I, James E Squires, hereby submit this original work as part of the requirements for the degree of Master of Science in Clinical and Translational Research.

It is entitled: Diagnostic and Predictive Value of Serum Biomarkers in Biliary Atresia

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Diagnostic and predictive value of serum biomarkers in biliary atresia

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by

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ABSTRACT

Purpose: Biliary atresia (BA) is a fibro-inflammatory obstruction of bile ducts that manifests as neonatal cholestasis. Gold standard for diagnosis is operative cholangiogram that distinguishes BA from neonatal intrahepatic cholestasis (IHC). After diagnosis, a hepatopanenterostomy (HPE) is performed to restore bile drainage, but the response is variable. The aim of this study was to identify biomarkers that differentiate BA from IHC, characterize subgroups of BA patients and predict which subgroups will respond favorably to HPE.

Methods: We obtained serum samples from 72 infants at the time of diagnosis of BA and 66 age-appropriate disease controls with IHC as part of an ancillary study to the Childhood Liver Disease Research and Education Network (ChiLDREN); 5 healthy age-matched infants served as normal controls. We used a multiplex assay to quantify the serum concentration of 33 cytokines, chemokines, VEGF and sICAM1 for all subjects. Bilirubin levels at 3 months post-HPE assessed response to HPE in the BA cohort. We used classification and regression tree (CART) analysis to see if biomarkers discriminate BA from IHC. We then applied CART and cluster analysis to determine if biomarker profiles sub-classify BA patients and predict response to HPE.

Results: Individually and as a group, serum biomarkers distinguished cholestatic infants from healthy controls but single biomarkers had a limited capacity to consistently discriminate BA from IHC. Applying CART analysis, we found that a combination of VEGF, sICAM1 and 11 cytokine/chemokines differentiated BA from IHC, with an area under the ROC curve (AUC) of 0.93 (CI 0.87, 0.99), sensitivity of 96% and specificity of 83%. Mining the biomarker profiles for only BA patients, CART and cluster analyses uncovered a unique expression profile of sICAM1 and 14 cytokines/chemokines that divided the BA group into subgroups of infants with biomarker levels similar to normal controls (N=51) and infants with higher levels (N=21) above controls. Most notably, high levels of biomarkers were associated with lower conjugated/direct bilirubin (<1 mg/dL) 3 months after HPE (high=81% vs. low=57%, odds ratio 3.22, 90% CI 1.16, 8.99, P=0.045 by Fisher’s test). Further independent CART analysis of biomarkers for BA patients found that IL15, MCP3, MDC, IL17a and MCP1 collectively identified those likely to achieve conjugated/direct bilirubin <1 mg/dL; (AUC of 0.912 [CI 0.801,1.00], sensitivity of 93% and specificity of 86%).

Conclusions: Quantification of serum cytokines, chemokines, VEGF and sICAM1 identifies a biomarker profile with discriminatory value for BA, with the potential to sub-classify BA patients and predict response to HPE.
ACKNOWLEDGEMENTS

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Background:

Biliary atresia (BA) is a progressive inflammatory and obstructing cholangiopathy of infants affecting roughly 1 in 5,000-8,000 live births (1, 2). Despite its distinctive anatomical abnormality, the clinical and biochemical presentation of children with BA is not substantially different from that of other infants with diseases of neonatal intrahepatic cholestasis (IHC) (3). Furthermore, its hallmark physical feature, jaundice, is often appreciated in the neonatal period of normally developing infants for a variety of reasons (4). Consequently, timely referral with appropriate surgical management can be delayed. The resulting delay is known to negatively impact the clinical outcomes of children with this devastating disease (5-9). To this end, improved diagnostic tests are needed to assist in the identification of children with BA thus facilitating timely referral and initiation of appropriate treatment interventions.

The current diagnostic gold standard for BA is an invasive operative cholangiogram demonstrating complete obstruction of segments or of the entire length of the extrahepatic bile ducts. Following diagnosis, a hepatopancreaticoenterostomy (HPE) is performed to restore bile drainage and prevent progressive hepatobiliary damage; however, the response is variable and the majority of infants progress and develop complications of biliary cirrhosis with end-stage liver disease. Thus, BA remains the most common indication for pediatric liver transplantation (10).

A growing body of literature describes the contributions of the innate and adaptive immune systems in the etiopathogenesis of BA (11-13). Recently, hepatic gene expression profiling has uncovered a distinctive signature distinguishing BA infants from disease controls with IHC (14). Additionally, liver histopathological scoring at diagnosis
has demonstrated sub-classification and predictive capabilities with regards to BA patient survival with native liver following HPE (15).

In the present study, we investigated the utility of non-invasive, serum biomarker profiles in differentiating BA from other causes of IHC. Our findings demonstrate that quantification of serum biomarkers display a discriminatory value for BA versus IHC. We also explored the effectiveness of serum biomarker profiles in characterizing subgroups of BA patients and predicting a favorable response following surgical HPE. In this regard, we illustrate that serum biomarker profiles can be used to sub-classify patients with BA into distinct groups of patients with elevated circulating biomarkers and patients with lower levels. Importantly, those patients with elevated serum biomarkers exhibit a more favorable response to surgical HPE. Finally, we discovered that biomarker profiles in the BA population could predict response to HPE independent of sub-classification.

Materials and Methods:

Patients.

Serum samples (100 µL) and clinical data were obtained from infants with both BA (n=77) and IHC (n= 71) enrolled into a prospective study [ClinicalTrials.gov Identifier: NCT00061828] of the NIDDK-funded Childhood Liver Disease Research and Education Network [www.childrennetwork.org]. Samples from normal controls (n=9) were obtained from infants being evaluated at Cincinnati Children’s Hospital Medical Center for conditions not associated with the liver or digestive system. Several subjects from each group were missing various portions of the evaluation and were excluded from the final analysis. Ultimately, 72 infants with BA, 66 age-matched disease controls with IHC, and
5 normal aged-matched controls were included in the final analyses (Table 1). The human research review boards of all participating institutions approved the study protocols.

**Cytokine Quantification.**

Cytokine concentrations in the sample supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using Milliplex™ Multiplex kits (Millipore, Billerica, MA). The protein multiplex is a bead-based assay that enables the quantification of a customized panel of cytokines simultaneously in small aliquots of serum. We performed the cytokine quantification according to manufacturer’s protocol. Briefly, in a 96 well multiscreen filter plate, 25µL sample in duplicate was incubated with 25µL antibody-coated beads overnight at 4°C on a plate shaker. Plates were then washed twice on a vacuum apparatus and 25µL of secondary antibody was added and incubated at room temperature for and additional 1 hour while shaking. Finally, 25µL of strept-avidin-RPE was added directly to the secondary antibody and incubated for 30 minutes at room temperature with shaking. Plates were then washed 2 more times and 100µL of sheath fluid was added. Plates were shaken for 5 minutes and then read using luminex technology on the Bio-Plex™ (Bio-Rad, Hercules, CA). Concentrations were calculated from standard curves using recombinant proteins and expressed in pg/mL. Using the protein multiplex technology we determined the concentration of the following 35 biomarkers (Th1, Th2 and Th17 cytokines and chemokines): IL8, IL1α, eotaxin, GM-CSF, IL1β, IL1-Rα, IL2, sIL-2Rα, IL4, IL5, IL6, IL10, IL12p40, IL12p70, IL13, IL15, IL17α, sCD40L, IFNα2, IFNγ, TNFα, IP10, MCP1, MCP3, CCL22, MIP1α, MIP1β and VEGF in Plate 1; and IL21, IL23, IL28a, IL33, LIF, SCF and TRAIL in Plate 2 (Table 2).
As noted, each plate requires 25 µL and duplicates were run, thus a total of 100 µL per subject was analyzed. The cytokine analysis was conducted with the assistance of the Cincinnati Children’s Research Flow Cytometry Core.

**Statistical Analysis.**

As an exploratory analysis, we first included all 35 serum biomarkers in a principal component analysis (PCA) to determine if there is a natural separation in first few components among the three groups of patients – BA, IHC and normal controls. To further investigate the utility of PCA to separate the two disease populations (BA and IHC), we performed ANOVA analysis controlling for false discovery rate (Benjamin & Hochberg’s method) on all biomarkers. A subsequent PCA was performed using only the 6 most significant cytokines identified after ANOVA.

Furthermore, we performed a classification and regression tree (6) analysis assessing the serum from both BA and IHC patients to determine if serum biomarkers could confidently differentiate between the disease populations.

We then examined only the serum of infants with BA. We first performed a cluster analysis looking at both the BA and healthy control populations in order to investigate if there is a subgroup of BA patients whose biomarker profiles mirrored those of normal controls. This analysis was based on the 15 most significant cytokines that differentiated BA patients and normal subjects. Subsequent application of CART analysis was performed in order to detect for the possibility that biomarker signatures could be used to predict which infants in each cluster were able to attain bilirubin normalization (defined as conjugated/direct bilirubin <1mg/dL) following HPE. Fisher’s exact test was used to determine the significance of the association between cluster and bilirubin
normalization. Finally, CART analysis was applied to all 35 biomarkers independent of clustering to determine if there was a signature that could predict the attainment of normal bilirubin levels following HPE.

The power calculations for the study were based on initial investigations of IL8 in differentiating BA from IHC (14) which used the Wilcoxon rank sum test with two-sided significance level set at 0.05 and 90% power. This was extended to the current study to allow for statistically powered classification and regression tree analyses at a significance level of 0.05.

All above analyses were realized in SAS® software Version 9.13. JMP package was used for a regression tree analysis to identify biomarkers that are predictive of 3-month bilirubin level.

Results:

**Principal Component Analysis (PCA) of Serum Biomarker Profiles Differentiates Cholestatic from Normal Controls.**

A PCA based on all 35 biomarkers showed that normal controls could be distinguished from both BA and IHC infants (Figure 1). However, the two disease groups did not separate well until the third principal component. To further investigate the utility of PCA in distinguishing between the two disease populations, we applied an analysis of variance (ANOVA) model that identified 6 biomarkers that maintained unadjusted p-values less than 0.05 (Table 3). Subsequent PCA with only these 6 biomarkers revealed a persistent, substantial overlap between the two disease populations (Figure 2). Taken together, these results suggest that PCA can distinguish normal controls from infants
with cholestatic liver diseases; however, its use alone is inadequate in discriminating infants with BA from infants with IHC.

**Classification and Regression Tree (CART) Analysis Differentiates Patients with BA from those with IHC.**

CART analysis identified a unique biomarker signature that distinguished BA from IHC. Analyzing ROC curves assessed discrimination. Investigation revealed that a combination of VEGF, sICAM and 11 additional cytokines and chemokines can categorize a patient with BA with an area under the receiver operating characteristics (AU ROC) curve of 0.93 (CI 0.87, 0.99), sensitivity of 96%, and specificity of 83% (Figure 3).

**CART and Clustering Analysis Can Sub-Classify BA patients and Predict Clinical Response to HPE.**

As has been previously reported, sub-classification schemes have been used with BA patients to assist in the prediction of clinical outcomes (15). Both the BA and healthy control cohorts were analyzed. Following ANOVA modeling, the 15 most significant cytokines were identified that when applied to a hierarchical clustering analysis uncovered 2 distinct cluster formations. Cluster 1 consisted of 51 BA subjects in addition to all analyzed normal control infants. The biochemical signature of cluster 1 was notable for its low levels of biomarker measures. In other words, the biomarker profiles of those BA patients in cluster 1 were most similar to the normal control population with the absence of substantial marker elevation. Cluster 2 consisted of the remaining 21 BA patients and these infants had biomarker signatures that consisted of markedly elevated levels compared to cluster 1 (Figure 4A). Most importantly, clinical
outcomes were significantly different depending on the cluster assignment. Only 57% (29 of 51) of the BA subjects in cluster 1 achieved a normalized direct/conjugated bilirubin (defined as <1 mg/dL) 3 months following HPE intervention. Remarkably, 81% (17 of 21) of BA infants in cluster 2 obtained a conjugated/direct bilirubin levels <1 mg/dL over the same time frame (Fisher's exact = p<0.05, OR 3.22, CI 1.16, 8.99) (Figure 4B). These findings were independent of age at HPE which is a well-known variable affecting the clinical outcomes of these children (5-9). The mean age at HPE for cluster 1 was 61.2 days compared to cluster 2 at 64.8 days (student’s t-test p=0.56). For those infants with BA on whom data was available (n=58), 2-year survival with native liver was strongly associated with normalization of bilirubin 3 months following HPE (p<0.001).

**Independent CART Analysis Predicts Response to HPE in BA.**

Further investigation uncovered a biomarker profile that predicted clinical response to HPE independent of clustering analysis. CART analysis identified 5 biomarkers from which a regression tree (4-fold cross-validated) was built to predict 3-month post-HPE bilirubin levels <1 mg/dL with a sensitivity of 93% and specificity of 86%, AUC of ROC 0.912 (CI 0.801, 1.00) (Figure 5). As above, the findings were unrelated to age at HPE. The mean age of those BA patients who attained a bilirubin level <1 mg/dL 3 months following surgical intervention had a mean age at HPE of 59.1 days while those who did not attain favorable bilirubin levels had a mean age of 64.4 days at the time of HPE (student’s t-test p=0.31).
Discussion:

Quantifying a collection of Th1, Th2 and Th17 cytokines and chemokines, we identified a serum biomarker profile that has a discriminatory value for infants with biliary atresia versus other neonatal cholestatic diseases. Furthermore, we assigned a diagnostic capacity to the biomarker profiles that demonstrate the ability to identify those patients with biliary atresia who are more likely to achieve normalization for bilirubin levels following HPE.

The medical community has long needed improved diagnostic tools to aid in distinguishing patients with biliary atresia in order to decrease referral times and optimize clinical outcomes following HPE. Hepatic gene expression of CD56 (16) as well as IL8 and LAMC2 (14) has been shown to have a discriminatory value in separating biliary atresia infants from infants with other forms of neonatal cholestasis. However, invasive biopsy is needed to obtain tissue on which these findings are based. Subsequently, non-invasive serum marker assessment has been investigated for potential usefulness. An early focus on elevations in gamma-glutamyl transpeptidase (GGT) led some investigators to suggest its use in identifying patients with biliary atresia (17). Indeed, in our population, GGT was significantly elevated in patients with biliary atresia at the time of diagnosis compare to disease controls (Table 1). However, it is well recognized that GGT elevations are found in children with other cholestatic diseases (18) and its use alone cannot be used to identify patients with biliary atresia. Recent publications have identified bile acids, microRNAs, plasma metabolomics and other serum markers that have discriminatory value in the diagnosis of infants with biliary atresia (19-23). Our findings are unique in that they build on the current
understanding of the immune systems’ contribution to the disease process (11-13) and add to this growing body of literature that suggests non-invasive circulatory markers can be used to correctly distinguish infants with biliary atresia from those with other diseases of neonatal cholestasis. Future work in this area will investigate if combinations of methods and markers result in maximized diagnostic capabilities.

Further exploring the utility of serum biomarker profiles in biliary atresia, we discovered that biomarkers could be used to categorize sub-groups of infants with biliary atresia and predict the clinical response following HPE. Current knowledge gaps regarding the etiology and pathogenic mechanisms of biliary atresia have resulted in very little variation or advancement in the clinical management of infants with this devastating disease. Clinical outcomes following HPE are surely affected by multiple factors, including center experience, coexistence of embryonic malformations and age of the infant at portoenterostomy (9, 24, 25). While age may be the most recognized predictor of clinical response to HPE, our findings indicate that the degree of progression across the continuum of inflammation may be a more important predictive factor. Thus, this novel strategy to use serum biomarker profiles to classify patients at presentation can serve as a prognostic tool to improve the care of infants with biliary atresia. Optimal therapeutic interventions are often directed at specific biological processes. Universal treatment interventions may not apply to all children with biliary atresia at diagnosis. For example, anti-inflammatory strategies may prove futile in infants whose disease has progressed beyond a primarily inflammatory stage and has begun to demonstrate changes consistent with chronicity, such as fibrosis and cirrhosis. Future efforts should build on this approach and investigate patient-specific characteristics such as clinical,
histological, molecular and biochemical qualities that can assist in more directed therapies.

In conclusion, we demonstrate the diagnostic and predicative capabilities of non-invasive, serum biomarker profiles in children with biliary atresia. Future studies will be needed to validate these exciting findings. Furthermore, this work provides foundational support to advance current clinical management of children with BA. The use of serum biomarker signatures to more accurately categorize an individuals' disease process and provide a more personalized therapeutic care plan may allow for new or alternative treatment strategies for select patients. Future efforts will build on this information to design more effective clinical trials with the ultimate goal of improving the health of all children with biliary atresia.
BIBLIOGRAPHY


## TABLES AND FIGURES

Table 1: Baseline Age and Laboratory Data

<table>
<thead>
<tr>
<th></th>
<th>Biliary atresia (n=72)</th>
<th>Intrahepatic cholestasis (n=66)</th>
<th>Normal control (n=5)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at lab draw, days (SD)</td>
<td>58.7 (22.2)</td>
<td>58.8 (49.7)</td>
<td>74.2</td>
<td>0.69*</td>
</tr>
<tr>
<td>Direct/conjugated bilirubin mg/dL (SD)</td>
<td>4.9 (1.9)</td>
<td>5.0 (3.1)</td>
<td></td>
<td>0.81**</td>
</tr>
<tr>
<td>Baseline GGT unit/L (SD)</td>
<td>643.5 (479.3)</td>
<td>169.7 (151.0)</td>
<td></td>
<td>&lt;0.001**</td>
</tr>
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</table>

* One-way ANOVA, ** Student’s T-test
SD - Standard Deviation

**Table 1.** Summary of baseline data regarding biliary atresia, intrahepatic cholestasis and normal control populations.

Table 2: Biomarkers

<table>
<thead>
<tr>
<th>Eotoxin</th>
<th>IFNγ</th>
<th>IL-1Rα</th>
<th>IL-4</th>
<th>IL-10</th>
<th>IL-13</th>
<th>IP-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>IL-1α</td>
<td>IL-2</td>
<td>IL-6</td>
<td>IL-12p40</td>
<td>IL-15</td>
<td>MCP-1</td>
</tr>
<tr>
<td>IFNα2</td>
<td>IL-1β</td>
<td>sIL-2RA</td>
<td>IL-8</td>
<td>IL-12p70</td>
<td>IL-17a</td>
<td>MCP-3</td>
</tr>
<tr>
<td>MDC</td>
<td>Mip-1β</td>
<td>VEGF</td>
<td>LIF</td>
<td>SCF</td>
<td>IL-28A</td>
<td>sICAM-1</td>
</tr>
<tr>
<td>Mip-1α</td>
<td>TNFα</td>
<td>IL-23</td>
<td>TRAIL</td>
<td>IL-21</td>
<td>IL-33</td>
<td>sCD40 Lig</td>
</tr>
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</table>

**Table 2.** Collection of 35 Th1, Th2 and Th17 cytokines and chemokines assessed
Table 3: Potentially Significant Biomarkers Differentiating BA and IHC

<table>
<thead>
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<th>Biomarker</th>
<th>Unadjusted p-value</th>
<th>False Discovery Rate p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>sICAM</td>
<td>0.00</td>
<td>0.13</td>
</tr>
<tr>
<td>MIP 1a</td>
<td>0.02</td>
<td>0.25</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0.03</td>
<td>0.25</td>
</tr>
<tr>
<td>TRAIL</td>
<td>0.03</td>
<td>0.25</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.04</td>
<td>0.25</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0.04</td>
<td>0.25</td>
</tr>
</tbody>
</table>

BA - Biliary Atresia, IHC - Intrahepatic Cholestasis

Table 3. Most significant biomarkers differentiating BA and IHC with ANOVA analysis. Note that the significance is lost after setting the false discovery rate at 0.05 (Benjamin & Hochberg’s method).

Figure 1. 35 Biomarker Principal Component Analysis. Using all 35 biomarkers, PCA demonstrates a clear separation between normal control (green) and disease populations (biliary atresia – blue; Intrahepatic cholestasis – red). However, the 2 disease populations do not separate well.
Figure 2. 6 Biomarker Principal Component Analysis. Biplot of PCA 1 vs PCA 3 using the 6 most significant biomarkers (after ANOVA analysis) differentiating BA (blue) from IHC (red). The 2 populations are unable to separate well.

<table>
<thead>
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<tr>
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<td>0.02</td>
<td>0.25</td>
</tr>
<tr>
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<tr>
<td>TRAIL</td>
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<tr>
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<td>0.25</td>
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</table>

Figure 3. Classification and Regression Tree (CART) Analysis Differentiates BA from IHC. CART Analysis demonstrates a combination of VEGF, sICAM, and 11 additional cytokines and chemokines (see box) can categorize a patient with BA with an area under the receiver operating characteristics (AU ROC) curve of 0.93 (CI 0.87, 0.99), sensitivity of 96%, and specificity of 83%.
Figure 4. CART and cluster analysis sub-classifies BA patients and predicts clinical response following HPE. A) Analysis using the 15 most significant cytokines differentiating BA from normal controls (See box) after ANOVA identifies 2 clusters of BA patients – cluster 1 with normal cytokine levels. Cluster 2 with elevated cytokine levels compared to normal controls. All normal controls (black) are grouped with cluster 1. B) Assessment of 3-month post HPE bilirubin levels demonstrates that only 57% (29 of 52) patients in cluster 1 were able to achieve adequate drainage with conjugated/direct bilirubin <1 mg/dL. Conversely, 81% (17 of 21) of BA patients in cluster 2 were able to attain adequate drainage. Fisher’s exact = p<0.05, OR 3.22, CI 1.16, 8.99.

Figure 5. Independent CART analysis predicts clinical response following HPE in BA. CART analysis identified a unique profile of 5 markers that was able to predict those patients likely to achieve 3-month post-HPE bilirubin levels < 1 mg/dL. AUC of ROC 0.912 (CI 0.801, 1.00) with a sensitivity of 93% and specificity of 86%.