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A Validation Study of Transferrin and I-FABP As Dried Blood Spot-Based Biomarkers of Environmental Enteric Dysfunction

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Abstract

Environmental Enteric Dysfunction (EED) is a subclinical condition characterized by increased intestinal permeability and impaired nutrient absorption, but current methods for assessing EED are invasive and resource intensive. Dried blood spot (DBS) biomarker sampling offers a minimally invasive method of detecting EED. We present a validation study of two DBS protocols for the measurement of transferrin, a biomarker of iron status, and I-FABP, a biomarker of intestinal permeability. Matched DBS from a fingerstick and venous whole blood samples from n=74 individuals were collected over a 3-week period. Whole blood was used to create additional DBS samples and then spun down to plasma for analysis. We found a weak linear association between plasma and DBS measurements of transferrin concentrations, but a strong linear association and high level of agreement between plasma and DBS measurements of I-FABP concentrations. These findings demonstrate the validity and feasibility of measuring I-FABP, but not transferrin, using DBS sampling.

Keywords

environmental enteric dysfunction, EED, dried blood spot, DBS, transferrin, I-FABP

Disciplines

Anthropology | Biological and Physical Anthropology

A VALIDATION STUDY OF TRANSFERRIN AND I-FABP AS DRIED BLOOD SPOT-BASED BIOMARKERS OF ENVIRONMENTAL ENTERIC DYSFUNCTION

By

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ABSTRACT

Environmental Enteric Dysfunction (EED) is a subclinical condition characterized by increased intestinal permeability and impaired nutrient absorption, but current methods for assessing EED are invasive and resource intensive. Dried blood spot (DBS) biomarker sampling offers a minimally invasive method of detecting EED. We present a validation study of two DBS protocols for the measurement of transferrin, a biomarker of iron status, and I-FABP, a biomarker of intestinal permeability. Matched DBS from a fingerstick and venous whole blood samples from n=74 individuals were collected over a 3-week period. Whole blood was used to create additional DBS samples and then spun down to plasma for analysis. We found a weak linear association between plasma and DBS measurements of transferrin concentrations, but a strong linear association and high level of agreement between plasma and DBS measurements of I-FABP concentrations. These findings demonstrate the validity and feasibility of measuring I-FABP, but not transferrin, using DBS sampling.

INTRODUCTION

Chronic malnutrition is a serious global health burden which increases young children's risk of poor cognitive development, lost adult productivity, chronic disease infection, and mortality. Environmental Enteric Dysfunction (EED), a subclinical gastrointestinal condition characterized by increased intestinal permeability and reduced nutrient absorption, plays a major role in the etiology of malnutrition. However, EED is a poorly understood disorder, in part due to various methodological constraints for diagnosing the disease in the low-resource and/or non-clinical settings in which it is most prevalent. Thus, there exists a need for validated, field-friendly techniques for detecting and studying EED.

Several blood-based biomarkers of EED hold the potential to be used with a minimally invasive, highly portable sampling technique known as dried blood spot (DBS) collection. In this procedure, five drops of blood are collected from a simple finger stick onto a standardized filter paper card and then dried. The present study contributes to ongoing efforts to identify and validate DBS-based biomarkers by investigating transferrin, a biomarker of blood iron levels, and the small intestinal fatty-acid binding protein (I-FABP), a biomarker of intestinal permeability. The validation of additional DBS-based biomarkers of EED will strengthen our ability to holistically evaluate this complex disorder.

BACKGROUND

Environmental Enteric Dysfunction (EED) is a subclinical gastrointestinal disorder caused by regular oral-fecal contamination that blunts the chorionic villi, weakening the intestinal mucosal barrier and leading to increased intestinal permeability (i.e., "leaky" gut membrane) (Korpe and Petri, 2012; Marie et al., 2018). This permeability facilitates translocation of bacteria and toxins into systemic circulation, and resultant systemic and

intestinal inflammation further increase an individual's susceptibility to other infections; in addition, the reduced surface area of the blunted villi limits nutrient absorption and perpetuates malnutrition (Kosek et al., 2014; Kosek et al., 2017; Tickell et al., 2019). These interdependent, synergistic effects of EED play a major role in the etiology of malnutrition, undermining supplementation-based interventions for malnutrition (Harper et al., 2018). Importantly, EED has been linked to linear growth stunting, oral vaccine failure, and diarrheal diseases, the last of which is estimated to affect 150 million children worldwide (Kosek et al., 2017; Owino et al., 2016; Naylor et al., 2015; Sullivan, 2021).

At present, primary methods for assessing EED are invasive, resource-intensive, time-consuming, and require significant laboratory infrastructure (Hoke et al., 2018). Using minimally invasive biomarkers to identify children at greatest risk for EED has been recognized as a powerful tool for clinical and research efforts (Kosek et al., 2013; Hoke et al., 2018; Harper et al., 2018; Tickell et al., 2019; Denson, 2021). However, detecting biomarkers through the collection of conventional biological samples (i.e. feces, urine, or whole blood) often pose several logistical constraints in the low-resource settings in which EED is most prevalent, such as limited laboratory access and discontinuous cold chains (Lim 2018; Hoke et al., 2018).

Dried blood spot (DBS) technology offers a minimally invasive and field-friendly alternative for studying EED (McDade et al., 2002; McDade et al., 2007). In this method, five drops of whole blood are collected onto standardized filter paper cards following a simple finger stick, and then left out to dry. The standard Whatman 903 Proteinsaver Card used in DBS collection is made up of nitrocellulose polymer networks with gap sizes specifically designed to capture and sequester proteins. Thus, these DBS samples are stable and highly portable, and can be transported over significant geographic distances. In the laboratory, the dried blood spots are

later extracted as sample discs via hole punch and reconstituted in solution using reagents specific to different enzyme-linked immunosorbent assay (ELISA) kits (McDade et al., 2002; McDade et al., 2007).

DBS sampling reduces the logistical burdens and costs of assessing this condition for researchers, especially in non-clinical settings (McDade et al., 2002; McDade et al., 2007). Additionally, this sampling method reduces the physiological burden on participants, particularly among infants, elderly individuals, or individuals with blood clotting disorders (Freeman et al. 2018). However, DBS may produce more imprecise measurements than its plasma or serum counterparts due to the hematocrit effect, which involves the variable interference of red blood cells in analyte extraction and measurement (Freeman et al., 2018). Because the volumetric percentage of red blood cells influences the viscosity of whole blood, too high or low hematocrit will result in a heterogeneous distribution of the sample and analyte on the filter paper card (Freeman et al., 2018). In addition, variability in blood drop volume, storage conditions, and sample reconstitution efficiency can also impede accurate detection of biomarker levels (McDade et al., 2002; McDade et al., 2007). Therefore, any candidate blood-based biomarkers of EED must first be validated for use with DBS sampling before field use.

To date, the only EED biomarker which has undergone rigorous assessment and validation for measurement from DBS is endotoxin-core antibodies (EndoCAb), a measure of microbial translocation which only captures one facet of EED (Hoke et al., 2018). However, EED is a highly complex disorder with several, interdependent mechanisms through which it impacts human health outcomes, and cannot be accurately diagnosed through one biomarker. In addition, though DBS offers significant advantages for researchers and participants, the greater variability of DBS sampling compared to venipuncture typically makes it more appropriate for

population-level research, rather than individual, absolute clinical assessments (Samuelsson et al., 2015). However, a combined evaluation of multiple EED biomarkers may increase the specificity and sensitivity of this method to more precisely determine an individual's EED status.

Transferrin, a family of glycoproteins that regulate iron transport throughout the blood plasma, is one potential biomarker of EED-related micronutrient deficiency (Ogun and Adebayo, 2020). As a key component of various metabolic processes, iron-deficiency anemia has been consistently linked to stunted growth among young children (Colston et al., 2019; McCormick et al. 2019). More recently, a mouse study by Mohan Kumar and colleagues (2020) indicated that severe anemia contributes to the destabilization of intercellular epithelial junctions, increasing intestinal permeability and further undermining nutrient absorption in individuals with EED. Validated DBS measures have been developed for transferrin receptors, but no such assessment of transferrin has been completed yet (Cook et al., 1998; McDade and Shell-Duncan, 2002). However, transferrin has been previously found to have a significant effect on short-term linear growth, warranting investigation of its measurement with DBS sampling and as a biomarker of EED (Colston et al., 2019).

Another potential EED biomarker is the small cytoplasmic intestinal fatty-acid binding protein (I-FABP), which is released into circulation following damage to the epithelial cells of the small intestinal mucosal barrier (Kanda et al., 1996; Uzun et al., 2014). Enterocyte damage is a direct precursor to increased intestinal permeability, one of EED's primary characteristics. Prendergast and colleagues (2014) previously found a significant relationship between high concentrations of plasma I-FABP and extensive intestinal damage among Zimbabwean infants. Though Prendergast et al. (2014) found no significant association between I-FABP and stunted growth, another study (Guerrant et al. 2016) conversely discovered a strong positive association

between I-FABP and stunted growth among Brazilian children. In addition, Uddin and colleagues (2016) found a positive association between I-FABP and anti-LPS (lipopolysaccharide) antibodies during intestinal infection in children with EED. Altogether, these findings suggest that enterocyte damage can be quantified by measuring I-FABP levels with commercially available human ELISA assay kits and act as a biomarker of EED (Funaoka et al., 2011; Uzun et al., 2014; Prendergast et al., 2015; Guerrant et al., 2016).

The following investigation therefore had two primary aims for each EED biomarker candidates. Aim 1: to optimize a protocol for the use of DBS samples and a commercially available human ELISA kit to examine transferrin and I-FABP concentrations. Aim 2: to measure levels of transferrin and I-FABP in matched plasma/DBS samples to evaluate the effectiveness and reliability of the DBS-optimized protocol in measuring biomarker levels compared to traditional plasma measurements. This study builds on ongoing efforts to validate minimally invasive, field-appropriate protocols for biomarker evaluation and incorporate biochemical perspectives into anthropological research.

METHODS

Aim 1: Protocol Optimization

Transferrin

Dried blood spot samples collected from thirteen healthy adults were used for the initial protocol validation of the commercially available human transferrin ELISA kit selected for this study (Invitrogen Human Transferrin ELISA Kit, ThermoFisher Scientific, Carlsbad, California). Based on estimates of normal concentrations of circulating transferrin, the amount present in a single 3.2 mm DBS disk, and the high rate of sample dilution required by the kit, it was determined that we would begin with the smallest possible sample amount. One 3.2 mm DBS

sample disc was eluted overnight in 80 µL of Assay Diluent D provided in the kit. Five µL of reconstituted sample was then diluted in 1.25 mL of Assay Diluent D, which yielded transferrin concentrations within the assay's detection range (1.029-750 ng/mL).

I-FABP

Dried blood spot samples collected from three healthy adults were used for the initial protocol validation of the commercially available human I-FABP ELISA kit selected for this study (Quantikine Human I-FABP Immunoassay, R&D Systems, Minneapolis, Minnesota). Because of the relatively lower concentrations in I-FABP in blood plasma, the kit recommended a limited dilution protocol for plasma samples. To ensure sufficient I-FABP presence in DBS samples, we experimented with using multiple DBS punches in different levels of assay diluent. The research team experimented with using 1, 2, 4, 6, and 8 DBS sample discs for each adult. Additionally, for samples with more than 1 DBS hole punch, the research team assessed whether 150 or 175 µL of Calibrator Diluent provided in the kit was necessary to elute the samples overnight (Table 1).

Though all dilution protocols yielded readings within the assay's detection range (0-1000 pg/mL), further experimentation using 1 DBS hole punch in 125 µL of Calibrator Diluent suggested high variability in sample reconstitution, with intra-assay CVs ranging from 0-59.3%. However, I-FABP readings did not linearly or predictably increase when over 2 DBS sample discs were used, which indicated a problematic variability in sample reconstitution when using too many DBS sample discs. Lastly, increasing the volume of Calibrator Diluent from 150 to 175 µL yielded no significant benefits. Thus, the final dilution protocol selected for Aim 2 of this study consisted of two 3.2 mm DBS hole punches eluted overnight in 150 µL of Calibrator Diluent.

Table 1

Protocol Optimization Dilution Combinations

Participant	Number of DBS Hole Punches	Volume of Calibrator Diluent (µL)	Average I-FABP Levels (pg/mL)
Adult 1	1	125	35.323
	2	150	64.677
	4	150	66.815
	6	150	79.353
	8	150	83.684
Adult 2	1	125	38.451
	2	175	47.353
	4	175	87.052
	6	175	72.376
	8	175	74.300
Adult 3	1	125	29.308
	2	175	31.233
	4	175	55.052
	6	175	62.511
	8	175	70.691

Aim 2: Matched Plasma/DBS Analysis

Approval for the study was obtained from the Institutional Review Board of the University of Pennsylvania. Healthy adults aged 18-45 from the University of Pennsylvania community were recruited via word-of-mouth, electronic mailing lists, and flyers posted throughout campus. Recruitment and data collection took place between September and October of 2021. All participants were first screened to confirm their eligibility, and all participants provided informed consent before data collection. People with known, mental or physical chronic conditions, liver diseases, kidney diseases, blood clotting disorders, and currently pregnant or breastfeeding were excluded from the study.

All participants arrived at the study site fasted for a minimum of three hours prior to data collection. Upon arrival, all participants reviewed and signed the informed consent document while research team members answered any remaining questions prior to proceeding with the study. To maintain confidentiality, all participants were assigned a study participant ID number which was used on all study materials (i.e., qualitative surveys, records of anthropometric data,

and biological samples). The research team provided snacks and water and monitored all participants for adverse reactions for a minimum of 15 minutes following data collection. All participants were fairly compensated for their time with an Amazon or Acme gift card of their choice.

Surveys and Anthropometric Data

The research team distributed qualitative surveys assessing participants' demographic and health backgrounds. For health backgrounds, the survey assessed factors known to impact biomarker levels and intestinal function (e.g. experiences of gastrointestinal distress or exposures to unsafe drinking water in the past 6 months). A research team member was present at all times during survey completion to answer any questions and/or clarify survey content. Following survey completion, trained research assistants used a portable stadiometer (Seca 213 Stadiometer, Seca, Hanover, Maryland) to measure participants' height and a scale (OMRON HBF-516B Body Composition Monitor and Scale, OMRON Healthcare, Inc., Lake Forest, Illinois) to measure participants' weight.

Dried Blood Spots

Trained research assistants collected five ~50 µL drops of blood from a fingerstick onto standardized filter paper cards (Whatman #903, GE Healthcare, Pascataway, New Jersey), labelled "fDBS" samples by the researchers. All fDBS cards were left out at room temperature to dry overnight, then stored at -25^{o}C until analysis.

Phlebotomy

A trained phlebotomist drew 4 mL of venous whole blood into BD Vacutainer tubes from participants' arms. Five 50 µL drops of venous blood were subsequently pipetted onto filter paper to make additional DBS cards (labelled "wbDBS"). All wbDBS cards were left out at

room temperature to dry overnight, then stored at -25ºC until analysis. The remaining whole blood was centrifuged at 1500g for 15 minutes to separate plasma as supernatant. Plasma samples were then aliquoted and stored at -80ºC until analysis.

Transferrin ELISA Assay Protocol

The following protocol is a modified version of a commercially available human transferrin ELISA kit designed for use with serum or plasma samples (Invitrogen Human Transferrin ELISA Kit, ThermoFisher Scientific, Carlsbad, California). All reagents provided in the kit were prepared and diluted according to the kit manual's specifications.

On the afternoon prior to the assay run, one 3.2 mm disc was removed from each DBS sample with a hole punch, transferred to a glass tube, and eluted overnight in 80 µL of Assay Diluent D provided in the kit at 5ºC. On the day of the run, samples were gently homogenized at room temperature for one hour at 300 RPM, after which 5 µL of the eluted samples were further diluted in 1.25 mL of Assay Diluent D. Two µL of thawed plasma was diluted in 998 µL of Assay Diluent D; 10 µL of diluted plasma was then further diluted in 390 µL of Assay Diluent D. All subsequent steps of the ELISA assay followed the protocol specified in the manufacturer's kit manual (see Appendix A).

I-FABP ELISA Assay Protocol

The following protocol is a modified version of a commercially available human I-FABP ELISA kit designed for use with serum or plasma samples (Quantikine Human I-FABP Immunoassay, R&D Systems, Minneapolis, Minnesota). All reagents provided in the kit were prepared and diluted according to the manufacturer's specifications.

On the afternoon prior to the run, two 3.2 mm diameter discs were extracted from the filter paper cards using a hydraulic hole punch into a glass tube, and then eluted overnight in 150

µL of Calibrator Diluent provided in the kit at 5°C. The morning of the assay run, samples were gently homogenized at room temperature for one hour at 300 RPM, while 50 µL of thawed plasma samples were diluted in 200 µL of Calibrator Diluent. All subsequent steps of the ELISA assay followed the protocol specified in the manufacturer's kit manual (see Appendix B).

Statistical Analyses

For all assays, three controls representing high, medium, and low biomarker concentrations were selected from available samples and included to calculate inter-assay coefficients of variation (CVs). For transferrin, all assay runs which yielded poor standard curves and individual samples which yielded an intra-assay CV greater than 10%, as specified in the manufacturer's kit manual, were re-analyzed in subsequent assay runs. For I-FABP, all assay runs which yielded poor standard curves and individual samples which yielded an intra-assay CV greater than 12% were re-analyzed in subsequent assay runs. Participant survey and anthropometric data variables were analyzed with standard descriptive summaries (i.e. means and standard deviations for continuous variables and percentages for categorical variables). Passing-Bablok regressions were used to assess the correlation between plasma and DBS measurements of biomarker levels. Bland-Altman analyses were used to assess the agreement between the plasma and DBS measurement methods. All statistical analyses were completed in Microsoft Excel and R Version 4.1.2 and are consistent with those used in previous biomarker validation studies (Eick et al., 2016; Hoke et al., 2018).

RESULTS

Out of 216 total respondents, 101 eligible participants were recruited for the study. Twenty-seven participants were withdrawn after exhibiting immediate, adverse reactions to the fingerstick or venous blood draw (e.g., dizziness, nausea, faintness), or due to an inability to

locate a vein after two attempts. For transferrin, 12 incomplete samples were excluded from all analyses due to limited biospecimen availability for assay re-runs. One additional sample was identified as an outlier and excluded from analyses. For I-FABP, 11 samples were excluded from all analyses due to dilution errors in one of the initial assay runs, while 2 were excluded from the analysis of the relationship between fDBS and plasma samples due to different collection dates of the biospecimens.

Transferrin

Sixty-one healthy adults between the ages of 18 and 45 were included for the analysis of the transferrin validation study. Final population demographics and anthropometric measurements for the sample are reported below (Table 2). The mean age of the sample was 22.15 years (SD = 4.60 years) and 68.85% of participants reported being biologically designated as "female" at birth. Most participants identified as White (55.74%) or Asian (39.34%), followed by Black (6.56%), Latinx (3.28%), Indigenous (1.64%), or Middle East / North African (1.64%). The average height of the participants was 1.69 m (SD =.08 m); the average weight was 65.21 kg (SD = 13.22 kg); and the average BMI was 22.85 kg/m^2 (SD = 3.69 kg/m^2).

Table 2

Descriptive Statistics of Participants Included for Transferrin Analysis

	Mean (Standard Deviation)
Age (yrs)	22.15 (4.60)
Height (m)	1.69 (.08)
Weight (kg)	65.21 (13.22)
BMI (kg/m^2)	22.85 (3.69)
	N (%)
Biological Sex	
Male	19 (31.15)
Female	42 (68.85)
Racial Identity	
Asian	24 (39.34)
Black	4 (6.56)
Indigenous*	1 (1.64)
Latinx	2 (3.28)
Middle East / North African	1 (1.64)
White	34 (55.74)

*self-reported

All inter-assay coefficients of variation (CVs) exceeded 12%, the acceptable threshold outlined by the assay kit manufacturer's specifications (Table 3). For the low, medium, and high controls, the inter-assay CVs were 23.20%, 18.50%, and 30.50%, respectively. Mean intra-assay CVs fell within the kit manufacturer's specifications of <10%; for fDBS, wbDBS, and plasma samples, the mean CVs were 9.10%, 8.92%, and 9.88%, respectively.

Table 3

Transferrin Assay Coefficients of Variability (CVs)

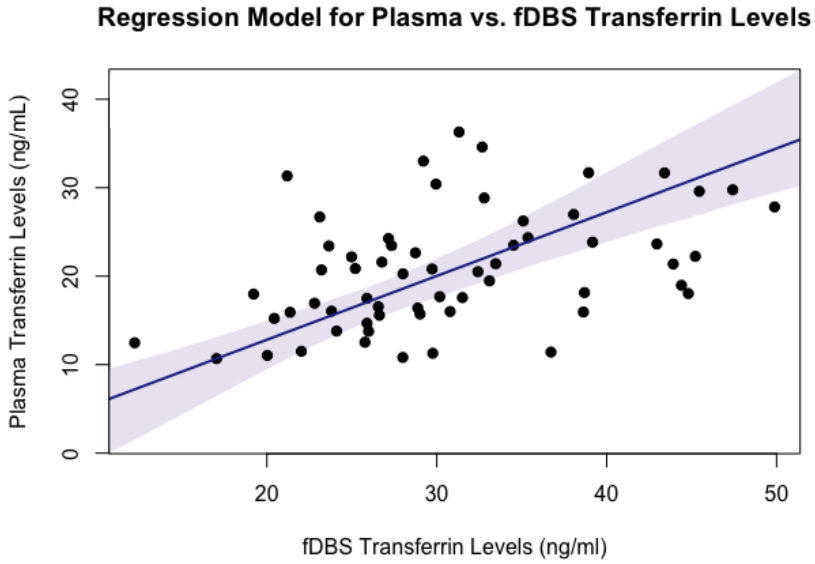
	%
Intra-Assay CVs	
fDBS	9.10
wbDBS	8.92
plasma	9.88
Inter-Assay CVs	
Control 1 (Low)	23.20
Control 2 (Medium)	18.50
Control 3 (High)	30.50

Matched DBS and Plasma Analysis

For fDBS vs. plasma samples, a Passing-Bablok regression yielded a model with slope 0.72. However, a Pearson's r (59) = 0.432, $p < .0005$ indicated that while a significant association existed, the average fDBS samples were not linearly and proportionally lower than average plasma samples (Figure 1).

Figure 1

Regression Model for Plasma vs. fDBS Transferrin Levels

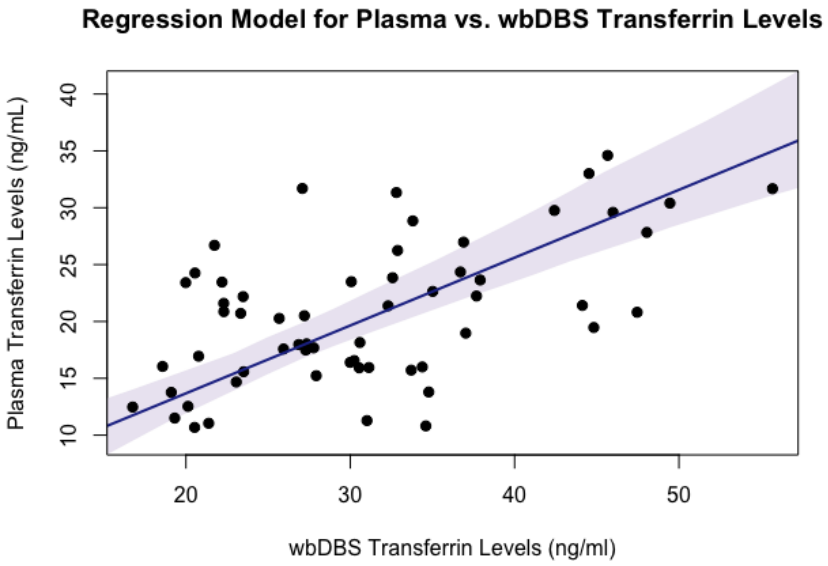


Note. The full equation generated from the Passing-Bablok regression fit is as follows: y = 0.72x – 1.60.

For wbDBS vs. plasma samples, a Passing-Bablok regression yielded a model with slope 0.60. However, a Pearson's r (59) = 0.463, $p < .0001$ indicated that while a significant association existed, the average wbDBS samples were not linearly or proportionally lower than average plasma samples (Figure 2).

Figure 2

Regression Model for Plasma vs. wbDBS Transferrin Levels



Note. The full equation generated from the Passing-Bablok regression fit is as follows: y = 0.60x + 1.74.

Given the poor strength of the linear relationship between DBS and plasma samples and the inconsistency across runs based on the interassay CVs for control samples, Bland-Altman analyses were not conducted.

I-FABP

Sixty-one healthy adults between the ages of 18 and 45 were included for final analysis of the relationship between fDBS and plasma samples, while sixty-three were included for final analysis of the relationship between wbDBS and plasma samples. Final population demographics and anthropometric measurements for the sample are reported below (Table 4). The mean age of the sample was 22.05 years (SD = 4.62 years) and 66.67% of participants were biologically designated as “female” at birth. Most participants identified as White (55.56%) or Asian (41.27%), followed by Black (6.35%), Latinx (3.18%), Indigenous (1.59%), or Middle East / North African (1.59%). The average height of the participants was 1.68 m (SD =.07 m); the

average weight was 65.01 kg (SD = 13.92 kg); and the average BMI was 22.92 kg/m^2 (SD = 3.83 kg/m^2).

Table 4

Descriptive Statistics of Participants Included for I-FABP Analysis

	Mean (Standard Deviation)
Age (yrs)	22.05 (4.62)
Height (m)	1.68 (.07)
Weight (kg)	65.01 (13.92)
BMI (kg/m^2)	22.92 (3.83)
	N (%)
Biological Sex	
Male	21 (33.33)
Female	42 (66.67)
Racial Identity	
Asian	26 (41.27)
Black	4 (6.35)
Indigenous*	1 (1.59)
Latinx	2 (3.18)
Middle East / North African	1 (1.59)
White	35 (55.56)

*self-reported

All calculated coefficients of variation (CVs) fell within an acceptable range outlined by the assay kit manufacturer's specifications (Table 5). For the low, medium, and high controls, the inter-assay CVs were 11.70%, 7.80%, and 9.10%, respectively. For fDBS, wbDBS, and plasma samples, the mean intra-assay CVs were 3.35%, 3.02%, and 1.88%, respectively.

Table 5

I-FABP Assay Coefficients of Variability (CVs)

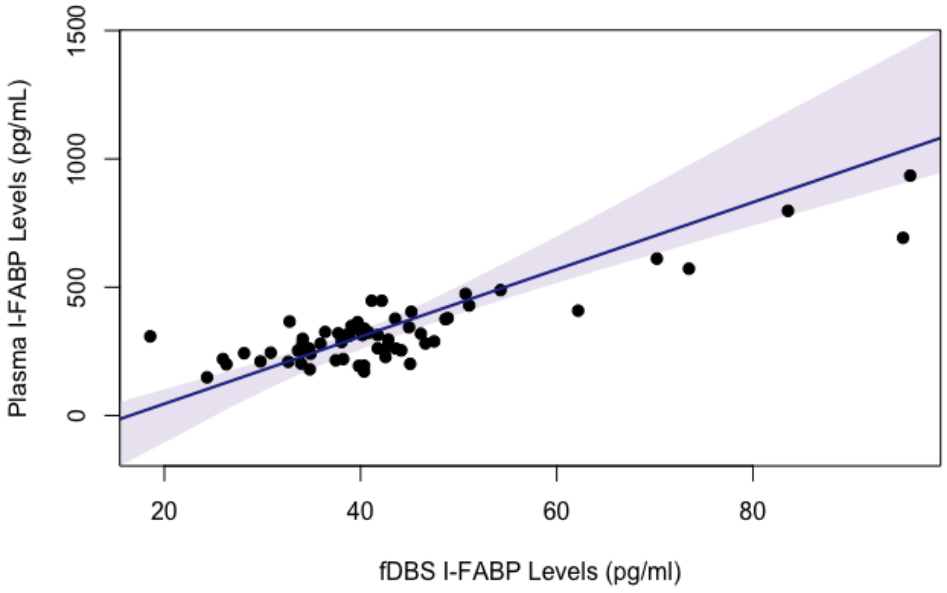
	%
Intra-Assay CVs	
fDBS	3.35
wbDBS	3.02
plasma	1.88
Inter-Assay CVs	
Control 1 (Low)	11.70
Control 2 (Medium)	7.80
Control 3 (High)	9.10

fDBS and Plasma Analysis

A Passing-Bablok regression yielded a model with slope 13.09, and a Pearson's r (59) = 0.873, $p < .0001$ indicated that average fDBS samples were linearly and proportionally lower than average plasma samples (Figure 3).

Figure 3

Regression Model for Plasma vs. fDBS I-FABP Levels

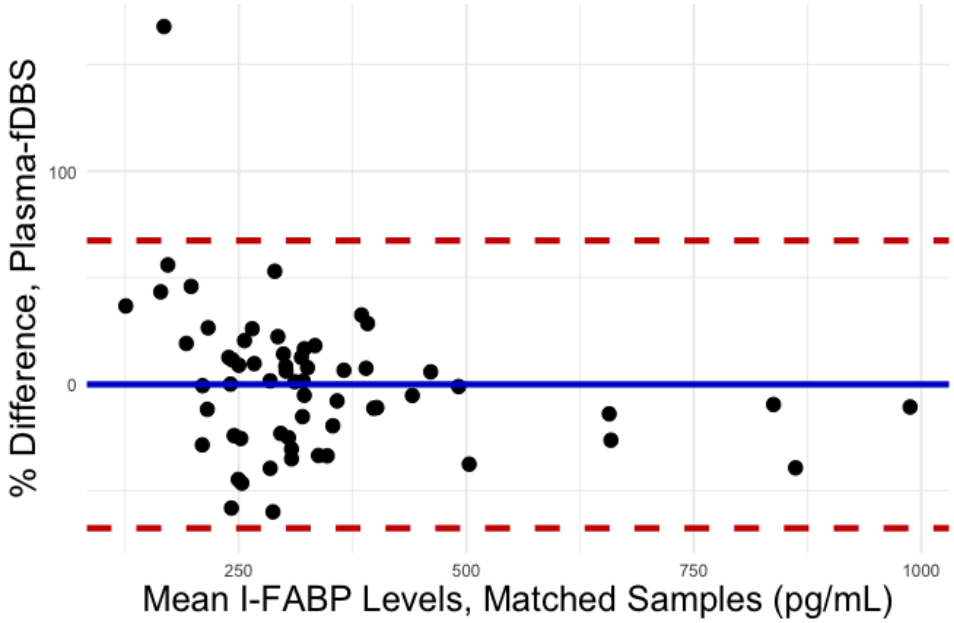


Note. The full equation generated from the Passing-Bablok regression fit is as follows: y = 13.09x – 216.07.

For the Bland-Altman analysis, fDBS values were then converted to their plasma equivalents using the linear relationship yielded from the Passing-Bablok regression. Assessment of the percentage difference between the two measurement methods revealed no significant proportional bias (% bias: -0.134%; 95% CI: -67.698% to 67.430%) (Figure 4).

Figure 4

Bland-Altman Plot for fDBS vs. Plasma

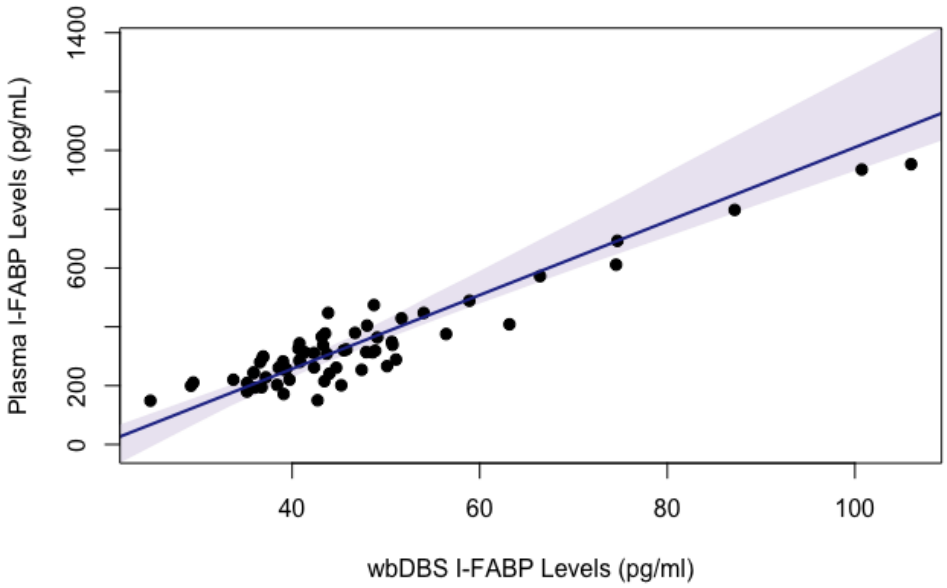


wbDBS and Plasma Analysis

A Passing-Bablok regression yielded a model with slope 12.56, and a Pearson's r (61) = 0.938, $p < .0001$ indicated that average wbDBS samples were linearly and proportionally lower than average plasma samples (Figure 5).

Figure 5

Regression Model for Plasma vs. wbDBS I-FABP Levels

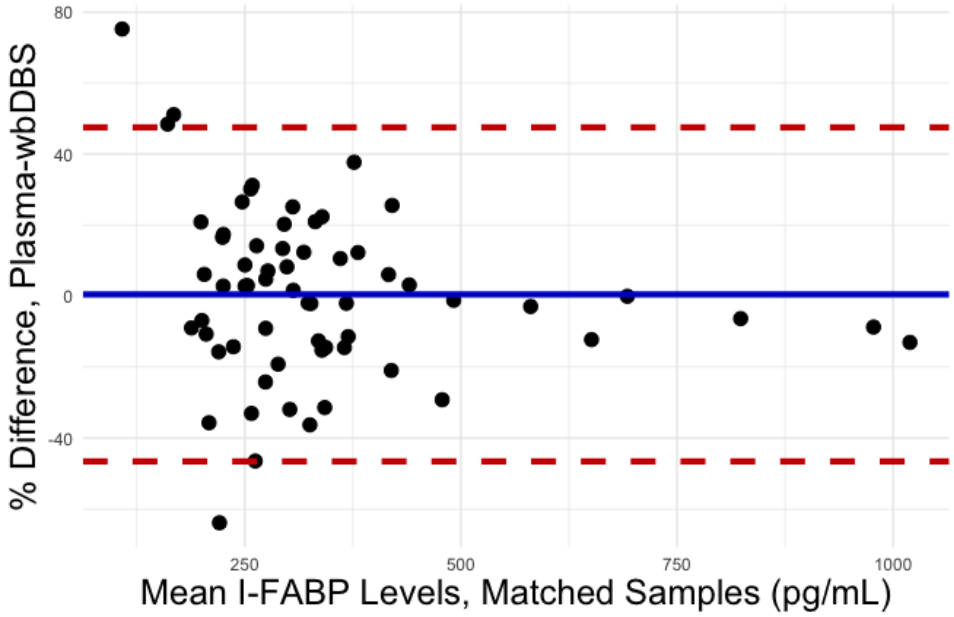


Note. The full equation generated from the Passing-Bablok regression fit is as follows: y = 12.56x – 245.17.

For the Bland-Altman analysis, wbDBS values were again converted to their plasma equivalents using the linear relationship yielded from the Passing-Bablok regression. Assessment of the percentage difference between the two measurement methods revealed no significant proportional bias (% bias: -0.471%; 95% CI: -46.583% to 47.526%) (Figure 6).

Figure 6

Bland-Altman Plot for wbDBS vs. Plasma



DISCUSSION

Transferrin

Statistical analyses of the DBS-based protocol revealed that transferrin is not compatible with the DBS sampling method. Though average wbDBS transferrin levels exhibited a slightly stronger correlation with plasma, the low Pearson's r values for both fDBS and wbDBS indicated that plasma and DBS concentrations of transferrin are not linearly and proportionally correlated. Thus, DBS transferrin levels could not be easily converted to plasma estimates, and Bland-Altman analyses could not be completed. The inter-assay CVs additionally suggested a poor consistency between individual assay runs, even though the same protocol was followed for every ELISA assay, and substantiated the poor viability of using DBS to measure transferrin.

Additionally, several limiting factors support the lack of validity of using DBS to evaluate transferrin status. Physiologically, a large amount of iron is necessary for red blood cell production and maintenance, meaning that erythrocyte uptake of transferrin is likely significant (Anderson and Frazer, 2017). Unlike plasma, which contains no erythrocytes, red blood cells are

often lysed during DBS whole blood collection, which may lead to an unpredictable release some transferrin during sampling. Tissues with a higher proliferative capacity, such as the distal capillaries punctured to collect fDBS samples, often also express greater transferrin receptors to meet their increased iron need (Anderson and Frazer, 2017). Therefore, capillary blood cells may uptake transferrin to a greater degree than venous blood cells, which may be one reason for the stronger correlation between wbDBS and plasma transferrin relative to the relationship between fDBS and plasma transferrin (additional reasons are discussed under I-FABP).

As for the protocol itself, all blood samples and reagents required significant dilution, with dilution factors ranging from 5 to 20,000. Given that blood sample quantities ranged from 1-10 µL, an error of even 1 mL could notably alter results, and account for the high inter-assay variability. Furthermore, we observed that high quantities of Assay Diluent D often caused the blood samples to become extremely viscous, especially the plasma samples. The increased viscosity made consistent pipetting extremely difficult, introduced a major source of intra-assay variability, and required biological samples to be analyzed multiple times during the study. As a result, these factors increased, rather than decreased, the burden of assessing transferrin levels in a timely and scientifically rigorous manner.

The primary limitation of focusing on iron deficiency and using transferrin as a biomarker for EED-related nutrient malabsorption is that low iron levels and anemia can be caused by multiple reasons. Iron levels are highly sensitive to numerous daily lifestyle factors, including menstruation in biological females, dietary intake, and physical activity. Iron sequestration, anemia, and elevated circulating transferrin also have anti-inflammatory effects against bacterial infections, another key feature of EED (Anderson and Frazer, 2017; Ardehali et al., 2004). Thus, while the role of iron-deficiency anemia in infant growth and intestinal health

warrants further investigation, disentangling all these influencing factors to identify EED-related micronutrient malabsorption cannot be directly measured through transferrin status alone.

While the present DBS transferrin protocol is not an appropriate measure of EED-related nutrient malabsorption, important future directions include the validation of minimally invasive techniques to assess EED-related malabsorption of other nutrients. In the 1970s, jejunal biopsies from a cohort of Peace Corps volunteers in Pakistan retrospectively suspected to have EED revealed abnormal carbohydrate absorptive function, which was eventually reversed 2-3 years after their return to the U.S. (Korpe and Petri, 2012). Several EED studies have since evaluated carbohydrate absorption through the urinary ratio of lactulose, a sugar which normally cannot cross the intestinal barrier, to mannitol, a sugar that can cross the intestinal barrier (Keusch et al., 2014). However, this method requires collecting multiple urine samples over the course of several hours, demonstrating a pressing need for more time-efficient, minimally invasive techniques for assessing EED-related nutrient malabsorption.

I-FABP

This validation study reveals that DBS can be used to measure I-FABP levels with acceptable reliability, precision, and sensitivity compared to plasma. Our analyses revealed a high level of correspondence between matched plasma and DBS readings of I-FABP in our sample, with wbDBS samples exhibiting a slightly stronger relationship with plasma than the fDBS samples. Though plasma samples consistently generated higher concentrations of I-FABP compared to their DBS counterparts, the regression model suggested there is a linear, proportional relationship which will allow researchers to convert between DBS and plasma I-FABP readings. Lastly, the Bland-Altman analyses indicated no significant, constant bias between the plasma- and DBS-based I-FABP measurement methods.

Differences between the fDBS and wbDBS samples must be considered when evaluating their linear relationships with plasma I-FABP readings. First, the wbDBS samples were created in ideal laboratory conditions: a pipette ensured that exactly five 50 µL drops of venous blood were collected and dried on DBS filter paper cards. Comparatively, the number and volume of blood drops collected via fingerstick often varied due to factors that impacted participants' distal blood circulation, such as level of physical activity or hydration prior to collection, and overall body temperature. Furthermore, collecting blood via fingerstick as opposed to pipetted blood is at greater risk for interference from detritus in the immediate environment, such as skin cells from the puncture. Second, the fDBS samples were collected from more distal finger capillaries, whereas the wbDBS and plasma samples were collected from the same, more proximal arm veins. I-FABP is specifically expressed in the epithelial cells of the small intestinal mucosal barrier and is released first into the intestinal circulation following enterocyte damage (Funaoka et al., 2011). Thus, circulating I-FABP levels are expected to vary in different parts of the body. Therefore, the stronger linear relationship between wbDBS and plasma I-FABP compared to the relationship between fDBS and plasma I-FABP was expected.

The validation of I-FABP introduces a new, salient DBS biomarker of EED and is an important step towards understanding the pathophysiology of this complex disorder. The small intestinal barrier is critical for maintaining homeostasis and promoting normal physiological development, and early detection of EED is crucial to prevent compounding adverse health effects among young children (Walrath et al., 2021; Harper et al., 2018). To our knowledge, this is the first study to validate I-FABP's measurement using minimally invasive sampling, and our findings build on a rapidly growing literature on DBS biomarker validation (Brindle et al., 2010; Crimmins et al., 2014; Eick et al., 2016; Eshghi et al., 2020; Lacher et al., 2013; Hoke et al.,

2018; Samuelsson et al., 2015). I-FABP's value as a biomarker of intestinal permeability and dysfunction has been widely acknowledged and investigated within the context of numerous diseases besides EED, ranging from enterocolitis to secondary organ failure (Coufal et al., 2020; Ng et al., 2013; Voth et al., 2015; Wiercinska-Drapalo et al., 2008). However, these studies relied on conventional biological sampling and were limited to clinical settings with extensive laboratory infrastructure. Logistically, this minimally invasive, field-friendly DBS protocol greatly expands the potential settings and populations in which intestinal permeability, and therefore intestinal dysfunction, can be assessed at the biochemical level.

Some limitations must be considered when evaluating this study. First, our study consists of a relatively small sample size compared to other DBS biomarker validation studies (Crimmins et al., 2014; Samuelsson et al., 2015). Second, the study has not yet completed a 'spike-and-retrieval' experiment, in which whole blood is spiked with known quantities of an analyte (McDade, 2014). Therefore, the absolute accuracy of the selected human I-FABP ELISA kit is unknown at the time of this writing. Third, the long-term stability of I-FABP in DBS samples is unknown; though analyte degradation to some degree is expected, the extent to which this impacts the accuracy of I-FABP measurement is unclear. Lastly, this study occurred in a setting with comprehensive, easily accessible laboratory infrastructure, but the development of this method is intended for use in low-resource and non-clinical field settings as well. I-FABP's stability in DBS samples in under different storage conditions is currently unknown and thus warrants further investigation. Despite these limitations, there is reasonable evidence supporting the validity of this I-FABP DBS protocol.

Immediate future directions therefore include the completion of a spike-and-retrieval experiment and the evaluation of I-FABP's stability in DBS samples over time and under

different storage conditions. The former involves spiking whole blood with different, pre-determined concentrations of Human FABP2 Standard solution provided in the kit. The spiked whole blood is used to create DBS samples, which are then analyzed for accuracy following the developed protocol (McDade, 2014). Long-term stability involves analyzing DBS samples at multiple time intervals and assessing the degree to the analyte degrades over time and across multiple freeze (McAllister et al., 2015). Exposing DBS cards to variable temperatures (e.g., 4^{o}C, room temperature, and 37^{o}C) or oscillating temperature patterns (e.g., 12 hours at 32^{o}C and 12 hours at 22^{o}C) is also necessary to evaluate analyte stability in possible, extreme field conditions in which this technique will be used (McAllister et al., 2015; McDade, 2014). Finally, researchers recommend comparing DBS samples which have never been thawed to DBS samples that have undergone multiple cycles of freezing and thawing; the same DBS samples are often used for multiple analyses, and analytes may degrade over multiple removals from the freezer during assay preparations (McDade, 2014; Eick et al., 2016; McDade et al., 2014).

Other future directions include measuring I-FABP in tandem with other biomarkers of EED, like EndoCAb, and validating additional candidate biomarkers for DBS sampling, including lipopolysaccharide-binding protein (LBP). LBP binds to bacterial cell walls and initiates a signaling cascade to mount an immune response to pathogens, and acts as a biomarker of systemic inflammation (Harper et al., 2018). Previous studies have indicated that elevated LBP levels are associated with villous blunting, intestinal permeability, and elevated I-FABP levels, making it a particularly promising EED biomarker (Amadi et al., 2017; Guerrant et al., 2016; Marie et al., 2018). Ongoing efforts to understand how EED impacts individual- and population-level health have thus far been impeded by unclear diagnostic criteria, and the

combined use of other EED biomarkers with I-FABP may elucidate biochemical mechanisms to clearly define the boundaries of this disorder (Harper et al., 2018).

Lastly, further validation of this DBS I-FABP protocol must be conducted in other populations, particularly among young children and infants who are most severely affected by EED. Our study population largely consisted of relatively young adults, with a mean age of approximately 22 years old. However, previous studies have observed variability in I-FABP levels in both infants and older adults, age ranges which were not included in this initial validation study (Shores et al., 2020; Walrath et al., 2021). Moreover, our study population had relatively healthy gut functioning, with only a handful of participants yielding higher than expected I-FABP levels. Given the imperfection of the linear correlations at the higher range of I-FABP levels (Figures 1 and 3) and the limited number of participants with increased intestinal permeability in this study, future studies in populations that experience greater intestinal distress are warranted.

CONCLUSION

This study adapted and validated the use of DBS sampling with a commercially available ELISA kit for the measurement of I-FABP but found DBS sampling of transferrin to be an invalid measurement method. There was a statistically significant linear correlation between DBS and plasma I-FABP levels and a high level of agreement between the two measurement methods; however, there was a statistically insignificant linear correlation between DBS and plasma transferrin levels. This method contributes to a growing body of DBS biomarker research and facilitates the study of EED and intestinal permeability in low-resource, non-clinical settings.

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Appendix A

Full Transferrin ELISA Protocol

Following sample preparation, 100 µL of all samples were loaded in duplicate to microtiter plate wells coated with transferrin antibodies. Samples were incubated for 2.5 hours with gentle shaking at room temperature, then washed using a plate washer as directed by the kit manual. Prepared biotin conjugate was added to the wells, which was then similarly incubated for an additional hour at room temperature and washed. Streptavidin-HRP solution was added to the wells and incubated for 45 minutes at room temperature. After the final wash, a solution of TMB substrate was added to each well and the enzyme reaction was allowed to proceed at room temperature for 30 minutes. Stop solution was then added, and optical densities were read at 450 nm within 30 minutes of adding the stop solution.

Appendix B

Full I-FABP ELISA Protocol

On the day of the assay run, 50 µL of Assay Diluent RD1-63 were first loaded into all microtiter plate wells coated with I-FABP antibodies, followed by 50 µL of all standards and samples in duplicate. Samples were incubated for 2 hours at room temperature on a horizontal orbital microplate shaker at 500 rpm, then washed with I-FABP Wash Buffer in a plate washer, as directed by the kit manual. Next, 200 µL of prepared Human I-FABP2 Conjugate was added to each well, and then similarly incubated for 2 hours and washed. Following this, 6 mL of Color Reagents A and B were used to prepare 12 mL of Substrate Solution; 200 µL of Substrate Solution was then added to each well, after which the plate was covered, incubated for 30 minutes, and washed. Last, 50 µL of prepared Stop Solution was added to each well, and the

plate was incubated for 15 minutes at room temperature at 200 rpm. Optical densities were read at 450 nm within 30 minutes of adding Stop Solution.