**Final Progress Report (November 30, 2020)_Lay version**

**Project Title:** Newborn Screening for Cystinosis
**Funding Period:** September 1, 2017 – August 31, 2019 (No Cost Extension November 2019 – August 2020)
**Grant Number:** CRFS-2017-006
**Principle Investigator:** Sihoun Hahn
**Application Organization:** Seattle Children's Hospital Research Institute

This final progress report summarizes the completion of milestones and activities during the funding period from September 2017 to August 2020. Below are the original milestones and our progress with regards to each.

**A. Summary of progress in achieving research goals, objectives, and aims.**
The primary objective of this project is to develop and validate a specific and quantitative assay for newborn screening (NBS) of cystinosis (CN) using dried blood spots (DBS). To date, we have been able to achieve progress in both of our proposed specific aims.

**Specific Aim 1:** *Enhance the sensitivity and specificity of the existing immuno-SRM assay for Cystinosis (CN) by adding additional marker peptides of cystinosin (CTNS).*

A high affinity monoclonal antibody (mAb) was successfully produced to the sequences CTNS 115 and SHPK 363. The CTNS 115 mAb enriches the target peptide sequence from DBS of both patients and healthy controls for quantification by liquid chromatography tandem mass spectrometry (LC-MS/MS). Because of the existence of a co-enriched contaminating peptide discussed below, additional mAb productions were undertaken. Peptides CTNS 120 and CTNS 194 failed to derive high affinity MAbs. The sequence CTNS 274 generated an antibody that successfully isolated standard peptides but failed to enrich endogenous CTNS 274 from DBS. This indicates that the sequences may be difficult to extract from human blood or digest into the target sequence.

When utilizing CTNS 115 mAb for enrichment of this sequence, a contaminating peak was consistently present in the analysis. This contaminant has an identical mass and a very similar retention time to the biomarker CTNS 115 sequence. This necessitated extended runtimes to allow for separation of the two peaks and accurate quantification. Changes to the enrichment process, including aggressive washing, salt washes, and heating, were unable to remove the contaminating sequence. A final antibody production is ongoing that will raise an mAb specifically to the CTNS 115_2 sequence is underway and discussed below under ***Ongoing Efforts***.

Important aspects of CTNS 115 immuno-SRM analysis have been studied over the course of the grant period. The limits of detection, intra-day (within day) coefficients of variation (CV), and inter-day (between day) variation were studied to determine whether they would allow for successful translation. Acceptable CVs are < 20%. Variations were measured by conducting triplicate full process assays (including DBS extraction, digestion, and capture) across five different days. Intra- and inter-day variations were < 11.4% and 4.83% across five days, respectively. The limits of detection and quantification for CTNS 115 were 3.4 and 7.8 pmol/L. All these parameters are within acceptable ranges for clinical translation.

**Specific Aim 2:** *Assess the ability of a multiplexed immuno-SRM assay to correctly identify patients with CN in a large set of clinical samples and proven carriers from a broad spectrum of mutations.*

Normal control DBS were screened to establish normal control ranges and tentative diagnostic cutoffs. When analyzed by immuno-SRM, over 95% of DBS samples from CN patients were below these

cutoffs. In the CTNS cohort, over 50% of patients had levels of CTNS 115 which were at or near the limits of detection, i.e., undetectable. Normal controls sample were collected by fingerstick while patient DBS were spotted from whole blood collected during routine clinical course. One third of patients with non-detectable SHPK were found to be homozygous for the 57-kb deletion mutation. In addition, SHPK levels were found to segregate patients with homozygous 57-kb deletions, heterozygous for the 57-kb deletion and another mutation, and heterozygous for two other distinct mutations. Combined, the results indicate the multi-plex quantification of CTNS and SHPK peptide in DBS effectively identify Cystinosis patients with high sensitivity and specificity.

During the grant period we discovered that the use of certain anticoagulants will significantly affect the measured concentrations of CTNS biomarker peptides. The use of Sodium Heparin as an anticoagulant for blood collection is comparable to the analysis of blood collected from fingerstick or with no anticoagulant. We found that in patient samples as well as controls, blood collection in K2EDTA reduced the measured CTNS 115 concentrations. This is not an issue for NBS as these DBS are collected by heel stick methods. In a clinical setting, however, it will now be important to collect CN DBS samples utilizing fingerstick or a specified anticoagulant.

**B. Ongoing Efforts:**

Our study opened the feasibility of newborn screening for Cystinosis using Immuno-SRM. Majority of confirmed patients presented with either absent or low signature target peptides and overall, the performance of the assay was acceptable for clinical use. The only but significant caveat was the interference peak which necessitated extended runtime to separate it from endogenous peak. This extended runtime makes the assay not yet suitable for high throughput newborn screening. Here, we are currently working on producing the antibodies that can help eliminate the contaminant in the sample preparation.

<u>Antibody production:</u>

Since the no cost extension, the peptide sequence contaminating CTNS 115 analysis has been identified. This allowed us to initiate an mAb production project that provides us multiple avenues to produce a successful NBS assay for CN. This project aims to generate antibodies against both the target sequence and the contaminating sequence. After this initial production, mAbs that bind both sequences will be removed by negative selection. This step should lead to mAb sequences that bind only CTNS 115_2 with no co-isolation. Successful production of a CTNS 115_2 specific antibody is an ideal solution that will be readily adaptable to our current workflows and eliminate the issue of interference. This assay will allow for rapid screening of CN patients as an extended analysis is no longer necessary to separate contaminants. Generation of an antibody against contaminant peptide gives the project a secondary solution. In this case, we can include depletion of contaminant peptide as an initial step in our analysis and utilize the current CTNS 115 mAb for isolation of CTNS 115 alone. This project gives two viable paths toward a rapid screening assay for CN.

<u>Patient sample collection:</u>

We continue to work with our collaborator (Dr. Gahl) at the NIH to collected patient samples in multiple anticoagulants so that the differences between them can be studied and appropriate diagnostic ranges can be established depending on the collection conditions.

**C. Podium Presentations:**

1. Proteomic Newborn Screening for Cystinosis. Cystinosis Research Foundation International Meeting. February 27-28, 2020. Newport Beach, CA, USA
2. Rapid Multiplexed Proteomic Screening: Wilson Disease, Primary Immunodeficiencies, and

Cystinosis. Pediatric Grand Round, Soonchunhyang University Hospital, September 18, 2018, Seoul Korea

3. Newborn Screening for Cystinosis. International Research Symposium. Cystinosis Research Foundation. March 1-2, 2018 Irvine, CA, U.S.A.

**[Poster]**

1. Application of targeted proteomics for potential newborn screening of primary Immunodeficiency disorders, Wilson disease and cystinosis. SSIEM, September 3-6, 2019, Rotterdam, Netherlands
2. Application of Proteolytic IMMUNO-SRM-MS/MS in Diagnosis of Wilson Disease, Primary Immunodeficiency Disorders, Cystinosis and Lysosomal Storage Diseases. Society of Inborn Metabolic Disorders, April 6-9, 2019, Bellevue, WA
3. Proteomic Peptide Screening of Dried Blood Spots for Cystinosis. Pediatric Academic Society, May 6-9, 2017, San Francisco, CA

**D. Patent/License:**

1. Non-Provisional IP (PCT/US2019/054856): Title of invention is “Newborn screening for primary immunodeficiencies, cystinosis and Wilson disease”.

**E. Funding:**

1. Multiplex Proteomic Analysis for Next-Generation Newborn Screening of Wilson Disease, Cystinosis, and Primary Immunodeficiencies (NIH R01HD098180: 2019-2013)