ten increased in acromegalic patients and decrease after pituitary surgery. S-Selectin as an endothelial cell-specific product might be useful in the assessment of cardiovascular risk in patients with acromegaly. An improvement in S-selectin concentrations may be related to decreased progression of macrovascular disease and normalization of long-term morbidity and mortality as observed after curative pituitary surgery (17–19).

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References


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High-Sensitivity Enzyme Immunoassay for C-Reactive Protein in Dried Blood Spots, Thomas W. McCade, James Burhop, and James Dohmally (1) Laboratory for Human Biology Research, 2 Department of Neurobiology and Physiology, and 3 Evanston Northwestern Healthcare Research Institute and the Feinberg School of Medicine, Northwestern University, Evanston, IL; * address correspondence to this author at Laboratory for Human Biology Research, Northwestern University, 1810 Hinman Ave, Evanston, IL 60208; fax 847-467-1778; e-mail t-mcdaed@northwestern.edu

C-Reactive protein (CRP), the prototypical acute-phase protein, is produced by liver hepatocytes and regulated by cytokines, particularly interleukin-6 (1, 2). Circulating concentrations of CRP indicate inflammatory activity, and the recent development of highly sensitive CRP assays (3–5) has led to the discovery that slight increases in CRP (>1–2 mg/L) are indicative of low-grade inflammatory processes that may be related to the pathophysiology of cardiovascular disease. More than a dozen population-based studies have demonstrated that increased CRP is an independent risk factor for future cardiovascular disease, with adjusted odds ratios >2.0 (6–9). The American Heart Association and the CDC have recommended measurements of CRP in clinical practice and called for additional population-based research (10).

A potential obstacle to the measurement of CRP (as well as other biomarkers) in large epidemiologic, community-based studies is the requirement for venous blood. Venipuncture is a relatively invasive procedure that must be performed by a trained phlebotomist (usually in a clinical setting), and it requires readily accessible facilities where blood samples can be promptly processed and stored under controlled conditions. Assays using whole blood dried on filter paper may provide a viable alternative: Several community-based applications have shown this to be a convenient and reliable means to facilitate sample collection, storage, and transportation, and laboratory methods have been validated for a growing number of analytes (11–16). "Guthrie papers" have been a core component of US hospital-based newborn-screening programs since the 1960s and are subject to a rigorous quality-control program (17).

Samples can be collected on filter paper easily by nonmedical personnel: The patient's finger is pricked with a sterile, disposable lancet (commonly used by diabetics), and up to five drops of blood (~50 µL per drop) are spotted onto standardized filter paper (no. 903; Schleicher and Schuell) that is certified to meet perfor-
mance standards for sample absorption and lot-to-lot consistency set by the NCCLS and by the Food and Drug Administration regulations for Class II Medical Devices. The samples are allowed to dry and then shipped by express or standard mail to the laboratory for freezer storage.

We describe a high-sensitivity enzyme immunoassay for CRP in dried blood spots. To minimize matrix differences and maximize comparability between calibrators and unknowns, dried-blood-spot calibrators were made by diluting delipidated human serum enriched with CRP [X0023 (standardized against the WHO International Reference Preparation); Dako] with washed erythrocytes, followed by application to filter paper. Washed erythrocytes were obtained as follows: (a) Whole blood was collected by venipuncture in 5-mL EDTA Vacutainer Tubes and centrifuged at 1500g for 15 min. (b) Plasma and buffy coat were removed and discarded. (c) Approximately 3 mL of saline (0.86 g of NaCl in 100 mL of deionized H₂O) was added. (d) Tubes were mixed gently for 5 min on a hematology rotor and centrifuged as before. Saline and any remaining buffy coat were removed, and steps c and d were repeated for a total of three washes. The CRP preparation was serially diluted in assay buffer (0.01 mol/L phosphate buffer, 0.5 mol/L NaCl, pH 7.2), diluted 1:2 with washed erythrocytes, and pipetted on filter paper in 50-μL drops. Final calibrator concentrations were 10.1, 5.07, 2.53, 1.27, 0.63, 0.32, 0.16, 0.08, and 0.00 mg/L.

Microtiter plates (cat. no. 439454; NUNC Maxisorp) were coated overnight with 100 μL/well rabbit anti-human CRP antibody (cat. no. A0073; Dako) at a concentration of 10 mg/L in coating buffer (0.01 mol/L phosphate buffer, 0.145 mol/L NaCl, 0.1 g/L Thimerosal, pH 7.2). One 3.2-mm disk from each blood spot sample, control, and calibrator was eluted overnight at 4 °C in 250 μL of wash/elution buffer (0.01 mol/L phosphate buffer, 0.5 mol/L NaCl, 1 mL/L Tween 20, pH 7.2) and rotated on a microplate shaker (cat. no. 51402; Cole-Parmer; 3 mm orbit) at 300 rpm at room temperature for 60 min the following day. Eluate (100 μL) from each disk was pipetted in duplicate into microtiter wells that had been blocked by incubation for 30 min with wash/elution buffer. Wells were washed after a 2-h incubation at room temperature, with rotation at 250 rpm. Detection antibody (peroxidase-conjugated rabbit anti-human CRP antibody; cat. no. P227; Dako) was diluted to 0.163 mg/L in wash/elution buffer, added to the wells (100 μL), and incubated for 2 h at room temperature. The wells were washed, and 100 μL of chromogenic substrate [14 mg of 1,2-phenylenediamine dihydrochloride (cat. no. S2045; Dako), 5 μL of 300 g/L H₂O₂, and 12 mL of deionized H₂O] were added for color development. Wells were incubated in the dark for 30 min before the addition of 100 μL of stop solution (0.5 mol/L H₂SO₄). The absorbance was read at 490 nm (BioTek EIAx809), and sample concentrations were calculated from the best-fit four-parameter logistic calibration curve (KCJunior; BioTek). Samples reading above the highest calibrator were reanalyzed at a higher dilution factor (i.e., disks were eluted in 500 μL of wash/elution buffer and the result was multiplied by 2).

The detection limit (defined as the concentration corresponding to the absorbance 2 SD above the mean of 10 replicates of the 0.00 mg/L [calibrator]) was 0.028 mg/L. Within-assay imprecision estimates (CV) at 1.01 and 5.09 mg/L were 5.1% and 6.4% (n = 10 determinations), respectively; between-assay CV were 9.5% and 6.9%, respectively (n = 10 determinations across 10 assays performed on different days). We investigated assay linearity by serially diluting two samples after elution. The observed values ranged from 94.5% to 109% of expected, with a mean of 103%. Control sera containing a known concentration of purified CRP (cat. no. X0025; Dako) were diluted 1:2 with washed erythrocytes and spotted on filter paper. Observed values for the low and high control samples were 102% and 93% of expected, respectively.

We compared CRP concentrations in 94 paired blood-spot and serum samples (Fig. 1) that were leftover patient samples. Serum samples were analyzed with a turbidimetric assay on the IMMAGE™ Immunochemistry System (Beckman Coulter, Inc.). The relationship between the blood spot and serum methods was linear, and the correlation was high. The regression equation provides a means for generating serum equivalents from blood-spot samples if desired (12), although a larger, more representative sample of paired blood-spot and serum samples should be enlisted to generate this equation before its application. We also compared serum and blood-spot results by calculating the ratio of serum CRP to blood-spot CRP and inspecting for differences or inconsistent variability across the measurement range (18). The mean (SD) ratio was 1.39 (0.48), with two values outside the 95% limits of agreement. These values belonged to samples with blood-spot CRP concentrations <0.5 mg/L and

![Graph](attachment:Fig_1.png)

**Fig. 1.** Relationship between blood-spot and serum CRP concentrations in 84 paired samples. The best-fit linear regression line is shown. Samples above the highest blood-spot calibrator (10.13 mg/L) are not included (n = 10).
reflect the slightly higher variability in the agreement between serum and blood spot results for samples <1.0 mg/L. There was no evidence of systematic differences in the serum: blood-spot CRP ratio across the assay range.

Hematocrit correction does not improve agreement between plasma and blood spot results for gonadotropins and is not necessary for samples with normal hematocrits (12). To confirm this for CRP, we added washed erythrocytes (in concentrations of 30%, 40%, and 50%) to three plasma samples and spotted them on filter paper. There was no consistent association between hematocrit and blood-spot CRP concentration.

Many epidemiologic analyses of the association between serum CRP and cardiovascular disease risk are categorical, with the distribution of CRP concentrations divided into tertiles or quartiles (7, 9). Recently, cutpoints of low risk (<1.0 mg/L), average risk (1.0–3.0 mg/L), and high risk (>3.0 mg/L) have been proposed that approximate the tertile distribution of serum/plasma CRP in a range of populations (10). We compared category assignments according to the blood-spot and serum methods in our 94 matched samples, with the distributions of blood-spot and serum CRP concentrations divided separately into tertiles. Eighty-seven of 94 individuals (93%) were assigned to the same category by both methods.

We evaluated the stability of CRP in dried blood spots by exposing nine samples sealed in plastic bags with two desiccant packs (cat. no. 61161-319; VWR) to the following temperature conditions for up to 14 days: 37 °C, room temperature (21–23 °C), and 4 °C. An additional variable-temperature condition was evaluated (12 h at 32 °C and 12 h at 22 °C) to simulate ambient temperature exposures in tropical environments. Samples were considered to remain stable as long as CRP concentrations remained within 2 SD of baseline values measured in samples stored at −30 °C immediately after collection. Six determinations were used to calculate the baseline mean (SD) for each sample: 1.11 (0.10), 1.58 (0.093), 1.60 (0.15), 2.46 (0.25), 2.65 (0.17), 4.28 (0.17), 4.57 (0.32), 6.40 (0.55), and 6.64 (0.44) mg/L. CRP concentrations remained stable in dried blood spots for 3 days at 37 °C, 3 days at 32/22 °C, and for at least 14 days at room temperature and 4 °C. The stability of CRP to repeated cycles of freezing and thawing was also evaluated, with no evidence of deterioration after five freeze–thaw cycles (1 h at room temperature, repeated over 5 different days).

Previous applications of whole-blood-spot methods have demonstrated performance characteristics similar to those for methods that rely on venipuncture (17). The ease of finger stick blood collection alleviates constraints associated with sampling in clinical settings, increases the frequency with which samples can be taken, and expands the methodologic options for population-level health research. In particular, our high-sensitivity CRP method could potentially be a useful tool for community-based, epidemiologic investigations of inflammation and cardiovascular risk.

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References

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Transferrin Enzyme Immunoassay for Quantitative Monitoring of Blood Contamination in Saliva, Eve B. Schwartz1* and Douglas A. Granger2* (Salimetrics LLC, State College, PA; 2Behavioral Endocrinology Laboratory, Department of Biobehavioral Health, Pennsylvania State University, University Park, PA; *address correspondence to this author at: Salimetrics LLC, 101 Innovation Blvd., Suite 302, State College, PA 16803; fax 814-234-1608, e-mail ebs@salimetrics.com)

When blood components are present in the oral mucosa, quantitative estimates of salivary hormone concentrations may be compromised (1). Blood and its components can