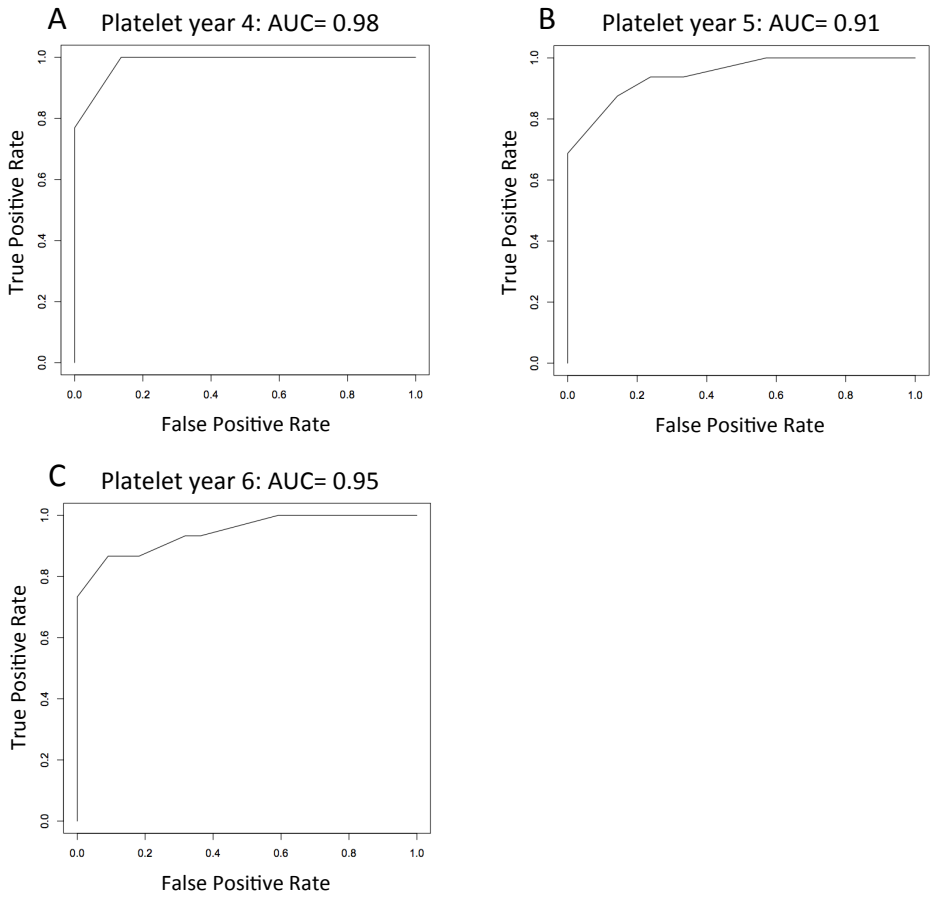

Figure 8: ROC curves with AUC for logistic regression models at 4, 5 and 6 years of age. ROC curves with false positive rates on the x-axis and true positive rates on the y-axis. Calculated AUC for each ROC curve was >0.9 suggesting the model including GGT at age 2 alone performed well at discriminating between the outcome (thrombocytopenia) at 4 years of age (A), 5 years of age (B) and 6 years of age (C).

FIGURE 9:
Stratification of platelet levels by GGT < or ≥ 100 U/L at age 2

Figure 9:

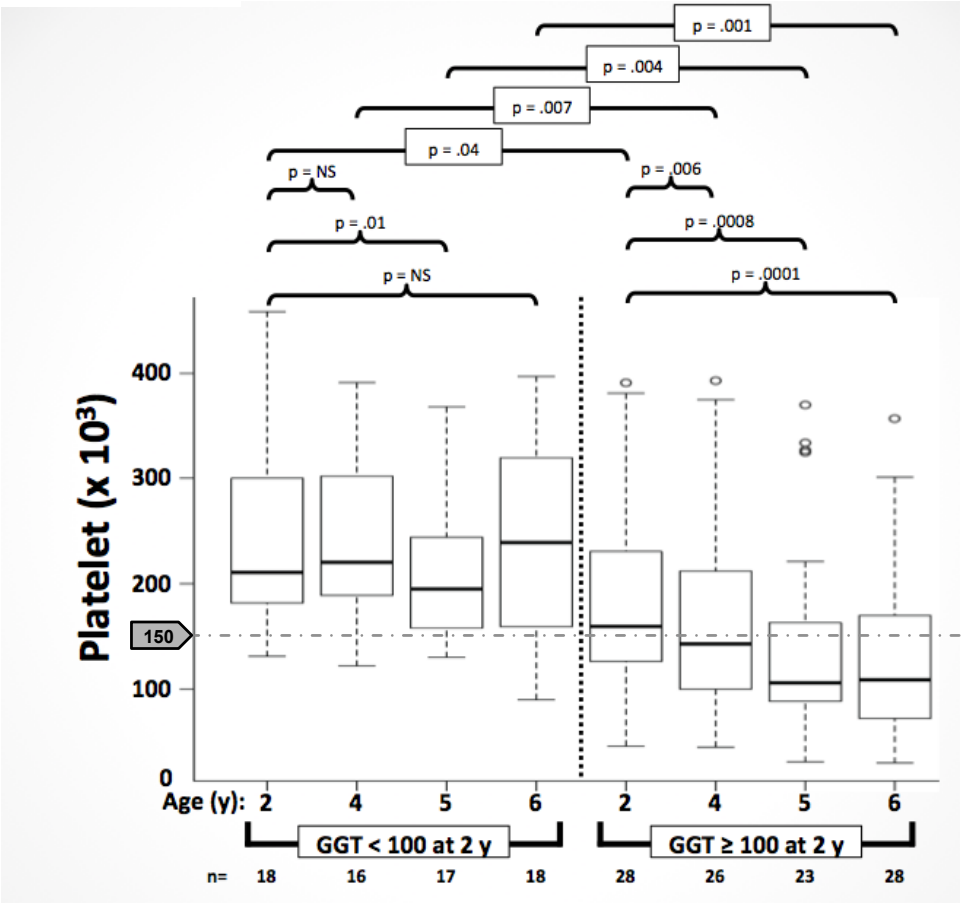

Figure 9: **Stratification of platelet levels by GGT < or ≥ 100 U/L at age 2**. At ages 2, 4, 5, 6, box and whisker plots (black bar represents median, box extends 25th-75th percentiles, and whiskers, 5th-95th percentiles) relating platelet levels at the indicated ages. ***Left*** represents those with GGT < 100 U/L at 2. ***Right***, GGT ≥ 100U/L at 2. n denotes subject numbers at each age group. Paired comparison p values by Fisher exact tests noted above the plot (within group, [braces], between groups [boxed] p values), although multiple comparisons not completed. The defining level for thrombocytopenia, 150,000/ml, is delineated by the texted block arrow and interrupted dashed gray horizontal line.