Biliary Atresia Study in Infants and Children (BASIC)

Protocol BASIC, Version 6,
Amendment 5

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For a list of sites, sponsor information and principal investigators; please refer to the study website: www.childrennetwork.org

ChiLDReN (Childhood Liver Disease and education Network)

Protocol Signature Sheet Instructions

ATTN: STUDY COORDINATOR

Print one copy of the protocol signature sheet on page 3. Original signature of the Principal Investigator and date of signature is required. Return a digital copy to the SDCC using the ChiLDReN-Monitors@arborresearch.org email address and the original signed copy ***must*** be maintained in your regulatory files.

ChiLDReN BASIC Amendment 5: Version 6

PROTOCOL SIGNATURE SHEET

Biliary Atresia Study in Infants and Children - BASIC

Protocol approved by ChiLDReN Steering Committee: Date of Protocol
Amendment 1: 12/19/06
Date of Protocol Amendment 2: 04/20/2010
Date of Protocol Amendment 3: 06/01/2013 Version 4.0
Date of Protocol Amendment 4: 11/03/2016 Version 5.0
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I hereby confirm that I have read and understand the Protocol **Amendment 5** and all its attachments and that these documents contain all the details necessary to perform the trial. Unclear passages were clarified in a discussion with the lead investigator of the study.

When necessary to delegate tasks, I undertake to delegate them only to qualified personnel, to inform the personnel about the study and their duties and to supervise the conduct of the study.

I agree that the NIDDK and their authorized representatives should have free access to all study documents at their request, to ascertain that the study is conducted in accordance with the protocol. This includes the informed consent.

I agree to the Protocol **Amendment 5** in all details and will perform the study in accordance with the amended protocol, the Declaration of Helsinki, the ICH Note on ‘Good Clinical Practice’, and local regulations.

Information related to the investigation should only be transferred to third persons after the written consent of the Childhood Liver Disease Research and Network Steering Committee has been obtained. This does not apply if the information transfer is mandatory (e.g. submission to ethical committee).

Despite the above, it is general policy of NIDDK to encourage publication of results from clinical investigations. Submission of manuscript for publication will be decided upon by the ChiLDReN Steering Committee.

_____ _____

(Signature of Primary Investigator) (Date)

_____

Print Name of Primary Investigator
Investigator's Copy (Original to filed in the Site Regulatory Binder)

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INSTITUTIONAL REVIEW BOARD APPROVAL REQUIRED	**YES**
REVISION OF INFORMED CONSENT REQUIRED	**YES**

1.0 Specific Aims

Biliary atresia (BA) is a progressive necroinflammatory process initially involving the extrahepatic biliary tree. As the disease progresses, there is loss of patency of the lumen and obstruction to bile flow. The result is cholestasis and chronic liver damage. With time, the intrahepatic biliary system becomes involved. BA occurs in one in 8,000 to 18,000 live births resulting in 250-400 new cases per year in the United States (1). Untreated, the disease leads to complete biliary obstruction with cirrhosis, and is uniformly fatal (2, 3). After a hepatoportoenterostomy (Kasai procedure), children have a variable disease progression with less than 20% surviving beyond the teen years without liver transplantation. Little is known about neither the factors that cause BA nor the factors that influence disease progression. A variety of genetic, autoimmune and environmental influences have been hypothesized to be important. Most studies to date have focused on the neonate and young child with BA, yet the older surviving child with BA can provide important information about genetics, as well as, natural history. The purpose of this database is to collect the pertinent clinical information, genetic material and body fluid samples to enable investigators to address the following hypotheses:

Specific Aim 1
To identify the gene or genes implicated in the etiology of BA.

> **Hypothesis 1:** A genetic defect is a likely causative factor for BA among children with BA and multiple congenital anomalies.

Specific Aim 2
To identify polymorphisms that may be important in disease susceptibility or progression, such as human leukocyte antigen (HLA) polymorphisms.

- ***i.*** To perform high resolution HLA-A, B, C, DRB1, DRB3 DRB4, DRB5, DQA1, DQB1, DPA1, and DPB1 typing on participants with BA.
- ***ii.*** To utilize a novel computer algorithm that permits screening large numbers of HLA alleles to detect shared epitopes in patients with BA.
- ***iii.*** To assess the role of HLA polymorphism in incidence and severity of BA using traditional analysis of allele frequency and a novel shared epitope algorithm.

> **Hypothesis 2:** Autoimmune factors are likely to contribute to disease progression or acquisition and can be identified by correlating HLA among children with BA to healthy controls and by comparison of those who develop early complications including, variceal bleed, ascites, and growth failure compared to those who do not.

Specific Aim 3
Characterize the natural history of the older, non-transplanted child with BA.

Hypothesis 3a: Sentinel events such as variceal bleeding, ascites and growth failure are earlier predictors of death or need for liver transplantation than the pediatric end-stage liver disease score (PELD).
Hypothesis 3b: Health-related quality of life will be impaired compared to healthy age-matched children and relate to severity of illness.
Hypothesis 3c: Growth failure as measured by anthropometrics and nutritional supplementation will be predictive of onset of sentinel events (ascites, variceal bleed, death, and transplant) in the following 24 months.

This study will be performed by the Childhood Liver Disease Research Network (ChiLDReN), a National Institute of Diabetes & Digestive and Kidney Diseases (NIDDK) funded network. Although ChiLDReN has the resources to collect the clinical data and the samples, the specific scientific studies described under Specific Aims 1 and 2 may require additional funding from other sources. However, these studies cannot be undertaken if the data and samples are not collected by ChiLDReN.

2.0 Background and Significance

Extrahepatic BA is a devastating condition of infancy in which there is obliteration or discontinuity of the hepatic or common bile ducts at any point from the porta hepatitis to the duodenum. When untreated, the condition results in severe liver injury and death in all cases. The development of the hepatoportoenterostomy procedure in 1959 by Kasai has permitted long-term survival in only 20% of the affected infants. The advent of liver replacement therapy has permitted long-term survival in many of the remaining infants, but not without cost and morbidity.

The estimated incidence of BA is one in 8,000 to 18,000 live births. Approximately 50 percent of all liver transplantations in children in the United States are for infants with BA—constituting 140-180 liver transplants per year. Despite the devastating nature of this illness and the high cost of its treatment, little is known about its pathogenesis or the factors implicated in disease progression. It is likely that BA is not a single disease, but rather a phenotype of several underlying specific disorders to which the infant liver responds in a stereotypic manner by a complex series of processes, including inflammation, bile duct proliferation, apoptosis and fibrogenesis. Improvement in outcomes will not occur until we have a better understanding of the mechanisms involved in these processes.

2.1 Genetics and Biliary Atresia

Specific Aim 1
Identify gene or genes implicated in the etiology of BA (funded D. Perlmutter).

The genetics of BA may be investigated on two levels. The first is to identify a group of patients whose etiology is a result of a genetic defect and the second is to examine the influence of genetics on disease acquisition.

A. Genetics as a causative factor

Several groups have suggested that BA is a heterogeneous condition. Desmet (4) and Schweizer (5) have suggested that there are two forms of BA: the embryonic or fetal type, found in 15-30% of patients, and the perinatal type, found in the remaining cases. The fetal type is characterized by early onset of cholestasis, absence of a jaundice-free period after physiological jaundice, absence of bile duct remnants in the hepatoduodenal ligament and presence of associated anomalies of laterality. The perinatal type is characterized by later onset, presence of a jaundice-free interval, presence of bile duct remnants in the hepatoduodenal ligament, and absence of associated congenital anomalies. Silveira and colleagues, on the basis of a review of 237 patients from Britain and Brazil (6, 7), have suggested that there may be at least four distinct etiopathogenic subgroups of BA. They subdivided patients into a prenatal form associated with malformations, a prenatal form associated with chromosomal abnormalities, a prenatal form in which there are neither malformations nor chromosomal abnormalities and a postnatal form which presumably overlaps with the perinatal type described by Desmet and Schweizer (4,5). It is likely that specific subgroups of children with BA have a heritable disorder, particularly those children with a fetal form or embryonic form of the disease.

The association of specific constellation of congenital anomalies with BA is well described. In most of these cases, the anomalies fall into the rubric of anomalies of laterality or situs determination, but there are clearly patients with other congenital anomalies that cannot be classified as situs anomalies. Interestingly, Silveira et al. examined eight of these patients for karyotype abnormalities and found specific gross defects in two of them, but did not follow up on the nature of these defects (8).

Several years ago, Yokoyama et al. described a transgenic mouse in which a recessive insertional mutation at the *inv* locus on chromosome 4 resulted in consistent situs inversus in the homozygous mutants (9). Anomalies included mirror-image left-right inversions of stomach, spleen, and liver. Some had polysplenia, preduodenal portal vein, intestinal malrotation, and dextrocardia. *Inv* mice became progressively jaundiced soon after birth, had cystic changes in their kidneys, grew poorly, and rarely lived beyond one week of age. Follow-up studies have shown that the inversin gene, a novel gene with tandem ankyrin-like repeat sequences expressed in liver, kidney, and other tissues early in embryonic life, is partially deleted in the *inv* mouse (10) (11). Jaundice has not been observed when other genes in the vertebrate left-right axis pathway are altered in mouse models of situs inversus, including the inv mouse, the FT mouse (12), the HFH-4 knockout mouse (13), the lefty-knockout mouse (14), the activin receptor IIB-knockout mouse (15), and the kinesin KIF3B-knockout mouse (16), or in human syndromes associated with situs anomalies including Kartagener's syndrome (17) or a syndrome linked to the gene for the Zic3 transcription factor on the X chromosome (18).

Perlmutter et al. examined the etiology of jaundice in the *inv* mouse to determine if this mouse also had an interruption of the extraheptic biliary tree and whether it represented an animal model of BA associated with situs inversus (19). The results show that these mice have cholestasis with conjugated hyperbilirubinemia, failure to excrete technetium-labeled mebrofenin from the liver into the small intestine, lack of continuity between the extrahepatic

biliary tree and the small intestine as demonstrated by Trypan blue cholangiography, and a liver histological picture indicative of extrahepatic biliary obstruction with negligible inflammation/necrosis within the hepatic parenchyma. Lectin histochemical staining of biliary epithelial cells in serial sections suggests the presence of several different anomalies in the architecture of the extrahepatic biliary system. These results suggest that the inversin gene plays an essential role in the morphogenesis of the hepatobiliary system and raise the possibility that alterations in the human orthologue of inversin account for some of the cases of BA in which there are also anomalies of situs determination. Indeed, the liver is one of the predominant sites of inversin gene expression, beginning very early in embryonic development (10, 11).

Many genes have now been implicated in the pathway for specification of the left- right axis. The pathway is thought to involve at least two genetically distinct steps: 1) generation of an asymmetric axis; and 2) conserved positioning of structures along this axis. Several genes, including inversin, HFH-4, the left-right dynein gene, which is mutated in the *iv* mouse, growth/differentiation factor-1, and the Zic3 gene, which is mutated in X-linked situs anomalies, appear to act early in the pathway and are, therefore, likely to be involved in the generation of the asymmetric axis (9, 10, 13, 18, 20). A number of other genes appear to act later in the pathway; transforming growth factor β molecules such as nodal, lefty-1, lefty-2, activin βB; molecules involved in transforming growth factor β signaling such as activin receptor 2a and 2b, Smad 2; hepatocyte nuclear factor 3β; sonic hedgehog; FGF8; Caronte; cWNT8; patched; and PitX2 (21-29).

Recent studies have suggested that situs is determined by the flow of extra- embryonic fluid in a critical location, the node, at a critical time in early embryonic development (30-32). The node, a cup-shaped cavity in the midline of the embryo, apparently uses anti-clockwise rotation of cilia to create directional flow of extraembryonic fluid. This nodal flow concentrates critical signaling molecules to one side of the node, in turn activating distinct downstream signaling pathways on each side. In the *iv* mouse, nodal cilia were immotile and nodal flow absent, resulting in random situs determination (31)(33). In the *inv* mouse, nodal cilia were motile but could only produce a very weak leftward nodal flow (31)(33). It is still unclear why this results in complete situs inversus in all of the homozygous progeny. It is also not known what role the inversin gene product plays in nodal flow, kidney and hepatobiliary development. Several recent studies have shown that *inv* complexes with nephrocystin, the protein mutated in one form of autosomal recessive polycystic kidney disease, and β-tubulin in the primary cilia of renal tubular cells (34). Together with left-right dynein, this complex plays a role in regulating the ciliary movement of the node that determines visceral laterality during embryogenesis (33, 35). Recent studies have also shown that one member of the nephrocystin gene family is mutated in a variant of polycystic kidney disease associated with congenital hepatic fibrosis (36). A number of other genes have been implicated in situs determination and ciliary function including DNAH5, DNAI1, Tg737, KIF3A, KIF3B (37) and RFX3 (38).

Positional/candidate gene cloning has been utilized to identify the genetic cause of laterality defect in several patients (39). To date, rare mutations in Zic3 (18), activin receptor ACVR2b (40), LeftyA (41), Cryptic Cfc1 (42), activin receptor ACVR1 and Smad2 have been found in humans with laterality defects, but together account for <10% of patients with heterotaxy. In a similar screen, no mutations were found in LeftyB, Nodal or PitX2. Although Schon et al. were unable to detect obviously pathological mutations in *Inv*, Lefty1, Lefty2, ACVR 2b, Nodal, Zic3 or Cfc1 in 65 patients with lateralization defect (43), Kosake et al. have identified mutations in *Inv* in 3 patients with congenital heart disease (40,41). Of the 65 patients examined by Schon et al. (43), seven had BA. In another study of 144 patients with laterality defects, Bamford et al. found heterozygous mutations of the Cfc1 in nine patients including one with BA (42). However, it is not clear whether this mutation can cause laterality defects or BA on its own.

A recent study by Ware et al. has shown pathogenic Zic3 mutations in two patients with BA and situs anomalies (44). The situs anomalies included characteristic congenital heart defects. Five other patients with congenital heart disease had Zic3 mutations. Zic3 is a zinc finger protein localized to the nucleus and therein is thought to be a transcription factor. The mutants described by Ware et al. lack transcriptional activating function *in* vitro and do not localize to the nucleus. In two families, an affected male had hypoplastic left heart syndrome. A priori these heart defects would not be considered as a part of the spectrum of anomalies of situs determination. An affected female with typical situs anomalies was also detected as a sporadic case. This indicates that Zic3 mutations may be a cause of sporadic situs inversus in both males and females. This is consistent with the observation that a proportion of Zic3-deficient carrier female mice have heart defects (45). It was also interesting to find Zic3 mutations in a patient with a diagnosis of VATER association and in a patient with a tentative diagnosis of Alagille syndrome, providing a basis for the idea that Zic3 mutations may overlap phenotypically with a number of complex malformation syndromes. Therefore, it is possible that mutations in genes which play a role in situs determination can cause anomalies of morphogenesis that are not necessarily characteristic of classical situs inversus phenotypes.

Although clinical experience and studies with larger numbers of BA patients have clearly excluded the possibility that BA could be caused by a simple Mendelian genetic mechanism, there are some families in which several siblings are affected with BA (46, 47), including twins. These data would suggest that there are genetic determinants of BA or, at least, genetic determinants of subtypes of BA.

B. Genetics as a modifying factor

Recent evidence suggests that missense mutations in *Jagged1* may increase susceptibility to BA. *Jagged1* is a ligand in the evolutionarily conserved, developmentally important Notch signaling pathway. Mutations in *Jagged1* have been shown to cause the dominant disorder Alagille syndrome, which is characterized by intrahepatic bile duct paucity, occurring with abnormalities of the heart, skeleton, eye, and characteristic facial features. Kohsaka et al., (48) studied 102 Japanese patients with Extrahepatic Biliary Atresia (EHBA), and identified missense mutations in 9 of the 102. These missense mutations have never been previously identified among the patients with Alagille syndrome, and have not been identified in a control

population to date. These studies have led to the hypothesis that missense mutations in JAG1 may be a predisposing factor to the development of EHBA. It is likely that other genes might be implicated as well. We intend to screen our population of BA patients for mutations in JAG1.

C. Genetic susceptibility to Biliary Atresia

Given the rarity of BA as articulated above, genetic approaches have most commonly and appropriately focused on children with laterality defects. Nevertheless, there remain broader clues to support the hypothesis that there is an underlying genetic susceptibility to BA. For instance, ethnicity specific rates of BA, consistent with population specific predisposing alleles, and certain HLA genotypes have been associated with BA (see below).

In the last few years, single nucleotide polymorphisms (SNPs) genome-wide association studies (GWAS) have identified at least 2,000 common variants associated with complex diseases or related traits (http://www.genome.gov/gwasstudies). Copy number variants (CNVs) consist of deletions and duplications that occur frequently in the population and these variable regions may contain many functional sequences, which may underlie genetic susceptibility to disease. The realization that the normal human genome contains regions that vary in copy number from individual to individual was an unexpected and exciting finding. Multiple investigators have suggested that this form of variation might be responsible for some of the variation in disease susceptibility. And hence, CNV in risk alleles may be a susceptibility factor for BA. Indeed, CNV has already been associated with susceptibility to complex diseases such as lupus glomerulonephritis and age-related macular degeneration. With this discovery of massive numbers of genetic markers and the development of better statistical tools, studies of genome-wide association (GWAS) have been used as an unbiased and powerful approach for identifying more elusive genes involved in complex phenotypes.

Although it is recognized that GWAS typically require a very large sample size to reach adequate statistical power, highly significant results have been obtained with small sample sizes in cases where the magnitude of the genetic risk is high (see Statistical Considerations). The combining of participants from both the BASIC study and the ChiLDReN PROBE study participant cohort (children with BA enrolled in the first six months of life) provides the opportunity to identify susceptibility genes for BA with the largest well-characterized cohort of BA participants gathered and available to date. Indeed, in response to RFA:PAR-09-247, “Ancillary studies to major ongoing clinical research studies to advance areas of scientific interest within the mission of the NIDDK”, a genome-wide association study is currently underway which is testing for association of both CNVs and SNPs to identify genes or genomic regions associated with BA disease susceptibility, with follow-up on regions and genes identified, via next generation sequencing (NGS), to identify causal variants. Identification of genetic susceptibility factors would be highly significant, by pointing to genes and pathways that are fundamentally important in biologic pathogenesis of BA, and providing crucial steps and accelerating the pace of research towards the development of more rational therapies and diagnostics.

2.2 Autoimmunity and Biliary Atresia

Specific Aim 2

Identify polymorphisms that may be important in disease progression.

Some forms of BA are thought to be a result of an autoimmune process. A novel study of HLA polymorphisms in BA has been developed by Drs. Mack, Sokol and Rosenthal with the expertise of Dr. Freed. The following aims have been proposed:

- ***i.*** To perform high resolution HLA-A, B, C, DRB1, DRB3 DRB4, DRB5, DQA1, DQB1, DPA1, and DPB1 typing on participants with BA.
- ***ii.*** To utilize a novel computer algorithm that permits screening large numbers of HLA alleles to detect shared epitopes in participants with BA.
- ***iii.*** To assess the role of HLA polymorphism in incidence and severity of BA using traditional analysis of allele frequency and our novel shared epitope algorithm.

Many studies support a role for immune dysfunction in BA. Like a number of autoimmune diseases, there appears to be a female predominance in BA and aberrant HLA expression in bile duct epithelium. It has been proposed that some insult to the fetus or neonate leads to abnormal expression of antigens in bile duct epithelium (1). The abnormal antigen expression triggers an autoimmune attack of bile duct epithelium leading to injury and obliteration. Support for such a theory comes from T-cell subsets ratios which are more suggestive of an immune or metabolic pathogenesis than infectious, for example being more akin to Alpha-1-antitrypsin deficiency than hepatitis B-related inflammation of the liver (49).

At present, there is evidence supporting a number of aspects of the immune cascade including antigen expression and presentation, T-cell activation, Kupffer cell activation, cytokine release and apoptosis. A number of HLA associations have been reported. Silveira reported an association with HLA-B12 and the haplotypes A9-B5 and A28-B35 (50). In a different ethnic group, Kobayashi reported associations with A33, B44, and DR6 (51). Several groups have reported aberrant expression of HLA-DR, a class II antigen, in biliary epithelium. As normally only major histocompatibility complex (MHC) class I antigens are expressed by bile duct epithelium, it is possible that, in some circumstances, biliary epithelium might act as an antigen presenting cell (APC) in the immune pathway and thus directly activate T- lymphocytes. The activation of T cells requires adhesion to the APC through intercellular adhesion molecules (ICAMs). ICAM expression by biliary epithelium in BA has been reported both by Broome (52) and Dillon (53). Lastly, Davenport et al. have demonstrated that activated and proliferating helper T-cells and natural killer cells are present in the liver and in bile ducts in BA (54). Taken together there is evidence for abnormal antigen expression in the livers of children with BA, and that T-cell activation and cytotoxicity play some role in causing injury. The damage may also be mediated through Kupffer cells. Recent histologic studies have demonstrated increased numbers and size of Kupffer cells in liver tissue of BA patients (55) and Davenport et al. have reported a poorer prognosis in children with increased CD68+ cells (Kupffer cells) in the biliary remnant (54). Expression of Fas ligand in bile duct epithium in BA patients has also been reported and may play a role in apoptotic injury (56).

The human MHC (HLA) is a region on chromosome 6 that contains more than 50 genes known to be involved in the immune response. The HLA class I genes (HLA-A, HLA-B and HLA-C) are single polypeptide chains that associate with β_2-microglobulin on the surface of most nucleated cells. HLA class I molecules present endogenous peptides to CD8+ T cells and mediate the generation of cytotoxic T cells capable of destroying infected targets. HLA class II molecules are heterodimers expressed predominantly on hematopoietic cells (B cells, macrophages, dendritic cells) and present exogenous peptides to CD4+ helper T cells. Helper T cells promote a variety of inflammatory responses, including allergy, graft rejection, delayed type hypersensitivity, and autoimmunity. As the name implies, helper T cells promote the functions of CD8+ T cells, B cells, and macrophages and thereby escalate the inflammatory reactions. From an anthropologic point of view, these proinflammatory responses are essential to host defense against viral, fungal, and parasitic infection. If allowed to proceed unchecked, these inflammatory responses would themselves be fatal to the host. However, the immune system has evolved a series of checks, including central deletion of autoreactive T cells and B cells, the requirement for multiple activation events, and cell cycle regulatory loops to halt proliferation of reactive cells. The system works with remarkable efficiency the vast majority of time. However, certain individuals exhibit inappropriate immune responses to self antigens (autoimmunity), to non-infectious substances (allergy) and environmental insults (berylliosis, metal hypersensitivity) with significant morbidity and mortality.

The underlying causes of these inappropriate immune responses are highly complex, involving both genetic susceptibility and changes in the environment. For example, the dramatic rise in asthma over the past 40 years cannot be accounted for by genetic drift in such a short period of time. Similarly, the rise in the incidence of chronic beryllium disease coincided with the use of beryllium in the computer and weapons industry. While avoidance of the offending immunogen is always the preferred practice, in many instances the immunogen has not been identified or cannot be eliminated from the environment for practical or economic reasons. Additionally, many instances of autoimmune disease, the initiating immunogen appears be an unavoidable self antigen. Thus, identification of the genetic susceptibility serves to identify at risk individuals as well as provide insight into the etiology of the disease.

Over the past 40 years, many studies have been conducted to elucidate the relationship between HLA and immune-mediated diseases. These diseases can be divided in three categories. In the first group, which includes rheumatoid arthritis, celiac disease, and type I diabetes, there is a strong correlation between specific HLA alleles and disease susceptibility. This group can benefit by HLA epitope analyses and research identifying the antigens that bind these epitopes. The second group, including multiple sclerosis and systemic lupus erythematosus (SLE), are immune-mediated diseases where the HLA link is poorly defined. In these cases, the HLA alleles may cluster with other disease susceptibility genes by linkage disequilibrium, or they be linked by shared epitopes on disparate HLA genes. The third group includes BA, sarcoidosis and emphysema, in which an HLA association has not been identified despite the compelling evidence that these diseases are immune-mediated.

It seems apparent that HLA contributes to varying degrees in different immune- mediated diseases. In some instances, certain HLA alleles present a clearly defined risk, while in others HLA shows only a weak association and may differ with the population studied. However, it is becoming increasingly clear that many of the weak HLA disease associations discovered over the past 40 years were due to an incomplete understanding of the MHC complex. The two most common errors were analysis of the 'wrong' locus and the lack of appreciation of 'shared epitopes' between disparate alleles. This can be illustrated by the advances in our understanding of chronic beryllium disease, an inflammatory lung disease in response to a simple metal (57). Initial studies suggested a link to HLA-DR that was subsequently determined to be incorrect. The actual HLA association is with the closely linked HLA-DP (57-59). Furthermore, T cells from patients with chronic beryllium disease respond to beryllium-treated antigen presenting cells expressing HLA-DPB1*0201, *0402, *0601, *0901, *1001 and *1701 alleles (60). These seemingly unrelated alleles all contain acidic amino acids in positions 55, 56 and 69, which correspond to two separate 'pockets' (encoded by hypervariable regions) that anchor peptides within the HLA groove. Acidic amino acids are believed to promote binding of the beryllium cation and thereby alter peptide binding. In contrast, alleles that confer resistance to berylliosis, such as HLA-DPB1*0301 and *0401, have neutral or basic amino acids in these positions. Thus, studies of HLA and disease susceptibility must include analyzing DNA sequences within the relevant alleles in order to define shared epitopes in peptide-binding pockets, rather than analyses limited to allele frequencies.

The University of Colorado Denver Health Sciences Center (UCDHSC) has a long history of research in immune-mediated diseases and is at the forefront of applying these techniques.

2.3 Natural History and Biliary Atresia

Specific Aim 3

Define the natural history of the older, non-transplanted child with BA.

Understanding the natural history of a disease is a prerequisite to interpreting disease severity, identifying patterns of illness, identifying early predictors of outcome and understanding the advantages or trade-offs of therapeutic interventions. As such, defining the natural history of BA is a focus of the mission of the ChiLDReN Network (www.ChildrenNetwork.org). ChiLDReN study PROBE, a prospective database of cholestatic infants, enrolls all children diagnosed with BA prior to six months of age. PROBE will provide information that defines the natural history of BA in the early years. However, approximately 50% of those enrolled will undergo transplantation by two years and it is expected that only 20% will remain transplant free by 20 years of age. Because of the rate of attrition and the length of time it will take to obtain information regarding participants 5, 10, and 20 years from time of diagnosis, BASIC was structured to study the natural history of the older BA participant. ChiLDReN study BASIC will provide detailed information to define the natural history of preschool, school-aged, and young adults with BA who have not undergone liver transplantation.

By recruiting older participants with BA and following each until the age of 20 or until transplanted, we will be able to combine multiple overlapping periods to enable us to model the natural history of the disease. Longitudinal modeling of chronic disease progression is not practical when each participant must be followed from the onset of disease to the final endpoint (death or transplantation). As a result, modelers of disease progression define a series of disease states that represent progression or onset of complications and then identify reports in the literature where one or more of the states are studied. Often this may be the control group in a clinical trial. By combining estimates of different parameters, each estimate potentially from a different study, the modelers are able to build a comprehensive model to estimate disease progression. For example, in diabetes the natural history consists of progression from normal glucose to impaired glucose tolerance, to overt diabetes, to death, as well as progression in its primary complications, such as retinopathy, neuropathy, and nephropathy. Modelers in this area combine estimates from many studies to construct their models (61-63). Similarly, in BA there is progression over time leading to complications, such as ascites and cholangitis, or to endpoints, such as transplant or death. By collecting data from prospective cohorts that start at various ages, it will be possible to estimate conditional probabilities of disease progression or onset of complications given the age at entry into the cohort. Combining these estimates longitudinally in time will allow a comprehensive model of progression of BA from infancy (PROBE) to age 20.

Most of the natural history of BA in toddler, school aged, and young adult patients has been ascertained from several large cross-sectional and mostly retrospective studies (64-73). These studies have provided information about quality of life, complications and survival. The most recent of these reports, by Lykavieris et al. presented the data of 271 patients operated on between 1968 and 1983 (74). Like previous studies, approximately 23% survived over 20 years without liver transplantation. Furthermore, the study reported seven women who gave birth to nine children and three men fathered six children. Complications were reported for the 63 survivors, but the critical information of how these symptoms progress (or not) is lacking. Pruritis, late cholangitis and gastrointestinal bleeding were all relatively common. Yet no information was provided relating onset of complications to subsequent clinical deterioration. Another recent study examined quality of life in BA participants from England and Japan. Conflicting information was found regarding the impact of the disease on quality of life; those in Japan reported impaired quality of life and those in England fell in the normal range (70). What was lacking from both studies was the ability to ascertain how disease progression and complications alter the functional status of patients affected by this disease.

Progressive liver disease due to BA can affect a variety of physiologic parameters all of which may be useful in defining the natural history. These parameters and the data that can be collected include:

- Growth and Nutrition: Height, Weight and Anthropometrics
- Hepatic Synthetic Function: Bilirubin, international normalized ratio (INR), albumin
- Hepatic Inflammation: alanine aminotransferase (ALT), GGTP
- Markers of fibrosis: hyaluronic acid, collagen markers

- Portal hypertension: liver texture, spleen size, ascites, variceal bleeding, encephalopathy, presence of portopulmonary hypertension or hepatopulmonary syndrome, platelet count
- PELD and MELD variables: Bilirubin, INR, albumin, creatinine, sodium

Prospective collection of these limited data at specific time points and correlation with defined clinical complications will provide the needed information to define the natural history of this disease.

Once this information has been obtained, it will provide the foundation for developing a disease staging system for BA and assessing studies involving therapeutic interventions. In the adult patient, the Child-Pugh classification has been useful to understand stages of cirrhotic liver disease and quality of life studies have been useful in understanding the trade-offs of chemotherapy in oncology and hepatitis C. No analogous staging system like Child-Pugh has been validated for older children with BA. For organ allocation, the PELD score is used, but to most clinicians in the field it has many shortcomings. PELD score was developed from the SPLIT database by analyzing factors that predicted those children most at risk for death or transfer to the intensive care unit (ICU) (as a surrogate marker for death) while awaiting liver transplantation. PELD is currently applied to all pediatric liver transplant candidates ≤12 years of age, while MELD is applied to children >12 years of age. Many of the medical complications that significantly impair a child's health are not factored into the organ allocation scores of PELD and MELD, such as signs and symptoms of portal hypertension (ascites, variceal bleeding and hypersplensim) and quality of life. In addition, PELD and MELD were designed to predict a high risk of death to assist with equitable allocation of organs for liver transplantation. They were not designed to assess the potentially more subtle stages of earlier disease progression.

A few studies have attempted to develop a disease-specific staging system in BA. A report in the European Journal of Pediatrics in 2003 by a group of Japanese physicians applied a retrospective analysis of 133 patients (75). With their system bilirubin, ALT, prothrombin time (PT), and signs and symptoms of portal hypertension were summed together to arrive at a final score that would predict transplant vs. non transplant. They reported a significant sensitivity and specificity of their scoring system. However, the study was limited by retrospective analysis and its limited application to only the infant. Another staging system was developed by the physician at King's College, United Kingdom and was presented at the American Association for the Study of Liver Diseases (AASLD) meetings in 2002. In this system three stratified groupings from mild to severe liver disease were defined using components of the PELD score. The study was also retrospective and never expanded nor validated in a larger prospective setting. (Personal communication with Drs. Hadzic, Mieli-Vergani and Bansal, King's College, UK)

BASIC will provide us with an adequate sample size to capture the spectrum of the disease process and explore development of a disease staging system.

3.0 Materials and Methods

Overview: Little is known about factors that cause BA or the factors that influence disease progression. A variety of genetic, autoimmune and environmental influences have been hypothesized to be important. Most studies to date have focused on the neonate and young child with BA, yet the older child with BA can provide important information about genetics, as well as, natural history. This project proposes to develop a database that contains appropriate clinical information, genetic material, and body fluid samples obtained from children and young adults with BA to address hypotheses aimed to identify genetic and clinical influences on etiology and outcome.

3.1 Inclusion Criteria

Participants need to have a confirmed diagnosis of BA determined by chart review including review of pertinent diagnostic biopsy reports, or radiologic reports and /or surgical reports (if surgery was performed). Participants need to be >6 months of age up to and equal to the age of 20 (participants enrolled at 20 years of age will have one visit). Participants either have their native liver or have a confirmed liver transplantation. Parent, guardian, or participant (if 18 years of age or older) is willing to provide informed consent and, when appropriate, the participant is willing to assent.

3.2 Exclusion Criteria

- Currently participating in the ChiLDReN study PROBE.
- Inability to confirm original diagnostic evaluation of BA.
- Inability or unwillingness of family or participant to participate in all scheduled visits.

3.3 Recruitment

Recruitment will be open to any eligible patient currently followed at the ChiLDReN clinical sites. The method of contacting the study participants will conform to IRB guidelines, but in general, the parents or guardians of all eligible patients at each ChiLDReN center, or the patients themselves if 18 years of age or older, will be approached to participate. New patients who are not participating in ChiLDReN study PROBE may be approached for study participation, but will not be eligible for enrollment until 6 months of age. The study will be listed on www.ClinicalTrials.gov, and the ChiLDReN website www.ChildrenNetwork.org. Clinical sites may advertise to attract self-referrals, subject to IRB approval.

3.4 Visits

i. Participants with a native liver will be seen at baseline and will continue to be followed at annual visits until 20 years of age (+/-6 months of age) or until transplant ed. If the participant undergoes a liver transplant, a collection of data and specimens should be obtained at the time of transplant.

ii. Participants that undergo a liver transplant while enrolled in the study (Native Liver Cohort) will no longer be seen at annual follow-up visits for data collection. Participants will remain active in the study until DNA collection is complete.

iii. Participants with a liver transplant before enrollment will complete a baseline visit only.

3.5 Measurements

The following two tables describe the evaluations to be performed at each visit: Table 3.5.1 Evaluations for participants enrolled with native liver and Table 3.5.2 Evaluations for participants recruited with a liver transplant. A visit may extend over more than one day. When possible, research visits will be scheduled to coincide with clinical visits.

Table 3.5.1 Evaluations for Participants Enrolled with Native Liver

Evaluations for participants enrolled with native liver	Baseline	Annual follow up	At transplant	Once (any visit)
Window for visit		± 6 months		
Informed consent & eligibility	X			
Lab Testing				
CBC	X	X	X	
Hepatic function	X	X	X	
PT/INR	X	X	X	
Vitamin D: 25-OH vitD	X	X		
Vitamin A with RBP	X	X		
Vitamin E	X	X		
Directed Physical Exam				
Weight	X	X	X	
Height	X	X	X	
Head circumference	X	X	X	
Vital signs/anthropometry	X	X	X	
Liver size & texture	X	X		
Spleen size & texture	X	X		
Skin	X	X		
Information				
Demographics (gender, race, DOB, ethnicity, #)	X			
Targeted Liver and medical history (SPLIT) (lifetime), PELD/MELD	X			
Detail of associated anomalies	X			
Medications/herbal remedies	X	X	X	
Targeted liver events		X	X	
Pregnancy questionnaires	X	X		
Specimens from Child				
Serum for repository	X	X	X	
Plasma for repository	X	X	X	
DNA for repository				X
Specimens from Parents				
DNA for repository				X

TABLE 3.5.2 Evaluations for participants recruited with liver transplant	
Visit Window: Complete data collection within 18 months	**Baseline**
Informed consent & eligibility	X
Information	
Demographics (gender, race, DOB, ethnicity)	X
Detail of associated anomalies	X
Target liver and medical /surgical history	X
Family medical history	X
Specimens from Participant	
DNA for repository	X
Specimens from Parents	
DNA for repository	X

Details of measures

After informed consent is obtained, participants will be seen at a ChiLDReN clinical site; a detailed interview will be conducted; laboratory results, physical exam information and quality of life assessments will be recorded in the research file. The data that will be obtained are described in the Tables 3.5.1 and 3.5.2 above which are self-explanatory except for the following:

Congenital Anomalies: Details of associated congenital anomalies will be obtained by chart review and verbal recollection from the primary caretaker. As part of the manual of operations we have compiled a complete list of which anomalies to detail. Our data collection forms have a checklist with all the anomalies of interest.

Brief Targeted Liver, Medical, and Surgical History: Targeted events will be queried by verbal history and review of medical records upon enrollment for all participants and at follow-up visits for participants living with their native liver. The information is obtained by query of cholangitis, sepsis, variceal bleeding, gastrointestinal bleeding, fractures, surgeries, and development of ascites. Female participants ≥18 years of child-bearing potential will be asked about their history of pregnancies and deliveries. Male participants ≥18 years will be asked about any children they may have fathered.

Laboratory tests: Routine tests for clinical care will be obtained at baseline and the yearly follow-up; these include: CBC, ALT, AST, GGT, total protein, albumin, alkaline phosphatase, retinol binding protein, PT/INR, creatinine, sodium, total bilirubin, direct bilirubin, vitamin A, 25-OH vitamin D and vitamin E, bile acids. The tests will be performed at specified intervals during outpatient follow-up. These tests will be analyzed in a CLIA and/or CAP-approved laboratory since clinical decisions will be made based on the results.

3.6 Specimens to be collected

3.6.1 Serum, plasma and/or saliva

These samples will be obtained at baseline. and at annual follow-up visits. When possible, they will also be obtained prior to liver transplant when the transplant is three months or more after an annual visit. The samples will be sent to a repository under contract with NIDDK.4 ml of whole blood will be drawn in two tubes:

1. 2 ml of whole blood to extract plasma: (~6 aliquots of 0.2 ml each)
2. 2 ml of whole blood to extract serum: (~6 aliquots of 0.2 ml each)

3.6.2 DNA

The following samples will be drawn once at a visit that is convenient. They may be drawn at a visit after the participant has a liver transplant.The samples will be sent to a facility (currently at Rutgers University) under contract with NIDDK. DNA may be collected via whole blood or, in the event that whole blood collection is not possible or contraindicated, by saliva.

From the biological mother and father:

1. 10ml of whole blood in one 10 ml NaEDTA vial to be sent to an NIDDK contract facility at Rutgers University for DNA extraction. OR
2. 2ml of saliva collected in a saliva collection kit.

From the participant, either:

1. 4 ml of whole blood in one 4 ml EDTA vial when the participant weighs less than 50 kg (110 lbs), OR
2. 10 ml of whole blood in one 10 ml NaEDTA vial for DNA extraction when the participant weighs 50 kg (110 lbs) or greater. OR
3. 2ml of saliva collected in a saliva collection kit.

NOTE: If an inadequate volume is collected, a redraw of blood sample may be requested.

3.6.3 Blood volume

Approximately, 6.5 ml of blood may be removed from the child at each visit to evaluate hepatic function, electrolytes, vitamin levels and blood count. In addition, 3 ml of blood may be removed to test vitamin levels. More blood may be withdrawn to perform additional clinically indicated laboratory tests.

When the participant is a patient at the ChiLDReN site, the blood for clinical tests will be drawn as part of the annual clinical evaluation of the participant. When the participant is a research participant and not a patient, this blood volume will be included in the total computation of blood volume for research use. However, this blood will not be drawn from research participants who are post-transplant.

3.6.4 Priority of Blood Sampling

When there is insufficient blood for all samples, the priority of blood samples are:

1) hepatic function, electrolytes and blood count*
2) other clinically indicated tests (non-research tests only)
3) vitamin levels
4) blood for DNA
5) plasma
6) serum

NOTE: Blood that is drawn specifically for research purposes will be obtained according to each study site's institutional guidelines.

- When the participant weighs less than 50 kg: 8ml of blood will be drawn for the repositories.
- When the participant weighs 50 kg or more: 14ml of blood will be drawn for the repositories.

In addition, 9.5 ml will be drawn for hepatic function, electrolytes, blood count, and vitamin levels.

3.6.5 Specimen Repositories

A central repository has been established by the NIDDK, a division of the National Institutes of Health, for long-term storage for blood, tissue specimens and a second repository has been established at Rutgers University for DNA extraction. Otherwise, samples will be shipped via licensed overnight carrier once every month to the NIDDK central repository.
All specimens are labeled with a de-identified study ID. A computer log will record all incoming samples at the central repository, the storage location, and the date, and the type of sample. Receipt of samples will be acknowledged to the originating center.

3.6.6 Specimen Use

The ChiLDReN Steering Committee has developed a policy for the approval of ancillary studies – studies that will require the use of samples in the repository. ChiLDReN investigators may propose such studies; non-ChiLDReN investigators may propose such studies only if they have a ChiLDReN investigator as a co-investigator. To be approved, these studies must relate to the specific aims of ChiLDReN, namely to study the pathogenesis and natural history of BA and neonatal hepatitis or to evaluate patterns of cellular gene and protein expression in tissue specimens and plasma by viral, genomic, and proteomic techniques. Examples of studies that have been proposed by ChiLDReN investigators (but as yet none are approved) are: screening for genetic mechanisms for pathogenesis and modifiers of BA; identifying novel antigens and T and B cell responses in BA; the association of HLA type and BA; and the identification of laterality genes associated with the "embryonic" form of BA.

These research studies are not related to clinical care; tests performed on anonymized samples will not be reported to the parents/guardians nor included in the medical record.

The goal of the NIDDK repositories is to make samples available for investigations that have not been specified. Until the funding for ChiLDReN terminates in May 2024and during any possible extension(s), all decisions about the use of the samples will be made by the ChiLDReN Steering Committee. After funding for ChiLDReN terminates, NIDDK will set up a peer review mechanism to determine the use of the remaining samples. All participant identifiers would have been removed from samples in the repositories (i.e., samples are de-identified).The study database will be transmitted to the NIDDK repository with all participant identifiers removed; e.g., dates will be converted to ages.

3.7 Costs and Payments to Participants

In addition to the collection of routinely obtained clinical data and the results of routine laboratory investigations, this research includes taking an extra blood sample (8-14mls depending on the visit and participant's weight), and samples of blood from parents. There will be no costs to the participant or their insurance for any research-related data collection, including the developmental assessments, and special laboratory investigation. The expenses for the storage and handling of the extra research blood are covered by the research.

For each scheduled follow-up visit the parents or guardians will receive a small stipend in the form of– cash, gift certificate, or check – to be determined by the clinical site and consistent with each clinical site’s regulatory authority to reimburse them for parking, meals, or other expenses that they may have related to the visit.

3.8. Number of Participants

A total of 781 post-transplant and 598 pre- transplant participants have been enrolled as of May 31, 2016. The ethnicity and racial categories of the participants are outlined in Table 3.8.1.

Table 3.8.1. Ethnicity and racial categories of the participants enrolled by May 31, 2016

	Post Transplant		Pre Transplant		Total	
	N	**PctN**	**N**	**PctN**	**N**	**PctN**
Total	**781**	**100.0%**	**598**	**100.0%**	**1379**	**100%**
Gender	489	62.6%	347	58.0%	836	60.6%
Female						
Male	289	37.0%	245	41.0%	534	38.7%%
Unknown	3	0.4%	6	1.0%	9	0.7%
Ethnicity	162	20.7%	89	14.9%	251	18.2%
Hispanic						
Non-Hispanic	614	78.6%	498	83.3%	1112	80.6%
Unknown	5	0.6%	11	1.8%	16	1.2%
Race	478	61.2%	376	62.9%	854	61.9%
White						
Black	118	15.1%	83	13.9%	201	14.6%
Asian	60	7.7%	57	9.5%	117	8.5%
Other	45	5.8%	28	4.7%	73	5.3%
Multiracial	57	7.3%	40	6.7%	97	7.0%
Unknown	23	2.9%	14	2.3%	37	2.7%

4.0 Statistical considerations

The expected number of participants (both pre- and post-transplant) for this study was originally expected to be 250. The table below provides the newly updated estimated number of 1,709 participants to be enrolled by the end of this funding period (June, 2019).

Table 4.0.1. Expected Enrollment Table

	Post-transplant	Pre-transplant	Total
	N	N	N
Total	934	775	1709
Gender	585	446	1031
Female			
Male	343	311	654
Unknown	6	18	24
Ethnicity	201	131	332
Hispanic			
Non-Hispanic	725	621	1346
Unknown	8	23	31
Race	577	508	1085
White			
Black	133	92	225
Asian	78	72	150
Other	45	28	73
Multiracial	66	43	109
Unknown	35	32	67

Specific Aim 1

Sequence analysis for the 19 candidate genes has been done on over 95 normal alleles for some of the genes and for 298 normal alleles for most genes by Dr. John Belmont at the Baylor College of Medicine in Houston, TX. That should be sufficient to provide a basis for distinguishing potential mutations from normal polymorphic variants. There are no data on a comparable population to form a basis for predicting the percentage of these participants that are likely to have a mutation of one of the 19 genes that are going to be sequenced. We have proposed a pilot study of 40 participants on the basis of the preliminary results of the Belmont lab showing pathogenic sequence variants in 61 of 238 amplicons from participants with situs defects of the heart. This means that a pilot study of 40 participants would identify

pathogenic sequence variants in at least 10 of the participants. The only data for chromosomal abnormalities is from Silveira et al. (8). Two of eight participants examined had chromosomal abnormalities.

We predict that approximately 15% of enrolled BA participants will have anomalies associated with classical situs inversus as well as anomalies that are not associated with classical situs inversus. The latter include anomalies that were found in the study of Silveira et al. that most extensively characterized anomalies in the single largest collection of BA patients in the literature (8). The rationale for including these anomalies comes from the study of Ware et al. (44) that found mutations in the *Zic3* gene in patients with congenital heart defects characteristic of classical situs inversus as well as in patients with congenital heart defects not associated with classical situs inversus.

Ancillary Study Sample Size Calculation: A reason to increase the number of total participants in this study (in this amendment) relates to the stated goal of BASIC to provide sufficient numbers of participants, human specimens and DNA to allow for Ancillary Studies (that are not detailed in this protocol) that will have the statistical power to perform genetic analyses and biomarker discovery investigations. For example, a GWAS Ancillary Study has been approved and funded to discover genetic susceptibility factors to BA by identifying genetic variants that are associated with the disease, by carrying out an association study to both SNPs and CNSv with follow-up by detailed sequencing of the regions identified (Principal Investigator: Nancy Spinner, PhD, Children's Hospital of Philadelphia). ChiLDReN Network will combine the group of PROBE participants who have BA and BASIC participants (all of whom have BA) to provide samples and data for this Ancillary Study. As the number of samples of non-white is small, and to minimize the risk of confounding due to population stratification, this initial analysis will focus on a discovery cohort of samples from self-reported white participants.

Based on various publications, it appears that for homogeneous populations (in terms of race/ethnicity) 800 cases and a comparable number of controls would be needed to detect a genome wide association on the order of a relative risk of 2.0 with respectable power (80-90%), and that far larger studies (on the order of 2,000-3,000 cases and a similar number of controls) would be needed for a smaller relative risk of 1.3-1.5 (76).

Based on most recent enrollment report (reviewed June 2016), BASIC and PROBE together will still have well under 2000 available samples at the end of this funding cycle. As of May 31, 2016, BASIC study had enrolled 1379 participants, which includes approximately 712 white children with BA with available DNA specimens. Together with PROBE, ChiLDReN has enrolled approximately 911 white children with BA with available DNA specimens, among which 80% (approximately 729) have isolated BA. Under the current enrollment trend, by the end of this funding period, we expect to enroll an additional 330 children in BASIC and 100 BA children in PROBE, which will yield approximately an additional 158 (according to the current trend, 61.9% and 57.3% participants are white, respectively, and 80% and 58% in BASIC and PROBE have DNA samples, respectively) white children with isolated BA and DNA specimens. Given the above calculation, at the end of this funding period, the number of available cases for a genome wide association study on white children with BA will be

729+158=887 approximately. We propose to continue enrollment into BASIC, in hopes of increasing the number of available participants and DNA samples to be combined with the PROBE BA population in order to have as large a population as possible for these genetic studies and to have adequate power for smaller relative risks, ideally in the 1.3-1.4 range.

Specific Aim 2

The unique aspect of the autoimmune data collection is the use of δ values to quantitate HLA data, thereby permitting the use of more powerful statistical tools. This method depends on selecting a 'disease epitope' against which to score all other epitopes, since an HLA allele has a numerical value only in comparison to other alleles. However, the project also involves a parallel analysis of HLA alleles in the traditional manner, and one final outcome of this project will be to compare the two methods. Dr. Freed and associates will look for the susceptible epitopes or HLA alleles within a group of patients who have the most severe form of the disease which is based on clinical criteria established for each of the diseases. We assume that this population is likely to have the most disease-prone epitopes, as well as the highest probability of being homozygous for susceptible HLA alleles and epitopes. We search all combinations of epitopes within the HLA-A, B, C, DRB1, DRB3, DRB4, DRB5, DQA1, DQB1, DPA1, and DPB1 loci. We then rank all possible epitopes and HLA alleles based on x^2 values, using several possible control populations to provide the expected frequencies.

The control frequencies will be determined by multiple sources the largest of which is the 6,600 cord blood units that have been typed at *ClinImmune Labs* since 1997. The cord blood units have low resolution HLA-A and B and high resolution HLA-DRB1. Because these were collected in the state of Colorado the percentages of African Americans and Asians are low, nevertheless they represent sizable numbers (n = 264 and 132, respectively) with which to determine allele or epitope frequency. The cord bank provides sufficient HLA- DRB1 alleles and epitopes to serve as a control for any study, but these individuals have not been high resolution typed for HLA-A, B, C, DQA1, DQB1, DPA1 or DPB1. Hence, a concurrent rheumatoid arthritis project will be developed as a control. The validity of this group as a control will be determined by comparing allele frequencies of HLA-A, B and DRB1 with the 6,600 cord blood units. Subsets of both of these control populations will be used to control from race and ethnicity.

The second phase of the analysis is to use those epitopes with the highest χ2 values to produce δ scores for all other participants with BA. Due to the fact that we have selected epitopes based on their prevalence within the disease population, the mean ± standard deviation for this Epitope 1 will, by definition, be very low (mean <10). Epitope 2, corresponding to the other HLA allele, could also be low in the patient population due to homozygosity for disease epitopes. However, Epitopes 1 and 2 in the control populations should be normally distributed. If the δ score is also low in the control population, it would indicate that the epitope is very common and the differences are not statistically significant. Thus, Dr. Freed's computer algorithm looks first for epitopes that are very common in the disease population, and then subsequently looks for those that have significantly lower δ scores than the control populations.

Due to the retrospective nature of epitope searching, there exists an increased possibility that we will identify shared epitopes with increased frequency and lower δ scores, but that are unrelated to the immune-mediated disease (Type I statistical error). From a statistical standpoint, this is a problem inherent in all retrospective analyses. However, as with most medical studies, the retrospective analysis must be performed to identify disease associations, and statistical validation follows with a second independent study. To minimize the chances of a Type I error, we set the significance value for differences in δ scores at $p<0.01$. However, BA may have many epitopes with significant differences in δ values between participants and controls, which can then be ranked from highest to lowest. Power calculations were used to determine the sample size required to find a statistically significant difference between frequency of an HLA antigen in the control group and each of the immune-mediated diseases, using a two-sided test with a power of 0.8 and a significance level of 0.01. SLE is the immune-mediated disease with the weak defined HLA association. If we use it as a model for BA, where the HLA association is unknown, we can estimate that we will need 131 participants to test our hypothesis.

The enrollment is mainly driven by the large sample size needed for the genetic studies in Aim 1. BA participants enrolled in both BASIC and PROBE and data collected on them will be used as a convenient sample for testing the hypothesis in Aim 2.

Specific Aim 3

We anticipate to have enrolled 775 pre-transplant participants by the end of the funding period (June, 2019). We assume with this expected number of participants, data from at least 100 participants will be available for disease modeling at each age, the standard error of an estimate of annual rate of transition (e.g., to liver transplant or of onset of cholangitis) will be no larger than 5%; the standard error will be 3% when the transition rate is 10% and 2% when the transition rate is 4%. This will enable us to model the conditional probability of liver transplant and other complications at each age.

In addition, according to the currently observed event rate, we expect 14% (approximately 74 participants) among the anticipated 527 pre-transplant participants will have died or had liver transplant by the end of the grant period. This allows us slightly more than 80% power to detect the relative risk of 1.887 as statistically significant (alpha=0.05, two-sided), for a factor that was present in approximately 50% of the population.

Finally, change over time in serum total bilirubin or other biochemical marker will be modeled longitudinally using a random effects model. These models will include data from all participants (~642) and will have 90% power to identify correlations as low as 0.15 using a two-sided test of significance.

5.0 References

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