Cincinnati Children's Hospital Medical Center
Division of Gastroenterology & Hepatology

Protocol Title: Pilot Study- Osmotic fragility in Red Blood Cells of Pediatric Patients with Cholestatic Liver Disease

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1. ABSTRACT

Background:

Cholestasis is a condition characterized by impairment of bile flow and/or formation. The condition can occur from either extrahepatic obstruction or intrahepatic hepatocyte bile secretory defects.

Cholestatic liver diseases are a well-recognized cause of dyslipidemia, and are closely associated with abnormal cholesterol and phospholipid metabolism. Cholesterol conversion to bile acids may be impaired, and apo-lipoprotein B binding to cholesterol may be impaired, resulting in the formation of lipoprotein X which contains apolipoprotein C. The end result of dyslipidemia in cholestatic patients is hypercholesterolemia and an increased membrane cholesterol to phospholipid ratio.

Elevated red cell membrane cholesterol to phospholipid ratios result in decreased osmotic fragility and decreased deformability of the red blood cell (RBC). When red cell membranes are compromised, and especially when membrane peroxidation is increased (due to exposure to an excessive oxidant load or due to reduction in antioxidants such as vitamin E), spur cells will form (1,2).

We hypothesize that even in the absence of spur cell formation, subclinical changes in osmotic fragility will appear and these can be measured by ektacytometry.

Ektacytometry is a tool to measure the deformability and osmotic fragility of red blood cells subjected to shear stress while exposed to an osmotic gradient (Figure 1). Ektacytometry can be used as a diagnostic tool to determine the presence of abnormalities in cell membrane structure. It does this by analyzing the RBC rheological behavior in a variety of physiologic and non-physiologic conditions.

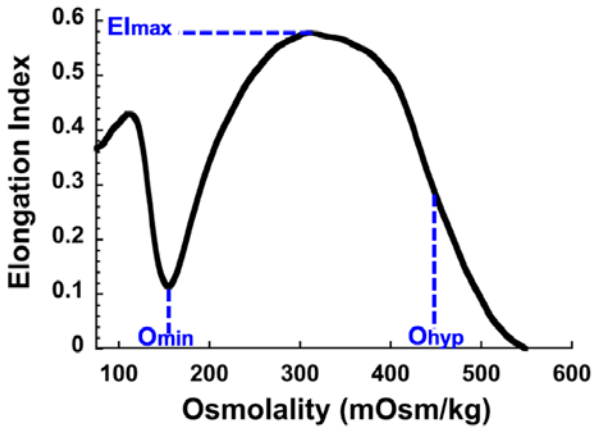

Figure 1. Normal RBC ektacytometry. The curve provides the following characteristics: **Elongation Index (Elmax):** Elmax corresponds to the maximum deformability of the erythrocyte. Its value depends mostly on the cytoskeleton mechanics. **Omin:** Omin corresponds to the value of the hypotonic osmolality where 50% of the cells hemolyze in an osmotic fragility assay and provides information on the initial surface to volume ratio of the cell sample. A shift to the right reflects a decrease in the surface area/volume ratio. **Ohyp:** The declining portion of the curve is represented by the Ohyp value. This is the osmolality value where the cell's maximum diameter is half of Elmax and correlates with the initial intracellular viscosity of the cell sample. A shift to the left reflects increased intracellular viscosity of the erythrocyte caused by increased intracellular concentration of hemoglobin and/or a dehydration state.

Objective:

We propose to perform ektacytometry on 20 pediatric patients over age one with cholestatic liver diseases and a direct bilirubin level of greater than 2 gm/dl. The most common diagnoses will be extrahepatic biliary atresia, progressive familial intrahepatic cholestasis, Alagille syndrome, autoimmune hepatitis, primary sclerosing cholangitis, and parenteral nutrition-associated cholestasis. We will correlate the osmotic fragility and deformability with their direct bilirubin levels, their serum cholesterol levels, their serum bile acid levels, and their vitamin E levels.

Design/Methods:

This pilot study will be a single center, prospective cross-sectional investigation of red blood cell ektacytometry in pediatric patients with extrahepatic cholestasis who are followed at Cincinnati Children's Hospital Medical Center. We will include all subjects with cholestasis regardless of the etiology in order to maximize the number of subjects. While the population will be heterogeneous, we will stratify patients according to diagnosis, recognizing that only a few patients may fall into each diagnostic category. Ektacytometry will be the method utilized to measure osmotic fragility and deformability of the RBC membrane. The ektacytometry of red cells from cholestatic patients will be compared to that of red cells obtained from contemporaneous age-matched controls recruited among patients without liver disease or red cell membrane defects undergoing blood sampling for evaluation of other entities including but not confined to functional abdominal pain.

2. PURPOSE OF STUDY

Primary Aim:

The primary aim is to determine if ektacytometry of red blood cells is abnormal in a population of pediatric patients over age one with cholestasis.

Secondary Aims:

The secondary aim is to ascertain the extent at which abnormalities in bilirubin levels, bile acid levels, vitamin E levels or cholesterol levels in cholestatic patients are related to changes in red blood cell ektacytometry in this patient population.

3. BACKGROUND

Cholestatic liver disease is an umbrella term for reduced bile flow from either extrahepatic obstruction of bile ducts or faulty hepatocyte bile secretion. Cholestasis is a well-recognized cause of dyslipidemia, and are closely associated with abnormal cholesterol and phospholipid metabolism (3). Cholesterol conversion to bile acids may be impaired, and apo-lipoprotein B binding to cholesterol may be impaired, resulting in the formation of lipoprotein X which contains apo-lipoprotein C (3). The end result of dyslipidemia in cholestatic patients is hypercholesterolemia and an increased membrane cholesterol to phospholipid ratio (3).

The lack of bile entering the digestive tract can lead to increased serum cholesterol, and abnormally high red cell membrane cholesterol: phospholipid ratios, resulting in altered red cell membrane structural integrity (4,5). To test for this phenomenon in patients with extrahepatic biliary atresia, 20 pediatric patients will be tested using ektacytometry to test the deformability and osmotic fragility of red blood cells.

Ektacytometry is a diagnostic tool used to measure the deformability of red blood cells on an osmotic gradient (6). More specifically it measures deformability due to shear stress, while the RBC are suspended in an osmotic medium (6,7). It can differentiate among several blood maladies such as hereditary spherocytosis, hereditary elliptocytosis, Southeast Asian ovalocytosis, and pyropoikilocytosis that are otherwise difficult to diagnose (8). It does this by analyzing their rheological behavior under constant shear stress in vitro and using photometric analysis of deformability. This is then quantified and provides a specific ektacytometry curve (Figure 1).

The ektacytometry curve will have several unique features depending on the red blood cell being analyzed. The Omin portion of the curve represents the value of the osmolality in which 50 percent of the red blood cells lyse. The Elmax is the value of the elongation index that reaches its maximum near physiologic osmolality (300 mOsm/Kg). And lastly the Ohyp, which represents the declining portion of the curve and correlates to the initial intracellular viscosity of the cell sample.

To date, ektacytometry has not been employed systematically to characterize potential red cell abnormalities in cholestatic patients. In our analysis the ektacytometry profiles of cholestatic patients will be compared to those of historical non-cholestatic age-matched controls. The degree of fragility will be correlated with serum direct bilirubin levels, serum cholesterol levels, and vitamin E levels.

4. STUDY DESIGN

This pilot study will be a single center, prospective cross-sectional investigation into the ektacytometry of red blood cells taken from 20 cholestatic pediatric patients followed at Cincinnati Children's Hospital. They are seen regularly for continued follow up in the hepatology clinic. Parents of all of these patients will be asked to participate in this investigation. We will also contemporaneously analyze ektacytometry of 20 age-matched controls recruited from the general GI clinics with neither cholestatic liver disease nor know red cell membrane defects.

5. DURATION

The study is anticipated to take approximately 6-12 months to complete. This time table includes 3-6 months to recruit patients and perform the assays and another 3-6 months to analyze the data and complete a manuscript.

6. SELECTION & RECRUITMENT OF PARTICIPANTS

- Inclusion Criteria

Disease Cohort
Age greater than one year old
Direct bilirubin level of >2 mg/dl.
No known congenital red cell membrane defect

Controls
Age matched to disease cohort
No evidence for cholestatic liver disease
No known congenital red cell membrane defect

- Exclusion Criteria.

Disease Cohort
Parental refusal
Age <1 year old.
Known congenital red cell membrane defect

Controls
Parental refusal
Age <1 year old.
Known congenital red cell membrane defect
Cholestatic liver disease

The study Principal Investigator (PI), PI designate, or research coordinator (research team) will have the responsibility for case finding and subject recruitment. Potential study participants will be identified by the PI and a further member of the research team will enroll the participants.

7. PROCESS OF OBTAINING CONSENT

The Research Team will discuss appropriateness for study inclusion with the clinical team, and contact the family, most likely during their hepatology outpatient clinic visit or an inpatient hospitalization. The study will enroll children, a vulnerable population, therefore patients and parents will be explicitly informed that choosing not to participate, will in no way affect the quality of the medical care that they will receive.

Details of the study will be reviewed with one or both parents prior to obtaining consent. All patients will be under the age of 18 years old and therefore only parental consent will be necessary along with the participant's assent if older than 11 years of age.

8. STUDY PROCEDURES

All patients are routinely seen in outpatient clinic by one of the hepatologists from Cincinnati Children's Hospital Medical Center. Control patients will be gastroenterology patients seen by one of the general gastroenterologists in the general gastroenterology clinics. The research team will approach the family in clinic or in the hospital and after informed consent will obtain a 500-1500 mcl aliquot left-over from blood that has been drawn into a lavender top (EDTA) tube for measurement of a complete blood count. or a yellow top tube for measurement of serum chemistries. To avoid research-only blood draws, blood will only be obtained if the patient is undergoing clinical laboratory testing. The team's PI, Dr. Samuel Kocoshis, and coinvestigators, Dr. Theodosia Kalfa and Mr. Theodore Kocoshis, will have access to the individual results of the assay.

All blood samples will be de-identified with the assigned research number.

In addition to providing a blood sample, we will record in a secure database the patient's demographics such as current age at time of blood draw, race and ethnicity, birth history, past medical and surgical history as well as contemporaneous direct bilirubin levels, hemoglobin levels, hematocrit levels, and red cell morphologic changes noted in the hemogram. Cholesterol levels, bile acid levels, and vitamin E levels which were obtained as close as possible to the ektacytometry analysis will also be recorded. Only the investigators at Cincinnati Children's will have access to this database.

Study Completion

Once the blood sample has been obtained, no additional study procedures will be performed and the remaining blood will be properly disposed once the assay is complete.

9. DATA ANALYSIS/METHODS

Sample size:
The sample size of 20 cholestatic patients and 20 controls is estimated based on the number of patients followed at CCHMC. We estimate a 75 % participation rate for the study. Knowing that we have a small patient pool within each diagnostic category of cholestatic patients, we will stratify patients according to diagnosis, but suspect that we will have only 3-5 patients within each diagnostic grouping, so it may be difficult to draw conclusions regarding relative differences in osmotic fragility and deformability within the overall cholestatic population. Because this is a pilot study we do not know what the difference in Omin or deformability between patients and controls will be. Hence we cannot conduct power analysis. However, we can utilize our preliminary data to facilitate power analysis for future studies.

Laboratory Methods:

Within 24 hours of blood sampling, we will conduct our ektacytometric analysis. Samples will be drawn into EDTA tubes. In the clinical hematology lab, hemoglobin, hematocrit, red cell indices, and RBC number will be measured by standard methods. A blood smear will be stained with a Giemsa stain and reviewed by clinical cytologists. Ektacytometry will be performed in the CCHMC Erythrocyte Diagnostic Lab per clinical testing SOP. Specifically, a 300 microliter sample will be injected into the Osmoscan LoRRca MaxSis (Mechatronics Instruments BV, Zwaag, The Netherlands) ektacytometer. Patients' RBCs deformation will be monitored while exposed to a constant shear stress of 30 Pa and an increasing osmotic gradient in

order to generate the ektacytometry curves. A positive result is seen as a divergence of the Omin, Elmax, and Ohyp of the sample compared to a concurrently-run normal control and the normal control range for the assay. Deformability will also be tested under an increasing shear stress for further investigation of the RBC cytoskeleton mechanics. The Erythrocyte Diagnostic Lab will then discard the remaining sample once a result is obtained.

Bilirubin levels, bile acid levels, lipid profiles, and vitamin E levels will be measured by standard laboratory methods employed by the CCHMC clinical laboratory. Bile acids will be measured by radioimmunoassay.

Statistical analysis
We will compare the Omin and EI max values between patients and controls by Student's t test of the means. The ektacytometry characteristics (mainly values of Omin and Elmax) will be correlated with direct bilirubin levels, serum cholesterol levels and vitamin E levels by linear regression analysis.

10. FACILITIES AND PERFORMANCE SITES

The research study, all patient recruiting and blood draws will be performed in either the inpatient hepatology unit, the hepatology clinic, the general GI unit, or one of the general GI clinics at CCHMC. Ektacytometery will be performed in the CCHMC Erythrocyte Diagnostic Laboratory and analyzed by Dr. Theodosia Kalfa. Bile acids will be analyzed within Dr. Miethke's laboratory.

11. POTENTIAL BENEFITS

There is minimal risk associated with participation in this study. The potential benefits of this study pertain to the advancement of scientific knowledge in the area red cell membrane changes among cholestatic patients.

There is unknown direct medical benefit for patients.

12. POTENTIAL RISKS, DISCOMFORTS, INCONVENIENCES AND PRECAUTIONS

There are no increased risks to participate in this study. The analyses will be conducted only upon blood that has been drawn for clinical purposes so there are no additional risks beyond what the patient would encounter for sampling conducted for clinical monitoring even if patients did not participate in the study. Blood will not be drawn specifically for this study, and a minimal amount of whole blood will be required.

All unanticipated problems and adverse events will be reported consistent with institutional policy.

13. PRIVACY AND CONFIDENTIALITY

All study material will be stored in a locked storage cabinet in the CCHMC Gastroenterology office that will remain locked when not in use by study personnel. Access to original data and study materials will be limited to the investigative team. Informed consent/assent documents will be stored in a locked storage cabinet separate from study data. All raw data and computer files containing data will have confidentiality protected with the use of unique assigned study identifier codes; computer files will also be password protected. As mentioned previously, only a few selected study team members will have access to the

database, completed forms and results of the assay. This dataset will only be used for research purposes. Each subject will be assigned a study identification code so that the study information will be confidential. All blood samples will be collected and processed in blood tubes containing the subject's unique identification code. All blood samples will be destroyed at the earliest opportunity as consistent with the conduct of the research, after all data on that patient has been collected and processed.

Information from this research study may be published. However, participants will not be identified in any publication. All subject identifiers (e.g. medical record number, date of birth) as defined by HIPAA will be removed, so that identities are not traceable within or outside the study. The privacy and confidentiality of patient information will be maintained in accordance with HIPAA regulations.

14. COST OF PARTICIPATION

There is no cost to the patient to participate in this study. The cost of performing ektacytometry will be borne by the Kalfa laboratory. The cost of performing bile acids will be borne by Dr. Miethke's laboratory. Other, clinically indicated testing, will be covered by the patients' insurers.

15. PAYMENT FOR PARTICIPATION

There is no payment to patients for participation in this study.

16. FUTURE USE

All data collected will be stored in a data repository for future analysis regarding similar research interests. All future projects will undergo separate IRB approval. The repository will be stored on a password protected file. There will be no linking information to patient identifier numbers and it will not be used as a means for future recruitment.

17. References

1. Cooper RA. Anemia with spur cells: a red cell defect acquired in serum and modified in the circulation. J Clin Invest. 1969 Oct; 48(10):1820-31.

2. Kohno M1, Murakawa K, Yasunari K, Yokokawa K, Horio T, Kano H, Minami M, Yoshikawa J. Improvement of erythrocyte deformability by cholesterol-lowering therapy with pravastatin in hypercholesterolemic patients. Metabolism. 1997 Mar; 46(3):287-91.

3. Corey KE, Cohen DE. Lipid and Lipoprotein Metabolism in Liver Disease. [Updated 2015 Jun 27]. In: De Groot LJ, Chrousos G, Dungan K, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000-.Available from: https://www.ncbi.nlm.nih.gov/books/NBK326742/

4. Cynamon HA, Isenberg JN, Gustavs LP, Gourley WK. Erythrocyte lipid alterations in pediatric cholestatic liver disease: spur cell anemia of infancy. J Pediatr Gastroenterol Nutr 1985;4:542-9

5. Allen DW, Manning N. Cholesterol Loading of Membranes of Normal Erythrocytes Inhibits Phospholipid Repair and Arachidonoyl-CoA: 1-Palmitoyl-sn-Glycero-3-Phosphocholine Acyl Transferase. A Model of Spur Cell Anemia. Blood 1996; 87:3489-3493

6. Bessis M, Mohandas N, Feo C. Automated Ektacytometry: a new method of measuring red cell deformability and red cell indices. Blood Cells 1980; 6(3):315-27

7. Mohandas N, Clark MR, Jacobs MS, Shohet SB. Analysis of factors regulating erythrocyte deformability. *J Clin Invest*. 1980; 66(3):563-573.

8. Da Costa L, Suner L, Galimand J, et al. Diagnostic tool for red blood cell membrane disorders: Assessment of a new generation ektacytometer. *Blood Cells Mol Dis*. 2016; 56(1):9-22.