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RESEARCH ARTICLE

Effect of Sample Processing Delays on the Values of Serum Based Biomarkers of Brain Injury Collected from the Umbilical Cord Blood of Neonates

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Abstract:

Background:

When a neonate is born with suspected brain injury, blood samples are often obtained from the umbilical cord blood but are not always processed immediately.

Objective:

Test the accuracy of brain injury biomarker assays on samples that experienced delayed processing.

Methods:

Healthy neonates who did not have risk factors for brain injury provided cord blood samples. Group 1 blood samples were centrifuged immediately, and the serum was removed and frozen at baseline, 4, and 8 hours. Group 2 had a baseline sample processed immediately and then blood samples remained in contact with the clotted portion until 4, and 8 hours and then were centrifuged. Enzyme-linked immunosorbent assays determined the concentrations of Ubiquitin C-Terminal Hydrolase L1 (UCH-L1) and Glial Fibrillary Acidic Protein (GFAP).

Results:

Group 1's average concentrations of GFAP were 62±47 pg/ml at 0 hours (n=32) with a mean increase of 3±14% and a decrease of 0.2±9% at 4 and 8 hours, respectively. UCH-L1 average concentrations were 3306±3093 pg/ml at 0 hours (n=37) with a mean increase of 3±10% at 4 hours and a mean decrease of 0.6±11% at 8 hours. Group 2's average GFAP concentrations were 104±111 pg/ml at 0 hours (n=9) with a mean decrease of 5±9% and 7±7% at 4 and 8 hours, respectively. UCH-L1 average concentrations were 3448±2456 pg/ml at 0 hour (n=8) with a mean increase of 9±6% and 6±18% at 4 and 8 hours, respectively.

Conclusion:

Delays in processing up to 8 hours did not significantly affect the concentration of UCH-L1 or GFAP.

Keywords: Hypoxic-ischemic Encephalopathy, Biomarkers, Neonates, GFAP, UCH-L1, Brain injury.

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1. INTRODUCTION

Hypothermia is the standard of care for neonates with Hypoxic-Ischemic Encephalopathy (HIE) [1]. Therapeutic hypothermia produces a normal developmental outcome in one neonate for every eight treated [2]. Researchers are working to develop synergistic neuroprotective agents that would act in concert with hypothermia. Several promising agents, including Xenon, erythropoietin, and melatonin, are being evaluated [3 - 6]. Currently, the bedside clinician is unable to differentiate a neonate who would respond to hypothermia alone or a neonate who would benefit from other neuroprotective therapies.

Biomarkers may assist the bedside clinician with dis-

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cerning neonates who respond to hypothermia versus non-responders. Several biomarkers have been explored including Ubiquitin C-Terminal Hydrolase L1 (UCH-L1) and Glial Fibrillary Acidic Protein (GFAP) [1, 7 - 11]. Biomarkers that predict injury at early time points may be the most clinically relevant [12].

Ideally, serum collected for biomarker analysis is processed soon after collection. The early time points, 0-6 hours after birth, are most likely to predict outcomes [12]. Approximately 55% of neonates born in the State of Florida who undergo therapeutic hypothermia are born at other facilities and transported for hypothermic treatment. Until real-time point-of-care devices are available for biomarkers, blood obtained at smaller facilities at 0-6 hours of life must be transported with the neonate and are not always centrifuged prior to transport. In Florida, transport times may be up to 4-6 hours. The samples obtained at the transferring facility would be transported to the hypothermia center and processed. The information from the 0-6 hour sample that is obtained around the time of the initiation of hypothermia may assist the bedside clinician in gauging the response to hypothermia with a rapid decrease in the biomarkers potentially representing a favorable long-term outcome [12]. Although the information may help the bedside clinician, the delay in processing the samples from transported neonates may affect the biomarker concentrations and make the test unreliable.

In this report, we tested the effect of processing time on UCH-L1 and GFAP concentrations. Umbilical cord blood was collected and processed with and without a delay to determine the effect of prolonged serum contact with Red Blood Cells (RBCs) and collection tube media. We hypothesized that the presence of the RBCs and the clotting process may induce changes in the measured concentrations of the serum biomarkers or interfere with the assay. As both umbilical venous and arterial blood were collected, we also examined the differences in UCH-L1 and GFAP in the arterial and venous samples.

2. MATERIAL AND METHODS

2.1. Sample Collection

The University of Florida Institutional Review Board approved the umbilical cord blood collection. Umbilical cord blood samples were obtained from healthy neonates who did not have known risk factors for HIE. The neonates had Apgar scores of 8 or higher at 1 minute and 5 minutes. In addition, the included neonates had normal neurological examinations and were admitted to the newborn nursery. A total of 40 subjects were enrolled with 48 samples collected. Seven subjects had multiple samples drawn in an attempt to obtain both arterial and venous blood. In these cases, at least one sample was positively identified as originating from the umbilical artery or vein. When there was doubt on the second specimen, it was labeled as a mixed sample. Samples were labeled as an umbilical artery (n=6), umbilical vein (n=12), or mixed (uncertain of vessel, n=30).

2.2. Blood Sample Processing

Two groups underwent analysis. Group 1 analysis was representative of ideal sample processing. Subjects in Group 1 (n=30 subjects, 38 samples-6 umbilical artery, 12 umbilical vein, 20 mixed) had umbilical cord blood (1 ml) collected using a 3.5 ml serum separator tube (BD Vacutainer® Serum Separator Tubes (SST) Plus Blood Collection Tube, Franklin Lakes, NJ). Samples were allowed to clot in an upright position at room temperature for 30 minutes in the processing lab (45 ± 15 minutes from time of collection), then centrifuged at 1200 RCF (g) at room temperature for 15 minutes in a fixed angle centrifuge rotor. Immediately following centrifugation, the serum was transferred from the SST tube using a disposable transfer pipette into three 2 ml cryovials with red cap inserts (USA Scientific, Orlando, FL). Then, one cryovial of separated serum was placed at −80°C at time points 0 (baseline), 4, and 8 hours. The serum samples were stored at 4°C between collection time points. A fiberboard cryogenic storage box (Fisher Part Scientific, Pittsburgh, PA) was used to store serum aliquots at −80°C until analysis for UCH-L1 and GFAP.

Group 2 was representative of delayed sample processing. Subjects in Group 2 (n=10 subjects, 10 samples- all labeled as mixed) had 3 ml umbilical cord blood collected. The 3 ml collected were portioned equally into 3 separate 3.5 ml Serum Separator Tubes (BD Vacutainer SST Plus Blood Collection Tube). Samples were allowed to clot in an upright position at room temperature for 30 minutes in the processing lab (45 ± 15 minutes from time of collection). One tube was processed at time point 0 (baseline) and centrifuged at 1200 RCF (g) at room temperature for 15 minutes in a fixed angle centrifuge rotor. The serum was transferred using a disposable transfer pipette into a 2 ml cryovial with a red cap insert (USA Scientific, Orlando, FL). A fiberboard cryogenic storage box (Fisher Scientific, Pittsburgh, PA) was used to store serum aliquots at −80°C until analysis for UCH-L1 and GFAP. Separate tubes were processed at 4 and 8 hours by repeating the above centrifugation and aliquot process. The 4- and 8-hour samples were stored at 4°C until processing.

2.3. Enzyme-Linked Immunosorbent Assay (ELISA)

Blinded serum samples were processed at Banyan Biomarkers, Inc.® (Alachua, FL) using proprietary sandwich Enzyme-Linked Immunosorbent Assays (ELISAs) to determine the concentrations and temporal profiles of UCH-L1 and GFAP in human serum. The ELISAs were carried out as previously published [13]. Briefly, Banyan Biomarkers, Inc.® produced in-house both mouse monoclonal capture antibody against recombinant UCH-L1 full length and partial protein and rabbit polyclonal detection antibody. Similarly, proprietary mouse monoclonal antibody for solid phase immobilization and a polyclonal rabbit detection antibody were used for ELISA to detect the levels of intact GFAP and its breakdown products. Such an approach allows more sensitive detection of GFAP analytes from patients' blood [13, 14]. Standard curves using recombinant proteins were generated for each assay, and quantitative determination of the biomarker levels in unknown samples were based on four-parameter non-linear regression analyses using SigmaPlot software (Systat, Chicago, IL).

2.4. Statistical Analysis

In the experimental design, each sample at time point 0 served as a baseline control. To understand the effect of delaying sample processing, differences between the baseline concentrations of each sample and the concentrations at 4 and 8 hours were analyzed. To accomplish the analysis, the following analysis was performed: (Figs. **1** and **2**).

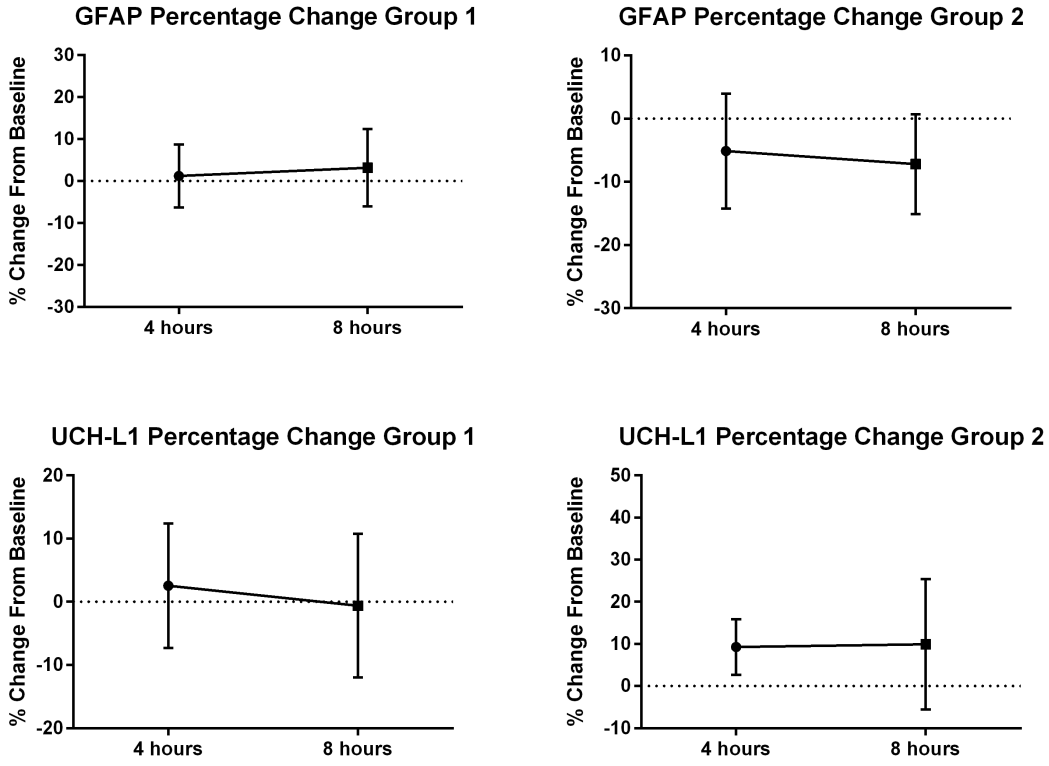

Fig. (1). The concentration of GFAP and UCH-L1 over time. The percentage change (averages with standard deviations) of GFAP and UCH-L1 in Group 1 (Panel 1A and C, respectively) and Group 2 (Panel 1B and D, respectively) from baseline to 4 and 8 hours.

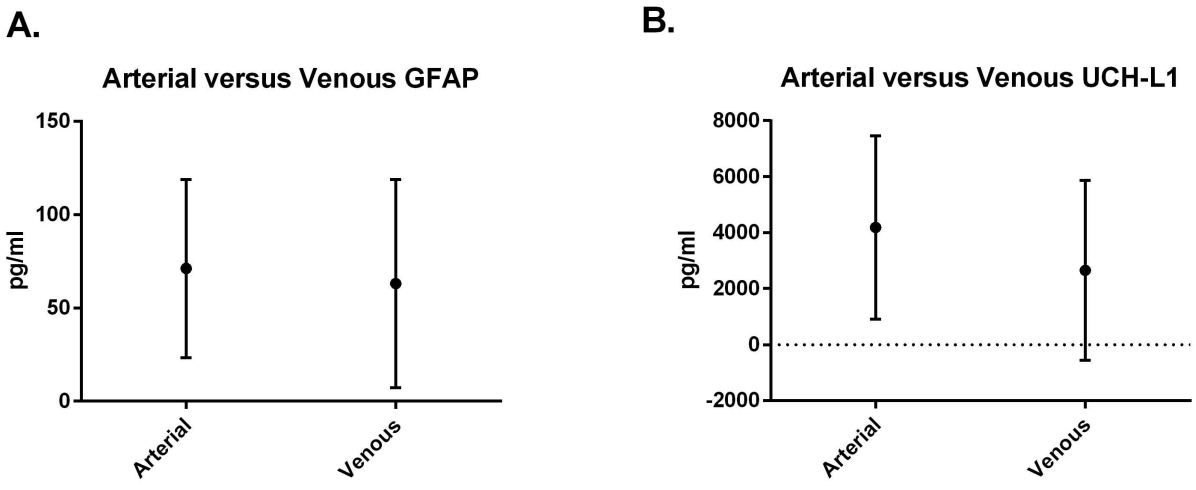

Fig. (2). The concentration of GFAP and UCH-L1 in umbilical cord blood A) The concentration of GFAP in arterial and venous umbilical cord blood (averages with standard deviations) B) The concentration of UCH-L1 in arterial and venous umbilical cord blood (averages with standard deviations).

Due to the longitudinal nature of our data (repeated measurements of the blood samples from the same patient), we employed a linear mixed statistical model. Concentrations of the biomarkers (UCH-L1 and GFAP) were used as response variables. For Group 1 data, the effects of time (0, 4 or 8 hours) and blood type sample (arterial, venous or mixed) and their interaction were quantified using the following random effects two-way ANOVA $Y_{ijk} = \alpha_i + \beta_j + \gamma_k + (\beta\gamma)_{jk} + \epsilon_{ijk}$, where Y_{ijk} is the response variable (biomarker concentration), α_i is the subject-specific random intercept, β_j is the main effect of blood type sample, γ_k is the effect of the hour, and $(\beta\gamma)_{jk}$ is the interaction term [15]. Model selection was performed via likelihood ratio testing for the significance of the interaction effect as well as the main effects of time and blood type sample.

Similarly, for Group 2 data where blood type was not differentiated, a submodel $Y_{ijk} = \alpha_i + \beta_j + \epsilon_{ijk}$ was used [15]. All analyses were carried out in the open source statistical environment R (R Core Team, 2016) using packages lme4 and mgcv.

3. RESULTS

3.1. UCH-L1 and GFAP Concentrations in Group 1: Removing Serum Immediately After Clotting from RBCs and Collecting at 0, 4, or 8 Hours

The baseline mean concentration of GFAP was 62±47 pg/ml at 0 hours (n=32). The mean change in the concentration of GFAP from baseline to 4 hours in each sample was an increase of 3±14%. The mean concentration of GFAP decreased 0.2±9% at 8 hours compared with the baseline concentrations in each individual sample. Overall, removing the serum and delaying the time to freezing at -80°C by 4 or 8 hours did not have a significant statistically effect on GFAP concentrations from the baseline concentrations ($p>0.05$).

Similarly, delaying the time to freezing at -80°C did not have a significant effect on the concentration of UCH-L1. The mean concentration of UCH-L1 was 3306±3093 pg/ml at 0 hours. The mean change in the concentration of UCH-L1 at 4 hours from baseline in each individual sample was an increase of 3±10% which was not a significant change from the baseline concentration at 0 hours ($p>0.05$). A delay in processing of 8 hours decreased the mean concentration by 0.6±11% in each individual sample from baseline ($p>0.05$).

3.2. UCH-L1 and GFAP Concentrations in Group 2: Collecting a Single Blood Specimen, Placing in Three Different Tubes and Processing at 0, 4, or 8 Hours

The effect of allowing the serum to clot and delaying the processing was examined. The GFAP mean concentration was 104±111 pg/ml at a baseline of 0 hours (n=9). The mean change in the concentration of GFAP from baseline to 4 hours in each sample was a decrease of 5±9% ($p>0.05$). A delay in sampling processing to 8 hours decrease in the mean concentration of GFAP by 7±7% in each sample ($p>0.05$).

Similarly, the mean concentration of UCH-L1 was not significantly different when processing was delayed. The mean concentration of UCH-L1 was 3448±2456 pg/ml at a baseline of 0 hours (n=8). At 4 hours, the mean concentration of UCH-L1 from baseline to 4 hours increased by a mean of 9±6% compared to each baseline concentration in each sample ($p>0.05$). Delaying processing to 8 hours, increased the mean concentration of UCH-L1 by 10+/-16% at 8 hours in each sample ($p>0.05$).

3.3. UCH-L1 and GFAP Concentrations in Umbilical Arterial *Versus* Umbilical Venous Blood Samples

For the analysis of differences in the concentrations of GFAP and UCH-L1 in the umbilical cord arterial and venous samples, only subjects with simultaneous umbilical cord arterial and venous samples were included in the analysis.

The average GFAP concentration of Group 1 at time 0 in the umbilical cord arterial serum sample was 57±16 pg/ml (n=4) compared to 47±18 pg/ml (n=4) in the umbilical cord venous serum samples. The measurements in the simultaneous samples from the umbilical artery and vein were not significantly different ($p>0.05$).

Similarly, the Group 1 UCH-L1 concentrations at time 0 were not significantly different between the umbilical cord arterial serum samples (4754±3273 pg/ml, n=4) and the umbilical cord venous serum samples (2114±1307 pg/ml, n=4) ($p>0.05$).

4. DISCUSSION

In this report, we demonstrated that the concentrations of UCH-L1 and GFAP do not change significantly with a delay in processing up to 8 hours. As the concentrations do not significantly change, samples can be drawn at smaller facilities at a very early time point and transported to a tertiary care facility for analysis. In addition, UCH-L1 and GFAP could be utilized in underdeveloped countries where processing may not occur immediately due to a lack of resources. To the best of our knowledge, a study examining the stability of protein biomarkers of brain injury has not been published.

The effect of a delay in sample processing was systemically examined. Our design allowed each sample to serve as its own control. The baseline samples from the two studied groups served as baseline controls and the 4 and 8 hour time points were the experimental groups. The design allowed for observation of changes over time in each of the two conditions. We chose to sample umbilical blood samples from normal term babies since it allowed for a large volume of blood for needed for our study. Ideally, neonates with HIE would be utilized. However, obtaining the volume of blood needed for the study was not possible and the umbilical cord blood from these neonates is currently being utilized at our center for a clinical trial (NCT02612155).

We sought to understand the role of RBCs and clotting on the concentration of the biomarkers UCH-L1 and GFAP. Group 1 in the study determined the effect of removing serum from the RBCs and storing at 4°C for 4 or 8 hours. The mean change in the concentration of GFAP and UCH-L1 at 4 hours from baseline was a decrease of 3±14% and an increase of 3±10%, respectively. Even at 8 hours, the average change was a decrease of 0.2±21% for GFAP and a decrease of 0.6±11%

for UCH-L1. Therefore, delaying the storage of samples at -80°C up to 8 hours following immediate spinning and removing the serum from the clotted RBCs did not affect the measured concentrations of either UCH-L1 or GFAP.

Group 2 allowed us to understand the effect on the concentrations of UCH-L1 and GFAP of leaving the serum in contact with the clotted RBCs. The GFAP concentration decreased 5±9% at 4 hours and decreased 7±7% at 8 hours. The changes were similar to Group 1. The UCH-L1 concentration increased 9±6% at 4 hours and increased 34±75% at 8 hours. Although the results at 8 hours were not statistically significant, the trend suggests that any further delay in processing could artificially increase the concentration of UCH-L1.

Biologic diversity may play a role when comparing biomarker concentrations between different subjects. Our design minimized biologic diversity by having each subject served as their own control. In our design, the baseline samples served as the control for each subject and the change from the baseline was measured and analyzed individually for each subject. Utilizing this approach, the effect of biologic diversity was minimized allowing for the study of the effect of delayed processing on the assay.

Similar studies have investigated the effect of delayed processing on serum analytes used in nutritional assessments. Similar to our design, a delay in processing for a period of up to 24 hours was evaluated [16]. Of the 36 analytes examined, only 5 analytes demonstrated changes, and the changes only occurred at 24 hours of delayed processing [16]. The study was performed to evaluate real-world conditions in remote testing environments such as the International Space Station. Similar to our Group 2, Zwart *et al*. examined 39 biochemistry analytes to evaluate whether clotting or proteins present with clotting affect the analyte concentrations [17]. The analytes were not affected if they were run on either whole blood or serum [17]. Our design differed in that we examined a delay in processing in addition to clotting. These practical studies are imperative to demonstrate the robustness of the assays used for measurement in real-world settings. To our knowledge, this study is the first to examine an ELISA-based biomarker assay in real-world conditions and the effect of these conditions on results. The effect of a delay in processing on ELISA-based biomarker assays was important to establish since proteomics studies have identified differences in assay results with delays in processing due to different stages of the coagulation and complement processes and the release of cell-derived products with changes in cell stability/activation such as platelet degranulation, or following synthesis of proteins upregulated during sample handling [18].

We also examined whether concentration differences existed in arterial and venous blood that was obtained simultaneously from the umbilical cord. This clinical question is critical because arterial samples are difficult to obtain. In addition, the source is not always identifiable, and therefore the sample is labeled as mixed. Although the blood from the fetus circulated through the placenta, the placenta does not appear to have an impact on the concentration of UCH-L1 and GFAP. UCH-L1 is a 24 kDa protein, which is a highly abundant neuronal protein thought to play a critical role in proteasome-mediated cellular protein degradation during normal and pathological conditions [19, 20]. GFAP is a 50 kDa type III intermediate filament that forms part of the cytoskeleton of mature astrocytes and other glial cells [21]. Proteins with molecular weights between 70-150 kDa, such as Alpha-fetoprotein (AFP), are able to pass from the fetal circulation into the maternal circulation [22]. The transfer of AFP is thought to be via a diffusional transport mechanism [22]. Although we did not measure maternal concentrations of UCH-L1 or GFAP, the umbilical arterial and umbilical venous samples were not different when obtained simultaneously. This similarity indicates that the concentrations of the biomarkers do not decrease as the blood circulates through the placenta. Furthermore, this observation suggests that either an arterial or venous sample reflects the concentration of UCH-L1 and GFAP in the neonate at the time of delivery. This steady level of biomarkers is particularly important for the real-world application of the biomarkers because the source of the umbilical cord blood is often unknown or is not identified clearly during sampling. To the best of our knowledge, this study is the first to examine potential differences. The umbilical artery and vein are often phenotypically similar, which makes correct identification difficult. This study shows that blood samples from either vessel will provide accurate levels of UCH-L1 and GFAP, which makes these tests practical for use at birth to identify brain injury. In addition, the sampling of the umbilical cord alleviates the need to perform a venipuncture on a neonate to obtain a sample.

5. LIMITATIONS OF THE STUDY

This study has several limitations. The number of subjects in Group 2 was smaller than Group 1. A majority of the collected samples were labeled as mixed samples, which greatly decreased the number of samples available for the analysis of the umbilical arterial versus venous samples. However, this limitation illustrates the real world challenges associated with identification of the sampling source. Furthermore, the study samples were stored at 4°C prior to processing in the delayed processing experiments which may not happen in the real world. This cooling procedure does mimic the transport of samples to a tertiary care center in an insulated bag with a cold pack.

CONCLUSION

Our data demonstrate that UCH-L1 and GFAP are robust biomarkers that are amenable to delays in processing. Until a point-of-care device is available, the robustness will allow umbilical cord blood samples to be collected at smaller referring facilities or in resource-poor environments and transferred for processing. Finally, the site of sampling from the umbilical cord, either arterial or venous, does not have an impact on the concentration of either GFAP or UCH-L1. This information assures clinicians that umbilical cord blood is a rapid, practical, and early site for biomarker sampling.

LIST OF ABBREVIATIONS

ELISAs	=	Enzyme-linked immunosorbent assays
GFAP	=	Glial Fibrillary Acidic Protein

HIE	=	Hypoxic-Ischemic Encephalopathy
RBCs	=	Red blood cells
UCH-L1	=	Ubiquitin C-Terminal Hydrolase L1

AUTHORS' CONTRIBUTION

Dr. Weiss supervised the study and wrote the manuscript. Dr. Hayes and Olena Glushakova performed the biomarker analysis. Melissa Huene and Nicole Copenhaver helped to consent families and obtain IRB approval. They also collected samples. Candace Rossignol processed all samples and shipped the samples to Banyan for analysis. Dr. Bliznyuk is a biostatistician and analyzed all data.

FUNDING

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the UF IRB on March 17, 2017.

HUMAN AND ANIMAL RIGHTS

No Animals were used in this research. All human research procedures followed were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

The umbilical cord blood was collected at the time of birth. Following the collection, all subjects' parents were consented for use of the umbilical cord blood by going through a consent form and then having a period of time to review the form. If the family chose not to sign the consent, the umbilical cord blood was destroyed and not used in the study.

CONFLICT OF INTERESTS

Dr. Hayes is the founder of Banyan Biomarkers, the company that ran the samples for this study.

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