Measurement of Cystatin C in Dried Blood Spot Specimens

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Abstract

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Dried blood spot (DBS) technologies can be used in a variety of clinical and research settings. Among other advantages, the use of DBS specimens facilitate the incorporation of biomarkers into demographic research, especially for large global studies conducted in remote areas with little or no laboratory infrastructure. Here, we report the results of an evaluation of a new method for measuring cystatin C (cysC) in DBS. We adapted a commercial ELISA kit designed to measure cysC in human plasma and were able to measure cysC concentrations across the physiologic range in DBS. Determinations from DBS were linearly correlated and near directly equivalent to cysC concentrations in DBS-matched plasma—intercept=-0.07 (95% confidence interval=-0.17to -0.01) and slope=0.98 (95% confidence interval=0.92 to 1.12), R=0.94. Bland Altman analysis of cysC concentrations in DBS vs. plasma revealed no bias and acceptable levels of agreement (Bias -0.02, 95% confidence interval=-0.21 to 0.17). The DBS-ELISA demonstrates utility as a screening tool; sensitivity and specificity for cysC concentrations outside the upper limit of the reference interval (0.51 mg/L to 1.02 mg/L) were 68% and 95% respectively; however, the DBS-ELISA had difficultly correctly assigning CKD stage from DBS. We further describe an intra-donor difference in cysC concentration between venous blood collected from venipuncture and capillary blood collected by fingerstick. The DBS-ELISA assay provides an opportunity to explore kidney function in large community-based studies.

Chapter 1

Introduction

1.1 Background

Drops of blood dried on filter paper have been used as samples in medical studies for decades [1]. The technique was pioneered by Dr. Robert Guthrie to measure phenylalanine in newborns for the detection for phenylketonuria, a genetic metabolic condition that can lead to mental retardation, seizures and other serious medical problems [2]. This method of collecting blood is used world-wide to screen newborns for debilitating congenital and inheritable metabolic diseases which can be reliably detected and treated. Today, state public health laboratories screen >95% of all newborns using DBS technologies [1] [3].

1.2 Advantages of Dried Blood Spots (DBS)

DBS collection is minimally invasive, relatively painless and can be conducted in a participant's home by interviewers, or even patients, who are not trained in phlebotomy. Unlike conventional venipuncture blood samples, DBS samples do not need to be centrifuged, be separated into blood components, or be frozen following collection [1]. Furthermore, DBS are generally stable at ambient temperatures and may be shipped via mail or commercial delivery services with minimal shipping label requirements [4].

The advantages afforded by DBS technologies ease the incorporation of biomarkers into demographic research, especially for large global studies performed in remote areas with little or no laboratory infrastructure. To date, biological information acquired using DBS has been added into a number of major data collection efforts in the United States as well as in international studies in an effort to understand how social, economic and community factors shape population health [1].

DBS technologies are based on filter paper discs punched from absorbent paper blood collection cards and simple extraction techniques. Typically, a disc of fixed diameter is punched from a DBS and

placed into a liquid to resuspend or dissolve the blood in the punch. The subsequent extract may be handled from this point as any other liquid sample.

1.3 Disadvantages of DBS

While the use of DBS offers solutions to many logistical concerns, these solutions are often offset by analytical challenges. Serum and plasma are generally preferred over whole blood for clinical determinations; the majority of standard laboratory protocols require serum or plasma due to the relative ease of sample handling and the absence of potentially confounding blood components—e.g. red and white blood cell membranes and intracellular material. From a clinical perspective DBS are non-standard samples for which new methods of analysis must be developed and validated.

In serum or plasma samples, the cellular component is removed by centrifugation. This allows a representative aliquot to be drawn from a homogenous sample—a requisite for accurate quantification. It is difficult or impossible to fulfill this condition with DBS. Differences in DBS sample matrix often result in analyte values that are not equivalent with those derived from serum or plasma [(4)]. Blood hematocrit, bloodspot size and type of filter paper all affect analyte recovery [(5)]. The diameter of a DBS created by a fixed volume of blood depends partly on the patient's hematocrit, which varies from person to person based on age, sex, nutrition and hydration status, as well as genetics—i.e. the amount of plasma present can differ between discs punched from DBS obtained from different donors. Additionally, if target sensitivity is a challenge using conventional methods, the issue will be exacerbated in DBS due to the limited amount of matrix.

Data generated by the University of Washington, Department of Laboratory Medicine indicates that plasma in the blood spotted onto the filter paper may not distribute homogenously across a DBS (Observations communicated by Mike Edenfield). Each analyte has a level of uncertainty from DBS to DBS, and likely punch to punch, in the amount of plasma available for analysis. Therefore, punches from different DBS can yield different analyte concentration estimates even if the

circulating concentrations in the DBS donors are the same. Despite this potential limitation, DBS analyses have been shown to be suitable for measuring many analytes.

1.4 DBS Assay for Cystatin C (cysC)

There is interest in developing an assay to evaluate kidney function using DBS. Glomerular filtration rate (GFR) is an important measure of kidney function. GFR describes the flow rate of filtered plasma through the kidney (6) and is used in the detection, evaluation and management of acute and chronic kidney disease (CKD) (6) (7). The "gold standard" methods for the determination of GFR are inulin clearance or [^{125}I] iothalamate clearance (6), both of which are expensive, cumbersome, and invasive. Most clinicians instead choose to estimate GFR from serum creatinine, usually determined by the Jaffe reaction (8) (9).

The non-specificity of the Jaffe reaction can cause falsely elevated creatinine concentrations when creatinine is measured in the presence of any of more than 30 substances including protein, glucose, and bilirubin (8) (10) (11). All of the substances present in whole blood would also be present in DBS; therefore, creatinine quantification by methods subject to these kinds of interferences—i.e. the Jaffe reaction—has limitations. Creatinine quantification from DBS has been unsuccessful to date. A DBS technology which separates plasma from cellular components would allow the measurement of creatinine; however, such technology may negate many of the attractive features of using DBS—e.g. adding additional expense or complexity to the collection process.

Measurement of cystatin C (cysC) provides an alternative estimate of kidney function. There is evidence that GFR is more closely correlated with serum levels of cysC than with levels of serum creatinine (7) (12). Development and validation of an enzyme-linked immunoassay (ELISA) DBS assay would provide a simple, convenient method of measuring cysC in large-scale epidemiology studies. An ELISA offers an important advantage in that results can be read on frequently available microtiter plate spectrophotometers. To date, we are not aware of any studies that have used ELISA to measure cysC in DBS.

Chapter 2

Biology of cystatin C

2.1 Cysteine proteases

Proteolysis is a ubiquitous mechanism by which the cell regulates the function of proteins, many of which are synthesized as inactive precursors that are converted into their active form by enzymatic cleavage [(13)]. Cystatin C, an inhibitor of many cysteine proteases such as papain and cathepsins B, H, K, L and S [(14)], is an integral member of the web of proteases and protease inhibitors that shape the proteome—the entire set of proteins expressed by the genome.

Cysteine proteinases comprise a group of enzymes that cleave peptide bonds by the use of a neutrophilic cysteine residue at the catalytic site [(15) (16)]. Intracellular cysteine proteinases participate in normal protein turnover, antigen and protein precursor processing, and apoptosis. Extracellularly, they are involved in tissue remodeling and turnover of the extracellular matrix, immune system function, and modulation or alteration of cell function [(17)]. Cysteine proteinases are classified into two major clans based on the structural organization of the active site: the CA clan, which shares structure and evolutionary history with papain; and the CD clan which includes legumains, caspases, lysosomal cathepsins B, H, and L, and dipeptidyl peptidase I [(18)].

The regulation of proteolytic activity is complicated but may be generalized as the control of the balance between the amounts of active enzyme present versus the amount of active enzyme inhibitor. Dysregulation of cysteine proteinases may initiate proteolytic cascades which can potentially lead to pathological damage. Destructive proteolysis by cysteine proteinases of host, bacterial and viral origin is prevented by the inhibitory properties of the cystatins [(14)].

2.2 Cystatin C Super Family

The Cystatin superfamily comprises the most abundant group of inhibitors of cysteine proteases [(17)]. Superfamily members are grouped into three families based on size, complexity and physiological

location (Table 1). Family 1 cystatins, also called stefins, are not synthesized as pre-proteins with signal peptides; this restricts them to the cytoplasm. Family 2 cystatins are non-glycosylated, translated with a secretory peptide leader sequence, and have two intra-chain disulfide bonds; they are considered to be extra-cellular. Family 3 cystatins are the kininogens, complex intravascular proteins present in the plasma and secretions—tears, milk, saliva, of mammalian species

Table 1: Human Cystatin superfamily (19) (20)

Family 1 Intracellular cystatins	**Family 2** Extracellular cystatins	**Family 3** Intravascular cystatins
Cystatin A	Cystatin C	LMW-kininogen
Cystatin B	Cystatin D	HMW-kininogen
	Cystatin E	
	Cystatin F	
	Cystatin S	
	Cystatin SA	
	Cystatin SN	

2.3 Biology of CysC

The amino acid sequence of human cysC was described in 1981 (21). It belongs to family 2 of the cystatin superfamily and contains 120 amino acid residues in a single non-glycosylated polypeptide chain with molecular mass between 13,343-13,359 Da, and has two intra-chain disulfide bonds (14) (19) (21). CysC inhibits cysteine proteinases belonging to the C1 superfamily, e.g. papain, ficin, and cathepsins B, C, H, K, L and S (14) (19) (22).

CysC is encoded by the CST3 gene, which is comprised of 3 exons with an overall size of approximately 4,500 base pairs. The gene has a promoter that has been identified as the housekeeping type and is ubiquitously expressed at moderate levels (23); consequently, the production rate of cysC has been found to be constant (24).

CysC pre-protein is synthesized with a hydrophobic signal peptide, suggesting that it is exported and that its functions as an inhibitor are primarily extracellular (23). The synthesis of cysC is not thought to be tissue specific and all nucleated cells express and constantly secrete cysC (14) (23), thus it is

present in most bodily fluids including blood plasma [14] [23]; however, concentrations vary considerably between body compartments (Table 2) [25]. In spinal fluid, cysC represents more than 90% of the total molar concentration of cysteine protease inhibitors, whereas in plasma it represents only a small fraction of the total protease inhibiting ability [25].

Table 2: Concentrations of cysteine proteinase inhibitors in extracellular fluids, mg/L [25]

	Cystatin A	Cystatin B	Cystatin C	Cystatin S	Kininogen
Blood Plasma	<0.2	<0.1	1.4	<0.1	472
Synovial Fluid	<0.2	<0.1	2.9	<0.1	327
Milk	<0.2	<0.1	3.4	<0.1	3.1
Saliva	3.8	<0.1	1.3	14	<0.5
Cerebrospinal Fluid	<0.2	<0.1	7.2	<0.1	2.4
Seminal Plasma	0.46	1.0	49	15	2.0
Amniotic Fluid	2.5	0.09	1.0	<0.1	20
Urine	<0.2	<0.1	<0.5	<0.1	3.9
Tears	1.0	0.05	2.4	33	1.0
Blood Plasma (Uremia)	<0.2	0.58	8.9	<0.1	504

2.4 Serum/Plasma CysC as an estimator for Glomerular Filtration Rate

An ideal estimator for glomerular filtration rate (GFR) would be a substance that appears endogenously in the plasma at a constant rate, is freely filtered at the glomerulus, neither reabsorbed nor secreted by the renal tubules, and undergoes no extra-renal elimination [26].GFR determination is the basis for detection and classification of CKD as it estimates the volume of blood which is cleared per minute by the kidneys, standardized to the body surface area of an average sized adult (1.73 m^2), and is usually expressed in mL/min/1.73m^2.

The measure of serum creatinine concentration is the most common test used to evaluate GFR; however, serum creatinine has several limitations and does not completely fulfill the characteristics of an ideal estimator of GFR [27]. While creatinine is small, circulates unbound to plasma proteins and is freely filtered at the glomerulus, it undergoes tubular secretion into the urinary space. Furthermore, tubular secretion of creatinine is not constant and varies, not only within an individual, but between individuals [26]. This renders creatinine measures insensitive to mild or moderate reductions in GFR [26]. Additionally, plasma creatinine concentrations are influenced by

muscle mass, diet, sex and age, which unless accounted for, lead to imprecision in estimates of GFR. Finally, many substances are known to influence creatinine assays—e.g. bilirubin, hemoglobin, ketones, high glucose, as well as several drugs [8] [10] [11].

An alternative measurement to serum creatinine is serum cysC; important characteristics are its small size and high isoelectric point (pI = 9.3)—which allows it to be freely filtered at the glomerulus, and its constant rate of production [19]. CysC plasma concentrations are not affected by muscle mass, diet or gender [14] and unlike creatinine, cysC has no known extra-renal routes of elimination. The main catabolic site of cysC is the kidney where the protein is almost completely cleared from the circulation by glomerular filtration [14]; the proximal tubular cells reabsorb cysC and transfer it into lysosomes for enzymatic degradation removing virtually all of the filtered cysC [14] [28] and preventing it from returning to circulation in its intact form. Thus, cysC meets many of the criteria of an ideal GFR estimator. Furthermore, only two circumstances have been identified that have an impact on cysC serum concentrations: high-dose glucocorticoid therapy and thyroid dysfunction [29].

Most studies have shown that serum cysC is more closely correlated with GFR than serum creatinine; however, studies that have compared serum cysC with GFR estimates based on serum creatinine, age, sex and races showed them to be comparable [7]. Serum creatinine estimates of GFR are typically calculated using Modification of Diet in Renal Disease (MDRD) study equation (eGFR $(\text{mL/min/1.73 m}^2) = 175 \times (S_{creatinine})^{-1.154} \times (\text{Age})^{-0.203} \times (0.742 \text{ if female}) \times (1.212 \text{ if African American})$). Conversely, many published cysC based estimating equations do not include terms for age, sex or race [7] [30] . Demographic information is not available for either plasma or DBS samples used in the following method comparison so GFR adjusted for age, sex and race will not be calculated.

Chapter 3

Materials and Methods

3.1 Sample population

The Clinical and Laboratory Standards Institute (CLSI) recommends that a minimum of 40 data points be collected with 50% of the points outside the reference interval [30] [31]. Correlations should involve comparison with an acceptable reference method.

DBS were created by pipetting 75 μL aliquots of EDTA-venipuncture blood, acquired from four groups of adult donors onto Whatman #903 filter paper (GE Healthcare, Cardiff, Wales). DBS were dried overnight at room temperature (RT), placed in resealable plastic bags with desiccant packs (Desiccare, Pomona, CA) and stored in a -70°C freezer until use. These DBS will be referred to as venipuncture dried blood spots (VP-DBS) in the following analyses.

Assay validation experiments for our DBS-ELISA were conducted using 141 VP-DBS samples analyzed over 7 days. The expected serum reference interval for cysC serum is 0.51 mg/L to 1.02 mg/L [14].Of the 141 samples we analyzed, the cysC concentration of 13% fell outside of the reference interval. While the range of cysC concentrations used in our evaluation is not ideal, it should be adequate to obtain good estimates of linear regression parameters.

Validation of our ELISA assay used for the quantification of cysC in DBS was performed using VP-DBS. In the field, however, DBS are collected by fingerstick, so the assay will ultimately be used on DBS created from fingerstick blood (FS-DBS). Fingerstick samples were collected as follows: the donor's finger was cleaned with isopropyl alcohol and then pricked with a sterile, disposable lancet. The first drop of blood was wiped away with gauze, and subsequent single blood drops were allowed to drop by force of gravity onto Whatman #903 filter paper. Samples were dried, placed in plastic bags with desiccant and stored in a -70°Cfreezer. Of the 141 VP-DBS used in the following method validation experiments, 132 had matching FS-DBS.

3.2 Creation of Standards and Controls

DBS assay calibrators and DBS quality control (QC) samples were created by spiking whole blood with recombinant human cysC and then applying the whole blood to filter paper. Three units (A, B and C) of outdated whole blood (WB) were purchased from Puget Sound Blood Center (Seattle, WA). An aliquot of each unit of WB was centrifuged and the plasma removed. The plasma cysC concentration for each unit was determined by a Beckman Coulter UniCelDxC 800 (DxC; Beckman Coulter, Indianapolis, IN) using cysC immunoassay reagent for Beckman Coulter UniCel Systems (Gentian, N-1509 Moss, Norway).

The Beckman Coulter method of cysC quantification is comparable to the Dade Behring BNII Nephelometer method [30] and was used during the creations of standard and QC samples due to convenience of access. Final cysC concentrations of DBS standards and QC samples were determined by the nephelometer.

Calculations used in creating DBS standards assumed a WB 50% hematocrit and no contribution of cysC by RBCs. An approximate 23 mL aliquot of WB from unit A with a plasma cysC concentration of 0.30 µg/mL was spiked with 890 µL of 100 µg/mL recombinant human cysC (RAB0105-1KT, Sigma Aldrich, St. Louis, MO) to create an WB DBS assay standard (assay high standard) with an approximate cysC plasma concentration of 8 µg/mL. Aliquots of the assay high standard were serially diluted with Unit A blood to create a set of potential assay standards (Standards A-F, Table 3).

Table 3: DBS Standard Creation

Standard	mL Unit A non-spiked WB (0.30µg/mL cysC)	mL Unit A spiked WB (8µg/mL cysC)	Calculated / Predicted cysC µg/mL WB in plasma
A	0.00	20.00	8.00
B	10.00	10.00	4.15
C	15.58	4.42	2.00
D	16.88	3.12	1.50
E	18.18	1.82	1.00
F	19.48	0.52	0.50

A 23 mL aliquot of Unit B with a plasma cysC concentration of 0.35 µg/mL was spiked with 175 µL of the recombinant cysC to create a high cysC QC sample with a 1.75 µg/mL calculated cysC concentration.

A 23 mL aliquot of Unit C (0.43 µg/mL cysC) was spiked with 41 µL of the recombinant cysC to create a low cysC QC sample with a 0.75 µg/mL calculated cysC concentration.

A zero standard was created. A 20 mL aliquot of unit A was centrifuged at 3000 rpm for 10 minutes, plasma and buffy coat were removed and discarded and a 0.9% saline solution was added to the residual red blood cells and mixed gently. Tubes were centrifuged as before then saline and any remaining buffy coat were removed. RBCs were washed three times and resuspended to an approximate 50% hematocrit with 5% human albumin (NDC 0053-7670-06, CSL Behring; Kankakee, IL).

Plasma was removed from an aliquot of the WB used to create each assay standard and QC sample, and the cysC concentrations were measured on the BNII Nephelometer. Standard A was eliminated because of the high coefficient of variation (%CV) among replicates when subsequently measured by ELISA. Each subsequent standard (B-E) was pipetted onto Whatman 903 filter paper in 75 µL drops and allowed to air dry at RT overnight to create a 6-point standard curve. Final (assigned) concentrations of the assay standards and QC samples as determined by the nephelometer are given in Table 4. We noted that the final concentration of cysC present in the standards was less than calculated—likely because our calculations assumed a WB 50% hematocrit whereas the hematocrit of the WB used was variable; however, the range was acceptable for our purposes.

Table 4: Final Standard Concentrations, Observed

Standard	Nephelometer cysC µg/mL
B	3.42
C	1.57
D	1.11
E	0.73
F	0.43
Zero	<0.05
LQC	0.94
HQC	1.47

3.3 Methods of Measure

We adapted the BioVendor Human CysC ELISA kit (RD191009100; BioVendor; Modrice, Czech Republic)—a sandwich ELISA was used for the quantitative measurement of cysC in plasma, as the basis for our DBS assay. The BioVendor Human CysC ELISA kit was selected based on its sensitivity. The standard curve ranges from 200 ng/mL (0.2 ng/µL) to 10,000 ng/mL (10 ng/µL). Per the kit protocol, each standard is diluted 400x and 100 µL is transferred to assay plate; an individual well on the assay plate will hold a csyC mass between 0.05 ng/well to 2.5 ng/well. Assuming that a typical 3.2 mm diameter DBS punch has 1 µL of plasma and a cysC concentration of 1 ng/µL (within the normal range [(14)]), we calculated that if this DBS were resuspended in 400 µL assay diluent, the resulting solution would have a cysC concentration of 0.0025 ng/µL. A transfer of 100 µL of eluent to the assay plate would result in a cysC mass of 0.25 ng/well—within the range of the kit standards. Thus, our expectation is that the ELISA that is capable of measuring the concentration of cysC expected to be typically found in a DBS punch.

CysC concentrations in DBS as determined by ELISA were compared to the cysC concentrations of matched plasma samples determined by a particle-enhanced immunonephelometric assay (N Latex Cystatin-C, Dade Behring Diagnostics, Marburg, Germany) conducted on a Siemens Dade Behring BNII Nephelometer (Dade Behring Diagnostics, Somerville, NJ). The assay employs anti-human cysC antibodies chemically coupled to polystyrene particles incubated with serum diluted 1:100 in assay buffer. Particles with bound cysC aggregate, causing a change in scattered light which is

directly proportional to the concentration of cysC present. A reference curve is created by multi-point calibration and cysC levels are evaluated against Dade Behring standards and controls. Plasma and serum samples are assumed to have identical cysC concentrations.

To permit analysis, a DBS sample must be eluted (i.e., the dried blood in the disc punched from a DBS must be "reliquified"). Typically, a 3.2 mm disc is punched from each DBS using a BSD Robotics BSD700 (BSD Robotics, Bundamba, Australia) semi-automatic puncher equipped with a 3.2 mm diameter punch head. The disc is placed into a microtiter plate with an elution buffer and shaken for one hour to create a hemolyzed liquid sample. The resulting extract is handled as any other liquid sample.

One 3.2 mm diameter disc was punched from each DBS standard, DBS quality control (QC) sample or DBS from a study participant into a deep-well microtiter plate (DWP) (Greiner Bio-One, Frickhausen, Germany). Punches were eluted for one hour at RT in 400 μL of reconstituted CysC Dilution Buffer (BioVendor) on a plate shaker at 1200 rpm. DBS eluent was transferred from the DWP to a Human CysC ELISA microtiter plate (BioVendor).

Each well of the BioVendor microtiter plate used in the ELISA was pre-coated with a polyclonal anti-human cysC antibody (solid phase immobilization). After addition of standards, QC samples and DBS samples, the assay plate was incubated for 1hrat RT with shaking at 300 rpm. The assay plate was washed 5 times after incubation with reconstituted cysC Wash Buffer Concentrate (BioVendor) to remove unbound material. A polyclonal anti-human cysC antibody, conjugated with horseradish peroxidase (enzyme-linked antibody) was added to each assay plate well (BioVendor). The assay plate was incubated for 30 min at RT with shaking at 300 rpm. The plate was washed 5 times to remove unbound antibody conjugate. A tetramethylbenzidine (TMB) and hydrogen peroxide (H_20_2) solution was added (BioVendor); peroxidase cleaves H_20_2, reacts with TMB and causes the solution to develop color.

The reaction was stopped by the addition of a 1.96% sulfuric acid solution after 10 min. Absorbance was measured on a spectrophotometer (Synergy HT; BioTek, Winooski, VT) The cysC concentration is directly proportional to the absorbance of the solution. Using the 5-parameter standard curve, the absorbance values of the QC samples and DBS samples were read as cysC concentrations. Acceptability of the assay was determined by comparing the cysC concentrations of the QC samples with their established values.

DBS measurements by nephelometry (DBS-Neph) were performed by the following protocol: Two 3.2mm diameter discs were punched from each DBS standard, QC DBS or study participant DBS. The two matched punches per DBS were individually eluted for 1hr at RT in 200µL of reconstituted Dilution Buffer (BioVendor). The DBS eluent was pooled by transferring 150µL from each of the two matched wells to a single 2.0mL microcentrifuge tube. For serum and plasma cysC analysis, the nephelometer automatically dilutes specimens 1:100. In order to accommodate the off-instrument dilution necessary to resuspend cysC from DBS, the nephelometer was programmed to recognize an external dilution of 1:20 and its automatic internal dilution was adjusted from 1:100 from 1:20. A standard curve of DBS with known plasma cysC concentrations accompanied each run and was used to generate plasma equivalent DBS cysC values.

3.4 Statistical Analyses

Statistical analyses were conducted using R (R Foundation for Statistical Computing, Vienna, Austria), GraphPad Prism 6 (GraphPad Software, Inc. La Jolla, CA) and Microsoft Excel 2010 (Microsoft, Redmond, WA). Correlation and linear regression analyses were used to evaluate the relationship between cysC concentrations in DBS versus in DBS-matched plasma samples. In addition, Bland-Altman plots were used to evaluate bias or inconsistent variability across the range of measurement.

Chapter 4

Results

4.1 Estimates of random error for both methods

DBS-ELISA assay precision, or intra-assay variation, was assessed by determining the mean cysC concentration in 3 DBS discs, punched and eluted separately, from 2 control samples analyzed across 13 runs. Reliability, or between-assay variation, was assessed by averaging control samples over 13 different days (Table 5).

For both intra-assay and inter-assay variation, the percent coefficient of variation was calculated for each control sample (%CV = 100 × SD/mean). A precision study for the DBS-ELISA demonstrated an acceptable intra-assay CV of 5.4% and inter-assay CV of 7.4%.

Estimates of nephelometer precision and reliability using DBS were determined from 2 control samples analyzed across 5 runs over 5 days. DBS analyzed on the Nephelometer showed an intra-assay CV of 4.2% and inter-assay CV of 6.9%—greater than manufacture claims listed on kit insert (intra-assay = 2.3%, inter-assay= 3.1%).

Table 5: Precision Assessment Using Control Specimens

	CLT 1 (Low QC)				**CLT2 (High QC)**			
Day	1	2	3	Mean	1	2	3	Mean
1	0.84	0.89	0.86	0.86	1.29	1.25	1.22	1.25
2	0.84	0.89	0.91	0.88	1.32	1.48	1.39	1.39
3	1.04	1.10	1.04	1.06	1.56	1.56	1.55	1.56
4	1.00	0.93	0.87	0.93	1.65	1.55	1.53	1.57
5	1.03	0.91	0.90	0.94	1.52	1.59	1.51	1.54
6	1.01	0.90	1.14	1.02	1.33	1.53	1.53	1.46
7	0.87	1.01	0.91	0.93	1.36	1.41	1.46	1.41
8	—	0.92	0.92	0.92	1.65	1.63	1.60	1.63
9	0.99	0.88	0.88	0.92	1.61	1.44	1.26	1.43
10	0.97	0.86	0.96	0.93	1.40	1.29	1.30	1.33
11	1.03	0.98	1.04	1.02	1.56	1.49	1.45	1.50
12	1.22	1.04	1.05	1.10	1.59	1.50	1.50	1.53
13	1.14	0.99	0.92	1.02	1.43	1.32	1.52	1.42
	Mean: 0.96, SD: 0.07				Mean: 1.46 — SD: 0.11			

4.2 Acceptance limits based on inherent imprecision of both methods

Based on our precision studies, the amount of analytical error between measurement systems that is allowable without compromising test interpretation was calculated. The inherent imprecision of both methods is calculated as $\sqrt{(CV^2_{method\ 1} + CV^2_{method\ 2})}$ [33][30]. As stated above, the nephelometer has an imprecision of 6.9% and the DBS-ELISA has an imprecision of 7.4%. The inherent imprecision of both methods is calculated as 10.1%. This means that if both methods were expected to measure identically then the difference between the two methods would be between the intervals of 0 +/- 1.96 x (10.1% x mean cysC concentration of the two methods) in 95% of measurements.

4.3 Evaluating performance; plotting the data

The purpose of the method comparison was to determine if ELISA and nephelometer determinations of cysC concentrations from DBS were equivalent. For this evaluation, cysC as measured by nephelometer in plasma was our "gold standard" reference method. However, due to the inherent variability in DBS we determined to judge acceptability based on DBS measurements from both systems.

We evaluated the performance of the DBS-ELISA against the nephelometer reference method. In figure 1, the data are plotted with the DBS-ELISA method on the *y*-axis and the nephelometer plasma method on the *x*-axis. The relationship is linear, highly correlated (R=0.94) and distributed around a line with a slope less than 1.0 (i.e. below the line of identity).

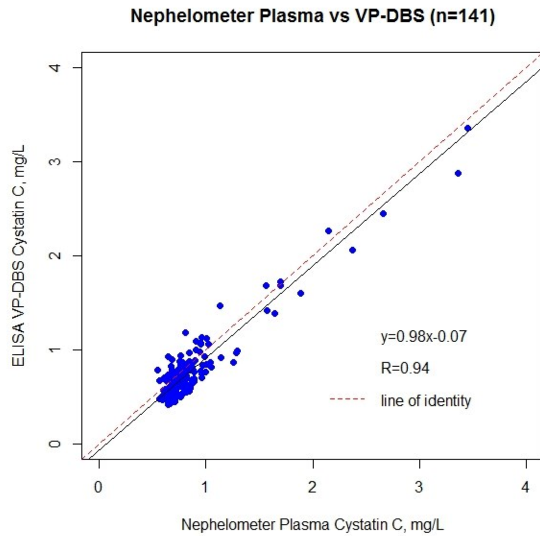

Figure 1: The dotted line represents the line of identity (*y*=*x*). Intercept = -0.07 (95% confidence interval = -0.17 to -0.01) and slope = 0.98 (95% confidence interval = 0.92 to 1.12).

Deming regression analyses indicated near equivalence between DBS-ELISA cysC determinations and plasma cysC measured by nephelometer; intercept of -0.07 (95% confidence interval= -0.17 to -0.01) and slope of 0.98 (95% confidence interval = 0.92 to 1.12). The slope is not significantly different than zero; however, the intercept shows a very slight constant bias. The regression equation (y=0.98x-0.07) provides a means for calculating the plasma-equivalent cysC concentration from the measured DBs cysC concentration.

We next checked the performance of the ELISA determinations of cysC in plasma against the reference method. In figure 2, the data are plotted with the ELISA method on the *y*-axis and the nephelometer plasma method on the *x*-axis. The relationship is linear, highly correlated (r=0.88) and generally distributed around the line of identity.

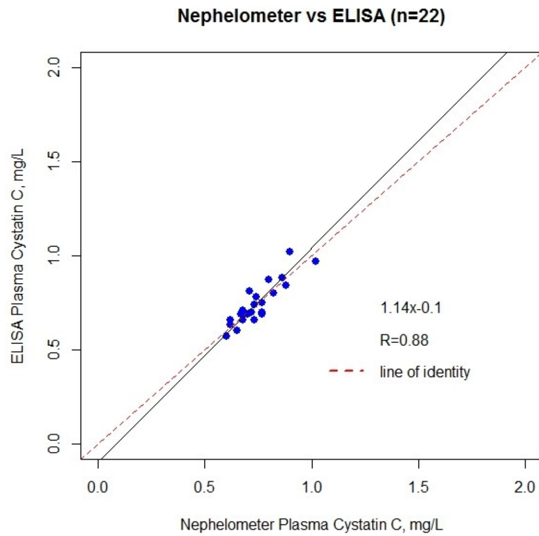

Figure 2: The dotted line represents the line of identity (*y*=*x*). Intercept=-0.10 (95% confidence interval = -0.39 to 0.07) and slope = 1.14 (95% confidence interval=0.90 to 1.52).

Deming regression analysis reveals an intercept of -0.10 (95% confidence interval = -0.39 to 0.07) and slope of 1.14 (95% confidence interval=0.90to 1.52). The intercept is not statistically significantly different from zero and the slope is not statistically different from 1.0, indicating equality between plasma cysC concentrations measured by nephelometry vs. cysC concentration measured by ELISA. The data provides evidence that the kit is performing per the manufacturer's claims.

To control for differences in sample matrix, we compared the cysC concentrations of 94 VP-DBS against DBS-matched plasma, both determined by nephelometry. In figure 3, the data are plotted with DBS-Neph cysC concentrations on the *y*-axis and the nephelometer plasma concentrations on the *x*-axis. The relationship is linear, highly correlated (r=0.89) and generally distributed around the line of identity.

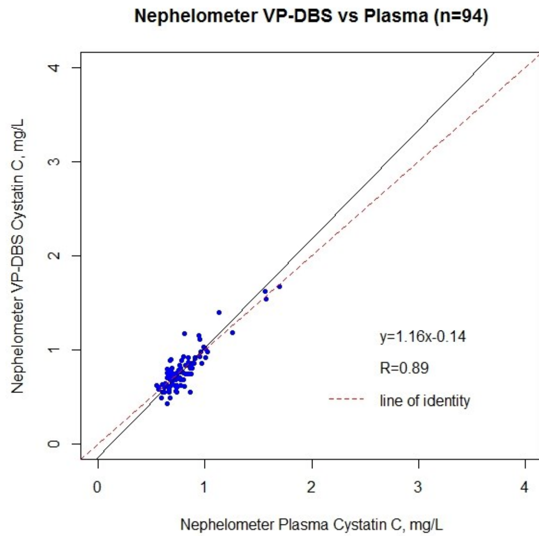

Figure 3: The dotted line represents the line of identity (*y=x*). Intercept=-0.14 (95% confidence interval=-0.34 to -0.05) and slope=1.16 (95% confidence interval=1.05 to 1.42).

Deming regression analysis reveals an intercept of-0.14 (95% confidence interval=-0.34to -0.05) and a slope of1.16 (95% confidence interval=1.05 to 1.42). There evidence of both proportional and constant bias; however, the confidence intervals for the slope and intercept overlap with what was observed in cysC measurements in DBS by DBS-ELISA suggesting that the two systems are performing similarly (Table6). A broader sample set—including more values in the upper range, may show closer agreement between cysC in DBS measured by nephelometry and the DBS-ELISA.

Table 6: Comparison of regression coefficients from Figure 1 and Figure 3

	Intercept	95% CI	Slope	95% CI
Nephelometer Plasma vs. DBS-ELISA (Figure 1)	-0.07	-0.17 to -0.01	0.98	0.92 to 1.12
Nephelometer VP-DBS vs. Nephelometer Plasma (Figure 3)	-0.14	-0.34 to -0.05	1.16	1.05 to 1.42

Finally, we assessed the performance of DBS-ELISA cysC determinations against DBS by nephelometry. In figure 4, the data are plotted with DBS-ELISA cysC concentrations on the *y*-axis and the nephelometer DBS concentrations on the *x*-axis. Again, the data are linear, highly correlated (r=0.91) and symmetrically distributed around the line of identity.

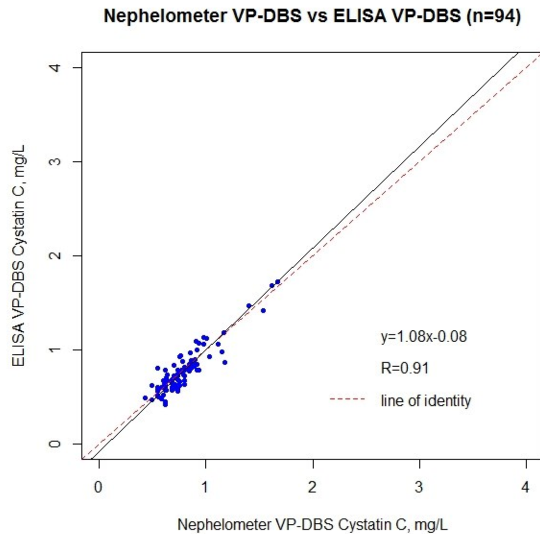

Figure 4: The dotted line represents the line of identity (*y*=*x*). Intercept=-0.08 (95% confidence interval=-0.17 to -0.01) and slope=1.08 (95% confidence interval=0.99 to 1.20).

Deming regression analysis reveals an intercept of -0.08 (95% confidence interval=-0.07 to -0.01) and a slope of 1.08 (95% confidence interval=0.99 to 1.20. The slope is not significantly different than zero; however, the intercept shows a very slight constant bias.

The relationship between cysC concentrations generated by DBS-ELISA and DBS or plasma by nephelometry is strongly linear and near equivalent. From these analyses we can conclude that the DBS-ELISA is capable of measuring cysC from DBS.

4.4 Judging acceptability

The analysis so far has revealed a very slight constant but no proportional bias. The next step is to judge whether the two methods are identical within inherent imprecision. A difference plot with the mean value of DBS-ELISA and nephelometer DBS measurements on the *x*-axis and the difference between the two methods on the *y*-axis was constructed (Figure 5).

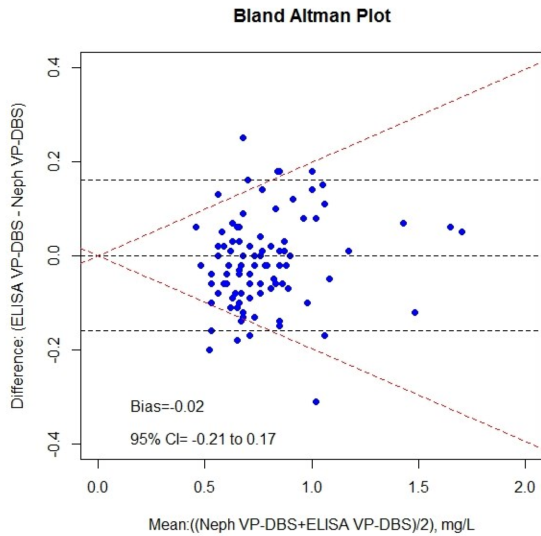

Figure 5: Difference plot (plasma cysC minus VP-DBS). Dotted lines represent 0 ± 1.96 · inherent imprecision of both methods (10.1%) Only 10 values of 94 measurements (11%) are outside the interval outlines by the dotted lines

As stated previously, the combined inherent imprecision (CV) as calculated above is 10.1%. Two lines representing, respectively, 0 + 1.96 X 10.1% and 0 − 1.96 X 10.1% are also inserted in the plot. While only 10 values of 94 measurements (11%) are outside the interval outlines defined by the dotted lines —this is greater than our expectation of 5% and likely represents additional variation inherent in DBS. Bland Altman analysis revealed a bias of -0.02 (95% confidence interval=-0.21 to 0.17). The bias was not statistically different from zero, indicating good agreement between the two methods.

4.5 Sensitivity and Specificity

A two by two table was constructed to determine the diagnostic accuracy of the DBS-ELISA cysC assay for the detection of abnormal kidney function. Sensitivity (e.g. the percentage of people with the disease who are correctly identified as having the condition) and specificity (e.g. the percentage of healthy people who are correctly identified as not having the condition) were calculated. For this evaluation, cysC as measured by nephelometry in plasma was our "gold standard" reference method. The expected serum reference interval for cysC serum is 0.51 mg/L to 1.02 mg/L [14]. The upper limit

of the reference interval (1.02 mg/L) was used as a cut point (Figure 6); any plasma with a cysC concentration greater than or equal to 1.02 mg/L measured by nephelometry was classified as disease, concentrations less than the cut point were classified as normal.

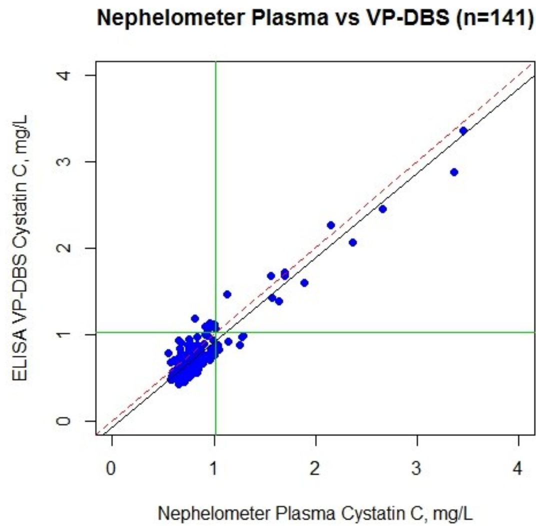

Figure 6: Data from Figure 1 plotted with intersecting lines denoting the upper limit of the reference interval (x=1.02, y= 1.02).

The results (Table 7) show that the DBS-ELISA assay is better at ruling out disease (specificity = 95%) then ruling in disease (sensitivity = 68%) in patients with circulating cysC concentrations near or outside the upper limit of the reference interval.

Table 7: Sensitivity and Specificity

	Nephelometer Positive	**Nephelometer Negative**		
DBS-ELISA Positive	13	6	68%	Positive predictive value (PPV)
DBS-ELISA Negative	6	116	95%	Negative predictive value (NPV)
	Sensitivity 68%	**Specificity 95%**		

The ability of the DBS-ELISA to correctly classify CKD by stage (Table 8) was examined. GFR was estimated from cysC concentrations in DBS by DBS-ELISA and plasma by nephelometry using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula ($76.7 \times cysC^{-1.19}$) [34][7] (Table 9) and compared. The results show that the classification of CKD stages from DBS was problematic; of the 141 samples analyzed by DBS-ELISA, 31% didn't agree with classifications by nephelometry from plasma; 23% were misclassified by 1 stage and 9% were misclassified by 2 stages. A scatter plot was created to visualize the position of misclassified points (Figure 7). Equations for the estimation of GFR from cysC exist that have terms for race and age are available and could correct for some— not likely all, of the discordance between CKD classifications; however, demographic information is not available.

Table 8: Severity of CKD.

CKD stage	Description	GFR (mL/min/1.73m^2)
Stage 1	Kidney damage with normal or ↑GFR	>= 90
Stage 2	Kidney damage with mild ↓ GFR	60-89
Stage 3	Moderate↓ GFR	30-59
Stage 4	Severe↓ GFR	15-29
Stage 5	Kidney failure	<15

Table 9: DBS-ELISA vs. Nephelometer Plasma Classification.

Classification Based on DBS-ELISA Assay of CysC in VP-DBS	CKD Classification Based on Nephelometric Plasma CysC: No Disease	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Total
No Disease	72	16	8	0	0	0	96
Stage 1	3	4	4	1	0	0	12
Stage 2	4	3	12	2	0	0	21
Stage 3	0	0	1	6	1	0	8
Stage 4	0	0	0	1	3	0	4
Stage 5	0	0	0	0	0	0	0
Total	79	23	25	10	4	0	141
Misclassified	**31%**						

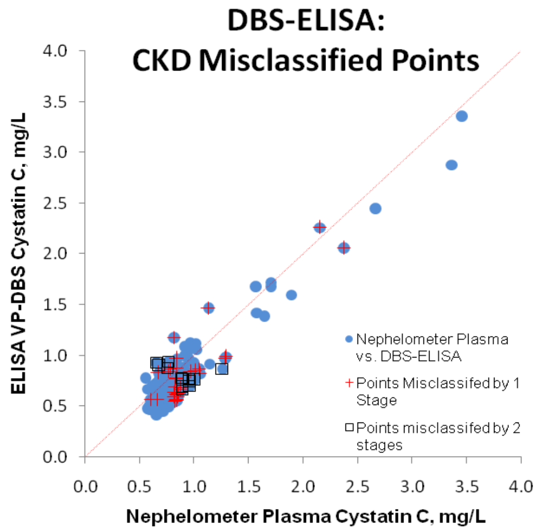

Figure 7: ELISA VP-DBS vs. Nephelometer Plasma cysC. Red crosses represent DBS which misclassify CKD by 1 stage black squares misclassify CKD by 2 stages.

4.6 Fingerstick CysC

Evaluation of DBS-ELISA assay was conducted on DBS created from venipuncture blood; however, fingerstick DBS (FS-DBS) are made from capillary blood. In figure 8, cysC concentrations in 132 FS-DBS by DBS-ELISA are compared to cysC in plasma by nephelometry; nephelometer plasma cysC are plotted on the *x*-axis and DBS-ELISA FS-DBS on the *x*-axis.

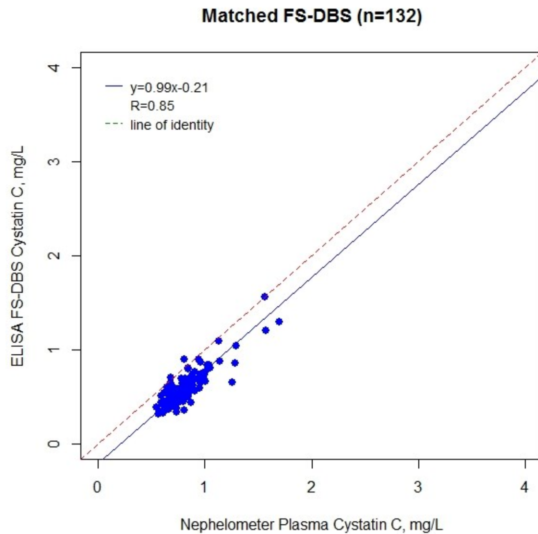

Figure 8: Intercept of -0.21 (95% confidence interval = -0.36 to -0.10) and a slope of 0.99 (95% confidence interval= 0.84 to 1.19).

Deming regression analysis of the data shows an intercept of -0.21 (95% confidence interval = -0.36 to -0.10) and a slope of 0.99 (95% confidence interval= 0.84 to 1.19). The slope is not significantly different than 1.0; however, the intercept shows a significant constant bias. The regression equation ($Y = 0.99*X-0.21$) provides a means for generating plasma equivalents from FS-DBS samples.

A paired t-test found the difference in means between VP-DBS and FS-DBS to be -0.14 (95% confidence interval= -0.16 to -0.11, $p<0.001$).

4.7 Difference between venous and capillary plasma as determined by nephelometer

To determine if the observed difference in cysC concentrations between VP-DBS and FS-DBS was a real phenomenon, we compared the cysC concentrations in plasma isolated from venous blood collected by venipuncture with plasma isolated from capillary blood collected by fingerstick. Venipuncture blood was collected in lithium heparin PSTs (BD, Franklin Lakes, NJ) from forty volunteers in a Harborview Medical Center clinical laboratory. Matched fingerstick plasma samples were collected concurrently as follows: the donor's finger was cleaned with isopropyl alcohol and then

pricked with a sterile, disposable lancet. The first drop of blood was wiped away with gauze, and 3 to 5 subsequent blood drops were allowed to drop by force of gravity into lithium heparin Microtainer® PST tubes (BD, Franklin Lakes, NJ). Prior to analysis both venipuncture and fingerstick plasma were pre-diluted 1:20 with phosphate buffered saline (137 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 and 2.7 mM KCl, pH 7.4) and stored in a -70°Cfreezer until analysis.

For our experiment the standard nephelometer cysC protocol was modified to accommodate pre-dilution. The instrument was programmed with an external dilution of 1:20 and adjusting the automatic dilution to 1:20 from 1:100.

In figure 9, plasma from 40 matched venipuncture and fingerstick samples are plotted with fingerstick on *y*-axis and venipuncture on the *x*-axis. The relationship is linear, highly correlated (r=0.97) and distributed around a line with a slope less than 1.0 (i.e. below the line of identity).

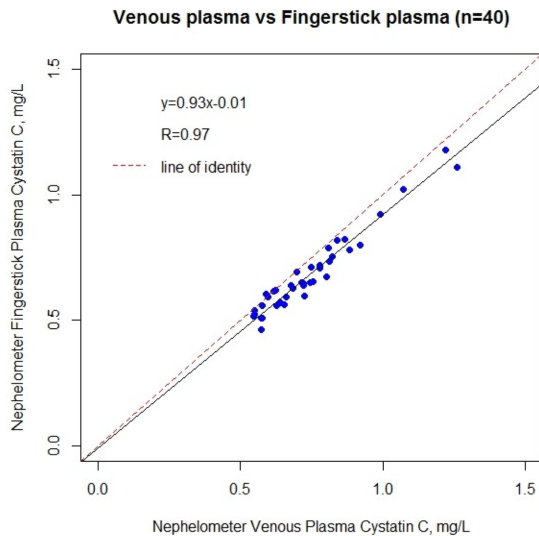

Figure 9: The dotted line represents the line of identity (*y*=*x*). Intercept=-0.01 (95% confidence interval=-0.07 to 0.05) and slope=0.93 (95% confidence interval=0.85 to 1.01).

Deming regression analysis reveals an intercept of -0.01 (95% confidence interval=-0.07 to 0.05) and a slope of 0.93 (95% confidence interval=0.85 to 1.01). Both the intercept and the slope are not

statistically significantly different from a line of identity; however, determining that there is not a difference between cysC concentrations based on the results of the Deming regression may be too conservative. A paired t-test revealed the difference between the mean cysC concentration in plasma from venous blood vs. plasma from capillary blood in this population to be -0.06 (95% confidence interval= -0.07 to -0.05, P<0.0001).

4.8 Effect of Hematocrit

Hematocrit is thought to be the single most important parameter influencing the spread of blood on DBS [34]; it impacts spot formation, spot size, drying time, homogeneity and ultimately the reproducibility of assays. The effect is most noticeable when a punch is taken from a DBS rather than using the whole spot.

In order to investigate if hematocrit was introducing uncertainty into the cysC concentration in DBS measured by ELISA, five aliquots of anonymized 7-day old EDTA blood were acquired from the Harborview hematology laboratory. Tubes were spun down and plasma removed; residual red cells were assumed to have a 100% hematocrit. Each sample's red cells were re-suspended in its corresponding reserved plasma to form a set of samples with approximate hematocrits of 30%, 40%, 50%, 60%, 70% and 80%, and then spotted onto filter paper. DBS were dried overnight at RT and analyzed using the DBS-ELISA method. For the purpose of this experiment, a 50% hematocrit was assumed to representative of a typical DBS. Concentrations of cysC in each hematocrit condition were evaluated against the concentration present in the DBS created from 50% hematocrit WB.

Results (Figure 10) are plotted as percent recovery of the concentration of the 50% hematocrit concentration (100 × observed/expected). A one-way ANOVA was used to test for differences in mean recovery between the 5 different levels as compared to the 50% hematocrit; means differed significantly across the 5levels, $F(5, 24) = 5.95$, $p<0.01$. However, it is not likely that we would receive a DBS created from an individual with a hematocrit greater than 60%. A one-way ANOVA of

mean recovery of 50% hematocrit in level 30%, 40% and 60% levels revealed that there was no statistically significant difference, $F(3,16)=0.42$, $p=0.75$.

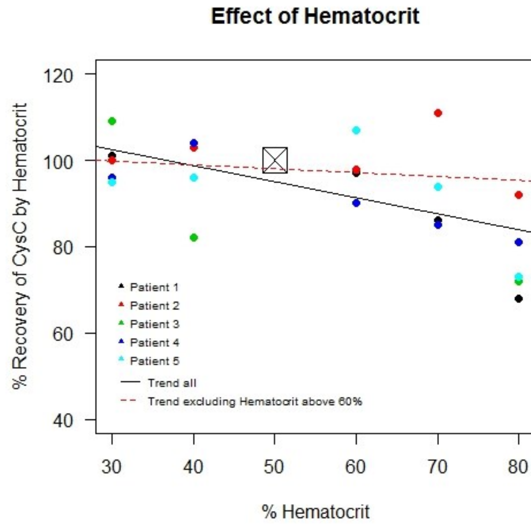

Figure 10: % recovery of the cysC concentration found in the 50% hematocrit (marked by the x-box). A one-way ANOVA was used to test for differences in mean recovery between the 6 different levels. Means differed significantly across the 6 levels, $F(5, 24) = 5.95$, $p=0.001$.

4.9 Linearity

Assay linearity was evaluated by serially diluting 4 DBS samples 1:1, 1:2, 1:4, and 1:8 in diluent buffer after elution (Figure 11). Mean recovery (100 × observed/expected) was 100%, and ranged from 95% to 103%, indicating a high degree of linearity across the assay range.

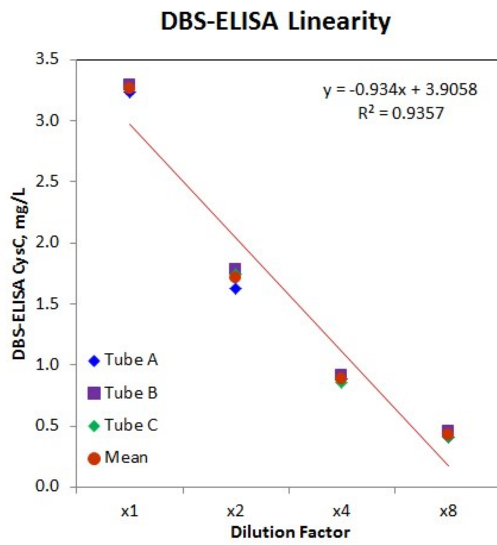

Figure 11: DBS-ELISA Linearity of serially diluted DBS samples.

4.10 Limit of Detection

The detection limit (defined as the concentration corresponding to the absorbance 2 SD above the mean of 24 replicates of the 0.00 mg/L calibrator) was 0.053 mg/L (data not shown), a value approximately 10% of the expected lower limit of the normal reference range.

Chapter 5

Discussion

Cystatin C (cysC) is a low molecular weight (12.8 kDa) protein synthesized by all nucleated cells; its physiological function is as a cysteine protease inhibitor. CysC is produced by all nucleated cells at constant rate and it is present in most bodily fluids. CysC is exclusively eliminated from the circulation by glomerular filtration—in the absence of significant tubular damage, cysC is reabsorbed and metabolized by the proximal tubular epithelial cells and is not returned to circulation. Furthermore, blood plasma concentrations of cysC are unaffected by muscle mass, diet or gender. These properties make cysC an excellent estimator of glomerular filtration rate.

There is interest in developing an assay to evaluate kidney function using dried blood spots (DBS). Currently, cysC concentrations may be measured in a hospital laboratory in serum or plasma by nephelometry with a high degree of precision and reliability; however, development and validation of ELISA assay using DBS would provide a simple, convenient method of measuring cysC in large-scale epidemiology studies.

The aim of typical method validation is to demonstrate that a particular method used for quantitative measurement of analytes in a particular biological matrix —e.g. blood, plasma, serum, or urine, is reliable and reproducible. Our method evaluation is atypical in that we intend to measure cysC concentrations in DBS by ELISA and compare them to cysC concentrations in plasma by nephelometry — a comparison across measurement systems and in different matrices. For this evaluation, cysC as measured by nephelometer in plasma was our "gold standard" reference method; however, due to the inherent variability in DBS we judged acceptability of the DBS-ELISA based on DBS measurements from both systems.

Deming regression was chosen for our analysis as it accounts for random error in both the comparative (X) and test method (Y)—unlike Ordinary Least Squares Regression (OLR) in which all

of the error is applied only to test method. Deming regression is superior to OLR in detecting systemic bias as OLR produces reliable estimates when the correlation coefficient is >0.975 [35]; however, standard Deming regression assumes that both the comparative and test methods have equal variance. Applying a weighted modification of the Deming procedure would be preferable but in order to do so we must obtain estimates the variation in the two comparators. The easiest way to attain an estimate is to use duplicate measurements in the method comparison study, allowing for calculation of analytical SDs and CVs —indeed, the use of duplicate sets of measurements in method comparison studies is generally recommended [35].

Experiments assessing the performance of the DBS-ELISA for cysC against the plasma nephelometer reference method showed near equivalence; i.e. no evidence of proportional, but a very slight constant bias (Figure 1). We determined that the BioVendor ELISA kit was performing per manufacturer specifications by comparing cysC concentrations in plasma determined by the kit against matched plasma by nephelometry (Figure 2). Measurements of cysC concentrations in VP-DBS compared with DBS-matched plasma, both determined by nephelometry, showed evidence of both constant and proportional bias (Figure 3); however, the confidence intervals for the slope and intercept overlap with what was observed in cysC measurements in DBS by DBS-ELISA (Table 6). To eliminate bias introduced by differences in sample matrix, we compared DBS-ELISA against nephelometry cysC concentrations, both in VP-DBS (Figure 4). Again, there was equivalence. Bland Altman analysis (Figure 5) of DBS-ELISA against DBS by nephelometer showed no bias and had acceptable levels of agreement (Bias -0.02, 95% CI=-0.21 to 0.17).

Hematocrit is thought to be the single most important parameter influencing the spread of blood on DBS as it impacts spot formation, spot size, drying time, homogeneity and ultimately the reproducibility of assays [34]. Experiments investigating the effect of hematocrit on cysC concentrations were inconclusive. A better idea of its impact could be obtained by better experimental design—e.g. spike and recovery experiments of cysC in blood of various hematocrit with known plasma cysC concentrations, and the use of triplicate measurements to reduce noise.

To our surprise, we observed that cysC concentrations in DBS created by fingerstick had a significant constant bias (-0.21, 95% confidence interval = -0.36 to -0.10) (Figure 8). Evidence of proportional bias in plasma collected from venipuncture and fingerstick by nephelometry was again observed (Figure 9); although the slope was not significantly different from 1.0, there was a significant difference ($p<0.0001$) between paired values by the more sensitive paired t-test statistic. We suspect that the bias is a real phenomenon, with the capillary and finger-stick concentration of cysC lower than the concentration in venous blood.

The lower concentrations of cysC found in blood collected by fingerstick may have been due to a dilutional effect caused by the mixing of blood with intracellular and interstitial fluid. This suggests that interstitial fluid might have a lower concentration of cysC than found in venous blood.

Another possibility is that venous and fingerstick bloods aren't constitutively identical and indeed, others have observed important differences between venous and capillary blood in the concentrations of glucose, potassium, total protein, and calcium [35]. In fingerstick samples, the contribution of arterial blood, due to increased pressure at the arteriolar segment of the capillary bed, is greater than that of the venules at the venous segment. The higher hydrostatic pressure on the arterial side of the capillary bed produces 'mix' of arterial and venous blood with a greater contribution of blood from the arteriole side. We hypothesized that if cysC concentrations were lower in arterial blood, then that possibility could account for lower observed concentrations. This possibility could be tested by acquiring simultaneous paired arterial and venous blood and analyzing for cysC. Analyzing mixed venous blood from the heart would further address this question, since it is possible that cysC concentrations vary in different venous blood sources. Intriguingly, if there is a difference in mixed venous vs. arterial concentrations of cysC, it might suggest that the lungs and/or heart have a role in clearance or consumption of cysC.

Future work should include increasing the number of VP-DBS and FS-DBS cysC samples outside of the reference interval in the dataset as additional high concentration cysC samples would allow for more accurate estimates of regression parameters. Both plasma and DBS samples should be run in

duplicate as the estimates of variation generated from the duplicates could be inputted as a parameter in weighted Deming regression analysis. Of additional interest would be to collect fingerstick blood and venipuncture blood from patients with known kidney disease to see if the observed difference between VP-DBS and FS-DBS holds true. Finally, spike and recovery experiments need to be conducted to help better understand DBS matrix effects and complete the suite of method validation studies recommended by University of Washington Department of Laboratory Medicine guidelines.

Using a commercially available ELISA kit we were able to measure cysC in DBS. Assay validation studies demonstrated that our DBS-ELISA method was able to determine cysC concentrations across the physiologic range in DBS created from both venipuncture and fingerstick blood. VP-DBS cysC concentrations by DBS-ELISA were strongly linear and near equivalent with cysC by nephelometry in plasma. (Intercept=-0.02, 95% confidence interval = -0.08 to 0.03; Slope=0.93, 95% confidence interval = 0.87 to 0.98.) Method evaluation demonstrated good correspondence between VP-DBS cysC concentrations determined by both DBS-ELISA and nephelometry. Bland Altman analysis of DBS-ELISA against DBS by nephelometer showed no bias and had acceptable levels of agreement (Bias -0.02, 95% CI=-0.21 to 0.17). The DBS-ELISA also demonstrated comparable precision and reliability (intra-assay 5.4%, inter-assay 7.4%) to DBS measured on a nephelometer (intra-assay 4.2%, inter-assay 6.9%). The DBS-ELISA demonstrates utility as a screening tool; sensitivity and specificity for cysC concentrations outside the upper limit of the reference interval (0.51 mg/L to 1.02 mg/L) were 68% and 95% respectively; however, the DBS-ELISA had difficultly correctly assigning CKD stages to cysC concentrations from DBS. Finally, this work has elucidated an interesting biological quirk, namely an intra-donor difference in cysC concentration between venous blood collected from venipuncture and capillary blood collected by fingerstick. Our DBS-ELISA assay provides an opportunity to determine kidney function based on cysC in larger community-based samples.

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